APPLICATION OF REAL-TIME QUANTITATIVE RT-PCR FOR IMPROVING THE

DIAGNOSIS, TREATMENT, AND CONTROL OF BOVINE ANAPLASMOSIS

by

JAMES BRANDON REINBOLD

B.S., University of Missouri-Columbia, 1999
D.V.M., University of Missouri-Columbia, 2003

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine and Pathobiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2009

Abstract

The Office International des Epizooties (OIE) Animal Health Code categorizes bovine anaplasmosis as a notifiable disease. Many species of the genus *Anaplasma* cause anaplasmosis. Co-infections with two or more *Anaplasma* spp. occur in cattle. A competitive ELISA is regarded as a reliable test for identifying *A. marginale*-infected cattle. However, cross-reactivity among related *Anaplasma* spp. has been reported when using cELISA. In the absence of effective treatment strategies and vaccine availability, anaplasmosis control strategies are primarily focused on disease identification and prevention and development of chemosterilization strategies. Four studies were completed to improve the diagnosis, treatment, and control of bovine anaplasmosis. In the first study, a real-time qRT-PCR was developed to detect as few as 100 copies of 16S rRNA of both *A. marginale* and *A. phagocytophilum* in the same reaction. This detection limit was equitable to the minimum infective unit of one *A. marginale* bacterium. In the second study, qRT-PCR results determined needle-free injection was superior to needle injection for controlling iatrogenic transmission of *A. marginale* in cattle. The qRT-PCR demonstrated 100% sensitivity by 21 days post-infection and 21 days prior to 100% sensitivity with cELISA. The third study determined the pharmacokinetic parameters of chlortetracycline in group fed, ruminating Holstein steers: volume of distribution (40.9 L∕kg); rate constant (0.0478 h^{-1}); dose-normalized area under the curve (0.29 h·µg∕L); clearance (1.8 L∕kg∕h); elimination half-life (16.2 h); maximum concentration/dose (4.5 ng∕mL); and time of maximum concentration (23.3 h). Dose linearity was confirmed for oral chlortetracycline dosages of 4.4, 11, and 22 mg/kg/day. The final study established an in *vivo* pharmacokinetic-pharmacodynamic relationship between chlortetracycline and anaplasmosis carrier clearance in bovine plasma (85.3 ng/mL). The qRT-PCR confirmed chemosterilization of all oral chlortetracycline-treated cattle within 49 days of treatment. Furthermore, qRT-PCR was an effective alternative to the subinoculation of splenectomized cattle for accurate and precise disease classification. The diagnosis, treatment, and control of anaplasmosis were enhanced through the application of qRT-PCR. Further studies are necessary for determining the mechanism of action between chlortetracycline binding to the 30S ribosome of *A. marginale* and carrier clearance.

APPLICATION OF REAL-TIME QUANTITATIVE RT-PCR FOR IMPROVING THE

DIAGNOSIS, TREATMENT, AND CONTROL OF BOVINE ANAPLASMOSIS

by

JAMES BRANDON REINBOLD

B.S., University of Missouri-Columbia, 1999
D.V.M., University of Missouri-Columbia, 2003

A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine and Pathobiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2009

Approved by:

Major Professor
Johann F. Coetzee BVSc, Cert CHP, PhD, DACVCP

Copyright

JAMES BRANDON REINBOLD

2009

Abstract

The Office International des Epizooties (OIE) Animal Health Code categorizes bovine anaplasmosis as a notifiable disease. Many species of the genus *Anaplasma* cause anaplasmosis. Co-infections with two or more *Anaplasma* spp. occur in cattle. A competitive ELISA is regarded as a reliable test for identifying *A. marginale*-infected cattle. However, cross-reactivity among related *Anaplasma* spp. has been reported when using cELISA. In the absence of effective treatment strategies and vaccine availability, anaplasmosis control strategies are primarily focused on disease identification and prevention and development of chemosterilization strategies. Four studies were completed to improve the diagnosis, treatment, and control of bovine anaplasmosis. In the first study, a real-time qRT-PCR was developed to detect as few as 100 copies of 16S rRNA of both *A. marginale* and *A. phagocytophilum* in the same reaction. This detection limit was equitable to the minimum infective unit of one *A. marginale* bacterium. In the second study, qRT-PCR results determined needle-free injection was superior to needle injection for controlling iatrogenic transmission of *A. marginale* in cattle. The qRT-PCR demonstrated 100% sensitivity by 21 days post-infection and 21 days prior to 100% sensitivity with cELISA. The third study determined the pharmacokinetic parameters of chlortetracycline in group fed, ruminating Holstein steers: volume of distribution (40.9 L∕kg); rate constant ($0.0478\ h^{-1}$); dose-normalized area under the curve (0.29 h·µg∕L); clearance (1.8 L∕kg∕h); elimination half-life (16.2 h); maximum concentration/dose (4.5 ng∕mL); and time of maximum concentration (23.3 h). Dose linearity was confirmed for oral chlortetracycline dosages of 4.4, 11, and 22 mg/kg/day. The final study established an in *vivo* pharmacokinetic-pharmacodynamic relationship between chlortetracycline and anaplasmosis carrier clearance in bovine plasma (85.3 ng/mL). The qRT-PCR confirmed chemosterilization of all oral chlortetracycline-treated cattle within 49 days of treatment. Furthermore, qRT-PCR was an effective alternative to the subinoculation of splenectomized cattle for accurate and precise disease classification. The diagnosis, treatment, and control of anaplasmosis were enhanced through the application of qRT-PCR. Further studies are necessary for determining the mechanism of action between chlortetracycline binding to the 30S ribosome of *A. marginale* and carrier clearance.

Table of Contents

List of Figures

List of Tables

Acknowledgements

Dedication

     I would like to dedicate this dissertation to my parents, Gary and Karen Reinbold; my sister, Kerri, and her children, Krissa, Chas, and Andrea; my wife, Misty Reinbold; my daughter, Tilly; and my veterinary mentors in private practice, Drs. Brian Darrow, Mel Falk, and Dennis Hood. Without the support of these special people in my life and the strength derived from my Saviour, Jesus Christ, none of this would have been possible. Moreover, this achievement would have been meaningless without them to share my success.

Preface

Anaplasmosis is a complex and challenging disease for stakeholders in the cattle industry, foreign policy and research communities alike. Anaplasmosis, caused by *Anaplasma marginale*, is one of the most prevalent tick-transmitted, rickettsial diseases of cattle worldwide (Dumler et al., 2001; Kocan et al., 2003; Uilenberg, 1995). Clinical disease symptoms in adult cattle acutely-infected with *A. marginale* include, but are not limited to, anemia, fever, icterus, lethargy, and death (Merck, 2008; Radostits et al., 2000). In 2003, anaplasmosis was estimated to cost the United States cattle industry over $300 million per year (Kocan et al., 2003). Cattle infected with anaplasmosis following natural infection and vaccination with live *Anaplasma* spp. remain lifelong carriers. Moreover, abortion, high mortality, reduced milk production, extensive treatment costs, and weight loss are key economic considerations of this disease.

Many of the current methods used for the diagnosis, treatment, eradication, and control of bovine anaplasmosis present many problems to the cattle industry. Historically, vaccination has been used to modulate disease severity. In some countries, naïve cattle are inoculated intravenously with bovine blood infected with *Anaplasma centrale* for reducing disease morbidity prior to infection with *A. marginale*. The appropriately-timed application of insecticides is recommended for reducing biological transmission by haematophagous arthropods (De Wall, 2000; Peter et al., 2005; Rodriguez-Vivas et al., 2004). Due to the lack of significant success with treatment strategies, vaccine availability, and problematic vector control, anaplasmosis control strategies should primarily concentrate on established methods for disease prevention.

The findings of this PhD program of study have the potential to significantly impact local, interstate, and international movement of cattle between endemic and non-endemic regions. A highly sensitive and specific quantitative RT-PCR (qRT-PCR) assay was developed for the detection of *A. marginale* and *A. phagocytophilum* in the same reaction. A needle-free injection system was validated by the qRT-PCR for the control of iatrogenic disease transmission. The application of the qRT-PCR assay was instrumental in characterizing the time of chemosterilization as well as establishing the *in vivo* dose-response relationship between plasma drug concentration and chemosterilization.

The following literature review is subdivided into eight sections. The primary focus of this PhD program has been centered on anaplasmosis caused by *A. marginale*. Therefore, the focus of this literature review will be directed towards the literature as it applies to *A. marginale*. Information may be included for both *A. centrale* and *A. phagocytophilum* when relevant to the discussion. Furthermore, certain topics will be covered without an in depth review of the literature for the sake of increasing the breadth of knowledge of both the author and the reader.

CHAPTER 1 - Literature Review

Etiology

Bovine anaplasmosis, caused by *Anaplasma marginale,* was first described by Sir Arnold Theiler in South Africa in 1909 (Kocan et al., 2000). *Anaplasma marginale* was detected along the intracellular margin of infected red blood cells by light microscopic examination of stained blood smears. However, *A. marginale* is not the exclusive species of the genus *Anaplasma* that is an etiologic agent of this hemoparasitic disease in cattle (Liu et al., 2005). The causative agents of bovine anaplasmosis (family *Anaplasmataceae)* are grouped by their distinction as obligate intracellular bacteria that replicate within a mammalian eukaryotic host cell membrane-derived vacuole (Dumler et al., 2001; Rikihisa, 1991). This categorization includes the species *A. bovis, A. centrale, A. marginale,* and *A. phagocytophilum.* Furthermore, anaplasmosis has been classified as a notifiable disease by the Office International des Epizooties (OIE) in the Terrestrial Animal Health Code 2.4.1 (OIE, 2009).

Anaplasma marginale receives the most attention from the cattle industry due to its socioeconomic impact and influence on international trade restrictions worldwide. However, the significance of *A. marginale* as an economically significant disease is frequently underestimated due to seasonal outbreaks and endemic stability (Rogers and Shiels, 1979). *Anaplasma bovis*, formerly known as *Ehrlichia bovis*, infects bovine mononuclear cells; however, these infected cells are rarely found in peripheral blood (Dumler et al., 2001). *Anaplasma centrale*, a less virulent cohabitant of the centralized intracellular region of the erythrocyte, is used as a live vaccine to reduce morbidity and mortality in cattle subsequently infected with *A. marginale* (Kocan et al., 2000). *Anaplasma phagocytophilum,* formerly *E. phagocytophilum*, infects myeloid cells (granulocytes and monocytes) of many mammals. Bovine anaplasmosis, cause by *A. phagocytophilum*, is typically reported as a self-resolving febrile disease of cattle (Pusterla and Braun, 1997). Even though *A. phagocytophilum* has been identified as an economically important disease in cattle (Hoar et al., 2008; Pusterla and Braun, 1997; Pusterla et al., 1997), the prevalence of this pathogen in the United States is unknown (Hoar et al., 2008). However, research interest has evolved for the development of disease models in cattle due to *A. phagocytophilum* being the etiologic agent of human granulocytic ehrlichiosis (de la Fuente et al., 2006).

