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Effects of carry-over of fluoroquinolones on the susceptibility of commensal *Escherichia coli* in the intestinal microbiota of poultry

Einfluss von Fluorchinolonen auf die Resistenzentwicklung kommensaler Escherichia coli im Darm beim Huhn

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Summary

Due to the frequent use of antibacterials in veterinary medicine as well as in human medicine the occurrence of antibacterial resistance rises worldwide. But independent of the usage of antimicrobials the microbiota from animals as well as from humans already harbour a diversity of resistance genes.
As a consequence of manufacturing animal production the treatment of livestock in case of illness is carried out via feed or drinking water. This automatically implies several risks. It has been demonstrated that an antibiotic treatment of livestock via feed or drinking water cause an accumulation of antibiotics and their metabolites in the direct environment of animals. This can lead to a carry-over or rather a resumption of the antimicrobials and their metabolites. Thus, the aim of this study was to determine the influence of carry-over of enrofloxacin as a representative of the fluoroquinolones on the development of bacterial resistance of commensal *E. coli* in the intestinal microbiota of poultry. Therefore four different treatment groups were provided and the minimal inhibitory concentrations (MICs) of commensal *E. coli* were measured: One group acted as untreated control, another one was therapeutically treated with the recommended dosage. The third and fourth group were exposed to different "carry-over dosages" for three weeks, 3% of the recommended dosage were applied to the third and 10% to the fourth group. To determine the influence of a therapeutic treatment on a prestressed microbiota, both groups were treated with the recommended dosage for five days.
The present study demonstrates that every kind of exposure of the commensal microbiota of poultry with enrofloxacin leads to an amplification and selection of resistant *E. coli*, which persist in the commensal microbiota. A long term exposure of gut microbiota, which already harbour non-wildtype *E. coli*, with high levels of carry-over of fluoroquinolones may lead to a development of high-level clinically resistant *E. coli* in the commensal microbiota. It has to be investigated to which extent antimicrobial leftovers occur in animal production.

Keywords: carry-over, fluoroquinolones, antimicrobial resistance, *E. coli*

Zusammenfassung

Weltweit steigen die Raten der Antibiotikaresistenz aufgrund des häufigen Einsatzes von Antibiotika sowohl in der Veterinär- als auch in der Humanmedizin. Jedoch beherbergen die Mikrobiome sowohl von Tier als auch Mensch unabhängig vom Antibiotikaeinsatz eine Vielfalt von Antibiotikaresistenzgenen.
Aufgrund der hohen Tierzahlen in der Intensivtierhaltung hat sich, der Durchführbarkeit der Behandlungen geschuldet, der Einsatz von Antiinfektiva über Futter und Trinkwasser etabliert. Dies ist mit einigen Risiken verbunden. Mehrere Studien haben gezeigt, dass eine Verteilung der antimikrobiellen Wirkstoffe und ihrer Metaboliten in der direkten Umgebung der behandelten Tiere stattfindet, Rückstände in Trinkwasserleitungen oder Futter wurden ebenfalls nachgewiesen. Dies kann zu einer Wiederaufnahme der Wirkstoffe führen. Ziel der vorliegenden

Studie war es, mithilfe von Verschleppungsszenarien den Einfluss verschleppter subtherapeutischer Konzentrationen von Fluorchinolonen am Modell von Enrofloxacin auf die Sensibilität kommensaler *E. coli* im Darm von Geflügel zu prüfen. Dazu wurden vier Tiergruppen in die Studie einbezogen: Eine Gruppe diente als unbehandelte Kontrolle, eine weitere wurde mit der empfohlenen Dosierung über fünf Tage behandelt. Die dritte und vierte Gruppe dienten zur Simulation von Verschleppungsszenarien und erhielten jeweils 3 bzw. 10 % der empfohlenen Dosierung über eine Dauer von drei Wochen. Um den Einfluss der therapeutischen Behandlung auf eine präexponierte Darm-Mikrobiota zu untersuchen, erhielten beide Gruppen im Anschluss an das jeweilige Verschleppungsszenario die empfohlene Dosierung über fünf Tage.
Die vorliegende Studie zeigt, dass jede Form der Exposition der Darm-Mikrobiota von Geflügel mit Enrofloxacin zu einer Amplifikation und Selektion resistenter kommensaler *E. coli* bzw. von Nicht-Wildtypen führt. Diese persistieren in der Darm-Mikrobiota.
Eine Langzeitexposition mit hohen Verschleppungskonzentrationen von Mikrobiomen, die schon *E. coli* beinhalten, die nicht dem Wildtyp entsprechen, kann zu einer Entwicklung von hochgradig klinisch resistenten *E. coli* (MHK ≥ 32 µg/ml) führen. Es bleibt zu prüfen, in welchem Ausmaß Verschleppungen antimikrobieller Substanzen in Intensivtierhaltungen vorkommen.

