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Running title: Fluoroquinolone resistance in *E. coli* in poultry

Synopsis

Objectives: Factors potentially contributing to fluoroquinolone resistance selection in commensal *E. coli* strains in poultry were studied through a series of *in vivo* experiments. The effect of the initial prevalence of enrofloxacin resistance in the *E. coli* gut microbiota, the effect of the bacterial fitness of the enrofloxacin-resistant strain and the effect of treatment with enrofloxacin (effect of dose and effect of route of administration) were assessed.

Methods: Four in vivo studies with broiler chickens were performed. Right after hatching, the chicks were inoculated either with a bacteriologically fit or a bacteriologically non-fit fluoroquinolone-

resistant strain either as a minority or a majority of the total *E. coli* population. Six days later, the chicks were treated for three consecutive days either orally or parenterally and using three different doses (under-, correct- and over-dose) of enrofloxacin. The faecal shedding of *E. coli* strains was quantified by plating on agar plates either supplemented or not supplemented with enrofloxacin. Linear mixed models were used to assess the effect of the aforementioned variables on the selection of enrofloxacin resistance.

Results: The factors that significantly contributed were treatment ($p<0.001$), bacterial fitness of the resistant donor strain ($p<0.001$), administration route ($p=0.052$) and the interactions between bacterial fitness and administration route ($p<0.001$).

Conclusions: In the currently used models, fluoroquinolone resistance selection was influenced by treatment, bacterial fitness of the inoculation strain, and administration route. The use of oral treatment seems to select more for fluoroquinolone resistance, especially in the model where a non-fit strain was used for inoculation.

Introduction

Antimicrobial resistance is a natural phenomenon dating back thousands of years before the use of antimicrobials.[1, 2] Nevertheless, the use of antimicrobials has contributed to the rise of antimicrobial resistance in bacterial pathogens of human and veterinary importance.[3-5] Fluoroquinolones constitute a critically important class of antimicrobial agents that directly inhibit DNA replication and transcription. In veterinary medicine, fluoroquinolones are widely used, especially in broiler production,[6] with the oral route being the preferred administration route. Despite their efficacy, the use of fluoroquinolones in veterinary medicine is controversial[7]. Concerns about the increasing resistance against enrofloxacin in poultry led to the withdrawal of its use in 2005 in the USA,[8] while in Australia its use has never been authorized. Nonetheless, in two later studies from USA[9] and Australia,[10] fluoroquinolone-resistant strains were detected in several broiler samples, prompting the

authors to suggest either a non-proper enforcement of the ban,[9] or co-selection caused by the use of other antimicrobial agents.[10]

Several studies have suggested a link between oral treatment with antimicrobial agents and selection of antimicrobial resistance in chickens.[11-14] Nevertheless, only a few studies compare parenteral and oral treatment protocols in broilers, and they focus solely on the pharmacokinetics of enrofloxacin and not on its effect on resistance selection.[15,16] Whether the effect of all contributing factors could be quantified, the optimal regimen (dose, route of administration, duration) could be improved to reduce resistance selection while maintaining clinical efficacy.

Antibiotic resistance mechanisms can induce a fitness cost to the bacterium. This cost is more considerable in chromosomal resistance mutations than in resistance acquired via horizontal gene transfer.[17] This cost is a key parameter in the spread and persistence of antimicrobial-resistant bacteria.[18] However, it is not yet known to what extent fitness influences the resistance selection. Studies supporting the reversibility of antibiotic resistance through minimizing antimicrobial use are available,[19-23] though other studies focus on concepts such as compensatory evolution and genetic co-selection that make reversibility less probable (even if a fitness cost is present) in real-life settings.[24-26] The potential for reversing antibiotic resistance through the reduction of antibiotic use will be dependent on the fitness cost of the resistance mechanism, the epidemic potential of the bacteria, and the transmission route of the species.[27]

Overall, there is insufficient information on the epidemiology of antimicrobial resistance, and this lack hampers efforts to provide appropriate and specific advice on measures that might reduce risks of resistance selection. In response, the present study aims at quantifying the effects of different factors on fluoroquinolone resistance in commensal *E.coli* in broilers, using well-defined and controlled experimental *in vivo* models. Four *in vivo* experiments were designed to study the influence of the: i) prevalence and ii) fitness of enrofloxacin-resistant strains in the early (one-day-

old) *E. coli* gut microbiota, iii) treatment dose and iv) route of administration of enrofloxacin on fluoroquinolone resistance selection in commensal *E. coli*.

Materials and methods

Ethics

In vivo experiments were compliant with all relevant institutional and European standards for animal care and experimentation. All experiments were approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University (EC2014/141, EC2015/33, EC2015/34, EC2015/61).

Bacterial strains

E. coli IA2 strain was obtained from a faecal sample of a healthy broiler chicken. The isolate was confirmed as being *E. coli,* and after being serotyped with the following monospecific antisera against 24 different somatic O antigens: O1, O2, O5, O6, O8, O9, O11, O12, O14, O15, O17, O18, O20, O35, O36, O45, O53, O78, O81, O83, O102, O103, O115, O116, it was deemed to be non-serotypable (identified and serotyped by CODA CERVA, Uccle, Belgium). Moreover, the strain was susceptible to all tested antibiotics (Table S1). On top of that, no plasmid mediated quinolone resistance (PMQR) genes were detected after using a protocol described by Robicsek *et al.* to detect for any *qnrA, qnrB, qnrS* determinants,[28] and by Park *et al.* to detect for the *aac(6')-Ib-cr* determinant.[29]

Using this strain, a spontaneous rifampicin-resistant mutant strain (*E. coli* IA31) was derived as previously described.[30] Using IA31 and the same technique, a bacteriologically non-fit (*E. coli* IA50) enrofloxacin-resistant strain and a bacteriologically-fit (*E. coli* IA66) enrofloxacin-resistant strain were created (Table 1). Bacterial fitness was assessed with *in vitro* growth competition assays between the resistant strain and the parental susceptible strain.[24] Equal densities of the enrofloxacin-susceptible and the enrofloxacin-resistant strain were mixed and incubated in antibiotic-free LB medium. Every

24h, 0.05 mL of the overnight culture was inoculated into 5 mL of new LB medium for growth. Aliquot parts of the same volume were plated, via a spiral plating technique, every 24h onto drug-free McConkey agar to count the number of colonies and onto McConkey agar plates containing enrofloxacin 1 mg/L to count the number of enrofloxacin-resistant strains. The number of parental enrofloxacin-susceptible colonies was calculated as the total number of bacterial cells minus the number of drug-resistant bacterial cells. The relative fitness was calculated as described.[31] All experiments were performed in triplicate with three independent cultures and a weighted mean was used for analysis.

Table 1. Strains (A.)and inoculums (B.) used in this paper. For all inoculums, the volume (dose per animal) was 0.2mL. and the concentration (cfu/mL inoculum) was ~ 10^8 cfu/mL.

