Evaluation of enrofloxacin and oxytetracycline to eliminate persistent *Anaplasma marginale* infection in cattle.

by

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Abstract

Bovine anaplasmosis is a tick-borne bacterial disease of cattle caused by the rickettsial pathogen *Anaplasma marginale.* Common clinical signs of anaplasmosis include anemia, lethargy, pyrexia, and death. Antimicrobial treatment of anaplasmosis has historically relied on tetracycline injectables such as oxytetracycline; however, a new antimicrobial injectable Baytril® 100-CA1 (enrofloxacin), in a different antimicrobial class (fluorquinolone), was recently granted conditional approval for the treatment of clinical anaplasmosis. In most cases, animals infected with *A. marginale* survive infection with or without treatment intervention and remain carriers of *A. marginale,* serving as infection reservoirs. In some operations, producers desire to eliminate *A. marginale* infection to reduce transmission risk, alleviate associated disease management costs, and improve chances for herd or individual animal (e.g. seed stock, embryo transfer animals) profitability. Despite increasing desire for protocols to clear cattle of *A. marginale* infection, no drugs currently have an indication for elimination of *A. marginale* infection. This study was designed to investigate the efficacy of enrofloxacin and oxytetracycline to eliminate *A. marginale* infection in persistently infected steers. Fourteen Holstein steers previously inoculated with the Virginia or KS2 *A. marginale* strain and confirmed positive using a PCR test targeting the *A. marginale* Major Surface Protein 5 gene were allocated to treatment groups receiving injections of enrofloxacin (Baytril® 100-CA1) or oxytetracycline (Bio-Mycin® 200). The recommended dosage for each treatment (Bio-Mycin® 200 at 4.5 mL/100 lb, 10 mL/injection site; and, Baytril® 100-CA1 at 5.7 mL/100 lb, 20 mL/injection site) was administered subcutaneously for five consecutive days (this repetitive dosing scheme is off-label and was used for experimental purposes only). Blood samples were collected daily during treatment and twice weekly following the last treatment to monitor *A. marginale* infection status

using the quantitative *msp5* PCR assay and serostatus using a commercial cELISA. All seven steers receiving oxytetracycline and five of the seven steers receiving enrofloxacin remained positive for *A. marginale* infection. Both steers in the enrofloxacin group where infection was cleared were infected with the *A. marginale* VA strain. Injection site reactions were observed for two steers treated with oxytetracycline. Further refinement of an antimicrobial-based *A. marginale* infection elimination protocol that considers drug, dosing intervals, and *A. marginale* strain diversity is needed to provide producers options to reduce anaplasmosis transmission potential in their herd and retain valuable stock.

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Chapter 1- Introduction

Bovine anaplasmosis (hereafter simply referred to as anaplasmosis) is a tick-borne rickettsial disease of cattle caused by the pathogen *Anaplasma marginale*. This disease has been identified worldwide in temperate, subtropical, and tropical regions with several domesticated and wild ruminants serving as susceptible hosts. Domestic cattle are the most common and most significant host species for this pathogen based on development of clinical disease in this species and disease-associated economic impact. Following is a review of the disease anaplasmosis and its prevalence, economic impact, transmission, diagnostics, and common management practices, including antimicrobial-based management practices. Currently, no validated protocol exists for antimicrobial-based elimination of *A. marginale*. Thus, the experimental portion of this report examines the efficacy of two drugs currently approved for the treatment of clinical anaplasmosis (oxytetracycline and enrofloxacin), administered using an experimental (off-label) repetitive dosing regimen, at eliminating *A. marginale* infection in persistently infected Holstein steers.

Disease

Anaplasmosis is an infectious disease of ruminants and cattle are a common species susceptible to this disease. The infectious agent, *A. marginale,* is an intracellular rickettsial pathogen that infects erythrocytes, visible microscopically on a stained blood smear (**Figure 1**). Commonly, clinical signs of anaplasmosis are more discernable in cattle two years or older. In animals under one year old, infection is mild and often goes unnoticed. Reduced appearance of clinical disease signs in younger animals is postulated to be associated with their ability to regenerate red blood cells more rapidly, avoiding critical acute anemia often observed when animals are first infected as adults. However, in adult cattle over the age of 2 years, acute clinical

disease can result in death with a mortality rate between 29 and 49% (Rickey 1991; Kocan 2003, Aubry 2011).

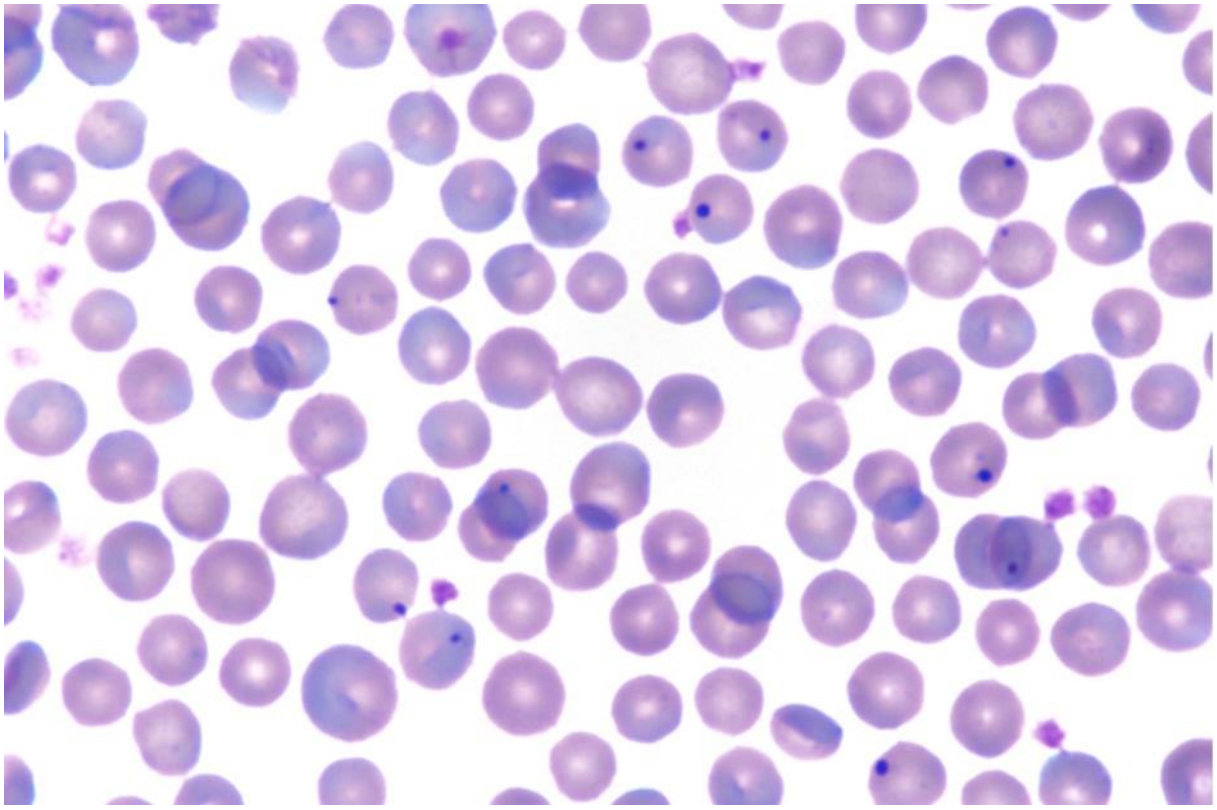

Figure 1. A stained blood smear of cattle erythrocytes (larger lilac colored cells) infected with *A. marginale* (small dark purple staining dots within the cattle erythrocyte – often near the margin of the erythrocyte).

Once infected, the disease incubation period ranges from 7 to 60 days, with an average of 28 days, at which point clinical signs are observed (Kocan 2010). During peak acute anaplasmosis, 70% or more red blood cells are infected. At this point the animal either succumbs to infection or (in most cases), the animal's immune system (reticuloendothelial cells specifically) recognizes and phagocytizes the *A. marginale* infected erythrocytes (Kocan 2003). Rapid destruction of erythrocytes leads to the hallmark clinical sign, anemia. The other common anaplasmosis clinical signs observed are directly related to this acute anemia and include lethargy, weight loss, pyrexia, abortion, and in extreme cases death. If clinical signs are present,

typically they last 4 to 9 days depending on intervention and acute disease management (i.e. fever or anemia treated with an injectable antimicrobial vs animal recovering naturally) (Gill 1994). However, recovery in terms of production losses like reduced weight gain, fertility issues, or decreased milk production can take up to several months or persist for several years until the end of that animal's production usefulness (Moore 2018). Common observable signs of an animal suffering from clinical anaplasmosis include identifying an anemic animal (e.g. pale or icteric mucous membranes reflecting a reduction in red blood cell concentration); or, identifying a hypoxic animal (e.g. increased respiration, unusual aggressiveness, or lethargy). However, diagnostic testing is required to confirm the presence of *A. marginale* as some signs could be attributed to a different cattle disease or underlying health issue.

Cattle often survive acute infection and serve as reservoirs (i.e. carriers) for subsequent transmission events as most cattle do not clear infection. These carrier cattle can remain persistently infected for the remainder of their life as *A. marginale* can evade the animal's immune response with emergence and replication of antigenic variants that circulate in a uniform cycle, with new variants emerging every 4 to 6 weeks (Aubry 2011). During persistent infection an immunocompetent animal rarely displays any clinical signs of anaplasmosis due to the lower bacteremia levels.

The recrudescence of clinical anaplasmosis does not commonly occur in healthy immunocompetent carrier animals as they often have premunition (infection-associated immunity) to reinfection. However, if an animal's immune system is compromised due to infection with other pathogens, other illness, steroid use, etc., there is a possibility of relapse and clinical signs may be observed (Moore 2018).

Prevalence

The full distribution and prevalence of anaplasmosis throughout the U.S. is not fully known; however, cases of anaplasmosis have been reported in almost every continental U.S. state. Regions in the U.S. that are considered endemic for anaplasmosis (high prevalence) include west central and southeast states (**Figure 2**). Over the last 20 years, a few studies of anaplasmosis prevalence among cattle or cattle herds have been published within specific states. In the majority of these studies, anaplasmosis 'status' was evaluated using a commercial, competitive enzyme linked immunosorbent assay (cELISA), the Anaplasma Antibody Test Kit (VMRD, Pullman, WA), to determine seroprevalence. Using this assay, cattle with a percent inhibition of ≥30% are classified as seropositive for *A. marginale* (see 'Diagnostics' section for more information about this assay). Because *A. marginale* infection commonly persists in cattle, cattle that are seropositive are often considered positive for active infection. Okafor et al. have performed anaplasmosis prevalence surveys in the following states: Kentucky, Texas, Georgia, and Mississippi. In each of these studies, data was obtained through active surveillance by collecting samples from slaughterhouse cull cattle, and/or passive surveillance by review of state Veterinary Diagnostic Laboratory (VDL) records. Following is a brief discussion of major findings for each of those studies published over the past 20 years (2000 to 2020). In Kentucky, samples tested with cELISA from 232 cows resulted in 10.78% seroprevalence and 2,573 VDL samples were 11.58% seropositive (Okafor 2018). In a Texas study of 215 cows and 15,156 VDL samples, seroprevalence was determined to be 13.49% and 15.91% (Okafor 2018a). In addition to serologic testing by cELISA, some VDL samples were also evaluated using the card test, complement fixation test (CFT), and quantitative PCR (qPCR). In a Georgia study, the anaplasmosis seroprevalence among 293 tested cows was 4.44% (Okafor 2019). The anaplasmosis seroprevalence in a Mississippi study of 207 cows and 5,182 VDL samples

identified a 28.99% and 16.72% seroprevalence, respectively (Okafor 2019). In addition, further evaluation of anaplasmosis prevalence in Texas cattle was completed by Hairgrove et al. where cELISA testing of 1,835 market cattle identified a 15.02% anaplasmosis seroprevalence (Hairgrove 2014). The evaluation of 11 cattle herds (size range of 25 to 200 animals), with cELISA and qPCR testing identified an anaplasmosis seroprevalence range of 0 to 88% and 0 to 82% among cattle within individual cattle herds (Hairgrove 2015). In Iowa, the cELISA testing of 659 feedlot calves from 31 consigners identified a 15.17% anaplasmosis seroprevalence (Coetzee 2010). More recently, herd-level anaplasmosis seroprevalence was evaluated in Kansas herds by Spare et al. In this study, 925 beef cow-calf production herds were tested by cELISA and 52.5% of the tested herds were found seropositive for *A. marginale* (Spare 2020).

Figure 2. Areas in the United States endemic for anaplasmosis (Beef 2020).

A summary of the major findings of each of these studies are presented in **Table 1**. Taken together, there is a variability of anaplasmosis (sero)prevalence among beef cattle in different U.S. states; and, the majority of studies rely on *A. marginale* serostatus to infer *A. marginale* infection status. Among surveyed states, the state with the lowest anaplasmosis seroprevalence among individually evaluated animals was Georgia (4.44%). The state with the greatest anaplasmosis seroprevalence amongst individually evaluated animals was Mississippi (28.99%). When comparing results across studies, sample size (number of samples evaluated), sample level (individual animal vs herd), representativeness (distribution of samples, e.g. all from same herd/area), location (endemic vs non-endemic region), animal demographics (bull, cow, calves, age, breed), and evaluation method (serologic versus molecular assay) should all be taken into consideration when interpreting results and drawing comparisons between studies.

