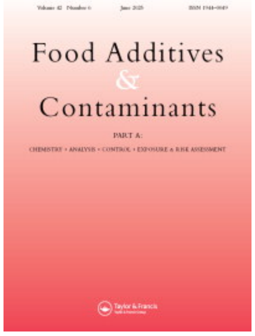

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The transmission of antimicrobial residues and occurrence of resistant bacteria after oxytetracycline treatment of veal calves in relation to animal keeping practices

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Wageningen Food Safety Research, Wageningen, The Netherlands

ABSTRACT

The use in animal husbandry of antimicrobials to treat bacterial infections remains unavoidable. However, the use of antimicrobials provokes antimicrobial resistance (AMR), therefore, there is a continuing effort to reduce veterinary antimicrobial use. A potential challenge is the transmission of residues and AMR from treated to untreated animals housed in the same environment. Taking this into consideration, alternative animal keeping practices should be explored. So far, there is little published research aiming to abate, especially the transmission of antimicrobial residues from treated to untreated animals and their environment. In this study, two animal husbandry practices were investigated, exploring the effect of physical separation on the transmission of oxytetracycline residues and the occurrence of antimicrobial resistance. Residue levels were analysed in internal (manure, urine, blood, and saliva) and external (wipes and hair) matrices. Additionally, antimicrobial resistance of *Escherichia coli* isolated from manure and the environment was assessed. When calves were housed in the same pen during treatment, residues were found not only in the treated animal but also observed in all tested matrices (internal and external) of the untreated pen-mate. When calves were physically separated during treatment to the end of the withdrawal period, no residues were detected in internal matrices of the untreated animals, indicating that transmission can be effectively prevented. Contamination of the pen and the exterior of the animals is also drastically reduced by the separation of animals during treatment and the withdrawal period. AMR results seem to indicate that tetracycline resistance levels return more or less to pre-treatment levels after the withdrawal period. In conclusion, it is recommended to physically separate calves for the duration of both treatment and the withdrawal period, in order to minimise and/or prevent transmission of oxytetracycline residues to untreated animals.

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Antimicrobial residues; antimicrobial resistance; transmission; barn environment; LC-MS/MS

Introduction

Currently, the use of antimicrobials to treat bacterial infections in animal husbandry remains inevitable. After treatment, antimicrobial residues will be present in various parts of the animal before they are excreted from the body *via*, e.g. urine or manure. In order to prevent exposure of the consumer to harmful levels of antimicrobial residues, maximum residue limits (MRL) are set for edible matrices ((EU) 37/2010 2010). Traditionally, enforcing maximum residue limits (MRL) in edible matrices was deemed sufficient for ensuring food safety. It cannot be ignored however that the excessive use of antimicrobials in humans and animals can lead to the selection of resistant bacteria (Ter Kuile et al. 2016; WHO 2023) and levels far below the minimum inhibitory concentration (MIC) can still cause an advantageous environment for resistant strains (Gullberg et al. 2011). Hence, there is a continuing effort to reduce veterinary antimicrobial treatment in order to constrain AMR (FAO and WHO 2023).

In the Netherlands, intensive collaboration between the government, veterinary professional

CONTACT Larissa J.M. Jansen larissa.jansen@wur.nl Wageningen Food Safety Research Akkermaalsbos 2, Wageningen, The Netherlands
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organisations, and the livestock sector, has led to a strong reduction of antimicrobial use since 2007 (SDa 2023). One of the measures taken to achieve this was the central registration of all prescribed and delivered antimicrobials on farms, enabling monitoring of antimicrobial consumption and enforcement of prudent use of antimicrobials (Speksnijder et al. 2015). This also led to the development of alternative monitoring strategies, no longer primarily focusing on MRL compliance, but also on tracking down unaccounted antimicrobial use (Berendsen et al. 2013, 2015; Dréano et al. 2023). These novel approaches are based on matrices like feathers, hair, and manure, in which antimicrobial residues persist longer than in edible tissues. Using these 'archive matrices' has shown that antimicrobial treatment can be detected after prolonged periods of time (Berendsen et al. 2018; Chiesa et al. 2018).

A potential challenge when enforcing the reduction of antimicrobial use rather than MRL compliance is the possible transmission of residues between treated and untreated animals housed in the same pen or barn. In general, not much is known about the transmission of residues to matrices from treated to untreated animals using different animal keeping practices. Transmission of residues occurs when treated and untreated animals are kept in the same pen, either by direct contact or more indirectly through environmental contamination. For instance, transmission of enrofloxacin residues from treated to untreated animals was observed in the urine of untreated pigs within the same barn, even though animals were in separate pens (Janssen et al. 2022). Environmental contamination was observed in studies where antimicrobial residues have even been shown to occur in deposited and airborne dust from animal housing (Hamscher et al. 2003; Stahl et al. 2016), in which they can persist for prolonged periods of time (Schulz et al. 2019). It is also known that some antimicrobials are persistent in manure (Berendsen et al. 2018) and can be transferred *via* this route.

If transmission of antimicrobial residues among animals occurs, this might have consequences for the reuse of animal byproducts, even of the untreated animals. Besides affecting the antimicrobial residue status of untreated co-housed animals or the products thereof, treatment of individual animals in a herd or flock has also been shown to affect microbial susceptibility patterns in untreated animals. This could either be caused by transmission of residues from treated to untreated animals, generating a selective environment, or by transmission of resistant bacteria originating from treated animals (Beyer et al. 2015; Janssen et al. 2022).

With the aim to reduce transmission of antimicrobial residues, transmission between treated and untreated veal calves after oxytetracycline treatment was explored using a study design comprising two different animal keeping practices; one representing direct contact (housing in the same pen) and the other representing physical separation (in a separate compartment) of animals during the treatment and withdrawal period. Residue analysis was performed on manure, urine, blood (using dried blood spot (DBS)), hair and (animal and environmental) surface wipe samples. Additionally, *E.coli* isolated from the manure of the animals and environmental dust wipes were tested for antimicrobial susceptibility, to monitor the effect of the different animal keeping practices on AMR occurrence. To the best of our knowledge this is the first study extensively comparing the effects of animal keeping practices on residue transmission and AMR in veal calves.

Materials and methods

Controlled animal study

An animal study was conducted following ethical approval by the Animal Tests Committee (DEC). The calves had unlimited access to feed (silage with concentrates) and drinking water. To comply with animal welfare standards, at least two calves were placed together within a single pen. Bedding consisted of sawdust, which was replenished regularly, and each pen was equipped with a brush.

Experimental set-up

For this study, 12 male veal calves around 12 weeks of age, originating from Dutch dairy farms were used, with weights 90.3 – 116.9 kg. During the rearing stage prior to the experimental study, the calves did not receive any antimicrobial treatment and were kept separately from animals not destined for the study.

On arrival at the experimental farm (nine days before the start of the study), animals were randomly allocated over six pens, with two pens housing four animals and two pens housing two animals. For animal ethics reasons, at least two calves must be housed in one pen. The negative control group consisted of four animals, which also served as a backup for replacement in case of unforeseen health issues. The experimental set-up of the animal study is shown in Figure 1. Calves are identified by animal numbers (no.) 1–12. Three phases can be distinguished: phase 1 (day 1 – 6) the adaptation phase, phase 2 (day 7 – 20) the treatment phase, including the withdrawal period, and phase 3 (day 21 – day 50) the washout phase, comprising the time between the end of the withdrawal period until the day of slaughter.