A. Strain	Parental strain	Bacteriological fitness (compared to its parental strain)	Resistance against enrofloxacin	Resistance against rifampicin (marker)
***E.coli* IA50**	*E. coli* IA31	Non-fit	Resistant	Resistant
***E.coli* IA66**	*E. coli* IA31	Fit	Resistant	Resistant
***E. coli* IA31**	***E. coli* IA2**	**Fit**	***Susceptible***	**Resistant**

B. Inoculum	1	2	3	4
Used at	Exp. 1	Exp. 2	Exp. 3	Exp. 4
Strains used	IA50 : IA31	IA66 : IA31	IA66 : IA31	IA50 : IA31
Ratio	**100 : 1**	**100 : 1**	**1 : 100**	**1 :100**

The MIC, MBC and mutant prevention concentration (MPC) of the reference and the resistant strains were determined as previously described.[32-35] *E. coli* ATCC 25922© was used as a control strain for the *in vitro* tests.

PCR amplification and DNA sequence analysis

For the PCR amplification and sequencing of *gyrA, gyrB, parC,* and *parE* genes, the respective primers were used[36-38] (Table S2). The amplification protocol was performed with a MasterCycler Gradient EP-S Thermal Cycler (Eppendorf AG, Hamburg, Germany). The PCR products for strains IA31, IA50 and IA66 were sequenced (Eurofins Genomics GmbH, Ebersberg, Germany) and the nucleotide sequences

obtained were analyzed for the presence of point mutations in the quinolone resistance determining regions (QRDR) using the BLAST search engine and the ClustalW multiple alignment tool[39] (Table 2).

Table 2. In vitro results. The topoisomerase mutations, the MIC, MBC and mutant prevention concentration(MPC)are presented. E.coli ATCC 25922 was used as a control strain to identify any amino acid changes when compared to E. coli IA31.

		***E. coli* ATCC 25922[a]**	***E. coli* IA31[b]**	***E .coli* IA50[c]**	***E .coli* IA66[d]**
***GyrA* changes**	nucleotides	167542- 166980			
	amino acid	5-191	NC[e]	83 (S->A (+)) 87 (D->G)	83 (S->L) 87 (D->G)
	accession nr.[f]	ref|WP_001281242.1|	KX525205	KX525206	KX525207
***GyrB* changes**	nucleotides	853200- 853572			
	amino acid	347-467	NC	NC	NC
	accession no.	ref|WP_000072067.1|	KX525208	KX525209	KX525210
***ParC* changes / Codon number**	nucleotides	1589650 – 1589896		78: GGC -> GAC	80: AGT -> AGA
	amino acid	53-133	NC	78 (G -> D)	80 (S -> R)
	accession no.	ref|WP_001281881.1|	KX525211	KX525212	KX525213
***ParE* changes**	nucleotides	1573843- 1574108			
	amino acid	412-499	NC	NC	NC
	accession nr	ref|WP_000195296.1|	KX525214	KX525215	KX525216
Enrofloxacin cut-off values	MIC (mg/L)	0.016	0.032	32	32
	MBC (mg/L)	0.016	0.047	64	32
	MPC (mg/L)	0.512	0.512	1024	1024

a: This is the in vitro control strain. All changes in the amino-acid level will be enumerated according to this strain b: This is the enrofloxacin-susceptible strain used in all in vivo experiments. c: Enrofloxacin-resistant non-fit mutant strain. d: Enrofloxacin-resistant fit mutant strain., e: NC: No changes took place. f: Accession no provided by GenBank (NCBI, Bethesda, MD, USA)

In vivo trials

Eggs, chickens, housing and welfare

Embryonated 17-day-old eggs were collected under aseptic conditions from a commercial poultry hatchery (Vervaeke-Belavi, Belgium). The eggs were disinfected with a gas formaldehyde mixture at the hatchery, but after transportation they were additionally dipped in 5% H_2O_2 for 10-15 seconds. After drying for 20-25 seconds, they were further incubated in three separate sanitized hatching cabinets. Each cabinet was placed in a separate decontaminated stable.

As soon as the chicks were hatched, they were orally inoculated (Table 1) and subsequently housed in groups (each group consisting of five chicks) in 1m^2 disinfected boxes in HEPA-filtered stables. Nine groups were used in each experiment and in total, 180 chickens were used in this study (45 per experiment). The birds received 16 hours of light daily, and had free access to autoclaved food and bottled water. Each bird was individually numbered. All birds were clinically examined on a daily basis and any clinical signs of disease were registered. Euthanasia was performed by intravenous injection with an overdose (10 mg/kg) of sodium pentobarbital 20% (Kela, Hoogstraten, Belgium).

Experimental setup

As soon as the chickens were hatched, they were all inoculated with a specific bacterial inoculum in accordance with the experiment performed (Table 1). The experimental setup, treatment and sampling procedure was identical for all experiments (Figure 1). All necessary biosecurity measures were taken to avoid any cross-contamination (feed, water, indirect contact, air-borne transmission) between groups. Each stable contained a control group (non-treated animals), a group that was treated with enrofloxacin (Baytril™ 2.5% inj. Solution, Bayer AG, Leverkusen, Germany) intramuscularly and a group that received enrofloxacin via drinking water (Baytril™ 10% oral solution, Bayer AG, Leverkusen, Germany). Water was provided *ad libitum*. Treatment period lasted three days (day 6 to day 8). Treatment doses (Table S3) were calculated on the basis of the daily average body weight and the average water consumption,[40] although water intake was not measured in detail.

In total, six faecal samplings took place in each experiment. Starting from day 3 of the experiment, there was one sampling every 3 days until day 15. Then, one additional sampling took place right before euthanasia on day 22. Each sample was collected from a single animal and consisted of ~ 1 g of faecal content. Upon collection, all fresh individual samples were placed in sterile tubes and immediately transported to the laboratory for bacteriological enumeration.

