

Shifts in the antibiotic susceptibility profiles of zoonotic enteric bacteria in response to long-term chlortetracycline usage for the control of bovine anaplasmosis

by

Naemi Pomwenepawa Bickmeier

B.V.M., Sokoine University of Agriculture, 2017
M.S., Kansas State University, 2019

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine/Pathobiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2025

Abstract

Antibiotic resistance is a global crisis with serious consequences for public health, animal health, food security, and economies. Prolonged antibiotic use is a major driver of antibiotic resistance. Bovine anaplasmosis, a globally prevalent, economically significant tick-borne disease of cattle, is commonly controlled through long-term use of chlortetracycline (CTC) medicated feed at dosages of 0.5-2.0 mg/lb BW/day for six months or more.

The goal of these studies was to determine if administering CTC at different dosages to control active anaplasmosis in steers promotes antibiotic resistance in off-target zoonotic bacteria, *E. coli* and *Enterococcus*. We hypothesized that feeding steers CTC for 120 days would increase the recovery of tetracycline (TET)-resistant *E. coli* and *Enterococcus* isolates, with resistance occurring more rapidly in groups receiving higher CTC doses. Additionally, we anticipated that TET resistance would co-select for resistance to other antibiotics not administered during the study.

Cattle chronically infected with *Anaplasma marginale* were assigned to one of four CTC treatment groups: 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day, and fed their respective CTC rations. Single *E. coli* (n=192) and *Enterococcus* (n=222) isolates were obtained from each steer 12 days prior to CTC administration, after 58 and 114 days of continuous CTC administration, and 21 days post-cessation of CTC administration. Antibiotic susceptibilities were assessed using Clinical & Laboratory Standards Institute (CLSI) or the National Antimicrobial Resistance Monitoring System (NARMS) breakpoint classifications. Data analyses were performed using linear mixed model analysis with $\alpha = 0.10$.

For *E. coli*, there was a significant CTC effect on tetracycline TET minimum inhibitory concentration (MIC) ($p = 0.089$). Against a panel of 2,688 (192 isolates * 14 antibiotics)

antibiotics tested, 18.0% were resistant. Resistance generally increased with higher CTC doses: 95, 116, 115, and 152 resistant tests in the 0.0, 0.5, 2.0, and 10.0 mg groups, respectively. Resistance increased during CTC administration and slightly decreased after withdrawal. Overall, 84% of isolates were resistant to at least one antibiotic, and 50.5% were multidrug-resistant. TET resistance was observed in 78.6% of isolates, with 58.8% of isolates resistant before CTC administration. While TET MIC values did not differ significantly among treatment groups ($p \leq 0.266$), the proportion of TET-resistant isolates increased during treatment. Significant CTC effects were also observed for chloramphenicol (CHL) and streptomycin ($p = 0.012$ and $p = 0.029$, respectively), with MIC values crossing breakpoint classifications during CTC administration and slightly decreasing post-cessation of CTC administration.

For *Enterococcus*, against a panel of antibiotics, the total number of resistant MIC tests was 71 (n=714), 64 (n=756), 70 (n=840), and 77 (n=738) for the groups 0.0-, 0.5-, 2.0- and 10.0 mg, respectively. Ninety-six isolates were resistant to at least one antibiotic, while 5.0% were multidrug-resistant. TET resistance was observed in 89.6% of isolates overall, with 93.0% resistant prior to CTC administration. No significant CTC effect was found on TET MIC values ($p \geq 0.1153$). Additionally, there was no evidence that CTC use promoted resistance to other antibiotics tested.

These studies demonstrate a high inherent TET resistance in both *E. coli* and *Enterococcus* isolates from steers. Furthermore, CTC administered at 0.5–2.0 mg/lb BW/day may promote resistance to non-tetracycline antibiotics in *E. coli*, but not in *Enterococcus*. Given that field use of CTC often exceeds 120 days, the resistance patterns observed here may be even more pronounced in real-world conditions. These results underscore the urgent need for non-

antibiotic alternatives to control bovine anaplasmosis, such as an effective, strain-independent vaccine.

Shifts in the antibiotic susceptibility profiles of zoonotic enteric bacteria in response to long-term
chlortetracycline usage for the control of bovine anaplasmosis

by

Naemi Pomwenepawa Bickmeier

B.V.M., Sokoine University of Agriculture, 2017
M.S., Kansas State University, 2019

A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine/Pathobiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2025

Approved by:

Co-Major Professor
Dr. Kathryn Reif

Approved by:

Co-Major Professor
Dr. Emily Reppert

Copyright

© Naemi Bickmeier 2025.

Abstract

Antibiotic resistance is a global crisis with serious consequences for public health, animal health, food security, and economies. Prolonged antibiotic use is a major driver of antibiotic resistance. Bovine anaplasmosis, a globally prevalent, economically significant tick-borne disease of cattle, is commonly controlled through long-term use of chlortetracycline (CTC) medicated feed at dosages of 0.5-2.0 mg/lb BW/day for six months or more.

The goal of these studies was to determine if administering CTC at different dosages to control active anaplasmosis in steers promotes antibiotic resistance in off-target zoonotic bacteria, *E. coli* and *Enterococcus*. We hypothesized that feeding steers CTC for 120 days would increase the recovery of tetracycline (TET)-resistant *E. coli* and *Enterococcus* isolates, with resistance occurring more rapidly in groups receiving higher CTC doses. Additionally, we anticipated that TET resistance would co-select for resistance to other antibiotics not administered during the study.

Cattle chronically infected with *Anaplasma marginale* were assigned to one of four CTC treatment groups: 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day, and fed their respective CTC rations. Single *E. coli* (n=192) and *Enterococcus* (n=222) isolates were obtained from each steer 12 days prior to CTC administration, after 58 and 114 days of continuous CTC administration, and 21 days post-cessation of CTC administration. Antibiotic susceptibilities were assessed using Clinical & Laboratory Standards Institute (CLSI) or the National Antimicrobial Resistance Monitoring System (NARMS) breakpoint classifications. Data analyses were performed using linear mixed model analysis with $\alpha = 0.10$.

For *E. coli*, there was a significant CTC effect on tetracycline TET minimum inhibitory concentration (MIC) ($p = 0.089$). Against a panel of 2,688 (192 isolates * 14 antibiotics)

antibiotics tested, 18.0% were resistant. Resistance generally increased with higher CTC doses: 95, 116, 115, and 152 resistant tests in the 0.0, 0.5, 2.0, and 10.0 mg groups, respectively. Resistance increased during CTC administration and slightly decreased after withdrawal. Overall, 84% of isolates were resistant to at least one antibiotic, and 50.5% were multidrug-resistant. TET resistance was observed in 78.6% of isolates, with 58.8% of isolates resistant before CTC administration. While TET MIC values did not differ significantly among treatment groups ($p \leq 0.266$), the proportion of TET-resistant isolates increased during treatment. Significant CTC effects were also observed for chloramphenicol (CHL) and streptomycin ($p = 0.012$ and $p = 0.029$, respectively), with MIC values crossing breakpoint classifications during CTC administration and slightly decreasing post-cessation of CTC administration.

For *Enterococcus*, against a panel of antibiotics, the total number of resistant MIC tests was 71 (n=714), 64 (n=756), 70 (n=840), and 77 (n=738) for the groups 0.0-, 0.5-, 2.0- and 10.0 mg, respectively. Ninety-six isolates were resistant to at least one antibiotic, while 5.0% were multidrug-resistant. TET resistance was observed in 89.6% of isolates overall, with 93.0% resistant prior to CTC administration. No significant CTC effect was found on TET MIC values ($p \geq 0.1153$). Additionally, there was no evidence that CTC use promoted resistance to other antibiotics tested.

These studies demonstrate a high inherent TET resistance in both *E. coli* and *Enterococcus* isolates from steers. Furthermore, CTC administered at 0.5–2.0 mg/lb BW/day may promote resistance to non-tetracycline antibiotics in *E. coli*, but not in *Enterococcus*. Given that field use of CTC often exceeds 120 days, the resistance patterns observed here may be even more pronounced in real-world conditions. These results underscore the urgent need for non-

antibiotic alternatives to control bovine anaplasmosis, such as an effective, strain-independent vaccine.

Table of Contents

List of Figures	xiv
List of Tables	xvi
Acknowledgements	xviii
Dedication	xx
Chapter 1 - Literature Review	1
Antibiotic Resistance Introduction	1
Impact of Antibiotic Resistance on Public Health	1
Impact of Antibiotic Resistance on Veterinary Health	2
Mechanisms of Antibiotic-Resistance	10
Horizontal Gene Transfer.....	11
Conjugation.....	11
Transformation.....	12
Transduction	13
1.3Co-selection of antibiotic resistance	15
Cross-resistance	16
Co-resistance.....	17
Spread of antibiotic-resistant bacteria between humans, animals, and the environment.....	18
Antibiotic Use in Production Animals.....	20
Indications for prevention of livestock diseases in the U.S.	20
Indications for treatment of livestock diseases in the U.S.	21
Indications for control of livestock diseases in the U.S.	21
Use of antibiotics for production purposes in the U.S.	22
The U.S. National Action Plan for Combating Antibiotic-resistant Bacteria.....	33
The FDA’s Actions in Response to the National Action Plans.	38
Selected actions of the FDA on addressing AMR	40
Importance of bovine anaplasmosis.....	44
Causative agent	45
Prevalence of bovine anaplasmosis.....	47
Impact of infection	51

Susceptible hosts	51
Clinical disease	51
Chronic disease	53
Challenges of bovine anaplasmosis control that necessitate long-term antibiotics	54
Ability for <i>A. marginale</i> to produce antigenic variants	54
<i>Anaplasma marginale</i> antibiotic resistance genes	56
Disadvantages of anaplasmosis control which necessitate antibiotics as a common control strategy	57
Maintaining an <i>A. marginale</i> -free herd	57
Preventing iatrogenic transmission	58
Using acaricides and insecticides.....	58
Vaccines	62
Antibiotics for <i>A. marginale</i> control.....	64
Summary	68
Chapter 2 - Antibiotic susceptibilities of <i>Escherichia coli</i> isolated from cattle hand-fed	
chlortetracycline for anaplasmosis control	71
Abstract	71
Introduction.....	72
Materials and Methods.....	75
Treatment Groups and Treatment Administration	75
Fecal Sample Collection	76
Isolation of <i>Escherichia coli</i>	77
Antibiotic Susceptibility Testing	77
Statistical Analysis	78
Results.....	79
Summary Statistics.....	79
Summary Statistics of Antibiotic Susceptibility for the Overall Study	79
Summary Statistics of Antibiotic Susceptibility for the 0.0 mg CTC Group	81
Summary Statistics of Antibiotic Susceptibility for the 0.5 mg CTC Group	86
Summary Statistics of Antibiotic Susceptibility for the 2.0 mg CTC Group	87
Summary Statistics of Antibiotic Susceptibility for the 10.0 mg CTC Group	89

Effect of Prolonged CTC Administration on <i>E. coli</i> Tetracycline Susceptibility	91
Effect of Prolonged CTC Administration on the Susceptibility of <i>E. coli</i> to Non-TET	
Antibiotics	100
Discussion	112
Conclusions	122
Chapter 3 - Antibiotic susceptibilities of <i>Enterococcus</i> isolated from cattle hand-fed	
chlortetracycline for anaplasmosis control	123
Abstract	123
Introduction	124
Materials and Methods	127
Treatment Groups and Treatment Administration	127
Fecal Sample Collection	128
Isolation and speciation of <i>Enterococcus</i> spp.	129
Antibiotic Susceptibility Testing	130
Statistical Analysis	131
Results	132
Summary Statistics	132
Summary Statistics for the Overall Study	132
Summary Statistics of Antibiotic Susceptibility for the 0.0 mg CTC Group	140
Summary Statistics of Antibiotic Susceptibility for the 0.5 mg CTC Group	142
Summary Statistics of Antibiotic Susceptibility for the 2.0 mg CTC Group	144
Summary Statistics of Antibiotic Susceptibility for the 10.0 mg CTC Group	146
Effect of Prolonged CTC Administration on <i>Enterococcus</i> Tetracycline Susceptibility ...	149
Effect of Prolonged CTC Administration on the Susceptibility of <i>Enterococcus</i> to Non-TET	
Antibiotics	155
Discussion	172
Chapter 4 - Concluding remarks	178
Background:	178
Knowledge gap:	179
Study aims:	179
Study hypothesis:	180

Summary of findings	180
<i>E. coli</i> study and comparative analysis with other studies:.....	180
<i>Enterococcus</i> and comparative analysis with other studies:	183
Combined findings from <i>E. coli</i> and <i>Enterococcus</i> studies:	185
Implications:	186
Strengths and limitations:	187
Future directions.	187
Summary:.....	188
Chapter 5 - References.....	189

List of Figures

Figure 1.1. The economic impact of antibiotic use in production animals and humans.....	9
Figure 1.2. Mechanisms of horizontal gene transfer.....	14
1.3Co-selection of antibiotic resistance	15
Figure 1.4. Co-selection of antibiotic resistance	17
Figure 1.5. Spread of antibiotic resistance	19
Figure 1.6. The classification of the Anaplasmataceae Family	46
Figure 1.7. <i>A. marginale</i> statewide prevalence within the last 20 years.....	50
Figure 1.8. (a) <i>A. marginale</i> infected red blood cells (b), Packed cell volume	53
Figure 1.9. <i>Anaplasma marginale</i> rickettssemia levels during replication in vertebrate hosts	56
Figure 1.10. Schematic diagram of <i>A. marginale</i> development in bovine erythrocytes and tick cells	60
Figure 2.1. Heatmap of the antibiotic resistance phenotypes of <i>E. coli</i> isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days	80
Figure 2.2. Proportions of <i>E. coli</i> isolates resistant to medically important antibiotics	85
Figure 2.3. Comparison of tetracycline MIC least mean square values from <i>E. coli</i> isolates collected from groups of steers administered different chlortetracycline dosages for 120 days	92
Figure 2.4. Comparison of <i>E. coli</i> antibiotic MIC least square mean values from isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days	94
Figure 3.1. Proportion of 222 <i>Enterococcus</i> isolates from cattle handfed chlortetracycline at varying doses for 120 consecutive days to a panel of 14 antibiotics	133
Figure 3.2. Resistant phenotypes from <i>Enterococcus</i> isolated from cattle administered CTC for bovine anaplasmosis control for 120 days for the entire study	134
Figure 3.3. Antibiotic-resistant <i>Enterococcus</i> isolates from cattle handfed chlortetracycline at varying doses for 120 consecutive days to a panel of 14 antibiotics	137
Figure 3.4. Antibiotic-resistant profiles of 222 total <i>Enterococcus</i> isolates from cattle handfed chlortetracycline at varying doses for 120 consecutive days	139

Figure 3.5. <i>Enterococcus</i> spp. tetracycline Minimum Inhibitory Concentration values from isolates collected from groups of steers administered different chlortetracycline dosages for 120 days	155
Figure 3.6. Comparison of <i>Enterococcus</i> antibiotic MIC least square mean values from isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days	159

List of Tables

Table 1-1. Results from previous studies of full and partial bans against using antibiotic feed additives in livestock production	7
Table 1-2. Indications for antibiotics in livestock	23
Table 1-3. Antibiotics marketed for food-producing animals in the U.S. in 2022, their indications, modes of action and mechanisms of resistance	24
Table 1-4. Statewide prevalence of <i>A. marginale</i>	48
Table 1-5. Historic antibiotic treatment regimens for bovine anaplasmosis control	64
Table 1-6. Current approved regimens for bovine anaplasmosis control	66
Table 2-1. Distributions of minimum inhibitory concentrations of 192 <i>E. coli</i> isolates from cattle handfed chlortetracycline at varying doses for 120 consecutive days to a panel of 14 antibiotics	81
Table 2-2. Distributions of minimum inhibitory concentrations of 48 <i>E. coli</i> isolates from cattle handfed 0.0 mg/lb BW/day chlortetracycline for 120 consecutive days to a panel of 14 antibiotics	83
Table 2-3. Animal IDs from where pan-susceptible <i>E. coli</i> was isolated	84
Table 2-4. Distributions of minimum inhibitory concentrations of 48 <i>E. coli</i> isolates from cattle handfed 0.5 mg/lb BW/day chlortetracycline for 120 consecutive days to a panel of 14 antibiotics	87
Table 2-5. Distributions of minimum inhibitory concentrations of 48 <i>E. coli</i> isolates from cattle handfed 2.0 mg/lb BW/day chlortetracycline for 120 consecutive days to a panel of 14 antibiotics	89
Table 2-6. Distributions of minimum inhibitory concentrations of 48 <i>E. coli</i> isolates from cattle handfed 10.0 mg/lb BW/day chlortetracycline for 120 consecutive days to a panel of 14 antibiotics	90
Table 2-7. Antibiotic minimum inhibitory concentrations of <i>E. coli</i> isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days	96

Table 3-1. Distributions of minimum inhibitory concentrations of 222 <i>Enterococcus</i> isolates from cattle handfed chlortetracycline at varying doses for 120 consecutive days to a panel of 14 antibiotics	135
Table 3-2. Animal IDs from where pan-susceptible <i>Enterococci</i> was isolated	136
Table 3-3. Distributions of minimum inhibitory concentrations of 51 <i>Enterococcus</i> isolates from cattle handfed 0.0 mg/lb BW/day chlortetracycline for 120 consecutive days to a panel of 14 antibiotics	141
Table 3-4. Distributions of minimum inhibitory concentrations of 54 <i>Enterococcus</i> isolates from cattle handfed 0.5 mg/lb BW/day chlortetracycline for 120 consecutive days to a panel of 14 antibiotics	144
Table 3-5. Distributions of minimum inhibitory concentrations of 60 <i>Enterococcus</i> isolates from cattle handfed 2.0 mg/lb BW/day chlortetracycline for 120 consecutive days to a panel of 14 antibiotics	146
Table 3-6. Distributions of minimum inhibitory concentrations of 57 <i>Enterococcus</i> isolates from cattle handfed 10.0 mg/lb BW/day chlortetracycline for 120 consecutive days to a panel of 14 antibiotics	148
Table 3-7. Antibiotic minimum inhibitory concentrations of <i>Enterococcus</i> isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days	151

Acknowledgements

I have been blessed with an incredible support system, family and friends whose encouragement and advice sustained me, even on days when I doubted, I would ever reach the finish line. First and foremost, I thank my husband, William “Bill” Bickmeier, and our mothers, Helena Lutombi and Jean Bickmeier. Your unwavering prayers, encouragement, and belief in me have been invaluable and kept me going.

I have also been fortunate to have wonderful friends who have provided a listening ear, allowed me to vent my frustrations, and made me laugh, helping me momentarily forget the challenges of the Ph.D. journey. Thank you, Barb and Ron Goodman, Moses Nyine, Carrie and Drew Fratecelli, Dominica Genda (Ferm), Jonathan Ferm, Tshegofatso Ngwaga (Brucken) and Dustin Brucken, Robert Mugabi, Kelly Shunje, and Catherine Obiribea Ofori-Bah.

I extend my heartfelt gratitude to my church communities: Vintage Faith Church, led by Pastor Jonathan Hastings in Manhattan, Kansas, and our small group, led by Kenan and Nada Ingalsbe. A special thank you as well to Grace Auburn Church in Auburn, Alabama, and our entire small group, led by Lisa and Pastor Jason Ingle, along with Morgan and Abby Bell. Your support, guidance, and prayers have meant the world to me.

I am also grateful to my fellow graduate and postdoctoral students who have helped me along the way, Dinali Perera, Changin Oh, Macy Flowers, Hulda Sang, Deepak Kumar, Rakshith Kumar, and Kamilyah Miller. Thank you for your advice, time, and willingness to assist, whether it was troubleshooting challenges or providing an audience for my presentations, including my preliminary exam preparations.

A special thank you to David George, Tippawan Anantatat, and Samir Elqaidi for your patience, kindness, and generosity in training me and teaching me new techniques. Your willingness to help me troubleshoot when needed has been greatly appreciated.

I would also like to express my sincere appreciation to my co-advisors, Dr. Kathryn Reif and Dr. Emily Reppert, as well as the rest of my committee, Dr. Waithaka Mwangi and Dr. Chika Okafor, for their invaluable guidance and support.

My deepest gratitude goes to Dr. Yunjeong Kim, my first Ph.D. advisor, for her mentorship, generosity, and continued support. Even after I transitioned from her lab, she graciously allowed me to use her lab space and resources. I will forever be grateful for your kindness.

Finally, a big thank you to the K-State College of Veterinary Medicine for providing unique opportunities, resources, and training that have enabled me to gain knowledge, refine my skills, and prepare for the post-graduation job market.

Dedication

To my husband and the head of our household, William “Bill” Bickmeier. You have been my steadfast anchor throughout this journey, as you are in all our journeys. I am truly blessed to have the gift of you.

Chapter 1 - Literature Review

Antibiotic Resistance Introduction

Antimicrobial resistance occurs when microorganisms such as bacteria, viruses, fungi, adapt to survive and grow in the presence of drugs that previously controlled them [1]. Specifically, resistance of bacteria to drugs is known as antibiotic resistance [2]. Infections caused by antibiotic-resistant bacteria can result in negative and, at times, severe outcomes, including prolonged treatment periods and, in some cases, death [3–5]. Furthermore, these infections may necessitate the use of alternative antibiotics, which can be more expensive, contraindicated, or, in some instances, nonexistent. Challenges in the discovery and approval processes for new antibiotics have further hindered the development of alternative antibiotic and non-antibiotic therapies [6].

Impact of Antibiotic Resistance on Public Health

Antibiotic resistance is a global public health threat that negatively impacts national economies [7–9]. A 2019 study involving 204 countries and territories aimed to determine the global burden of bacterial antimicrobial resistance [10]. The study reported that antibiotic resistance was associated with more than 5 million deaths in 2019. According to the Centers for Disease Control’s (CDC) 2019 Antibiotic Resistance Threat Report, antibiotic resistance in the U.S. accounts for approximately 2.8 million antibiotic-related infections and about 35,000 deaths per year [11]. Additionally, a U.S. cost estimate study based on Department of Veterans Affairs records found that antibiotic-resistant infections resulted in \$4.1–5.1 billion in healthcare costs in 2017 [9]. These costs included direct medical expenses such as hospital stays, intensive care unit care, surgeries, and mechanical ventilation. However, the study did not account for healthcare

costs incurred by patients' post-hospitalization, such as those related to long-term illnesses, nor did it consider economic impacts such as loss of work time and mortalities. In the European Union (EU) and European Economic Area, antibiotic resistance was responsible for more than 800,000 illnesses and more than 35,000 deaths in 2020 [12]. The economic burden of antibiotic resistance in the EU (based on data from 2002 to 2007) was estimated to be around €1.5 billion annually, with costs attributed to healthcare expenses and reduced workforce participation [13].

According to a forecast from the EcoAMR series (Health and Economic Impacts of AMR in Humans and Food-Producing Animals), which is funded by the UK Department of Health and Social Care's Fleming Fund, antimicrobial resistance (AMR) is projected to cause 38.5 million deaths between 2025 and 2050 [14]. This report, which takes a One Health approach to provide current estimates of AMR's economic burden in both humans and livestock, also predicts that AMR-related healthcare costs will reach \$159 billion per year by 2025.

Impact of Antibiotic Resistance on Veterinary Health

While the burden of antibiotic resistance in animals is comparable to that in humans, it has received relatively little attention in the literature [15]. Most research on antibiotic resistance in animals focuses primarily on its potential risks to human health [15]. One of the few studies addressing the burden of antibiotic resistance specifically in animals is the EcoAMR series [14]. This series projects that, by 2050, annual livestock production losses under a very low resistance scenario/reference scenario (5%) could equal the consumption needs of approximately 746 million people, whereas losses under a more pessimistic scenario (antibiotic use disease burden attributed to AMR doubles in all regions and faster than in reference scenario) could equate to the needs of 2 billion people. The report further estimates that the cumulative GDP loss due to

AMR in livestock from 2025 to 2050 will be \$575 billion under the reference scenario and \$953 billion under a pessimistic scenario. Additionally, the spillover impact of AMR in livestock on human populations, reflected in reduced labor productivity, is projected to reach \$1.1 trillion under the reference scenario and \$5.2 trillion under the pessimistic scenario.

Antibiotics play a critical role in production systems by serving three main purposes: prevention/prophylaxis (administered to at-risk animals to prevent infections and reduce the likelihood of clinical disease), treatment (administered to alleviate suffering and treat existing infections), and control (administered to infected animals to lessen clinical symptoms, limit disease spread, and prevent transmission to other animals) of bacterial diseases [16–18]. Several bacterial diseases significantly affect production systems. For instance, respiratory and enteric diseases are common in intensive production systems like feedlots, while mastitis is a major concern in dairy operations [15].

Antibiotic-resistant pathogens exacerbate these challenges, leading to diseases that are potentially life-threatening, require prolonged and costly treatments, or lack effective alternatives. This can result in substantial production losses, including direct animal losses, weight loss or reduced weight gain due to illness, and discarded products such as milk during infection and antibiotic therapy. A notable example is a mastitis outbreak in a closed herd caused by multidrug-resistant *Staphylococcus aureus*. This outbreak, which lasted 18 months, resulted in significant reductions in milk quantity and quality. Moreover, the cull rate of infected cows matched the incidence rate at approximately 2.6%, shortening the lifespan of affected cows. [19]. The persistence of antibiotic-resistant *S. aureus* in the environment further prolonged the outbreak, exacerbating milk losses and highlighting the enduring challenges posed by resistant pathogens.

The burden of antibiotic resistance on animals includes significant economic costs. However, the direct economic impact of antibiotic resistance in animals has not been extensively documented in published literature. Challenges in estimating these costs arise from difficulties in determining the exact reasons for antibiotic administration, the dosages used, and the costs associated with non-antibiotic-responsive infections, such as those resulting in extended treatment, production losses, and veterinary expenses [20]. This issue is particularly pronounced in many developing countries where veterinary oversight of antibiotic use is often lacking, allowing producers to purchase antibiotics over the counter and administer them to animals without guidance or oversight [21,22]. In the U.S., the regulation of antibiotics is overseen by the Food and Drug Administration (FDA). Currently, the best available estimates of antibiotic use in U.S. production animals are derived primarily from annual sales data published by the U.S. Department of Agriculture (USDA).

The economic burden of antibiotic resistance in production animals impacts both producers and consumers. For producers, the economic burden primarily stems from lost income due to reduced productivity (e.g., decreased milk production, decreased weight gain, and high culling rates) and increased treatment costs [15,23,24]. To compensate for these losses, producers may raise their prices, which in turn places a financial burden on consumers through higher food costs. This reduction in productivity at a large scale threatens global food security. Additionally, antibiotic resistance poses risks of trade restrictions due to trade bans aimed at reducing the spread of antibiotic resistance [24]. Decreased exports can also result from lower production levels and concerns over importing livestock and livestock products from countries with high rates of antibiotic resistance. A 2017 World Bank Group report on the economic and developmental impacts of AMR projected that antibiotic resistance could lead to a global

reduction in livestock and livestock product exports by 1.1% under a very low resistance scenario/reference scenario (5%) and by 3.8% a pessimistic scenario (antibiotic use disease burden attributed to AMR doubles in all regions and faster than in reference scenario) [24].

Because direct estimates of antibiotic resistance in livestock production systems are lacking, the economic burden of antibiotic resistance can be assessed by estimating the cost of not using antibiotics in these systems. Historically, antibiotics have been used in U.S. livestock production to promote animal growth, with demonstrated benefits in weight gain and feed efficiency. For instance, a review in Doane's Agricultural Report: Reference Volume noted that antibiotic use in steers and heifers increased weight gain by 6% and improved feed efficiency by 4% [25,26]. In swine, antibiotics enhanced weight gain by 10% and feed efficiency by 5%, while in dairy cattle, they supported weight gains of up to 60 [26,27]. The cost of foregoing antibiotics for growth promotion can thus be estimated by calculating the price per pound of carcass weight and factoring in the weight gains attributable to antibiotic use.

Estimates of the economic burden of banning antibiotic feed additives on producers and consumers have been summarized in a 2001 report by the USDA (Table 1-1. Results from previous studies of full and partial bans against using antibiotic feed additives in livestock production) [26]. For instance, swine production reached 19.278 billion pounds in 1999. If antibiotic feed additives were associated with a 5% increase in weight gain, then approximately 229 million pounds of the total swine production was attributed to these additives. Consequently, the estimated production loss without antibiotic additives would translate to a net loss of about \$45.5 million for the U.S. national economy [26]. However, a different study indicated that the net economic gains from using antibiotic feed additives are influenced by the management practices of farming systems [28]. According to this study, advanced farming systems with good

management practices such as hygiene and biosecurity, showed negligible economic gains from the use antibiotic feed additives. In contrast, farming systems with less advanced practices, such as those in Brazil and China, experienced significant economic benefits from these additives. This disparity may be attributed to the fact that antibiotic feed supplements helped mitigate production losses stemming from poor hygiene and sanitary conditions.

Although there are benefits to using antibiotics for growth promotion, their use has been restricted in some countries [29–33]. For instance, in 2006, Europe banned the use of all antibiotics for growth promotion in production animals, and in 2017, the U.S. prohibited the use of all medically important antibiotics for the same purpose.

Table 1-1. Results from previous studies of full and partial bans against using antibiotic feed additives in livestock production

Study	Year of publication	Aggregate costs per year (million dollars)	Aggregate costs per year (adjusted to 1998 million dollars)
Allen and Burbee; and Gilliam et al ¹	1972; 1973	5,660	12,707
Dworkin (Beef and pork only) ¹	1976	2,140	4,804
Mann and Paulsen ¹	1976	580	1,302
Headley ¹ :	1978		
i. Ban penicillin and tetracycline		1,440	3,233
ii. Ban nitrofurans and sulfa compounds		4,680	10,507
USDA II (moderate efficiency, penicillin, tetracyclines, sulfa drugs, and nitrofurans banned) ¹	1978	1,240	2,784
Wade and Barkley (4% decrease in Supply; Supply elasticity of 0.40; 5% increase in Demand; Demand elasticity of -2.50)	1992		
i. Swine (loss in consumer surplus, 1987 dollars)		11.74	16.85
ii. Swine (loss in producer surplus, 1987 dollars)		12.59	18.07
National Research Council/Institute of Medicine	1998		
i. Partial ban		1,261	1,281
ii. Total ban		2,527	2,566
Hayes et al.	1999		
i. Losses to producers		160.3	160.3
ii. Losses to consumers		748	748

Source: Mathews K.H 2001 [26]

*¹Data from table 45 of the 1981 CAST report (Council for Agricultural Science and Technology, Antibiotics in Animal Feeds, Report No. 88): 5-year totals divided by 5 years.

The economic impact of forgoing antibiotics for disease prevention, control, and treatment can also be estimated, and several studies have examined this issue. One such study evaluated the economic consequences of not using antibiotics for disease prevention and control, or avoiding antibiotics entirely, in a U.S. feedlot scenario involving 100 steers [34]The analysis

considered factors such as average daily weight gain (based on literature), feed and operational costs, veterinary expenses, and transportation costs. The study focused on common feedlot diseases, including Bovine Respiratory Disease (BRD) and liver abscesses, and accounted for the costs of antibiotics used to prevent, control, and treat these conditions. Results showed that abstaining from antibiotics for disease prevention and control led to a net revenue loss of \$66 per animal, while complete elimination of antibiotics resulted in a loss of \$96 per animal. Both scenarios also resulted in higher incidences of BRD and liver abscesses, with the highest rates observed when antibiotics were not used at all.

Another study evaluated the cost of banning antibiotic use in U.S. dairy systems for both producers and consumers [35]. This study found that banning antibiotics in a dairy herd of 1,000 cows would increase milk prices from \$0.423 per liter to \$0.425 per liter, resulting in a predicted annual loss of \$152 million. Furthermore, prohibiting antibiotic use in such a system is expected to lead to a loss of 354 million kgs of milk, which represents 0.4% of the total milk produced in the U.S. between 2012 and 2016. The total increased cost to consumers in this scenario is estimated to be \$232 million due to higher milk prices. In contrast, producers are estimated to offset their losses by raising milk prices, experiencing a net loss of only \$38 million. The study noted that costs would vary depending on the level of antibiotic use on a farm, with heavy antibiotic users experiencing more significant effects.

Although antibiotic use promotes antibiotic resistance, with serious consequences for human, animal, and economic health, it remains essential for preventing, controlling, and treating diseases and thus contributes significantly to socioeconomic gains (Figure 1.1. The economic impact of antibiotic use in production animals and humans) [15,36]. Rather than banning antibiotic use in production animals, efforts should be made to minimize its contribution to

antibiotic resistance. Efforts to achieve this are already underway in several countries, including the U.S. The U.S. Food and Drug Administration (FDA), which regulates antibiotics, is dedicated to combating antibiotic resistance by preserving the efficacy of current antibiotics and encouraging the development of new antibiotics to reduce the emergence and spread of resistant bacteria [37].

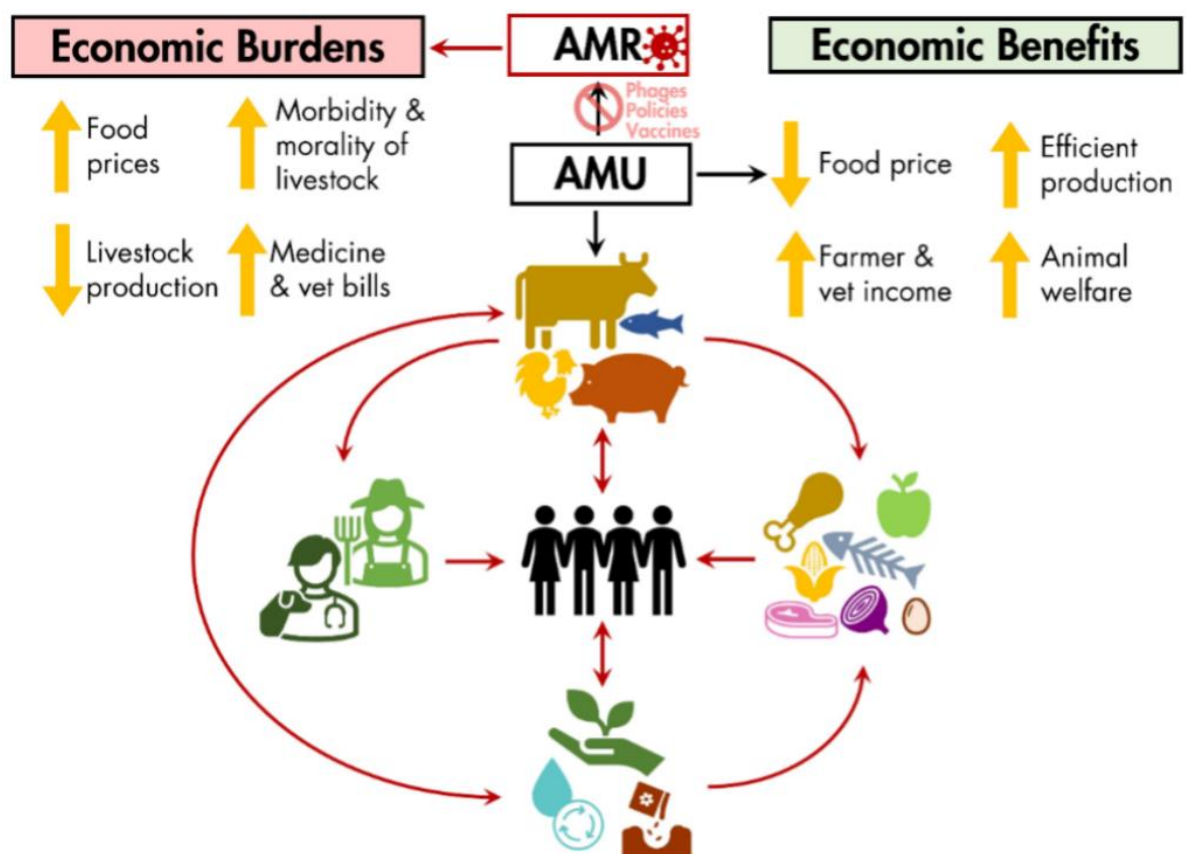


Figure 1.1. The economic impact of antibiotic use in production animals and humans

The economic impact of antibiotic use (AMU). Right side: Economic benefits when AMU results in increased production, increased animal welfare, increased producer income, and decreased food prices for consumers. Left side: Economic burdens when AMU promotes antibiotic resistance (AMR), which results in increased livestock morbidity and mortality, increased cost of treatment, increased food prices, and decreased production.

Source: <https://www.rand.org/randeurope/research/projects/2023/ecoamr.html> [36].

Mechanisms of Antibiotic-Resistance

Bacteria develop antibiotic resistance through various mechanisms, including modification of antibiotic target sites, active efflux of antibiotics from their cells, inactivation of antibiotics, and reduction of antibiotic permeability into bacteria cells (reviewed in [38]).

Resistance can develop via natural or acquired mechanisms.

Natural resistance includes intrinsic and induced resistance. In intrinsic resistance, the resistance to antibiotics is consistently expressed and universally shared within a bacterial species. For example, gram-negative bacteria possess intrinsically reduced outer membrane permeability, which limits antibiotic entry into their cells (reviewed in [38]). In contrast, induced resistance occurs when naturally resistant genes are expressed only after exposure to antibiotics [38,39]. An example of this is the presence of efflux pumps in some bacteria which actively pump antibiotics out of the cells.

In acquired resistance, antibiotic resistance develops through genetic mutations of antibiotic gene targets or via horizontal gene transfer of antibiotic-resistant genes in response to exposure to antibiotics [39]. Genetic mutations contributing to antibiotic resistance have been demonstrated for several antibiotics, including rifampicin, streptomycin, and fluoroquinolone (reviewed in [40,41]).

Horizontal gene transfer enables bacteria to develop resistance to multiple antibiotics, as genetically linked resistance genes can be acquired simultaneously. Resistance genes can be transferred between and across bacterial species. Consequently, the potentiation of antibiotic resistance through horizontal gene transfer is a major concern in the antibiotic resistance crisis and will be discussed in more detail below [41,42].

Horizontal Gene Transfer

Antibiotic resistance resulting from horizontal gene transfer occurs by one of three mechanisms: conjugation, transformation, and transduction (Figure 1.2. Mechanisms of horizontal gene transfer).

Conjugation

Conjugation is the most extensively studied mechanism of horizontal gene transfer. It involves the direct transfer of DNA between cells, facilitated by conjugation machinery encoded by genetic elements such as plasmids and transposons [42,43]. It begins with the formation of a pilus from the donor bacterium, which facilitates the transfer of genetic material to the recipient bacterium. The requirement for direct cell-to-cell contact protects the DNA, which is why conjugation is often considered the most common and most efficient mechanism of horizontal gene transfer (reviewed in [42]).

The transfer of antibiotic-resistant genes via conjugation has been reported across various bacterial species, including those from distant taxa, highlighting its significance in disseminating antibiotic-resistant genes. For example, the prevalence of the tetracycline-resistant gene *tetQ* in *Bacteroides* species isolated from healthy volunteers and clinical samples increased from 30% to 80% between 1980 and 2000 [44]. In that study, the DNA sequences of the *tetQ* alleles were 96%-100% identical between both study populations, suggesting horizontal gene transfer within the population. Southern blotting analysis of samples from both study populations indicated that gene transfer occurred via conjugation involving a transposon.

Another example is the demonstration of intraspecies conjugal transfer between *Enterococcus* isolates in a series of in vivo and in vitro experiments [45]. In the in vitro studies,

donor *Enterococcus faecium* harboring the *aadE*, *aphA-3*, and *erm(B)* genes on a single transposon were co-cultured with recipient isolates on agar plates. This resulted in a mean transfer frequency of 2×10^{-5} transconjugants/donor genes, confirming the transfer of resistant genes between the *Enterococcus* isolates. In the in vivo studies, mice were orally inoculated with the recipient strain, *E. faecium* 64/3RFS, which is resistance to streptomycin. Five days later, colonization of the mouse gut by donor strain, *E. faecium* 160/00, resistant to multiple antibiotics (erythromycin, streptomycin, kanamycin, penicillin, tetracycline and gentamycin, was observed. By day ten, transconjugates of the recipient strain were detected in the mice, which were also resistant to erythromycin, kanamycin, penicillin, tetracycline and gentamycin. Resistance gene transfer was confirmed in both experiments using PCR, pulsed-field gel electrophoresis, plasmid profiling, and Southern hybridization.

Transformation

Gene transfer via transformation involves the uptake and integration of naked DNA by bacteria [46]. The recipient cell drives this process and encodes all the required proteins. Currently, only a few transformation-competent bacteria are known, making transformation the least common method of horizontal gene transfer [47]. According to a 2018 study, only 82 bacterial species were known to be naturally transformable [47]. These include certain *Staphylococcus* species (e.g., *S. aureus* Mu50), some *Bacillus* species (e.g., *B. licheniformis* DSM 13), select *Streptococcus* species (e.g., *S. pneumoniae* R6), and *E. coli* K-12 (which is only competent when *sxy* is expressed).

For example, a study examined *Acinetobacter baumannii*, a human pathogen with high genetic plasticity that frequently acquires antibiotic-resistance genes through transformation [48].

In this study, DNA from two carbapenem-resistant *Klebsiella pneumoniae* strains (VA360 and Kb18), *Providencia rettgeri* strain M15758, and a methicillin-resistant *Staphylococcus aureus* ‘Cordobes’ clone was mixed and cultured with the carbapenem-susceptible *A. baumannii* A118 strain. Transformation was evaluated through antibiotic susceptibility testing and confirmed by whole-genome sequencing of the recipient strain. The study found that mobile genetic elements, antibiotic resistance genes, and metabolism-related operons were frequently acquired by *Acinetobacter baumannii* through transformation.

Transduction

Gene transfer via transduction utilizes bacteriophages and phage-related elements to acquire genetic material from bacteria they infect, which may provide advantages for their survival [49]. Examples of antibiotic-resistant genes transferred via transduction include ceftazidime, imipenem, and aztreonam resistance genes in *Pseudomonas aeruginosa*, mediated by AP-1 and AP-12 phages (reviewed in [49]). Similarly, P1-like phages are known to carry extended β -lactamase genes in *E. coli* [50].

One study demonstrated that bacteriophages isolated from chicken could transfer antibiotic resistance genes to *E. coli* isolates [51]. In this study, fifty antibiotic-resistant *E. coli* isolates were obtained from chicken meat products collected from street markets, supermarkets, and butcher shops, and phage lysates were extracted from these isolates. Transduction experiments were then conducted by incubating the phage lysates with commercially sourced *E. coli* donor cultures (ATCC 13706). The presence of resistance genes in transduced *E. coli* isolates was verified via PCR, and antibiotic susceptibility was assessed through susceptibility testing. Results showed that a quarter of the phages from chicken products were capable of

transducing antibiotic resistance genes for at least one of four tested antibiotics, tetracycline, ampicillin, chloramphenicol, and kanamycin, to the donor *E. coli* strains.

Transduction-mediated antibiotic resistance occurs more frequently than previously believed and has been documented across various bacterial species and ecosystems, including water bodies (e.g., rivers and sewage), soil, humans, and animals (reviewed in [42,51]). Consequently, antibiotic resistance via transduction is thought to play a significant role in the spread of resistance among bacteria of food origin [51].

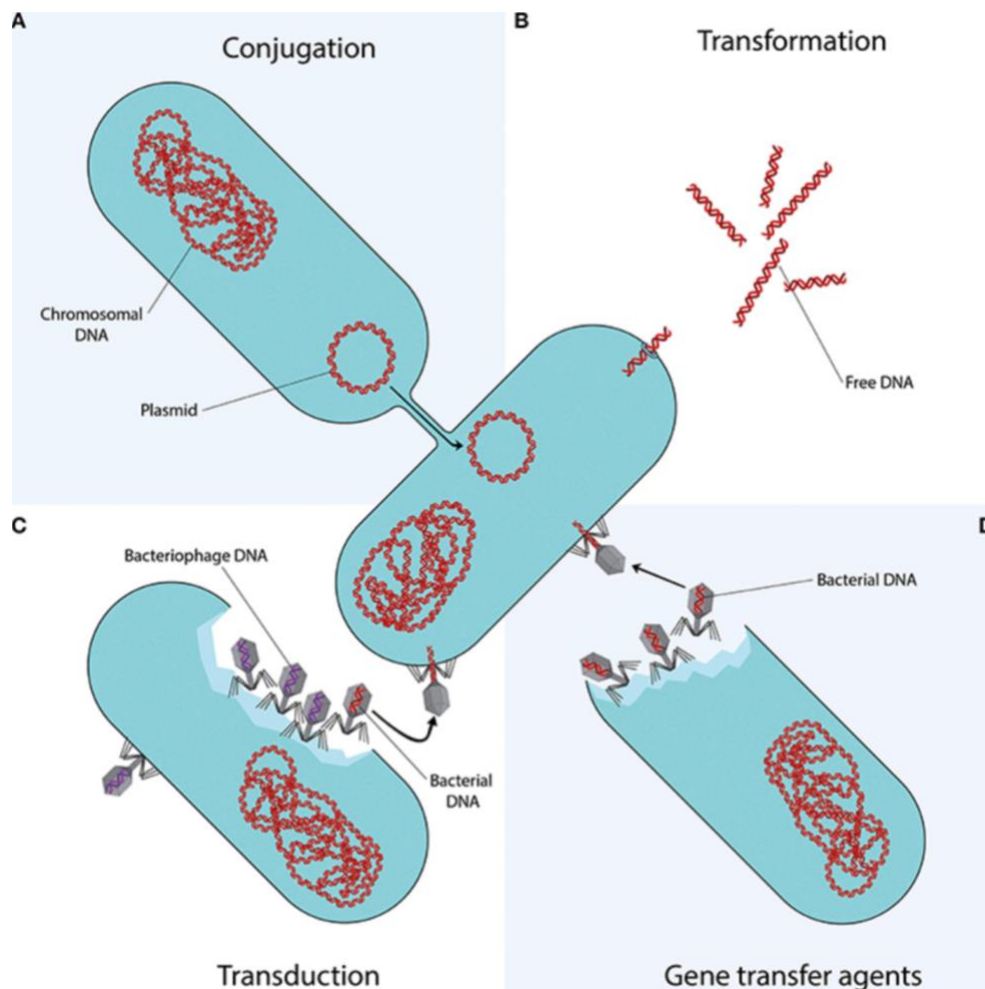


Figure 1.2. Mechanisms of horizontal gene transfer

Each of the four sections represents a form of horizontal gene transfer. A) Conjugation: Plasmids expressing DNA transfer between cells and require cell-to-cell contact. B) Transformation: The uptake of naked DNA from the environment. C) Transduction: The transfer of bacterial DNA by bacteriophages from a previously infected bacterial cell. D) Transfer of genes by bacteriophage-like agents from previously infected bacteria.

Source : Wintersdorff et al. 2016 [42]

11.311.3 Co-selection of antibiotic resistance

Co-selection of antibiotic resistance occurs when exposure to one antibiotic facilitates simultaneous resistance to multiple antibiotics. This phenomenon has been demonstrated in numerous studies. For instance, a randomized study investigating the effects of feeding pigs subtherapeutic levels of chlortetracycline found a higher prevalence of *Salmonella* strains resistant to tetracycline, ampicillin, gentamicin, and ceftriaxone in pigs receiving chlortetracycline compared to the untreated control group [52]. These results suggest selection of tetracycline resistance may co-select for resistance to these non-tetracycline antibiotics.

Similarly, another study examined the relationship between antibiotic use on pig farms and the prevalence of antibiotic-resistant *E. coli* [53]. In this study, *E. coli* isolates from 297 apparently healthy pigs, across different farms, with antibiotic histories documented up to six months prior. Of these pigs, 189 had received one or more antibiotics, while 356 had not received antibiotics in the past six months. The primary antibiotics administered were tetracyclines (n=66), penicillins (n=34), a methoprim-sulfonamide combination (n=33), and a penicillin-streptomycin combination (n=22). A total of 66, 34, 33, and 22 *E. coli* were obtained from pigs treated with penicillins, trimethoprim-sulfonamide and a penicillin-streptomycin combination, respectively. Antibiotic susceptibility was determined using antibiotic susceptibility tests. Comparative analysis revealed an association between tetracycline treatment and resistance to ampicillin, dihydrostreptomycin, oxytetracycline, kanamycin, and

trimethoprim. Additionally, penicillin use was associated with resistance to both penicillin and trimethoprim.

Cross-resistance

Co-selection of resistance can occur through mechanisms known as cross-resistance and co-resistance. In cross-resistance, there exists a positive association between resistance to multiple antibiotics (**Error! Reference source not found.**) [54,55]. This phenomenon commonly occurs when antibiotics share the same bacterial targets, such as genes and target pathways, particularly among antibiotics within the same class. For example, because quinolone antibiotics target the gyrase and topoisomerase enzymes, resistance often arises from mutations in these target genes [56]. A mutation in the gyrase enzyme, due to selection pressure from quinolone treatment, can confer resistance not only to the selecting antibiotic but also to other antibiotics that target the same gene.

For instance, an experimental study identified a point mutation, A87G, in the *Salmonella* *GyrA* gene (confirmed via sequencing) [57]. Susceptibility testing showed that the mutant strains developed resistance to various quinolone antibiotics including fluoroquinolone, ciprofloxacin, and nalidixic acid, as well as to the non-quinolone compound, triclosan. Similarly, in another study, *E. coli* isolates exposed to increasing doses of one of twelve antibiotics over approximately 336 generations exhibited mutations, including the same A87G mutation, in the *gyrA* gene which was confirmed through whole-genome sequencing. The antibiotic susceptibility phenotype of these *E. coli* isolates indicated resistance not only to fluoroquinolones but also to ampicillin, kanamycin, tobramycin, and ceftiofur [58].

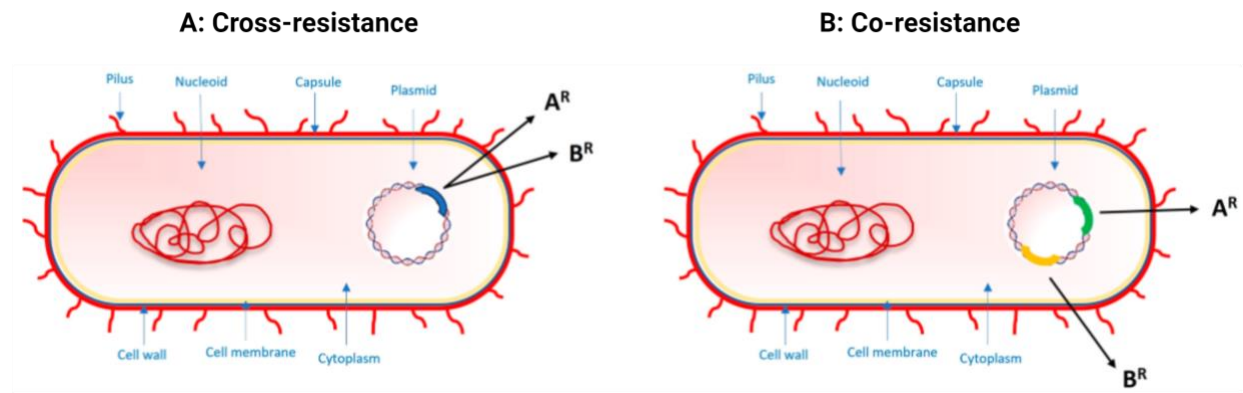


Figure 1.4. Co-selection of antibiotic resistance

Co-selection of antibiotic resistance. A) Cross-resistance: A single mechanism, e.g., resistance gene, confers resistance to multiple antibiotics. B) Co-resistance: Multiple antibiotic resistance genes physically present on the same plasmid.

Source : Carresi C. et al. 2024. [59].

Co-resistance

Co-selection via co-resistance occurs when multiple resistance genes are located on the same genetic element, such as a plasmid [54,55]. This allows the simultaneous inheritance of resistance to several antibiotics (**Error! Reference source not found.**). For example, a study found that *E. coli* isolates from wastewater plants could transfer the fluoroquinolone resistance gene (*qnrS*) and beta-lactam resistance genes (*bla_{TEM-1}*, *bla_{SHV-11}*, and *bla_{CTX-M-15}*) to recipient *E. coli* strains [60]. These resistance genes were located on a shared plasmid, wwA8.

In another study, researchers isolated *E. coli* and *Klebsiella pneumoniae* from neonates in an Indian intensive care unit [61]. Using PCR, they identified 29 isolates carrying both fluoroquinolone (*Aac(6')-Ib-cr*) and beta-lactam (*bla_{NDM}*) resistant genes. Through conjugation assays, twelve of these isolates were shown to transfer these resistant genes via IncFIIK, IncA/C, and IncN plasmids to recipient *E. coli* J53.

Spread of antibiotic-resistant bacteria between humans, animals, and the environment

Zoonotic antibiotic-resistant bacteria can be transmitted between animals and humans, as well as to the environment (Figure 1.5. Spread of antibiotic resistance). Specifically, carcasses and meat products may become contaminated with antibiotic-resistant bacteria of animal or human origin during the slaughter process or post-slaughter processing. If not cooked thoroughly, these bacteria can be consumed and cause antibiotic-resistant infections in humans. Additionally, both animals and humans can shed bacteria (including antibiotic-resistant bacteria) into the environment, contaminating water sources and vegetation. This contamination can, in turn, affect other animals and humans through the ingestion of contaminated vegetation and water. Contamination of food products in retail stores has also been reported. For example, a Turkish study on the incidence of *S. aureus* contamination in retail lamb, beef and chicken products found that, of the recovered *S. aureus* isolates, 67.5% were methicillin-resistant, 87.5% were bacitracin resistant, and 53.8% were penicillin G-resistant [62]. Similarly, another study isolated multiple bacterial species, including *Klebsiella pneumoniae*, from retail meats in Oklahoma [63]. In that study, all *K. pneumoniae* isolates were phenotypically and genetically resistant to ampicillin, tetracycline, streptomycin, gentamycin, and kanamycin.

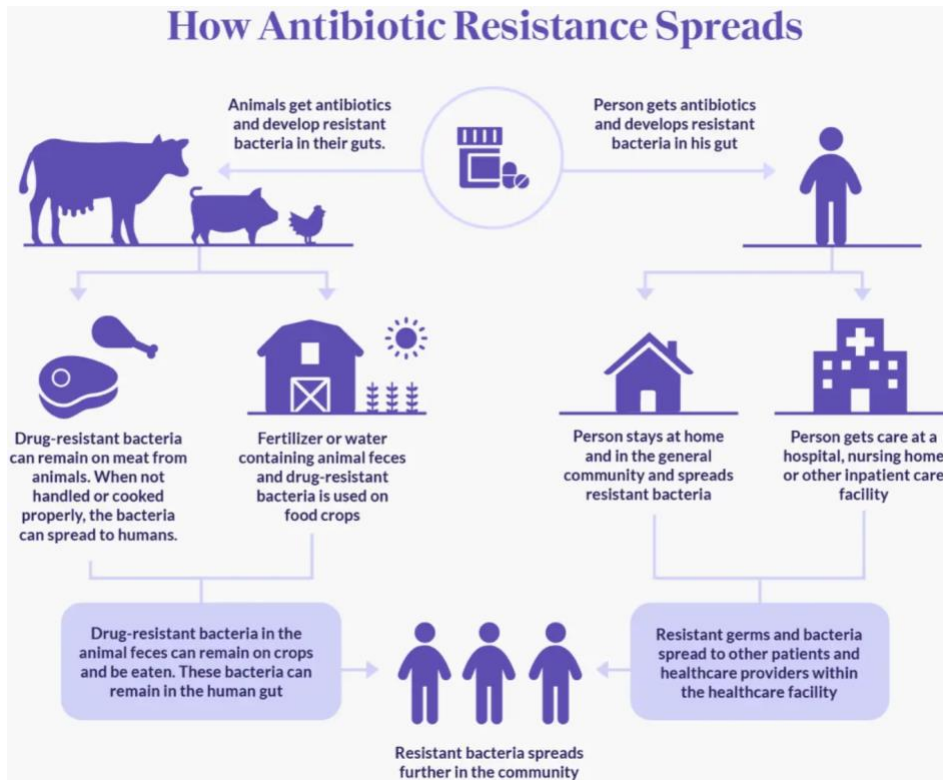


Figure 1.5. Spread of antibiotic resistance

Antibiotic use in animals and humans may promote antibiotic resistance in bacteria in these species. Animals and humans may shed antibiotic resistant bacteria into the environment and may contaminate meat, food crops, and water sources. Native animals and humans may acquire antibiotic resistant bacteria from contaminated food sources.

Source: <https://healthmatch.io/blog/antimicrobial-resistance-next-health-crisis> [64].

Because animals and humans share antibiotics or use antibiotics from the same class, antibiotic-resistant bacteria from one can infect and cause antibiotic-resistant infections in the other. The transmission of resistant bacteria from animals typically occurs through the shedding of these bacteria, which leads to the contamination of the environment (e.g., water sources and fresh produce) and animal meat products. Humans then acquire these resistant bacteria by consuming contaminated food. The acquisition of antibiotic-resistant bacteria through food consumption has been experimentally demonstrated [65]. For example, one study investigated the transfer of antibiotic resistance genes from *E. coli* isolated from chickens to humans [66]. In

that study, researchers used a Mucosal Simulator of the Human Intestinal Microbial Ecosystem (M-SHIME), which mimics the human intestinal environment. The colon vessels of the M-SHIME were inoculated with fecal sample slurries from a human volunteer who had not been exposed to antibiotics for five consecutive years. This fecal slurry provided the gut microbial population (recipient bacteria). The M-SHIME was then inoculated with donor *E. coli* harboring plasmids expressing resistant genes. The transfer of these plasmids to the recipient bacteria was confirmed via PCR. The donor *E. coli* transferred plasmids expressing resistance genes to the recipient bacteria as early as two hours after the donor isolates were introduced. By 24 hours, 96% of selected recipient coliforms had acquired the resistance plasmids. These results suggest that humans can quickly acquire resistant genes from animal commensal bacteria, such as *E. coli*, by ingesting contaminated food products.

Antibiotic Use in Production Animals

Antibiotics are used in animals for various purposes, including prevention/prophylaxis, treatment, and disease control. Additionally, antibiotics may be administered for production purposes, such as growth promotion, improved feed efficiency, and estrus suppression. Antibiotics can be administered at both the individual and group levels.

Indications for prevention of livestock diseases in the U.S.

When used for prevention, antibiotics are administered to non-infected, at-risk animals to prevent infection and reduce the appearance of clinical disease in the event of exposure [16,67]. The determination of an animal being at risk of infection is based on history, clinical judgment, or epidemiological knowledge of the anticipated disease [16]. These antibiotics can be

administered at either the individual or group level. The term “metaphylaxis” is used when antibiotics are also given to healthy animals within a group that includes infected or diseased animals receiving treatment [68]. Antibiotics for prevention are typically administered through feed or water over prolonged periods. For example, in the U.S., oxytetracycline is labeled for preventing coccidiosis in poultry to either prevent infection or reduce clinical signs of disease **(Error! Reference source not found.)**.

Indications for treatment of livestock diseases in the U.S.

When used for treatment, antibiotics are administered to individual clinically sick animals to treat or alleviate suffering from specific bacterial infections [18]. Antibiotics for therapeutic purposes are generally given in high doses for short, defined durations [18]. The most common routes of administration for therapeutic antibiotics are injection or water medication, particularly in poultry. Common conditions where antibiotics are used include respiratory and enteric infections of bacterial origin, as well as other conditions such as foot rot, mastitis, and clinical bovine anaplasmosis. Examples of antibiotics labeled for treatment in the U.S. are provided in **(Error! Reference source not found.)**.

Indications for control of livestock diseases in the U.S.

For disease control, antibiotics are given to individual animals or the entire group. This helps reduce clinical symptoms, reduce disease spread, and limit transmission to other animals **(Error! Reference source not found.)** [16]. Antibiotics for control are often administered in feed. For example, in the U.S., chlortetracycline is used in medicated feed to control active bovine anaplasmosis [69].

Use of antibiotics for production purposes in the U.S.

In the U.S., only non-medically important antibiotics are approved use as growth promoters. These antibiotics aim to enhance weight gain or improve feed conversion efficiency in livestock. These are typically administered at the group level through feed or water, often over extended periods (**Error! Reference source not found.**). While the exact mechanisms behind growth promotion remain unclear, most researchers agree that these effects are primarily linked to the antibiotics' antimicrobial properties [70]. The influence of specific antibiotics used for growth promotion on gut flora has been reviewed [71]. For instance, bambarmycin has been shown to reduce *Clostridium perfringens* in chicken feces, while having no significant effect on bacteria such as enterococci, lactobacilli, and coliforms. Similarly, virginiamycin and avilamycin were found to reduce *C. perfringens* in chicken intestines. In pigs, bambarmycin decreased the presence of *Escherichia coli* and *Enterococcus faecalis* in feces, but it did not significantly alter the total number of enterococci.

Several hypotheses have been proposed to explain how these antibiotics promote growth. These include: (1) improved nutrient absorption due to a thinner intestinal epithelium; (2) a reduction in gut bacteria that compete for nutrients, increasing nutrient availability; (3) decreased presence of gut bacteria responsible for subclinical infections; and (4) lower levels of bacterial toxins and growth-suppressing metabolites [18,70,71].

Table 1-2. Indications for antibiotics in livestock

Indication	Description	Example
------------	-------------	---------

Prevention/prophylaxis	To administer antibiotics to prevent infection to animals at risk. Done at individual or population level.	Individual level: Administering 100 mg/gal/day bacitracin to broiler chickens to prevent infection with <i>Clostridium perfringens</i> . Population-level: Infusing 100,000 units of penicillin G in the mammary glands of dry cows at drying off.
Treatment	To provide episodic, therapeutic doses of antibiotic agents to treat individual clinically or diagnostically diseased animals.	Individual level: Administering 5 mg/lb BW/day oxytetracycline to cattle with bovine anaplasmosis. Population: Administering 113.5-11,350 g/ton (to provide 10 mg/kg BW/day amprolium in feed to cattle for coccidiosis treatment.
Control	To administer antibiotics to subclinical animals to reduce the incidence of disease or spread of infection to other organs. Done at an individual or group level.	Individual level: Administering 3-5 mg/kg BW ceftiofur to pigs to control infection caused by <i>Actinobacillus pleuropneumoniae</i> . Population level: Administering 0.5-2.0 mg/lb BW/day chlortetracycline to cattle with active bovine anaplasmosis.
Growth promotion/increased feed	To administer antibiotics to healthy animals for prolonged periods to improve production.	Population level: Administering 50-480 mg/head/day monensin in feed to cattle for improved feed efficiency.