Transmission

Horizontal transmission

Horizontal transmission occurs between anaplasmosis carriers to susceptible animals. The foremost recognized mode of horizontal transmission of bovine anaplasmosis is mediated by tick vectors. The tick vectors include the genera *Argas*, *Boophilus*, *Dermacentor*, *Hyalomma*, *Ixodes*, *Ornithodoros*, and *Rhipicephalus*. Biological transmission of *A. marginale* by ticks occurs after extensive multiplication in tick gut and salivary tissues (Kocan et al., 2000).The tick life cycle aids in the transmission of anaplasmosis. However, ticks do not acquire anaplasmosis transovarially. In general, ticks are multi-host feeders during development from the larval, nymphal, and adult stages (Vredevoe, website accessed October 1, 2009). Once a tick feeds on a carrier animal, tick transmission of anaplasmosis occurs intra- and transstadially. Once a female tick develops to the adult stage, she must engorge on a blood meal prior to mating with a male tick. The female tick then releases from the parasitized animal for oviposition. After oviposition, the female tick may die (hard ticks) or attach to another host for feeding (soft ticks). Male ticks are purported as the most problematic vector (Kocan et al., 2000; Stiller and Coan, 1995). This is due to the shorter feeding period of ticks and disease transmission during multiple feedings. The percentage of ticks infected during feeding is related to the circulating parasitemia (Eriks et al., 1989; Eriks et al., 1993). However, a key clarification to the discussion is that not all isolates of *A. marginale* are tick-transmissible (Morley and Hugh-Jones, 1989a; Scoles et al., 2005).

Anaplasma marginale differs from other *Anaplasma* spp. by the ability to be transmitted mechanically by haematophagous arthropods and blood-contaminated fomites (Kocan et al., 2000). In a review by Ewing, 12 species of Muscidae, 8 species of Tabanidae, and 3 species of Culicidae were reported as mechanical vectors of *A. marginale* (Ewing, 1981). Mosquitos may also play a less significant role in transmission (Richey, 1991). In one study, tabanids were capable of *A. marginale* transmission from infected calves to splenectomized calves in as few as 10 bites over a 2 hour time period (Hawkins et al., 1982). However, the role of tabanids as an anaplasmosis vector may be overemphasized due to this study being conducted on calves with parasitemias of 35 to 87%. In controlled studies typical of field infection, ticks have been

demonstrated to be more efficient vectors of anaplasmosis than Tabanidae and Muscidae on the order of >240-fold and >300-fold, respectively (Scoles et al., 2005; Scoles et al., 2008).

Iatrogenic transmission

Iatrogenic transmission of *A. marginale* by blood-contaminated fomites has been demonstrated to occur during routine animal husbandry procedures (as reviewed by Dikmans, 1948). A non-exhaustive list of blood-contaminated fomites that may spread *A. marginale* are needles, dehorning equipment, nose tongs, tattooing tools, ear tag equipment, and castration devices (De Wall, 2000; Kocan et al., 2000; Rodriguez-Vivas et al., 2004). In an iatrogenic transmission study (Reeves and Swift, 1977), a cow was identified as an anaplasmosis carrier through serologic evaluation with the complement fixation test; additionally, 8 heifers were determined seronegative. The seropositive cow followed by 5 seronegative heifers were loaded into an alley system. The carrier cow was then vaccinated intramuscularly with physiologic saline by an automatic syringe (a conventional needle injection technique) followed by the serial vaccination of 5 heifers at 1 minute intervals with the same needle. The order of injection was recorded. The remaining heifers served as non-injected controls. At the end of a 60 day study, only the first heifer receiving an intramuscular injection was iatrogenically infected. These results are similar to the iatrogenic transmission study described in Chapter 3. However, a comparison was made in this study between conventional needle injection and a needle-free injection system. Furthermore, the iatrogenic transmission model described in Chapter 3 was a more robust evaluation of iatrogenic transmission with each injection technique. Following 10 replicates of a sham intramuscular vaccination with each technique without disinfection or replacement of injection equipment, needle-free injection was found to be superior to conventional needle injection for the prevention of iatrogenic transmission of *A. marginale*.

Due to all cattle not being castrated, dehorned, ear-tagged, or tattooed, it is likely that vaccination is the leading cause of iatrogenic transmission of *A. marginale*. However, disease prevention strategies during vaccination have been considered impractical, uneconomical, or potentially deleterious to the procedure (Andrews and Lamport, 1985; Makoschey and Beer, 2004). In a survey of fifty-five large animal veterinary practices, 32% did not exchange hypodermic needles between each cow (Anderson and Silviera, 2008). Resources have been dedicated to the development of needle-free injection devices for improving the processing and

throughput of cattle during vaccination. Needle-free injection techniques have proven to be efficacious for the delivery of vaccines in cattle (Hollis et al., 2005; Huang et al., 2005; Manoj et al., 2004). However, research has suggested the potential for blood product transfer during the use of needle-free injectors for consecutive injections (Sweat et al., 2000).This potential was not significant enough to cause iatrogenic transmission of *A. marginale* during needle-free injection as described in Chapter 3.

Vertical transmission

Vertical transmission in cattle is the transfer of disease from the dam to her gestating calf *in utero*. *Anaplasma marginale* causes abortion in acutely infected cattle (Radostits et al., 2000). However, abortion is neither absolute in acutely infected cows nor in carrier cows. The relationship between *in utero* transmission and trimester of pregnancy was described in the literature (Zaugg, 1985). Six pregnant, susceptible cows were inoculated with a Virginia isolate of *A. marginale* during each trimester of pregnancy. Through subinoculation of splenectomized calves with blood samples from fetuses at various stages of development and pre-colostrally into intact calves, it was determined that *in utero* transmission occurs during the second and third trimesters of pregnancy. However, abortion occurred in one cow 22 days after inoculation during the third trimester. Unfortunately, the findings of this study were confounded by the potential for inadvertent contamination of fetal blood samples collected during the surgery and inability to infect susceptible calves after birth.

The incidence of *in utero* transmission has been documented in observational studies in which surgical collection of blood did not occur (Bird, 1973; Potgieter and van Rensburg, 1987; Zaugg and Kuttler, 1984). Potgieter and van Rensburg reported an *in utero* transmission rate of 7.7% in calves born to both acutely infected and carrier cows. Therefore, acute infection of the dam is not necessary for vertical transmission to the fetus. The *in utero* transmission rate of *A. centrale* in acutely infected, splenectomized cows was 30.8%. Furthermore, transplacental transmission of *A. marginale* and *A. centrale* was possible in each trimester of pregnancy. The *A. marginale* transmission rate is consistent with a prevalence study in 58 calves born to carrier cows (6.7%, data unpublished). This *in utero* transmission rate was as determined by the qRT-PCR described in Chapter 2, not by serologic methods.

Pathogenesis

The progression of anaplasmosis, caused by *A. marginale,* is typified by four stages of variable duration; (1) peracute/incubation, (2) acute/developmental, (3) convalescent, and (4) carrier (Radostits et al., 2000; Richey, 1991).The peracute phase (1) is described as the time from infection of a susceptible animal until a detectable parasitemia occurs (1%). The duration (4-6 weeks) of this subclinical stage of infection is influenced by the isolate and number of infective *A. marginale* bacteria. The start of the acute phase (2), which coincides with the first signs of clinical disease, is signaled by an increase in body temperature. The end of the acute phase is signaled by the first signs of reticulocytes in the circulation. The appearance of reticulocytes (erythropoiesis) occurs much earlier in the acute phase in younger cattle due to a regenerative anemia; however, a paradoxical non-regenerative anemia is typical in older cattle. This non-regenerative anemia observed during the acute phase is the reason why cattle > 1 year of age display clinical signs of anaplasmosis and have high mortality rates. The duration of the acute phase is generally 4-9 days. Clinical signs appear during the acute phase. Until clinical signs develop, the need for clinical intervention is inapparent (Alfonso et al., 1996). The period typified by the convalescent phase (3) is from the release of reticulocytes until normal blood values return. Upon the return of normal blood values, the carrier state (4) begins and continues until death or chemosterilization.

A cyclic rickettsemia is a characteristic of the carrier state (Kieser et al., 1990). In the carrier state, the number of infected erythrocytes ranges from 10^4-10^7 per milliliter at 5 week intervals (Eriks et al., 1993). This steady rise and rapid decline parallels the rickettsemias level variation seen in the acute phase. The host immune response to *A. marginale* infection depends upon the production of anti-parasitic and anti-erythrocytic antibodies inducing the erythrophagocytosis of parasitized erythrocytes (Jatkar and Kreier, 1969). The mechanism by which anaplasmosis eludes the host immune response is unknown; however, the lack of conservation of gene sequences encoding Major Surface Protein (MSP) antigens of *Anaplasma* spp. has been the focus of research (de la Fuente et al., 2005). The analysis of the outer membrane fraction of *A. marginale* has led to the characterization of 6 major polypeptides: MSP-1a, MSP-1b, MSP-2, MSP-3, MSP-4, and MSP-5 (as reviewed by Palmer and McElwain, 1995; Tebele et al, 1991).

1

Clinical findings

A seasonal distribution of cattle presenting with clinical disease during the summer and fall is typical of infection with *A. marginale*. However, infection and the subsequent need for medical intervention is common year-round in tropical regions when naïve cattle are introduced into endemically stable herds. Additionally, iatrogenic transmission also necessitates the need for diligent attention to the symptoms of this disease and subsequent potential for year-round treatment.

A differential diagnosis list includes infectious and non-infectious diseases that cause anemia and icterus (Table 1.1). All ages of cattle are susceptible to infection with *A. marginale* (Bird, 1973; Zaugg, 1985; Zaugg and Kuttler, 1984). However, clinical disease and mortality rates increase with age (Richey, 1991). Typically, cattle less than 1 year of age become infected without exhibiting any clinical signs of disease. As the age of cattle increases, morbidity and mortality rates rise during the acute phase of disease. A febrile response, which coincides with a detectable parasitemia, is typically the first sign of infection. Unless cattle present with clinical disease supported by a diagnosis of anaplasmosis caused by *A. marginale*, treatment is not warranted (Alfonso et al., 1996). Abortion caused by *A. marginale* occurs in acutely infected and carrier cows (Correa et al., 1978; Zaugg, 1985; Zaugg and Kuttler, 1984).

Anaplasmosis, caused by *A. phagocytophilum*, is typified by a much shorter incubation period of 6.3 + 1.2 days (Pusterla and Braun, 1997). *Anaplasma phagocytophilum* bodies are first observed between 4-8 days of infection. The duration of the detectable parasitemia is approximately 10 days. Clinical signs include pyrexia (40.2 - 47.1°C), decreased milk production, respiratory distress, abnormal locomotion, and nasal discharge. However, these clinical signs resolve without treatment in adult cattle. Abortion is reported to occur in the last 2 months of gestation. A list of clinical signs has been prepared for comparison between *A. marginale* and *A. phagocytophilum* (Table 1.2).

Disease caused by *A. centrale* is typically subclinical. Additionally, splenectomized and intact cows that were both acutely and chronically infected with *A. centrale* did not abort during acute infection or the carrier state (Potgieter and van Rensburg, 1987). Therefore, disease caused by *A. centrale* will not be discussed.

Diagnosis

Necropsy

Post-mortem findings are typical of death caused by anemia. Generalized tissue pallor, an enlarged, red-brown discolored spleen, and enlarged liver and gall bladder are common findings. Icterus may be present depending on the time of death during the acute phase of disease. Thin blood film preparations can be made from the incised surface of the spleen and liver for detection of parasitized erythrocytes.

