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Antimicrobial susceptibility in faecal *Escherichia coli* from pigs after enrofloxacin administration in an experimental environment

Empfindlichkeit fäkaler Escherichia coli von Schweinen gegenüber Antibiotika nach Enrofloxacinverabreichung in einer experimentellen Umgebung

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Summary

The study objective was to evaluate the effect of oral (OT) and parenteral (PT) administration of enrofloxacin to weaners on untreated contact animals. We assessed a) fluoroquinolone occurrence in the blood serum of untreated contact animals (COT, CPT); b) resistance to (fluoro)quinolones in commensal *Escherichia coli (E. coli)* in OT, PT, COT and CPT compared to the control (CON), and c) resistance to other antimicrobials in *E. coli* in OT, PT, COT and CPT compared to the initial situation before the treatment in these groups. Five groups of 14 weaners each were housed in three separate rooms (OT with COT, PT with CPT, CON alone). OT and PT were treated with enrofloxacin for five days. Rectal swabs and blood samples were taken before, during and until 51 days after treatment. Enro- and ciprofloxacin were detected in all treated, all COT and half of the CPT pigs. Neither through selective isolation nor by susceptibility testing of one random non-selectively isolated faecal *E. coli* per sample, resistance to ciprofloxacin (metabolite of enrofloxacin) and nalidixic acid was detected in both treatment and contact groups during and short after treatment. However, a transient increase of *E. coli* resistant to antimicrobials other than quinolones followed the treatment in isolates from OT and COT (e.g. ampicillin $p < 0.05$). In conclusion, animals in contact with treated animals are exposed to and can intake antimicrobials. Animals in contact with orally treated animals show occurrence of antibiotic resistant *E. coli*. Further studies are needed to show whether these preliminary findings can be confirmed under different conditions and with more sensitive detection methods.

Keywords: Fluoroquinolone, resistance, route, transfer

Zusammenfassung

Ziel der Studie war, den Effekt der oralen (OT) und parenteralen (PT) Verabreichung von Enrofloxacin an Aufzuchtschweine auf unbehandelte Kontakttiere zu untersuchen. Untersucht wurde a) Fluorchinolonpräsenz im Blutserum von unbehandelten Kontakttieren (COT, CPT); b) Resistenz gegen (Fluor-)Chinolone in kommensalen *Escherichia coli (E. coli)* in OT, PT, COT und CPT im Vergleich zur Kontrollgruppe (CON) und c) Resistenz gegenüber anderen Antibiotika in *E. coli* in OT, PT, COT und CPT im Vergleich zur Ausgangssituation vor der Behandlung in

diesen Gruppen. Fünf Gruppen von jeweils 14 Absetzern wurden in drei getrennten Räumen untergebracht (OT mit COT, PT mit CPT, CON alleine). OT und PT wurden mit Enrofloxacin für fünf Tage behandelt. Rektaltupfer und Blutproben wurden vor, während und bis 51 Tage nach der Behandlung genommen. Enro- und Ciprofloxacin wurden in allen behandelten, allen COT und der Hälfte der CPT Schweine nachgewiesen. Es wurde keine Resistenz gegenüber Ciprofloxacin (Metabolit von Enrofloxacin) und Nalidixinsäure in beiden Behandlungs- und ihren Kontaktgruppen während und kurz nach der Behandlung nachgewiesen – weder durch selektive Isolierung noch durch Sensibilitätstestung eines zufällig, nicht-selektiv isolierten fäkalen *E. coli* pro Probe. Allerdings folgte auf die Behandlung ein vorübergehender Anstieg resistenter *E. coli* gegenüber anderen Antibiotika als Chinolonen in den Isolaten aus OT und COT (z. B. Ampicillin, $p < 0,05$). Wir schlussfolgern, dass die Kontakttiere behandelter Tiere Antibiotika ausgesetzt sind und diese aufnehmen und Kontakttiere oral behandelter Tiere antibiotisch resistente *E. coli* aufweisen können. Weitere Untersuchungen sind notwendig, um zu zeigen, ob diese ersten Ergebnisse unter anderen Bedingungen und mit erweiterten Nachweismethoden bestätigt werden können.

Schlüsselwörter: Fluorchinolon, Resistenz, Verabreichungsweg, Übertragung

Introduction

Fluoroquinolones are highest priority critically important antimicrobials for veterinary and human medicine (FAO/WHO/OIE, 2008; WHO, 2017). Enrofloxacin is approved for the oral and parenteral treatment of respiratory diseases, urinary and digestive tract infections and septicaemia in pigs (e. g. in Germany; VETIDATA, 2016). However, fluoroquinolone use and resistance of Enterobacteriaceae [including commensal, *Escherichia (E.) coli*] are correlated and counts of resistant bacteria are higher the more antimicrobial is used (Nguyen et al., 2012; Chantziaras et al., 2014; ECDC, EFSA and EMA, 2017). Hence, preservation of its effectiveness is a political target (WHO, 2015).

Oral group treatment is common (97.7% of applications to pigs) in German pig production (2.3% of applications to pigs are parenteral; year 2011; VetCAb-Bericht, 2013). Bio-availability of enrofloxacin is similar after its oral and parenteral administration (Baytril®; VETIDATA, 2016). Because treated animals shed a part of the antimicrobial, its active metabolites and resistant bacteria with urine and faeces (Nguyen et al., 2012), untreated animals in contact with treated ones or their environment potentially get exposed (Kietzmann et al., 1995; Wiuff et al., 2003). However, information on bio-availability in contact pigs is not available yet. Furthermore, the impact of treatment route and contact exposure on antimicrobial resistance (AMR) in a long-term perspective is unclear for pigs.

Our objectives were to evaluate the effect of oral (OT) and parenteral (PT) administration of enrofloxacin on contact animals by assessing a) fluoroquinolone occurrence in the blood serum of untreated contact animals (COT, CPT); b) resistance to (fluoro)quinolones and c) other antimicrobials in commensal *E. coli* in OT, PT, COT and CPT. Our hypotheses were that a) fluoroquinolones can be detected in COT and CPT animals; b) frequency of faecal *E. coli* resistant to (fluoro)quinolone temporarily increases in OT, PT, COT and CPT compared to untreated weaners without contact (CON); c) AMR to other agents in *E. coli* temporarily increases compared to initial values.

Materials and Methods

Animals, study groups and housing

The study was approved by legal authorities of the Regional Office for Health and Social Affairs Berlin (LAGeSo, G 0332/13). All applicable institutional and national guidelines for the care and use of animals were followed. Experimental treatments of animals were classified as to lead to no worse than minor discomfort in the animals due to low pain of very short duration (e. g. injection and handling) and were approved by LAGeSo.