1. Sources: Adapted from [72]
2. supplementary information from [17].
3. Antibiotic indications [73,74]

Table 1-3. Antibiotics marketed for food-producing animals in the U.S. in 2022, their indications, modes of action and mechanisms of resistance

Antibiotic class	Approved Use Species: <u>Indication</u> (causative agent(s)).	Mechanism of action [source(s)].	Method of Antibiotic Resistance Development
Active ingredient			
Aminocoumarins^{3, E}		Inhibit DNA gyrase and topoisomerase IV, enzymes essential for DNA replication (bacteriostatic) [75,76].	1. Accumulation of point mutations in target genes (esp. <i>gyrB</i>) [77,78]. Horizontal gene transfer documented in <i>Rhodobacter capsulatus</i> via transduction [79].
Novobiocin	BOV: <u>Treatment</u> (<i>Staphylococcus</i> spp.). AVI: <u>Treatment</u> (<i>Staphylococcus</i> spp., and <i>Pasteurella</i> spp.).		
Aminoglycosides^{1, B}		Bind to 30s ribosomal subunit, inhibiting polypeptide synthesis, and resulting in truncated or non-functional proteins (bactericidal) [80–82].	1. Enzymatic modification and inactivation of drug. 2. Increased efflux. 3. Decreased permeability. 4) Modification of target site [83–85]. Horizontal gene transfer documented [85]. E.g., Gentamicin via conjugation [86]
Dihydrostreptomycin	BOV: <u>Treatment</u> (<i>Leptospira pomona</i>). SUI: <u>Treatment</u> (<i>Leptospira</i> spp.).		
Gentamicin	SUI: <u>Treatment</u> (<i>Escherichia coli</i> , <i>Treponema hyodysenteriae</i> , <i>Brachyspira hyodysenteriae</i> , <i>Arizona Hinshaw</i> , <i>Salmonella</i> spp. and <i>Mycoplasma meleagridis</i>). AVI: <u>Prevention</u> of mortality (<i>Escherichia coli</i> , <i>Salmonella typhimurium</i> and <i>Pseudomonas aeruginosa</i>). BOV: <u>Treatment</u> (<i>Mycoplasma bovis</i>).		
Neomycin	AVI: <u>Control</u> (<i>Escherichia coli</i> , <i>Mycoplasma</i> spp., <i>Pasteurella multocida</i> and <i>Histomonas meleagridis</i>). CAP: <u>Treatment</u> and/or <u>control</u> (<i>Escherichia coli</i>). OVI: <u>Treatment</u> and/or <u>control</u> (<i>Escherichia coli</i> and <i>Pasteurella multocida</i>).		

BOV: Treatment and/or control
(*Escherichia coli*, *Mycoplasma bovis* and *Pasteurella multocida*).

SUI: Treatment and/or control
(*Escherichia coli*, *Salmonella choleraesuis*, *Pasteurella multocida* and *Leptospira Pomona*).

Amphenicols^{1, C}		Bind to 50s subunit of ribosomes and disrupts protein synthesis (bacteriostatic) [87].	1. Enzymatic inactivation of drug. 2. Efflux systems. 3. Modification of target site. 4. Decreased permeability [88].
Florfenicol	SUI: <u>Treatment</u> (<i>Streptococcus suis</i>, <i>Actinobacillus pleuropneumoniae</i>, <i>Salmonella choleraesuis</i>, and <i>Bacterioides bronchiseptica</i>). BOV: <u>Treatment</u> (<i>Mannheimia haemolytica</i>, <i>Pasteurella multocida</i>, <i>Histophilus somni</i>, <i>Mycoplasma bovis</i>, <i>Fusobacterium. necrophorum</i> and <i>Bacteroides melaninogenicus</i>). AQU: <u>Control</u> (<i>Edwardsiella ictaluri</i>, <i>Flavobacterium</i> spp., <i>Streptococcus</i> spp., and <i>Aeromonas salmonicida</i>).		Horizontal gene transfer documented via conjugation [89,90]
1st Gen. Cephalosporins^{2, C}		Inhibit bacterial wall synthesis by binding to bacterial penicillin-binding protein (PBP) (important for strengthening bacterial cell wall cross-linking peptidoglycan units) (bactericidal) [91,92].	1. Modification or acquiring beta-lactam insensitive PBP. 2. Reduced permeability. 3. Active efflux. 4. Drug inactivation [92–94].
Cephapirin			Horizontal gene transfer documented via conjugation [95].
3rd Gen. Cephalosporins^{1, A}		Inhibit bacterial wall synthesis by binding to bacterial PBP (β-lactam resistance determinants) (bactericidal) [96,97].	1. Modification or acquiring β-lactam insensitive PBP. 2. Reduced permeability. 3. Active efflux. 4. Drug inactivation [92–94].
Ceftiofur	SUI: <u>Treatment</u> and/or <u>control</u> (<i>Actinobacillus pleuropneumoniae</i>, <i>Pasteurella multocida</i>, <i>Haemophilus. parasuis</i>, <i>Streptococcus sui</i>, <i>Salmonella Choleraesuis</i>). BOV: <u>Treatment</u> and/or <u>control</u> (<i>M. haemolytica</i>, <i>Pasteurella multocida</i>, <i>Histophilus somni</i>, <i>Escherichia coli</i>, <i>Fusobacterium</i>		Horizontal gene transfer documented via conjugation [98].

	<i>necrophorum</i>, <i>Bacteroides melaninogenicus</i>, <i>Staphylococcus</i> spp. and <i>Streptococcus</i> spp.). OVI and CAP: <u>Treatment</u> (<i>Mannheimia haemolytica</i> and <i>Pasteurella multocida</i>). AVI: Control (<i>Escherichia coli</i>).		
Diaminopyrimidines^{1, C}		Interfere with folic acid metabolism by inhibiting reduction of dihydrofolic acid to tetrahydrofolic acid (a co-factor of amino acid and nucleic acid synthesis), therefore inhibiting DNA and protein synthesis (bacteriostatic) [56,99].	1. Active efflux. 2. Modification of target gene [100,101].
Ormetoprim	AVI: <u>Prevention</u> (<i>Eimeria</i> spp., <i>Heterakis gallinarum</i>, <i>Escherichia coli</i> and <i>Pasteurella multocida</i>). AQU: <u>Control</u> (<i>Aeromonas salmonicida</i> and <i>Edwardsiella ictaluri</i>).		Horizontal gene transfer of resistant genes documented FOR trimethoprim via conjugation [102].
Fluoroquinolones^{1, A}		Inhibit bacterial DNA gyrase and topoisomerase IV (critical for DNA replication and cell division) (bactericidal) [103–105].	1. Modification of target genes. 2. Efflux pumps. 3. Reduced permeability [106,107].
Danofloxacin	BOV: <u>Treatment</u> (<i>Mannheimia haemolytica</i> and <i>Pasteurella multocida</i>).		Horizontal gene transfer of resistant genes documented via conjugation [108].
Enrofloxacin	BOV: <u>Treatment</u> (<i>Mannheimia haemolytica</i>, <i>Pasteurella multocida</i>, <i>Histophilus somni</i> and <i>Mycoplasma bovis</i>). SUI: <u>Treatment</u> (<i>Actinobacillus pleuropneumoniae</i>, <i>Pasteurella multocida</i>, <i>Haemophilus parasuis</i>, <i>Streptococcus suis</i>, <i>Bordetella bronchiseptica</i> and <i>Mycoplasma hyopneumoniae</i>).		
Lincosamides^{2, C}		Bind to 50s ribosome, inhibiting protein synthesis ([109,110].	1. Modification of target site. 2. Efflux pumps. 3. Drug inactivation [[111–113].
Lincomycin	SUI: <u>Treatment</u> and/or <u>control</u> (<i>Brachyspira</i> spp., <i>Mycoplasma</i> spp., <i>Lawsonia intracellularis</i>). <u>Prevent</u> establishment and migration (<i>Ascaris suum</i> and <i>Oesophagostomum</i> spp.). AVI: <u>Control</u> (<i>Clostridium</i> spp.). <u>Prevent</u> (<i>Eimeria</i> spp.).		Horizontal gene transfer of resistant genes documented via conjugation [114].
Pirlimycin	BOV: <u>Treatment</u> (<i>Staphylococcus</i> spp. and <i>Streptococcus</i> spp.).		

Macrolides^{1, B}		Bind to 50s ribosome, inhibiting protein synthesis (bacteriostatic) [[56,115–117].	1. Modification of target site. 2. Efflux pumps. 3. Drug inactivation [113,118].
Gamithromycin	BOV: <u>Treatment and/or control</u> (<i>Mannheimia haemolytica</i> , <i>Pasteurella multocida</i> , <i>Histophilus somni</i> and <i>Mycoplasma bovis</i>).		Horizontal gene transfer of resistant genes documented via conjugation [119,120].
Tildipirosin	BOV: <u>Treatment</u> (<i>Mannheimia haemolytica</i> , <i>Pasteurella multocida</i> , and <i>Histophilus somni</i>).		
Tilmicosin	SUI: <u>Control</u> (<i>Mycoplasma hyopneumoniae</i> , <i>Actinobacillus pleuropneumoniae</i> and <i>Pasteurella multocida</i>). BOV: <u>Control and/ or prevent</u> (<i>Pasteurella multocida</i> , <i>Histophilus somni</i> , <i>Mannheimia haemolytica</i> and <i>Eimeria</i> spp.). OVI: <u>Treatment</u> (<i>Mannheimia</i> (P.) <i>haemolytica</i>)		
Tulathromycin	BOV: <u>Treatment and/or control</u> (<i>Mannheimia haemolytica</i> , <i>Pasteurella multocida</i> , <i>Histophilus somni</i> , <i>Mycoplasma bovis</i> , <i>Fusobacterium necrophorum</i> and <i>Porphyromonas levii</i>). SUI: <u>Treatment</u> (<i>Pasteurella multocida</i> , <i>Bordetella bronchiseptica</i> , <i>Haemophilus parasuis</i> and <i>Mycoplasma hyopneumoniae</i>).		
Tylosin	AVI: <u>Treatment and/or control</u> (<i>Mycoplasma</i> spp. and <i>Clostridium perfringens</i>). SUI: <u>Treatment and/or control</u> (<i>Brachyspira hyodysenteriae</i> , <i>Brachyspira bronchyseptica</i> , <i>Mycoplasma hyosynoviae</i> , <i>Pasteurella multocida</i> , <i>Erysipelothrix rhusiopathiae</i> , <i>Treponema hyodysenteriae</i> , <i>Lawsonia intracellularis</i> , <i>Arcanobacterium pyogenes</i> and <i>Streptococcus</i> spp.). <u>Prevent</u> migration (<i>Ascaris suum</i> , and <i>Oesophagostomum</i> spp.). BOV: <u>Treatment</u> (<i>Pasteurella multocida</i> , <i>Arcanobacterium</i>		

	<i>pyogenes</i> and <i>Fusobacterium necrophorum</i>). <u>Weight gain</u>. <u>Improve</u> feed efficiency. <u>Suppression</u> of estrus. <u>Prevent</u> (<i>Eimeria</i> spp.). <u>Reduce</u> ammonia gas emission.		
Tylvasolin	SUI: <u>Control</u> (<i>Lawsonia intracellularis</i>, <i>Bordetella bronchiseptica</i>, <i>Glaesserella (Haemophilus) parasuis</i>, <i>Pasteurella multocida</i>, <i>Streptococcus suis</i>, and <i>Mycoplasma hyopneumoniae</i>).		
Penicillins^{1, C}			
Amoxicillin	BOV: <u>Treatment</u> (<i>Escherichia coli</i>, <i>Pasteurella</i> spp., <i>Haemophilus</i> spp., <i>Staphylococcus</i> spp. and <i>Streptococcus</i> spp.). SUI⁵: Treatment (<i>Escherichia coli</i>)	Inhibit cell wall peptidoglycan layer by binding to PBP and (bactericidal) [121–124].	1. Drug inactivation by bacterial β-lactamase [125,126]. Horizontal gene transfer of resistant genes documented. For amoxicillin via conjugation [86].
Ampicillin	BOV: <u>Treatment</u> (<i>Escherichia coli</i>, <i>Aerobacter</i> spp., <i>Klebsiella</i> spp., <i>Staphylococcus</i> spp., <i>Streptococcus</i> spp. and <i>Pasteurella multocida</i>). SUI: Treatment (<i>Escherichia coli</i>)		
Cloxacillin	BOV: <u>Treatment</u> (<i>Staphylococcus aureus</i> and <i>Mycoplasma agalactiae</i>).		
Penicillin	AVI: <u>Treatment</u> (<i>Erysipelothrix rhusiopathiae</i>). BOV: <u>Treatment</u> (<i>Streptococcus</i> spp., <i>Staphylococcus</i> spp., <i>Pasteurella multocida</i>, and <i>Actinomyces pyogenes</i>). OVI: <u>Treatment</u> (<i>Pasteurella multocida</i>). SUI: <u>Treatment</u> (<i>Erysipelothrix rhusiopathiae</i>).		
Streptogramins^{3, C}			
Virginiamycin	AVI: <u>Prevention</u> (<i>Clostridium perfringens</i> and <i>Eimeria</i> spp.). SUI: <u>Treatment</u> and <u>control</u> of swine dysentery.	Binds to 50s ribosome, inhibiting protein synthesis (bacteriostatic) [56,127].	1. Active efflux, 2. modification of drug target and 3. Drug inactivation [128]. Horizontal gene transfer of resistant genes via conjugation [129].

BOV: <u>Reduction</u> of liver abscess incidences.			
Sulfonamides^{2, C}		Interferes with folic acid pathway by inhibiting bacterial and protozoan dihydropteroate synthase therefore inhibiting DNA and protein synthesis (bacteriostatic) [130–132].	1. Modification of target gene, 2. Acquisition of foreign target genes insensitive to drug, 3. Decreased permeability [133–135]. Horizontal gene transfer of resistant genes documented via conjugation [136].
Sulfadimethoxine	AVI: <u>Treatment</u> (<i>Eimeria</i> spp., <i>Heterakis gallinarum</i> and <i>Escherichia coli</i>). BOV: <u>Treatment</u> (<i>Pasteurella</i> spp. and <i>Fusobacterium necrophorum</i>). AQU: Control (<i>Aeromonas salmonicida</i> and <i>Edwardsiella ictaluri</i>).		
Sulfamethazine	SUI: <u>Prevention</u> and/or <u>treatment</u> (<i>Escherichia coli</i> , <i>Salmonella choleraesuis</i> , <i>Bordetella bronchiseptica</i> , <i>Brachyspira hyodysenteriae</i> , <i>Arcanobacterium pyogenes</i> , and <i>Pasteurella</i> spp.). AVI: <u>Control</u> (<i>Eimeria</i> spp., <i>Pasteurella multocida</i> , <i>Salmonella pullorum</i> , <i>Avibacterium paragallinarum</i>). BOV: <u>Treatment</u> (<i>Pasteurella</i> spp., <i>Escherichia coli</i> , <i>Fusobacterium necrophorum</i> , <i>Streptococcus</i> spp., <i>Eimeria</i> spp.).		
Sulfaquinoxaline	AVI: <u>Treatment</u> and/or <u>control</u> (<i>Eimeria</i> spp., <i>Pasteurella multocida</i> and <i>Salmonella gallinarum</i>). BOV: <u>Treatment</u> (<i>Eimeria</i> spp.)		
Tetracyclines^{2, C}		Binds to 30s ribosomal subunit thereby, interfering with protein synthesis (bacteriostatic) [56,116,137]	1. Active efflux, 2. Target modification, 3. Drug modification [138–140]. Horizontal gene transfer of resistant genes documented via conjugation [141].
Chlortetracycline	SUI: <u>Control</u> and/or <u>treatment</u> (<i>Pasteurella</i> spp., <i>Haemophilus</i> spp., <i>Klebsiella</i> spp., <i>Escherichia coli</i> , <i>Actinobacillus pleuropneumonia</i> , <i>Streptococcus</i> spp., <i>Leptospira Pomona</i> , <i>Lawsonia intracellularis</i> , <i>Brachyspira hyodysenteriae</i> and <i>Salmonella choleraesuis</i>). <u>Weight gain</u> . Improved feed efficiency. BOV: <u>Control</u> and/or <u>treatment</u> (<i>Escherichia coli</i>), <i>Pasteurella</i>		

	spp., <i>Actinobacillus pleuropneumonia</i>, <i>Haemophilus</i> spp., <i>Klebsiella</i> spp., <i>Salmonella</i> spp., <i>Eimeria</i> spp. and <i>Anaplasma marginale</i>). <u>Reduction</u> of liver abscesses. <u>Improved</u> feed efficiency. <u>Weight gain</u>. <u>Suppression</u> of estrus. AVI: <u>Control</u> (<i>Mycoplasma</i> spp., <i>Hexamita meleagridis</i>, <i>Salmonella typhimurium</i>, <i>Pasteurella multocida</i>, and <i>Escherichia coli</i>). <u>Prevent</u> (<i>Eimeria</i> spp.). OVI: <u>Reduction</u> (<i>Campylobacter fetus</i>).
Oxytetracycline	BOV: <u>Treatment</u> and/or <u>control</u> (<i>Escherichia coli</i>, <i>Haemophilus</i> spp, <i>Salmonella typhimurium</i>, <i>Pasteurella</i> spp., <i>Fusobacterium necrophorum</i>, <i>Leptospira pomona</i>, <i>Actinobacillus lignieresii</i>, <i>Staphylococcus</i> spp., <i>Streptococcus</i> spp., <i>Histophilus</i> spp., <i>Klebsiella</i> spp., <i>Bacillus anthracis</i>, <i>Moraxella bovis</i>, <i>Anaplasma marginale</i>). AVI: <u>Prevention</u>, <u>treatment</u> and/or <u>Control</u> (<i>Mycoplasma</i> spp., <i>Escherichia coli</i>, <i>Pasteurella multocida</i>, <i>Eimeria</i> spp. and <i>Hexamita meleagridis</i>). SUI: <u>Control</u> and/or <u>treatment</u> (<i>Escherichia coli</i>, <i>Salmonella</i> spp. <i>Pasteurella multocida</i> and <i>Leptospira pomona</i>) OVI: <u>Control</u> and/or <u>treatment</u> (<i>Escherichia coli</i>, and <i>Pasteurella multocida</i>) AQU: <u>Control</u> (<i>Haemophilus piscium</i>, <i>Aeromonas</i> spp., <i>Pseudomonas</i> spp., <i>Flavobacterium</i> spp. and <i>Aerococcus viridans</i>).
Tetracycline	BOV: <u>Treatment</u> and/or (<i>Escherichia coli</i>), bacteria pneumonia (<i>Pasteurella</i> spp., <i>Hemophilus</i> spp., and <i>Klebsiella</i> spp.).

Glycolipids^{4, E}		Inhibits cell wall peptidoglycan by binding to penicillin-binding protein and (bactericidal) [142].	Not applicable. Only indicated for growth promotion.
Bambermycin (flavomycin)	AVI, SUI, BOV: <u>Weight gain</u> and <u>improved feed efficiency</u> .		
Ionophores^{2, E}		Bind to and transport cations across cell membranes, therefore, disrupting intracellular pH and increasing ATP demand (bactericidal) [143–147].	1. Efflux system, 2. Drug degradation in aerobic conditions, 3. Cell wall permeability [148,149]. Horizontal gene transfer of resistant genes documented via conjugation [150].
Laidlomycin	BOV: <u>Weight gain</u> and <u>improved feed efficiency</u>		
Lasalocid	AVI: <u>Prevent</u> (<i>Eimeria</i> spp.). <u>Weight gain</u> and <u>improved feed efficiency</u> . BOV: <u>Control</u> (<i>Eimeria</i> spp.). <u>Weight gain</u> and <u>improved feed efficiency</u> . OVI: <u>Prevent</u> of coccidiosis (<i>Eimeria</i> spp.).		
Monensin	AVI: <u>Prevent</u> (<i>Eimeria</i> spp.). <u>Weight gain</u> and <u>improved feed efficiency</u> . <u>Control</u> of transmissible enteritis. BOV: <u>Prevent</u> and/or <u>control</u> (<i>Eimeria</i> spp.) <u>Improved feed efficiency</u> . <u>Increased milk production</u> . <u>Weight gain</u> . OVI: <u>Prevent</u> (<i>Eimeria</i> spp.).		
Narasin	AVI: <u>Prevent</u> (<i>Eimeria</i> spp.). <u>Increased milk production</u> . <u>Weight gain</u> . SUI: <u>Improve</u> feed efficiency. <u>Weight gain</u> .		
Salinomycin	AVI: <u>Prevent</u> (<i>Eimeria</i> spp. and <i>Clostridium</i> spp.).		
Orthosomycins^E		Binds to the 50s ribosomal subunit thereby, interfering with protein synthesis (bactericidal) [151].	1. Target modification [152].
Avilamycin	AVI: <u>Prevent</u> mortality caused by necrotic enteritis (<i>Clostridium perfringens</i>) and coccidiosis (<i>Eimeria</i> spp.).		Horizontal gene transfer of resistant genes not documented.

	SUI: <u>Reduce</u> incidence and severity (pathogenic <i>Escherichia coli</i>).		
Pleuromutilins^{2, E}		Bind to 50s subunit of ribosomes, inhibiting protein synthesis (bactericidal) [153].	1. Target modification [154]. Horizontal gene transfer of resistant genes not documented.
Tiamulin	SUI: <u>Treatment</u> and/or <u>control</u> (<i>Brachyspira</i> spp. <i>Lawsonia intracellularis</i> and <i>Actinobacillus pleuropneumoniae</i>).		
Polypeptides^{2, D}		Binds to the C55-isoprenyl pyrophosphate (IPP), a carrier of peptidoglycan with pentapeptide across the membrane therefore blocking cell wall synthesis (bacteriostatic or bactericidal, depending on dose and microorganism) [155,156].	1. Efflux transporters [156–158]. Horizontal gene transfer of resistant genes documented via conjugation [159].
Bacitracin	AVI: <u>Treatment</u> and/or <u>prevent</u> (<i>Clostridium</i> spp.). <u>Weight gain</u> . <u>Improved</u> feed efficiency. SUI: <u>Treatment</u> (<i>Brachyspira hyodysenteriae</i>). <u>Weight gain</u> . <u>Improved</u> feed efficiency. BOV: <u>Reduction</u> of liver abscesses.		
Quinoxalines^{3, E}		Cause generation of reactive oxidative species and hydroxyl causing oxidative damage of bacterial DNA in anaerobic conditions (bactericidal) [160].	1. Active efflux, 2. Target modification [161]. Horizontal gene transfer of resistant genes documented via conjugation and transduction [162,163].
Carbadox	SUI: <u>Control</u> and/or <u>prevent</u> (<i>Salmonella</i> spp.). <u>Prevent</u> establishment and migration (<i>Ascaris suum</i> and <i>Oesophagostomum</i>). <u>Improved</u> feed efficiency. <u>Weight gain</u> .		

¹: World Organization for Animal Health classification (WOAH) veterinary critically important antibiotics

²: WOAH veterinary highly important antibiotics

³: WOAH veterinary important antibiotics

⁴: No WOAH classification

⁵: Species not on 21 CFR Part 556 list

^A: WHO (World Health Organization) medically important (highest priority critically important antimicrobial)

^B: WHO medically important (critically important antimicrobial)

^C: WHO medically important (highly important antimicrobial)

^D: WHO medically important (important antimicrobial)

^E: WHO Not medically important antibiotic

AVI: Chickens, turkeys, and/or ducks.

BOV: Cattle

CAP: Goats

OVI: Sheep

SUI: Pigs

AQU: Fish and lobsters

NB: Indications for bees, rabbits not included.

Sources:

4. Antibiotics marketed for animal use in 2022 [164]
5. Antibiotic indications [73,74]
6. Veterinary important antibiotics [165]
7. Medically important antimicrobials [166]

The U.S. National Action Plan for Combating Antibiotic-resistant Bacteria

In 2013, the CDC released its first report on the threats and burdens posed by antibiotic resistance to human health, titled Antibiotic Resistance Threats Report [167] . This report underscored how infections caused by antibiotic-resistant bacteria strain the healthcare system. Such infections often require prolonged and more expensive treatments, extended hospital stays, additional doctor visits, and can lead to increased disability and death. The report estimated that antibiotic-resistant bacteria caused at least 2,049,442 illnesses and 23,000 deaths annually in the United States. It also highlighted an economic burden due to antibiotic resistance of approximately \$20 billion in excess direct healthcare costs and \$35 billion in productivity losses per year (based on a 2008 study). The report categorized antibiotic-resistant bacteria into three priority levels: urgent threats (e.g., drug-resistant *Neisseria gonorrhoeae* and carbapenem-resistant *Enterobacteriaceae*), serious threats (e.g., vancomycin-resistant *Enterococcus* and extended-spectrum β -lactamase-producing *Enterobacteriaceae*), and concerning threats (e.g., vancomycin-resistant *Staphylococcus aureus* and erythromycin-resistant Group A *Streptococcus*). Finally, the report proposed four key action plans to combat antibiotic resistance: (1) preventing infections and the spread of antibiotic-resistant bacteria, (2) tracking resistant bacteria, (3) improving the use of antibiotics, and (4) promoting the development of new antibiotics and diagnostic tools for resistant bacteria.

In response to the CDC's Antibiotic Resistance Threats Report, the White House released the National Strategy for Combating Antibiotic-Resistant Bacteria in 2014 [168] . The strategy aimed to identify, prioritize, and coordinate investments to prevent, detect, and control outbreaks caused by antibiotic-resistant pathogens. Its goals included ensuring the continued availability of effective antibiotics for treating bacterial infections and strengthening the ability to detect and control new antibiotic-resistant bacteria emerging in humans and animals.

The strategy outlined five interrelated goals:

1. Slow the development of resistant bacteria and prevent the spread of resistant infections. This required the judicious use of antibiotics in healthcare and agricultural settings. Achieving this goal involved cooperation among healthcare providers, pharmaceutical companies, veterinarians, the agricultural industry, healthcare leaders, and patients.
2. Strengthen National One-Health Surveillance Efforts to Combat Resistance. This involved enhancing existing surveillance programs to detect antibiotic-resistant bacteria, monitor their emergence, and identify at-risk populations. Key programs included the National Healthcare Safety Network (NHSN), which monitors resistance in healthcare systems; the National Antimicrobial Resistance Monitoring System (NARMS), which tracks resistance in agricultural settings; and the Emerging Infections Program (EIP), which addresses resistance across healthcare and community environments.
3. Advance Development and Use of Rapid and Innovative Diagnostic Tests for Identification and Characterization of Resistant Bacteria. This goal aimed to create

- and widely distribute affordable, point-of-care tests to quickly identify and profile resistant pathogens.
4. Accelerate Basic and Applied Research and Development for New Antibiotics, Other Therapeutics, and Vaccines. Efforts focused on discovering new antibiotic classes, identifying novel targets, and exploring alternative therapies to reduce antibiotic use in agriculture.
 5. Improve International Collaboration and Capacities for Antibiotic Resistance Prevention, Surveillance, Control, and Antibiotic Research and Development. Strengthening global efforts would ensure early detection and containment of antibiotic resistance, limiting its spread to other regions.

The implementation of these action plans required collaboration among various federal agencies, including the Department of Health and Human Services (HHS), the Department of Defense (DOD), the Department of Veterans Affairs (VA), the U.S. Department of Agriculture (USDA), the Environmental Protection Agency (EPA), the U.S. Agency for International Development (USAID), and the Department of State. The milestones outlined in the White House's National Strategy for Combating Antibiotic-Resistant Bacteria formed the foundation of the U.S. Government's National Action Plan for Combating Antibiotic-Resistant Bacteria, which was designed to be achieved over five years (2015–2020). Progress reports were released annually from Year 1 to Year 4, and several challenges associated with the action plan have been reviewed [169] .

In 2020, a second action plan, the National Action Plan for Combating Antibiotic-Resistant Bacteria, 2020-2025, was introduced [170] . This plan aimed to build on the goals of

the first action plan while placing greater emphasis on the One Health approach. Additionally, it sought to incorporate new strategies to combat antibiotic resistance. The plan outlined five overarching goals, each supported by three to four specific objectives:

1. Slow the emergence of antibiotic-resistant bacteria and prevent the spread of antibiotic-resistant infections.

Objectives included:

- i. Expanding the capacity of national, regional, and state systems to detect, contain, and prevent antibiotic-resistant infections.
- ii. Enhancing education, training, and communication campaigns on responsible antibiotic use, preventing resistance, and preventing life-threatening conditions such as sepsis.
- iii. Develop and implement policies and practices which promote the responsible use of antibiotics
- iv. Creating evidence-based policies and practices to prevent infections and combat resistance within a One Health framework.

2. Strengthen national One Health surveillance efforts.

Objectives included:

- i. Enhancing testing and training capabilities, improving data harmonization and integration, and expanding federal antibiotic resistance laboratory networks across the One Health spectrum.
- ii. Improving access to specimen and data repositories for research and innovation.

- iii. Strengthening infrastructure for antibiotic use and resistance surveillance through standardized terminology, improved timeliness, and better data utility
 - iv. Enhancing the national infrastructure for antibiotic use surveillance data across One Health with standardized terminology and improved capacity.
3. Advance development and use of rapid and innovative diagnostic tests for identification and characterization of resistant bacteria.

Objectives included:

- i. Developing and validating new diagnostic tools.
 - ii. Supporting research to determine the appropriate use of diagnostics.
 - iii. Promoting the adoption and effective use of diagnostics.
4. Accelerate basic and applied research and development for new antibiotics, other therapeutics and vaccines.

Objectives included:

- i. Expanding basic and applied interdisciplinary research to understand the emergence, spread and persistence of antibiotic resistance and to develop mitigation strategies across One Health.
- ii. Intensifying basic, translational, and clinical research to support the discovery and development of new treatments, including antibiotics, non-traditional therapeutics, and optimized treatment regimens.

- iii. Advancing basic, translational, and clinical research to support the discovery and development of new preventative products and strategies such as vaccines
- iv. Promoting sustainability in the commercial market for new antibiotic products.

5. Improve International Collaboration and Capacities for Antibiotic-resistance Prevention, Surveillance, Control, and Antibiotic Research and Prevention.

Objectives included:

- i. Enhancing U.S. leadership in the global fight against antibiotic resistance.
- ii. Promoting awareness and capacity-building in other countries to address and slow the spread of antibiotic resistance.
- iii. Generating consistent, actionable global data on antibiotic resistance by extending the CDC's Antibiotic Resistance Lab Network to global sites.
- iv. Increase international collaborations to support basic, translational, and clinical research into understanding the causes of antibiotic resistance and the development of countermeasures.

The National Action Plan for Combating Antibiotic-Resistant Bacteria, 2020-2025 is also being implemented by the same federal agencies: HHS, USDA, DoD, DoI, DoS, EPA, USAID and VA.

The FDA's Actions in Response to the National Action Plans.

As part of the HHS, the FDA collaborates with domestic and international partners to address antimicrobial resistance (AMR). Its efforts focus on preserving the effectiveness of

existing antimicrobial drugs and fostering the development of new medical products through the following initiatives:

1. Facilitating the development of new antimicrobials, diagnostic tests, and vaccines:
 - The FDA prioritizes and fast-tracks the review and approval of drugs for serious or life-threatening conditions.
 - It offers incentives, such as a five-year extension for the development of infectious disease products targeting serious bacterial or fungal infections.
 - Current FDA-supported developments include bacteriophages, vaccines, live biotherapeutic products (containing live organisms to prevent, treat, or cure diseases), microbiome therapies, and immune modulators.
 - Additionally, the FDA encourages the creation of innovative in vitro diagnostic devices to detect AMR.
2. Promoting the appropriate and responsible use of antimicrobials and disseminating information, promoting interventions that slow the development of resistance. The FDA collaborates with local and international stakeholders to encourage judicious antibiotic use in veterinary and human healthcare settings.
 - In veterinary contexts, the FDA ensures animal drugs are safe and effective for their approved uses and coordinates regulations and policies for antimicrobial use in animals.
 - In human healthcare, the FDA supports policies designed to preserve antimicrobial effectiveness through responsible use.

3. Supporting development and enhancement of tools for conducting surveillance of antimicrobial use and resistance to facilitate tracking, treatment or response to AMR outbreaks.
 - The FDA works with the CDC and USDA to track AMR in foodborne bacteria affecting humans, retail meats, and food-producing animals through the National Antimicrobial Resistance Monitoring System (NARMS).
 - It funds data collection on antimicrobial use in U.S. animal agriculture and requires drug sponsors to annually report the quantities of antimicrobials sold or distributed for food-producing animals.

Advancing regulatory science to develop tools, standards and approaches to facilitate the translation of breakthrough discoveries in science and technology into innovative, safe and effective medical products.

Selected actions of the FDA on addressing AMR

- 1980's

All new medically important antibiotics have been approved with veterinary oversight.

- 1993 to present

New applications for medically important antimicrobials used in food-producing animals have been approved only under Veterinary Feed Directive (VFD) or prescription status, requiring veterinary involvement in decisions regarding their use.

- 1996

The FDA, in collaboration with the USDA and CDC, established the National Antimicrobial Resistance Monitoring System (NARMS) to track antimicrobial resistance in foodborne bacteria.

- 2003

FDA established industry guidance for assessing antimicrobial resistance risks as part of the drug approval process (Guidance for Industry #152) [171] .

- 2010

Launched efforts to promote the judicious use of antimicrobials with the release of draft Guidance for Industry #209 [172] .

Published the first annual report summarizing antimicrobials sold or distributed for use in food-producing animals.

- 2012

Published final Guidance for Industry #209, establishing a framework to end production uses of medically important antimicrobials (e.g., for growth promotion and feed efficiency) in food-producing animals and to place all remaining therapeutic uses under veterinary oversight.

Published draft VFD proposed rule.

- 2013

Published final Guidance for Industry #213 and proposed VFD rule.

- 2014

Implemented whole genome sequencing, making the data publicly accessible through the National Center for Biotechnology Information (NCBI) database.

- 2015

The FDA issued the Veterinary Feed Directive (VFD) final rule, which regulates how veterinarians authorize the use of VFD-designated antimicrobial drugs in animal feed.

- 2016

Approved Imrestor, the first alternative to an antimicrobial drug. Imrestor marked a significant milestone as it was the first animal drug for use in food-producing animals to be simultaneously reviewed and approved in both the United States and Canada.

- 2017

The FDA finalized the implementation of Guidance for Industry (GFI) #213, transitioning all medically important antimicrobial drugs used in the feed or drinking water of food-producing animals from over the counter (OTC) status to either Veterinary Feed Directive (VFD) or prescription (Rx) status, thereby requiring veterinary oversight. Additionally, this process eliminated the use of these drugs for production purposes, such as growth promotion.

Of the 292 new animal drug applications initially affected by GFI #213:

Of the 208 remaining applications:

- ✓ 84 were completely withdrawn

- ✓ 93 applications for oral dosage form products intended for use in water were converted from over the counter to prescription status
- ✓ 115 applications for products intended for use in feed were converted from over the counter to veterinary feed directive status
- ✓ All 31 applications with production-related indications, such as growth promotion, had those indications removed.

- 2018

The Center for Veterinary Medicine (CVM) released a Five-Year Plan for Supporting Antimicrobial Stewardship in Veterinary Settings. This plan built upon earlier efforts to eliminate the production uses of medically important antimicrobials (those critical for treating human diseases) and ensure that all remaining therapeutic uses were conducted under the oversight of licensed veterinarians. The plan emphasized the judicious use of antimicrobials in food-producing animals, guided by the principle that these drugs should only be used when necessary for the treatment, control, or prevention of specific diseases.

- 2023

CVM announced the successful implementation of guidance for industry (GFI) #263.

One of the antibiotic stewardship practices the FDA is currently working towards defining durations of use for all medically important antibiotics [173]. Of the 280 medically important FDA-approved antibiotics for use in food-producing animals, 97 currently lack a defined duration. These include aminoglycosides (neomycin), lincosamides (lincomycin),

macrolides (tylosin), streptogramins (virginiamycin), sulfonamides (sulfadimethoxine, sulfamethazine, and sulfaquinoxaline), tetracyclines (chlortetracycline and oxytetracycline), ionophores (monensin, lasalocid, narasin and laidlomycin), and polypeptides (bacitracin) [174].

An example of an approved indication with no defined duration of use is chlortetracycline in” beef cattle and nonlactating dairy cattle: As an aid in the control of active infection of anaplasmosis caused by *Anaplasma marginale* susceptible to chlortetracycline”.

Chlortetracycline is administered through feed at the herd level for extended periods. *Anaplasma marginale*, the causative agent of bovine anaplasmosis, is found worldwide and across all continental U.S. states. Given the economic impact and high prevalence of bovine anaplasmosis, controlling it is essential. Furthermore, there are no effective non-antibiotic control strategies, making long-term use of chlortetracycline a common option. However, prolonged antibiotic use may promote resistance not only in the target bacteria but also in off-target species, including zoonotic bacteria, which further exacerbates the issue of antibiotic resistance. In addition, antibiotics used to target one bacterial population will have broader implications on non-target bacterial populations.

The significance of bovine anaplasmosis and the challenges associated with its control will be explored in the following sections.

Importance of bovine anaplasmosis

Bovine anaplasmosis is an economically significant hematopoietic disease of cattle, present in nearly all cattle-producing regions worldwide [175–177]. The disease leads to considerable economic losses. In the United States, the clinical cost of bovine anaplasmosis is

estimated at \$660 per affected animal, with 66% of the costs attributed to death, 16% to treatment, 11% to chronic infection, and 7% to abortions [178]. Clinical infection is marked by anemia and fever, which can lead to reduced production outcomes such as weight loss, decreased milk yield, and reproductive issues (e.g., abortions, stillbirths, transient infertility) [179–181]. In severe cases, the disease can be fatal.

Cattle that recover from clinical infection become chronically infected. Though they may appear healthy, they are often less productive than non-infected cattle. Additionally, they serve as reservoirs of infection for naïve cattle and can experience clinical disease again if they become immunosuppressed [182,183].

Beyond its economic impact, controlling bovine anaplasmosis poses significant challenges, necessitating the use of long-term antibiotic treatments. These challenges include a high prevalence of the disease, impact of disease including establishment of persistent infections, and the limited effectiveness of existing control strategies, including vaccines and management practices. These issues will be explored further in the sections below.

Causative agent

Anaplasma marginale belongs to the family *Anaplasmataceae* and order Rickettsiales, which it shares with five other families: *Rickettsiaceae*, *Holosporaceae*, *Candidatus Midichloriceae*, and *Coxiellaceae* [184]. The *Anaplasmataceae* family includes six genera: *Aegyptianella*, *Anaplasma*, *Candidatus Neoehrlichia*, *Ehrlichia*, and *Wolbachia* (Figure 1.1) (reviewed in [184,185]). The genus *Anaplasma* contains eight species capable of causing disease in domestic animals and humans: *A. marginale* (causes bovine anaplasmosis), *A. bovis* (causes monocytic anaplasmosis), *A. capra* (a zoonotic pathogen causing mild disease in goats and deer),

A. centrale (causes mild anaplasmosis in cattle), *A. odocoilei* (causes infection in deer), *A. ovis* (causes ovine anaplasmosis), *A. phagocytophilum* (causes human granulocytic anaplasmosis), and *A. platys* (causes cyclic thrombocytopenia in dogs) [186–188]. All of these species are transmitted by ticks.

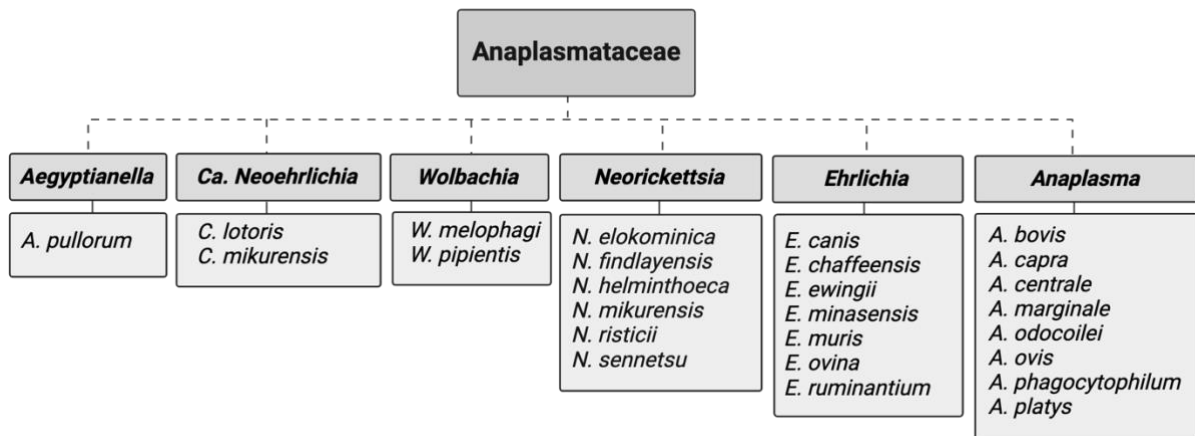


Figure 1.6. The classification of the Anaplasmataceae Family

Anaplasmataceae family, member genera, and respective species. Created with BioRender.com.

The *A. marginale* genome is circular and estimated to range between 1.2 to 1.6 Mb, with size variation due to the variable number of tandem repeat amino acid sequences in the major surface protein 1a (*msp1a*) coding region across different strains [189–191]. The genome contains 949 predicted coding DNA sequences (CDSs), including genes for major surface proteins (MSP1a, MSP1b, MSP2, MSP3, MSP4, and MSP5), some of which are immunogenic and have been extensively studied [191,192].

Prevalence of bovine anaplasmosis

Anaplasma marginale was first reported in South Africa in 1910 and has since been found on almost every continent except Antarctica. [187]. The movement and trade of cattle have contributed to its global spread. The widespread presence of *A. marginale*, combined with limited effective control strategies, makes bovine anaplasmosis a significant challenge to profitable cattle production. This is particularly true in tropical and subtropical regions, where *A. marginale* is endemic, and the duration of vector activity can last all year. While *A. marginale*'s geographic prevalence is tied to cattle movement and presence of biological vectors, some strains, like the Florida strain, are not tick transmissible. This emphasizes the role of mechanical transmission in the spread of anaplasmosis.

In the U.S., *A. marginale* has been reported in nearly every state except Hawaii and is endemic in most Gulf Coast states, western and midwestern states, and much of the southern Atlantic region [175,177,193]. Surveys in major beef-producing states highlight the disease's widespread presence in the U.S. cattle industry. For example, estimated *A. marginale* seroprevalence rates are 15.9% in Texas, 35.2% in Missouri, 9.4% in Kentucky, 2.6% in Georgia, and 29.0% in Mississippi (Error! Reference source not found.) [194–198]. A study of 434 cattle from 11 herds across eight Texas counties found a within-herd seroprevalence ranging from 0% to 88% [199]. Additionally, a southeastern U.S. seroprevalence study involving 65,328 samples from eight states reported within-herd prevalence rates between 2.4% and 35.2% [198]. In Kansas, a study showed that 52.2% of the 925 cow-calf herds tested were infected with *A. marginale*, with herd prevalence ranging from 19.2% to 87.3% [200].

Table 1-4. Statewide prevalence of *A. marginale*

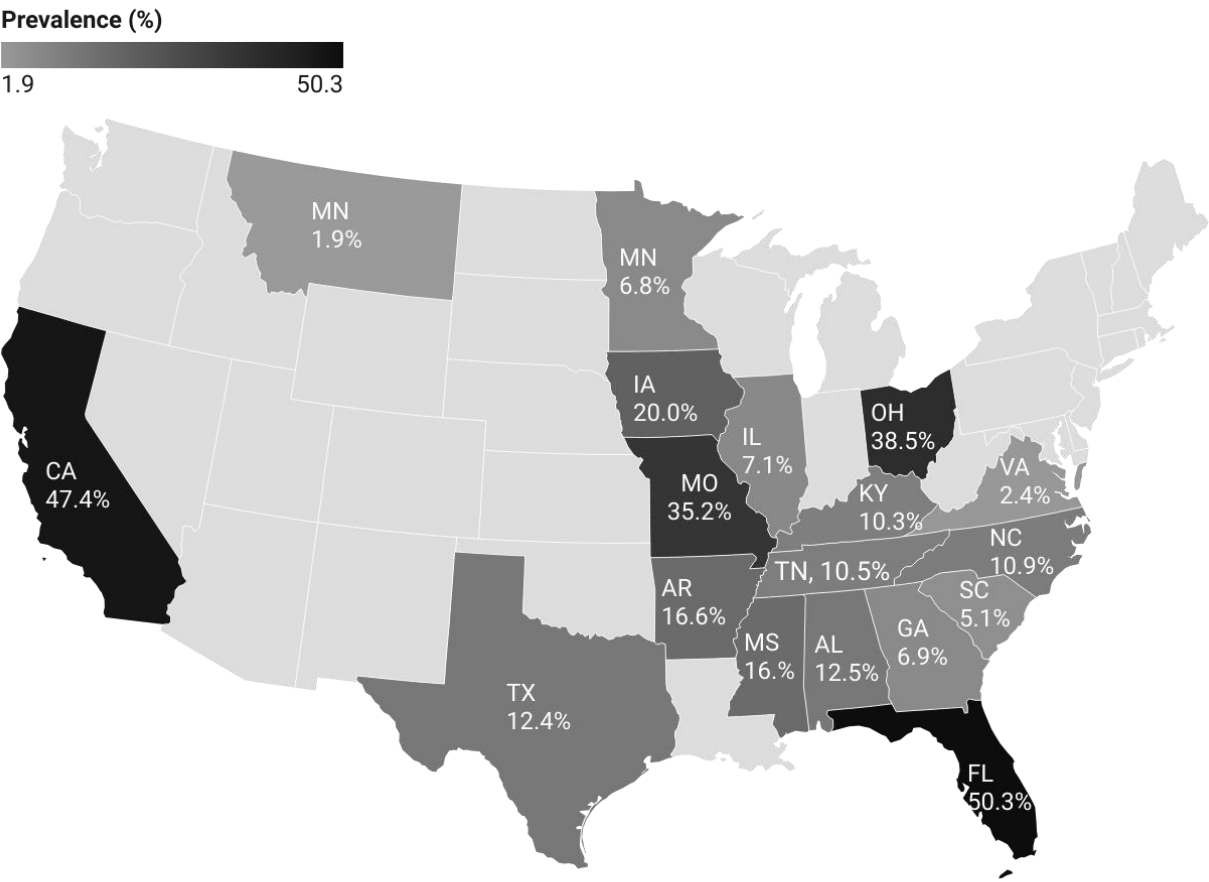
State	Year	Sampling Unit	Sample Size (Counties Tested/Total Counties))	Diagnostic Method and Prevalence	Ref:
Texas	2014	Active slaughter survey	215 (19/254)	cELISA = App: 13.49%, True: 12.35%	[195]
	2002- 2012	Texas A&M VDL records	15, 156 (n/a)	App: 15.91%	
	2002- 2012	Necropsy data from AAVLD laboratories	20, 866: (n/a)	App: 14.4%	[198]
Georgia	2013- 2014	Beef cows at slaughterhouses	293 (6/159)	cELISA = App: 4.44%; True: 2.63%	[197]
	2018- 2019	Convenient beef cattle	1,095 (24/159)	cELISA = App: 8.4%; True: 6.9%	[201]
Mississippi	2013- 2014	Active slaughter surveillance	207 (16/82)	cELISA= App: 28.99%; True: 29.02%	[196]
	2002- 2018	Mississippi VDL records	5, 150 (n/a)	App: 16.72%	
	2002- 2012	Necropsy data from AAVLD laboratories	402 (n/a)	App: 27.6%	[198]
	2013	Beef cattle at slaughter plants	111 (n/a)	cELISA = App: 32.48%	
Arkansas	2002- 2012	Necropsy data from AAVLD laboratories	1, 848: (n/a)	App: 16.6%	[198]
Illinois	1986	Market cattle	4, 994 (97/102)	MCAT = App: 7.6%; True 7.1% to 10.7% * ¹	[202]

Ohio	2020-2021	Beef herds	327 (4/88)	cELISA = 38.53%	[203]
Alabama	2002-2012	Necropsy data from AAVLD laboratories	17, 755 (n/a)	App: 7.9%	[198]
	2013	Beef cattle at slaughter plants	24 (n/a)	cELISA = App: 12.50%	
Kentucky	2002-2012	Necropsy data from AAVLD laboratories	232 (19/120)	cELISA = App: 10.78%; True: 9.44%	[194]
	2013	Beef cattle at slaughter plants	2, 573 (64/120)	cELISA= App: 11.58%; True: 10.3%	
North Carolina	2002-2012	Necropsy data from AAVLD laboratories	1, 0537 (n/a)	App: 10.9%	[198]
	2013	Beef cattle at slaughter plants	24 (n/a)	cELISA = App: 16.67%	
South Carolina	2002-2012	Necropsy data from AAVLD laboratories	467 (n/a)	App: 5.1%	[198]
Tennessee	2002-2012	Necropsy data from AAVLD laboratories	10, 550: (n/a)	App: 56.0%)	[198]
	2013	Beef cattle at slaughter plants	247 (n/a)	cELISA = App: 10. 53%	
Missouri	2013	Beef cattle at slaughter plants	54 (n/a)	cELISA = App: 35.18%	[198]
Virginia	2013	Beef cattle at slaughter plants	41 (n/a)	cELISA = App: 2.44%	[198]
Minnesota	2001-2010	Minnesota VDL data	438,407	cELISA (n= 226,923) = App: 6.8% CFT (n= 211,484) = App: 2.4%	[204]

Iowa	2002	Feedlot cattle	659	cELISA = APP: 15.7%	[205]
	2017-2022	ISU DVL	5, 307 (93/99)	cELISA and PCR= 20%	[206]

Statewide prevalence within the last 20 years.
 *¹ True prevalence after adjustment based on MCAT sensitivity and specificity

A. *marginal*e prevalence in the U.S.



Map: Naemi Bickmeier • Created with Datawrapper

Figure 1.7. A. *marginal*e statewide prevalence within the last 20 years
*Anaplasma marginal*e statewide prevalence summarized from **Error! Reference source not found.**

Impact of infection

Bovine anaplasmosis infection causes significant economic losses to the cattle industry, \$660 per head [178]. These losses stem from both animal mortality and decreased production efficiency due to infection. Moreover, the disease often leads to persistent infections, necessitating long-term control strategies to manage its impact.

Susceptible hosts

A wide range of hosts are susceptible to *Anaplasma marginale* infection, including cattle, bison, water buffalo, red deer, and African antelopes [175,177,207–209]. The pathogen has also been detected in other domestic ruminants, such as goats and sheep [210–212]. Although white-tailed deer can be experimentally infected, natural infections have not been documented, excluding these animals as potential reservoirs of infection [213–215].

In susceptible hosts, *A. marginale* typically causes persistent infections that may present as mild or inapparent. However, cattle, particularly those over two years of age, are more likely to develop clinical disease, which can be severe and sometimes fatal (reviewed in [176,216]).

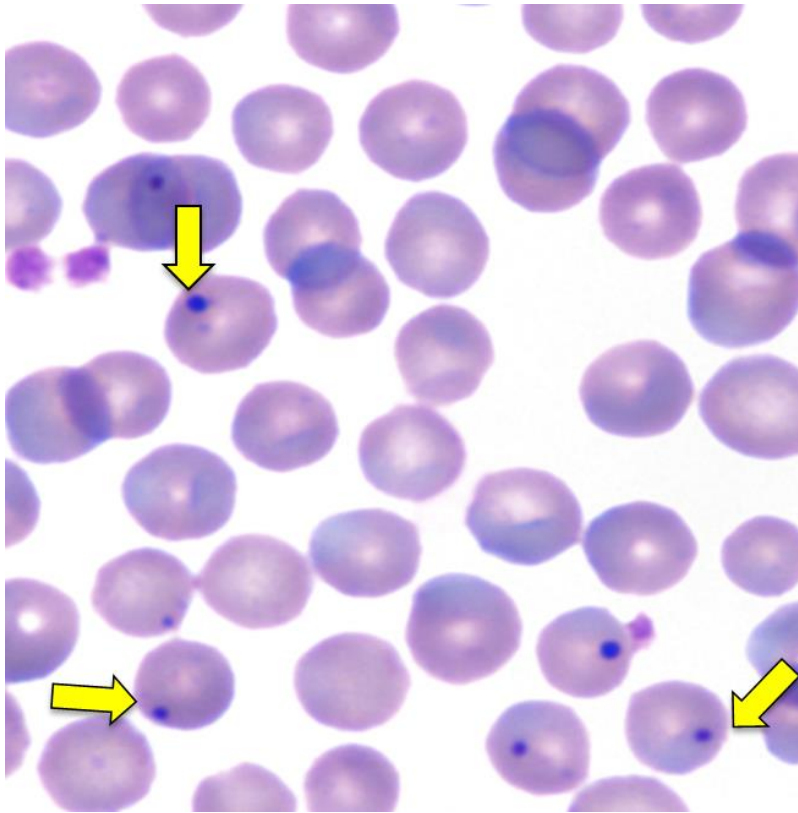
Clinical disease

The incubation period of *A. marginale* in cattle averages 28 days (ranging from 7 to 60 days), depending on the virulence of the infecting strain and dose (reviewed in [188]). During the initial infection, *A. marginale* multiplies rapidly, infecting up to 70% of circulating red blood cells. These infected erythrocytes are then phagocytosed by the reticuloendothelial system. The clearance of infected red blood cells results in anemia, the hallmark of clinical anaplasmosis, which presents with pale mucous membranes, jaundice, and the absence of hemoglobinuria

(reviewed in [176]. Anemia is often accompanied by other clinical signs, such as fever (around 41°C), which peaks alongside parasitemia [181].

Infected cattle may experience a loss of appetite, depression, general weakness, and fatigue. They may lag behind the herd, lie down frequently, and have difficulty rising. These symptoms can lead to weight loss and a drop in milk production [217–220]. Additionally, clinical bovine anaplasmosis can reduce reproductive efficiency: bred females may fail to conceive, and those infected late in pregnancy may experience abortions or stillbirths [221,222]. Infected bulls may suffer from transient infertility, characterized by decreased scrotal circumference and poor sperm quality [181]. In severe cases, death may occur, particularly in cattle over two years old, as they have a reduced ability to rapidly regenerate red blood cells[179,219].

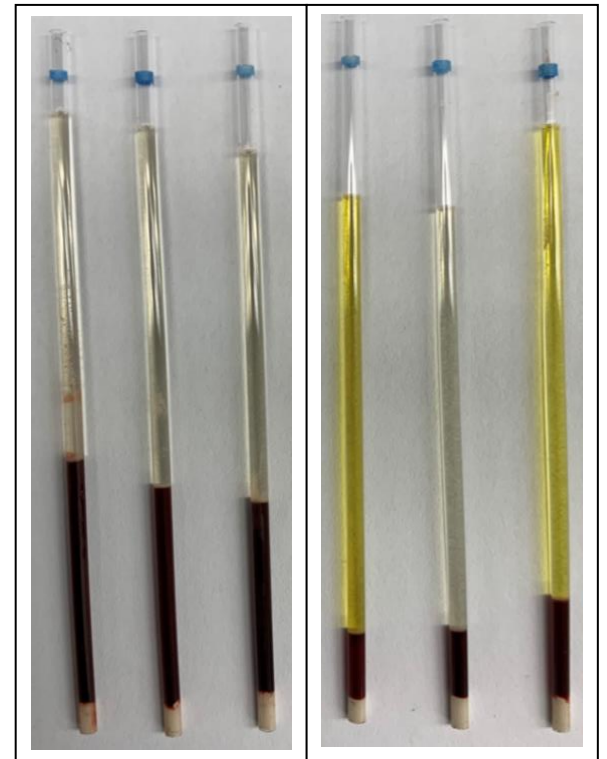
During clinical disease, *A. marginale* can be observed on thin blood smears as small points on the margins of red blood cells (Figure 1.8. (a) *A. marginale* infected red blood cells (b), Packed cell volume). Additionally, the packed cell volume of clinically infected cattle decreases as infected red blood cells are phagocytosed [181].



(a)

Non-infected

Acutely infected



(b)

Figure 1.8. (a) *A. marginale* infected red blood cells (b), Packed cell volume

Anaplasma marginale infects red cells, seen as black dots on the margins of red blood cells (yellow arrow) in panel (a). The hallmark clinical sign of bone anaplasmosis is anemia, which is represented by reduced packed volume in panel (b), the last three microcapillary tubes compared no normal packed cell volume of non-infected cattle, panel (b) the first three microhematocrit tubes.

Chronic disease

Most cattle recover from clinical anaplasmosis and transition into a chronic, persistent phase of infection [223,224]. While chronically infected cattle generally appear healthy, their production levels are often lower compared to non-infected animals [217]. These cattle are at risk of developing recrudescent clinical anaplasmosis if they become immunocompromised, such as during co-infections or pregnancy [183]. Additionally, chronically infected cattle act as reservoirs of infection, posing a risk to naïve animals [225,226].

Due to its high prevalence, economic significance and ability to establish persistent infections, bovine anaplasmosis requires long-term control strategies.

Challenges of bovine anaplasmosis control that necessitate long-term antibiotics

The two main factors that necessitate the use of long-term chlortetracycline (CTC) for controlling active anaplasmosis are the ability of the causative agent, *A. marginale*, to produce new variants, thereby evading clearance by the host's immune system, and the lack of nationwide alternative control strategies.

Ability for *A. marginale* to produce antigenic variants

One of the characteristics of *A. marginale* that complicates control efforts is its ability to undergo antigenic variation in its immunodominant proteins, creating new variants. Two of the most immunodominant proteins, MSP2 and MSP3 are coded by multigene superfamily genes, allowing them to undergo antigenic reassortment during replication in vertebrate hosts [227–229]. This ability to generate new variants approximately every fifth replicative cycle enables *A. marginale* to evade host immune responses and establish persistent infections [230].

In cattle infected with *A. marginale*, bacteremia levels exhibit cyclic rickettsemia. During clinical infection, rickettsemia peaks at around 10^9 bacteria per milliliter of blood by 2.5 weeks, then decreases [175]. This initial decline marks the end of the acute phase and usually the clinical disease. However, recovered cattle remain persistently infected. Persistent infection is driven by the ability of immunodominant proteins, MSP2 and MSP3 to undergo antigenic reassortment and variation, producing new variants that evade host immune recognition (Figure

1.9. *Anaplasma marginale* rickettsemia levels during replication in vertebrate hosts)

[176,182,229,230]. As new variants emerge, rickettsemia rises again, peaking at around 10^6 bacteria per milliliter of blood. This cyclic fluctuation occurs approximately every fifth replication cycle, with each rise corresponding to the host's immune system preparing to mount a response against the newly created variants, followed by a drop once the immune response is effective.

The ability of two of *A. marginale*'s immunodominant proteins to undergo antigenic variation complicates control efforts. This unpredictability makes it difficult to forecast which variants will emerge, and different variants can appear even within the same herd infected by the same *A. marginale* strain. As a result, *A. marginale* evades complete clearance by the host's immune system and establishes persistent infections which require long term-control strategies [182,231].

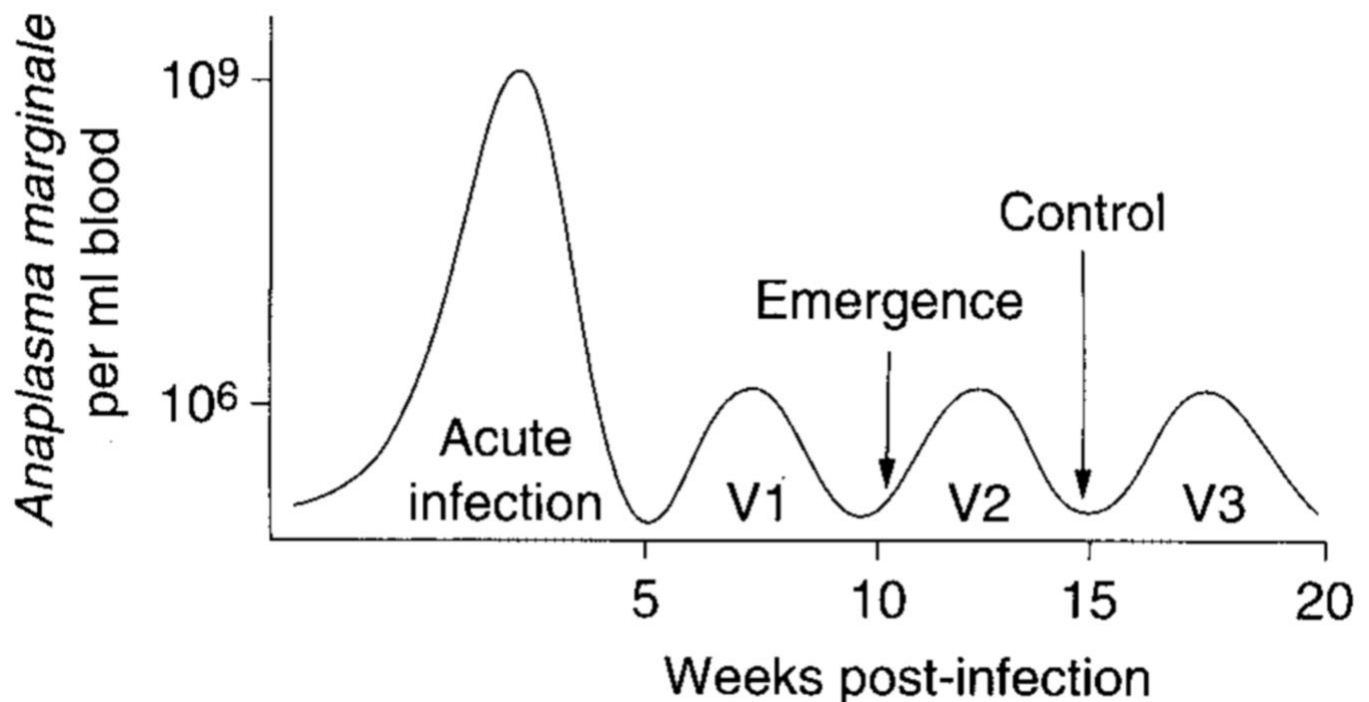


Figure 1.9. *Anaplasma marginale* rickettsemia levels during replication in vertebrate hosts

During acute infection, rickettsemia levels increase to greater than 10^9 pathogens/mL. However, these levels drop after the primary immune response. Once antigenic variants of *A. marginale* are produced at about the 5th cycle, the Rickettsemia levels rise again to peak at about 10^6 pathogens/mL. Subsequent immune responses are specific to previous variants, allowing for personal infection.