First generation diagnostic methods

Since first discovered by Theiler in 1909, the foundation for diagnosis of anaplasmosis is based on light microscopy, a first generation diagnostic method. First generation diagnostic methods rely on the growth or visualization of the organism of interest. These methods have limited sensitivity and lack adequate specificity to differentiate between morphologically similar pathogens, normal structures, and stain artifacts. A diagnosis of A. *marginale* infection by light microscopic examination of stained blood smears is made from blood collected in anticoagulant.

A method for staining blood smears and performing percent parasitized erythrocyte counts is included in Chapter 3. The "feathered edge" of the blood smear is examined for parasitized erythrocytes. *Anaplasma marginale* are found along the intracellular margin of erythrocytes. These dense, basophilic bacteria are 0.3-1.0 μm in diameter. A minimum of 500 erythrocytes should be examined at 1000X magnification to detect a parasitemia. If parasitized erythrocytes are observed, a percent parasitized erythrocyte count (PPE) should be calculated according to the method in Chapter 3. *Anaplasma phagocytophilum* infection is also detected in Giemsa stained blood smears at 1000X magnification. A minimum of 500 leukocytes (neutrophils, eosinophils and monocytes) should be examined to detect a parasitemia. Cytoplasmic inclusions of parasitized leukocytes are typically first observed between 5.7 + 1.0 days post-infection and up to 15.1 + 2.8 days post-infection. The average duration of a detectable parasitemia is 10.4 + 2.8 days (Pusterla and Braun, 1997).

Failure to observe blood cells parasitized with *A. marginale* or *A. phagocytophilum* does not rule out disease (Pusterla et al., 1997; Richey, 1991). When screening cattle for *A. marginale*, the disease incidence determined by light microscopy was only 8.9% as compared to a serologic

method (indirect fluorescent antibody) that determined incidence to be 68% (Akinboade and Dipeolu, 1984). Due to cyclic rickettsemias in carrier cattle (Eriks et al., 1989; French et al., 1998), disease classification by light microscopic examination of stained blood smears is only reliable during the acute phase of infection (Gale et al., 1996a). As described in Chapter 3, the sensitivity of light microscopy is maximized during peak parasitemia of the acute phase of infection (Reinbold et al., 2009b).

The subinoculation of naïve, splenectomized calves with up to 500 mL of whole blood from cattle of unknown disease status has served as the gold standard test for disease detection. However, this gold standard method is neither cost-effective nor is animal welfare supported. Therefore, the determination of *A. marginale* disease status has depended upon several second generation methods (Amerault and Roby, 1968; Barry et al., 1986; Duzgun et al., 1988; Goff et al., 1985; Schuntner and Leatch, 1988; Shkap et al., 1990; Winkler et al., 1987).

Second generation diagnostic methods

Second generation methods, which rely on the identification of cell components, metabolic products, or detection of antigenic components, are currently the most commonly used techniques for disease classification in clinical medicine and research. Several serologic tests have been developed with an *A. marginale* antigen preparation to detect antibody in carrier cattle (Gale et al., 1996a). Such methods include capillary tube agglutination, rapid card agglutination, indirect fluorescent antibody detection, complement fixation, and competitive enzyme-linked immunosorbent assay (cELISA). The complement fixation, rapid card agglutination, and cELISA are the most common methods used throughout the world (Coetzee et al., 2007; De Wall, 2000). Serial comparisons are often necessary to confirm or refute a diagnosis due to significant test sensitivity and specificity deficiencies (Boulanger et al., 1966; Coetzee et al., 2007; Dreher et al., 2005; Strik et al., 2007). Furthermore, a distinction cannot be made between current infection and vaccination (Gale et al., 1996a).

The cELISA (Anaplasma Antibody Test Kit, cELISA, VMRD Inc.; Pullman, WA) is the only second generation method used during this program of study. Therefore, only the cELISA will be discussed. The cELISA uses a 19-kDa antigen, recombinant major surface protein 5 (MSP5) that is highly conserved among different strains of *A. marginale*, *A. centrale*, and *A. phagocytophilum* (Knowles et al., 1996). A common epitope is shared between the native protein

and a recombinant MSP5 fused to a maltose binding protein. This epitope is recognized by the monoclonal antibody AnaF16C1. The cELISA is based on the serum antibody inhibition of AnaF16C1 binding to MSP5 in *A. marginale* infected cattle.

The common measurements of performance for test validation are sensitivity and specificity (Greiner and Gardner, 2000). The performance of the cELISA has been evaluated with varying degrees of agreement and refutation. The focus of this disparity is due, in part, to the % inhibition used for the negative cut-off value. The current negative cut-off recommendation is 30% inhibition (OIE, 2009). However, a 42% inhibition is recommended as the negative cut-off value in Canada (CFIA, 2006; Van Donkersgoed et al., 2006). Beyond the effects of the cut-off value as well as random and systematic error, differences in sensitivity and specificity are attributable to the reference population(s) and sampling strategy. Therefore, conflicting reports of method performance should be critically evaluated. The reference populations tested should be selected based upon varying levels of prevalence of the disease of interest as well as the potential for co-infection with closely related pathogens. Additionally, methods should be tested in animals with equitable stages of infection.

The following studies describe the performance of the cELISA with variable negative cut-off values, cattle naturally and experimentally infected, and disease prevalence ranging from 0-100%. The first evaluation of cELISA occurred during the development of the assay (Knowles et al., 1996). In 17 calves experimentally infected with a Florida strain of *A. marginale*, cELISA sensitivity was 88.2% using a 25% inhibition cut-off value. When using a 30% inhibition cut-off, the sensitivity was 82.4%. At 42% inhibition, the sensitivity was only 35.3%. Notably, the values reported were from samples collected between 22-31 days post-infection. As discussed in Chapter 3, cELISA peak sensitivity was observed at 41 days post-infection using a 30% inhibition cut-off. Furthermore, the false positive rate of the data in Chapter 3 would have decreased to 0% had a 42% inhibition cut-off been used.

In 268 serum specimens collected from cattle located in non-endemic regions, specificity was demonstrated at 100% using a 25% inhibition cut-off value. In a separate study of 235 serum samples collected from cattle located in endemic regions, the sensitivity and specificity of the cELISA was evaluated with a nested PCR (Torioni de Echaide et al., 1998). The sensitivity and specificity of the cELISA were 96 and 95%, respectively, using a 28% inhibition cut-off value. No further interpretation could be made using other cut-off values due to the manner in which

the data was presented. Regardless of the negative cut-off value used to optimize sensitivity and specificity, cELISA has replaced the complement fixation and card agglutination tests as the accepted basis for testing cattle prior to interstate and international movement (OIE, 2009). Even though cELISA is the more robust test of all second generation methods, other methods are still offered by diagnostic laboratories (Coetzee et al., 2007; De Wall, 2000).

In 40 steers experimentally infected with an Oklahoma isolate of *A. marginale*, the sensitivity of the cELISA was 94.8% when using a negative cut-off value of 30% inhibition (Coetzee et al., 2007). Study results were confirmed through the inoculation of blood into susceptible, splenectomized calves. Specificity was not determined due to all cattle being infected. The time to peak sensitivity (13 days post-infection) was shorter than the study described in Chapter 3 (41 days post-infection). This may be attributed to the high infective dose used. A decreased time of onset for a detectable parasitemia has been reported for increasingly high challenge doses (Eriks et al., 1989; Eriks et al., 1993; Gale et al., 1996b).

The cELISA has been used to evaluate *A. marginale* chemosterilization strategies using oxytetracycline (Coetzee et al., 2005). In this study, 2 different preparations and 3 different treatment regimens using a long-acting oxytetracycline were administered to 40 steers persistently infected with an Oklahoma isolate of *A. marginale*. Samples were collected at 31 and 60 days post-treatment. Chemosterilization failure was confirmed through the subinoculation of susceptible, splenectomized calves. The sensitivity of the cELISA and disease prevalence were 100%; therefore, cELISA could not be evaluated as a tool to detect chemosterilization. In Chapter 5, cELISA confirmed chemosterilization success in a chlortetracycline chemosterilization study. However, this did not occur until anti-MSP5 antibodies had declined below a 30% inhibition cut-off. This confirmation was not achieved for up to 54 days after the time of chemosterilization confirmed by the qRT-PCR described in Chapter 2.

Serologic cross-reactivity among closely related *Anaplasma* spp. has been previously described (Dreher et al., 2005; Strik et al., 2007). A comparison was made between the MSP5 amino acid sequences of 1 isolate of *A. centrale*, 3 isolates of *A. marginale*, and 1 isolate of *A. phagocytophilum* (Dreher et al., 2005). The percent similarity between the *A.centrale* isolate and the *A. marginale* isolates was > 92%. The percent similarity between the *A. centrale* isolate and the *A. phagocytophilum* isolate was 65%. The percent similarity between the *A. phagocytophilum* isolate and the *A. marginale* isolates ranged from 63-64%. The high percent similarity of the

cELISA has also enabled its use for detecting antibody in cattle vaccinated with *A. centrale* with a sensitivity of 93.3% (Molad et al., 2006). In contrast, the *A. phagocytophilum* MSP5 was characterized and used to study the extent of cross-reactivity with *A. marginale* (Strik et al., 2007). Their study results illustrated no serologic distinction could be made between *A. phagocytophilum* and *A. marginale*. Therefore, the results of the cELISA (Knowles et al., 1996; VMRD) must be interpreted carefully for screening field samples in areas where infection with *A. centrale*, *A. marginale*, and *A. phagocytophilum* may occur.

The use of cELISA for screening neonatal calves post-colostrum consumption may be problematic. In an unpublished study, the prevalence of antibody in calves born to carrier cows was 66.7%. However, the prevalence was determined to be only 6.7% by the qRT-PCR described in Chapter 2. Pre-colostrum consumption samples were not analyzed. Therefore, the utility of the cELISA for screening calves for anaplasmosis is uncertain.

Third generation diagnostic methods

Third generation methods have evolved through the elucidation of genomic DNA and RNA sequences. The selection of an appropriate target for the accurate and precise diagnosis of disease is critical to the successful development of third generation methods. The analysis of genomic sequences has led to the development of nucleic acid-based techniques for the classification of disease status. In turn, nucleic acid-based techniques have extensively contributed to the understanding of anaplasmosis disease processes. Third generation methods offer superior sensitivity over first and second generation methods through the ability to detect pathogens down to the minimum biological unit for infection (Zarlenga and Higgins, 2001). Inadequate specificity due to cross-reactivity is not a disadvantage when using third generation methods. Additionally, the specificity of molecular methods has led to the development of multiplex methods for detecting co-infection of clinically relevant pathogens.

The cyclic parasitemia of *A. marginale* in carrier cattle was first described with the use of a nucleic acid probe for detecting infected erythrocytes (Eriks et al., 1989). An *A. marginale* specific probe accurately hybridized with as few as 0.1 ng of genomic DNA and 500-1000 parasitized erythrocytes in 500 mL of blood. The findings of this sensitive and specific method were correlated to the parasitemia level. It was determined that the parasitemia level among

carrier cattle was highly variable. Furthermore, this led to the hypothesis that the ability to transmit disease may fluctuate with parasitemia level.