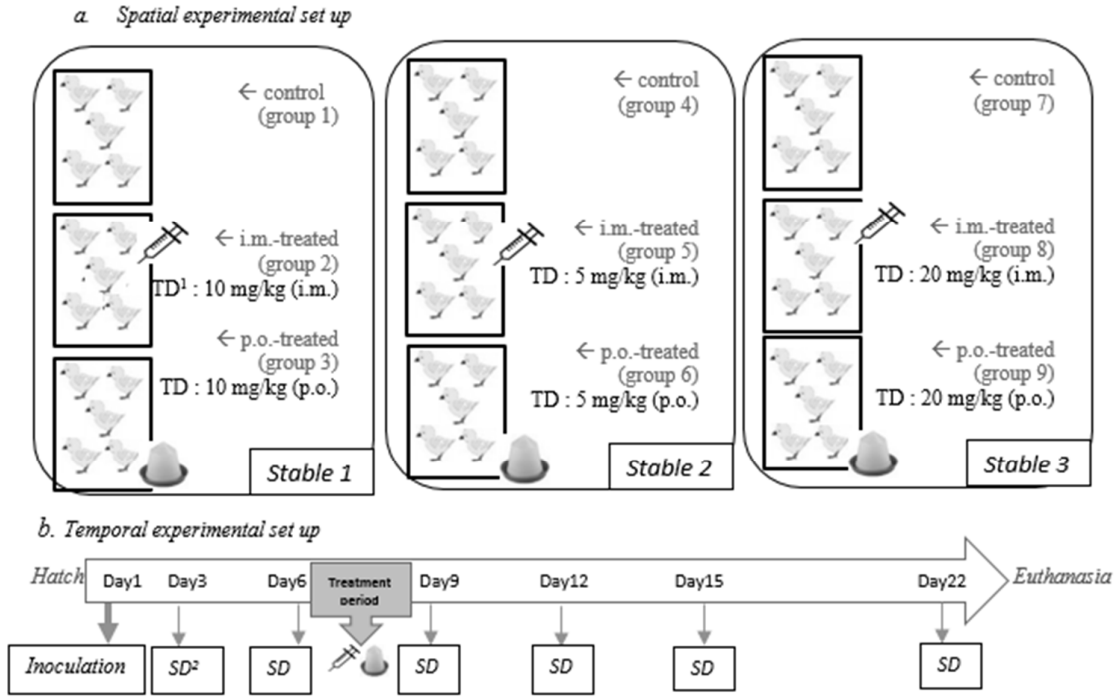

Figure 1. Schematic plan of the experiments (same for each experiment performed). As soon as the chicks were hatched, they were inoculated with a specific bacterial inoculum according to the experiment performed. Each stable contained a control group (non-treated animals), a group that was treated with Baytril™ 2.5% inj. solution intramuscularly and a group that received oral treatment (Baytril™ 10% oral solution). Treatment period lasted 3 days (day 6 to day 8). The treatment started right after the second sampling took place. The treatment dose schemes (Stable 1: proper-, Stable 2: half- and Stable 3:double- dose) were calculated based on the recommended therapeutic protocol of the company (Bayer AG, Leverkusen, Germany). The drinking water medication was prepared daily. one sampling every 3 days until day 15. Then, one additional sampling took place right before euthanasia on day 22. [1] : Treatment dose, [2] : Sampling day

Bacteriological enumeration in faecal samples

The faecal content was serially ten-fold diluted in phosphate buffered saline solution (10^{-1} to 10^{-4}). The spiral plating technique was used to enumerate the *E. coli* population (Eddy Jet, IUL Instruments, Barcelona, Spain).

All serial dilutions were plated on both i) rifampicin-supplemented (100 mg/L) McConkey agar plates and ii) enrofloxacin-supplemented (0.25 mg/L) and rifampicin-supplemented (100 mg/L) McConkey agar plates. Since the enrofloxacin-susceptible strain cannot grow on the enrofloxacin-supplemented plates, these plates were used to differentiate the inoculated strains and allow for the calculation of the ratio of resistant strains .

After the grafting of each plate, they were placed in an incubator set at 37.0°C ± 1°C and examined after 24h ± 3h for the presence of typical colonies of *E.coli*. The colonies per plate were counted on plates ideally having 20 - 200 colonies per plate and the number of colony forming units (cfu)/g of faeces was calculated. In the exceptional case where less than five colonies per plate were counted in the lowest dilution, the sample was omitted.

Statistical analysis

In all the following analyses, linear mixed models were used (IBM SPSS Statistics for Windows, version 23.0, Armonk, NY). Each animal was listed as 'subject', and the sampling as 'repeat'. An autoregressive covariance matrix of the first order was used for the repeated covariance structure. To correct for the interdependency of chicks within a given pen, 'pen' was included as a random variable. The dependent variable used was the ratio of the number of enrofloxacin-resistant colonies to the total number of retrieved *E.coli*.

Assessment of treatment

The proportion of resistant strains over time was compared between animals that received treatment and those that did not. Observations for day 3 and day 6 (prior treatment) were similar within each respective experiment and in order to focus on the intervention effect of the treatment only observations from day 9 onwards (after treatment) were included.

Assessment of prevalence (inoculum ratio), fitness of enrofloxacin-resistant strains, treatment dose and route of administration

All animals that received treatment were included in the model. The fixed factors that were studied were the following: inoculum ratio (highly resistant, highly susceptible), bacteriological fitness (fit, non-fit), treatment dose (proper-, half-, double- treatment schemes) and route of enrofloxacin administration (oral, parenteral (i.m.)). All potential fixed factors were first tested univariately. Only variables with a P-value < 0.2 were selected to be included in a mixed (multivariate) linear model.

The model was built according to a stepwise forward selection procedure. In the final mixed linear model, two-way interactions between significant variables were also evaluated. The main effects from the fixed factors were compared and Bonferroni correction was used to adjust confidence intervals. Throughout the entire analysis, the significance level was set at $P \leq 0.05$.

Results and Discussion

In vitro characterization of strains

The DNA sequencing of the QRDR's of *gyrA, gyrB, parC and parE* are shown in Table 2. The reference strain showed no codon mutations resulting in amino acid changes when compared with *E. coli ATCC 25922*. For *gyrA*, two point mutations at codons 83 and 87 took place in both IA50 and IA66 when compared to the IA31 strain. Furthermore, for *parC*, a mutation at codon 78 occurred in the non-fit isolate, whereas a mutation at codon 80 in the fit strain was seen. In various studies,[41-46] mutations in *parC* accompanied the mutations from *gyrA* in the vast majority of the clinically-resistant isolates that were tested. In these studies the predominant mutation in *parC* was found in codon 80, accompanied in some isolates by a mutation in codon 84. When comparing the strains in these studies with the strains of this paper, the fit strain had a mutation in codon 80, while the mutation in the non-fit strain occurred in codon 78. To the authors' knowledge, only two strains with a mutation in codon 78 (for *parC*) have been reported previously in the literature,[45,47] and for IA50, the exact combination of codon changes here reported is described for the first time. It is not clear whether these mutations could be linked with the strain's inferior bacterial fitness.[48] In an *in vitro* study in which the fitness of isogenic resistant strains was assessed,[49] the strains with similar mutations with IA66 were also found to be bacteriologically fit when compared with their parental strains.

The enrofloxacin MIC levels of the isogenic resistant strains were both 32 mg/L, and for the (parental) susceptible strain 0.032 mg/L. Likewise, the MPC of enrofloxacin for the susceptible strain was 0.512 mg/L (corresponding to a 16-fold increase in comparison with the MIC), while the MPC for

the resistant strains was 1024 mg/L (Table 2). In a study from Devreese *et al.*,[15] a validated liquid chromatography-tandem mass spectrometry method for the quantification of enrofloxacin in the intestinal content of broiler chickens was described. There it was shown that after the administration of 10 mg/kg enrofloxacin (p.o. and i.m.), the intestinal microbiota in cecum and colon was exposed to significant levels of enrofloxacin (21–130 µg/g). Therefore it can be assumed that in the *in vivo* experiments of the present study, the gut concentrations of enrofloxacin were much higher than the MPC of the susceptible strain. Although selection for additional resistance in the susceptible strain cannot be excluded, the observed changes in the proportions of susceptible and resistant strains were interpreted as being the result of the multiplication of the already present (inoculation) resistant or susceptible stains. As a consequence, the effects described are caused by resistance selection rather than by the emergence of new resistance. This is further supported by the fact that the enrofloxacin resistance of the resistant strain is located in the chromosome (non-mobile), indicating that only the fluoroquinolone resistant strains were spreading under the selection pressure of the treatment (MIC value of 32 mg/L). Inclusion of a control group inoculated with a fully fluoroquinolone susceptible inoculum would have provided further information on the selection for additional resistance in the susceptible strain, but due to the limitations of the experimental setup, this was not feasible.