Schlüsselwörter: Verschleppung, Fluorchinolone, antimikrobielle Resistenz, *E. coli*

Introduction

Due to the frequent use of antibacterials in veterinary medicine as well as in human medicine the occurrence of antibacterial resistance rises worldwide (Ewers et al., 2012; Schaberle and Hack, 2014). Looft et al. (2012) and Moore et al. (2013) reported that microbiota from animals and humans who have not been in contact with antibiotics before, like healthy pediatric clinic patients at the age of one month, already harbour diverse and novel antibiotic resistance genes.

Fluoroquinolones are synthetic bactericidal antimicrobials used in human and veterinary medicine, which are characterized by inhibiting DNA-gyrase and DNA-topoisomerase IV (Drlica and Zhao, 1997). Both enzymes are crucially involved in the procedures of replication and transcription of the DNA or rather bacterial cell growth and proliferation (Drlica and Zhao, 1997; Gross et al., 2003). The exhibition of a broad spectrum of antimicrobial activity, the good bioavailability after oral as well as after intravenous application, the adequate distribution in tissue in association with high plasma levels makes them to nearly ideal substances (Andersson and MacGowan, 2003). Thus, fluoroquinolones are commonly used in human medicine as well as in veterinary medicine.

The fluoroquinolone enrofloxacin was exclusively developed for veterinary use (Bauditz, 1987). As a consequence of the manufacturing animal production and the high number of animals the treatment of livestock via feed and drinking water in case of illness plays a major role (Kamphues, 1996). Based on its high oral bioavailability of almost 90% and the good solubility, the application of enrofloxacin via drinking water is approved and formulations for use via drinking water are available. Enrofloxacin is applied for the treatment of colibacillosis, pasteurellosis and infections with *Mycoplasma* in poultry (Bauditz, 1987). It is partly metabolized to ciprofloxacin by N-deethylation in the liver, a fluoroquinolone used in human medicine (Tyczkowska et al., 1989).

Using antimicrobials via drinking water, it has to be considered that complications like the shortfall of efficient levels of the active ingredient in the medicated animals and the risk of lasting medications in water such as leftovers in drinking systems can occur (Kamphues, 1996; Kogelheide, 2007). Additionally, several studies show that enrofloxacin is mainly excreted as unmetabolized enrofloxacin and its metabolite ciprofloxacin (Wu et al., 2005; Janusch, 2014), whereat ciprofloxacin represents only a small fraction (Anadon et al., 1995; Knoll et al., 1999). In a previous study we could determine a distribution of enrofloxacin and ciprofloxacin in the direct environment of poultry under the recommended treatment with 10 mg enrofloxacin/kg body weight (b.w.) over a period of five days via drinking water (Scherz, 2013). The distribution of different antibiotics in the surrounding of treated livestock was shown before in several studies including monitoring studies as well as experimental designs (Hamscher et al., 2003; Zessel, 2012). Furthermore an ingestion of carry-over of antimicrobial residues in the direct environment of untreated swine was also determined (Elliot et al., 1994; Kietzmann et al., 1995). Subtherapeutic concentrations of antimicrobials are under strong suspicion to be involved in the development of antimicrobial resistance (Schuurmans et al., 2010; Gullberg et al., 2011).

Due to our previous findings of distributed enrofloxacin and its metabolite ciprofloxacin in the direct environment of treated animals, leftovers of antibiotics in drinking water as well as the risk of the shortfall of efficient levels, the aim of this study was to determine the influence of carry-over of enrofloxacin on the development of antibacterial resistance of commensal *E. coli* in the intestinal microbiota of poultry. Furthermore, the influence of a therapeutic treatment on a prestressed microbiota was monitored and compared to a microbiota, which was not exposed to enrofloxacin before.

Material and Methods

Experimental Design

The experiments were designed to determine the influence of carry-over of fluoroquinolones on the development of antibacterial resistance of commensal *E. coli* in the intestinal microbiota of chickens. Therefore, different schedules of contamination of the environment in comparison to a therapeutic treatment of poultry with enrofloxacin as test substance were simulated to ascertain changes in the sensitivity of *E. coli* (Fig. 1):

Four different treatment groups (n=10 chicken) were provided for the treatment schedule of enrofloxacin applied via drinking water. The first one A) acted as untreated control, the second one B) was treated with the recommended dosage (10 mg/kg) for 5 days, the third C) and the fourth D) group were exposed to subtherapeutic dosages [C) 0.3 mg/kg; D) 1.0 mg/kg] for 21 days to determine the influence of a simulated carry-over simulation for example during a mast period. Both carry-over simulations were followed by a treatment with the recommended dosage (10 mg/kg) for 5 days to evaluate the effect of a therapeutic treatment on a prestressed microbiota.