Seventy clinically healthy weaners of German Landrace (28 females, 42 males) were obtained from a single animal breeding unit. The weaners were selected by the breeder for neither the piglets nor their dams having been treated with antimicrobials and for being as homogenous in body weight and condition as possible. At the age of four weeks, the pigs were weaned from their dams and transported with institutional, wet cleaned and disinfected (Calgonit sterizid Kokzi PRO®, 1% cresol, Calvatis GmbH, Ladenburg, Germany) vehicles 226 km to the experimental facilities at the German Federal Institute for Risk Assessment in Berlin. The pigs originated from eleven dams and four sires and were allocated randomly into five groups of 14 animals each using a randomized block design to minimize the potential effect of the dam. Sample size was calculated by comparing proportions (Dohoo et al., 2009), based on 5.9% risk before/without treatment (reference value), 54% risk after treatment, 95% confidence interval and 80% power. This resulted in 13 necessary animals per group. One animal was added per group to account for potential loss.

The pigs were group-housed in three separate rooms (rooms 1–3). Each room was equipped with air-filtration, waste water disinfection, a separate hygiene sluice, a water stub (no connection between rooms nor to hand washbasins in the hygiene sluice) and separate feed supply. The groups OT and COT were housed mixed in room 1, the groups PT and CPT were housed mixed in room 2 and CON was housed in room 3. Room 2 was located between rooms 1 and 3. Rooms 1–3 were

accessed from the same alley but a door separated the part of the alley leading to room 3. Animals were fed two types of commercial pelleted feed applied in two-phase-feeding. Each type originated from one batch and was filled and traded in 25kg-bags (Trede und von Pein, Itzehoe, Germany). The bags were distributed randomly among all rooms. The feed was applied in feeders, refilled every morning. The pigs had permanent access to water via nipple drinkers and were offered toys for environmental enrichment. The stocking density was 0.6–0.7 animals per square meter.

Study period and handling procedure

The trial was carried out from September to October, 2014. Already at the end of August, the weaners were assigned to the groups, moved to the experimental facilities and had seven days to get settled within the groups and to get accustomed to the facility and the caretakers. During that week, pigs were also drenched with water to train the drenching procedure used for treatment in the trial. The study started when the pigs were five weeks old, ran for 56 days and ended when the pigs were 13 weeks old.

On sampling days, the pigs were handled in the morning after feeding. Each pig was manually restrained, visually checked for its health status, submitted to its group specific procedure (generally COT before OT and CPT before PT) and weighed on a scale before returning to its pen.

Antimicrobial treatment

Enrofloxacin was applied to non-fasted pigs of the groups OT and PT for five consecutive days according to the manufacturer's instructions (2.5 mg enrofloxacin per kg bodyweight and day). Dosage was adjusted daily based on the bodyweight of the previous day. Animals in PT were treated with 2.5% enrofloxacin solution (Baytril®, 2.5% ad us vet injectable solution, Bayer HealthCare AG, DE) by intramuscular injection at the base of the ear on alternating sides from day to day. In OT, enrofloxacin solution (Baytril®, 0.5% oral solution, Bayer HealthCare AG, DE) was administered with a drencher using strokes of 1.5 ml each (three strokes for the smallest pig (7.7 kg) on day 1 and five strokes for the biggest pig (13.5 kg) on day 5). As the pigs reacted with strong salivation immediately after receiving the first stroke, the dose in OT was increased by one additional stroke (dosage increase of 20–33%) to assure sufficient uptake of the drug. From study day 2–5, all pigs of all groups received a sugar solution as an oral treat after their group specific procedure. All treatments and sampling were carried out on each pig separately in a separate room in the same hygienic unit to avoid cross-contamination and contamination of the pen with drugs during the handling procedure as far as possible.

Faecal sampling

Rectal swabs were taken from all pigs on study days 1 to 5, 7, 14, 28, 42 and 56. Sampling on days 1 to 5 was done directly before enrofloxacin treatment. The sample of study day 1, taken before first treatment, was used as a reference for comparisons in later analyses. Within one hour after collection, the swabs were transferred into cryo-tubes containing Luria-Bertani-broth (Merck, Germany) with 20% glycerol (Carl Roth GmbH, Germany) and stored at –80°C until further processing.

Blood sampling

Blood samples of 5 ml were collected into monovettes from the vena jugularis or the cranial vena cava of all pigs in groups OT and PT on study days 1, 3, 5 and 7. Samples taken before the first treatment on study day 1 served as a reference for comparisons in further analyses. Blood samples from all pigs in COT, CPT and CON were taken on study day 5 (no reference from study day 1). Samples on study days 3 and 5 in OT and PT were taken 1.5–2 hours after enrofloxacin treatment. Samples on study day 5 in COT, CPT and CON and on day 7 in OT and PT were taken immediately during handling procedures. The samples were centrifuged at 1,500 g for 10–15 min at room temperature. Serum was stored at –20°C until further processing.

Health and weight check

All pigs were visually checked for their health status every morning around feeding. All pigs were weighed eight, three and one days before first treatment, on study days 1–7 and subsequently once a week until the end of the study period. Body condition and feeding behaviour of the pigs in all groups and local reactions at the injection site in PT were observed and scored on a 0-1-2-scale using a standardized clinical examination protocol (accessible via corresponding author) on study days 1–7.

Biosecurity measures and study rules

Before enrolment of the weaners, the rooms had been cleaned, disinfected and checked for absence of Enterobacteriaceae by using dip slides (Roti-DipSlide VRBD, Carl Roth GmbH, Germany) on the floor, wall, door, ceiling, ventilation, feeder, drinker, barrier and pipe.

While the pigs were housed, the rooms and equipment were wet cleaned every morning. In the daily routines, the groups were either cared for by different persons working in parallel or by the same person in a given sequence (CON first, then PT/CPT and OT/COT last). Personnel were not allowed to go back the same day from OT/COT to PT/CPT or to CON without showering and changing clothes first. Each room system was entered wearing a new single-use overall (covering also the head), disposable gloves, a face mask and disinfected rubber boots that remained in the respective hygiene sluice during the whole study period.

Pigs showing signs of disease were dealt with according to a predefined protocol. Pigs showing minor health and welfare disturbances were observed more frequently and if necessary treated within their group. Antimicrobial treatment, other than required for the study, was not permitted. If necessary, the animal was separated within the room remaining in contact with its group. Pigs that were seriously ill without perspective to recover were removed from the trial and euthanized.

