Administration of chlortetracycline to beef cattle: evaluation of antimicrobial activity, resistance selection, duration of detection, and efficacy

by

Alyssa Rae Toillion

B.S., University of Nebraska at Lincoln, 2013
MPH, Kansas State University, 2017

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine/Pathobiology
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Abstract

Chlortetracycline (CTC) is one of the most widely used antimicrobials in food-animal production. Approved routes of administration for CTC in cattle are through the feed or water. This dissertation evaluates the antimicrobial activity, resistance selection, duration of detection, and efficacy of chlortetracycline administered to beef cattle.

A systematic review was conducted to determine the effect of in-feed tetracyclines on the antimicrobial resistance of enteric bacteria in cattle. Results of the review demonstrated that regardless of antimicrobial exposure, tetracycline resistant *E. coli* were prevalent in bovine feces. Additionally, environmental factors play a major role in harboring and disseminating antimicrobial-resistance genes. In the second study, the susceptibly profile of *E. coli* isolates was investigated in anaplasmosis-endemic pastured cattle herds provided four different free-choice formulations (700, 5000, 6000, 8000 g/ton) of CTC-medicated mineral for five consecutive months to control anaplasmosis. Most animals, pre- and post- CTC exposure, harbored *E. coli* isolates with CTC MIC values > 4 µg/ml, falling in the CSLI categories of intermediate or resistant. No pattern or clear development of resistance was observed in *E. coli* isolated from CTC-treated cattle.

The third study explored the antimicrobial activity of CTC and its transformation products (ECTC and ICTC) against enteric and environmental bacteria via microbroth dilution. Resistance associated with CTC has been extensively researched, but little has been reported on resistance associated with its transformation products 4-epi-CTC (ECTC) and iso-CTC (ICTC). Upon examination our study reported antimicrobial activity of chlortetracycline in *Bacillus subtilis, Campylobacter jejuni, Pseudomonas aeruginosa, Clostridium perfringens,* and

Staphylococcus aureus. In contrast, 4-epi-CTC, displayed antimicrobial activity against *B. subtilis* and *C. jejuni*. No activity was displayed for iso-CTC in any of the bacteria tested.

The fourth study investigated plasma and urine elimination characteristics of three regimens (Group A: 22 mg/kg of bodyweight daily, Group B: 350 mg/head per day, and Group C: 70 mg/head per day) of chlortetracycline administered through the feed to beef cattle. Mean ± standard deviation values for plasma T ½ (for the period up to 6 days following the last day of administration) for groups A, B, and C were 1.1 ± 1.5, 1.3 ± 1.4, and 1.3 ± 1.3 days, respectively. Terminal urine T ½ values (from 23 to 79 days post administration) were 35.4 ± 1.5, 51.7 ± 1.9, and 64.7 ± 1.9 days for groups A, B, and C, respectively. The persistence of CTC in the urine and the long-reported urine half-lives suggest chlortetracycline remains in the animal for a prolonged period of time.

Finally, a pharmacokinetic approach was utilized to investigate two in-feed CTC regimens (Group A: 22 mg/kg of bodyweight daily and Group B: 350 mg/head per day). The dose-adjusted total drug 24-hr mean (± SD) AUC values were 4.181 ± 1.724 µg·hr/ml and 0.302 ± 0.064 µg·hr/ml for treatment groups A and B, respectively. Treatment group A achieved >90% target (AUC_{24}/MIC of 25) attainment at an MIC of 0.06 µg/ml, whereas treatment group B displayed only 12.6% target attainment at the lowest MIC evaluated (0.015 µg/ml). These results suggest a very low potential for efficacy against common bovine pathogens.

Taken together, these studies help define resistance selection pressure from administration of CTC in the mineral of pasture cattle, compare antimicrobial effects of CTC and two common degradation products, characterize the duration of detection of CTC after administration in both plasma and urine, and determine the potential for efficacy from two commonly used in-feed CTC regimens.

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Approved by:

Co-Major Professor
Dr. Raghavendra Amachawadi

Approved by:

Co-Major Professor
Dr. Mike Apley

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Dedication

I would like to dedicate and give all the glory of this work to God. “Whatever you do, work at it with all your heart, as working for the Lord.” (Colossians 3:23) His plans for me have proven to be greater than I could ever imagine.

I would also like to dedicate this to my parents. Both of you have sacrificed, endured, and supported me through it all. This is as much your accomplishment as it is mine.

Chapter 1 - The effect of in-feed tetracyclines on the antimicrobial resistance of enteric bacteria in cattle: A systematic review of published studies, 2000-2022

Alyssa R Toillion[*], Michael D Apley[*¶], and Raghavendra G Amachawadi[*]

[*]Department of Clinical Sciences, Kansas State University, Manhattan, KS, 66506, USA; toillion@vet.k-state.edu, mapley@vet.k-state.edu, agraghav@vet.k-state.edu

[¶]Corresponding author: Michael D Apley, Department of Clinical Sciences, College of Veterinary Medicine, Kansas State University, 1620 Denison Avenue, Manhattan, KS, 66506, USA. Email: mapley@vet.k-state.edu

ABSTRACT

This systematic review was conducted to evaluate antimicrobial resistance of enteric bacteria associated with in-feed tetracycline use in cattle. Three different databases (PubMed, CAB Direct, and Agricola) were searched along with a previously compiled topic-related reference list. A total of 26 full-text articles were eligible for data extraction. A collective finding of this systematic review was that antimicrobial exposure is not essential for the maintenance of resistance; however, it does increase the prevalence of resistance. Additionally, factors other than antimicrobial use need to be considered when analyzing resistant bacteria.

KEYWORDS: Antimicrobial resistance, AMR, Beef, Cattle, Chlortetracycline, CTC, Tetracyclines

INTRODUCTION

Antimicrobial resistance (AMR) is an increasingly serious global public health threat. Resistant bacteria circulate among humans, animals, and the environment (Berendonk et al., 2015). Therefore, a multi-faceted effort from all sectors is required to effectively combat AMR. In veterinary medicine, important steps are being taken to change how important antimicrobials

can be legally used in-feed or water for food-producing animals. As of January 2017, the final implementation of Food and Drug Administration Center for Veterinary Medicine (FDA CVM) Guidance for Industry (GFI) #213 resulted in all labels of medically important antimicrobials intended for use in or on animal feed requiring the supervision of a licensed veterinarian as authorized through a veterinary feed directive (GFI #213). The start of this major change occurred with the finalization of FDA CVM GFI #209 in 2012, which not only set the initial parameters for GFI #213, but also established that any use of medically important antimicrobials for growth promotion (improvements in rate of gain or feed conversion) were to be phased out (GFI #209). This phase out occurred at the same time as the transition to VFD labels in 2017.

The FDA CVM is on record as considering prevention and control uses of medically important antimicrobials as therapeutic uses, and therefore judicious use (GFI #209). Others propose that delivering antibiotics to food animals for reasons other than treatment of clinically diagnosed disease, especially via free-choice feeding methods, poses an unnecessary public health risk (Love et al., 2011). A large proportion of the medically important antimicrobials used for prevention, control, and treatment through the feed are the tetracyclines. In 2020, tetracyclines were estimated to be 66% of all medically important antimicrobial drugs approved for use in food-producing animals and 62% of all medically important drugs that are administered in the feed as represented by kilograms of drug (2020 FDA Summary Report). An evaluation of antimicrobial use in 20 U.S. beef feedyards found that 14.2% of total milligrams administered, only 3.8% of regimens consisted of chlortetracycline in the feed (Apley et al., 2023 *in press*). These results underscore the importance of understanding the nuances of different reporting metrics in considering relative uses of antimicrobials in populations.

Tetracyclines are employed in-feed to cattle at different dosage regimens labeled for bovine respiratory disease (BRD), enteritis, anaplasmosis, and reduction in liver abscesses. Tetracyclines are not only used in veterinary medicine, but are widely used in human medicine and are considered highly important antimicrobials by the World Health Organization (WHO 2018; *proposed draft* GFI #152). The practice of adding antimicrobials in feed (or milk) creates selective pressure that could affect both animal and human health through future reference; reflected in the prevalence of resistance among indicator bacteria, such as *E. coli*. This systematic review was conducted to evaluate antimicrobial resistance of enteric bacteria associated with in-feed tetracycline use in cattle. We hypothesized that orally administered tetracyclines would increase the prevalence of resistant enteric bacteria compared to cattle that were not treated.

MATERIALS AND METHODS

Literature search

This review was conducted in accordance with PRISMA (Preferred Reporting for Items for Systematic Reviews and Meta-Analyses) reporting standards (Page et al., 2021).

On November 18, 2022 we conducted a systematic search in PubMed, CAB, and Agricola databases from 2000 to 2020 for retrieval of topics relevant to the objective using the search terms “bovine”, “cattle”, “cow”, “cows”, “calves”, “calf”, “tetracyclines”, “anti-infective agents”, “drug resistance, microbial”, “animal feed”, “chlortetracycline”, “CTC”, “in-feed”, “antimicrobial resistance”, and “AMR” in combinations. Only studies published in English were included. An initial search revealed a total of 46 (PubMed), 16 (CAB Direct), and 9 (Agricola).

On December 5, 2022 a subsequent search was conducted in the same three databases, however we expanded our search to include not only in-feed CTC, but also in-feed

oxytetracycline and tetracycline from 2000-2022. This search revealed a total of 142 (PubMed), 61 (CAB Direct), and 33 (Agricola). These searches can be seen in Appendix A (Supplementary Material).

Study screening

Two reviewers (AT, MA) independently screened studies at the title and abstract level. The following screening questions were used to determine whether the study met the eligibility criteria:

1. Does the title/abstract describe a primary research study (experimental as opposed to observational or a review)?
2. Does the title/abstract describe the use of in-feed chlortetracycline, oxytetracycline, or tetracycline or in-combination products in cattle?
3. Does the title/abstract include the outcome of phenotypic antimicrobial resistance (minimum inhibitory concentration or selective plating) or genetic resistance (any specific antimicrobial resistance genes) in enteric bacteria?

Studies that did not meet all eligibility criteria were excluded. Studies that passed the preliminary title and abstract screening were reviewed at the full-text level using the same inclusion criteria. If any of the eligibility requirements were not fulfilled, the study was excluded.

Data extraction

From each eligible publication, we extracted the following information: first author, year of publication, year of study, study location (country and continent), production type (beef, dairy, and mixed), age (calves and adults), study design, intervention details, laboratory procedure, bacteria investigated, sample size, number of pens, number of samples tested, number of

sampling occasions, prevalence of resistant isolates, general results and conclusions, and any bias reported or observed. The extracted information was entered into a Microsoft Excel spreadsheet.

STATISTICAL ANALYSIS

Data were entered into a software package (Microsoft Excel 2019, Microsoft Corporation; Redmond, WA).

RESULTS

A flow chart showing the systematic literature search procedure is shown in Figure 1.1. A total of 307 records were retrieved from three search engines (PubMed, CAB Direct, and Agricola. The initial search on 11/18/22 identified 71 records from 3 databases. A secondary revised search on 12/5/22 identified 236 records from the same 3 databases. One of the co-authors (MA) provided their previously compiled topic-related reference list containing 348 records. After deduplicating references from the search engines, 210 publications were retained for screening. After relevance screening of the titles and abstracts, 28 articles were retained. Following full-text evaluation 2 more studies were excluded resulting in 26 eligible full-text articles for data extraction and analysis.

Studies that met the inclusion criteria are detailed in Table 1.1. Briefly, twenty-two studies isolated *Escherichia coli*, five studies isolated *Salmonella* spp., two studies isolated *Campylobacter* spp., and one study isolated *Enterococcus* species. Phenotypic resistance was examined via: Sensititre (four studies), agar dilution (six studies), disk diffusion (four studies), and microbroth dilution (two studies). Genotypic resistance was examined via: polymerase chain reaction (PCR) (twelves studies) and pulsed-field gel electrophoresis (PFGE) (five studies). Lastly, shotgun metagenomics, whole genome sequencing, and LC-MS/MS used for drug

analysis were methodologies each conducted once. Twenty-four studies analyzed fecal samples with two of those studies being fecal deposits. The remaining two studies sampled manure (Sharma et al., 2009; Xu et al., 2018). Blood was sampled in two studies: (1) for the detection and quantification of CTC by LC-MS/MS (Toillion et al., 2021) and (2) to detect the presence of inhibitory substances via a serum inhibition assay (Berge et al., 2006). Other sample types collected included: environment (six studies), feed (one study), water (one study), carcasses (two studies), air (one study), and ground beef (one study). The studies in relation to their in-feed dosages are displayed in Table 1.2. Seven studies evaluated oxytetracycline (Alali, 2004; Checkley, 2010; Kaneene, 2008; Kaneene, 2009; Khachatryan, 2004; Khachatryan, 2006; Pereira, 2014). One study evaluated medicated milk replacer containing tetracycline hydrochloride at concentrations of 22 mg/kg of bodyweight per day (Berge et al., 2006). The remaining 18 studies evaluated in-feed administration of CTC, with one of those being CTC-medicated mineral (Toillion et al., 2021). The sample sizes of the various studies were as follows: 99 animals or below (10 studies), 100-199 animals (9 studies), and 200 animals and above (7 studies). Only 5 of the studies enrolled yearlings or older cattle, while the remaining studies utilized calves. Three studies assessed the antimicrobial effects of CTC withdrawal. In Alexander et al. (2010), antibiotics were withdrawn 28 days prior to slaughter. In Alexander et al. (2008) and Mirzaagha et al. (2011), both studies fed CTC for 61 days, discontinued for 86 days and then reintroduced for an additional 42-day period. The four studies with the longest duration of treatment were as follows: 197 consecutive days of CTC at 44 ppm (Alexander 2009, 2011) and 197 consecutive days of CTC at 350 mg/animal per day (Sharma 2008, 2009). A comparison table of the different in-feed dosages for each study included in this review are detailed in Table 1.2.

DISCUSSION

Our systematic review assessed antimicrobial resistance associated with the use of in-feed tetracyclines on enteric bacteria of cattle. The majority of studies indicated an increase in tetracycline-resistant bacteria, primarily *Escherichia coli*. Studies that categorically reported phenotypic resistance as pan-susceptible, resistant to a single antimicrobial, or multi-drug resistant (MDR) are displayed in Table 1.3. All studies that stated their findings in this format reported the highest proportion of isolates in the MDR category (Table 1.3). With the exception of *Campylobacter*, the proportion of isolates (*E. coli* and *Salmonella*) representing pan-susceptible demonstrated increasing MDR isolates following antimicrobial exposure. The association between chlortetracycline administration and reduced susceptibility to tetracyclines was found to be transient in multiple studies. Minimum inhibitory concentrations (MICs) returned to or approached previous values 17 days post-treatment (Platt, 2008; Kanwar, 2013). Evaluation of the in-feed CTC dosage of 22 mg/kg of bodyweight for 5 days, reported a temporary increase of fecal and pen surface Tet(r) *E. coli* concentrations (Agga et al., 2016). In another study, changes related to CTC administration were concluded to have minimal short-term impact and no long-term impact on AMR levels shed by cattle at commercial feedlots (Miller et al., 2018).

To determine the effect of antibiotic withdrawal on bacterial resistance, in-feed CTC and CTC in combination with sulfamethazine were discontinued for 86 days in both Alexander et al. (2008) and Mirzaagha et al. (2011). Both studies found that removing antibiotics did not significantly alter the prevalence of the cattle shedding resistant bacteria and had minimal impact on AMR in *E. coli*. Removal of chlortetracycline from the diet was not a viable strategy for reducing the level of tetracycline-resistant *E. coli* (Alexander et al., 2008). Likewise, despite

antimicrobial withdrawal 28 days prior to slaughter, populations of Amp(r) and Tet(r) *E. coli* remained higher in feces of treated animals compared to control animals (Alexander et al., 2010).

Several of the evaluated studies would suggest that tetracycline resistance determinants have already established a steady state in *E. coli* populations, indicating their prevalence is not necessarily directly related to antimicrobial usage in the study. This observation is supported by studies reporting resistance prior to antimicrobial exposure. Alexander, et al., (2008) reported that more than 40% of the steers shed tetracycline-resistant *E. coli* on the first sampling day (day 15) after arrival at the feedlot. Similarly, although cattle from the control group were not fed antimicrobials, their fecal deposits had a relatively high number of Tet(r) *E. coli* on day 0 (Alexander et al., 2009). Similar results have been described for feedlot cattle (Sharma 2008) and dairy calves (Khachatryan et al., 2004) that have not been fed antimicrobials. There is a level of inherent resistance within the microbial community independent of CTC exposure (Toillion et al., 2021).

A collective finding of this systematic review is that antimicrobial exposure is not essential for the maintenance of resistance; however, it does increase the prevalence of resistance. Prior to antimicrobial exposure, Tet(r) *E. coli* were prevalent; although, treatment resulted in Tet(r) *E. coli* concentrations higher in the treatment group compared to the control group (Agga, 2016; Alexander, 2009). Similarly, animals were 115 times more likely to have 1 or more fecal Tet(r) *E. coli* on day 15 than day 0, when oxytetracycline was used in the feed (Checkley et al., 2010).

Concerns extend beyond resistance to ‘‘co-selection’’ for resistance to other classes of antimicrobials. Several studies found genetic linkages among resistance determinants (Berge, 2016; Alexander, 2008, 2009, 2010, 2011; Kanwar, 2013; Miller, 2008; Xu, 2018). Similar to

resistance determinants, even in the absence of selective pressure, genetic linkages between genes are able to be maintained. In studies that evaluated genetic relatedness (Alexander, 2008; 2009, 2010; Sharma, 2008; Khachatryan, 2004) there was a clear relationship between treatment and the genetic diversity of resistant *E. coli*. Animals treated over time showed increasingly similar genotypes. Based on the observed shift in Amp(r) *E. coli* diversity after environmental exposure, Alexander et al. (2009) hypothesized that some genotypes may survive better in secondary environments. Therefore, a key factor for the establishment of resistance within bacterial communities is the ability for resistant-genes to persist in the environment.

A limited number of studies reviewed resistance associated with the use of medicated milk replacers. Feeding calves milk with residual concentrations of antimicrobial drugs had an important effect on the frequency of AMR in pre-weaned dairy calves (Pereira et al., 2014). Studies by Kaneene et. al (2008, 2009) found that discontinuing feeding medicated milk replacer increased tetracycline susceptibility and reduced MDR in *E. coli* and *Salmonella*. A recurring theme among studies, including studies focused on medicated milk replacers, was transient resistance. Supplementation of milk replacer with antibiotics may increase the probability of *E. coli* O157:H7 shedding in dairy calves, but the effect seems to be of low magnitude and short duration (Alali et al., 2004). In contrast, prevalence of Tet(r) *E. coli* was slightly higher for calves that received supplement without oxytetracycline as opposed to calves that did receive oxytetracycline ($P = 0.039$), indicating drug use was not required to maintain a high prevalence of resistant *E. coli* (Khachatryan et al., 2004). In agreement with the previous study, oxytetracycline had no apparent additive effect on the prevalence of streptomycin, sulfadiazine, and tetracycline (SSuT) strains relative to the group receiving the unmedicated supplement (Khachatryan et al., 2006). In accordance with the in-feed studies, Berge et al. (2006)

demonstrated that calves not receiving in-milk antimicrobials, but being treated for clinical disease, transiently shed more resistant *E. coli* populations than untreated calves.

Following in-feed CTC administration, Platt et al. (2008) reported a transient reduction in the prevalence of fecal 3rd generation cephalosporins-resistant (3GC(r)) *E. coli*, whereas Kanwar et al. (2013) reported an increase in 3GC(r) *E. coli* as a result of ceftiofur followed by in-feed CTC administration. In contrast to both studies, Agga et al. (2016) reported fecal swab 3GC(r) *E. coli* prevalence did not differ significantly ($P \geq 0.90$) between groups on any sampling occasion and factors other than in-feed CTC treatment had a greater impact on Tet(r) and 3GC(r) *E. coli* shed by feedlot cattle. By graphing each group's (CTC, control, empty) pen surface generic and Tet(r) *E. coli* concentrations, the author's concluded the principal factor contributing to the increase in Tet(r) *E. coli* concentrations were the deposition of manure (feces and urine) in occupied pens (control and CTC groups) compared to unoccupied pens. Therefore, it is possible, independent of treatment, pen surface soil is re-inoculating cattle with resistant bacteria. These findings are consistent with other studies that found an increase in antimicrobial-resistant genes and bacteria in soils from pens holding cattle that had not received antibiotics and soils fertilized with manure from dairy cattle that had not received antibiotics (Liu, 2016; Udikovic-Kolic, 2014; Agga, 2016).

Beyond administering tetracyclines directly into the feed, other acquisition sources of Tet(r) *E. coli* should be considered: fecal matter, water sources, herd cohorts, pen surface materials, diet, and wildlife. Each of these acquisition sources play a critical role in the survivability and dissemination resistant determinants. The half-life for tetracycline, chlortetracycline, and oxytetracycline being in stored feedlot manure is 17.2, 13.5, and 31.1 days, respectively (Storteboom, 2007; Masse, 2014). In Alexander et al. (2009) and (2011), resistance

determinants were quantifiable in feces after 175 days. Likewise, Alexander et al. (2008) reported the survival of *E. coli* to occur up to 150 days in fecal pats on range land (Sinton et al., 2007). Even after a considerable period of time, fecal material remains as a source of antimicrobial resistant bacteria.

Dietary factors have been implicated in the development of antimicrobial resistant *E. coli* populations in ruminants (Alexander, 2008; Kachatryan, 2006). The one study included in this review that analyzed feed and water samples found temporal increases of generic, Tet(r), and 3GC(r) *E. coli* in both feed and water samples (Agga et al., 2016). Feedlot steers fed a grain-based diet including chlortetracycline and sulfamethazine in combination displayed a higher prevalence of tetracycline-resistant and ampicillin-resistant *E. coli* (Alexander et al., 2008). The change in diet may create a selective advantage for some resistant bacteria perhaps through a decrease in the pH of the rumen or perhaps feed has been implicated as a source of antimicrobial-resistant genes (Dargatz, 2002; Checkley, 2010).

Laboratory detection methods have evolved over time. Since this review only went as far back as 2000, the methodologies employed were somewhat similar across most of the studies. Cultivation-based techniques used to grow and isolate bacteria were consistent throughout. Molecular techniques such as 16S RNA and whole genome sequencing were used. Chemical analysis of liquid chromatography mass spectrometry, specifically ultra-high performance, is a newer technique that hasn't been used much in the context of this research topic. Therefore, the articles reviewed lacked verifying drug quantification. Only one study quantified the amount of CTC in the animals (Toillion et al., 2021). Generally, studies evaluate the presence or absence of resistant bacteria and/or genes but fail to incorporate the actual amount of antibiotic consumed.

CONCLUSION

We identified 26 studies that investigated the effects of in-feed tetracyclines on AMR in enteric bacteria. The main conclusions found this in the review are (i) bovine feces serve as a long-term reservoir of resistance determinants (ii) withdrawal of CTC from the diet has limited impact for decreasing resistant bacteria (iii) prevalence of Tet(r) *E. coli* may be independent of antimicrobial exposure in a group of animals, and (iv) environmental factors play a role in harboring and disseminating resistance. Factors other than antimicrobial use need to be considered when analyzing resistant bacteria.

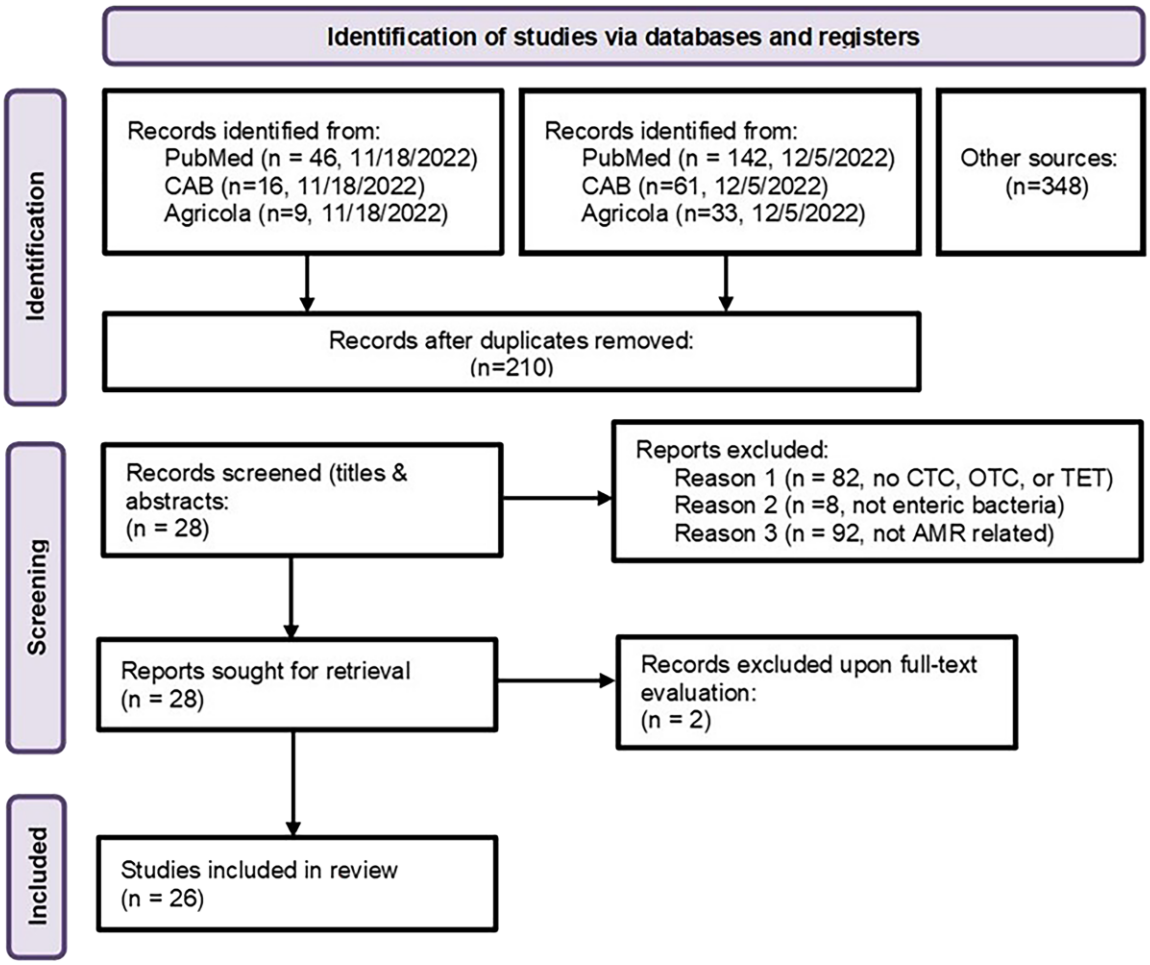

Figure 1.1. PRISMA flow diagram of the search and selection process.

Reference First Author, Year	Location	Sample Type	Bacteria Investigated	Methodology	Intervention Groups	Duration of Treatment	Main Outcome
Agga et al. 2016	Clay Center, NE	Fecal swabs, pen surface material, feed, and water samples	*Escherichia coli*	Multiplex PCR targeting *lac*Y, *lac*Z, *cyd*, and *uid*A genes to confirm presumptive *E. coli*	10 mg/lb of bodyweight per day	5 days	CTC administered in-feed for 5-days to feedlot beef cattle temporarily increased fecal and pen surface Tet(r) *E. coli* concentrations and did not impact fecal or pen surface 3GC(r) *E. coli* prevalence.
Alali et al. 2004	Manhattan, KS	Fecal samples, post-necropsy: gut contents and tissue samples	*Escherichia coli* O157:H7	Enumeration and/or detection of NA-resistant *E. coli* O157:H7. Confirmed by latex agglutination.	**2 treatments:** (i) milk replacer with no antibiotic (ii) milk replacer with **OTC (200 mg/kg)** and neomycin (400 mg/kg)	not specified	Supplementation of milk replacer with antibiotics may increase the probability of *E. coli* O157:H7 shedding in dairy calves, but the effect seems to be of low magnitude and short duration.
Alexander et al. 2008	Lethbridge, Alberta	Fecal samples	*Escherichia coli*	AST: agar dilution and PFGE	**6 treatments:** (i) CON (ii) CTC + sulfamethazine (each at 44 ppm) (iii) CTC at 44 ppm (iv) monensin at 25 ppm (v) tylosin phosphate at 11 ppm (vi) virginiamycin at 31 ppm	61 days then discontinued for 86 days and then reintroduced for an additional 42-day period	Subtherapeutic administration of CTC + sulfamethazine increased the prevalence of Amp(r) and Tet(r) *E. coli* in cattle. Resistance may be related to additional environmental factors such as diet.

Reference First Author, Year	Location	Sample Type	Bacteria Investigated	Methodology	Intervention Groups	Duration of Treatment	Main Outcome
Alexander et al. 2009	Lethbridge, Alberta	Fecal deposits	*Escherichia coli*	PFGE and PCR for select genes encoding **tetracycline** (*tet*A, *tet*B, *tet*C, *tet*L, *tet*M, *tet*O, and *tet*W), **ampicillin** (*bla*OXA1, *bla*PSE1, and *bla*TEM1), and **sulfonamide** (*sul*1 and *sul*2) resistance.	**3 treatments:** i) CON (ii) CTC + sulfamethazine (each at 44 ppm) (iii) CTC at 44 ppm	197 days	Fecal deposits remain a source of resistant *E. coli* even after a considerable period of environmental exposure.
Alexander et al. 2010	Lethbridge, Alberta	Fecal, environmental abattoir, hides and carcasses, air, ground beef, and intestinal digesta	*Escherichia coli*	AST: disc diffusion, and pulsed-field gel electrophoresis (PFGE)	**2 treatments:** (i) CON (ii) CTC + sulfamethazine (each at 44 ppm)	28 days	Feeding subtherapeutic antimicrobials to feedlot cattle increased the proportion of Amp(r) and Tet(r) *E. coli* in the feces of animals.
Alexander et al. 2011	Lethbridge, Alberta	Fecal deposits	*tet*, *sul*, and *erm* resistance genes	Conventional and quantitative real-time PCR, and denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S-rRNA.	**4 treatments:** (i) CON (ii) CTC + sulfamethazine (each at 44 ppm) (iii) CTC at 44 ppm (iv) tylosin phosphate at 11 ppm	197 days	The abundance of genes coding for antimicrobial resistance in bovine feces can be affected by inclusion of antibiotics in the feed. Resistance genes can persist in feces from cattle beyond 175 days with concentrations of some genes increasing with time.