Table 1. Prevalence studies completed in the United States.

State	Sample type (unit)	Sample collection year(s)	Sample Size	Evaluation Methods	Results	Reference
Kentucky	Beef cattle- cull cows at slaughterhouses,	2013	232 cows (from 19 of 35 KY counties)	cELISA	10.78% (25/232)	Okafor 2018
	University of Kentucky VDL records	2002-2012	2,573 VDL samples		11.58% (247/2,573)	
Texas	Beef cattle- cull cows at slaughterhouses	2014	215 cows from 19 of TX 254 counties	cELISA	13.49% (29/215)	Okafor 2018a
	Texas A&M VDL records	2002-2012	15,156 VDL samples	card test (439), CFT (7752), cELISA (6799), qPCR (166)	15.91% (2412/15,156)	
Georgia	Beef cattle- cull cows at slaughterhouses	2013 & 2014	293 cows from 6 of GA 159 counties	cELISA	4.44% (13/293)	Okafor 2019
Mississippi	Beef cattle- cull cows at slaughterhouses	2013 &2014	207 cows from 16 of MS 82 counties	cELISA	28.99% (60/207)	Okafor 2019

	VDL records	2002-2018	5182 VDL samples		16.72%	
Texas	Texas cattle in 23 cattle auctions	2011	1,835 market cattle	cELISA	15.02%	Hairgrove 2014
Texas	Texas cattle herds in 8 counties	2012 to 2014	11 herds (herd size: 25 to 200 animals; 434 cows tested)	cELISA and RT-qPCR	cELISA- 0 to 88%* RT-qPCR- 0 to 82%*	Hairgrove 2015
Iowa	Feedlot cattle	2002	659 feedlot calves	cELISA	15.17% (100/659)	Coetzee et. al. 2010
Kansas	Kansas cow-calf herds	2016 & 2017	925 herds (10 cows tested per herd)	cELISA	52.5%* (486/925)	Spare 2020

Economic Impact

Anaplasmosis causes direct profit loss to producers due to cattle death (more common in adult cattle), costs associated with treatment and prevention measures, culling of infected animals, and abortion/fertility issues. Anaplasmosis is roughly estimated to cost U.S. cattle producers $300 million annually (Kocan 2003). A severe clinical case of anaplasmosis can cost up to $400 per animal (Coetzee 2017). Anaplasmosis control measures, such as use of the conditionally-licensed anaplasmosis vaccine manufactured by University Products LLC (Baton Rouge, LA) costs approximately $20 per head for the initial prime-booster immunization and $10 per head for annual booster immunizations thereafter. Diagnostic testing costs range based on choice of diagnostic test with serologic tests ranging $6 to $14 per test and molecular tests ranging $27 to $36 per test (ISUVDL and KSUVDL 2021). For treatment of cattle exhibiting clinical signs of anaplasmosis, an oxytetracyline injectable product is often used, for which the approximate cost for a 1,000 lb animal for three days would be roughly $4.50 to $6.50 (Whittier

1996). Today's common oxytetracycline products include Bio-Mycin® 200 and Liquamycin® Oxytetracycline Injection (Zoetis) LA-200. The cost for a 1,000 lb animal treated with a single dose Bio-Mycin® 200 would be roughly $5.60; and, the cost for a 1,000 lb animal treated with a single dose of Liquamycin® Oxytetracycline Injection would be $7.09. The newly-approved enrofloxacin clinical anaplasmosis treatment, Baytril® 100-CA1, has a recommended single dose therapy and would cost $37.20 for a 1,000 lb animal. For fly/tick control; a common pour-on insecticide, Ultra Boss® Pour-On Insecticide (Merck Animal Health Kenilworth, NJ) can cost $1.03 per administration for a 1,000 lb animal (treatment frequency up to once every two weeks); or, for the recommended 2 tags per cow of insecticide ear tags, CyLence Ultra® (Bayer Animal Health Shawnee, KS) costs $5.30 per animal (**Table 2**). Use of an FDA approved free-choice CTC medicated mineral (or similarly approved CTC-medicated feed product) at a dose of 0.5 to 2.0 mg/ lb/d is commonly fed during the duration of vector season to control anaplasmosis. The estimated intake of mineral for a cow on pasture is 0.25 lb/day. The cost of non-medicated mineral is approximately $0.111 hd/d and $3.33 per month. For CTC-medicated mineral, 4,800g/ton is $0.133 hd/d and $3.98 per month, 6,000g/ton is $0.138 hd/d and $4.14 per month (Justin Axman, written communication). Providing CTC-medicated mineral for an average vector season (180 days/6 months), the cost per head for non-medicated would be $19.98 and CTC-medicated $23.88 (4,800g/ton) or $24.84 (6,000g/ton). The cost difference for some of these common products used for prevention and control of anaplasmosis may not seem substantial on their own but can be significant when calculating costs versus return per animal.

Table 2. Examples of per animal costs for products commonly used for anaplasmosis prevention and control. The estimated cost is based on a 1,000 lb animal.

Product	Dose (ml/lb)	Cost per dose ($)	# of treatments	Total ($)

Ultra Boss ® pour-on	3ml/100 lb	$1.03	Twice	$2.06
CyLence Ultra® Insecticide Cattle Ear Tag	2 ear tags per cow	$5.30	Once	$5.30
Bio-Mycin® 200	4.5ml/100 lb	$5.60	Single Dose	$5.60
Liquamycin® LA-200	4.5ml/100 lb	$7.09	Single dose	$7.09
Baytril® 100 CA1	5.7ml/100 lbs	$18.60	Single dose	$18.60
CTC medicated mineral	0.5 to 2.0 mg/lb/d	$0.133 hd/d	Daily (vector season)	$23.88

According to the USDA, maintaining a healthy and profitable cattle industry is important to the overall success of the U.S. agriculture economy. Cattle production is the most important agricultural industry in the U.S. and is forecasted to represent 17% of the $391 billion total cash receipts for agricultural commodities in 2021 (USDA ERS 2021). In a biannual report released by the USDA on Jan. 29, 2021, the total number of all cattle and calves in the U.S. was 93.6 million head. For 2021, all cattle and calves cash receipts total $65,968,605,000 (USDA ERS 2021). In 2021, Kansas (ranked 3rd nationally in overall cattle number), has 6.5 million head of cattle (U.S. total 40.69 million head), of which 1.65 million head are cows and heifers that calved. The 2020 calf crop in Kansas totaled 1.43 million head (U.S. total 35.1 million head) (USDA NASS 2021). The top five U.S. states for cattle production are: Texas 13.1 million head, Nebraska 6.85 million head, Kansas 6.5 million head, Oklahoma 5.3 million head, and California 5.15 million head. All of these states are endemic for anaplasmosis.

The total number of U.S. cattle and calf operations is 882,692, with the vast majority of those operations (802,317) being cow/calf and stocker/backgrounder productions. Evaluating cattle production systems, cow-calf operations have the greatest opportunity to be negatively impacted by anaplasmosis. Compared to stocker and feedlot operations, on average, cow-calf animals remain on the premises longer. Cows can remain in the herd for the longevity of their production period (8 to 12 years), heifers are retained for breeding stock, and bulls are often kept for multiple breeding seasons. It is common for bulls to be outsourced for genetic diversity and to emphasize desired breed traits. Anaplasmosis disease and infection risk is positively correlated with cattle age such that older cattle are more likely to be infected with *A. marginale*. The longer an animal remains in a herd the greater the number of opportunities for *A. marginale* exposure and infection, and infected animals often remain persistently infected carriers and transmission reservoirs for life.

The introduction of anaplasmosis into an uninfected herd is estimated to result in a 3.6% reduction in calf crop, a 30% increase in cull rate, and a 30% mortality rate in adult cattle with clinical disease (Alderink 1982). Movement of cattle between herds within an operation or between operations is common and creates opportunities for introduction of *A. marginale* into a naïve herd or introduction of novel *A. marginale* strains into an endemic herd. Because cattle are not routinely tested for *A. marginale*, inadvertent movement of *A. marginale* among and between cattle herds is likely very common. Examples of animals that may be introduced into a herd include: new bull(s) into an established cow herd for the breeding season (a bull >2 years of age can service 30 cows in a 60-70 day breeding season); cows or heifers to increase cow/calf production; and, calves bought from sale barn/another producer to put on grass.

Death of cows due to anaplasmosis can exert a significant economic cost. Costs associated with death of an adult cow can include: sale cost of the animal, loss of money invested in feed costs for that animal, costs associated with additional veterinary care, and loss of potential yearly calf sales. According to the Texas A&M AgriLife Standardized Performance Analysis (SPA) database, the annual cost of maintaining a mature cow for the average Texas producer is $796 per year. The magnitude of cattle operations varies so not everyone is "average". The lowest-cost-quartile producers only spent $618 per cow per year, while the highest-quartile producers spent $1,261 per cow per year (Carpenter 2019). In cases where anaplasmosis does not cause death, anaplasmosis can result in morbidity-associated costs, including: reduced animal performance, lower average daily gain (ADG), reduced milk production, and fertility/abortion issues with cows having a greater chance of remaining open or not producing a calf to weaning (Kocan 2003, Curtis 2021).

Transmission

There are three common routes of *A. marginale* transmission among cattle: biological, mechanical, and transplacental. Several species of ixodid ticks are biological vectors for *A. marginale*, as this pathogen can efficiently replicate in the tick midgut and salivary glands (**Figure 3**). A competent tick vector becomes infected via blood-feeding on an infected animal and ingesting *A. marginale* infected erythrocytes. The *A. marginale* bacteria first colonize and replicates in the tick midgut and then disseminates to and replicates in the tick salivary glands. Transmission of *A. marginale* to a naïve animal occurs via tick saliva during the tick's next bloodmeal on a naïve host. Male ticks transmit *A. marginale* intrastadially (within the same life stage). Infected ticks act as a reservoir for *A. marginale* and have the potential to infect multiple animals if they have to opportunity to feed on multiple naïve hosts (Kocan 2003).

Multiple tick species have been identified as competent *A. marginale* transmission vectors. In the U.S., *Dermacentor* species of ticks, primarily *D. andersoni*, *D. variabilis*, and *D. occidentalis* transmit *A. marginale*. (Aubry 2011). Although the majority of *A. marginale* are tick-transmissible, there are a few interesting strains that are not and these non-tick transmissible strains are primarily transmitted by mechanical methods through biting fly mouth parts or unproperly decontaminated processing equipment. In most tropical and subtropical regions of the world, *Rhipicephalus* (*Boophilus*) *microplus* and *R.* (*B.*) *annulatus* are the dominant tick vectors of *A. marginale*.

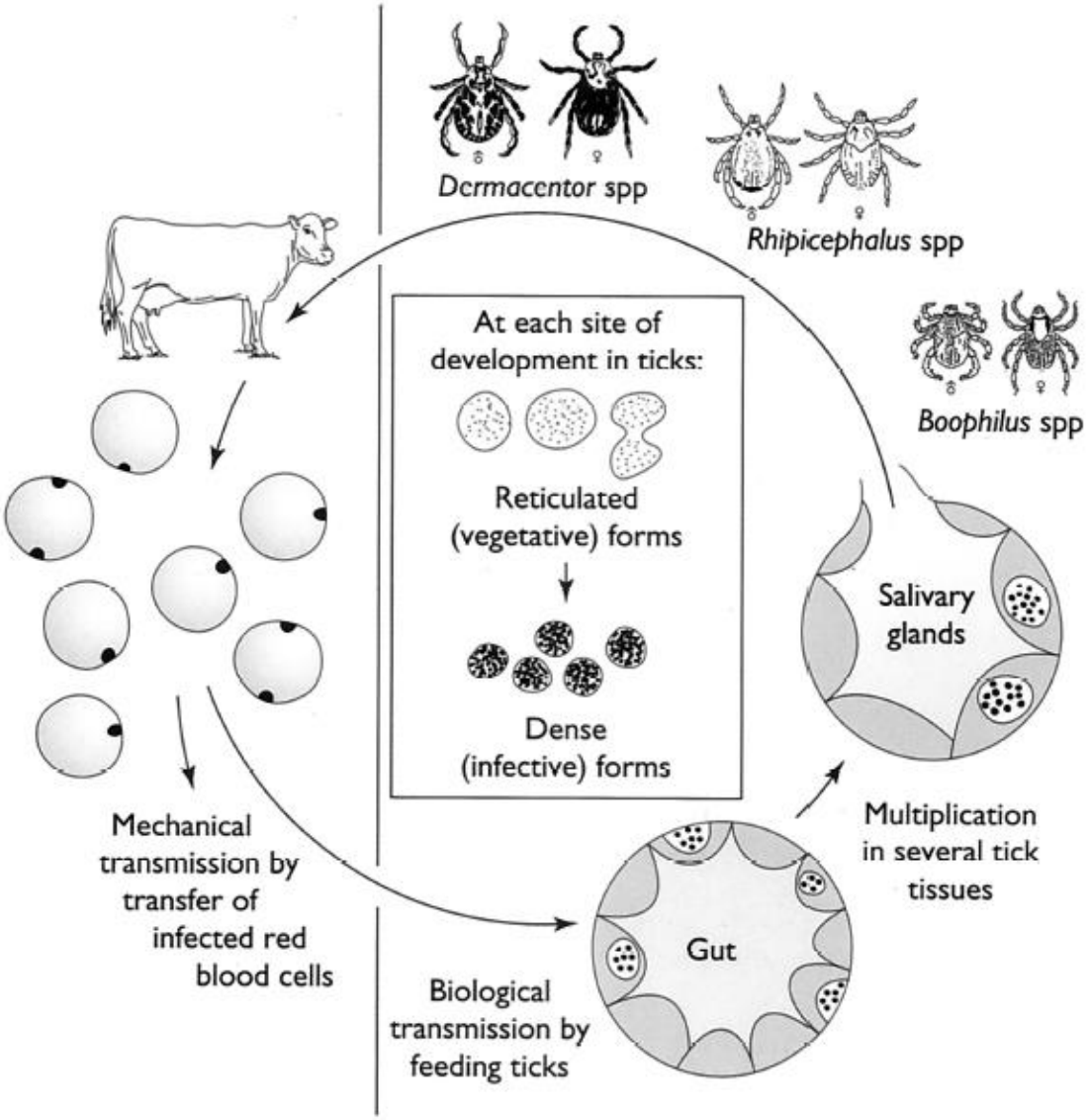

Figure 3. The development cycle of *A. marginale* in ticks and cattle (Kocan 2003).