Evaluation of blood serum concentration of enro- and ciprofloxacin

Concentrations of enrofloxacin and its metabolite ciprofloxacin in blood serum were determined with a modified method based on L 06.00-66 (German Federal Office of Consumer Protection and Food Safety, 2015). Depending on the expected concentration of enro- and ciprofloxacin, 100 µl or 500 µl of blood serum were used. They were mixed with 10 ml of ethylenediaminetetraacetic acid (Serva Electrophoresis GmbH, DE) and McIlvaine-buffer (Merck, DE) solution (0.1 *M*; pondus

Hydrogenii, pH, value 4) (McIlvaine, 1921) by vortexing and ultrasonication. For recovery studies or matrix calibration, the sample was spiked with appropriate volumes of a methanolic enrofloxacin or ciprofloxacin standard mix solution (1 µg ml^{-1}, Sigma-Aldrich, DE). OASIS Hydrophilic-Lipophilic-Balanced cartridges (6 ml, 200 mg, Waters, DE) were used for clean-up by solid-phase extraction. Analytes were eluted with 6 ml of methanol. Finally, the eluate was evaporated to dryness and redissolved in 500 µl water/acetonitrile (hypergrade for liquid chromatography-mass spectrometry, Merck, DE; 90/10, vol/vol; with 0.2% formic acid). The final extract was filtered using a 0.45 µm nylon membrane (Phenex-NY, 4-mm syringe filter, Phenomenex, DE).

Liquid chromatographic separation (Agilent 1260 Infinity LC, Agilent, DE) on 18 C (octadecyl) column (Thermo Hypersil Gold, 150 × 2.1 mm, 3 µm, Phenomenx security guard C18) was performed with 0.2% formic acid (Merck, DE) in deionized water as first mobile phase (P1) and 0.2% formic acid in acetonitrile as second mobile phase (P2). The following gradients were used: 90% P1 and 10% P2 for the first minute, 40% P1 and 60% P2 from 1st to 12th min, 40% P1 and 60% P2 from 12th to 15th min, 90% P1 and 10% P2 from 15th to 16th min and 90% P1 and 10% P2 from 16th to 25th min (flow: 0.3 ml min^{-1}, oven temperature: 30°C, injection volume: 10 µl, auto sampler temperature: 4°C). Transitions were monitored by tandem mass spectrometry (Sciex, 6500 QTrap mass spectrometer DE; ionization mode: electrospray ionization in positive ion mode; scan type: multiple reaction monitoring) for ciprofloxacin: charge ratio (m/z) = 332 to 314 and to 245 and for enrofloxacin: m/z = 360 to 316 and to 245. The quantification was conducted by external matrix calibration (2, 5, 10, 30, 100 and 300 µg l^{-1} enrofloxacin or ciprofloxacin) with verification by standard addition.

The validation of the whole procedure, including extraction and analysis, was performed applying a factorial design according to Commission Decision 2002/657/EC using the software InterVAL (quodata GmbH, DE) for calculation. The relevant factors that may influence the analysis were operator (experienced vs. inexperienced), solid phase extraction cartridges (two different batches), species (pig and chicken) and kind of serum (commercially available vs. centrifugated blood of untreated animals). For eight runs, test samples of enrofloxacin- and ciprofloxacin-free pig and chicken serum were spiked with 0, 2.5, 5, 10, 30 and 50 µg l^{-1} and calculated against a matrix calibration of 0, 2, 5, 10, 20, 40 and 60 µg l^{-1}. The decision limit of the lowest concentration level at which a method can discriminate with a statistical certainty of $1 - \alpha$ whether the particular analyte is present ($CC\alpha$) were 4.40 µg l^{-1} for enrofloxacin and 5.50 µg l^{-1} for ciprofloxacin. The detection capability of lowest concentration at which a method is able to detect truly contaminated samples with a statistical certainty of $1 - \beta$ ($CC\beta$) were 5.91 µg l^{-1} for enrofloxacin and 8.13 µg l^{-1} for ciprofloxacin (according to Commission Decision 2002/657/EC). At 7.5 µg l^{-1} of enrofloxacin and ciprofloxacin, the repeatability was 10.4 and 13.1%, the in-house reproducibility was 10.8 and 16.7% and the mean recovery was 103.0 and 114.1%. Based on this validation the method was considered to be fit for purpose.

Isolation of *E. coli* and susceptibility testing

The broth from the faecal swab samples was defrosted and cultured on MacConkey-agar (McC, Merck, DE) and on MacConkey-agar with 0.12 µg ml^{-1} ciprofloxacin (McC + CIP, Sigma-Aldrich, DE) to increase the chance of detecting resistant isolates (Gunn et al., 2008). For *E. coli* quantification, 100 µl of the 10^0, 10^{-1} and 10^{-3} from a serial dilution were plated in logarithmic mode and in duplicate by using a spiral plater resulting in a detection limit of 10^2 cfu ml^{-1} and an upper quantification limit of 10^8 cfu ml^{-1}. The plates were incubated for 18–20 hours at 37°C. In parallel, non-specific enrichment was performed in buffered peptone water (500 µl sample material in 4.5 ml peptone water, for 18 to 20 hours, at 37°C).

Colonies of *E. coli* were counted on the McC- and McC + CIP-plates for a possible statement on the ratio of resistant to total *E. coli* in the sample. One single colony first detected with typical *E. coli* morphology (mid-sized, pink with precipitation zone) per sample from McC and McC + CIP plates was picked, and species was confirmed using Matrix Assisted Laser Desorption Ionization combined with Time of Flight analysis (Microflex Biotyper, Bruker, USA). Isolates confirmed as *E. coli* were preserved at –80°C in Luria-Bertani-broth (Merck, DE) with 20% glycerol (Carl Roth, DE). If no *E. coli* grew on the McC-medium (with or without ciprofloxacin) 100 µl of the enrichment broth was plated on McC (18–20 hours, 37°C) to increase the detection rate.

Determination of the minimum inhibitory concentration (MIC) was performed by broth microdilution following guidelines from Clinical and Laboratory Standards Institute (2012). Commercial test plates (Sensititre, TREK Diagnostic Systems, UK) were used containing 14 antimicrobial agents (including the quinolones ciprofloxacin and nalidixic acid) in accordance with Decision 2013/652/EU on the monitoring and reporting of AMR in zoonotic and commensal bacteria (European Commission, 2013). Minimum inhibitory concentrations were evaluated against the epidemiological cut-off values provided by EUCAST (2015). In this regulation resistance to ciprofloxacin is used as an indicator for fluoroquinolone resistance due to its cross resistance to enrofloxacin. Resistance to nalidixic acid is used as indicator for quinolone resistance. For ciprofloxacin *E. coli* with MIC values > 0.06 µg ml^{-1} were characterized as resistant, for nalidixic acid the cut-off value was > 16 µg ml^{-1}.

Clonality analyses

The XbaI digestion and subsequent pulsed-field gel electrophoresis (PFGE) of selected ciprofloxacin resistant isolates were performed following the protocols of PulseNet International (2013). Patterns of PFGE were analysed with dice (0.5% optimization, 2% tolerance) using the unweighted pair group method with arithmetic mean by BioNumerics 7.1 (Applied Maths, BE).