Reference First Author, Year	Location	Sample Type	Bacteria Investigated	Methodology	Intervention Groups	Duration of Treatment	Main Outcome
Berge et al. 2006	California	Fecal, blood, and environmental samples	*Escherichia coli*	AST: disk diffusion and serum inhibition bioassay	**4 treatments:** (i) no antimicrobials in milk and therapeutic treatments only consisted of nonantimicrobial alternatives (ii) same as above (iii) no antimicrobials in milk but received individual therapy for clinical disease (iv) medicated milk replacer containing **tetracycline hydrochloride (22 mg/kg)** and neomycin sulfate (22 mg/kg)	28 days	In calves not receiving in-feed antimicrobials, older calves had higher levels of resistance compared to day-old calves. Individual antimicrobial therapy increased resistance in these calves but appeared to be transient.
Checkley et al. 2010	Western Canada	Fecal samples	*Escherichia coli*	AST: agar dilution	**3 treatments:** (i) no antimicrobials on arrival (ii) **oxytetracycline at 110 g/kg** (iii) long-acting **oxytetracycline 20 mg/kg BW** SQ on day 0	14 days	There was no difference in the proportion of animals with *E. coli* isolates resistant to tetracycline between the treatment groups preslaughter. However, there were significantly more animals with Tet(r) *E. coli* at preslaughter than at arrival.

Reference First Author, Year	Location	Sample Type	Bacteria Investigated	Methodology	Intervention Groups	Duration of Treatment	Main Outcome
Kaneene et al. 2008	Michigan and New York	Fecal and environmental samples	*Salmonella*, *Campylobacter*, and *Escherichia coli*	AST: Sensititre	**2 treatments:** (i) medicated milk replacer containing **oxytetracycline** and neomycin (ii) same brand of milk replacer without antimicrobials	90 days	Tetracycline susceptibility increased in intervention herds for the first 3 months after switching to non-medicated milk replacer, but declined in subsequent months.
Kaneene et al. 2009	not stated	Fecal and environmental samples	*Salmonella, Campylobacter, and Escherichia coli*	AST: Sensititre	**2 treatments:** (i) medicated milk replacer containing **oxytetracycline** and neomycin (ii) same brand of milk replacer without antimicrobials	90 days	Results demonstrated that the intervention did reduce multidrug resistance in these bacteria, but that other factors also influenced multidrug resistance.
Kanwar et al. 2013	Canyon, TX	Fecal samples	non-type-specific *E. coli* *tet*(A), *tet*(B), and *bla*CMY	AST: Sensititre and duplex PCR (to detect both *tet*(A) and *tet*(B))	**4 treatments:** (i) All-CCFA group / No CTC (ii) 1-CCFA / No CTC (iii) 1-CCFA & **CTC group 22 mg/kg** (iv) All-CCFA & **CTC group 22 mg/kg**	3 separate 5-day periods	Results indicate that CTC can exacerbate ceftiofur resistance following CCFA therapy and therefore should be avoided, especially when considering their use in sequence.
Kanwar et al. 2014	Texas	Fecal samples	*Escherichia coli*	Genotypic quantification of *bla*CMY-2, *bla*CTX-M, *tet*(A), *tet*(B), and 16S rRNA genes by qPCR	**4 treatments:** (i) All-CCFA group / No CTC (ii) 1-CCFA / No CTC (iii) 1-CCFA & **CTC group 22 mg/kg** (iv) All-CCFA & **CTC group 22 mg/kg**	3 separate 5-day periods	Chlortetracycline is contraindicated when attempting to avoid expansion of resistance to critically important third-generation cephalosporins.

Reference First Author, Year	Location	Sample Type	Bacteria Investigated	Methodology	Intervention Groups	Duration of Treatment	Main Outcome
Khachatryan et al. 2004	Pullman, Washington	Fecal samples	*Escherichia coli*	AST: agar dilution, PCR (*tet*A and *tet*B, *str*A and *str*B, and *sul*2 and *sul*2), and PFGE	**2 treatments:** (i) milk supplement with **oxytetracycline (Terramycin; TM-50)** (ii) supplement with no no OTC	12 weeks	Active selection for traits linked to the SSuT phenotype are responsible for maintaining drug-resistant *E. coli* in this population of dairy calves.
Khachatryan et al. 2006	Pullman, Washington	Fecal samples	*Escherichia coli*	AST: agar dilution	**3 treatments:** (i) supplement without OTC (ii) supplement with **OTC (110 g/kg)** (iii) no supplement	not specified	Results were consistent with selection and maintenance of SSuT *E. coli* due to environmental components independent of antibiotic selection.
Miller et al. 2018	US	Fecal swabs and pen surface materials	10 resistance genes: aac(6′)-Ie-aph(2″), *bla*CMY-2, *bla*CTX-M, *bla*KPC-2, *mec*A, aadA1, *erm*(B), *tet*(A), *tet*(B), and *tet*(M)	Metagenomic DNA isolation and qPCR	10 mg/lb of bodyweight per day	5 days	The results suggest that a single 5-d in-feed CTC prophylaxis of beef cattle to prevent BRD has a negligible impact on the abundances of resistance genes.

Reference First Author, Year	Location	Sample Type	Bacteria Investigated	Methodology	Intervention Groups	Duration of Treatment	Main Outcome
Mirzaagha et al. 2011	Lethbridge, Alberta	Fecal samples	*Escherichia coli*	AST: agar dilution, PFGE, and multiplex PCR	**4 treatment groups:** (i) controls (ii) CTC + sulfamethazine (each at 44 ppm) (iii) CTC at 11 ppm (iv) virginiamycin at 31 ppm	61 days then discontinued for 86 days and then reintroduced for an additional 42-day period	Factors other than, or in addition to subtherapeutic administration of antibiotics influence the establishment and transmission of AMR *E. coli* among feedlot cattle.
O'Connor et al. 2002	Ontario, CA	Fecal samples	*Escherichia coli*	AST: agar dilution	**Chlortetracycline** was added to the ration at a daily dose of **8 mg/lb BW for approximately 8 d** and then **3 mg/lb BW for approximately 8 d** (sick animals were treated with SC long-acting **oxytetracycline 9 mg/lb BW**)	16 days	This study reports changes in the prevalence of resistance to antibiotics in commensal *Escherichia coli* from cattle receiving either SQ oxytetracycline in addition to in-feed chlortetracycline or only in-feed chlortetracycline.

Reference First Author, Year	Location	Sample Type	Bacteria Investigated	Methodology	Intervention Groups	Duration of Treatment	Main Outcome
Ohta et al. 2017	Canyon, Texas	Fecal samples	*Salmonella*	AST: Sensititre and WGS	**4 treatments:** (i) All-CCFA group / No CTC (ii) 1-CCFA / No CTC (iii) 1-CCFA & **CTC group 22 mg/kg** (iv) All-CCFA & **CTC group 22 mg/kg**	3 separate 5-day periods	The selection pressures of a 3rd generation cephalosporin and chlortetracycline during the feeding period contribute to dynamic population shifts between antimicrobial susceptible and resistant *Salmonella*.
Ohta et al. 2019	Canyon, Texas	Fecal samples	*Salmonella* and *E. coli*	Spiral plating (colony counting) and qPCR	**4 treatments:** (i) All-CCFA group / No CTC (ii) 1-CCFA / No CTC (iii) 1-CCFA & **CTC group 22 mg/kg** (iv) All-CCFA & **CTC group 22 mg/kg**	3 separate 5-day periods	The results of this study demonstrate that antibiotic use decreases the overall quantity of *Salmonella* in cattle feces in the short term; however, the overall quantities of antimicrobial-resistant NTS *E. coli* and *Salmonella* tend to remain at a constant level throughout.

Reference First Author, Year	Location	Sample Type	Bacteria Investigated	Methodology	Intervention Groups	Duration of Treatment	Main Outcome
Pereira et al. 2014	Ithaca, NY	Fecal samples	*Escherichia coli*	AST: disk diffusion	**2 treatments:** (i) raw milk no drug residues (ii) raw milk with drug residues by adding ceftiofur, penicillin, ampicillin, and **oxytetracycline** at final concentrations in the milk of 0.1, 0.005, 0.01, and 0.3 mg/ml, respectively.	Twice a day for 6 weeks	Feeding calves milk with residual concentrations of antimicrobial drugs from birth to weaning resulted in a significantly greater proportion of *E. coli* resistant to multiple antimicrobial drugs when compared to isolates in non-treated calves.
Platt et al. 2008	not stated	Fecal samples	*Escherichia coli* and *Enterococcus* spp.	AST: microbroth dilution	**2 treatments:** (i) **Chlortetracycline (22 mg/kg)** in cottonseed meal (ii) CON (cottonseed meal only)	3 separate 5-day periods	Exposure to chlortetracycline was associated with a temporary increase in the likelihood of recovering resistant bacteria. Exposure to chlortetracycline decreased the likelihood of recovering ceftiofur-resistant *E. coli* isolates, even though isolates were co-resistant to tetracycline.

Reference First Author, Year	Location	Sample Type	Bacteria Investigated	Methodology	Intervention Groups	Duration of Treatment	Main Outcome
Sharma et al. 2008	Lethbridge, AB	Fecal samples	*Escherichia coli*	AST: disk diffusion, genotyping by PFGE, and multiplex PCR	**3 treatments:** (i) CON (ii) **350 mg/h CTC**, (iii) **CTC + sulfa at 350 mg/h**	197 days	Antimicrobial administration was not found to be essential for the maintenance of inherently Amp(r) and Tet(r) *E. coli* in control animals; however, higher Tet(r) *E. coli* shedding was observed in animals subjected to the two treatments.
Sharma et al. 2009	not stated	Manure	*Escherichia coli*	Conventional and RT-PCR	**3 treatments:** i) CON (ii) tylosin phosphate at 11 mg/kg (iii) **CTC + sulfa at 350 mg/h**	197 days	These results indicate that even though composting reduces antimicrobial resistant *E. coli*, *tet* and *erm* genes could still be detected.
Toillion et al. 2021	Manhattan, KS	Fecal and blood samples	*Escherichia coli*	AST: microbroth dilution and CTC quantification by LC-MS	**5 treatments:** free-choice **CTC medicated mineral (0, 700, 5000, 6000, 8000 g CTC/ton)**	5 months	No pattern or clear development of resistance was observed in *E. coli* isolated from CTC-treated cattle.

Reference First Author, Year	Location	Sample Type	Bacteria Investigated	Methodology	Intervention Groups	Duration of Treatment	Main Outcome
Weinroth et al. 2018	Canyon, TX	Fecal samples	*Salmonella* and *E. coli*	Shotgun metagenomics	**4 treatments:** (i) **22 mg/kg CTC** for 23 days and all cattle treated on day 0 with CCFA (ii) **CTC in feed**, only 1 of 11 steers in each pen was treated on day 0 with CCFA (iii) No CTC, all cattle treated on day 0 with CCFA (iv) **Treated with CTC** and only 1 of the 11 steers in each pen was treated on day 0 with CCFA.	3 separate 5-day period	Cattle fed chlortetracycline had a significant increase in relative abundance of tetracycline resistance genes.
Xu et al. 2018	Lethbridge, AB	Manure	*tet*, *sul*, and *erm* resistance genes	Quantitative PCR (qPCR) and 16S rDNA	**4 treatments:** (i) CON (ii) CTC + sulfamethazine (each at 44 ppm) (iii) CTC at 44 ppm (iv) tylosin phosphate at 11 ppm	up to the 3 months	Oral administration conferred more time for resistant bacterial populations to become established in manure, resulting in greater persistence of resistance genes during subsequent composting.

Table 1.1. Characteristics of studies included in the systematic review.

	In-feed dosages		
Study	**Oxytetracycline**	**Tetracycline**	**Chlortetracycline**
Agga et al., 2016			10 mg/lb bw
Alali et al., 2004	200 mg/kg*+		
Alexander et al., 2008			11ppm and 44ppm+
Alexander et al., 2009			44ppm and 44ppm+
Alexander et al., 2010			44ppm+
Alexander et al., 2011			44ppm and 44ppm+
Berge et al., 2006		22 mg/kg*+	
Checkley et al., 2010	Terramycin-50 (110 g/kg)		
Kaneene et al., 2008	unknown*+		
Kaneene et al., 2009	unknown*+		
Kanwar et al., 2013			22 mg/kg+
Kanwar et al., 2014			22 mg/kg+
Khachatryan et al., 2004	Terramycin-50*		
Khachatryan et al., 2006	Pennox-50*		
Miller et al., 2018			10 mg/lb bw
Mirzaagha et al., 2011			11ppm and 44ppm+
O'Connor et al., 2002			8 mg/lb bw
Ohta et al., 2017			22 mg/kg+
Ohta et al., 2019			22 mg/kg+
Pereira et al., 2014	0.3 ug/ml*+		
Platt et al., 2008			22 mg/kg
Sharma et al., 2008			350 mg/h and 350 mg/h+
Sharma et al., 2009			350 mg/h+
Toillion et al., 2021			700, 5000, 6000, 8000 g/ton^
Weinroth et al., 2018			22 mg/kg+
Xu et al., 2018			44ppm and 44ppm+

*medicated milk replacer, +in combination, ^intended to provided 0.5 - 2.0 mg CTC /lb bodyweight per day

Table 1.2. In-feed dosages for each study included in this review.

Study	Pan-Susceptible % Susceptible (# of isolates)	Resistant to a Single Antimicrobial % Resistant (# of isolates)	Multi-Drug Resistant % Resistant (# of isolates)
Berge et al., 2006	39% (357)	6% (55)	55% (412)
Pereira et al., 2014	6% *	N/A	84% *
Pereira et al., 2014	46% **	N/A	37% **
Platt et al., 2008	24.4% (128)	75.6% (397) ^	90.2% (330)
Platt et al., 2008	9.6% (35) #	18% (66) #	72% (265)
Kaneene et al., 2009	9.9% (142) *E. coli*, 15.5% (25) *Salmonella*, and 12.2% (10) *Campylobacter*	N/A	88.5% (1,273) *E. coli*, 84.5% (136) *Salmonella*, and 11% (9) *Campylobacter*
Kanwar et al., 2013	32.4% (340)	67.6% (710) ^	N/A
Ohta et al., 2017	100% (35) +	N/A	22.2% (18) +
Ohta et al., 2017	100% (33) ++	N/A	100% (29) ++

* treatment group, ** control group, ^ resistant to at least one antimicrobial, #*Enterococcus* spp., +treatment group (All CCFA and all CTC) (Ohta et al., 2017), ++control group (1-CCFA/no CTC) (Ohta et al., 2017)

Table 1.3. Percentage prevalence of susceptible, resistant, and multi-drug resistant *E. coli* isolates.

APPENDIX A

Databases

All relevant articles published in English language and indexed in the following databases will be searched: PubMed, Google Scholar, and Science Direct. The electronic search strategy will be as follows:

Library/Database: PubMed

Date of Search: 11/18/2022

Search String: (((((((bovine) OR (cow)) OR (cows)) OR (calves)) OR (calf)) AND ((((Anti-Infective Agents) OR (antimicrobial)) OR (antimicrobial resistance)) OR (Drug Resistance, Microbial))) AND ((Animal Feed) OR (in-feed))) AND ((chlortetracycline) OR (CTC)) **Filters:** from 2000 - 2020

 # Hits: 46

Library/Database: PubMed

Date of Search: 12/5/2022

Search String: (((((((bovine) OR (cow)) OR (cows)) OR (calves)) OR (calf)) AND ((((Anti-Infective Agents) OR (antimicrobial)) OR (antimicrobial resistance)) OR (Drug Resistance, Microbial))) AND ((Animal Feed) OR (in-feed))) AND ((tetracycline) OR (oxytetracycline) OR (OTC) OR (chlortetracycline) OR (CTC)) Filters: from 2000 - 2022

 # Hits: 142

Library/Database: CAB Direct

Date of Search: 11/18/2022

Search String: ((feed additives) OR (feeds)) AND (chlortetracycline) AND (cattle) AND (anti-infective agents)) Filters: from 2000 - 2020

 # Hits: 16

Library/Database: CAB Direct

Date of Search: 12/5/2022

Search String: ((oxytetracycline) OR (tetracycline) OR (chlortetracycline)) AND ((feeds) OR ((feed additives)) AND ((cattle) AND ((Drug Resistance, Microbial))) Filters: from 2000 - 2022

 # Hits: 61

Library/Database: Agricola

Date of Search: 11/18/2022

Search String: (((((((bovine) OR (cow)) OR (cows)) OR (calves)) OR (calf)) AND ((((Anti-Infective Agents) OR (antimicrobial)) OR (antimicrobial resistance)) OR (Drug Resistance, Microbial))) AND ((Animal Feed) OR (in-feed))) AND ((chlortetracycline) OR (CTC)) **Filters:** from 2000 - 2020

 # Hits: 9

Library/Database: Agricola

Date of Search: 12/5/2022

(((((((bovine) OR (cow)) OR (cows)) OR (calves)) OR (calf)) AND ((((Anti-Infective Agents) OR (antimicrobial)) OR (antimicrobial resistance)) OR (Drug Resistance, Microbial)))

AND ((Animal Feed) OR (in-feed))) AND ((tetracycline) OR (oxytetracycline) OR (OTC) OR (chlortetracycline) OR (CTC)) **Filters:** from 2000 – 2022

 # Hits: 33

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Chapter 2 - Effect of protracted free-choice chlortetracycline-medicated mineral for anaplasmosis control on *Escherichia coli* chlortetracycline resistance profile from pastured beef cattle

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Alyssa R. Toillion[1], Emily J. Reppert[2], Raghavendra G. Amachawadi[2], K. C. Olson[3], Johann F. Coetzee[4], Qing Kang[5], and Kathryn E. Reif[1*]

[1]Department of Diagnostic Medicine/Pathobiology, Kansas State University, Manhattan, KS 66506, USA; toillion@vet.k-state.edu, kreif@vet.k-state.edu

[2]Department of Clinical Sciences, Kansas State University, Manhattan, KS 66506, USA; erepper@vet.k-state.edu (E.J.R.); agraghav@vet.k-state.edu (R.G.A.)

[3]Department of Animal Sciences and Industry, Kansas State University, Manhattan, KS 66506, USA; kcolson@ksu.edu

[4]Department of Anatomy and Physiology, Kansas State University, Manhattan, KS 66506, USA; jcoetzee@vet.k-state.edu

[5]Department of Statistics, Kansas State University, Manhattan, KS 66506, USA; qkang@k-state.edu

*Correspondence: kreif@vet.k-state.edu; Tel.: +1-785-532-5552

ABSTRACT

Anaplasmosis is an economically-significant, hemolytic, tick-borne disease of cattle caused by *Anaplasma marginale* which can cause clinical anemia and death. Current control options are limited, and FDA-approved antimicrobial control options do not have a defined duration of use. A practical and routinely used anaplasmosis control method involves feeding free-choice chlortetracycline (CTC)-medicated mineral to pastured cattle for several months. Constant antimicrobial use poses the risk of expediting the development and dissemination of antimicrobial resistance in off-target commensal bacteria in the bovine gastrointestinal tract. The

objective of this study was to determine the CTC-susceptibility of *Escherichia coli* isolated from anaplasmosis endemic beef cattle herds provided different FDA-approved free-choice CTC-medicated mineral formulations, all intended to provide cattle a dosage of 0.5 to 2.0 mg CTC/lb bodyweight per day. A closed-herd, comprised of Hereford-Angus cows, naturally endemic for anaplasmosis, were grazed in five different pastures with one herd serving as an untreated control group. The other cattle herds were randomly assigned one of four FDA-approved CTC-medicated mineral formulations (700, 5000, 6000, and 8000 g CTC/ton) labeled for "the control of active anaplasmosis" and provided their respective CTC-medicated mineral formulation for five consecutive months. Fecal samples were collected monthly from a subset of cows (n = 6 or 10) per pasture. Fecal samples were cultured for *E. coli* isolates and the minimal inhibitory concentration of CTC was determined. Baseline CTC-susceptibility of *E. coli* was variable among all treatment and control groups. The susceptibility of *E. coli* isolates was significantly different between study herds over the treatment period ($p = 0.0037$ across time and 0.009 at the final sampling time). The interaction between study herds and treatment period was not significant ($p = 0.075$).

KEYWORDS: *Anaplasma marginale*; AMR; antimicrobial resistance; CTC; *E. coli*

INTRODUCTION

The use of medically important antimicrobials in food-producing animals has raised concerns about promoting antimicrobial resistance (AMR) [1]. In 2019, the total amount of antimicrobial drugs actively marketed was approximately 11.4 million kilograms [2]. Of the total drugs marketed, 6 million kilograms were medically important antimicrobial drugs sold and distributed for food-producing animals, of which 2.5 million kilograms were for use in cattle [2]. Tetracycline antimicrobials are considered highly important and have consistently been the

largest portion (67%) of all antimicrobials sold in the U.S. for food-producing animals, including in cattle [2]. There is strong evidence that antimicrobial-resistant bacteria can be transferred from livestock to humans [3,4]. A systematic literature review reported that reducing antibiotic use in food-producing animals decreased the prevalence of antibiotic-resistant bacteria in animals by about 15% and multidrug-resistant bacteria by 24–32% [5]. In many places, antibiotics are overused and misused in animals and people, and often given without professional oversight [6,7]. With a limited number of antimicrobials available, improper use can promote AMR and reduce the effective lifespan of the antimicrobial. As a means to reduce AMR in the U.S., all in-feed medically important antimicrobial drugs for use in food-producing animals have recently been put under veterinary oversight [8]. Despite an increased regulatory structure, some currently approved in-feed antimicrobial indications for food-producing animals do not have a defined duration of use. For example, chlortetracycline (CTC) indicated for "control of active anaplasmosis" does not have a current limit on the duration of use as long as a producer has a valid Veterinary Feed Directive (VFD).

Bovine anaplasmosis (hereafter referred to simply as anaplasmosis) is a globally occurring tick-borne disease of cattle [9]. Clinical disease is most commonly observed in mature cattle. Anaplasmosis is a disease of economic importance in the U.S., conservatively estimated to cost the U.S. cattle industry greater than $300 million per year [10,11]. The causative agent, *Anaplasma marginale*, invades and colonizes red blood cells (RBCs); mass destruction of infected RBCs can lead to anemia, the hallmark of clinical anaplasmosis. Other clinical anaplasmosis signs include icterus, lethargy, fever, aggression, abortions, and death. Infected cattle may never show signs of disease, especially if infected when young, and most animals (treated or untreated) will recover from disease and serve as infection reservoirs. Subsequent

transmission events can occur via ticks, blood-contaminated biting fly mouthparts, or blood-contaminated equipment such as needles, dehorning, and castration equipment. Increased cattle movement has facilitated the spread of anaplasmosis into almost every continental state. In a Kansas seroprevalence study (n = 925 herds), 52.54% (486/925) of sampled cow-calf herds tested seropositive for *A. marginale* [12]. In other previous U.S. anaplasmosis seroprevalence studies, cELISA testing from a 2013–2014 slaughter survey (n = 215) in Mississippi found a seroprevalence of 29.02% (95% CI: 22.74–36.07%) [13]. In Texas, results from an active slaughter survey (n = 215) performed between August and December 2014 as well as reviewing Texas A&M Veterinary Medical Diagnostic Laboratory records of specimens submitted for anaplasmosis testing from January 2002 to June 2012 (n = 15,460) found the estimated seroprevalence of anaplasmosis in Texas to be 15.91% (95% CI: 15.34–16.50%) [14]. Samples taken from a Georgia (n = 293) auction barn and abattoir from 2013–2014 found the estimated seroprevalence to be 4.44% (95% CI: 2.61–7.44%) [15].

In beef production, antimicrobials are important to maintain or improve animal health towards increasing productivity and economic viability [16,17]. The most common antimicrobial-based anaplasmosis control for pastured cattle is CTC delivered in-feed or via medicated mineral. Currently, the FDA allows producers to provide CTC-medicated feed products for anaplasmosis control with no limits on the duration of use as long as the producer maintains a valid VFD [8]. In-feed or mineral supplementation of CTC is indicated for "control of active anaplasmosis" and can be administered free-choice or hand-fed. "Hand-fed" CTC-medicated feeds are provided and consumed daily (0.5 mg CTC/lb of BW daily) whereas "free-choice" CTC-medicated feeds are kept constantly available to the animal and intend to provide 0.5 to 2.0 mg CTC/lb of BW daily. The free-choice feeding method presumes that the animal

will balance its own diet based on individual nutritional needs. There are two public (6000 g CTC/ton) and three proprietary (700, 5000, 8000 g CTC/ton) FDA-approved free-choice CTC-medicated feed formulations indicated for the control of anaplasmosis. All of these formulations are intended to deliver a dosage of CTC that will fall within the approved range when consumed in a free-choice manner (0.5 to 2.0 mg CTC/lb body weight/day). In a statewide survey of Kansas cattle producers, CTC-medicated feed products were used by 25.3% (109/431) of respondents, of which 76.1% (83/109) reported year-round use and only 23.9% (26/109) reported CTC use in the spring and summer months [12].

As antimicrobials have broad activity against multiple bacterial species, the use of antimicrobials to control one disease (i.e., CTC to control anaplasmosis) may have unintended consequences on other microbial community members (i.e., AMR development in off-target microbes such as *Escherichia coli*). Fecal shedding of resistant *E. coli* is common in cattle and is a public health concern due to the risk of foodborne transmission that can result in severe, or even fatal, disease in people [18]. Tetracycline resistance among *E. coli* in cattle is relatively common [19–22]. Previous studies demonstrated that exposure to in-feed CTC was associated with a temporary increase in the likelihood of recovering resistant bacteria [23,24]. In feedlot cattle, in-feed CTC administration (10 mg CTC /lb of body weight/day) for 5-days temporarily increased fecal tetracycline-resistant *E. coli* but did not impact long-term resistance in the *E. coli* population [19]. In a 314-day study where feedlot steers received CTC as a top-dress (11 ppm Aureomycin), 47.1% (3413/7184) of the *E. coli* isolates were found to be resistant [25]. Long-term use of medically-important antimicrobials such as CTC for anaplasmosis control may contribute to expediting the development of CTC-resistant bacteria in off-target commensal bacteria species (i.e., *E. coli*) in the bovine gastrointestinal tract. Therefore, the objective of this

study was to determine the CTC susceptibility of *E. coli* isolated from anaplasmosis endemic beef cattle herds provided different FDA-approved free-choice CTC-medicated mineral formulations in a pasture setting. Regardless of CTC-medicated mineral formulation, the susceptibly profile of *E. coli* was hypothesized to decrease as treatment length increased, with little to no difference among approved CTC formulations intended to provide the same CTC dosages. Critical evaluation of antimicrobial treatment outcomes is not only important for the intended microbe target but off-target susceptible microbes as well. Understanding off-target implications of broad-acting drugs are important when developing drug use protocols and policy to both protect drug effectiveness and minimize broader unintended impacts.

MATERIALS AND METHODS

The Kansas State University Institutional Animal Care and Use Committee reviewed and approved all animal handling and animal care practices used in this study (IACUC #: 3858.1). All procedures were conducted in accordance with the Guide for the Care and Use of Animals in Agricultural Research and Teaching [26].

Experimental Design

Study herd

A portion of the Kansas State University Cow-Calf herd (n = 245), a closed-herd, comprised of Hereford-Angus cows, naturally endemic for anaplasmosis, was used in this study. Animals were grazed in five different pastures: Goheen (G), Texas Hog (TH), Shane Creek (SC), South Konza (SK), and North Konza (NK) located in the Konza Prairie research facility (39.1069° N, 96.6091° W) (May-Oct) 5 miles south of Manhattan, KS, USA. Cow age ranged from 1 to 16 years old. Pastures were stocked at a rate of 3.24 hectares per cow for 150 days.

Pastures were dominated by big bluestem (*Andropogon gerardii*), little bluestem (*Schizachyrium scoparium*), Indian grass (*Panicum virgatum*), and sideoats grama (*Boutelua curtipendula*) [27].

Treatment groups

Treatment groups were randomly assigned by pasture allocation. Pasture location and allocation of animals into their respective pastures were determined based on their involvement in a concurrent unrelated study. Therefore, animal movement amongst the pastures to correct for an unequal number of cows per treatment group was prohibited. Chlortetracycline-medicated mineral formulations (700, 5000, 6000, 8000 g CTC/ton) were randomly assigned to pastures TH, SC, SK, and NK, respectively (i.e., 700 g CTC/ton Aureo Anaplaz C700 Pressed [Sweetlix Livestock Supplements, Mankato, MN, USA]; 5000 g CTC/ton, Purina Anaplasmosis Block [Purina Animal Nutrition, Gray Summit, MO, USA]; 6000 g CTC/ton, Stockmaster Aureo FC C6000 Mineral [Hubbard Feeds, Mankato, MN, USA]; 8000 g CTC/ton, MoorMan's Special Range Minerals AU 168XFE [ADM Animal Nutrition, Quincy, IL, USA]; Table 2.1). Pasture G served as the untreated control, receiving a non-medicated free-choice mineral supplement (i.e., 0 g CTC/ton). The remaining herds, in their respective pastures, were provided one of the four approved free-choice CTC-medicated mineral formulations *ad libitum* from early June through late October in 2017. The animals in the study had not been fed CTC for at least 17 months prior to this study; undetectable plasma-CTC concentrations were confirmed in cows prior to the initiation of this study. Details of CTC-medicated mineral administration to these cattle herds have previously been published [27]. Briefly, cattle were acclimated to non-medicated mineral for 14 days prior to sample collection. Mineral feeders were monitored and filled once a week. One mineral feeder was provided for every 10 cows in individual pastures.