In order to study the effects of the different animal keeping practices on the transmission of oxytetracycline (OTC) residues, the experimental design comprised four physically separated compartments in the same building. Each compartment had a sliding door to enter and pens were built from wooden plates at the bottom and waist-high fencing all around. Animals were housed in the pens either in pairs or quadruplets. Animals in compartment 1

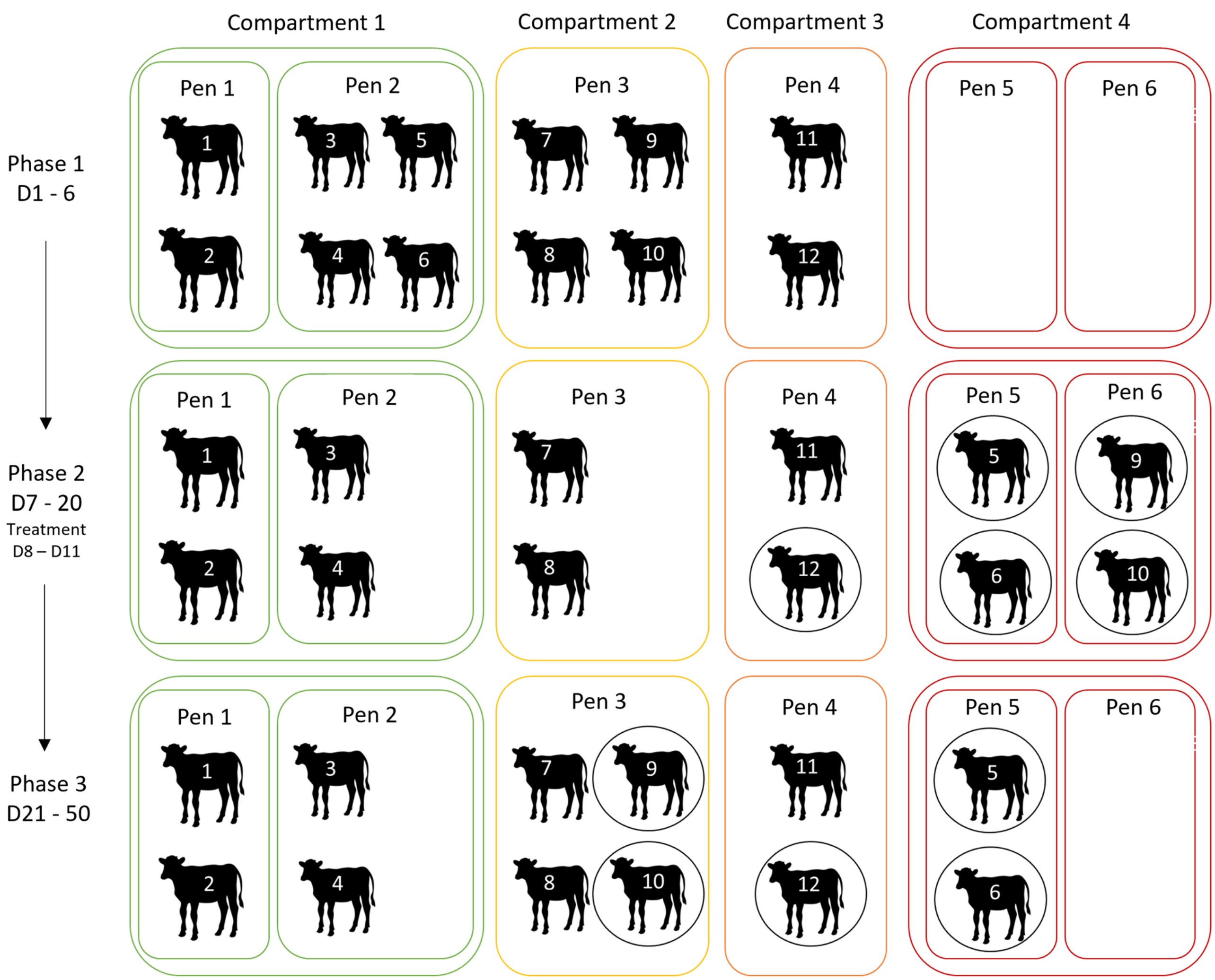

Figure 1. Schematic overview of the set-up and time path of the animal study. Animals in compartment 1 served as controls, compartment 2 allowed studying animal keeping practice 2 and compartment 3 for animal keeping practice 1. Compartment 4 was used for housing treated animals (positive controls). After the initial adjustment phase (Phase 1), treatment with oxytetracycline (day 8 – 11) was started in phase 2 (day 7 – 20). Treated animals are indicated with a circle; one animal in pen 4 and two animals from pen 2 and 3, after having been moved to compartment 4 (pen 5 and 6). After phase 2 (treatment + withdrawal period, day 8–20), the treated animals (pen 6) were reunited with their pen mates (pen 3) for the wash out period (Phase 3).

served as untreated and unexposed controls, compartment 2 allowed studying the effect of physical separation during treatment and withdrawal period (animal keeping practice 2), while compartment 3 was intended for studying the effects of treatment without physical separation (animal keeping practice 1). Compartment 4 was used for housing treated animals (positive controls).

After the adaptation phase, on day 7 (one day before the start of the treatment), calves 5 and 6, as well as calves 9 and 10, were moved from their initial pen (respectively 2 and 3), to compartment 4 (pen 5 and 6). Also, one of the calves in compartment 3 (calf 12) was selected for treatment. These five calves (indicated with a circle in Figure 1) were treated with oxytetracycline hydrochloride 1000 mg kg^{-1} (Dopharma REG NL 9617) starting at day 8. To ensure administration of the full dose, animals were bottle fed with a dose of 10 mg kg^{-1} at 12 h intervals during five consecutive days (day 8 – 12). After the withdrawal period of eight days (on day 21), calves 9 and 10 were moved back to their original pen 3, while calves 5 and 6 remained in compartment 4 until the end of the study period. All calves were sacrificed at day 50.

To prevent carryover of residues between different compartments caused by animal caretaking and sampling activities, fixed walking routes were set. All activities were carried out starting from the lowest contaminated area (compartment 1) to the highest contaminated area (compartment 4), and in case of pens housing treated as well as untreated animals (compartment 2 and 3), sampling started with the untreated animal, after which the treated animal was sampled. Furthermore, before entering a new compartment shoes and clothes were changed, and between each sampling, gloves were replaced.

Sampling

Samples were taken on day 1, 9, 12, 15, 18, 22, 29, 36, 43 and just before slaughter on day 50. Hair samples were obtained by shaving a surface of approximately 1 dm^2 on the upper part of the shoulder or leg of the calf with a regular hair clipper. The hair clipper was cleaned after every individual sample collection. At each sampling time point, an adjacent area was shaved. Hair samples were stored at −18 °C until analysis.

Manure samples were taken rectally and stored at −18 °C until residue analysis was performed. For each sampling time point except day 15, a separate aliquot of the manure sample was kept at 4–6 °C for microbial analysis.

Animal wipe samples were collected by wiping over the length of the back of the calf with a cotton pad wetted with 70% ethanol. The cotton pad was then immediately placed in a 50 mL PP tube (Greiner Bio-One, Alphen aan den Rijn, The Netherlands) and stored at −18 °C until analysis.

Urine was sampled at each time point by attaching a collection container to the calf in the morning, after the animals stood up. In most cases, urine could be collected immediately or within 30 min after placing the collecting vessel. The urine was then transferred into a sample jar and stored at −18 °C until analysis.

Blood was sampled by puncturing the ear of the calf and collecting the blood in a sterile tube. The blood was then immediately applied to Dried Blood Spot (DBS) AutoCollect™ cards (Ahlstrom, Helsinki, Finland), which were stored in separate bags at 4–6 °C until analysis.

Saliva was collected by letting the calf chew on an absorbent rope (Richmond Dental & Medical, Texas, USA) until it was saturated with saliva. The dental rope was then immediately transferred to a 50 mL PP tube, which was stored at −18 °C until analysis.