Mechanical transmission of *A. marginale* can occur by other blood-feeding arthropods and processing equipment. In the mechanical transmission routes, it is important to note that *A. marginale* does not replicate on the surfaces of equipment or in/on flies. Thus, these transmission routes do not amplify *A. marginale* bacterial numbers. The main arthropod-associated mechanical transmission routes are the mouthparts of blood-feeding flies that can move infected blood between cattle. However, transmission by fly mouthpart contamination is only likely when flies feed on an animal with a high *A. marginale* bacterial level (i.e. during acute clinical disease,

lasts about 1-2 weeks) (Kocan 2003). Transmission by flies also requires multiple flies simultaneously flying between and feeding on acutely infected and naïve cattle. Use of fly control products can help reduce risk of *A. marginale* mechanical transmission by biting flies.

Mechanical transmission routes via processing equipment contaminated with *A. marginale* infected blood include: castration tools, dehorning implements, ear taggers, and multi-use needles. To reduce *A. marginale* transmission by these methods, sterilizing or disinfecting processing equipment between use on different animals and eliminating the re-use of needles between animals is recommended.

Transplacental transmission occurs when an *A. marginale* infected dam transmits the pathogen to her calf via the placenta. This route of transmission is less studied and the frequency by which transmission via this route occurs is not well understood. In a study evaluating transplacental *A. marginale* transmission, a 15.6% incidence of *A. marginale* in utero transmission was observed (Potgieter 1987). In addition, a study investigating inoculating dams with *A. marginale* at different trimesters during gestation reported infective blood recovered from two fetuses (one from a dam inoculated in the second trimester and the other from a dam inoculated during the third trimester) (Zaugg 1985). However, in anaplasmosis endemic herds, the prevalence of *A. marginale* in animals under one or two years of age is low and indicates that transplacental transmission does not appear to be a prominent source of maintaining infection within a herd.

Diagnostics

Anaplasmosis can circulate within cattle herds unsuspected until clinical signs present or confirmation by diagnostic testing. However, clinical signs are not always easily detected in

cattle with acute anaplasmosis. For example, in persistently infected carrier cattle, clinical signs are rarely observed, which further hinders prompt identification and diagnosis. Cattle suffering from clinical anaplasmosis can display abnormal behaviors such as: not coming to the bunk/off feed, standing away from the herd/lethargic, or uncommon aggressiveness for that animal while being handled. Additional clinical signs that a producer may observe include: high rectal temperature and pale or icteric mucous membranes, the unexplained death of a cow(s), greater number of cows open/aborting, or more instances of animals off feed/"not acting right" are situations that could turn a producer towards testing their herds for anaplasmosis. Factors that influence choice of diagnostic assay choice include: accessibility (often requires a veterinarian to collect samples and submission of the sample to a diagnostic laboratory), cost, turnover time for results, and reliability or working knowledge of how to interpret test results. Not only is reliable determination of infection status important to producers, the collection of epidemiologic information is key to maintaining the free-trade policy between anaplasmosis endemic and non-endemic countries (Reinbold 2010).

To evaluate an animal's anaplasmosis status serologically, the most common assay used is a competitive enzyme-linked immunosorbent assay (cELISA) that identifies cattle seropositive for *A. marginale* by detecting serum antibodies that target the major surface protein 5 (MSP5) surface protein of *Anaplasma* spp. (Aubry 2011). More specifically, data indicates the detection of antibodies against the MSP5 epitope, specifically defined by the monoclonal antibody (Mab) ANAF16C1 (this epitope is broadly conserved among *Anaplasma* species and isolates) (Knowles 1996). The diagnostic quality of a cELISA test to identify serologically positive cattle depends on the set inhibition cut-off value at 30% inhibition to determine the animal's status (cattle are considered seropositive if they are at or above 30% inhibition). The test has a sensitivity of 95%

and a specificity of 98% using a cut-off point of 30% inhibition (%I) (VMRD, Aubry 2011). The limitations of this diagnostic method include: low sensitivity for detection of early infection, cross-reactivity with other *Anaplasma* species, and insufficient specificity for evaluating treatment success when trying to reduce or clear *A. marginale* infection from an animal (Aubry 2011).

By detecting an *A. marginale*-specific antibody, the commercial cELISA confirms the immune system of animal was presented with that antigen or specific epitope at some time; however, it does not necessarily mean the pathogen is present at the time the test is performed (Tana-Hernández 2017). The time period between infection and development of the anti-MSP5 antibody response (seroconversion) can lead to a failure to identify infected cattle during acute anaplasmosis (Reinbold 2010, Coetzee 2007, Reinbold 2010). A study found that during the first 10 days post-inoculation, the sensitivity of the commercial cELISA was <50% (Coetzee 2007). This is a recognized limitation of cELISA testing and is clinically relevant when developing a diagnostic plan for cattle in the early stages of anaplasmosis. However, after 156-day post-inoculation, the overall sensitivity was 94.8% with a majority of post-inoculation collection periods at 100% sensitivity (Coetzee 2007). In cattle herds where *A. marginale* is endemic, the cELISA assay has a high sensitivity and specificity at determining individual animal status and aids in monitoring infected animal movement into non-endemic regions (de Echaide 1998). Determining seroprevalence of calves with cELISA is less helpful and less definitive as calves receive maternal antibodies from the colostrum of their dam that can cause a positive result if the dam is infected with *A. marginale*. The cELISA test is commonly used by producers testing their herds due to affordability, accuracy, and rapid results.

Molecular detection and quantification of *A. marginale* DNA in cattle blood is completed by assays such as quantitative polymerase chain reaction (qPCR) testing. Similar to the serologic assays for anaplasmosis, the molecular diagnostic assays often target a portion of the conserved MSP5 protein gene (*msp5*). Several molecular diagnostic assays have been developed targeting *msp5*, and a positive result is highly indicative that the animal is actively infected, and most of these assays have been validated as highly specific for *A. marginale* with no cross-reactions detected with other haemoparasites of ruminants (Aubry 2011). A study evaluating the sensitivity of one of these assays identified that the referenced PCR assay was able to detect as few as 10^1 DNA copies $10\mu l^{-1}$ of standard DNA and $3x10^1$ *Anaplasma*-infected erythrocytes ml^{-1} (Carelli 2007).

Other types of molecular assays using different chemistries, platforms, and targets have also been developed for detection of *A. marginale* in bovine blood including: nested PCR, conventional PCR, real-time PCR with intercalating dyes (i.e. SYBR Green 1 reagent), real-time PCR with TaqMan probe, real-time reverse transcriptase PCR, and loop-mediated isothermal amplification (LAMP) (Kovalchuk 2020). Not every assay is the same and the sensitivity, specificity, and repeatability of each assay depends on the target gene (i.e. *msp5*, 16S rRNA), primer sequences, reagents, instruments, cycling parameters, and internal controls (not all have) (Kovalchuk 2020).

As acute anemia is a hallmark of clinical anaplasmosis, evaluating packed cell volume (PCV) is commonly used to determine the severity of anemia – caused by destruction of *A. marginale* infected red blood cells. The normal PCV range for cattle is 24 to 46 percent and an animal experiencing acute anaplasmosis can have a PCV of 20 or less (Navarre 2007). PCV evaluation can be used to evaluate or compare treatment efficacy in animals experiencing clinical

anaplasmosis. In our acute infection studies, animals that develop a PCV <17% in the face of treatment are considered treatment failures. This is also the PCV where we typically initiate treatment to avoid the animal potentially dying in controlled *A. marginale* infection experiments.

Direct visualization of *A. marginale* in red blood cells is another way to diagnosis clinical anaplasmosis. During acute clinical anaplasmosis, the *A. marginale* pathogen is visible microscopically on a stained blood smear as basophilic-staining colonies inside red blood cells. The percent parasitized erythrocytes (PPE) is a measure of the percentage of red blood cells that are infected with *A. marginale*. Infected red blood cells are visible roughly 2 to 6 weeks after infection (Merck Manual). An animal during the acute infection phase has a higher level of bacteremia and evaluating infection level by PPE is possible and definitive for acute infection. Direct visualization of *A. marginale*-infected red blood cells is not reliable when observing blood smears from pre-symptomatic or carrier animals as parasitemia is at low levels (Aubry 2011).

Non-antimicrobial-based anaplasmosis management practices:

There are several non-antimicrobial based management practices producers can implement to prevent and control anaplasmosis within their herds. Interaction of cattle with vectors (flies or ticks) can be prevented by using insecticides or acaricides, available as pour-ons or impregnated ear tags. Pesticide resistance should be taken into consideration and managed with application timing, appropriate dose administration, and alternating insecticides. The maintenance of cattle pasture and feeding grounds by removing manure and feed waste can help reduce potential mechanical vector fly breeding grounds. In addition, controlled pasture burning can also help reduce vector habitat and population. A record keeping system on all cattle is important and a herd's anaplasmosis status can be monitored with diagnostics. In addition,

knowing the anaplasmosis status of animals entering the herd can help control infection and inform anaplasmosis management strategies.

The development of a broadly effective anaplasmosis vaccine to prevent infection and/or limit clinical disease in the U.S. is still being researched. Currently, there is no fully approved anaplasmosis vaccine available to cattle producers in the U.S. The two main types of vaccines that have been previously used to control clinical anaplasmosis are: live and killed vaccines. Both of these vaccine strategies are intended to reduce or prevent clinical disease, but they do not prevent cattle from becoming initially or persistently infected. The live vaccine involves a less pathogenic isolate of *A. marginale* called *A. centrale* (originally thought to be a subspecies of *A. marginale* but is now recognized as a separate species). Use of the live *A. centrale* live vaccine is not allowed in the U.S. The primary killed anaplasmosis vaccine was developed in the U.S. but removed from the market in 1999 (Kocan 2003). In 2000, a new manufacturer (University Products LLC., Baton Rouge, LA) began preparing the discontinued vaccine under a conditional USDA license. This conditionally approved vaccine is commonly used today, however, no data on the efficacy of this vaccine is available. In addition, there is currently research focusing on developing a subcutaneous ear implant vaccine to provide long-lasting protection against clinical anaplasmosis (Curtis 2021). Development of an anaplasmosis vaccine that would both prevent infection and clinical anaplasmosis in cattle would be ideal to reduce transmission and infection rates.

Antimicrobial-based anaplasmosis control and treatment

Antibiotics are important to both livestock and human medicine, and the FDA has labeled specific antimicrobial drugs, including tetracyclines, as medically important. The FDA seeks to

promote the judicious use of medically important antimicrobials in food animals by removing their use for production purposes (e.g. improve feed efficiency, enhance growth) and restricting and refining their therapeutic use(s) (e.g. treat, control, prevent diseases) to approved indications requiring oversight by a licensed veterinarian (FDA 2018). Tetracyclines are labeled as medically important antimicrobials and examples of other medically important antimicrobials commonly used in food animals are: fluoroquinolones, penicillin, macrolides (e.g. tylosin; to reduce incidence of liver abscesses), sulfonamides (e.g. sulfadmethoxine; for treatment of foot rot, calf diphtheria, and BRDC in dairy cattle), and cephalosporins (e.g. ceftiofur and cephapirin; for treatment of intramammary infusion in dairy cattle). Tetracyclines are the only FDA fully-approved antimicrobial to treat and control anaplasmosis. When clinical signs of anaplasmosis (i.e. fever, anemia, lethargy) are observed in an animal, injectable oxytetracycline is commonly administered to alleviate and avoid animal health decline. The control of active anaplasmosis caused by infection with *A. marginale* is commonly managed with chlortetracycline (CTC)-medicated feed products. Use of tetracyclines for treatment and control of anaplasmosis is described below in more detail.