Sample Collection and Processing

Fecal collection

Study animals were gathered from their grazing pasture and fecal samples were collected approximately monthly from June 2017 to October 2017. Data were collected at six time points, including 6/5/17 (Baseline), 6/26–6/28 (JUN), 7/27/17 (JUL), 8/30/17 (AUG), 9/25/17 (SEP), and 10/23–10/31 (OCT). At Baseline, fecal samples were collected from six (n = 6) or ten (n = 10) randomly selected cattle per treatment group (Supplemental Table A.1). The same subset of cattle was utilized at each time for subsequent fecal collections and blood draw. Some time points did not have all ten fecal samples collected because the cattle could not be feasibly gathered and restrained. After collection, aliquots of individual fecal samples were resuspended in glycerol in two 10 mL tubes at an approximate 1:1 ratio of the fecal matter: glycerol and stored at −80 °C.

Escherichia coli isolation

Fecal samples were cultured for *E. coli* isolates using our previously published procedures [28,29]. In brief, approximately 1 g of fecal sample was homogenized with 10 mL of phosphate-buffered saline. Then, 50 µL of the fecal suspension was plated onto a MacConkey Agar and incubated at 37 °C for 24 h. For each fecal sample, two putative lactose-fermenting isolates (i.e., biological replicates) were selected from each plate for further characterization. Each isolate was individually streaked onto a blood agar plate (Remel, Lenexa, KS, USA) and incubated at 37 °C for 24 h. The isolates were confirmed as *E. coli* via the Spot Indole Test (described below). For each fecal sample, two *E. coli* isolates were identified and tested for CTC susceptibility (described below).

Spot indole test

Cultured blood agar plates were removed from the incubator after 24 h. Filter paper placed in a petri dish was saturated with James' reagent (bioMérieux, St. Louis, MO, USA). A bacteriologic loop was used to remove a small portion of the bacterial isolate from the agar surface and the sample was rubbed onto the filter paper. A bacterial isolate is positive for indole if the filter paper turns a pink color within 30 s (*E. coli* positive). No color development is considered negative for indole production (*E. coli* negative). All confirmed indole positive *E. coli* isolates were stored in cryoprotectant beads CryoBeads™ (KEY Scientific Products, Stanford, TX, USA) at −80 °C. Two *E. coli* isolates were stored for each sample.

Chlortetracycline (CTC) susceptibility testing of Escherichia coli isolates

The micro-broth dilution method was used to determine the minimal inhibitory concentrations (MIC) of *E. coli* isolates in response to CTC. The *E. coli* isolates were tested as previously described [30,31] using a modified version of the CLSI guidelines [32]. The modification was using a slightly different two-fold dilution scheme (100, 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39, and 0.195 µg/mL) instead of (128, 64, 32, 16, 8, 4, 2, 1, 0.5, and 0.25 µg/mL). Each isolate stored in cryoprotectant beads was streaked onto a blood agar plate and incubated at 37 °C for 24 h. Individual colonies were suspended in 10 mL Mueller-Hinton II (MH) Broth and the turbidity was adjusted to 0.5 McFarland turbidity standards. A 1:100 dilution of culture was prepared by adding 50 µL (0.05 mL) of the isolate culture into 5 mL of MH broth.

Next, a micro-dilution was prepared in a 96-well U-bottom plate. The first column of the plate served as a bacterial growth control (no antibiotic), while the second column served as antibiotic control (no bacterial inoculum). Forty microliters of the CTC stock solution (100 µg/mL) was added to the plate beginning in column #2. This was mixed well by repeat pipetting

and a serial dilution was achieved by mixing and transferring 100 µL from column #2 to column #3 and so on until the last column. The remaining 100 µL from the last column was then discarded. Each column except column #2 were inoculated with 100 µL of dilute culture. Lastly, 100 µL of MH broth was added to column #2 (antibiotic control). The final concentrations of CTC in each well are as follows: 100, 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39, and 0.195 µg/mL. Plates were incubated at 37 °C for 18 to 24 h.

The MIC for each *E. coli* isolate was reported as the lowest concentration of the antimicrobial that inhibited visible bacterial growth. For this study, MIC refers to the geometric mean of MICs for two isolates derived from the same sample. For *E. coli*, isolates are classified as susceptible (≤4 µg/mL), intermediate (8 µg/mL), or resistant (≥16 µg/mL) to CTC, based on guidelines established by the CLSI [32].

CTC quantification

Blood was drawn from each animal at the same five, approximately monthly, post-treatment initiation time points when fecal samples were collected. At each time point, approximately 6 mL of blood was collected via coccygeal venipuncture directly into a lithium heparin tube from each cow (Vacuette, Greiner Bio-One North America Inc., Monroe, NC, USA). Blood samples were spun down, plasma collected, and stored at −80 °C until plasma-CTC analysis. Plasma-CTC concentrations for this sample set were previously reported in Reppert et al. (2020) [27].

Statistical Analysis

For the purpose of statistical modeling, MIC of fecal isolates that fell above the upper limit of detection (50 µg/mL) were replaced by twice the detection limit (100 µg/mL); blood

plasma CTC concentration levels that fell below the lower limit of detection (1 ng/mL) were replaced by half of the detection limit (0.5 ng/mL).

The data were subjected to natural-log transformation before linear mixed model analysis to better achieve the model assumptions.

The linear mixed model for log-transformed MIC contained the fixed effects of treatment group (0 g CTC/ton (G), 700 g CTC/ton (TH), 5000 g CTC/ton (SC), 6000 g CTC/ton (SK) 8000 g CTC/ton (NK)), time point (Baseline, JUN, JUL, AUG, SEPT, OCT) and their interaction. Random effects of the model included animal ID (the error term vector corresponding to repeated measurement over time) and animal-ID-by-time-point (the error term vector corresponding to subsampling of two isolates). The variance-covariance structure of animal ID was taken as compound symmetry according to the model fitting criteria. The variance-covariance structure of animal-ID-by-time-point was taken as variance components. The age of cattle served as a covariate. Baseline MIC did not serve as the covariate because it was not available for four cattle. Instead, the present statistical approach considered Baseline as one of the time point levels and adjusted for the Baseline effect via customizing the linear functions of model fixed effects during hypotheses testing and parameter estimation.

The linear mixed model for log-transformed CTC plasma concentration contained the fixed effects of treatment group, time point (JUN, JUL, AUG, SEPT, OCT), and their interaction. Random effects of the model included animal ID (error term vector corresponding to repeated measurement over time). The variance-covariance structure of animal ID was taken as first-order autoregressive according to the model fitting criteria. The age of cattle served as a covariate.

The treatment effect was assessed via back-transforming least squares means (corresponding to medians on the original scale), their standard error (SE), and mean differences (corresponding to ratios of medians on the original scale). Pairwise comparisons were carried out using the 2-sided test. Disregarding results of the test for treatment-by-time point interaction, treatment groups were compared within each time point (Table 2.2); time points were compared within each treatment group; main effect of treatment was also reported and compared (Table 2.3). No multiplicity adjustment was applied. SAS Statistical analysis was executed via Statistical Analysis Software (SAS version 9.4; Cary, NC, USA) PROC MIXED with option DDFM = KR.

RESULTS

Experimental Group Demographics and Sampling

Study animals consisted of cows and heifers from a natural anaplasmosis-endemic cow-calf herd routinely maintained on pasture from May to October. Cattle ranged in age from 1 to 16, however, the animals in the untreated control group (G) were all heifers (Table 2.1). While there was considerable age variability within treatment groups and between the treatment and control groups, there was no statistical difference between age and CTC-medicated mineral formulation or MIC values. Fecal samples were collected from six (n = 6) or ten (n = 10) randomly selected cows per treatment group (Supplemental Table A.1.). The same cattle were utilized each time for subsequent fecal collections and blood draw. Some time points did not have all ten (or six for group NK) fecal samples collected because the cattle could not be feasibly gathered and restrained on pasture (Supplemental Table A.1.).

Evaluation of changes in E. coli susceptibility in cattle offered different free-choice CTC-medicated feed products

Free-choice administration of CTC is frequently used to control anaplasmosis in grazing cattle; however, long-term antimicrobial use may promote the development of AMR in off-target microbial species such as *E. coli* in the bovine gastrointestinal tract. To evaluate the effect of extended CTC administration for anaplasmosis control on off-target microbes, CTC susceptibility was evaluated in *E. coli* isolated from cattle herds provided different FDA-approved, free-choice CTC-medicated feed formulations for five consecutive months. Specifically, the susceptibility of *E. coli* isolates to CTC was evaluated by determining the MIC monthly over the course of CTC administration. The median, minimum, and maximum MIC values for all study groups at each sampling time point are provided in Supplemental Table A.2. Overall, *E. coli* isolates from the NK group (8000 g CTC/ton) had the highest median MIC at four of the six time points, with five of the median MIC values falling into the CLSI resistant (≥16 μg/mL) category. In contrast, *E. coli* isolates from the G group (0 g CTC/ton) demonstrated the most susceptibility to CTC, with the median MIC at two, four, and one time point(s) falling into the CLSI susceptible (<4 μg/mL), intermediate (8 μg/mL), and resistant category (≥16 μg/mL), respectively.

The MIC was evaluated in response to the following effects: animal age ($p = 0.578$), treatment group ($p = 0.037$), time ($p = 0.090$), and treatment group by time point ($p = 0.075$). The only significant effect was treatment group ($p = 0.037$) (Table 2.2) where an overall significant difference between median treatment group MICs was observed in August ($p = 0.027$) and October ($p = 0.009$) (Table 2.3). In August, the median MIC in the 700 g/ton pasture was 10.20 times as high as that in the 6000 g/ton pasture ($p = 0.002$) (Table 2.3). In October, the median MIC value in the 700 g CTC/ton pasture was 8.21 times as high as that in the 5000 g CTC/ton pasture ($p = 0.006$) (Table 2.3). The median MIC data for *E. coli* isolated from cattle

treated with different CTC-medicated mineral formulations over time (Baseline to October) is presented in Figure 2.1. Baseline adjustments were made so each treatment and control group had the same starting MIC value for comparison purposes, with Baseline median MIC values falling in the intermediate (8 µg/mL) CLSI category. Median MIC remained in the intermediate CLSI category throughout the study period with a few exceptions where transient differences were observed (Figure 2.1, Table 2.3). Study group TH (700 g CTC/ton) cows had consistently higher median MICs compared to the other groups (Figure 2.1). While the variability of MIC values was evident across all treatment groups and sampling time points, no distinct pattern (i.e., the trend towards greater resistance as treatment continued) was observed.

Collectively, from study groups provided a CTC-medicated feed product (TH, SC, SK, NK), the distribution of *E. coli* isolate CTC MIC values prior to initiation of CTC treatment and after five months of treatment are presented in Supplemental Figure A.1. Pre-treatment MIC levels (Baseline) ranged from 0–50 µg/mL with approximately half of the animals with a MIC between 6.25–50 µg/mL (intermediate to resistant CLSI category) and approximately 15% with a MIC value > 25 µg/mL (Supplemental Figure A.1.). Post-treatment MIC levels (October) ranged from 1.56–50 µg/mL but were more consolidated with no *E. coli* isolates observed to have a MIC < 1.56 µg/mL (compared to approximately 13% at Baseline) and less than 5% of isolates with MIC values above 25 µg/mL (compared to approximately 15% at Baseline) (Supplemental Figure A.1.). Even though *E. coli* isolates with extremely high and low MIC values were less prevalent in October, overall *E. coli* isolate MIC values had a uniform distribution at both Baseline (May) and the final time point (October) with isolates falling within susceptible to resistant CLSI guideline categories.

The MIC50 and MIC90 were calculated from the cohort of animals used in this study from each treatment group provided a CTC-medicated feed product (TH, SC, SK, NK). Among study animals, their cumulative Baseline MIC50 was 10.7 µg/mL and their cumulative MIC90 was 50 µg/mL. After being provided a CTC-medicated feed product, their cumulative MIC50 and MIC90 remained similar at 12.5 µg/mL and decreased to 25 µg/mL, respectively.

Association/relationship between MIC and plasma CTC levels

In a free-choice administration system, individual animals balance their own diet and choose whether and how much to feed on provided medicated product. Therefore, some animals may consume more or less medicated product on a more or less frequent basis while other animals may consume none. The daily amount of CTC-medicated feed product consumed by individual animals in each study group was not calculated as these cattle were maintained on pasture throughout the study period. Because of expected heterogeneity in CTC-medicated mineral consumption and the half-life of CTC, *E. coli* isolate MIC values were compared among study animals based on the frequency of having detectable plasma CTC from matched blood samples.

The raw plasma CTC concentration data, previously published in Reppert et al., 2020 [27], was re-evaluated to investigate the association between plasma CTC level and changes in MIC collectively among animals provided a CTC-medicated feed product (TH, SC, SK, NK). Plasma CTC concentration data was evaluated in relation to age, treatment group, time, and treatment effect by time point. The only significant effect of plasma CTC concentration was the treatment effect by time point (p = value 0.036). Within time point, there was a significant difference in plasma CTC concentration among treatment groups at two of the five time points: June (p = 0.0011) and October (p = 0.0389) (Supplemental Figure A.2.). The median herd-wide

plasma CTC concentrations, among all cattle with detectable plasma CTC, ranged between 3.9 to 45.3 ng/mL over the treatment period. Of the cohort of cattle evaluated in the present study (n = 36 total from groups TH, SC, SK, NK), plasma CTC was detectable in 137 out of 180 possible samples (36 animals × 4 CTC-treatment groups × 5 time points). Despite the expected intake variability between animals, plasma CTC concentrations remained similar throughout the study.

To further investigate potential changes in *E. coli* CTC susceptibility as a result of offering CTC for a prolonged period of time, *E. coli* isolate MIC was compared from a subset of animals (n = 21) that had detectable plasma CTC concentrations during at least two time points (suggests a greater likelihood for routine CTC-medicated mineral consumption). Frequency of detectable plasma CTC is the number of sampling time points an individual animal had a detectable amount (>1 ng/mL) of CTC in their plasma out of the five post-treatment sampling time points (June-October). For these 21 animals, *E. coli* isolate MIC was compared at Baseline and after five months of continuous CTC provision (October). The majority of these 21 animals harbored *E. coli* isolates with some degree of resistance prior to CTC being administered (Figure 2.2), with 71% (15/21) having Baseline MIC value >4 µg/mL (intermediate CLSI category) (Figure 2.2). Though not statistically analyzed, a positive association between the frequency of detecting plasma CTC and *E. coli* isolate MIC was observed (Figure 2.2).

DISCUSSION

Antimicrobial use in food-producing animals is a critical and unavoidable practice for the treatment of diseases in which vaccines or alternative therapies are not available. Antimicrobial resistance is a by-product that needs to be carefully considered when treating a large number of animals for an extended period of time, as it threatens the effective lifespan of these drugs to treat both animal and human diseases. This study investigated changes in CTC susceptibly of *E.*

coli isolated from pastured cattle provided different free-choice CTC-medicated mineral formulations for the control of anaplasmosis. Anaplasmosis occurs in temperate, tropical, and subtropical regions worldwide and has been reported in almost every state in the U.S. [9]. Pastured cattle frequently receive continuous CTC-medicated feed products for extended periods of time to control anaplasmosis; however, the impact of this antimicrobial usage on other microbial species carried by cattle, such as *E. coli*, is unknown.

There are currently four FDA-approved free-choice CTC-medicated mineral formulations (700, 5000, 6000, 8000 g/ton) labeled for "the control of active anaplasmosis". Regardless of formulation, all are intended to deliver in the target dose range of 0.5 to 2.0 mg CTC per lb of body weight per animal per day. Free-choice medicated mineral is a common method of antimicrobial-based anaplasmosis control used for pastured cattle. Feeding free-choice mineral assumes each individual animal uses its natural instinctive ability to self-regulate intake of the medicated product to satisfy nutrient requirements. With no limit on the duration of use, approved CTC-medicated feed products may be fed for an extended period of time (months to all year), as long as the producer has a valid VFD. In this study, we addressed: (1) the impact of CTC-medicated mineral formulation on the susceptibility profile of *E. coli*; and (2) how the duration of exposure to the mineral product may facilitate the expansion of resistant *E. coli*.

The median *E. coli* isolate MIC values were compared among study groups of pastured cattle provided no CTC (pasture G, untreated control) or one of four different formulations of CTC-medicated mineral from prior to CTC exposure through five consecutive months of CTC exposure (Table 3). Changes in MIC over the treatment period (Baseline-Oct) were evaluated by Baseline adjusting all MIC values to fall in the intermediate CLSI category for *E. coli* isolated from individual fecal samples collected pre-treatment (Figure 2.1). The MIC in response to

treatment ($p = 0.037$) was statistically significant; specifically driven by differences in the median MICs among the five study groups in August ($p = 0.027$) and October ($p = 0.009$) (Table 3). The plotted MIC values only represent a subset of the population ($n = 6$ or $n = 10$) from each pasture; therefore, higher MIC values may be a result of fewer data points skewing the data. While the variability of MIC values was evident across all treatment groups and sampling time points, no distinct pattern (i.e., the trend towards greater resistance as treatment continued) was observed. Despite some differences between individual groups at specific time points, most evaluated *E. coli* isolates remained in the intermediate (8 μg/mL) CLSI category (Figure 2.1).

The physical form of CTC-medicated feed products varied between treatment groups. Pastures TH and SC that had access to 700 g/ton and 5000 g/ton, respectively, received supplemental CTC-medicated mineral in block form, while pastures SK and NK that had access to 6000 g/ton and 8000 g/ton, respectively, received supplemental CTC-medicated mineral in granular form. The block-style supplements are formulated with molasses products to facilitate increased palatability. Therefore, animals in the 700 g/ton pasture may have consumed more CTC, subsequently affecting the MIC values of the animals in that pasture. We can theorize, block-style supplements formulated with molasses may have encouraged greater or more regular consumption, which could drive *E. coli* resistance to CTC. Similar to our findings, Reppert et al., 2020 [27] found that cows exposed to 700 g/ton CTC medicated mineral block had significantly higher dose-adjusted plasma CTC concentrations compared with other pasture groups ($p < 0.0001$). This linear relationship of plasma CTC level and MIC could indicate that exposure to CTC increases the likelihood of subsequent CTC resistance.

The cumulative effect of exposure to CTC and subsequent *E. coli* susceptibility over the treatment period was evaluated by comparing *E. coli* isolates derived from animals prior to CTC

treatment (Baseline) compared to animals that had access to CTC for the previous five months (October) (Supplemental Figure A.1.). As expected, we saw a slight increase in *E. coli* resistance to CTC over the treatment period (Supplemental Figure A.1.). A previous study addressing the concern of tetracycline-resistant *E. coli* following 5-days of in-feed CTC administration found a temporary increase in fecal tetracycline-resistant *E. coli* [19]. This is consistent with our findings, where approximately 12% of the isolates from the Baseline initially in the susceptible category (0, 1.56 µg/mL) were absent during the final sampling time point, suggesting reduced population susceptibility to CTC. However, this increase in resistance is small and may be attributed to the limited sample size (May: 32 animals, October: 25 animals). In addition to sample size, the two isolates used for testing may not provide a clear representation of the entire *E. coli* bacterial population in the bovine fecal microbiome.

For all treatment groups (TH, SC, SK, NK), at Baseline the MIC50 was 10.7 µg/mL and the MIC90 was 50 µg/mL. Similarly, for all treatment groups, the final time point in October the MIC50 was 12.5 µg/mL and the MIC90 was 25 µg/mL. Although all animals in each treatment group had access to one of the medicated mineral formulations, this does not guarantee each animal regularly consumed a consistent amount of CTC-medicated product (discussed below). Furthermore, MIC50 and MIC90 are metrics commonly used in antimicrobial susceptibility analyses for large populations; however, due to our limited sample size (May: 32 animals, October: 25 animals), the value of these metrics decreases.

Dose-adjusted plasma CTC concentrations were previously reported in Reppert et al., 2020 [27]. The raw plasma CTC concentration data was re-evaluated in this study to investigate the association frequency of detectable CTC level and *E. coli* isolate MIC. A range of variability in plasma CTC concentrations was expected, since the CTC-medicated mineral product is

provided in a free-choice manner, allowing individual animals to consume varying amounts based on individual needs. However, all CTC-medicated mineral formulations are intended to deliver the same dosage (0.5 to 2.0 mg CTC/lb of BW daily) of CTC. Despite variable intake expected in a pasture setting, CTC concentrations remained overall similar between treatment groups (Supplemental Figure A.2.). The mean plasma CTC concentrations determined in this study are consistent with a previous study where cattle were administered hand-fed CTC-medicated product on pasture. The mean plasma CTC concentration in cattle on pasture ranged from <0.1–51.1 ng/mL [33]. As samples (fecal and blood) were collected only once per month; individual animal mineral consumption patterns, including the timing of last medicated mineral consumption prior to sample collection, may impact fecal *E. coli* isolate MIC and blood plasma CTC level. In steers that were hand-fed CTC in a feedlot setting, the mean half-life of CTC was determined to be 16.2 h [34]. Using this half-life, in an animal that consumed CTC-medicated mineral 24 h prior to sampling collection, the plasma CTC concentration would be near elimination. Universal and uniform consumption of free-choice mineral produced by individuals in a herd should not be assumed [35].

While each treatment group was provided one of the FDA-approved CTC-medicated mineral formulations (all intended to deliver 0.5 to 2.0 mg CTC/lb of BW daily), this does not ensure all the study animals in each treatment pasture equally consumed the medicated mineral. Variation in mineral intake can be attributed to environmental conditions, pasture topography and size, soil fertility, climate changes, location of water and shade, access/location of mineral feeders, frequency of sampling, and more. To address this likelihood of variable medicated mineral intake and its impact on *E. coli* isolate MIC values, a subset of animals (n = 36) were evaluated based on the percentage of sampling time points with where they had detectable CTC

levels (Figure 2). Unsurprisingly, most animals at Baseline (prior to any exposure to CTC), harbored *E. coli* isolates with CTC MIC values >4 µg/mL (Figure 2.2). According to CLSI guidelines, this breakpoint indicates the majority of the isolates at Baseline were in either the intermediate or resistance categories. Tetracycline resistance among *E. coli* in cattle is relatively common [23]. This is consistent with our findings displayed at Baseline in Figure 2.2, there is a level of inherent resistance within the microbial community independent of CTC exposure. These findings support previous research showing that resistance to tetracycline is widespread [36] and prevalent in the ruminant intestinal microflora even when animals are not fed antibiotics [25]. Additionally, an upward trend in (MIC) resistance relative to the frequency of detectable CTC was observed in the *E. coli* isolates (Figure 2.2).

Measuring drug intake and evaluating drug efficacy based on intake in pastured cattle provided free-choice medicated feed can be challenging due to sampling access as well as a multitude of biotic and abiotic variables that can influence consumption of the medicated product [37]. Most commonly, producers will have their herd in a pasture with access to *ad libitum* forage and supply a free-choice mineral supplement, as was done in this study. The inherent variability of CTC intake makes it challenging to explore the linkage between CTC consumption and antimicrobial resistance in *E. coli*. Future studies are needed to evaluate the off-target impact of long-term antimicrobial-based anaplasmosis, control on other microbial species, in different production settings, among cattle of various signalments, with greater sample sizes, and more sampling time points.

CONCLUSION

The susceptibly profile of *E. coli* isolates was investigated in anaplasmosis-endemic pastured cattle herds provided different free-choice formulations of CTC-medicated mineral for

five consecutive months to control anaplasmosis. No pattern or clear development of resistance was observed in *E. coli* isolated from CTC-treated cattle; however, variations in medicated mineral consumption patterns among sampled cattle may have affected the results.

While the limitations discussed above hinder our ability to state definitive conclusions, the aspects of the study align with real-world scenarios. Antimicrobial-based anaplasmosis treatment and control options are limited and continuously becoming more regulated. To preserve the efficacy and longevity of medically-important antimicrobials, it is critical to periodically (re)evaluate their effectiveness against their intended targets (i.e., *A. marginale*) as well as investigate potential off-target implications (i.e., promoting antimicrobial resistance in off-target *E. coli*). Additional studies evaluating the off-target impact of long-term antimicrobial-based anaplasmosis control in different production settings, with greater sample sizes and sampling time points are needed. The relationship between continuous CTC administration and development of antimicrobial resistance of off-target species is complicated and requires continued investigation.

Treatment Group (CTC)[1]	Control-or-Medicated Feed	Pasture	# of Grazing Animals (3.24 ha/cattle)	Max # of Animals Sampled (fecal samples)[2]
0 g/ton (G)	Control	Goheen	51	10
700 g/ton (TH)	700 g/ton	Texas Hog	50	10
5,000 g/ton (SC)	5,000 g/ton	Shane Creek	59	10
6,000 g/ton (SK)	6,000 g/ton	South Konza	56	10
8,000 g/ton (NK)	8,000 g/ton	North Konza	29	6

[1] FDA-approved free-choice CTC-medicated feed formulations. All are intended to deliver 0.5 to 2.0 mg CTC/lb body weight/day.

[2] Fecal samples were collected from a subset of randomly selected cattle per treatment group. The same cattle were utilized each time for subsequent fecal collections.

Table 2.1. Treatment groups and the number of fecal samples collected for analysis.

p-value for Testing Overall Treatment Effect	Treatment	MIC (µg/ml) Baseline Adj. Median+/-SE		Ratio (*p*-value) Comparison Between Groups			
				700 g/ton (T)	5000 g/ton (SC)	6000 g/ton (SK)	8000 g/ton (NK)
0.037	0 g/ton (G)	5.5 +/- 1.2	A	0.17 (0.003)	0.56 (0.290)	0.73 (0.585)	0.50 (0.276)
	700 g/ton (TH)	31.8 +/-18.0	B	--	3.24 (0.038)	4.25 (0.014)	2.89 (0.104)
	5000 g/ton (SC)	9.8 +/- 5.3	A	--	--	1.31 (0.630)	0.89 (0.856)
	6000 g/ton (SK)	7.5 +/- 4.2	A	--	--	--	0.68 (0.553)
	8000 g/ton (NK)	11.0 +/- 6.9	AB	--	--	--	--

Table 2.2. Across-time comparison of median MIC values in treatment groups of pastured cattle provided four different FDA-approved formulations of CTC-medicated mineral products. Treatment groups with the same letter were not significantly different in their pairwise comparison.

Time	*p*-value for Testing Overall Treatment Effect	Treatment	MIC (µg/mL) Baseline-Adj. Median+/-SE		Ratio (*p*-value) Comparison* To			
					700 g/ton (TH)	5,000 g/ton (SC)	6,000 g/ton (SK)	8,000 g/ton (NK)
Baseline	--	--	9.3 +/- 3.5		--	--	--	--
Jun	0.113	0 g/ton (G)	5.9 +/- 2.4	A	0.18 (0.023)	0.48 (0.295)	1.10 (0.899)	0.59 (0.519)
		700 g/ton (TH)	32.3 +/-21.3	B	--	2.63 (0.177)	6.03 (0.016)	3.22 (0.163)
		5,000 g/ton (SC)	12.3 +/- 7.5	AB	--	--	2.29 (0.233)	1.22 (0.801)
		6,000 g/ton (SK)	5.3 +/- 3.4	A	--	--	--	0.53 (0.445)
		8,000 g/ton (NK)	10.0 +/- 7.5	AB	--	--	--	--
Jul	0.287	0 g/ton (G)	3.1 +/- 1.4	A	0.21 (0.045)	0.23 (0.069)	0.41 (0.250)	0.41 (0.324)
		700 g/ton (TH)	14.8 +/- 9.8	B	--	1.08 (0.919)	1.98 (0.360)	1.99 (0.431)
		5,000 g/ton (SC)	13.7 +/- 9.6	AB	--	--	1.83 (0.443)	1.83 (0.504)
		6,000 g/ton (SK)	7.5 +/- 4.8	AB	--	--	--	1.00 (0.996)
		8,000 g/ton (NK)	7.5 +/- 5.9	AB	--	--	--	--
Aug	0.027	0 g/ton (G)	7.5 +/- 3.2	A	0.15 (0.014)	0.40 (0.206)	1.54 (0.570)	0.60 (0.535)
		700 g/ton (TH)	49.4 +/-32.4	B	--	2.66 (0.173)	10.20 (0.002)	3.98 (0.091)
		5,000 g/ton (SC)	18.6 +/-11.3	AB	--	--	3.84 (0.062)	1.50 (0.603)
		6,000 g/ton (SK)	4.8 +/- 3.2	A	--	--	--	0.39 (0.250)
		8,000 g/ton (NK)	12.4 +/- 8.9	AB	--	--	--	--
Sep	0.251	0 g/ton (G)	16.0 +/- 5.8	AB	0.38 (0.190)	2.18 (0.294)	1.39 (0.644)	1.20 (0.823)
		700 g/ton (TH)	42.4 +/-28.7	B	--	5.75 (0.027)	3.68 (0.088)	3.17 (0.177)
		5,000 g/ton (SC)	7.4 +/- 4.9	A	--	--	0.64 (0.556)	0.55 (0.484)
		6,000 g/ton (SK)	11.5 +/- 7.4	A	--	--	--	0.86 (0.857)
		8,000 g/ton (NK)	13.4 +/-10.0	AB	--	--	--	--

Time	*p*-value for Testing Overall Treatment Effect	Treatment	MIC (µg/mL) Baseline-Adj. Median+/-SE		Ratio (*p*-value) Comparison* To			
					700 g/ton (TH)	5,000 g/ton (SC)	6,000 g/ton (SK)	8,000 g/ton (NK)
Oct	0.009	0 g/ton (G)	2.3 +/- 1.0	A	0.07 (<.001)	0.57 (0.462)	0.22 (0.050)	0.17 (0.096)
		700 g/ton (TH)	32.5 +/-21.9	C	--	8.21 (0.006)	3.09 (0.145)	2.51 (0.378)
		5,000 g/ton (SC)	4.0 +/- 2.5	AB	--	--	0.38 (0.186)	0.31 (0.244)
		6,000 g/ton (SK)	10.5 +/- 6.9	BC	--	--	--	0.81 (0.839)
		8,000 g/ton (NK)	12.9 +/-12.4	AB	--	--	--	--

Table 2.3. Within-time comparison of median MIC values in treatment groups of pastured cattle provided four different FDA-approved formulations of CTC-medicated mineral throughout five months continuous CTC treatment. Treatment groups with the same letter were not significantly different in their pairwise comparison.

