Characterization of contemporary *Anaplasma marginale* strains for assessing antimicrobial-based anaplasmosis control strategies

by

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Abstract

Bovine anaplasmosis is a tick-borne bacterial disease caused by *Anaplasma marginale*, a global pathogen estimated to cost the U.S. cattle industry >$300 million per year. Anaplasmosis treatment and control strategies rely heavily on tetracycline antibiotics; however, variations in susceptibility to this antibiotic class among *A. marginale* strains have been documented. Use of characterized contemporary strains is important when evaluating or re-evaluating antimicrobial management strategies. The first objective of our work was to locate and propagate two isolates of *A. marginale* actively circulating in Kansas cattle herds and characterize the progression of infection and clinical disease associated with these contemporary isolates in adult beef cattle. Adult beef cows naturally infected with contemporary *A. marginale* strains not previously isolated or studied were identified from the Kansas State University Cow-Calf herd, a herd naturally endemic for anaplasmosis. Blood samples containing these uncharacterized *A. marginale* isolates (KS1 and KS2) were collected and sub-inoculated into splenectomized calves for isolate propagation. To characterize the virulence and infection kinetics of these isolates, adult beef cows were inoculated with stabilates of *A. marginale* isolates 'KS1' and 'KS2', and clinical disease and isolate infection kinetics were monitored using packed cell volume and polymerase chain reaction assays, respectively. Animals challenged with KS1 reached clinical anaplasmosis by 35 days post-inoculation (dpi), approximately 3 days earlier than KS2-challenged animals. Animals challenged with KS1 reached a peak bacteremia of 3.43×10^6 bacteria/mL blood, whereas KS2-challenged animals reached 9.46×10^8 bacteria/mL blood. Both isolates caused clinical anasplasmosis in challenged animals that required treatment intervention; however, KS1 and KS2 had distinct infection kinetic characteristics. These isolates will be used in future studies to evaluate or re-evaluate the efficacy of antimicrobial-based anaplasmosis

treatment and control strategies. Collectively, the work presented in this thesis will contribute to the need for data-driven recommendations for effective and judicious antimicrobial-based anaplasmosis management strategies.

Key words: *Anaplasma marginale*, anaplasmosis, antimicrobial, genetic diversity

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List of Acronyms

AAALAC: Association for Assessment and Accreditation of Laboratory Animal Care

ANOVA: analysis of variance

AMR: antimicrobial resistance

AMU: antimicrobial use

BQA: Beef Quality Assurance

cELISA: competitive inhibition enzyme-linked immunosorbent assay

CTC: chlortetracycline

DMSO: dimethyl sulfoxide

DNA: deoxyribose nucleic acid

DR25: *A. marginale* isolate DR25

dpi: days post-inoculation

EDTA: ethylenediamine tetraacetic acid

FDA: Food and Drug Administration

IACUC: Institutional Animal Care and Use Committee

KS1: *A. marginale* isolate KS1

KS2: *A. marginale* isolate KS2

Mab: monoclonal antibody

Msp: major surface protein

Msp1a: major surface protein 1a

Msp2: major surface protein 2

Msp4: major surface protein 4

Msp5: major surface protein 5

OK: Oklahoma strain of *A. marginale*

OTC: oxytetracycline

PBS: phosphate buffer solution

PCR: polymerase chain reaction

PCV: packed cell volume

PPE: percent parasitized erythrocyte

qPCR: quantitative polymerase chain reaction

rRNA: ribosomal ribonucleic acid

USDA: United States Department of Agriculture

VA: Virginia strain of *A. marginale*

VCPR: veterinary client patient relationship

VFD: veterinary feed directive

Glossary

******Terms are defined in reference to Anaplasma marginale or anaplasmosis.***

Acute phase: phase of disease when clinical anaplasmosis symptoms are typically experienced and greatest levels of *A. marginale* infection levels are observed.

Antimicrobial susceptibility: susceptibility of a bacteria to antibiotics.

Bacteremia: quantification of bacteria in the blood.

Biological transmission: mode of transmission in which pathogen replication occurs; most effective and efficient mode of transmission.

Contemporary strains: actively circulating *A. marginale* field strains that have been exposed to natural and management-driven selection pressures.

Endemic: an area where a disease/pathogen is regularly identified.

Genotype: for *A. marginale*, the sequential arrangement of individual tandem repeats in the Msp1a gene.

Historical strains: strains of *A. marginale* isolated and characterized decades ago that have not been exposed to the same intensity and frequency of selection pressures as contemporary strains.

Infection kinetics: changes in *A. marginale* bacterial levels in cattle during the course of infection.

Inoculation: act of introducing the infective pathogen into the host.

Isolate: refers to the source of *A. marginale*, which may contain multiple *A. marginale* strains/genotypes.

Mechanical transmission: mode of transmission in which no pathogen replication occurs; movement of infected material from an infected host to a naïve host; less efficient.

PCV nadir: the lowest observed packed cell volume record for an animal.

Persistent phase: phase of disease characterized by cyclical fluctuations of *A. marginale* bacteremia during which signs of clinical disease are rarely observed.

Phenotype: observable characteristics of an individual *A. marginale* isolate or strain.

Propagation: method of replicating *A. marginale* isolates or strains, usually in an immunocompromised animal.

Seroprevalence: percentage of animals with pathogen-specific antibodies.

Splenectomized: an animal that has had its spleen removed; a procedure performed to culture/grow high levels of *A. marginale*.

Stabilate: infectious material produced from propagation of *A. marginale* that is commonly used as the inoculum in experimental animal infections.

Strain: in this study, defined by the *A. marginale* Msp1a genotype.

Vector: an object or tick that transmits the pathogen from one animal to another.

Virulence: the severity of disease.

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Chapter 1 - Review of Literature

Overview of Anaplasmosis

Bovine anaplasmosis, caused by the obligate intracellular rickettsial pathogen *Anaplasma marginale*, is an economically important hemolytic disease of domesticated cattle. First discovered by Smith and Kilbourne in 1893, *A. marginale* was mistakenly thought to be part of the life cycle of the protozoal pathogen *Babesia bigemina* (Smith and Kilbourne, 1893). In the early 1900's, Sir Arnold Theiler was able to differentiate between babesiosis and anaplasmosis. Animals that were previously inoculated with South African redwater (*Babesia bigemina*) later developed disease when injected with blood from animals infected with the then unknown *A. marginale*. Sir Arnold Theiler concluded that due to its longer incubation period, its prolonged duration, and the observation of coccus-like marginal points, this disease was different from babesiosis. Theiler coined the name "anaplasma" and initially believed this to be a protozoan pathogen (Theiler, 1911). As Theiler conducted experiments on this disease, he found the pathogen located in marginal points within red blood cells in some animals and located more central points within red blood cells in others. Theiler also noticed that the distinct configurations displayed unique disease properties. To distinguish between the two "anaplasms", he designated one *Anaplasma marginale* (**Figure 1.1**), and the other *Anaplasma marginale* (variety *centrale*).

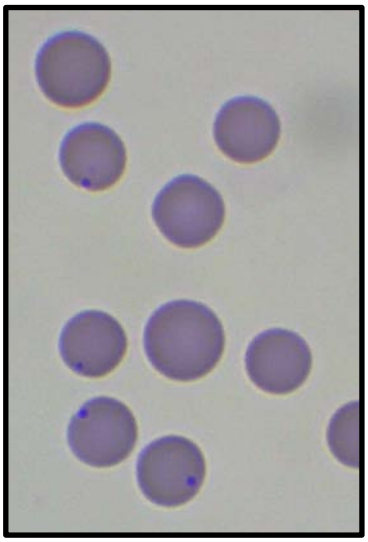

Figure 1.1 – *Anaplasma marginale* (basophilic-staining bodies) infected bovine erythrocytes

All ages of cattle are susceptible to *A. marginale*, but mature animals develop more severe disease. The following list of clinical symptoms of anaplasmosis was part of an in-depth review by Jones and Brock (1966). The first recorded sign of disease was an

increased temperature. The successive clinical symptoms resulted from the onset of anemia, a condition in which there are not enough healthy red blood cells to adequately supply body tissues with oxygen. When the red blood cell count (hematocrit) was decreased by 40 to 50% (**Figure 1.2**), clinical signs were more consistently recognized, and animals displayed lethargy, depression, weight loss, decreased milk production, pale mucous membranes (**Figure 1.2**), and thin watery blood. Some animals became restless and excited when forced to move, as cardiac rate and output increased, and cerebral anoxia set in. Abortion was also observed due to infection during advanced stages of pregnancy, as well as temporary infertility in breeding bulls. Icterus (**Figure 1.2**) was seen during the early stages of convalescence but those that did not convalesce died from complications associated with severe anemia. The animals that survived acute disease became persistently infected and served as transmission reservoirs for the pathogen (Jones and Brock, 1966).

Figure 1.2 – Clinical symptoms of anaplasmosis. Hematocrit of animal suffering severe anemia (left), pale mucous membranes from onset of anemia (center), icteric vulva (Hashem et al., 2018) (right).

The role and mechanics of persistent *A. marginale* infection in host animals was largely unknown until 1989 when Eriks et al. developed a nucleic-acid probe that was more sensitive to

detecting low levels of parasitemia in carrier cattle. Using this hybridization assay, they observed highly variable parasitemia levels among carrier cattle ranging from greater than 0.0025 to less than 0.000025% infected erythrocytes. The observed range of parasitemia levels implied that a smaller percentage of infected erythrocytes was associated with lesser risk of pathogen transmission (Eriks et al., 1989). Kieser et al. (1990) further characterized persistent *A. marginale* infection and demonstrated a logarithmic variation of bacteremia in persistently infected cattle. They noted that the cyclical fluctuation (**Figure 1.3**) from less than 10^3 to greater than 10^5 infected erythrocytes/mL of whole blood could be consistent with the development of antigenic variations that the host's immune system did not recognize (Kieser et al., 1990).

The fact that the immune system was capable of preventing development of high parasitemia levels observed during acute infection but was incapable of clearing a low-level persistent infection suggested a mechanism of escape from the immune response (Palmer et al., 2000). One such method of escape by *A. marginale* was found to be related to natural antigenic variation among two immunogenic surface proteins: major surface protein 2 (Msp2) and major surface protein 3 (Msp3). It was found that before complete clearance of variants by the immune system, new variants emerged that were not recognized by the immune response (Palmer et al., 2000). The new Msp2 escape variants were expressed in each new bacteremic cycle, which were about six weeks in durations. Using this rate of variant expression over a 7-year period, greater than 240 different variants would have potentially challenged the host's immune system. Each cycle during persistent infection resulted in an increased level of parasitemia that quickly decreased, likely due to the immune response recognizing most, but not all, of the Msp2 variants expressed (French et al., 1998). The increased level of parasitemia reached during each cycle of persistent infection was lower than that reached during initial infection and rarely resulted in clinical

manifestations (**Figure 1.3**). In contrast, if an animal became immunosuppressed and unable immunologically to counter the emergence of a new variant, that animal developed recrudescent clinical anaplasmosis.