Tetracycline mechanism of action: Tetracyclines are bacteriostatic antimicrobials that inhibit bacterial protein synthesis by preventing the association of aminoacyl-tRNA with the bacterial ribosome. To work, the tetracycline drug enters the *A. marginale*-infected erythrocyte, passes through the outer-membrane of the *A. marginale* bacteria, diffuses through the inner cytoplasmic membrane by becoming an uncharged molecule, and reversibly binds to the 30S ribosome to inhibit protein synthesis (Chopra 2001, Reinbold 2010) The bacteriostatic aspect prevents the pathogen from replicating and gives the animal's immune system time to respond but does not normally directly kill (eliminate) the bacteria itself.

Antimicrobial-based anaplasmosis control: CTC is used in medicated feeds for the control of active anaplasmosis caused by susceptible strains of *A. marginale*. "A number of applications for use of chlortetracycline (CTC) in Type C medicated feeds (including complete medicated feeds and several free-choice medicated feed formulations) are approved for control of active infection of anaplasmosis" (FDA 2021). An approved free-choice or hand-fed mineral or feed formulation containing CTC is placed in pasture or pen, respectively, and is usually initiated before the onset of vector season. The FDA requires a Veterinary Feed Directive (VFD) to feed CTC-medicated feed products as hand-fed or free-choice based upon an established veterinarian-client-patient relationship (VCPR). The hand-fed method constitutes medicated feed that is fed to a group of animals on a daily (24-hour period) basis in an amount that provides the entire approved daily drug dose. Free-choice is medicated feed products placed in a pasture setting for animals to freely consume and balance their own diet (FDA 2021). The hand-fed dose of CTC for control of active anaplasmosis is 0.5 mg/lb of BW daily and the free-choice dose of CTC for control of active anaplasmosis is 0.5-2.0 mg/lb of BW daily. However, there is no limit on the duration of time/days CTC can be fed which raises concerns for antimicrobial resistance. In addition, there is no withdrawal period for CTC when used according to the label. Use of CTC medicated feed products are not approved for the 'treatment', 'prevention', or 'clearance' of *A. marginale*.

Antimicrobial-based anaplasmosis treatment: There are two antimicrobials available for the treatment of clinical anaplasmosis: oxytetracycline and enrofloxacin. Historically, and still most common today, oxytetracycline injectables are used to treat clinical anaplasmosis. The use of an oxytetracycline intended for the treatment of anaplasmosis must be indicated on the label or specifically ordered by a licensed veterinarian: "Federal law restricts this drug to use by or on

the order of a licensed veterinarian" (FDA 2021). Despite their common use for this purpose, no oxytetracycline injectable products has a specific label indication for the treatment of clinical anaplasmosis. As approved by a veterinarian, this product can be used off-label to treat cattle displaying signs of clinical anaplasmosis. A study evaluated an oxytetracycline product for the treatment of anaplasmosis in seven cows acutely infected and presenting with several clinical signs of disease, including: pale mucous membranes, inappetence, weakness, and increased rectal temperature. The presence of *A. marginale* was confirmed on blood smears and hemoglobin estimation showed severe anemia. Treatment was administered intravenously (10 mg/kg) daily for 3 days and one-week post treatment clinical signs resolved, and blood parameters increased for all treated animals (Kumar 2015).

Recently, enrofloxacin (Baytril® 100-CA1, Elanco US Inc, Greenfield, IN) was granted conditional approval for the treatment of anaplasmosis in cattle with the following label indication: "Indicated for the treatment of clinical anaplasmosis associated with *Anaplasma marginale* in replacement dairy heifers <20 months of age and all classes of beef except beef calves <2 months and beef bulls of any age intended for breeding". The Baytril® 100-CA1 dosage regimen is: administered as a single dose for treatment of clinical anaplasmosis; administered, by subcutaneous injection at a single dose of 12.5 mg/kg of body weight (5.7 mL/100 lb).

Evaluating antimicrobial risk factors, it was determined in the approval of the Baytril® 100-CA1 application "by all estimates performed, the number of cattle with clinical anaplasmosis on an annual basis is lower than 310,000. Therefore, the FDA concluded that the use of enrofloxacin to treat clinical anaplasmosis in cattle in the U.S. constitutes a minor use, and the product and indication are eligible for conditional approval." (FDA FOI Summary, 2020). In

addition, the extra-label use of fluoroquinolones is prohibited by law (21 CFR 530.21) in food-producing animals and enrofloxacin will be a prescription only drug (FDA FOI Summary 2020).

Enrofloxacin is a fluoroquinolone antimicrobial that inhibits bacterial DNA gyrase preventing DNA supercoiling and decantenation of original chromosomes and replicates. A newer fluoroquinolone at optimal concentrations can cause susceptible organisms to lose viability within 20 minutes of exposure (Boothe 2015). Fluoroquinolones are broad-spectrum antimicrobials and have strict usage guidelines in food animals as they are important to both human and animal medicine. The approval of enrofloxacin to treat clinical anaplasmosis was based upon evaluated effectiveness across a variety of study designs that included differences in: dose, frequency, duration, route of administration, animal class and age, infection method and product formulation. It is a prescription only drug that includes parameters to determine if cattle are eligible to receive enrofloxacin for the treatment of anaplasmosis and the FDA states this additional use will result in only a minor increase in enrofloxacin use (FDA FOI Summary 2020). In addition, there is a 28-day withdrawal period for enrofloxacin. The bactericidal properties of enrofloxacin support evaluating elimination of *A. marginale* infection.

A study tested the efficacy of enrofloxacin (Baytril® 100) against severe (>25% PPE) experimental *A. marginale* infections in splenectomized calves (Coetzee 2006). Six Holstein calves at the age of 8 months were splenectomized and confirmed anaplasma free by cELISA. Two calves were inoculated with West Coast (St. Maries) isolate, three calves with Oklahoma (OK) isolate, and one calf with Virginia (VA) isolate. Two calves with the OK isolate were deemed control and untreated. The remaining 4 calves infected with either VA, OK, or St. Maries strain received two subcutaneous injections of enrofloxacin at 12.5 mg/kg, 48 hr apart. PPE, PCV, and cELISA were used for post treatment monitoring. Both calves in the control

group became moribund when the PCV dropped below 10% (3-6 days following peak parasitemia) and were euthanized. The four treated animals survived and *A. marginale* infection and clinical diseases signs were reduced (less severe drop in PCV, no animals died or needed to be euthanized). Infection was not eliminated in any of the treated calves. Recrudescence of *A. marginale* parasites was observed in all animals within 30 days following treatment. However, subsequent rickettsemias were less severe and self-limiting (Coetzee 2006). The study demonstrated the administration of enrofloxacin was successful in reducing parasitemia and improving clinical signs in calves challenged with two different U.S. strains of *A. marginale*.

Antimicrobial-based clearance of *A. marginale* infection

Several previous research studies have provided evidence that antimicrobials can be used to successfully treat clinical anaplasmosis signs and help prevent death due to disease, as well as, control infection in endemic herds, but they do not include consistent evidence of *A. marginale* infection elimination. Currently, no drugs are approved to clear *A. marginale* infection in cattle during either the acute or chronic disease stage. For several production operations, infection clearance is preferred over culling infected animals. A few studies have investigated whether any of the current drugs approved for anaplasmosis treatment or control result in clearance of *A. marginale* infection or could be modified slightly to achieve infection clearance.

Initial research studies stated results of successful *A. marginale* infection clearance in carrier animals. In one study, a long-acting injection of oxytetracycline (LA-200) given at 20 mg/kg IM once every 7 days eliminated *A. marginale* infection in 12 yearling cattle in all three treatment groups: treatment twice (n=4), treatment 3 times (n=4), and treatment 4 times (n=4). Subsequent inoculation of blood from treated calves 83 days post treatment into a naïve calf and complement-fixation tests confirmed elimination for all treated animals (Roby et al. 1978). In

another study, *A. marginale* infection was eliminated with a long-acting oxytetracycline (Swift 1983). Treatment was administered to 16 naturally infected carrier cows at 20 mg/kg, 3-day intervals of 3 injections (n=8) or 4 injections (n=8). An additional 4 seropositive cows served as untreated controls. Over a 130-day post treatment period, rapid card agglutination and complement fixation tests were performed 5 times and confirmed infection elimination in all treated cows (Swift 1983). In both of these early studies, confirmation of infection clearance using the serological tests available at the time must be interpreted cautiously as the complement fixation tests have a sensitivity of only 20% (Kocan 2010).

In a more recent study evaluating the efficacy of a three-dose regimen of injectable long-acting oxytetracycline at three-day intervals to clear *A. marginale* infection, this study concluded this regimen was not effective for eliminating the carrier state of *A. marginale* from naturally infected animals (Wallace 2007). In this study, the sample size was 29 naturally infected, mature beef cows receiving treatment of: three daily consecutive doses at 5 mL/100 lb of long-acting, injectable oxytetracycline product containing 200 mg/mL of oxytetracycline. It was confirmed not effective for eliminating the carrier state of *A. marginale* by PCR testing with 25 of 29 (86%) treated animals remaining PCR positive both after treatment and 2 months post-treatment (Wallace 2007).

In another study with the goal of eliminating *A. marginale* infection, three oxytetracycline regimens were compared (Coetzee 2005). In this study, 40 cross-bred beef steers were all inoculated with an Oklahoma *A. marginale* strain. The three oxytetracyline treatment groups were: A=300 mg/ml of oxy (Tetradure LA-300) at 30 mg/kg IM on day 0 (n=10), B= same as treatment A but on day 0 and again on day 5 (n=10), C= 200 mg/mL of oxy (Liquamycin LA-200) at 22 mg/kg IV q24h for 5 days (n=10) (Treatment C is the current OIE

recommended treatment regimen for persistent *A. marginale* infection). The fourth group was untreated infected control cattle (n=10). At 60 days post treatment all treated cattle tested positive by nested PCR and cELISA and infection was confirmed by subincoulation of blood from treated cattle into a splenectomized Holstein calf. Based on the results of this study, the authors suggested re-evaluation of the current OIE recommendations are needed for elimination of *A. marginale* in cattle (Coetzee 2005).

Finally, a study compared the efficacy of different drugs of different antimicrobial classes (enrofloxacin - ENRO, imidocarb dipropionate - IMD, and oxytetracycline - OTC) to eliminate *A. marginale* infection (Coetzee 2006). The oxytetracyline regimen chosen was proposed by the World Organization for Animal Health for treatment of persistent *A. marginale* infections in cattle. In this study, 12 Holstein calves were infected with an Oklahoma, Virginia, or West Coast (St. Maries) isolate. Infection was monitored with PPE, PCV, and cELISA serology and clearance was evaluated by subinoculating blood from treated calves into naïve calves. Treatment groups included: ENRO (100 mg/ml) at 5 mg/kg IV q24h for 5 days (n=4, strain- 2 OK and 2 VA), IMD (120 mg/ml) at 5 mg/kg IM 7 days apart (n=4, strain- 1 OK, 2 VA, 1 St. Maries), and OTC (LA 200) 22 mg/kg IV q24h for 5 days (n=4, strain- 3 VA and 1 OK). Again, the OTC treatment is the current OIE recommendation for treating persistent infection. None of the calves in the ENRO treatment group were cleared of infection. Two antimicrobial treated calves failed to infect their recipient splenectomized calf following blood subinoculation: one calf in the IMD group infected with an OK isolate, and one calf in the OTC group, infected with a VA isolate. At 44 days post treatment, both became cELISA negative but the imidocarb treated calf remained PCR positive. This study demonstrated likely *A. marginale* elimination in one of the four oxytetracycline treated calves which had a negative nested PCR 80 days post treatment

and blood subinoculated into a splenectomized calf that did not result in parasitemia. This calf was one of three in that group infected with the VA strain. This suggested that antimicrobial susceptibility may differ between different *A. marginale* strains (Coetzee 2006).

Developing and continuing to refine antimicrobial-based treatment and control regimens against *A. marginale* while preserving drug efficacy is important. There is still interest in identifying an antimicrobial-based treatment regimen to eliminate *A. marginale* infection. As described above, several studies have explored different treatment regimens to achieve this goal with variable success. Important considerations when interpreting the results of these studies are: *A. marginale* strain, diagnostic method, study animal (splenectomized, acutely infected, persistently infected), treatment product, treatment timing, duration of treatment, and time points post treatment analyzed. In this Report, we investigate whether repeated administration of either enrofloxacin or oxytetracyline at current FDA-approved dosages is effective at clearing persistent *A. marginale* infection caused by a historic *A. marginale* strain (Virginia – isolated in 1978) and contemporary *A. marginale* strain (KS2 – isolated in 2018). Identifying a broadly effective antimicrobial-based treatment regimen to clear cattle of persistent anaplasmosis caused by diverse *A. marginale* strains would address an important and current stakeholder need.