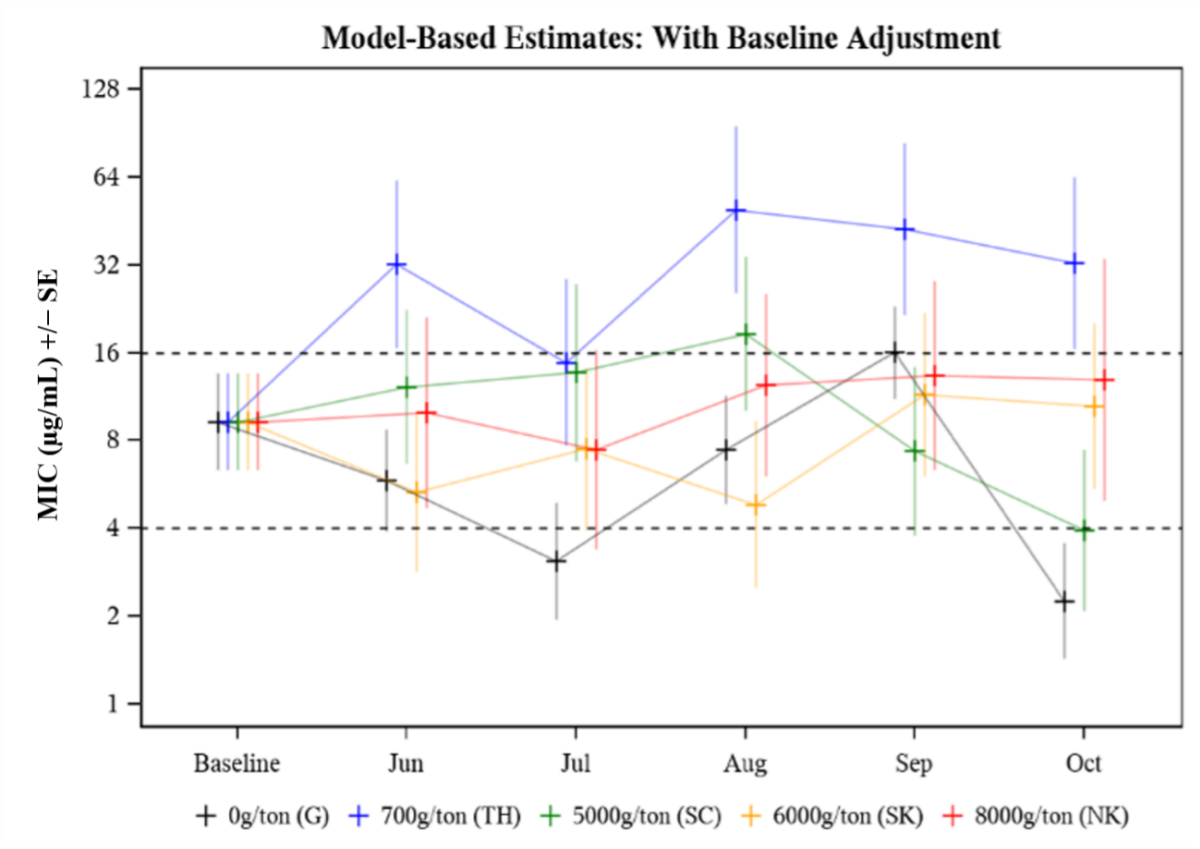


Figure 2.1. Model-based estimates of median minimum inhibitory concentration (MIC) for *E. coli* isolated from cattle herds provided different FDA-approved CTC-medicated mineral formulations to control *A. marginale*. Baseline adjusted MIC for *E. coli* recovered from cattle treated with different CTC-medicated mineral formulations for six months.

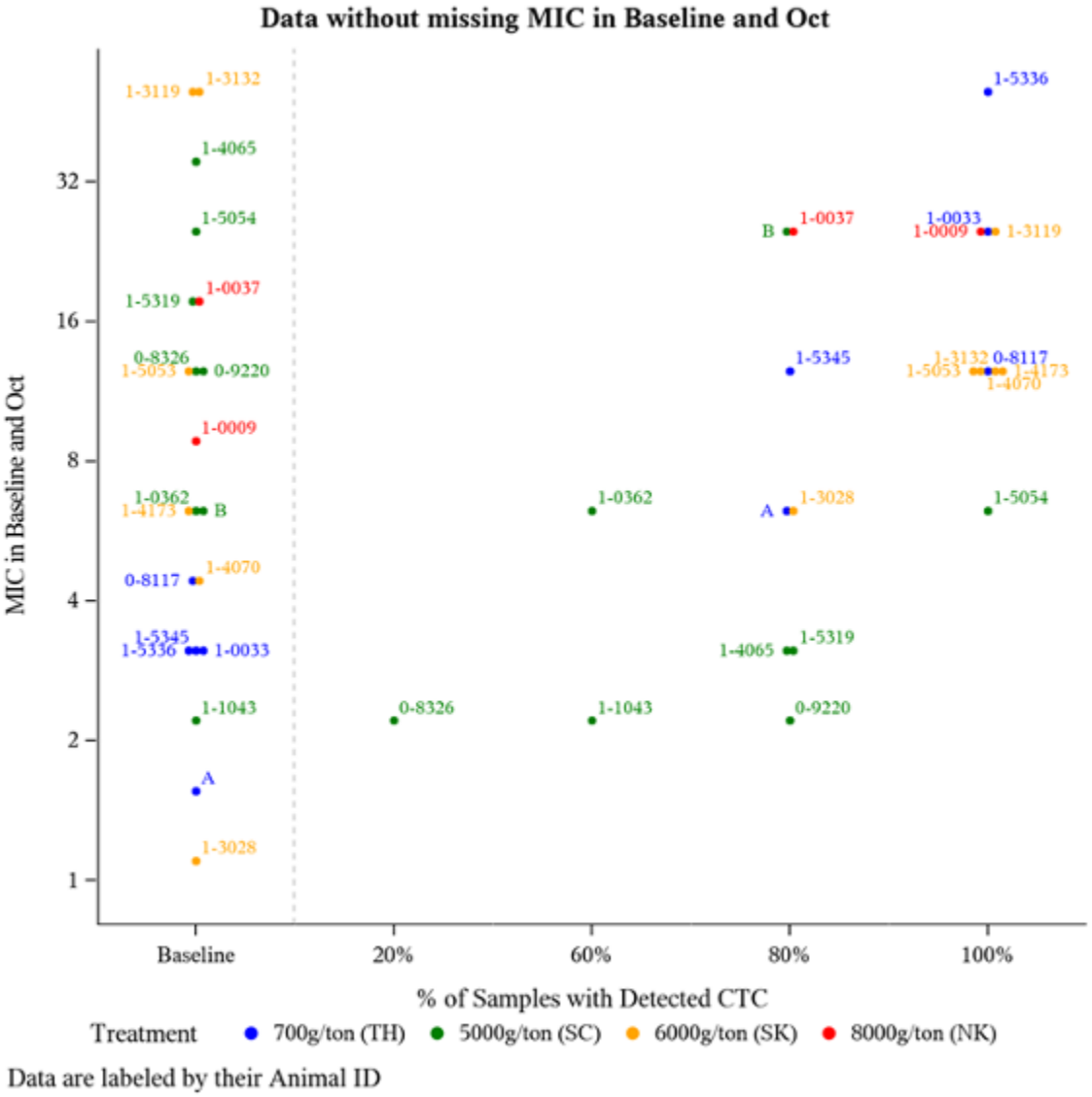


Figure 2.2. Distribution of *E. coli* isolate MIC levels pre-CTC treatment versus post-CTC treatment relative to the frequency of detectable plasma CTC levels. Of the 36 animals with detectable plasma CTC concentration at a minimum of 2 sampling time points, 21 animals had a MIC measurement at both Baseline (May) and the final time point in October.

APPENDIX A - SUPPLEMENTARY MATERIALS

Pasture	Treatment Group[1]	# of Animals Fecal Sampled at Each Time Point						Total # of fecal samples tested per pasture
		Baseline[2]	June	July	Aug	Sept	Oct	
Goheen	0 g/ton	9	9	8	8	10	7	51
Texas Hog	700 g/ton	10	9	8	8	7	8	50
Shane Creek	5,000 g/ton	10	10	9	10	9	8	56
South Konza	6,000 g/ton	10	10	10	10	10	9	59
North Konza	8,000 g/ton	6	6	4	6	5	2	29
								245

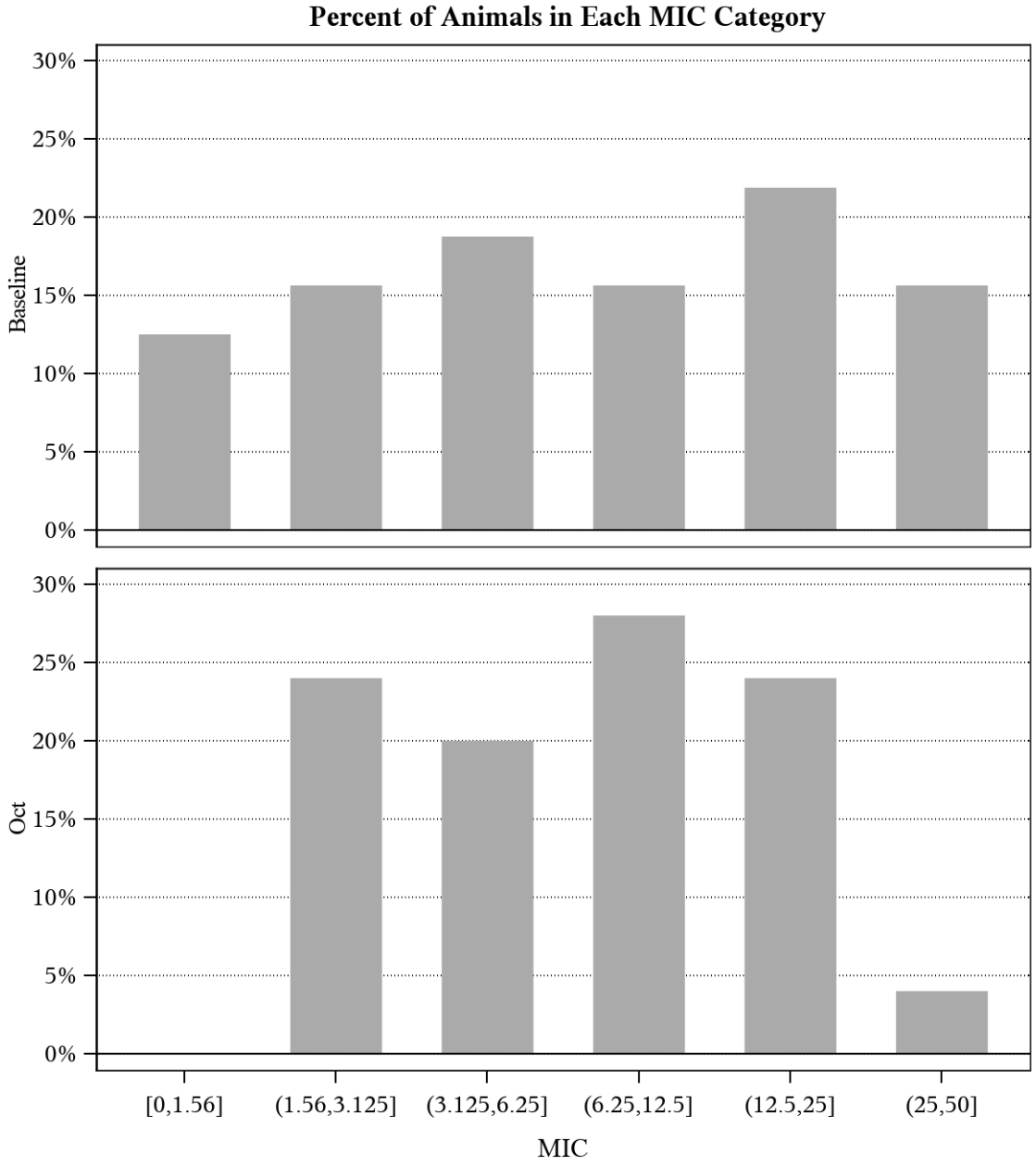
[1] All formulations are intended to deliver 0.5 to 2.0 mg CTC/lb body weight/day.

[2] Baseline is prior to any exposure to CTC.

Supplemental Table A.1. # of animals fecal sampled within each treatment group at each time point.

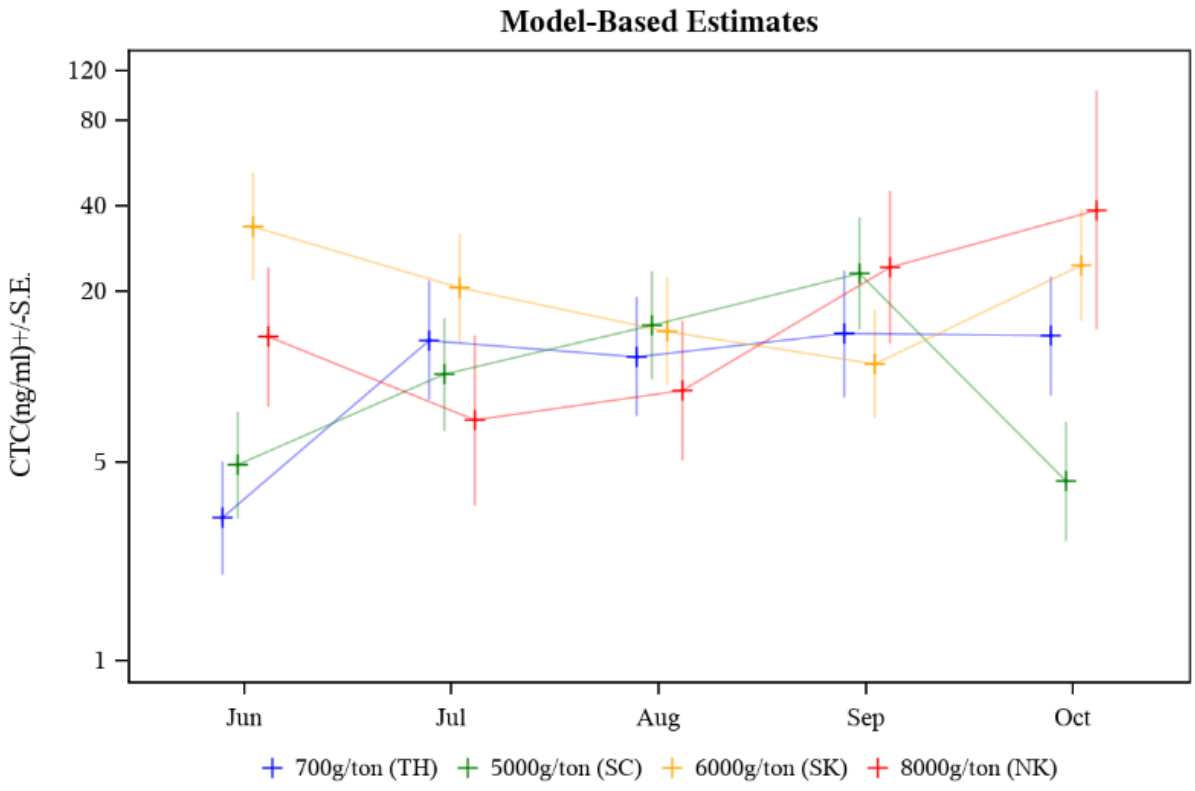
		MIC: Geometric Mean of 2 Isolates				
		# of Animals	Median	Min	Max	# of Isolates >50
Baseline	0g/ton (G)	9	8.839	0.552	50	0
	700g/ton (TH)	8	3.125	1.56	50	0
	5000g/ton (SC)	10	12.5	1.56	35.36	1
	6000g/ton (SK)	8	9.375	1.103	50	0
	8000g/ton (NK)	6	17.68	8.839	50	0
Jun	0g/ton (G)	8	6.247	1.56	35.36	0
	700g/ton (TH)	8	21.34	0.78	>50	2
	5000g/ton (SC)	10	18.75	2.208	50	0
	6000g/ton (SK)	10	6.247	0.39	25	0
	8000g/ton (NK)	5	25	12.5	35.36	0
Jul	0g/ton (G)	6	3.125	1.56	6.25	0
	700g/ton (TH)	8	6.627	0.552	50	0
	5000g/ton (SC)	5	17.68	2.208	50	0
	6000g/ton (SK)	9	6.25	0.78	50	0
	8000g/ton (NK)	4	12.5	12.5	25	0
Aug	0g/ton (G)	7	6.25	1.56	25	0
	700g/ton (TH)	8	21.34	6.25	50	0
	5000g/ton (SC)	10	25	4.419	50	0
	6000g/ton (SK)	8	5.333	1.56	50	1
	8000g/ton (NK)	6	25	12.5	50	0
Sep	0g/ton (G)	10	26.52	0.78	>50	2
	700g/ton (TH)	7	25	3.125	50	0
	5000g/ton (SC)	6	8.839	1.103	25	0
	6000g/ton (SK)	9	12.5	3.122	>50	1
	8000g/ton (NK)	5	25	25	35.36	0

Supplemental Table A.2. Median, minimum, and maximum MIC values for all study groups at each sampling time point.



MIC refers to the geometric mean of MICs for two isolates

Supplemental Figure A.1. Distribution of MIC values from *E. coli* isolates derived from study groups provided a CTC-medicated feed product at Baseline (prior to CTC exposure) and October (5 months continuous treatment). This data was derived from a subset of the animals that had a MIC value available at baseline or the final sampling time point (Baseline: 32 animals, October: 25 animals). CLSI breakpoints for *E. coli* susceptible (≤ 4 μg/ml), intermediate (8 μg/ml), or resistant (≥ 16 μg/ml) to CTC.



Supplemental Figure A.2. Model-based estimates of median plasma CTC values in each treatment group over the treatment period.

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Chapter 3 - Antimicrobial activity of chlortetracycline, and its transformation products 4-epi chlortetracycline and iso-chlortetracycline, against enteric and environmental bacteria

Alyssa R Toillion[*], Raghavendra G Amachawadi[*¶], and Michael D Apley[*]

[*]Department of Clinical Sciences, Kansas State University, Manhattan, KS, 66506, USA; toillion@vet.k-state.edu, agraghav@vet.k-state.edu, mapley@vet.k-state.edu

[¶]Corresponding author: Raghavendra G Amachawadi, Department of Clinical Sciences, College of Veterinary Medicine, Kansas State University, 1620 Denison Avenue, Manhattan, KS, 66506, USA. Email: agraghav@vet.k-state.edu

ABSTRACT

Chlortetracycline (CTC) is a widely used antimicrobial in food animal production and is actively excreted via feces and urine. Widespread use and frequent detection of CTC in the environment have raised concerns regarding the emergence, amplification, and transmission of resistance. Resistance associated with CTC has been extensively researched, but little has been reported on resistance associated with its transformation products 4-epi-CTC (ECTC) and iso-CTC (ICTC). As an initial step in addressing this knowledge gap, the micro-broth dilution method was used to determine the minimal inhibitory concentrations (MIC) of 5 bacterial species in response to CTC, ECTC, and ICTC. Upon examination our study reported antimicrobial activity of chlortetracycline in *Bacillus subtilis, Campylobacter jejuni, Pseudomonas aeruginosa, Clostridium perfringens,* and *Staphylococcus aureus.* In contrast, 4-epi-CTC, displayed antimicrobial activity against *B. subtilis* and *C. jejuni*. No activity was displayed for iso-CTC in any of the bacteria tested.

KEYWORDS: Antimicrobial Resistance, Bacteria, Chlortetracycline, Iso-CTC, 4-Epi-CTC, Minimum Inhibitory Concentration

INTRODUCTION

Chlortetracycline (CTC) is a tetracycline antibiotic, isolated in the late 1940s from *Streptomyces aureofaciens* (Chopra et al., 2001). The antimicrobial properties of CTC and its transformation products are influenced by chemical structural modifications. These modifications are driven by changes in pH, temperature, ionic strength, and chelation. Under fairly weak acidic and alkaline conditions, an epimer of CTC, 4-Epi-chlortetracycline (ECTC) and the structural isomer, iso-CTC (ICTC) are formed (Soeborg et al., 2014). ECTC is reversibly formed from the modification of the C4 dimethyl amino group and/or in the presence of anions such as phosphate and citrate ions (Lee et al., 2020). Whereas, ICTC is irreversibly formed by cleaving of the C6 hydroxyl group (Chen & Huang, 2009) (Figure 3.1). The antimicrobial activity associated with CTC and other tetracyclines has been extensively researched, but little has been reported on antimicrobial activity associated with ECTC and ICTC. A previous study evaluating the biological activity of tetracycline derivatives demonstrated antimicrobial activity of CTC against *B. subtilis* but no activity from 4-epi-CTC against tested bacteria (Kwon et al., 2011). The objective of this study was to determine the *in-vitro* antimicrobial activity of CTC, ECTC, and ICTC against multiple enteric and environmental bacteria by microbroth dilution.

MATERIALS AND METHODS

Chemicals

Chlortetracycline (CTC) (Sigma Aldrich, St. Louis, MO), iso-chlortetracycline hydrochloride (ICTC) (Cayman Chemical, Ann Arbor, MI), and 4-epi-chlortetracycline (ECTC) (Clearsynth, Mumbai, India) were utilized in this study. Stock solutions of these compounds (1 mg/mL) were prepared in sterilized water for CTC, and 50% DMSO for ECTC and ICTC, and stored at −20°C until further use. Solvent control plates were analyzed for each bacterial *genus*

by replacing 50% DMSO with the antimicrobial (CTC, ECTC, ICTC) in the dilution scheme. The 50% DMSO had no impact on the antimicrobial activity of any bacterial *genus* analyzed.

Bacteria

The bacterial isolates utilized in this study are listed in Table 3.1. Five different strains were analyzed for *P. aeruginosa*, *C. perfringens*, and *S. aureus*; two strains each were analyzed for *B. subtilis* and *C. jejuni*.

Micro-Broth Dilution

Unless otherwise mentioned, all the bacterial isolates stored in cryoprotect beads were cultured using a blood agar plate (Remel Inc., Lenexa, KS) in aerobic condition at 37 °C for 24 h. The *Campylobacter jejuni* isolates were grown in microaerophilic conditions created by an AnaeroPack™ MicroAero Gas Generator (Remel Inc., Lenexa, KS) at 42°C for 24 h. The *Clostridium perfringens* isolates were grown in anaerobic conditions created by an AnaeroPack™ (Remel Inc., Lenexa, KS) at 37 °C for 24 h.

Individual colonies were suspended in 10 mL cation-adjusted Mueller-Hinton II (MH) broth (BD Difco™) and the turbidity was adjusted to 0.5 McFarland turbidity standards (Remel Inc., Lenexa, KS). Bacterial suspensions were vortexed and aliquoted into a 96-well plate (Falcon Corning Brand). The final concentrations of antibiotic in each well were as follows: 100, 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39, and 0.195 µg/mL. Plates were incubated at 37 °C for 20 to 24 h and then manually read by two independent readers. Each bacterium was tested with a biological and technical replicate, with the technical replicate consisting of different times when susceptibility testing was conducted and the biological replicate consisting of two tests within each technical replicate. This resulted in a total of 4 tests per bacterial isolate for each compound. In the text and within Table 3.2, results are reported with an MIC value and the number of

observations for that MIC value within the 4 total replicates (2 technical x 2 biological for each technical replicate). All procedures were conducted in a biological safety hood. Two appropriate ATCC (American Type Culture Collection, Manassas, VA) quality control strains: *Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 27853 were used as reference standards. The minimum inhibitory concentration (MIC) for each isolate was reported as the lowest concentration of the antimicrobial (CTC, ECTC, ICTC) that inhibited visible bacterial growth. Isolates were tested in duplicate on the same 96-well plate (i.e. bacterial replicate) as well as on another 96-well plate (i.e. technical replicate).

STATISTICAL ANALYSIS

Data were entered into a software package (Microsoft Excel 2019, Microsoft Corporation; Redmond, WA) for analysis of values.

RESULTS

Reference standards *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 25923 were tested and found to be within the acceptable QC ranges.

Homogeneity among biological and technical replicates was displayed for all bacteria analyzed. There was only one instance with CTC and one with ECTC where there was a one dilution factor difference in the reported MICs between biological replicates. There were seven differences in MICs between technical replicates for CTC and only one difference between technical replicates for ECTC; each of these was within one dilution factor difference.

Minimum inhibitory concentrations (μg/mL) for each antimicrobial/bacterial combination are shown in Table 3.2. As displayed in the table, the MICs reported in this section will be displayed as individual MIC values with the number of replicates with that respective MIC in parenthesis.

Iso-CTC displayed no activity for any isolate analyzed. *Bacillus subtilis* had MICs of 25 (2), 50 (2), 100 (2), and >100 (2) μg/mL for CTC. Whereas, both *B. subtilis* strains had MICs of 50 (8) μg/mL for ECTC. *Campylobacter jejuni* had MICs of 6.25 (8) μg/mL for CTC and 6.25 (3), 12.5 (5) μg/mL for ECTC. *Pseudomonas aeruginosa* had the following MICs: 6.25 (2), 12.5 (4), 25 (2), 50 (4), 100 (4), >100 (4) μg/mL for CTC. In contrast, the MICs for *P. aeruginosa* to ECTC were: 25 (4) and >100 (16) μg/mL. *Clostridium perfringens* had the following MICs of: <0.195 (2), 0.39 (2), 6.25 (4), 12.5 (2), 25 (2), >100 (8) μg/mL for CTC and had MICs >100 μg/mL for ECTC and ICTC. Lastly, each strain of *Staphylococcus aureus* except for one, reported MICs >100 μg/mL for CTC, ECTC, and ICTC. One *S. aureus* strain had MICs of <0.195 (2), 0.39 (1), and 0.78 (1) μg/mL for CTC. As an outlier among the other *S. aureus* strains analyzed, this particular strain reporting lower MIC values was re-tested and confirmed.

DISCUSSION

Chlortetracycline is a widely used antimicrobial in food animal production to prevent and or treat enteric and systemic diseases. In feed rations for cattle, chlortetracycline is indicated for treatment and control of bacterial pneumonia caused by *Pasteurella multocida,* treatment of bacterial enteritis caused by *Escherichia coli*, control of active infection of anaplasmosis caused by *Anaplasma marginale* and reduction of liver abscesses. In this study, the micro-broth dilution method was used to determine the minimal inhibitory concentrations (MIC) of bacteria in response to CTC and its transformation products: 4-epi-CTC (ECTC), and iso-CTC (ICTC).

The basic structure of tetracyclines are four linearly fused carbon rings, to which different functional groups are attached. For transformation products to retain antimicrobial activity, the fundamental skeletal structure of specific chains of carbon and functional groups must be maintained (Blackwood & English, 1970). In the past, modifications to position C4 (4-epi-CTC)

resulted in reduced antimicrobial activity, whereas modifications at the C6 position (i.e. iso-CTC) proved to result in high antimicrobial activity (Blackwood & English, 1970). Furthermore, a recent study by Tariq et al. (2018) concluded that a dimethylamine group at C4 in ring A, is necessary to show antibiotic properties. As displayed in Figure 3.1, both transformation products (ECTC and ICTC) maintain the necessary C4 dimethylamine group for antimicrobial activity. However, earlier work reported antimicrobial activity may be decreased by epimerization of the natural 4S isomer of C4-dimethylamino group to its 4R isomer (Doerschuk et al., 1995). Evaluation of the antimicrobial activity of CTC and 4-epi-CTC in chicken droppings reported inhibition of bacterial growth at every sampling point for both CTC and 4-epi CTC (Cornejo et al., 2018).

Upon examination of the antimicrobial activities of CTC and its transformation products, our study describes antimicrobial activity of chlortetracycline against *B. subtilis*, *C. perfringens*, *P. aeruginosa*, *C. jejuni* and in one strain of *S. aureus* (Table 3.2). Whereas for ECTC, antimicrobial activity was displayed in *B. subtilis* and *C. jejuni* and in one strain of *P. aeruginosa* (Table 3.2). No antimicrobial activity was displayed for iso-CTC in any of the bacteria tested (Table 3.2). Since CTC has been extensively utilized and undergone years of selection pressures, it is a plausible hypothesis that epimers and isomers may have been previously active have progressed to being microbiologically inactive along with the parent compound.

As a widely used feed additive and probiotic, *Bacillus* spp. are a growing public health concern as microbes used as dietary supplements are potential reservoirs and sources for the transfer of antibiotic resistance genes (Adimpong, 2012; Gevers, 2003; Witte, 2000). Despite this growing concern, there is a limited amount of information available on the antimicrobial susceptibility profiles of *Bacillus* spp. While the enteric pathogens *B. subtilis* and *C. jejuni*

showed some susceptibly to CTC and ECTC, it's important to address that only two strains for each bacterium were analyzed. Of the environmental bacteria analyzed, one strain of *Staphylococcus aureus* reported lower MICs of <0.195, 0.39, and 0.78 µg/mL (CTC) in comparison to its strain counterparts; the four remaining *S. aureus* strains reported MICs of >100 µg/mL for CTC, ECTC, and ICTC. Although it may differ depending on strain, in this study, the antimicrobial activity appeared be consistent among bacteria. In comparison to the enteric bacteria, the higher MICs reported among the environmental bacteria in this study highlights the potential impact of the development and dissemination among antibiotic-resistant bacteria. Similar to other tetracyclines, CTC is not metabolized (Eisner & Wulf, 1963). Cattle excrete a significant proportion of CTC (approx. 75%) directly into the urine and feces unchanged or as an active product (epimers or isomers) of the parent compound (Elmund, 1971; Mackie, 2006; Masse, 2014; Arikan, 2008). Chlortetracycline has been shown to be persistent and prevalent in the environment. Following 10 days of CTC medication, concentrations of CTC, ECTC, and ICTC in pig feces were 281,000 µg/kg, 157,000 µg/kg, and 67,000 µg/kg (Grote, 2004; Arikan, 2009). Similarly, CTC was detected in cattle manure slurry and pig manure slurry at concentrations of 11 µg/kg and 1,435 µg/kg, respectively (Hamscher, 2000; Arikan, 2008). A limitation of this study was the unknown history of the isolates utilized. It is possible that the isolates obtained were previously exposed and therefore displayed more resistance than expected. Additionally, while the limited number of isolates per strain analyzed in this study serve as a guidance for more extensive future research, they should not be interpreted as a representative sample of the bacterial community.