Phenotyping of E. coli from in vivo experiments

No animal showed any signs of disease throughout the duration of the experiments. The *E. coli* strains that were used, successfully colonized the gastrointestinal tract of the animals (Figures 2-5). In several studies examining the gastrointestinal microbiota of hatched chickens,[50,51] it has been found that after reaching optimal growth within the first 2 days of life, the *E. coli* population decreased rapidly at 8-9 days and was hardly found at 14 days. Hence, while the very young chick is quite a good and permissive host for *E. coli,*[52] it is quickly colonized by other types of bacteria. Although this decline was also observed in the present *in vivo* experiments (data not shown), a

sufficient number of *E. coli* colonies were nevertheless retrieved in the majority of the samples until the end of the experiment, thus allowing for a full evaluation of the effect. Only in Experiment 2, on the last sampling day and for the samples from orally-treated animals (half dose group: 5mg/kg), were no *E. coli* cfu found on the McConkey plates (Figure 3).

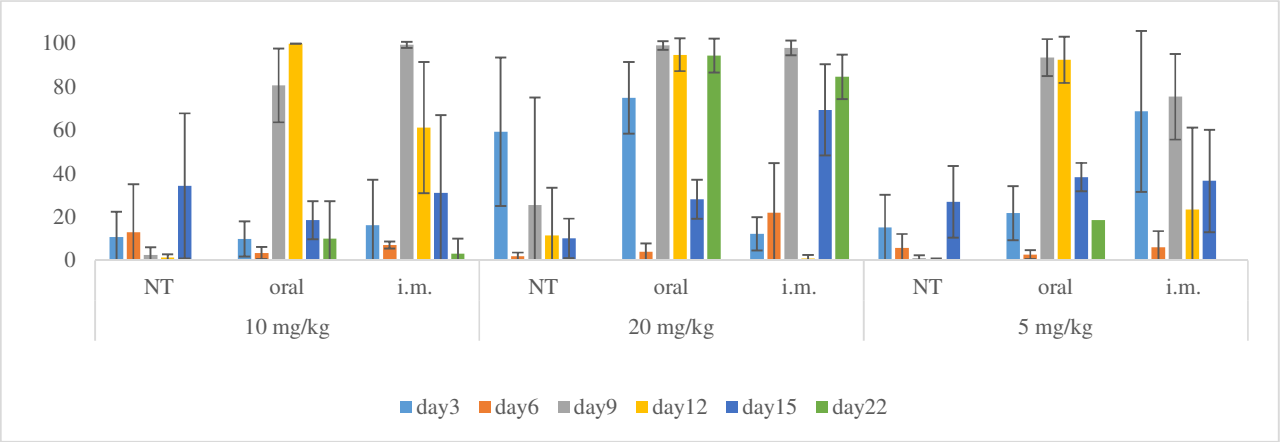

Figure 2. Experiment 1 (inoculation with a bacteriologically non-fit resistant strain and a bacteriologically fit susceptible strain in a 100:1 ratio) results. The y-axis presents the average percentage of resistant strains compared to the total of inoculated strains (retrieved from individual droppings) per group per sampling day. All NT (No treatment, control) groups did not receive enrofloxacin treatment. All treated animals received enrofloxacin treatment for 3 successive days (day 6 to day 8) and the groups are allocated according to the route of administration (oral, i.m.) and treatment dose (proper-, double-, half-dose). NT: No treatment, control group.

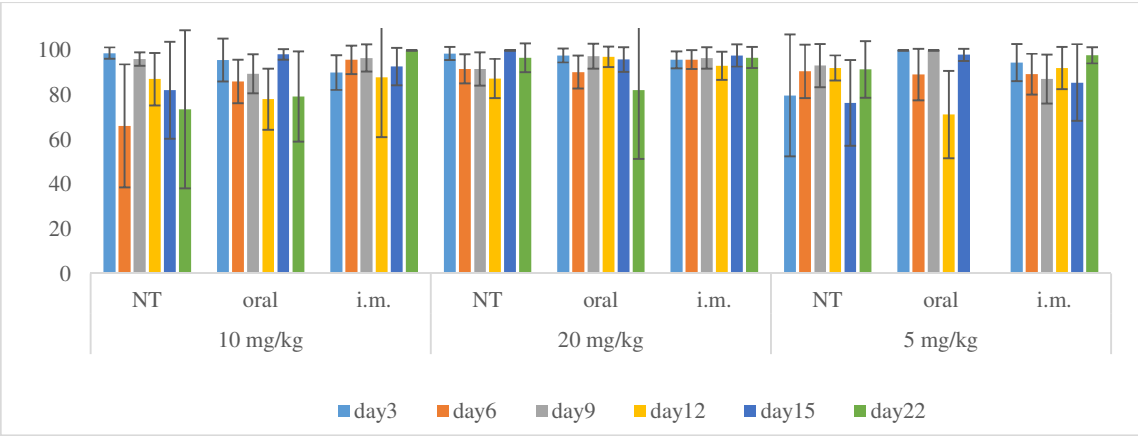

Figure 3. Experiment 2 (inoculation with a bacteriologically fit resistant strain and a bacteriologically fit susceptible strain in a 100:1 ratio) results. The y-axis presents the average percentage of resistant strains compared to the total of inoculated strains (retrieved from individual droppings) per group per sampling day. All NT (No treatment, control) groups did not receive enrofloxacin treatment. All treated animals received enrofloxacin treatment for 3 successive days (day 6 to day 8) and the groups are allocated according to the route of administration (oral, i.m.) and treatment dose (proper-, double-, half-dose). On Experiment 2, at the last sampling day (Day 22) and for the samples from orally-treated animals (half dose group: 5mg/kg) no E. coli cfu grew on the McConkey plates. NT: No treatment, control group.

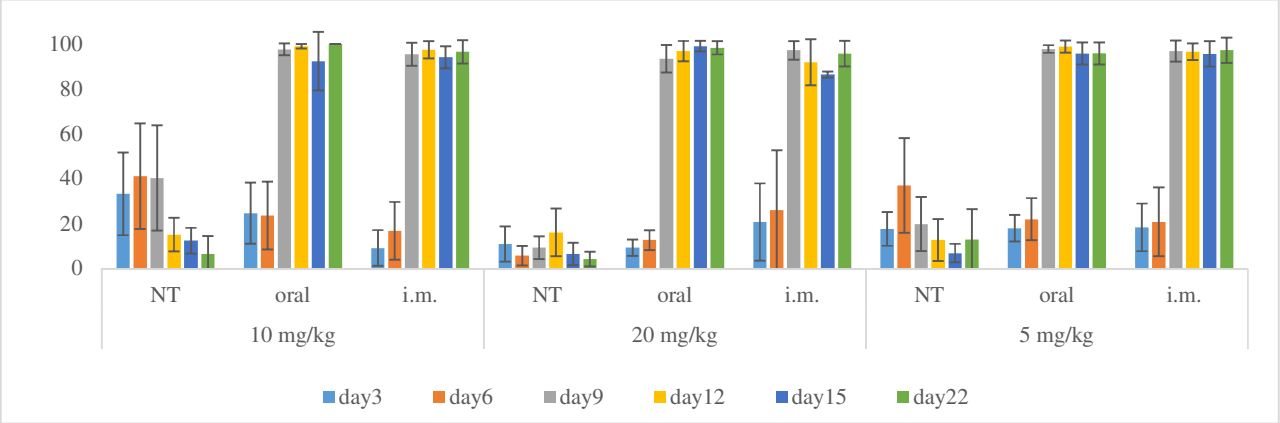

Figure 4. Experiment 3 (inoculation with a bacteriologically fit resistant strain and a bacteriologically fit susceptible strain in a 1:100 ratio) results. The y-axis presents the average percentage of resistant strains compared to the total of inoculated strains (retrieved from individual droppings) per group per sampling day. All NT (No treatment, control) groups did not receive enrofloxacin treatment. All treated animals received enrofloxacin treatment for 3 successive days (from day 6 (after sampling) until day 8) and the groups are allocated according to the route of administration (oral, i.m.) and treatment dose (proper-, double-, half-dose). NT: No treatment, control group.