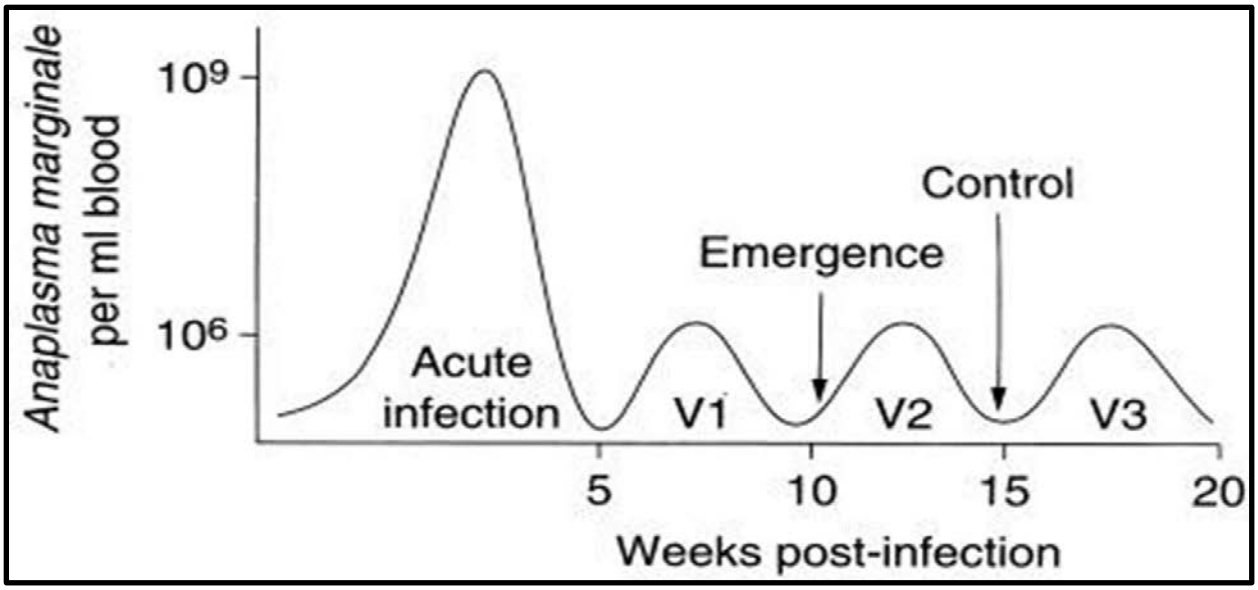

Figure 1.3 – Infection cycle of *A. marginale*. Increased bacteremia during acute infection followed by cyclical fluctuations reaching bacteremia levels lower than initial infection through persistent infection (Kocan et al., 2003).

Multiple diagnostic tests have been developed to diagnose *A. marginale* infection or exposure. For the purposes of this thesis, the three diagnostic tests used in our lab (i.e., light microscopy, cELISA, and PCR) will be reviewed. Light microscopy of stained blood smears was the first diagnostic test used to identify and diagnose *A. marginale* infection of bovine erythrocytes (Smith and Kilbourne, 1893; Theiler, 1911). This diagnostic method was most useful during acute clinical disease when the bacteremia was greater than 1×10^7 *A. marginale*/mL of blood. At lesser bacteremia levels, including during persistent infection in carrier animals, blood smear observation of infection is often not practical or possible. Consequently, this diagnostic method was most effective when animals were suffering clinical symptoms, as a result of the onset of anemia. In severely anemic animals, this diagnostic method

may also be of limited utility due to spleen-removal of most of the infected erythrocytes from the bloodstream (Potgieter and Stoltsz, 1994).

A competitive inhibition enzyme-linked immunosorbent assay (cELISA) was developed in 1996 by Knowles et al. to determine the presence of bovine antibodies against *Anaplasma* major surface protein 5 (Msp5). Detection of these antibodies indicated that the animal being sampled was exposed to and possibly infected with *A. marginale*. This assay utilized a monoclonal antibody (Mab) ANAF16C1 that recognized Msp5 among *Anaplasma* spp (Knowles et al., 1996). This diagnostic assay is the most practical means of evaluating large groups of animals due to price, speed, and limited diversity among *Anaplasma* spp infecting cattle in the United States. As animals commonly become persistently infected with *A. marginale*, animals with a positive serologic test result are often interpreted as actively infected with *A. marginale*.

A third diagnostic method, quantitative polymerase chain reaction (qPCR), detects the pathogen itself (specifically pathogen genetic material), including trace amounts of pathogen in carrier animals. By amplifying certain fragments of *A. marginale*-specific nucleic acid, qPCR assays can detect as little as 10 *A. marginale* bacteria/mL of blood. A recently developed assay demonstrated acceptable sensitivity and specificity by targeting 16S rRNA transcript copies of *A. marginale* in blood (Reinbold et al., 2010c). For detection of *A. marginale* in infected animals, primers targeting Msp4 and Msp1a genes have been used to distinguish among different *Anaplasma* species and strains. (de la Fuente, 2001a). Another common target was the highly conserved Msp5 gene; however, in areas where multiple *Anaplasma* spp existed, there can be challenges with cross-reactively among related species (Visser et al., 1992). However, in the United States, only *A. marginale* is known to infect cattle; therefore, the necessity of discriminating between multiple *Anaplasma* spp. is of limited importance.

Epidemiology and Strain Diversity

Bovine anaplasmosis occurs worldwide in tropical and subtropical regions on six continents and throughout the United States (de la Fuente et al., 2007; Hove et al., 2018). Bovine anaplasmosis is endemic in the southeastern part of the United States but it has also been identified in every state but Hawaii (McCallon, 1973). The widespread nature of this disease is likely due to the frequent and extensive transportation of cattle across the country and the expansive distribution of competent tick vectors.

Anaplasmosis was first recognized in the United States by P.B. Darlington in 1926 when he reported that southeastern Kansas cattle "had a febrile disease that usually occurs in the late summer and fall of the year" (Darlington, 1926). A herd-level *A. marginale* infection prevalence survey performed in Kansas from 2016-2017 found that herd-level *A. marginale* seroprevalence ranged from 19.8 to 34.4%, 44.2 to 57.3%, and 76.9 to 87.3% in the western, central, and eastern thirds of the state, respectively (Spare et al., 2020). Another recent seroprevalence survey was performed in Texas that evaluated individual animals by examining 1835 serum samples collected from 23 sale barns across the state. The results yielded a statewide *A. marginale* seroprevalence of 15.02% with the greatest seroprevalence, 20-38%, in the western portion of the state (Hairgrove et al., 2014). The previously mentioned seroprevalence of *A. marginale* in Texas was supported by Okafor and co-workers who found a seroprevalence of 13.25% among samples collected from a Texas slaughter facility and from samples submitted to the Texas A&M Veterinary Medical Diagnostic Laboratories between January 2002 and June 2012 (Okafor et al., 2018b). In a similarly constructed survey, Okafor and co-workers later found the *A. marginale* seroprevalence in Mississippi to be 28.99% (Okafor et al., 2019b). Statewide *A. marginale* seroprevalences of approximately 4 and 11% were also documented for Georgia and Kentucky,

respectively (Okafor et al., 2018a and 2019a). These surveys collectively described different risk factors for *A. marginale* prevalence in beef cattle including, distribution of known vectors due to geographical location (Hairgrove et al., 2014; Spare et al., 2020), density of cattle population (Okafor 2018a,b and 2019b), animal age (Okafor et al., 2019a), and season (Okafor 2018a and 2019a).

***Anaplasma marginale* Seroprevalence**						
Location	**Prevalence (%)**	**Active/Retrospective**	**Operation type**	**# and type of sample**	**Diagnostic type**	**Reference**
Georgia	4.44	Active	Sale barn/slaughter	293 cows	cELISA	Okafor et al., 2019a
Mississippi	28.99	Active	Slaughter facility	207 cows	cELISA	Okafor et al., 2019b
	22.11	Retrospective	VDLs	5182 records	cELISA	
Texas	13.49	Active	Slaughter facility	215 cows	cELISA	Okafor et al., 2018a
	13.02	Retrospective	VDL	15,460 records	cELISA	
Texas	15.02	Active	Sale barn	1835 cattle	cELISA	Hairgrove et al., 2014
Kentucky	10.78	Active	Slaughter facility	232 cows	cELISA	Okafor et al., 2018b
	11.58	Retrospective	VDL	2573 records	cELISA	
Kansas	51.7	Active	Cow/calf	**925 herds**	cELISA	Spare et al. 2020

Table 1.1 – Statewide *A. marginale* seroprevalence surveys. Contemporary (post-2000) U.S. anaplasmosis seroprevalence surveys. Some surveys examine individual animal seroprevalence and some examine herd-level seroprevalence.

Hundreds of strains of *A. marginale* have been identified from multiple geographical regions around the world. Structural differences in major surface proteins (Msp) can influence the ability of different *A. marginale* strains to be transmitted and cause infection. Six Msp's were identified by Palmer et al. (1999) as being useful to discriminate between strains; Msp1a was proposed as being most useful because it is only found in *A. marginale* and not other *Anaplasma* spp. Strain diversity among *A. marginale* is most commonly defined by the organization of the tandem repeat region of Msp1a. Individual repeats range in length from 28-29 amino acids and are given a unique alpha or alpha-numeric name. The sequential arrangement of these repeats

(**Figure 1.4**) in the tandem repeat region are used to define the Msp1a genotype (de la Fuente et al., 2001a). Major surface protein 1a was shown to be an adhesion for tick cells and bovine erythrocytes (de la Fuente et al., 2005) and to contribute to *A. marginale* infection immunity in cattle (Kocan et al., 2003). The Msp1a genotype is frequently used to define 'strain', such that 'strain' is often synonymous with 'Msp1a genotype' for many *A. marginale* 'strains'. Multiple *A. marginale* genotypes may co-exist within a host (bovine or tick); moreover, some *A. marginale* isolates contain multiple *A. marginale* Msp1a genotypes/strains.