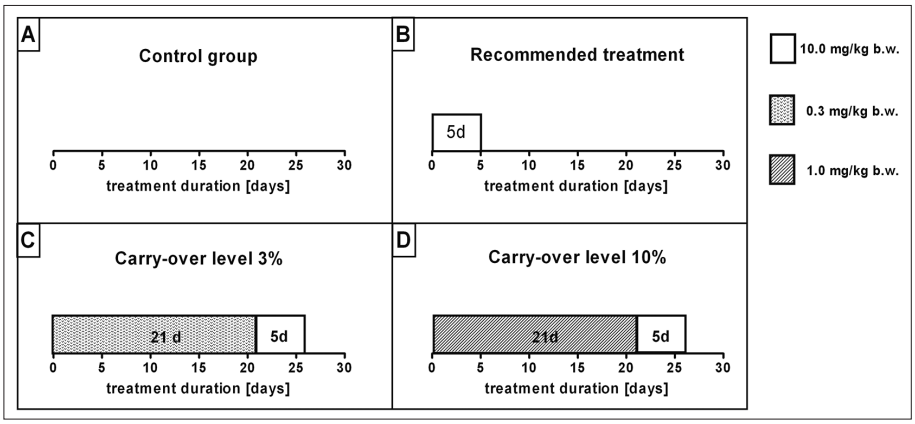


FIGURE 1: *Experimental design of the treatment schedule of simulated contamination compared to a recommended treatment: A) untreated control, B) recommended dosage (10.0 mg/kg b.w.), C) subtherapeutic dosage (0.3 mg/kg b.w.) followed by the recommended one, D) another subtherapeutic dosage (1.0 mg/kg b.w.) followed by the recommended one.*

To monitor the development of antibacterial resistance of commensal *E. coli* of the intestinal microbiota, cloacal swabs were taken directly prior to the treatment start, during and after the medication of the animals (time of sampling see results Fig. 2 and 3).

Animals

40 hens in the age of 20 weeks were obtained from a poultry farm (Zahrte GmbH & Co. KG, Wrestedt, Germany). They were kept in groups of 10 animals in a barn system in the stables of the Clinic for Poultry, University of Veterinary Medicine, Foundation, Hannover. Each group of birds was housed in a separated stable with a restricted area of 3.6 m² to approach conditions in commercial poultry farms. The separated stables were built identically and each room was featured with an appropriate ventilation. A lighting program of 14 hours of light and 10 hours of dark was used. The animals had free access to feed and tap water from bell drinkers. All animals were clinically healthy during the entire experiment. The study was authorized by the Lower Saxony State Office for Consumer Protection and Food Safety, Niedersachsen, Germany, reference number 33.9-42502-04-11/0338.

Antibiotics

Enrofloxacin was added to the drinking water based on the dosage per kg weight, therefore Baytril® 10% Oral Solution (Bayer Animal Health GmbH, Leverkusen, Germany) was used. The particular medication of