Environmental wipe samples for residue analysis were collected from each pen at two different locations; one from the fence at approximately 50 cm height from the ground and one from the inner side of the feeding trough, at a position where contact with the calf was expected. Additionally, a wipe sample was taken from the ventilation grid in each compartment. Samples were taken by wiping a surface of approximately 1 dm^2 with a cotton pad wetted with 70% ethanol. With respect to pen sampling, at each time point, a surface area adjacent to the previous sampling area was wiped, to allow for the determination of cumulative deposition of residues. For the ventilation grids, the same surface area was sampled for all time points. In addition, on day 1, 22 and 50, two surface samples of the pen (at a location opposite of the wipe for residue analysis) were taken with sterile wipes (Sterile cloth, SodiBox, Pont C'Hoat 29920 Nevez France) for the isolation of (resistant) *E. coli* bacteria.

Standards, solvents and reagents

A stock solution of oxytetracycline.$2H_2O$ (Sigma-Aldrich, Missouri, United States) was prepared at 1000 mg L^{-1} in methanol (MeOH, Actu-All Chemicals, Oss, The Netherlands). A stock solution of the labelled standard oxytetracycline-$^{13}C_{22}$-$^{15}N_2$ (Toronto Research Chemicals, Toronto, ON, Canada) was prepared by dissolving the entire product (2.5 µg) in 1 mL of MeOH, yielding a stock solution of 2.5 mg L^{-1}.

McIlvain-ethylenediaminetetraacetic acid (EDTA) buffer was prepared by dissolving 74.4 g EDTA disodium (Titriplex® III, VWR International, Darmstadt, Germany) into 500 mL of 0.1 M citric acid (citric acid monohydrate, VWR International) and 280 mL of 0.2 M phosphate buffer (disodium hydrogen phosphate dihydrate, VWR International). Then 1 L of MilliQ water was added whereafter the pH was adjusted to 4.0 (if necessary) by adding 0.1 M citric acid or 0.2 M phosphate buffer. Finally the total volume was adjusted to 2 L with MilliQ water.

Milli-Q water was prepared using a Milli-Q system with a resistivity of at least 18.2 MΩ.cm, (Merck Millipore). In all cases where water is mentioned, this refers to MilliQ water.

Acetonitrile ULC/MS grade (ACN) was also purchased at Actu-All Chemicals. Formic acid (FA) was purchased from VWR International and trifluoroacetic acid (TFA) was purchased at Sigma-Aldrich.

Sample analysis

After sample extraction and clean-up, chemical sample analysis was carried out by Ultra-High-Performance Liquid Chromatography – Triple Quadrupole Mass Spectrometry (UHPLC-MS/MS) analysis. Extraction, sample clean-up and analyses are described below per matrix.

Hair

The hair samples were 2 – 3 cm in length and 0.5 grams of the sampled hair was weighed into a 50 mL PP tube. The material was extracted 3 times with 10 mL of 0.125% TFA in methanol using a head-over-head apparatus (Heidolph REAX-2, Schwabach, Germany). The extracts were combined and a 3 mL aliquot was diluted with 27 mL of McIlvain EDTA buffer. Sample clean-up was done using solid phase extraction (SPE). A reversed phase 6 mL, 200 mg SPE cartridge (Strata-X, Phenomenex, Torrance, CA, USA) was sequentially conditioned with 5 mL MeOH and 5 mL McIlvain-EDTA buffer. Then the entire extract was brought onto the cartridge. After rinsing with 5 mL of water, extracts were eluted with 5 mL of MeOH. The MeOH was evaporated under a gentle flow of nitrogen and samples were reconstituted in 500 µL of methanol/water (40/60% V/V%).

An aliquot of 5 µL extract was injected into a Shimadzu Nexera UHPLC system (Shimadzu Corporation, Kyoto, Japan) coupled to a QTRAP 6500 LC-MS/MS system (Sciex, Framingham, Massachusetts, USA). Chromatographic separation for all matrices except manure was achieved using an Acquity HSS-T3 C18 analytical column of 2.1 × 100 mm, 1.8 µm (Waters Corporation, Milford, Massachusetts) placed in a column oven at 40 °C. The mobile phase consisted of 2 mM ammonium formate and 0.016% FA in water (Solvent A) and 2 mM ammonium formate and 0.016% FA in MeOH (Solvent B). The gradient was: 0–1.0 min, 0% mobile phase B, 1.0–2.5 min, linear increase to 25% B, 2.5–5.4 linear increase to 70% B, and 5.4–5.5 min linear increase to 100% with a final hold of 1.0 min. The gradient is returned to its initial conditions within 0.1 min and equilibrates for 0.9 min before the next injection is initiated, resulting in a total run time of 7.5 min. The flow rate was 0.4 mL min^{-1}.

Detection was carried out using positive electrospray ionisation (ESI) mode. The operating parameters of the QTRAP 6500 LC-MS/MS system were the following: the Turbo Spray IonDrive operated with an IonSpray Voltage of 5500 and a temperature of 500 °C. The general collision gas was set at 'medium', the ion source gasses to 60, the entrance potential to 10 and the curtain gas to 35. Fragmentation was carried out by collision-induced dissociation (CID). The SRM transitions were; for OTC m/z 461.0 to 426.0 and 154.0 and for OTC-$^{13}C_{22}$-$^{15}N_2$ m/z 485.2 to 449.2. The settings for compound specific parameters declustering potential (DP), collision energy (CE) and Collision Cell Exit Potential (CXP) were: DP of 61 V for all transitions, CE of 27 V for all transitions, CXP of 12 V for OTC *m/z* 461.0 to 426.0, of 37 V for OTC *m/z* 461.0 to 154.0 and of 24 V for OTC-$^{13}C_{22}$-$^{15}N_2$ *m/z* 485.2 to 449.2. The detection limit for OTC in hair that can be achieved with this method was 10 μg kg^{-1}.

Manure

Manure samples were prepared according to Jansen et al. (2019); 2 g of manure was extracted with 4 mL of 0.125% TFA in acetonitrile and subsequently 4 mL of McIlvain EDTA buffer using a head-over-head apparatus. After adding 2 mL of lead acetate solution (200 g/L), samples were centrifuged, and the acetonitrile was evaporated under a nitrogen stream at 40 °C. After evaporation of the acetonitrile, 13 mL of 0.2 M EDTA solution was added to the remaining extract. Sample clean-up was then done using the SPE method as described for hair. The samples were reconstituted in 500 μL of methanol/water (20/80 V/V%).

Aliquots of 5 μL extract were injected into the UHPLC-MS/MS system as described for hair, using the same MS/MS conditions and mobile phases. Chromatographic separation for manure was carried out using a Kinetex column, 1.7 μ C18 100 A, 100*2.1 mm (Phenomenex) placed in a column oven at 40 °C. The gradient for manure extracts started slightly different, to avoid matrix effects: 0-0.5 min, 1% mobile phase B, 0.5–2.5 min, linear increase to 25% B and continued the same as for hair. To avoid matrix-specific interferences, the flow rate for manure was 0.3 mL min^{-1}. The detection limit that can be achieved for OTC in manure is 12.5 μg kg^{-1}.

Animal wipe samples

Wipe samples, already enclosed in a 50 mL PP tube during sampling, were extracted using 12 mL of McIlvain EDTA buffer. The wipes used as blanks were dry and therefore extracted using 16 mL of McIlvain EDTA buffer. Samples were extracted during 15 min using a head-over-head apparatus, ultrasonicated during 10 min and centrifuged for 10 min at 3500 × g. Clean-up was done using SPE as described for hair. After evaporation, samples were reconstituted in 500 μL methanol/water (20/80 V/V%).