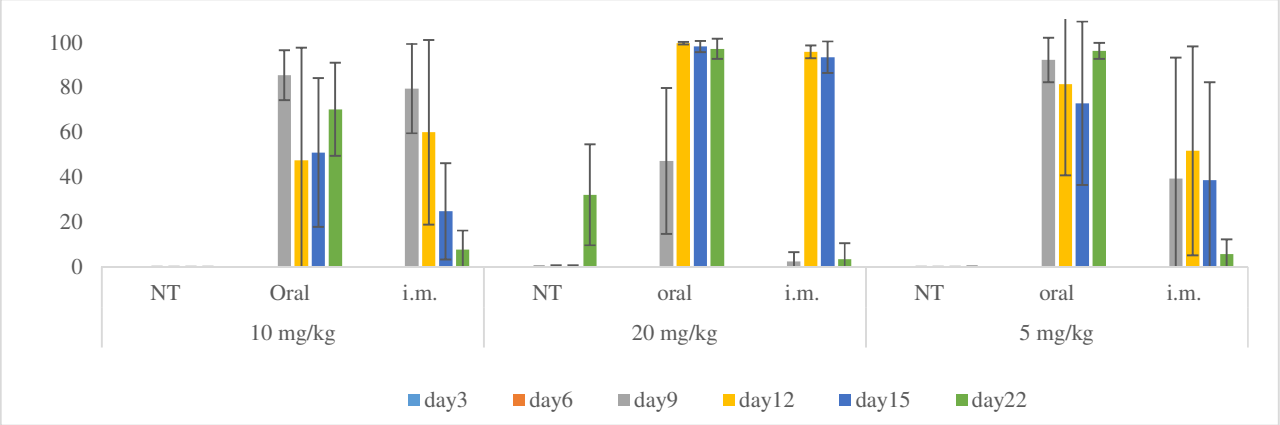

Figure 5. Experiment 4 (inoculation with a bacteriologically non-fit resistant strain and a bacteriologically fit susceptible strain in a 1:100 ratio) results. The y-axis presents the average percentage of resistant strains compared to the total of inoculated strains (retrieved from individual droppings) per group per sampling day. All NT (No treatment, control) groups did not receive enrofloxacin treatment. All treated animals received enrofloxacin treatment for 3 successive days (day 6 to day 8) and the groups are allocated according to the route of administration (oral, i.m.) and treatment dose (proper-, double-, half-dose). NT: No treatment, control group.

A study of the data from the control groups of all four experiments (Figures 2-5) revealed significant differences between the experiments. These differences, which were due to the different inoculums used, were verified by statistical analysis ($p < 0.001$). However, no significant difference ($p = 0.304$)

was found between the control groups over time, indicating that within each experiment the control groups behaved relatively alike. This indicates that likely no cross-contamination occurred between the treated and the control groups, which suggests that the experimental setup worked.

Effect of strain in the absence of treatment

The results from all non-treated animals (Figures 3-4) show that the animals that were inoculated either with a 100:1 ratio of the fit resistant/fit susceptible strain (Experiment 2) or with the opposite ratio (1:100) (Experiment 3) did not show any signs of reversion of antimicrobial resistance towards susceptibility, which suggests that for this strain there appears not to be a fitness cost due to resistance. This finding is in agreement with findings *of in* vitro[53] and *in vivo*[54] mutational resistance studies focusing, respectively, on *Mycobacterium* spp. and on *Campylobacter spp.* By contrast, three weeks (Day 23) after inoculation, the non-fit resistant strain could no longer be isolated from the samples taken from non-treated animals that had been inoculated with the non-fit resistant strain either in a 100:1 (Figure 2) or in a 1:100 (Figure 5) ratio.

Effect of treatment

A comparison of the temporal fluctuations of the results from each treated group per experiment (Figures 2-5) shows that there were considerable fluctuations in experiments 1 and 4. This could be attributed to the reduced fitness of the strain that was predominantly inoculated, since the growth potential of bacteriologically non-fit bacteria is difficult to predict *in vivo.*[31]

After the treatment period, the control groups from experiments 1, 3 and 4 showed significantly lower proportions of resistant strains compared to the respective treated groups (Table 3). The results of experiments 1 and 4 suggest that when animals are inoculated with non-fit strains, treatment resulted in the persistence of a strain that would normally die out before the end of the experiment (no enrofloxacin-resistant *E. coli* isolates were found on day 22 in all three control

groups). The results from experiment 3 suggest that when the inoculum consists of a small proportion of fit resistant strains, the treatment clearly promotes this fit strain, thus allowing it to become the dominant strain. These results are in agreement with the findings of other studies[14,55], where significant differences in resistance rates persisted between the intestinal *E. coli* of the enrofloxacin orally-treated groups and the non-treated groups.

Table 3. Linear mixed models performed per experiment and overall to assess the effect of treatment. Each animal was listed as subject, and sampling as repeated. The repeated covariance type was first-order autoregressive. The dependent variable used was the proportion of the enrofloxacin-resistant colonies to the sum of the resistant and the susceptible colonies.

	Univariate analysis		
Categorical variable	**Estimate**	**St. Error**	**P-value**
Experiment 1			**.001**
Intercept	55.09	4.56	<.001
No treatment	-45.81	7.96	.001
Treatment (ref)	0	0	.
Experiment 2			**.313**
Intercept	89.07	1.99	<.001
No treatment	-3.45	3.38	.313
Treatment (ref)	0	0	.
Experiment 3			**<.001**
Intercept	96.02	1.20	<.001
No treatment	-82.42	2.09	.004
Treatment (ref)	0	0	.
Experiment 4			**.004**
Intercept	59.73	7.69	.002
No treatment	-56.86	13.31	.004
Treatment (ref)	0	0	.
Overall			**<.001**
Intercept	74.50	3.12	<.001
No treatment	-47.14	5.40	<.001
Treatment (ref)	0	0	.

In contrast, for experiment 2, the findings from the control and the treated groups were similar up to the end of the experiment (p=0.313). The inoculum was largely composed of the fit resistant strain, which apparently colonized the gut of the animals successfully and remained prevalent up to the end of the experiment, irrespective of treatment. Accordingly, Austin *et al.*[56] suggested that once the resistance prevalence reaches a certain level, antimicrobial use no longer plays a role in the resistance selection. Handel *et al.*[57] reported that small changes in the volumes of antimicrobials used in a population with a low level of antimicrobial resistance lead to much larger changes in resistance when compared with changes in antimicrobials used at a high level of resistance. Similar

effects were seen when comparing antimicrobial use with antimicrobial resistance levels for several classes of antibiotics using data from seven European countries.[58]

Fitness of the resistant strain and route of administration

All potential effects were tested univariately and the statistically significant effects of strain ($p<0.001$) and administration route ($p=0.044$) were selected and further included in a multivariate linear mixed model. The factors that were finally selected were bacterial fitness of the resistant strain ($p<0.001$) and route of administration ($p=0.052$), as well as the interaction between bacterial fitness and administration route $p<0.001$ (Table 4). Regarding fitness, the inoculation with a fit resistant strain clearly resulted in significantly higher proportions of resistant *E.coli.* When comparing the administration routes, oral administration selected more for resistance. When looking at the interactions, it becomes clear that the combination of oral treatment and a non-fit strain had a significantly larger influence on the outcome. No similar studies have been performed for broiler chickens, but in a somewhat comparable study focusing on pigs and using *Salmonella* enrofloxacin-susceptible and enrofloxacin-resistant strains,[59] selection for resistance among the artificially introduced *Salmonella* was also higher for oral administration than for intramuscular.