Source: Source: Kocan et al. 2003. [176]

***Anaplasma marginale* antibiotic resistance genes**

Although presence of antibiotic resistance genes does not always mean bacteria are resistant to that antibiotic, two multi-drug resistance genes whose functions are unknown have been identified in the *A. marginale* genome [191]. Additionally, two tetracycline resistance genes, *otrA* and *otrB*, were identified in a separate study [232]. Tetracyclines inhibit bacterial protein synthesis by preventing aminoacyl-tRNA from interacting with bacterial ribosomes [233]. The *otrA* gene codes for a ribosomal protection protein, which reduces the efficacy of

tetracyclines, while the *otrB* gene codes for efflux pumps that export oxytetracycline (OTC) from bacterial cells [233].

The discovery of multi-drug and tetracycline resistance genes in *A. marginale* isolates could suggest that *A. marginale* may have some level of resistance to tetracyclines. This would be not entirely surprising, given that tetracyclines have been the drug of choice for treating and controlling *A. marginale* since its discovery by Sir Arnold in the early 1910's. This may also explain reports from some producers claiming that CTC is no longer effective in controlling active anaplasmosis.

Disadvantages of anaplasmosis control which necessitate antibiotics as a common control strategy

Bovine anaplasmosis control strategies include maintaining an *A. marginale*-free herd, preventing iatrogenic transmission, using acaricides and insecticides and vaccines. All these control strategies have disadvantages which have allowed CTC to be the common anaplasmosis control method in the U.S.

Maintaining an *A. marginale*-free herd

One bovine anaplasmosis control strategy is preventing introduction of the pathogen in the herd or maintaining an *A. marginale*-free herd. This involves quarantining and testing new cattle before introducing them to the herd. However, diagnostic tools like clinical signs and PCR are often ineffective during the incubation period or when rickettsemia levels are low in chronically infected cattle. Testing every animal is also costly and impractical. Additionally, in

endemic regions, maintaining a disease-free herd may not be desirable, as it creates a large number of cattle susceptible to severe disease, which could lead to significant losses if an outbreak occurs.

Preventing iatrogenic transmission

Anaplasma marginale can be iatrogenically transmitted to naïve cattle through contact with contaminated blood. For example, dehorning cattle with instruments contaminated with *A. marginale* can lead to mechanical transmission, especially during clinical infections when blood cells are heavily infected. To prevent this, sanitary practices such as disinfecting veterinary instruments between uses, not re-using needles, and other measures to limit the transfer of contaminated blood should be implemented [234]. However, these practices can be challenging, especially in large-scale operations, and using a new needle for each animal can become costly for producers.

Using acaricides and insecticides

Vector transmission of *A. marginale* can occur through the bite of a biological or mechanical vector. More than 20 vector-competent ticks for *A. marginale* have been identified, with *Dermacentor* ticks being the primary vectors in the U.S. [176,235,236]. *Anaplasma marginale* replicates within the vacuoles of infected cattle's red blood cells. Uninfected adult ticks on pastures in search of a host attach to cattle and acquire infected red blood cells with a bloodmeal [237]. In the tick, the gut epithelium becomes infected first, and *A. marginale* undergoes development and replication (Figure 1.10. Schematic diagram of *A. marginale* development in bovine erythrocytes and tick cells).

Male ticks, after feeding, search for female ticks to mate with on the same host or nearby hosts, guided by attractant sex pheromones secreted by females [238]. As they search for female ticks, and in between mating sessions, male ticks take several feedings (reviewed in [239]). During the subsequent feeding, tick salivary gland cells become infected [236,240]. Within salivary glands, additional *A. marginale* development and replication occurs, increasing the bacterial load [241,242]. Naïve cattle become infected when bitten by these infected ticks during subsequent feedings [236,237,240,242,243]. In tick cells, *A. marginale* first develops into a reticulated (vegetative) form and then into a dense (infective) form [236].

Due to their intermittent feeding behavior, greater likelihood of moving between hosts in search of mates, and their preference for large mammal like cattle, male adult ticks are considered the most epidemiologically relevant tick life stage for *A. marginale* transmission (reviewed in [239]). Like cattle, ticks can develop persistent infections and serve as a source of infection for naïve cattle [236].

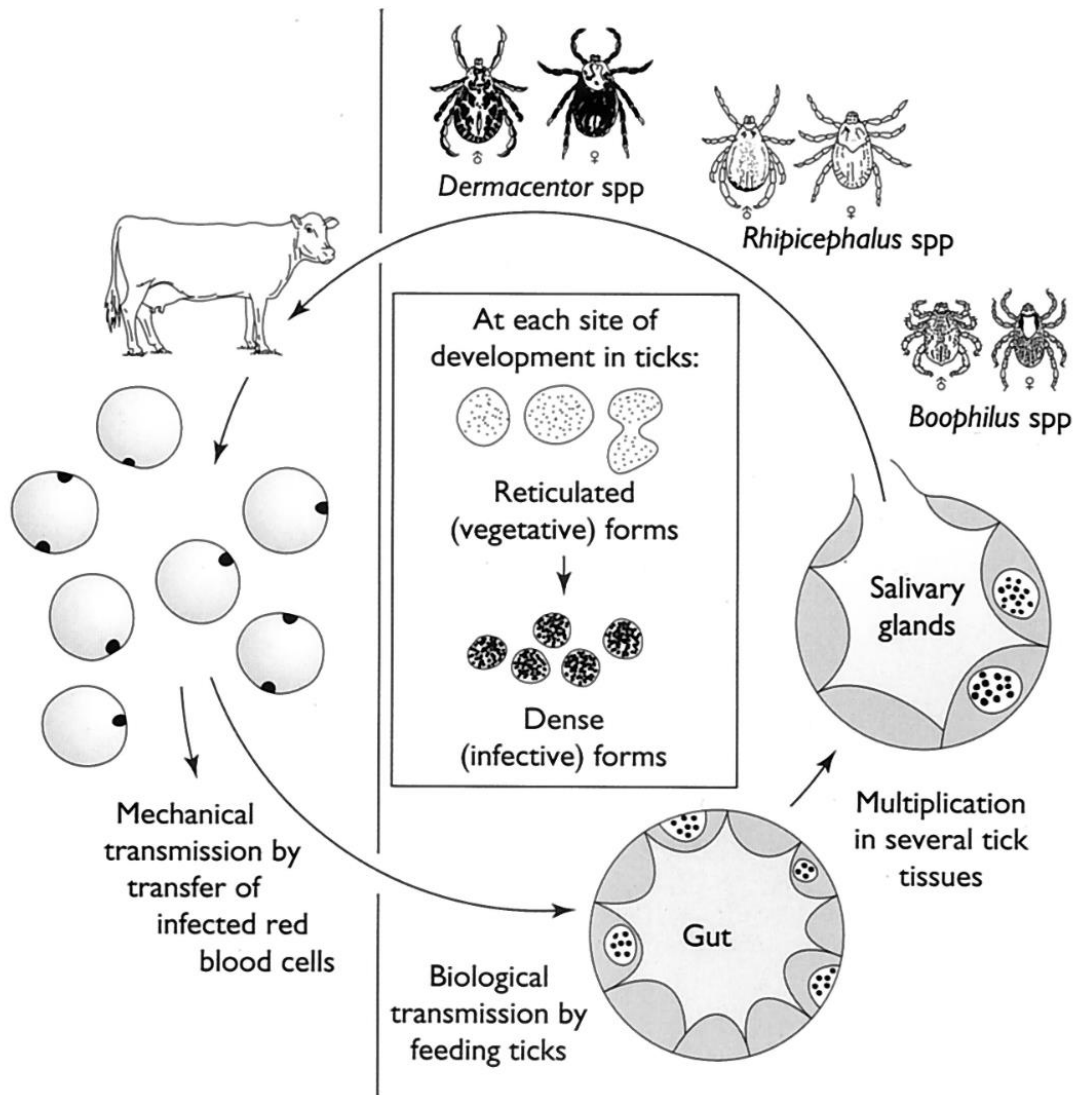


Figure 1.10. Schematic diagram of *A. marginale* development in bovine erythrocytes and tick cells

Schematic development cycle of *A. marginale* in ticks and cattle. Ticks ingest red blood cells with a blood meal and *A. marginale* replicates at the first site of infection, tick guts cells. At the second feed, other organs, including the salivary, become infected, and *A. marginale* is transmitted to cattle being fed on. Source: Kocan et al. 2003 [176].

Mechanical vectors of *A. marginale* include biting flies, such as tabanids (horse flies) and *Stomoxys* (stable flies). Unlike biological vectors, *A. marginale* does not develop or replicate within mechanical vectors. As a result, mechanical transmission is most efficient during acute

infection when bacteremia levels are high [244,245]. For transmission to occur, intact red blood cells must remain alive and infective, so the time between contamination of the fly's mouthparts and transfer to naïve cattle must be short before the blood cells desiccate. Biting flies exhibit interrupted feeding behavior which is further influenced by cattle's defensive movements in response to painful bites [246].

When feeding on *A. marginale*-infected cattle, biting flies ingest contaminated blood, contaminating their mouthparts in the process [244,245,247]. During subsequent feedings on nearby naïve cattle, the contaminated red blood cells on the fly's mouthparts are injected into the naïve cattle, facilitating transmission [246].

Anaplasma marginale can be controlled by using acaricides and insecticides during periods of high vector activity. Several formulations exist, including pour-on treatments, drenches, and acaricide-coated ear tags. In some endemic regions, particularly in developing countries, vector control aims to reduce tick populations rather than eliminate them. This approach allows younger cattle, which are less susceptible to severe disease, to become infected with *A. marginale*, thereby establishing persistently infected herds that are more resistant to severe disease.

However, there are disadvantages of using acaricides and insecticides. Residues may persist in meat and meat products, potentially posing health risks to consumers and contaminating the environment. Additionally, the regularly use of these chemicals exerts selection pressure on vectors, leading to acaricide and insecticide resistance. Control is even more challenging in areas with three-host ticks, such as *Dermacentor* species, which have a broad host range and feed on different hosts at various life stages. Acaricides used on cattle primarily target adult ticks, leaving younger life stages uncontrolled. Other limitations include

the high cost of these products and the need for multiple, repeated applications, making the process labor-intensive.

Vaccines

The history of *A. marginale* vaccination dates back to the early 1910s, pioneered by Sir Arnold Theiler in South Africa. Sir Arnold observed that *A. centrale* was less pathogenic than *A. marginale* and noted that prior infection with *A. centrale* reduced the severity of clinical disease when cattle were later infected with *A. marginale* [187].

Several live and attenuated vaccine strategies have explored, including inoculating cattle with fresh whole blood or stabilate infected with less pathogenic strains of *A. marginale*, or with *A. centrale* in certain countries [248–250]. However, challenges arose with live or blood product vaccines, such as the risk of transmitting other pathogens, concerns about introducing new strains like *A. centrale* into regions where they were not previously present, and the occurrence of neonatal isoerythrocytosis (hemolytic anemia and jaundice in calves born to vaccinated dams) [251–256]. These issues prompted efforts to further purify live vaccines to remove red blood cell contaminants [254]. The inoculation of whole blood or stabilate was prohibited in some countries due to fears that vaccine strains might revert to virulence. In these cases, attenuation through passaging in non-cattle hosts and/or irradiation was employed [257–259].

The only commercial modified live attenuated vaccine in the U.S. was Anavac® (Poultry Health Laboratories), which was licensed only in California [260]. However, this vaccine was later withdrawn. The first killed vaccine in the U.S. was used from the early 1960s until 1999, when it was discontinued due to company restructuring (reviewed in [176]). Another inactivated vaccine, Anaplaz ® (Fort Dodge), was marketed in 1965, but it was contaminated with

erythrocytes and caused neonatal isoerythrocytosis in some vaccinated animals [271,276–279]. Anaplaz[®] was also discontinued. With no other vaccines available in the U.S., a petition led to the resumption of Plazvax[®] production by University Products L.L.C. under an experimental vaccine status [256,261–264]. The University Products anaplasmosis vaccine is currently conditionally licensed in 15 states. This vaccine reportedly uses the same antigen preparation and purification methods as Plazvax[®], but without the association with neonatal isoerythrocytosis.

Other vaccine platforms, such as sub-unit vaccines and recombinant vaccines based on immunodominant MSPs, and more recently, T4SS proteins, have also been investigated; however, none have been commercialized. Examples of investigated *A. marginale* vaccine targets include MSP1a, MSP1b, MSP2, and MSP3 [229,265]. Other T4SS proteins such as VirB2, VirB9-2, VirB10, and CTP, have also been studied [266–268]. More recently, a target mutagenesis approach was reported, leading to the development of an attenuated *A. marginale* vaccine that successfully reduced *A. marginale* growth and prevented infection in challenged cattle. In this study, the *A. marginale* phage head-to-tail connector protein gene (*phtcp*), believed to be important in the bacterium's ability to acquire metal ions, was deleted [269]. This deletion resulted in growth attenuation and provided protection against disease, regardless of the infecting *A. marginale* strain.

A major limitation to vaccine development is the presence of many diverse *A. marginale* strains driven by variations in the MSP1a region and the antigenic diversity resulting from the ability of two most immunodominant proteins, MSP2 and MSP3, to undergo antigenic variation [223,230]. This leads to unpredictable bacterial heterogeneity, even within a single host. As a result, a universal *A. marginale* vaccine based on traditional methods is unlikely to be effective.

While the more recent attenuated vaccine demonstrated the ability to prevent clinical disease in cattle, the duration of protection remains unknown.

Antibiotics for *A. marginale* control

In the U.S., antibiotics, primarily tetracyclines, have been used to treat clinal bovine anaplasmosis and control chronic anaplasmosis (Table 1-5. Historic antibiotic treatment regimens for bovine anaplasmosis control). Other drugs, such as imidocarb, along with less effective treatments including antimalarials and arsenicals, have been used in different parts of the world (reviewed in [177]). Historically, antibiotics have also been employed to prevent disease and eliminate carrier states (Table 1-5. Historic antibiotic treatment regimens for bovine anaplasmosis control). Tetracyclines have been the most commonly used, and more recently, enrofloxacin was approved for use in the U.S., though it has since been withdrawn.

Table 1-5. Historic antibiotic treatment regimens for bovine anaplasmosis control

Drug and Use	Route	Dose (mg/lb BW/day)	Frequency
Prevention			
Chlortetracycline *	Oral	0.10-0.25	Daily year-round
Chlortetracycline	Oral	0.50-2.0	Daily during vector season
Oxytetracycline (50-100 mg/ml) *	IV or IM	3.0-5.0	Every 28 days during vector season
Oxytetracycline (LA-200) *	IM	9.0	Every 28 days during vector season
Carrier Elimination			
Chlortetracycline *	Oral	0.50	Daily for 120 days
Chlortetracycline*	Oral	5.0	Daily for 60 days
Oxytetracycline (50-100 mg/ml) *	IV or IM	5.0	Daily for 10 days
Oxytetracycline (50-100 mg/ml) *	IV or IM	10.0	Daily for 5 days

Oxytetracycline (LA-200) *	IM	9.0	4 RX at 3-day intervals
Treatment of sick animals			
Oxytetracycline (50-100 mg/ml) *	IM	5.0	Usually, one treatment
Oxytetracycline (LA-200) *	IM	9.0	One treatment
Enrofloxacin (100-CA) *	SC	12 mg/kg	One treatment
Temporary Protection During Outbreaks			
Oxytetracycline (50-100 mg/ml) *	IM	5.0	One treatment
Oxytetracycline (LA-200) *	IM	9.0	One treatment
Prolonged Protection During Outbreaks			
Oxytetracycline (50-100 mg/ml) *	IM	5.0	Every 28 days during vector season
Oxytetracycline (LA-200) *	IM	9.0	Every 28 days during vector season
Chlortetracycline	Oral	0.50	Daily for 60 days

Adapted from “Anaplasmosis in beef cattle” by Ron Gill; Texas Agricultural Extension Service and Brandt Skinner, master’s thesis.

* Regimens now considered illegal.

Today, antibiotics for managing bovine anaplasmosis in the U.S. are labeled only for treating clinical disease and controlling active infection (Table 1-6. Current approved regimens for bovine anaplasmosis control). Clinical cases are treated with parenteral administration of oxytetracycline (OTC) 200 mg/ml with a single dose of 5.0 mg/lb BW/day [175,270]. Active anaplasmosis is controlled by administering type C medicated feeds, either as hand-fed options or as free-choice medicated minerals or salt blocks [271]. The hand-fed options are designed to deliver the full 0.5 mg/lb BW/day CTC to a group of cattle [272,273]. Free-choice medicated feed products are intended to provide CTC at doses ranging from 0.5 to 2.0 mg/lb BW/day [272–274]. These products include feed blocks, mineral salt mixes, and liquid feed supplements placed in feeding or grazing areas for cattle to consume at will, which results in no control over the exact amount consumed.

Approved free-choice mineral salt mixes include Aureomycin® (NADA 048-761) and Deracin® (ANADA 200-510), both containing 6,000 g/ton of CTC to provide 0.5 to 2.0 mg/lb BW/day, with published Blue Bird labels detailing the formulation and conditions of use for the medicated feed products. Minerals with published formulations can be manufactured at any feed mill. However, there are other approved free-choice medicated feed products with proprietary formulations, which can only be manufactured at licensed mills. These proprietary formulations are available in concentrations of 700 g/ton, 5,000 g/ton and 8,000 g/ton, providing 0.5 to 2.0 mg/lb BW/day of CTC.

The use of tetracyclines for anaplasmosis control requires a Veterinary Feed Directive (VFD) from a veterinarian who has determined that active anaplasmosis, with or without apparent clinical disease, is present in the herd. The VFD is valid for six months and cannot be re-filled, but a new VFD can be issued if necessary [272]. A common practice is to administer CTC-medicated feeds throughout the entire vector season, when new infections and clinical diseases is likely to occur. In this context, CTC is essentially being used as a preventive measure, even though it is not approved for that purpose. In some areas, the vector season can last year-round, leading to extended periods of CTC use.

Table 1-6. Current approved regimens for bovine anaplasmosis control

Drug and Use	Route	Dose (mg/lb BW/day)	Frequency
Control of active anaplasmosis			
Chlortetracycline	Oral-hand fed	0.5	Daily year-round
Chlortetracycline	Oral-free choice	0.5-2.0	Daily year-round
Treatment of sick animals			
Oxytetracycline (200 mg/ml)	IM	5.0	One treatment

Source: FDA List of 21 CFR 522.1660a and 21 CFR 558.128. [69,275]

Efficacy studies on antibiotic regimens for treating and controlling bovine anaplasmosis have been conducted (reviewed in [175]). The effectiveness of administering 5.0 mg/lb of OTC to treat clinical anaplasmosis was evaluated. One study demonstrated that administering short-acting OTC at 10.0 mg/kg (approximately 5.0 mg/lb) intramuscularly, twice with 24-hour intervals, effectively reduced the appearance of clinical disease in treated calves compared to untreated controls [276]. Similar results were reported in another study conducted during an outbreak, where administering 10.0 mg/kg OTC intramuscularly for three consecutive days significantly reduced the severity of clinical disease [277].

The effect of CTC in controlling active anaplasmosis has also been studied. In one study, chronically infected cattle were administered 1.1 mg/kg (approximately 0.5 mg/lb) of CTC for 70 days. The results showed that *A. marginale* bacteremia levels in these cattle remained similar to baseline levels prior to CTC administration. Furthermore, the bacteremia levels in the CTC-treated cattle were comparable to those of untreated controls [278]. Additionally, there is anecdotal evidence from producers suggesting that CTC, at currently approved doses, is no longer effective for controlling active anaplasmosis.

Chlortetracycline medicated feed products to provide a dose of 0.5 mg/lb BW per day was approved as early as 1988 [279]. Until today (about 40 years later) the effectiveness of CTC 0.5-2.0 mg/lb BW/day to control anaplasmosis has not been re-evaluated. Considering how long CTC for anaplasmosis control is administered, how common the control strategy is and how long this regimen has been used, it is possible that a CTC dose of 0.5-2.0 mg/lb BW/day is no longer effective at controlling active anaplasmosis. If this is the case, the current CTC doses for

anaplasmosis may be contributing to antibiotic resistance without controlling anaplasmosis as they are intended for.

Summary

Bovine anaplasmosis is a significant cattle disease with global prevalence and a high occurrence in the U.S., posing a substantial economic threat to cattle production. Clinically infected cattle develop clinical disease, leading to production inefficiencies and, in some cases, death. Additionally, cattle that recover from clinical disease remain persistently infected, serving as reservoirs of infection for naïve cattle. Chronically infected cattle are also at risk of developing clinical anaplasmosis again, further complicating control efforts. The ability for *A. marginale* to establish chronic infections necessitates long-term strategies for its control. Control measures for anaplasmosis include maintenance of an *A. marginale*-free herd, preventing iatrogenic transmission, use of acaricides and insecticides for vector control and vaccination. However, each of these measures has significant limitations which have resulted to long-term antibiotics being the common method of control in the U.S.

Ideally, a vaccine would be the most effective control strategy. However, the most immunodominant proteins, MSP2 and MSP3, undergo antigenic variation, producing new variants during replication. This means that sub-unit vaccines targeting these proteins, as well as whole-killed vaccines, are unlikely to be effective against heterologous *A. marginale* strains. The whole-killed, single-strain University Products L.L.C vaccine is the only vaccine currently used in the U.S., but a key drawback is the lack of published efficacy data. Furthermore, it is based on a single strain of *A. marginale*, making it potentially ineffective against heterologous strain challenges. The newer investigated vaccine, based on targeted mutagenesis approach, is

promising advancement as it is the first vaccine to inhibit *A. marginale* replication in the host and completely prevent disease occurrence. In addition, its efficacy is independent of strain diversity. However, this vaccine could create naïve herds, placing them at high risk of fatalities during outbreaks in regions where competent vectors are endemic.

In the absence of effective alternative control strategies, CTC is used to control anaplasmosis. Chlortetracycline is administered as medicated feed at doses of 0.5 to 2.0 mg/lb BW/day for six months or longer, with or without the presence of clinical signs. However, several disadvantages accompany the use of antibiotics for anaplasmosis control. Long-term use of sub-therapeutic antibiotics is known to contribute to antibiotic resistance. In addition, 0.5-2.0 mg/lb BW/day CTC was approved for anaplasmosis control about 40 years ago and the effectiveness of the dose to control anaplasmosis has not been re-evaluated [279]. This is especially concerning given that tetracyclines have been the primary drug of choice for the prevention, treatment, and control of anaplasmosis since its discovery, and they are also used for other purposes in both animals and humans [191,232]. The potential for CTC to promote antibiotic resistance in *A. marginale* is supported by the discovery of multi-drug resistance genes and tetracycline resistance genes, *otrA* and *otrB*. Furthermore, some producers report that the current CTC regimen for controlling active anaplasmosis is no longer effective.

The overuse of tetracyclines could also select for resistance in off-target bacterial species, such as enteric bacteria like *E. coli* and *Enterococcus* species. Both *E. coli* and *Enterococcus* can be pathogenic and cause antibiotic-resistant infections. There is also concern that tetracycline resistance could lead to co-selection for resistance to non-tetracycline antibiotics, including those that are medically important. Antibiotic-resistant bacteria can cause infections that are unresponsive to treatment and contribute to antibiotic resistance-related deaths. The global

antibiotic resistance crisis has resulted in millions of illnesses and thousands of related deaths world, including in the U.S. [11].

It is currently unknown whether CTC to control active anaplasmosis promotes antibiotic resistance in off-target zoonotic bacteria, such as *E. coli* and *Enterococcus* spp.

Chapter 2 - Antibiotic susceptibilities of *Escherichia coli* isolated from cattle hand-fed chlortetracycline for anaplasmosis control

Abstract

Bovine anaplasmosis is commonly managed by protracted use of chlortetracycline (CTC)-medicated feed. However, prolonged antibiotic use for anaplasmosis control may inadvertently promote antibiotic-resistant bacteria. The objective of this study was to evaluate changes in *E. coli* antibiotic susceptibilities from cattle provided CTC for anaplasmosis control. Cattle with chronic anaplasmosis were blocked by weight, randomly allocated to study groups, and hand-fed CTC-medicated feed (0.0, 0.5, 2.0, or 10.0 mg/lb BW/day) for 120 days. *Escherichia coli* isolated from rectally collected fecal samples were evaluated with Sensititre™ NARMS Gram Negative Plates for antibiotic susceptibility using CLSI or NARMS breakpoints. There was a significant CTC dose effect on *E. coli* tetracycline (TET) minimum inhibitory concentration (MIC) least square mean value (p-value = 0.089). The number of TET-resistant isolates increased during CTC administration; however, MIC values were not significantly different between groups (p-values ≥ 0.266). Significant CTC dose and study day effects were observed for chloramphenicol (p-value = 0.012 and <0.001 , respectively) and streptomycin (p-value = 0.029 and <0.001 , respectively), and MIC values significantly increased during CTC administration, sometimes crossing breakpoint classifications. A similar trend, but to a lesser degree, was observed for sulfisoxazole, trimethoprim/sulfamethoxazole and ampicillin (p-values for CTC dose effects ranged between <0.001 and 0.013). Cefoxitin, azithromycin, gentamicin, nalidixic acid, amoxicillin/clavulanic acid 2:1 and ceftiofur MIC least square mean values did

not or only minimally changed (p-values for CTC dose effects ranged between 0.066 and 0.603). This study indicates that long-term antibiotic use for anaplasmosis control can influence the antibiotic susceptibility of concurrently occurring bacterial species, highlighting the need for judicious antibiotic use.

Introduction

Bovine anaplasmosis is an economically significant cattle disease found around the world where cattle production occurs, including in the United States (U.S.) [175–177]. This disease is caused by the rickettsial pathogen, *A. marginale*, which is naturally transmitted by vector-competent tick species or mechanically transmitted through infected blood contaminated fomites (e.g., needles, biting flies) [234,235,280]. In the U.S., anaplasmosis is endemic in several western and midwestern states, and Atlantic and Gulf Coast states [175–177]. Clinical signs of anaplasmosis include anemia, fever, weight loss, decreased milk yield, infertility, and death in severe cases [179–181]. Animals that recover from clinical disease remain persistently infected, making them epidemiologically important as infection reservoirs [182]. Additionally, cattle chronically infected with *A. marginale* may become clinically ill again if immunosuppressed [183]. Control of active anaplasmosis in endemic herds is commonly managed using chlortetracycline (CTC)-medicated feed products at dosages of 0.5-2.0 mg CTC/lb body weight (BW)/day for free-choice medicated blocks or mineral salt mixes or 0.5 mg CTC/lb BW/day for hand-fed medicated feed. Of note, there is no current limit on the duration of CTC use for active anaplasmosis control as long as the producer has a valid Veterinary Feed Directive (VFD) [281]. In anaplasmosis-endemic areas, it is common for producers to administer CTC-medicated feed products for six to twelve months per year.

Long-term use of antibiotics can promote antimicrobial resistance (AMR) [282,283]. Antimicrobial resistance threatens food security and public health by rendering antibiotics ineffective at treating bacterial infections. In 2019, the Centers for Disease Control and Prevention (CDC) estimated that AMR in the U.S. annually contributes to over 2.8 million infections and 35,000 deaths [1,11]. Antibiotic resistance in humans has been linked to antibiotic use in agricultural animals [284–286]. In food-producing animals, antibiotics are used to prevent and treat disease and, in some parts of the world, as growth promoters (use of antibiotics to promote growth was banned in the U.S. in 2017) [22,287–289]. Of all the antibiotics sold for animal use in 2022, 56% (6,245,720 kg out of 11,170,242 kg) were medically important antibiotics (important for treatment of human disease) [290]. Of these medically important drugs, 41% (2,569,381 kg out of 6,245,720 kg) were marketed for cattle, and 63% (3,960,767 kg out of 6,245,720 kg) were administered in-feed. In addition, of the tetracyclines sold for animal use, 41% (1,765,839 kg out of 4,056,479 kg) were marketed for cattle.

Zoonotic bacteria can enter the public food chain through contaminated animal carcasses or contaminated produce [291–293]. For example, a study of 280 randomly sampled porcine carcasses at a slaughterhouse, evaluated in pools of five carcasses, found 58.9% of pleural cavity pools and 44.6% of distal colon and rectum pools were contaminated with *Campylobacter* spp. [294]. Another study of various food products (n = 1,648), including beef and pork, poultry, and miscellaneous products (fresh fruit, vegetables, fermented cheeses, and dry salami) from various retail stores in the Minneapolis-St. Paul area identified *E. coli* contamination in 24% of all samples, 92% of poultry samples, and 69% of meats (beef or pork) samples, and 9% of all miscellaneous produce samples [295]. A similar study of 767 produce products found 16% of samples contaminated with *E. coli* and 78% contaminated with *Enterococcus* spp. [296].

Importantly, bacterial species contaminating food chain products may be resistant to medically important antibiotics. Antibiotic resistance in bacteria can occur by natural mutations during replication or by transfer of resistance genes via mobile genetic components (e.g., plasmids, transposons), both within and across bacterial species [41,297,298]. Regardless of the mechanism, maintenance of antibiotic-resistant bacteria can be promoted through selection pressure driven by antibiotic misuse practices (e.g., prolonged use, sub-optimal dose administration, overuse) [299–301]. Additionally, co-selection of resistance genes within and across antibiotic classes may arise when resistance genes located on the same mobile genetic elements are transferred between bacteria [298,302,303]. Co-selection of resistance has been reported for several antibiotics. For example, a study in Australia where fluoroquinolones are not used in production animals identified fluoroquinolone-resistant *E. coli* isolates, which were additionally resistant to antibiotics including amoxicillin, tetracycline, trimethoprim/sulfamethoxazole, and gentamicin [304]. These fluoroquinolone-resistant isolates all had the same chromosomal mutations, suggesting horizontal co-transfer of fluoroquinolone resistance elements with other antibiotic resistance elements.

The primary purpose of antibiotic use in food animals is for disease control. Typically, antibiotics are administered to control a specific bacterial pathogen or treat a specific disease state; however, broad-acting antibiotics may also affect other microbial species existing within the same treated host community. In addition, chlortetracycline use has been associated with decreasing rumen pH and consequently altering rumen microbial populations resulting into reduced rumen digestion by these microbes [305,306]. Understanding the potential off-target implications of antibiotic use, including how a dosing regimen may contribute to the broad selection of antibiotic-resistant microbes, is essential when defining judicious antibiotic use

protocols. To help preserve antibiotic efficacy and promote good antibiotic use stewardship, the off-target implications of antibiotic dose regimens, especially those with open-ended use durations should be investigated. Whether protracted CTC use for active anaplasmosis control promotes antibiotic resistance in off-target bovine-associated zoonotic bacterial species is unknown. Therefore, the objective of this study was to evaluate changes in antibiotic susceptibilities of *E. coli* isolated from rectally collected fecal samples of cattle treated with different dosages of CTC for 120 consecutive days for anaplasmosis control to a panel of different antibiotics. *Escherichia coli* was specifically chosen for evaluation in this study because it is a zoonotic pathogen and a common member of the gastrointestinal microbiome of cattle. We hypothesize that prolonged CTC treatment will result in increased recovery of tetracycline-resistant *E. coli*, with resistance occurring more quickly in cattle treated with greater CTC dosages. In addition, prolonged selection pressure to tetracycline will also co-select for resistance to non-study antibiotics.

Materials and Methods

Treatment Groups and Treatment Administration

Forty-eight Holstein-Jersey cross cattle (approximately 8-month-old steers, single-sourced) chronically infected with *A. marginale* were blocked by weight and allocated to one of four study groups (12 steers/group). Cattle in the same treatment group were housed together in dry lot pens. Each treatment group had its own feeding trough and ad libitum access to water. Steers were limit fed a complete ration once daily (custom blended by Wildcat Feeds, Topeka, KS). The daily ration was administered in two parts to ensure complete consumption of the medicated ration. First, the medicated ration (with the entire daily CTC dose) was administered,

and immediately after complete consumption of the medicated ration, steers were provided the second half of their daily ration unmedicated. Limit feeding was used to encourage and efficiently monitor steer consumption of the CTC-medicated ration. Complete consumption of the daily ration occurred within one hour of administration.

Hand-fed CTC medicated ration was administered for 120 days to each study group as follows: the first group received no CTC in feed (0.0 mg/lb BW/day) (untreated control), the second group received CTC at the current FDA-approved hand-fed dose (0.5 mg/lb BW/day), the third group received CTC at the high end of the current FDA-approved free-choice dose (2.0 mg/lb BW/day), and the fourth group received an experimentally high dose (10.0 mg/lb BW/day). The 0.5, and 2.0 mg CTC groups received their respective CTC-containing ration daily. To match another FDA-approved CTC dosing regimen (for respiratory infections [272], the 10.0 mg group received CTC for five consecutive days (Monday through Friday) with two days off medicated feed (Saturdays and Sundays) during the 120-day CTC administration period. The untreated control group was fed in the same two-part ration administration as the CTC-treated groups.

Fecal Sample Collection

Fecal samples were collected directly from the rectum using individual rectal palpation sleeves, and four time points were used for downstream analyses: 02 July 2019 (12 days before CTC administration began) (baseline); 10 September 2019 (58 days of continuous CTC administration); 05 November 2019 (114 days of continuous CTC administration) and 03 December 2019 (21 days after CTC administration had stopped). Fecal samples were transported to the lab in a cooler box with ice packs. To preserve fecal samples for *E. coli* isolation, aliquots

of feces were resuspended in 10 mL glycerol at a 1:1 ratio (glycerol: fecal matter) and stored at -80 °C.

Isolation of *Escherichia coli*

Identification of *E. coli* was done using biochemical tests as follows: To culture and isolate *E. coli*, approximately 1.0 g of fecal sample was homogenized in 10.0 mL phosphate-buffered saline and 50.0 µL of fecal suspension was plated on MacConkey agar plates. After incubation of the inoculated plates at 37°C for 24 hours, a single lactose fermenting colony was picked and individually inoculated on a blood agar plate (Remel, Lenexa KS) and incubated at 37°C for 24 hours (the sample size was determined with the advice of a field expert). The resulting colonies were then subjected to a Spot Indole test, and indole-positive colonies were stored on CryoBeads™ (KEY Scientific Products, Stanford, TX) at -80°C. A single colony was isolated from each of the 12 fecal samples collected from each of the four-time points and, for each of the four CTC groups, which resulted in a total of 192 colonies.

Antibiotic Susceptibility Testing

CryoBeads™ (KEY Scientific Products) containing individual *E. coli* isolates were thawed and subcultured on blood agar plates (Remel, Lenexa KS) and incubated at 35°C. After 18 hours of incubation, a single colony per sample were suspended in demineralized water to a standardized density of 0.5 Polymer McFarland Standard (Thermo Scientific, Waltham, MA). This was followed by inoculation of 10.0 µL of the suspension into Mueller-Hinton Broth (Thermo Scientific) and inoculating Sensititre™ NARMS Gram Negative (CMV3AGNF, Thermo Scientific) with 50.0 µL of the suspension per well. The plates were incubated at 35°C

for 18 hours. The *E. coli* strain (Migula) Castellani and Chalmers (ATCC 25922) was used as a quality control strain for antibiotic susceptibility testing.

The minimum inhibitory concentration (MIC) values were determined for antibiotics: ceftiofur (XNL), gentamycin (GEN), streptomycin (STR), ampicillin (AMP), amoxicillin/clavulanic acid (AUG2), ceftriaxone (AXO), azithromycin (AZI), nalidixic acid (NAL), ciprofloxacin (CIP), chloramphenicol (CHL), trimethoprim/sulfamethoxazole (SXT), sulfisoxazole (FIS), ceftiofur (FOX), and tetracycline (TET) were determined using the 2021 Clinical & Laboratory Standards Institute (CLSI) breakpoints classifications [307]. The XNL breakpoints were determined using the 2011 NARMS breakpoint classifications [308]. The antibiotic three-letter abbreviations used are as they appear on the Sensititre (CMV3AGNF) assay plate, which match the CLSI antibiotic abbreviations except for amoxicillin/clavulanic acid, whose CLSI abbreviation is AUG2.

Statistical Analysis

For each of the fourteen antibiotics, MIC data was summarized with descriptive statistics by CTC administration group at each selected time point. Due to the lack of variability, MICs of AXO and CIP were excluded from the statistical analysis.

Data underwent natural log transformation before statistical modeling to better achieve model assumptions. The log-transformed post-treatment MIC of the remaining twelve antibiotics was subjected to linear mixed model analysis. Fixed effects of the model included CTC groups, study day, and their interaction. Baseline log-transformed MIC served as the covariate. The random effect was animal ID (i.e., the error term vector corresponding to repeated measurement over the study day). The variance-covariance structure of animal ID was taken as either

compound symmetry, first-order autoregressive, or unstructured according to the model fitting criteria. The treatment effect was assessed via back-transformed least squares means and SEM. Comparisons were carried out using the 2-sided test at the 0.1 level. Tukey's multiplicity adjustment was applied for pairwise comparisons of CTC groups at a given study day and pairwise comparisons of study days within a given CTC group. Statistical analysis was performed using Statistical Analysis Software (SAS version 9.4; Cary, NC) PROC MIXED with option DDFM=KR in the MODEL statement.

Results

Summary Statistics

Summary Statistics of Antibiotic Susceptibility for the Overall Study

Out of the 192 isolates in the study, 163 (85%) were resistant to at least one antibiotic, and 97 (51%) isolates were resistant to three or more antibiotics (Figure 2.1. Heatmap of the antibiotic resistance phenotypes of *E. coli* isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days). Twenty-nine isolates (15%) were pan-susceptible (susceptible to all antibiotics tested) (Figure 2.1. Heatmap of the antibiotic resistance phenotypes of *E. coli* isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days). The most common antibiotics the isolates were resistant to were TET (151, 79% of isolates), STR (112, 58% of isolates), CHL (94, 49%, of isolates), AMP (69, 36% of isolates), and SXT (28, 15% of isolates) (Figure 2.1. Heatmap of the antibiotic resistance phenotypes of *E. coli* isolates collected from groups of steers administered

different chlortetracycline (CTC) dosages for 120 days and **Error! Reference source not found.**

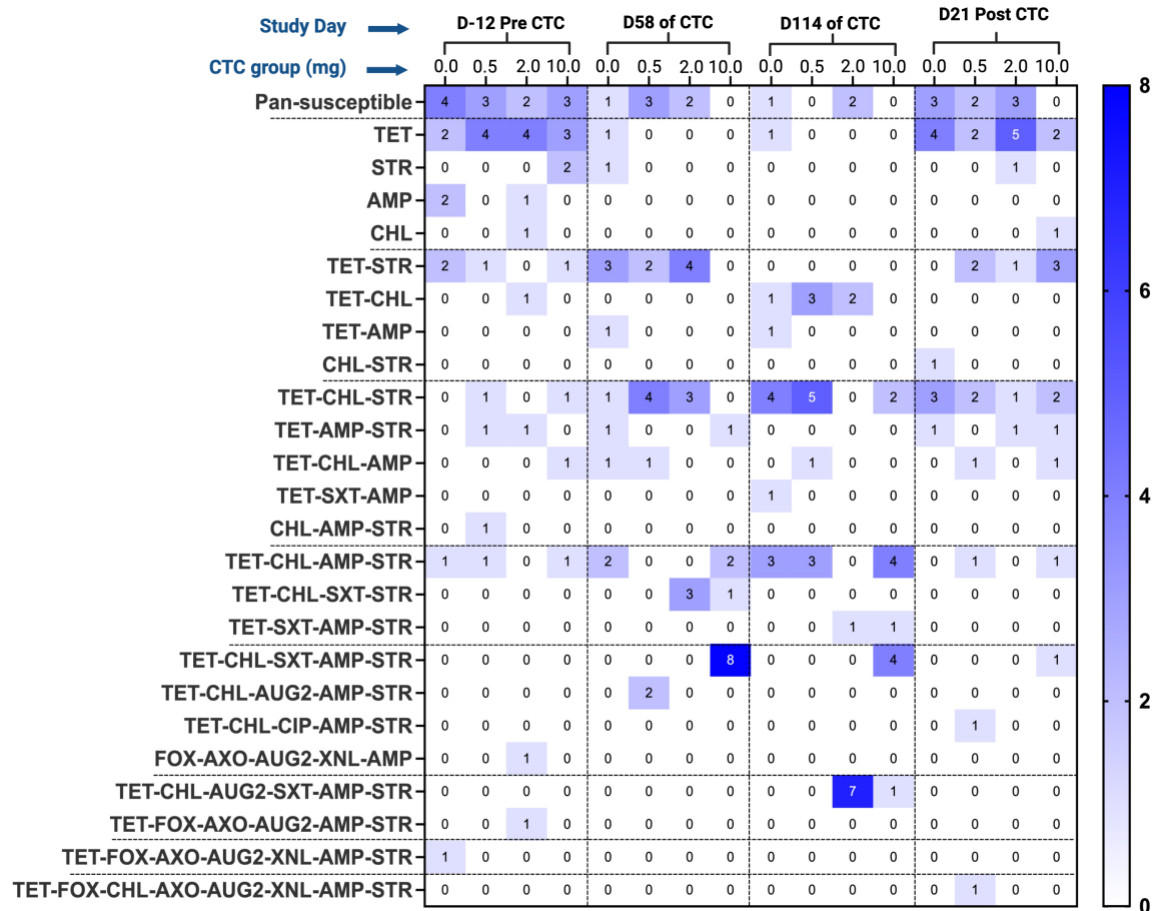


Figure 2.1. Heatmap of the antibiotic resistance phenotypes of *E. coli* isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days

Isolates of *E. coli* from groups of steers provided CTC for 120 consecutive days at dose rates of 0, 0.5, 2.0, or 10.0 mg CTC/lb BW/day (*received 10.0 mg CTC for five consecutive days, followed by two days off medicated feed). The isolates were assessed for antibiotic susceptibility at 12 days pre-CTC administration, after 58 and 114 days of consecutive CTC administration, and 21 days post-cessation of CTC administration (12 *E. coli* isolates/study group/time point). Antibiotic susceptibility was determined using the 2021 Clinical & Laboratory Standards Institute (CLSI) breakpoints for all antibiotics except ceftiofur, which was categorized using the 2011 NARMS breakpoints. Antibiotics that made up the resistant phenotypes include TET (tetracycline), STR (streptomycin), AMP (ampicillin), CHL (chloramphenicol), SXT (trimethoprim/sulfamethoxazole 1:19), AUG2 (amoxicillin/clavulanic acid 2:1), CIP (ciprofloxacin), FOX (cefoxitin), AXO (ceftriaxone), and XNL (ceftiofur). The columns represent the study days: 12 days pre-CTC administration, 58 and 11 days of continuous CTC administration, and 21 days post-cessation of CTC administration. A dotted line separates them. Each study day contains values from each CTC group: 0.0 mg, 0.5 mg, 2.0 mg, and 10.0 mg. The rows are the resistant phenotypes observed, and the dotted lines separate the resistance phenotypes based on the number of antibiotics each phenotype is made from.

Table 2-1. Distributions of minimum inhibitory concentrations of 192 *E. coli* isolates from cattle handfed chlortetracycline at varying doses for 120 consecutive days to a panel of 14 antibiotics

Antibiotic ¹	% Resistant ²	Distribution (%) of <i>E. coli</i> isolates within antibiotic MIC dilution series ³															MIC 95% CI ⁴	
		0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256		
TET	78.6								15.1	6.3	11.5	19.3	47.9				22.83-26.04	
CHL	50.0								0.0	10.4	33.3	7.3	17.7	31.3				18.19-21.64
AUG2	7.3	1.6							4.2	50.5	34.4	2.1	6.3	1.0				6.49-8.58
FIS	0.0																141.60-173.73	
SXT	14.6				70.3	8.3	2.6	2.1	2.1	3.1	11.5	24.0	15.1	0.5	4.7	12.0	43.8	0.57-0.96
AMP	35.9	2.6							6.8	47.4	5.7	1.6	10.4	25.5				12.35-16.18
STR	58.3								0.0	4.2	33.3	4.2	10.4	19.8	28.1			33.74-41.26
NAL	0.0						0.5	1.0	71.9	26.6	0.0	0.0	0.0				2.38-2.64	
FOX	2.1						0.0	0.5	0.5	57.3	34.9	4.7	0.5	1.6				5.84-7.19
XNL	1.6				0.5	0.5	81.8	14.6	0.5	0.5	1.6						0.57-0.85	
AZI	0.0				0.0	0.0	0.0	0.0	3.6	82.3	13.0	1.0					4.31-4.83	
GEN	0.0					0.0	25.0	70.8	3.6	0.5	0.0	0.0						0.87-0.98
CIP	0.5	93.8	5.7	0.0	0.5	0.0	0.0	0.0	0.0								0.012-0.018	
AXO	2.1					93.2	3.1	1.6	0.0	0.0	1.0	1.0	0.0	0.0				0.26-0.77

¹Antibiotics evaluated: TET = tetracycline, CHL = chloramphenicol, AUG2 = amoxicillin/clavulanic acid, FIS = sulfisoxazole, SXT = trimethoprim/sulfamethoxazole, AMP = ampicillin, STR= streptomycin, NAL = nalidixic acid, FOX = ceftiofur, XNL = ceftiofur, AZI = azithromycin, GEN = gentamycin, CIP = ciprofloxacin and AXO = ceftriaxone.

²The percent of 192 total *E. coli* isolates resistant to the tested antibiotic.

³Antibiotic MIC dilution series for evaluated by Sensititre CMV3AGNF plate. The white area indicates the available evaluation range for a given antibiotic and the percentage of *E. coli* isolates at that MIC. Isolates with MIC values outside the range are in the gray areas of the distribution area. Absolute MIC values for 28 isolates from all CTC groups, and all isolated prior to CTC administration were not taken, and any MIC values outside the range of the Sensititre plate were assigned the nearest MIC value evaluated by the Sensititre plate. Vertical black lines within an antibiotic dilution series indicate the consensus resistance breakpoint for the CLSI or NARMS (for XNL). Amoxicillin/clavulanic acid is shown at clavulanic acid at 1/2X the concentration that of amoxicillin. Trimethoprim/sulfamethoxazole is shown at 19X concentration that of trimethoprim.

⁴The MIC 95% confidence interval of the minimum inhibitory concentrations.

Summary Statistics of Antibiotic Susceptibility for the 0.0 mg CTC Group

Of the 48 isolates in the 0.0 mg CTC group, 39 (81%) were resistant to at least one antibiotic, and 19 (40%) isolates were resistant to three or more antibiotics (Figure 2.1. Heatmap of the antibiotic resistance phenotypes of *E. coli* isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days and **Error! Reference source not found.**). Overall, nine (19%) *E. coli* isolated isolates recovered from nine different

animals were pan-susceptible (Figure 2.1. Heatmap of the antibiotic resistance phenotypes of *E. coli* isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days and Table 2-3. Animal IDs from where pan-susceptible *E. coli* was isolated). Four of these were isolated on day 12 prior to CTC administration, two isolates were recovered during the CTC administration period, one isolate on each of the two days and, three isolates recovered on day 21 post-cessation of CTC administration (Table 2-3. Animal IDs from where pan-susceptible *E. coli* was isolated). Nineteen (40%) isolates were MDR. The most common antibiotics the isolates were resistant to were TET (35, 73% of isolates), STR (23, 48% of isolates), CHL (17, 35% of isolates), and AMP (15, 31% of isolates), followed by FOX, AXO, AUG2, XNL, and SXT (1, 2.1% of isolates each) (**Error! Reference source not found.** and **Error! Reference source not found.**). Prior to CTC administration, the isolates were resistant to a total of 19/168 (11.3%) antibiotics. The number of antibiotics the isolates were resistant to increased to 26/168 (15.5%) and 32/168 (19.0%) on days 58 and 114 of continuous CTC administration, respectively. On day 21 post-cessation of CTC administration, the number of antibiotic resistances decreased to 18/168 (10.7%).

Table 2-2. Distributions of minimum inhibitory concentrations of 48 *E. coli* isolates from cattle handfed 0.0 mg/lb BW/day chlortetracycline for 120 consecutive days to a panel of 14 antibiotics

Antibiotic ¹	% Resistant ²	Distribution (%) of <i>E. coli</i> isolates within antibiotic MIC dilution series ³															MIC 95% CI ⁴
		0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	
TET	72.9								20.8	6.3	18.8	20.8	33.3				18.19-25.15
CHL	35.4							0.0	10.4	43.8	10.4	12.5	22.9				13.53-20.30
AUG2	2.1							0.0	4.2	64.6	29.2	0.0	0.0	2.1			4.41-6.93
FIS	0.0											22.9	25.0	0.0	4.2	8.3	106-35-172.99
SXT	2.1				83.3	4.2	4.2	2.1	4.2	2.1							0.12-0.51
AMP	31.3							0.0	12.5	47.9	4.2	4.2	18.8	12.5			9.36-16.97
STR	47.9								0.0	8.3	41.7	2.1	8.3	31.3	8.3		24.19-39.85
NAL	0.0						0.0	0.0	85.4	14.6	0.0	0.0	0.0				2.08-2.50
FOX	2.1						0.0	0.0	00.0	66.7	31.3	0.0	0.0	2.1			4.58-7.08
XNL	2.1				0.0	0.0	81.3	16.7	0.0	0.0	2.1						0.42-1.06
AZI	0.0				0.0	0.0	0.0	0.0	8.3	87.5	2.1	2.1					3.61-4.73
GEN	0.0					0.0	29.2	68.8	2.1	0.0	0.0	0.0					0.79-0.96
CIP	0.0	93.8	6.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0							0.015-0.017
AXO	2.1					97.9	0.0	0.0	0.0	0.0	0.0	2.1	0.0	0.0			-0.08-1.24

¹Antibiotics evaluated: TET = tetracycline, CHL = chloramphenicol, AUG2 = amoxicillin/clavulanic acid, FIS = sulfisoxazole, SXT = trimethoprim/sulfamethoxazole, AMP = ampicillin, STR= streptomycin, NAL = nalidixic acid, FOX = ceftiofur, XNL = ceftiofur, AZI = azithromycin, GEN = gentamycin, CIP = ciprofloxacin and AXO = ceftriaxone.

²The percent of 48 total *E. coli* isolates that were resistant to the tested antibiotic.

³Antibiotic (Minimum Inhibitory Concentration) MIC dilution series for evaluated by Sensititre CMV3AGNF plate. The white area indicates the available evaluation range for a given antibiotic and the percentage of *E. coli* isolates at that MIC. Isolates with MIC values outside the range are in the gray areas of the distribution area. Absolute MIC values for seven isolates from all CTC groups, and all isolated prior to CTC administration were not taken and any MIC values outside the range of the Sensititre plate were assigned the nearest MIC value evaluated by the Sensititre plate. Vertical black lines within an antibiotic dilution series indicate the CLSI or NARMS (for XNL) consensus resistance breakpoint. Amoxicillin/clavulanic acid is shown at clavulanic acid at 1/2X concentration that of amoxicillin. Trimethoprim/sulfamethoxazole is shown at 19X concentration that of trimethoprim.

⁴The MIC 95% confidence interval of the minimum inhibitory concentrations.

Table 2-3. Animal IDs from where pan-susceptible *E. coli* was isolated

Study Day			
D12 Pre CTC	D58 of CTC	D114 of CTC	D21 Post CTC
0.0 mg			
3	65	86	26
7			73
16			98
50			
0.5 mg			
48	2		29
81	19		47
47	29		
0.5 mg			
46	45	14	8
115	46	45	14
			33
10.0 mg			
68			
101			
88			

The columns represent CTC days: 12 days prior to CTC administration (D12 pre-CTC), days 58 and 114 of continuous CTC administration (D58 of CTC and D114 of CTC, respectively), and 21 days post-cessation of CTC administration (D21 Post CTC).

The rows are divided into four study groups (0.0 mg, 0.5 mg, 2.0 mg, and 10.0 mg).

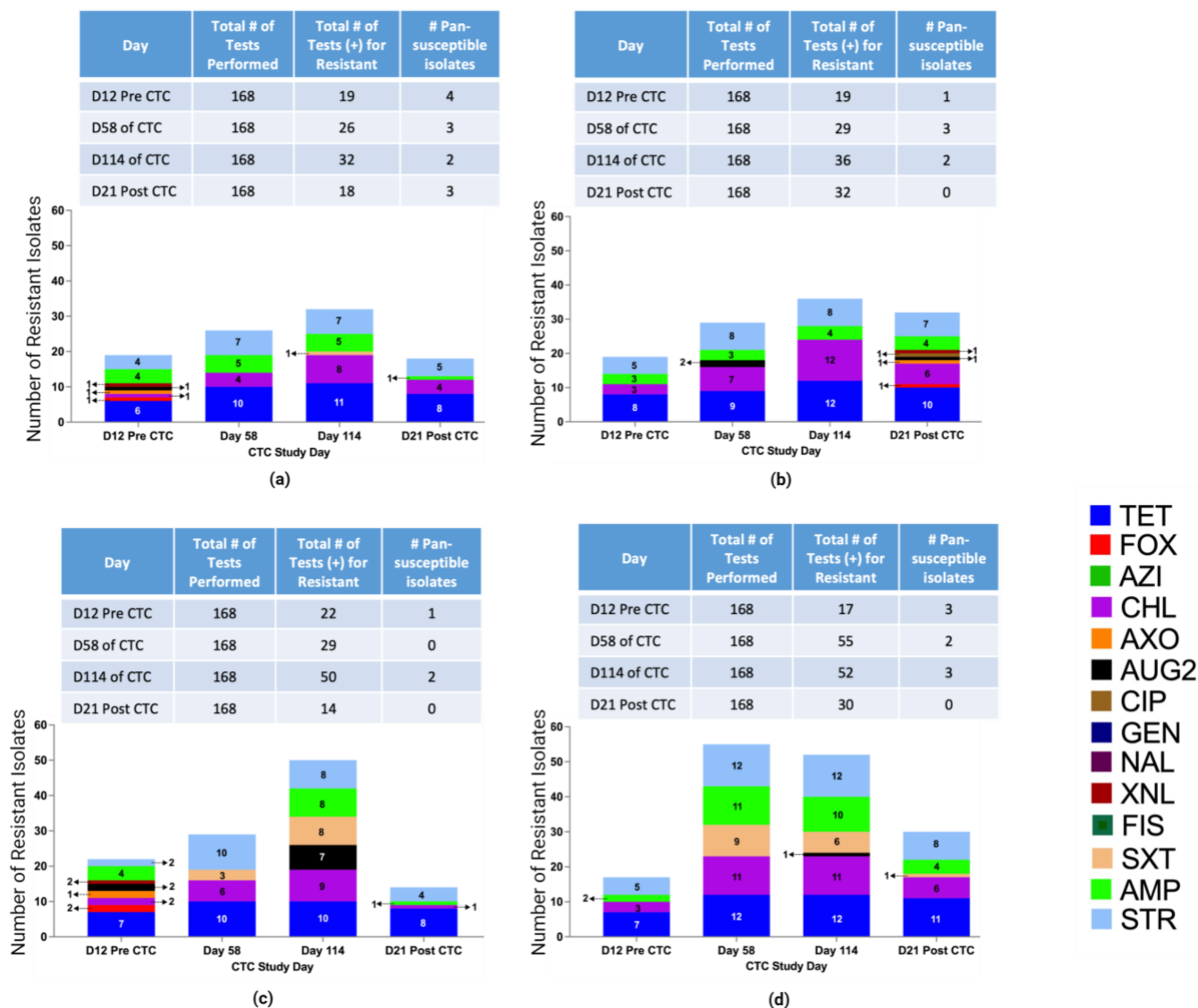


Figure 2.2. Proportions of *E. coli* isolates resistant to medically important antibiotics

The isolates were collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 consecutive days at dose rates of 0, 0.5, 2.0, or 10.0 mg CTC/lb BW/day (*received 10.0 mg CTC for five successive days followed two days off medicated feed). The isolates were assessed for antibiotic susceptibility at 12 days pre-CTC administration, after 58 and 114 days of consecutive CTC administration, and 21 days post-cessation of CTC administration (12 *E. coli* isolates/study group/time point). Antibiotic susceptibility was determined using the 2021 Clinical & Laboratory Standards Institute (CLSI) breakpoints for all antibiotics except ceftiofur, which was categorized using the 2011 NARMS breakpoints. The antibiotics are color coded and include tetracycline (TET), cefoxitin (FOX), azithromycin (AZI), chloramphenicol (CHL), ceftriaxone (AXO), amoxicillin/clavulanic acid 2:1 (AUG2), ciprofloxacin (CIP), gentamycin (GEN), nalidixic acid (NAL), ceftiofur (XNL), sulfisoxazole (FIS), trimethoprim/sulfamethoxazole 1:19 (SXT), ampicillin (AMP) and streptomycin (STR). (a) Proportion of antibiotic-resistant *E. coli* isolates for CTC group 0.0 mg for each study day; (b) Proportion of antibiotic-resistant *E. coli* isolates for CTC group 0.5 mg for each study day; (c) Proportion of antibiotic-resistant *E. coli* isolates for CTC group 2.0 mg for each study day; (d) Proportion of antibiotic-resistant *E. coli* isolates for CTC group 10.0 mg for each study day.

Summary Statistics of Antibiotic Susceptibility for the 0.5 mg CTC Group

Of the 48 isolates from the 0.5 mg CTC group, 40 (83%) were resistant to at least one antibiotic, and 26 (54%) isolates were resistant to three or more antibiotics (**Error! Reference source not found.**). Eight isolates (17%) were pan-susceptible (Figure 2.1. Heatmap of the antibiotic resistance phenotypes of *E. coli* isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days and Table 2-3. Animal IDs from where pan-susceptible *E. coli* was isolated). The only isolates recovered from the same calve were two isolates recovered from the same calve prior to CTC administration and on day 21 post-cessation of administration and two more isolates recovered from the same calve on days 58 of continuous CTC administration and on day 21 post-cessation of administration (Table 2-3. Animal IDs from where pan-susceptible *E. coli* was isolated). Overall, 26 (54%) isolates were MDR. The most common antibiotics the isolates were resistant to were TET (39, 81% of isolates), CHL, and STR (28, 58% isolates each), AMP (14, 29% isolates), and AUG2 (3, 6% isolates) (Figure 2.1. Heatmap of the antibiotic resistance phenotypes of *E. coli* isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days and **Error! Reference source not found.**). Prior to CTC administration, the isolates were resistant to a total number of 19/168 (11.3%) antibiotics. The total number of antibiotics resistances increased to 29/168 (17.3%) and 36/168 (21.4%) on days 58 and 114 of continuous CTC administration, respectively. The number total number of antibiotic resistances decreased to 32/168 (19.0%) on day 21 post-cessation of CTC administration.

Table 2-4. Distributions of minimum inhibitory concentrations of 48 *E. coli* isolates from cattle handfed 0.5 mg/lb BW/day chlortetracycline for 120 consecutive days to a panel of 14 antibiotics

Antibiotic ¹	% Resistant ²	Distribution (%) of <i>E. coli</i> isolates within antibiotic MIC dilution series ³															MIC 95% CI ⁴
		0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	
TET	81.3									16.7	2.1	16.7	22.9	41.7			20.90-27.43
CHL	58.3								0.0	10.4	27.1	4.2	29.2	29.2			18.36-25.47
AUG2	6.3							0.0	4.2	52.1	35.4	2.1	6.3				5.33-9.33
FIS	0.0										16.7	8.3	14.6	2.1	0.0	20.8	125.69-192.97
SXT	0.0				131.3	22.9	0.0	2.1	0.0	0.0							0.13-0.21
AMP	29.2							2.1	2.1	56.3	10.4	0.0	16.7	12.5			8.78-16.18
STR	58.3								0.0	2.1	31.3	8.3	16.7	18.8	22.9		28.58-43.28
NAL	0.0					2.1	2.1	64.6	31.3	0.0	0.0	0.0	0.0				2.27-2.86
FOX	2.1					0.0	0.0	2.1	58.3	31.3	6.3	0.0	2.1				5.11-7.98
XNL	2.1				0.0	0.0	85.4	10.4	2.1	0.0	2.1						0.42-1.06
AZI	0.0				0.0	0.0	0.0	0.0	2.1	79.2	16.7	2.1					4.22-5.53
GEN	0.0					0.0	27.1	68.8	4.2	0.0	0.0	0.0					0.81-0.10
CIP	2.1	89.6	8.3	0.0	2.1	0.0	0.0	0.0	0.0								0.014-0.023
AXO	2.1					91.7	0.0	6.3	0.0	0.0	2.1	0.0	0.0	0.0			0.13-0.79

¹Antibiotics evaluated: TET = tetracycline, CHL = chloramphenicol, AUG2 = amoxicillin/clavulanic acid, FIS = sulfisoxazole, SXT = trimethoprim/sulfamethoxazole, AMP = ampicillin, STR= streptomycin, NAL = nalidixic acid, FOX = ceftiofur, XNL = ceftiofur, AZI = azithromycin, GEN = gentamycin, CIP = ciprofloxacin and AXO = ceftriaxone.

²The percent of 48 total *E. coli* isolates that were resistant to the tested antibiotic.

³Antibiotic (Minimum Inhibitory Concentration) MIC dilution series for evaluated by Sensititre CMV3AGNF plate. The white area indicates the available evaluation range for a given antibiotic and the percentage of *E. coli* isolates at that MIC. Isolates with MIC values outside the range are in the gray areas of the distribution area. Absolute MIC values for twelve isolates from all CTC groups, and all isolated prior to CTC administration were not taken and any MIC values outside the range of the Sensititre plate were assigned the nearest MIC value evaluated by the Sensititre plate. Vertical black lines within an antibiotic dilution series indicate the CLSI or NARMS (for XNL) consensus resistance breakpoint. Amoxicillin/clavulanic acid is shown at clavulanic acid at 1/2X concentration that of amoxicillin. Trimethoprim/sulfamethoxazole is shown at 19X concentration that of trimethoprim.