Aliquots of 5 μL of the extract were injected into the UHPLC-MS/MS system as described for hair, using the same chromatographic and MS/MS conditions. The detection limit that can be achieved for OTC in wipes with this method is 5 ng per wipe.

Urine

After mixing thoroughly, 1 mL of urine was pipetted into a 12 mL PP tube. Samples were extracted with 2 mL of McIlvain EDTA buffer using a head-over-head apparatus. After adding the buffer, the pH was checked and, if necessary, adjusted to pH 4.0 using 37% hydrochloric acid. Sample clean-up was done using SPE as described for hair and finally samples were reconstituted in 1 mL of methanol/water (20/80% V/V%).

Aliquots of 5 μL extract were injected into an Acquity UPLC System (Waters Corporation, Milford, Massachusetts) coupled to a Xevo TQ-S MS/MS (Waters) system. The chromatographic conditions and flow rate remained the same as described for the UHPLC system used for hair. Detection was carried out using positive electrospray ionisation (ESI) mode. The operating parameters of the Xevo TQ-S system were: capillary voltage 3.0 kV, cone voltage 25 V, desolvation temperature 550 °C, desolvation gas 650 L $hour^{-1}$, source temperature 130 °C and the gain value was set to 0.5. The SRM transitions were; for OTC *m/z* 460.9 to 426.0 and 282.9 and for OTC-$^{13}C_{22}$-$^{15}N_2$ *m/z* 485.2 to 449.0. The compound specific parameter CE was respectively set to 18 V, 16 V and 18 V. The detection limit that could be achieved for OTC in urine using this method was 10 μg L^{-1}.

Dried blood spots

Analysis of blood was performed automatically using a Dried Blood Spot autosampler™ (Spark Holland, Emmen, The Netherlands) coupled to an in-line SPE module (Spark Holland) and LC-MS/MS (Spark Holland LC pump and Waters Xevo TQ-S MS/MS system).

One DBS card (Ahlstrom Autocollect™) was used for each animal. This card was placed in the DBS autosampler system. The sample spot is desorbed at a temperature of 50 °C with 2 mL of 0.1% formic acid containing internal standard solution at a concentration of 250 µg L^{-1}. This desorption fluid is then trapped on a pre-conditioned in-line SPE cartridge. The $CHRO_{SPE}$ C18HD 10 x 1 mm ID cartridge (Axel Semrau, Sprockhövel, Germany) was pre-conditioned (before trapping the desorption fluid) with sequentially 1 mL of methanol and 1 mL of 0.1% formic acid solution in water. After trapping the desorption fluid, the cartridge was rinsed using 250 µL of 1% TFA in water and subsequently 500 µL of water. The cartridge is eluted using the UHPLC gradient, with 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). The gradient was: 0–1.0 min, 0% mobile phase B, 1.0–2.5 min, linear increase to 25% B, 2.5–5.4 linear increase to 70% B, and 5.4–5.5 min linear increase to 100% with a final hold of 3.0 min. The gradient is returned to its initial conditions within 0.1 min and equilibrates for 3.4 min before the next SPE-elution is initiated, resulting in a total run time of 12 min. The flow rate was 0.4 mL min^{-1}. The SPE eluate was brought directly onto the analytical column, an Acquity UPLC BEH C18 2.1 x 100 mm, 1.7 µm (Waters), and into the MS/MS-system. The MS/MS detection parameters were as described for urine. The limit of detection for OTC with this method was 12.5 µg L^{-1}.

Saliva

Saliva was retrieved from the absorbent rope by centrifuging for 10 min at 4000 g. A volume of 100 µL saliva was transferred into a 12 mL PP tube and extracted by adding 250 µL of ACN and vortexing for 30 s. The samples were centrifuged during 10 min at 10,000 × g. The supernatant was transferred into a 1.5 mL vial and evaporated (N_2, 40 °C). Samples were reconstituted in methanol/water (10/90% V/V%).

Five µL of the extracts was injected into the UHPLC-MS/MS system as described for hair, using the same chromatographic and MS/MS conditions. Due to the fast depletion that the first measurements showed, saliva sampling was performed until day 22 (the start of phase 3). The detection limit that can be achieved for OTC in saliva with this method was 10 µg kg^{-1}.

Data processing and calculations

Processing of the UHPLC-MS/MS data was done using MultiQuant Software (Sciex) or QuanLynx Software (Waters), depending on the type of system used. Samples were all quantified using a calibration curve prepared in matrix by adding a standard solution of OTC. In addition, OTC-$^{13}C_{22}{}^{15}N_2$ (Biopure, Massachusetts, United States) was used as an internal standard to correct for variations in sample preparation and LC-MS/MS analysis.

With exception of DBS and wipes, the samples taken on June 8 (day 1) were used as blank material for the calibration line, as no treatment had taken place yet at this time, and no residues were detected in these samples. For DBS, blood of calf no. 1 was taken during slaughter to be used as blank. A large range of concentrations was used for the matrix matched calibration curves; for hair 10–5000 µg kg^{-1}, for manure 12.5–2000 µg kg^{-1}, for wipes 5 –1000 ng per wipe, for urine 10 – 10000 µg L^{-1}, for DBS 12.5–1250 µg L^{-1} and for saliva 10–2500 µg L^{-1}. Concentrations above the highest calibration point were extrapolated.

Isolation and susceptibility testing of E. coli

The isolation of *E. coli* from manure and surface wipes was initiated within 48 h after sampling. One gram of manure was mixed with 9 mL buffered peptone water (BPW, Biotrading), while wipes were submerged in 225 mL of BPW. Samples were incubated for 18–22 h at 37 °C. A loop full of material (approximately 10 µl) was transferred onto Tryptone Bile X-glucuronide (TBX, Biotrading) chromogenic agar plates and incubated for another 18–22 h at 44 °C. Typical colonies (blue/green appearance) were streaked on Columbia Blood Agar (CBA, Biotrading), incubated for 20–24 h at 37 °C, and analysed with MALDI-TOF (Biotyper, Bruker) for species confirmation. One *E. coli* isolate per sample was subjected to antimicrobial susceptibility testing (AST), which was performed by broth microdilution according to ISO 20776-1:2020 using commercial Sensititre EUVSEC plates (Thermo Fisher Scientific). See (Supplementary information Table S1) for the antimicrobials, their concentration ranges, and the applied cut-off values for resistance.

Results and discussion

During the study, all animals except one (no. 9) maintained good health (no abnormal conditions or clinical symptoms were reported). Calf no. 9 displayed signs of abdominal breathing in the period before treatment. The animal was selected for oxytetracycline treatment and was additionally monitored during the study by measuring its temperature (which decreased from 39.2 to 38.5 °C), and by checking its appetite, which did not reduce. No further complications occurred during the animal study.

Control samples

In this study, the transmission of OTC residues to different matrices of untreated animals was examined after treatment of animals in animal keeping practice 1 (calves kept together in a pen) and animal keeping practice 2 (calves kept separated during treatment and withdrawal period). Additionally, OTC residues were analysed in the matrices of four blank control animals and two positive control animals. Figure 2 shows the average concentrations of OTC residues in the negative control calves (no. 1–4) and the positive control calves (no. 5 and 6) separated into internal (manure, urine, blood and saliva) and external (hair, wipes) samples.

The negative control samples of the internal samples remained blank for the entire duration of the study. For DBS, for one sample (calf no. 3 on day 43) no concentration could be determined. For the external samples, incidentally, relatively low amounts (< 50 ng per wipe/<20 µg kg^{-1} hair) of residues were found in wipes of the blank animals (on day 18 and day 36) and (on day 29) also in hair. These results are unexpected, but are low and therefore distinguishable from positive samples at the same time points.