Statistical analyses

The statistical analyses were carried out using SAS version 9.4 (North Carolina). Study groups were nominally scaled variables, treatment as well as resistance to antimicrobials was coded as a binary variable (presence/absence, resistance/susceptibility). In all analyses, $p < 0.05$ was chosen as the threshold for significance.

Body weight was compared between study groups and between sexes using ANOVA (MIXED Procedure) and non-parametric tests (Mann-Whitney for sex and Kruskall-Wallis for study group; npar1way Procedure).

The association of the presence of enro- and ciprofloxacin in serum with the groups COT or CPT and CON

(nominal scaled variable; hypothesis a) on study day 5 was evaluated using Fisher's exact test on two by two tables. The effect of study group (COT, CPT and CON) on the probability for presence of enro- and ciprofloxacin in the serum was evaluated using logistic analyses (LOGISTIC Procedure) with study group (including COT, CPT and CON) as fixed factor. In total four analyses were run, testing presence of enro- and ciprofloxacin at concentrations above a cut-off at 0 µg l^{-1} and above a cut-off at CCα. The null hypothesis that the slope is not significantly different from zero was tested.

The relationship between resistance to ciprofloxacin and nalidixic acid (based on selective testing) and the study groups (nominal scaled variable; hypothesis b) was evaluated using Fisher's exact test. The probability of ciprofloxacin and nalidixic acid resistance in an *E. coli* isolate of a pig was analysed between study groups (fixed factor) for single study days using a logistic analysis model (LOGISTIC Procedure). Specification of the model (example enrofloxacin) is logit (*P*) = *a* + $Group_i$ where logit (*P*) is the logit transformation of the probability *P* for enrofloxacin resistance in *E. coli* of the pig, *a* is the overall intercept and Group *i* is the fixed effect of the *i*th level of Group (*I* = 1: OT, 2: COT or 3: PT, 4: CPT or 5: CON). The where-statement was used to specify the respective study day. The null hypothesis that no significant difference occurs between respective study groups was tested.

The relationship of resistance to antimicrobial agents other than ciprofloxacin (based on non-selective testing) between different study days (nominal scaled variable) was evaluated within study groups using McNemar's test. The probability for AMR was analysed per study group for the study day (fixed factor) showing a peak of resistance frequency compared with the group's initial value of study day 1 (hypothesis c) using a logistic analysis model (GENMOD Procedure). Specification of the model (example ampicillin; 5^{th} vs. 1^{st} study day; OT) is logit (*P*) = *a* + DAY_i where logit (*P*) is the logit transformation of the probability *P* for ampicillin resistance in *E. coli* of the pig in OT, *a* is the overall intercept and DAY_i is the fixed effect of the *i*th level of DAY (*I* = 1: 5^{th} study day, 2: 1^{st} study day). The where-statement was used to specify the respective study group. The null hypothesis that the slope is not significantly different from zero was tested.

Results

Animals, health and weight check

Of the 70 weaners included in the study, 68 passed through the full experimental period. Two pigs in COT were euthanized, one for severe lameness/inflammation of the carpal joint on study day 7 (after faecal sampling) and the other for suspected enterotoxaemia on study day 9. Therefore, these pigs were only included in the analyses of data from study days 1 to 7 and were missing in the data analyses for the remaining study days. Another three pigs were temporarily separated from the group but remained within the same room with physical contact through fences. Of those, two animals in OT were lame (separated for seven days; separated for three days (whereof two days were before study start and one day was during enrofloxacin treatment) and later for another 14 days), one was in PT with umbilical hernia (separated for two days). The two euthanized pigs and the two lame pigs were treated with analgetics/anti-inflammatory drugs (meloxicam or dexamethasone).

Each study group contained five to seven females and seven to nine males. The mean body weight of all pigs was 9.2 ± 1.2 kg on study day 1 and 49.1 ± 5.7 on study day 56 and did not differ ($p > 0.05$) between study groups, or between sexes.

Enrofloxacin and ciprofloxacin in the blood serum (hypothesis a)

On study day 1, enrofloxacin and its metabolite ciprofloxacin were not present above the detection limit in the serum of pigs from OT and PT. All samples from groups OT and PT on days 3 and 5 (during treatment) were positive for enro- and ciprofloxacin. The concentration (mean ± SD) of enrofloxacin on study days 3 and 5 was 370 ± 240 µg l^{-1} and 375 ± 217 µg l^{-1} in OT and 948 ± 233 µg l^{-1} and 987 ± 226 µg l^{-1} in PT. The concentration of ciprofloxacin was 132 ± 83 µg l^{-1} and 127 ± 71 µg l^{-1} in OT and 287 ± 112 µg l^{-1} and 252 ± 98 µg l^{-1} in PT. The concentration varied widely within the groups on both days. On study day 7, i.e. two days after the last treatment, the concentrations in OT and PT had decreased to about 1% of those on treatment days.

Enrofloxacin and ciprofloxacin were detected in the blood serum of all COT weaners (median 4.86 µg l^{-1}) on day 5. For enrofloxacin, values were below the CCα of 4.40 µg l^{-1} in seven samples. In the other seven samples, the values were above the CCα. For ciprofloxacin all 14 values were > 0 but below the CCα of 5.50 µg l^{-1} (median 2.7 µg l^{-1}). The substances were also detected in 8/14 (enrofloxacin, median 1.3 µg l^{-1}) and 7/14 (ciprofloxacin, median 0.6 µg l^{-1}) CPT weaners. However, all positive samples had values below the CCα. Enrofloxacin or ciprofloxacin was not detected in the serum of the CON group on study day 5. The proportion of enro- and ciprofloxacin positive samples (cut-off at 0 µg l^{-1}) differed between the groups COT and CPT compared to CON on study day 5 ($p < 0.01$; non-directional Fisher's exact test). With the cut-off at CCα, enrofloxacin presence in COT and CON differed ($p < 0.01$) but neither presence of enrofloxacin in CPT, nor ciprofloxacin presence in COT and CPT differed from CON (all $p > 0.05$). Directional Fisher's exact tests proved higher proportions of drug presence in the contact groups. Due to quasi-complete separation of data points, the validity of the model fit in the logistic analysis was questionable and results are not presented.

Isolation of *E. coli*

Escherichia coli were isolated from 635 of 692 samples, using the non-selective culture (McC), and from 57 samples using the selective culture (McC + CIP). The number of *E. coli* positive samples (reflected by isolated *E. coli* from McC) decreased in OT and PT from 14 isolates per group on the first and second study day to 6–12 isolates between study days 3 and 7 (38 of 112 samples without *E. coli* detection). After day 7, detection rates on McC returned to 100% of the samples.