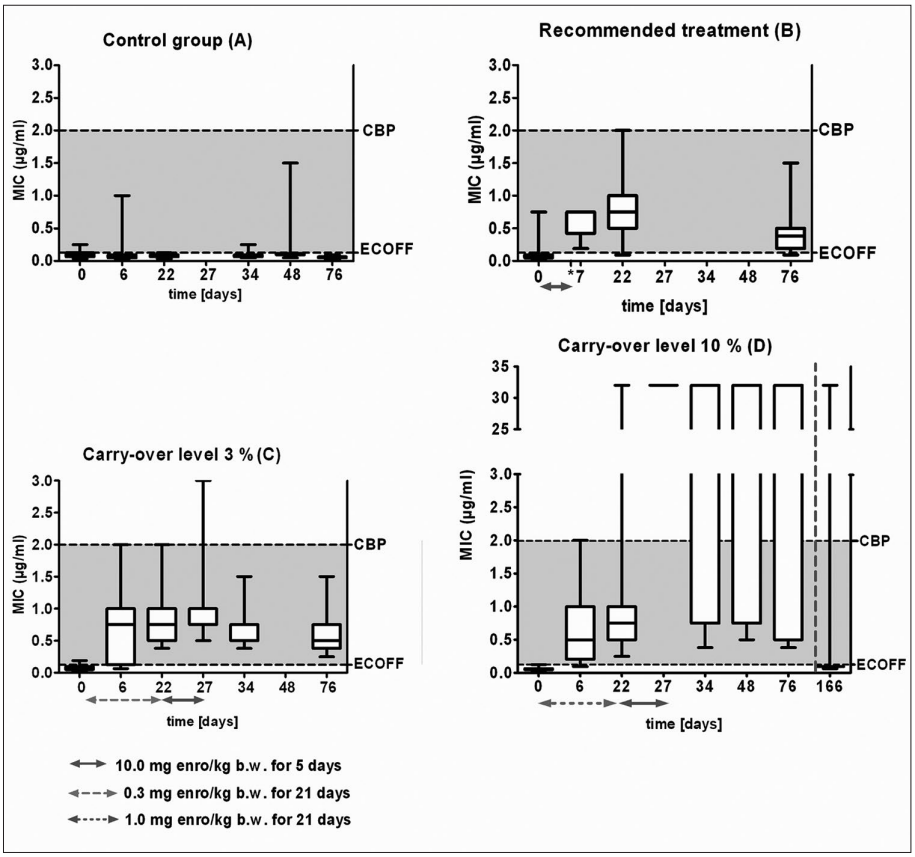


FIGURE 2: *MIC-distributions of 10 E. coli colonies per animal and sampling day from 6 animals per group (n = 60) in boxplots. Treatment schedule: A) untreated control, B) recommended dosage, C and D) simulation of different carry-over levels followed by the therapeutic treatment. The range between the epidemiological Cut-Off (ECOFF) and the clinical breakpoint (CBP) is shaded in grey. * On day 6 no E. coli colonies could be isolated.*

the animals via drinking water was prepared daily and calculated on the basis of the total body weight of the group and the daily water consumption. The body weight of the animals was determined one day before treatment with the particular dosage started.

Isolation and MIC-measurements of non-type-specific *E. coli*

Ten animals per group were sampled by using two cloacal swabs per chicken and sampling. Either of them was directly smeared on an Endo agar plate, a selective medium for members of the coliform group and incubated at 37°C for 20–24 hours. *E. coli* colonies were identified by the red colour combined with a permanent red sheen (fuchsine sheen). Ten colonies per plate and animal were picked randomly. The isolated *E. coli* colonies of six randomly chosen animals were used to determine the minimal inhibitory concentration (MIC) of the particular colony by using the Etest® (BioMèrieux SA, Marcy-l'Etoile, France). The colonies of the other four animals were stored in skim milk and frozen at –80°C.

To confirm the tested bacteria as *E. coli*, bacterial cells were incubated in LMX-broth modified by Manafi and Ossmer at 37°C for 24 hours. Then the suspension was visually tested for a colour change to blue, a blue fluorescence under 364 nm UV-light and the indole reaction by adding Kovacs' reagent. The total of colour change (X-Gal reaction), the fluorescence under long wave UV-light (MUG-reaction) and the red indole ring identify the inoculated bacteria as *E. coli*.

The second cloacal swab was enriched in 9 ml Luria-Bertani-Bouillon at 37°C for 24 hours, afterwards 30 µl of the cell suspension were streaked onto Endo agar plates supplemented with 2 µg/ml enrofloxacin. The inoculated agar plates were incubated at 37°C for 24 hours. Thereafter the plates were visually checked for *E. coli* colonies.

Statistical analysis

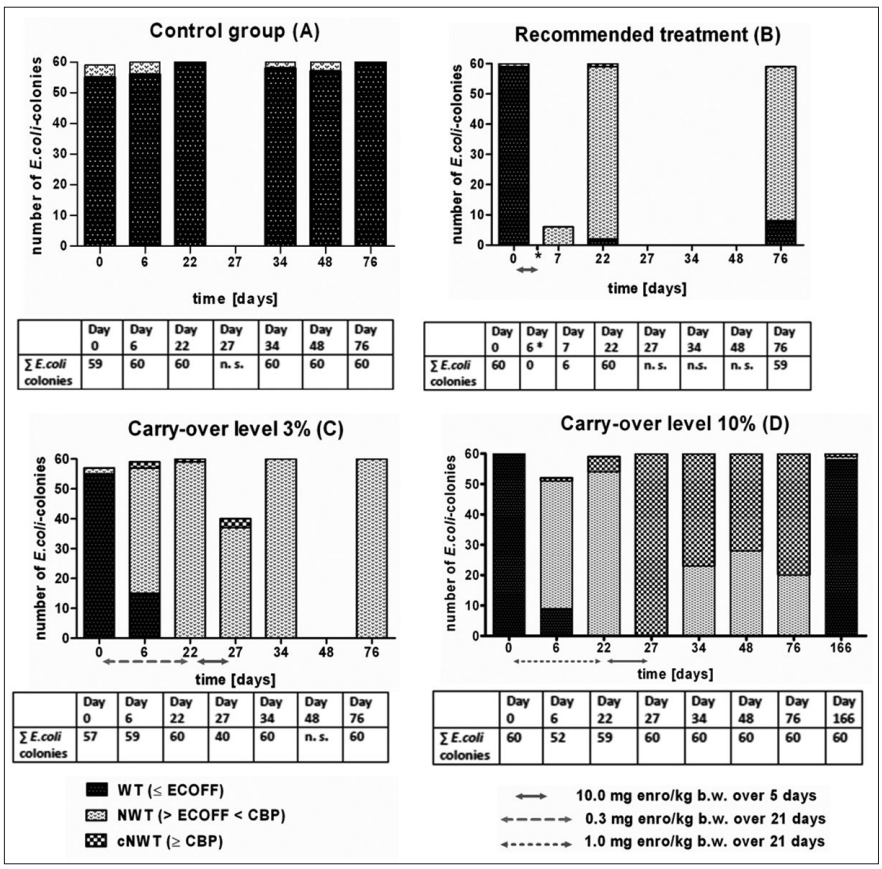
The statistical evaluation of the data was carried out by using SAS 9.3 software. At each point of time the different treatment groups were compared among each other via single factor variance analyses for independent samples with subsequent multiple mean comparison via Tukey-test. Values in the range of $P < 0.05$ indicate statistical significance.