CONCLUSION

We demonstrated similar activity of 4-epi-chlortetracycline and chlortetracycline in *Bacillus subtilis*, *Campylobacter jejuni*, and in one of 4 isolates of *Pseudomonas aeruginosa*. No antimicrobial activity of 4-epi-chlortetracycline was demonstrated in the other 4 isolates of *Pseudomonas aeruginosa* or in *Clostridium perfringens* or in *Staphylococcus aureus*. No antimicrobial activity was demonstrated for iso-chlortetracycline against any of the isolates tested. Future studies are needed to expand this research by including more isolates with known treatment histories.

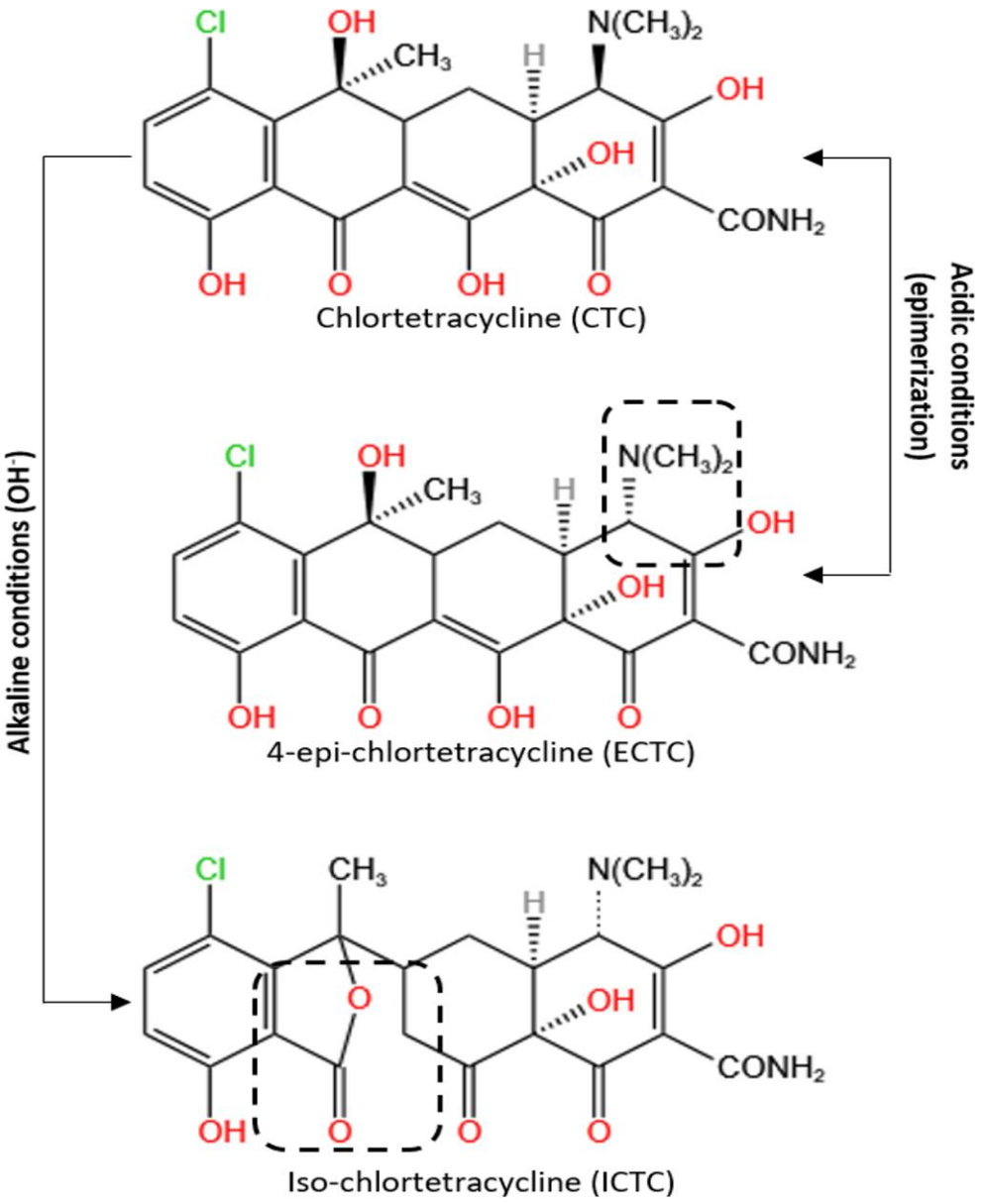

Figure 3.1. Chemical structures of CTC, epimer (ECTC) and isomer (ICTC).
(Created using KingDraw)

Enteric/Environmental	**Bacteria**	**Strain ID**	**Source**
Enteric	*Bacillus subtilis*	*Bacillus subtilis* ATCC 6051	ATCC
		Bacillus subtilis ATCC 23857	
Enteric	*Campylobacter jejuni*	D19-070801-VDL	KSVDL
		D20-005400-VDL	
Environmental	*Pseudomonas aeruginosa*	D22-075287-VDL	KSVDL
		D22-075204-VDL	
		D22-069851-VDL	
		D22-069172-VDL	
		D22-062672-VDL	
Environmental	*Clostridium perfringens*	D22-078022-VDL	KSVDL
		D22-080331-VDL	
		D22-080094-VDL	
		D23-002906-VDL	
		D23-000254-VDL	
Environmental	*Staphylococcus aureus*	D22-076281-VDL	KSVDL
		D22-076073-VDL	
		D22-069968-VDL	
		D22-069837-VDL	
		D22-067748-VDL	

Table 3.1. Enteric and environmental bacterial isolates obtained and tested.

Bacteria Name (No. of strains)	CTC MIC (µg/mL)	ECTC MIC (µg/mL)	ICTC MIC (µg/mL)
Bacillus subtilis (n=2)	**25 (2) 50 (2)**	**50 (4)**	**>100 (4)**
	100 (2) >100 (2)	**50 (4)**	**> 100 (4)**
Campylobacter jejuni (n=2)	**6.25 (4)**	**6.25 (3) 12.5 (1)**	**>100 (4)**
	6.25 (4)	**12.5 (4)**	**>100 (4)**
Pseudomonas aeruginosa (n=5)	**100 (4)**	**>100 (4)**	**>100 (4)**
	25 (2) 12.5 (2)	**25 (4)**	**>100 (4)**
	>100 (4)	**>100 (4)**	**>100 (4)**
	12.5 (2) 6.25 (2)	**>100 (4)**	**>100 (4)**
	50 (4)	**>100 (4)**	**>100 (4)**
Clostridium perfringens (n=5)	**>100 (4)**	**>100 (4)**	**>100 (4)**
	<0.195 (2) 0.39 (2)	**>100 (4)**	**>100 (4)**
	>100 (4)	**>100 (4)**	**>100 (4)**
	6.25 (4)	**>100 (4)**	**>100 (4)**
	12.5 (2) 25 (2)	**>100 (4)**	**>100 (4)**
Staphylococcus aureus (n=5)	**>100 (4)**	**>100 (4)**	**>100 (4)**
	>100 (4)	**>100 (4)**	**>100 (4)**
	<0.195 (2) 0.39 (1) 0.78 (1)	**>100 (4)**	**>100 (4)**
	>100 (4)	**>100 (4)**	**>100 (4)**
	>100 (4)	**>100 (4)**	**>100 (4)**

Table 3.2. Minimum inhibitory concentrations (µg/mL) for each antimicrobial/bacterial combination. These values are reported as individual MIC values with the number of replicates for each isolate with that respective MIC in parenthesis.

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Chapter 4 - Plasma and urine elimination characteristics of three regimens of chlortetracycline administered through the feed to beef cattle

Alyssa R Toillion[*], Michael D Apley[*¶], Johann F Coetzee[§], Kushan Kompalage[$], Nora Schrag[+]

[*]Department of Clinical Sciences, Kansas State University, Manhattan, KS, 66506, USA; toillion@vet.k-state.edu, mapley@vet.k-state.edu

[§]Department of Anatomy and Physiology, Kansas State University, Manhattan, KS, 66506, USA; jcoetzee@vet.k-state.edu

[$]Department of Chemistry, Kansas State University, Manhattan, KS, 66506, USA; kushanpa@ksu.edu

[+]Livestock Veterinary Resources, LLC, Olsburg, KS, 66520, USA; livestockvets@gmail.com

[¶]Corresponding author: Michael D Apley, Department of Clinical Sciences, College of Veterinary Medicine, Kansas State University, 1620 Denison Avenue, Manhattan, KS, 66506, USA. Phone: 785-532-2153. Email: mapley@vet.k-state.edu

ABSTRACT

Thirty crossbred heifers were stratified by initial body weight and randomly assigned into one of four treatment groups. Treatment group (A) received oral chlortetracycline 22 mg/kg of bodyweight daily for 5 consecutive days (n=8), group (B) received 350 mg/head per day (1.5 ± 0.2 mg/kg based on actual bodyweights) for 7 consecutive days (n=8), group (C) received 70 mg/head per day (0.3 ± 0.03 mg/kg based on actual bodyweights) for 7 consecutive days (n=8); and group (D) served as untreated controls receiving no CTC (n=6). On the day after drug administration ceased, 2 animals from group D were added to each of groups A, B, and C, as sentinels. Blood and urine samples were collected at selected time points throughout the 86-day study. Samples were analyzed by an ultrahigh performance liquid chromatography-mass spectroscopy/mass spectroscopy analytical method. Chlortetracycline urine concentrations were

detected for all treatment groups through the end of the study, 79 days post-CTC cessation. Terminal urine T ½ values were 35.4 ± 1.5, 51.7 ± 1.9, and 64.7 ± 1.9 days for groups A, B, and C, respectively. The long-reported urine half-lives suggest chlortetracycline may continue to be detectable in urine for a prolonged period beyond 79 days after ceasing in-feed administration.
KEYWORDS: Cattle, Chlortetracycline, Plasma, Residues, Urine

INTRODUCTION

A recent article noted that chlortetracycline (CTC) was detected in the urine of animals participating in a program designated as "Raised Without Antibiotics" (RWA). The study tested for antibiotics in urine from 312 lots supplied by 33 different RWA-certified feedyards which represented approximately 12% of the total RWA beef production program during a seven-month sampling period. The twenty-six urine samples that tested positive on an initial lateral-flow test, were then analyzed by LC/MS to identify, and quantify specific antimicrobials. Out of the 26 samples analyzed by LC/MS, 21 of the samples were CTC positive with concentrations ranging from 0.2 – 42.8 ng/mL. The authors concluded that "some beef cattle processed for the RWA market have been administered antibiotics," with the majority in their study being confirmed as an exposure to CTC. A key piece of information missing in evaluating these CTC values is the long-term characteristics of CTC elimination in the urine of cattle.

The elimination characteristics of CTC in urine has been previously reported in pigs (Apley, National Pork Board). This swine study evaluated CTC concentrations in multiple tissues as well as urine after 22 mg/kg of body weight CTC per day in feed for 14 days. Detection of CTC in urine and plasma was possible through the end of the study at 28 days post-exposure; Day 21 and 28 samples were very close in concentrations, suggesting a prolonged terminal elimination half-life although there were not sufficient time points to calculate an

elimination halftime. This study also analyzed bone samples, with substantial concentrations of CTC detected in the bone at 28 days, ranging from 375 to 1120 ppb. This disposition of tetracyclines in bone has also been demonstrated in rats (Buyske et al., 1960).

These findings of prolonged presence of CTC in the urine and accumulation in bone, along with the concentrating effect of the kidneys, led to the hypothesis that CTC would be detectable in the urine of cattle for a prolonged period of time beyond detectable plasma concentrations. To investigate persistence in both plasma and urine, the current study utilized three different CTC dosages intended to represent both intentional and incidental exposures. The objectives of this study were to: (1) determine the plasma and urine elimination characteristics of three CTC regimens and, (2) determine environmental exposure of sentinel animals housed with cattle recently receiving different doses of CTC. A third objective of describing the plasma Area Under the Concentration curve (AUC) and relating this to efficacy against common bovine pathogens is reported in a companion paper (Toillion dissertation, Chapter 5).

MATERIALS AND METHODS

This study was approved by the Kansas State University (KSU) Institutional Animal Care and Use Committee (protocol #4740).

Experimental Design

Thirty crossbred heifers of Hereford or Hereford cross breeding, approximately 6 to 10 months old and weighing 246 ± 23 kg (542 ± 50 lbs) were leased from a commercial facility located in Olsburg, KS. The study animals were transported 5 miles by trailer from the commercial facility to the study facility and housed together in a dry lot pen for a 24-hour acclimation period. The heifers were stratified by initial body weight and randomly assigned into one of four treatment groups. Group (A) received CTC at 22 mg/kg of bodyweight daily for 5

consecutive days (n=8); group (B) received CTC at 350 mg/head per day (1.5 ± 0.2 mg/kg) for 7 consecutive days (n=8); group (C) received CTC at 70 mg/head per day (0.3 ± 0.03 mg/kg) for 7 consecutive days (n=8); and group (D) served as untreated controls receiving no CTC (n=6) (Table 4.1). The mg/kg dose for groups B and C based on individual animal bodyweights at the start of feeding were 1.5 ± 0.2 and 0.3 ± 0.03 mg/kg, respectively. In comparison, group A had a mean dose of 22.1 ± 1.5 mg/kg based on the distribution of individual animal bodyweights at the time of treatment initiation. Each treatment group was individually housed in their respective pen after randomization and provided a total mixed ration (TMR) with CTC (Supermix Aureo 10-gram crumbles; Purina, St. Louis, MO) mixed to provide the target dose. The respective CTC dose was mixed into the ration prior to consumption each day.

Initial administration of CTC to treatment groups B and C was designated as study day 1, continuing for 7 consecutive days. Treatment group A (22 mg/kg of bodyweight daily) received CTC beginning on study day 3 and was included in the daily ration for 5 consecutive days, the maximum duration on the label. This allowed all treatment groups to complete their CTC dosage regimen concurrently on study day 7. Using the 16 hr elimination half-life of CTC reported in Reinbold et al. (2010), 97% of steady state would be achieved at 80 hrs (3.3 days) and 99.9% of steady state would be achieved at 112 hours (4.7 days). By feeding treatment group A for 5 consecutive days (the maximum allowed by the label) and groups B and C for 7 days, this gave a reasonable probability that the animals were at or near steady state at the end of the feeding period. Heifers assigned to the control group D were housed separately through day 7 and had no access to medicated feed at any time during the study. On study day 8, these six sentinel animals were randomly allocated and housed with treatment groups A, B, and C (2 sentinel animals per treatment group) for study days 8 through 16.

Each treatment group was housed separately in their respective pen for the first 16 days. Pens were situated so any potential runoff would come from the control group or a 'lower exposure' treatment group. On day 16, all study animals were relocated back to the commercial facility and comingled in a larger dry lot pen for once weekly sampling for the duration of the study. Once a plasma or urine sample was below the assay's level of detection (LOD) twice in a row, then testing of samples from that animal were discontinued. Animals were monitored daily by the investigators or cattle operations staff and received veterinary care as required. No tetracycline class antibiotic was administered to the cattle during the study.

Feeding

The heifers were fed once daily in a manner that ensured that all feed given was consumed each day. Animals were housed in dry lot pens and once a day received a Total Mixed Ration (TMR) consisting of corn silage, ground prairie hay, ground alfalfa and a grain mix consisting of 80% distillers and 20% corn combined with a premix (RU1200 from Suthers containing monensin sodium). The diet was fed at approximately 2% of body weight (dry matter) given as a once a day feeding. The feed was mixed at the commercial facility and transported to the study facility on study days 0 - 16. At the study facility, each pen was provided water *ad libitum* in a separate water source for each treatment group, preventing any cross contamination. For all remaining study days, feed was mixed on site at the commercial facility and water was supplied *ad libitum.*

Chlortetracycline (CTC) preparation

A commercial chlortetracycline product (Supermix Aureo 10-gram crumbles; Purina, St. Louis, MO) was used as a Type B medicated feed. The amount of crumbles delivered to each pen were calculated based on the average bodyweight for treatment group A and based on the

daily amount from groups B and C. Animals in treatment group A (22 mg/kg of bodyweight per day) were re-weighed on study day 3 prior to beginning their CTC treatment regimen to ensure the most accurate CTC dosing on a per pound bodyweight basis. To ensure a uniform distribution of CTC throughout the feed, soybean meal (500 grams) was used as a carrier for the 350 mg/head per day, and 70 mg/head per day treatment groups due to the relatively small amount of pellets to be disbursed in the feed. Immediately after the daily feed ration was delivered to the bunk, the appropriate amount of CTC was manually spread over the surface of the feed and then manually mixed in the feed. The heifers were kept away from the feed bunk until the CTC was thoroughly mixed in with the feed. The length of each feed bunk was adequate to ensure space for all heifers in a pen to eat at the same time. Heifers were fed once daily in the morning. For the first 16 days of the study, feed bunks were inspected and found to be clean by 9 pm each evening. When handling was necessary for sample collections, the animals were individually restrained in a squeeze chute, with use of a rope halter at the time of jugular venipuncture. On days when sample collection coincided with scheduled treatment (CTC feeding), samples were collected prior to feeding of the medicated feed.

Sample Collection

Water samples

Pre-CTC exposure water samples were collected on day -1 at the commercial facility and on day 0 at the study facility. During CTC exposure water samples were collected on days 3 and 6 at the study facility. Post-CTC exposure water samples were collected on day 13 at the study facility, and days 30 and 79 at the commercial facility. Each sample was collected from the respective treatment pens' water tank. However, on day 79, in addition to the pen water tank

sample, the water tank close to the chute where animals came up for sample collection was also sampled.

Water samples were collected via 50 mL conical tubes (ThermoFisher, Waltham, MA, USA), immediately placed on ice, and transported to the lab. Using disposable transfer pipettes, each water sample was aliquoted into five ultra-low temperature cryotubes (Greiner Bio-One, Monroe, NC, USA) and stored at -20°C until further analysis.

Blood samples

Baseline blood samples were collected from all animals in treatment groups A, B, C, and sentinels (n=30) on day 0. Prior to daily CTC feeding, trough plasma samples were collected on days 4, 5, and 6 for treatment groups A and B (n=16). On study days 8, 9, 10, 13, and 16, blood was collected from all animals in treatment groups A, B, and C which now included the sentinel animals placed with each of these groups (n=30). Sampling then occurred once a week for all animals in treatment groups A, B, and C until study completion.

Blood samples were collected via jugular venipuncture into a vacutainer tube containing sodium heparin (BD Vacutainer, Franklin Lakes, NJ, USA). All blood samples were immediately placed on ice and transported to the lab. Samples were centrifuged at 3,000 X g for 10 minutes. Using disposable transfer pipettes, plasma was aliquoted into duplicate ultra-low temperature cryotubes (Greiner Bio-One, Monroe, NC, USA) and stored at -80°C until analysis.

Urine samples

Baseline urine samples were collected from all animals in treatment groups A, B, C, and sentinels (n=30) on day 0. Post-CTC exposure, urine was collected from all animals in treatment groups A, B, and C, which now included the sentinel animals, (n=30) on days 8, 9, 10, 13, and

16. Then sampling occurred once a week for all animals in treatment groups A, B, and C until study completion.

Urine samples were collected in 4.5 oz sterile specimen containers (Medline Industries, Oceanside, CA, USA) by manual stimulation of the area just below the vulva. If manual stimulation was unsuccessful, the heifers were brought back through the chute after a brief rest period and manual stimulation was attempted again. If again unsuccessful, a single furosemide injection (Salix®, Merck Animal Health, Rahway, NJ) dosed at 1 mg/lb of bodyweight was administered intramuscularly using an 18 gauge, 1 ½ inch needle. All urine samples were immediately placed on ice and transported to the lab. Using disposable transfer pipettes, urine was aliquoted into duplicate ultra-low temperature cryotubes (Greiner Bio-One, Monroe, NC, USA) and stored at -80°C until analysis.

Precautions were taken to ensure no cross-contamination during lab processing and handling of samples. Urine samples collected from sentinel animals were handled separately from the remaining samples to ensure no cross-contamination occurred.

Sample Analysis

All matrix samples were analyzed for CTC content using ultrahigh performance liquid chromatography–mass spectroscopy/mass spectroscopy (UPLC–MS/MS). Once a plasma or urine sample was below the assay's level of detection (LOD) twice in a row, sample analysis was discontinued for that animal.

Chemicals and reagents

Chlortetracycline (CTC) hydrochloride and the internal standard (IS) oxytetracycline (OTC) hydrochloride were purchased from Sigma-Aldrich (St. Louis, MD). Optima® LC/MS grade water, methanol, acetonitrile, formic acid, and phosphoric acid were obtained from Fisher

Scientific (Waltham, MA). Untreated bovine serum was purchased from Innovative Research (Novi, MI). Untreated bovine urine was collected from a university donor cow that had never received antimicrobials and was confirmed CTC negative via direct infusion into the Xevo TQ-S mass spectrometer.

Chlortetracycline analysis

Chlortetracycline and OTC were dissolved in water-methanol (1:1) at a concentration of 9.30 mg/mL for each of the respective analytes and stored at -20 °C. Working stocks of CTC at 1 μg/mL and OTC at 50 ng/mL were prepared fresh daily. Quality controls (QCs) were initially prepared and then further diluted in plasma, urine, or LC/MS grade water to the final CTC concentrations: 0.475, 0.95, 4.75, 47.5, 95, and 475 ng/mL. Calibration standards were prepared daily from the 1 μg/mL CTC stock solution at the following concentrations in aqueous 4% phosphoric acid: 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 25, 50, 100, 250, 500, and 1000 ng/mL.

Standards and samples were prepared as previously described (Schrag et al., 2020). In brief, initial dilutions for the calibration standards and QCs were prepared in 1.5 mL Eppendorf tubes and then transferred to an untreated 96-well plate (Fisher Scientific, Waltham, MA). The calibration standards, IS, negative control, QCs, and samples were vortexed separately and then transferred to the Oasis HLB Prime μElution 96-well plates for solid-phase extraction (SPE) following manufacturer specifications. The target compounds (CTC and OTC) within each sample were washed with water-methanol (95:5), eluted with 50 μL of acetonitrile-methanol (90:10), followed by the addition of 50 μL of formic acid (0.2%) and added directly into the collection plate (Waters Co, Milford, MA).

Liquid chromatography

Chlortetracycline concentrations were determined using an Ultra High-Performance Liquid Chromatography system (Acquity UPLC; Waters Corporation, Milford, MA) combined with a Xevo TQ-S mass spectrometer (MS/MS) utilizing a Vici DBS Mistral EVO-40 nitrogen generator. Chromatographic separation was performed with a Waters Acquity HSS T3 column (1.8 µm, 50 x 2.1 mm) maintained at 40 °C during analysis. Mobile phase A and B consisted of formic acid (0.1%) in LC/MS grade water and acetonitrile-methanol mix (90:10), respectively. Separation was achieved at a flow rate of 0.5 mL/min. The following gradient program was used: mobile phase B was increased linearly from 2% to 100% in the first 3 min, kept at 100% from 3.0 to 4.0 min, changed to 2% at 4.01 min, and held constant until the run ended. The total run time was 6 minutes. The autosampler compartment was set at 8 °C and the injection volume was 2 µL.

Mass spectrometry

The analytes were detected using a triple quadrupole mass spectrometer (Xevo TQ-S MS/MS) from Waters Co. (Milford, MD) equipped with an electrospray ionization (ESI) probe operating in positive mode with the following operating parameters: the capillary voltage was 0.5 kV, source temperature 150 °C, desolvation temperature 500 °C, desolvation (Nitrogen) gas flow 1000 L/h, cone gas flow rate 150 L/h, and collision gas (Argon) flow rate of 0.15 mL/min.

Chlortetracycline fragment ions were monitored in the multiple reaction monitoring (MRM) mode with transitions for CTC at m/z 479 → 444 (quantifier) and m/z 479 → 154 (qualifier) with a cone voltage of 16V and collision voltages of 20 and 26V, respectively for the quantifier and qualifier voltages. Oxytetracycline (IS) fragment ions were monitored for transitions at m/z 461 → 426 with a cone voltage of 36V and collision voltage of 18V. Data

acquisition and data quantification were performed using the Waters MassLynx 4.1 and TargetLynx software 4.2 (Waters Corp).

Sample CTC concentrations above the calibration curve linear range were diluted appropriately and re-analyzed. The reproducibility of the data was assessed by running a standard curve each run, multiple sets of QCs, repeated samples, and confirmed clean washes between samples. The LOD and LOQ were determined by inter- and intra- precision and accuracy, following the FDA Bioanalytical Validation guidelines.

Plasma CTC drug concentration analysis

Plasma drug concentrations were determined from whole blood samples collected with evacuated tubes containing sodium heparin. The calibration curve was linear from 0.1 to 1000 ng/mL with a correlation coefficient (R^2) of at least 0.99. The accuracy of this assay was 99% and the precision (CV%) was 5.6%. This was determined by measuring three replicates for each of the QC concentrations (0.475, 0.95, 4.75, 47.5, 95, and 475 µg/mL). Recoveries ranged from 95 to 102%. The limit of detection (LOD) and limit of quantification (LOQ) of the method were 0.25 and 0.5 ng/mL, respectively.

Urine CTC drug concentration analysis

Urine drug concentrations were determined from urine samples collected in 4.5 oz sterile specimen containers. The calibration curve was linear from 0.5 to 1000 ng/mL with a correlation coefficient (R^2) of at least 0.99. The accuracy of this assay was 96% and the precision (CV%) was 7.6%. This was determined by measuring three replicates for each of the QC concentrations (0.95, 4.75, 47.5, 95, and 475 µg/mL). Recoveries ranged from 95 to 98%. The limit of detection (LOD) and limit of quantification (LOQ) of the method were 0.5 and 2.5 ng/mL, respectively.

Due to higher CTC concentrations detected in urine, samples above 1000 ng/mL were diluted appropriately.

Water CTC drug concentration analysis

Water drug concentrations were determined from water samples collected in 50 mL conical tubes. The calibration curve was linear from 0.5 to 1000 ng/mL with a correlation coefficient (R^2) of at least 0.99. The accuracy of this assay was 106% and the precision (CV%) was 6.5%. This was determined by measuring three replicates for each of the QC concentrations (0.95, 4.75, 47.5, 95, and 475 µg/mL). Recoveries ranged from 97 to 112%. The limit of detection (LOD) and limit of quantification (LOQ) of the method were 0.1 and 0.5 ng/mL, respectively.

Feed CTC drug concentration analysis

A sample of 200 grams of the medicated feed product was collected directly from the 50 lb bag of commercial chlortetracycline product (Supermix Aureo 10-gram crumbles; Purina, St. Louis, MO) and sent to the Iowa State Diagnostic Lab for CTC quantification analysis using an LC-MS/MS technique. A concentration of 16,393 ppm of CTC was detected, equating to 74.4% (7.44 grams per lb) of the expected 10 grams per pound was in the product.

STATISTICAL ANALYSIS

Data were entered into a software package (Microsoft Excel 2019, Microsoft Corporation; Redmond, WA). Descriptive statistics were calculated in both Excel and then verified in the statistical program JMP 16 (SAS Institute Inc., Cary). Non-compartmental analysis used the software R Studio Version 1.1.456 (User interface for R statistical software, R Core Team, 2022; R Foundation for Statistical Computing, Vienna, Austria). Data cleaning, calculations, and graphics were produced using R and packages tidyverse (Wickham et al., 2017)

and pracma (Borchers et al., 2022). Linear trendlines were calculated by simple linear regression (geom_smooth, method = ‘lm’). Values below LOQ and above LOD were replaced by the median value between LOQ and LOD. A linear regression model (method = ‘lm’) in base R was used for plotting each phase for each individual animal (Figures 4.1, 4.2, and 4.3) and for half-life calculations (Table 4.2). Two distinct phases of elimination were established via visual evaluation (Figures 4.1, 4.2, and 4.3). Phase I represents study days 8 to 13 and phase II represents days 16 to 86. The brevity of plasma values for treatment groups B and C did not allow for calculation of a phase II T ½.

A mixed model fit by REML ['lmerMod'] was used as a prediction model with time as a fixed effect and individual animal as a random effect. Treatment group was not significant and made no meaningful difference in the predictions; therefore, it was excluded from the final model. This model allows for both a random slope and intercept for each animal. The simulation stopped at 600 days and filtered the result for concentrations that are ≥ to the urine LOD (0.5 ng/mL).

RESULTS

Concentration results in this paper are presented as determined through the assays and are not adjusted back to the target regimens due to the lower than advertised concentration in the medicated feed product. Based on our findings of dose linearity in this study and others, the reader may extrapolate plasma and urine to reflect concentrations that might be detected at the actual target doses. The plasma samples for treatment group A were analyzed to the end of the study (day 86) for 7 out of the 8 animals. For treatment groups B and C, by study day 44 all animals met the criterion for discontinuing analysis. All urine samples (treatment groups A, B,

and C) were analyzed through the end of the study (day 86), as none of the samples from any animal met the criterion for discontinuation.