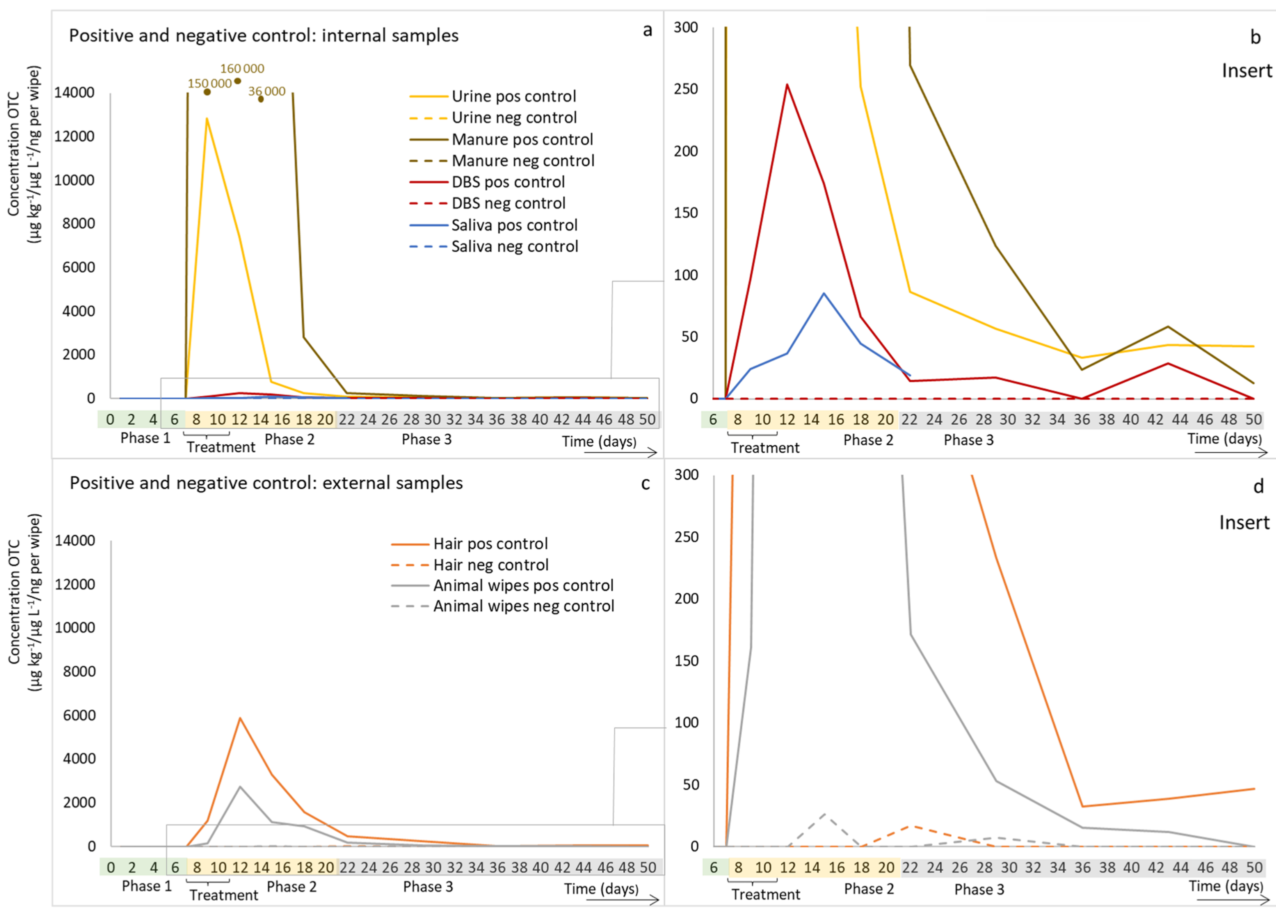

Figure 2. Average oxytetracycline concentration in the analysed matrices on the different sampling time points of calves of the positive control (no. 5 and 6, continuous line), and the calves serving as negative control (no. 1 – 4, dashed line). The insert b and d are added to demonstrate the lower concentrations in the indicated range.

Occurrence of OTC residues in calves of animal keeping practice 1

The aim of the study was to explore the effects of physical separation on the transmission of oxytetracycline residues and occurrence of antimicrobial resistance. Animal keeping practice 1 represents the situation in which the treated (no. 12) and untreated calf (no. 11) were not physically separated during treatment (compartment 3/pen 4 in Figure 1). Figure 3 shows the concentrations of OTC residues detected in the different types of samples over time.

High concentrations of OTC (up to 300 mg kg^{-1}) were found in the manure of the treated animal (no. 12) after the start of the treatment. Also, the urine of this animal contained considerable levels of OTC (up to 13 mg kg^{-1}). The transmission of OTC residues immediately becomes apparent from the high concentration of OTC in the manure of the untreated animal (no. 11) at days 9 and 12 (approximately 5% of the OTC level found in the treated animal). Also, substantial concentrations of OTC could be observed in the urine of this animal (approximately 5% of the OTC level in the treated animal). OTC was only detected at significant levels (0.88 mg kg^{-1}) in the saliva of the treated animal during the actual treatment. Because residue concentrations in saliva were already close to, or diminished below, the detection limit prior to day 22, sample collection was terminated after this time point. Nevertheless, transmission to the untreated calf was observed for saliva as well (approximately 4% of the peak at day 9).

Also, OTC levels in the blood of the treated animal were low (0.33 mg kg^{-1}), and transmission was seen to the blood of the untreated animal (approximately 4% of the peak at day 9). Residue concentrations in blood are usually represented by plasma or serum, because whole blood in itself is hard to keep or analyse. DBS is a promising monitoring tool which enables the sampling, storing and analysis of whole blood. However, compared to OTC levels in plasma reported in literature, the levels measured in blood by DBS analysis are much lower (Meijer et al. 1993). The results nevertheless show that this type of sampling is a feasible approach for showing the presence of OTC residues during treatment and withdrawal.