Results

To determine the influence of enrofloxacin on the development of antibacterial resistance of commensal *E. coli*, the epidemiological cut-off (ECOFF) ≤0.125 µg enrofloxacin/ml (EUCAST, 2013a) and the clinical breakpoint ≥2.0 µg/ml for poultry and turkey (CLSI, 2008) were used as critical parameters for interpretation. Isolates with minimal inhibitory concentrations (MICs) below the ECOFF are susceptible and categorized as wildtypes (WT) with regard to enrofloxacin. Isolates which exceed the ECOFF are classified as resistant or non-wildtypes (NWTs) (EUCAST, 2013b). *E. coli* strains with MICs equivalent to or higher as the clinical breakpoint are categorized as clinically resistant non-wildtypes (cNWT). Figure 2 shows the MIC-distributions of the isolated *E. coli* strains in the different treatment groups as a function of time in boxplots. Figure 3 represents the fractions of WTs, NWTs and cNWTs of the isolated amount of *E. coli* colonies per sampling day.

Control group (A)

The animals of the antibiotic free control group were sampled over a period of 76 days. The majority of the 60 isolated *E. coli* colonies (10 isolates each from 6 chickens) per sampling day, had MICs under the ECOFF of 0.125 µg/ml and were identified as WTs before treatment started (day 0). Only single colonies showed MICs between the ECOFF (≤0.125 µg/ml) and the clinical breakpoint (≥2.0 µg/ml), which are categorized as NWTs. No clinical resistance could be determined by an additional enrichment of the cloacal swabs and streaking the cell suspension on Endo agar plates supplemented with 2.0 µg enrofloxacin/ml.



Control group (A)	Day 0	Day 6	Day 22	Day 27	Day 34	Day 48	Day 76
∑ *E.coli* colonies	59	60	60	n. s.	60	60	60

Recommended treatment (B)	Day 0	Day 6 *	Day 7	Day 22	Day 27	Day 34	Day 48	Day 76
∑ *E.coli* colonies	60	0	6	60	n. s.	n.s.	n. s.	59

Carry-over level 3% (C)	Day 0	Day 6	Day 22	Day 27	Day 34	Day 48	Day 76
∑ *E.coli* colonies	57	59	60	40	60	n. s.	60

Carry-over level 10% (D)	Day 0	Day 6	Day 22	Day 27	Day 34	Day 48	Day 76	Day 166
∑ *E.coli* colonies	60	52	59	60	60	60	60	60

FIGURE 3: *Number of E. coli colonies which are categorized as wildtypes (WT) and which show resistance separated in two groups: Non-wildtypes (NWT) with minimal inhibitory concentrations (MIC) below the epidemiological Cut-Off (ECOFF) and non-wildtypes with clinical resistance (cNWT) with MICs above the clinical breakpoint (CBP) as a function of time. Treatment schedule: A) untreated control, B) recommended dosage, C and D) simulation of different carry-over levels followed by the therapeutic treatment. The tables represent the total number of isolated E. coli colonies per sampling day. n.s. = no sampling. * On day 6 no E. coli colonies could be isolated.*

Treatment with the recommended dosage (B)
To evaluate the effect of a therapeutic treatment on the commensal microbiota of chickens, group B was treated with the recommended dosage of 10.0 mg enrofloxacin/kg b.w. for five days. Equivalent to the other groups the majority of the isolates represented WTs with MICs below the ECOFF before treatment started (day 0). But the commensal microbiota also harboured single resistant colonies or rather NWTs with MICs between the ECOFF and the CBP. After exposition of the chickens to the therapeutic treatment no *E. coli* colonies could be isolated (day 6), one day later (day 7) only a total of six resistant NWT-*E. coli* colonies was determined. After two weeks without antimicrobial treatment the majority of the isolates of the group also represented resistant NWT-*E. coli*, which had MICs between the ECOFF and the CBP (day 22). The situation did not change after ten weeks without antimicrobial treatment (day 76).