Plasma CTC Concentrations

Prior to CTC exposure, no study animals in treatment groups A, B, and C had quantifiable (> 0.5 ng/mL) plasma CTC concentrations. Concentrations peaked on day 7 at 676.5 ± 177.8 ng/mL and 35.3 ± 6.3 ng/mL, for treatment groups A and B, respectively. For treatment group C, plasma CTC concentrations peaked on day 8 at 5.5 ± 1.3 ng/mL. On study day 51, one animal in treatment group A met the criteria for ceasing analysis by testing below LOD twice in a row. The remaining 7 animals in group A had detectable concentrations for the remainder of the study. In comparison, six of the eight animals in group B were below LOD twice in a row by day 37, and by the next sampling (day 44) the remaining two animals met the criterion. In treatment group C, five of the eight treatment animals were below LOD twice in a row by day 37, and the remaining three animals met the study criterion for analysis discontinuation on the next sampling (day 44). Mean plasma concentration at 72 days past the last CTC administration period was 0.5 ± 0.1 ng/mL for treatment group A, with the other two groups having already met the study criterion and below LOD.

Concentrations approaching steady state were evaluated by trough concentrations (prior to feeding) collected on the mornings of study days 4, 5, and 6. Treatment group A had mean plasma CTC trough concentrations of 91.2 ± 23.2, 193.1 ± 89.5, and 144.7 ± 45.3 ng/mL for days 4, 5, and 6, respectively. These day 4, 5, and 6 values were 40%, 84%, and 63%, respectively, of the mean day 7 trough concentration of 228.7 ± 106.7 ng/mL. Treatment group B had plasma CTC trough concentrations at 12.8 ± 4.2, 13.8 ± 7.5, and 14.1 ± 5.2 ng/mL for days 4, 5, and 6, respectively. These day 4, 5, and 6 values were 76%, 82%, and 83%, respectively of

the mean day 7 trough concentration of 16.9 ± 6.5 ng/mL. These results suggest that the study animals were approaching steady state by study day 7.

At baseline (study day 0), two of the six sentinels had detectable (> 0.25 ng/mL) plasma CTC concentrations. Incidentally, those two animals were the sentinels which were later randomly allocated to treatment group A; these sentinels maintained the highest plasma CTC concentrations of all the sentinels throughout the study. Only sentinels in group A reached 1 ng/mL. Sentinel animals added in treatment groups B and C were only quantifiable on day 9, and by study day 30 concentrations were undetectable.

Plasma elimination half-lives

The plasma half-life phases for each treatment group are displayed in Table 4.2. Mean ± standard deviation values for plasma T ½ in the initial phase (up to 6 days following the last day of administration) for groups A, B, and C were 0.9 ± 0.1, 1.1 ± 1.1, and 1.6 ± 1.2 days, respectively (Table 4.2). Only group A had sufficient plasma concentrations during the period 9 to 79 days after the last oral administration to allow for plasma T ½ calculation, resulting in a value of 59.6 ± 1.5 days (Table 4.2). The brevity of phase II for treatment groups B and C did not allow for calculation of a phase II T ½.

Urine CTC Concentrations

Prior to CTC exposure in this study, CTC was quantifiable in the urine of all animals in groups A, B, and C with a range of 2.8 to 53.8 ng/mL. For both treatment groups A and B, study days 8, 9, and 10 had high average urine CTC concentrations and by day 13 CTC concentrations began to decline. On study day 8, urine CTC concentrations for treatment group A ranged from 121.3 to 74,928.1 ng/mL. For treatment group B study day 8 urine CTC concentrations ranged from 445.3 to 1,645.2 ng/mL. For treatment group C study day 8 urine CTC concentrations

ranged from 60.1 to 600.6 ng/mL. Mean urine concentrations at 72 days past the last CTC administration period were 7.1 ± 3.4, 2.6 ± 2.9, and 4.1 ± 2.9 days for groups A, B, and C, respectively. These concentrations were lowest for groups A and B, and for treatment group C were lowest the previous week (day 72). Then, urine CTC concentrations spiked for all groups on study day 86 (Figure 4.4, 4.5, 4.6).

At baseline, sentinel animals had urine CTC concentrations ranging from 4.6 to 20.5 ng/mL (12.0 ± 6.5) (Figure 4.8). On study day 65, sentinel urine concentrations were 10.0 and 112.2 ng/mL, 17.0 and 16.0 ng/mL, and 9.4 and 13.7 ng/mL for treatment groups A, B, and C, respectively. By study day 86, sentinel urine concentrations were 62.0 and 23.9 ng/mL, 2.6 and 13.7 ng/mL, and 9.9 and 13.9 ng/mL for treatment groups A, B, and C, respectively.

Urine elimination half-lives

The urine half-life phases for each treatment group are displayed in Table 4.2. Mean ± standard deviation values for urine T ½ in the initial phase (up to 6 days following the last day of administration) for groups A, B, and C were 1.1 ± 1.5, 1.3 ± 1.4, and 1.3 ± 1.3 days, respectively (Table 4.2). Terminal T ½ values for urine were 35.4 ± 1.5, 51.7 ± 1.9, and 64.7 ± 1.9 days for groups A, B, and C, respectively (Table 4.2).

Plasma and Urine Comparisons

Post-CTC exposure (day 8), urine:plasma ratios (calculated by comparing urine mean values to plasma mean values) were as follows: 65.6, 53.6, and 91.4 for treatment groups A, B, and C, respectively. The last sampling day when all animals were analyzed for both plasma and urine (day 44) displayed urine:plasma ratios as: 62.6, 78.4, and 101.1 for treatment groups A, B, and C, respectively.

Dose Linearity

On a mg/kg basis, treatment group A received approximately 15 times the amount of CTC than treatment group B, treatment group B received approximately 5 times the amount of CTC than treatment group C, and treatment group A received approximately 76 times the amount of CTC than treatment group C. Dose linearity (ratios 15, 5, 76) was evaluated across treatment groups. On study day 8, mean plasma CTC drug concentrations were 280.6 ± 84.6, 18.7 ± 4.8, and 3.6 ± 1.3 ng/mL for treatment groups A, B, and C, respectively resulting in an A-B ratio of 15.1, a B-C ratio of 5.2, and an A-C ratio of 78.2.

Water CTC Concentrations

There was no detectable (> 0.5 ng/mL) CTC in any of the water samples collected throughout the duration of the study.

Animals Treated for Concurrent Disease or with Furosemide for Urine Collection

Eighteen animals were treated for infectious bovine keratoconjunctivitis and one for infectious pododermatitis, all with a macrolide.

Fourteen instances throughout the study required a second manual attempt to collect urine; half of those were from repeat animals. There were thirteen instances throughout the study that required a single furosemide injection (Salix®, Merck Animal Health, Rahway, NJ) to stimulate urination. Urine was collected from each of these animals within 8 minutes from injection. Upon evaluation, there was no identifiable pattern among animals requiring a furosemide injection and their respective urine CTC concentrations.

DISCUSSION

The objectives of this study were to: (1) determine the plasma and urine elimination characteristics of three CTC regimens and, (2) determine environmental exposure of sentinel animals housed with cattle recently receiving different doses of CTC.

Plasma Elimination

As previously mentioned, two distinct elimination phases were evident in urine for all groups and in plasma for treatment group A. The two phases are more prominently displayed in urine concentration curves as compared to plasma. Figures 4.1, 4.2, and 4.3 shows individual animal urine concentration curves for each treatment group and their respective phases. The first distribution phase (phase I) suggests elimination rates primarily influenced by plasma clearance by organs of elimination. In contrast, the second displayed plasma elimination phase by treatment group A is suggestive of a flip-flop pharmacokinetic situation where the apparent T ½ is more heavily influenced by release from a depot of the drug than by the plasma clearance rate.

Oral administration of CTC at a dose of 22 mg/kg of bodyweight per day in the current study, showed a comparable phase I plasma elimination half-life of approximately 1 day (24 h) compared to Holstein steers administered a twice daily feeding of the same dose for 80 days (elimination half-life 16.2 h), as previously described in Reinbold et al., (2010). Additionally, the observed Cmax was similar between studies. In Reinbold et al. (2010), the reported Cmax was 485.9 ng/mL (22 mg/kg dose) as compared to the equivalent dose in the current study, which reported a similar mean Cmax of 497.3 ± 32.2 ng/mL.

The previously mentioned swine study detected CTC in plasma 28 days post-cessation of CTC administration (22 mg/kg) in 3 of the 5 animals at a range of 2.3 to 3.9 ng/mL (Apley, National Pork Board). In comparison, the current study found CTC concentrations in cattle

ranging from 0.46 to 0.92 ng/mL 30 days post-cessation (day 37) in all 8 animals in treatment group A. This increased duration of exposure in the former study may be a contributing factor to the slightly higher reported CTC concentrations in associations with species differences. It should be noted, the swine study fed 22 mg/kg of bodyweight per day for 14 consecutive days, the maximum approved duration in pigs, in comparison to the current study that fed CTC the maximum legally approved duration of 5 consecutive days in cattle.

Urine Elimination

By evaluating urine residues resulting from differing CTC concentrations and duration since exposure, it is possible to better understand previous exposures and elimination characteristics of these regimens. We hypothesized that CTC would be detectable in urine for a prolonged period of time beyond detectable plasma concentrations. As displayed in Figures 4.4, 4.5, and 4.6, substantial inter-subject variability was observed. Chlortetracycline urine concentrations were detected through the end of the study for all treatment groups (study day 86), 79 days post-CTC cessation. On the final sampling (day 86), mean ± SD urine CTC concentrations were as follows: group A 34.9 ± 17.3 ng/mL; group B 21.2 ± 7.3 ng/mL; and group C 15.7 ± 8.9 ng/mL. It can be misleading to extrapolate beyond the available data; however, evaluating these terminal concentrations in light of the calculated phase II elimination half-lives for each treatment group may be informative as to the potential for extended detection in the urine, especially when designing studies to further investigate the duration of urine residues. These half-lives were 35.4 ± 1.5, 51.7 ± 1.9, and 64.7 ± 1.9 days for treatment groups A, B, and C, respectively (Table 4.2). The estimated recovery of CTC in the urine extrapolated beyond the available data (expressed as mean estimate in days [95% prediction interval]) were 254-281 days [163-490], 285-387 days [143-598], and 302-459 days [115-600] for treatment

groups A, B, and C, respectively. The data derived from this model are intended to assist future researchers who study CTC elimination in the urine of cattle and who wish to extend sampling in order to achieve negative results.

Without extrapolation of values beyond observed data, the urine analysis for treatment group C detected CTC through the end of the study (day 86). After a low exposure of 70 mg per day for 7 days, this study shows that urine CTC may be detected for up to 79 days after the exposure, with the terminal phase T ½ of 64.7 ± 1.9 days, suggesting continued urine detection well beyond this period. The distribution of the observed urine half-lives is displayed in Figure 4.7. A negative T ½ was considered to have an infinite number of half-life days; indicated by the error bars reaching the top of the graph. As displayed, in such low concentration environments, a small difference in concentration trajectory can result in a large change in T ½.

There were insufficient data for calculation of a terminal T ½ of urine in a swine study after feeding 22 mg/kg of bodyweight for 14 days, but concentrations were detected in 3 of the 5 pigs with urine samples at 14 days post-administration (252, 253, and 340 ng/mL) (Apley, National Pork Board). On day 21, there was one pig with a urine concentration (561 ng/mL) due to samples being available in the bladder at slaughter, and on day 28, urine concentrations were detected in 4 of the 5 pigs (37, 128, 371, and 397 ng/mL) (Apley, National Pork Board). These concentration data appear to be relatively consistent across the sample times, suggesting a prolonged T ½ value. Tissue elimination half-lives for the parent CTC molecule were determined in this swine study for muscle, liver, and kidney after feeding 22 mg/kg of bodyweight for 14 days, yielding 18.0, 19.6, and 24.7 days, respectively. Tissue depletion of CTC in the swine study suggest prolonged presence at low concentrations, similar to the phase II half-lives reported here in heifers for plasma and urine.

Drug molecules with a high affinity for bone, such as CTC, become fixed on newly formed bone and are able to be maintained for extended periods. Tetracyclines rapidly accumulate at the site of active bone remodeling by chelating with free calcium. When CTC was quantified in bone in a swine study 28 days after the feeding of 22 mg/kg per day for 14 days, concentrations ranged from 375 to 1120 ng/g (Apley, National Pork Board). Chlortetracycline persistence in bone has also been previously described in rats (Buskey et al., 1960). Long-term consumption in rats, at a rate of 1% CTC in their diet for two-years, found CTC bone concentrations as high as 560 ug/g (Buyske et al., 1960). In the same study, following a single intravenous administration of 50 mg/kg of tetracycline in rats it was discovered that the bone chelation phenomenon is an immediate consequence of the presence of tetracycline in the bloodstream and physical contact of the antibiotic with the bone; as early as 1 min after injection, bone concentrations were found to be 24 to 34 ug/g in the femora of rats. A study in growing pigs fed a diet containing 800 mg/kg CTC for either a 28- or 56- day duration (Guillot et al., 2010). The risk of bone discoloration increased with dosing duration and decreased with withdrawal time, suggesting that designing a proper dosing regimen may prevent bone discoloration. These results suggest bones serve as a reservoir, contributing to persistent low-level urine CTC concentrations.

As previously mentioned there was a noticeable spike in both plasma and urine concentrations between days 79 and 86. This spike was temporally associated with a premix formulation change implemented during that time. Nutritional differences are known to impact physiological changes of bone remodeling specifically affecting the rate and amount of bone resorption. While the spike was evident in plasma for treatment group A (the only group still being analyzed at this time point), it was more pronounced in urine concentrations (Figures 4.4,

4.5, and 4.6). Additionally, there was a sudden decline in all urine CTC concentrations between days 65 and 72. While changes in the diet during this time were present, it is known that physiological changes and environmental influences can affect the rate of drug elimination, and possibly bone metabolism. Assay outputs during these days were re-evaluated and confirmed to be accurate. Interestingly, one animal in treatment group A, consistently had the highest plasma and urine CTC concentrations throughout the entire study. High inter- and intra-individual variability was observed in both plasma and urine concentrations. Probable contributions to this variation include feed/water consumption patterns, individual animal bioavailability, plasma clearance, and bone matrix metabolism.

Baseline chlortetracycline urine concentrations

Mean urine CTC concentrations before any treatments were administered (day 0) were 16.3 ± 16.3, 10.6 ± 6.2, and 15.3 ± 5.9 ng/mL for treatment groups A, B, and C, respectively. The highest baseline urine CTC concentration was 53.8 ng/mL. At baseline (day 0), sentinel animals had comparable mean urine CTC concentrations of 12.0 ± 6.5 ng/mL (4.6 – 20.5 ng/mL). When first detected, day 0 samples were re-analyzed as well as testing the assay against animals known to not have been exposed to CTC. All results confirmed the detected amounts in day 0 urine samples.

The positive baseline (day 0) values in urine were surprising, but in light of the prolonged presence of CTC in urine demonstrated in this study, these may well be the results of prior exposure. Treatment records for the study animals prior to being purchased by the study collaborator, including administration of chlortetracycline in the feed or mineral, were unavailable. The study animals had been held at the commercial facility for at least 30 days prior

to entering the study; CTC is not used at this location and has not been used since construction of the pens in which the study animals were housed.

The administration of chlortetracycline through the mineral to cattle on pasture is a common industry practice, as is administration of either the 22 mg/kg of bodyweight or 350 mg/head regimen at the time of weaning. In the case of medicated mineral, it is intended for consumption by the cows; however, it is likely their calves are also consuming mineral. A calf consuming approximately $1/16^{th}$ of a pound, or 1 oz of 6,000 g/ton CTC-medicated mineral is equivalent to 187 mg of CTC per day, twice the mg dose of treatment group C in this study. Consuming 2 oz of mineral per day would be equivalent to a 375 mg dose, slightly more than treatment group B in this study. Based on current management practices and these study results, it is reasonable to hypothesize that the study calves consumed and/or were exposed to CTC prior to their purchase and enrollment in the study.

Environmental Exposure of Sentinel Animals Added to the Treatment Groups the Day After the Last Feeding of CTC.

The purpose of housing sentinel animals with each treatment group following oral CTC administration was to evaluate the potential for environmental exposure, including through shared water tanks within each treatment group. The potential for exposure through shared water sources within each treatment group was discarded due to no CTC being detected (LOD > 0.5 ng/mL) in any of the water samples.

Interestingly, sentinel animals co-housed with treated animals displayed urine CTC concentrations throughout the entire study. It is possible the observed concentrations were partly due to the previous exposure documented on study day 0 (baseline) concentrations in the urine. The continued CTC concentrations in sentinel animals, albeit low concentrations, supports either

some degree of low-level exposure or from long-term elimination from being previously exposed prior to the study. Based on the current study, we cannot definitely attribute these urine concentrations to exposure solely during our study. Future studies are needed to address this topic and utilize cattle with no previous exposure from direct administration or from indirectly being exposed to an environment that may have previously had antimicrobial usage.

A known study limitation was brief exposure once a week to a water tank shared by other non-study animals, many of which had just been purchased with unknown CTC exposure. This exposure occurred after the cattle were moved back to the commercial facility of origin and occurred for the weekly period required to sample all of the animals, varying based on sampling order (which was random). While this created a potential reservoir for CTC exposure once a week when animals were brought up to the chute for sample collection, no CTC was detected. Although there was no CTC detected in any of the water samples from this study, tetracyclines are one of the most abundantly detected environmental antimicrobials. Residuals of CTC in the environment have been well-characterized (Alexander 2008, 2009, 2011; Benedict, 2015; Holman, 2019; Platt, 2008). As noted above, no CTC has been administered at the commercial facility since construction of the pens, where the cattle were housed before study initiation, and for the final portion of the study during weekly sampling. However, cattle of unknown CTC administration history have been purchased and housed in these pens. At the study location for CTC feeding and sampling over study days 0 to 16, no cattle had been on the location from 1992 through 2006, and since that time period no CTC has been administered at any time to pasture cattle at this site.

When related back to the RWA program and testing for antimicrobials such as CTC, it would be difficult to pinpoint whether these concentrations are related to an intentional exposure

or a result of unintentionally being exposed to the environment or to other animals. For example, on study day 65, treatment group A had a mean ± SD urine CTC concentration of 104.3 ± 42.5 ng/mL, group B 32.1 ± 3.17 ng/mL, and group C 25.9 ± 12.8 ng/mL. These concentrations are reflective of animals that are not being routinely exposed; this is 58 days after CTC treatment, and they maintained these high of urine concentrations throughout the study. In regard to sentinels on that same day (day 65), treatment group A sentinels (n=2) had urine CTC concentrations of 112.2 and 10.0 ng/mL, group B sentinels (n=2) had concentrations of 17.0 and 16.0 ng/mL, and group C sentinels (n=2) had concentrations of 9.4 and 14.1 ng/mL. The current study along with the RWA study, suggests CTC persists within the animal for many months following exposure. While 70 mg/head per day is the approved dose in the United States for reduction in liver abscesses, this dose also correlates well with a potential incidental exposure. For example, if a 2,272 kg (5,000 lb) load of feed was mixed to provide 22 mg/kg of bodyweight of CTC per day to 225 kg (495 lb) calves consuming 6.8 kg (15 lbs) of feed per day as fed, the grams of CTC per ton of feed (as fed) would be 660 g/ton. If 90.9 kg (200 lbs) of this ration remained as residual in the mixer and was incorporated in the next 2,272 kg batch of feed, the total amount of CTC contributed to the next 2,362.9 kg of feed in the mixer would be 66 grams, or 25.4 g/ton. If this unintentionally contaminated ration were fed to 636 kg steers (1400 lbs) eating 2.6% of their bodyweight as dry matter (of a 70% dry matter ration), they would consume 23.6 kg (52 lbs) as fed. When the 25.4 g/ton in this ration is considered, each steer would consume 660 mg, or 0.96 mg/kg. For comparison, the mean mg/kg doses for treatment groups B and C in this study were 1.5 ± 0.2 and 0.3 ± 0.03 mg/kg, respectively.

The scenario could be changed to the same contaminated feed (25.4 g/ton) being fed to another group of calves with no intended CTC exposure. If the calves were the same weight with

the same as-fed feed consumption as the calves being fed CTC in the example above, the 25.4 g/ton would result in a mean individual animal dose of 190 mg, or 2.7 times the dose administered to treatment group C in this study. From these calculations, it is apparent that urine concentrations of CTC could be detected, at or above the reported concentrations from the urine residue studies in no-antibiotic cattle, for prolonged periods after incidental exposures.

Ideally, the duration of this study could have been extended, as CTC urine concentrations were detectable in all animals throughout the study. It is unknown exactly how long CTC remains in the urine. If bone remodeling serves as a continual source of prolonged low-level drug exposure, it is likely the reported half-lives are unable to capture the full extent of how long CTC remains in the body. Treatment groups B and C allowed us to compare urine concentrations when plasma concentrations were no longer detectable. On study day 23, all animals in treatment group B had detectable CTC plasma concentrations; however, by the next sampling timepoint (day 30) all but two animals were below the LOD. In comparing plasma concentrations with the respective urine concentrations, on day 23 the mean urine CTC concentration was 23.1 ± 13.2 ng/mL and by day 79 declined to 2.6 ± 2.9 ng/mL. Similarly, treatment group C on study day 23 is the last timepoint all animals have detectable plasma CTC concentrations. In comparison, mean urine CTC concentrations were 21.2 ± 8.4 ng/mL and by day 79 decline to 4.1 ± 2.9 ng/mL. The persistence of CTC in the urine and the long-reported urine half-lives suggest further investigations on CTC bone residue concentrations are needed

CONCLUSION

In summary, CTC administered in-feed to beef heifers at 22 mg/kg of bodyweight daily for 5 days, and 350 mg/head or 70 mg/head for 7 days showed chlortetracycline can be detected in the urine of cattle for a prolonged period of time after administration of common regimens.

Due to low-level CTC concentrations in the urine of sentinel animals at baseline and throughout the study, we cannot make determinations on environmental exposure.

Treatment Group	Target CTC Dose	Duration of Treatment	Actual CTC Dose Based on Bodyweight	Approved Indication
Treatment Group A	22 mg/kg of bodyweight per day[1]	5 days	22.1 ± 1.5 mg/kg of bodyweight per day	Treatment of bacterial enteritis caused by *Escherichia coli* and bacterial pneumonia caused by *Pasteurella multocida* organisms susceptible to chlortetracycline.[2]
Treatment Group B	350 mg/head per day[1]	7 days	1.5 ± 0.2 mg/kg of bodyweight per day	Control of bacterial pneumonia associated with shipping fever complex caused by *Pasteurella* spp. susceptible to chlortetracycline.[2]
Treatment Group C	70 mg/head per day[1]	7 days	0.3 ± 0.03 mg/kg of bodyweight per day	Reduction of liver condemnation due to liver abscesses.[2]
Treatment Group D	0 mg/head per day	0	N/A	N/A

[1]Chlortetracycline (Supermix Aureo 10-gram crumbles; Purina, St. Louis, MO) orally administered.
[2]21 C.F.R § 588.128 (2023).

Figure 4.1. Dose regimens for each treatment group. For comparison purposes, the target dose for groups B and C were calculated as mg/kg based on individual bodyweights (expressed as mean ± SD). Eight heifers received chlortetracycline in feed for each of three different regimens (n=24). Two control heifers were added to each treatment group after CTC administration ended (n=6).

	Half-Lives	22 mg/kg of bodyweight per day	350 mg/head per day	70 mg/head per day
Plasma	Phase I T ½ (d)	0.9 ± 1.1	1.1 ± 1.1	1.6 ± 1.2
	Phase II T ½ (d)	59.6 ± 1.5	--	--
Urine	Phase I T ½ (d)	1.1 ± 1.5	1.3 ± 1.4	1.3 ± 1.3
	Phase II T ½ (d)	35.4 ± 1.5	51.7 ± 1.9	64.7 ± 1.9

Figure 4.2. Plasma and urine half-lives reported in days (geometric mean ± geometric standard deviation) for chlortetracycline administered orally at three different dosing regimens (n=24). Phase I represents study day 8 to 13. Phase II represents study day 16 to 86. The brevity of plasma values for treatment groups B and C did not allow for calculation of a phase II T ½.

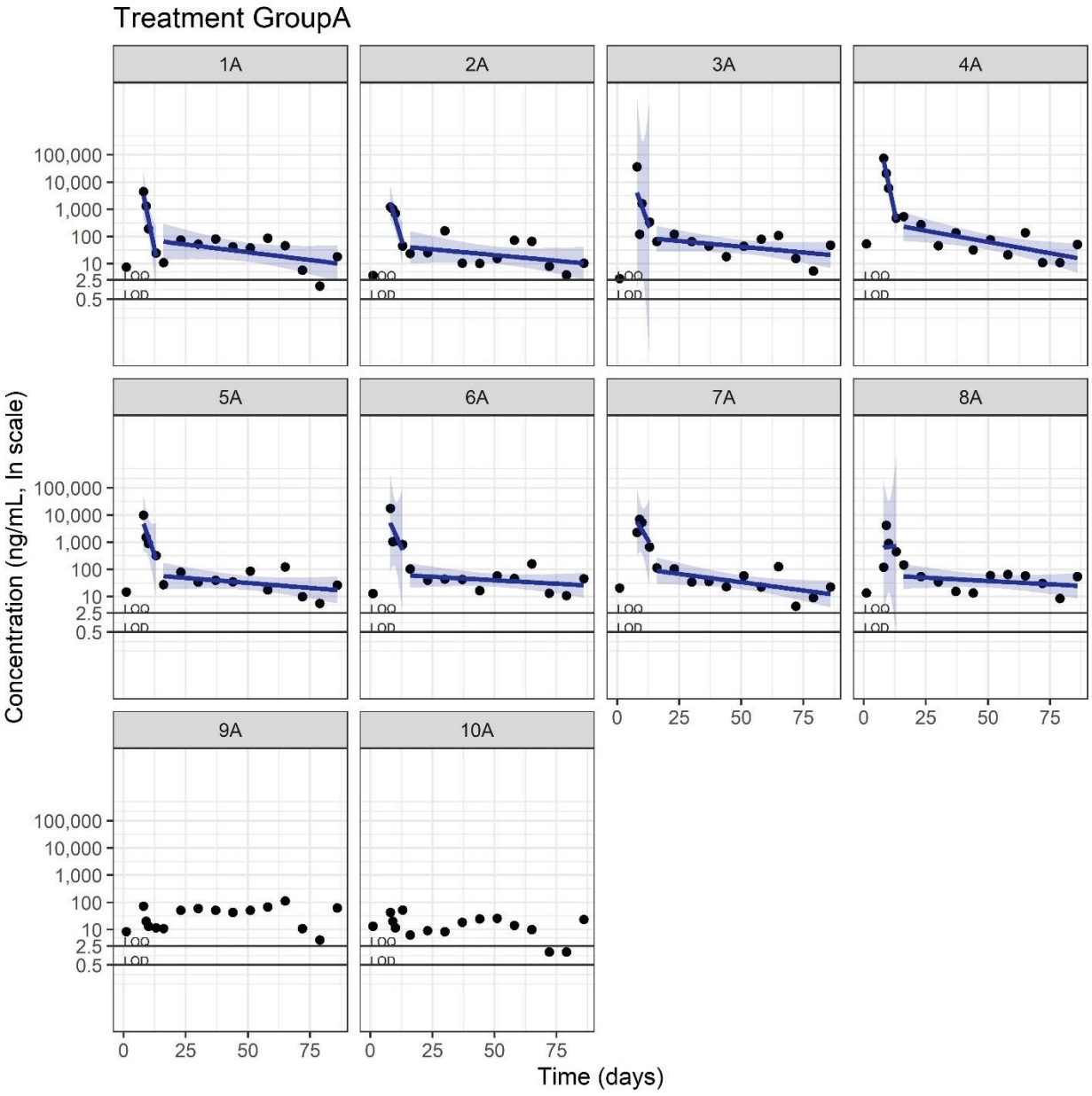

Figure 4.3. Treatment group A (22 mg/kg) individual animal urine CTC concentration (ng/mL) curves in ln scale. Each half-life phase is represented by the two shaded lines on each graph: phase I (days 8 to 13) and phase II (days 16 to 86). The two horizontal lines signify the limit of detection and limit of quantification (LOD 0.5, LOQ 2.5 ng/mL). Sentinel animals are '9A' and '10A'.

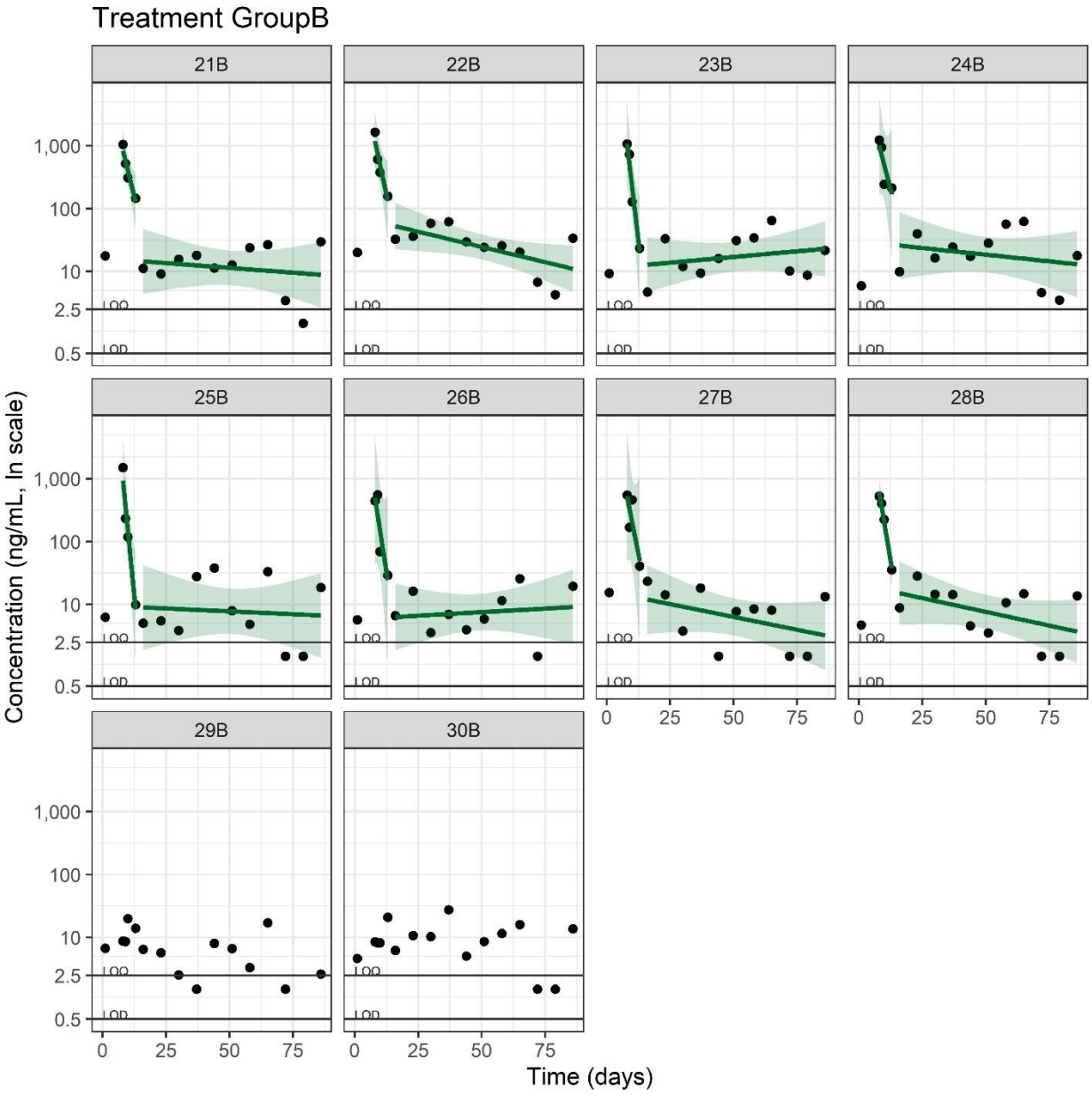

Figure 4.4. Treatment group B (350 mg/h) individual animal urine CTC concentration (ng/mL) curves in ln scale. Each half-life phase is represented by the two shaded lines on each graph: phase I (days 8 to 13) and phase II (days 16 to 86). The two horizontal lines signify the limit of detection and limit of quantification (LOD 0.5, LOQ 2.5 ng/mL). Sentinel animals are '29B' and '30B'.