Chapter 2- Materials and Methods

Animals

Fourteen, approximately one-year-old Holstein steers, persistently-infected with *A. marginale* were enrolled in this study. Average steer weight at the start of the study ranged from 633 to 859 pounds. Each steer had a unique (alpha) numeric identification tag in its ear that served as the Animal ID. Seven months prior to this study, the steers were challenged with the historic *A. marginale* Virginia (VA) strain (isolated in 1978) (n=7) or the contemporary KS2 *A. marginale* strain (isolated from an endemic eastern Kansas beef cattle herd in 2018 and is still actively circulating today) (n=7). For the KS2 strain inoculation, animals DP694, T4963, T4970 were needle inoculated and animals DP696, JS460, JS463, and T4968 were infected via tick from a previous tick transmission study (**Table 3**). Animals DP695, DP697, JS461, JS464, T4962, T4965, and T4971 were needle inoculated with the VA strain (**Table 3**). Despite differing routes of *A. marginale* inoculation, all animals were in the persistent phase of disease. Persistent *A. marginale* infection in each steer was confirmed using a quantitative real-time Polymerase Chain Reaction (qPCR) assay targeting a portion of the *A. marginale* Major Surface Protein 5 gene (*msp5*). Steers in the same treatment group were housed together in an outdoor dry lot pen. All steers were fed a standard grain ration (Wildcat Feeds, Topeka, KS) with hay and water at libitum.

This study protocol and all animal use and animal procedures were approved by Kansas State University Institutional Animal Care and Use Committee and the Kansas State University Institutional Biosafety Committee. All procedures were conducted in accordance with the Guide for the Care and Use of Animals in Agricultural Research and Teaching (FASS 2010).

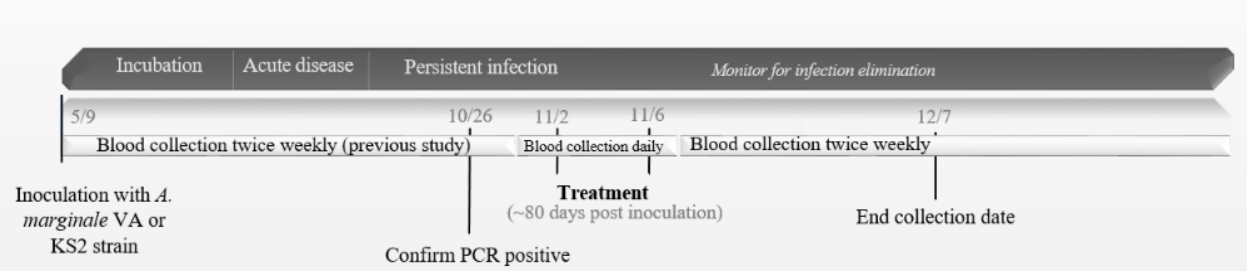

Figure 4. Study timeline. The start of the timeline with inoculation (5/9/2020) depicts the time period these steers went through to develop persistent infection. The clearance study started with treatment on 11/2/2020 for 5 consecutive days and concluded approximately 30 days after the final treatment (12/7/2020).

Experimental Design

Steers were randomly allocated into two treatment groups: Group A steers (n=7) were treated with 200 mg/mL oxytetracyline (Bio-Mycin® 200, Boehringer Ingelheim Animal Health USA Inc., St. Joseph, MO) at a dose of 4.5 mL per 100 lb body weight, administered subcutaneously at a volume of 10 mL per injection site, for 5 consecutive days. Group B steers (n=7) were treated with 100 mg/mL enrofloxacin (Baytril® 100 CA1, Elanco US Inc, Greenfield, IN) at a dose of 5.7 mL per 100 lb body weight, administered subcutaneously at a volume of 20 mL per injection site, for 5 consecutive days. The dose administered to each animal was per label instructions, calculated based on body weight (**Table 3**). The frequency of treatment for both products used in this study were being evaluated for experimental purposes only and is off-label for both products. Blood samples were collected prior to treatment initiation to establish baseline *A. marginale* infection levels; daily during the treatment period (blood sample collected before administration of the daily treatment); and, twice weekly after the final treatment to monitor *A. marginale* infection level. Health checks were performed daily to monitor for any adverse reactions to the treatment regimen.

Table 3. Steers in Group A received 4.5 mL per 100 lb Bio-Mycin® 200 for five consecutive days and steers Group B received 5.7 mL per 100 lb Baytril® 100-CA1 for five consecutive days.

Treatment Group	*A. marginale* strain	Infection Route	Animal ID	Weight (lbs)	Daily treatment dose volume (mL)
A	KS2	Tick	DP696	761	39
A	KS2	Needle	T4963	795	36
A	KS2	Tick	T4968	673	34
A	KS2	Needle	T4970	735	33
A	VA	Needle	DP695	858	32
A	VA	Needle	DP697	713	30
A	VA	Needle	T4971	673	30
B	KS2	Needle	DP694	817	47
B	KS2	Tick	JS460	721	41
B	KS2	Tick	JS463	633	36
B	VA	Needle	JS461	859	49
B	VA	Needle	JS464	751	43
B	VA	Needle	T4962	687	39
B	VA	Needle	T4965	677	39

Antimicrobial Treatment Administration

Both Bio-Mycin® 200 and Baytril® 100 CA1 treatments were administered subcutaneously. The preferable region to administer subcutaneous injections to cattle is a triangular region on the neck framed by the vertebrae with the nuchal ligament running from the withers to the back of the neck, a few inches up from the bottom of the neck from the esophagus and jugular vein, and in front of the shoulder blade. The calculated dose for daily treatments required multiple injections to accommodate administration of the full dose; thus, treatment sites for individual days were alternated to the right or left side of the neck avoiding areas where previous injection were administered as much as possible. Steers were monitored for signs of treatment reaction (described below).

Blood Collection

Low stress cattle handling techniques were used to move steers from their group pen into an indoor working facility. In the working facility, steers were briefly restrained in a cattle chute (Preifert Manufacturing, Mount Pleasant, TX) and their heads secured. Blood samples were collected from the jugular on either the right or left side of the neck using an 18 G, 1 ½ inch vacutainer needle. From each steer, blood was collected into 10 mL vacuette EDTA, lithium heparin, and/or no anticoagulant collection tubes. Each blood tube was pre-labeled with the steer's individual animal ID. Collected samples were brought back to the lab and processed immediately. Serum (from blood collected into tubes with no anticoagulant) and plasma (from blood collected into tubes with lithium heparin) were spun down in a table-top centrifuge (Thermo Fisher Scientific Inc., Waltham, MA) at 2,000 rpm for 10 minutes. Two, 700 µL aliquots of serum and plasma were prepared for each sample. Both serum and plasma samples were stored at -80°C. Whole blood (from blood collected into EDTA tubes) was used for packed cell volume analysis (PCV) (described below); 100 µL was reserved for DNA extraction (described below), and 1 mL was reserved for long-term storage. Whole blood samples were stored at -20°C.

Competitive Enzyme Linked Immunosorbant Assay (cELISA)

Serum samples from all steers were submitted to the Iowa State University Veterinary Diagnostic Laboratory for serological testing to evaluate their specific *A. marginale* antibody immune response. A commercial cELISA test that detects antibodies specific for *A. marginale* major surface protein 5 (MSP-5) was performed per manufacturer recommendations (VMRD Inc., Pullman, WA.). Time points evaluated by cELISA included: pre-treatment, 48 hours post

final treatment, two weeks post final treatment, and four weeks post final treatment (last collection date).

Packed Cell Volume (PCV) Determination

PCV evaluation was performed at each blood collection from blood collected into EDTA vacutainer tubes. Briefly, whole blood samples were drawn up into 75 µL capillary tubes via capillary action and then plugged at one end with sealant. Capillary tubes were spun at 12,000 rpm in a microhematocrit centrifuge (Unico, Dayton, NJ), and the percent of packed red blood cells manually determined using a hematocrit slide reader (**Figure 5**).

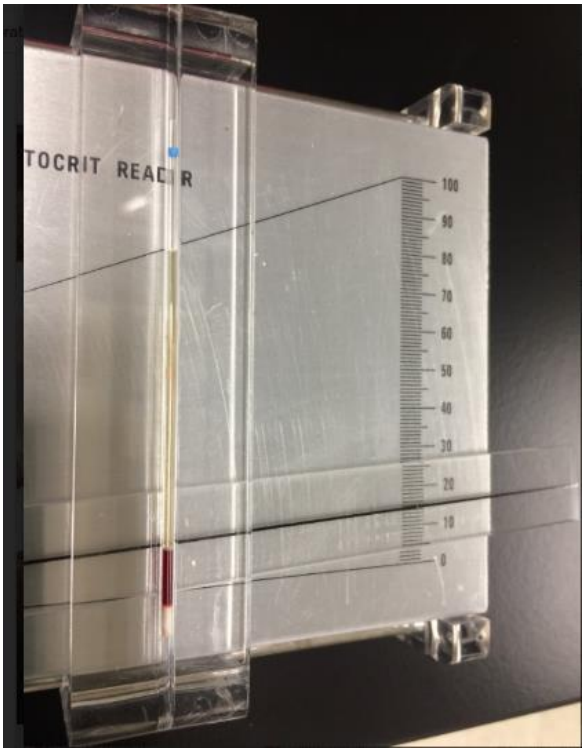

Figure 5. Example of a PCV reading on a hematocrit slide reader. The shown sample has a PCV of about 15%.

DNA Extraction

Genomic DNA (gDNA) was extracted from whole blood using Quick-DNA Miniprep kit (Zymo Research, Irvine, CA) following manufacturer instructions. Briefly, 100 µL of whole blood is aliquoted into a 1.7 mL DNAse free microcentrifuge tube to which 400 µL of Genomic Lysis Buffer was added, completely mixed by vortexing for 15 seconds, and incubated at room temperature for 15 minutes. The lysed blood sample was then pipetted into a Zymo-spin^{TM} IIC Column in a collection tube and centrifuged at 12,000 rpm for 1 minute. The collection tube containing the flow-through waste was discarded and the column placed in a new collection tube. To the column, 200 µL of DNA Pre-Wash buffer was added and then centrifuged at 12,000 rpm for 1 minute after which the column is transferred to a new 1.7 mL DNase free microcentrifuge tube. To the column, 35 µL of pre-warmed DNA Elution buffer was added, allowed to incubate for 5 minutes at room temperature, and then centrifuged at 12,000 rpm for 1 minute to collect the eluted DNA. If not used immediately, the eluted gDNA was stored at minus 20°C.

Quantitative Real-time Polymerase Chain Reaction (qPCR)

Quantitative real-time PCR (qPCR) targeting a conserved portion of the *A. marginale* Major Surface Protein 5 gene (*msp5*) was used to monitor *A. marginale* infection levels in steers. The qPCR mastermix was prepared in a template-free clean working station located in a separate room from where DNA extractions are performed and were thermocyclers are located to reduce chances of incidental contamination. The volume of the mastermix was calculated based on the number of samples, with the per sample volume of each component being as follows: 7.2 µL nuclease-free water, 10 µL SsoAdvance Universal SYBR (Bio-Rad Laboratories, Hercules, CA), 0.4 µL 10 µM Am msp5 F (sequence (5'- 3') ATA CCT GCC TTT CCC ATT GAT GAG GTA CAT), 0.4 µL 10 µM Am msp5 R (sequence (5'- 3') AGG CGA AGA AGC AGA CAT AAA

GAG CGT), for a total volume of 18 µL per sample (**Table 4**). The mastermix is vortexed to thoroughly mix and 18 µL is aliquoted to individual wells on a 96 well PCR plate.

Table 4. PCR reaction mixture. A PCR reaction master mix showing 1 reaction and 100 reactions to set up for a 96 well plate. There is a 5% extra reaction calculated to compensate for pipetting error while preparing the plate.

No.	Component	1 reaction	100 reactions	Final conc.
1	Water	7.2 µL	720µL	
2	SsoAdvance Universal SYBR (Bio-Rad, Cat#172-5271)	10 µL	1000µL	1X
3	10µM Am msp5 F	0.4 µL	40µL	0.2 µM
4	10µM Am msp5 R	0.4 µL	40µL	0.2 µM
	Total	18 µL	1800µL	
5	DNA	2 µL		

Sample addition and plate setup were as follows: 2 µL *msp5* positive control (pCR™4-TOPO plasmid containing target *msp5* amplicon) are added to the first two wells; 2 µL of nuclease free water is added to the last well (non-template control - NTC); and, 2 µL of gDNA sample is added into their assigned well. Plates were sealed with an optically clear plastic seal, securely pressed over the wells to seal all individual wells and prevent cross contamination of DNA. The sealed plate was then centrifuged at 2,000 rpm for 30 seconds to collect the mixture in each well to the bottom. Thermal cycling was performed using a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). The cycling conditions used were: initial denaturation at 98°C, 2 minutes for 1 cycle; 40 cycles of 98°C for 5 seconds (denaturation), 60°C for 5 seconds (annealing), 74°C for 15 seconds; and, a final melt curve analysis with temperature step down from 65°C to 95°C in 0.5°C increments at 2 seconds per

step. The CFX Maestro Software (Bio-Rad Laboratories, Hercules, CA) was used to view results (**Figure 6**). The resulting amplification curve of each sample was compared against the known concentration of the positive control to quantify the number of *A. marginale* DNA copies within our given sample. The resulting melt curve (dissociation) of each sample that depicts the reaction temperature at which the double-stranded DNA melts into a single-stranded DNA, was compared against the positive control to confirm amplicon specificity.