Treatment dose

The treatment dose did not result in significant differences ($p = 0.764$) with regard to the resistance ratio. This is in agreement with Jurado *et al.*[14], who found no significant differences among different enrofloxacin dosage schemes that were administered orally to broiler chickens. Although various studies have showed that dosage can have an effect on the emergence of resistance,[34,35,60,61] the results of the present study suggest that it does not have an effect on the selection and spread of resistance. Grouping the referred studies with regards to commensal versus pathogenic *E. coli* isolates, the mutation frequencies of the strains were compared. The mutation frequencies of pathogenic *E. coli* isolates resulted mostly in MPC:MIC ratios of 8 or 16[34,61,62] and were comparable with the mutation frequencies of commensal *E. coli* isolates – including those in this study (ratio of

16). However, it must be noted that the phenomenon of emergence of resistance was not studied in the current *in vivo* experiments. *De novo* resistance mutations could not be assessed in this study design because no fully susceptible inoculum was included.

Table 4. Linear mixed models performed for all experiments, including data from 120 chickens after they had received enrofloxacin treatment. After assessing univariately the main effects of strain, prevalence of resistance before treatment (inoculum ratio), treatment dose, and administration route, a multivariate model tested the effects of fitness of strain and administration route. In the final mixed linear model, two-way interactions between significant variables were also evaluated (with the significance level set at P < 0.05). In all models, to correct for the interdependency of chicks within a pen, pen was included as a random variable.

	Univariate analysis			***Multivariate analysis with interactions***		
Categorical variable	*Estimate*	*Std. Error*	*P-value*	*Estimate*	*Std. Error*	*P-value*
Intercept				92.60	3.97	<.001
Bacterial strain			**<.001**			**<.001**
Intercept	92.69	3.97	<.001			
Non-fit	-35.31	3.08	<.001	-48.10	4.13	<.001
Fit (ref.)	0	0	.	0	0	.
Administration route			**.044**			**.052**
Intercept	67.94	3.26	<.001			
Oral	13.38	4.62	.044	.06	5.63	.992
Parenteral (ref.)	0	0	.	0	0	.
Inoculum ratio			**.223**			
Intercept	77.16	4.18	<.001			
100res:1sens	-5.16	4.22	.223			
1res:100sens (ref)	0	0	.			
Treatment dose			**.764**			
Intercept	72.33	7.44	.002			
10 mg/kg	-0.87	10.50	.994			
20 mg/kg	6.92	10.49	.557			
5 mg/kg	0	0	.			
Interactions strain * administration route						**<.001**
non-fit * oral				25.86	5.88	<.001
Non-fit * parenteral				0	0	.
Fit &* oral				0	0	.
Fit * parenteral				0	0	.

In accordance with the European Medicines Agency (EMA) guidelines, we used the most commonly recommended dose for chickens and turkeys (10mg enrofloxacin/kg bodyweight per day for 3-5 consecutive days), and, based on this, we calculated the half and the double dose. However, this dose range does not cover the full range of doses available in leaflets across Europe (from 2.5 mg/kg to 20 mg/kg from 2 to 10 days), albeit for different animal species and indications.[63] We selected the current dose variation as a starting point for assessing the effect of dosing because deviations from

doses below the authorized dosages, together with plasmid mediated resistance, have been found to enhance resistance.[64-66]

Final remarks and further use

The use of isogenic strains allowed for direct comparisons between all *in vivo* experiments since differences between the fit and non-fit strains can be attributed to the point mutations leading to resistance. To our knowledge, this is the first time such *in vivo* experiments have been performed to measure selection for resistance taking into account the prevalence of enrofloxacin resistance in the initial gut microbiota, bacterial fitness of the resistant strain, route of administration and treatment dose. However, the results could have been different if a strain carrying PMQR genes had been used. Although these genes are quite rare in commensal *E. coli* strains isolated from chickens,[67-69] the transfer rate of resistance is expected to be higher in the presence of such plasmids.[70] Phenomena such as plasmid loss[71] and plasmid incompatibility[72] should also be considered.

The wild-type strain did not show increased MIC levels against enrofloxacin. The enrofloxacin-resistant strain was created *in vitro* via a chromosomal mutation of the parental wild-type strain. *In vivo*, we took all necessary biosecurity measures to prevent the introduction of other strains (via feed, water, air-borne, etc.). Given the fact that the ratio of the resistant strains in the control groups after treating the other groups was not affected (in favour of the resistant strains) in any group and in any experiment, this provided an *in vivo* illustration of the effectiveness of the experimental setting. Thus, the presence of PMQR genes was ruled out.

Overall, the experimental setup made it possible to study and assess several effects concerning the selection of fluoroquinolone resistance. This study provides a basis for selecting and further investigating relevant research topics. By studying the benefits and the limitations of each experiment, one could select the appropriate experimental setting in accordance with the specific research question. For example, one study setup could be selected to focus on treatment effect and its administration patterns (use of non-fit enrofloxacin-resistant strain, oral administration of

enrofloxacin and test various treatment schemes), a different setup could focus on selection of antimicrobial resistance (treated animals, use of bacteriologically-fit enrofloxacin-resistant strains and testing inoculums of increasing prevalence), and a third setup could focus on characteristics of reversion of antimicrobial resistance (non-treated animals inoculated with strains of different bacteriological fitness and testing for reversibility of antimicrobial resistance).

Conclusions

For the purposes of this study, a standardized *in vivo* model was developed that can be used to investigate resistance selection in commensal *E. coli* in poultry. Gut colonization with a bacteriologically-fit enrofloxacin-resistant strain and oral administration of enrofloxacin selected more for antimicrobial resistance than colonization with a non-fit resistant strain and parenteral treatment respectively. This novel protocol made it possible to study various factors both selectively and collectively, and to identify the advantages and disadvantages in each case, thus providing insights into treatment strategies using enrofloxacin.

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Transparency declarations

None to declare

Supplementary data

Tables S1 to S3 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References

1. D'Costa VM, King CE, Kalan L *et al.* Antibiotic resistance is ancient. *Nature* 2011; **477**: 457-61.

2. Wright GD, Poinar H. Antibiotic resistance is ancient: implications for drug discovery. *Trends Microbiol* 2012; **20**: 157-9.

3. Soulsby L. Antimicrobials and animal health: a fascinating nexus. *J Antimicrob Chemother* 2007; **60** Suppl 1: i77-8.

4. Snary EL, Kelly LA, Davison HC *et al.* Antimicrobial resistance: a microbial risk assessment perspective. *J Antimicrob Chemother* 2004; **53**: 906-17.

5. Wegener HC. Antibiotics in animal feed and their role in resistance development. *Curr Opin Microbiol* 2003; **6**: 439-45.