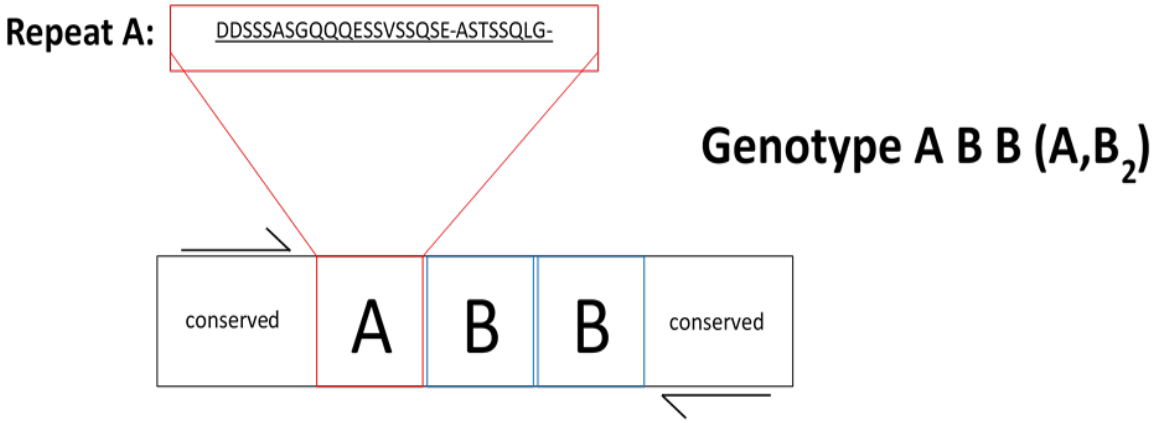

Figure 1.4 – Schematic representation of Msp1a tandem repeat region used for strain genotyping. Schematic organization of Msp1a variable tandem repeat region where "A" and "B" represent individual tandem repeats such that the Msp1a genotype of the example depicted above would be A B B (A, B_2).

Worldwide geographical diversity of *A. marginale* was recently reviewed with records for over 350 *A. marginale* Msp1a genotypes (de la Fuente et al., 2007; Catanese et al., 2016; Hove et al., 2018). In China, 61 differing *A. marginale* genotypes were identified based on Msp1a genotyping (Yang et al., 2017). A total of 190 different genotypes of *A. marginale*, 188 of them being unique, were later identified in South Africa alone (Hove et al., 2018). Catanese et al. (2016) reviewed identification of 43 unique *A. marginale* genotypes in the United States. At least

11 of the 43 identified genotypes of *A. marginale* in the United States were identified in a single herd at Kansas State University (Palmer et al., 2004).

Genetically unique strains of *A. marginale* differ in transmissibility, disease characteristics, antigenicity, and susceptibility to antimicrobial treatments. One strain of *A. marginale* from Florida was not transmissible by *Dermacentor variabilis* ticks, an uncommon characteristic of *A. marginale*, whereas an Oklahoma strain was transmissible in the same experiment (de la Fuente et al., 2001b). Coetzee et al. (2006) assessed antimicrobial efficacy of three antimicrobial products against two *A. marginale* strains, Virginia and Oklahoma. They reported greater susceptibility of the Virginia strain to an oxytetracycline antimicrobial treatment regimen, compared with the Oklahoma strain (Coetzee et al., 2006). In an experiment designed to produce a viable vaccine candidate, Hammac et al. (2013) documented differing infection kinetics between an *A. centrale* strain and the St. Maries *A. marginale* strain, with *A. centrale* producing less severe clinical symptoms.

Transmission

Transmission of *A. marginale* from an infected animal to a susceptible animal can be achieved through ticks and blood-contaminated fomites; however, tick transmission is more efficient and effective due to the replication and propagation of the pathogen within the tick. Ticks ingest infected erythrocytes while feeding on an infected host animal. In the tick, *A. marginale* first colonizes the midgut epithelium and then disperses to the salivary glands. When the tick takes another bloodmeal, this signals *A. marginale* to continue replicating in the salivary glands and, while the tick is still feeding, *A. marginale* is dispensed via tick saliva into the new host (Kocan et al., 1992). Lohr et al. (2002) found that *A. marginale* levels in the salivary glands can reach 10^4 to 10^5 bacteria / salivary gland. About 20 species of tick were reported to be

competent vectors of *A. marginale* (Kocan et al., 2004), but the two most prominent tick species found to transmit *A. marginale* in the United States were *D. variabilis* (**Figure 1.5**) and *D. andersoni* (**Figure 1.6**; Scoles et al., 2005). Male ticks appear to be the primary vector for transmission as they intermittently feed, first to finish sexually maturing and then later after locating and mating with a female tick. If the male tick first feeds on an infected host and then finds a female tick on a naïve host, transmission of *A. marginale* occurs when the male tick attaches and feeds on the naïve host after mating (Stiller et al., 1989).

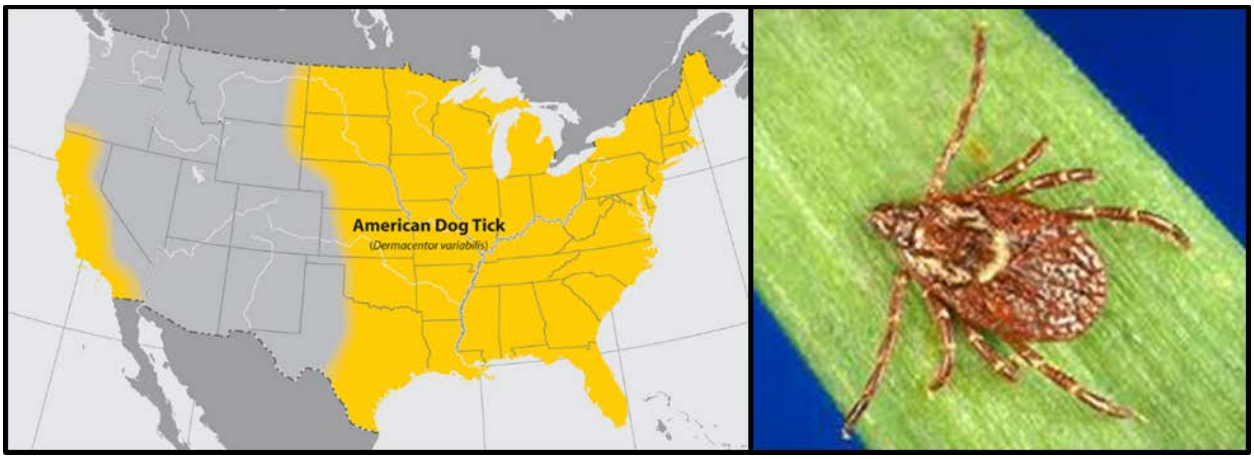

Figure 1.5 – Distribution and image of *D. variabilis* in the U.S. Images (distribution map-left; female *D. variabilis* tick-right) modified from CDC.

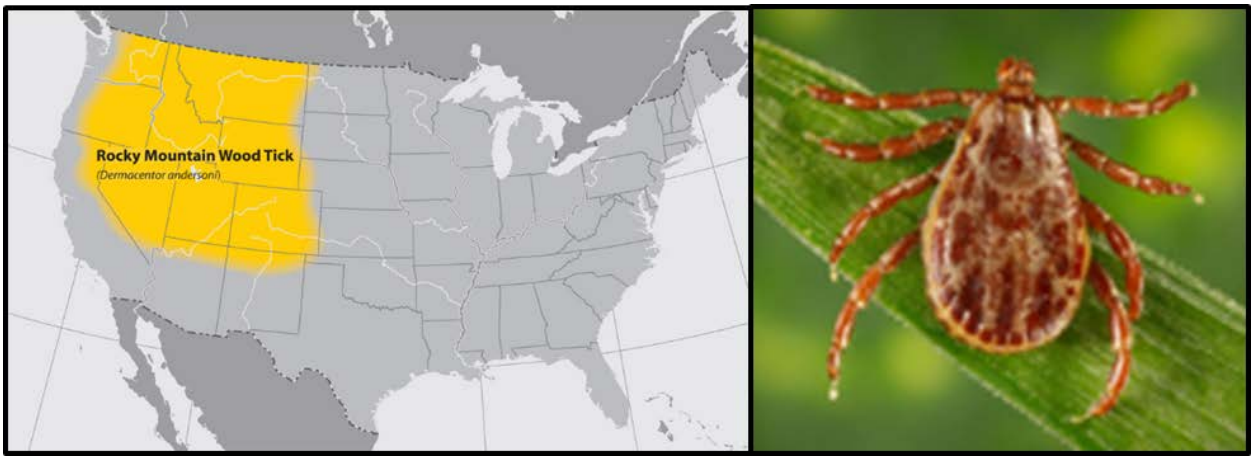

Figure 1.6 – Distribution and image of *D. andersoni* in the U.S. Images (distribution map-left; male *D. andersoni* tick-right) modified from CDC.

Kocan et al. (1990) confirmed transmission competence of *D. andersoni* for *A. marginale* when they acquisition-fed ticks on calves with ascending parasitemia for seven days. The ticks were removed, held for another seven days in a humidity chamber, then placed on naïve calves. The naïve calves developed clinical disease after an incubation period of about 25 days. Subsequently, *D. andersoni* and *D. variabilis* ticks were collected from an area in Canada where cattle were free of anaplasmosis and fed them on *A. marginale* infected animals. The ticks were then placed on naïve splenectomized calves that later developed *A. marginale* infection, confirming the competence of different geographic populations of *D. andersoni* and *D. variabilis* ticks to transmit *A. marginale* (Lankester et al., 2007). In a later experiment, acquisition-fed *D. andersoni* ticks on mammalian hosts known to be superinfected with two genetically distinct strains of *A. marginale* were found to be coinfected with both strains. These researchers reported subsequent successful transmission of both strains from tick to susceptible calves (Leverich et al., 2008).

Biological transmission through ticks is the primary mode of transmission of *A. marginale* as ticks can acquire and efficiently transmit the pathogen from animals in the acute or persistent phase of infection, but mechanical transfer of infected erythrocytes from an infected animal to a naïve animal has also been documented. Mechanical transmission can occur through blood contaminated fomites such as needles, dehorning equipment, castration equipment, ear taggers, tattoo equipment, or any other management practice that exposes naïve animals to erythrocytes from an infected animal. Mechanical transmission is less effective as there is no pathogen replication.