An *A. marginale*-specific nested PCR (nPCR), currently offered for use by the Washington Animal Disease Diagnostic Laboratory at Washington State University, was used to support the licensing of the cELISA by the United States Department of Agriculture described previously (Knowles et al., 1996; Torioni de Echaide et al., 1998; Valdez et al., 2002; VMRD-2; VMRD). This type of PCR uses a nucleic acid based probe to recognize a nucleotide sequence amplified during the PCR reaction. Identification was based upon the recognition of amplified *A. marginale* MSP5 DNA sequences from blood samples. The nPCR was able to detect as few as 30 parasitized erythrocytes in 1 mL of blood. This detection sensitivity is greatly enhanced over the nucleic acid probe described previously. The nPCR was also used to determine chemosterilization success (Coetzee et al., 2005). However, the utility of the nPCR under these conditions was not realized during this study due to all steers not clearing infection. In a separate chemosterilization study, nPCR was determined to be an unreliable method for determining success of chemosterilization in calves due to a lack of sensitivity (Coetzee 2006). Furthermore, this nPCR is not recommended by the OIE due to the potential for non-specific amplification.

Several polymerase chain reaction (PCR) methods have been developed to identify DNA of *A. marginale* (Bekker et al., 2002; Eriks et al., 1989; French et al., 1998; Gale et al., 1996b; Ge et al., 1995; Molad et al., 2006). However, conventional methods rely on the visualization of amplified product on agarose gels without the ability to accurately estimate the initial quantity of genetic template. The use of real-time molecular methods that combine PCR chemistry and fluorescent probe detection of amplified product have greatly enhanced the ability to diagnose disease. Real-time methods are reliably used to accurately correlate assay results to the quantity of initial template through regression analysis. Furthermore, these methods are capable of detecting more than one pathogen through the development of multiplex assays.

Numerous real-time assays have been developed for the detection of *A. marginale* alone or in combination with other pathogens of tick-borne diseases. Simplex assays that target the DNA gene sequences encoding for a major surface protein (MSP1b) and groEL of *A. marginale* have been described (Carelli 2007 and Decaro 2008). The high analytical sensitivity of both methods provided for the detection of 10 DNA copies or 10 infective *A. marginale* bacteria. The assay described by Carelli in 2007 was proven highly specific, due to the absence of cross-

reactivity observed with other *Anaplasma* spp., such as *A. centrale*, *A. bovis*, *A. ovis*, *A. phagocytophilum*, *B. bovis*, *B. bigemina*, *T. annulata*, and *T. buffeli*. A similarity was observed in the development of TaqMan based systems using the fluoresecent probe, FAM, and quencher molecule, BHQ, TaqMan as the simplex qRT-PCR assay described in Chapter 2. However, the simplex qRT-PCR assay described in Chapter 2 was only able to detect as few as 100 copies of 16S rRNA. It is noteworthy that this inferior detection limit is equivalent to the minimum infective unit of a single *A. marginale* bacterium.

A review of the literature found reports of methods for the detection of *A. phagocytophilum* that have been limited primarily to the diagnosis of disease in humans and canines by multiplex real-time methods (Courtney et al., 2004; Sirigireddy and Ganta, 2005). However, a real-time simplex reverse-transcriptase polymerase chain reaction (RT-PCR) assay was previously described in cattle for detecting 16S ribosomal RNA (16S rRNA) of *A. phagocytophilum* (Pusterla et al., 1999). Gene sequence analysis has identified highly conserved and specific regions of the 16S rRNA gene segment of the family *Anaplasmataceae*. The analytical sensitivity of this assay is extraordinary based upon the detection of as few as ten 16S rRNA molecules in a reaction. The selection of 16S rRNA was critical for the development of this simplex assay. Due to their role in translation of the genetic code, ribosomes, as well as rRNA, are present in high copy numbers. Therefore, the analytical sensitivity of the assay described by Pusterla et al. in 1999 was enhanced due to the high ratio of 16S rRNA:16S DNA (Sirigireddy and Ganta, 2005). This is a similar advantage of the qRT-PCR described in Chapter 2.

When developing a multiplex real-time method, the assay must be able to detect non-equivalent molar ratios. Non-equivalent molar ratios are encountered due to differences in the circulating parasitemia, infection of different types of blood cells (erythrocytes versus leukocytes), and chronic versus acute infection. The multiplex method described in Chapter 2 is capable of detecting equivalent molar ratios as few as one hundred 16S rRNA molecules of *A. marginale* and *A. phagocytophilum* in the same reaction tube. However, the ability to detect non-equivalent molar ratios up to 100-fold may be a limitation of the assay for simultaneous screening of samples for these *Anaplasma* spp. (Courtney et al., 2004; Sirigireddy and Ganta, 2005). However, this could not be accurately assessed during this study due to not detecting *A. phagocytophilum* in the field samples screened. However, a simplex assay for the detection of *A.*

phagocytophilum has been described and applied in this study for the screening of samples whose duplex results did not agree with that of the cELISA as in a similar study (Sirigireddy and Ganta, 2005). Regardless, the qRT-PCR described in Chapter 2 is the only reported method for detecting *A. marginale* and *A. phagocytophilum* in the same reaction tube.

The final frontier for real-time method use is in the determination of *A. marginale* chemosterilization success. However, this application had not been performed until the study described in Chapter 5 (Reinbold et al., 2009c). In 18 steers treated with oral chlortetracycline for 80 days under experimental conditions, the qRT-PCR was used to determine chemosterilization success in all steers by 49 days of treatment. Furthermore, the qRT-PCR validated a chemosterilization strategy using a single treatment of injectable oxytetracycline and oral chlortetracycline for 30 days. The qRT-PCR assay results were confirmed through the subinoculation of susceptible, splenectomized steers. When these chemosterilization strategies were applied in the field under conditions of natural infection, a limitation of the qRT-PCR was illustrated (unpublished data). This limitation was due to the suppression of the circulating parasitemia being less than one *A. marginale* bacteria in a 250 μL of plasma-free blood sample. However, the qRT-PCR was used to illustrate significant differences in the number of circulating bacteria prior to the start of treatment, end of treatment, and following a drug washout period. Therefore, this information may be used in studies testing the hypothesis that a lower level parasitemia may affect the ability to transmit disease.

Treatment

The treatment of acute anaplasmosis caused by *A. marginale* was primarily restricted to the supportive care of clinically ill cattle prior to the discovery and use of antimicrobials. However, supportive care alone is largely ineffective for reducing the mortality rate. Over time, antimicrobials have been used alone or in combination in anaplasmosis treatment regimens. Currently, there are no approved formulations for the treatment of anaplasmosis in the United States or Canada. Pharmacotherapeutic intervention with a carbanilide derivative (imidocarb dipropionate at 3 mg/kg) or tetracycline antibiotics (particularly long-acting formulations of oxytetracycline at 20 mg/kg bodyweight administered intramuscularly or subcutaneously) have decreased the mortality rate in acutely infected cattle when administered prior to the onset of an increasingly severe anemia (Kuttler, 1980; Radostits et al., 2000). However, the development of

a carrier state commonly occurs in spite of treatment and recovery from infection. The treatment of acutely infected cattle was not a part of this program of study. Therefore, treatment of clinically-ill cattle will not be discussed further in this dissertation.

Chemosterilization

Cattle infected with anaplasmosis following natural infection and vaccination with live *Anaplasma* spp. remain lifelong carriers (Kocan et al., 2003). Carriers are responsible for horizontal, iatrogenic, and vertical transmission of anaplasmosis to naïve cattle by providing a reservoir of infective blood for biological, mechanical, and *in utero* infection (Futse et al., 2003; Norton et al., 1983). The inability to clear the carrier state of infection of anaplasmosis in cattle is due to an ineffective immune response subsequent to disease challenge and lack of an established pharmacokinetic-pharmacodynamic relationship between plasma drug concentration and carrier clearance in validated chemosterilization strategies.

Immune evasion

The host immune response to *A. marginale* infection depends upon the production of anti-parasitic and anti-erythrocytic antibodies inducing the erythrophagocytosis of parasitized erythrocytes (Jatkar and Kreier, 1969). The mechanism by which anaplasmosis eludes the host immune response is unknown; however, the lack of conservation of gene sequences encoding major surface protein antigens of *Anaplasma* spp. has been the focus of research (de la Fuente et al., 2005). Six types of major surface proteins have been identified: MSP-1a, MSP-1b, MSP-2, MSP-3, MSP-4, and MSP-5. Evidence exists for a rapid decrease in antigen-specific T cells and immunologic memory following infection of cattle pre-immunized with the major surface protein 1a (Han et al., 2008). Other research has shown a second exposure prior to and during treatment did not facilitate the chemosterilization process immunologically (Kuttler, 1983). Furthermore, the study in Chapter 5 and the findings by others (Magonigle and Newby, 1984; Renshaw et al., 1976) have illustrated a loss of immunologic memory through the loss of anti-parasitic antibody and re-infection of cattle previously chemosterilized.

Mode of action of tetracycline antibiotics

The absorption of tetracycline into the erythrocyte has been characterized as a simple diffusion process (DeLoach and Wagner, 1984). Once inside the erythrocyte, drug passes as a

cation through porin channels of the outer membrane of Gram negative bacteria into the periplasm, becomes an uncharged molecule to diffuse through the inner cytoplasmic membrane and reversibly binds to the 30S ribosome to inhibit protein synthesis (Chopra and Roberts, 2001). Tetracyclines are also known to interact with the 16S rRNA subunit (Moazed and Noller, 1987). However, this did not affect qRT-PCR assay performance during the study in Chapter 5.

Efflux and ribosomal protection proteins, as well as enzymatic inactivation, are mechanisms of resistance to counteract the efficacy of tetracycline antibiotics during treatment. These mechanisms are driven by numerous resistance genes found in commensal and pathogenic bacteria today (Chopra and Roberts, 2001). The ability to define tetracycline resistance is difficult due to the frequent occurrence of mutants that are impermeable to drug uptake (Moazed and Noller, 1987). Furthermore, no apparent difference is distinguishable between resistance genes of the tetracycline family of antibiotics.

Chlortetracycline pharmacokinetic data

Chlortetracycline was discovered in a soil sample collected from Sanborn Field at the University of Missouri in Columbia, MO in 1945. In 1954, CTC was approved for use in feed for improving feed efficiency, growth promotion, and the treatment of CTC-sensitive pathogens (NADA, 065-440). Today, the label indications, based on multiple dosing regimens, are for increased rate of weight gain, improved feed efficiency, control of bacterial pneumonia associated with shipping fever complex, control of active infection of anaplasmosis, reduction of liver condemnation because of liver abscessation, and treatment of bacterial enteritis (2009 Feed Additive Compendium). The 1999 NAHMS feedlot study reported that 51.9% of all feedlots surveyed administer CTC as a health or production management tool to 18.2% of cattle fed. Operations with capacities of 1,000–7,999 head, as well as those feeding cattle weighing <318.2 kg, tend to administer CTC to cattle more often than operations feeding >8,000 head and cattle weighing >318.2 kg. On average, CTC is fed 8.6 and 7.7 days to cattle weighing <318.2 and >318.2 kg, respectively.