⁴The MIC 95% confidence interval of the minimum inhibitory concentrations.

Summary Statistics of Antibiotic Susceptibility for the 2.0 mg CTC Group

Thirty nine (81%) isolates from the 2.0 mg CTC group, 39 (81%) were resistant to at least one antibiotic, and 19 (40%) isolates were resistant to three or more antibiotics (Figure 2.1. Heatmap of the antibiotic resistance phenotypes of *E. coli* isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days and **Error! Reference source not found.**). Nine isolates (19%), two recorded from each study days except

on day 21 post cessation of CTC administration where three pan-susceptible isolates were recovered (Figure 2.1. Heatmap of the antibiotic resistance phenotypes of *E. coli* isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days and Table 2-3. Animal IDs from where pan-susceptible *E. coli* was isolated). Two isolates, one recovered prior to CTC administration and the other from day 58 of continuous CTC administration were recovered from the same calve (Table 2-3. Animal IDs from where pan-susceptible *E. coli* was isolated). Similarly, two isolates, one recovered on day 58 of continuous CTC administration and the other from day 114 of continuous CTC administration were recovered from the same calve. And likewise, two isolates, one recovered on day 114 of continuous CTC administration and the other from 21 days post-cessation CTC administration were recovered from the same calve. There were 19 (40%) MDR isolates. Tetracycline (35, 73% of isolates), STR (24, 50% of isolates), CHL (18, 38% of isolates), AMP (13, 27% of isolates) and SXT (11, 23% of isolates) were the most common antibiotics the isolates were resistant to (**Error! Reference source not found.** and **Error! Reference source not found.**). Prior to CTC administration, the total number of antibiotics the isolates were resistant to were 22/168 (13.1%). The total number of antibiotic resistances decreased to 14/168 (8.3%) on day 21 post-cessation of CTC administration.

Table 2-5. Distributions of minimum inhibitory concentrations of 48 *E. coli* isolates from cattle handfed 2.0 mg/lb BW/day chlortetracycline for 120 consecutive days to a panel of 14 antibiotics

Antibiotic ¹	% Resistant ²	Distribution (%) of <i>E. coli</i> isolates within antibiotic MIC dilution series ³															MIC 95% CI ⁴
		0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	
TET	72.9																20.24-27.26
CHL	37.5																13-60-20.57
AUG2	18.8																7.05-13.33
FIS	0.0																99.21-165.46
SXT	22.9																0.54-1.50
AMP	27.1																7.60-15.03
STR	50.0																26.51-42.66
NAL	0.0																2.25-2.79
FOX	4.2																5.71-9.33
XNL	2.1																0.49-1.18
AZI	0.0																4.12-5.05
GEN	0.0																0.85-1.17
CIP	0.0																0.014-0.016
AXO	4.2																0.04-1.50

¹Antibiotics evaluated: TET = tetracycline, CHL = chloramphenicol, AUG2 = amoxicillin/clavulanic acid, FIS = sulfisoxazole, SXT = trimethoprim/sulfamethoxazole, AMP = ampicillin, STR= streptomycin, NAL = nalidixic acid, FOX = ceftiofur, XNL = ceftiofur, AZI = azithromycin, GEN = gentamycin, CIP = ciprofloxacin and AXO = ceftriaxone.

²The percent of 48 total *E. coli* isolates that were resistant to the tested antibiotic.

³Antibiotic (Minimum Inhibitory Concentration) MIC dilution series for evaluated by Sensititre CMV3AGNF plate. The white area indicates the available evaluation range for a given antibiotic and the percentage of *E. coli* isolates at that MIC. Isolates with MIC values outside the range are in the gray areas of the distribution area. Absolute MIC values for four isolates from all CTC groups, and all isolated prior to CTC administration were not taken and any MIC values outside the range of the Sensititre plate were assigned the nearest MIC value evaluated by the Sensititre plate. Vertical black lines within an antibiotic dilution series indicate the CLSI or NARMS (for XNL) consensus resistance breakpoint. Amoxicillin/clavulanic acid is shown at clavulanic acid at 1/2X concentration that of amoxicillin. Trimethoprim/sulfamethoxazole is shown at 19X concentration that of trimethoprim.

⁴The MIC 95% confidence interval of the minimum inhibitory concentrations.

Summary Statistics of Antibiotic Susceptibility for the 10.0 mg CTC Group

Of the total number of isolated from the 10.0 mg CTC group and 45 (94%) were resistant to at least one antibiotic, and 33 (69%) isolates were resistant to three or more antibiotics (Figure 2.1. Heatmap of the antibiotic resistance phenotypes of *E. coli* isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days and **Error!**

Reference source not found.). Three isolates (6%), all recovered prior to CTC administration from three different calves were pan-susceptible (Table 2-3. Animal IDs from where pan-susceptible *E. coli* was isolated). Overall, 33 (69%) isolates were MDR. The most common

antibiotics the isolates were resistant to were TET (42, 88% of isolates), STR (37, 77% of isolates), CHL (31, 65% of isolates), AMP (27, 56% of isolates), and SXT (16, 33% of isolates) (**Error! Reference source not found.** and **Error! Reference source not found.**). The total number of antibiotics the isolates were resistant to prior to CTC administration was 17/168 (10.1%). During CTC administration, the total number of antibiotics the isolates were resistant to increased to 55/168 (32.7%) and 52/168 (31.0%) on days 58 and 114 of continuous CTC administration, respectively. On day 21 post-CTC administration, the total number of antibiotics the isolates were resistant to decreased to 30/168 (17.9%).

Table 2-6. Distributions of minimum inhibitory concentrations of 48 *E. coli* isolates from cattle handfed 10.0 mg/lb BW/day chlortetracycline for 120 consecutive days to a panel of 14 antibiotics

Antibiotic ¹	% Resistant ²	Distribution (%) of <i>E. coli</i> isolates within antibiotic MIC dilution series ³															MIC 95% CI ⁴	
		0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256		
TET	87.5								4.2	8.3	4.2	12.5	70.8				25.60-30.74	
CHL	64.6							0.0	6.3	22.9	6.3	25.0	39.6				20.42-27.08	
AUG2	2.1						0.0	2.1	47.9	41.7	6.3	2.1					5.55-8.38	
FIS	0.0																171.20-227.47	
SXT	33.3			47.9	6.3	4.2	4.2	4.2	4.2	29.2		12.6	8.3	0.0	6.3	16.7	56.3	1.03-2.07
AMP	56.3						2.1	0.0	35.4	4.2	2.1	4.2	52.1				16.11-24.10	
STR	77.1							0.0	0.0	18.8	4.2	12.5	14.6	50.0			40.68-54.32	
NAL	0.0					0.0	0.0	66.7	33.3	0.0	0.0	0.0					2.39-2.94	
FOX	0.0					0.0	0.0	0.0	54.2	41.7	4.2	0.0					5.34-7.00	
XNL	0.0			0.0	2.1	89.6	8.3	0.0	0.0	0.0							0.49-0.58	
AZI	0.0			0.0	0.0	0.0	0.0	0.0	83.3	16.7	0.0						4.23-5.10	
GEN	0.0					0.0	20.8	77.1	2.1	0.0	0.0	0.0					0.84-0.99	
CIP	0.0	95.8	4.2	0.0	0.0	0.0	0.0	0.0	0.0								0.145-0.016	
AXO	0.0					100	0.0	0.0	0.0	0.0	0.0	0.0	0.0				n/a	

¹Antibiotics evaluated: TET = tetracycline, CHL = chloramphenicol, AUG2 = amoxicillin/clavulanic acid, FIS = sulfisoxazole, SXT = trimethoprim/sulfamethoxazole, AMP = ampicillin, STR= streptomycin, NAL = nalidixic acid, FOX = cefoxitin, XNL = ceftiofur, AZI = azithromycin, GEN = gentamycin, CIP = ciprofloxacin and AXO = ceftriaxone.

²The percent of 48 total *E. coli* isolates that were resistant to the tested antibiotic.

³Antibiotic (Minimum Inhibitory Concentration) MIC dilution series for evaluated by Sensititre CMV3AGNF plate. The white area indicates the available evaluation range for a given antibiotic and the percentage of *E. coli* isolates at that MIC. Isolates with MIC values outside the range are in the gray areas of the distribution area. Absolute MIC values for five isolates from all CTC groups, and all isolated prior to CTC administration were not taken and any MIC values outside the range of the Sensititre plate were assigned the nearest MIC value evaluated by the Sensititre plate. Vertical black lines within an antibiotic dilution series indicate the CLSI or NARMS (for XNL) consensus resistance breakpoint. Amoxicillin/clavulanic acid is shown at clavulanic acid at 1/2X concentration that of amoxicillin. Trimethoprim/sulfamethoxazole is shown at 19X concentration that of trimethoprim.

⁴The MIC 95% confidence interval of the minimum inhibitory concentrations.

^{n/a}All AXO MIC values were the same and therefore 95% CI could not be determined.

Effect of Prolonged CTC Administration on *E. coli* Tetracycline Susceptibility

Prior to CTC administration, the mean TET MIC was within the intermediate category for CTC group 0.0 mg (<16.0 µg/mL and >4.0 µg/mL) and in the resistant breakpoint for the rest of the CTC groups ≥16.0 µg/mL (Figure 2.3. Comparison of tetracycline MIC least mean square values from *E. coli* isolates collected from groups of steers administered different chlortetracycline dosages for 120 days and **Error! Reference source not found.**). There was a significant CTC dose effect on *E. coli* TET MIC least square mean value (p-value = 0.089); however, there was no significant study day effect (p-value 0.106). Although the CTC dose effect on TET MIC least square mean values was not significant on days 58 (p-value = 0.270) and 114 (p-value = 0.630) of continuous CTC administration, there was a subtle increase in TET MIC least square mean values in all study groups, with MIC least square mean values in the 10.0 mg group reaching 32±6.6 µg/mL (the highest possible Sensititre assay dilution, 32.0 µg/mL) (Figure 2.4. Comparison of *E. coli* antibiotic MIC least square mean values from isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days (a), first panel from the top). The MIC least square mean values between groups had a downward trend on day 21 post-cessation of CTC administration; however, the CTC dose effect on MIC least square mean values was not significant (p-value = 0.266). Isolates of *E. coli* within the intermediate susceptible category (MIC values greater than 4.0 µg/mL but less than 16.0 µg/mL) were only observed prior to CTC and 21 days post-cessation of CTC administration (**Error! Reference source not found.**).

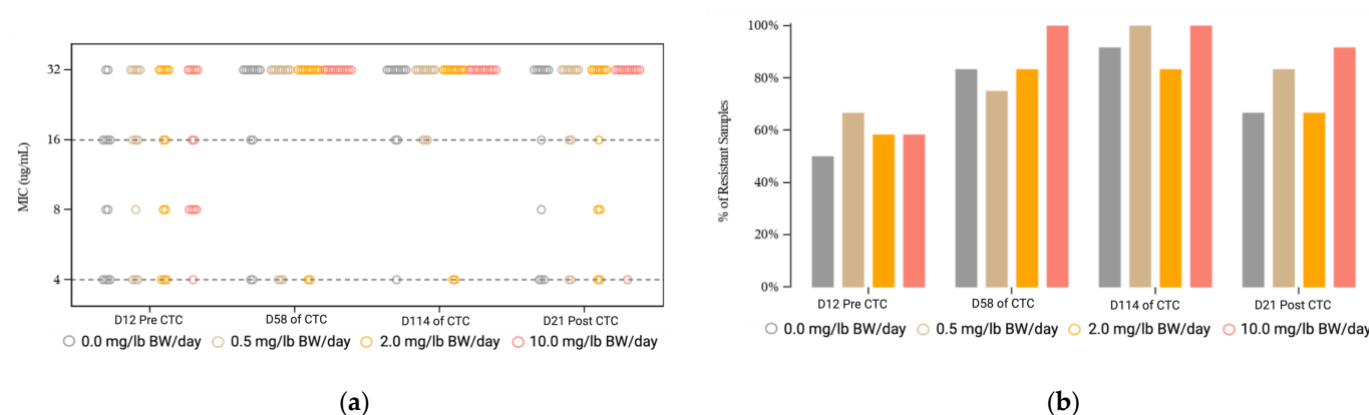
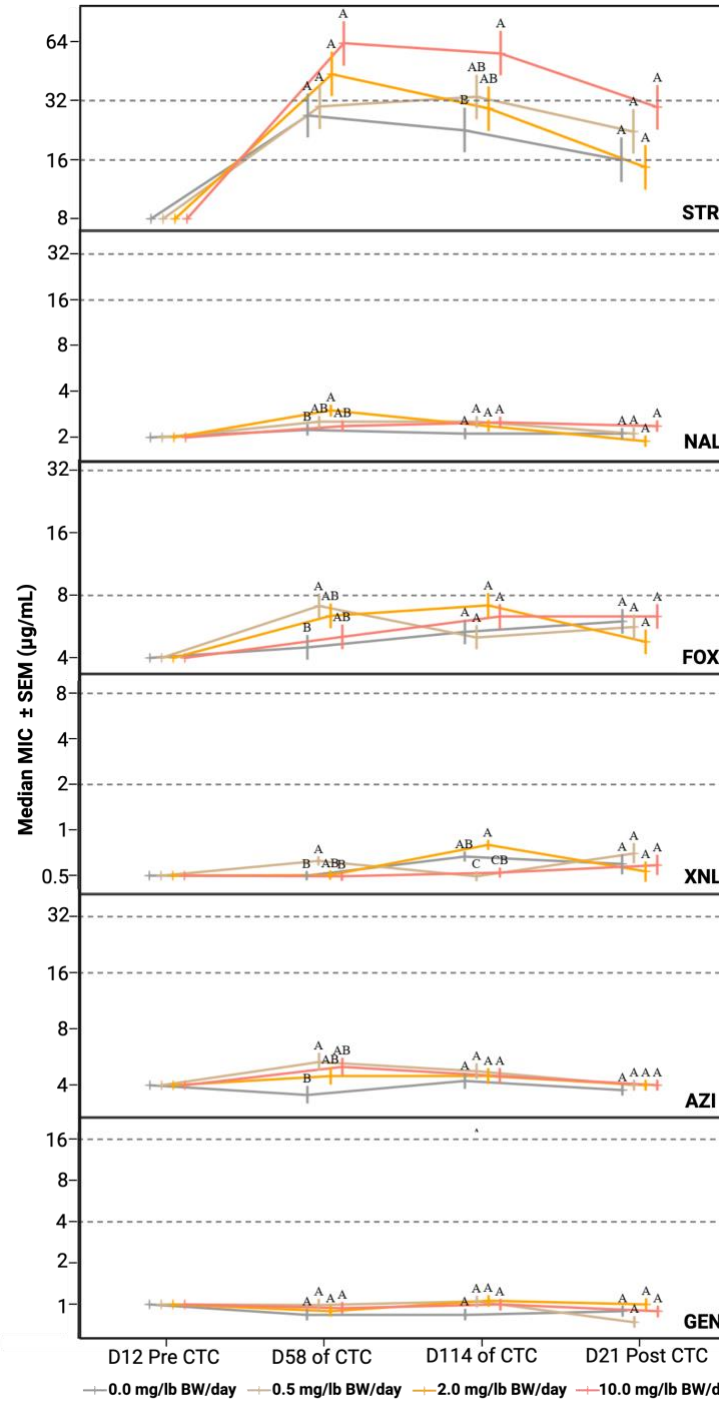
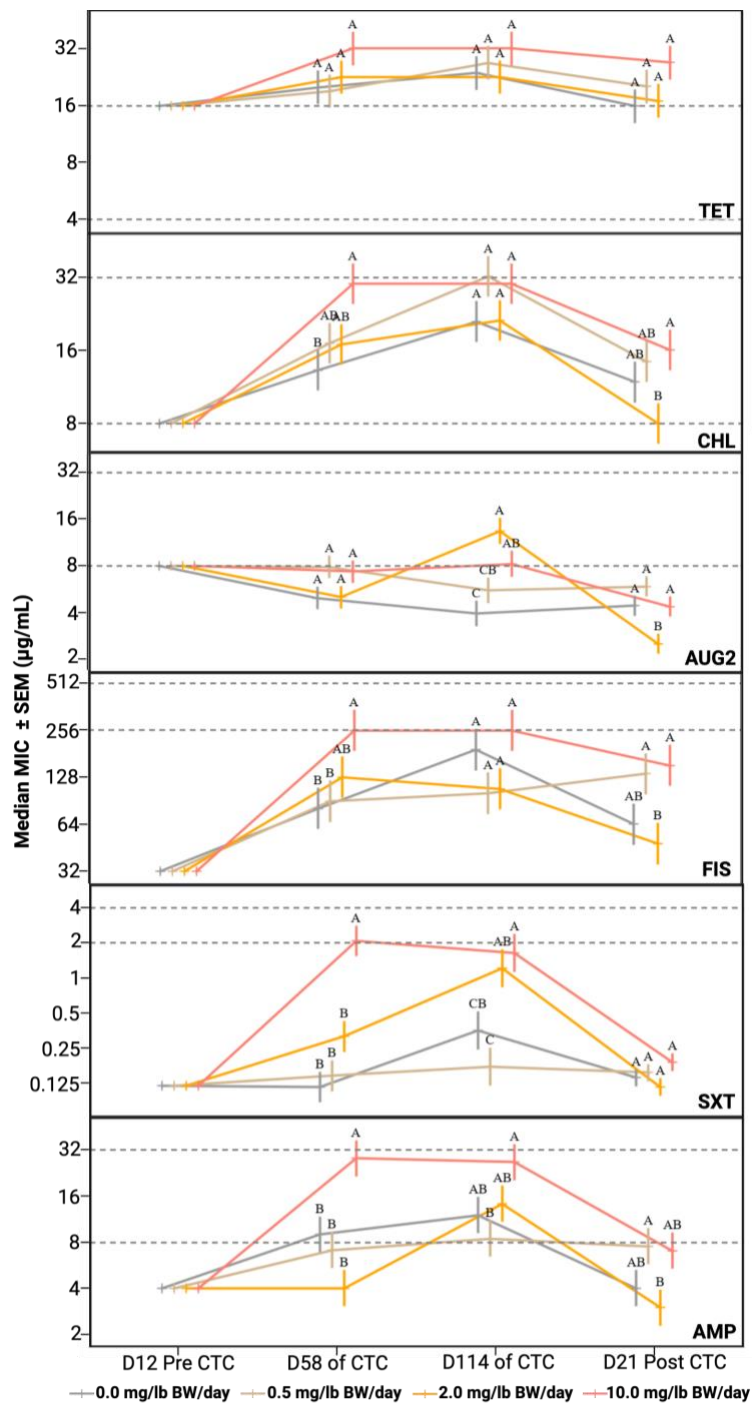


Figure 2.3. Comparison of tetracycline MIC least mean square values from *E. coli* isolates collected from groups of steers administered different chlortetracycline dosages for 120 days

Isolates of *E. coli* from groups of steers provided CTC for 120 consecutive days at dose rates of 0, 0.5, 2.0, or 10.0 mg CTC/lb BW/day (*received 10.0 mg CTC for five consecutive days followed by two days off medicated feed) and assessed for susceptibility to tetracycline (TET). (a) Presented are the TET minimum inhibitory concentration (MIC) least mean square values for *E. coli* isolates collected at 12 days pre-CTC administration, after 58 and 114 days of consecutive CTC administration, and 21 days post-cessation of CTC administration (12 *E. coli* isolates/study group/time point). Susceptibility of *E. coli* to TET was determined using the 2021 Clinical & Laboratory Standards Institute (CLSI) breakpoints. The lower dashed line represents the CLSI TET susceptibility cut-off and isolates with MIC values at or below the dashed line are considered susceptible to TET. The upper dashed line represents the CLSI TET resistance cut-off and isolates with MIC values at or above the cut-off value are considered resistant to TET. Isolates with MIC values between the lower and upper dashed lines are in the intermediate susceptible category; (b) Percentage of TET-resistant isolates (MIC > 16 $\mu\text{g/mL}$) before, during, and after CTC administration.

Most isolates were already TET-resistant prior to CTC administration (**Error! Reference source not found.**, Figure 2.3. Comparison of tetracycline MIC least mean square values from *E. coli* isolates collected from groups of steers administered different chlortetracycline dosages for 120 days, and **Error! Reference source not found.**). After 58 and 114 days of consecutive CTC administration, an overall increase in TET-resistant *E. coli* was observed for all study groups (including the study group that did not receive CTC), with 100% (12/12) of *E. coli* isolates from the 10.0 mg group on day 58 and from the 0.5 mg and 10.0 mg CTC groups on day

114. On day 21, after cessation of CTC administration, the percentage of TET-resistant isolates slightly decreased for both groups.



(a)

(b)

Figure 2.4. Comparison of *E. coli* antibiotic MIC least square mean values from isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days

Isolates of *E. coli* from groups of steers provided CTC for 120 consecutive days at dose rates of 0, 0.5, 2.0, or 10.0 mg CTC/lb BW/day (*received 10.0 mg CTC for five consecutive days followed by two days off medicated feed). The isolates were assessed for antibiotic susceptibility at 12 days pre-CTC administration (D12 Pre CTC, after 58 and 114 days of consecutive CTC administration, and 21 days post-cessation of CTC administration (12 *E. coli* isolates/study group/time point). Antibiotic susceptibility was determined using the 2021 Clinical & Laboratory Standards Institute (CLSI) breakpoints for all antibiotics except ceftiofur, which was categorized using the 2011 NARMS breakpoints: **(a)** MIC least square mean values for tetracycline (TET), chloramphenicol (CHL), amoxicillin/clavulanic acid 2:1 (AUG2) (only the MIC values for amoxicillin are displayed) and sulfisoxazole (FIS), trimethoprim/sulfamethoxazole 1:19 (SXT) (only the MIC values for trimethoprim are displayed) and ampicillin (AMP); **(b)** MIC least square mean values for streptomycin (STR), nalidixic acid (NAL), ceftiofur (FOX), ceftiofur (XNL), azithromycin (AZI) and gentamicin (GEN). Study groups with different letters are significantly different from each other within a given time point. In each graph, the lower dashed line represents the CLSI or NARMS antibiotic susceptibility cut-off and isolates with MIC values at or below the dashed line are considered susceptible to that antibiotic. The upper dashed line represents the CLSI antibiotic resistance cut-off value, and MIC values at or above that line are considered resistant to that antibiotic. Isolates with MIC values between the lower and upper dashed lines are in the intermediate susceptible category.

Table 2-7. Antibiotic minimum inhibitory concentrations of *E. coli* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days

CTC Study Groups	D12 Pre CTC		D58		D114		D21 Post CTC	
	Mean	% Resistant Isolates (Number)	LSMean (SEM) or Mean	% Resistant Isolates (Number)	LSMean (SEM) or Mean	% Resistant Isolates (Number)	LSMean (SEM) or Mean	% Resistant Isolates (Number)
TET MIC (ug/mL)								
0.0 mg	12.0	50% (6)	20.0±4.1	83% (10)	23.8±4.9	92% (11)	15.9±3.3	67% (8)
0.5 mg	16.0	67% (8)	19.1±3.9	75% (9)	27.0±5.5	100% (12)	20.2±4.1	83% (10)
2.0 mg	16.0	58% (7)	22.6±4.6	83% (10)	22.6±4.6	83% (10)	17.0±3.5	67% (8)
10.0 mg	16.0	58% (7)	32.1±6.6	100% (12)	32.1±6.6	100% (12)	27.0±5.5	92% (11)
CHL MIC (ug/mL)								
0.0 mg	8.0	8% (1)	13.3±2.6	33% (4)	21.1±4.1	67% (8)	11.8±2.3	33% (4)
0.5 mg	8.0	25% (3)	17.2±3.3	58% (7)	32.4±6.3	100% (12)	14.4±2.8	50% (6)
2.0 mg	8.0	17% (2)	16.9±3.3	50% (6)	21.3±4.1	75% (9)	8.0±1.5	8% (1)
10.0 mg	8.0	25% (3)	30.3±5.8	92% (11)	30.3±5.8	92% (11)	16.0±3.1	50% (6)
AUG2 MIC (ug/mL)								
0.0 mg	8.0	8% (1)	5.0±0.8	0	3.9±0.8	0	4.4±0.7	0
0.5 mg	4.0	0	7.9±1.3	17% (2)	5.6±1.1	0	5.9±0.9	8% (1)
2.0 mg	8.0	17% (2)	5.0±0.9	0	13.4±2.6	58% (7)	2.5±0.4	0
10.0 mg	4.0	0	7.4±1.2	0	8.3±1.6	8% (1)	4.4±0.7	0
FIS MIC (ug/mL)								
0.0 mg	24.0	0	81±25	0	192±58	0	64±20	0
0.5 mg	32.0	0	90±27	0	101±31	0	135±41	0
2.0 mg	16.0	0	128±39	0	108±33	0	48±15	0
10.0 mg	32.0	0	256±78	0	256±78	0	152±46	0
SXT MIC (ug/mL)								
0.0 mg	0.12	0	0.12±0.04	0	0.36±0.13	8% (1)	0.14±0.02	0
0.5 mg	0.12	0	0.15±0.04	0	0.17±0.07	0	0.15±0.03	0
2.0 mg	0.12	0	0.32±0.10	25% (3)	1.21±0.45	67% (8)	0.12±0.02	0
10.0 mg	0.12	0	2.08±0.64	75% (9)	1.65±0.62	50% (6)	0.19±0.03	8% (1)

CTC Study Groups	D12 Pre CTC		D58		D114		D21 Post CTC	
	Mean	% Resistant Isolates (Number)	LSMean (SEM) or Mean	% Resistant Isolates (Number)	LSMean (SEM) or Mean	% Resistant Isolates (Number)	LSMean (SEM) or Mean	% Resistant Isolates (Number)
AMP MIC (ug/mL)								
0.0 mg	4.0	33% (4)	9.0±2.4	42% (5)	12.0±3.3	42% (5)	4.0±1.1	8% (1)
0.5 mg	4.0	25% (3)	7.1±1.9	25% (3)	8.5±2.3	33% (4)	7.6±2.0	33% (4)
2.0 mg	4.0	33% (4)	4.0±1.1	0	14.3±3.9	67% (8)	3.0±0.8	8% (1)
10.0 mg	4.0	17% (2)	28.2±7.7	92% (11)	26.7±7.2	83% (10)	7.1±1.9	33% (4)
STR MIC (ug/mL)								
0.0 mg	8.0	33% (4)	27.0±7.0	58% (7)	22.7±5.9	58% (7)	16.0±4.2	42% (5)
0.5 mg	8.0	42% (5)	29.9±7.8	67% (8)	33.5±8.8	67% (8)	22.4±5.8	58% (7)
2.0 mg	8.0	17% (2)	43.9±11.5	83% (10)	29.3±7.7	67% (8)	14.6±3.8	33% (4)
10.0 mg	12.0	42% (5)	62.9±16.5	100% (12)	56.1±14.7	100% (12)	29.7±7.8	67% (8)
NAL MIC (ug/mL)								
0.0 mg	2.0	0	2.25±0.20	0	2.12±0.19	0	2.12±0.19	0
0.5 mg	2.0	0	2.52±0.22	0	2.52±0.22	0	2.12±0.19	0
2.0 mg	2.0	0	3.00±0.26	0	2.38±0.21	0	1.89±0.17	0
10.0 mg	3.0	0	2.37±0.21	0	2.51±0.22	0	2.37±0.21	0
FOX MIC (ug/mL)								
0.0 mg	4.0	8% (1)	4.5±0.6	0	5.3±0.7	0	6.0±0.8	0
0.5 mg	4.0	0	7.1±1.0	0	5.0±0.7	0	5.6±0.8	8% (1)
2.0 mg	4.0	17% (2)	6.4±0.9	0	7.2±1.0	0	4.8±0.7	0
10.0 mg	4.0	0	5.0±0.7	0	6.3±0.9	0	6.3±0.9	0
XNL MIC (ug/mL)								
0.0 mg	0.5	8% (1)	0.50±0.03	0	0.67±0.05	0	0.60±0.09	0
0.5 mg	0.5	0	0.63±0.04	0	0.50±0.04	0	0.70±0.11	8% (1)
2.0 mg	0.5	8% (1)	0.50±0.03	0	0.80±0.06	0	0.53±0.08	0
10.0 mg	0.5	0	0.50±0.03	0	0.53±0.04	0	0.59±0.09	0

CTC Study Groups	D12 Pre CTC		D58		D114		D21 Post CTC	
	Mean	% Resistant Isolates (Number)	LSMean (SEM) or Mean	% Resistant Isolates (Number)	LSMean (SEM) or Mean	% Resistant Isolates (Number)	LSMean (SEM) or Mean	% Resistant Isolates (Number)
AZI MIC (ug/mL)								
0.0 mg	4.0	0	3.6±0.4	0	4.2±0.4	0	3.8±0.3	0
0.5 mg	4.0	0	5.3±0.6	0	4.8±0.5	0	4.0±0.3	0
2.0 mg	4.0	0	4.5±0.5	0	4.5±0.4	0	4.0±0.3	0
10.0 mg	4.0	0	5.0±0.5	0	4.5±0.4	0	4.0±0.3	0
GEN MIC (ug/mL)								
0.0 mg	1.0	0	0.84±0.08	0	0.84±0.08	0	0.89±0.09	0
0.5 mg	0.5	0	0.99±0.10	0	1.05±0.10	0	0.75±0.07	0
2.0 mg	1.0	0	0.89±0.09	0	1.06±0.10	0	1.00±0.10	0
10.0 mg	0.75	0	0.94±0.09	0	1.00±0.10	0	0.89±0.09	0
CIP MIC (ug/mL)								
0.0 mg	0.015	0	0.015	0	0.015	0	0.015	0
0.5 mg	0.015	0	0.015	0	0.015	0	0.015	8% (1)
2.0 mg	0.015	0	0.015	0	0.015	0	0.015	0
10.0 mg	0.015	0	0.015	0	0.015	0	0.015	0
AXO MIC (ug/mL)								
0.0 mg	0.25	8% (1)	0.25	0	0.25	0	0.25	0
0.5 mg	0.25	0	0.25	0	0.25	0	0.25	8% (1)
2.0 mg	0.25	17% (2)	0.25	0	0.375	0	0.25	0
10.0 mg	0.25	0	0.25	0	0.25	0	0.25	0

Isolates of *E. coli* from groups of steers provided CTC for 120 consecutive days at dose rates of 0, 0.5, 2.0, or 10.0 mg CTC/lb BW/day (*received 10.0 mg CTC for five consecutive days followed two days off medicated feed) and assessed for susceptibility to t14 antibiotics on days: 12 days prior to CTC administration, 58 and 114 days of continuous CTC administration, and 21 days post cessation of CTC administration.

D12 Pre CTC was used as a covariate, and CIP and AXO MIC values were not statistically analyzed as they lacked variation; therefore, their least square mean values were not calculated.

The susceptibility of antibiotics was determined using the 2021 Clinical & Laboratory Standards Institute (CLSI) breakpoints for all antibiotics except ceftiofur, which was categorized using the 2011 NARMS breakpoints.

Effect of Prolonged CTC Administration on the Susceptibility of *E. coli* to Non-TET Antibiotics

Although not administered leading up to or during this study, the susceptibility profile of *E. coli* isolates were determined across a broad spectrum of non-TET antibiotics. The MIC least square mean values for CHL, AUG2, FIS, SXT, AMP, AZI, and STR varied significantly between CTC study groups. There was a significant CTC dose effect on MIC least square mean values for AZI, CHL, AUG2, FIS, SXT, AMP, and STR over the study period and a significant study day effect on MIC least square mean values for AZI, CHL, AUG2, NAL, XNL, FIS, SXT, AMP and STR. Detailed descriptions of non-TET antibiotic MIC least square mean value differences between study groups and over the CTC administration period are presented below.

Chloramphenicol (CHL). There was a significant CTC dose effect (p-value = 0.012) and study day effect (p-value <0.001) on CHL MIC least square mean values over the study period. The MIC least square mean values for CHL prior to, during, and after continuous CTC administration for all groups were within the susceptible category (≤ 8.0 $\mu\text{g/mL}$) (Table 2-7. Antibiotic minimum inhibitory concentrations of *E. coli* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days). On day 58 of continuous CTC administration, the CHL MIC least square means increased into the intermediate susceptible category for all groups (Figure 2.4. Comparison of *E. coli* antibiotic MIC least square mean values from isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days (a), second

panel from the top). On that day, the CHL MIC least square means for the 10.0 mg CTC group was significantly higher than the 0.0 mg CTC group (p-value 0.017) and almost reached the CHL resistant breakpoint value ($\geq 32.0 \mu\text{g/mL}$). On day 114 of continuous CTC administration, the CHL MIC least square means for the 0.0, 0.5, and 2.0 mg CTC groups continued to increase and were all statistically similar (p-value = 0.254). On day 21, post-cessation of CTC administration, the CHL MIC least square mean values for all groups had a downward trend. The percentage of CHL-resistant isolates per group increased from below 50% resistant isolates per group prior to CTC administration to at least 50% during the CTC administration period for CTC groups 0.5-, 2.0, and 10 mg (**Error! Reference source not found.** and Table 2-7. Antibiotic minimum inhibitory concentrations of *E. coli* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days). On day 21 post CTC administration, the number of resistant isolates decreased per group to 33% (4/12), 50% (6/12), 8% (1/12), and 50% (6/12) for the same groups, respectively.

Amoxicillin/clavulanic acid (AUG2). The CTC dose and study day effects on AUG2 MIC least square mean values throughout the study were significant (p-value = 0.034 and 0.001, respectively). The AUG2 mean MIC values prior to CTC administration for all groups were in the susceptible category ($\leq 8.0/4.0 \mu\text{g/mL}$) (Table 2-7. Antibiotic minimum inhibitory concentrations of *E. coli* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days). Although an upward trend in the AUG2 MIC least square mean values for all groups was observed on day 58 of continuous CTC administration, they remained statistically similar (p-value = 0.120) (Figure 2.4. Comparison of *E. coli* antibiotic MIC least square mean values from isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days (a), third panel from the

top). On day 114 of continuous CTC administration, the MIC least square mean values for the 2.0 and 10.0 mg CTC groups had an upward trend. During that time, the 2.0 mg CTC group MIC least square mean value was statistically greater than that of the 0.0 and 0.5 mg CTC group AUG2 MIC values (p -values $\leq$ to 0.012) and had crossed the susceptible breakpoint value into the intermediate susceptible category, while the 10.0 CTC mg group AUG2 MIC least square mean value was statistically greater than that of the 0.0 mg CTC group (p -value = 0.042). On day 21 post-cessation of CTC administration, the AUG2 MIC least square mean values for all groups decreased with that of the 2.0 mg CTC group returning to the intermediate susceptible category and statistically lower than the rest of the groups (p -values less than or equal to 0.056). The percent of AUG2-resistant isolates throughout the study only varied slightly: 8.3% (1/12) to 16.7% (2/12) except on day 58 of continuous CTC administration, where the percent of AUG2-resistant isolates was 58.3% (7/12) for CTC group 2.0 mg (**Error! Reference source not found.** and Table 2-7. Antibiotic minimum inhibitory concentrations of *E. coli* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days).

Sulfisoxazole (FIS). There was a significant CTC dose effect (p -value = 0.013) and study day effect (p -value = 0.028) on FIS MIC least square mean values throughout the study period. The MIC least square mean values for all groups prior to CTC administration were less than or equal to 16.0 $\mu\text{g/mL}$ (Table 2-7. Antibiotic minimum inhibitory concentrations of *E. coli* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days). On day 58 of continuous CTC administration, the FIS MIC least square mean values for all groups had increased in a dose-dependent manner, with that of the 10.0 mg CTC group being significantly higher than that of the 0.0 mg (p -value = 0.043) and

0.5 mg (p-value = 0.079) CTC groups, respectively (Figure 2.4. Comparison of *E. coli* antibiotic MIC least square mean values from isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days (a), fourth panel from the top). At this time, the 10.0 mg CTC group reached the susceptible breakpoint value ($\geq 256.0 \mu\text{g/mL}$), the highest possible Sensititre assay dilution for the plate used. On day 114 of continuous CTC administration, there was a significant CTC dose effect on FIS MIC least square mean values (p-value 0.091), and an upward trend in MIC least square mean values for groups 0.0, 0.5 and 2.0 mg was observed. However, pairwise comparisons did not find differences between MIC least square mean values between groups to be statistically different (p-values between 0.143 and 0.999). On day 21 post cessation of CTC administration, the FIS MIC least square mean values for the 0.0, 2.0, and 10.0 mg CTC groups decreased. There were no FIS-resistant isolates prior to, during, or after the cessation of CTC administration (**Error! Reference source not found.** and Table 2-7. Antibiotic minimum inhibitory concentrations of *E. coli* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days).

Trimethoprim/sulfamethoxazole (SXT). There was a significant CTC dose effect (p-value < 0.001) as well as a study day effect (p-value < 0.001) on SXT MIC least square mean values during the study. On days 58 of continuous CTC administration, the MIC least square mean values 0.5, 2.0, and 10.0 mg study groups increased in a dose-dependent manner, with that for the 10.0 mg group being significantly higher than the rest of the groups (p-values < 0.001) and reached the susceptible breakpoint value ($\leq 2.0 \mu\text{g/mL}$) (Figure 2.4. Comparison of *E. coli* antibiotic MIC least square mean values from isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days (a), fifth panel from the

top). On day 114 of continuous CTC administration, the SXT MIC least square mean values for 0.0, 0.5, and 2.0 mg CTC groups continued to increase, and for the 2.0 mg group) was statistically greater than that of the 0.5 mg CTC group (p-value = 0.003) while that of the 10.0 mg CTC group was statistically higher than that of the 0.0 and 0.5 mg CTC groups (p-values less than or equal to 0.029). Although the CTC dose effect on day 21 post cessation of CTC administration was not significant (p-value= 0.292), SXT MIC least square mean values for all study groups had a downward trend. There were no SXT-resistant isolates in any group prior to CTC administration (**Error! Reference source not found.** and Table 2-7. Antibiotic minimum inhibitory concentrations of *E. coli* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days). The number of SXT-resistant isolates increased during the CTC administration period for CTC groups 2.0 and 10.0 mg, while only the 10 mg group had a resistant isolate on day 21 post CTC administration.

Ampicillin (AMP). Significant CTC dose and study day effects (p-values <0.001) occurred during the study period. Prior to CTC administration, all isolates were in the susceptible category for AMP (≤ 8.0 $\mu\text{g/mL}$) (Table 2-7. Antibiotic minimum inhibitory concentrations of *E. coli* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days). On day 58 of CTC administration, the MIC least square mean values increased for all groups, except for the 2.0 mg group and that for the 10.0 mg group was significantly higher than the rest of the groups (p-values between <0.001 and 0.019) and almost reached the resistant breakpoint value (≥ 32.0 $\mu\text{g/mL}$) (Figure 2.4. Comparison of *E. coli* antibiotic MIC least square mean values from isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days (a), bottom panel). On day 114 of continuous CTC administration, the MIC least square mean values for groups 0.0 and 0.5

mg had increased and were now also in the intermediate susceptible category. On that day, the MIC least square mean values for the 10.0 mg group were only statistically higher than that of the 0.5 mg group (p-value = 0.018). On day 21 post-cessation of continuous CTC administration, the AMP MIC least square mean values for all groups decreased back to below the susceptible breakpoint value. During the CTC administration, the percent of AMP-resistant isolates increased for CTC groups 0.0 and 10.0 mg on day 58, and for all CTC groups on day 114 of continuous CTC administration (**Error! Reference source not found.** and Table 2-7. Antibiotic minimum inhibitory concentrations of *E. coli* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days). On day 21 p, post-CTC administration, the percent of resistant isolates for CTC 0.0, 2.0, and 0.0 mg CTC groups decreased.

Streptomycin (STR). There was a significant CTC dose effect (p-value = 0.029) and study day effect (p-value <0.001) on STR MIC least square mean over the study period. The STR MIC least square mean values for all CTC groups were in the susceptible category (≤ 16 $\mu\text{g/mL}$) (Table 2-7. Antibiotic minimum inhibitory concentrations of *E. coli* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days). On day 58 of continuous CTC administration, there was a significant CTC dose effect (p-value = 0.092) on the MIC least square mean values for all groups, which increased in a dose-dependent manner (Figure 2.4. Comparison of *E. coli* antibiotic MIC least square mean values from isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days(b), first panel from the top). At this time point, the MIC values moved into the intermediate susceptible category (>16 $\mu\text{g/mL}$ and < 32 $\mu\text{g/mL}$) for CTC groups 0.0 mg and 0.5 mg, and into the resistant category ($\text{MIC} \geq 32$ $\mu\text{g/mL}$) for CTC

groups 2.0 mg and 10.0 mg. However, pairwise comparisons found MIC values for all groups to be statistically similar p-values (p-values between 0.107 and 0.992). On day 114 days of continuous CTC administration, the MIC least square mean values for the 0.5 mg CTC group further increased into the resistant category. Although the CTC group effect on MIC values was not significant on that day (p-value = 0.101), the MIC least square mean value for the 10.0 mg CTC group was statistically greater than that of the 0.0 mg CTC group (p-value = 0.075). On day 21 post-cessation of CTC administration, all group MIC values had a downward trend and were statistically similar p-value of 0.212. At this point, the STR MIC least square mean values decreased into the intermediate susceptible category for the 0.5 mg and 10.0 mg groups and into the susceptible category for the 0.0 mg and 2.0 mg groups. The percent of STR-resistant isolates for each group increased during the CTC administration period with all isolates from the CTC group 10.0 being STR-resistant (**Error! Reference source not found.** and Table 2-7. Antibiotic minimum inhibitory concentrations of *E. coli* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days). The percent of resistant isolates for all groups decreased on day 21 post-cessation CTC administration.

Nalidixic acid (NAL). There was no significant CTC dose effect on NAL MIC least square mean values for the overall study (p-value = 0.500). However, there was a significant study day effect (p-value = 0.020). On day 58 of continuous CTC administration, the NAL MIC least square mean values for all groups were similar to the baseline values. Although the CTC effect for that day was not significant (p-value = 0.110), pairwise comparisons for that day revealed the MIC least square means for the 2.0 mg CTC group was statically greater than the 0.0 mg CTC group (p-value = 0.097) (Figure 2.4. Comparison of *E. coli* antibiotic MIC least

square mean values from isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days (b), second panel from the top). On day 114 of continuous CTC administration and day 21 post-cessation of CTC administration, the CTC dose effect on NAL MIC least square mean values was not significant (p-value = 0.462 and 0.340, respectively). The NAL MIC least square mean values for all CTC groups were within the susceptible category (≤ 16.0 $\mu\text{g/mL}$) for all CTC groups (Table 2-7. Antibiotic minimum inhibitory concentrations of *E. coli* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days). There were no NAL-resistant isolates prior to, during, or after the cessation of CTC administration period (Table 2-7. Antibiotic minimum inhibitory concentrations of *E. coli* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days).

Cefoxitin (FOX). There was no significant CTC dose effect (p-value = 0.603) or study day effect (p-value = 0.875) on FOX MIC values for the overall study. On day 58 of continuous CTC administration, the FOX MIC least square mean values slightly increased for all groups, with the MIC least square mean value for the 0.5 mg CTC group being higher than the rest of the groups but only statically higher than the 0.0 mg CTC group (p-value = 0.089) (Figure 2.4. Comparison of *E. coli* antibiotic MIC least square mean values from isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days (b), third panel from the top). On day 114 days continuous CTC administration, the FOX MIC least square mean value for all groups had a subtle increase however, they remained statistically similar to each other (p-value = 0.250). On day 21, post-cessation of CTC administration, the MIC least square mean values for all groups had only subtle changes and the CTC dose effect on was not

significant (p-value = 0.505). The MIC values for all groups remained within the susceptible category (≤ 8.0 $\mu\text{g/mL}$) throughout the study. Prior to CTC administration, there were only one (8.3%) and two (16.7%) FOX-resistant isolates in CTC groups 0.0 mg and 2.0 mg, respectively (**Error! Reference source not found.**). There were no FOX-resistant isolates throughout the rest of the study except on day 21 post cessation of CTC administration; CTC group 0.5 mg had one (8.3%) FOX-resistant isolate.

Ceftiofur (XNL). There was no significant CTC dose effect on XNL MIC values (p-value = 0.500); however, there was a significant study day effect (p-value = 0.025). There was a slight MIC least square mean value increase for some CTC groups during the CTC administration period (Table 2-7. Antibiotic minimum inhibitory concentrations of *E. coli* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days): On day 58 of continuous CTC administration, the XNL MIC least square mean value for the 0.5 mg CTC group had a slight increase and was statistically greater than the 0.0 mg CTC group (p-value = 0.088) and the 10.0 mg CTC group (p-value = 0.066) (Figure 2.4. Comparison of *E. coli* antibiotic MIC least square mean values from isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days (b), fourth panel from the top). On day 114 of continuous CTC administration, the XNL MIC least square mean value slightly increased for the 0.0 mg and the 2.0 mg groups, with that of the 2.0 mg CTC group being statistically higher than the 0.5 mg CTC (p-value < 0.001) and the 10.0 mg (p-value = 0.002) CTC groups. On day 21, post-cessation of CTC administration, the MIC least square mean value for all groups had slight MIC value changes and became statistically similar (p-value 0.653). The MIC values for all groups remained within the susceptible category throughout the study. Prior to CTC administration, there were only one (8.3%) XNL-resistant isolates in both

CTC groups 0.0 mg and 2.0 mg (**Error! Reference source not found.** and Table 2-7. Antibiotic minimum inhibitory concentrations of *E. coli* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days). There were no XNL-resistant isolates throughout the rest of the study except on day 21 post cessation of CTC administration were CTC group 0.5 mg had one (8.3%) XNL-resistant isolate.

Azithromycin (AZI). There was a significant CTC dose effect (p-value = 0.066) and a significant study day effect (p-value = 0.041) on AZI MIC least square mean values for the overall study. There were only slight AZI MIC changes throughout the study period (Table 2-7. Antibiotic minimum inhibitory concentrations of *E. coli* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days): On day 58 of continuous CTC administration, the MIC least square mean values for 0.5 mg, 2.0 mg and 10.0 mg groups slightly increased, and that for the 0.5 mg CTC group was statistically higher than that of the 0.0 mg CTC group (p-value = 0.052) (Figure 2.4. Comparison of *E. coli* antibiotic MIC least square mean values from isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days (b), fifth panel from the top). On day 144 of continuous CTC administration, the MIC least square mean values had slight changes; however, they were statistically similar among all CTC groups (p-value = 0.874). Similarly, on day 21 post-cessation of CTC administration, the MIC least square mean values changed slightly but remained statistically similar between the groups (p-value = 0.907). The MIC least square mean values remained within the susceptible breakpoint classification (≤ 16.0 $\mu\text{g/mL}$). There were no AZI-resistant isolates before, during, or after cessation of CTC administration (**Error! Reference source not found.** and **Error! Reference source not found.**).

Gentamicin (GEN). There was no significant CTC dose effect (p-value = 0.463) or study day effect (p-value = 0.196) on GEN MIC least square mean values for the overall study. The MIC least square mean values had subtle changes throughout the study (Table 2-7. Antibiotic minimum inhibitory concentrations of *E. coli* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days). In addition, they were statistically similar between groups (p-values = 0.667, 0.308, and 0.197 for days 58 and 114 of continuous CTC administration and 21 days post-cessation of CTC administration, respectively) (Figure 2.4. Comparison of *E. coli* antibiotic MIC least square mean values from isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days (b), bottom panel). The MIC values for all groups remained within the susceptible category (≤ 4.0 $\mu\text{g/mL}$) throughout the study. There were no resistant isolates before CTC administration, during CTC administration, or after cessation of CTC administration (**Error! Reference source not found.** and Table 2-7. Antibiotic minimum inhibitory concentrations of *E. coli* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days).

Ciprofloxacin (CIP). The mean CIP MIC values for all CTC groups were within the susceptible category (≤ 0.06 $\mu\text{g/mL}$), and this remained unchanged throughout the study period (Table 2-7. Antibiotic minimum inhibitory concentrations of *E. coli* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days). As a result of this lack of variation, CIP MIC data could not be statistically analyzed. There was only CIP-resistant isolate throughout the study except for one (8.3%) isolate for CTC group 0.5 mg (**Error! Reference source not found.** and Table 2-7. Antibiotic

minimum inhibitory concentrations of *E. coli* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days).

Ceftriaxone (AXO). The mean MIC values for AXO prior to CTC administration were within the susceptible category (1.0 µg/mL), and this remained fairly unchanged throughout the study period (Table 2-7. Antibiotic minimum inhibitory concentrations of *E. coli* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days). As a result of this lack of variation, AXO MIC data could not be statistically analyzed. Prior to CTC administration, there were only one (8.3%) and two (16.7%) AXO-resistant isolates for CTC groups 0.0 mg and 2.0 mg, respectively **Error! Reference source not found.** and Table 2-7. Antibiotic minimum inhibitory concentrations of *E. coli* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days). There were no AXO-resistant isolates throughout the rest of the study except on day 21 post cessation of CTC administration where CTC group 0.5 mg had one (8.3%) AXO-resistant isolate.

Discussion

The goal of this study was to examine if prolonged use of CTC to control active anaplasmosis influences the susceptibility of commensal *E. coli* to TET and, more broadly, to non-TET antibiotic classes. In this study, *E. coli* antibiotic susceptibility to fourteen medically important antibiotics was evaluated throughout the course of CTC administration for anaplasmosis control using CLSI guidelines and breakpoint classifications. Of the 192 *E. coli* isolates whose antibiotic susceptibility was characterized, 163 (85%) were resistant to at least

one antibiotic, and 97 (51%) isolates were resistant to three or more antibiotics (Figure 2.1. Heatmap of the antibiotic resistance phenotypes of *E. coli* isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days). The total number of antibiotics the isolates were resistant to increased for each group during the CTC administration period, with the most increases being in the higher dose groups compared to lower dose groups and decreased after CTC administration. This observation was also observed in the control group without CTC administration. Changes in *E. coli* susceptibility to specific antibiotics clustered into three categories: significant increases in MIC least square means over the CTC administration period, minimal but no significant changes in MIC least square means over the CTC administration period, or no change in MIC least square means over the CTC administration period Figure 2.4. Comparison of *E. coli* antibiotic MIC least square mean values from isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days). Significant increases in *E. coli* MIC least square mean values were observed in CHL, FIS, SXT, AMP, and STR over the CTC administration period, especially for *E. coli* isolates from the 2.0 and 10.0 mg CTC groups, with some MIC least square mean values crossing or approaching breakpoint classifications. Minimal but no meaningful MIC least square means changes to FOX, AZI, GEN, NAL, AUG2, and XNL were observed; however, they were not always correlated with CTC administration or did not cross/approach CLSI breakpoints. The MIC least square mean values for TET also fell within this category, but likely for a separate reason: many isolates were already at or just below the maximum antibiotic concentration evaluated by the Sensititre assay (Figure 2.4. Comparison of *E. coli* antibiotic MIC least square mean values from isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days and Table 2-7. Antibiotic minimum inhibitory

concentrations of *E. coli* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days). No MIC least square mean values changes were observed for CIP and AXO over the CTC administration period (Table 2-7.

Antibiotic minimum inhibitory concentrations of *E. coli* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days).

In previous studies, CTC administration resulted in a decrease in susceptibility to tetracyclines [302,309,310]. The present study's TET MIC least square mean values had an upward trend during CTC administration. However, they did not meet statistical significance (p-value 0.270 and 0.630 on study days 58 and 144 of continuous CTC administration, respectively), likely attributable to the fact that most *E. coli* isolates were already TET resistant prior to CTC administration and had MIC values near or already at the maximum concentration quantifiable on the Sensititre plates (Figure 2.3. Comparison of tetracycline MIC least mean square values from *E. coli* isolates collected from groups of steers administered different chlortetracycline dosages for 120 days and Table 2-7. Antibiotic minimum inhibitory concentrations of *E. coli* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days). Similar to the present study, a high starting incidence of TET resistance isolates with high TET MIC values have been observed [302,309–311]. An observational study investigating antibiotic use and the prevalence of resistance in pig farms that commonly use in-feed antibiotics also reported no association between TET use and TET resistance. They reported that these results could have been due to a lack of statistical power since both TET use and TET-resistance prevalence were high in their study [312]. Similarly, in the present study, 28 of the 48 *E. coli* isolates (58%) were already TET

resistant prior to CTC administration (Figure 2.3. Comparison of tetracycline MIC least mean square values from *E. coli* isolates collected from groups of steers administered different chlortetracycline dosages for 120 days and Table 2-7. Antibiotic minimum inhibitory concentrations of *E. coli* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days). Although an increased TET MIC was not observed in the present study (likely due to assay limitations), an increase in the percent of TET-resistant isolates was observed during the CTC administration period (85% and 94% at days 58 and 114 of continuous CTC administration) and in the groups receiving the highest CTC doses (2.0 and 10.0 mg CTC groups) **Error! Reference source not found.** and Table 2-7. Antibiotic minimum inhibitory concentrations of *E. coli* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days).

Antibiotics provide selection pressure for bacteria to develop resistance, which may come at a fitness cost that may be lost once the pressure is removed. This transient resistance to antibiotics has been demonstrated for *E. coli* isolated from cattle fed CTC in cottonseed meal on days 0 to 4, 6 to 10, and 12 to 16 which resulted in a transient increase in TET MIC that returned to pre-exposure levels on day 33 [302]. Similarly, in this study, TET-resistant *E. coli* isolates decreased to 77% by day 21 post-cessation of CTC (**Error! Reference source not found.** and Table 2-7. Antibiotic minimum inhibitory concentrations of *E. coli* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days). Removal of CTC for 21 days resulted in a downward trend of TET MIC values of *E. coli* to near pre-treatment TET MIC values; however, these changes were not statistically significant between groups (p-value = 0.266) (Figure 2.3. Comparison of tetracycline

MIC least mean square values from *E. coli* isolates collected from groups of steers administered different chlortetracycline dosages for 120 days and Table 2-7. Antibiotic minimum inhibitory concentrations of *E. coli* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days). A similar downward trend was observed by others [312].

Co-selection of resistance to different antibiotics can occur when resistance genes to different antibiotics are linked on the same genetic element. This has been shown in cases where the use of one antibiotic was associated with resistance to another antibiotic [313–315]. For example, amoxicillin use was associated with trimethoprim and phenicol, and the presence of resistance genes to these different antibiotics were observed on the same genetic element [304]. In the present study, treatment of cattle with CTC for 120 days was associated with changes in *E. coli* susceptibility to other antibiotics. A significant effect of CTC use was observed based on study group and study period for CHL (p-values = 0.012 and <0.001 respectively), AUG2 (p-values = 0.034 and 0.001 respectively), FIS (p-values = 0.013 and 0.028 respectively), SXT (p-values = <0.001 and <0.001 respectively), AMP (p-values = <0.001 and <0.001 respectively) and STR (p-values = 0.029 and <0.001 respectively), as well as, a significant effect on study period on AZI (p-value = 0.041), NAL (p-value = 0.020) and XNL (p-value = 0.025).

Resistance to TET has previously been associated with promotion of and co-selection of resistance to third generation cephalosporins (including XNL and AXO evaluated in this study) because *bla*_{CMY-2} and *tet(A)* genes are located on the same plasmid [309]. However, another study which administered 10.0 mg CTC/lb BW/day for five days and found no association between CTC administration and recovery of *E. coli* isolates that expressed both *bla*_{CMY-2} and *tet(A)* genes, although this was determined by the absence of resistance genes and not by evaluating the

antibiotic susceptibility to these antibiotics as was done in the present study [316]. Similarly in the present study, AXO MIC least square mean values for all groups throughout the study did not vary (Table 2-7. Antibiotic minimum inhibitory concentrations of *E. coli* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days). Although a significant effect of TET use on study day for XNL was observed (p-value = 0.025), the MIC least square mean changes were inconsistent with CTC dose or group, changing with no particular pattern between groups and across days that may suggest it is not due to co-selection (Figure 2.4. Comparison of *E. coli* antibiotic MIC least square mean values from isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days (b), fourth panel from the top); however, this warrants further investigation.

In another study, where steers were administered 22.0 mg/kg CTC (10.0 mg CTC/lb BW) at three time periods, each consisting of five days with a one-day break between treatment pulses, CTC use led to a decrease in XNL-resistant *E. coli* isolates (p-value = 0.01) [302]. In contrast, another study reported that CTC administration was associated with the recovery of XNL-resistant *E. coli* isolates; however, in that study, some steers were also given XNL prior to CTC administration. Based on study groups, the provision of XNL and/or CTC may explain the discrepant results [309]. These studies also evaluated the susceptibility of *E. coli* to antibiotics, including GEN, STR, AMP, AUG2, FOX, AXO, NAL, SXT, and CHL. However, they did not report an association of CTC use with any of them (except XNL, discussed above).

In our study, we found a significant association between TET administration and AMP MIC least square mean changes between study groups and across study days (p-values <0.001). This was mainly reflected in the 10.0 mg group on days 58 and 114 of continuous CTC

administration (Figure 2.4. Comparison of *E. coli* antibiotic MIC least square mean values from isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days (a) bottom panel). After 21 days of cessation of CTC administration, the MIC least square mean values decreased. In addition, the percentage of AMP-resistant isolates increased from 33% prior to CTC administration to 92% and 83% on days 58 and 114 of continuous CTC administration. It decreased back to 33% 21 days after cessation of CTC administration. These results contrast to a previous study involving 300 steers administered 11 ppm (4.9 mg/lb BW/day) TET mixed with 5 kg diet shared between 30 steers weighing 198 ± 20 kg for 61 days followed by discontinuation for 86 days and resumption of treatment for 42 days [311].

That study found an association between CTC administration and AMP resistance only after antibiotic administration had ceased but not during. Their data partly aligns with the present study's 2.0 mg CTC group data; however, it contrasts with data from 10.0 mg CTC group which found a significant MIC value increases during CTC administration (Figure 2.4. Comparison of *E. coli* antibiotic MIC least square mean values from isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days (b), bottom panel).

The different results could be because the 10.0 mg CTC group in the present study received twice the CTC concentration than the group in the referenced study. The differences in TET doses, which were low in that study compared to ours (0.5 mg CTC/lb to 10.0 mg CTC/lb). The lower dose of TET used in that study may explain the discrepant results of the present study. In furtherance of these results, another study identified an association of TET use to AMP resistance when steers were given a provided 44 ppm TET mixed in 5 kg of feed but in combination with sulfamethazine; however, the influence of sulfamethazine cannot be ruled out [311]. Our previous study evaluated the effect of long-term CTC use on *E. coli* susceptibility to

CTC in pastured beef cattle. Although no significant association between CTC use and the development of *E. coli* resistance to CTC was found, an increasing trend in CTC MIC was observed. Possible reasons why no statistical significance may have been influenced by the method of CTC administration (as free-choice medicated minerals) and, therefore, there was no way of ensuring each study animal consumed the intended daily CTC dose [317]. Ampicillin is a broad-spectrum antibiotic indicated to treat a wide range of gram-negative and positive bacteria in humans, and therefore, AMP-resistant bacteria are a concern [318]. Although warranting further investigation, this study's result suggests the use of 10.0 mg CTC/lb BW may promote AMP resistance in *E. coli*.

Throughout the study, the STR MIC least square mean values had subtle changes between study days and remained statistically similar between all groups. Although, on day 114 of continuous CTC administration the MIC least square means for 10.0 mg CTC group was statistically higher than that of the 0.0 mg CTC group (p-value = 0.075) (Figure 2.4. Comparison of *E. coli* antibiotic MIC least square mean values from isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days (b), top panel). Of note, however, was that the MIC least square mean values moved *E. coli* isolates from an intermediate susceptibility phenotype into a resistant phenotype. This change was more evident in *E. coli* isolated from cattle receiving higher CTC doses (10.0 mg and 2.0 mg CTC groups). The observed subtle decrease in *E. coli* susceptibility to STR was also transient, with MIC least square mean values in all groups decreasing on day 21 post-CTC administration. The fact that a transient reduction in STR MIC was also seen in the group that did not receive CTC makes co-selection of TET with STR less likely. A similar transient decrease in susceptibility to CHL was also observed (Figure 2.4. Comparison of *E. coli* antibiotic MIC least square mean values from

isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days (a), second panel from the top), suggesting other environmental variables might further influence antibiotic susceptibility. In the U.S., CHL is not approved for use in agricultural animals; however, our study and several others have reported CHL-resistant bacteria from agricultural animals [319].

For example, one observational study reported that 90% of CHL-resistant *E. coli* was also resistant to TET. Similarly, in the present study, 95.7% (90/94) of CHL-resistant isolates were also TET-resistant (Table 2-7. Antibiotic minimum inhibitory concentrations of *E. coli* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days). Although we observed increases in CHL MIC least square mean values over time, the observed increase occurred in all groups, including the 0.0 mg CTC group. The only statistical difference for CHL MIC values was only observed between the 0.0 and 10.0 mg CTC groups after days 58 of continuous CTC administration (p-value = 0.017) and between the 2.0 and 10.0 mg CTC groups at day 21 post-cessation of CTC administration (p-value = 0.058) (Figure 2.4. Comparison of *E. coli* antibiotic MIC least square mean values from isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days (a), second panel from the top).

Although MIC changes were also observed control group, our results suggest that the use of CTC for the control of active anaplasmosis at the current FDA-approved dose of 2.0 mg CTC/lb BW/day could broadly influence off-target *E. coli* antibiotic susceptibility to different antibiotic classes, including other medically important antibiotics. The greatest changes in antibiotic MIC values were observed in the 10.0 mg CTC study group. It is also important to note that this selection could be more significant in cattle operations where CTC is used for

anaplasmosis control for more than 120 days. In addition, for each study group per time point, only a single isolate was evaluated per steer (24 isolates/group/time point), which may not fully represent the *E. coli* population within those study cohorts. The 10.0 mg CTC/lb BW/day dosing regimen is not approved for controlling active anaplasmosis; however, administering this dose for five days is approved for treating bacterial pneumonia [272]. Additional studies are needed to investigate if *A. marginale* is still effectively controlled by the current FDA-approved CTC regimens. If anaplasmosis control at currently approved CTC dosing regimens is minimally or no longer effective, care should be taken to broadly understand the implications of approving higher dosages without a use duration limit. Results from the present study suggest that increasing the CTC dose for anaplasmosis control may more quickly and broadly accelerate the selection of multi-drug resistance in commensal *E. coli* populations.