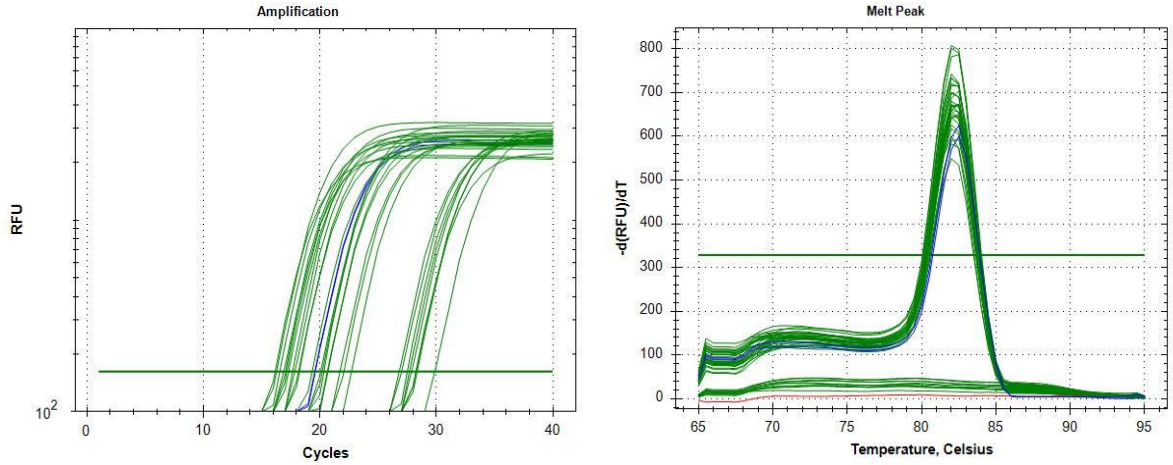

Figure 6. Example of representative amplification and melt curve for the *msp5* qPCR assay. Amplification curve (left). The curve rising across the threshold line ranging from 15 to 28 cycles indicates a given Ct (threshold cycle number) value to used to determine the number of copies of *msp5* target in a positive sample. Melt curve (right). Dissociation curve used to confirm specificity of amplified product. The melt curve of a true positive sample should overlay the positive control.

Health Assessments

All steers were checked daily for general health and signs of clinical anaplasmosis (although no signs of clinical anaplasmosis were anticipated because clinical signs are rare in otherwise healthy persistently infected cattle). The health parameters evaluated when observing steers for common signs of clinical anaplasmosis included increased respiration, depression, or a steer separating itself or not coming to feed. If a steer displayed any abnormal sign/behavior it was brought into the working facility for further evaluation, determination of issue, treatment (if

necessary) and veterinary consultation (if necessary). In addition, steers were monitored for any signs of non-anaplasmosis-related issues, including diarrhea, pink eye, lameness, or bloat.

Assessment of Antimicrobial Treatment Reactions

Steers were monitored for reactions to oxytetracycline or enrofloxacin treatment. Per drug label indications, both oxytetracyline and enrofloxacin were subcutaneously administered to steers in their respective treatment group. The consecutive days of treatment, treatment volume per dose, and the number of injection sites required per dose were factors that could influence development of injection site reactions. Evidence of injection site reactions included evidence of tenderness to touch at or around treatment site(s), swelling, focal alopecia, and abscess formation. Treatment was discontinued in any steer displaying severe injection site reactions and when repeated treatment administration was advised against by the overseeing veterinarian.

Statistical Analysis

Descriptive statistics (mean, median, and range) for *A. marginale* bacterial levels were calculated using a Microsoft Excel spreadsheet. For comparison of bacterial levels at certain time points between strains and group, a student's t-test was performed. Analyses results where the p-value ≤ 0.05 were considered significant.

Chapter 3- Results

Baseline steer and *A. marginale* infection parameters pre-antimicrobial treatment initiation

Persistent *A. marginale* infection was confirmed in all steers by qPCR prior to the first treatment. The KS2 *A. marginale* strain had a mean starting bacterial level of 5.49E+06 *A. marginale*/mL of blood (median= 4.64E+06 *A. marginale*/mL of blood, range = 2.49E+06 to 1.19E+07 *A. marginale*/mL of blood) (**Table 5**) The VA strain had a mean staring bacterial level of 3.57E+05 *A. marginale*/mL of blood (median=1.08E+03 *A. marginale*/mL of blood, range= 0.00E+00 to 2.37E+06 *A. marginale*/mL of blood) (**Table 5**). One steer, JS464, infected with the VA strain, had a non-detectable level the first day of treatment but was positive during the date of initial enrollment (this steer fluctuated at low levels throughout the study). There was a significant difference in starting bacterial levels depending on whether steers were infected with the KS2 and VA strain (p-value = 0.004) even though steers had been infected for a similar period of time (approximately 5 months).

Table 5. Description of starting and lowest *A. marginale* infection levels. The starting *A. marginale* bacterial level in steers and lowest achieved *A. marginale* bacterial level in response to antimicrobial treatment are presented. Regardless of treatment group, steers infected with the KS2 strain had significantly greater starting *A. marginale* bacterial levels.

Treatment Group	*A. marginale* strain	Infection Route	Animal ID	Starting copies (*A. marginale*/mL blood)	Lowest copies (*A. marginale*/mL blood)
A	KS2	Tick	DP696	6.12E+06	2.86E+04
A	KS2	Needle	T4963	4.64E+06	2.59E+02
A	KS2	Tick	T4968	5.77E+06	3.92E+04
A	KS2	Needle	T4970	4.22E+06	1.34E+03
A	VA	Needle	DP695	1.23E+05	5.17E+02
A	VA	Needle	DP697	1.08E+03	0.00
A	VA	Needle	T4971	2.37E+06	5.23E+03
B	KS2	Needle	DP694	2.49E+06	2.49E+04

B	KS2	Tick	JS460	3.28E+06	2.38E+03
B	KS2	Tick	JS463	1.19E+07	6.14E+03
B	VA	Needle	JS461	8.31E+02	0.00
B	VA	Needle	JS464	0.00	0.00
B	VA	Needle	T4962	3.95E+02	0.00
B	VA	Needle	T4965	2.42E+03	4.79E+02

The mean steer weight for Group A was 744 pounds (median = 735 pounds, range = 673 to 858 pounds). For Group B, the mean steer weight was 735 pounds (median = 721 pounds, range = 633 to 859 pounds). The steer cohort assigned to each treatment groups did not significantly differ in starting weight (p-value = 0.8) (weight is a parameter that informs treatment dosing and required number of injections per dose).

The mean starting PCV for Group A steers was 33% (median = 32%, range = 30 to 40%). Similarly, Group B had mean starting PCV of 32% (median= 33%, range= 30 to 38%). The steer cohort assigned to each treatment groups did not significantly differ in starting PCV (p-value = 0.4). All steers had starting PCV values within the normal healthy range which indicates that all steers had successfully recovered from any anemia experienced during acute anaplasmosis that occurred months earlier.

All steers at the start of the study were in good overall health. There were no signs of clinical anaplasmosis observed in any steer and no underlying health issues were present. None of the 14 steers had received any antimicrobial treatment up to 5 months previous to enrollment in this attempted *A. marginale* infection elimination study.

Treatment with oxytetracycline and enrofloxacin reduces but rarely eliminates *A. marginale* infection

Holstein steers, persistently infected with the VA or KS2 strain of *A. marginale* were divided into two groups and treated daily for five consecutive days with oxytetracycline (4.5 mL/100 lb, subcutaneously) or enrofloxacin (5.7 mL/100 lb, subcutaneously) in an attempt to eliminate *A. marginale* infection. Changes in *A. marginale* bacterial level and overall infection status were evaluated by qPCR over the treatment period and at weekly time points for 4 weeks post-cessation of treatment. The *A. marginale* bacterial level for steers in each treatment group were compared between treatment groups and between *A. marginale* strains.

Overall, at 30 days post-treatment 12 of the 14 steers remained PCR positive for *A. marginale* infection despite antimicrobial treatment. The starting *A. marginale* bacterial level and lowest *A. marginale* bacterial level observed in each steer is presented in Table 5. For all steers infected with the KS2 strain, treatment with oxytetracycline or enrofloxacin resulted in a temporary reduction of *A. marginale* bacterial level (described in more detail below). For steers infected with the VA strain, treatment with oxytetracycline or enrofloxacin had variable results due to the varied starting levels of this strain in steers (mean = 3.57E+05 *A. marginale*/mL blood, median = 1.08E+03 *A. marginale*/mL blood, range = 0.00E+00 to 2.37E+06 *A. marginale*/mL blood) (described in more detail below).

Treatment Group A (oxytetracycline)

In Treatment Group A (oxytetracycline) all 7 steers remained positive for *A. marginale* infection. The oxytetracycline treatment regimen resulted in a significant decrease in *A. marginale* bacterial levels, with the lowest bacterial levels observed 2 to 3 weeks post-treatment.

The KS2 strain had an average peak reduction of 5.17E+06 *A. marginale*/mL blood (median = 5.19E+06 *A. marginale*/mL blood range = 4.22E+06 to 6.09E+06 *A. marginale*/mL blood). The average rebound infection level (final sample point) for KS2 steers was 5.20E+05 *A. marginale*/mL blood (median = 4.71E+05 *A. marginale*/mL blood, range = 4.10E+04 to 1.10E+06 *A. marginale*/mL blood). There was a significant difference of starting infection level versus final infection level for the KS2 steers (p-value = 0.0005); however, if the observation period had been continued bacterial levels would likely have reached pre-treatment levels as these rebounding bacterial levels had a positive trajectory.

The VA average peak reduction was 8.29E+05 *A. marginale*/mL blood (median = 1.22E+05 *A. marginale*/mL blood, range = 1.08E+03 to 2.36E+03 *A. marginale*/mL blood). There was not a significant difference in starting infection level versus peak low infection level for the VA strain (p-value = 0.394), and *A. marginale* infection was not eliminated in any VA infected steer treated with the oxytetracycline regimen. For VA steers, infection level rebound to pre-treatment levels was not observed and remained moderately low in these steers (Figure 7). The average final infection level (final sample point) for VA steers was 2.00E+03 *A. marginale*/ml blood (median = 5.17E+02 *A. marginale*/mL blood, range = 2.37E+02 to 5.23E+03 *A. marginale*/mL blood). There was not a significant difference of starting infection level vs final infection level for the VA steers (p-value = 0.39).

There was a significant difference in starting infection level versus peak low infection level for the KS2 strain (p-value = 0.0014); however, *A. marginale* infection was not eliminated in any KS2 infected steer treated with the oxytetracycline regimen. Comparing the KS2 and VA strains; KS2 steers infection levels returned to near pre-treatment levels by 30 days post-treatment (Figure 7).

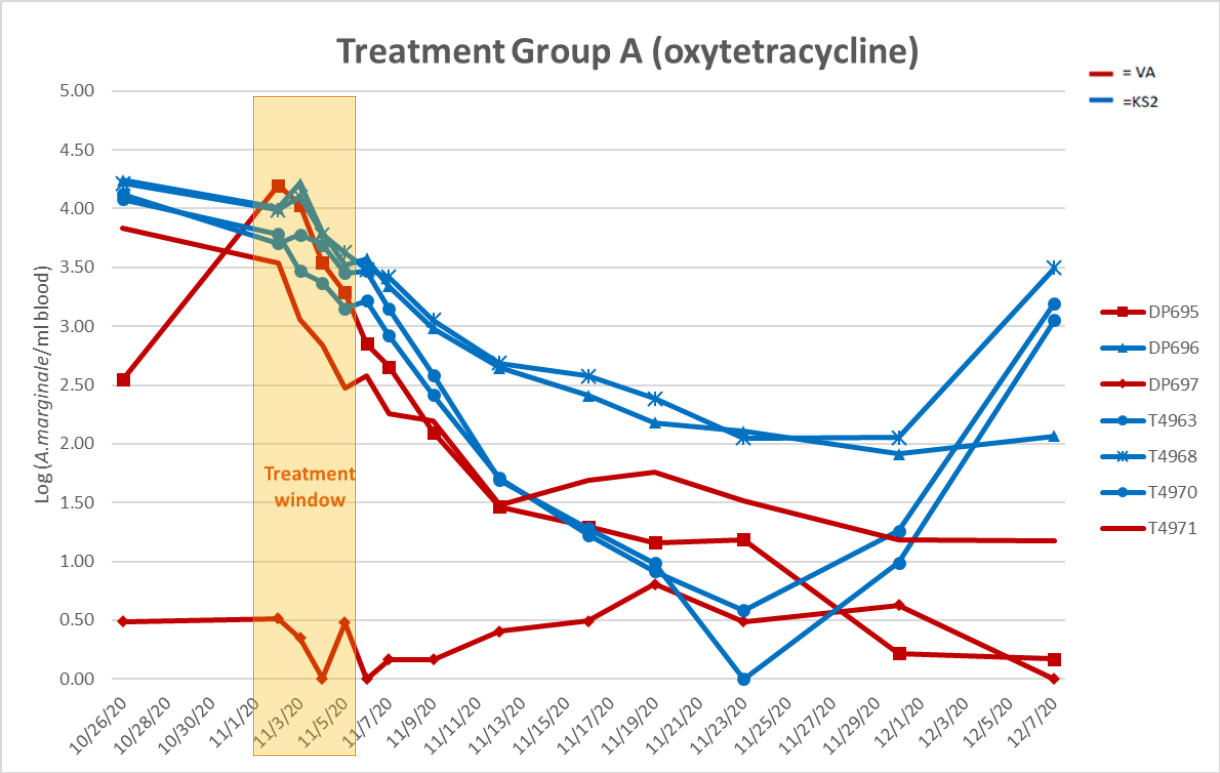

Figure 7. Changes in *A. marginale* bacterial level in steers treated with oxytetracycline. All seven steers treated with oxytetracyline for 5 consecutive days remained infected with *A. marginale*. A significant decrease in infection levels was observed for all treated steers; however, *A. marginale* infection levels eventually returned to near pre-treatment levels.