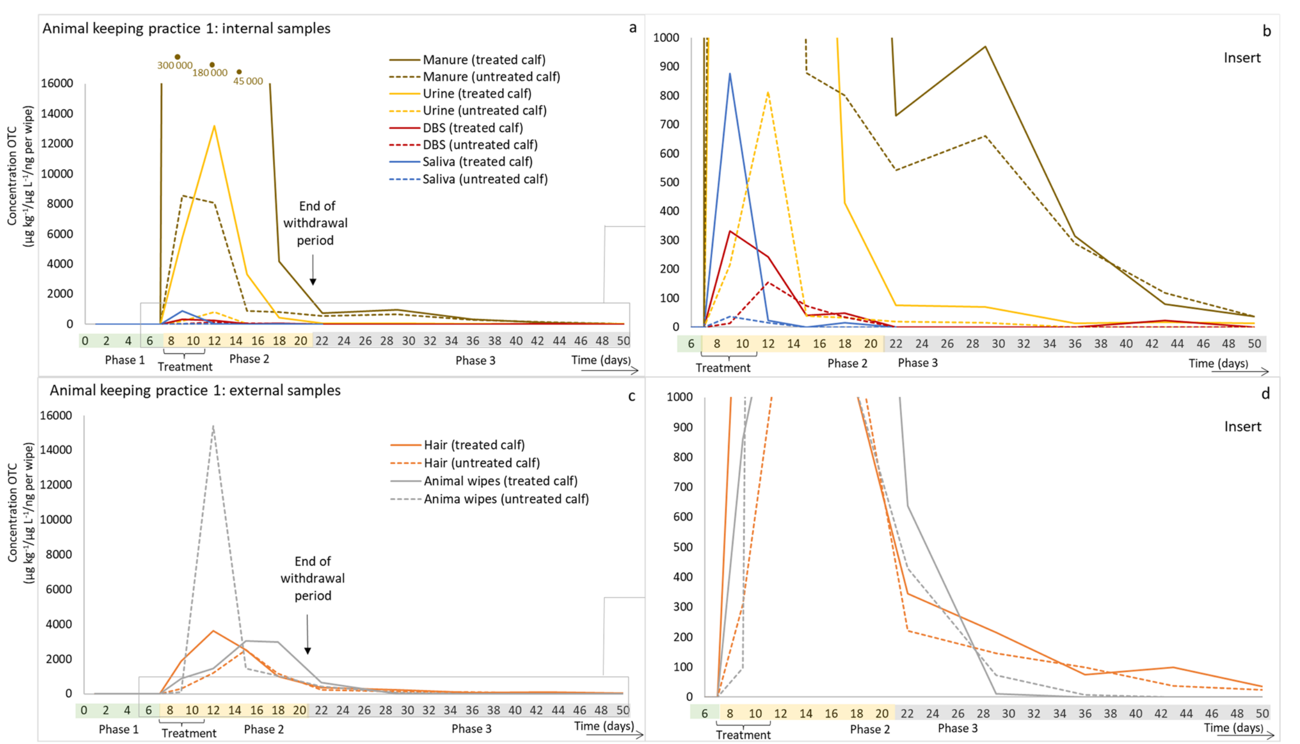

Figure 3. Oxytetracycline concentration in the internal (panel a and insert b) and external (panel c and insert d) samples of treated (no. 12, continuous line) and untreated (no. 11, dashed line) calves on different sampling time points in animal keeping practice 1. The inserts b and d are added to provide a better view at the lower concentration range.

For the internal samples, there is a clear distinction between treated and untreated up to the end of the withdrawal period (day 22) for manure and urine. After that period, the residue concentrations start to overlap. For the other internal matrices, saliva and blood this already takes place at day 15.

In addition, the external samples show significant levels of OTC residues. A remarkably high concentration was observed for the wipe of the untreated animal at day 12. Possibly this is the result of a local peak concentration originating from intensive recent contact with the treated penmate or excreta thereof. OTC concentrations in the hair samples of the treated and untreated animals were found to be similar. Concentrations rapidly decrease during the withdrawal period; however detectable concentrations remain in the hair samples of both the treated and untreated animal up to the day of slaughter.

In conclusion, OTC residues were found in the untreated animal in all matrices (internal and external), showing extensive transmission of residues from the treated animal to its pen mate.

Occurrence of OTC residues in calves of animal keeping practice 2

Animal keeping practice 2 represents the situation in which the treated animals (no. 9 and 10) were physically separated from the non-treated pen mates (no. 7 and 8) by moving them to compartment 4; pen 6 (Figure 1) during the treatment and withdrawal period, and moving them back to their original pen 2 in compartment 2 afterwards. Figure 4 shows the average ($n=2$) concentrations of OTC residues detected in the different types of internal and external samples over time.

The depletion of residues in the treated calves in animal keeping practice 2 was comparable to the treated calves in animal keeping practice 1 for all internal matrices (Figure 4a). Since the untreated calves were not in contact with the treated calves, no residues were detected in the internal samples taken from them during phase 2. After reunion (start of phase 3), the manure and urine of the treated calves contained respectively 230 µg kg^{-1} and 140 µg kg^{-1} OTC, while their saliva and blood concentrations had dropped below 50 µg kg^{-1} (Figure 4b). No OTC residues were detected in the

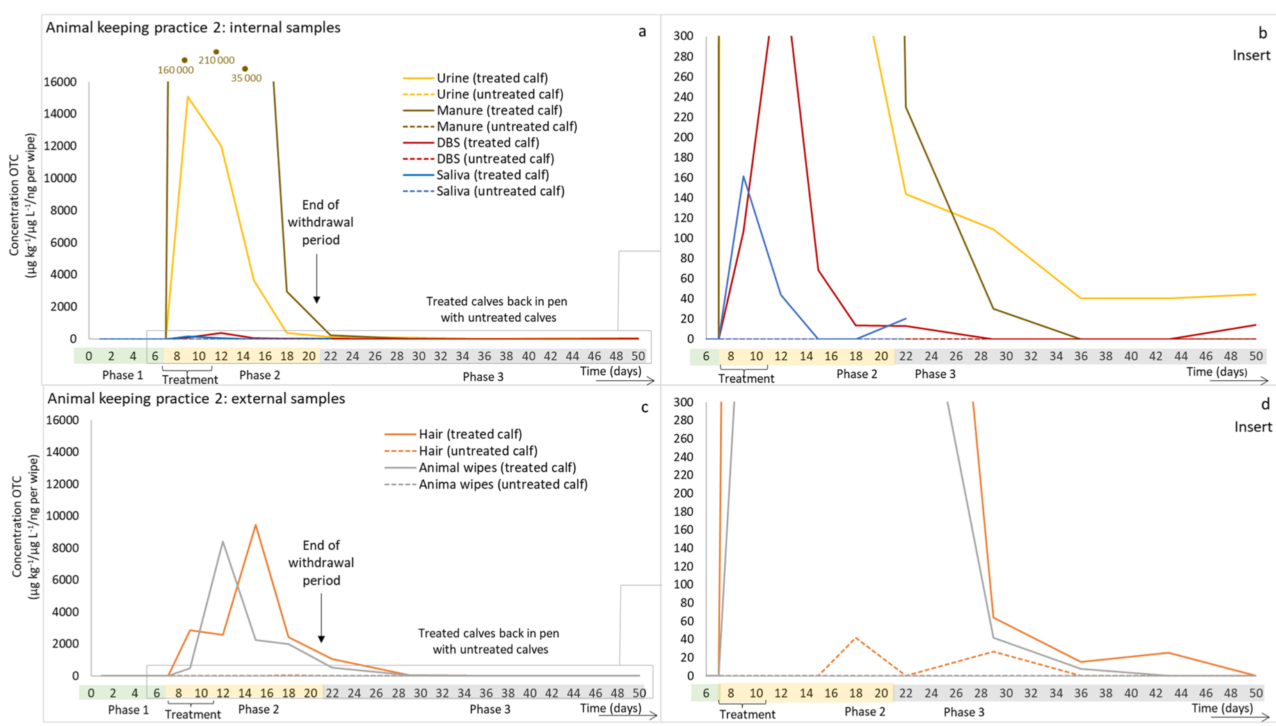

Figure 4. Average oxytetracycline concentration in the internal (panel a and insert b) and external (panel c and insert d) matrices on the different sampling time points of treated (no. 9 and 10, continuous line) and untreated (no. 7 and 8, dashed line) calves in animal keeping practice 2. The inserts b and d are added to demonstrate the lower concentrations in the indicated range.

internal matrices of the untreated calves. The physical separation of the calves during treatment and the withdrawal period appears to be effective to prevent (detectable) uptake of residues in internal matrices of the untreated pen mate.

The external matrices hair and animal wipes are successful in detecting use after a longer period of time as also seen in animal keeping practice 1. However, it should be noted that the depletion pattern for the external matrices shows a larger variation in concentrations between the different calves (Figure 4c), which is to be expected due to the inhomogeneous distribution of residues caused by external factors (e.g. wipe surface, contact with the other calf, manure, barn dust). The hair and animal wipes of the treated animals of animal keeping practice 2 still contained on average, respectively 1100 μg kg^{-1} and 530 ng per wipe of OTC after reunion at the beginning of phase 3 (day 22). Measurable concentrations were observed in the hair sample of one of the untreated animals at day 29. However, in the same calf, residues were also detected during phase 2 at day 18, raising the question whether the residues found at day 29 are the result of transmission. No residues could be detected in the hair of the other untreated calf. Since the wipes and hair of treated animals still contained relatively high concentrations of residues, the transmission to one of the untreated calves due to physical contact with a 'hot spot' (an area containing a high concentration of residues) cannot be ruled out based on these results. Nevertheless, no residues were detected above the detection limit in the animal wipes of the untreated calves.