In this study, MIC values for the group that received no CTC followed a similar but lower trend to the groups that received CTC (Figure 2.4. Comparison of *E. coli* antibiotic MIC least square mean values from isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days), this finding similarly reported in previous studies [302,309]. Factors that may influence the percent of TET-resistant isolates recovered, or TET MIC values could include abiotic (temperature, humidity, other environmental conditions) or biotic (contamination of pens with cattle feces or runoff from other pens; personnel; individual animal characteristics) factors not evaluated in this study [320–323].

A great limitation of the study is the small sample size (n=192) where only a single isolate was recovered from 48 fecal samples recovered from 12 cattle for each of the four CTC groups. However, it is worth noting that despite of this limitation, we did find significant MIC changes as a result of CTC administration. This study provides evidence that prolonged use of

CTC in cattle at doses approved to control active anaplasmosis can impart a selection pressure that can broadly influence antibiotic resistance in off-target commensal *E. coli*. Broad changes in *E. coli* susceptibility to medically important antibiotics included increased MIC least square mean values for AMP and STR. These antibiotics are used for the treatment of life-threatening diseases. Long-term use of antibiotics, for example, CTC use to control active anaplasmosis, should be refined to ensure the most judicious antibiotic use protocol to manage the target disease and critically evaluated to understand the broader implications this indication may have for co-occurring bacterial species of public health concern. These findings may be even more concerning in developing countries, including African countries like South Africa, and Asian countries, including India and Thailand, which use in-feed chlortetracycline for bovine anaplasmosis control for the entire vector season and where there is poor regulatory control for antibiotic use [324,325].

The exact expectation for CTC in anaplasmosis control is unspecified but is likely intended to reduce chances for recrudescence clinical signs and minimize production losses associated with chronic anaplasmosis (the approved CTC regimens are not indicated to cure infection or prevent new infection). Additionally, strains of *A. marginale* may differ in antibiotic susceptibility, and co-infection of individual animals and herds with multiple strains may complicate the determination of an effective CTC dosing regimen for anaplasmosis control [175].

Conclusions

Antibiotic resistance is a global problem that reduces antibiotic options for disease treatment, threatening economic security, food security, and public and animal health. Protracted

antibiotic administration, such as that used to control active anaplasmosis in fattening steers, may inadvertently promote the development of antibiotic resistance in *E. coli* recovered from these steers and should be avoided when possible. In addition, alternative approaches are needed to control this prevalent and highly economically taxing cattle disease.

Chapter 3 - Antibiotic susceptibilities of *Enterococcus* isolated from cattle hand-fed chlortetracycline for anaplasmosis control

Abstract

In the U.S., chronic bovine anaplasmosis is commonly managed by protracted use of chlortetracycline (CTC)-medicated feed products, with no limit on duration of use. Prolonged antibiotic use may have unintended consequences, including development of antimicrobial resistance in off-target microbes. The objective of this study was to evaluate changes in *Enterococcus* antibiotic susceptibilities from cattle provided CTC for chronic anaplasmosis control. Holstein-Jersey cross cattle with chronic anaplasmosis were blocked by weight, randomly allocated to one of the CTC study groups (0.0, 0.5, 2.0, and 10 mg/lb BW/day), and fed their respective CTC doses for 120 days. *Enterococcus* species were isolated from fecal samples collected pre-administration after 58 and 114 days of consecutive administration and 21 days post-administration cessation. The isolates were speciated using Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS). Antibiotic susceptibilities were determined using the Sensititre™ NARMS Gram Negative (CMV4AGNF) plate and assessed using Clinical and Laboratory Standards Institute (CLSI) breakpoint classifications or National Antimicrobial Resistance Monitoring System (NARMS) interpretation criteria. Eight *Enterococcus* species were isolated: *E. hirae* (60.8%), *E. casseliflavus* (19.8%), *E. durans* (6.3%), *E. faecium* (5.9%), *E. mundtii* (5.0%), *E. gallinarum* (1.4%), *E. avium* (0.5 %) and *E. aquimarinus* (0.5%). Of these 222 isolates, 96.4% were resistant to at least one antibiotic, 5.0% were resistant to three or more antibiotics, and 5.4% were pan-susceptible. The proportion of tetracycline-resistant isolates was 89.6%, and most isolates (93.0%) were tetracycline-resistant prior to CTC administration. No overall CTC effect on TET MIC values was observed (p-value 0.8370) or CTC effect on TET MIC values on any of the study days (p-values ≥ 0.1153). Although overall CTC effects on ciprofloxacin (p-value = 0.0434), vancomycin (p-value =

0.0072), and chloramphenicol (p-value = 0.0072) were observed, changes in MIC values were not constant with CTC administration dose or study day. Under the conditions of this study, CTC administration to control bovine anaplasmosis did not result in decreased susceptibility of *Enterococcus* species to tetracycline and non-tetracycline antibiotics analyzed in the study.

Introduction

Bovine anaplasmosis is an economically important hematopoietic, vector-borne disease of cattle [235,280]. The causative agent, *A. marginale*, is an intracellular rickettsial pathogen reported on almost every continent. In the U.S., *A. marginale* has been reported on all continental states and is endemic in several Midwest, Gulf Coast, and Atlantic states [175–177]. The pervasiveness of anaplasmosis has been demonstrated in prevalent studies in major beef-producing states. For example, the estimated seroprevalences in Mississippi, Texas, Kentucky, and Georgia were 29.0%, 15.9%, 9.4%, and 2.6%, respectively [194–197]. In addition, the herd level prevalence in cow-calf operations in Kansas and Arkansas was 52.5% and 51.9%, respectively [200,326]. Once infected, cattle may suffer clinical disease characterized by anemia and may be accompanied by reduced production efficiencies [179–181]. Cattle that recover from clinical disease became persistently infected, appearing healthy but serving as reservoirs of infection to naïve cattle and potentially producing sub-optimally [182]. In addition, chronically infected cattle can become clinical again if immunocompromised [183]. Therefore, in endemic areas in the U.S., bovine anaplasmosis is commonly controlled by administering CTC-medicated feed products as free-choice mineral mixes or salt blocks at doses of 0.5-2.0 mg/lb BW/day or as handfed medicated feed products at a 2.0 mg/lb BW/day dose. There is currently no limit on the duration of CTC to control chronic anaplasmosis as long as the producer has a valid veterinary

feed directive [281]. Therefore, producers commonly administer CTC to cattle for prolonged periods, six to twelve months per year.

Antibiotic resistance (AMR) is a major public health concern attributing to significant illnesses and death. In a 2019 report, the Centers for Diseases and Control (CDC) estimated that over 2.8 million illnesses and 35,000 deaths in the U.S. were attributed to AMR [1,11]. In addition, it is estimated that AMR will be attributed to about 300 million deaths by the year 2050 and a global economic loss between \$60 to \$100 trillion [327]. Antibiotic use, especially its misuse (i.e., sub-optimal dosing and prolonged usage), provides a selection pressure for bacteria to develop and maintain antibiotic resistance [282,283]. The 2022 CDC report on antibiotics sales revealed that 41% (2,569,381 kg out of 6,245,720 kg) of the medically important antibiotics sold in 2022 were marketed for cattle and that 63% (3,960,767 kg out of 6,245,720 kg) of these were administered in feed [290]. In addition, 41% (1,765,839 kg out of 4,056,479 kg) of tetracyclines were marketed for cattle.

Bacteria harboring resistance genes contaminate food products and transfer resistance genes to neighboring bacteria and can be a source of resistance genes to pathogenic bacteria in humans [291–293,328,329]. *Enterococcus* spp. are ubiquitous bacteria, present in the environment and inhabiting the gut of animals and humans and are increasingly being isolated in nosocomial infections [330,331]. *Enterococcus faecalis* amounts to about 90% of all clinical isolates, *E. faecium* amounts to 5-15%, and other species amount to about 5% or less of clinical isolates [332]. Vancomycin-resistant *Enterococci*, which is inherently resistant to other antibiotics, is especially of concern. Antibiotic resistance in *Enterococcus* spp., as in other bacteria, has been shown to also occur through mobile genetic elements within *Enterococcus* spp. and across other bacteria when resistant genes are present on the same genetic element

[41,333–337]. A study demonstrated horizontal transfer of antibiotic-resistance genes between *Enterococcus* isolates on ready-to-eat food [328]. Antibiotic resistance in humans has been linked to antibiotic use in production animals [284–286]. Humans may acquire these bacteria of animal origin from contaminated food products, including carcasses and fresh produce. For example, a study sampled 891 raw meat and meat products from 263 grocery stores in Iowa. That study reported 97% and 100% *Enterococcus* contamination of the pork and beef samples, respectively [338]. A similar study isolated 300 *Enterococcus* isolates from 316 packages of raw chicken purchased from supermarkets across 26 states harboring resistance genes to a wide range of medically important antibiotics, with more resistance genes being isolated from samples obtained from producers that commonly used antibiotics [339]. A similar study involving 13 farms and five packing sheds in southwestern United States isolated multidrug-resistant *Enterococcus* from 304 fresh produce (including leafy greens and herbs) [340]. The antibiotics the isolates were resistant to includes medically important antibiotics.

A global response to curb AMR is to adopt antimicrobial stewardship practices, which in the U.S. includes veterinary oversight of antibiotic use in production animals. However, bovine anaplasmosis remains one of the few diseases controlled by prolonged administration of antibiotics. This practice may be promoting antibiotic resistance not only in target but also in off-target bacterial species. Therefore, antimicrobial stewardship efforts should include evaluating and understanding the potential effects of antibiotic use (especially with open-ended use durations) on antibiotic susceptibilities of co-infecting bacterial populations, especially those with zoonotic potential, such as *Enterococcus* spp. Whether protracted use of CTC to control chronic bovine anaplasmosis promotes antibiotic resistance in *Enterococcus* spp. is unknown. Therefore, this study aimed to evaluate changes in antibiotic susceptibilities of *Enterococcus* spp.

isolated from cattle hand-fed different CTC doses for 120 consecutive days for the control of bovine anaplasmosis to a panel of 14 antibiotics. *Enterococcus* was chosen because it is a common zoonotic and gastrointestinal bacterium of cattle. We hypothesize that prolonged CTC administration will result in recovery of TET-resistant *Enterococcus* spp., with resistance occurring faster in groups given higher CTC doses. In addition, prolonged exposure to CTC will increase the recovery of *Enterococcus* isolates resistant to antibiotics not administered in the study.

Materials and Methods

Treatment Groups and Treatment Administration

The study involved fifty-eight chronically infected *A. marginale* Holstein-Jersey cross steers of approximately eight months old from a single source. The steers were blocked weight and randomly allocated to four study groups, each consisting of 12 steers. Each group of cattle was housed together in dry lot pens, and each pen had its feeding trough. The cattle also had access to water ad libitum. Each day, the steers were hand-fed a complete ration (custom blended by Wildcat Feeds, Topeka, KS), which was administered in two parts to ensure consumption: The first ration administered had the entire daily CTC dose. In contrast, the second part of the ration was unmedicated and was administered immediately after. Limited feeding was used to further encourage complete consumption of rations and for ease of monitoring. The rations were completely consumed within an hour of administering.

The CTC-mediated feeds were administered to the four study groups the following way: The untreated control group received no CTC (0.0 mg/lb BW/day), two groups received the current FDA-approved hand-fed doses with one group receiving (0.5 mg/lb BW/day) while the

other group received (2.0 mg/lb BW/day). These three groups received their CTC daily rations for 120 consecutive days. The fourth group received an FDA-approved dose for the treatment of bovine respiratory infections [272], 10.0 mg/lb BW/day for five consecutive days (Monday to Friday), with two days off feed for the same 120-day CTC administration period.

Fecal Sample Collection

The fecal samples were collected weekly directly from the rectum with hands individually gloved with palpation sleeves and transported in a cooler box containing ice packs. Glycerol stocks were made by resuspending feces in 10.0 mL glycerol at a glycerol: fecal matter ratio of 1:1 and stored at -80 °C for future use. Samples from four time points: 02 July 2019 (12 days before CTC administration began) (baseline); 10 September 2019 (58 days of continuous CTC administration); 05 November 2019 (114 days of continuous CTC administration) and 03 December 2019 (21 days after CTC administration had stopped) were selected for a downstream assay.

Isolation and speciation of *Enterococcus* spp.

To culture and isolate *Enterococcus* spp., about 1.0 g of fecal sample was homogenized in 10.0 mL phosphate-buffered saline, and 50.0 µL of fecal suspension was plated on mEnterococcus agar plates and incubated at 37°C for 48 hours. Individual *Enterococcus* suspect colonies were picked, and subcultured blood agar plates (Remel, Lenexa KS). *Enterococcus* colonies were subjected to Esculin hydrolysis tests. Briefly, a single colony was picked from blood agar plates, dispensed, and mixed with 100.0 µL of mEnterococcus broth in 96 well plates. The sample size was determined with the advice of a field expert. The plates were incubated at

37°C for 6 hours, and esculin hydrolyzing colonies, as assessed by their abilities to turn the broth brown or black, were recorded. Esculin hydrolyzing colonies were on CryoBeads™ (KEY Scientific Products, Stanford, TX) at -80°C. A single colony was isolated from the 12 fecal samples collected from the four time points and for each CTC group. *Enterococcus* isolates could only be isolated from three time-points in ten cattle, which resulted in a total of 222 colonies.

To confirm and speciate suspect *Enterococcus* isolates, isolates previously stored on CryoBeads™ (KEY Scientific Products, Stanford, TX) at -80 °C were subcultured on blood agar (Remel, Lenexa KS, USA), two plates per isolate. Briefly, CryoBeads were thawed, and sterile 5-200 µL graduated low binding barrier tips (MIDSCI, St. Louis, MO) were used to extract a single bead from the CryoBeads tube and do the initial streaking onto the blood agar plates. The rest of the agar streaking was done using reusable calibrated inoculating loops (R503153, Thermo Scientific™ Remel™), sterilized between streaks in a BACTI-CINERATOR IV Inoculating Loop and Needle Sterilizer (Leica Biosystems Richmond Inc, Richmond IL, USA) for 20 secs. The plates were incubated at 37° C for 24 hours. One plate for each isolate was sent to the Kansas State Veterinary Diagnostic Laboratory (KSVDL) for speciation using Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS). The second plate Of each isolate was subcultured onto fresh agar plates and used for antibiotic susceptibility testing once speciation was complete.

Antibiotic Susceptibility Testing

The *Enterococcus*-containing CryoBeads™ (KEY Scientific Products) were thawed, subcultured onto blood agar plates (Remel, Lenexa KS), and incubated for 12 hours at 35°C. A

single colony suspension was made using demineralized water, and its density was standardized using a 0.5 Polymer McFarland Standard (Thermo Scientific, Waltham, MA). This was followed by incubating 10 mL of the suspension into Mueller-Hinton Broth (Thermo Scientific) and then inoculating 50 mL of that suspension into each well of the Sensititre™ NARMS Gram Negative Plate (CMV4AGNF, Thermo Scientific). The plates were incubated at 35°C for 18 hours.

The minimum inhibitory concentrations (MIC) values were determined for tetracycline (TET), tigecycline (TGC), daptomycin (DAP), erythromycin (ERY), gentamycin (GEN), ciprofloxacin (CIP), vancomycin (VAN), ampicillin (AMP), quinupristin/dalfopristin (SYN), chloramphenicol (CHL), streptomycin (STR), linezolid (LZD), nitrofurantoin (NIT), avilamycin (AVL) using the Sensititre™ Manual Viewbox (Thermo Scientific). Breakpoint classifications for TET, DAP, ERY, GEN, CIP, VAN, AMP, SYN, CHL, STTR, LZD, NIT, and AVL were determined using the 2023 Clinical & Laboratory Standards Institute (CLSI) breakpoints classifications [341]. No CLSI breakpoint classification has been defined for TGC, GEN, STR, AVL. As a result, the National Antimicrobial Resistance Monitoring System (NARMS) 2021 Interpretation Criteria were used. Because the NARMS 2021 Interpretation Criteria only describes TGC susceptible breakpoint, an MIC greater than or equal 0.5 µg/mL was considered resistant.

The breakpoint classifications were as follows: TET (≤ 4 µg/mL = susceptible (S), 8 µg/mL = intermediate (I), ≥ 16 µg/mL = resistant (R)); TGC (≤ 0.25 µg/mL = S, ≥ 0.5 µg/mL = R). DAP for *E. faecium* only (≤ 4 µg/mL = S, ≥ 4 µg/mL = R); DAP for other *Enterococcus* spp. (≤ 2 µg/mL = S, 4 µg/mL = I, ≥ 8 µg/mL = R); ERY (≤ 0.5 µg/mL = S, 1-4 µg/mL = I, ≥ 8 µg/mL = R); GEN (≤ 500 µg/mL = S, 4 µg/mL = I, > 500 µg/mL = R); CIP (≤ 1 µg/mL = S, 2 µg/mL = I, ≥ 4 µg/mL = R); VAN (≤ 4 µg/mL = S, 8-16 µg/mL = I, ≥ 32 µg/mL = R); AMP (≤ 8 µg/mL =

S, ≥ 16 $\mu\text{g/mL}$ = R); SYN (≤ 1 $\mu\text{g/mL}$ = S, 2 $\mu\text{g/mL}$ = I, ≥ 4 $\mu\text{g/mL}$ = R); CHL (≤ 8 $\mu\text{g/mL}$ = S, 16 $\mu\text{g/mL}$ = I, ≥ 32 $\mu\text{g/mL}$ = R); STR (≤ 16 $\mu\text{g/mL}$ = S, ≥ 32 $\mu\text{g/mL}$ = R); LZD (≤ 2 $\mu\text{g/mL}$ = S, 4 $\mu\text{g/mL}$ = I, ≥ 8 $\mu\text{g/mL}$ = R); NIT (≤ 32 $\mu\text{g/mL}$ = S, 64 $\mu\text{g/mL}$ = I, ≥ 128 $\mu\text{g/mL}$ = R); AVL (≥ 16 $\mu\text{g/mL}$ = R). *Enterococcus faecalis* (Andrewes and Horder) Schleifer and Kilpper-Balz strain (ATCC 29212) was used as the quality control organism.

Statistical Analysis

For each of the 14 antibiotics, MIC data was summarized with appropriate descriptive statistics on each study day. Gentamycin data could not be statistically analyzed due to a lack of variation in MIC values.

The data were baseline log transformed prior to analysis to achieve model assumptions. The log-transformed MIC values were then subjected to analysis of variance (ANOVA) where the CTC group, study day, and their interaction served as fixed effects, baseline log-transformed MIC values as the covariate, and animal ID as a random effect. The CTC effects on antibiotic MIC values for each study day were determined using a separate model with CTC group as the fixed effect and baseline log-transformed MIC values as the covariate. Statistical analyses were performed using SAS (v9.4).

Results

Summary Statistics

Summary Statistics for the Overall Study

The 222 *Enterococcus* isolates analyzed represented eight species: *E. hirae* (135, 60.8% of isolates), *E. casseliflavus* (44, 19.8% of isolates), *E. durans* (14, 6.3% of isolates), *E.*

faecium (13, 5.9% of isolates), *E. mundtii* (11, 5.0% of isolates), *E. gallinarum* (3, 1.4% of isolates), *E. aquimarinus* (1, 0.5% of isolates) and *E. avium* (1, 0.5% of isolates) (Figure 3.1. Proportion of 222 *Enterococcus* isolates from cattle handfed chlortetracycline at varying doses for 120 consecutive days to a panel of 14 antibiotics). Of these, 210 (94.6%) isolates were resistant to at least one antibiotic, and 11 (5.0%) were resistant to three or more antibiotics (Figure 3.2. Resistant phenotypes from *Enterococcus* isolated from cattle administered CTC for bovine anaplasmosis control for 120 days for the entire study). Twelve (5.4%) isolates were susceptible to all antibiotics tested (pan-susceptible). Overall, 281/3108 (9.0%) antibiotic resistances were rerecorded (**Error! Reference source not found.**). Only *E. mundtii* (6, 2.7% isolates), *E. casseliflavus* (5, 2.3% isolates), and *E. gallinarum* (1, 0.5% of isolates) had at least one pan-susceptible isolate (Table 3-2. Animal IDs from where pan-susceptible *Enterococci* was isolated and Figure 3.3. Antibiotic-resistant *Enterococcus* isolates from cattle handfed chlortetracycline at varying doses for 120 consecutive days to a panel of 14 antibiotics). *Enterococcus casseliflavus* (4, 1.8% isolates), *E. hirae* (4, 1.8% isolates), *E. aquimarinus* (1, 0.5% isolates), *E. durans* (1, 0.5% isolates) and *E. faecium* (1, 0.5% isolates) had multidrug-resistant isolates (Figure 3.2. Resistant phenotypes from *Enterococcus* isolated from cattle administered CTC for bovine anaplasmosis control for 120 days for the entire study).

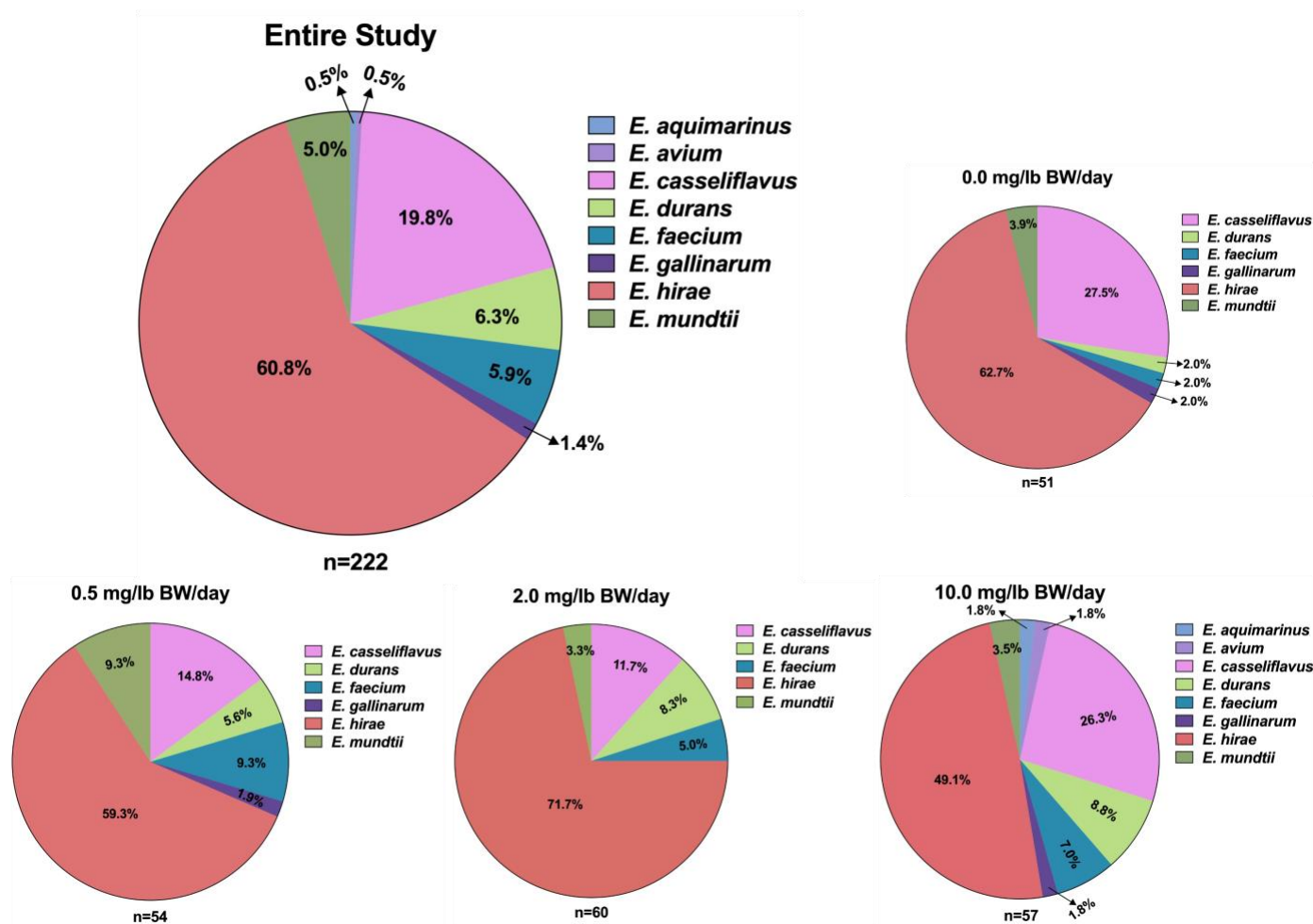


Figure 3.1. Proportion of 222 *Enterococcus* isolates from cattle handfed chlortetracycline at varying doses for 120 consecutive days to a panel of 14 antibiotics

Fecal samples were obtained from steers from each group (0.0 mg/lb BW/day, 0.5 mg/lb BW/day, 2.0 mg/lb BW/day, and 10.0 mg/lb BW/day). *Enterococci* were identified using biochemical tests and confirmed and speciated using Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS). Eight *Enterococcus* species were identified during the entire study and are represented by different colors on each pie chart.

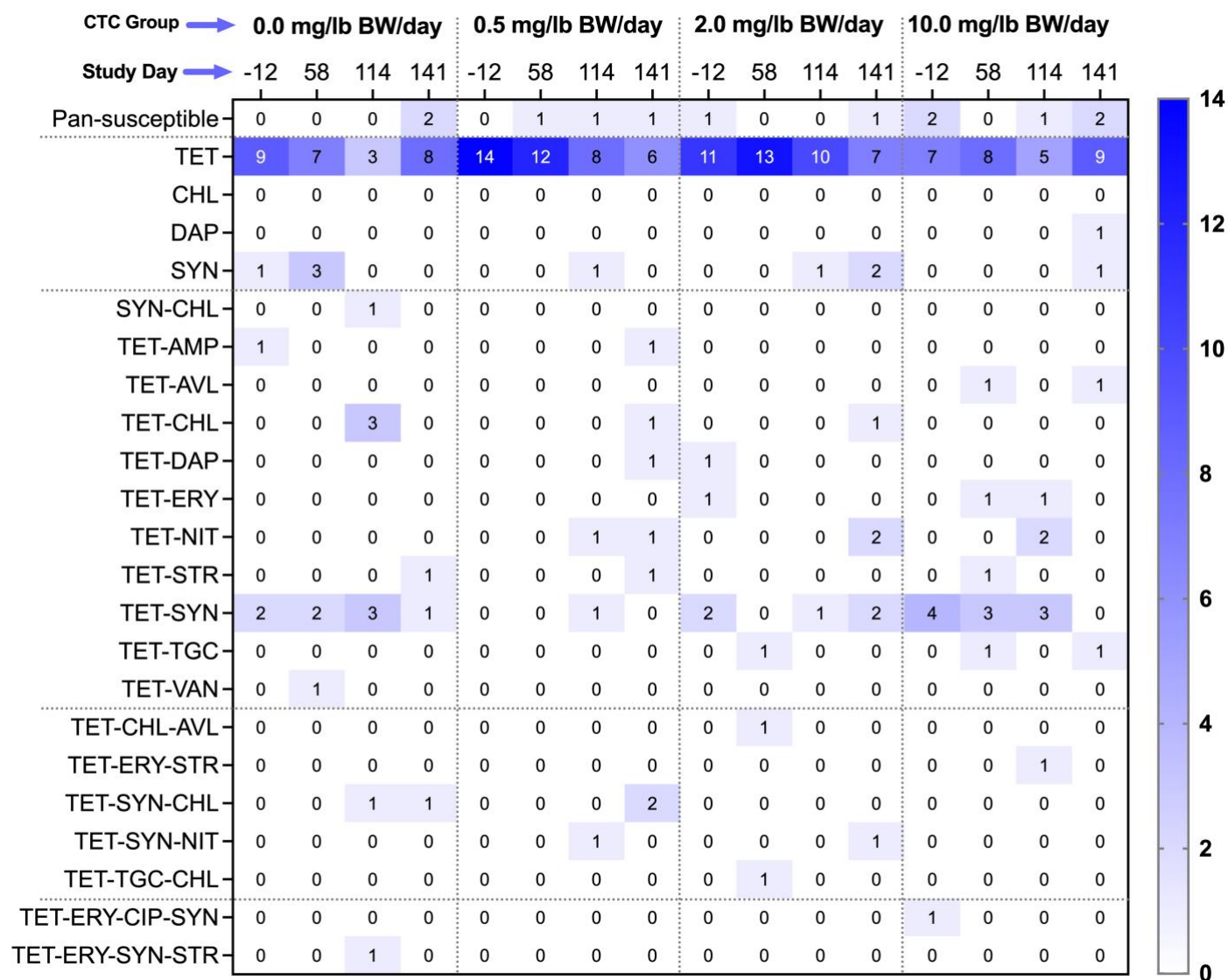


Figure 3.2. Resistant phenotypes from *Enterococcus* isolated from cattle administered CTC for bovine anaplasmosis control for 120 days for the entire study

Columns: CTC Groups (0.0 mg group, 0.5 mg group, 2.0 mg group and 10.0 mg group) and Study Day (12 days prior to CTC administration, 58 and 114 days of consecutive CTC administration and 21 days post-cessation of CTC administration). Rows: Resistant phenotypes (TET = Tetracycline. TCG = Tigecycline. DAP = Daptomycin. ERY = Erythromycin. GEN = Gentamicin. CIP = Ciprofloxacin. VAN = Vancomycin. AMP = Ampicillin. SYN = Quinupristin / Dalfopristin. CHL = Chloramphenicol. STR = Streptomycin. LZD = Linezolid. NIT = Nitrofurantoin. AVL = Avilamycin.)

Table 3-1. Distributions of minimum inhibitory concentrations of 222 *Enterococcus* isolates from cattle handfed chlortetracycline at varying doses for 120 consecutive days to a panel of 14 antibiotics

Antibiotic ¹	% Resistant ²	Distributions (%) of <i>Enterococcus</i> isolates within antibiotic MIC dilution series ³																		MIC 95% CI ⁴
		0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	2048	
TET	199							5.0	0.9	1.8	2.7	5.9	1.4	82.4						26.88-29.33
TGC	4	0.0	2.7	14.0	42.3	39.6	0.5	0.9												0.16-0.18
DAP	3					3.7	9.0	29.3	47.7	9.0	0.9	0.5								1.61-2.00
ERY	6					66.7	9.9	7.7	8.6	4.5	1.4	1.4								0.66-1.06
GEN	0											100	0.0	0.0	0.0	0.0	0.0	0.0		n/a
CIP	1				0.9	41.0	34.2	19.8	3.6	0.5	0.0	0.0								0.50-0.62
VAN	1					14.9	38.7	12.2	25.2	8.6	0.0	0.0	0.5							1.04-1.65
AMP	2					23.5	50.0	20.7	5.0	0.0	0.0	0.0	0.5	0.5						0.51-1.30
SYN	42						34.3	9.5	37.4	10.4	2.3	2.3	0.9	3.2						2.41-4.12
CHL	12								1.4	44.1	47.3	1.8	0.5	1.8	3.2					7.48-10.88
STR	5													94.2	1.8	0.5	1.4	1.4	0.0	73.47-132.37
LZD	0					0.9	62.6	36.0	0.5	0.0										1.30-1.44
NIT	8							0.0	2.3	8.1	40.5	38.7	6.8	2.3	1.4	0.0				25.85-34.72
AVL	3									95.1	3.6	0.9	0.5							4.07-4.68

¹Antibiotics evaluated: TET (tetracycline), TGC (tigecycline), DAP (daptomycin), ERY (erythromycin), GEN (gentamycin), CIP (ciprofloxacin), VAN (vancomycin), AMP (ampicillin), SYN (quinupristin/dalfopristin), CHL (chloramphenicol), STR (streptomycin), LZD (linezolid), NIT (nitrofurantoin) and AVL (avilamycin).

²The percent of 222 total *Enterococcus* isolates resistant to the tested antibiotic.

³Antibiotic (Minimum Inhibitory Concentration) MIC dilution series evaluated by Sensititre CMV4AGNF plate. The white area indicates the available evaluation range for a given antibiotic and the percentage of *Enterococcus* isolates at that MIC. Isolates with MIC values outside the range are in the gray areas of the distribution area. Vertical black lines within an antibiotic dilution series indicate the CLSI or FDA consensus resistance breakpoint.

⁴The MIC 95% confidence interval of the minimum inhibitory concentrations.

^{n/a}All GEN MIC values were the same, and therefore 95% CI could not be determined.

Table 3-2. Animal IDs from where pan-susceptible *Enterococci* was isolated

Study Day			
D12 Pre CTC	D58 of CTC	D114 of CTC	D21 Post CTC
0.0 mg			
			98 (<i>E. casseliflavus</i>)
			3 (<i>E. casseliflavus</i>)
0.5 mg			
	48 (<i>E. mundtii</i>)	48 (<i>E. mundtii</i>)	31 (<i>E. mundtii</i>)
2.0 mg			
60 (<i>E. mundtii</i>)			60 (<i>E. casseliflavus</i>)
10.0 mg			
		68 (<i>E. gallinarum</i>)	32 (<i>E. casseliflavus</i>)
32 (<i>E. mundtii</i>)			95 (<i>E. casseliflavus</i>)
77 (<i>E. mundtii</i>)			

The columns represent CTC days: 12 days prior to CTC administration (D12 pre-CTC), days 58 and 114 of continuous CTC administration (D58 of CTC and D114 of CTC, respectively) and 21 days post-cessation of CTC administration (D21 Post CTC).

The rows are divided into four study groups (0.0 mg, 0.5 mg, 2.0 mg and 10.0 mg).

Enterococcus species names are in brackets next to the animal IDs.



Figure 3.3. Antibiotic-resistant *Enterococcus* isolates from cattle handfed chlortetracycline at varying doses for 120 consecutive days to a panel of 14 antibiotics

Fecal samples were obtained from steers from each group (0.0 mg/lb BW/day, 0.5 mg/lb BW/day, 2.0 mg/lb BW/day and 10.0 mg/lb BW/day) and *Enterococci* were identified using biochemical test and confirmed and speciated using Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS). Eight *Enterococcus* species were identified during the entire study and are represented by individual bars. The number of antibiotic resistances for each species is represented by the different colors that make up the bars. (a) CTC group 0.0 mg, (b) CTC group 0.5 mg, (c) CTC group 2.0 mg and (d) CTC group 10.0 mg.

The most common antibiotics the isolates were resistant to were TET (199, 89.6% of isolates), SYN (42, 18.9% of isolates), and CHL (12, 5.4% of isolates) (Figure 3.2. Resistant phenotypes from *Enterococcus* isolated from cattle administered CTC for bovine anaplasmosis control for 120 days for the entire study, Figure 3.3. Antibiotic-resistant *Enterococcus* isolates from cattle handfed chlortetracycline at varying doses for 120 consecutive days to a panel of 14 antibiotics, **Error! Reference source not found.** and **Error! Reference source not found.**). Overall, seven multidrug-resistant phenotypes were observed: TET-SYN-CHL (4, 1.8% isolates), TET-SYN-NIT (2, 0.9% isolates), TET-CHL-AVL (1, 0.5% isolates), TET-ERY-STR (1, 0.5% isolates), TET-TGC-CHL (1, 0.5%isolates), TET-ERY-CIP-SYN (1, 0.5% isolates) and TET-ERY-SYN-STR (1, 0.5% isolates) (Figure 3.2. Resistant phenotypes from *Enterococcus* isolated from cattle administered CTC for bovine anaplasmosis control for 120 days for the entire study). All *E. hirae* (n=135), *E. durans* (n=14), *E. faecium* (n=13), *E. avium* (n=1) and *E. aquimarinus* (n=1) isolates were TET-resistant (Figure 3.2. Resistant phenotypes from *Enterococcus* isolated from cattle administered CTC for bovine anaplasmosis control for 120 days for the entire study).

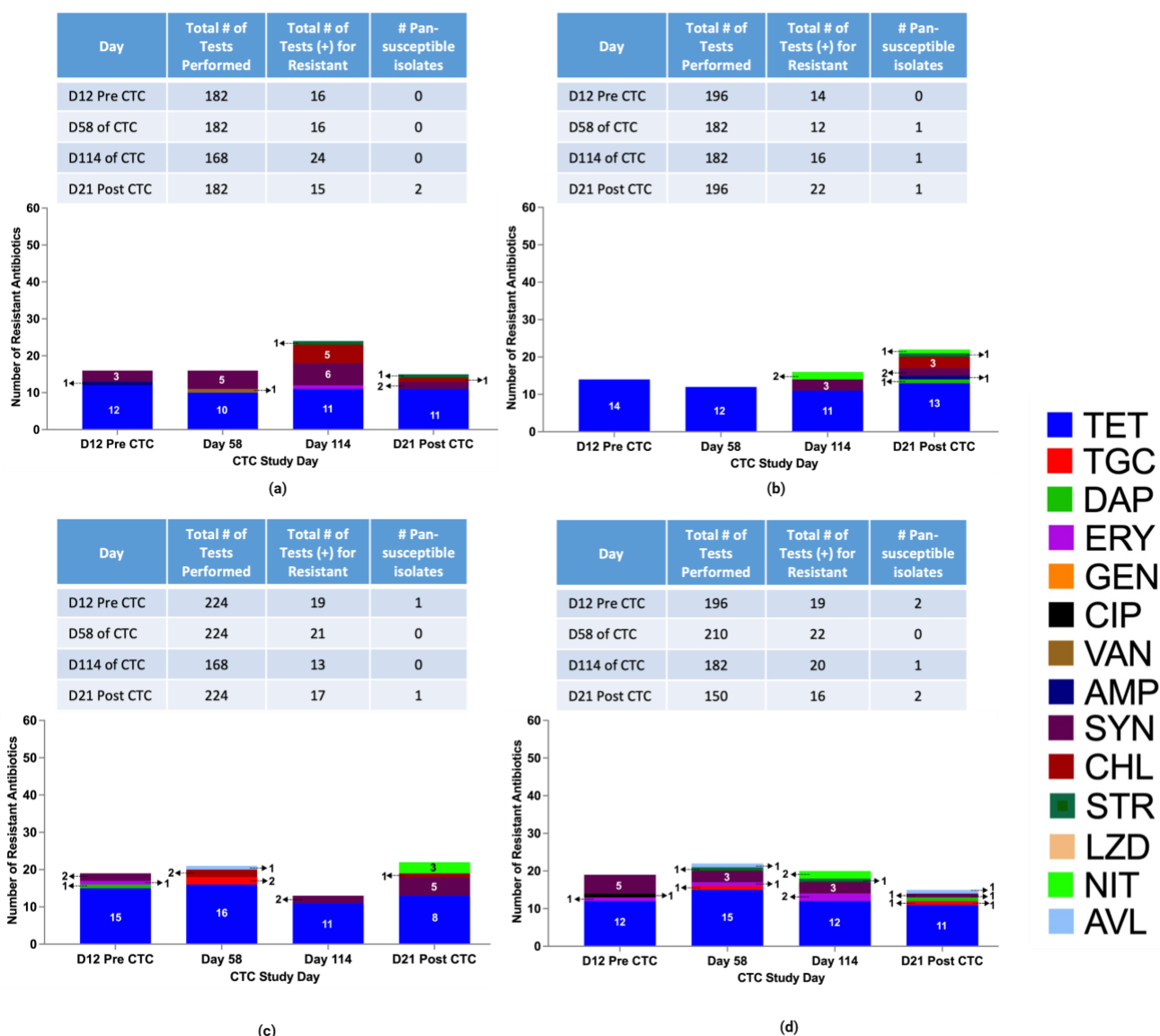


Figure 3.4. Antibiotic-resistant profiles of 222 total *Enterococcus* isolates from cattle handfed chlortetracycline at varying doses for 120 consecutive days

Fecal samples were obtained from steers from each group, and the antibiotic susceptibility profiles of *Enterococcus* spp. were assessed using the NARMS CMV4AGNF plate with breakpoint classifications according to the 2023 Clinical & Laboratory Standards Institute (CLSI) breakpoints or FDA Antibacterial Susceptibility Test Interpretive Criteria for tigecycline were MIC values $0.5 \geq$ were considered resistant. The antibiotics evaluated are represented by different colors on the bars, and each bar represents a study day. The antibiotics evaluated include tetracycline (TET), tigecycline (TGC), daptomycin (DAP), erythromycin (ERY), gentamycin (GEN), ciprofloxacin (CIP), vancomycin (VAN), ampicillin (AMP), quinupristin/dalfopristin (SYN), chloramphenicol (CHL), streptomycin (STR), linezolid (LZD), nitrofurantoin (NIT) and avilamycin (AVL). The arrows and numbers on the bars represent the number of isolates resistant to each antibiotic. (a) the 0.0 mg group, (b) 0.5 mg group, (c) 2.0 mg group and (d) 10.0 mg group.

Summary Statistics of Antibiotic Susceptibility for the 0.0 mg CTC Group

Six *Enterococcus* species amounting to a total of 51 isolates from the 0.0 mg group were analyzed: *E. hirae* (32, 62.7% isolates), *E. casseliflavus* (14, 27.5% isolates), *E. mundtii* (2, 3.9% isolates), *E. durans* (1, 2.0% of isolates), *E. faecium* (1, 2.0% of isolates) and *E. gallinarum* (1, 2.0% of isolates) (Figure 3.1. Proportion of 222 *Enterococcus* isolates from cattle handfed chlortetracycline at varying doses for 120 consecutive days to a panel of 14 antibiotics). Forty-nine of these (96.1%) isolates were resistant to at least one antibiotic, and three (5.9%) isolates were resistant to three or more antibiotics (Figure 3.2. Resistant phenotypes from *Enterococcus* isolated from cattle administered CTC for bovine anaplasmosis control for 120 days for the entire study). Two of these (3.9%) isolates, all *E. casseliflavus* and isolated on day 21 post-cessation of CTC administration were pan-susceptible (Table 3-2. Animal IDs from where pan-susceptible *Enterococci* was isolated). Overall, 71/714 (9.9%) antibiotic resistances were recorded (**Error! Reference source not found.**, (a). Prior to CTC administration and throughout the study period, the isolates were resistant to 7.1% of the antibiotics tested: Prior to CTC administration (13/182), on day 58 of continuous CTC administration (13/182), on day 114 of continuous CTC administration (12/168) and on day 21 post-cessation of CTC administration (13/182) (**Error! Reference source not found.** (a).

The most common antibiotics the isolates were resistant to were TET (44, 86.3% isolates), SYN (16, 31.4% isolates), and CHL (6, 11.8% isolates) (**Error! Reference source not found.** (a) and **Error! Reference source not found.**). Only *E. casseliflavus* (2, 3.9% isolates) had at least one pan-susceptible isolate, and *E. hirae* (2, 3.9% of all isolates), *E. casseliflavus* (1, 2.0% of all isolates) had multidrug-resistant isolate (Figure 3.3. Antibiotic-resistant *Enterococcus* isolates from cattle handfed chlortetracycline at varying doses for 120 consecutive

days to a panel of 14 antibiotics (a) and Table 3-2. Animal IDs from where pan-susceptible *Enterococci* was isolated). Two multidrug-resistant phenotypes were observed: the resistant phenotype to three antibiotics was TET-SYN-CHL (2, 3.9% of isolates), while that of four antibiotics was TET-ERY-SYN-STR (1, 2.0% of isolates) (Table 3-2. Animal IDs from where pan-susceptible *Enterococci* was isolated).

Table 3-3. Distributions of minimum inhibitory concentrations of 51 *Enterococcus* isolates from cattle handfed 0.0 mg/lb BW/day chlortetracycline for 120 consecutive days to a panel of 14 antibiotics

Antibiotic ¹	% Resistant ²	Distributions (%) of <i>Enterococcus</i> isolates within antibiotic MIC dilution series ³																		MIC 95% CI ⁴	
			0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024		2048
TET	44							5.9	0.0	3.9	3.9	3.9	2.0	80.4							24.67-30.35
TGC	0	0.0	2.0	7.8	45.1	45.1	0.0														0.15-0.19
DAP	0					5.9	7.8	21.6	56.9	7.8	0.0	0.0									1.47-1.98
ERY	1					58.9	13.7	5.9	15.7	3.9	0.0	2.0									0.52-1.29
GEN	0											100	0.0	0.0	0.0	0.0	0.0	0.0			n/a
CIP	0			0.0	37.3	29.4	29.4	3.9	0.0	0.0	0.0										0.49-0.73
VAN	1				5.9	29.4	19.6	27.5	15.7	0.0	0.0	2.0									0.91-3.41
AMP	1				27.5	43.1	21.6	5.9	0.0	0.0	0.0	2.0									0.00-2.49
SYN	16					33.3	7.8	27.5	13.7	7.8	3.9	2.0	3.9								2.30-6.66
CHL	6							0.0	29.4	54.9	3.9	2.0	3.9	5.9							8.18-18.02
STR	2												90.2	0.0	0.0	5.9	2.0	0.0	2.0		58.26-237.90
LZD	0					0.0	60.8	39.2	0.0	0.0											1.25-1.53
NIT	0							0.0	3.9	7.8	47.1	39.2	2.0	0.0	0.0	0.0	Zoom				19.02-25.21
AVL	0								98.0	2.0	0.0	0.0									3.92-4.24

¹Antibiotics evaluated: TET (tetracycline), TGC (tigecycline), DAP (daptomycin), ERY (erythromycin), GEN (gentamycin), CIP (ciprofloxacin), VAN (vancomycin), AMP (ampicillin), SYN (quinupristin/dalfopristin), CHL (chloramphenicol), STR (streptomycin), LZD (linezolid), NIT (nitrofurantoin) and AVL (avilamycin).

²The percent of 51 total *Enterococcus* isolates that were resistant to the tested antibiotic.

³Antibiotic (Minimum Inhibitory Concentration) MIC dilution series for evaluated by Sensititre CMV4AGNF plate. The white area indicates the available evaluation range for a given antibiotic and the percentage of *Enterococcus* isolates at that MIC. Isolates with MIC values outside the range are in the gray areas of the distribution area. Vertical black lines within an antibiotic dilution series indicate the CLSI or FDA consensus resistance breakpoint.

⁴The MIC 95% confidence interval of the minimum inhibitory concentrations.

^{n/a}All GEN MIC values were the same and therefore 95% CI could not be determined.

Summary Statistics of Antibiotic Susceptibility for the 0.5 mg CTC Group

A total of six *Enterococcus* spp. amounting to 54 isolates were isolated from the 0.5 mg group: *E. hirae* (32, 59.3% isolates), *E. casseliflavus* (8, 14.8% isolates), *E. faecium* (5, 9.3% isolates), *E. mundtii* (5, 9.3% isolates), *E. durans* (3, 5.6% isolates) and *E. gallinarum* (1, 1.9% of isolates) (Figure 3.1. Proportion of 222 *Enterococcus* isolates from cattle handfed chlortetracycline at varying doses for 120 consecutive days to a panel of 14 antibiotics). Fifty-one of these isolates (94.4%) were resistant to at least one antibiotic, while three isolates (5.6%) were resistant to three or more antibiotics (Figure 3.2. Resistant phenotypes from *Enterococcus* isolated from cattle administered CTC for bovine anaplasmosis control for 120 days for the entire study). Three isolates (5.6%), all *E. mundtii* were pan-susceptible (Table 3-2. Animal IDs from where pan-susceptible *Enterococci* was isolated). Two of these isolates, one isolates on day 58 of continuous CTC administration and the other on day 114 of continuous CTC administration were isolated from the same study animal (Animal ID 48). Overall, 64/756 (7.1%) antibiotic resistances were recorded (Figure 3.2. Resistant phenotypes from *Enterococcus* isolated from cattle administered CTC for bovine anaplasmosis control for 120 days for the entire study). Prior to CTC administration, the isolates were resistant to a total number of 14/196 (7.1%) antibiotics (**Error! Reference source not found.** (b). On days 58 and 114 of continuous CTC administration and on day 21 post-cessation of CTC administration, the isolates were resistant to a total number of 12/182 (6.6%), 12/182 (6.6%) and 13/196 (6.6%), respectively.

The antibiotics the isolates were most resistant to were TET (50, 92.6% isolates), SYN (5, 9.3% isolates), and CHL and NIT (3, 5.6% isolates) each (**Error! Reference source not found.**). All *E. hirae* (32, 59.3%) isolates, *E. casseliflavus* (8, 14.8%) isolates, *E. faecium* (5, 9.3%) isolates and *E. durans* (3, 5.6%) were TET-resistant (Figure 3.3. Antibiotic-resistant

Enterococcus isolates from cattle handfed chlortetracycline at varying doses for 120 consecutive days to a panel of 14 antibiotics). Two multidrug-resistant phenotypes were observed: TET-SYN-CHL (2, 3.7% of isolates) and TET-SYN-NIT (1, 1.9% of isolates) (Figure 3.2. Resistant phenotypes from *Enterococcus* isolated from cattle administered CTC for bovine anaplasmosis control for 120 days for the entire study).

Table 3-4. Distributions of minimum inhibitory concentrations of 54 *Enterococcus* isolates from cattle handfed 0.5 mg/lb BW/day chlortetracycline for 120 consecutive days to a panel of 14 antibiotics

Antibiotic ¹	% Resistant ²	Distributions (%) of <i>Enterococcus</i> isolates within antibiotic MIC dilution series ³																MIC 95% CI ⁴
		0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	
TET	50							7.4	0.0	0.0	0.0	7.4	1.9	83.3				26.08-30.95
TGC	0	0.0	1.9	9.3	48.1	40.7	0.0											0.15-0.19
DAP	1				1.9	9.3	42.6	38.9	5.6		1.9	0.0						1.29-1.96
ERY	0				66.7	14.8	1.9	11.1	5.6	0.0								0.44-0.97
GEN	0											100	0.0	0.0	0.0	0.0	0.0	n/a
CIP	0			1.9	46.3	31.5	18.5	1.9	0.0	0.0	0.0							0.40-0.59
VAN	0				13.0	48.1	9.3	27.8	1.9	0.0	0.0	0.0						0.78-1.22
AMP	1				22.3	46.3	27.8	3.7	0.0	0.0	0.0	0.0	0.0	2.1				0.06-2.40
SYN	5					38.9	16.7	35.2	1.9	1.9	1.9	0.0	0.0	3.7				1.07-4.47
CHL	3							1.9	61.1	29.6	1.9	0.0	0.0	5.6				4.95-12.45
STR	1												96.3	1.9	0.0	0.0	1.9	47.27-118.66
LZD	0				0.0	75.9	24.1	0.0	0.0									1.12-1.36
NIT	3							0.0	3.7	11.1	40.7	29.6	9.3	1.9	3.7	0.0		21.56-48.07
AVL	0								96.3	3.7	0.0	0.0						3.94-4.36

¹Antibiotics evaluated: TET (tetracycline), TGC (tigecycline), DAP (daptomycin), ERY (erythromycin), GEN (gentamycin), CIP (ciprofloxacin), VAN (vancomycin), AMP (ampicillin), SYN (quinupristin/dalfopristin), CHL (chloramphenicol), STR (streptomycin), LZD (linezolid), NIT (nitrofurantoin) and AVL (avilamycin).

²The percent of 54 total *Enterococcus* isolates that were resistant to the tested antibiotic.

³Antibiotic (Minimum Inhibitory Concentration) MIC dilution series for evaluated by Sensititre CMV4AGNF plate. The white area indicates the available evaluation range for a given antibiotic and the percentage of *Enterococcus* isolates at that MIC. Isolates with MIC values outside the range are in the gray areas of the distribution area. Vertical black lines within an antibiotic dilution series indicate the CLSI or FDA consensus resistance breakpoint.

⁴The MIC 95% confidence interval of the minimum inhibitory concentrations.

n/a All GEN MIC values were the same and therefore 95% CI could not be determined.

Summary Statistics of Antibiotic Susceptibility for the 2.0 mg CTC Group

From CTC group 2.0 mg, five *Enterococcus* spp. amounting to a total of 60 isolates were analyzed. These included *E. hirae* (43, 71.7% isolates), *E. casseliflavus* (7, 11.7% isolates), *E. durans* (5, 8.3%), *E. faecium* (3, 5.0% isolates) and *E. mundtii* (2, 3.3% isolates) (Figure 3.1. Proportion of 222 *Enterococcus* isolates from cattle handfed chlortetracycline at varying doses for 120 consecutive days to a panel of 14 antibiotics). Fifty-eight (96.7%) isolates were resistant to at least one antibiotic while two (3.3%) isolates were pan-susceptible (Figure 3.3. Antibiotic-resistant *Enterococcus* isolates from cattle handfed chlortetracycline at varying doses for 120 consecutive days to a panel of 14 antibiotics). The pan-susceptible isolates, one *E. mundtii* and the other *E. casseliflavus* were isolated from the same study animal (Animal ID 60) prior to CTC administration and on day 21 pos-cessation of CTC administration (Table 3-2. Animal IDs from where pan-susceptible *Enterococci* was isolated). Overall, there were 75/840 (8.9%) antibiotic resistances recorded (Figure 3.2. Resistant phenotypes from *Enterococcus* isolated from cattle administered CTC for bovine anaplasmosis control for 120 days for the entire study). Prior to CTC administration and on day 21 post-cessation of CTC administration, the isolates were resistant to 15/225 (6.7%) of the antibiotics (Figure 3.3. Antibiotic-resistant *Enterococcus* isolates from cattle handfed chlortetracycline at varying doses for 120 consecutive days to a panel of 14 antibiotics (c)). During the CTC administration period, the isolates were resistant to 7.1% of the antibiotics (16/224 and 12/168, respectively).

The most common antibiotics the isolates were resistant to were TET (55, 91.7% isolates), SYN (9, 15.0% isolates), and CHL and NIT (3, 5.0% isolates each) (**Error! Reference source not found.**). Three (5%) multidrug-resistant isolates were recorded, two of which were *E. casseliflavus* and the third, *E. durans* (Figure 3.2. Resistant phenotypes from *Enterococcus* isolated from cattle administered CTC for bovine anaplasmosis control for 120 days for the

entire study). The multidrug-resistant phenotypes observed were TET-CHL-AVL (1, 1.7% isolates), TET-SYN-NIT (1, 1.7% isolates) and TET-TGC-CHL (1, 1.7% isolates). All *E. hirae* (43, 71.7%), *E. durans* (5, 8.3%), and *E. faecium* isolates were TET-resistant (Figure 3.3. Antibiotic-resistant *Enterococcus* isolates from cattle handfed chlortetracycline at varying doses for 120 consecutive days to a panel of 14 antibiotics, c).

Table 3-5. Distributions of minimum inhibitory concentrations of 60 *Enterococcus* isolates from cattle handfed 2.0 mg/lb BW/day chlortetracycline for 120 consecutive days to a panel of 14 antibiotics

Antibiotic ¹	% Resistant ²	Distributions (%) of <i>Enterococcus</i> isolates within antibiotic MIC dilution series ³																			MIC 95% CI ⁴
			0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	2048	
TET	55	1.7 3.3 1.7 1.7 3.3 1.7 86.7																			26.92-31.24
TGC	2	0.0 3.3 13.3 41.7 40.0 0.0 1.7																			0.15-0.20
DAP	1	1.7 8.3 25.0 51.7 11.7 1.7 0.0																			1.60-2.25
ERY	1	75.0 8.3 6.7 5.0 3.3 0.0 1.7																			0.34-0.98
GEN	0	100 0.0 0.0 0.0 0.0 0.0 0.0																			n/a
CIP	0	0.0 48.3 43.3 8.3 0.0 0.0 0.0																			0.37-0.48
VAN	0	23.4 40.0 6.7 26.7 3.3 0.0 0.0																			0.76-1.22
AMP	0	25.0 56.7 13.3 5.0 0.0 0.0																			0.48-0.68
SYN	9	31.6 8.3 45.0 11.7 0.0 0.0 3.3																			1.23-4.12
CHL	3	0.0 45.0 50.0 0.0 3.3 1.7																			5.67-12.33
STR	0	95.0 3.3 1.7 0.0 0.0 0.0																			62.33-76.34
LZD	0	0.0 63.3 36.7 0.0 0.0																			1.24-1.49
NIT	3	0.0 0.0 6.7 33.3 48.3 6.7 3.3 1.7 0.0																			24.63-43.64
AVL	1	90.0 8.3 1.7 0.0																			4.05-5.02

¹Antibiotics evaluated: TET (tetracycline), TGC (tigecycline), DAP (daptomycin), ERY (erythromycin), GEN (gentamycin), CIP (ciprofloxacin), VAN (vancomycin), AMP (ampicillin), SYN (quinupristin/dalfopristin), CHL (chloramphenicol), STR (streptomycin), LZD (linezolid), NIT (nitrofurantoin) and AVL (avilamycin).

²The percent of 60 total *Enterococcus* isolates that were resistant to the tested antibiotic.

³Antibiotic (Minimum Inhibitory Concentration) MIC dilution series for evaluated by Sensititre CMV4AGNF plate. The white area indicates the available evaluation range for a given antibiotic and the percentage of *Enterococcus* isolates at that MIC. Isolates with MIC values outside the range are in the gray areas of the distribution area. Vertical black lines within an antibiotic dilution series indicate the CLSI or FDA consensus resistance breakpoint.

⁴The MIC 95% confidence interval of the minimum inhibitory concentrations.

n/a All GEN MIC values were the same and therefore 95% CI could not be determined.

Summary Statistics of Antibiotic Susceptibility for the 10.0 mg CTC Group

Eight *Enterococcus* spp. amounting to 57 isolates were isolated from the 10.0 mg group: *E. hirae* (28, 49.1% isolates), *E. casseliflavus*, (15, 26.3% isolates), *E. durans* (5, 8.8% isolates), *E. faecium* (4, 7.0% isolates), *E. mundtii* (2, 3.5% of isolates), *E. aquimarinus* (1, 1.8% isolates), *E. avium* (1, 1.8% isolates) and *E. gallinarum* (1, 1.8% isolates) (Figure 3.1. Proportion of 222 *Enterococcus* isolates from cattle handfed chlortetracycline at varying doses for 120 consecutive days to a panel of 14 antibiotics). Overall, the isolates were resistant to 76/798 (9.6%) antibiotics (Figure 3.2. Resistant phenotypes from *Enterococcus* isolated from cattle administered CTC for bovine anaplasmosis control for 120 days for the entire study, (d)). Prior to CTC administration, the isolates were resistant to 12/196 (6.1%) antibiotics (Figure 3.2. Resistant phenotypes from *Enterococcus* isolated from cattle administered CTC for bovine anaplasmosis control for 120 days for the entire study, (d)). On days 58 and 114 of continuous CTC administration, the isolates were resistant to 15/210 (7.1%) and 12/182 (6.6%) antibiotics, respectively. On day 21 post-cessation of CTC administration, the isolates were resistant to 13/150 (8.7%) antibiotics.

The most common antibiotics the isolates were resistant to were TET (50, 87.7%), SYN (12, 21.0%), and ERY (4, 7.0%) (**Error! Reference source not found.**). Fifty-two (91.2%) of the isolates were resistant to at least one antibiotic, two (3.5%) isolates, one *E. casseliflavus* and the other *E. aquimarinus* were multidrug-resistant. Five (8.8%) isolates; two *E. casseliflavus*, two *E. mundtii*, and one *E. gallinarum* were pan-susceptible and were isolated from five different study animals (Table 3-2. Animal IDs from where pan-susceptible *Enterococci* was isolated). Two (3.5%) multidrug-resistant phenotypes from two isolates were observed: TET-ERY-STR and TET-ERY-CIP-SYN (Figure 3.2. Resistant phenotypes from *Enterococcus* isolated from cattle administered CTC for bovine anaplasmosis control for 120 days for the entire study). All *E. hirae* (28, 49.1%), *E. durans* (5, 8.8%), *E. faecium* (4, 7.0%), *E. aquimarinus* (1, 1.8%) and *E.*

avium (1, 1.8%) isolates were TET-resistant (Figure 3.3. Antibiotic-resistant *Enterococcus* isolates from cattle handfed chlortetracycline at varying doses for 120 consecutive days to a panel of 14 antibiotics, (d)).

Table 3-6. Distributions of minimum inhibitory concentrations of 57 *Enterococcus* isolates from cattle handfed 10.0 mg/lb BW/day chlortetracycline for 120 consecutive days to a panel of 14 antibiotics

Antibiotic ¹	% Resistant ²	Distributions (%) of <i>Enterococcus</i> isolates within antibiotic MIC dilution series ³																			MIC 95% CI ⁴
			0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	2048	
TET	50							5.3	0.0	1.8	5.3	8.8	0.0	78.9							24.61-29.81
TGC	2	0.0	3.5	24.6	35.1	33.3	1.8	1.8													0.13-0.19
DAP	1					5.3	10.5	28.1	43.9	10.5	0.0	1.8									1.36-2.50
ERY	4					64.9	3.5	15.8	3.5	5.3	5.3	1.8									0.63-1.73
GEN	0											100	0.0	0.0	0.0	0.0	0.0	0.0			n/a
CIP	1				1.8	31.6	31.6	24.6	8.8	1.8	0.0	0.0									0.55-0.91
VAN	0					15.8	36.8	14.0	19.3	14.0	0.0	0.0	0.0								0.98-1.64
AMP	0					21.1	52.6	21.1	5.3	0.0	0.0	0.0	0.0								0.52-0.74
SYN	12					33.4	5.3	40.4	14.0	0.0	3.5	1.8	1.8								1.62-4.93
CHL	0							3.5	40.4	54.4	1.8	0.0	0.0								5.66-6.97
STR	2													94.8	1.8	0.0	0.0	1.8	0.0	1.8	39.88-193.67
LZD	0					3.5	50.9	43.9	1.8	0.0											1.31-1.64
NIT	2							0.0	1.8	7.0	42.1	36.8	8.8	3.5	0.0	0.0					22.89-35.64
AVL	2									96.5	0.0	1.8	1.8								3.64-5.77

¹Antibiotics evaluated: TET (tetracycline), TGC (tigecycline), DAP (daptomycin), ERY (erythromycin), GEN (gentamycin), CIP (ciprofloxacin), VAN (vancomycin), AMP (ampicillin), SYN (quinupristin/dalfopristin), CHL (chloramphenicol), STR (streptomycin), LZD (linezolid), NIT (nitrofurantoin) and AVL (avilamycin).