Pharmacokinetic parameters for orally administered CTC are available for preruminating calves, poultry, and swine (Bradley et al., 1982; Dyer, 1988; Kilroy et al., 1990; Luthman and Jacobsson, 1983; Nielsen and Gyrd-Hansen, 1996; Pollet et al., 1983; Wanner et al., 1991). Remarkably, similar information for ruminating cattle is unknown in spite of the widespread use

of CTC. Pharmacokinetic parameters were determined for a single, 22 mg/kg dose of CTC by noncompartmental analysis in two-week-old, Holstein calves fed a starter concentration at 2% body weight plus ad libitum alfalfa hay (Bradley et al., 1982). Parameters derived for the conventionally fed calves were $AUC_{0\text{-}LOQ}$ (7.5 hr·µg/mL), $t_{1/2\ \lambda z}$ (17.75 h), Cl/F (1.3 L/kg/hr), and V_z/F (40.9 L/kg). These parameters are dissimilar to the mean parameters derived by noncompartmental analysis parameters for ruminating, Holstein steers that are discussed in Chapter 4 (Reinbold et al., 2009a); however, a separate pharmacokinetic analysis technique, nonlinear mixed effects modeling, reported similar results to the 1982 study by Bradley et al. The dissimilarity parameters derived by noncompartmental analysis is likely caused by significant differences in anatomy, metabolism, physiology of the test cattle, method of administration, and compartmental vs. noncompartmental analysis between the studies.

Chlortetracycline and A. marginale chemosterilization

Chlortetracycline is the only antimicrobial that has a labeled indication for anaplasmosis in the United States. Chlortetracycline is labeled for the "control of active infection of anaplasmosis caused by *A. marginale* susceptible to CTC in cattle in the United States" (2009 Feed Additive Compendium). Several reports exist for the use of CTC in successful chemosterilization strategies. These chemosterilization regimens were assessed using antigen/antibody-mediated second generation diagnostic methods, such as capillary agglutination and complement fixation, and subinoculation of splenectomized calves (Table 5.1) (Brock et al., 1959; Franklin et al., 1966; Franklin et al., 1967; Franklin et al., 1965; Richey et al., 1977; Twiehaus, 1962). These studies were: (1) performed in calves and adult cattle; (2) study animals were either intact or splenectomized; (3) regimens were validated in experimental and natural *A. marginale* infection; (4) treatments were either group or individualized; and (5) the average dose and duration were 5.21 mg/kg and 54.1 days, respectively. A key difference among these reports was only 5 of these regimens were confirmed through the subinoculation of susceptible, splenectomized calves. The remaining regimens either relied on the sensitivity and specificity of serological methods to determine disease status or the splenectomy of study animals. Overall, these regimens were similar to the study described in Chapter 5 in regard to use of a serologic method for determining disease status and the subinoculation of susceptible, splenectomized calves for confirmation of carrier clearance. However, the qRT-PCR assay definitively defined

the time of carrier clearance and aided in the establishment of an *in vivo* minimum inhibitory concentration (MIC) for *A. marginale* carrier clearance when using chlortetracycline.

The Clinical and Laboratory Standards Institute (CLSI) has set the susceptible minimum inhibitory concentration (MIC) breakpoint of tetracycline antibiotics (200 mg/mL injectable oxytetracycline product) at 2 μg/mL when treating bovine bacterial infection caused by *Mannheimia haemolytica, Pasteurella multocida,* and *Histophilus somni* (CLSI, 2008); yet, a similar pharmacokinetic-pharmacodynamic relationship does not exist for the treatment of bacterial infection caused by *A. marginale*. This is in part due to the inability to sustain *in vitro* cultures of *A. marginale* in erythrocytes (Coetzee et al., 2006; Palmer and McElwain, 1995). However, the continuous *in vitro* cultivation of *A. marginale* is possible in tick cell lines. The response of *A. marginale* to enrofloxacin, imidocarb, and oxytetracycline in short-term erythrocyte cultures was evaluated. Enrofloxacin was shown to inhibit *A. marginale* in a dose-dependent manner; the effectiveness of imidocarb declined with increasing drug concentration; and oxytetracycline was the least effective (Coetzee et al., 2006).

Due to the widespread use of CTC for the control of anaplasmosis in cattle, it is essential for an *in vivo* pharmacokinetic-pharmacodynamic relationship be established between plasma drug concentrations and carrier clearance to prevent imprudent use of antimicrobials. Furthermore, treatment regimens are needed to offer an alternative strategy for minimizing animal handling and elimination of individual treatment. The study described in Chapter 5 illustrated an oral CTC dose of 4.4 mg/kg of bodyweight for 49 days resulted in a minimum inhibitory plasma drug concentration of 85.3 ng/mL. Furthermore, higher oral dosages did not shorten the duration of therapy.

The mechanism for the reason why extensive duration of treatment is necessary for chemosterilization success is not understood. One reason for the extended duration of treatment is the reliance on serology to determine chemosterilization success and failure in previous studies. This may have been avoided if a diagnostic method, such as qRT-PCR, was used to detect carrier clearance on a real time basis. However, the expense of this would be cost-prohibitive under field conditions. Other reasons that may be key contributors are the reversible binding of chlortetracycline to the 30S ribosome and the extensive lifespan of erythrocytes (160 days) (Tebele et al., 1991). To my knowledge, the lifespan of a parasitized erythrocyte is currently unknown. The concentration of drug achieved in parasitized erythrocytes may be

inadequate for a bactericidal effect. Another theory would be attributable to the bacteriostatic effect of tetracycline antibiotics, and this theory is supported based upon the findings of the Coetzee et al. article in 2006. The mode of action of enrofloxacin is the inhibition of DNA gyrase function; therefore, it kills bacteria quickly and classifies enrofloxacin as bactericidal. In contrast, tetracycline antibiotics inhibit protein synthesis by reversible binding to the 30S ribosomal RNA subunit; therefore, the growth of the bacteria is suppressed and classifies tetracycline antibiotics as bacteriostatic. Finally, the inhibition of protein synthesis may prevent the infection of non-parasitized erythrocytes; however, parasitized erythrocytes must still be removed from circulation by erythrophagocytosis in the spleen.

The missing link is the mechanism responsible for the absence of an immune response. It may be possible that the inhibition of protein synthesis impairs antigenic variation of MSP's. This would allow the bovine immune system to recognize a conserved MSP epitope and mount an immune response. Ultimately, carrier clearance is influenced by an extensive drug absorption process, reversible binding of the tetracycline antibiotic to the 30S ribosome, an inadequate host immune response, and erythrophagocytosis of parasitized erythrocytes.

Due to the duration of treatment being critical to successful chemosterilization, it is unknown if this use will permanently select for or facilitate the distribution of resistance determinants in bacterial species present in cattle production systems (Stevens et al., 1993). Two multidrug resistance pumps were identified in *A. marginale* (Brayton et al., 2005). A significant change was reported in antimicrobial susceptibility of enteric bacteria from cattle fed chlortetracycline in three consecutive, five day pulse treatment regimens at 22 mg/kg (Platt et al., 2008). However, this change was only temporary as values returned to pre-exposure levels within 33 days. Due to length of treatment being critical to successful chemosterilization, it is unknown if this return to pre-exposure levels would be similar in situations where the duration of therapy is more extensive.

Prevention

An anaplasmosis disease control program is defined by the interaction of four key criteria: (1) diagnosis of disease in the herd, (2) the culling of less valuable cattle that are anaplasmosis carriers, (3) the treatment of cattle that are anaplasmosis carriers with

13

chlortetracycline, and (4) the prevention of disease entry or, in some cases, re-entry after a successful chemosterilization program (Figure 1.1).

Vector control

The success of a vector control program for reducing biological and mechanical transmission of anaplasmosis by haematophagous arthropods is based upon the appropriately-timed application of insecticides such as arsenicals, chlorinated hydrocarbons, organophosphates, carbamates, formamidines, pyrethroids, and macrocyclic lactones, directly on the animal or into the environment (De Wall, 2000; Peter et al., 2005; Rodriguez-Vivas et al., 2004). Furthermore, the success of a vector control program relies on arthropod susceptibility and managed application of chemicals. The continuous and frequent application of insecticides is problematic in modern cattle production systems due to due to environmental, target resistance, economic, and toxicity concerns. In the event that an ideal parasiticide and vector control program is developed, vertical transmission should not be overlooked in programs concentrating solely on vector control to reduce the prevalence of anaplasmosis over time (Potgieter and van Rensburg, 1987; Stiller and Coan, 1995).

Vaccination

Historically, vaccination (also referred to as premunition) with the antigenically similar pathogen and less pathogenic organism, *A. centrale*, is a common tool used in *A. marginale*-enzootic regions (Palmer and McElwain, 1995). Cattle are inoculated intravenously with bovine blood infected with live *A. centrale*. Premunized cattle develop mild or inapparent infection with *A. centrale*. The infection progresses from the acute phase of disease into a persistent carrier state. The carrier state does not prevent subsequent infection with *A. marginale*. Rather, the *A. centrale* carrier status only prevents or reduces clinical disease upon challenge exposure with *A. marginale* (Kocan et al., 2000). Premunition is limited by (i) the potential for concomitant transmission of other blood-borne disease, (ii) transmission of emergent pathogens, (iii) virulence variability among geographically-diverse regions, (iv) immune response failure due to loss of infectivity during field transport of live *A. centrale* vaccine, (v) premunition-induced immune-mediated hemolytic anemia (Palmer and McElwain, 1995).

Premunition has been used in many parts of the world, including Israel and South Africa, for more than 75 years (Kocan et al., 2000). However, this strategy is not recommended due to

the potential for transfer of other blood-borne pathogens and production of erythrocytic isoantibodies. Live vaccines that consist of bovine erythrocytes infected with *Anaplasma* spp. have been reported to cause an immune-mediated hemolytic anemia in calves born to dams vaccinated with an anaplasmosis vaccine of bovine blood origin. This disease is characterized by anemia and icterus in calves following consumption of colostrum (Dennis et al., 1970).

This phenomenon was prevented with use of a killed vaccine, licensed under the name Plazvax (Schering Plough; Kenilworth, NJ, formerly Mallinckrodt), through the harvest of *A. marginale* from infected erythrocytes of cattle. However, it is only currently available under special permission through a state veterinarian in restricted areas of the United States (Luther, 1999). Due to immune evasion, the need for repeated immunizations, and loss antigen-specific memory, the use of this serotype-specific vaccine is not advocated.

Chemoprohylaxis

Chlortetracycline is labeled for the control of active infection of anaplasmosis caused by *A. marginale* susceptible to CTC in cattle (2009 Feed Additive Compendium). The continuous feeding of CTC to naïve cattle in high risk areas is advocated during the vector season (Brock et al., 1957). Chlortetracycline is added to free-choice cattle feeds such as feed blocks or salt-mineral mixes. The rate of inclusion depends on the type and weight of cattle with special use provisions in each scenario (Table 1.3) In an unpublished chemosterilization study of cattle naturally infected with *A. marginale*, circulating parasitemia levels were significantly decreased through the continuous feeding of CTC for 30 days at 4.4 mg/kg of bodyweight/day as well as weekly subcutaneous injections of a long-acting oxytetracycline at 20 mg/kg of bodyweight. A key finding in another study was the percentage of ticks infected during feeding is related to the circulating parasitemia (Eriks et al., 1989; Eriks et al., 1993). Therefore, the interaction between chlortetracycline-suppressed parasitemia in carrier cattle and the transmission of *A. marginale* deserves further attention.