In an experimental setting, needle inoculation was shown to result in *A. marginale* transmission in 60% of animals stuck with a needle immediately after the needle had been used

on an acutely *A. marginale*-infected animal (Reinbold et al., 2010a). Though the percentage of transmission was high in that experiment, note should be taken that the needle was injected into the acutely infected animal each time before subsequent injection into naïve animals. This experiment demonstrated the likelihood of a naïve animal being infected if the animal being injected before is acutely *A. marginale*-infected and needles are not changed between animals. The Beef Quality Assurance (BQA) training program available to beef producers suggests that needles be changed every five animals, or immediately after damage to or dulling of needle. The previously mentioned experiment did not assess the likelihood of needle transmission of *A. marginale* from persistently infected animals to naïve animals, and as most *A. marginale*-infected animals exist in the persistent phase of infection, risk of needle transmission in cattle production is largely unknown.

Mechanical transmission of *A. marginale* may also be facilitated by certain biting/sucking fly species. In experimental settings, *Tabanus fuscicostatus* (horse fly) and *Stomoxys calcitrans* (stable fly), two of the more important fly species in bovine health, have been implicated in transmission of *A. marginale*. An early fly-transmission experiment demonstrated the competence of certain species of horse flies to transmit *A. marginale* (Howell et al., 1941); however, this experiment was conducted under a laboratory setting using an unnaturally large number of flies. In a more recent series of *A. marginale* fly-transmission experiments by Scoles et al. (2005 and 2008), *T. fuscicostatus* and *S. calcitrans* were unable to transmit *A. marginale*. These researchers compared the efficiencies of transmission between *D. andersoni* ticks and stable flies and reported that stable flies were not competent vectors of *A. marginale* at a parasitemia 300-fold greater than the appreciated infectious dose. Conversely, *D. andersoni* ticks were successfully able to acquire and transmit *A. marginale* between these same animals (Scoles

et al., 2005). Horse fly transmission competence was later compared to *D. andersoni* transmission competence by the same research group (Scoles et al., 2008). They report a failure of fly-borne mechanical transmission from an animal in acute disease while *D. andersoni* successfully transmitted from the same animal once it had reached a persistent phase of infection. Though these experiments noted failure of fly-borne *A. marginale* transmission, there may be differences in mechanical transmission competence among genetically differing strains of *A. marginale*. Recent experiments by Scoles et al. (2005 and 2008) did not take into account multiple days of consistent feeding on acutely infected animals that can take place in a natural setting. Despite conflicting results, it seems that if fly-transmission were to commonly occur, then the prevalence of *A. marginale* within infected herds would be much greater due to the number of flies that can be found on animals throughout the vector season.

Prevention, Treatment, and Control

Prevention and control of *A. marginale* transmission is complex and difficult. Control of vector transmission relies on limiting the risk of exposure to ticks, biting flies, and blood-contaminated processing equipment. Flies can be controlled through use of insecticides in pour-on and drip products, feed-through products, and coated ear tags. Maintenance and upkeep of cattle living and feeding areas, which are favorable habitats for flies, are another important practice for limiting exposure of animals to fly populations. Ticks, the biological vectors of *A. marginale*, are not as easily eradicated or controlled (Jongejan and Uilenberg, 2004). Use of acaricides and pesticides for tick and fly control are an option, but vector resistance to these products should be considered. Application of these products during vector season is labor-intensive and requires frequent retreatment to remain effective. This is not ideal for producers as

animals are not gathered for other management practices often enough to meet the demand of the product.

Implementation of management practices such as disinfection and cleaning of production instruments between animals can greatly reduce the risk of *A. marginale* transmission within a herd by lysing the red blood cell which effectively kills the pathogen. The work of Reinbold et al. (2010) highlighted the importance of implementing a single-use needle practice when administering immunizations, antimicrobials, or other medications for management purposes. Implementing bio-secure production practices greatly reduces the risk of mechanically transmitting *A. marginale* within a herd. These practices may be the easiest to implement and add minimal extra time and cost during processing.

Antimicrobial-based anaplasmosis treatment and control is permitted in the United States with the use of tetracyclines. **Table 1.2** contains an earlier list of antibiotic treatment regimen options for management of anaplasmosis in beef cattle (Richey and Palmer, 1990). This table contains recommendations that were historically accepted and utilized by veterinary professionals and producers. With increased concern of antimicrobial resistance (AMR), restrictions on antimicrobial usage (AMU), especially when delivered in feed products, have been put in place. Many of these treatment regimens are now extra-label and considered illegal. The currently approved antimicrobial treatment and control regimens are listed in **Table 1.3**. The United States Food and Drug Administration (FDA) has approved the use of oxytetracycline (OTC; FDA: 21 CFR 522.1660a) for treatment of anaplasmosis and chlortetracycline (CTC; FDA: 21 CFR 558.128) for control of active infection of anaplasmosis caused by *A. marginale*. Injectable oxytetracycline for the treatment of anaplasmosis is approved at 5-9 mg/lb of BW. For a single treatment of 9 mg/lb of BW, an animal could receive up to 100 mg of oxytetracycline in

a single injection. Per BQA suggestions of 10 mg per injection site, this animal would need to be needle-penetrated 10 different times in 10 different locations to fully-administer the treatment. This is not ideal as risk of injection site lesions increases. Use of CTC for control of active infection of anaplasmosis is approved at two dosages. It can be hand-fed at 0.5 mg/lb of BW daily or fed free-choice at 0.5-2.0 mg/lb of BW daily.

Antibiotic Treatment Regimens for Anaplasmosis Management			
Use & Drug	**Route**	**Dose (mg/lb. BW)**	**Fequency of Treatment**
Prevention			
Chlortetracycline*	Oral	0.10-0.25	Daily year-round
Chlortetracycline	Oral	0.50	Daily during vector season
Oxytetracycline (50-100 mg/ml)*	IV or IM	3.0-5.0	Every 28 days during vector season
Oxytetracycline (LA-200)*	IV	9.0	Every 28 days during vector season
Carrier Elimination			
Chlortetracycline*	Oral	0.5	Daily for 120 days
Chlortetracycline*	Oral	5.0	Daily for 60 days
Oxytetracycline (50-100 mg/ml)*	IV or IM	5.0	Daily for 10 days
Oxytetracycline (50-100 mg/ml)*	IV or IM	10.0	Daily for 5 days
Oxytetracycline (LA-200)*	IM	9.0	4 RX at 3 day intervals
Treatment of Sick Animals			
Oxytetracycline (50-100 mg/ml)	IM	5.0	Usually one treatment
Oxytetracycline (LA-200)	IM	9.0	One treatment
Temporary Protection During Outbreaks			
Oxytetracycline (50-100 mg/ml)*	IM	5.0	One treatment
Oxytetracycline (LA-200)*	IM	9.0	One treatment
Prolonged Protection During Outbreaks			
Oxytetracycline (50-100 mg/ml)*	IM	5.0	Every 28 days during vector season
Oxytetracycline (LA-200)*	IM	9.0	Every 28 days during vector season
Chlortetracycline	Oral	0.50	Daily for 60 days

Table 1.2 - Historic antibiotic treatment regimens. Adapted from "Anaplasmosis in beef cattle" by Ron Gill; Texas Agricultural Extension Service. (*regimens are now extra-label and considered illegal.)

Although CTC fed free-choice is easy to implement, it is a costly management practice for producers (see *Economic impact*). Implementation of CTC into a management program also requires a Veterinary Feed Directive (VFD; FDA 21 CFR 558.6) approved and written by a producer's partnering veterinarian. A veterinary-client-patient-relationship (VCPR) is necessary

when considering the aforementioned antimicrobial usages for treatment and control. Depending on operation type and production goals, implementation of a specific antimicrobial treatment plan may differ between producers. There are no specifically approved drugs for clearance of *A. marginale* infection at the time of this writing. Several experiments investigating whether OTC or CTC treatment regimens were capable of clearing *A. marginale* infection have been conducted with mixed results that may or may not be representative of contemporary circulating strains of the pathogen (Kuttler et al., 1980; Coetzee et al., 2005 and 2006; Reinbold et al., 2010b). Tetracycline antimicrobials have been used increasingly over the last 50 years and susceptibility differences to these antimicrobials between some *A. marginale* strains have been documented. Pathogen diversity and continuous tetracycline use in cattle have likely further increased selection pressure on *A. marginale* strains to become more resistant to these antimicrobials. Continuous selection pressure and AMR concerns point out the need for research on contemporary strains to evaluate their susceptibility to approved antimicrobial treatment regimens.

Antibiotic Treatment Regimens for Anaplasmosis Management			
Use & Drug	**Route**	**Dose (mg/lb. BW)**	**Fequency of Treatment**
Control of active *A. marginale* infection			
Chlortetracycline	Oral - Hand fed	0.5	Daily year-round
Chlortetracycline	Oral - Free Choice**	0.5-2.0	Daily year-round
Treatment of Sick Animals			
Oxytetracycline (50-100 mg/ml)	IM	5.0	Usually one treatment, no more than 4 consecutive days
Oxytetracycline (LA-200)	IM or SQ	9.0	One treatment
** - Must be manufactured in accordance with a Blue Bird Label			

Table 1.3 – Current FDA-approved antibiotic treatment regimens. List of current approved anaplasmosis-based antimicrobial treatment (21 CFR 522.1660a**)** and control (21 CFR 558.128) strategies (FDA).

Work has also been conducted to develop an effective vaccine for prevention of infection and acute disease; however, the genetic diversity of *A. marginale* presents a significant obstacle

to developing a broadly effective vaccine capable of protecting animals against multiple *A. marginale* strains. Vaccine work for *A. marginale* was first initiated by Sir Arnold Theiler in 1911 when he made the distinction between what he called "*A. marginale* proper" and "*A. marginale* (variety *centrale*)". He questioned whether *A. marginale* (variety *centrale*) would confer immunity against an '*A. marginale* proper' infection. Theiler experimented with this hypothesis and concluded that "the previous inoculation of *A. marginale* (variety *centrale*) gave sufficient immunity to protect the animals from a severe attack of anaplasmosis" (Theiler, 1911). The practice of vaccinating animals with a live vaccine is widely utilized in few countries across the world but is not currently approved for use in the United States (Hammac et al., 2013).

There are no fully USDA-licensed vaccines for anaplasmosis at the time of this writing; however, an experimentally licensed vaccine that used an inactivated strain of *A. marginale* (i.e., Mississippi) became available in 2007 that claims to provide protection against clinical bovine anaplasmosis (Luther, 2007). Efficacy data on this vaccine is not available. In lieu of a fully USDA-approved vaccine, beef producers are left to rely largely on the use of antimicrobials for treatment and control.