²The percent of 60 total *Enterococcus* isolates that were resistant to the tested antibiotic.

³Antibiotic (Minimum Inhibitory Concentration) MIC dilution series for evaluated by Sensititre CMV4AGNF plate. The white area indicates the available evaluation range for a given antibiotic and the percentage of *Enterococcus* isolates at that MIC. Isolates with MIC values outside the range are in the gray areas of the distribution area. Vertical black lines within an antibiotic dilution series indicate the CLSI or FDA consensus resistance breakpoint.

⁴The MIC 95% confidence interval of the minimum inhibitory concentrations.

n/a All GEN MIC values were the same and therefore 95% CI could not be determined.

Effect of Prolonged CTC Administration on *Enterococcus* Tetracycline

Susceptibility

Overall, no CTC dose effect (p-value = 0.8370) or study day effect (p-value = 0.4648) on TET least square means and standard error of the means (LSMean (SEM)) were found. In addition, there was no CTC and study day interaction effect (p-value = 0.2218).

The median and standard deviation (SD) TET MIC values prior to CTC administration were above the resistant breakpoint value (≥ 16 $\mu\text{g/mL}$) for all study groups (Table 3-7. Antibiotic minimum inhibitory concentrations of *Enterococcus* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days). The LSMean (SEM) only changed slightly during and after continuous CTC administration period and remained above the resistant breakpoint (Figure 3.5. *Enterococcus* spp. tetracycline Minimum Inhibitory Concentration values from isolates collected from groups of steers administered different chlortetracycline dosages for 120 days). There was no significant CTC effect on day 141 of continuous CTC administration (p-value = 0.6833) and on day 21 post-cessation of CTC administration (p-value = 0.7373). Although there was no CTC effect on day 58 of continuous CTC administration (p-value = 0.1153), pairwise comparisons indicated the LSMean (SEM) of CTC groups 2.0 mg (p-value = 0.0502) and 10.0 mg (p-value = 0.0603) were statistically higher than that of the 0.0 mg group (Figure 3.5. *Enterococcus* spp. tetracycline Minimum Inhibitory Concentration values from isolates collected from groups of steers administered different chlortetracycline dosages for 120 days, (a)).

Prior to CTC administration, at least 85% of the isolates from each group were TET-resistant (Table 3-7. Antibiotic minimum inhibitory concentrations of *Enterococcus* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for

120 consecutive days and Figure 3.5. *Enterococcus* spp. tetracycline Minimum Inhibitory Concentration values from isolates collected from groups of steers administered different chlortetracycline dosages for 120 days, (b)). The number of resistant isolates changed slightly in no orderly fashion during the CTC administration period, and after cessation of CTC administration however, most isolates per group remained TET-resistant (**Error! Reference source not found.**, (b))

Table 3-7. Antibiotic minimum inhibitory concentrations of *Enterococcus* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days

CTC Study Groups (mg)	D12 Pre CTC		D58		D114		D21 Post CTC	
	Mean (SD)	Number Resistant (%)	LSMean (SEM)	Number Resistant (%)	LSMean (SEM)	Number Resistant (%)	LSMean (SEM)	Number Resistant (%)
TET MIC (ug/mL)								
0.0	28.39 (9.34)	12/13 (92%)	16.88 (4.04)	10/13 (77%)	28.29 (7.06)	11/12 (92%)	22.03 (5.28)	11/13 (85%)
0.5	30.86 (4.28)	14/14 (100%)	21.76 (5.24)	12/13 (92%)	18.94 (4.56)	11/13 (85%)	23.88 (5.54)	13/14 (93%)
2.0	28.13 (8.84)	15/16 (94%)	32.03 (6.9)	16/16 (100%)	23.56 (5.87)	11/12 (92%)	21.69 (4.69)	13/16 (81%)
10.0	25.29 (11.78)	12/14 (86%)	31.85 (7.40)	15/15 (100%)	26.51 (6.65)	12/13 (92%)	15.92 (3.70)	11/15 (73%)
TGC MIC (ug/mL)								
0.0	0.17 (0.07)	5/13 (38%)	0.21 (0.03)	10/13 (77%)	0.15 (0.01)	4/12 (33%)	0.11 (0.01)	4/13 (32%)
0.5	0.19 (0.07)	7/14 (50%)	0.13 (0.01)	3/13 (23%)	0.20 (0.03)	9/13 (69%)	0.11 (0.01)	3/14 (21%)
2.0	0.18 (0.07)	8/16 (50%)	0.19 (0.02)	11/16 (69%)	0.16 (0.02)	4/12 (33%)	0.10 (0.01)	2/16 (13%)
10.0	0.15 (0.09)	6/14 (43%)	0.18 (0.02)	8/15 (53%)	0.156 (0.01)	5/13 (39%)	0.09 (0.01)	2/15 (13%)
DAP MIC (ug/mL)								
0.0	1.44 (0.66)	0/13 (0%)	1.18 (0.2)	0/13 (0%)	1.89 (0.35)	0/12 (0%)	1.63 (0.29)	0/13 (0%)
0.5	1.43 (0.51)	0/14 (0%)	1.02 (0.18)	0/13 (0%)	1.39 (0.24)	0/13 (0%)	1.75 (0.30)	1/14 (7%)
2.0	2.19 (1.97)	1/16 (6%)	1.68 (0.2)	0/16 (0%)	1.54 (0.28)	0/12 (0%)	1.75 (0.28)	0/16 (0%)
10.0	0.80 (0.33)	0/14 (0%)	1.61 (0.28)	0/15 (0%)	1.34 (0.25)	0/13 (0%)	1.96 (0.35)	1/15 (7%)
ERY MIC (ug/mL)								
0.0	0.83 (1.15)	0/13 (0%)	0.65 (0.17)	0/13 (0%)	0.52 (0.14)	1/12 (8%)	0.38 (0.10)	0/13 (0%)
0.5	0.29 (0.09)	0/14 (0%)	0.78 (0.21)	0/13 (0%)	0.48 (0.13)	0/13 (0%)	0.33 (0.08)	0/14 (0%)
2.0	0.78 (1.93)	1/16 (6%)	0.39 (0.09)	0/16 (0%)	0.45 (0.12)	0/12 (0%)	0.36 (0.08)	0/16 (0%)
10.0	1.09 (2.06)	1/14 (7%)	0.47 (0.12)	1/15 (7%)	0.69 (0.19)	2/13 (15%)	0.47 (0.12)	0/15 (0%)
CIP MIC (ug/mL)								
0.0	0.58 (0.36)	0/13 (0%)	0.54 (0.09)	0/13 (0%)	0.62 (0.10)	0/12 (0%)	0.44 (0.07)	0/13 (0%)
0.5	0.30 (0.11)	0/14 (0%)	0.67 (0.11)	0/13 (0%)	0.39 (0.06)	0/13 (0%)	0.36 (0.05)	0/14 (0%)
2.0	0.30 (0.10)	0/16 (0%)	0.45 (0.06)	0/16 (0%)	0.41 (0.07)	0/12 (0%)	0.38 (0.05)	0/16 (0%)

CTC Study Groups (mg)	D12 Pre CTC		D58		D114		D21 Post CTC	
	Mean (SD)	Number Resistant (%)	LSMean (SEM)	Number Resistant (%)	LSMean (SEM)	Number Resistant (%)	LSMean (SEM)	Number Resistant (%)
10.0	0.70 (0.99)	1/14 (7%)	0.57 (0.09)	0/15 (0%)	0.77 (0.13)	0/13 (0%)	0.54 (0.08)	0/15 (0%)
VAN MIC (ug/mL)								
0.0	1.00 (1.08)	0/13 (0%)	1.71 (0.42)	1/13 (8%)	1.41 (0.36)	0/12 (0%)	1.31 (0.32)	0/13 (0%)
0.5	0.71 (0.55)	0/14 (0%)	1.17 (0.29)	0/13 (0%)	0.62 (0.15)	0/13 (0%)	0.74 (0.17)	0/14 (0%)
2.0	0.55 (0.43)	0/16 (0%)	0.73 (0.1)	0/16 (0%)	1.06 (0.27)	0/12 (0%)	0.70 (0.15)	0/16 (0%)
10.0	0.89 (0.67)	0/14 (0%)	0.71 (0.16)	0/15 (0%)	0.99 (0.25)	0/13 (0%)	1.28 (0.30)	0/15 (0%)
AMP MIC (ug/mL)								
0.0	3.21 (8.67)	1/13 (8%)	0.56 (0.01)	0/13 (0%)	0.42 (0.01)	0/12 (0%)	0.50 (0.08)	0/13 (0%)
0.5	0.64 (0.29)	0/14 (0%)	0.36 (0.06)	0/13 (0%)	0.72 (0.12)	0/13 (0%)	0.82 (0.13)	1/14 (7%)
2.0	0.52 (0.21)	0/16 (0%)	0.44 (0.06)	0/16 (0%)	0.48 (0.08)	0/12 (0%)	0.59 (0.09)	0/16 (0%)
10.0	0.55 (0.460)	0/14 (0%)	0.70 (0.11)	0/15 (0%)	0.47 (0.08)	0/13 (0%)	0.55 (0.09)	0/15 (0%)
SYN MIC (ug/mL)								
0.0	1.85 (2.27)	3/13 (23%)	2.22 (0.66)	5/13 (39%)	3.77 (1.17)	6/12 (50%)	1.45 (0.43)	2/13 (15%)
0.5	0.79 (0.43)	0/14 (0%)	1.52 (0.45)	0/13 (0%)	1.69 (1.35)	3/13 (23%)	1.32 (0.3)	2/14 (14%)
2.0	1.56 (1.17)	2/16 (13%)	1.05 (0.28)	0/16 (0%)	1.59 (0.4)	2/12 (17%)	2.28 (0.61)	5/16 (31%)
10.0	4.14 (8.15)	5/14 (36%)	1.93 (0.56)	3/15 (20%)	2.15 (0.67)	3/13 (23%)	1.07 (0.31)	1/15 (7%)
CHL MIC (ug/mL)								
0.0	6.462(2.03)	0/13 (0%)	6.90 (1.29)	0/13 (0%)	18.11 (3.52)	5/12 (42%)	7.28 (1.36)	1/13 (8%)
0.5	5.43 (1.99)	0/14 (0%)	4.91 (0.91)	0/13 (0%)	5.46 (1.02)	0/13 (0%)	7.92 (1.42)	3/14 (21%)
2.0	6.25 (2.05)	0/16 (0%)	8.42 (1.41)	2/16 (13%)	5.33 (1.03)	0/12 (0%)	6.49 (1.09)	1/16 (6%)
10.0	5.43 (1.99)	0/14 (0%)	7.17 (1.29)	0/15 (0%)	7.09 (1.37)	0/13 (0%)	4.16 (0.75)	0/15 (0%)
STR MIC (ug/mL)								
0.0	64.00 (0.00)	0/13 (0%)	88.13 (15.1)	0/13 (0%)	84.84 (15.1)	1/12 (8%)	92.96 (15.9)	1/13 (8%)
0.5	64.00 (0.00)	0/14 (0%)	63.85 (10.9)	0/13 (0%)	67.38 (11.5)	0/13 (0%)	78.02 (12.8)	1/14 (7%)

CTC Study Groups (mg)	D12 Pre CTC		D58		D114		D21 Post CTC	
	Mean (SD)	Number Resistant (%)	LSMean (SEM)	Number Resistant (%)	LSMean (SEM)	Number Resistant (%)	LSMean (SEM)	Number Resistant (%)
2.0	64.00 (0.00)	0/16 (0%)	64.00 (9.88)	0/16 (0%)	67.92 (12.)	0/12 (0%)	72.88 (11.2)	0/16 (0%)
10.0	64.00 (0.00)	0/14 (0%)	78.02 (12.8)	1/15 (7%)	87.39 (15.5)	1/13 (8%)	67.25 (11.1)	0/15 (0%)
LZD MIC (ug/mL)								
0.0	1.54 (0.52)	0/13 (0%)	1.09 (0.10)	0/13 (0%)	1.56 (0.15)	0/12 (0%)	1.15 (0.10)	0/13 (0%)
0.5	1.29 (0.47)	0/14 (0%)	1.32 (0.12)	0/13 (0%)	1.12 (0.10)	0/13 (0%)	1.12 (0.10)	0/14 (0%)
2.0	1.25 (0.45)	0/16 (0%)	1.44 (0.12)	0/16 (0%)	1.36 (0.13)	0/12 (0%)	1.26 (0.10)	0/16 (0%)
10.0	1.43 (0.51)	0/14 (0%)	1.55 (0.14)	0/15 (0%)	1.39 (0.13)	0/13 (0%)	1.10 (0.10)	0/15 (0%)
NIT MIC (ug/mL)								
0.0	19.08 (7.69)	0/13 (0%)	16.09 (3.38)	0/13 (0%)	21.74 (4.74)	0/12 (0%)	23.37 (4.91)	0/13 (0%)
0.5	21.71 (7.96)	0/14 (0%)	19.06 (3.99)	0/13 (0%)	24.63 (5.16)	2/13 (15%)	27.53 (5.56)	1/14 (7%)
2.0	26.00 (12.90)	0/16 (0%)	29.06 (5.53)	0/16 (0%)	20.28 (4.41)	0/12 (0%)	31.69 (6.04)	3/16 (19%)
10.0	18.29 (7.96)	0/14 (0%)	25.20 (5.12)	0/15 (0%)	34.62 (7.54)	2/13 (15%)	20.67 (4.2)	0/15 (0%)
AVL MIC (ug/mL)								
0.0	4.00 (0.00)	0/13 (0%)	4.21 (0.30)	0/13 (0%)	3.99 (0.30)	0/12 (0%)	3.99 (0.29)	0/13 (0%)
0.5	4.00 (0.00)	0/14 (0%)	3.99 (0.2)	0/13 (0%)	4.44 (0.3)	0/13 (0%)	3.99 (0.28)	0/14 (0%)
2.0	4.25 (1.00)	0/16 (0%)	4.58 (0.30)	1/16 (6%)	4.27 (0.32)	0/12 (0%)	4.38 (0.29)	0/16 (0%)
10.0	4.00 (0.00)	0/14 (0%)	4.41 (0.31)	1/15 (7%)	4.00 (0.30)	0/13 (0%)	4.63 (0.32)	1/15 (7%)

Isolates of *Enterococcus* from groups of steers provided CTC for 120 consecutive days at dose rates of 0, 0.5, 2.0, or 10.0 mg CTC/lb BW/day (*received 10.0 mg CTC for five consecutive days followed two days off medicated feed) and assessed for susceptibility to 14 *medically important* antibiotics on days: 12 days prior to CTC administration, 58 and 114 days of continuous CTC administration, and 21 days post-cessation of CTC administration.

D12 Pre CTC was used as a covariate and *GEN* MIC values were not statistically analyzed as they lacked variation therefore, their least square mean values were not calculated.

The susceptibility of antibiotics were determined using the 2023 Clinical & Laboratory Standards Institute (CLSI) breakpoints or the 2011 NARMS breakpoints.

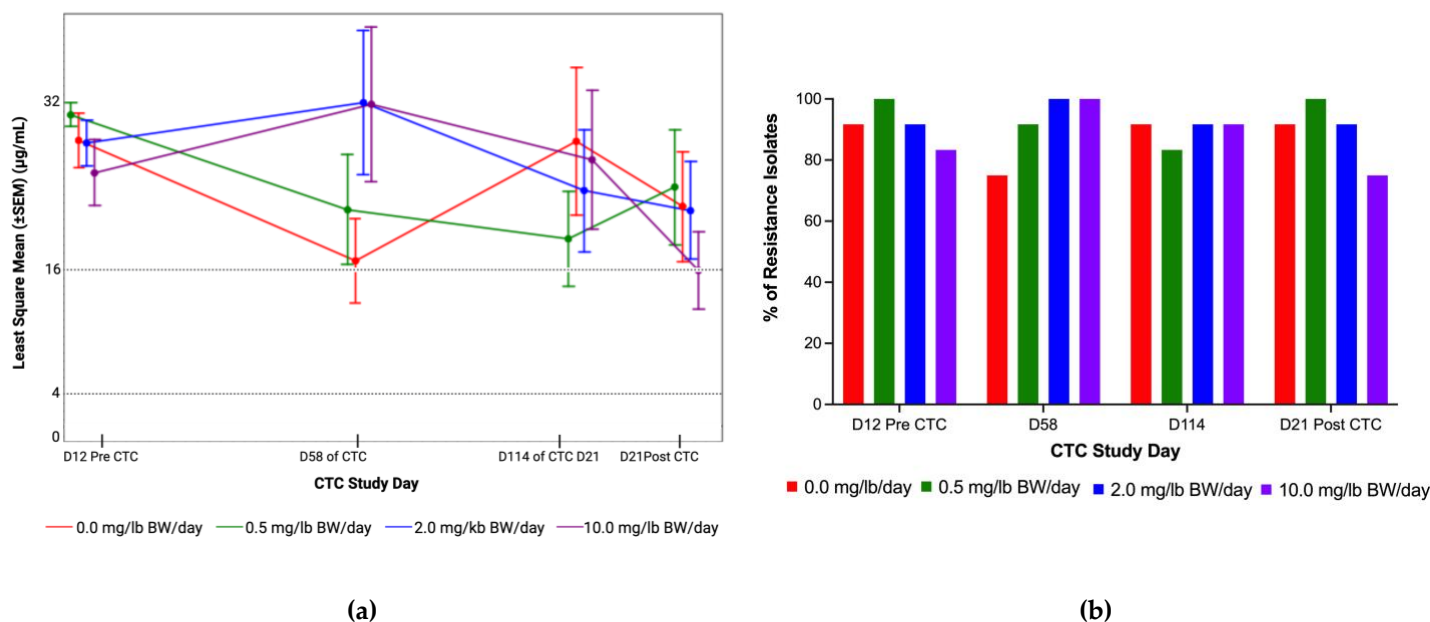


Figure 3.5. *Enterococcus* spp. tetracycline Minimum Inhibitory Concentration values from isolates collected from groups of steers administered different chlortetracycline dosages for 120 days

Isolates of *Enterococcus* from groups of steers provided CTC for 120 consecutive days at dose rates of 0, 0.5, 2.0, or 10.0 mg CTC/lb BW/day (*received 10.0 mg CTC for five successive days followed by two days off medicated feed) and assessed for susceptibility to tetracycline (TET). (a) Presented are *Enterococcus* TET minimum inhibitory concentration (MIC) values for isolates collected 12 days pre-CTC administration (Pre Tx), after 58 (D58 Tx) and 114 (D112 Tx) days of consecutive CTC administration, and 21 days post-cessation of CTC administration (D21 Post Tx) (12 *Enterococcus* isolates/study group/time point). The susceptibility of *Enterococcus* spp. to TET was determined using the NARMS CMV4AGNF plate with breakpoint classifications according to the 2023 Clinical & Laboratory Standards Institute (CLSI) breakpoints. NARMS Antibacterial Susceptibility Test Interpretive Criteria was used for tigecycline susceptible breakpoint, and an MIC value ≥ 0.5 was considered resistant. The lower dashed line represents the CLSI TET susceptibility cut-off and isolates with MIC values at or below the dashed line are considered susceptible to TET. The upper dashed line represents the CLSI TET resistance cut-off and isolates with MIC values at or above the cut-off value are considered resistant to TET. Isolates with MIC values between the lower and upper dashed lines are in the intermediate susceptible category; (b) Percentage of TET-resistant isolates (MIC ≥ 16 µg/mL) before, during, and after CTC administration.

Effect of Prolonged CTC Administration on the Susceptibility of *Enterococcus* to Non-TET Antibiotics

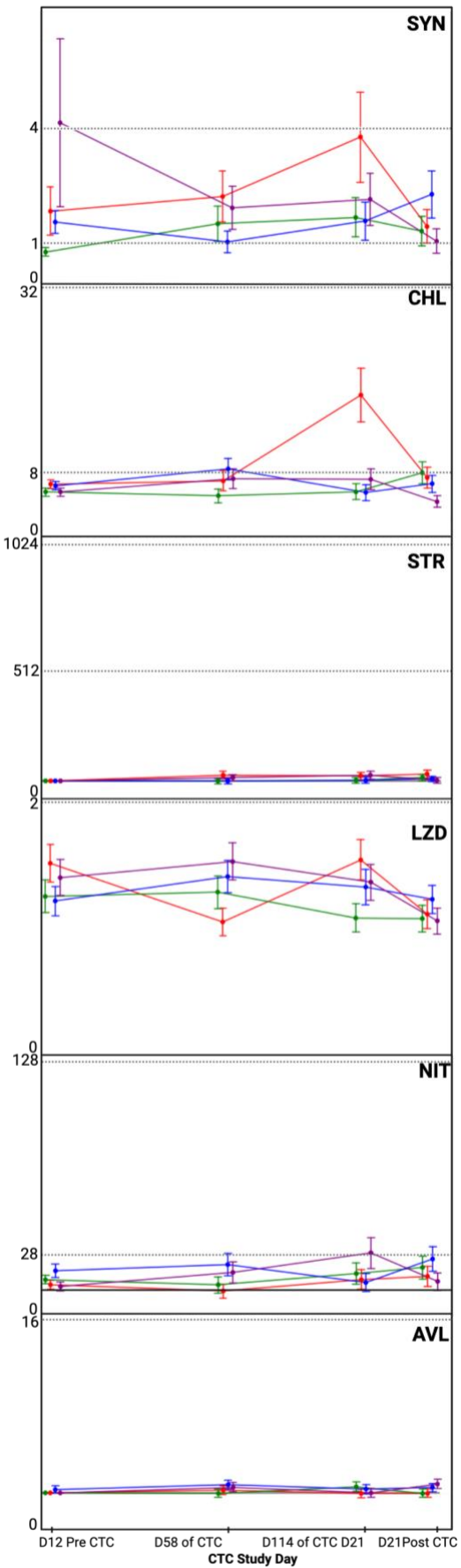
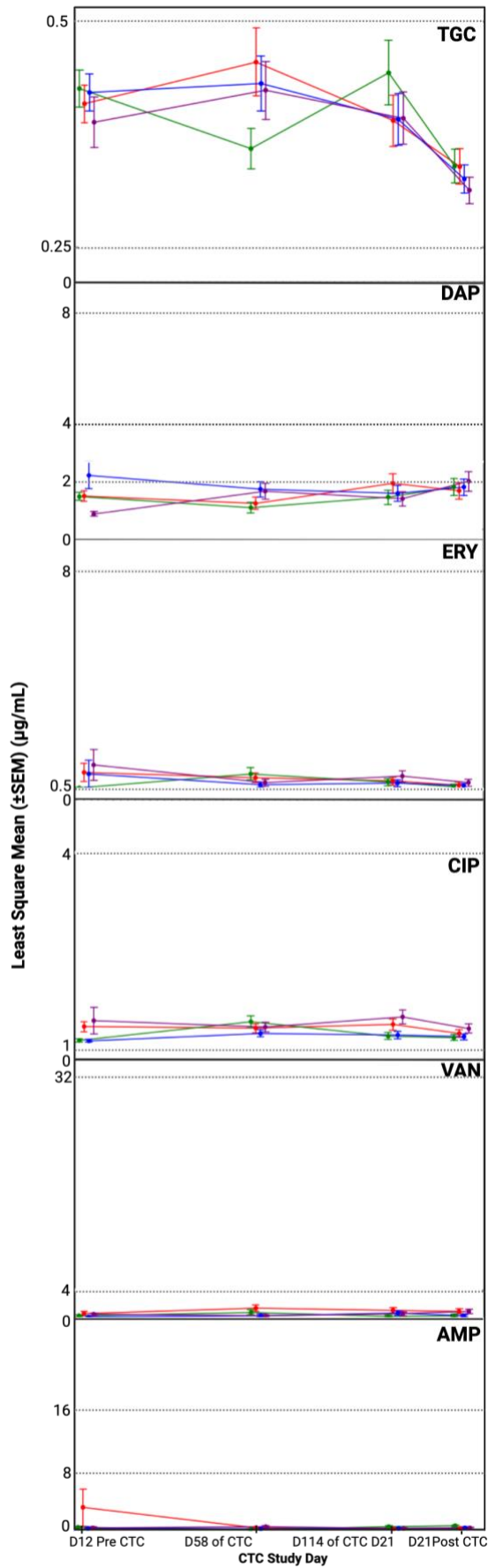
The susceptibility profiles of *Enterococcus* isolate to non-TET antibiotics were also assessed. There was an overall CTC dose effect on LSMean (SEM) for CIP, VAN, and CHL; a study day effect on TGC, DAP, ERY, CIP, and LZD and an interaction effect for AMP, CHL,

and LZD on LSMean (SEM) MIC values was observed. Detailed descriptions of median MIC values between CTC study groups and across the CTC administration period are presented below.

Tigecycline (TGC). Overall, there was no CTC effect on TGC LSMean (SEM) MIC values (p-value = 0.8674); however, there was a study day effect (p-value <0.0001). No CTC and study day interaction effect was observed (p-value = 0.1222).

Prior to CTC administration, median (SD) TGC MIC values for each group were below the resistant breakpoint ($\geq 0.5 \mu\text{g/mL}$) (Table 3-7. Antibiotic minimum inhibitory concentrations of *Enterococcus* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days). During the entire study, the LSMean (SEM) for CTC groups 0.0 mg, 0.5 mg, and 2.0 mg changed slightly in no orderly fashion. In comparison, those for the 10.0 mg group increased slightly during the CTC administration period and decreased slightly post-cessation of CTC administration. There was no significant CTC effect on day 114 of continuous CTC administration (p-value = 0.2187) and day 21 post-cessation of CTC administration (p-value = 0.7542). Similarly, no significant CTC effect was observed on day 58 of continuous CTC administration however, pairwise comparisons revealed the LSMean (SEM) for CTC group 0.0 mg was significantly higher than the 0.5 mg group (p-value = 0.0226), the LSMean (SEM) for the 2.0 mg group was significantly higher than the 0.5 mg group (p-value = 0.0550) and the LSMean (SEM) for 0.5 mg group was significantly higher than the 10.0 mg group (p-value = 0.0938) (Figure 3.6. Comparison of *Enterococcus* antibiotic MIC least square mean values from isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days, (a), top panel).

Prior to CTC administration, the percentage of TGC-resistant isolates for the study groups ranged between 38% and 50% (Table 3-7. Antibiotic minimum inhibitory concentrations of *Enterococcus* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days). During CTC administration and after continuous administration CTC, the percentage of TGC-resistant isolates changed (increased or decreased) in no orderly fashion.



(a)

(b)

Figure 3.6. Comparison of *Enterococcus* antibiotic MIC least square mean values from isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days

Isolates of *Enterococcus* from groups of steers provided CTC for 120 consecutive days at dose rates of 0, 0.5, 2.0, or 10.0 mg CTC/lb BW/day (*received 10.0 mg CTC for five consecutive days followed by two days off medicated feed). The isolates were assessed for antibiotic susceptibility at 12 days pre-CTC administration (D12 Pre CTC, after 58 and 114 days of consecutive CTC administration, and 21 days post-cessation of CTC administration (12 *Enterococcus* isolates/study group/time point). Antibiotic susceptibility was determined using the 2021 Clinical & Laboratory Standards Institute (CLSI) breakpoints or 2011 NARMS breakpoints: (a) MIC least square mean values for tigecycline (TGC), daptomycin (DAP), erythromycin (ERY), ciprofloxacin (CIP), vancomycin (VAN) and ampicillin (AMP); (b) MIC least square mean values for quinupristin/dalfopristin (SYN), chloramphenicol (CHL), streptomycin (STR), linezolid (LZD), nitrofurantoin (NIT) and avilamycin (AVL). In each graph, the lower dashed line represents the CLSI or NARMS antibiotic susceptibility cut-off and isolates with MIC values at or below the dashed line are considered susceptible to that antibiotic. The upper dashed line represents the CLSI antibiotic resistance cut-off value, and MIC values at or above that line are considered resistant to that antibiotic. DAP has 2 upper dashed lines depending on species. Isolates with MIC values between the lower and upper dashed lines are in the intermediate susceptible category.

Daptomycin (DAP). Overall, no CTC effect on DAP LSMean (SEM) MIC values was observed (p-value = 0.5614) however, there was a study day effect (p-value = 0.0723). There was no interaction effect on DAP LSMean (SEM) MIC values (p-value = 0.3857).

Prior to CTC administration, median (SD) DAP MIC values were all below the resistant breakpoint value (≥ 8.0 $\mu\text{g/mL}$) (Table 3-7. Antibiotic minimum inhibitory concentrations of *Enterococcus* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days). The LSMean (SEM) MIC value for the 0.0 mg group had subtle increases during the CTC administration period and a slight decrease on day 21 post-cessation of CTC administration. The rest of the study groups experienced slight increases or decreases during the CTC administration period and slight increases or decreases on day 21 post-cessation of CTC administration. No significant CTC effects were observed on study days 58 (p-value = 0.2270) and 114 of continuous CTC administration. (p-value = 0.5539), or on day 21 post-CTC administration (p-value = 0.8896). However, on day 58 of continuous CTC

administration, the LSMean (SEM) MIC for CTC groups 2.0 mg and 10.0 mg were significantly higher than that of the 0.5 mg group (p-values = 0.0428 and 0.0787, respectively) (Figure 3.6. Comparison of *Enterococcus* antibiotic MIC least square mean values from isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days, (a) second panel from the top). The LSMean (SEM) MIC values remained below the resistant breakpoint value throughout the rest of the study.

Prior to CTC administration, only a single isolate from the 2.0 mg group was DAP-resistant (Table 3-7. Antibiotic minimum inhibitory concentrations of *Enterococcus* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days). There were no DAP-resistant isolates during CTC administration period. However, there was one DAP-resistant isolate in group 0.5. mg and one in group 10.0 mg on day 21 post-cessation of CTC administration.

Erythromycin (ERY). Overall, there was no CTC effect on ERY LSMean (SEM) MIC values (p-value = 0.4981); however, there was a stud day effect (p-value = 0.0987). There was also no interaction effect on ERY LSMean (SEM) MIC values (p-value = 0.6180).

Prior to CTC administration and throughout the rest of the study, the median (SD) ERY MIC values for all study groups were within the intermediate susceptible category (1.0-4.0 µg/mL) (Figure 3.6. Comparison of *Enterococcus* antibiotic MIC least square mean values from isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days, (a) third panel from the top). Throughout the study period, subtle changes in LSMean (SEM) MIC values were observed for each study group. No CTC effects were observed on either the study days: day 58 of continuous CTC administration (p-value 0.31.91), day 114 of continuous CTC administration (p-value = 0.8504), and on day 21 post-cessation of CTC

administration (p-value = 0.6824). However, on day 58 of continuous CTC administration, pairwise comparisons showed that the LSMean (SEM) MIC value for CTC group 0.5 mg was significantly higher than that for the 2.0 mg group (p-value = 0.0595) (Figure 3.6. Comparison of *Enterococcus* antibiotic MIC least square mean values from isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days (a), third panel from the top).

Group 0.5 mg had no ERY-resistant isolates throughout the study (Table 3-7. Antibiotic minimum inhibitory concentrations of *Enterococcus* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days). However, CTC groups 0.0 mg and 2.0 mg each had a single ERY-resistant isolate, isolated on day 114 of continuous CTC administration and prior to CTC administration, respectively. The CTC group 10.0 mg had at least one ERY-resistant isolate prior to CTC administration and during CTC administration but none on day 21 post-cessation of CTC administration.

Ciprofloxacin (CIP). There was an overall CTC and study day effect on CIP LSMean (SEM) MIC values (p-values = 0.0434 and 0.0451, respectively). No interaction effect was found (p-value = 0.2379).

Prior to, during, and after continuous CTC administration, the median (SD) or LSMean (SEM) CIP MIC values were all below the susceptible breakpoint value (≤ 1.0 $\mu\text{g/mL}$) (Table 3-7. Antibiotic minimum inhibitory concentrations of *Enterococcus* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days). Slight increases in LSMean (SEM) MIC values were observed on one or both days of CTC administration for all study groups (Table 3-7. Antibiotic minimum inhibitory

concentrations of *Enterococcus* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days and Figure 3.6. Comparison of *Enterococcus* antibiotic MIC least square mean values from isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days (a), fourth panel from the top). On day 58 of continuous CTC administration, there was no significant CTC effect (p-value = 0.2631) on the LSMean (SEM) MIC value; however, pairwise comparisons revealed the LSMean (SEM) MIC value for CTC group 0.5 was statistically higher than that of the 2.0 mg group (p-value = 0.0714). On day 114 of continuous CTC administration, a significant CTC effect was observed (p-value = 0.0399), and the LSMean (SEM) for CTC group 0.0 mg was statistically higher than that for the 0.5 mg group (p-value = 0.0713). In addition, the LSMean (SEM) for CTC group 10.0 mg was statistically higher than those for the 0.5 and 2.0 mg groups (p-values = 0.0072 and 0.0138, respectively). On day 21 post cessation of CTC administration, no significant CTC effect was observed (p-value = 0.5404). On that day, there was a slight decrease in LSMean (SEM) MIC value for all study groups, and the LSMean (SEM) MIC value for CTC group 10.0 mg was significantly higher than that for the 0.5 mg group (p-value = 0.0904) (Figure 3.6. Comparison of *Enterococcus* antibiotic MIC least square mean values from isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days (a) fifth panel from the top and Table 3-7. Antibiotic minimum inhibitory concentrations of *Enterococcus* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days).

Throughout the study, there were no CIP-resistant isolates except prior to CTC administration and a single CIP-resistant isolate in the study group 10.0 mg (Table 3-7. Antibiotic minimum inhibitory concentrations of *Enterococcus* isolates from cattle handfed

chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days).

Vancomycin (VAN). Overall, there was a CTC effect on VAN LSMean (SEM) MIC values (p-value = 0.0072). However, there was no study day effect or CTC and study day interaction effect on VAN LSMean (SEM) MIC values (p-values = 0.9662 and 0.2267, respectively).

Prior to CTC administration and throughout the rest of the study, the median (SD) or LSMean (SEM) VAN MIC values were all below the susceptible breakpoint value (≤ 4.0 $\mu\text{g/mL}$) (Table 3-7. Antibiotic minimum inhibitory concentrations of *Enterococcus* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days). There were subtle changes (increases and decreases) in LSMean (SEM) CIP MIC values throughout the study for all CTC groups in no orderly fashion (Figure 3.6. Comparison of *Enterococcus* antibiotic MIC least square mean values from isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days (a), fifth panel from the top and Table 3-7. Antibiotic minimum inhibitory concentrations of *Enterococcus* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days). Although no CTC effect was observed on day 58 of continuous CTC administration (p-value = 0.2631), pairwise comparisons found the LSMean (SEM) VAN MIC values for CTC group 0.0 mg to be statistically higher than those for the 2.0 and 10.0 mg groups (p-values = 0.0136 and 0.0122, respectively). Likewise, no CTC effect was observed on day 114 of continuous CTC administration (p-value = 0.0399); however, pairwise comparisons revealed that the LSMean (SEM) MIC values for CTC group 0.5 mg were statistically higher than those for the 0.0 mg group (p-value = 0.0232). On day 21 ,post-cessation

of CTC administration, there was a significant CTC effect on LSMean (SEM) VAN MIC values (p-values = 0.0199). On that day, the LSMean (SEM) MIC for CTC group 0.0 mg was statistically higher than that for the 2.0 mg group. (p-value = 0.674) while that for the 10.0 mg group was statistically higher than that for the 2.0 mg group (p-values = 0.0704).

Throughout the study period, only a single VAN-resistant isolate was found in the 0.0 mg group on day 114 of continuous CTC administration (Table 3-7. Antibiotic minimum inhibitory concentrations of *Enterococcus* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days).

Ampicillin (AMP). Overall, there were no CTC or study day effect on AMP LSMean (SEM) MIC values (p-values = 0.5178 and 0.1661, respectively). However, there was a CTC and study day interaction effect (p-value = 0.0080).

Throughout the study period, the median (SD) or LSMean (SEM) AMP MIC values were all below the susceptible breakpoint value (≤ 8.0 $\mu\text{g/mL}$) (Table 3-7. Antibiotic minimum inhibitory concentrations of *Enterococcus* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days). There were increases and decreases in LSMean (SEM) MIC values in all study groups during and after the CTC administration period, and in no orderly fashion (Table 3-7. Antibiotic minimum inhibitory concentrations of *Enterococcus* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days and Figure 3.6. Comparison of *Enterococcus* antibiotic MIC least square mean values from isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days (a), bottom panel). A significant CTC effect was observed on day 58 of continuous CTC administration (p-value = 0.0118), and the LSMean (SEM) MIC value for CTC group 0.5 mg was significantly higher than

that of the 0.0 mg group (p-value = 0.797). In addition, the LSMean (SEM) MIC for the CTC group 10.0 mg was statistically higher than that for the 0.5 and 2.0 mg groups (p-values = 0.0069 and 0.0394, respectively). Similarly, there was a significant CTC effect on day 114 of continuous CTC administration (p-value = 0.0236), and the LSMean (SEM) MIC value for CTC group 0.5 mg was significantly higher than that of the 0.0, 0.5, and 10.0 mg groups (p-values = 0.336, 0.0996 and 0.0934, respectively). There was no significant CTC effect on day 21 post-cessation of CTC administration (p-value = 0.4252); however, pairwise comparisons found the LSMean (SEM) MIC value for CTC group 0.5 mg to be statistically higher than that of the 0.0 and 10.0 mg groups (p-values = 0.0461 and 0.0926, respectively).

Throughout the study period, only two AMP-resistant isolates were found: one from the 0.0 mg group prior to CTC administration and the second from CTC group 0.5 mg on day 21 post-cessation of CTC administration (Table 3-7. Antibiotic minimum inhibitory concentrations of *Enterococcus* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days).

Quinupristin/dalfopristin (SYN). Overall, no CTC, study day, or their interaction effect was observed on SYN LSMean (SEM) MIC values (p-values = 0.2266, 0.1925 and 0.1919, respectively).

Prior to CTC administration, the median (SD) SYN MIC values were below the resistant breakpoint value (≤ 4.0 $\mu\text{g/mL}$) for all groups except for CTC group 10.0 mg (Table 3-7. Antibiotic minimum inhibitory concentrations of *Enterococcus* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days). Least square Mean (SEM) MIC value increases were observed during the CTC administration period for most groups (Table 3-7. Antibiotic minimum inhibitory

concentrations of *Enterococcus* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days). There were no CTC effects on study days 58 and 114 of continuous CTC administration or on day 21 post-CTC administration (p-values = 0.1959, 0.1260, and 0.3741, respectively). However, pairwise comparisons found the LSMean (SEM) MIC value for CTC group 0.0 mg to be statistically higher than that for the 2.0 mg group (p-value = 0.0641) on study day 58 of continuous CTC administration and statistically higher than those for CTC groups 0.5 and 2.0 mg (p-values = 0.0656 and 0.0513, respectively) on day 114 of continuous CTC administration (Figure 3.6. Comparison of *Enterococcus* antibiotic MIC least square mean values from isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days (b), top panel). On day 21 post-cessation of CTC administration, decreases in LSMean (SEM) MIC values were observed for some groups; however, the only statistical LSMean (SEM) MIC value differences were observed between CTC groups 2.0 mg and 10.0 mg (p-value = 0.0578) with that for the 2.0 mg group being higher.

Throughout the study period, SYN-resistant isolates were isolated from all groups (except from the 0.5 mg group prior to CTC administration), and these changed in no orderly fashion (Table 3-7. Antibiotic minimum inhibitory concentrations of *Enterococcus* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days).

Chloramphenicol (CHL). Overall, a CTC effect on CHL LSMean (SEM) MIC values was observed (p-value = 0.0072), but no study day effect (p-value = 2381). Additionally, there was a CTC and study day interaction effect on CHL LSMean (SEM) MIC values (p-value = 0.0005).

Prior to CTC administration, the median (SD) CHL MIC values for each group were below the susceptible breakpoint value ($\leq 8.0 \mu\text{g/mL}$) (Table 3-7. Antibiotic minimum inhibitory concentrations of *Enterococcus* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days). There was a significant CTC effect on day 58 of continuous CTC administration Figure 3.6. Comparison of *Enterococcus* antibiotic MIC least square mean values from isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days, (b) second panel from the top). On that day, LSMean (SEM) MIC values for CTC groups 0.0, 2.0, and 10.0 mg slightly increased, with that for the 2.0 mg group crossing the susceptible breakpoint value and being significantly higher than that for the 0.5 mg group (p-value = 0.0349). A more significant CTC effect was observed on study day 114 of continuous CTC administration (p-value = 0.0001). On that day, the LSMean (SEM) MIC values for groups 0.0 and 0.5 mg further increased, and those for the 0.0 mg group were above the intermediate susceptible breakpoint value ($16.0 \mu\text{g/mL}$) and significantly higher than that for the 0.5, 0.5 and 2.0 mg groups (p-values < 0.0001, <0.0001 and 0.0010, respectively). On that day, the LSMean (SEM) MIC values for the rest of the groups slightly decreased. No CTC effect was observed on day 21 post-CTC administration (p-value = 0.1445). On that, the LSMean (SEM) MIC value for CTC groups 0.0 and 10.0 mg had a slight decrease, and pairwise comparisons showed that the LSMean (SEM) MIC value for CTC group 10.0 mg was significantly lower than those for the 0.0, 0.5 and 2.0 mg groups (p-values = 0.0343, 0.0131 and 0.0750, respectively). On that day, all LSMean (SEM) MIC values for all CTC groups were below the susceptible breakpoint value.

There were no CHL-resistant isolates prior to CTC administration. On day 58 of continuous CTC administration, there were 13% CHL-resistant isolates in CTC group 2.0 mg

and 42% in CTC group 0.0 mg on day 114 of continuous CTC administration (Table 3-7. Antibiotic minimum inhibitory concentrations of *Enterococcus* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days). On day 21 post-cessation of CTC administration, each group had at least one CHL-resistant isolate, except for the CTC group 10.0 mg.

Streptomycin (STR). Overall, no CTC, study day, or their interaction effect on STR LSMean (SEM) MIC values were observed (p-values = 0.3193, 0.8504, and 0.8699, respectively).

Prior to CTC administration, the median (SD) STR MIC values for all CTC groups was 64.0 µg/mL, below the susceptible breakpoint value (≤ 512.0 µg/mL) (Table 3-7. Antibiotic minimum inhibitory concentrations of *Enterococcus* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days). During the CTC administration period and on day 21 post-cessation of CTC administration, there were increases in LSMean (SEM) MIC for most of the CTC groups, and all remained below the susceptible breakpoint value. There was no CTC effect on LSMean (SEM) MIC values throughout the study (p-values = 0.3100, 0.7278 and 0.5672, respectively) (Figure 3.6. Comparison of *Enterococcus* antibiotic MIC least square mean values from isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days, (b) third panel from the top). There were no STR-resistant isolates prior to CTC administration (Table 3-7. Antibiotic minimum inhibitory concentrations of *Enterococcus* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days). On day 58 of continuous CTC administration, there was only a single STR-resistant isolate, which was in the 10.0 mg group. On day 114 of continuous

CTC administration, there was a single STR-resistant in groups 0.0 and 10.0 mg. On day 21, post-cessation of CTC administration, there was a single STR-resistant isolate in groups 0.0 and 0.5 mg.

Linezolid (LZD). Overall, there was no significant CTC effect on LZD LSMean (SEM) MIC values (p-value = 0.2711) however, there was a study day effect and a CTC and study day interaction effect (p-values = 0.0137 and 0.0892, respectively).

Prior to, during, and after CTC administration, the median (SD) or LSMean (SEM) STR MIC values for all CTC groups were below the susceptible breakpoint value (≤ 2.0 $\mu\text{g/mL}$) (Table 3-7. Antibiotic minimum inhibitory concentrations of *Enterococcus* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days). Throughout the study period, there were LSMean (SEM) MIC value changes in no orderly fashion (Table 3-7. Antibiotic minimum inhibitory concentrations of *Enterococcus* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days). There was a significant CTC effect on day 58 of continuous CTC administration (p-value = 0.0321), and the LSMean (SEM) MIC values for CTC groups 2.0 and 10.0 mg were significantly higher than that for the 0.0 mg group (p-values = 0.0350 and 0.0089, respectively) while that for the 0.0 mg group was significantly higher than that for the 0.5 mg group (p-value = 0.0175) (Figure 3.6. Comparison of *Enterococcus* antibiotic MIC least square mean values from isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days, (b) fourth panel from the top). There were no significant CTC effects on study day 114 of continuous CTC administration and on day 21 post-CTC administration (p-values = 0.1657 and 0.7429, respectively).

There were no LZD-resistant isolates throughout the study (Table 3-7. Antibiotic minimum inhibitory concentrations of *Enterococcus* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days).

Nitrofurantoin (NIT). Overall, there was no CTC, study day, or an interaction effect on NIT LSMean (SEM) MIC values (p-values = 0.2291, 0.5480 and 0.3264, respectively).

Prior to CTC administration, the median (SD) NIT MIC values were below the susceptible breakpoint value (≤ 32.0 $\mu\text{g/mL}$) (Table 3-7. Antibiotic minimum inhibitory concentrations of *Enterococcus* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days). There was a CTC effect on day 58 of continuous CTC administration (p-value = 0.0928) however, the LSMean (SEM) MIC values between the groups were statically similar (Table 3-7. Antibiotic minimum inhibitory concentrations of *Enterococcus* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days). The LSMean (SEM) MIC values changed in no orderly fashion during the CTC administration period. The LSMean (SEM) MIC value for CTC group 10.0 mg crossed the susceptible breakpoint value during this period. On day 21, post-cessation of CTC administration, the LSMean (SEM) MIC values changed in no orderly fashion (some increased and some decreased) and were all below the susceptible breakpoint value. No CTC effects were observed on days 114 of continuous CTC administration and on day 21 post-cessation of CTC administration (p-values = 0.3095 and 0.5279, respectively) (Figure 3.6. Comparison of *Enterococcus* antibiotic MIC least square mean values from isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days, (b) fifth panel from the top).

There were no NIT-resistant isolates prior to CTC administration or on day 58 of continuous CTC administration (Table 3-7. Antibiotic minimum inhibitory concentrations of *Enterococcus* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days). On days 114 of continuous CTC administration, there were 15% NIT-resistant isolates in both CTC groups 0.5 and 10.0 mg and 7% and 19% NIT-resistant isolates in CTC groups 0.5 and 2.0 mg, respectively on day 21 post-cessation of CTC administration.

Avilamycin (AVL). Overall, there was no CTC, study day, or an interaction effect on AVL LSMean (SEM) MIC values (p-values = 0.4427, 0.8674 and 0.7000, respectively).

Prior to, during, and after cessation of CTC administration, the median (SD) or LSMean (SEM) AVL MIC values were all below the resistant breakpoint value (≤ 16.0 $\mu\text{g/mL}$) (Table 3-7. Antibiotic minimum inhibitory concentrations of *Enterococcus* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days). There were no CTC effects throughout the study period (p-values = 0.6311, 0.3245, and 0.5020, respectively), and only subtle LSMean (SEM) MIC value changes were observed (Table 3-7. Antibiotic minimum inhibitory concentrations of *Enterococcus* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days).

Prior to CTC administration and on day 114 of continuous CTC administration, there were no AVL-resistant isolates (Table 3-7. Antibiotic minimum inhibitory concentrations of *Enterococcus* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days). Table 3-7. Antibiotic minimum inhibitory concentrations of *Enterococcus* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-,

2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days). On day 58 of continuous CTC administration, there were two AVL-resistant isolates, one from CTC group 2.0 and the other from 10.0 mg. On day 21 post-cessation of CTC administration, there was a single AVL-resistant isolate from CTC group 10.0 mg.

Discussion

This study aimed to assess the effects of long-term use of chlortetracycline to control bovine anaplasmosis (doses 0.5 mg/lb BW/day to 2.0 mg/lb BW/day) on the antibiotic susceptibility profiles of *Enterococcus* species to a panel of 14 medically important antibiotics.

In the present study, CTC administration was not associated with increased isolation of TET-resistant *Enterococcus* isolates (Table 3-7. Antibiotic minimum inhibitory concentrations of *Enterococcus* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days and

Figure 3.6. Comparison of *Enterococcus* antibiotic MIC least square mean values from isolates collected from groups of steers administered different chlortetracycline (CTC) dosages for 120 days). It is, however, important to note that most isolates were TET-resistant prior to CTC administration, and most MIC values were already beyond what the Sensititre assay could capture (Table 3-7. Antibiotic minimum inhibitory concentrations of *Enterococcus* isolates from cattle handfed chlortetracycline (CTC) at 0.0-, 0.5-, 2.0-, and 10.0 mg/lb BW/day CTC doses for 120 consecutive days). These results were different from those reported in another study where beef calves were administered about 10.0 mg/lb BW/day CTC for 15 days, broken up into three administration periods consisting of five consecutive days, and interspaced by one day isolates, CTC administration was associated with increased recovery of TET resistance in *Enterococcus*

isolates in a transient manner [302]. However, this observation was made in the CTC exposed and unexposed control group and no significant difference in TET-resistance between the exposed and control groups was observed. The small sample size in the present study (222 isolates) or that only a single isolated from each sample was analyzed, may have attributed to the differences observed.

Although not statistically analyzed due to the small sample size, TET resistance was more common in *E. hirae* (135, 100% isolates), *E. durans* (14, 100% isolates), and *E. casseliflavus* (13, 100% isolates) than in *E. mundtii* (29, 66% isolates) and *E. casseliflavus* (29, 66% isolates) (Figure 3.3. Antibiotic-resistant *Enterococcus* isolates from cattle handfed chlortetracycline at varying doses for 120 consecutive days to a panel of 14 antibiotics). This general observation was also true at a CTC group level, where only *E. casseliflavus* and *E. hirae* were assessed due to a small sample size of the other species at a CTC group level. However, there was an exception for CTC group 0.5 mg where all *E. hirae* and *E. casseliflavus* isolates where all isolates (100%) were TET-resistant (Figure 3.3. Antibiotic-resistant *Enterococcus* isolates from cattle handfed chlortetracycline at varying doses for 120 consecutive days to a panel of 14 antibiotics). The same study reported a species variation in TET resistance (p-value = 0.01) [302]. However, the number of isolates per species in that study: *E. durans* (9, 100% isolates), *E. hirae* (35, 39.3% isolates), *E. faecium* (25, 25.8% isolates) and *E. casseliflavus* (8, 10.1% isolates), were considerably larger than those in the present study where species other than *E. hirae* were severely underrepresented (Figure 3.1. Proportion of 222 *Enterococcus* isolates from cattle handfed chlortetracycline at varying doses for 120 consecutive days to a panel of 14 antibiotics) [302].

Overall, two non-TET MIC change patterns were observed throughout the study: MIC values that did not change or only slightly changed throughout the study (DAP, ERY, CIP, VAN, AMP, STR, AVL, and GEN) and MIC values that had intermediate MIC changes (TGC, SYN, CHL, LZD, and NIT) (Figure 5). Of these, overall CTC effects were only observed for CIP (p-value = 0.0434), VAN (p-value = 0.0072), and CHL (p-value = 0.0072). The rest of the antibiotics had significant CTC effects only one study day, and the p-values ranged between 0.0118 and 0.928. Some CTC groups that did not have any CTC effects had significant differences in pairwise comparisons. However, these may have been a result of study day effects. All of the observed significant non-TET MIC changes were inconsistent with CTC administration dose or administration days. In other words, we expected MIC values to increase transiently, with increases being observed during the CTC administration period and decreases post-cessation of CTC administration. Moreover, we expected these changes to be more pronounced in groups that received higher CTC doses. Instead, we found random CTC effects on mostly one of the CTC administration days, and high MIC values observed in lower CTC groups than in higher dose CTC groups. Therefore, the observed CTC effects may have occurred by chance. A possible reason for this could be the limited sample size. The small sample size was worsened even more by the presence of different *Enterococcus* species that have been shown to vary in their antibiotic susceptibilities [342]. In addition, number of antibiotics the isolates were resistant to did not significantly vary between CTC groups or between study days (**Error! Reference source not found.**).

Overall, eight *Enterococcus* species were isolated: *E. hirae* (135, 60.8% isolates), *E. casseliflavus* (44, 19.8% isolates), *E. durans* (14, 6.3% isolates), *E. faecium* (13, 5.9% isolates), *E. mundtii* (11, 5.0% isolates), *E. gallinarum* (3, 1.4% isolates), *E. aquimarinus* (1, 0.5%

isolates) and *E. avium* (1, 0.5% isolates) (Figure 3.1. Proportion of 222 *Enterococcus* isolates from cattle handfed chlortetracycline at varying doses for 120 consecutive days to a panel of 14 antibiotics). Similar species and general proportions of *Enterococcus* species have been documented by others with minor differences: For example, an *Enterococcus* species distribution and antibiotic resistance observational study on dairy cattle reported *E. hirae* as being the most prevalent species, *E. mundtii* and *E. casseliflavus* having an intermediate prevalence and *E. avium* and *E. gallinuram* being the least prevalent [343]. In that study however, they also isolated the *Enterococcus* species not isolated in the current study. These include *E. faecalis* and *E. falesccens*. These differences could be a result of the differences in bacterial isolation used in that study compared to the current study. In that study, all *Enterococcus* isolates were evaluated as opposed to the present study, where only a single *Enterococcus* colony per sample was isolated. The slight differences could also be influenced by the age and type of cattle (type of production). Dairy cattle and beef cattle may have slight differences in *Enterococcus* species distribution. In the current study, the proportion of *Enterococcus* species isolated per CTC group did not vary much (Figure 3.3. Antibiotic-resistant *Enterococcus* isolates from cattle handfed chlortetracycline at varying doses for 120 consecutive days to a panel of 14 antibiotics). However, the CTC group 10.0 mg had more species isolated (eight) than the rest of the groups, which had six, six, and five species isolated for CTC groups 0.0 mg, 0.5 mg, and 2.0, respectively (Figure 3.1. Proportion of 222 *Enterococcus* isolates from cattle handfed chlortetracycline at varying doses for 120 consecutive days to a panel of 14 antibiotics). In the present study, 210 (94.6%) *Enterococcus* isolates were resistant to at least one antibiotic. This proportion did not vary greatly between the CTC groups: 0.0 mg (49/51, 96.1% isolates), 0.5 mg (51/54, 94.4% isolates), 2.0 mg (48/60, 96.7% isolates) and 10.0 mg group (52, 91.2% of

isolates) (**Error! Reference source not found.**). This was similar to another study where beef calves were administered about 10.0 mg/lb BW/day CTC for 15 days [302]. That study reported that 330 (90.2%) isolates were resistant to at least one antibiotic, and that they found no significant difference between the number of *Enterococcus* isolates resistant to at least one antibiotic from the CTC exposed group (170, 90.9% isolates) and non-exposed group (160, 89.4% isolates).

Seven multidrug-resistant phenotypes were observed in the present study. Three of these comprised three antibiotic combinations: TET-CHL-AVL (1, 0.5% isolates), TET-ERY-STR (1, 0.5% isolates), TET-SYN-CHL (4, 1.8% of isolates), TET-SYN-NIT (2, 0.9% isolates) and TET-TGC-CHL (1, 0.5% isolates) (**Error! Reference source not found.**). The remaining three multidrug-resistant phenotypes comprised four antibiotic combinations: TET-ERY-CIP-SYN (1, 0.5% isolates) and TET-ERY-SYN-STR (1, 0.5% isolates). This percentage of multidrug resistance is considerably less than what was observed in a similar study [302]. That study isolated 112 (30.6%) *Enterococcus* isolates resistant to three antibiotics, 42 (11.5%) *Enterococcus* isolates resistant to four antibiotics, and 31 (5.8%) *Enterococcus* isolates resistant to five or more antibiotics. The proportion of multidrug-resistant profiles in the present study was not dissimilar between groups: CTC group 0.0 mg (3, 5.9% isolates), CTC group 0.5 mg (3, 5.6% isolates) and CTC group 2.0 mg (3, 5.0% isolates) but was slightly lower for CTC group 10.0 mg (2, 3.5% isolates) (Table 2). One isolate (0.5%), *E. casseliflavus*, was VAM-resistant. This was similar to that study, which found three (0.8%) VAN-resistant, two of which were *E. gallinarum* and one was *E. casseliflavus* [302].

A significant limitation of the study is the small sample size, which comprised of several *Enterococcus* species. The diversity in *Enterococcus* species might have further worsened the

sample size as different *Enterococcus* species have been reported to vary in their antibiotic susceptibilities [302].

Chapter 4 - Concluding remarks

Background:

Antibiotic resistance is a global crisis that negatively impacts animal and human health and threatens food security and national economies [7–9]. While antibiotic resistance is a natural bacterial defense mechanism, the use of antibiotics creates selection pressure that accelerates resistance development [344]. Bacteria can share resistance traits through mobile genetic elements, further spreading resistance [42]. Additionally, resistance to one antibiotic can co-select for resistance to others, compounding the spread of antibiotic resistance [42].

Antibiotic use in veterinary medicine, particularly in production animals, has been heavily linked to the antibiotic resistance crisis [345]. As a result, global efforts have aimed to curb resistance by restricting antibiotic use in livestock. In the U.S., such efforts include banning medically important antibiotics for growth promotion and establishing defined durations for their use [29,174]. Currently, 97 medically important antibiotic formulations, including chlortetracycline for bovine anaplasmosis control, lack defined usage durations [174].

Bovine anaplasmosis is a tick-borne disease affecting cattle worldwide, including in the U.S., with an estimated economic burden of \$300 million annually on the U.S. cattle industry [175,176]. Clinical anaplasmosis, characterized by anemia, can lead to decreased productivity, such as reduced milk production and infertility [181,247]. Cattle that recover enter a chronic, persistently infected state, serving as reservoirs of infection and remaining less productive than naïve cattle [182,183].

Control strategies for bovine anaplasmosis include management practices, acaricides, insecticides, and a conditionally approved vaccine available in 15 states [175]. However, these methods are generally ineffective. In the absence of a nationally available, strain-independent control strategy, anaplasmosis in the U.S. is often managed with chlortetracycline (CTC) in feed at doses of 0.5–2.0 mg/lb BW/day for extended periods, sometimes six months or longer. This prolonged, sub-therapeutic use of CTC may promote antibiotic resistance in both target and non-target bacterial species, including foodborne pathogens, and may co-select for resistance to other non-tetracycline antibiotics.

Knowledge gap:

It is currently unknown whether using chlortetracycline (CTC) to control active anaplasmosis promotes antibiotic resistance in enteric zoonotic bacteria from cattle.

Study aims:

This research aimed to evaluate changes in antibiotic susceptibilities in *E. coli* and *Enterococcus* isolated from cattle handfed CTC at 0.5–2.0 mg/lb BW/day to control active anaplasmosis, with an additional experimental group receiving 10.0 mg/lb BW/day for 120 consecutive days. *E. coli* and *Enterococcus* were selected for study because they are common gut bacteria in cattle, capable of becoming pathogenic. Additionally, they are zoonotic and can serve as reservoirs for antibiotic-resistant genes that may transfer to humans.

Study hypothesis:

We hypothesized that prolonged administration of CTC would lead to an increased recovery of tetracycline (TET)-resistant *E. coli*, with resistance developing more rapidly in groups receiving higher CTC doses. Furthermore, we expected that prolonged selection pressure from TET would co-select for resistance to antibiotics not administered in this study.

Summary of findings

***E. coli* study and comparative analysis with other studies:**

The proportion of TET-resistant isolates increased during the CTC administration period for all study groups, including the control group. However, these differences between groups were not statistically significant (overall CTC effect, $p = 0.089$; daily p -values ranging from 0.266 to 0.630). This result may reflect the high baseline TET resistance (58.8%) in *E. coli* isolates before CTC administration, potentially confounding the analysis. Additionally, our assay could not determine whether CTC use to control active anaplasmosis promotes TET resistance in *E. coli* isolated from cattle hand-fed CTC for 120 days as most isolates had MIC values at or above the highest dilution of the assay.

Similar results have been reported in other studies. One study with 20 steers in a feedlot setting administered 22.0 mg/kg (about 10.0 mg/lb) of CTC in a cottonseed meal for three five-day periods, with one-day intervals between feedings. CTC was administered on days 0–4, 6–10, and 12–16 [302]. The CTC concentrations in that study were similar to those of our 10.0 mg group, though our study extended CTC administration to 17 five-day periods with two-day intervals. The feedlot study reported a significant effect of CTC on TET MIC values on days -7 (p -value = 0.02), 2 (p -value 0.03), day 8 (p -value = 0.02) and 14 (p -value = 0.01) [302]. In contrast, our

study found no overall CTC effect on MIC values (p -value = 0.089), regardless of group or study day.

One possible explanation for the difference in significance is our smaller sample size. The feedlot study included 525 *E. coli* isolates from 176 fecal samples (approximately three isolates per sample), whereas our study had only 192 isolates evenly split across four groups (48 isolates per group). Thus, our study may have lacked the statistical power to detect significance, even though both studies observed similar trends in MIC values.

The same feedlot study assessed *E. coli* susceptibility to 13 additional antibiotics, although only findings on ceftiofur (XNL) were reported [302]. They observed a significant interaction between study day and CTC treatment on XNL resistance (p = 0.02). Cattle in the CTC-exposed group had significantly higher XNL MIC values than controls on days 6, 8, and 12 (p = 0.01), which are the days of CTC administration but no significant differences afterward. Additionally, this study found that *E. coli* from the non-CTC-exposed group were more likely to be resistant to XNL than the CTC-exposed group (p = 0.01). Similarly, we found a significant interaction between CTC group and study day (p <0.001), although this effect may have been primarily driven by study day (p -value = 0.025) as the CTC effect on XNL MIC values was not significant (p -value = 0.500).

Furthermore, the same study found that 81.0% (173/213) of *E. coli* isolates from the CTC-exposed group were resistant to one or more of the 14 antibiotics tested, compared to 70.2% (129/184) in the control group (p -value= 0.04) [302]. In our study, more isolates in the 10.0 mg group were resistant to at least one antibiotic (93.8%, 45/48 isolates) compared to the control group (81.3%, 9/48), though this difference was not statistically analyzed.

Overall, we found a significant effect of CTC on MIC values for several non-tetracycline antibiotics, including chlortetracycline (CHL), amoxicillin/clavulanic acid (AUG2), sulfisoxazole (FIS), trimethoprim/sulfamethoxazole (SXT), ampicillin (AMP), and streptomycin (STR), with p-values ranging from <0.001 to 0.034. MIC values for CHL, FIS, SXT, AMP, and STR increased during the CTC administration period and decreased slightly post-cessation. However, AUG2 did not follow a logical trend, suggesting no apparent resistance development to AUG2 due to CTC.