6. Gouvea R, Dos SFF, Aquino MHCD *et al.* Fluoroquinolones in Industrial Poultry Production, Bacterial Resistance and Food Residues: a Review. *Braz J Poultry Sci* 2015; **17**: 1-10.

7. Landoni MF, Albarellos G. The use of antimicrobial agents in broiler chickens. *Vet J* 2015; **205**: 21-7.

8. FDA 2005. *Withdrawal of enrofloxacin for poultry*. http://www.fda.gov/animalveterinary/safetyhealth/recallswithdrawals/ucm042004.htm .

9. Love DC, Halden RU, Davis MF *et al.* Feather Meal: A Previously Unrecognized Route for Reentry into the Food Supply of Multiple Pharmaceuticals and Personal Care Products (PPCPs). *Environ Sci Technol* 2012; **46**: 3795-802.

10. Ingram PR, Rogers BA, Sidjabat HE *et al.* Co-selection may explain high rates of ciprofloxacin non-susceptible *Escherichia coli* from retail poultry reared without prior fluoroquinolone exposure. *Journal Med Microbiol* 2013; **62**: 1743-6.

11. Simoneit C, Burow E, Tenhagen BA *et al.* Oral administration of antimicrobials increase antimicrobial resistance in *E. coli* from chicken - A systematic review. *Prev Vet Med* 2015; **118**: 1-7.

12. Li L, Jiang ZG, Xia LN *et al.* Characterization of antimicrobial resistance and molecular determinants of beta-lactamase in *Escherichia coli* isolated from chickens in China during 1970-2007. *Vet Microbiol* 2010; **144**: 505-10.

13. Kaesbohrer A, Schroeter A, Tenhagen BA *et al.* Emerging antimicrobial resistance in commensal *Escherichia coli* with public health relevance. *Zoonoses Public Health* 2012; **59** Suppl 2: 158-65.

14. Jurado S, Medina A, Ruiz-Santa-Quiteria JA *et al.* Resistance to non-quinolone antimicrobials in commensal *Escherichia coli* isolates from chickens treated orally with enrofloxacin. *Jpn J Vet Res* 2015; **63**: 195-200.

15. Devreese M, Antonissen G, De Baere S *et al.* Effect of administration route and dose escalation on plasma and intestinal concentrations of enrofloxacin and ciprofloxacin in broiler chickens. *Bmc Vet Res* 2014; **10**.

16. Bugyei K, Black WD, McEwen S. Pharmacokinetics of enrofloxacin given by the oral, intravenous and intramuscular routes in broiler chickens. *Can J Vet Res* 1999; **63**: 193-200.

17. Vogwill T, MacLean RC. The genetic basis of the fitness costs of antimicrobial resistance: a meta-analysis approach. *Evol Appl* 2015; **8**: 284-95.

18. Sandegren L, Lindqvist A, Kahlmeter G *et al.* Nitrofurantoin resistance mechanism and fitness cost in *Escherichia coli*. *J Antimicrob Chemother* 2008; **62**: 495-503.

19. Levin BR, Perrot V, Walker N. Compensatory mutations, antibiotic resistance and the population genetics of adaptive evolution in bacteria. *Genetics* 2000; **154**: 985-97.

20. Andersson DI. The biological cost of mutational antibiotic resistance: any practical conclusions? *Curr Opin Microbiol* 2006; **9**: 461-5.

21. Levin BR, Lipsitch M, Perrot V *et al.* The population genetics of antibiotic resistance. *Clin Infect Dis* 1997; **24**: S9-S16.

22. Levin BR. Models for the spread of resistant pathogens. *Neth J Med* 2002; **60**: 58-64.

23. Græsbøll K, Nielsen SS, Toft N *et al.* How fitness reduced, antimicrobial resistant bacteria survive and spread: a multiple pig-multiple bacterial strain model. Plos One 2014; 9: e100458.

24. Andersson DI, Hughes D. Antibiotic resistance and its cost: is it possible to reverse resistance? *Nat Rev Microbiol* 2010; **8**: 260-71.

25. Sundqvist M, Geli P, Andersson DI *et al.* Little evidence for reversibility of trimethoprim resistance after a drastic reduction in trimethoprim use. *J Antimicrob Chemother* 2010; **65**: 350-60.

26. Kunz AN, Begum AA, Wu H *et al.* Impact of fluoroquinolone resistance mutations on gonococcal fitness and *in vivo* selection for compensatory mutations. *J infect Dis* 2012: jis277.

27. Sundqvist M. Reversibility of antibiotic resistance. *Ups J Med Sci* 2014; **119**: 142-8.

28. Robicsek A, Strahilevitz J, Sahm DF *et al. qnr* Prevalence in ceftazidime-resistant Enterobacteriaceae isolates from the United States. Antimicrob Agents Chemother 2006; **50**: 2872-4.

29. Park CH, Robicsek A, Jacoby GA *et al.* Prevalence in the United States of *aac(6')-Ib-cr* encoding a ciprofloxacin-modifying enzyme. *Antimicrob Agents Chemother* 2006; **50**: 3953-5.

30. Tóth I, Csík M, Emçdy L. Spontaneous antibiotic resistance mutation associated pleiotropic changes in *Escherichia coli* O157: H7. *Acta Vet Hung* 2003; **51**: 29-44.

31. Petersen A, Aarestrup FM, Olsen JE. The *in vitro* fitness cost of antimicrobial resistance in *Escherichia coli* varies with the growth conditions. *FEMS Microbiol Lett* 2009; **299**: 53-9.

32. Haritova AM, Rusenova NV, Parvanov PR *et al.* Integration of pharmacokinetic and pharmacodynamic indices of marbofloxacin in turkeys. *Antimicrob Agents Chemother* 2006; **50**: 3779-85.

33. Zhao X, Drlica K. Restricting the selection of antibiotic-resistant mutant bacteria: measurement and potential use of the mutant selection window. *J Infect Dis* 2002; **185**: 561-5.

34. Olofsson SK, Marcusson LL, Komp Lindgren P *et al.* Selection of ciprofloxacin resistance in *Escherichia coli* in an *in vitro* kinetic model: relation between drug exposure and mutant prevention concentration. *J Antimicrob Chemother* 2006; **57**: 1116-21.

35. Zhao X, Drlica K. Restricting the selection of antibiotic-resistant mutants: a general strategy derived from fluoroquinolone studies. *Clin Infect Dis* 2001; **33 Suppl 3**: S147-56.

36. Weigel LM, Steward CD, Tenover FC. *gyrA* mutations associated with fluoroquinolone resistance in eight species of Enterobacteriaceae. *Antimicrob Agents Chemother* 1998; **42**: 2661-7.

37. Vila J, Ruiz J, Marco F *et al.* Association between double mutation in *gyrA* gene of ciprofloxacin-resistant clinical isolates of *Escherichia coli* and MICs. *Antimicrob Agents Chemother* 1994; **38**: 2477-9.

38. Everett MJ, Jin YF, Ricci V *et al.* Contributions of individual mechanisms to fluoroquinolone resistance in 36 *Escherichia coli* strains isolated from humans and animals. *Antimicrob Agents Chemother* 1996; **40**: 2380-6.