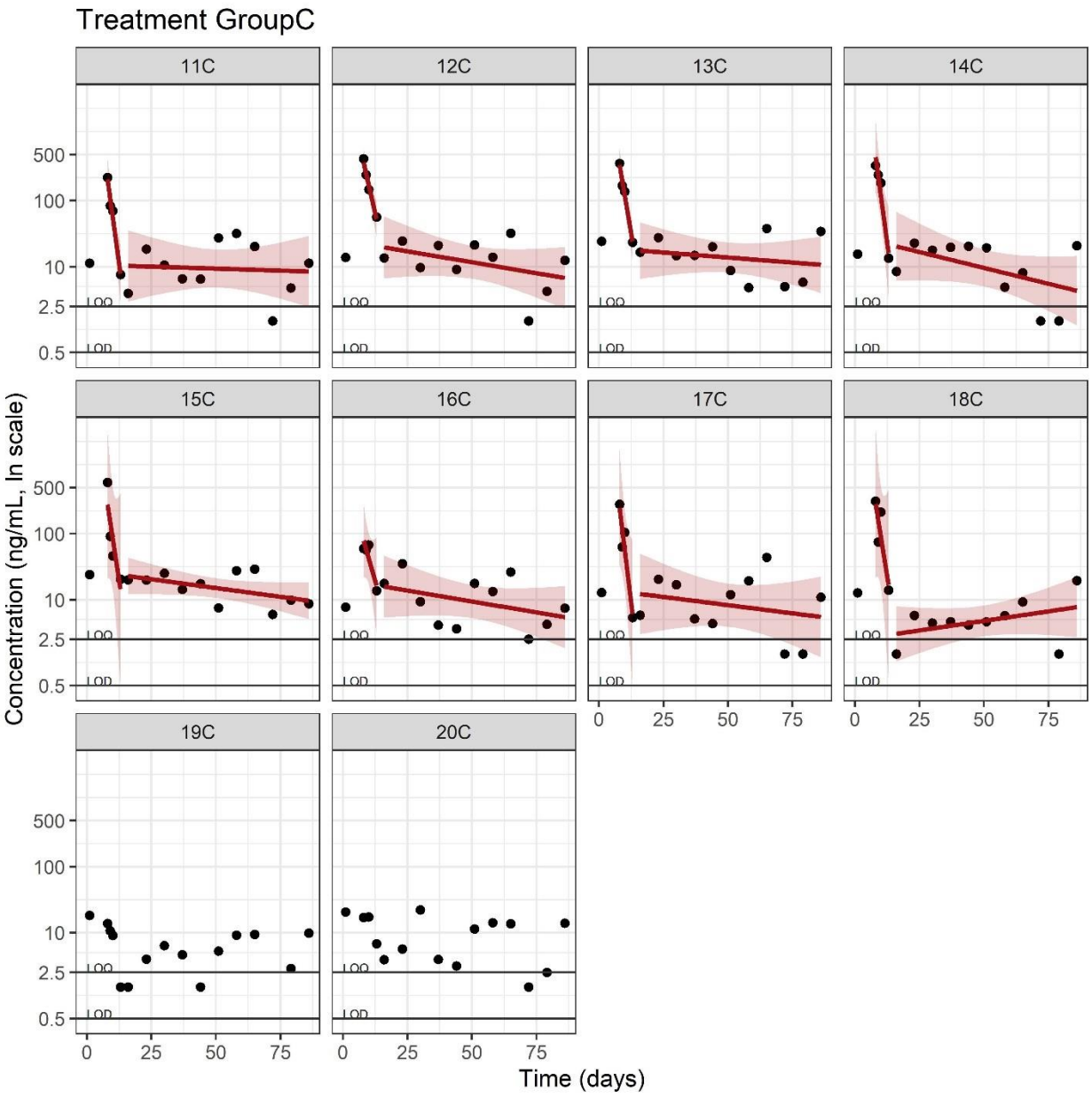

Figure 4.5. Treatment group C (70 mg/h) individual animal urine CTC concentration (ng/mL) curves in ln scale. Each half-life phase is represented by the two shaded lines on each graph: phase I (days 8 to 13) and phase II (days 16 to 86). The two horizontal lines signify the limit of detection and limit of quantification (LOD 0.5, LOQ 2.5 ng/mL). Sentinel animals are '19C' and '20C'.

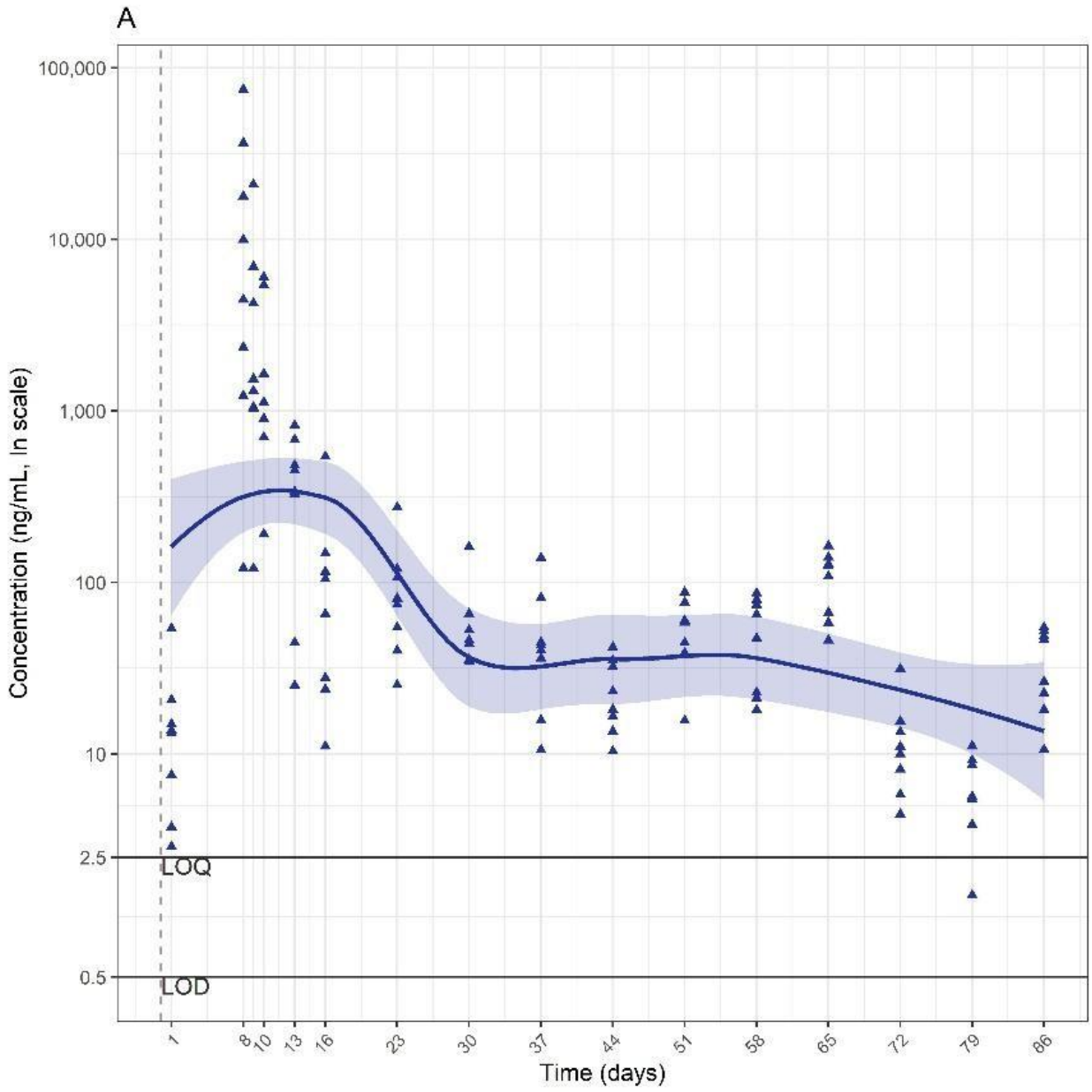

Figure 4.6. Urine CTC concentrations (ng/mL) in ln scale for treatment group A (22 mg/kg). The limit of detection and limit of quantification are displayed on each graph (LOD 0.5, LOQ 2.5 ng/mL). The shaded (blue) area demonstrates a 95% prediction interval. Values below LOQ and above LOD were replaced by the median value between LOQ and LOD.

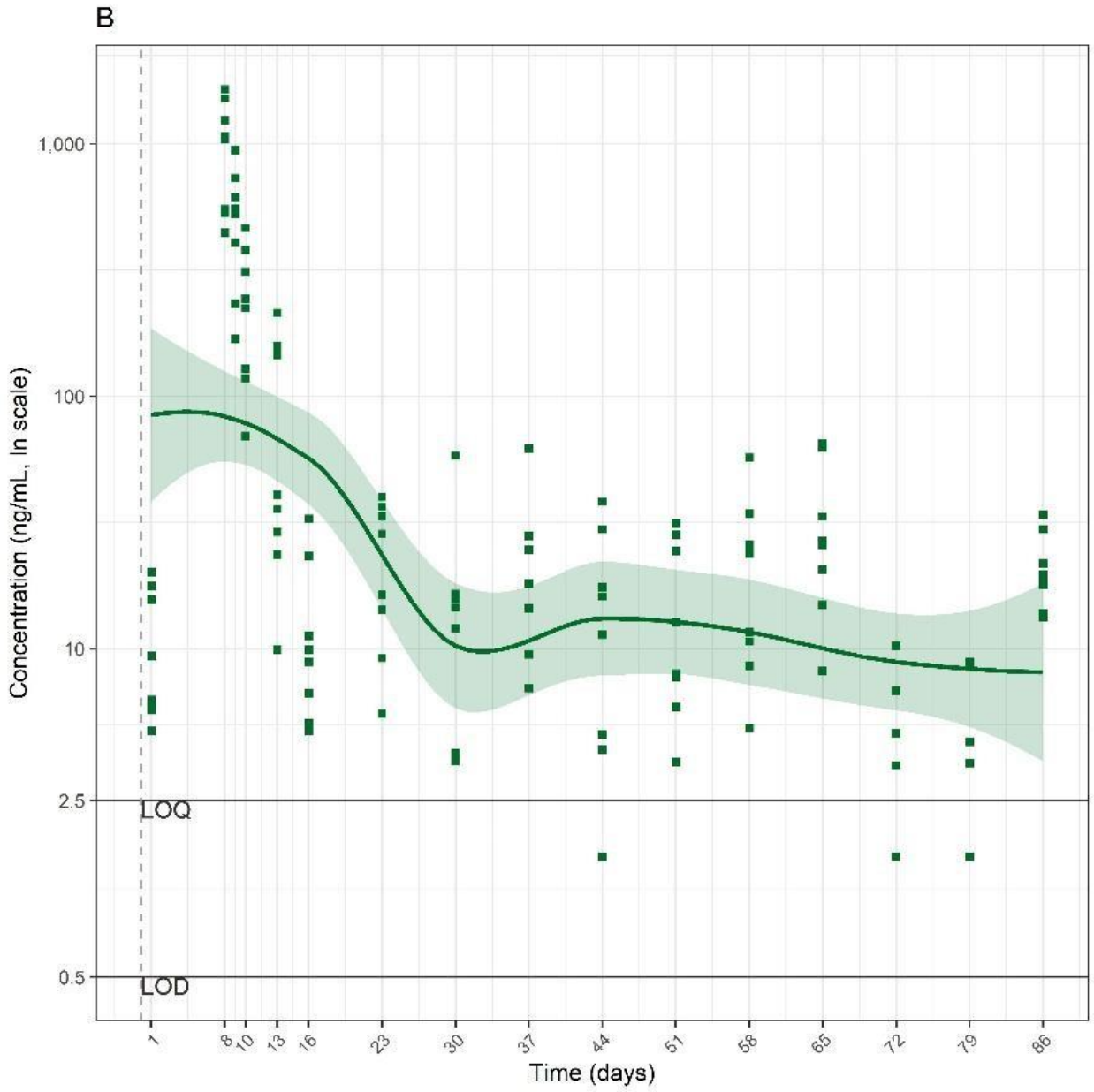

Figure 4.7. Urine CTC concentrations (ng/mL) in ln scale for treatment group B (350 mg/h). The limit of detection and limit of quantification are displayed on each graph (LOD 0.5, LOQ 2.5 ng/mL). The shaded (green) area demonstrates a 95% prediction interval. Values below LOQ and above LOD were replaced by the median value between LOQ and LOD.

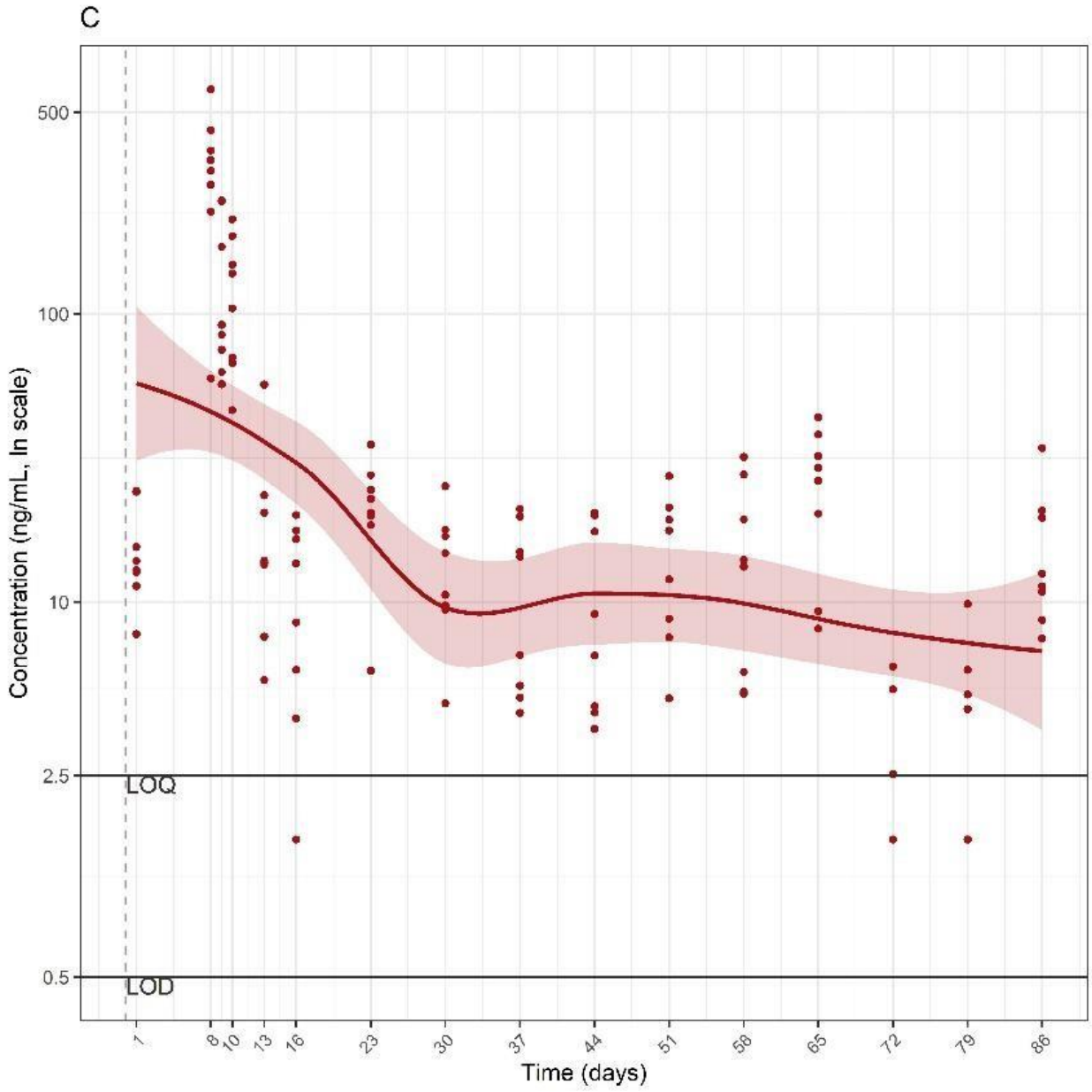

Figure 4.8. Urine CTC concentrations (ng/mL) in ln scale for treatment group C (70 mg/h). The limit of detection and limit of quantification are displayed on each graph (LOD 0.5, LOQ 2.5 ng/mL). The shaded (red) area demonstrates a 95% prediction interval. Values below LOQ and above LOD were replaced by the median value between LOQ and LOD.

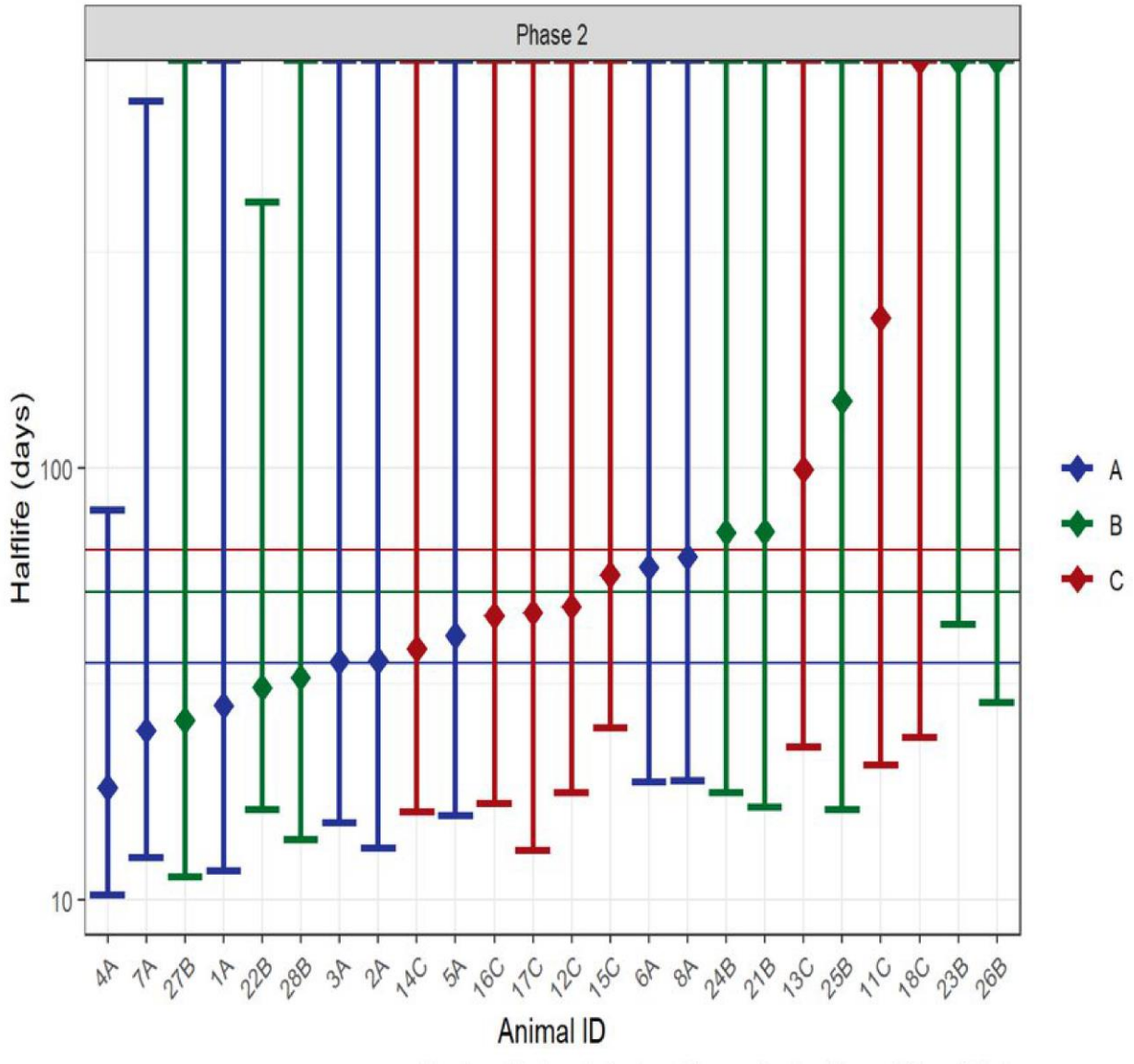

Figure 4.9. Individual animal phase II urine half-lives (in days), colored by treatment group. Phase II represents study days 16 to 86. Each diamond represents the calculated half-life (ln2/k). Error bars that reach the top of the graph extend beyond the axis limit. The colored horizontal lines are the geometric mean for each treatment group. Animals on the x-axis are ordered according to duration of half-life (shortest to longest).

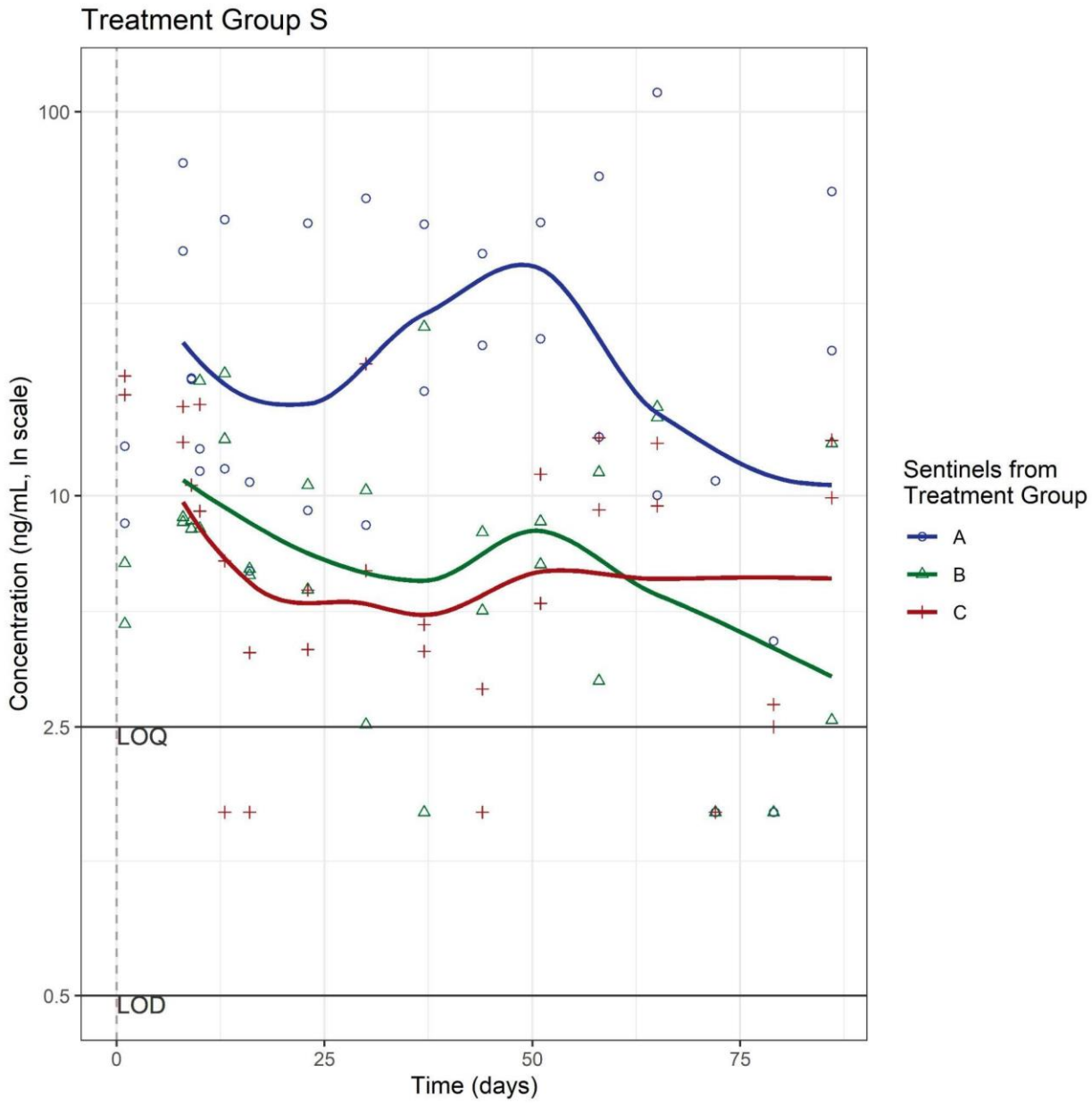

Figure 4.10. Urine CTC concentrations (ng/mL) in ln scale for sentinel animals, colored by treatment group. The limit of detection and limit of quantification are displayed on each graph (LOD 0.5, LOQ 2.5 ng/mL). Values below LOQ and above LOD were replaced by the median value between LOQ and LOD.

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Chapter 5 - Pharmacokinetics and pharmacodynamics of two orally administered chlortetracycline regimens provided to beef cattle

Alyssa R Toillion*, Michael D Apley*[¶], Johann F Coetzee[§], Kushan Kompalage[$]

*Department of Clinical Sciences, Kansas State University, Manhattan, KS, 66506, USA; toillion@vet.k-state.edu, mapley@vet.k-state.edu

[§]Department of Anatomy and Physiology, Kansas State University, Manhattan, KS, 66506, USA; jcoetzee@vet.k-state.edu

[$]Department of Chemistry, Kansas State University, Manhattan, KS, 66506, USA; kushanpa@ksu.edu

[¶]Corresponding author: Michael D Apley, Department of Clinical Sciences, College of Veterinary Medicine, Kansas State University, 1620 Denison Avenue, Manhattan, KS, 66506, USA. Phone: 785-532-2153. Email: mapley@vet.k-state.edu.

ABSTRACT

Sixteen crossbred Hereford heifers were stratified and randomly assigned by initial body weight into two treatment groups. Treatment group (A) received chlortetracycline (CTC) at a target dose of 22 mg/kg of bodyweight daily (22.1 ± 1.5 mg/kg based on actual bodyweights) for 5 consecutive days (n=8) and group (B) received CTC at 350 mg/head per day (1.5 ± 0.2 mg/kg based on actual bodyweights) for 7 consecutive days (n=8). Blood samples were collected in 4-hr increments for a 24-hr period on the 5th or 7th day of administration for groups A and B, respectively. Plasma CTC analysis was performed using ultrahigh performance liquid chromatography–mass spectroscopy/mass spectroscopy. Non-compartmental analysis was used to calculate plasma area under the concentration curve. The mean observed (± SD) AUC values

were 4.18 (± 1.72) µg/ml and 0.30 (± 0.06) µg/ml for treatment groups A and B, respectively. The probability of target attainment for AUC_{24}/MIC values of 25 and 12.5 was modeled using Monte Carlo simulations based on unbound plasma AUC values over the MIC range of 0.015 to 2 µg/ml. Treatment group A achieved >90% target attainment (AUC_{24}/MIC of 25) at an MIC of 0.06 µg/ml, whereas treatment group B displayed only 12.6% target attainment (AUC_{24}/MIC of 25) at the lowest MIC evaluated (0.015 µg/ml). Based on this study and using selected bacteria from multiple sources, both in-feed CTC regimens failed to obtain a reasonable target attainment rate in light of expected MIC distributions of potential pathogens.

KEYWORDS: Cattle, Chlortetracycline, Monte Carlo, Pharmacodynamics, Pharmacokinetics, Plasma

INTRODUCTION

In 2021, nearly 1.7 million kg of tetracycline antimicrobials were sold and distributed for use in cattle (FDA, 2022). Use of in-feed tetracyclines is a common therapeutic practice, accounting for 55% of all medically important antimicrobials in food production animals as measured by kg of product sold (FDA, 2022). A 2016-2017 survey assessing antimicrobial use in beef cattle from 22 beef feedyards reported 1.6 million medically important treatment regimens, with 19.4% (393,676) attributable to in-feed tetracyclines (Hope et al., 2020). Likewise, national surveillance data reported 40.4% of all feedyards in 2016 gave cattle chlortetracycline in feed. Of that 40.4%, the majority (85.7%) of chlortetracycline was used to control or treat respiratory disease (USDA NARMS data). Chlortetracycline is fed to control bovine respiratory disease at 350 mg/head/day or to treat respiratory disease at 22 mg/kg of bodyweight per day for up to 5 days. The latter dosage is also indicated for the treatment of bacterial enteritis caused by *Escherichia coli*. The 350 mg/head per day regimen has no limit on duration, the 22 mg/kg of bodyweight per day regimen may be fed up to 5 days, and neither regimen requires a slaughter

withdrawal period. Both regimens require authorization by a veterinarian through a veterinary feed directive.