In conclusion, no residues were detected in internal matrices of the untreated animals, indicating that transmission can be effectively prevented. Contamination of the pen and the exterior of the animals is also drastically reduced by the separation of animals during treatment and the withdrawal period.

Occurrence of OTC residues in the environment

Environmental transmission to the compartments was analysed by taking wipe samples from the pens and exhaust ventilation grids. Results are shown in Figure 5. Environmental contamination was found to be extensive in the compartments holding the calves for treatment (compartment 3 and 4), but shows a steady decrease after the end of the treatment. Although both compartment 3 and 4 are used for treatment, the amount of OTC found in the environmental wipes varies. Considerable variation is also observed between the duplicate samples at some time points (compartment 3 at day 9 and day 12). This can be explained by the fact that transmission will not be distributed homogeneously, as it is mostly results from contact with the calf itself or its faeces or urine. Also, these environmental samples show that the physical separation of animals during treatment and the withdrawal period is an effective strategy to drastically reduce the occurrence of residues not only in the untreated animals but also in the entire compartment. The detection of low concentrations of OTC residues on untreated calves (Figure 2, d) and the pens in compartment 1 (Figure 5, b) shows that, despite the strict precautionary measures, transmission of residues could not be totally prevented.

The results of this study show that after treatment with antimicrobials, there is transmission of antimicrobial residues to the barn environment and untreated calves housed in this environment. Other research has shown that antimicrobial residues can persist in regular keeping practices and pile up in barn dust (Hamscher et al. 2003; Schulz et al. 2016) and can be considered a large reservoir for antimicrobial resistance genes (Luiken et al. 2022). This transmission adds to the many known pathways in which humans can be exposed to antimicrobial residues and antimicrobial resistance genes (Khalifa et al. 2024). The physical separation of animals for treatment enables transmission to take place in a more contained environment, whereafter thorough cleaning should be performed. Additionally, having a separate contaminated area can enhance awareness about possible exposure risks.

Susceptibility of E. coli isolates

Tetracycline susceptibility of the *E. coli* isolated from the manure of the calves and the surface wipes (one isolate per sample) is shown in Table

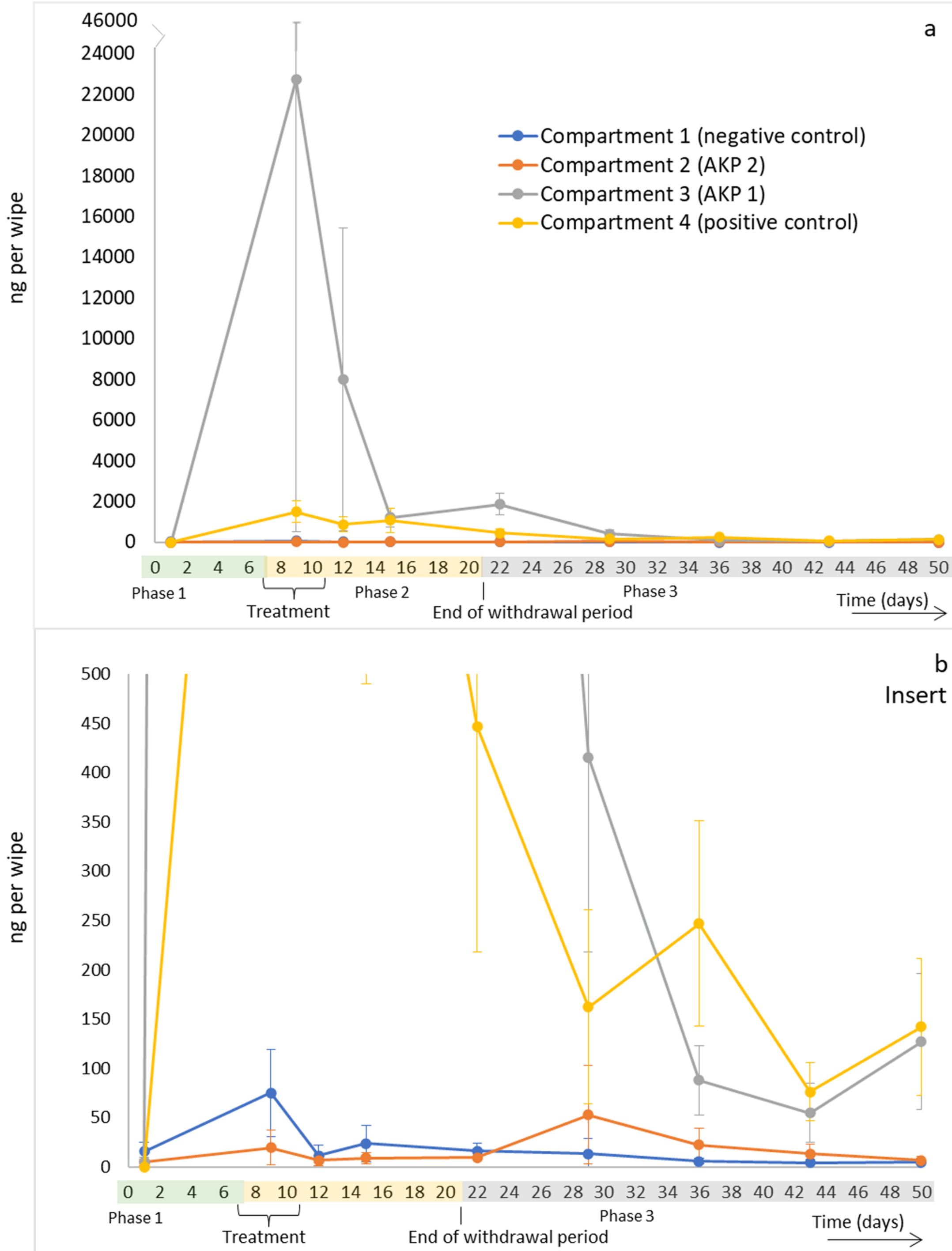

Figure 5. Average oxytetracycline amount (ng per wipe) in the environmental wipes ($n = 2$) from the pens and the ventilation grids in the different compartments, where AKP is animal keeping practice.

1. Overall, 31 out of 108 (29%) of the *E. coli* isolated from manure and 7 out of 26 (37%) of the *E. coli* isolated from surface wipes were found to be tetracycline resistant.

As expected, only tetracycline-resistant resistant *E.coli* isolates were found in manure samples taken during treatment (5 out of 5 on both day 9 and 12) and also during the withdrawal period (on day 18) 4 out of 5 isolates showed a resistant phenotype. Selective pressure of the administered OTC appears to decrease fairly soon upon termination of the treatment, since on day 22, the isolates from 3 out of 5 treated animals show a susceptible phenotype again. On the other hand, at this time point all of the surface wipes from the pens in which treatment took place still yielded resistant isolates, while on the final day of the animal trial only susceptible isolates were retrieved from the pens surface. Since only one representative *E. coli* isolate per animal per sampling point was analysed for antimicrobial susceptibility, these results only provide an indicative representation of the prevalence of tetracycline resistance, precluding any statistically substantiated conclusions.