Economic Impact

Calculating a precise economic impact of tick-borne disease on livestock production worldwide has proven difficult, due to the complexity of the diverse parameters used in the calculations and the number of variables that influence tick-borne pathogen treatment and control. To understand the magnitude of the economic cost of ticks and tick-borne diseases on livestock, it was estimated that 80% of the world's cattle are infected or at risk of tick-borne pathogens and that the annual cost to the global cattle industry approaches $19 billion per year (Mack, 2016). Of the tick-borne pathogens that affect cattle, *A. marginale* is the most common

and widespread, infecting cattle on six continents. For anaplasmosis specifically, economic loss encompasses the cost of treatments, losses due to mortality and abortions, reduction in milk production, and reduced weight gain (Kocan et al., 2003).

In 1973, B.R. McCallon stated that the original estimated annual economic loss to the beef industry in the United States of $35 million “was no longer realistic”. He concluded by adding that he “would hazard a guess” that anaplasmosis was costing the U.S. beef industry about $100 million annually (McCallon, 1973). In 2003, Kocan et al., updated McCallon’s original cost estimate of bovine anaplasmosis in the United States beef industry to be greater than $300 million annually (Kocan et al., 2003). In Latin America, an $800 million annual loss to the cattle industry from bovine anaplasmosis was calculated (Lombardo, 1975). Efforts were made to calculate more accurate estimations for smaller samples sizes. In Texas, a survey estimated an individual clinical case of anaplasmosis to cost Texas cattle producers about $425. When this number was considered across the state for the span of a year, it was estimated the total annual economic cost to Texas cattle producers was approximately $8.96 million (Alderink and Dietrich, 1983). A similar survey conducted in California found that beef producers in that state suffered an annual loss in 1976 of about $5.25 million (Goodger et al., 1979). All these surveys considered the losses resulting from mortality, morbidity, and treatment costs.

Today, the most commonly used practice for control of anaplasmosis is a free-choice medicated mineral. Providing free-choice mineral to grazing cattle is inherently expensive and with the addition of CTC, it becomes more expensive. Medicated mineral for grazing cows costs an average $0.12 more per pound than non-medicated mineral. A grazing animal consumes approximately 0.25 lbs of mineral per day during an average summer grazing season of 150 days. Seasonally, this represents approximately $4.50/head in added costs (B. Greenwood, oral

communication, 2020). This is a relatively small added cost when considering total cow cost per year, but any additional costs added to production management decreases profit upon realization.

As antimicrobials are currently the primary method of treatment and control for active *A. marginale* infection, emphasis has been placed on producer education. A survey performed with Tennessee cattle producers investigated attitudes and knowledge of producers regarding antimicrobial use (AMU) and antimicrobial resistance (AMR) (Ekakoro et al., 2019). The survey asked four questions: What are the opinions on factors driving AMU? What are the opinions on alternatives to antimicrobials? What is the level of knowledge and perceptions regarding AMU and AMR? What are the preferred avenues for receiving information on prudent AMU? What they found was that profitability of the operation was the driving factor in their decision to use antimicrobials. Most producers also noted that additional training on infection prevention and implementation of farm-level biosecurity programs would reduce AMU in operations. About 80% of producers reported being at least moderately familiar with AMR and only about 25% believed there was an over-use of antimicrobials in beef production. The last question revealed that only 36% of producers preferred educational seminars as the route for receiving information prudent to AMU (Ekakoro et al., 2019).

Bovine anaplasmosis continues to be an important hemolytic disease in cattle across the world. The difficulty in recognizing clinical symptoms without laboratory diagnostics and the genetic diversity of *A. marginale* strains multiplies the complexities of trying to treat, control, and prevent disease. Taking into consideration risk factors such as geographical location, climate, cattle density, vector populations, and age of cattle, producers can implement management strategies specific to their operations. Use of injectable oxytetracycline and feed-grade chlortetracycline remain the primary method for treatment and control for active *A.*

marginale infection, as there are currently no fully FDA-approved vaccines. The historical and intensive use of tetracyclines in beef production, added to the complex genetic diversity of *A. marginale* strains, have likely placed selection pressures upon the pathogen. Experiments evaluating contemporary *A. marginale* strains and their antimicrobial susceptibility are important to determine the efficacies of the currently approved antimicrobial treatments. In the following chapter, we isolate and characterize two contemporary *A. marginale* strains from a Kansas beef cow herd. These strains will be used to investigate the antimicrobial susceptibility of contemporary *A. marginale* strains, and ultimately contribute to data-driven antimicrobial-based anaplasmosis management strategies.

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Chapter 2 - Isolation and characterization of two *Anaplasma marginale* isolates from a Kansas beef cattle herd

Abstract

Bovine anaplasmosis is a tick-borne bacterial disease caused by *Anaplasma marginale*, a global pathogen estimated to cost the U.S. cattle industry >$300 million per year. Treatment and control strategies rely heavily on tetracycline antimicrobials; however, susceptibility differences among *A. marginale* strains to this antimicrobial class have been documented. Changes in susceptibility to tetracycline antimicrobials have likely increased from selective pressures associated with the intensive tetracycline use in cattle production over the last half century. Therefore, use of characterized contemporary strains are essential when evaluating or re-evaluating antimicrobial-based anaplasmosis management strategies. The objective of this experiment was to locate and propagate two contemporary isolates of *A. marginale* actively circulating in Kansas cattle and characterize their virulence and infection kinetics in adult beef cows. Contemporary *A. marginale* strains were identified from naturally infected adult beef cows in the Kansas State University Cow-Calf herd. Blood samples containing these unique isolates (KS1 and KS2), were collected, propagated in splenectomized calves, and stabilates were prepared as challenge material for adult beef cows. Virulence of isolates was characterized by progression of anemia whereas infection kinetics of isolates was evaluated by progression of bacteremia. Isolate KS1-challenged animals reached peak clinical anaplasmosis by 35 days post-inoculation (dpi), ~3 days earlier than isolate KS2-challenged animals. KS1-challenged animals reached a peak bacteremia of 3.43×10^6 bacteria/mL of blood while isolate KS2-challenged animals reached a significantly greater level of 9.46×10^8 bacteria/mL of blood. Although KS1

and KS2 had different infection kinetic profiles, both isolates produced clinical anasplasmosis in challenged animals that required treatment intervention. Results from this experiment demonstrate the virulence and infection kinetic profiles of two *A. marginale* isolates from different Msp1a genotype families. Characterized contemporary *A. marginale* isolates, such as KS1 and KS2, will be useful for future experiments seeking to evaluate or re-evaluate antimicrobial-based anaplasmosis management strategies.

Introduction

Economic losses due to bovine anaplasmosis and implementation of anaplasmosis management strategies conservatively cost the U.S. cattle industry more than $300 million/year (McCallon 1973; Kocan et al., 2003). The causative agent of anaplasmosis is *Anaplasma marginale*, an obligate-intracellular rickettsial pathogen of several ruminant species. Found in cattle throughout six continents, *A. marginale* is likely the most prevalent tick-transmitted livestock disease worldwide (de la Fuente et al., 2007; Hove et al., 2018). In the United States, *Dermacentor* species ticks are the natural biological vectors of *A. marginale* (Kocan et al., 1992; Scoles et al., 2008). Mechanical transmission of *A. marginale* can also occur by blood-contaminated fomites such as needles, dehorning and castration equipment, and biting flies (Hawkins et al., 1982; Scoles et al., 2005; Reinbold et al., 2010).

Following transmission, *A. marginale* parasitizes bovine erythrocytes. After an incubation period of 6-70 days, the spleen rapidly destroys infected erythrocytes resulting in severe anemia, the hallmark of clinical disease (Kocan et al., 2003). Other symptoms of clinical disease can include fever, weight-loss, abortion, lethargy, and death in animals over 2 years of age (Kocan et al., 2003). Cattle that survive acute disease develop persistent infection and serve as reservoirs for subsequent transmission events. Antimicrobial treatment and control strategies

in the U.S. are limited to the use of injectable and feed-grade tetracyclines. The most common anaplasmosis management strategy used by cattle producers is chlortetracycline-medicated, free-choice mineral offered to animals during seasons in which vectors are active. Other management strategies include using new needles for each animal, fly and tick management, herd biosecurity, disinfecting processing tools between animals, and implementing a diseased-animal culling plan. At this time, an anaplasmosis vaccine that is fully licensed by the FDA is not available.

Hundreds of genetically differing strains of *A. marginale* have been identified around the world. Genetic diversity of *A. marginale* is most-frequently characterized by examining the arrangement of short, tandemly-arranged repeat sequences in the variable region of the Major Surface Protein 1a (Msp1a) gene (*msp1a*), a putative surface protein involved with adhesion of *A. marginale* to host cells (de la Fuente et al., 2001a; 2003a). A recent review of *A. marginale* diversity identified over 350 Msp1a genotypes from around the world. The diversification of Msp1a may be attributed to long-term, continuous selection pressure from both human and natural sources (de la Fuente et al., 2003b); however, in repeated passage experiments between ticks and cattle, this gene remained a stable genetic *A. marginale* strain marker.

Because *A. marginale* diversity is commonly defined by Msp1a genotype, the terms *genotype* (referring to Msp1a genotype) and *strain* are used synonymously in this manuscript. Strains can have important phenotypic differences that influence anaplasmosis disease dynamics and management strategies, such as differences in tick-transmissibility, antigenic characteristics, and antimicrobial susceptibility (Palmer et al., 2000; de la Fuente et al., 2001b; Coetzee et al., 2006a,b). For example, the *A. marginale* Oklahoma strain was determined to be less susceptible than the Virginia strain when these pathogen strains were treated with different antimicrobial products during both *in vitro* and *in vivo* experiments (Coetzee et al., 2006a,b).

Intensive reliance on tetracycline antimicrobials to manage anaplasmosis, as well as several other cattle diseases, over the past half century has put enormous selection pressure on pathogens to evolve antimicrobial-resistance mechanisms. In addition, of all cattle diseases with tetracycline-use indications, only for anaplasmosis is there no legal limit on treatment duration. Thus, cattle may be treated with low-dose chlortetracycline year-round, provided that a cattle producer has a valid Veterinary Feed Directive. Heavy reliance on the use of tetracycline antimicrobials, especially feed-grade chlortetracycline to control *active* anaplasmosis and the notable diversity of *A. marginale*, highlight the necessity of having characterized, contemporary *A. marginale* strains for research aimed at evaluating or re-evaluating antimicrobial-based anaplasmosis control options.