Treatment Group B (enrofloxacin)

In Treatment Group B (enrofloxacin), *A. marginale* infection was eliminated in 2 of the 7 steers (JS464, T4962). There was a decrease in infection levels for all steers infected with the KS2 strain (Figure 8). The KS2 average peak reduction was 5.88E+06 *A. marginale*/mL blood (median = 3.28E+06 *A. marginale*/mL blood, range = 2.47E+06 to 1.19E+07 *A. marginale*/mL blood). There was not a significant difference in starting infection level versus peak low infection level for the KS2 strain (p-value = 0.19). *A. marginale* infection was not eliminated in any KS2 infected steer treated with the enrofloxacin regimen. The average rebound infection level (final sample point) for KS2 steers was 1.81E+06 *A. marginale*/mL blood (median =

8.94E+05 *A. marginale*/mL blood, range = 6.83E+05 to 3.86E+06 *A. marginale*/mL blood). There was not a significant difference of starting infection level vs final infection level for the KS2 steers (p-value = 0.31).

In steers infected with the VA strain, the average peak reduction was 7.92E+02 *A. marginale*/mL blood (median = 6.13E+02 *A. marginale*/mL blood, range = 0.00E+00 to 1.94E+03 *A. marginale*/mL blood). There was not a significant difference in starting infection level versus peak low infection level for the VA strain (p-value = 0.23). *A. marginale* infection was eliminated in two VA infected steers treated with the enrofloxacin regimen. For VA steers the infection level rebound to pre-treatment levels was not observed and remained moderately low (**Figure 8**). The average rebound infection level (final sample point) for VA steers was 2.92E+02 *A. marginale*/mL blood (median = 7.75E+01 *A. marginale*/mL blood, range = 0.00E+00 to 1.01E+03 *A. marginale*/mL blood). There was not a significant difference of starting infection level versus final infection level for the VA steers (p-value = 0.35).

Comparing the success of the enrofloxacin treatment regimen for steers infected with different *A. marginale* strains, the steers infected with the VA strain had significantly lower starting infection levels compared to the KS2 steers (**Figure 8**). Comparing the KS2 and VA strains; KS2 steers infection levels returned to near pre-treatment levels by 30 days post-treatment. Comparing the infection level decrease in response to treatment between groups, for the KS2 strain; Group B (enrofloxacin) had a greater infection level mean decrease, 5.88E+06 *A. marginale*/mL blood, while Group A (oxytetracycline) had mean decrease of 5.17E+06 *A. marginale*/mL blood. For the VA strain; Group A had a greater infection level mean decrease, 8.29E+05 *A. marginale*/mL blood and Group B with 7.92E+02 *A. marginale*/mL blood.

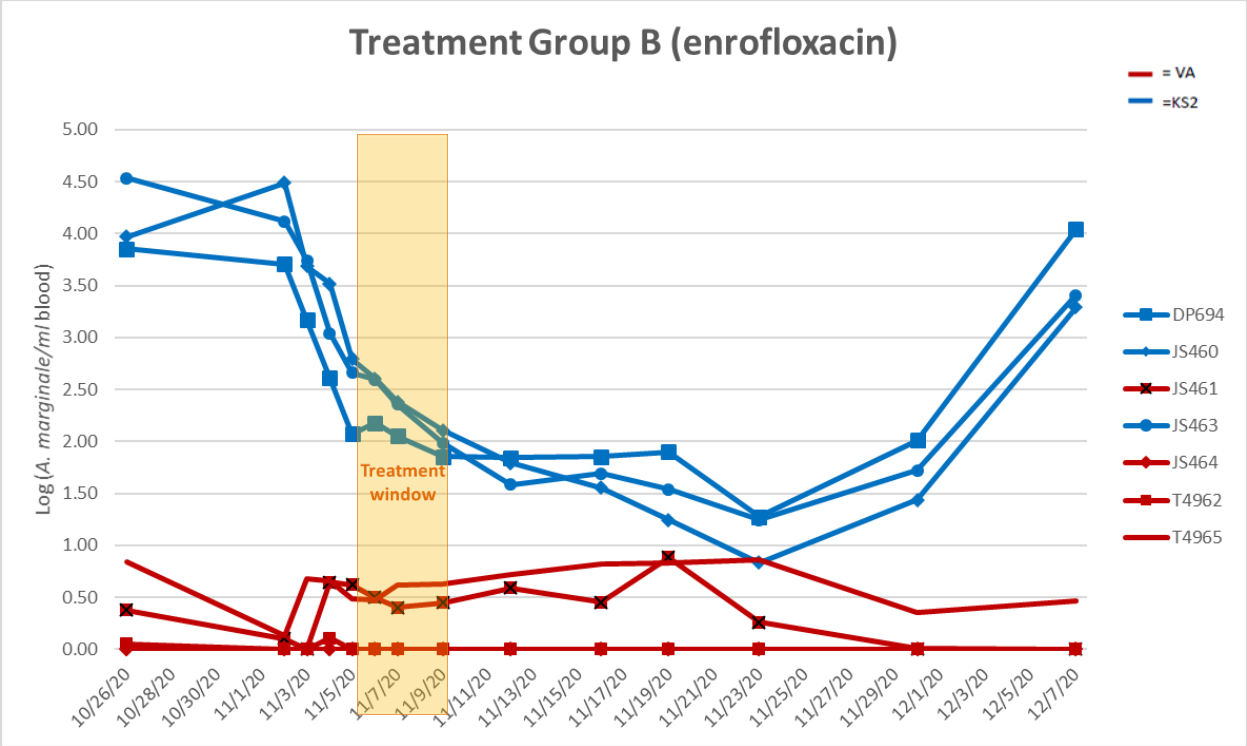

Figure 8. Changes in *A. marginale* bacterial level in steers treated with oxytetracycline. In two steers (JS464, T4962), *A. marginale* infection was eliminated after treatment with enrofloxacin for 5 consecutive days. A significant decrease in infection levels was observed in all steers infected with the *A. marginale* KS2 strain; however, *A. marginale* bacterial levels eventually returned to near pre-treatment levels.

Occurrence of antimicrobial treatment site reactions

Using the respective drug label doses, steers were administered large volumes of the respective drug based on their treatment group on each treatment day (dosed based on each animal's individual bodyweight). The steers in the oxytetracycline group were subcutaneously injected with a mean dose of 33 mL (median = 33 mL, range = 30 to 39 mL) of Bio-Mycin® 200. At a recommended 10 mL per injection site, a mean of 4 injection sites were required to fully treat steers (median = 4 injection sites, range = 3 to 4 injection sites). The steers in the enrofloxacin group were subcutaneously injected with a mean dose of 42 mL (median = 41 mL, range = 36 to 49 mL) of Baytril® 100-CA1. At a recommended 20 mL per injection site, a mean

of 3 injection sites were required to fully treat steers (median = 3 injection sites, range = 2 to 3 injection sites).

The mean required volume of 42 mL for Baytril® 100 CA1 was greater than the 33 mL of Bio-Mycin® 200. However, Baytril® 100-CA1 only required a mean of 3 injection sites with 20 mL per injection site. The mean of 4 injection sites for Bio-Mycin® 200 was greater due to the recommended 10 mL per injection site.

With the large volume of drug administered for consecutive days, signs of reaction to treatment was monitored throughout the treatment period and until resolution when treatment site reactions were observed. In Treatment Group A (oxytetracycline), two steers (DP695 and DP697) developed noticeable swelling at the treatment injection site after the first and second administration of Bio-Mycin® 200 (**Figure 9**). Approximately 24-hours after the first treatment, steer DP695 had developed severe swelling at the treatment site and with the steer's welfare in mind, treatment was ceased. Steer DP697 had a mild treatment site reaction; however, due to this initial reaction, the remainder of this steer's treatments were administered intravenously (approved on-label, alternative treatment administration route). No additional treatment reactions were observed in steer DP697. No treatment site reactions were observed for any Treatment Group B (enrofloxacin – Baytril® 100-CA1) steer at any study point.

Figure 9. Injection-site reaction to treatment administration. Two steers treated with Bio-Mycin® 200 developed significant injection site swelling (circled).

Clinical signs of anaplasmosis are not observed during attempted A. marginale infection elimination

Immunologically sound cattle persistently infected with *A. marginale* usually do not demonstrate any signs consistent with clinical anaplasmosis. As anemia is the hallmark of clinical anaplasmosis, steers were monitored over the study period for signs of anemia by monitoring changes in PCV. The steers in Treatment Group A had a mean PCV of 33% at the beginning of the study and a mean PCV of 35% at the end of the study. The steers in Treatment Group B had a mean PCV of 33% at the beginning of the study and a mean PCV of 34% at the end of the study. Throughout the study, all steers remained above a 30% PCV which is within the parameters of a normal PCV.

Steers were also monitored for other common signs of clinical anaplasmosis such as: pyrexia, lethargy, pale mucous membranes, increased respiration rate, and loss of appetite; none of which were observed in any steer throughout the study.

The experimental antimicrobial-based *A. marginale* treatment regimen rarely alters steer serostatus for *A. marginale*

Serostatus is frequently used as an indication of infection status, because *A. marginale* infection commonly persists in cattle, steer serostatus was monitored by cELISA at the following study time points: pre-treatment (10/26/2020), 48-hours post final treatment (11/9/2020), two weeks post final treatment (11/23/2020), and four weeks post final treatment (12/7/2020). To be considered seropositive using this assay, steers must have a percent inhibition value ≥30%. Of the 14 steers, two steers (DP697 and T4965) started and remained below the 30% inhibition cutoff for the duration of the study. Steer DP697 was in Treatment Group A (**Figure 10**) and steer T4965 in Group B (**Figure 11**); both steers were initially infected with the VA strain. In Treatment Group A (oxytetracycline), all steers infected with the KS2 strain remained seropositive for *A. marginale* throughout the study; however, the percent inhibition was reduced by the end of the study for some KS2 infected steers. The two VA strain infected steers that started off seropositive for *A. marginale* in the oxytetracycline group, remained seropositive for *A. marginale* throughout the study.

In Treatment Group B (enrofloxacin), all steers infected with the KS2 strain remained seropositive for *A. marginale* throughout the study with percent inhibition values for these steers remaining above 80% throughout the study. The three VA strain infected steers that started off

seropositive for *A. marginale* in the enrofloxacin group became seronegative by the final study time point.

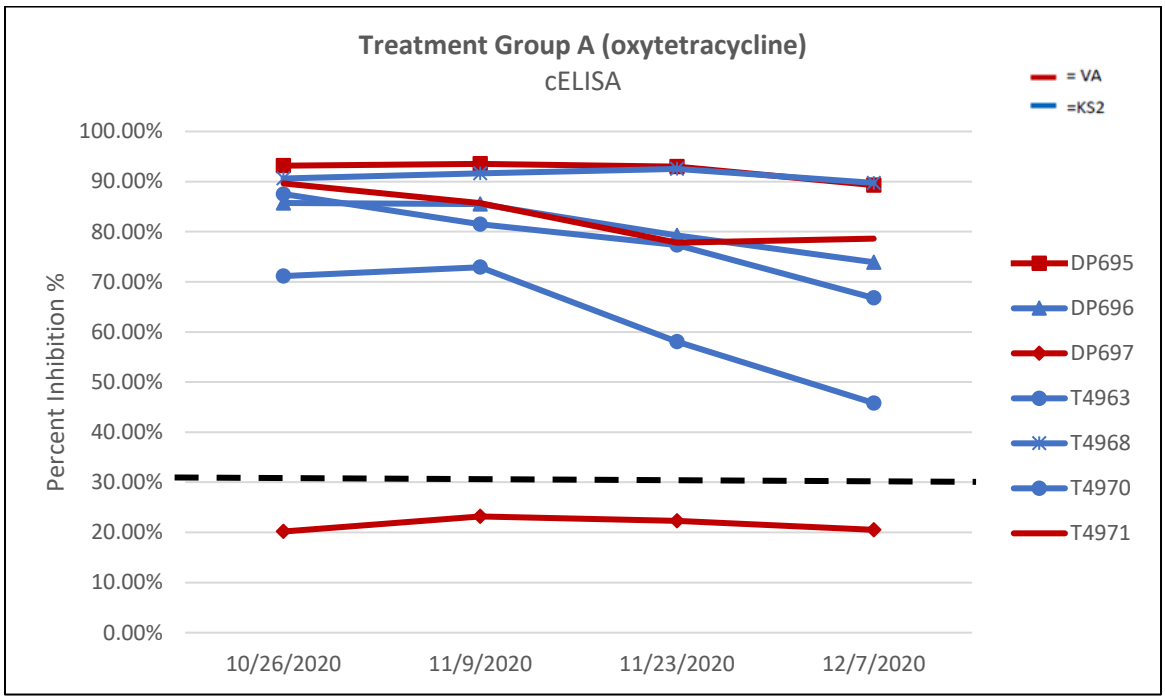

Figure 10. cELISA results Treatment Group A. The dashed line indicates the 30% inhibition cut-off value to be considered seropositive.