(Fluoro)Quinolone resistance (hypothesis b)

Of the 635 *E. coli* isolates from McC, 2 (0.3%) showed a MIC of 2 µg ml^{-1} for ciprofloxacin (i.e. above the ECOFF cut off 0.06 µg ml^{-1}): one isolate was from group OT

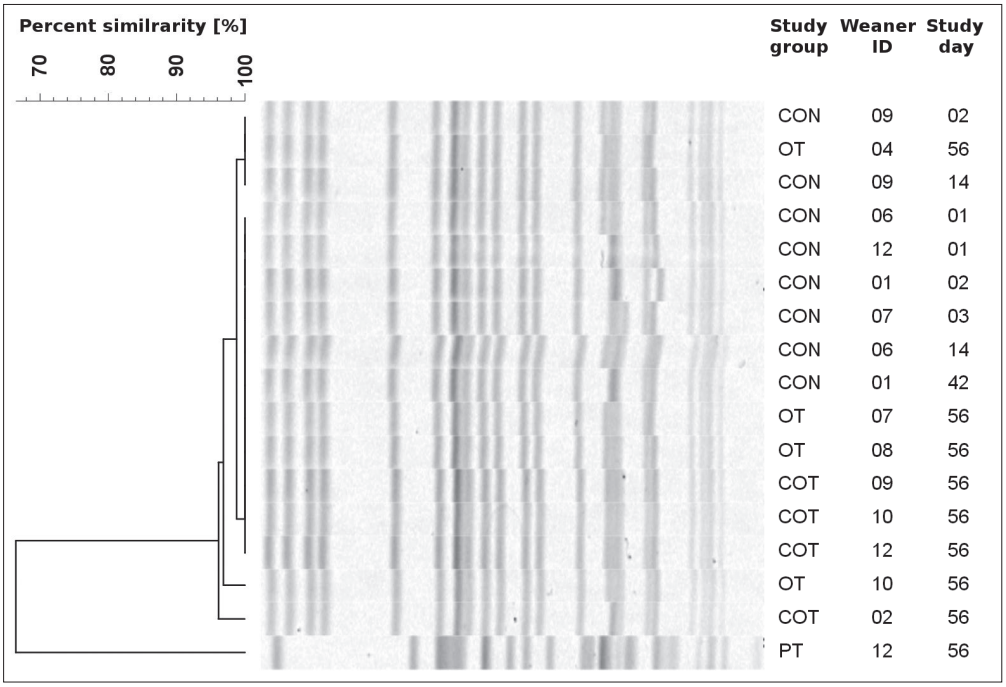

FIGURE 1: *Patterns of pulsed-field gel electrophoresis after xbaI macrorestriction of 8 strains from orally treated weaners (OT) and untreated contact weaners of OT (COT; study day 56), a selection of eight strains from untreated control (CON, study day 1–42) and 1 strain from a parenterally treated weaner (PT; study day 56) analysed by using BioNumerics 7.1 (band based comparison; dice: optimization 0.5%, tolerance 2%; unweighted pair group method with arithmetic mean).*

on study day 56, the other isolate from group CON on study day 14. Both isolates were also resistant to nalidixic acid (MIC: > 128 µg ml^{-1}). Both samples with (fluoro)quinolone resistant *E. coli* detection from McC were also identified with the McC + CIP selective isolation.

Of the 57 *E. coli* isolates from McC + CIP, 55 (96.5%) were confirmed by MIC testing, i.e. displayed MICs of > 0.06 µg ml^{-1} (Table 1), of those, 47 isolates originated from group CON on study days 1–42. In this group, ciprofloxacin-resistant *E. coli* were isolated on two to six study days per animal. The probability of detecting an *E. coli* resistant to ciprofloxacin (or nalidixic acid) via selective cultivation was significantly ($p < 0.05$) higher in CON compared to OT, PT, COT and CPT on study days 1, 3, 5, 7 and 14. In OT and COT, four *E. coli* isolates per group were detected on McC + CIP in samples on the last study day (56; Table 1). All eight isolates had MICs well beyond the cut-off value. According to Fisher's exact test, the proportions of detecting an *E. coli* resistant to ciprofloxacin and nalidixic acid via selective cultivation significantly differed between COT and CON ($p = 0.0331$) but not between OT and CON ($p = 0.0978$) on day 56. The directional Fisher's exact test (OT > CON, COT > CON) proved higher proportion of resistant isolates in COT ($p = 0.0331$) and also OT ($p = 0.0489$). In the logistic analysis, data points were quasi-completely separated and therefore, the validity of the model fit was questionable and results are not presented.

Two isolates from PT and COT grown on McC + CIP were not confirmed to be ciprofloxacin resistant by MIC-testing and excluded from further analyses.

Clonality analyses

The eight *E. coli* strains with ciprofloxacin resistance from OT and COT and a selection of eight resistant strains from CON were compared using pulsed-field gel electrophoresis (Fig. 1). The strains from CON were selected from five pigs originating from different dams and for three of these pigs, the isolates of study days with first and last resistance detection (susceptibility test) were picked. The eight strains from OT and COT on study day 56 showed closely related patterns (> 95% similarity) to the 8 strains from CON that were sampled between study day 1–42. The strain from PT (day 56), that grew on McC + CIP but was not confirmed by MIC-testing differed from the resistant isolates (similarity 66.4%).

Resistance to other antimicrobial agents (hypothesis c)

The proportion of resistant *E. coli* among all detected *E. coli* from McC per study group changed in a similar way for ampicillin (Fig. 2), sulfamethoxazole, trimethoprim and tetracycline. The orally treated and its contact group peaked with up to 75 and 46% resistant isolates on study days 5 and 7. In contrast, no resistant *E. coli* were detected in PT and CPT on these days. All other resistance proportions ranged between 0 and 21% in all groups during whole study period. According to McNemar test, the proportions of detecting an *E. coli* resistant to ampicillin, sulfamethoxazole and trimethoprim did not significantly differ in OT (e.g. ampicillin: value 2.000, $p = 0.1573$ and 3.000, $p = 0.0833$; 8 isolated *E. coli* on both days) but in COT (e.g. ampicillin: 5.444, $p = 0.0196$ and 4.5000, $p = 0.0339$; 13 and 12 isolated *E. coli*) at the study days 5 and 7 compared to day 1. In the logistic analysis, the risk differed in OT (e.g. ampicillin: odds ratio day 5: 2.1, CI 1.6 to 2.9, $p < 0.0001$ and odds ratio day 7: 1.9, CI 1.3 to 2.6, $p < 0.0001$) and COT (e.g. ampicillin: odds ratio day 5: 1.4, CI 1.1 to 1.8, $p = 0.0012$ and odds ratio day 7: 1.5, CI 1.1 to 1.9; $p = 0.0008$) on study days 5 and 7 compared to day 1. Afterwards, the odds decreased and there were no significant ($p > 0.05$) differences within the groups anymore on study day 56 compared to day 1. Minimum inhibitory concentrations differed by at least four dilution steps between susceptible and resistant isolates within study groups on study days 5 and 7 and between susceptible and resistant isolates from individual animals on study days 5 and 7 vs.