Anaplasmosis prevalence in the U.S. has been a subject of recent alarm to the nation's beef producers; this alarm has, in part, been fueled by anecdotal reports of anaplasmosis treatment and control failures. Some anaplasmosis management failures may be due to *A. marginale* becoming tolerant to current legal antimicrobial management options. To evaluate or re-evaluate antimicrobial-based anaplasmosis management strategies, well-characterized contemporary *A. marginale* strains are necessary. Therefore, the objective of our experiment was to locate, identify, and propagate, two contemporary *A. marginale* field isolates and characterize their virulence and infection kinetics in naïve adult beef cows. We hypothesized that these *A. marginale* isolates, each from different Msp1a genotype families, would exhibit unique virulence and infection kinetics as measured by PCV and PCR, respectively. The data from our experiment will serve as a baseline for future research aimed at evaluating antimicrobial-based, anaplasmosis-management strategies.

Materials and Methods

Animal use

The Kansas State University Institutional Animal Care and Use Committee and the Institutional Biosafety Committee reviewed and approved all animal handling and animal care practices used in our experiment. All animal procedures were conducted in accordance with the Guide for the Care and Use of Animals in Agricultural Research and Teaching (FASS, 2010).

Anaplasma marginale isolate identification and collection

Two adult beef cows (1-1018 and 1-0307) (**Table 2.1**) from the Kansas State University Cow-Calf Unit herd previously identified as naturally infected with different *A. marginale* Msp1a genotypes (Reif et al. unpublished) were selected as the source animals for *A. marginale* strain isolation. Cow 1-0307 was infected with Msp1a genotype B B, referred to hereafter as *A. marginale* isolate KS1. Cow 1-1018 was infected with Msp1a genotypes D D E, (K1) D D D E, and (K1) D D D D D E, referred to hereafter as *A. marginale* isolate KS2. Cows were restrained in squeeze chutes and ~60 mL of fresh blood was collected via jugular venipuncture into blood bags containing heparin for subsequent inoculation into splenectomized calves for *A. marginale* isolate propagation.

Propagation of *A. marginale* isolates

Two Holstein bull calves (**Table 2.1**), negative for *A. marginale* infection by PCR and cELISA, weighing approximately 57 kg, were splenectomized to propagate *A. marginale* field isolates KS1 and KS2. Isolates were propagated by inoculating splenectomized calves 15836 and 15855 with 60 mL of freshly collected blood from cows 01-0307 and 01-1018, respectively. Calves were monitored daily for signs of clinical anaplasmosis (see *Clinical virulence monitoring*). Once *A. marginale* bacteremia peaked, or when clinically indicated, calves were

anesthetized under a general plane of anesthesia and 3 to 5 L of whole blood was harvested from the jugular vein into blood bags containing heparin sulfate at a concentration of 5 U heparin/mL blood. Immediately following blood collection and while still under anesthesia, animals were humanely euthanized. Blood was transported immediately on ice and stored at 4°C for stabilate preparation.

Animal ID	Animal Type	Strain	Infection Source	Genotype Analysis	# Msp1a Genotypes
1-0307	Am strain source cow	KS1	natural	B B;	1
15836	splenectomized calf	KS1	blood (1-0307)	B B;	1
7379	challenged beef cow	KS1	stabilate (An15836)	B B;	1
7362	challenged beef cow	KS1	stabilate (An15836)	B B;	1
7096	challenged beef cow	KS1	stabilate (An15836)	B B;	1
7155	challenged beef cow	KS1	stabilate (An15836)	B B;	1
1-1018	Am strain source cow	KS2	natural	D D E; K1 D D D E; K1 D D D D D E	3
15855	splenectomized calf	KS2	blood (1-1018)	K1 D D D D D E; K1 D D D E; K1 D D E	3
7338	challenged beef cow	KS2	stabilate (An15855)	K1 D D D D D E; K1 D D D E; K1 D D E	3
7311	challenged beef cow	KS2	stabilate (An15855)	D D E; K1 D E; K1 D D D E; K1 E	4
503	challenged beef cow	KS2	stabilate (An15855)	K1 D D D D D E; K1 D D D E; K1 D E; K1 E	4
7151	challenged beef cow	KS2	stabilate (An15855)	K1 D D D D D E; K1 D D E; K1 D E; K1 E	4

Table 2.1 – List of animals used in Chapter 2. List of animals used in the experiment, their purpose, which *A. marginale* strain they were infected with, what the source of infection was, and the Msp1a genotypes contained within each strain. (Am: *Anaplasma marginale*).

Stabilate preparation

Whole blood collected for stabilate production was aliquoted and centrifuged for 30 min at 1,000 × G to separate serum and red blood cells. Serum and buffy coat were removed, and the

remaining red blood cells were washed three times with PBS and centrifuged for 30 min at 1,000 × G following each wash. After the final wash, the packed red blood cells were mixed with a stabilate buffer (31.2% DMSO in 1× PBS) in a 1:1 ratio. The mixture was then aliquoted into cryotubes and stored in liquid nitrogen.

Anaplasma marginale field-isolate challenge

Ten adult, spleen-intact beef cows between the ages of two and five years were purchased and confirmed *A. marginale* negative by qPCR and cELISA. Cows were assigned randomly to two groups (n=4/group) with the remaining two animals serving as sentinels (**Table 2.1**). Cows in Group 1 were inoculated via jugular with 4 mL of the KS1 isolate stabilate diluted in equal parts with serum previously collected from the respective cow. Cows in Group 2 were inoculated with the KS2 isolate as described above. Blood samples were collected via coccygeal venipuncture at least weekly to monitor *A. marginale* bacteremia. The two main study parameters tracked were bacteremia and packed cell volume (PCV). Bacteremia was monitored over time to evaluate the infection kinetics of each field isolate; PCV was monitored also over time as the primary disease measurement and used to determine onset of anemia, hematocrit nadir, and need for treatment intervention. During sampling, cows with an observed rectal temperature over 104°F or a PCV below 14% were treated with subcutaneously administered oxytetracycline (9 mg/lb body weight; Bio-Mycin, Boehringer Ingelheim, Duluth, GA).

Clinical virulence monitoring

Animals challenged with *A. marginale* were monitored for overt clinical signs of anaplasmosis (e.g. elevated rectal temperatures, elevated respiration rates, depression, and anorexia). Blood samples were collected at least twice weekly from each animal via coccygeal venipuncture into 4 mL vacuette tubes containing EDTA to monitor the development of

bacteremia (see *Pathogen detection*) and anemia. The development of anemia was monitored by evaluating the PCV of each animal after every blood sample collection. Briefly, whole blood samples were drawn into capillary tubes, centrifuged in a micro-hematocrit centrifuge for 5 min and the percent of packed red cells in the samples were recorded.

Quantitative PCR (qPCR)

The development of bacteremia in *A. marginale*-challenged animals was monitored by qPCR. To determine bacteremia, DNA was extracted from 100 µL of whole blood using the Quick-gDNA™ Miniprep Kit (Zymo Research, Irvine, CA) per manufacturer recommendations. Resulting DNA was eluted in 35 µL DNA Elution Buffer. Each qPCR reaction included: water, 1X SsoAdvance Universal SYBRsupermix (Bio-Rad Laboratories, Hercules, CA), 10µM each of primer (*Am* msp5F and *Am* msp5R, Hammac 2013), and 2 µL of DNA. Samples were analyzed using a CFX Connect™ Real-Time System (Bio-Rad Laboratories, Hercules, CA) with the following cycling conditions: 98°C for two min, 40 cycles of 98°C for 5 sec, 60°C for 5 sec, and 74°C for 15 sec; a final melt curve was included (65-95°C in 0.5°C increments). The CFX Maestro Software (Bio-Rad Laboratories, Hercules, CA) was used to display results.

Percent parasitized erythrocyte (PPE) analysis

Bacteremia was monitored by evaluating PPE. To estimate PPE, blood smears were prepared and stained using Hema 3™ stain (Fisherbrand, Pittsburgh, PA). At least two microscopic field views with a minimum of 100 red blood cells per field were evaluated to determine PPE. In each microscopic field view, the number of parasitized erythrocytes were divided by the total number of erythrocytes within the field view and multiplied by 100 to get the percentage of infected erythrocytes. An average percent of infected erythrocytes was calculated between the two microscopic field views for each sample.

Competitive enzyme-linked immunosorbent assay (cELISA)

Serum samples from *A. marginale*-challenged animals were evaluated by cELISA for development of an *A. marginale*-specific immune response. Serum samples were submitted to the Iowa State University Veterinary Diagnostic Laboratory for evaluation using a commercial cELISA that detected antibodies specific to *A. marginale* Msp5 (VMRD Inc., Pullman, WA). The assay was conducted per manufacturer instructions and samples with a percent inhibition value greater than 30% were considered seropositive.

Anaplasma marginale Msp1a genotype analysis

To confirm the intended *A. marginale* strain/isolate was the source of disease in challenged cows, Msp1a genotyping was performed on blood from each challenged cow. Briefly, the amino terminus of the Msp1a gene was amplified as previously described (Palmer et al., 2004). The resulting PCR product was cloned into a pCR™2.1-TOPO cloning vector and transformed into DH5α™-TOP10 *E. coli*, according to manufacturer instructions (Invitrogen, Carlsbad, CA). A minimum of 10 resultant clones were screened for presence of the insert and a minimum of five positive clones were submitted for rolling cycle amplification (RCA) and 5' and 3' sequencing using T3 or T7 as sequencing primers (MC Lab, South San Francisco, CA).

Statistical analysis

Statistical analyses were performed using GraphPad Prism v7.01 (San Diego, CA). Two-way analysis of variance (ANOVA) with Sidak correction for multiple comparisons was used to compare PCV, bacteremia, and PPE between KS1- and KS2-challenged animals. Linear regression was used to compare ascending bacteremia slopes between KS1 and KS2 inoculated animals. Unpaired, two-tailed t-tests with Welch's correction were used to compare greatest hematocrit change, time to PCV nadir, peak bacteremia, and time to peak bacteremia between

KS1- and KS2-challenged animals. When protected by a significant *F*-test (i.e., < 0.05), differences were considered significant when $P < 0.05$.

Sequences were analyzed both manually and using RepeatAnalyzer (Catanese et al., 2016) to identify Msp1a genotypes. Only sequences containing the full tandem repeat region were included in the analysis.

Results

Propagation of *A. marginale* isolates in splenectomized calves

Isolates KS1 and KS2 were inoculated into two splenectomized calves (**Table 2.1**) to propagate the strains in order to characterize their infection kinetics and virulence in adult beef cows. During isolate propagation, both calves maintained rectal temperatures above 102.6°F; calf 15836 spiked to 105.4° F on the day of euthanasia. Immediately prior to inoculation, starting PCV values for splenectomized calf 15836 and calf 15855 were 39% and 37%, respectively. Calf 15836 was inoculated with KS1 and was euthanized 23 days post-infection (dpi) with a final PCV value of 18%, 25.8% PPE, and a bacteremia of 1.05×10^7 bacteria/mL (**Table 2.2**). Calf 15855 was inoculated with KS2 and was euthanized 39 dpi with a final PCV value of 18%, 18.3% PPE, and a bacteremia of 2.96×10^9 bacteria/mL (Table 2.2). Although the infection process was slower for calf 15855, bacteremia was first detected by qPCR in both calves at 8 dpi.