To the authors knowledge, the pharmacokinetic and pharmacodynamic characterization of the 350 mg/head per day regimen, and pharmacodynamic characterization of the 22 mg/kg per day regimen, have not been previously described. Such evaluation has been conducted for injectable oxytetracycline in cattle against respiratory pathogens by the generic breakpoint working group of the Clinical and Laboratory Standards Institute Veterinary Antimicrobial Susceptibility Testing Subcommittee (CLSI VAST). These interpretive criteria do not apply to any tetracycline uses other than injectable oxytetracycline.

To support the pharmacokinetic and pharmacodynamic evaluation of the two CTC in-feed regimens reported here, this study utilized plasma CTC concentration data collected in conjunction with a companion study (Toillion dissertation, Chapter 4) to support Monte Carlo simulation of Area Under the Concentration Curve (AUC) related to selected MICs to determine the percent of the population reaching the AUC/MIC ratio target for each MIC.

MATERIALS AND METHODS

This study was approved by the Kansas State University (KSU) Institutional Animal Care and Use Committee (protocol #4740).

Animals and Treatments

Sixteen crossbred Hereford heifers were stratified by weight and randomly assigned to two treatment groups. Treatment group (A) received CTC at 22 mg/kg of bodyweight daily (22.1 ± 1.5 mg/kg based on actual bodyweights) for 5 consecutive days (n=8) and group (B) received CTC at 350 mg/head per day (1.5 ± 0.2 mg/kg based on actual bodyweights) for 7 consecutive days (n=8). Each treatment group was individually housed and provided a total mixed ration

(TMR) with CTC (Supermix Aureo 10-gram crumbles; Purina, St. Louis, MO). The concentration of this product was analyzed via CTC quantification analysis at the Iowa State University Diagnostic Laboratory. The actual resulting concentration was reported as 7.44 grams per lb of product, which is 74.4% of the advertised 10 grams per pound concentration. In response, plasma AUC_{24} values are reported both as measured and as adjusted to reflect the concentrations expected of feeding the actual target doses. This adjustment is supported by our demonstration of dose linearity in a companion paper (Toillion dissertation, Chapter 4) and also by Reinbold, et al. (2010).

On study day 3, treatment group A animals were re-weighed prior to beginning their CTC 5-day treatment regimen, the maximum duration on the label. Chlortetracycline was included in the daily ration to treatment group B beginning on study day 1 for 7 consecutive days. This allowed both treatment groups to complete their CTC dosage regimens concurrently. Using the 16 hr elimination half-life of CTC reported in Reinbold et al. (2010), 97% of steady state would be achieved at 80 hrs (3.3 days) and 99.9% of steady state would be achieved at 112 hours (4.7 days). By feeding treatment group A for 5 consecutive days (the maximum allowed by the label) and group B 7 days, this gave a reasonable probability that the animals were at or near steady state at the end of the feeding period. Animals were fed a daily feed ration each morning. The appropriate amount of CTC was manually spread over the surface of the feed and then manually mixed in. Both treatment groups were provided water *ad libitum* from separate water sources. Additional details on study conduct are available in a companion paper (Toillion dissertation, Chapter 4).

Sampling

Study day 7 was the final day when both groups concluded their respective CTC regimens. On that day, blood samples were collected in 4-hr increments for a 24-hr period: 7:30am, 11:30am, 3:30pm, 7:30pm, 11:30pm, 3:30am, and 7:30am. The study animals were fed their final treatment on study day 7 at approximately 9 am.

Chlortetracycline Analysis

Detection and quantification of CTC in plasma was conducted using ultrahigh performance liquid chromatography–mass spectroscopy/mass spectroscopy (UPLC–MS/MS).

Standards and samples were prepared as previously described in our companion study (Toillion dissertation, Chapter 4). In brief, CTC and OTC were solubilized in water-methanol (1:1) and stored at -20 °C. Working stocks were prepared fresh daily of CTC at 1 µg/ml and OTC at 50 ng/mL. For plasma analysis, the calibration curve was linear from 0.1 to 1000 ng/ml with a correlation coefficient (R^2) of at least 0.99. The accuracy of this assay was 99% and the precision (CV%) was 5.6%. Accuracy and precision were determined by measuring three replicates for each of the QC concentrations (0.475, 0.95, 4.75, 47.5, 95, and 475 µg/mL). Recoveries ranged from 95 to 102%. Following the FDA Bioanalytical Validation guidelines, the limit of detection (LOD) and limit of quantification (LOQ) of the method were 0.25 and 0.5 ng/ml, respectively.

PHARMACOKINETIC AND PHARMACODYNAMIC (PK/PD) ANALYSIS

Plasma Area Under the Curve

Non-compartmental analysis was performed to estimate area under the plasma concentration curve (AUC). Area under the curve (AUCs) were calculated by averaging the

concentrations over a specified time interval (AUC = ½ (C_1 + C_2) / (t_2-t_1)) and then summing all those intervals together, to conclude total drug exposure from the first time point to the last. The AUCs were extrapolated to the target dose by dividing each AUC value by 0.744 to account for the low concentration of the crumble product. Following extrapolation, dose adjusted AUCs were divided by 0.5 to account for a reported protein binding of chlortetracycline of 47-50% in cattle (Riviere & Papich, 2018).

AUC/MIC Target

A ratio of 25 for plasma free (unbound) drug concentration over a 24-h period to minimum inhibitory concentration of the target pathogen (AUC_{24}/MIC) has been established as target PK/PD index for the tetracyclines. This has been reported by multiple authors (Hnot, 2015; Andes & Craig, 2007; Toutain, 2021). One paper is cited as supporting a free doxycycline AUC_{24}/MIC ratio of 12 against *Staphylococcus aureus* (LaPlante et al., 2008). Therefore, an AUC_{24}/MIC ratio of 25 was modeled along with evaluation of the effect of halving this ratio to 12.5.

MIC Distributions

The MIC distribution (0.015 to 2 µg/ml) used in this study was selected based on observing the wild-type populations of *Escherichia coli, Mannheimia haemolytica,* and *Pasteurella multocida* as presented in international aggregate distributions (Figure 5.2) by the European Union Committee on Antimicrobial Susceptibility Testing (EUCAST, 2023). The MIC distributions were truncated at a maximum of 2 µg/ml based on reasonable expectations for target attainment.

Monte Carlo Simulations

A 5,000 iteration Monte Carlo simulation (Oracle Crystal Ball; Release 11.1.2.4.850) was conducted for each antimicrobial regimen and MIC pair. The mean and standard deviation for each treatment group formed the basis for population AUC distributions. Percent target attainment represents what proportion of the modeled population will meet or exceed the target AUC/MIC of 25 or 12.5 at a specific MIC. Investigators have recommended that the probability of target attainment should be ≥ 90% to define the susceptible breakpoint (Frei, 2008; Zelenitsky, 2011; Maaland, 2013). The normal distributions for each treatment group were truncated after 3 standard deviations and at zero (which only affected treatment group A).

RESULTS

The AUC values used for Monte Carlo simulation modeling were adjusted for the reduced amount in the medicated feed product as compared to the advertised concentration, and to account for protein binding. The extrapolated mean AUCs for plasma, as adjusted to the target doses based on analysis of the actual content of the medicated feed added to the ration, were 8,362 ± 3,447 ng*hr/ml and 605 ± 128 ng*hr/ml for treatment groups A and B, respectively (Table 5.4). After accounting for protein binding, the free drug 24-hour plasma AUC values used in Monte Carlo simulations for treatment group A were 4,181 ± 1,724 ng*hr/ml, and for treatment group B 302 ± 64 ng*hr/ml (Table 5.4).

Figure 5.1 represents MIC values (μg/ml) versus percent attainment of the free drug dose adjusted AUC_{24}/MIC targets (12.5 and 25). The accompanying simulation summary data for each orally administered CTC regimen (22 mg/kg bodyweight daily and 350 mg/head per day) are displayed in Tables 5.2 and 5.3, respectively. As displayed, higher MIC values increase the probability that 0% of the population will attain the desired PK/PD target.

For treatment group A, 94.9% of the animals attained an AUC/MIC ratio of 25 at an MIC of 0.06 μg/ml (Table 5.2). In comparison, 94.5% of the animals in treatment group A attained an AUC/MIC ratio of 12.5 at an MIC of 0.125 μg/ml. For treatment group B, the attainment rate did not reach 90% with an AUC/MIC ratio of 25; however, 96.3% of the animals attained an AUC/MIC ratio of 12.5 at an MIC of 0.015 μg/ml (Table 5.3).

DISCUSSION

Monte Carlo simulation was used in this study to model target attainment based on target AUC_{24}/MIC ratios of 25 and 12.5 for two orally administered CTC regimens. The question arises as to the sensitivity of % target attainment predictions based on altering the selected AUC_{24}/MIC ratio. Typically, MICs are established using a two-fold serial dilution. Therefore, if the target AUC/MIC ratio is halved or doubled the target attainment curve moves down or up one MIC dilution, respectively. For example, halving the AUC/MIC ratio moves the same percentage target attainment value down one dilution factor (a one dilution factor lower MIC value now equates to the same percentage target attainment value). This mathematical relationship suggests that discussion about whether the correct value for the AUC/MIC ratio was selected only pertains to moving the 90% target attainment MIC one or perhaps two dilutions either direction.

The predictive utility of PK/PD modeling has been well described in human and veterinary applications in seminal papers by Craig and Papich, respectively, associated with a large branch of antimicrobial literature dedicated to pharmacodynamics (Craig, 1998; Papich, 2014). Fundamentally, each of the PK/PD indices is an attempt to link a summary PK measure of exposure (AUC_{24}/MIC) with a measure of the *in vitro* "potency" of the antibiotic as provided by the MIC (Landersdorfer & Nation, 2021).

A limitation of PK/PD modeling is the extrapolation of pharmacodynamic indices, and target values for these indices, across different naturally occurring and modeled diseases in different species with different pathogens. The work on the pharmacodynamics of first generation tetracyclines (oxytetracycline, tetracycline, and chlortetracycline) is limited in relation to other classes and generations. These authors recognize the complexity of applying pharmacodynamic parameters to a different member of the tetracycline class in a different disease with a different pathogen, yet propose this approach reasonably frames the potential for efficacy against beef cattle pathogens. Our evaluation applies only to systemic infections which are appropriate for predictions based on plasma concentrations (e.g., pneumonia, soft tissue infections); there is no application to enteric infections.

An evaluation of potential efficacy through PK/PD modeling can only be conducted in light of expected MIC values of the potential pathogens. A source of MIC distribution compilations for tetracycline is the European Union Committee on Antimicrobial Susceptibility Testing (EUCAST) which provides compilations of reported MIC distributions for multiple bacterial pathogens. When sufficient data are available, EUCAST also provides a calculated epidemiological cutoff (ECOFF) which partitions the MIC range into wild type and non-wild type populations, with the non-wild type population considered to have a high likelihood for having resistance genes for the tested antimicrobial. As stated on the EUCAST website, “The accepted distributions include studies from a wide variety of sources and time periods and some on purpose, include high and others low, proportions of resistant organisms, so the distributions must not be used to represent rates of resistance to any agent and cannot be used to compare resistance rates among agents, resistance over time or resistance in different geographic locations.” As reported here, these distributions are presented for the purpose of illustrating the

potential range of MICs for the selected bacteria as reported from multiple sources in the literature and other contributions to the database; they are not to be construed as indicating a proportion of isolates addressed by the regimens evaluated here. However, it is noteworthy if a regimen fails to obtain a reasonable target attainment rate for any reported MIC of a potential pathogen. Evaluating the 90% target attainment MIC values (or lack of target attainment), it is notable that these MIC values for both regimens are below the lowest reported MIC values for *Mannheimia haemolytica* and *Pasteurella multocida*. The 90% target attainment MIC values are also below all reported MICs in the *Escherichia coli* distribution, although plasma concentrations are likely not well related to efficacy in an *E. coli* enteric infection.

The use of chlortetracycline in feed for the purpose of treatment of BRD using the regimen of 22 mg/kg CTC/kg bodyweight fed daily has been evaluated in clinical studies. A trial evaluating 5 days of in-feed CTC, at 22 mg/kg CTC/kg bodyweight fed daily, found that while the control group had over 25% (38/150) develop illness and require treatment, only 1.3% of the animals in the CTC group required treatments (Agga et al., 2016). In Kreikemeier et al. (1996), feeding CTC (22 mg/kg per day) for 5 consecutive days reduced morbidity from 81% to 60%. No mention of subjective evaluators being masked to treatment was found in either of these studies. Other studies either report no effect or are confounded with other drugs. A study by Szasz et al. (2019) in calves administered tulathromycin for control of respiratory disease, reported no statistically significant reduction in morbidity when CTC was administered at 22 mg/kg BW concurrently with the tulathromycin treatment as compared to tulathromycin alone. This study lacked a negative control to evaluate the effect of CTC by itself. Another study evaluated the effects of CTC plus decoquinate (DEQ) in a 220-day study against untreated controls (Thomson et al., 2014); the administration of 22 mg CTC/kg of bodyweight fed daily for

5 consecutive days reduced respiratory morbidity (17% vs 23%, $P < 0.01$) in steers in the CTC+DEQ groups as compared to those in the CON group.

These studies suggest a clinical effect of feeding 22 mg/kg per day of CTC to calves is possible. Such data are lacking for the 350 mg/head per day regimen. The logical question is why do we have clinical efficacy shown in some studies for the regiment used in group A in light of our dire PK/PD predictions? Four possibilities could be considered for this discrepancy.

- The studies showing positive results reflect positive publication bias and are not a consistent outcome.
- There may be effects on the growth rate of bacterial populations below the MIC determined in the laboratory (Landersdofer & Nation, 2021). The determination of an MIC is not a re-creation of the antimicrobial/pathogen interaction in the animal. It is a repeatable, quality-controlled laboratory determination which is linked to clinical efficacy through clinical studies.
- The tetracyclines have been documented to have other activity in addition to their antimicrobial effect, such as anti-inflammatory effects. Enzymes produced by inflammatory cells called matrix-metalloproteinases (MMP) are known to be inhibited by tetracyclines (Weinberg, 2005; Orylska-Ratynska, 2022; Golub, 1991; Golub, 1998). It is unknown whether these anti-inflammatory properties are contributing to the clinical success reported in previously published studies. Before concluding that anti-inflammatory effects of chlortetracycline are responsible for efficacy against bovine respiratory disease in the absence of antimicrobial activity, consideration should also be given to the lack of demonstrated differences in treatment outcomes in the therapy of

acute bovine respiratory disease when non-steroidal anti-inflammatory drugs are added to the regimen (Francoz, 2012; Apley, 2021).

- The AUC_{24}/MIC ratio for addressing clinical disease may be different than the ratio required for reducing morbidity in a population. This has been demonstrated for other AUC/MIC antimicrobials, such as the fluoroquinolones, where the AUC/MIC has been shown to vary substantially depending on the therapeutic goal (USCAST, 2018; Qu, 2015).

CONCLUSION

Both in-feed CTC regimens evaluated in this study failed to obtain a reasonable target attainment rate for published distributions of potential pathogen MIC values. When considering the dissonance between these findings and published studies demonstrating efficacy in reducing morbidity due to bovine respiratory disease for the regimen in treatment group A, it could be that these studies are not representative of the overall effect of CTC in the cattle population, the target AUC_{24}/MIC ratio used for modeling is not appropriate for changing the course of pre-clinical disease (too high), or that other factors besides antimicrobial effects could contribute to reduced incidence of BRD.

In consideration of treatment group B (350 mg/head per day) the Monte Carlo simulation results are so far removed from any values associated with potential efficacy that clinical trials are highly recommended to avoid the ultimate failure of antimicrobial stewardship, administering an antimicrobial with no therapeutic benefit.

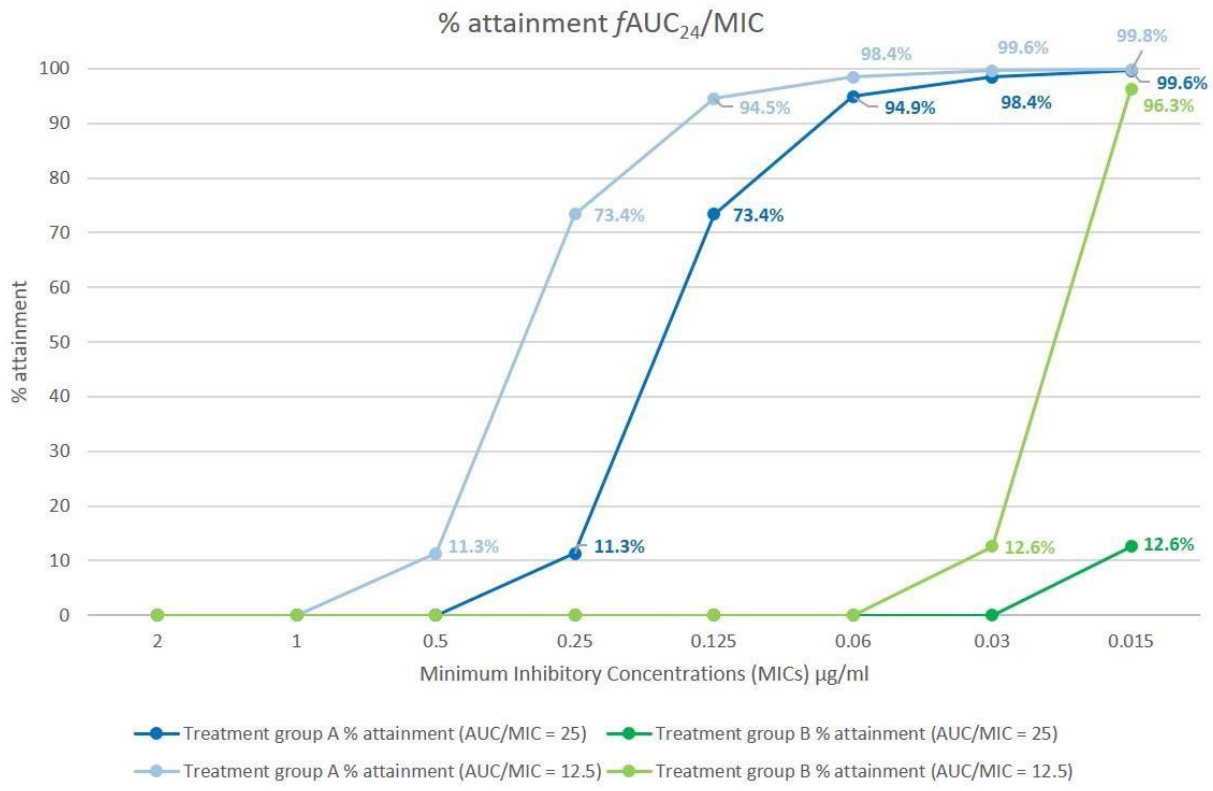

Figure 5.1. Monte Carlo simulation of free drug dose adjusted AUC_{24}/MIC percent attainment for wild-type populations of *Escherichia coli*, *Mannheimia haemolytica*, and *Pasteurella multocida* after oral administration of chlortetracycline. [Treatment group A (22 mg/kg bodyweight per day) and group B (350 mg/head per day)]. The plot shows MIC values (µg/ml) versus percent attainment of the specified AUC_{24}/MIC targets (12.5 and 25).

Treatment Group A (22 mg/kg bodyweight daily)				
MIC (µg/ml)	**AUC_{24}/MIC mean ± SD**	**AUC_{24}/MIC 10th Percentile (90% above)**	**% Attainment AUC/MIC = 25**	**% Attainment AUC/MIC = 12.5**
0.015	280.13 ± 110.80	137.31	99.6	99.8
0.03	140.06 ± 55.40	68.66	98.4	99.6
0.06	70.03 ± 27.70	34.33	94.9	98.4
0.125	33.62 ± 13.30	16.48	73.4	94.5
0.25	16.81 ± 6.65	8.24	11.3	73.4
0.5	8.40 ± 3.32	4.12	0	11.3
1	4.24 ± 1.66	2.06	0	0
2	2.10 ± 0.83	1.03	0	0

Table 5.1. Monte Carlo simulation summary data based on target AUC_{24}/MIC ratios of 25 and 12.5 for an orally administered CTC regimen of 22 mg/kg bodyweight daily.

Treatment Group B (350 mg/head per day)				
MIC (µg/ml)	**AUC_{24}/MIC mean ± SD**	**AUC_{24}/MIC 10th Percentile (90% above)**	**% Attainment AUC/MIC = 25**	**% Attainment AUC/MIC = 12.5**
0.015	20.14 ± 4.24	14.61	12.6	96.3
0.03	10.07 ± 2.12	7.31	0	12.6
0.06	5.03 ± 1.06	3.65	0	0
0.125	2.42 ± 0.51	1.75	0	0
0.25	1.21 ± 0.25	0.88	0	0
0.5	0.60 ± 0.13	0.44	0	0
1	0.30 ± 0.06	0.22	0	0
2	0.15 ± 0.03	0.11	0	0

Table 5.2. Monte Carlo simulation summary data based on target AUC_{24}/MIC ratios of 25 and 12.5 for an orally administered CTC regimen of 350 mg/head per day.

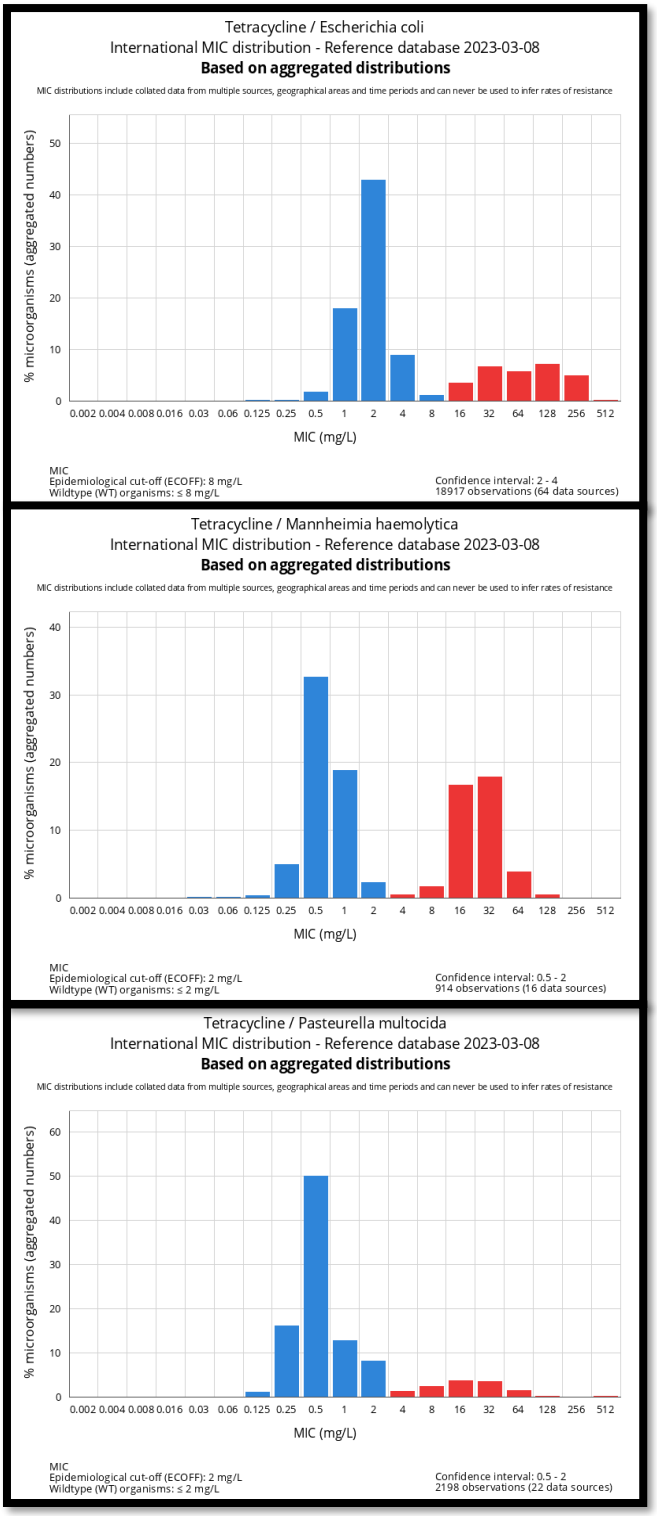

Figure 5.2. Minimum inhibitory concentrations (mg/L) as presented in international aggregate distributions provided by EUCAST for tetracyclines and three relevant bacteria species.

Treatment Group A (µg·hr/mL)			
Animal #	**AUC (measured total)**	**AUC Adjusted to Target Dose**	**AUC Free Drug (50%)**
1	3.629	4.878	2.439
2	5.119	6.881	3.440
3	5.170	6.949	3.475
4	12.087	16.246	8.123
5	5.794	7.787	3.894
6	4.769	6.410	3.205
7	6.486	8.717	4.359
8	6.719	9.031	4.515
Mean	**6.222**	**8.362**	**4.181**
Std Dev	**2.565**	**3.447**	**1.724**

Treatment Group B (µg·hr/mL)			
Animal #	**AUC (measured total)**	**AUC Adjusted to Target Dose**	**AUC Free Drug (50%)**
10	0.555	0.746	0.373
11	0.352	0.473	0.236
12	0.447	0.601	0.301
13	0.600	0.807	0.403
14	0.423	0.569	0.285
15	0.312	0.419	0.210
16	0.446	0.600	0.300
17	0.464	0.624	0.312
Mean	**0.450**	**0.605**	**0.302**
Std Dev	**0.095**	**0.128**	**0.064**

Table 5.3. Twenty-four hour plasma AUC values as measured, as adjusted to target dose (divided by 0.744), and then adjusted to free drug (multiplied by 0.5) for each treatment animal (n=16).

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Chapter 6 - Research conclusions and implications for further research

A collective finding in the systematic review (Chapter 1) was that antimicrobial exposure is not essential for the maintenance of Tet(r) *E. coli*; however, it does temporarily increase the prevalence of resistance. Additionally, antimicrobial withdrawal did not prove to be a viable route for reducing the level of tetracycline resistance in *E. coli* (Alexander, 2008; Alexander, 2009; Mirzaagha 2011). Beyond administering tetracyclines directly into the feed, other acquisition sources play a central role in the dissemination of resistant determinants. The degradation half-lives for tetracycline, chlortetracycline, and oxytetracycline in stored feedlot manure are 17.2, 13.5, and 31.1 days, respectively (Storteboom, 2007; Masse, 2014). Therefore, even after a considerable period of time, CTC in manure may remain as a source of antimicrobial selection pressure. The implications of this prolonged selection pressure in the environment should be further explored.

The second chapter in this dissertation demonstrated that despite variable mineral intake expected in a pasture setting, plasma CTC concentrations remained overall similar between cattle herds provided different four FDA-approved free-choice CTC-medicated mineral formulations (700, 5000, 6000, 8000 g CTC/ton). Resulting plasma CTC concentrations ranged between 3.9 to 45.3 ng/mL over the treatment period. These values were consistent (<0.1 - 51.1 ng/mL) with a previous study by Schrag et al. (2020) where cattle were administered hand-fed CTC-medicated product on pasture. Additional studies evaluating the off-target impact of long-term antimicrobial-based anaplasmosis control in different production settings and with greater sample sizes and sampling time points are needed.

While resistance associated with CTC has been extensively researched, little has been reported on resistance associated with its transformation products 4-epi-CTC (ECTC) and iso-CTC (ICTC). Upon examination (Chapter 3) our data provided evidence of antimicrobial activity of ECTC. These findings were contradictory to findings from previous studies which found modifications to the C4 position actually resulted in reduced antimicrobial activity (Blackwood & English, 1970). It is possible the epimers and isomers that were previously active have progressed to being microbiologically inactive along with the parent compound. Future studies are needed to expand this research to include more isolates with known treatment histories.

The study presented in Chapter 4 characterized the duration of detection of CTC after administration in both plasma and urine for three in-feed regimens (22 mg/kg of bodyweight daily, 350 mg/head per day, and 70 mg/head per day). Oral administration of CTC at a dose of 22 mg/kg of bodyweight per day showed a comparable phase I plasma elimination half-life of approximately 1 day (24 h) compared to Holstein steers administered a twice daily feeding of the same dose for 80 days (elimination half-life 16.2 h), as previously described in Reinbold et al., (2010). Terminal T ½ values for urine were 35.4 ± 1.5, 51.7 ± 1.9, and 64.7 ± 1.9 days for groups A, B, and C, respectively. Previous knowledge regarding tetracyclines affinity to bind to calcium suggests bones serve as a reservoir, contributing to persistent low-level urine CTC concentrations. The persistence of CTC in the urine and the long-reported urine half-lives in this study suggest further investigations on CTC bone residue concentrations are needed. This has major implications for the potential of unintentional feed contamination to cause prolonged urine concentrations, and potentially disqualification of cattle from no-antibiotic programs.

Lastly in Chapter 5, a pharmacokinetic approach was utilized to investigate two in-feed CTC regimens (22 mg/kg of bodyweight daily and 350 mg/head per day) for beef cattle. This

evaluation through PK/PD modeling in light of expected MIC values reveals discordance between the AUC/MIC ratio and some of the clinical study data. Based on the results, both in-feed CTC regimens failed to obtain a reasonable target attainment rate using a combined MIC distribution from published data for potential pathogens. Future studies should consider the sub-MIC effects of CTC transformation products such as ECTC which displayed antimicrobial activity in Chapter 3. In addition, challenge studies are needed to evaluate if these pre-determined AUC_{24}/MIC ratios are therapeutic in a clinical setting.

To conclude, ongoing research is needed to further define the efficacy and resistance patterns associated with in-feed administration of chlortetracycline to cattle, and to further characterize the duration of detectable urine concentrations after intentional and incidental exposure.

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