Regardless, these chemoprohylaxis practices may have the potential to inadvertently disrupt endemic stability by chemosterilizing infected cattle and select for or facilitate the distribution of resistance determinants in bacterial species present (Stevens et al., 1993). However, these hypotheses were not substantiated by the findings of the unpublished study or by others. A significant change in antimicrobial susceptibility of enteric bacteria has been reported

in cattle fed chlortetracycline in three consecutive, five day pulse treatment regimens at 22 mg/kg (Platt et al., 2008). However, these values returned to pre-exposure levels within 33 days. Due to length of the vector season, it is unknown if a similar return to pre-exposure levels would be observed when the duration of therapy is more extensive. The development and liberal application of improved animal husbandry practices, such as needle-free injection during vaccination (Chapter 3), as well as establishment of an endemically stable herd (Figueroa et al., 1998), could considerably reduce the need for tetracycline antibiotics when managing anaplasmosis in cattle.

Animal husbandry

The introduction of anaplasmosis into a previously naïve herd may lead to a 3.6% reduction in calf crop due to abortion, a 30% increase in cull rate from a loss of production characterized by herd performance benchmarks, and a 30% mortality rate in acutely infected cattle (Alderink and Dietrich, 1982). Current estimates of the realized economic loss of this scenario are unknown. However, this was estimated to be over $400 per animal over 3 decades ago (Alderink and Dietrich, 1982; Goodger et al., 1979).

The repeated inter-animal use of hypodermic needles is not recommended. The needle-free injection technique described in Chapter 3 has been shown to prevent the iatrogenic transmission of *A. marginale*. However, this system may not fit into every production system. Therefore, there are other techniques for preventing iatrogenic transmission. These include the use and exchange of single use needles and cleansing of needles via insertion into foam impregnated with biocide. However, these disease prevention strategies associated with vaccination have been considered impractical, uneconomical or are potentially deleterious to the success of the procedure (Andrews and Lamport, 1985; Makoschey and Beer, 2004).

Susceptibility of cattle by species

Breed differences are reported for peak change in packed cell volume, parasitemia level, and tick susceptibility (Bock et al., 1999; Jonsson et al., 2008; Wilson et al., 1980). However, all cattle are susceptible to infection with *A. marginale*. Serologic survey studies based upon the complement fixation test have reported a significantly greater prevalence of reactors in *Bos taursus* cattle than *Bos indicus* (Rogers and Shiels, 1979). Furthermore, prevalence was significantly influenced by age, stocking density (Rodriguez-Vivas et al., 2004), and level of *B.*

microplus tick infestation. However, sex of the animal had no influence on prevalence. These findings may be biased by an over-representation of *Bos taurus*–type cattle that are more susceptible to *B. microplus* infestation. Additionally, higher stocking densities are associated with higher prevalence and increased chance of transfer of developing ticks

Endemic stability

In the Unites States, 48 states are endemic for anaplasmosis. Current national disease prevalence is unknown. However, seroprevalence has been previously established in California (15% in 1989), Illinois (10.7% in 1986), Louisiana (11.2% in 1989), Montana (1.35% in 2003), Oklahoma (9.2% in 1991), and Texas (0.3% in 1982) (Alderink and Dietrich, 1982; Behymer et al., 1991; Hungerford and Smith, 1997; Morley and Hugh-Jones, 1989b; Rodgers et al., 1994; Van Donkersgoed et al., 2006). These seroprevalence reports may or may not be accurate. Nonetheless, the key point is that the vast majority of cattle located in these regions are susceptible to infection. Reports of seropositive status following colostrum ingestion by suckling beef calves is as high as 82% (Maas et al., 1986). Even though carrier cows may be protected from clinical disease, colostral antibody ingestion by calves was not protective in seropositive, splenectomized neonatal calves subinoculated with 5 mL of whole blood collected from a carrier cow (Zaugg and Kuttler, 1984). The ingestion of colostral antibodies only lengthened the prepatent period and delayed both the climax of parasitemia and onset of anemia. Therefore, anti-anaplasma antibodies provide no lasting protection in suckling calves. If infection was established early in the life of cattle, an inapparent infection would occur with lifelong, acquired immunity. Over time, this would prevent clinical disease and the subsequent economic losses endured by the cattle industry.

A major disadvantage is that an endemically stable herd does not permit the comingling of cattle of unknown disease status or reduce trade restrictions between endemic and non-endemic countries. Furthermore, the establishment of an endemically stable herd would have no effect on tick vectors. The feeding of ticks on *A. marginale*-immune cattle does not affect tick infection rates (Kocan et al., 1996). Finally, it should be noted that wild ruminants are reservoirs of *Anaplasma* spp. (Arens et al., 2003; Kuttler, 1984; Maas et al., 1981; Smith et al., 1982). Due to the potential interaction of domestic cattle with ticks that have previously fed on wild

ruminants, sporadic outbreaks of disease may still occur in anaplasmosis-free herds. The occurrence of this scenario may be sparse, but it should not be ignored.

Figures and Tables

Figure 1.1 Elements of an anaplasmosis control program.
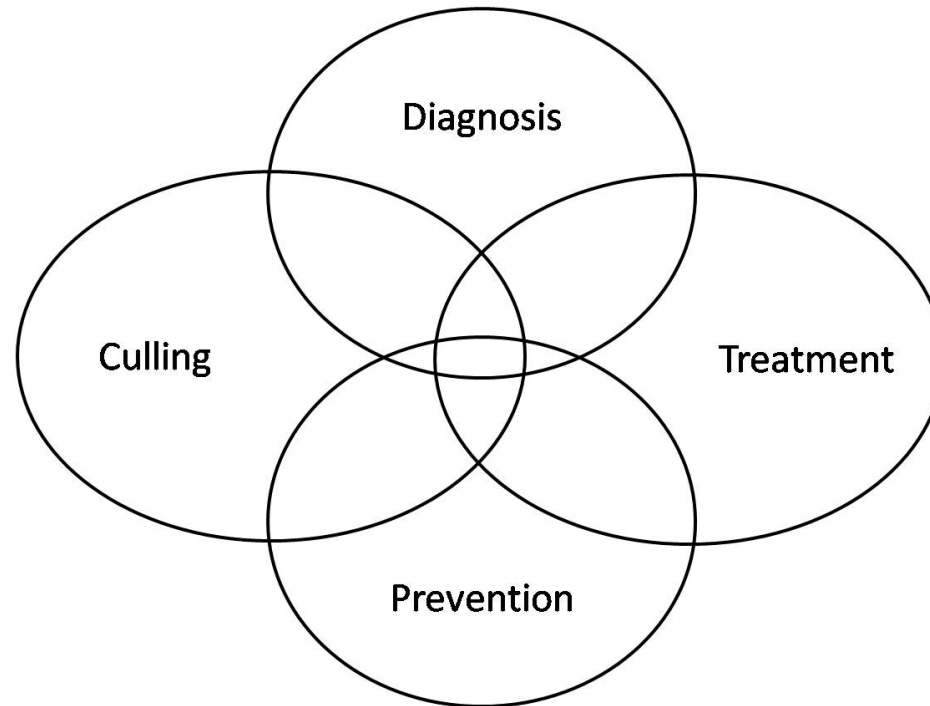
Table 1.1 Differential diagnosis list of diseases associated with similar presenting clinical signs as *Anaplasma marginale* and *Anaplasma phagocytophilum* (Merck, 2008; Radostits et al., 2000).

A. marginale		*A. phagocytohilum*	
anemia	babesiosis	lymphadenopathy	bovine leukemia virus
	blood transusion reaction		lymphosarcoma
	cold water hemolysis		tuberculosis
	drug-induced		actinobacillosis
	leptospirosis		
	theileriasis	respiratory disease	infectious bovine rhinotracheitis
			parainfluenza
icterus	bacillary hemoglobinuria		bovine viral diarrhea virus
	hepatotoxic plant poisoning		bovine respiratory syncitial virus
	leptospirosis		allergic rhinitis

Table 1.2 Comparison of clinical signs observed with anaplasmosis caused by *Anaplasma marginale* and *Anaplasma phagocytophilum* (Merck, 2008; Pusterla and Braun, 1997; Radostits et al., 2000).

	Clinical sign	Pathogen	
		A. marginale	*A. phagocytophilum*
Subjective	mucous membrane pallor	Y	N
	muscle weakness	Y	Y
	depression	Y	Y
	anorexia	Y	Y
	respiratory distress	Y	N
	irritable	Y	N
	grossly thin and watery blood	Y	N
	icterus	Y	N
	nasal discharge	N	Y
	abnormal lung sounds	N	Y
	cough	N	Y
	abnormal locomotion	N	Y
Objective	pyrexia	up to 41°C	up to 41°C
	dehydration	Y	Y
	tachycardia	Y	Y
	tachypnea	Y	Y
	lymphadenopathy	N	Y
	hematocrit change	PCV 5-15%	PCV < 28%
	hemoglobinuria	N	Y
	total RBC count (10/mm)	1.5-4.0	N/A
	bilirubin (mg/100 mL)	0.25-7.0	N/A
	decreased milk production	Y	Y
	abortion	all trimesters	3rd trimester
	treatment indicated	Y	N
	mastitis	Y	Y
	limb edema	N	Y

Table 1.3 Labeled indications and rate of inclusion of chlortetracycline in free-choice cattle feeds in the United States (2009 Feed Additive Compendium).

Dose	Cow type	Chlortetracycline inclusion rate
1	beef cattle <318.2 kg[a]	350 mg/hd/day
2	beef cattle >318.2 kg[a]	1.1 mg/kg of bodyweight/day
3	beef and non-lactating dairy cattle[b]	1.1 - 4.4 mg/kg of bodyweight/day
4	cattle <318.2 kg fed in confinement for slaughter[c]	25-100 g/ton of feed with 10 - 30 g/ton of Lasalocid
5	beef cattle <318.2 kg fed in confinement for slaughter[d]	25-42.2 g/ton of feed with 25 - 30 g/ton of Lasalocid
6	beef cattle <318.2 kg[e]	25 - 2,800 g/ton with 30 - 181.8 g/tom of Lasalocid
7	Pasture cattle <318.2 kg (slaughter, stocker, feeder cattle, beef replacement heifers)[f]	25-700 g/ton of feed with 30 - 600 g/ton of Lasalocid
8	Pasture cattle >318.2 kg (slaughter, stocker, feeder cattle, beef replacement heifers)[f]	25-1,100 g/ton of feed with 30 - 600 g/ton of Lasalocid

| 9 | Pasture cattle (slaughter, stocker, feeder cattle, beef replacement heifers)[g] | 500 - 4,000 g/ton of feed with 30 - 600 g/ton of Lasalocid |

[a]Note: Withdrawal times vary by manufacturer. Free-choice feed must be manufactured under a feed mill license using an FDA-approved formulation. [b]Limitations: In free-choice cattle feeds such as feed blocks or sal-mineral mixes; Free-choice feed must be manufactured from Aureomycin Type A medicated articles, under a feed mill license using an FDA-approved formulation. [c]Limitations for use: Feed continuously in complete feed at a rate of 350 mg CTC and not less than 100 mg nor more than 360 mg of lasalocid sodium activity per head per day. [d]Limitations for use: Feed continuously in complete feed at a rate of 350 mg CTC and not less than 250 mg nor more than 360 mg of lasalocid sodium activity per head per day. [e]Limitations for use: Hand feed continuously at a rate of 350 mg CTC per head per day and 1 mg of lasalocid per 4.4 kg bodyweight per day with a maximum of 360 mg lasalocid per head per day. [f]Limitations for use: Hand feed continuously at a rate of 350 mg CTC and not less than 60 mg nor more than 300 mg of lasalocid sodium activity per head per daily in atleast 2.2 kg of feed. [g]Limitations for use: Hand feed continuously for not more than 5 days to provide 4.5 mg CTC per kg bodyweight per day and not less than 60 mg nor more than 300 mg of lasalocid sodium activity per head per daily in at least 2.2 kg of feed.