TABLE 1: *Characteristics of weaners and their ciprofloxacin-resistant[1] Escherichia coli isolates from ciprofloxacin-selective media per study group and study day (in total 55 E. coli)*

Study group[2]	Weaner ID[3]	Sex[4]	Weaner's dam ID[3]	Weaner's sire ID[3]	Minimum inhibitory concentration, µg mL^{-1} per study day								
					1	2	3	4	5	7	14	42	56
OT	04	æ	01	02									1
	07	æ	03	01									2
	08	‘	06	04									2
	10	æ	07	02									2
COT	02	æ	01	02									2
	09	æ	06	04									2
	10	‘	07	02									2
	12	æ	10	02									2
CON	01	‘	02	02		2		1	2	2	2	2	
	03	‘	08	04		2	2	2					
	04	‘	05	01						2			
	05	æ	03	01			2	2	2	2	2		
	06	æ	03	01	2	2				2	2		
	07	æ	06	04			2	2					
	08	æ	07	02		2	2	2		2	2		
	09	æ	07	02		2	2	2	2	2	2		
	10	æ	09	01	2		2			2	2		
	11	æ	09	01				2		2			
	12	æ	10	02	2	2	2	2	2	2			
	13	æ	10	02						2	2		
	14	æ	01	01			2						
Total number of *E. coli* isolates per study day					**3**	**6**	**8**	**8**	**4**	**10**	**7**	**1**	**8**

[1] Minimum inhibitory concentration > 0.06 µg ml^{-1}

[2] Study groups: OT = orally treated group; COT = untreated contact group of OT; CON = untreated control

[3] ID = identification number

[4] Sex: æ = male, ‘ = female

day 1. The proportion of *E. coli* resistant to tetracycline was already high on study day 1 but decreased after study day 6 similarly as for ampicillin, sulfamethoxazole and trimethoprim.

Discussion

Blood serum concentration of enro- and ciprofloxacin (hypothesis a)

Enrofloxacin and its metabolite ciprofloxacin could be detected in blood serum of most contact animals. This indicates that contact animals in both treatment groups were exposed to the antimicrobials without being treated themselves. The proportion of positive blood samples was significantly higher in COT and CPT compared to CON.

Maximum intestinal tissue concentrations of enrofloxacin and ciprofloxacin did not differ and resorption rates are reported as being similar between animals treated orally or parenterally (Wiuff et al., 2002; VETIDATA, 2016). However, Devreese et al. (2014) found higher enro- and ciprofloxacin concentrations in the cloaca of broiler chickens after parenteral compared to oral administration. Literature provides no information whether pigs shed higher concentrations of drugs in their faeces after parenteral than after oral administration. Orally treated pigs may additionally have shed a certain amount of substance with the saliva and may also have done this in the housing area, while for PT pigs this is unlikely. The experimental setup was designed to minimize direct contamination of the animal environment with the drug by performing treatment outside the stable in a separate treatment room. However, spillage of the substance via salivation may have continued after returning the drenched pigs to their group. Decontamination of the skin of treated pigs did not appear appropriate as contamination caused by salivation may also happen under commercial farming conditions and ongoing salivation might have led to re-contamination anyway. We decided for increasing the dosage by one additional stroke of the drencher (20–33%) per OT pig to assure the intake of the desired treatment dose. We fed a sugar solution at the end of each handling procedure on day 2–5 to end the salvation reaction. Just recently, taste-masking was developed for oral enrofloxacin formulations to increase the intake of enrofloxacin-medicated feed (Liu et al., 2017). Such a kind of drug would be preferable in future studies. Altogether, the most relevant pathway of the antimicrobial from PT to CPT pigs seems to be the transmission via faeces or urine. Even though this study was not designed to perform pharmacodynamic or pharmacokinetic analyses of the substance, in future studies, measurement of the concentration of enrofloxacin/ciprofloxacin in the urine and faeces in treated animals would be of additional interest to investigate the amount and dominating route of exposure of contact animals (Post et al., 2003). In conclusion, oral and even parenteral treatment may lead to transmission of antimicrobials to untreated animals housed together with treated ones. The transmission may have potential consequences for the selection for antimicrobial resistance in these contact animals as Nguyen et al. (2012, 2014) demonstrated for pigs receiving low dosages of ciprofloxacin.

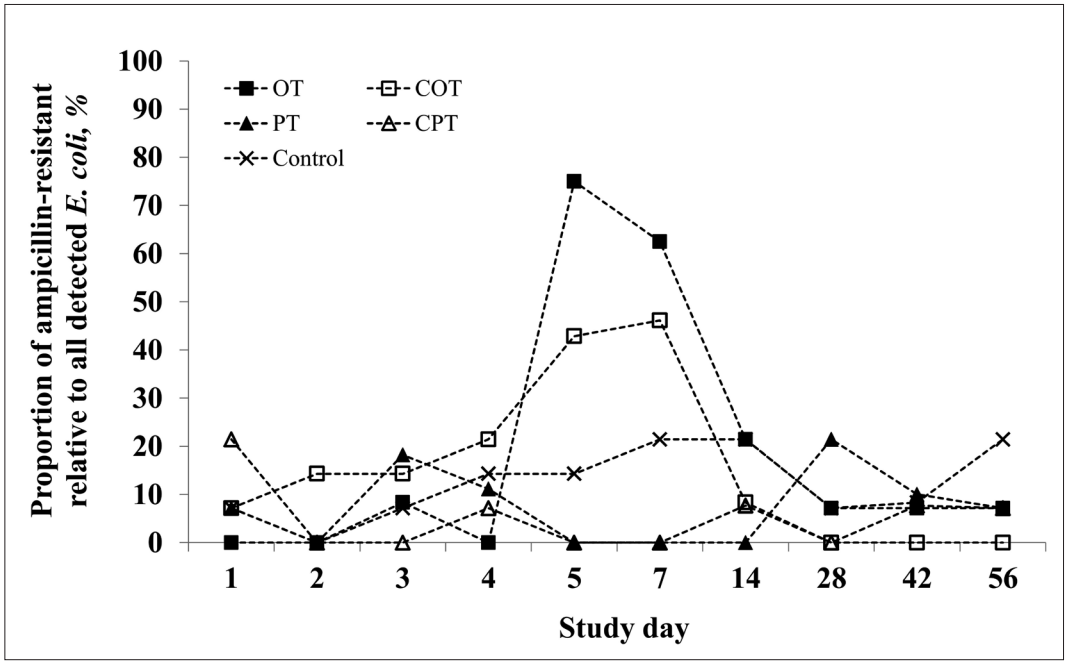

FIGURE 2: *Frequency of ampicillin-resistant (minimum inhibitory concentration > 8 µg ml^{-1}) Escherichia coli isolates from non-selective media per study day and the study groups orally treated (OT), contact to orally treated (COT), parenterally treated (PT), contact to parenterally treated (CPT) and control; number of all detected E. coli = 635; in the logistic analysis, the risk for an E. coli to be resistant was higher on day 5 and 7 compared to day 1 in OT (odds ratio 2.1, p < 0.0001 and 1.9, p < 0.0001) and COT (odds ratio 1.4, p = 0.0012 and 1.5, p = 0.0008) and decreased to non-significant differences (p > 0.05) on day 56.*