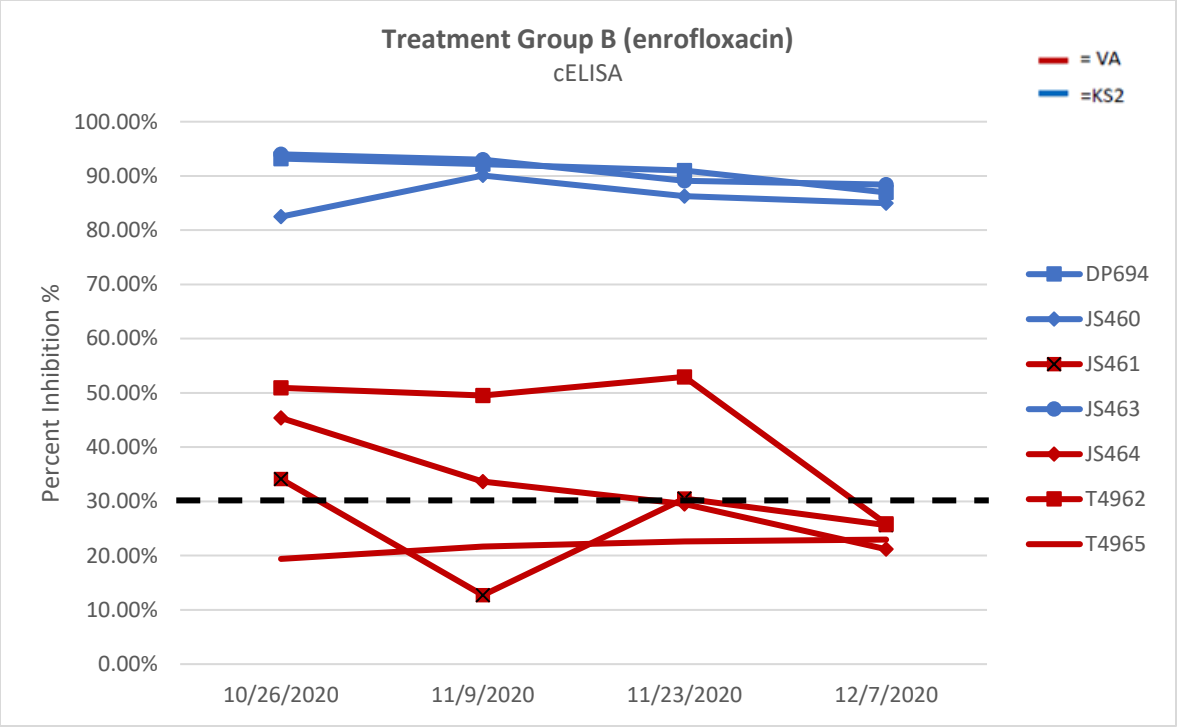

Figure 11. cELISA results Treatment Group B. The dashed line indicates the 30% inhibition cut-off value to be considered seropositive.

Chapter 4- Discussion

A treatment protocol to eliminate *A. marginale* infection is desired by many producers who seek to retain valuable cattle rather than cull them due to *A. marginale* infection. Repetitive treatment with antimicrobials commonly used to treat clinical anaplasmosis have had mixed success in eliminating *A. marginale* infection (Coetzee 2005, 2006; Wallace 2007). To be useful, a reliable antimicrobial-based *A. marginale* elimination protocol should include: antimicrobials already approved for anaplasmosis treatment or control; broadly effective at achieving infection elimination regardless of the infecting *A. marginale* strain; and, should be consistent with judicious antimicrobial use practices. Because cattle rarely clear *A. marginale* infection naturally, the most common anaplasmosis disease state among cattle is chronic anaplasmosis. Cattle with chronic anaplasmosis rarely display clinical signs of disease; however, this disease state may contribute to reduced production outcomes (e.g. weight gain, milk production, and calving success) and exacerbate other underlying conditions or diseases. Additionally, there needs to be an appreciation of the cyclical infection cycle of *A. marginale* in which bacteremia levels fluctuate over time in response to the pathogen's ability to continually evade the immune system. Lack of clinical signs in chronically infected animals and cyclical bacteremia can make identifying chronically infected cattle challenging; therefore, subsampling herds or testing incoming animals using serologic or molecular diagnostic tests is a good way to know herd or individual animal anaplasmosis status. For production operations seeking to remain anaplasmosis-free the only current option of eliminating *A. marginale* infection is culling infected animals from the herd; however, identification of a reliable method to eliminate *A. marginale* infection would provide producers an option to retain valuable stock.

In this study, two common injectable antimicrobials currently approved for the treatment of anaplasmosis, oxytetracyline and enrofloxacin, were evaluated in an extended treatment regimen to eliminate *A. marginale* from steers persistently infected with different strains of *A. marginale*. Treatment was administered subcutaneously for 5 consecutive days; oxytetracycline (Bio-Mycin® 200) at 4.5 mL/100 lb and enrofloxacin (Baytril® 100-CA1) at 5.7 mL/100 lb. The elimination of *A. marginale* infection was observed in only two steers treated with enrofloxacin (Baytril® 100-CA1). Both steers where persistently infected *A. marginale* was eliminated were infected with the VA strain (JS464, T4962). However, all steers persistently infected with the *A. marginale* VA strain had significantly lower starting infection levels than the other study steers infected with the KS2 strain. Additionally, in one of the steers (JS464) where *A. marginale* was eliminated, it appeared that this steer was naturally going to eliminate infection as *A. marginale* bacterial levels were below the limit of detection for the qPCR assay on the first day of treatment (infection was confirmed for initial enrollment into the study), and initiation of treatment likely prevented any infection recrudescence.

Treatment-associated *A. marginale* infection level decreases were observed in steers from both study groups. Comparing the infection level decrease in response to treatment between groups, for the KS2 strain; Group B (enrofloxacin) had a greater infection level mean decrease than Group A (oxytetracycline). However, for the VA strain; Group A had a greater infection level mean decrease than Group B. The observed decreases in infection levels were expected with both antimicrobials. As the starting infection levels for KS2 were greater than VA, specifically in Group B, a more significant infection level reduction was anticipated with KS2 as it had a significantly greater range to decrease. The infecting *A. marginale* strain may influence elimination success but further evaluation is needed to determine the difference in strains

susceptibility to antimicrobials. In both groups, the *A. marginale* VA strain may have increased susceptibility to tetracycline antimicrobials naturally (i.e. genetically-based) or due to lack of sustained selection pressure as this strain was isolated over 40 years ago with stocks of this isolate maintained free of antimicrobial selection pressure. In contrast, the KS2 strain was only recently isolated (2018) and may have increased decreased susceptibility naturally (i.e. genetically-based) or as a result of greater selection pressure (degree of possible selection pressure for this strain is unknown but as a field strain it has had greater opportunities for antimicrobial exposure, especially tetracycline antimicrobials).

Ideally, an antimicrobial-based *A. marginale* infection elimination protocol would include drugs already approved for use to treat anaplasmosis infection. Although the treatment protocol evaluated in this study did not reliably eliminate *A. marginale* infection, daily treatment with Bio-Mycin® 200 and Baytril® 100-CA1 for 5 consecutive days significantly reduced *A. marginale* infection levels; however, most steers rebounded to near pre-treatment infection levels within 30 days post-treatment and infection was eliminated from a few steers infected with the *A. marginale* VA strain. The frequency for both treatments (5 consecutive days) was for experimental use only; the dosage of 4.5 mL/100 lb for Bio-Mycin® 200 and 5.7 mL/100 lb for Baytril® 100-CA1is labeled only for single treatment. Results of lowered bacteremia levels for a period of 30 days post treatment suggest that varying treatment timing to support the downward infection level momentum toward infection elimination is possible.

The efficacy of enrofloxacin was previously evaluated in splenectomized calves experimentally infected with West Coast (St. Maries), OK, and VA *A. marginale* strains. The treated animals had suppressed *A. marginale* infection and reduced clinical signs of disease (less severe anemia, no death), but infection was not eliminated and a recurrence of *A. marginale* was

observed 30 days post treatment (Coetzee and Apley 2006), similar to our study. Another study using calves persistently infected with OK, VA, and St. Maries *A. marginale* strains resulted in enrofloxacin not clearing infection at a dose of 5 mg/kg IV q24h for 5 days. However, the oxytetracycline regimen (22 mg/kg IV q24h for 5 days) cleared infection in one VA infected calf (Coetzee 2006). Our present study had steers infected with the VA strain not clear infection with our oxytetracyline regimen (4.5 mL/100 lb for 5 days) but two VA infected steers did clear infection with our enrofloxacin regimen (5.7 ml/100 lb for 5 days).

The number of injections per treatment and approved routes of treatment administration are factors veterinarians and producers consider when selecting an antimicrobial. Two steers treated with Bio-Mycin® 200 developed significant injection site swelling. The label for Bio-Mycin® 200 states under Adverse Reacitons: "Reports of adverse reactions associated with oxytetracycline administration include injection site swelling, restlessness, ataxia, trembling, swelling of eyelids, ears, muzzle, anus and vulva (or scrotum and sheath in males), respiratory abnormalities (labored breathing), frothing at the mouth, collapse and possibly death". Observing steer behavior while treatment was administered subcutaneously, the Bio-Mycin® 200 seemed to cause more discomfort to the animal than Baytril® 100-CA1 with shaking of the head and agitated body movement but no vocal distress was expressed by the steers. Additionally, two steers treated with Bio-Mycin® 200 developed extensive swelling at the injection site; one steer (DP695) to the point where treatment was discontinued. However, Bio-Mycin® 200 is advertised to be "smooth and easy on your cattle" and "reduces tissue irritation and cattle discomfort." (Boehringer Ingelheim). The steer where subcutaneous treatment was discontinued and treatment route switched to intravenous administration of Bio-Mycin® 200 tolerated the new treatment administration route well with no other adverse reactions noted. The Baytril® 100-CA1 label

states “No adverse reactions were observed during Baytril® 100 clinical trials” and there were none observed with its use in this study. There is no label indication for the intravenous administration of Baytril® 100-CA1. Individual animals may experience different sensitivities to drug treatments. In these cases, treatment route or type should be changed as directed by a licensed veterinarian.

Further directions towards developing a reliable antimicrobial regimen to eliminate *A. marginale* infection should evaluate varying the number of total treatments, treatment timing, or antimicrobial product. This infection elimination protocol would also need to be evaluated across multiple *A. marginale* strains to ensure that the protocol is broadly effective regardless of differences in *A. marginale* strain antimicrobial susceptibility. To confirm infection elimination, two common diagnostic methods are used; molecular qPCR detects and quantifies *A. marginale* DNA in cattle blood (pathogen detection, positive= highly indicative animal is actively infective) and the serologic assay cELISA that detects antibodies that target a specific *Anaplasma* protein (antibody detection, positive = doesn’t mean pathogen present at time of testing). Some steers post treatment were qPCR positive but below the $\leq$30% inhibitory cutoff for cELISA. Steers with low levels of infection would occasionally fluctuate between being qPCR positive and negative based on the sensitivity of this assay. Overall, the diagnostic method recommended to evaluate if *A. marginale* infection was successfully eliminated would be a molecular assay (e.g. *msp5* qPCR) to confirm that *A. marginale* nucleic acid is no longer present.

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Glossary of Terms

Acute infection: onset phase of anaplasmosis when *A. marginale* is most abundant in erythrocytes (70% or more erythrocytes may become infected).

Bacteremia: quantity/concentration of bacteria in the blood.

Bactericidal: antimicrobial kills the bacteria.

Bacteriostatic: antimicrobial prevents growth (keeps the bacteria in the stationary phase of growth).

Clinical disease: stage where characteristic signs of anaplasmosis can be observed and in most cases an animal will survive.

Clinical signs: pyrexia, weight loss, abortion, lethargy, icterus, and in extreme cases death.

Cull: selectively removing an animal from the herd.

Endemic: an area or population where *A. marginale* is prevalent.

Isolates/Strain: identification of *A. marginale* based on genotype and geographic origin.

Molecular prevalence: percent of population *A. marginale* is detected in with quantification of A. marginale DNA.

Serological prevalence: percent of population that have specific *A. marginale* antibodies.