Tetracyclines exert their effects by binding to the 30S ribosomal subunit, inhibiting protein synthesis. Mechanisms of TET resistance include active efflux, drug modification, and target modification [137,138,140]. These resistance mechanisms differ from those of CHL, AUG2, FIS, SXT, AMP, and STR, suggesting that co-selection with TET is likely due to co-resistance, where resistance genes for multiple antibiotics are located on the same mobile genetic element. Co-selection of TET with STR, for example, is common and has been frequently documented. TET resistance spread via conjugation is also widespread [141]. A retrospective study of 1,792 *E. coli* isolates from human and food samples in the U.S. found that 29.7% were co-resistant to TET and STR, and 18.8% were resistant to both TET and AMP [325]. Likewise, an antibiotic resistance study of 290 *E. coli* isolates from healthy feedlot cattle in South Korea found co-selection rates of 82.2% for TET and STR, 45.3% for TET and AMP, 28.8% for TET and CHL, and 25.3% for TET and SXT [116]. In that study, the most common multidrug resistance phenotype was TET-STR-AMP (13.7%).

All of these antibiotics play essential roles in veterinary and human medicine. Tetracyclines (e.g., TET), sulfonamides (e.g., FIS), aminopenicillins (e.g., AMP), aminoglycosides (e.g., STR), sulfonamides with diaminopyrimidines (e.g., SXT), and

amphenicols (e.g., CHL) are classified as critically important veterinary antimicrobials (CVIA) [142]. However, CHL use is restricted in food-producing animals due to its potential to cause aplastic anemia in humans [355]. In human medicine, tetracyclines (e.g., TET) are categorized by the WHO as highly important C1 antibiotics, often the only effective treatment for serious infections [143]. Amphenicols (e.g., CHL), aminopenicillins (e.g., AMP), sulfonamides (e.g., FIS), and sulfonamides with diaminopyrimidines (e.g., SXT) are also highly important C2, used to treat infections transmitted from non-human sources or associated with resistance genes from such sources. Aminoglycosides (e.g., STR) are critically important, classified as C1, C2, and prioritization factor 2 (PF2) for treating life-threatening infections with documented transmission of resistance from non-human sources [143].

***Enterococcus* and comparative analysis with other studies:**

A total of 222 *Enterococcus* isolates representing eight different species were identified in this study: *E. hirae* (60.8%), *E. casseliflavus* (19.8%), *E. durans* (6.3%), *E. faecium* (5.9%), *E. mundtii* (5.0%), *E. gallinarum* (1.4%), *E. aquimarinus* (0.5%), and *E. avium* (0.5%). These species were similar to those isolated in a study where 10.0 mg/day CTC was administered to feedlot steers in three separate five-day periods, with one-day intervals between periods [308]. That study reported 366 *Enterococcus* isolates from six species. Two species, *E. casseliflavus* (11.6%) and *E. durans* (2.5%), were present in similar proportions to ours. However, they observed higher proportions of *E. faecium* (26.5%) and *E. gallinarum* (24.9%) and lower proportions of *E. hirae* (24.3%). Additionally, they identified *E. columbae* (0.3%), which was not present in our study.

No significant CTC effect on *Enterococcus* TET MIC was observed in our study (p-value = 0.8370). Similarly, the proportion of TET-resistant isolates did not increase during CTC administration. The study with 10.0 mg/day CTC did not report a CTC effect on *Enterococcus* TET MIC values, although it did find a significant interaction between treatment and study day (p-value = 0.02) [308]. They also reported significant CTC effects on TET MIC values, with control group isolates showing lower geometric means compared to exposed group isolates on study days 2, 6, 8, 12, 14, and 22. In contrast, our study did not find a significant interaction effect between CTC and study day (p-value = 0.2218), and CTC effects on study days 58, 114, and 21 days post-cessation were also not significant (p-values ranged between p-value 0.1153 and 0.7373). One reason for this disparity may be that most of our isolates were already TET-resistant, potentially confounding our results. Additionally, many of these isolates were at or above the assay's detection limit, making it challenging to assess whether CTC use promotes TET resistance in these isolates.

While not statistically analyzed, differences in TET-resistance were noted between *Enterococcus* species: *E. hirae* (100%), *E. casseliflavus* (65.9%), *E. durans* (100%), *E. faecium* (100%), *E. mundtii* (45.5%), *E. gallinarum* (33.3%), *E. aquimarinus* (100%), and *E. avium* (100%). However, these were not statistically analyzed. The feedlot study administering 10.0 mg/day CTC found significant differences in TET resistance across steers (p-value 0.01): *E. hirae* (33.9%), *E. casseliflavus* (10.1%), *E. durans* (100%), *E. faecium* (25.8%), *E. gallinarum* (42.8%), and *E. columbae* (0%) [308].

Our study observed significant CTC effects on MIC values for ciprofloxacin (CIP), vancomycin (VAN), and chlortetracycline (CHL), with p-values of 0.0434, 0.0072 and 0.0072, respectively. However, these MIC values were inconsistent across study days and CTC doses.

Therefore, under the conditions of this study, CTC use for anaplasmosis control did not promote selection of non-TET antibiotics in *Enterococcus* isolates. While the feedlot study also evaluated *Enterococcus* susceptibility to 13 non-TET antibiotics, their discussion did not include these results. In our study, one *E. casseliflavus* isolate (0.5%) was VAN-resistant, whereas the feedlot study identified three VAN-resistant isolates (0.8%): two *E. gallinarum* and one *E. casseliflavus* [308].

No evidence of co-selection was found in the *Enterococcus* study. However, this may be due to the lack of species-specific analysis, as different *Enterococcus* species have varying antibiotic susceptibilities [356].

Combined findings from *E. coli* and *Enterococcus* studies:

Based on the conditions of these studies, we accept the primary null hypothesis: CTC use for controlling active anaplasmosis does not promote TET resistance in *E. coli* and *Enterococcus* isolated from fattening steers. However, it is important to note that most *E. coli* and *Enterococcus* isolates were already TET-resistant before CTC administration. Additionally, isolates with MIC values above the assay's detection limit were assigned the highest MIC value in the Sensititre assays, which may have introduced some bias.

We reject our minor hypothesis that CTC use for anaplasmosis control does not promote resistance to non-TET antibiotics in *E. coli*, though we accept it for *Enterococcus* isolates. In the *E. coli* study, CTC administration increased the recovery of isolates resistant to CHL, FIS, SXT, AMP, and STR, suggesting co-selection of resistance. Resistance magnitude, as indicated by MIC values, was highest in groups receiving higher CTC doses. During CTC administration, MIC values for TET, CHL, FIS, SXT, AMP, and STR increased across all study groups,

including the 0.0 mg group, then decreased after cessation of CTC. The reasons for this trend are unclear but may involve factors not assessed in this study. A similar phenomenon was observed in a previous study administering 10.0 mg CTC to feedlot cattle [308].

Implications:

To our knowledge, this is the first report examining the effect of CTC use on the antibiotic susceptibilities of zoonotic bacteria relevant to veterinary and human medicine. Our findings emphasize the negative impact of using CTC to control anaplasmosis on the susceptibility of *E. coli*, a zoonotic bacterium capable of transmitting resistance genes for antibiotics crucial in both animals and humans, including some that are among the few treatment options for critical human infections. Although the 10.0 mg/lb BW/day dose is not used for CTC control, it is employed in cattle to treat pneumonia caused by *Pasteurella multocida* and enteric infections caused by *E. coli* and *Eimeria* species [150]. Results from the *E. coli* study suggest that increasing CTC doses might accelerate co-selection for resistance to CHL, FIS, SXT, AMP, and STR, resulting in higher MIC values.

Because *A. marginale* is a highly prevalent and economically important pathogen, discontinuing antibiotics without an alternative control strategy is not feasible. Given the limited effectiveness of other control strategies, such as environmental management, acaricide and insecticide use, and a single-strain killed vaccine in some states, these findings underscore the need for a more effective non-antibiotic control strategy that is independent of strain diversity. A promising option could be a vaccine based on newer technology.

Strengths and limitations:

A major strength of these studies, particularly the *E. coli* study, is that despite a relatively small sample size, we observed significant effects of CTC on *E. coli* susceptibility to non-TET antibiotics. However, a key limitation is that only single isolates of *E. coli* and *Enterococcus* were analyzed from each animal on each study day, which may not accurately represent the antibiotic susceptibility profiles for each steer. A more representative susceptibility profile from each steer would have come from isolating multiple isolates from each study steer per study day. Additionally, in the *Enterococcus* study, sample size determinations did not account for the variation in antibiotic susceptibilities among different *Enterococcus* species, potentially confounding the data. It is possible that focusing on a single species with a high sample size might have yielded different results.

Another limitation is that our assays could not determine whether CTC use to control active anaplasmosis promotes TET resistance in *E. coli* and *Enterococcus*, as most isolates were already TET-resistant before CTC administration. Additionally, all isolates with MIC values above the highest dilution on the Sensititre assay were assigned that maximum value, potentially biasing the results. Including genotypic analyses of resistance genes, especially for co-selection data, would have provided further insight.

Future directions.

These studies were conducted in controlled settings using steers of similar age, from a single source, and all of the same gender. Additionally, the steers received no other antibiotics. In field settings, however, herds often consist of animals from diverse sources with varying

antibiotic histories. Field conditions also typically involve longer CTC administration periods, which may amplify the effects observed in *E. coli* resistance.

Future studies should therefore investigate antibiotic susceptibilities of bacteria isolated from cattle in different field operations. This would provide a more representative understanding of CTC's impact on antibiotic resistance in varied real-world conditions.

Summary:

Antibiotic resistance is a global threat that limits treatment options for animal and human diseases, threatening food security and national economies. Long-term antibiotic use, such as for controlling active anaplasmosis in fattening steers, may promote resistance to antibiotics critical in both veterinary and human medicine, especially in zoonotic enteric pathogens like *E. coli*. Given the prevalence and economic significance of bovine anaplasmosis, discontinuing CTC use without alternative control strategies is not feasible. Thus, there is an urgent need for a more effective non-antibiotic control strategy, such as a vaccine based on newer technology that is independent of strain diversity.

Chapter 5 - References

- [1] P. Dadgostar, Antimicrobial Resistance: Implications and Costs, *Infect Drug Resist* 12 (2019) 3903–3910. <https://doi.org/10.2147/idr.s234610>.
- [2] H. Y, G. N, Antibiotic Resistance - StatPearls - NCBI Bookshelf, (2024). <https://www.ncbi.nlm.nih.gov/books/NBK513277/> (accessed July 20, 2024).
- [3] G.M. Eliopoulos, S.E. Cosgrove, Y. Carmeli, The Impact of Antimicrobial Resistance on Health and Economic Outcomes, *Clin. Infect. Dis.* 36 (2003) 1433–1437. <https://doi.org/10.1086/375081>.
- [4] S.E. Cosgrove, G. Sakoulas, E.N. Perencevich, M.J. Schwaber, A.W. Karchmer, Y. Carmeli, Comparison of Mortality Associated with Methicillin-Resistant and Methicillin-Susceptible *Staphylococcus aureus* Bacteremia: A Meta-analysis, *Clin. Infect. Dis.* 36 (2003) 53–59. <https://doi.org/10.1086/345476>.
- [5] Y. Cetinkaya, P. Falk, C.G. Mayhall, Vancomycin-Resistant Enterococci, *Clin. Microbiol. Rev.* 13 (2000) 686–707. <https://doi.org/10.1128/cmr.13.4.686-707.2000>.
- [6] L.J. Piddock, The crisis of no new antibiotics—what is the way forward?, *Lancet Infect. Dis.* 12 (2012) 249–253. [https://doi.org/10.1016/s1473-3099\(11\)70316-4](https://doi.org/10.1016/s1473-3099(11)70316-4).
- [7] R.C. Founou, L.L. Founou, S.Y. Essack, Clinical and economic impact of antibiotic resistance in developing countries: A systematic review and meta-analysis, *PLoS ONE* 12 (2017) e0189621. <https://doi.org/10.1371/journal.pone.0189621>.
- [8] F. Prestinaci, P. Pezzotti, A. Pantosti, Antimicrobial resistance: a global multifaceted phenomenon, *Pathog. Glob. Heal.* 109 (2015) 309–318. <https://doi.org/10.1179/2047773215y.0000000030>.
- [9] R.E. Nelson, K.M. Hatfield, H. Wolford, M.H. Samore, R.D. Scott, S.C. Reddy, B. Olubajo, P. Paul, J.A. Jernigan, J. Baggs, National Estimates of Healthcare Costs Associated With Multidrug-Resistant Bacterial Infections Among Hospitalized Patients in the United States, *Clin. Infect. Dis.* 72 (2021) S17–S26. <https://doi.org/10.1093/cid/ciaa1581>.
- [10] C.J.L. Murray, K.S. Ikuta, F. Sharara, L. Swetschinski, G.R. Aguilar, A. Gray, C. Han, C. Bisignano, P. Rao, E. Wool, S.C. Johnson, A.J. Browne, M.G. Chipeta, F. Fell, S. Hackett, G. Haines-Woodhouse, B.H.K. Hamadani, E.A.P. Kumaran, B. McManigal, S. Achalapong, R. Agarwal, S. Akech, S. Albertson, J. Amuasi, J. Andrews, A. Aravkin, E. Ashley, F.-X. Babin, F. Bailey, S. Baker, B. Basnyat, A. Bekker, R. Bender, J.A. Berkley, A. Bethou, J. Bielicki, S. Boonkasidecha, J. Bukosia, C. Carvalheiro, C. Castañeda-Orjuela, V. Chansamouth, S. Chaurasia, S. Chiurchiù, F. Chowdhury, R.C. Donatien, A.J. Cook, B. Cooper, T.R. Cressey, E.

Criollo-Mora, M. Cunningham, S. Darboe, N.P.J. Day, M.D. Luca, K. Dokova, A. Dramowski, S.J. Dunachie, T.D. Bich, T. Eckmanns, D. Eibach, A. Emami, N. Feasey, N. Fisher-Pearson, K. Forrest, C. Garcia, D. Garrett, P. Gastmeier, A.Z. Giref, R.C. Greer, V. Gupta, S. Haller, A. Haselbeck, S.I. Hay, M. Holm, S. Hopkins, Y. Hsia, K.C. Iregbu, J. Jacobs, D. Jarovsky, F. Javanmardi, A.W.J. Jenney, M. Khorana, S. Khusuwan, N. Kissoon, E. Kobeissi, T. Kostyaney, F. Krapp, R. Krumkamp, A. Kumar, H.H. Kyu, C. Lim, K. Lim, D. Limmathurotsakul, M.J. Loftus, M. Lunn, J. Ma, A. Manoharan, F. Marks, J. May, M. Mayxay, N. Mturi, T. Munera-Huertas, P. Musicha, L.A. Musila, M.M. Mussi-Pinhata, R.N. Naidu, T. Nakamura, R. Nanavati, S. Nangia, P. Newton, C. Ngoun, A. Novotney, D. Nwakanma, C.W. Obiero, T.J. Ochoa, A. Olivas-Martinez, P. Oliaro, E. Ooko, E. Ortiz-Brizuela, P. Ounchanum, G.D. Pak, J.L. Paredes, A.Y. Peleg, C. Perrone, T. Phe, K. Phommasone, N. Plakkal, A. Ponce-de-Leon, M. Raad, T. Ramdin, S. Rattanavong, A. Riddell, T. Roberts, J.V. Robotham, A. Roca, V.D. Rosenthal, K.E. Rudd, N. Russell, H.S. Sader, W. Saengchan, J. Schnall, J.A.G. Scott, S. Seekaew, M. Sharland, M. Shivamallappa, J. Sifuentes-Osornio, A.J. Simpson, N. Steenkeste, A.J. Stewardson, T. Stoeva, N. Tasak, A. Thaiprakong, G. Thwaites, C. Tigoi, C. Turner, P. Turner, H.R. van Doorn, S. Velaphi, A. Vongpradith, M. Vongsouvath, H. Vu, T. Walsh, J.L. Walson, S. Waner, T. Wangrangsimaikul, P. Wannapinij, T. Wozniak, T.E.M.W.Y. Sharma, K.C. Yu, P. Zheng, B. Sartorius, A.D. Lopez, A. Stergachis, C. Moore, C. Dolecek, M. Naghavi, Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis, *Lancet* 399 (2022) 629–655. [https://doi.org/10.1016/s0140-6736\(21\)02724-0](https://doi.org/10.1016/s0140-6736(21)02724-0).

[11] M. Craig, Antibiotic Resistance Threats in the United States, 2019, U.S. Department of Health and Human Services, Atlanta, GA, 2019. <https://www.cdc.gov/antimicrobial-resistance/data-research/threats/index.html> (accessed August 12, 2024).

[12] E.C. for D.P. and Control, Assessing the health burden of infections with antibiotic-resistant bacteria in the EU/EEA, 2016-2020, Stockholm: ECDC, 2022. <https://www.ecdc.europa.eu/sites/default/files/documents/Health-burden-infections-antibiotic-resistant-bacteria.pdf> (accessed September 17, 2024).

[13] E.C. for D.P. and Control, The bacterial challenge: time to react, Stockholm, 2009. https://www.ecdc.europa.eu/sites/default/files/media/en/publications/Publications/0909_TER_The_Bacterial_Challenge_Time_to_React.pdf (accessed September 17, 2024).

[14] A. B, A. HT, A. M, B. H, H. M, H. S, J. M, K. D, K. S, K. ND, O. F, R. I, S. SJ, Y. E, Y.-M. J, Z. S., Forecasting the Fallout from AMR: Economic Impacts of Antimicrobial Resistance in Food-Producing Animals – A report from the EcoAMR series., World Organisation for Animal Health and World Bank, Paris (France) and Washington, DC (United States of America), 2024.

[15] B. Bengtsson, C. Greko, Antibiotic resistance—consequences for animal health, welfare, and food production, *Upsala J. Méd. Sci.* 119 (2014) 96–102. <https://doi.org/10.3109/03009734.2014.901445>.

[16] A.V.M. Association, Definitions of antimicrobial use for treatment, control, and prevention, (n.d.). <https://www.avma.org/resources-tools/avma-policies/avma-definitions-antimicrobial-use-treatment-control-and->

[prevention#:~:text=Antimicrobial%20prevention%20of%20disease%20\(synonym,clinical%20judgement%2C%20or%20epidemiological%20knowledge.](#) (accessed October 19, 2024).

[17] D.R. Smith, P.S. Gaunt, P.J. Plummer, H.M. Cervantes, P. Davies, V.R. Fajt, E. Frey, M.T. Jay-Russell, T.W. Lehenbauer, M.G. Papich, T.M. Parker, E.C. Phipps, J.M. Scheftel, L.V. Schnabel, R.S. Singer, J.E. Whaley, J.K. Wishnie, L.R. Wright, M. Costin, The AVMA's definitions of antimicrobial uses for prevention, control, and treatment of disease, *J. Am. Vet. Méd. Assoc.* 254 (2019) 792–797. <https://doi.org/10.2460/javma.254.7.792>.

[18] M. Peng, S. Salaheen, D. Biswas, *Encyclopedia of Agriculture and Food Systems*, Anim. Heal.: Artic. Titles: A (2014) 346–357. <https://doi.org/10.1016/b978-0-444-52512-3.00187-x>.

[19] T.H. Smith, L.K. Fox, J.R. Middleton, Outbreak of mastitis caused by one strain of *Staphylococcus aureus* in a closed dairy herd., *J. Am. Vet. Méd. Assoc.* 212 (1998) 553–6.

[20] N.A. of Sciences, *Combating Antimicrobial Resistance and Protecting the Miracle of Modern Medicine*, The National Academies Press., Washington, DC, 2021. <https://doi.org/10.17226/26350>.

[21] R.A.D. Silva, N.E. Arenas, V.L. Luiza, J.A.Z. Bermudez, S.E. Clarke, Regulations on the Use of Antibiotics in Livestock Production in South America: A Comparative Literature Analysis, *Antibiotics* 12 (2023) 1303. <https://doi.org/10.3390/antibiotics12081303>.

[22] T.T.H. Van, Z. Yidana, P.M. Smooker, P.J. Coloe, Antibiotic use in food animals worldwide, with a focus on Africa: Pluses and minuses, *J. Glob. Antimicrob. Resist.* 20 (2020) 170–177. <https://doi.org/10.1016/j.jgar.2019.07.031>.

[23] D.C.J. MAIN, M.C. APPLEBY, D.B. WILKINS, E.S. PAUL, Essential veterinary education in the welfare of food production animals: -EN- -FR- Les fondamentaux de l'enseignement vétérinaire dans le domaine du bien-être des animaux d'élevage destinés à l'alimentation -ES- Enseñanza veterinaria básica en materia de bienestar de los animales para consumo humano, *Rev. Sci. Tech. l'OIE* 28 (2009) 611–616. <https://doi.org/10.20506/rst.28.2.1900>.

[24] W. Bank, *Drug-Resistant Infections: A Threat to Our Economic Future*, Washington, DC: World Bank, Washington, DC., 2017. https://documents.worldbank.org/en/publication/documents-reports/documentdetail/323311493396993758/final-report?_gl=1*_95qxsp*_gcl_au*MTYyMTM1OTIwMy4xNzI2NTE3OTA0 (accessed September 17, 2024).

[25] G.L. Cromwell, WHY AND HOW ANTIBIOTICS ARE USED IN SWINE PRODUCTION, *Anim. Biotechnol.* 13 (2002) 7–27. <https://doi.org/10.1081/abio-120005767>.

- [26] Mathews, K.H, Antimicrobial Drug Use and Veterinary Costs in U.S. Livestock Production, 2001. https://www.ers.usda.gov/webdocs/publications/42375/17768_aib766_1_.pdf?v=4794.2 (accessed September 17, 2024).
- [27] D.A. Service, Doane's Agricultural Report: Reference Volume, Doane Agricultural Service, University of Illinois at Urbana-Champaign, n.d.
- [28] M. Ryan, Evaluating the economic benefits and costs of antimicrobial use in food-producing animals, OECD Food, Agriculture and Fisheries Papers (2019). <https://doi.org/10.1787/f859f644-en>.
- [29] U.S.F. and D. Administration, FDA Announces Implementation of GFI #213, Outlines Continuing Efforts to Address Antimicrobial Resistance, (2021). <https://wayback.archive-it.org/7993/20190423131636/https://www.fda.gov/AnimalVeterinary/NewsEvents/CVMUpdates/ucm535154.htm> (accessed September 20, 2024).
- [30] I. Phillips, Withdrawal of growth-promoting antibiotics in Europe and its effects in relation to human health, Int. J. Antimicrob. Agents 30 (2007) 101–107. <https://doi.org/10.1016/j.ijantimicag.2007.02.018>.
- [31] F. and D. Administration, D. of H. and H. Services, 520.445 Chlortetracycline and sulfamethazine powder., (2016). <https://www.ecfr.gov/current/title-21/part-520/section-520.445> (accessed September 21, 2024).
- [32] F. and D. Administration, D. of H. and H. Services, 558.128 Chlortetracycline., (2024). <https://www.ecfr.gov/current/title-21/part-558/section-558.128> (accessed September 21, 2024).
- [33] F. and D. Administration, D. of H. and H. Services, 558.140 Chlortetracycline and sulfamethazine, (2021). <https://www.ecfr.gov/current/title-21/section-558.140> (accessed September 21, 2024).
- [34] G. Lhermie, P. Sauvage, L.W. Tauer, L.V. Chiu, K. Kanyiamattam, A. Ferchiou, D. Raboisson, H.M. Scott, D.R. Smith, Y.T. Grohn, Economic effects of policy options restricting antimicrobial use for high risk cattle placed in U.S. feedlots, PLoS ONE 15 (2020) e0239135. <https://doi.org/10.1371/journal.pone.0239135>.
- [35] G. Lhermie, L.W. Tauer, Y.T. Gröhn, An assessment of the economic costs to the U.S. dairy market of antimicrobial use restrictions, Prev. Vet. Med. 160 (2018) 63–67. <https://doi.org/10.1016/j.prevetmed.2018.09.028>.
- [36] R. Europe, The economic impacts of antimicrobial resistance in livestock (EcoAMR), (n.d.). <https://www.rand.org/randeurope/research/projects/2023/ecoamr.html> (accessed September 18, 2024).

- [37] U.S. FDA, FDA's role in antimicrobial resistance (AMR) preparedness and response, and information about AMR, (2024). <https://www.fda.gov/emergency-preparedness-and-response/mcm-issues/antimicrobial-resistance> (accessed November 11, 2024).
- [38] W.C. Reygaert, D. of B.S. USA Oakland University William Beaumont School of Medicine, Rochester, MI, An overview of the antimicrobial resistance mechanisms of bacteria, *AIMS Microbiol.* 4 (2018) 482–501. <https://doi.org/10.3934/microbiol.2018.3.482>.
- [39] G. Cox, G.D. Wright, Intrinsic antibiotic resistance: Mechanisms, origins, challenges and solutions, *Int. J. Méd. Microbiol.* 303 (2013) 287–292. <https://doi.org/10.1016/j.ijmm.2013.02.009>.
- [40] N. Woodford, M.J. Ellington, The emergence of antibiotic resistance by mutation, *Clin. Microbiol. Infect.* 13 (2007) 5–18. <https://doi.org/10.1111/j.1469-0691.2006.01492.x>.
- [41] J.M. Munita, C.A. Arias, Mechanisms of Antibiotic Resistance, *Microbiol. Spectr.* 4 (2016). <https://doi.org/10.1128/microbiolspec.vmbf-0016-2015>.
- [42] C.J.H. von Wintersdorff, J. Penders, J.M. van Niekerk, N.D. Mills, S. Majumder, L.B. van Alphen, P.H.M. Savelkoul, P.F.G. Wolffs, Dissemination of Antimicrobial Resistance in Microbial Ecosystems through Horizontal Gene Transfer, *Front. Microbiol.* 7 (2016) 173. <https://doi.org/10.3389/fmicb.2016.00173>.
- [43] R.A.F. Wozniak, M.K. Waldor, Integrative and conjugative elements: mosaic mobile genetic elements enabling dynamic lateral gene flow, *Nat. Rev. Microbiol.* 8 (2010) 552–563. <https://doi.org/10.1038/nrmicro2382>.
- [44] N.B. Shoemaker, H. Vlamakis, K. Hayes, A.A. Salyers, Evidence for Extensive Resistance Gene Transfer among *Bacteroides* spp. and among *Bacteroides* and Other Genera in the Human Colon, *Appl. Environ. Microbiol.* 67 (2001) 561–568. <https://doi.org/10.1128/aem.67.2.561-568.2001>.
- [45] C.H. Lester, N. Frimodt-Moller, A.M. Hammerum, Conjugal transfer of aminoglycoside and macrolide resistance between *Enterococcus faecium* isolates in the intestine of streptomycin-treated mice, *FEMS Microbiol. Lett.* 235 (2004) 385–391. <https://doi.org/10.1111/j.1574-6968.2004.tb09614.x>.
- [46] C. Johnston, B. Martin, G. Fichant, P. Polard, J.-P. Claverys, Bacterial transformation: distribution, shared mechanisms and divergent control, *Nat. Rev. Microbiol.* 12 (2014) 181–196. <https://doi.org/10.1038/nrmicro3199>.
- [47] O. Johnsborg, V. Eldholm, L.S. Håvarstein, Natural genetic transformation: prevalence, mechanisms and function, *Res. Microbiol.* 158 (2007) 767–778. <https://doi.org/10.1016/j.resmic.2007.09.004>.

- [48] G.M. Traglia, K. Place, C. Dotto, J.S. Fernandez, S. Montaña, C. dos S. Bahiense, A. Soler-Bistue, A. Iriarte, F. Perez, M.E. Tolmasky, R.A. Bonomo, R.G. Melano, M.S. Ramírez, Interspecies DNA acquisition by a naturally competent *Acinetobacter baumannii* strain, *Int. J. Antimicrob. Agents* 53 (2019) 483–490. <https://doi.org/10.1016/j.ijantimicag.2018.12.013>.
- [49] M. Brown-Jaque, W. Calero-Cáceres, M. Muniesa, Transfer of antibiotic-resistance genes via phage-related mobile elements, *Plasmid* 79 (2015) 1–7. <https://doi.org/10.1016/j.plasmid.2015.01.001>.
- [50] T. Billard-Pomares, S. Fouteau, M.E. Jacquet, D. Roche, V. Barbe, M. Castellanos, J.Y. Bouet, S. Cruveiller, C. Médigue, J. Blanco, O. Clermont, E. Denamur, C. Branger, Characterization of a P1-Like Bacteriophage Carrying an SHV-2 Extended-Spectrum β -Lactamase from an *Escherichia coli* Strain, *Antimicrob. Agents Chemother.* 58 (2014) 6550–6557. <https://doi.org/10.1128/aac.03183-14>.
- [51] A. Shousha, N. Awaiwanont, D. Sofka, F.J.M. Smulders, P. Paulsen, M.P. Szostak, T. Humphrey, F. Hilbert, Bacteriophages Isolated from Chicken Meat and the Horizontal Transfer of Antimicrobial Resistance Genes, *Appl. Environ. Microbiol.* 81 (2015) 4600–4606. <https://doi.org/10.1128/aem.00872-15>.
- [52] J.A. Funk, J.T. Lejeune, T.E. Wittum, P.J. Rajala-Schultz, The Effect of Subtherapeutic Chlortetracycline on Antimicrobial Resistance in the Fecal Flora of Swine, *Microb. Drug Resist.* 12 (2006) 210–218. <https://doi.org/10.1089/mdr.2006.12.210>.
- [53] K. Harada, T. Asai, M. Ozawa, A. Kojima, T. Takahashi, Farm-Level Impact of Therapeutic Antimicrobial Use on Antimicrobial-Resistant Populations of *Escherichia coli* Isolates from Pigs, *Microb. Drug Resist.* 14 (2008) 239–244. <https://doi.org/10.1089/mdr.2008.0836>.
- [54] C. Baker-Austin, M.S. Wright, R. Stepanauskas, J.V. McArthur, Co-selection of antibiotic and metal resistance, *Trends Microbiol.* 14 (2006) 176–182. <https://doi.org/10.1016/j.tim.2006.02.006>.
- [55] R. Cantón, P. Ruiz-Garbajosa, Co-resistance: an opportunity for the bacteria and resistance genes, *Curr. Opin. Pharmacol.* 11 (2011) 477–485. <https://doi.org/10.1016/j.coph.2011.07.007>.
- [56] A. Baran, A. Kwiatkowska, L. Potocki, Antibiotics and Bacterial Resistance—A Short Story of an Endless Arms Race, *Int. J. Mol. Sci.* 24 (2023) 5777. <https://doi.org/10.3390/ijms24065777>.
- [57] M.A. Webber, V. Ricci, R. Whitehead, M. Patel, M. Fookes, A. Ivens, L.J.V. Piddock, Clinically Relevant Mutant DNA Gyrase Alters Supercoiling, Changes the Transcriptome, and Confers Multidrug Resistance, *MBio* 4 (2013) e00273-13. <https://doi.org/10.1128/mbio.00273-13>.
- [58] V. Lázár, I. Nagy, R. Spohn, B. Csörgő, Á. Györkei, Á. Nyerges, B. Horváth, A. Vörös, R. Busa-Fekete, M. Hrtyan, B. Bogos, O. Méhi, G. Fekete, B. Szappanos, B. Kégl, B. Papp, C. Pál,

Genome-wide analysis captures the determinants of the antibiotic cross-resistance interaction network, *Nat. Commun.* 5 (2014) 4352. <https://doi.org/10.1038/ncomms5352>.

[59] C. Carresi, R. Marabelli, P. Roncada, D. Britti, Is the Use of Monensin Another Trojan Horse for the Spread of Antimicrobial Resistance?, *Antibiotics* 13 (2024) 129. <https://doi.org/10.3390/antibiotics13020129>.

[60] Q. Li, W. Chang, H. Zhang, D. Hu, X. Wang, The Role of Plasmids in the Multiple Antibiotic Resistance Transfer in ESBLs-Producing *Escherichia coli* Isolated From Wastewater Treatment Plants, *Front. Microbiol.* 10 (2019) 633. <https://doi.org/10.3389/fmicb.2019.00633>.

[61] S. Mitra, S. Mukherjee, S. Naha, P. Chattopadhyay, S. Dutta, S. Basu, Evaluation of co-transfer of plasmid-mediated fluoroquinolone resistance genes and bla_{NDM} gene in Enterobacteriaceae causing neonatal septicaemia, *Antimicrob. Resist. Infect. Control* 8 (2019) 46. <https://doi.org/10.1186/s13756-019-0477-7>.

[62] N. Gundogan, S. Citak, N. Yucel, A. Devren, A note on the incidence and antibiotic resistance of *Staphylococcus aureus* isolated from meat and chicken samples, *Meat Sci.* 69 (2005) 807–810. <https://doi.org/10.1016/j.meatsci.2004.10.011>.

[63] S.-H. Kim, C.-I. Wei, Y.-M. Tzou, H. An, Multidrug-Resistant *Klebsiella pneumoniae* Isolated from Farm Environments and Retail Products in Oklahoma, *J. Food Prot.* 68 (2005) 2022–2029. <https://doi.org/10.4315/0362-028x-68.10.2022>.

[64] HealthMatch, V. Wise, Is Antimicrobial Resistance The Next Health Crisis?, (2022). <https://healthmatch.io/blog/antimicrobial-resistance-next-health-crisis> (accessed September 21, 2024).

[65] S. Schjørring, K.A. Krogfelt, Assessment of Bacterial Antibiotic Resistance Transfer in the Gut, *Int. J. Microbiol.* 2011 (2011) 312956. <https://doi.org/10.1155/2011/312956>.

[66] E. Lambrecht, E.V. Coillie, E.V. Meervenne, N. Boon, M. Heyndrickx, T.V. de Wiele, Commensal *E. coli* rapidly transfer antibiotic resistance genes to human intestinal microbiota in the Mucosal Simulator of the Human Intestinal Microbial Ecosystem (M-SHIME), *Int. J. Food Microbiol.* 311 (2019) 108357. <https://doi.org/10.1016/j.ijfoodmicro.2019.108357>.

[67] F. and D. Administration, D. of H. and H. Services, 558.450 Oxytetracycline, (2024). <https://www.ecfr.gov/current/title-21/part-558/section-558.450> (accessed October 4, 2024).

[68] S. Sneeringer, J. MacDonald, N. Key, W. McBride, K. Mathews, Economics of Antibiotic Use in U.S. Livestock Production, U.S. Department of Agriculture, Economic Research Service, 2015. <https://www.ers.usda.gov/webdocs/publications/45485/err-200.pdf> (accessed October 19, 2024).

[69] F. and D. Administration, D. of H. and H. Services, 558.128 Chlortetracycline, (2024). <https://www.ecfr.gov/current/title-21/section-558.128> (accessed October 4, 2024).

- [70] S.D. Feighner, M.P. Dashkevicz, Subtherapeutic levels of antibiotics in poultry feeds and their effects on weight gain, feed efficiency, and bacterial cholytaurine hydrolase activity, *Appl. Environ. Microbiol.* 53 (1987) 331–336. <https://doi.org/10.1128/aem.53.2.331-336.1987>.
- [71] P. Butaye, L.A. Devriese, F. Haesebrouck, Antimicrobial Growth Promoters Used in Animal Feed: Effects of Less Well Known Antibiotics on Gram-Positive Bacteria, *Clin. Microbiol. Rev.* 16 (2003) 175–188. <https://doi.org/10.1128/cmr.16.2.175-188.2003>.
- [72] S.E. Katz, MD, Use of Antibiotics in Animal Agriculture: Implications for Pediatrics: Technical Report | Pediatrics | American Academy of Pediatrics, American Academy of Pediatrics, Committee on Infectious Diseases, Council on Environmental Health and Climate Change, 2024. <https://publications.aap.org/pediatrics/article/154/4/e2024068467/199441/Use-of-Antibiotics-in-Animal-Agriculture> (accessed October 5, 2024).
- [73] F. and D. Administration, D. of H. and H. Services, 21 CFR Part 556, (2019). <https://www.ecfr.gov/current/title-21/chapter-I/subchapter-E/part-556?toc=1> (accessed September 20, 2024).
- [74] F.A.R.A.& D. Program, Food Animal Residue Avoidance Databank, (n.d.). <http://www.farad.org/> (accessed September 20, 2024).
- [75] J.A. Burlison, L. Neckers, A.B. Smith, A. Maxwell, B.S.J. Blagg, Novobiocin: Redesigning a DNA Gyrase Inhibitor for Selective Inhibition of Hsp90, *J. Am. Chem. Soc.* 128 (2006) 15529–15536. <https://doi.org/10.1021/ja065793p>.
- [76] F. Collin, S. Karkare, A. Maxwell, Exploiting bacterial DNA gyrase as a drug target: current state and perspectives, *Appl. Microbiol. Biotechnol.* 92 (2011) 479–497. <https://doi.org/10.1007/s00253-011-3557-z>.
- [77] M. Fujimoto-Nakamura, H. Ito, Y. Oyamada, T. Nishino, J. Yamagishi, Accumulation of Mutations in both *gyrB* and *parE* Genes Is Associated with High-Level Resistance to Novobiocin in *Staphylococcus aureus*, *Antimicrob. Agents Chemother.* 49 (2005) 3810–3815. <https://doi.org/10.1128/aac.49.9.3810-3815.2005>.
- [78] A.A. Vickers, I. Chopra, A.J. O'Neill, Intrinsic Novobiocin Resistance in *Staphylococcus saprophyticus*, *Antimicrob. Agents Chemother.* 51 (2007) 4484–4485. <https://doi.org/10.1128/aac.00708-07>.
- [79] R. Bernelot-Moens, J.T. Beatty, DNA Gyrase Inhibitors Increase the Frequency of Bacteriophage-like RcGTA-Mediated Gene Transfer in *Rhodobacter capsulatus*, *Genes* 13 (2022) 2071. <https://doi.org/10.3390/genes13112071>.
- [80] J.E. Davies, STUDIES ON THE RIBOSOMES OF STREPTOMYCIN-SENSITIVE AND RESISTANT STRAINS OF *ESCHERICHIA COLI**, *Proc. Natl. Acad. Sci.* 51 (1964) 659–664. <https://doi.org/10.1073/pnas.51.4.659>.

- [81] V. N, K. C, Neomycin, StatPearls [Internet] (2023).
<https://www.ncbi.nlm.nih.gov/books/NBK560603/> (accessed September 7, 2024).
- [82] C. BJ, T. P, Gentamicin, StatPearls [Internet] (2023).
<https://www.ncbi.nlm.nih.gov/books/NBK557550/> (accessed September 7, 2024).
- [83] Y. Doi, J. Wachino, Y. Arakawa, Aminoglycoside Resistance The Emergence of Acquired 16S Ribosomal RNA Methyltransferases, *Infect. Dis. Clin. North Am.* 30 (2016) 523–537.
<https://doi.org/10.1016/j.idc.2016.02.011>.
- [84] S. Garneau-Tsodikova, K.J. Labby, Mechanisms of resistance to aminoglycoside antibiotics: overview and perspectives, *MedChemComm* 7 (2015) 11–27.
<https://doi.org/10.1039/c5md00344j>.
- [85] W. Yang, F. Hu, Research Updates of Plasmid-Mediated Aminoglycoside Resistance 16S rRNA Methyltransferase, *Antibiotics* 11 (2022) 906.
<https://doi.org/10.3390/antibiotics11070906>.
- [86] T.J. Coffey, C.G. Dowson, M. Daniels, J. Zhou, C. Martin, B.G. Spratt, J.M. Musser, Horizontal transfer of multiple penicillin-binding protein genes, and capsular biosynthetic genes, in natural populations of *Streptococcus pneumoniae*, *Mol. Microbiol.* 5 (1991) 2255–2260.
<https://doi.org/10.1111/j.1365-2958.1991.tb02155.x>.
- [87] D.G. White, C. Hudson, J.J. Maurer, S. Ayers, S. Zhao, M.D. Lee, L. Bolton, T. Foley, J. Sherwood, Characterization of Chloramphenicol and Florfenicol Resistance in *Escherichia coli* Associated with Bovine Diarrhea, *J. Clin. Microbiol.* 38 (2000) 4593–4598.
<https://doi.org/10.1128/jcm.38.12.4593-4598.2000>.
- [88] S. Schwarz, C. Kehrenberg, B. Doublet, A. Cloeckaert, Molecular basis of bacterial resistance to chloramphenicol and florfenicol, *FEMS Microbiol. Rev.* 28 (2004) 519–542.
<https://doi.org/10.1016/j.femsre.2004.04.001>.
- [89] P. Li, T. Zhu, D. Zhou, W. Lu, H. Liu, Z. Sun, J. Ying, J. Lu, X. Lin, K. Li, J. Ying, Q. Bao, T. Xu, Analysis of Resistance to Florfenicol and the Related Mechanism of Dissemination in Different Animal-Derived Bacteria, *Front. Cell. Infect. Microbiol.* 10 (2020) 369.
<https://doi.org/10.3389/fcimb.2020.00369>.
- [90] E.P. on B.H. (BIOHAZ), K. Koutsoumanis, A. Allende, A. Alvarez-Ordóñez, D. Bolton, S. Bover-Cid, M. Chemaly, R. Davies, A.D. Cesare, L. Herman, F. Hilbert, R. Lindqvist, M. Nauta, G. Ru, M. Simmons, P. Skandamis, E. Suffredini, D.I. Andersson, V. Bampidis, J. Bengtsson-Palme, D. Bouchard, A. Ferran, M. Kouba, S.L. Puente, M. López-Alonso, S.S. Nielsen, A. Pechová, M. Petkova, S. Girault, A. Broglia, B. Guerra, M.L. Innocenti, E. Liébana, G. López-Gálvez, P. Manini, P. Stella, L. Peixe, Maximum levels of cross-contamination for 24 antimicrobial active substances in non-target feed. Part 7: Amphenicols: florfenicol and thiamphenicol, *EFSA J.* 19 (2021) e06859. <https://doi.org/10.2903/j.efsa.2021.6859>.

- [91] C.P. Weiner, C. Buhimschi, *Drugs for Pregnant and Lactating Women* (Second Edition), (2009) 120–251. <https://doi.org/10.1016/b978-1-4160-4013-2.00003-x>.
- [92] B. T, P. P, P. CV, Cephalosporins, StatPearls [Internet] (2024). [//www.ncbi.nlm.nih.gov/books/NBK551517/](https://www.ncbi.nlm.nih.gov/books/NBK551517/) (accessed September 9, 2024).
- [93] Z. Wangu, K.K. Hsu, *Principles and Practice of Pediatric Infectious Diseases*, (2023) 776-782.e2. <https://doi.org/10.1016/b978-0-323-75608-2.00126-9>.
- [94] C. Carson, X.-Z. Li, A. Agunos, D. Loest, B. Chapman, R. Finley, M. Mehrotra, L.M. Sherk, R. Gaumond, R. Irwin, Ceftiofur-resistant *Salmonella enterica* serovar Heidelberg of poultry origin – a risk profile using the Codex framework, *Epidemiology Infect.* 147 (2019) e296. <https://doi.org/10.1017/s0950268819001778>.
- [95] A. Kimura, M. Yossapol, S. Shibata, T. Asai, Selection of broad-spectrum cephalosporin-resistant *Escherichia coli* in the feces of healthy dogs after administration of first-generation cephalosporins, *Microbiol. Immunol.* 61 (2017) 34–41. <https://doi.org/10.1111/1348-0421.12466>.
- [96] M.G. Papich, *Saunders Handbook of Veterinary Drugs* (Fourth Edition), (2016) 137–139. <https://doi.org/10.1016/b978-0-323-24485-5.00146-7>.
- [97] Pharmacompass, Ceftiofur, (n.d.). <https://www.pharmacompass.com/chemistry-chemical-name/ceftiofur> (accessed September 9, 2024).
- [98] S.D. Alcaine, S.S. Sukhnanand, L.D. Warnick, W.-L. Su, P. McGann, P. McDonough, M. Wiedmann, Ceftiofur-Resistant *Salmonella* Strains Isolated from Dairy Farms Represent Multiple Widely Distributed Subtypes That Evolved by Independent Horizontal Gene Transfer, *Antimicrob. Agents Chemother.* 49 (2005) 4061–4067. <https://doi.org/10.1128/aac.49.10.4061-4067.2005>.
- [99] Z. Inc., PRIMOR- sulfadimethoxine and ormetoprim tablet, (n.d.). <https://dailymed.nlm.nih.gov/dailymed/fda/fdaDrugXsl.cfm?setid=c74215f7-382f-4082-a950-2e38c3a15d56&type=display> (accessed September 9, 2024).
- [100] G.M. Eliopoulos, P. Huovinen, Resistance to Trimethoprim-Sulfamethoxazole, *Clin. Infect. Dis.* 32 (2001) 1608–1614. <https://doi.org/10.1086/320532>.
- [101] G.B. Michael, P. Butaye, A. Cloeckaert, S. Schwarz, Genes and mutations conferring antimicrobial resistance in *Salmonella*: an update, *Microbes Infect.* 8 (2006) 1898–1914. <https://doi.org/10.1016/j.micinf.2005.12.019>.
- [102] Keen, P. L, *Antimicrobial Resistance in the Environment*, 1st ed., Hoboken, N.J. : Wiley-Blackwell, 2011. <https://onlinelibrary.wiley.com/doi/book/10.1002/9781118156247#page=430> (accessed October 23, 2024).

- [103] I. Sartini, B. Łebkowska-Wieruszewska, A. Lisowski, A. Poapolathep, M. Giorgi, Danofloxacin pharmacokinetics and tissue residues in Bilgorajska geese, *Res. Vet. Sci.* 136 (2021) 11–17. <https://doi.org/10.1016/j.rvsc.2021.01.017>.
- [104] V. Anderson, N. Osheroff, Type II Topoisomerases as Targets for Quinolone Antibacterials Turning Dr. Jekyll into Mr. Hyde, *Curr. Pharm. Des.* 7 (2001) 337–353. <https://doi.org/10.2174/1381612013398013>.
- [105] Ł. Grabowski, L. Gaffke, K. Pierzynowska, Z. Cyske, M. Choszcz, G. Węgrzyn, A. Węgrzyn, Enrofloxacin—The Ruthless Killer of Eukaryotic Cells or the Last Hope in the Fight against Bacterial Infections?, *Int. J. Mol. Sci.* 23 (2022) 3648. <https://doi.org/10.3390/ijms23073648>.
- [106] J.M. Blondeau, Fluoroquinolones: mechanism of action, classification, and development of resistance, *Surv. Ophthalmol.* 49 (2004) S73–S78. <https://doi.org/10.1016/j.survophthal.2004.01.005>.
- [107] D.C. Hooper, Mechanisms of Action and Resistance of Older and Newer Fluoroquinolones, *Clin. Infect. Dis.* 31 (2000) S24–S28. <https://doi.org/10.1086/314056>.
- [108] S.S. Manoharan-Basil, N. González, J.G.E. Laumen, C. Kenyon, Horizontal Gene Transfer of Fluoroquinolone Resistance-Confering Genes From Commensal *Neisseria* to *Neisseria gonorrhoeae*: A Global Phylogenetic Analysis of 20,047 Isolates, *Front. Microbiol.* 13 (2022) 793612. <https://doi.org/10.3389/fmicb.2022.793612>.
- [109] K.H. Plumlee, Clinical Veterinary Toxicology, PART THREE: Cl. Toxic. (2004) 282–336. <https://doi.org/10.1016/b0-32-301125-x/50027-3>.
- [110] P.D. Constable, K.W. Hinchcliff, S.H. Done, W. Gruenberg, Veterinary Medicine (Eleventh Edition), 2016. <https://doi.org/10.1016/b978-0-7020-5246-0.00006-1>.
- [111] J. Spížek, T. Řezanka, Lincosamides: Chemical structure, biosynthesis, mechanism of action, resistance, and applications, *Biochem. Pharmacol.* 133 (2017) 20–28. <https://doi.org/10.1016/j.bcp.2016.12.001>.
- [112] M.C. Roberts, Environmental Macrolide–Lincosamide–Streptogramin and Tetracycline Resistant Bacteria, *Front. Microbiol.* 2 (2011) 40. <https://doi.org/10.3389/fmicb.2011.00040>.
- [113] R. Leclercq, Mechanisms of Resistance to Macrolides and Lincosamides: Nature of the Resistance Elements and Their Clinical Implications, *Clin. Infect. Dis.* 34 (2002) 482–492. <https://doi.org/10.1086/324626>.
- [114] Y. Yang, S. Xie, F. He, Y. Xu, Z. Wang, A. Ihsan, X. Wang, Recent development and fighting strategies for lincosamide antibiotic resistance, *Clin. Microbiol. Rev.* 37 (2024) e00161-23. <https://doi.org/10.1128/cmr.00161-23>.

- [115] H. Wyns, E. Meyer, E. Plessers, A. Watteyn, S.D. Baere, P.D. Backer, S. Croubels, Pharmacokinetics of gamithromycin after intravenous and subcutaneous administration in pigs, *Res. Vet. Sci.* 96 (2014) 160–163. <https://doi.org/10.1016/j.rvsc.2013.11.012>.
- [116] M.G. Papich, *Saunders Handbook of Veterinary Drugs (Fourth Edition)*, (2016) 789–791. <https://doi.org/10.1016/b978-0-323-24485-5.00557-x>.
- [117] M. Elbadawy, M. Aboubakr, A. Abugomaa, Pharmacokinetics of Tylvalosin in Broiler Turkeys (*Meleagris Gallopavo*) After Single Intravenous and Oral Administration, *Front. Vet. Sci.* 6 (2019) 355. <https://doi.org/10.3389/fvets.2019.00355>.
- [118] C. Fyfe, T.H. Grossman, K. Kerstein, J. Sutcliffe, Resistance to Macrolide Antibiotics in Public Health Pathogens, *Cold Spring Harb. Perspect. Med.* 6 (2016) a025395. <https://doi.org/10.1101/cshperspect.a025395>.
- [119] M. Barlow, Horizontal Gene Transfer, *Genomes in Flux*, *Methods Mol. Biol.* 532 (2009) 397–411. https://doi.org/10.1007/978-1-60327-853-9_23.
- [120] M.C. Roberts, Update on macrolide–lincosamide–streptogramin, ketolide, and oxazolidinone resistance genes, *FEMS Microbiol. Lett.* 282 (2008) 147–159. <https://doi.org/10.1111/j.1574-6968.2008.01145.x>.
- [121] E. J, H. M, W. M, Amoxicillin Clavulanate, (2024). [//www.ncbi.nlm.nih.gov/books/NBK538164/](https://www.ncbi.nlm.nih.gov/books/NBK538164/).
- [122] P. BV, B. H, G. M, Ampicillin, StatPearls [Internet] (2023). [//www.ncbi.nlm.nih.gov/books/NBK519569/](https://www.ncbi.nlm.nih.gov/books/NBK519569/) (accessed September 9, 2024).
- [123] N.C. for B. Information, PubChem Compound Summary for CID 6098, Cloxacillin, (2024). <https://pubchem.ncbi.nlm.nih.gov/compound/Cloxacillin>.
- [124] Y. DW, G. V, Penicillin, StatPearls [Internet] (2024). [//www.ncbi.nlm.nih.gov/books/NBK554560/](https://www.ncbi.nlm.nih.gov/books/NBK554560/) (accessed September 9, 2024).
- [125] L.A. Dever, T.S. Dermody, Mechanisms of Bacterial Resistance to Antibiotics, *Arch. Intern. Med.* 151 (1991) 886–895. <https://doi.org/10.1001/archinte.1991.00400050040010>.
- [126] A.M. Geddes, K.P. Klugman, G.N. Rolinson, Introduction: historical perspective and development of amoxicillin/clavulanate, *Int. J. Antimicrob. Agents* 30 (2007) 109–112. <https://doi.org/10.1016/j.ijantimicag.2007.07.015>.
- [127] P. Vannuffel, C. Cocito, Mechanism of Action of Streptogramins and Macrolides, *Drugs* 51 (1996) 20–30. <https://doi.org/10.2165/00003495-199600511-00006>.
- [128] T.A. Mukhtar, G.D. Wright, Streptogramins, Oxazolidinones, and Other Inhibitors of Bacterial Protein Synthesis, *Chem. Rev.* 105 (2005) 529–542. <https://doi.org/10.1021/cr030110z>.

- [129] A.M. Hammerum, L.B. Jensen, F.M. Aarestrup, Detection of the *satA* gene and transferability of virginiamycin resistance in *Enterococcus faecium* from food-animals, *FEMS Microbiol. Lett.* 168 (1998) 145–151. <https://doi.org/10.1111/j.1574-6968.1998.tb13267.x>.
- [130] N.C. for B.I. (2024), PubChem Compound Summary for CID 5327, Sulfamethazine, (n.d.). <https://pubchem.ncbi.nlm.nih.gov/compound/Sulfamethazine> (accessed September 9, 2024).
- [131] M.V. Smith, *Textbook of Rabbit Medicine*, (2023) 100–137. <https://doi.org/10.1016/b978-0-7020-8403-4.00003-x>.
- [132] M.G. Papich, *Papich Handbook of Veterinary Drugs*, (2021) 865–866. <https://doi.org/10.1016/b978-0-323-70957-6.00507-0>.
- [133] P. Huovinen, L. Sundström, G. Swedberg, O. Sköld, Trimethoprim and sulfonamide resistance, *Antimicrob. Agents Chemother.* 39 (1995) 279–289. <https://doi.org/10.1128/aac.39.2.279>.
- [134] M. Venkatesan, M. Fruci, L.A. Verellen, T. Skarina, N. Mesa, R. Flick, C. Pham, R. Mahadevan, P.J. Stogios, A. Savchenko, Molecular mechanism of plasmid-borne resistance to sulfonamide antibiotics, *Nat. Commun.* 14 (2023) 4031. <https://doi.org/10.1038/s41467-023-39778-7>.
- [135] J.M.T. Hamilton-Miller, Mechanisms and distribution of bacterial resistance to diaminopyrimidines and sulphonamides, *J. Antimicrob. Chemother.* 5 (1979) 61–73. https://doi.org/10.1093/jac/5.supplement_b.61.
- [136] M.E. Poey, M.F. Azpiroz, M. Laviña, On sulfonamide resistance, *sul* genes, class 1 integrons and their horizontal transfer in *Escherichia coli*, *Microb. Pathog.* 135 (2019) 103611. <https://doi.org/10.1016/j.micpath.2019.103611>.
- [137] D.G. Waller, A.P. Sampson, *Medical Pharmacology and Therapeutics (Fifth Edition)*, Sect. 11: Chemother. (2018) 581–629. <https://doi.org/10.1016/b978-0-7020-7167-6.00051-8>.
- [138] E. Sheykhsaran, H.B. Baghi, M.H. Soroush, R. Ghotaslou, An overview of tetracyclines and related resistance mechanisms, *Rev. Méd. Microbiol.* 30 (2019) 69–75. <https://doi.org/10.1097/mrm.000000000000154>.
- [139] B.S. Speer, N.B. Shoemaker, A.A. Salyers, Bacterial resistance to tetracycline: mechanisms, transfer, and clinical significance, *Clin. Microbiol. Rev.* 5 (1992) 387–399. <https://doi.org/10.1128/cmr.5.4.387>.
- [140] F. Nguyen, A.L. Starosta, S. Arenz, D. Sohmen, A. Dönhöfer, D.N. Wilson, Tetracycline antibiotics and resistance mechanisms, *Biol. Chem.* 395 (2014) 559–575. <https://doi.org/10.1515/hsz-2013-0292>.

- [141] S.W. Shin, M.K. Shin, M. Jung, K.M. Belaynehe, H.S. Yoo, Prevalence of Antimicrobial Resistance and Transfer of Tetracycline Resistance Genes in *Escherichia coli* Isolates from Beef Cattle, *Appl. Environ. Microbiol.* 81 (2015) 5560–5566. <https://doi.org/10.1128/aem.01511-15>.
- [142] H.L. Wang, M. Shi, X. Xu, L. Pan, P.F. Zhao, X.K. Ma, Q.Y. Tian, X.S. Piao, Effects of Flavomycin, *Bacillus licheniformis* and Enramycin on Performance, Nutrient Digestibility, Gut Morphology and the Intestinal Microflora of Broilers, *J. Poult. Sci.* 53 (2015) 128–135. <https://doi.org/10.2141/jpsa.0150077>.
- [143] D. Łowicki, A. Huczyński, Structure and Antimicrobial Properties of Monensin A and Its Derivatives: Summary of the Achievements, *BioMed Res. Int.* 2013 (2013) 742149. <https://doi.org/10.1155/2013/742149>.
- [144] H.D. Chapman, T. Rathinam, Focused review: The role of drug combinations for the control of coccidiosis in commercially reared chickens, *Int. J. Parasitol.: Drugs Drug Resist.* 18 (2022) 32–42. <https://doi.org/10.1016/j.ijpddr.2022.01.001>.
- [145] H. Zhang, L. Zhang, H. Si, X. Liu, X. Suo, D. Hu, Early Transcriptional Response to Monensin in Sensitive and Resistant Strains of *Eimeria tenella*, *Front. Microbiol.* 13 (2022) 934153. <https://doi.org/10.3389/fmicb.2022.934153>.
- [146] W. Markiewicz, D. Barski, E. Tomaszewska, A. Burmańczuk, Toxicity of salinomycin and narasin in turkeys, *J. Elem.* (2014). <https://doi.org/10.5601/jelem.2014.19.1.567>.
- [147] T. Koltai, S.J. Reshkin, F. Baltazar, L. Fliegel, Prostate Cancer Metabolism, (2021) 369–385. <https://doi.org/10.1016/b978-0-323-90528-2.00002-3>.
- [148] J.B. Russell, A.J. Houlihan, Ionophore resistance of ruminal bacteria and its potential impact on human health¹, *FEMS Microbiol. Rev.* 27 (2003) 65–74. [https://doi.org/10.1016/s0168-6445\(03\)00019-6](https://doi.org/10.1016/s0168-6445(03)00019-6).
- [149] A. Wong, Unknown Risk on the Farm: Does Agricultural Use of Ionophores Contribute to the Burden of Antimicrobial Resistance?, *MSphere* 4 (2019) 10.1128/msphere.00433-19. <https://doi.org/10.1128/msphere.00433-19>.
- [150] A.-O. Naemi, H. Dey, N. Kiran, S.T. Sandvik, J.S. Slettemeås, L.L. Nesse, R. Simm, NarAB Is an ABC-Type Transporter That Confers Resistance to the Polyether Ionophores Narasin, Salinomycin, and Maduramicin, but Not Monensin, *Front. Microbiol.* 11 (2020) 104. <https://doi.org/10.3389/fmicb.2020.00104>.
- [151] H. Wolf, Avilamycin, an inhibitor of the 30 S ribosomal subunits function, *FEBS Lett.* 36 (1973) 181–186. [https://doi.org/10.1016/0014-5793\(73\)80364-3](https://doi.org/10.1016/0014-5793(73)80364-3).
- [152] C.B. Kofoed, B. Vester, Interaction of Avilamycin with Ribosomes and Resistance Caused by Mutations in 23S rRNA, *Antimicrob. Agents Chemother.* 46 (2002) 3339–3342. <https://doi.org/10.1128/aac.46.11.3339-3342.2002>.

- [153] S. Gatadi, J. Gour, S. Nanduri, Natural product derived promising anti-MRSA drug leads: A review, *Bioorg. Med. Chem.* 27 (2019) 3760–3774. <https://doi.org/10.1016/j.bmc.2019.07.023>.
- [154] J. Bøsling, S.M. Poulsen, B. Vester, K.S. Long, Resistance to the Peptidyl Transferase Inhibitor Tiamulin Caused by Mutation of Ribosomal Protein L3, *Antimicrob. Agents Chemother.* 47 (2003) 2892–2896. <https://doi.org/10.1128/aac.47.9.2892-2896.2003>.
- [155] R. Nguyen, N.R. Khanna, A.O. Safadi, P. Patel, Y. Sun, Bacitracin Topical, StatPearls [Internet] (2024). <https://www.ncbi.nlm.nih.gov/books/NBK536993/> (accessed October 22, 2024).
- [156] Z. Podlesek, A. Comino, B. Herzog-Velikonja, M. Grabnar, The role of the bacitracin ABC transporter in bacitracin resistance and collateral detergent sensitivity, *FEMS Microbiol. Lett.* 188 (2000) 103–106. <https://doi.org/10.1111/j.1574-6968.2000.tb09176.x>.
- [157] Z. Podlesek, A. Comino, B. Herzog-Velikonja, D. Žgur-Bertok, R. Komel, M. Grabnar, *Bacillus licheniformis* bacitracin-resistance ABC transporter: relationship to mammalian multidrug resistance, *Mol. Microbiol.* 16 (1995) 969–976. <https://doi.org/10.1111/j.1365-2958.1995.tb02322.x>.
- [158] J. Ma, J. Liu, Y. Zhang, D. Wang, R. Liu, G. Liu, H. Yao, Z. Pan, Bacitracin resistance and enhanced virulence of *Streptococcus suis* via a novel efflux pump, *BMC Vet. Res.* 15 (2019) 377. <https://doi.org/10.1186/s12917-019-2115-2>.
- [159] J.M. Manson, S. Keis, J.M.B. Smith, G.M. Cook, Acquired Bacitracin Resistance in *Enterococcus faecalis* Is Mediated by an ABC Transporter and a Novel Regulatory Protein, BcrR, *Antimicrob. Agents Chemother.* 48 (2004) 3743–3748. <https://doi.org/10.1128/aac.48.10.3743-3748.2004>.
- [160] F. Xu, G. Cheng, H. Hao, Y. Wang, X. Wang, D. Chen, D. Peng, Z. Liu, Z. Yuan, M. Dai, Mechanisms of Antibacterial Action of Quinoxaline 1,4-di-N-oxides against *Clostridium perfringens* and *Brachyspira hyodysenteriae*, *Front. Microbiol.* 7 (2016) 1948. <https://doi.org/10.3389/fmicb.2016.01948>.
- [161] F. Perez, S.D. Rudin, S.H. Marshall, P. Coakley, L. Chen, B.N. Kreiswirth, P.N. Rather, A.M. Hujer, P. Toltzis, D. van Duin, D.L. Paterson, R.A. Bonomo, OqxAB, a Quinolone and Olaquinox Efflux Pump, Is Widely Distributed among Multidrug-Resistant *Klebsiella pneumoniae* Isolates of Human Origin, *Antimicrob. Agents Chemother.* 57 (2013) 4602–4603. <https://doi.org/10.1128/aac.00725-13>.
- [162] B.L. Bearson, H.K. Allen, B.W. Brunelle, I.S. Lee, S.R. Casjens, T.B. Stanton, The agricultural antibiotic carbadox induces phage-mediated gene transfer in *Salmonella*, *Front. Microbiol.* 5 (2014) 52. <https://doi.org/10.3389/fmicb.2014.00052>.