(Fluoro)Quinolone resistance (hypothesis b)

In contrast to our expectations, no resistance to ciprofloxacin and nalidixic acid was detected via selective isolation of ciprofloxacin resistant *E. coli* in both treatment and their contact groups during the treatment period and until day 42 after the onset of treatment. Although we may have missed the detection of resistant bacteria due to the testing of only one colony from the non-selective plates, the fact that no single suspicious colony could be detected from the selective media strongly supports that no resistance development occurred during the selective pressure. In line with that, the detection rate of *E. coli* from McC was decreased during and shortly after treatment in OT and PT indicating growth inhibition of the bacteria by the treatment. Resistant isolates in the treated groups were only detected with selective isolation on day 56. Also the non-selective resistance determination in one random *E. coli* isolate per sample showed no increase in the ciprofloxacin resistance. Weaners in COT and CPT were expected to acquire resistant *E. coli* either by selection through the detected levels of fluoroquinolones in the blood or by uptake of resistant bacteria from OT and PT. As no quinolone-resistant *E. coli* were found in the treated groups until day 42, neither by non-selective nor by selective testing, could one assume that none of the scenarios occurred. Anyhow, at least excretion of quinolone-resistant *E. coli* did not occur on detectable levels in single random *E. coli* isolates from the 14 contact animals in each group.

The overall detection rate might be lowered by the freezing of the samples before bacteriological testing instead of cultivating the fresh swab. Nevertheless, isolation of *E. coli* on non-selective media was possible in 92% of the samples. Only from 38 samples during and shortly after the treatment, isolation of *E. coli* failed even after pre-enrichment, indicating the ciprofloxacin susceptibility of the *E. coli* in the microbiota of our study population. Lautenbach et al. (2008) found a higher recovery rate for fluoroquinolone-resistant (91%) compared to -susceptible (83%) *E. coli* after freezing (based on 23 samples).

In general, analysing only one random colony per swab sample is not representative for the whole excreted *E. coli* population of the gut microbiota of an animal. Therefore, picking only one *E. coli* could limit detection rate for resistance development. Brun et al. (2002) and Persoons et al. (2011) found the resistance prevalence among the isolates of a faeces sample to vary much less within one animal at one day (virtually nil) compared to between animals (0.14–2.42) and between days (0.85–1.79). Hence, we used a relatively high sample size of animals compared with e.g. Wiuff et al. (2003), allowing detection of expected differences in proportions of resistant *E. coli* between groups. However, further confirmation with a more comprehensive testing scheme is needed. Studies of Vieira et al. (2008) and Bosman et al. (2012) suggest evaluation of more than one isolate per animal and day. Picking and analysing several colonies per sample (Humphry and Gunn, 2014) or even to do a metagenome-analysis (Humphry et al., 2002) would have increased sensitivity to better reflect the total bacterial population and would have enabled to reflect the variety of potential clones in the faeces (Schierack et al., 2007; Bednorz et al, 2013; Dixit et al, 2004).

Moreover, sampling a specific amount of faeces per animal and day would have allowed for quantification of the concentration of bacteria and comparison of bacterial counts per gram between samples. Thereby, also the chance of resistance-detection, even of few existing resistant *E. coli*, may have increased. For the study groups, low numbers of resistant *E. coli* could have been overgrown by non-resistant *E. coli* on non-selective agar and the amount of faeces tested could have been too low for the detection of very low numbers of resistant bacteria, even on selective agar. However, the methods used proved effective in finding such bacteria on day 56 and in the control group. Anyway, a specific amount and comparison of quantification of the concentration of bacteria would be preferable.

Overall, the effect of contact of untreated with treated animals on the composition of their microbiota and on a possible resistance development in excreted bacteria

needs further investigation involving expanded detection methods.

Anyway, as the selective isolation approach is more sensitive than random testing of one *E. coli* isolate (Gunn et al., 2008), it is not likely that another detection method would have led to a completely opposite result for resistance occurrence in *E. coli* from the treatment and contact groups.

However, the findings of the present preliminary study are in contrast to Wiuff et al. (2003) who used the same dosage and treatment duration and found increased resistance to ciprofloxacin immediately after the first enrofloxacin-treatment of pigs compared to before treatment or an untreated control (4 pigs per study group). They reported around 100% resistance in isolated *E. coli* in orally and parentally treated weaners during the treatment period. High resistance rates in their study persisted for up to five days and then decreased down to 1–20% at the last measurement 13 days after withdrawal. In another study, the increased resistance to enrofloxacin, flumequine and nalidixic acid persisted for about two months (Belloc et al., 2005). Callens et al. (2015) reported that the risk of enrofloxacin resistance increased by an odds ratio of 26.8 if enrofloxacin was used in pigs. However, those studies did not report to have used previously untreated pigs and their pig groups may have harboured resistant isolates before treatment. Pigs and their dams in the present study were chosen based on the breeder's declaration not to have treated them previously and resistant isolates were absent in the two treated groups even using selective isolation. Nguyen et al. (2012) orally administered ciprofloxacin to pigs similarly dosed as the enrofloxacin in the present study and additionally using a tenfold dose. They found higher concentrations of the substance and resistant counts in faeces after the high dosage treatment. We do not know whether a higher dosage would have evoked resistant bacteria in the present study. We decided to use the licenced dosage rather than an unrealistic dosage. Altogether, the findings of this preliminary study indicate that enrofloxacin-treatment at therapeutic dosage does not necessarily evoke ciprofloxacin- and nalidixic acid-resistance in susceptible *E. coli* under experimental conditions.

Hygiene standards were high in the trial facility with daily wet cleaning of rooms and equipment. Moreover, group sizes of 14 to 28 animals per room were small compared to commercial conditions. Therefore, a higher chance of resistance occurrence under commercial conditions would be possible due to a potentially higher bacterial load in the environment and more animals per room. We only investigated resistance development in *E. coli* as it is the established indicator bacterial species for such studies and ignored other bacterial species. However, overall under these experimental conditions, neither treatment with enrofloxacin nor contact to treated weaners did evoke detectable levels of ciprofloxacin-resistance in faecal *E. coli* from any of the studied pigs.