It has to be noted that a considerable occurrence of tetracycline-resistant *E. coli* isolates is observed in the untreated/unexposed calves (no. 1–4, Table 1) during the animal trial. Although it cannot be excluded that this is caused by some unaccountable carry-over event, it is more likely to represent a background level of tetracycline resistance present in the gut microbiome of the calves. This is supported by the fact that one of the isolates obtained prior to the treatment period (calf 3) already showed a tetracycline-resistant phenotype. Moreover, the antimicrobial resistance profiles of the tetracycline-resistant isolates showed high diversity (Table 2), which is unlikely to have arisen from oxytetracycline treatment only. Many of the resistant *E. coli* isolates showed a multidrug resistant phenotype. The observed types of resistance are in line with what is commonly found in Dutch veal calves (MARAN 2023). Only one of the tetracycline susceptible isolates was found to display a non-pansusceptible phenotype. This concerned an ampicillin resistant isolate retrieved from the surface wipe of pen 3 at the start of the animal trial.

Several risk factors for AMR prevalence in pre-weaned calves have been identified. Obviously, the treatment of calves is an important risk factor (Jarrige et al. 2020; Formenti et al. 2021), but feeding with (waste) milk from cows treated with antimicrobials was also identified as a risk factor in several studies (Maynou et al. 2017; Jarrige et al.

Table 2. Resistance profiles of the tetracycline resistant isolates. Resistance was observed to tetracycline (TET), sulfamethoxazole (SMX), trimethoprim (TMP), chloramphenicol (CHL), ampicillin (AMP), azithromycin (AZI), ceftazidime (TAZ), gentamicin (GEN) and ciprofloxacin (CIP). Between brackets is the number of different calves in which the specific (multi)drug resistant phenotype was observed.

Resistance profile	number of isolates (number of individual calves)
TET	7 (4)
TET-SMX	5 (2)
TET-SMX-TMP	5 (3)
TET-SMX-CHL	3 (3)
TET-SMX-AMP	2 (2)
TET-SMX-CHL-AMP	3 (3)
TET-SMX-CHL-TMP	2 (2)
TET-SMX-TMP-AZI	1 (1)
TET-SMX-TMP-TAZ	1 (1)
TET-SMX-TMP-CHL-GEN	1 (1)
TET-TMP-CHL-AMP-CIP	1 (1)

Table 1. Tetracycline susceptibility of the *E. coli* isolated from manure and surface wipes. Values represent the Minimum Inhibitory Concentration (MIC (µg mL^{-1})) observed for tetracycline. Isolates with an MIC >8 are considered resistant (indicated in bold). Behind the calf number, negative controls (neg), animal keeping practice (AKP) 1 and 2 and positive controls (pos) are indicated. For the compartments (comp.), wipes taken n=1 for day 1 and n=2 for day 22 and 50. Treatment took place in compartments 3 and 4 on calves no. 5, 6, 9, 10 and 12.

	Day 1	Day 9	Day 12	Day 18	Day 22		Day 29	Day 36	Day 43	Day 50	
Animal											
Calf 1 (neg)	<= 2	<= 2	<= 2	<= 2	<= 2		<= 2	<= 2	<= 2	= 4	
Calf 2 (neg)	<= 2	**= 64**	<= 2	<= 2	<= 2		= 4	<= 2	= 4	= 4	
Calf 3 (neg)	**= 64**	<= 2	**= 64**	<= 2	**= 64**		**> 64**	<= 2	<= 2	<= 2	
Calf 4 (neg)	<= 2	<= 2	= 4	<= 2	<= 2		**> 64**	<= 2	**> 64**	<= 2	
Calf 5 (pos)	<= 2	**> 64**	**> 64**	<= 2	<= 2		<= 2	<= 2	<= 2	<= 2	
Calf 6 (pos)	<= 2	**> 64**	**> 64**	**> 64**	= 4		<= 2	<= 2	<= 2	**> 64**	
Calf 7 (AKP 2)	<= 2	<= 2	<= 2	<= 2	<= 2		<= 2	<= 2	<= 2	<= 2	
Calf 8 (AKP 2)	<= 2	<= 2	**> 64**	**> 64**	<= 2		**> 64**	<= 2	<= 2	<= 2	
Calf 9 (AKP 2)	<= 2	**= 64**	**> 64**	**= 32**	**> 64**		<= 2	<= 2	<= 2	<= 2	
Calf 10 (AKP 2)	<= 2	**> 64**	**> 64**	**> 64**	**= 64**		= 4	<= 2	<= 2	= 4	
Calf 11 (AKP 1)	<= 2	**> 64**	**= 64**	**> 64**	<= 2		<= 2	**> 64**	<= 2	<= 2	
Calf 12 (AKP 1)	<= 2	**= 64**	**= 64**	**> 64**	<= 2		<= 2	<= 2	= 4	= 4	
Compartment											
Comp. 1, pen 1 (neg)	<= 2	–	–	–	**> 64**	<= 2	–	–	–	<= 2	<= 2
Comp. 1, pen 2 (neg)	= 4	–	–	–	<= 2	<= 2	–	–	–	<= 2	<= 2
Comp. 2, pen 3 (AKP2)	<= 2	–	–	–	<= 2	= 4	–	–	–	<= 2	<= 2
Comp. 3, pen 4 (AKP1)	<= 2	–	–	–	**= 64**	**= 64**	–	–	–	<= 2	<= 2
Comp. 4, pen 5 (pos)	–	–	–	–	**= 16**	**> 64**	–	–	–	<= 2	<= 2
Comp. 4, pen 6 (pos)	–	–	–	–	**> 64**	**> 64**	–	–	–	–	–

2020; Weber et al. 2021). It was also suggested that the environment is an important source of colonisation (Formenti et al. 2021), as well as general health/fitness of the animals and quality of farm hygiene (Weber et al. 2021). As a consequence, exposure to selective antimicrobial concentrations during treatment is likely to result in a rapid proliferation of these pre-existing bacterial clones. Since the genes coding for AMR are often located on plasmids, this will cause the collateral dissemination (and maintenance) of other types of AMR, which can explain the prevalence of AMR to classes of antimicrobials rarely (or not at all) used for treatment of veal calves (amphenicols, fluoroquinolones, cefalosporins) (MARAN 2023). The fact that the number of *E. coli* analysed in this study was limited to one representative isolate per sample, combined with a considerable background resistance level, precludes the drawing of definite conclusions with respect to the effects of the different management regimes on AMR prevalence. Overall, however, results seem to indicate that the tetracycline resistance level returns more or less to pre-treatment occurrence after the withdrawal period.

Conclusions

In conclusion, physical separation of animals during treatment and the withdrawal period is an effective measure to minimise the transfer of residues to untreated animals and the environment. It successfully prevents the transmission of (measurable) residue concentrations to internal matrices like manure, urine, blood and saliva. Moreover, it minimises the transmission of residues to external matrices, hair and animal wipes. This prevention and reduction of the transmission of residues in a barn environment will help to improve the animal production system.

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Authors' contribution

Larissa J.M. Jansen: Writing – original draft, Formal analysis, Visualisation, Resources; Mariël G. Pikkemaat: Writing – original draft, Visualisation; Michel Rapallini: Writing – review & editing, Investigation, Formal analysis, Visualisation; Maura Arends: Investigation, Formal analysis; Laura L.M. van Asseldonk: Investigation, Formal analysis; Robbert van den Beld: Investigation, Formal analysis; Ron J. Berentsen: Investigation, Formal analysis; Coen V.M. Nibbeling: Investigation, Formal analysis; Michael G. Schoeman: Investigation, Formal analysis; Stanislava Hoijtink-Vonsovic: Investigation, Formal analysis; Bjorn J.A. Berendsen: Writing – review & editing, Conceptualisation, Project administration, Supervision, Resources; Milou G.M. van de Schans: Writing – review & editing, Conceptualisation, Funding acquisition, Project administration, Supervision, Resources

Disclosure statement

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