Simulation of carry-over situations
Carry-over level 3% (C)
When chickens were exposed to the carry-over dosage of 3% for 21 days the composition of the different susceptible types of *E. coli* changed over time. Equivalent to the other groups the majority of the isolates represented susceptible WTs with MICs below the ECOFF, but a minority of resistant isolates with MICs exceeding the ECOFF were detectable on day 0. Under the exposition with the carry-over dosage of enrofloxacin the proportions reversed and at the end only resistant NWTs with MICs exceeding the ECOFF and a minority of clinically resistant isolates (cNWTs) were isolated (day 22). The following therapeutic treatment did not significantly change the situation and during a period of eight weeks without antimicrobial exposition only resistant isolates with MICs exceeding the ECOFF were detected (day 76).

Carry-over level 10% (D)
When chickens were exposed to the carry-over dosage of 10% for 21 days all isolates had MICs exceeding the ECOFF and some were even clinically resistant with MICs ≥32 µg/ml (day 22). Due to the highly increased MICs the following therapeutic treatment selected for the clinically resistant isolates (cNWTs), which is in contrast with the solely therapeutically treated group, where no *E. coli* strains could be isolated after treatment on day 6. In the present group the entire amount of isolates had high-level resistances with MICs ≥32 µg/ml (day 6). Within the next seven weeks after therapeutic treatment the amount of isolates consisted of resistant (NWT) and clinically resistant (cNWT) *E. coli* strains but no susceptible WTs were detected (days 34, 48, 76). After 20 weeks without antibiotic exposure the population seemed to be returned to the initial situation, the majority of the isolates represented susceptible WTs with MICs below the ECOFF (day 166). But one highly clinically resistant isolate with a MIC ≥32 µg/ml and one resistant isolate with a MIC between the ECOFF and the CBP were isolated. The additionally enriched cloacal swabs, which were streaked on Endo agar plates supplemented with 2 µg/ml enrofloxacin, confirm that each animal of the group still harboured a minority of highly clinically resistant *E. coli* strains. These results show that even when the majority of the population returns to control levels, the intestinal microbiota still harbours single highly clinically resistant strains.

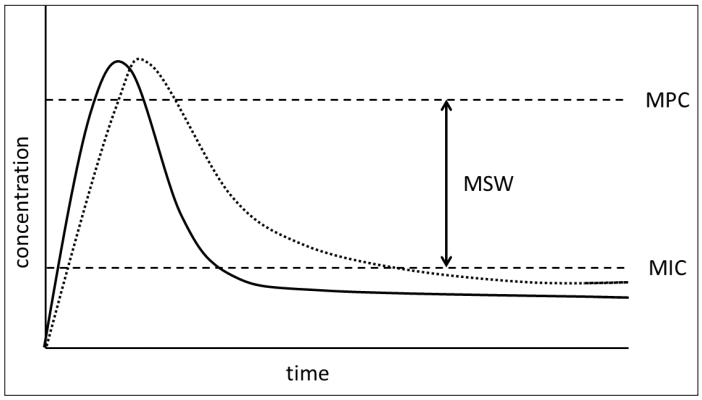


FIGURE 4: *The Mutant Selection Window. Modified according to Drlica (2003). The curves represent a hypothetical concentration of an antimicrobial as a function of time. Mutant prevention concentration = MPC; Mutant Selection Window = MSW; Minimal Inhibitory Concentration = MIC. The antimicrobial represented by the dashed curve needs a longer time period to cross the MSW than the antimicrobial represented by the continuous curve.*

Discussion

Before treatment started (day 0) WT-*E. coli* strains were predominantly isolated in each group. Anyway, the commensal intestinal microbiota of the animals in all groups harboured a minority of NWT-*E. coli* with MICs between the ECOFF and the CBP. After treatment of group B, which was solely medicated with the recommended dosage for five days, no *E. coli* colonies were isolated, neither susceptible WT-*E. coli* nor resistant NWT-*E. coli*. With regard to the Mutant Prevention Concentration (MPC) hypothesis (Fig. 4) including the Mutant Selection Window (MSW) this might not surprise.

The acquisition of chromosomal mediated fluoroquinolone resistance occurs in a stepwise manner (Belland et al., 1994; Pan et al., 1996). As described before fluoroquinolones act bactericidal by inhibiting DNA-gyrase and DNA-topoisomerase IV (Drlica and Zhao, 1997). In gram-negative bacteria DNA-gyrase is the main target, while DNA-topoisomerase IV represents the main target in gram-positive bacteria (Hooper, 2001, 2002; Intorre et al., 2007). Chromosomal mediated resistance is manifested by mutations in the Quinolone Resistance Determining Regions (QRDR) in the subunits of the particular target enzyme (Drlica and Zhao, 1997). This induces a decreased antimicrobial binding affinity (Blondeau, 2004). Single mutations in one of the target enzymes (first-step mutants) often cause intermediate or rather low-level resistance, in some cases the strains remain susceptible (Weigel et al., 1998; Bagel et al., 1999; Lim et al., 2003; Pletz et al., 2006). Non-target resistances, which do not develop in the target enzymes DNA-gyrase and Topoisomerase IV, also cause low-level resistance (Zhou et al., 2000). High-level or rather complete fluoroquinolone resistance requires mutations in both target enzymes (Everett et al., 1996; Bagel et al., 1999; Lim et al., 2003; Pletz et al., 2006).