KS1 and KS2 Strain Propagation				
Animal ID	**Strain**	**Final PCV (%)**	**Final PPE (%)**	**Final bacteremia**
15836	KS1	16	25.8	1.05×10^7
15855	KS2	18	18.3	2.96×10^9

Table 2.2 – Strain propagation final values. Characteristics of infected blood used to prepare strain stabilates to be used as challenge material.

Assessment of *A. marginale* KS1 and KS2 isolate virulence in adult beef cows

Adult beef cows were inoculated with prepared stabilates of *A. marginale* KS1 and KS2 isolates (**Table 2.1**). Virulence of *A. marginale* KS1 and KS2 isolates was assessed by monitoring PCV and evaluating: i) time to initiation of anemia, ii) PCV nadir; iii) time to PCV nadir; and, iv) requirement for treatment intervention. Other virulence-related clinical parameters which were measured but not statistically analyzed due to sample size and high individual animal variability were rectal temperature, overt signs of respiratory distress, depression, and anorexia. Initial onset of anemia began 29 dpi and 33 dpi for cows challenged with KS1 and KS2, respectively. Overall mean hematocrit reduction (based on PCV data; **Figure 2.1**, **Table 2.3**) was similar for KS1-challenged (mean hematocrit reduction = 49.7%, SD ±4.9%) and KS2-challenged (mean hematocrit reduction = 50.6%, SD ±10.8%) animals; animals in both groups experienced an average of 50% hematocrit reduction. Time to PCV nadir was significantly longer for KS2-challenged animals (mean = 37.5 dpi, SD ±1.0 dpi), compared with KS1-challenged (mean = 35.3 dpi, SD ±1.3 dpi) animals. Three of four animals challenged with KS1 and KS2 reached experiment-mandated parameters for oxytetracycline-treatment intervention. The ultimate virulence endpoint – death – was not evaluated in this study and animals were rescue-treated with injectable oxytetracycline when their PCV reached 18% or their temperature was >104.5°F. Rectal temperatures remained relatively normal throughout the experiment. Exceptions were cows 7379, 7338, and 503, that had one-time rectal temperatures over 103.0 F. No overt signs of respiratory distress, depression or anorexia were observed for any cow, even during peak of acute disease.

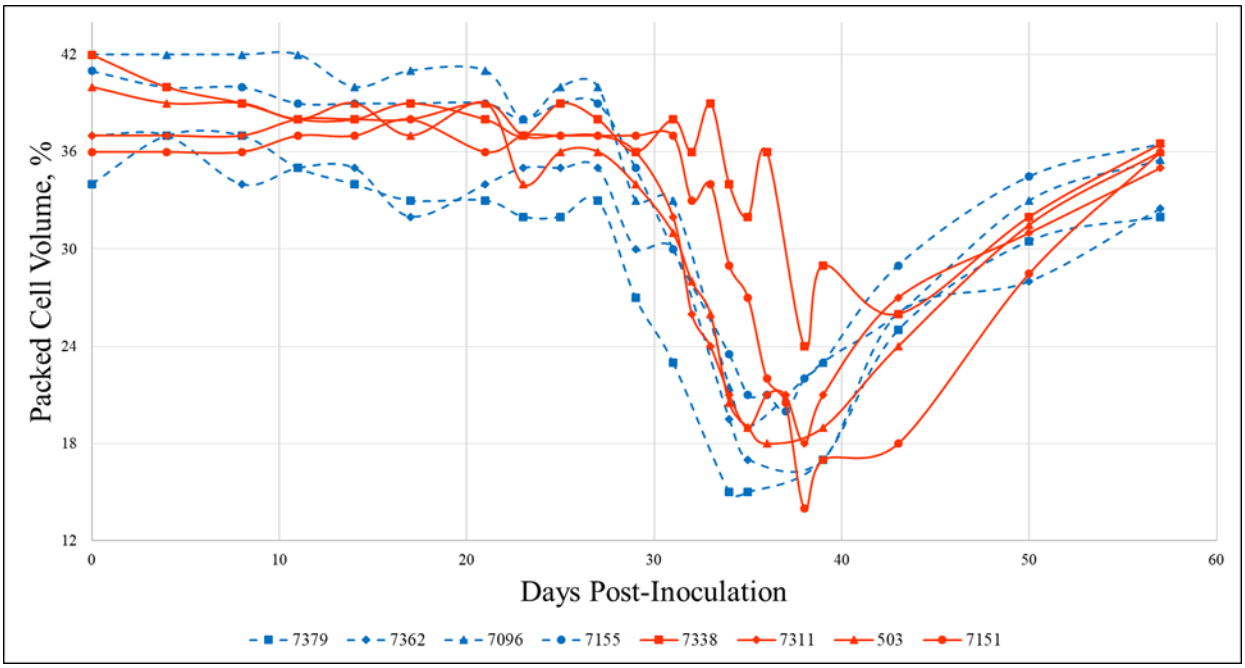

Figure 2.1 – PCV of *A. marginale*-challenged animals. Changes in packed cell volume (PCV) over time where KS1-challenged animals are represented by dashed lines and KS2-challenged animals are represented by solid lines.

Assessment of *A. marginale* KS1 and KS2 isolate virulence					
Animal ID	**Isolate**	**Time to initiation of anemia (dpi)**	**PCV nadir (%)**	**Time to PCV nadir (dpi)**	**Treatment Intervention?**
7379	KS1	27	15	34	Yes
7362	KS1	27	17	35	Yes
7096	KS1	27	19	35	Yes
7155	KS1	27	23	39	Yes*
7338	KS2	36	24	38	Yes*
7311	KS2	29	18	38	Yes
503	KS2	27	18	36	Yes
7151	KS2	31	14	38	Yes

Table 2.3 – *Anaplasma marginale* virulence. Isolate virulence assessed using individual animal packed cell volume (PCV) values. (*treated with OTC despite not reaching experiment-mandated treatment requirements.)

Assessment of *A. marginale* KS1 and KS2 isolate infection kinetics in adult beef cows

Infection kinetics of KS1 and KS2 *A. marginale* isolates were assessed (**Table 2.4**; **Figure 2.2**) by evaluating: i) dpi to detectable infection by qPCR, ii) rate of ascending bacteremia; iii) peak bacteremia; and, iv) time to peak bacteremia. The percentages of parasitized

erythrocytes were evaluated as an additional measure of *A. marginale* infection but was not statistically assessed. In KS1- and KS2-challenged animals, all cows were qPCR positive for *A. marginale* by 14 dpi. From the earliest point of qPCR detection, KS2 maintained approximately 1-log greater bacterial levels in cows compared with KS1. The slope of ascending bacteremia was significantly greater for KS2 (1.27×10^7 $\pm4.23\times10^6$) compared to KS1 (7.81×10^4 $\pm2.29\times10^4$; F=5.179, DFn = 1, DFd = 25, *P* 0.0317). Bacteremia levels were greater in KS2-challenged animals compared to KS1-challenged animals. Although the time to reach peak was longer for KS2-challenged animals (mean = 35, SD ±1.5 dpi; median = 36 dpi) compared with KS1-challenged animals (mean = 29, SD ±1.9 dpi; median = 28 dpi), KS2-challenged animals reached significantly greater peak bacteremia (mean = 9.46×10^8, SD ± 7.05×10^8 bacteria/mL blood) than KS1-challenged animals (mean = 3.43×10^6, SD ± 1.91×10^6 bacteria/mL blood).

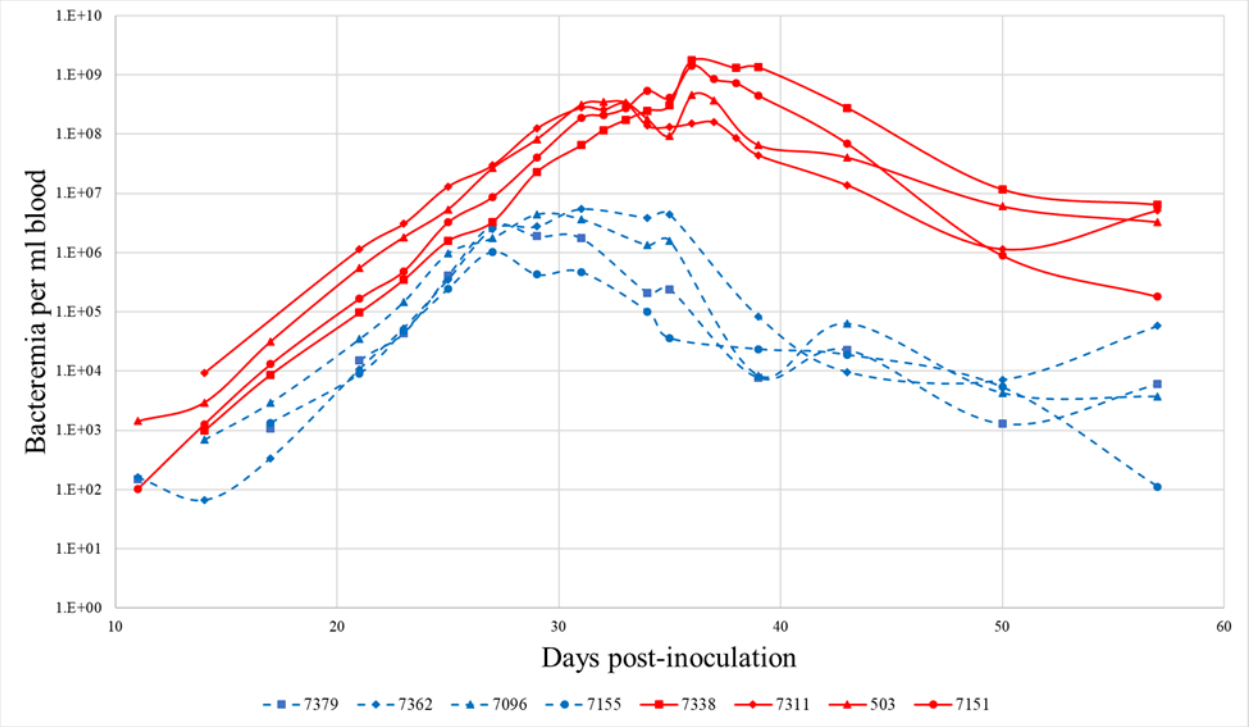

Figure 2.2 – *Anaplasma marginale*-challenged cow bacteremia. Change in bacteremia over time calculated using polymerase chain reaction (PCR) where KS1-challenged animals are represented by dashed lines and KS2-challenged animals are represented by solid lines.