- [163] W. Guo, H. Hao, M. Dai, Y. Wang, L. Huang, D. Peng, X. Wang, H. Wang, M. Yao, Y. Sun, Z. Liu, Z. Yuan, Development of Quinoxaline 1, 4-Dioxides Resistance in *Escherichia coli* and Molecular Change under Resistance Selection, *PLoS ONE* 7 (2012) e43322. <https://doi.org/10.1371/journal.pone.0043322>.
- [164] F. and D.A. (FDA), 2022 Summary Report On Antimicrobials Sold or Distributed for Use in Food-Producing Animals, U.S. Department of Health and Human Services, Rockville, MD, 2021. <https://www.fda.gov/animal-veterinary/antimicrobial-resistance/2022-summary-report-antimicrobials-sold-or-distributed-use-food-producing-animals> (accessed September 20, 2024).
- [165] W.O. for A.H. (WOAH), OIE LIST OF ANTIMICROBIAL AGENTS OF VETERINARY IMPORTANCE, 2021. <https://www.woah.org/app/uploads/2021/06/a-oie-list-antimicrobials-june2021.pdf> (accessed September 20, 2024).
- [166] W.H. Organization, WHO's List of Medically Important Antimicrobials: a risk management tool for mitigating antimicrobial resistance due to non-human use, Geneva, 2024. https://cdn.who.int/media/docs/default-source/gcp/who-mia-list-2024-lv.pdf?sfvrsn=3320dd3d_2 (accessed September 20, 2024).
- [167] C. for D.C. and Prevention, Antibiotic Resistance Threats in the United States, 2013, 2013. <https://www.cdc.gov/antimicrobial-resistance/media/pdfs/ar-threats-2013-508.pdf> (accessed November 26, 2024).
- [168] T.W. House, National strategy for combating antibiotic-resistant bacteria, Washington, DC, 2014. <https://www.ncbi.nlm.nih.gov/books/NBK577283/#pz348-8> (accessed November 26, 2024).
- [169] P. GH, B. GJ, eds., The National Action Plan for Combating Antibiotic-Resistant Bacteria, in: Combating Antimicrobial Resistance and Protecting the Miracle of Modern Medicine., National Academies Press (US), Washington (DC), 2021. <https://www.ncbi.nlm.nih.gov/books/NBK577283/#pz348-8> (accessed November 26, 2024).
- [170] F.C. (Federal T.F. on C.A.-R. Bacteria), National action plan for combating antibiotic-resistant bacteria 2020-2025, Office of the Assistant Secretary for Planning and Evaluation, Department of Health and Human Services, Washington, DC, 2020. <https://www.ncbi.nlm.nih.gov/books/NBK577283/#pz347-5> (accessed November 27, 2024).
- [171] F. and D. Administration, Evaluating the Safety of Antimicrobial New Animal Drugs with Regard to their Microbiological Effects on Bacteria of Human Health Concern, 2003. <https://www.fda.gov/media/69949/download> (accessed November 27, 2024).
- [172] F. and D. Administration, The Judicious Use of Medically Important Antimicrobial Drugs in Food-Producing Animals, 2012.

- [173] U.S.F. and D. Administration, CVM GFI #273 Defining Durations of Use for Approved Medically Important Antimicrobial Drugs Fed to Food-Producing Animals, 2023. <https://www.fda.gov/media/172362/download> (accessed October 19, 2024).
- [174] C. for V. Medicine, List of Approved Medically Important Antimicrobial Drugs Administered in the Feed of Food-Producing Animals that Lack a Defined Duration of Use, FDA (2023). <https://www.fda.gov/animal-veterinary/antimicrobial-resistance/list-approved-medically-important-antimicrobial-drugs-administered-feed-food-producing-animals-lack>.
- [175] P. Aubry, D.W. Geale, A Review of Bovine Anaplasmosis, *Transboundary and Emerging Diseases* 58 (2011) 1–30. <https://doi.org/10.1111/j.1865-1682.2010.01173.x>.
- [176] K.M. Kocan, J. de la Fuente, A.A. Guglielmone, R.D. Meléndez, Antigen and Alternatives for Control of *Anaplasma marginale* Infection in Cattle, *Clin. Microbiol. Rev.* 16 (2003) 698–712. <https://doi.org/10.1128/cmr.16.4.698-712.2003>.
- [177] K.M. Kocan, J. de la Fuente, E.F. Blouin, J.F. Coetzee, S.A. Ewing, The natural history of *Anaplasma marginale*, *Vet. Parasitol.* 167 (2010) 95–107. <https://doi.org/10.1016/j.vetpar.2009.09.012>.
- [178] A.F. Railey, T.L. Marsh, Economic Benefits of Diagnostic Testing in Livestock: Anaplasmosis in Cattle, *Front. Vet. Sci.* 8 (2021) 626420. <https://doi.org/10.3389/fvets.2021.626420>.
- [179] A.M. Fernández, J.A. Ortiz, A. Jabbar, A. Ghafar, A. Cabezas-Cruz, G. de la Fuente, J. de la Fuente, I.G.F. de Mera, Fatal cases of bovine anaplasmosis in a herd infected with different *Anaplasma marginale* genotypes in southern Spain, *Ticks and Tick-Borne Diseases* 13 (2022) 101864. <https://doi.org/10.1016/j.ttbdis.2021.101864>.
- [180] P. Parodi, M.T. Armúa-Fernández, L.G. Corbellini, R. Rivero, C. Miraballes, F. Riet-Correa, J.M. Venzal, Description of bovine babesiosis and anaplasmosis outbreaks in northern Uruguay between 2016 and 2018, *Veterinary Parasitology: Regional Studies and Reports* 29 (2022) 100700. <https://doi.org/10.1016/j.vprsr.2022.100700>.
- [181] A.C. Lovett, E.J. Reppert, J.R. Jaeger, Q. Kang, M.R. Flowers, N.P. Bickmeier, T. Anantat, S.C. O’Day, C.L. Armstrong, K.E. Reif, Satisfactory breeding potential is transiently eliminated in beef bulls with clinical anaplasmosis, *BMC Vet Res* 18 (2022) 381. <https://doi.org/10.1186/s12917-022-03470-7>.
- [182] S.T. Kieser, I.S. Eriks, G.H. Palmer, Cyclic rickettsemia during persistent *Anaplasma marginale* infection of cattle, *Infection and Immunity* 58 (1990) 1117–1119. <https://doi.org/10.1128/iai.58.4.1117-1119.1990>.
- [183] R.A. Valdez, T.C. McGuire, W.C. Brown, W.C. Davis, J.M. Jordan, D.P. Knowles, Selective In Vivo Depletion of CD4+ T Lymphocytes with Anti-CD4 Monoclonal Antibody

during Acute Infection of Calves with *Anaplasma marginale*, *Clinical and Vaccine Immunology* 9 (2002) 417–424. <https://doi.org/10.1128/cdli.9.2.417-424.2002>.

[184] J.S. Dumler, A.F. Barbet, C.P. Bekker, G.A. Dasch, G.H. Palmer, S.C. Ray, Y. Rikihisa, F.R. Rurangirwa, Reorganization of genera in the families Rickettsiaceae and Anaplasmataceae in the order Rickettsiales: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and designation of *Ehrlichia equi* and “HGE agent” as subjective synonyms of *Ehrlichia phagocytophila*, *Int J Syst Evol Microbiol* 51 (2001) 2145–2165. <https://doi.org/10.1099/00207713-51-6-2145>.

[185] L. Pruneau, A. Moumène, D.F. Meyer, I. Marcelino, T. Lefrançois, N. Vachiéry, Understanding Anaplasmataceae pathogenesis using “Omics” approaches, *Front. Cell. Infect. Microbiol.* 4 (2014) 86. <https://doi.org/10.3389/fcimb.2014.00086>.

[186] S.M. Chen, J.S. Dumler, J.S. Bakken, D.H. Walker, Identification of a granulocytotropic *Ehrlichia* species as the etiologic agent of human disease, *J. Clin. Microbiol.* 32 (1994) 589–595. <https://doi.org/10.1128/jcm.32.3.589-595.1994>.

[187] A. Theiler, Further investigations into anaplasmosis of South African cattle, Pretoria : Government Printer and Stationery Office, 1911. <https://repository.up.ac.za/handle/2263/11310>.

[188] M. Battilani, S.D. Arcangeli, A. Balboni, F. Dondi, Genetic diversity and molecular epidemiology of *Anaplasma*, *Infect., Genet. Evol.* 49 (2017) 195–211. <https://doi.org/10.1016/j.meegid.2017.01.021>.

[189] D.R. Allred, T.C. McGuire, G.H. Palmer, S.R. Leib, T.M. Harkins, T.F. McElwain, A.F. Barbet, Molecular basis for surface antigen size polymorphisms and conservation of a neutralization-sensitive epitope in *Anaplasma marginale*., *Proceedings of the National Academy of Sciences* 87 (1990) 3220–3224. <https://doi.org/10.1073/pnas.87.8.3220>.

[190] J. de la Fuente, J.C. Garcia-Garcia, E.F. Blouin, K.M. Kocan, Characterization of the functional domain of major surface protein 1a involved in adhesion of the rickettsia *Anaplasma marginale* to host cells, *Vet. Microbiol.* 91 (2003) 265–283. [https://doi.org/10.1016/s0378-1135\(02\)00309-7](https://doi.org/10.1016/s0378-1135(02)00309-7).

[191] K.A. Brayton, L.S. Kappmeyer, D.R. Herndon, M.J. Dark, D.L. Tibbals, G.H. Palmer, T.C. McGuire, D.P. Knowles, Complete genome sequencing of *Anaplasma marginale* reveals that the surface is skewed to two superfamilies of outer membrane proteins, *Proc Natl Acad Sci U S A* 102 (2005) 844–849. <https://doi.org/10.1073/pnas.0406656102>.

[192] N. Viseshakul, S. Kamper, M.V. Bowie, A.F. Barbet, Sequence and expression analysis of a surface antigen gene family of the rickettsia *Anaplasma marginale*, *Gene* 253 (2000) 45–53. [https://doi.org/10.1016/s0378-1119\(00\)00241-9](https://doi.org/10.1016/s0378-1119(00)00241-9).

[193] B.R. McCallon, Prevalence and Economic Aspects of Anaplasmosis, in: 1973.

- [194] C.C. Okafor, S.L. Collins, J.A. Daniel, B. Harvey, X. Sun, J.F. Coetzee, B.K. Whitlock, Factors associated with Seroprevalence of *Anaplasma marginale* in Kentucky cattle, *Veterinary Parasitology: Regional Studies and Reports* 13 (2018) 212–219. <https://doi.org/10.1016/j.vprsr.2018.07.003>.
- [195] C.C. Okafor, S.L. Collins, J.A. Daniel, B. Harvey, J.F. Coetzee, B.K. Whitlock, Factors associated with seroprevalence of bovine anaplasmosis in Texas, *Veterinary Parasitology: Regional Studies and Reports* 14 (2018) 32–40. <https://doi.org/10.1016/j.vprsr.2018.08.004>.
- [196] C.C. Okafor, S.L. Collins, J.A. Daniel, J.F. Coetzee, B.K. Whitlock, Factors associated with seroprevalence of bovine anaplasmosis in Mississippi, USA, *Veterinary Parasitology: Regional Studies and Reports* 17 (2019) 100301. <https://doi.org/10.1016/j.vprsr.2019.100301>.
- [197] C.C. Okafor, S.L. Collins, J.A. Daniel, J.F. Coetzee, B.K. Whitlock, Seroprevalence of bovine Anaplasmosis in Georgia, *Veterinary Parasitology: Regional Studies and Reports* 15 (2019) 100258. <https://doi.org/10.1016/j.vprsr.2018.100258>.
- [198] B.K. Whitlock, J.A. Daniel, B.S. Harvey, J.K. Johnson, J.F. Coetzee, Seroprevalence of bovine anaplasmosis in the southern US, *Am. Assoc. Bov. Pr. Conf. Proc.* (2014) 157. <https://doi.org/10.21423/aabppro20143739>.
- [199] T. Hairgrove, M.E. Schroeder, C.M. Budke, S. Rodgers, C. Chung, M.W. Ueti, M.A. Bounpheng, Molecular and serological in-herd prevalence of *Anaplasma marginale* infection in Texas cattle, *Prev. Vet. Med.* 119 (2015) 1–9. <https://doi.org/10.1016/j.prevetmed.2015.02.006>.
- [200] M.R. Spare, G.A. Hanzlicek, K.L. Wootten, G.A. Anderson, D.U. Thomson, M.W. Sanderson, R.R. Ganta, K.E. Reif, R.K. Raghavan, Bovine anaplasmosis herd prevalence and management practices as risk-factors associated with herd disease status, *Veterinary Parasitology* 277 (2020) 100021. <https://doi.org/10.1016/j.vpoa.2019.100021>.
- [201] A.L. Jones, 1, A.A. Kalantari, B. Credille, H.K. Naikare, B. Heins, J.T. Saliki, R.P. Wilkes, Seroprevalence and molecular detection of *Anaplasma marginale* infected beef herds in Georgia, USA, *The Bovine Practitioner* 2 (2022).
- [202] L.L. Hungerford, R.D. Smith, Variations in Seroprevalence and Host Factors for Bovine Anaplasmosis in Illinois, *Vet. Res. Commun.* 21 (1997) 9–18. <https://doi.org/10.1023/b:verc.0000009696.68224.a8>.
- [203] A. Eleftheriou, D. Cole, J. Kieffer, R. Pesapane, Molecular prevalence of *Anaplasma marginale* and associated risk factors in beef cattle herds from Ohio: a cross-sectional study, *J. Am. Vet. Méd. Assoc.* 260 (2022) 1839–1843. <https://doi.org/10.2460/javma.22.05.0204>.
- [204] DURRANI, ANEELA, A retrospective study of *Anaplasma* in Minnesota cattle, *Turkish Journal of Veterinary & Animal Sciences* 36 (2012). <https://doi.org/10.3906/vet-1012-632>.

- [205] J.F. Coetzee, P.L. Schmidt, A.M. O'Connor, M.D. Apley, Seroprevalence of *Anaplasma marginale* in 2 Iowa feedlots and its association with morbidity, mortality, production parameters, and carcass traits., *Can. Vet. J. Rev. Veterinaire Can.* 51 (2010) 862–8.
- [206] Geoffroy, L. M, Hindman, M.S, Distribution trends of bovine *Anaplasma marginale* in Iowa, based upon veterinary diagnostic lab submissions from 2017-2022., *The Bovine Practitioner* 2 (2024) 34–42. <https://doi.org/10.21423/bpj20249021>.
- [207] A.H.L. Nguyen, S. Tiawsirisup, M. Kaewthamasorn, Molecular detection and genetic characterization of *Anaplasma marginale* and *Anaplasma platys*-like (Rickettsiales: Anaplasmataceae) in water buffalo from eight provinces of Thailand, *BMC Vet. Res.* 16 (2020) 380. <https://doi.org/10.1186/s12917-020-02585-z>.
- [208] R. Mwale, M. Mulavu, C.S. Khumalo, A. Mukubesa, K. Nalubamba, B. Mubemba, K. Changula, E. Simulundu, S. Chitanga, B. Namangala, L. Mataa, V.C. Zulu, M. Munyeme, W. Muleya, Molecular detection and characterization of *Anaplasma* spp. in cattle and sable antelope from Lusaka and North-Western provinces of Zambia, *Vet. Parasitol.: Reg. Stud. Rep.* 39 (2023) 100847. <https://doi.org/10.1016/j.vprsr.2023.100847>.
- [209] J. de la Fuente, J. Vicente, U. Höfle, F. Ruiz-Fons, I.G.F. de Mera, R.A.V.D. Bussche, K.M. Kocan, C. Gortazar, *Anaplasma* infection in free-ranging Iberian red deer in the region of Castilla-La Mancha, Spain, *Vet. Microbiol.* 100 (2004) 163–173. <https://doi.org/10.1016/j.vetmic.2004.02.007>.
- [210] N.B. da Silva, N.S. Taus, W.C. Johnson, A. Mira, L. Schnittger, J.D.M. Valente, O. Vidotto, H.E. Masterson, T.S.W.J. Vieira, M.W. Ueti, R.F.C. Vieira, First report of *Anaplasma marginale* infection in goats, Brazil, *PLoS ONE* 13 (2018) e0202140. <https://doi.org/10.1371/journal.pone.0202140>.
- [211] I.C. Barbosa, M.R. André, R.B. do Amaral, J.D.M. Valente, P.C. Vasconcelos, C.J.B. Oliveira, M.M.G. Jusi, R.Z. Machado, T.S.W.J. Vieira, M.W. Ueti, R.F.C. Vieira, *Anaplasma marginale* in goats from a multispecies grazing system in northeastern Brazil, *Ticks Tick-Borne Dis.* 12 (2021) 101592. <https://doi.org/10.1016/j.ttbdis.2020.101592>.
- [212] S. Ali, M. Hasan, A.S. Ahmad, K. Ashraf, J.A. Khan, M.I. Rashid, Molecular prevalence of *Anaplasma marginale* in ruminants and *Rhipicephalus* ticks in northern Pakistan, *Trop. Biomed.* 40 (2023) 7–13. <https://doi.org/10.47665/tb.40.1.005>.
- [213] M.K. Keel, W.L. Goff, W.R. Davidson, AN ASSESSMENT OF THE ROLE OF WHITE-TAILED DEER IN THE EPIZOOTIOLOGY OF ANAPLASMOSIS IN THE SOUTHEASTERN UNITED STATES, *J. Wildl. Dis.* 31 (1995) 378–385. <https://doi.org/10.7589/0090-3558-31.3.378>.
- [214] U.G. Munderloh, C.M. Tate, M.J. Lynch, E.W. Howerth, T.J. Kurtti, W.R. Davidson, Isolation of an *Anaplasma* sp. Organism from White-Tailed Deer by Tick Cell Culture, *J. Clin. Microbiol.* 41 (2003) 4328–4335. <https://doi.org/10.1128/jcm.41.9.4328-4335.2003>.

- [215] V.A. Lobanov, A.A. Gajadhar, B. Al-Adhami, H.M. Schwantje, Molecular Study of Free-ranging Mule Deer and White-tailed Deer from British Columbia, Canada, for Evidence of *Anaplasma* spp. and *Ehrlichia* spp., *Transbound. Emerg. Dis.* 59 (2012) 233–243. <https://doi.org/10.1111/j.1865-1682.2011.01259.x>.
- [216] K.L. Kuttler, ANAPLASMA INFECTIONS IN WILD AND DOMESTIC RUMINANTS: A REVIEW, *J. Wildl. Dis.* 20 (1984) 12–20. <https://doi.org/10.7589/0090-3558-20.1.12>.
- [217] A.K. Curtis, J.F. Coetzee, Assessment of within-herd seroprevalence of *Anaplasma marginale* antibodies and associated decreased milk production in an Iowa dairy herd, *Appl. Anim. Sci.* 37 (2021) 126–131. <https://doi.org/10.15232/aas.2020-02110>.
- [218] J.B. da Silva, A.H. da Fonseca, Risk factors for anaplasmosis in dairy cows during the peripartum, *Trop. Anim. Heal. Prod.* 46 (2014) 461–465. <https://doi.org/10.1007/s11250-013-0514-0>.
- [219] M. Aktas, S. Özübek, Outbreak of anaplasmosis associated with novel genetic variants of *Anaplasma marginale* in a dairy cattle, *Comp Immunol Microbiol Infect Dis* 54 (2017) 20–26. <https://doi.org/10.1016/j.cimid.2017.07.008>.
- [220] R.Z. Machado, J.B. da Silva, M.R. André, L.R. Gonçalves, C.A. Matos, D. Obregón, Outbreak of anaplasmosis associated with the presence of different *Anaplasma marginale* strains in dairy cattle in the states of São Paulo and Goiás, Brazil, *Rev. Bras. Parasitol. Veterinária* 24 (2015) 438–446. <https://doi.org/10.1590/s1984-29612015078>.
- [221] L.C. Henker, M.P. Lorenzetti, R. Fagundes-Moreira, A.G.C. Dalto, L. Sonne, D. Driemeier, J.F. Soares, S.P. Pavarini, Bovine abortion, stillbirth and neonatal death associated with *Babesia bovis* and *Anaplasma* sp. infections in southern Brazil, *Ticks Tick-Borne Dis.* 11 (2020) 101443. <https://doi.org/10.1016/j.ttbdis.2020.101443>.
- [222] D. Fowler, B.L. Swift, Abortion in cows inoculated with *Anaplasma marginale*, *Theriogenology* 4 (1975) 59–67. [https://doi.org/10.1016/0093-691x\(75\)90106-5](https://doi.org/10.1016/0093-691x(75)90106-5).
- [223] D.M. French, W.C. Brown, G.H. Palmer, Emergence of *Anaplasma marginale* Antigenic Variants during Persistent Rickettsemia, *Infect. Immun.* 67 (1999) 5834–5840. <https://doi.org/10.1128/iai.67.11.5834-5840.1999>.
- [224] A.C. Jablinski, E.J. Reppert, S. Huser, B.D. Robért, J.R. Jaeger, Q. Kang, R. Liu, T. Anantatat, C.L. Armstrong, K.E. Reif, Kansas beef bulls with chronic anaplasmosis demonstrate satisfactory breeding soundness outcomes at breeding soundness examination, *J. Am. Vet. Méd. Assoc.* 262 (2023) 1–8. <https://doi.org/10.2460/javma.23.05.0292>.
- [225] H.E.G. Grau, N.A. da Cunha, F.G. Pappen, N.A. da R. Farias, Transplacental transmission of *Anaplasma marginale* in beef cattle chronically infected in southern Brazil, *Rev. Bras. Parasitol. Veterinária* 22 (2013) 189–193. <https://doi.org/10.1590/s1984-29612013000200038>.

- [226] I.S. Eriks, D. Stiller, G.H. Palmer, Impact of persistent *Anaplasma marginale* rickettsemia on tick infection and transmission, *J. Clin. Microbiol.* 31 (1993) 2091–2096. <https://doi.org/10.1128/jcm.31.8.2091-2096.1993>.
- [227] A.R. Alleman, G.H. Palmer, T.C. McGuire, T.F. McElwain, L.E. Perryman, A.F. Barbet, *Anaplasma marginale* major surface protein 3 is encoded by a polymorphic, multigene family, *Infect Immun* 65 (1997) 156–163. <https://doi.org/10.1128/iai.65.1.156-163.1997>.
- [228] G.H. Palmer, G. Eid, A.F. Barbet, T.C. McGuire, T.F. McElwain, The immunoprotective *Anaplasma marginale* major surface protein 2 is encoded by a polymorphic multigene family, *Infection and Immunity* 62 (1994) 3808–3816. <https://doi.org/10.1128/iai.62.9.3808-3816.1994>.
- [229] A.F. Barbet, J. Yi, A. Lundgren, B.R. McEwen, E.F. Blouin, K.M. Kocan, Antigenic Variation of *Anaplasma Marginale*: Major Surface Protein 2 Diversity during Cyclic Transmission between Ticks and Cattle, *Infect Immun* 69 (2001) 3057–3066. <https://doi.org/10.1128/iai.69.5.3057-3066.2001>.
- [230] K.A. Brayton, G.H. Palmer, A. Lundgren, J. Yi, A.F. Barbet, Antigenic variation of *Anaplasma marginale* msp2 occurs by combinatorial gene conversion, *Mol. Microbiol.* 43 (2002) 1151–1159. <https://doi.org/10.1046/j.1365-2958.2002.02792.x>.
- [231] D.M. French, W.C. Brown, G.H. Palmer, Emergence of *Anaplasma marginale* Antigenic Variants during Persistent Rickettsemia, *Infect Immun* 67 (1999) 5834–5840. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC96963/>.
- [232] P. Shahbazi, S.N. Gharajalar, K. Mohebbi, J. Taeb, H.H. Farhang, A.A. Nikvand, R. Norouzi, First Survey on the Presence and Distribution of Oxytetracycline-Resistance Genes in *Anaplasma* Species, *Acta Parasitol.* 66 (2021) 501–507. <https://doi.org/10.1007/s11686-020-00306-y>.
- [233] I. Chopra, M. Roberts, Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance, *Microbiol Mol Biol Rev* 65 (2001) 232–260 ; second page, table of contents. <https://doi.org/10.1128/membr.65.2.232-260.2001>.
- [234] J.B. Reinbold, J.F. Coetzee, L.C. Hollis, J.S. Nickell, C.M. Riegel, J.A. Christopher, R.R. Ganta, Comparison of iatrogenic transmission of *Anaplasma marginale* in Holstein steers via needle and needle-free injection techniques, *Am J Vet Res* 71 (2010) 1178–1188. <https://doi.org/10.2460/ajvr.71.10.1178>.
- [235] Z. Zivkovic, A.M. Nijhof, J. de la Fuente, K.M. Kocan, F. Jongejan, Experimental transmission of *Anaplasma marginale* by male *Dermacentor reticulatus*, *BMC Veterinary Research* 3 (2007) 32. <https://doi.org/10.1186/1746-6148-3-32>.
- [236] K.M. Kocan, W.L. Goff, D. Stiller, P.L. Claypool, W. Edwards, S.A. Ewing, J.A. Hair, S.J. Barron, Persistence of *Anaplasma marginale* (Rickettsiales: Anaplasmataceae) in male

Dermacentor andersoni (Acari: Ixodidae) transferred successively from infected to susceptible calves., J. Méd. Entomol. 29 (1992) 657–68. <https://doi.org/10.1093/jmedent/29.4.657>.

[237] K.M. Kocan, D. Stiller, W.L. Goff, P.L. Claypool, W. Edwards, S.A. Ewing, T.C. McGuire, J.A. Hair, S.J. Barron, Development of *Anaplasma marginale* in male *Dermacentor andersoni* transferred from parasitemic to susceptible cattle, Am J Vet Res 53 (1992) 499–507. <http://www.ncbi.nlm.nih.gov/pubmed/1586018>.

[238] A.E. Kiszewski, F.-R. Matuschka, A. Spielman, MATING STRATEGIES AND SPERMIOGENESIS IN IXODID TICKS, Annu. Rev. Entomol. 46 (2001) 167–182. <https://doi.org/10.1146/annurev.ento.46.1.167>.

[239] G. Földvári, P. Široký, S. Szekeres, G. Majoros, H. Sprong, *Dermacentor reticulatus*: a vector on the rise, Parasites Vectors 9 (2016) 314. <https://doi.org/10.1186/s13071-016-1599-x>.

[240] K.M. Kocan, D. Stiller, W.L. Goff, P.L. Claypool, W. Edwards, S.A. Ewing, T.C. McGuire, J.A. Hair, S.J. Barron, Development of *Anaplasma marginale* in male *Dermacentor andersoni* transferred from parasitemic to susceptible cattle., Am. J. Vet. Res. 53 (1992) 499–507. <http://www.ncbi.nlm.nih.gov/pubmed/1586018>.

[241] Yu.S. Balashov, Bloodsucking Ticks (Ixodoidea)—Vectors of Diseases of Man and Animals, Entomological Society of America's Thomas Say Books, 1972. <https://doi.org/10.4182/hktd2318.8-5.167>.

[242] N.-L. Ge, K.M. Kocan, E.F. Blouin, G.L. Murphy, Developmental Studies of *Anaplasma marginale* (Rickettsiales: Anaplasmataceae) in Male *Dermacentor andersoni* (Acari: Ixodidae) Infected as Adults by Using Nonradioactive In Situ Hybridization and Microscopy, Journal of Medical Entomology 33 (1996) 911–920. <https://doi.org/10.1093/jmedent/33.6.911>.

[243] K.M. Kocan, S.J. Barron, S.A. Ewing, J.A. Hair, Transmission of *Anaplasma marginale* by adult *Dermacentor andersoni* during feeding on calves, Am J Vet Res 46 (1985) 1565–1567.

[244] G.A. Scoles, J.A. Miller, L.D. Foil, Comparison of the Efficiency of Biological Transmission of *Anaplasma marginale* (Rickettsiales: Anaplasmataceae) by *Dermacentor andersoni* Stiles (Acari: Ixodidae) with Mechanical Transmission by the Horse Fly, *Tabanus fuscicostatus* Hine (Diptera: Muscidae), J. Méd. Entomol. 45 (2008) 109–114. <https://doi.org/10.1093/jmedent/45.1.109>.

[245] G.A. Scoles, A.B. Broce, T.J. Lysyk, G.H. Palmer, Relative efficiency of biological transmission of *Anaplasma marginale* (Rickettsiales: Anaplasmataceae) by *Dermacentor andersoni* (Acari: Ixodidae) compared with mechanical transmission by *Stomoxys calcitrans* (Diptera: Muscidae), J Med Entomol 42 (2005) 668–675. [https://doi.org/10.1603/0022-2585\(2005\)042\[0668:reobto\]2.0.co;2](https://doi.org/10.1603/0022-2585(2005)042[0668:reobto]2.0.co;2).

- [246] F. Baldacchino, V. Muenworn, M. Desquesnes, F. Desoli, T. Charoenviriyaphap, G. Duvallet, Transmission of pathogens by Stomoxys flies (Diptera, Muscidae): a review, Parasite 20 (2013) 26. <https://doi.org/10.1051/parasite/2013026>.
- [247] G.D. Rodrigues, M. Lucas, H.G. Ortiz, L. dos S. Gonçalves, E. Blodorn, W.B. Domingues, L.S. Nunes, A. Saravia, P. Parodi, F. Riet-Correa, A. Menchaca, V.F. Campos, T.K. Krolow, R.F. Krüger, Molecular of Anaplasma marginale Theiler (Rickettsiales: Anaplasmataceae) in horseflies (Diptera: Tabanidae) in Uruguay, Sci. Rep. 12 (2022) 22460. <https://doi.org/10.1038/s41598-022-27067-0>.
- [248] L.H. Miller, J.A. Pino, J.J. McKelvey, L.L. Callow, Vaccination Against Bovine Babesiosis, in: Immunity to Blood Parasites of Animals and Man, Springer US, Boston, MA, 1977: pp. 121–149. http://link.springer.com/10.1007/978-1-4615-8855-9_9.
- [249] L.L. Callow, L.T. Mellors, A NEW VACCINE FOR BABESIA ARGENTINA INFECTION PREPARED IN SPLENECTOMISED CALVES, Australian Vet J 42 (1966) 464–465. <https://doi.org/10.1111/j.1751-0813.1966.tb14476.x>.
- [250] R.A. Todorovic, E.F. Gonzalez, O. Garcia, Immunization against anaplasmosis and babesiosis. Part III. Evaluation of immunization under field conditions in the Cauca River Valley, Tropenmed Parasitol 30 (1979) 43–52. <http://www.ncbi.nlm.nih.gov/pubmed/442200>.
- [251] C.M. Brizuela, C.A. Ortellado, E. Sanabria, O. Torres, D. Ortigosa, The safety and efficacy of Australian tick-borne disease vaccine strains in cattle in Paraguay, Vet Parasitol 76 (1998) 27–41. [https://doi.org/10.1016/s0304-4017\(97\)00047-2](https://doi.org/10.1016/s0304-4017(97)00047-2).
- [252] E. Pipano, E. Mayer, M. Frank, Comparative response of Friesian milking cows and calves to Anaplasma centrale vaccine, British Veterinary Journal 141 (1985) 174–178. [https://doi.org/10.1016/0007-1935\(85\)90148-4](https://doi.org/10.1016/0007-1935(85)90148-4).
- [253] J.L. Zaugg, K.L. Kuttler, Bovine anaplasmosis: in utero transmission and the immunologic significance of ingested colostral antibodies, Am J Vet Res 45 (1984) 440–443. <http://www.ncbi.nlm.nih.gov/pubmed/6711971>.
- [254] J.R. Budden, G.T. Dimopoulos, Finite purification of Anaplasma marginale: serologic inactivity of the anaplasma body, Am J Vet Res 38 (1977) 633–636. <http://www.ncbi.nlm.nih.gov/pubmed/879562>.
- [255] C.K. Dimmock, K. Bell, Haemolytic Disease of the Newborn in Calves, Australian Veterinary Journal 46 (1970) 44–47. <https://doi.org/10.1111/j.1751-0813.1970.tb05029.x>.
- [256] R A Dennis, P J O'Hara, M F Young, K D Dorris, Neonatal Immuno-hemolytic Anemia and Icterus of Calves, 156 (1970) 1861–1869.
- [257] M. Ristic, S. Sibinovic, C.J. Welter, An attenuated Anaplasma marginale vaccine, Proc Annu Meet U S Anim Health Assoc 72 (1965) 56–69.

- [258] M.B. Osorno, P.M. Solana, J.M. Perez, T.R. Lopez, Study of an attenuated *Anaplasma marginale* vaccine in Mexico natural challenge of immunity in an enzootic area, *Am J Vet Res* 36 (1975) 631–633. <http://www.ncbi.nlm.nih.gov/pubmed/1137209>.
- [259] S.P. Sharma, G.C. Bansal, Immune responses in cattle vaccinated with gamma-irradiated, *Indian Veterinary Research Institute* 56 (1986) 490–493.
- [260] Anaplasmosis Vaccines, *Veterinary Clinics of North America: Food Animal Practice* 6 (1990) 247–250. [https://doi.org/10.1016/s0749-0720\(15\)30927-0](https://doi.org/10.1016/s0749-0720(15)30927-0).
- [261] Searl C R., Use of an anaplasma vaccine as related to neonatal isoerythrolysis [Heifers], VM/SAC. *Veterinary Medicine and Small Animal Clinician (USA)* (1980). https://scholar.google.com/scholar_lookup?title=Use+of+an+anaplasma+vaccine+as+related+to+neonatal+isoerythrolysis+%5BHeifers%5D&author=Searl%2C+R.C.&publication_year=1980.
- [262] W.E. Brock, C.C. Pearson, I.O. Kliwer, A Vaccine for Anaplasmosis, Oklahoma State University, Experiment Station, 1965. <https://books.google.com/books?id=JAKnAQAAAMAJ>.
- [263] Searl C R., Report on use of inactivated anaplasmosis vaccine., in: *Proceedings of the California Anaplasmosis Conference*, Sacramento, Calif, 1972: pp. 28–31.
- [264] C. Stormont, Etiology of Bovine Neonatal Isoerythrolysis, *Bov. Pract.* (1977) 22–28. <https://doi.org/10.21423/bovine-vol1977no12p22-28>.
- [265] G.H. Palmer, A.F. Barbet, G.H. Cantor, T.C. McGuire, Immunization of cattle with the MSP-1 surface protein complex induces protection against a structurally variant *Anaplasma marginale* isolate, *Infection and Immunity* 57 (1989) 3666–3669. <https://doi.org/10.1128/iai.57.11.3666-3669.1989>.
- [266] J.E. Lopez, G.H. Palmer, K.A. Brayton, M.J. Dark, S.E. Leach, W.C. Brown, Immunogenicity of *Anaplasma marginale* Type IV Secretion System Proteins in a Protective Outer Membrane Vaccine, *Infect Immun* 75 (2007) 2333–2342. <https://doi.org/10.1128/iai.00061-07>.
- [267] E.L. Suttén, J. Norimine, P.A. Beare, R.A. Heinzen, J.E. Lopez, K. Morse, K.A. Brayton, J.J. Gillespie, W.C. Brown, *Anaplasma marginale* Type IV Secretion System Proteins VirB2, VirB7, VirB11, and VirD4 Are Immunogenic Components of a Protective Bacterial Membrane Vaccine, *Infect Immun* 78 (2010) 1314–1325. <https://doi.org/10.1128/iai.01207-09>.
- [268] M.C. Vidotto, E.J. Venâncio, O. Vidotto, Cloning, sequencing and antigenic characterization of rVirB9 of *Anaplasma marginale* isolated from Paraná State, Brazil, *Genet Mol Res* 7 (2008) 460–466. <https://doi.org/10.4238/vol7-2gmr416>.
- [269] P. Hove, S. Madesh, A. Nair, D. Jaworski, H. Liu, J. Ferm, M.D. Kleinhenz, M.A. Highland, A.K. Curtis, J.F. Coetzee, S.M. Noh, Y. Wang, D. Genda, R.R. Ganta, Targeted mutagenesis in *Anaplasma marginale* to define virulence and vaccine development against

bovine anaplasmosis, PLoS Pathog 18 (2022) e1010540.
<https://doi.org/10.1371/journal.ppat.1010540>.

[270] J.F. Coetzee, M.D. Apley, Efficacy of enrofloxacin against severe experimental *Anaplasma marginale* infections in splenectomized calves., Vet. Ther. : Res. Appl. Vet. Med. 7 (2006) 319–28.

[271] eCFR :: 21 CFR Part 558 -- New Animal Drugs for Use in Animal Feeds, (n.d.).
<https://www.ecfr.gov/current/title-21/chapter-I/subchapter-E/part-558> (accessed February 9, 2024).

[272] F. and D. Administration, D. of H. and H. Services, 21 CFR Part 558, (2024).
<https://www.ecfr.gov/current/title-21/part-558> (accessed February 9, 2024).

[273] FDA, Questions and Answers: FDA Approved Free-Choice Feeding Options for Anaplasmosis Control in Cattle, (2024). <https://www.fda.gov/animal-veterinary/development-approval-process/questions-and-answers-fda-approved-free-choice-feeding-options-anaplasmosis-control-cattle> (accessed August 5, 2024).

[274] F. and D. Administration, D. of H. and H. Services, 21 CFR 510.455, (2024).
<https://www.ecfr.gov/current/title-21/part-510/section-510.455> (accessed August 5, 2024).

[275] F. and D. Administration, D. of H. and H. Services, 522.1660a Oxytetracycline solution, 200 milligrams/milliliter, (n.d.). <https://www.ecfr.gov/current/title-21/part-522/section-522.1660a> (accessed September 30, 2024).

[276] D. Stewart, C.G. G. P., The comparative efficiency of a short and a long acting Oxytetracycline for the treatment of *Anaplasma marginale* in splenectomized calves, Journal of the South African Veterinary Association 50 (1979).

[277] T. Kumar, N. Sindhu, G. Charaya, A. Kumar, P. Kumar, G. Chandratere, D. Agnihotri, R. Khurana, Emerging status of anaplasmosis in cattle in Hisar, Vet. World 8 (2015) 768–771.
<https://doi.org/10.14202/vetworld.2015.768-771>.

[278] A.K. Curtis, M.D. Kleinhenz, T. Anantatat, M.S. Martin, G.C. Magnin, J.F. Coetzee, K.E. Reif, Failure to Eliminate Persistent *Anaplasma marginale* Infection from Cattle Using Labeled Doses of Chlortetracycline and Oxytetracycline Antimicrobials, Vet. Sci. 8 (2021) 283.
<https://doi.org/10.3390/vetsci8110283>.

[279] O. of the F. Register, Federal register, 1988. <https://www.govinfo.gov/content/pkg/FR-1988-08-18/pdf/FR-1988-08-18.pdf> (accessed January 16, 2025).

[280] J. de la Fuente, J.C. Garcia-Garcia, E.F. Blouin, B.R. McEwen, D. Clawson, K.M. Kocan, Major surface protein 1a effects tick infection and transmission of *Anaplasma marginale*, International Journal for Parasitology 31 (2001) 1705–1714. [https://doi.org/10.1016/s0020-7519\(01\)00287-9](https://doi.org/10.1016/s0020-7519(01)00287-9).

- [281] C. for V. Medicine, Questions and Answers: FDA Approved Free-Choice Feeding Options for Anaplasmosis Control in Cattle, FDA (2024). <https://www.fda.gov/animal-veterinary/development-approval-process/questions-and-answers-fda-approved-free-choice-feeding-options-anaplasmosis-control-cattle>.
- [282] S.B. Levy, The Challenge of Antibiotic Resistance, *Scientific American* 278 (1998) 46–53. <https://www.jstor.org/stable/26057703>.
- [283] E.Y. Furuya, F.D. Lowy, Antimicrobial-resistant bacteria in the community setting, *Nat Rev Microbiol* 4 (2006) 36–45. <https://doi.org/10.1038/nrmicro1325>.
- [284] F. Ma, S. Xu, Z. Tang, Z. Li, L. Zhang, Use of antimicrobials in food animals and impact of transmission of antimicrobial resistance on humans, *Biosafety and Health* 3 (2021) 32–38. <https://doi.org/10.1016/j.bsheal.2020.09.004>.
- [285] I. Chantziaras, F. Boyen, B. Callens, J. Dewulf, Correlation between veterinary antimicrobial use and antimicrobial resistance in food-producing animals: a report on seven countries, *Journal of Antimicrobial Chemotherapy* 69 (2014) 827–834. <https://doi.org/10.1093/jac/dkt443>.
- [286] F.J. Angulo, V.N. Nargund, T.C. Chiller, Evidence of an association between use of antimicrobial agents in food animals and anti-microbial resistance among bacteria isolated from humans and the human health consequences of such resistance, *J Vet Med B Infect Dis Vet Public Health* 51 (2004) 374–379. <https://doi.org/10.1111/j.1439-0450.2004.00789.x>.
- [287] C. Manyi-Loh, S. Mamphweli, E. Meyer, A. Okoh, Antibiotic Use in Agriculture and Its Consequential Resistance in Environmental Sources: Potential Public Health Implications, *Molecules* 23 (2018) 795. <https://doi.org/10.3390/molecules23040795>.
- [288] S. Pokharel, P. Shrestha, B. Adhikari, Antimicrobial use in food animals and human health: time to implement ‘One Health’ approach, *Antimicrob. Resist. Infect. Control* 9 (2020) 181. <https://doi.org/10.1186/s13756-020-00847-x>.
- [289] U.F. and D. Administration, Guidance for industry #213, new animal drugs and new animal drug combination products administered in or on medicated feed or drinking water of food-producing animals: Recommendations for drug sponsors for voluntarily aligning product use conditions with GFI #209, (2013). <https://www.fda.gov/media/83488/download> (accessed February 9, 2024).
- [290] Food and Drug Administration (FDA). 2022 Summary Report On Antimicrobials Sold or Distributed for Use in Food-Producing Animals., Rockville, MD: U.S. Department of Health and Human Services (2022). <https://www.fda.gov/animal-veterinary/antimicrobial-resistance/2022-summary-report-antimicrobials-sold-or-distributed-use-food-producing-animals> (accessed February 6, 2024).

- [291] A.A. Salyers, A. Gupta, Y. Wang, Human intestinal bacteria as reservoirs for antibiotic resistance genes, *Trends in Microbiology* 12 (2004) 412–416. <https://doi.org/10.1016/j.tim.2004.07.004>.
- [292] A.E. van den Bogaard, E.E. Stobberingh, Epidemiology of resistance to antibiotics: Links between animals and humans, *International Journal of Antimicrobial Agents* 14 (2000) 327–335. [https://doi.org/10.1016/s0924-8579\(00\)00145-x](https://doi.org/10.1016/s0924-8579(00)00145-x).
- [293] A. Kirbis, M. Krizman, Spread of Antibiotic Resistant Bacteria from Food of Animal Origin to Humans and Vice Versa, *Procedia Food Sci.* 5 (2015) 148–151. <https://doi.org/10.1016/j.profoo.2015.09.043>.
- [294] H.S. Hurd, J. Brudvig, J. Dickson, J. Mirceta, M. Polovinski, N. Matthews, R. Griffith, Swine Health Impact on Carcass Contamination and Human Foodborne Risk, *Public Heal. Rep.* 123 (2008) 343–351. <https://doi.org/10.1177/003335490812300314>.
- [295] J.R. Johnson, M.A. Kuskowski, K. Smith, T.T. O'Bryan, S. Tatini, Antimicrobial-resistant and extraintestinal pathogenic *Escherichia coli* in retail foods, *J Infect Dis* 191 (2005) 1040–1049. <https://doi.org/10.1086/428451>.
- [296] E.C. AILES, J.S. LEON, L.-A. JAYKUS, L.M. JOHNSTON, H.A. CLAYTON, S. BLANDING, D.G. KLEINUM, L.C. CKER, C.L. MOE, Microbial Concentrations on Fresh Produce Are Affected by Postharvest Processing, Importation, and Season, *J. Food Prot.* 71 (2016) 2389–2397. <https://doi.org/10.4315/0362-028x-71.12.2389>.
- [297] T.L. Poole, T.R. Callaway, K.N. Norman, H.M. Scott, G.H. Loneragan, S.A. Ison, R.C. Beier, D.M. Harhay, B. Norby, D.J. Nisbet, Transferability of antimicrobial resistance from multidrug-resistant *Escherichia coli* isolated from cattle in the USA to *E. coli* and *Salmonella* Newport recipients, *Journal of Global Antimicrobial Resistance* 11 (2017) 123–132. <https://doi.org/10.1016/j.jgar.2017.08.001>.
- [298] H. Hasman, F.M. Aarestrup, *trcB*, a gene conferring transferable copper resistance in *Enterococcus faecium*: occurrence, transferability, and linkage to macrolide and glycopeptide resistance, *Antimicrob Agents Chemother* 46 (2002) 1410–1416. <https://doi.org/10.1128/aac.46.5.1410-1416.2002>.
- [299] E. Mateu, M. Martin, Why is Anti-Microbial Resistance a Veterinary Problem As Well?, *Journal of Veterinary Medicine, Series B* 48 (2001) 569–581. <https://doi.org/10.1111/j.1439-0450.2001.00475.x>.
- [300] S.N. Rollo, B. Norby, P.C. Bartlett, H.M. Scott, D.L. Wilson, V.R. Fajt, J.E. Linz, C.E. Bunner, J.B. Kaneene, J.C. Huber, Prevalence and patterns of antimicrobial resistance in *Campylobacter* spp isolated from pigs reared under antimicrobial-free and conventional production methods in eight states in the Midwestern United States, *Journal of the American Veterinary Medical Association* 236 (2010) 201–210. <https://doi.org/10.2460/javma.236.2.201>.

- [301] B. Spellberg, The Maturing Antibiotic Mantra: “Shorter Is Still Better,” *J Hosp Med* 13 (2018) 361.362. <https://doi.org/10.12788/jhm.2904>.
- [302] T.M. Platt, G.H. Loneragan, H.M. Scott, B. Norby, D.U. Thomson, M.S. Brown, S.E. Ives, M.M. Brashears, Antimicrobial susceptibility of enteric bacteria recovered from feedlot cattle administered chlortetracycline in feed, *Am J Vet Res* 69 (2008) 988–996. <https://doi.org/10.2460/ajvr.69.8.988>.
- [303] M.D. Weinroth, H.M. Scott, B. Norby, G.H. Loneragan, N.R. Noyes, P. Rovira, E. Doster, X. Yang, D.R. Woerner, P.S. Morley, K.E. Belk, Effects of Ceftiofur and Chlortetracycline on the Resistomes of Feedlot Cattle, *Appl Environ Microbiol* 84 (2018) e00610-18. <https://doi.org/10.1128/aem.00610-18>.
- [304] P.R. Ingram, B.A. Rogers, H.E. Sidjabat, J.S. Gibson, T.J.J. Inglis, Co-selection may explain high rates of ciprofloxacin non-susceptible *Escherichia coli* from retail poultry reared without prior fluoroquinolone exposure, *J. Méd. Microbiol.* 62 (2013) 1743–1746. <https://doi.org/10.1099/jmm.0.062729-0>.
- [305] R.E. Hungate, D.W. Fletcher, I.A. Dyer, Effects of Chlortetracycline Feeding on Bovine Rumen Microorganisms, *J. Anim. Sci.* 14 (1955) 997–1002. <https://doi.org/10.2527/jas1955.144997x>.
- [306] Y. Yu, X. Li, Z. Liu, Y. Xu, Y. Shen, G. Li, X. Huang, Effects of Chlortetracycline Rumen-Protected Granules on Rumen Microorganisms and Its Diarrhea Therapeutic Effect, *Front. Vet. Sci.* 9 (2022) 840442. <https://doi.org/10.3389/fvets.2022.840442>.
- [307] C. and L.S.I. (CLSI), M100-Performance Standards for Antimicrobial Susceptibility Testing, 31st Edition, 31st ed., Clinical and Laboratory Standards Institute (CLSI), USA, 2021.
- [308] FDA, National Antimicrobial Resistance Monitoring System – Enteric Bacteria (NARMS): 2011 Executive Report, U.S. Department of Health and Human Services, Food and Drug Administration, Rockville, MD, 2013.
- [309] N. Kanwar, H.M. Scott, B. Norby, G.H. Loneragan, J. Vinasco, M. McGowan, J.L. Cottell, M.M. Chengappa, J. Bai, P. Boerlin, R. Schuch, Effects of Ceftiofur and Chlortetracycline Treatment Strategies on Antimicrobial Susceptibility and on tet(A), tet(B), and blaCMY-2 Resistance Genes among *E. coli* Isolated from the Feces of Feedlot Cattle, *PLoS ONE* 8 (2013) e80575. <https://doi.org/10.1371/journal.pone.0080575>.
- [310] R. Sharma, K. Munns, T. Alexander, T. Entz, P. Mirzaagha, L.J. Yanke, M. Mulvey, E. Topp, T. McAllister, Diversity and distribution of commensal fecal *Escherichia coli* bacteria in beef cattle administered selected subtherapeutic antimicrobials in a feedlot setting, *Appl Environ Microbiol* 74 (2008) 6178–6186. <https://doi.org/10.1128/aem.00704-08>.
- [311] T.W. Alexander, L.J. Yanke, E. Topp, M.E. Olson, R.R. Read, D.W. Morck, T.A. McAllister, Effect of Subtherapeutic Administration of Antibiotics on the Prevalence of

Antibiotic-Resistant *Escherichia coli* Bacteria in Feedlot Cattle, *Appl. Environ. Microbiol.* 74 (2008) 4405–4416. <https://doi.org/10.1128/aem.00489-08>.

[312] H.T. Akwar, C. Poppe, J. Wilson, R.J. Reid-Smith, M. Dyck, J. Waddington, D. Shang, S.A. McEwen, Associations of antimicrobial uses with antimicrobial resistance of fecal *Escherichia coli* from pigs on 47 farrow-to-finish farms in Ontario and British Columbia, *Can J Vet Res* 72 (2008) 202–210. <http://www.ncbi.nlm.nih.gov/pubmed/18505211>.

[313] K.B. Pouwels, R. Freeman, B. Muller-Pebody, G. Rooney, K.L. Henderson, J.V. Robotham, T. Smieszek, Association between use of different antibiotics and trimethoprim resistance: going beyond the obvious crude association, *J. Antimicrob. Chemother.* 73 (2018) 1700–1707. <https://doi.org/10.1093/jac/dky031>.

[314] K.B. Pouwels, B. Muller-Pebody, T. Smieszek, S. Hopkins, J.V. Robotham, Selection and co-selection of antibiotic resistances among *Escherichia coli* by antibiotic use in primary care: An ecological analysis, *PLoS ONE* 14 (2019) e0218134. <https://doi.org/10.1371/journal.pone.0218134>.

[315] L.T.M. Vien, N.N.Q. Minh, T.C. Thuong, H.D. Khuong, T.V.T. Nga, C. Thompson, J.I. Campbell, M. de Jong, J.J. Farrar, C. Schultsz, H.R. van Doorn, S. Baker, The Co-Selection of Fluoroquinolone Resistance Genes in the Gut Flora of Vietnamese Children, *PLoS ONE* 7 (2012) e42919. <https://doi.org/10.1371/journal.pone.0042919>.

[316] E. Miller, A. Vikram, G.E. Agga, T.M. Arthur, J.W. Schmidt, Effects of In-Feed Chlortetracycline Prophylaxis in Beef Cattle on Antimicrobial Resistance Genes, *Foodborne Pathog. Dis.* 15 (2018) 689–697. <https://doi.org/10.1089/fpd.2018.2475>.

[317] A.R. Toillion, E.J. Reppert, R.G. Amachawadi, K.C. Olson, J.F. Coetzee, Q. Kang, K.E. Reif, Effect of Protracted Free-Choice Chlortetracycline-Medicated Mineral for Anaplasmosis Control on *Escherichia coli* Chlortetracycline Resistance Profile from Pastured Beef Cattle, *Microorganisms* 9 (2021) 2495. <https://doi.org/10.3390/microorganisms9122495>.

[318] W.H. Organization, Critically important antimicrobials for human medicine : 6th revision, 2019. <https://www.who.int/publications/i/item/9789241515528> (accessed February 9, 2024).

[319] D.A. Tadesse, S. Zhao, E. Tong, S. Ayers, A. Singh, M.J. Bartholomew, P.F. McDermott, Antimicrobial drug resistance in *Escherichia coli* from humans and food animals, United States, 1950-2002, *Emerg Infect Dis* 18 (2012) 741–749. <https://doi.org/10.3201/eid1805.111153>.

[320] A.R. Khachatryan, T.E. Besser, D.D. Hancock, D.R. Call, Use of a nonmedicated dietary supplement correlates with increased prevalence of streptomycin-sulfa-tetracycline-resistant *Escherichia coli* on a dairy farm, *Appl Environ Microbiol* 72 (2006) 4583–4588. <https://doi.org/10.1128/aem.02584-05>.

[321] P. Collignon, J.J. Beggs, T.R. Walsh, S. Gandra, R. Laxminarayan, Anthropological and socioeconomic factors contributing to global antimicrobial resistance: a univariate and

multivariable analysis, *The Lancet Planetary Health* 2 (2018) e398–e405.
[https://doi.org/10.1016/s2542-5196\(18\)30186-4](https://doi.org/10.1016/s2542-5196(18)30186-4).

[322] S.R. Zwenger, T. Welsch, E.T. Gillock, Bacteria Resistant to Ciprofloxacin, Chloramphenicol and Tetracycline Isolated from Western Kansas Feedlots, *Transactions of the Kansas Academy of Science* (1903-) 111 (2008) 125–135.
<https://www.jstor.org/stable/20476348>.

[323] D.V. Hoyle, C.M. Yates, M.E. Chase-Topping, E.J. Turner, S.E. Davies, J.C. Low, G.J. Gunn, M.E.J. Woolhouse, S.G.B. Amyes, Molecular epidemiology of antimicrobial-resistant commensal *Escherichia coli* strains in a cohort of newborn calves, *Appl Environ Microbiol* 71 (2005) 6680–6688. <https://doi.org/10.1128/aem.71.11.6680-6688.2005>.

[324] D.T.D. WAAL, Anaplasmosis Control and Diagnosis in South Africa, *Ann. N. York Acad. Sci.* 916 (2000) 474–483. <https://doi.org/10.1111/j.1749-6632.2000.tb05327.x>.

[325] G.M. Malijan, N. Howteerakul, N. Ali, S. Siri, M. Kengganpanich, O.-D.S. Group, R. Nascimento, R.D. Booton, K.M.E. Turner, B.S. Cooper, A. Meeyai, A scoping review of antibiotic use practices and drivers of inappropriate antibiotic use in animal farms in WHO Southeast Asia region, *One Heal.* 15 (2022) 100412.
<https://doi.org/10.1016/j.onehlt.2022.100412>.

[326] G.L. Apple, L.R. Thomas, H.M. Ward, K.E. Reif, T. Anantatat, M. Flowers, A.C. White, J.G. Powell, Surveillance of *Anaplasma marginale* in Arkansas beef cattle herds, *American Association of Bovine Practitioners Conference Proceedings* (2020) 372–372.
<https://doi.org/10.21423/aabppro20208116>.

[327] J. O'Neill, Antimicrobial resistance: tackling a crisis for the health and wealth of nations, *Rev. Antimicrob. Resist.* (2014). <https://cir.nii.ac.jp/crid/1370857593729357568>.

[328] W. Chajęcka-Wierzchowska, A. Zadernowska, U. Zarzecka, A. Zakrzewski, J. Gajewska, Enterococci from ready-to-eat food – horizontal gene transfer of antibiotic resistance genes and genotypic characterization by PCR melting profile, *J. Sci. Food Agric.* 99 (2019) 1172–1179.
<https://doi.org/10.1002/jsfa.9285>.

[329] C.H. Lester, N. Frimodt-Møller, T.L. Sørensen, D.L. Monnet, A.M. Hammerum, In Vivo Transfer of the vanA Resistance Gene from an Enterococcus faecium Isolate of Animal Origin to an E. faecium Isolate of Human Origin in the Intestines of Human Volunteers, *Antimicrob. Agents Chemother.* 50 (2006) 596–599. <https://doi.org/10.1128/aac.50.2.596-599.2006>.

[330] I. Kühn, A. Iversen, L.G. Burman, B. Olsson-Liljequist, A. Franklin, M. Finn, F. Aarestrup, A.M. Seyfarth, A.R. Blanch, X. Vilanova, H. Taylor, J. Caplin, M.A. Moreno, L. Dominguez, I.A. Herrero, R. Möllby, Comparison of enterococcal populations in animals, humans, and the environment - a European study, *Int. J. Food Microbiol.* 88 (2003) 133–145.
[https://doi.org/10.1016/s0168-1605\(03\)00176-4](https://doi.org/10.1016/s0168-1605(03)00176-4).

- [331] I. Kühn, A. Iversen, M. Finn, C. Greko, L.G. Burman, A.R. Blanch, X. Vilanova, A. Manero, H. Taylor, J. Caplin, L. Domínguez, I.A. Herrero, M.A. Moreno, R. Möllby, Occurrence and Relatedness of Vancomycin-Resistant Enterococci in Animals, Humans, and the Environment in Different European Regions, *Appl. Environ. Microbiol.* 71 (2005) 5383–5390. <https://doi.org/10.1128/aem.71.9.5383-5390.2005>.
- [332] S. Gordon, J.M. Swenson, B.C. Hill, N.E. Pigott, R.R. Facklam, R.C. Cooksey, C. Thornsberry, W.R. Jarvis, F.C. Tenover, Antimicrobial susceptibility patterns of common and unusual species of enterococci causing infections in the United States. Enterococcal Study Group, *J. Clin. Microbiol.* 30 (1992) 2373–2378. <https://doi.org/10.1128/jcm.30.9.2373-2378.1992>.
- [333] D.B. Clewell, Movable genetic elements and antibiotic resistance in enterococci, *Eur. J. Clin. Microbiol. Infect. Dis.* 9 (1990) 90–102. <https://doi.org/10.1007/bf01963632>.
- [334] G.M. Dunny, D.B. Clewell, Transmissible toxin (hemolysin) plasmid in *Streptococcus faecalis* and its mobilization of a noninfectious drug resistance plasmid, *J. Bacteriol.* 124 (1975) 784–790. <https://doi.org/10.1128/jb.124.2.784-790.1975>.
- [335] A.E. Jacob, S.J. Hobbs, Conjugal Transfer of Plasmid-Borne Multiple Antibiotic Resistance in *Streptococcus faecalis* var. *zymogenes*, *J. Bacteriol.* 117 (1974) 360–372. <https://doi.org/10.1128/jb.117.2.360-372.1974>.
- [336] P.M. Courvalin, C. Carlier, O. Croissant, D. Blangy, Identification of two plasmids determining resistance to tetracycline and to erythromycin in group D *Streptococcus*, *Mol. Gen. Genet. MGG* 132 (1974) 181–192. <https://doi.org/10.1007/bf00269391>.
- [337] P.M. Courvalin, C. Carlier, Y.A. Chabbert, Plasmid-linked tetracycline and erythromycin resistance in group D “*streptococcus*”, *Ann. l’Inst. Pasteur* 123 (1972) 755–9.
- [338] J.R. Hayes, L.L. English, P.J. Carter, T. Proescholdt, K.Y. Lee, D.D. Wagner, D.G. White, Prevalence and Antimicrobial Resistance of *Enterococcus* Species Isolated from Retail Meats, *Appl. Environ. Microbiol.* 69 (2003) 7153–7160. <https://doi.org/10.1128/aem.69.12.7153-7160.2003>.
- [339] A.L. Manson, D.V. Tyne, T.J. Straub, S. Clock, M. Crupain, U. Rangan, M.S. Gilmore, A.M. Earl, Chicken Meat-Associated Enterococci: Influence of Agricultural Antibiotic Use and Connection to the Clinic, *Appl. Environ. Microbiol.* 85 (2019). <https://doi.org/10.1128/aem.01559-19>.
- [340] L.M. Johnston, L.-A. Jaykus, Antimicrobial Resistance of *Enterococcus* Species Isolated from Produce, *Appl. Environ. Microbiol.* 70 (2004) 3133–3137. <https://doi.org/10.1128/aem.70.5.3133-3137.2004>.
- [341] CLSI, Performance Standards for Antimicrobial Susceptibility Testing, 33rd ed., 2023.

- [342] P. Butaye, L.A. Devriese, F. Haesebrouck, Differences in Antibiotic Resistance Patterns of *Enterococcus faecalis* and *Enterococcus faecium* Strains Isolated from Farm and Pet Animals, *Antimicrob. Agents Chemother.* 45 (2001) 1374–1378. <https://doi.org/10.1128/aac.45.5.1374-1378.2001>.
- [343] C.R. Jackson, J.E. Lombard, D.A. Dargatz, P.J. Fedorka-Cray, Prevalence, species distribution and antimicrobial resistance of enterococci isolated from US dairy cattle, *Lett. Appl. Microbiol.* 52 (2011) 41–48. <https://doi.org/10.1111/j.1472-765x.2010.02964.x>.
- [344] L.A. Richardson, Understanding and overcoming antibiotic resistance, *PLoS Biol.* 15 (2017) e2003775. <https://doi.org/10.1371/journal.pbio.2003775>.
- [345] A. Caneschi, A. Bardhi, A. Barbarossa, A. Zaghini, The Use of Antibiotics and Antimicrobial Resistance in Veterinary Medicine, a Complex Phenomenon: A Narrative Review, *Antibiotics* 12 (2023) 487. <https://doi.org/10.3390/antibiotics12030487>.