References

2009 Feed Additive Compendium, Chlortetracycline. *Feedstuffs* 46, 223-229.

Akinboade, O.A., Dipeolu, O.O., 1984. Comparison of blood smear and indirect fluorescent antibody techniques in detection of haemoparasite infections in trade cattle in Nigeria. *Vet Parasitol* 14, 95-104.

Alderink, F.J., Dietrich, R.A., 1982. Economic and epidemiological implications of anaplasmosis in Texas beef cattle herds. *86th Annual Meeting of the United States Animal Health Association*, 66-75.

Alfonso, J., Medina, R., Fazzino, F., Caballero, H., 1996. Clinical and hematological changes in calves infected with *Anaplasma marginale*. *Acta Cient Venez* 47, 50-57.

Amerault, T.E., Roby, T.O., 1968. A rapid card agglutination test for bovine anaplasmosis. *J Am Vet Med Assoc* 153, 1828-1834.

Anderson, D.E., Silviera, F., 2008. Survey of Large Animal Veterinarians' Biosecurity Practices. *Proceedings 41st Annual Convention AABP* in Charlotte, NC, 244.

Andrews, A.H., Lamport, A., 1985. A practical method of reducing spread of disease by hypodermic needles. *Vet Rec* 116, 185-186.

Arens, M.Q., Liddell, A.M., Buening, G., Gaudreault-Keener, M., Sumner, J.W., Comer, J.A., Buller, R.S., Storch, G.A., 2003. Detection of *Ehrlichia* spp. in the blood of wild white-tailed deer in Missouri by PCR assay and serologic analysis. *J Clin Microbiol* 41, 1263-1265.

Barry, D.N., Parker, R.J., De Vos, A.J., Dunster, P., Rodwell, B.J., 1986. A microplate enzyme-linked immunosorbent assay for measuring antibody to *Anaplasma marginale* in cattle serum. *Aust Vet J* 63, 76-79.

Brayton, K.A., Kappmeyer, L.S., Herndon, D.R., Dark, M.J., Tibbals, D.L., Palmer, G.H., McGuire, T.C., Knowles, D.P., Jr., 2005. Complete genome sequencing of *Anaplasma marginale* reveals that the surface is skewed to two superfamilies of outer membrane proteins. *Proc Natl Acad Sci USA* 102, 844-849.

Behymer, D.E., Riemann, H.P., Utterback, W., C, D.E., Franti, C.E., 1991. Mass screening of cattle sera against 14 infectious disease agents, using an ELISA system for monitoring health in livestock. *Am J Vet Res* 52, 1699-1705.

Bekker, C.P., de Vos, S., Taoufik, A., Sparagano, O.A., Jongejan, F., 2002. Simultaneous detection of *Anaplasma* and *Ehrlichia* species in ruminants and detection of *Ehrlichia ruminantium* in *Amblyomma variegatum* ticks by reverse line blot hybridization. *Vet Microbiol* 89, 223-238.

Bird, J.E., 1973. Neonatal anaplasmosis in a calf. *J S Afr Vet Med Assoc* 44, 69-70.

Bock, R.E., Kingston, T.G., De Vos, A.J., 1999. Effect of breed of cattle on innate resistance to infection with *Anaplasma marginale* transmitted by *Boophilus microplus*. *Aust Vet J* 77, 748-751.

Boulanger, P., Bannister, G.L., Avery, R.J., Gray, D.P., Barrett, B.B., Ruckerbauer, G.M., 1966. Anaplasmosis: comparison of complement fixation methods and study of the cattle population of southern Alberta. *Can J Comp Med Vet Sci* 30, 102-106.

Bradley, B.D., Allen, E.H., Showalter, D.H., Colaianne, J.J., 1982. Comparative pharmacokinetics of chlortetracycline in milk-fed versus conventionally fed calves. *J Vet Pharmacol Ther* 5, 267-278.

Brock, W.E., Pearson, C.C., Kliewer, I.O., 1959. Anaplasmosis control by test and subsequent treatment with chlortetracycline. *Proc US Livestock San Assoc* 62, 66-70.

Brock, W.E., Pearson, C.C., Staley, E.E., Kliewer, I.O., 1957. The prevention of anaplasmosis by feeding chlortetracycline. *J Am Vet Med Assoc* 130, 445-446.

CFIA, Canadian Food Inspection Agency. 2006. Consultation paper: An overview of anaplasmosis and assessing the risks for Canada (http://www.inspection.gc.ca/english/anima/heasan/disemala/anaplasmos/consult2007/indexe.shtml) (Nepean, Ontario Canada, Canadian Food Inspection Agency).

CLSI, 2008. *Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; Approved standard - Third edition*. CLSI document M31-A3. Table 2. 28, 65-72.

Coetzee, J.F., Apley, M.D., Kocan, K.M., Jones, D.E., 2006. Flow cytometric evaluation of selected antimicrobial efficacy for clearance of *Anaplasma marginale* in short-term erythrocyte cultures. *J Vet Pharmacol Ther* 29, 173-183.

Coetzee, J.F., Apley, M.D., Kocan, K.M., Rurangirwa, F.R., Van Donkersgoed, J., 2005. Comparison of three oxytetracycline regimens for the treatment of persistent *Anaplasma marginale* infections in beef cattle. *Vet Parasitol* 127, 61-73.

Coetzee, J.F., Schmidt, P.L., Apley, M.D., Reinbold, J.B., Kocan, K.M., 2007. Comparison of the complement fixation test and competitive ELISA for serodiagnosis of *Anaplasma marginale* infection in experimentally infected steers. *Am J Vet Res* 68, 872-878.

Correa, W.M., Correa, C.N., Gottschalk, A.F., 1978. Bovine abortion associated with *Anaplasma marginale*. *Can J Comp Med* 42, 227-228.

Courtney, J.W., Kostelnik, L.M., Zeidner, N.S., Massung, R.F., 2004. Multiplex real-time PCR for detection of *Anaplasma phagocytophilum* and *Borrelia burgdorferi*. *J Clin Microbiol* 42, 3164-3168.

de la Fuente, J., Ayoubi, P., Blouin, E.F., Almazan, C., Naranjo, V., Kocan, K.M., 2006. Anaplasmosis: focusing on host-vector-pathogen interactions for vaccine development. *Ann N Y Acad Sci* 1078, 416-423.

de la Fuente, J., Lew, A., Lutz, H., Meli, M.L., Hofmann-Lehmann, R., Shkap, V., Molad, T., Mangold, A.J., Almazan, C., Naranjo, V., Gortazar, C., Torina, A., Caracappa, S., Garcia-Perez, A.L., Barral, M., Oporto, B., Ceci, L., Carelli, G., Blouin, E.F., Kocan, K.M., 2005. Genetic diversity of *Anaplasma* species major surface proteins and implications for anaplasmosis serodiagnosis and vaccine development. *Anim Health Res Rev* 6, 75-89.

De Wall, D.T., 2000. Anaplasmosis control and diagnosis in South Africa. *Ann N Y Acad Sci* 916, 474-483.

Dennis, R.A., O'Hara, P.J., Young, M.F., Dorris, K.D., 1970. Neonatal immunohemolytic anemia and icterus of calves. *J Am Vet Med Assoc* 156, 1861-1869.

Dikmans, G., 1948. Anaplasmosis. *Abstr Int Congr Trop Med Malar* 56, 112.

Dreher, U.M., de la Fuente, J., Hofmann-Lehmann, R., Meli, M.L., Pusterla, N., Kocan, K.M., Woldehiwet, Z., Braun, U., Regula, G., Staerk, K.D., Lutz, H., 2005. Serologic cross-reactivity between *Anaplasma marginale* and *Anaplasma phagocytophilum*. *Clin Diagn Lab Immunol* 12, 1177-1183.

Dumler, J.S., Barbet, A.F., Bekker, C.P., Dasch, G.A., Palmer, G.H., Ray, S.C., Rikihisa, Y., Rurangirwa, F.R., 2001. Reorganization of genera in the families *Rickettsiaceae* and *Anaplasmataceae* in the order *Rickettsiales*: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and designation of *Ehrlichia equi* and 'HGE agent' as subjective synonyms of *Ehrlichia phagocytophila*. *Int J Syst Evol Microbiol* 51, 2145-2165.

Duzgun, A., Schuntner, C.A., Wright, I.G., Leatch, G., Waltisbuhl, D.J., 1988. A sensitive ELISA technique for the diagnosis of *Anaplasma marginale* infections. *Vet Parasitol* 29, 1-7.

Dyer, D.C., 1988. Pharmacokinetics of chlortetracycline in the turkey: evaluation of biliary secretion. *Am J Vet Res* 49, 36-37.

Eriks, I.S., Palmer, G.H., McGuire, T.C., Allred, D.R., Barbet, A.F., 1989. Detection and quantitation of *Anaplasma marginale* in carrier cattle by using a nucleic acid probe. *J Clin Microbiol* 27, 279-284.

Eriks, I.S., Stiller, D., Palmer, G.H., 1993. Impact of persistent *Anaplasma marginale* rickettsemia on tick infection and transmission. *J Clin Microbiol* 31, 2091-2096.

Ewing, S.A., 1981. Transmission of *Anaplasma marginale* by arthropods. *Proceedings of the Seventh National Anaplasmosis Conference*, 425-434.

Figueroa, J.V., Alvarez, J.A., Ramos, J.A., Rojas, E.E., Santiago, C., Mosqueda, J.J., Vega, C.A., Buening, G.M., 1998. Bovine babesiosis and anaplasmosis follow-up on cattle relocated in an endemic area for hemoparasitic diseases. *Ann N Y Acad Sci* 849, 1-10.

Franklin, T.E., Cook, R.W., Anderson, D.J., 1966. Feeding chlortetracycline to range cattle to eliminate the carrier state of anaplasmosis. *Proc US Livestock San Assoc* 66, 85-90.

Franklin, T.E., Cook, R.W., Anderson, D.J., Kuttler, K.L., 1967. Medium and low level feeding of chlortetracycline with comparisons to anaplasmosis CF and CA tests. *Southwest Vet* 20, 101-104.

Franklin, T.E., Huff, J.W., Grumbles, L.C., 1965. Chlortetracycline for elimination of anaplasmosis in carrier cattle. *J Am Vet Med Assoc* 147, 353-356.

French, D.M., McElwain, T.F., McGuire, T.C., Palmer, G.H., 1998. Expression of *Anaplasma marginale* major surface protein 2 variants during persistent cyclic rickettsemia. *Infect Immun* 66, 1200-1207.

Futse, J.E., Ueti, M.W., Knowles, D.P., Jr., Palmer, G.H., 2003. Transmission of *Anaplasma marginale* by *Boophilus microplus*: retention of vector competence in the absence of vector-pathogen interaction. *J Clin Microbiol* 41, 3829-3834.