Assessment of *A. marginale* KS1 and KS2 isolate infection kinetics				
Animal	**Time to detect infection**	**Rate of ascending bacteremia**	**Peak bacteremia**	**Time to peak bacteremia**
7379	14 dpi	$6.70 \times 10^4 \pm 2.29 \times 10^4$	2.89×10^6	27 dpi
7362	14 dpi	$1.15 \times 10^5 \pm 3.95 \times 10^4$	5.41×10^6	31 dpi
7096	14 dpi	$1.09 \times 10^5 \pm 3.39 \times 10^4$	4.41×10^6	29 dpi
7155	14 dpi	$2.03 \times 10^4 \pm 7.50 \times 10^3$	1.03×10^6	27 dpi
KS1 avg	***14 dpi***	$\boldsymbol{7.81 \times 10^4 \pm 2.29 \times 10^4}$	$\boldsymbol{3.43 \times 10^6}$	***29 dpi***
7338	14 dpi	$1.62 \times 10^7 \pm 8.44 \times 10^6$	1.74×10^9	36 dpi
7311	14 dpi	$7.01 \times 10^6 \pm 1.78 \times 10^6$	3.30×10^8	33 dpi
503	14 dpi	$9.42 \times 10^6 \pm 2.53 \times 10^6$	4.53×10^8	36 dpi
7151	14 dpi	$1.80 \times 10^7 \pm 6.73 \times 10^6$	1.44×10^9	36 dpi
KS2 avg	***14 dpi***	$\boldsymbol{1.27 \times 10^7 \pm 4.23 \times 10^6}$	$\boldsymbol{9.46 \times 10^8}$	***35 dpi***

Table 2.4 – Isolate KS1 and KS2 bacteremia. Infection kinetics as evaluated by individual animal and isolate average bacteremia values.

The secondary method used to monitor infection kinetics in *A. marginale*-challenged animals was PPE (**Table 2.5**). PPE values greater than 1% were observed for all KS1-challenged animals by 27 dpi and PPE values greater than 1% were observed for all KS2-challenged animals by 31 dpi. Peak PPE values were similar between KS1-challenged (mean = 23.5%, SD ±12.6%, range = 13.1 to 40.3%) and KS2-challenged (mean = 28.6%, SD ±13.2%, range = 15.7 to 43.4%) animals. Time to reach peak PPE value was also similar between KS1- (mean = 34.5, SD ±0.6 dpi) and KS2-challenged (mean = 34.8, SD ±1.5 dpi) animals.

Assessment of *A. marginale* infection kinetics (PPE)			
Animal	**Time to detect >1% PPE**	**Peak PPE**	**Time to peak PPE**
7379	27 dpi	14.6	34 dpi
7362	27 dpi	40.3	35 dpi
7096	27 dpi	25.9	35 dpi
7155	27 dpi	13.1	34 dpi
KS1 avg	***27 dpi***	***23.5***	***34.5 dpi***
7338	32 dpi	35.8	36 dpi
7311	29 dpi	15.7	33 dpi
503	31 dpi	19.4	34 dpi
7151	31 dpi	43.4	36 dpi
KS2 avg	***31 dpi***	***28.6***	***34.8 dpi***

Table 2.5 – Isolate KS1 and KS2 PPE. Infection kinetics as evaluated by individual animal and isolate average percent parasitized erythrocyte (PPE) values.

Genotype analysis of *A. marginale* isolates

To confirm that cows challenged with KS1 and KS2 stabilates maintained the same Msp1a genotypes identified in the original donor animals (cow 1-0307 and cow 1-1018, respectively), the variable region of Msp1a was amplified, cloned, and sequence from each KS1- and KS2-challenged cow. The Msp1a genotypes identified from KS1- and KS2-challenged cows were consistent with the genotypes identified from the original donor cow and the splenectomized calf used to prepare the respective stabilates (**Table 2.1**). Animals challenged with KS1 contained a single Msp1a genotype (B B), whereas KS2-challenged animals contained multiple Msp1a genotypes in the '(K1) D^x E' family.

Discussion

Tetracycline antimicrobials have been used intensively in cattle production for the past half century to increase growth and as therapeutics. The FDA restricted use of medically important antimicrobials, such as tetracyclines, to therapeutic indications only in 2017. In addition, a Veterinary Feed Directive was required for any in-feed administration of a legally indicated, medically important antimicrobial (FDA: 21 CFR 558.6).

Anaplasmosis is the major tick-transmitted disease of cattle in the U.S., endemic to all major cattle producing states. Antimicrobials are the primary means used by cattle producers to manage anaplasmosis in infected herds. Currently, only tetracyclines have legal use indications for anaplasmosis management. Reports on increasing anaplasmosis incidence in major cattle-producing states and anecdotal reports of management difficulties have increased awareness of this economically costly cattle disease.

Multiple strains of *A. marginale* are known to exist in the U.S. and previous reports have demonstrated that antimicrobial susceptibility can differ between strains. Whether intensive

tetracycline use in cattle production is actively selecting for more antimicrobial-tolerant *A. marginale* strains or actively driving diversification of *A. marginale* toward strains less susceptible to tetracycline antimicrobials is unclear. To address questions on the evolution of *A. marginale* antimicrobial susceptibility requires well-characterized contemporary *A. marginale* strains. Historic or laboratory *A. marginale* strains, not under similar sustained selective pressures as contemporary field-strains, may be of limited value in reflecting the susceptibility of contemporary *A. marginale* strains.

In our experiment, two *A. marginale* field-isolates were isolated, propagated, and their virulence and infection kinetics characterized in adult beef cows. Virulence was assessed by monitoring the progress of anemia and temperature and by monitoring other overt clinical signs of disease (e.g., tachypnea, depression, anorexia). Both isolates caused a similar reduction in hematocrit but time to PCV nadir was significantly longer for KS2-challenged animals. In each group, three of the four challenged-animals reached experiment-mandated parameters requiring treatment intervention. Infection kinetics of the isolates were assessed by evaluating bacteremia and the propagation tendencies. Both isolates developed detectable bacteremia by 14 dpi and KS2 maintained at least ~1 log higher bacteremia than KS1 from that point forward. Though KS2-challenged animals took longer to reach peak bacteremia, they reached a significantly greater bacterial load. The percentage of parasitized erythrocytes was measured as a secondary method of disease assessment but was not statistically analyzed. PPE values followed a similar trend as PCV values with PPE values for KS2-challenged animals above 1% being detected about 4 days later than KS1-challenged animals. Peak PPE values and time to reach peak PPE were numerically similar between animal groups.

The virulence of KS1 and KS2 *A. marginale* isolates was described by assessing the severity of clinical disease in challenged animals. As anemia is the hallmark of clinical anaplasmosis, virulence was described by monitoring the PCV of challenged animals for the onset of anemia. Other clinical symptoms occurred subsequent to the onset of anemia, so evaluating PCV nadir and time to PCV nadir were valuable also for documenting clinical disease. Rectal temperature, tachypnea, depression, and anorexia, all symptoms of clinical anaplasmosis, were monitored as virulence-related clinical parameters. These are symptoms that would be easily recognized by producers; however, none of the KS1- or KS2-challenged cows displayed overt outward signs of clinical disease. Lack of easily observable signs of clinical anaplasmosis is one of the major barriers to identification of animals experiencing acute anaplasmosis in time to intervene before death.

Incubation periods of 29 and 33 dpi for isolates KS1 and KS2, respectively, were similar to those of other *A. marginale* strains, with the average incubation period being ~28 days (Kocan et al., 2003). Mean hematocrit reductions of 49.7 and 50.6% for KS1- and KS2-challenged animals, respectively, were also similar to previously published data which indicated that animals experienced a 40 to 50% reduction in red blood cells (Jones and Brock, 1966). As our experiment was conducted, virulence intensity was not found to differ between KS1 and KS2 isolates, but it was unknown if either isolate would have been more likely to result in death as animals that reached the experiment-defined PCV intervention level were rescue-treated.

Infection kinetics focus more on the characteristics of pathogen propagation than on animal symptoms. By utilizing qPCR and stained blood smears we were able to quantify and monitor the pathogen itself. Peak PPE calculations for KS1- and KS2-challenged animals, 23.5 and 28.6% respectively, fell into the previously documented range of 10 to 90% parasitized

erythrocytes (Aubry and Geale, 2011). Although PPE determination was based upon visual recognition of the pathogen in red blood cells, this diagnostic was not the most accurate or effective in estimating bacteremia because the number of *A. marginale* in a single red blood cell can range as the colony expands (Kocan et al., 2003). Another limitation of monitoring anaplasmosis by blood smear is that infected red blood cells are only detectable for a few days around the peak of clinical disease. Molecular methods of detection such as qPCR allow for more accurate quantification of bacteremia and better understanding of infection kinetics. Animals challenged with KS1 and KS2 reached distinctly different peaks in bacteremia, but both fell within the ranges reported by other researchers (Palmer et al., 1999).

Differences in antimicrobial susceptibility have been documented between historical strains that have not been exposed to the same selection pressures as contemporary strains (Coetzee et al., 2006a,b). Considering the intensive and frequent exposure of some *A. marginale* contemporary strains to tetracyclines, it seems possible that these strains could have become resistant over time. With the potential that strains are becoming more resistant to tetracycline antimicrobials, it is important that *A. marginale* contemporary strains are considered when evaluating or re-evaluating antimicrobial efficacy.

In summary, we isolated and characterized the virulence and infection kinetics of two *A. marginale* isolates from different Msp1a genotype families that had not been previously characterized. Observed PCV values indicated that virulence intensity did not differ between isolates; however, the time to reach PCV nadir differed between isolates. Infection kinetics were observed to be different among isolates as KS2-challenged animals developed bacteremia levels 2-log greater than those developed by KS1-challenged animals. These results confirmed the understanding that strains with different Msp1a genotypes can differ in important disease

phenotypes related to development of disease and infection kinetics. Antimicrobial efficacy experiments against strains of *A. marginale* have historically been performed using laboratory strains that have not been under the same selection pressures as contemporary strains. To reassess other important *A. marginale* strain phenotypes, such as antimicrobial susceptibility, it is important to use well-characterized contemporary strains to see how these important phenotypes may have changed following extensive selection pressure. This experiment serves to identify and characterize contemporary strains to be used in future research designed to evaluate or re-evaluate the efficacy of approved antimicrobial treatment regimens.

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