In contrast to the treated animals and their contact animals, ciprofloxacin-resistant strains were detected in the untreated group CON and remained prevalent throughout the trial period. Even though this finding was based on only one isolate per animal and day, not being representative for whole excreted *E. coli* population of the gut microbiota of the animals, resistant isolates were repeatedly detected in this group. All pigs originated from the same herd, had been transported together on the same transport vehicles which had been wet cleaned and disinfected before transport. All rooms and equipment had been disinfected and tested free for *E. coli* before enrolling the pigs. All 70 pigs were offered feed originating from the same batch. Piglets from different litters were purposefully mixed when assigning the piglets to the study groups by random allocation using sampling intervals. Therefore, detection of resistance to ciprofloxacin only in the control group was highly unexpected. The original source of the resistant strain remains unknown. The resistant isolates from group CON showed nearly identical PFGE-patterns which indicates horizontal spread of the bacteria rather than spread of mobile genetic elements harbouring resistance genes.

The late occurrence of resistant isolates in OT and COT using selective testing on study day 56 was most probably not linked to the treatment because there was no exposure to antimicrobials at that time which could have induced the occurrence of the strain. An effect of treatment was expected during or shortly after treatment because of the selective pressure induced as described in the literature (Wiuff et al., 2003; Belloc et al., 2005). It was not expected on day 56. No sampling took place between study day 42, when no resistant isolates were found, and study day 56, leaving 14 days without information. The PFGE pattern of the resistant strains in OT and COT were highly concordant with the patterns of the resistant strains found in CON until day 42 (> 95% similarity), and the resistant *E. coli* isolates had the same ciprofloxacin MIC level (1 to 2 µg ml^{-1}). Therefore, it can be assumed that – despite strict biosecurity measures – the resistant *E. coli* clone was transferred from room 3 to room 1 at some point of the experiment. The resistant strain appeared equally distributed between animals from OT and COT on study day 56 which is in line with the even distribution of this resistant *E. coli* among CON pigs throughout the study. In a study of Andraud et al. (2011), contact animals of pigs infected with resistant bacteria developed resistance after two days. Considering our findings and the published data, a transmission from room 3 to room 1 between study day 42 and 56 appears likely although the transmission route remains unknown.

The housing rooms each had a hygiene sluice and air-filtration system and its own food and water supply and all rooms were separated by several doors. Feed was taken from the same batch for all animals. The personnel entering a room, was wearing room-specific protective clothing. There may anyway be a residual risk for transfer of resistant strains by e. g. air or personnel. According to the biosecurity measures and study rules, personnel going from CON to another room only had to change single use clothes/rubber shoes and wash hands but not to shower the whole body and change underwear as was the rule when changing from rooms 1 or 2 (containing treated animals) to another room. These circumstances may have increased the residual risk of transmission from CON slightly.

The assumed transmission from room 3 to room 1 underlines the risk of transfer of bacteria due to undetected hygienic breaks and the importance of strictly separating groups during treatment if the resistance spread is to be evaluated. It also underlines that bacteria

that are resistant to ciprofloxacin may persist in the population and may be transferred between groups that are separated by insufficient hygiene barriers even without selection pressure. In the study by Wiuff et al. (2003), the untreated control group showed a significant development of ciprofloxacin resistance during the experiments indicative of undesired resistance transfer between study groups. In that study temporary use of the same environment was the likely route of transmission.

Dunlop et al. (1998) identified building separation to reduce risk of antimicrobial resistance in pigs. Visitors without pig contact within last two days (Taylor et al., 2009) and protective clothes for visitors (Sternberg Lewerin et al., 2015) were found to reduce the risk of resistance and enteric disease in pig herds. In our study, the groups PT and CPT were often visited directly after CON after changing protective clothing, gloves, boots etc. and no ciprofloxacin resistant *E. coli* were detected in these groups. Nevertheless, the absence of ciprofloxacin resistant *E. coli* in the treatment and contact groups until study day 42 may indicate a constantly effective biosecurity system for these groups. However, the assumed transfer of resistant *E. coli*, even under experimental conditions emphasizes the relevance of cross-contamination in the spread of resistant bacteria and the importance of strict hygiene management in commercial pig production.

Resistance to other antimicrobial agents (hypothesis c)

The proportion of *E. coli* resistant to ampicillin, sulfamethoxazole and trimethoprim increased in OT and COT after enrofloxacin treatment on study days 5 and 7 based on one random isolate per sample. Very similar to our findings, Beraud et al. (2008, evaluating three isolates per sample) reported a decrease of susceptibility to ampicillin, and trimethoprim-sulfamethoxazole after i. m. administration of enrofloxacin to four to eight weeks-old pigs. However, in contrast to our study, they also observed an increase in resistance to quinolones. More generally, Akwar et al. (2008) described that use of antimicrobials in pigs selects for AMR among faecal *E. coli* within and between classes of antimicrobials. Antimicrobial treatment influences the gut microbiota (Sommer and Dantas, 2011). Despite the ciprofloxacin-treatment with therapeutic dosage we were able to isolate susceptible *E. coli* from the faeces of most of the PT and OT animals, showing that not all intestinal bacteria were reached by an inhibitory drug concentration. Nevertheless, it is likely that the treatment shifted the proportions of different *E. coli* strains, in our case to strains expressing resistance to certain antimicrobials. The underlying mechanisms that allowed these strains to become more dominant were not further investigated in this study. One could compare resistant with non-resistant isolates of individual animals with molecular biological methods in future research. Still, our results indicate that the use of a fluoroquinolone may select for resistance to other antimicrobial classes, even though strains are not resistant to quinolones. Further investigations are necessary to confirm the result applying expanded detection methods and to analyse the reasons of these changes.

Nearly all quinolone resistant isolates were detected using the selective media, while the chance of randomly picking a ciprofloxacin resistant strain from non-selective media was less than 1%. This was expected (Gunn et al., 2008) and our reason for using selective media along with non-selective media. It indicates that even if a number of *E. coli* are tested in a population harbouring resistant isolates as in our CON group, resistance to ciprofloxacin may be missed without selective media. Even using selective media, resistance to ciprofloxacin was not observed on day 28 although, based on the identical PFGE patterns of the resistant bacteria before and after day 28, it was likely present in the population. This further underlines the challenges of AMR detection in populations, which might be important if resistance patterns are included e. g. in improved biosecurity protocols of breeding herds.

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Conflict of interest

There are no protected, financial, professional or other personal interests in a product, service and/or company that could influence the content or opinions expressed in the above manuscript.

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