The Mutant Prevention Concentration (MPC) is defined as the concentration of an antimicrobial agent, which avoids the growth of first-step mutants, based on the testing of bacterial inocula $>10^{10}$ cells, which are

larger than those associated with infections (Dong et al., 1999; Blondeau, 2009).

The Mutant Selection Window (MSW) is defined as the range between the particular MIC and the MPC, within which first-step mutants are selectively amplified and enriched (Drlica et al., 2009).

With regard to the present study the NWT-*E. coli* strains with MICs between the ECOFF and the CBP, which were occasionally isolated in all groups at the beginning of the experiments (day 0), represented at least first-step mutants.

The fact that no *E. coli* strains could be isolated in the solely therapeutically treated group (group B) after treatment (day 6) indicates the achievement of the MPC or a higher concentration, which was sufficient to eradicate the resistant NWT-*E. coli* strains. But that raises the question why NWT-*E. coli* with low-level or rather moderate resistances with MICs exceeding the ECOFF but below the CBP were isolated the next day. With regard to the MSW (Fig. 4) it is shown that the concentration of the antibiotic automatically crosses the MSW after the end of treatment. That means that the commensal *E. coli* were exposed to concentrations of enrofloxacin in the MSW (Fig. 4) over a variable time period, so that de novo occurring bacterial mutants had a selection advantage. It may be hypothesized, that the rate of de novo mutations depends on the time period, within which the bacteria are exposed to antimicrobial concentrations in the MSW (Fig. 4). One reason for the development of de novo mutants might be the initiation of the SOS-Regulon. Enrofloxacin induces double-strand breaks of the bacterial DNA, which initiate a signaling cascade to induce the repair by recombination (Howard et al., 1993). Cirz et al. (2005) demonstrated, that the occurrence of ciprofloxacin resistant *E. coli* is connected with the SOS-Regulon triggered by the bactericidal effect of ciprofloxacin. The carry-over simulation with 3% (0.3 mg enrofloxacin/kg b.w.) of the recommended dosage applied for 21 days (group C) was chosen to reflect a realistic situation. Antibiotic left-overs of 2.5% are known to occur in drinking systems (van der Horst et al., 2013). Thereafter a treatment with the recommended dosage followed for five days to evaluate the influence of a therapeutic therapy in case of illness on a prestressed intestinal microbiota. When chickens were exposed to the carry-over dosage of 3% only NWT-*E. coli* strains with MICs exceeding the ECOFF were isolated (day 22). With regard to the MPC and MSW hypothesis it seems like the carry-over level of 3% reached plasma concentrations inside the MSW. Thus NWT-*E. coli* strains were amplified and selected, while the above MIC concentration was lethal for WT-*E. coli*. After the following treatment with the recommended dosage the number of isolated colonies was limited, they were consistently categorized as NWTs including a minority of cNWTs. There is again evidence that the MPC was not reached, so that first-step mutants were selected. Thus, no sufficient concentrations of enrofloxacin were achieved in the animals to eradicate the NWT-strains.

According to group B, which was solely treated with the recommended dosage, the resistance situation did not change significantly in the next 6 weeks without antibiotics and only NWTs were isolated (days 34 and 76). One explanation might be, that to that point of time no cleaning of the stables was carried out. In a previous study we could show that under a therapeutic treatment with the recommended dosage of enrofloxacin (10 mg enrofloxacin/kg b.w.) for five days, enrofloxacin and its active metabolite ciprofloxacin are distributed in the direct environment of the animals (Scherz, 2013). In case of group C the previous carry-over simulation (3%) must be considered additionally. It is likely that enrofloxacin and ciprofloxacin were distributed in the stable. Some kinds of mutations lead to fitness costs like large decreases concerning the growth rates of the particular strains and with regard to the study of Gullberg et al. (2011) it is possible that the permanent intake of minimal antibacterial concentrations led to a majority or rather a long-term persistence of NWT-*E. coli* by balancing the fitness costs of the resistant, and reducing the growth rates of the susceptible bacteria. The animals of group D were exposed to 10% (1.0 mg enrofloxacin/kg b.w.) of the recommended dosage for 21 days, the development was similar to the carry-over simulation with 3% of the recommended dosage, with the exception that highly clinically resistant *E. coli* with MICs ≥32 µg/ml occurred for the first time. From the beginning of the study on, cloacal swabs were enriched and streaked on supplemented Endo agar plates (2.0 µg enrofloxacin/ml) to identify clinically resistant *E. coli*. The first positive result was achieved concurrently to the first identification of cNWTs with high-level resistances via Etest® in eight out of ten animals in the group on day 22. That proves that further mutations occurred de novo and that the carry-over dosage of 10% was not sufficient to reach the MPC to eradicate the NWT-*E. coli* the animals harboured before treatment started. Gillespie et al. (2003) demonstrated that once a mutation occurred a second mutation arises readily. The following therapeutic treatment selected for the highly clinically resistant *E. coli*, and out of each animal of the group clinically resistant *E. coli* strains were isolated (day 27). It is a debatable point whether each animal had *E. coli* with further occurring mutations or whether only some animals developed highly clinically resistant *E. coli*, which were transmitted from animal to animal.