39. NCfBI. *Escherichia coli str. K-12 subst. MG1655*, complete genome. 2016. http://www.ncbi.nlm.nih.gov/nuccore/U00096

40. Bayer Business Group Animal Health D-L, Germany. Balancing the dose. 2016. https://animalhealth.bayer.com/ah/fileadmin/media/baycox/balancing_Folder.pdf

41. Johnning A, Kristiansson E, Fick J *et al.* Resistance Mutations in *gyrA* and *parC* are Common in *Escherichia* Communities of both Fluoroquinolone-Polluted and Uncontaminated Aquatic Environments. *Front Microbiol* 2015; **6**.

42. Lysnyansky I, Gerchman I, Mikula I *et al.* Molecular characterization of acquired enrofloxacin resistance in *Mycoplasma synoviae* field isolates. *Antimicrob Agents Chemother* 2013; **57**: 3072-7.

43. Morgan-Linnell SK, Boyd LB, Steffen D *et al.* Mechanisms Accounting for Fluoroquinolone Resistance in *Escherichia coli* Clinical Isolates. *Antimicrob Agents Chemother* 2009; **53**: 235-41.

44. Vicca J, Maes D, StakeMborg T et al. Resistance mechanism against fluoroquinolones in *Mycoplasma hyopneumoniae* field isolates. *Microb Drug Resist* 2007; **13**: 166-70.

45. Jurado S, Orden JA, Horcajo P *et al.* Characterization of fluoroquinolone resistance in *Escherichia coli* strains from ruminants. *J Vet Diagn Invest* 2008; **20**: 342-5.

46. Zayed AAF, Essam TM, Hashem AGM *et al.* 'Supermutators' found amongst highly levofloxacin-resistant *E. coli* isolates: a rapid protocol for the detection of mutation sites. *Emerg Microbes Infec* 2015; **4**.

47. Heisig P. Genetic evidence for a role of parC mutations in development of high-level fluoroquinolone resistance in *Escherichia coli*. *Antimicrob Agents Chemother* 1996; **40**: 879-85.

48. Park M, Sutherland JB, Kim JN *et al.* Effect of fluoroquinolone resistance selection on the fitness of three strains of *Clostridium perfringens*. *Microb Drug Resist* 2013; **19**: 421-7.

49. Marcusson LL, Frimodt-Moller N, Hughes D. Interplay in the Selection of Fluoroquinolone Resistance and Bacterial Fitness. *Plos Pathog* 2009; **5**.

50. Shaufi MAM, Sieo CC, Chong CW *et al.* Deciphering chicken gut microbial dynamics based on high-throughput 16S rRNA metagenomics analyses. *Gut Pathog* 2015; **7**.

51. Lu JR, Idris U, Harmon B *et al.* Diversity and succession of the intestinal bacterial community of the maturing broiler chicken. *Appl Environ Microb* 2003; **69**: 6816-24.

52. Baron S, Jouy E, Touzain F *et al.* Impact of the administration of a third-generation cephalosporin (3GC) to one-day-old chicks on the persistence of 3GC-resistant *Escherichia coli* in intestinal flora: An in vivo experiment. *Vet Microbiol* 2016; **185**: 29-33.

53. Sander P, Springer B, Prammananan T *et al.* Fitness cost of chromosomal drug resistance-conferring mutations. *Antimicrob Agents Chemother* 2002; **46**: 1204-11.

54. Luo ND, Pereira S, Sahin O *et al.* Enhanced in vivo fitness of fluoroquinolone-resistant *Campylobacter jejuni* in the absence of antibiotic selection pressure. *Proc Natl Acad Sci USA* 2005; **102**: 541-6.

55. Miranda JM, Vazquez BI, Fente CA *et al.* Evolution of resistance in poultry intestinal *Escherichia coli* during three commonly used antimicrobial therapeutic treatments in poultry. *Poultry Sci* 2008; **87**: 1643-8.

56. Austin DJ, Kristinsson KG, Anderson RM. The relationship between the volume of antimicrobial consumption in human communities and the frequency of resistance. *Proc Natl Acad Sci USA* 1999; **96**: 1152-6.

57. Handel A, Regoes RR, Antia R. The role of compensatory mutations in the emergence of drug resistance. *PLoS Comput Biol* 2006; **2**: e137.

58. Chantziaras I, Boyen F, Callens B *et al.* Correlation between veterinary antimicrobial use and antimicrobial resistance in food-producing animals: a report on seven countries. *J Antimicrob Chemother* 2014; **69**: 827-34.

59. Wiuff C, Lykkesfeldt J, Svendsen O *et al.* The effects of oral and intramuscular administration and dose escalation of enrofloxacin on the selection of quinolone resistance among Salmonella and coliforms in pigs. *Res Vet Sci* 2003; **75**: 185-93.

60. Olofsson SK, Cars O. Optimizing drug exposure to minimize selection of antibiotic resistance. *Clin Infect Dis* 2007; **45** Suppl 2: S129-36.

61. Ozawa M, Asai T. Relationships between mutant prevention concentrations and mutation frequencies against enrofloxacin for avian pathogenic *Escherichia coli* isolates. *J Vet Med Sci* 2013; **75**: 709-13.

62. Marcusson LL, Komp Lindgren P, Olofsson SK *et al.* Mutant prevention concentrations of pradofloxacin for susceptible and mutant strains of *Escherichia coli* with reduced fluoroquinolone susceptibility. *Int J Antimicrob Agents* 2014; **44**: 354-7.

63. EMA. Enrofloxacin_Annex I : List of the names, pharmaceutical forms, strengths of the veterinary medicinal products, animal species, routes of administration, applicants/marketing authorisation holders in the Member States. http://www.ema.europa.eu/docs/en_GB/document_library/Referrals_document/2014/04/WC500164988.pdf.

64. Canton R, Morosini MI. Emergence and spread of antibiotic resistance following exposure to antibiotics. *FEMS Microbiol Rev* 2011; **35**: 977-91.

65. Macia MD, Perez JL, Molin S *et al.* Dynamics of mutator and antibiotic-resistant populations in a pharmacokinetic/pharmacodynamic model of Pseudomonas aeruginosa biofilm treatment. *Antimicrob Agents Chemother* 2011; **55**: 5230-7.

66. Couce A, Blazquez J. Side effects of antibiotics on genetic variability. *FEMS Microbiol Rev* 2009; **33**: 531-8.

67. Oh J-Y, Kwon Y-K, Tamang MD *et al.* Plasmid-Mediated Quinolone Resistance in *Escherichia coli* Isolates from Wild Birds and Chickens in South Korea. *Microbial Drug Resistance* 2016; **22**: 69-79.

68. Abdi-Hachesoo B, Asasi K, Sharifiyazdi H. Farm-level evaluation of enrofloxacin resistance in *Escherichia coli* isolated from broiler chickens during a rearing period. *Comp Clin Path* 2017: 1-6.

69. Yue L, Jiang HX, Liao XP *et al.* Prevalence of plasmid-mediated quinolone resistance *qnr* genes in poultry and swine clinical isolates of *Escherichia coli*. *Vet Microbiol* 2008; **132**: 414-20.

70. Robicsek A, Jacoby GA, Hooper DC. The worldwide emergence of plasmid-mediated quinolone resistance. *Lancet Infect Dis* 2006; **6**: 629-40.

71. Sanchez MB, Martinez JL. Differential Epigenetic Compatibility of *qnr* Antibiotic Resistance Determinants with the Chromosome of *Escherichia coli*. *Plos One* 2012; **7**.

72. Carattoli A. Plasmids and the spread of resistance. *Int J Med Microbiol* 2013; **303**: 298-304.