In the following seven weeks after treatment (days 27–76) the ratio of NWTs without clinical resistance increased, while the fraction of clinically resistant *E. coli* decreased. Komp Lindgren et al. (2005) determined in vitro and in vivo that first-step *E. coli* mutants resistant to the fluoroquinolone norfloxacin had little or no differences in fitness relative to a WT-parental strain. But a few second-step and most of the third-, fourth-, and fifth-step mutants suffered from large decreases in fitness cost concerning growth rates (Komp Lindgren et al., 2005). The requirement for bacteria to develop clinical resistance to fluoroquinolones is the achievement of several mutations in the subunits GyrA and ParC of the target enzymes. With regard to the study from Komp Lindgren et al. (2005), it has to be considered that the clinically resistant isolates in the present study suffer from larger fitness costs than the NWT-*E. coli* without clinical resistance. This can partially explain the decrease of the clinically resistant and the increase of the NWTs without clinical resistance. Furthermore it is likely that the animals had an intake of leftovers of enrofloxacin and its metabolite ciprofloxacin from the environment, so that the commensal intestinal bacteria were permanently exposed to subinhibitory concentrations. It seems like the highly clinically resistant bacteria need higher concentrations of enrofloxacin to balance fitness costs than the NWTs. In the next five months

(day 76–166) the number of NWTs with and without clinical resistance decreased and WT-*E. coli* occurred as the majority. Only one clinically resistant isolate was determined by directly streaking the cloacal swabs, but after enrichment highly clinically resistant *E. coli* strains were determined from each animal. That demonstrates that clinically resistant *E. coli* outlast in the intestinal microbiota even over months without antibiotic medication. Komp Lindgren et al. (2005) determined a partial restoration of fitness in association with accumulation of late mutation-steps, what might be the explanation for the permanent clinical resistance. Independent of dosage or duration of exposure with enrofloxacin NWT-*E. coli* exceeding the ECOFF occurred or were rather amplified and selected in the intestinal microbiota of poultry. Only the animals of group D, which were exposed to 10% of the recommended dosage for 21 days, developed high-level clinically resistant *E. coli* (MIC ≥32 µg/ml) which were amplified and selected by the following recommended treatment. Due to the fact that the animals as well as the human microbiota already harbour diverse resistance genes (Looft et al., 2012; Moore et al., 2013), a long-time exposure with carry-over levels of enrofloxacin may lead to an accumulation of further mutations, which leads to clinically resistant commensal *E. coli*. In the present study the animals already harboured low-level resistances before treatment started. That finding is in accordance to the present situation. Own randomized investigations of several flocks of poultry, including broilers and laying hens, show that the intestinal microbiota of many animals already harbour commensal NWT-*E. coli* up to clinically resistant NWT-*E. coli* relating to enrofloxacin (unpublished data). That was also confirmed by Miranda et al. (2008), who determined different levels of resistance from susceptible strains over low-level up to clinical resistance in commensal *E. coli* of poultry as well. The resistant strains also seemed to be amplified and selected under selective pressure by treatment with enrofloxacin.

These findings raise the question, where the resistant *E. coli* strains in the untreated microbiota come from. It may be hypothesized that a vertical transfer from parental animals to the progenies happens for example via the egg shell, while the young chickens are hatching. Otherwise an exposition of the commensal microbiota of poultry with carry-over of fluoroquinolones may occur in some cases.

In conclusion, the present study demonstrates that every kind of exposure of the commensal microbiota of poultry with enrofloxacin leads to an amplification and selection of resistant *E. coli*, which persist in the commensal microbiota. A long term exposure of microbiota which already harbour NWT-*E. coli* with high levels of carry-over of fluoroquinolones may lead to a development of high-level clinically resistant *E. coli* in the commensal microbiota. Thus, it has to be investigated whether leftovers of that range occur in the direct environment of treated animals.

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