Gale, K.R., Dimmock, C.M., Gartside, M., Leatch, G., 1996a. *Anaplasma marginale*: detection of carrier cattle by PCR-ELISA. *Int J Parasitol* 26, 1103-1109.

Gale, K.R., Leatch, G., DeVos, A.J., Jorgensen, W.K., 1996b. *Anaplasma marginale*: effect of challenge of cattle with varying doses of infected erythrocytes. *Int J Parasitol* 26, 1417-1420.

Ge, N.L., Kocan, K.M., Murphy, G.L., Blouin, E.F., 1995. Detection of *Anaplasma marginale* DNA in bovine erythrocytes by slot-blot and in situ hybridization with a PCR-mediated digoxigenin-labeled DNA probe. *J Vet Diagn Invest* 7, 465-472.

Goff, W.L., Johnson, W.C., Kuttler, K.L., 1985. Development of an indirect fluorescent antibody test, using microfluorometry as a diagnostic test for bovine anaplasmosis. *Am J Vet Res* 46, 1080-1084.

Goodger, W.J., Carpenter, T., Riemann, H., 1979. Estimation of economic loss associated with anaplasmosis in California beef cattle. *J Am Vet Med Assoc* 174, 1333-1336.

Greiner, M., Gardner, I.A., 2000. Epidemiologic issues in the validation of veterinary diagnostic tests. *Prev Vet Med* 45, 3-22.

Han, S., Norimine, J., Palmer, G.H., Mwangi, W., Lahmers, K.K., Brown, W.C., 2008. Rapid deletion of antigen-specific CD4+ T cells following infection represents a strategy of immune evasion and persistence for *Anaplasma marginale*. *J Immunol* 181, 7759-7769.

Hawkins, J.A., Love, J.N., Hidalgo, R.J., 1982. Mechanical transmission of anaplasmosis by tabanids (Diptera: Tabanidae). *Am J Vet Res* 43, 732-734.

Hoar, B.R., Nieto, N.C., Rhodes, D.M., Foley, J.E., 2008. Evaluation of sequential coinfection with *Anaplasma phagocytophilum* and *Anaplasma marginale* in cattle. *Am J Vet Res* 69, 1171-1178.

Hollis, L.C., Smith, J.F., Johnson, B.J., Kapil, S., Mosier, D.A., 2005. A comparison of serological responses when modified- live infectious bovine rhinotracheitis virus vaccine and *Mannheimia haemolytica* bacterin-toxoid are administered with needle-free versus conventional needle-based injection in yearling feedlot steers. *Bovine Practitioner* 39, 106-109.

Huang, Y., Babiuk, L.A., van Drunen Littel-van den Hurk, S., 2005. Immunization with a bovine herpesvirus 1 glycoprotein B DNA vaccine induces cytotoxic T-lymphocyte responses in mice and cattle. *J Gen Virol* 86, 887-898.

Hungerford, L.L., Smith, R.D., 1997. Variations in seroprevalence and host factors for bovine anaplasmosis in Illinois. *Vet Res Commun* 21, 9-18.

Jatkar, P.R., Kreier, J.P., 1969. Pathogenesis of anaemia in anaplasma infection II--Auto-antibody and anaemia. *Indian Vet J* 46, 560-566.

Jonsson, N.N., Bock, R.E., Jorgensen, W.K., 2008. Productivity and health effects of anaplasmosis and babesiosis on *Bos indicus* cattle and their crosses, and the effects of differing intensity of tick control in Australia. *Vet Parasitol* 155, 1-9.

Kieser, S.T., Eriks, I.S., Palmer, G.H., 1990. Cyclic rickettsemia during persistent *Anaplasma marginale* infection of cattle. *Infect Immun* 58, 1117-1119.

Kilroy, C.R., Hall, W.F., Bane, D.P., Bevill, R.F., Koritz, G.D., 1990. Chlortetracycline in swine--bioavailability and pharmacokinetics in fasted and fed pigs. *J Vet Pharmacol Ther* 13, 49-58.

Knowles, D., Torioni de Echaide, S., Palmer, G., McGuire, T., Stiller, D., McElwain, T., 1996. Antibody against an *Anaplasma marginale* MSP5 epitope common to tick and erythrocyte stages identifies persistently infected cattle. *J Clin Microbiol* 34, 2225-2230.

Kocan, K.M., Blouin, E.F., Barbet, A.F., 2000. Anaplasmosis control. Past, present, and future. *Ann N Y Acad Sci* 916, 501-509.

Kocan, K.M., Blouin, E.F., Palmer, G.H., Eriks, I.S., Edwards, W.L., Claypool, P.L., 1996. Preliminary studies on the effect of *Anaplasma marginale* antibodies ingested by *Dermacentor andersoni* ticks (Acari:Ixodidae) with their blood meal on infections in salivary glands. *Exp Appl Acarol* 20, 297-311.

Kocan, K.M., de la Fuente, J., Guglielmone, A.A., Melendez, R.D., 2003. Antigens and alternatives for control of *Anaplasma marginale* infection in cattle. *Clin Microbiol Rev* 16, 698-712.

Kuttler, K.L., 1980. Pharmacotherapeutics of drugs used in treatment of anaplasmosis and babesiosis. *J Am Vet Med Assoc* 176, 1103-1108.

Kuttler, K.L., 1983. Influence of a second *Anaplasma* exposure on the success of treatment to eliminate *Anaplasma* carrier infections in cattle. *Am J Vet Res* 44, 882-883.

Kuttler, K.L., 1984. Anaplasma infections in wild and domestic ruminants: a review. *J Wildl Dis* 20, 12-20.

Liu, Z., Luo, J., Bai, Q., Ma, M., Guan, G., Yin, H., 2005. Amplification of 16S rRNA genes of *Anaplasma* species in China for phylogenetic analysis. *Vet Microbiol* 107, 145-148.

Luther, D.G. 1999. anaplasmosisvaccine.com.

Luthman, J., Jacobsson, S.O., 1983. The availability of tetracycline in calves. *Nordisk veterinarmedicin* 35, 292-299.

Maas, J., Buening, G.M., Porath, W., 1981. Serologic evidence of *Anaplasma marginale* infection in white-tailed deer (*Odocoileus virginianus*) in Missouri. *J Wildl Dis* 17, 45-47.

Maas, J., Lincoln, S.D., Coan, M.E., Kuttler, K.L., Zaugg, J.L., Stiller, D., 1986. Epidemiologic aspects of bovine anaplasmosis in semiarid range conditions of south central Idaho. *Am J Vet Res* 47, 528-533.

Magonigle, R.A., Newby, T.J., 1984. Response of cattle upon reexposure to *Anaplasma marginale* after elimination of chronic carrier infections. *Am J Vet Res* 45, 695-697.

Makoschey, B., Beer, M., 2004. Assessment of the risk of transmission of vaccine viruses by using insufficiently cleaned injection devices. *Vet Rec* 155, 563-564.

Manoj, S., Griebel, P.J., Babiuk, L.A., van Drunen Littel-van den Hurk, S., 2004. Modulation of immune responses to bovine herpesvirus-1 in cattle by immunization with a DNA vaccine encoding glycoprotein D as a fusion protein with bovine CD154. *Immunology* 112, 328-338.

Merck, 2008. *The Merck Veterinary Manual, 9th Edition*. Merck & Co., Inc., Whitehouse Station, NJ.

Molad, T., Mazuz, M.L., Fleiderovitz, L., Fish, L., Savitsky, I., Krigel, Y., Leibovitz, B., Molloy, J., Jongejan, F., Shkap, V., 2006. Molecular and serological detection of *A. centrale-* and *A. marginale*-infected cattle grazing within an endemic area. *Vet Microbiol* 113, 55-62.

Morley, R.S., Hugh-Jones, M.E., 1989a. Seroepidemiology of *Anaplasma marginale* in white-tailed deer (*Odocoileus virginianus*) from Louisiana. *J Wildl Dis* 25, 342-346.

Morley, R.S., Hugh-Jones, M.E., 1989b. Seroprevalence of anaplasmosis in the Red River Plains and south-east areas of Louisiana. *Vet Res Commun* 13, 287-296.

NADA, 065-440. Freedom of Information Summary http://www.fda.gov/cvm/FOI/552.htm.

Nielsen, P., Gyrd-Hansen, N., 1996. Bioavailability of oxytetracycline, tetracycline and chlortetracycline after oral administration to fed and fasted pigs. *J Vet Pharmacol Ther* 19, 305-311.

Norton, J.H., Parker, R.J., Forbes-Faulkner, J.C., 1983. Neonatal anaplasmosis in a calf. *Aust Vet J* 60, 348.

OIE 2009. Office International des Épizooties, Manual of standards for diagnostic tests and vaccines for terrestrial animals. Chapter 2.4.1 17th ed. 2008. Accessed July 13, 2009. http://www.oie.int/eng/normes/mmanual/2008/pdf/2.04.01_BOVINE_ANAPLASMOSIS.pdf

Palmer, G.H., McElwain, T.F., 1995. Molecular basis for vaccine development against anaplasmosis and babesiosis. *Vet Parasitol* 57, 233-253.

Peter, R.J., Van den Bossche, P., Penzhorn, B.L., Sharp, B., 2005. Tick, fly, and mosquito control--lessons from the past, solutions for the future. *Vet Parasitol* 132, 205-215.

Platt, T.M., Loneragan, G.H., Scott, H.M., Norby, B., Thomson, D.U., Brown, M.S., Ives, S.E., Brashears, M.M., 2008. Antimicrobial susceptibility of enteric bacteria recovered from feedlot cattle administered chlortetracycline in feed. *Am J Vet Res* 69, 988-996.

Pollet, R.A., Glatz, C.E., Dyer, D.C., Barnes, H.J., 1983. Pharmacokinetics of chlortetracycline potentiation with citric acid in the chicken. *Am J Vet Res* 44, 1718-1721.

Potgieter, F., van Rensburg, L., 1987. The persistence of colostral *Anaplasma* antibodies and incidence of in utero transmission of *Anaplasma* infections in calves under laboratory conditions. *Onderstepoort J Vet Res* 54, 557-560.

Pusterla, N., Braun, U., 1997. Clinical findings in cows after experimental infection with *Ehrlichia phagocytophila. Zentralbl Veterinarmed* A 44, 385-390.

Pusterla, N., Huder, J., Wolfensberger, C., Braun, U., Lutz, H., 1997. Laboratory findings in cows after experimental infection with *Ehrlichia phagocytophila. Clin Diagn Lab Immunol* 4, 643-647.

Pusterla, N., Huder, J.B., Leutenegger, C.M., Braun, U., Madigan, J.E., Lutz, H., 1999. Quantitative real-time PCR for detection of members of the *Ehrlichia phagocytophila* genogroup in host animals and *Ixodes ricinus* ticks. *J Clin Microbiol* 37, 1329-1331.

Radostits, O.M., Gay, C.C., Blood, D.C., Hinchcliff, K.W., 2000. *Veterinary Medicine: A textbook of the diseases of cattle, sheep, pigs, goats and horses, 9th Edition.* W. B. Saunders Company Ltd, St. Louis.

Reeves, J.D., 3rd, Swift, B.L., 1977. Iatrogenic transmission of *Anaplasma marginale* in beef cattle. *Vet Med Small Anim Clin* 72, 911-914.

Reinbold, J.B., Coetzee, J.F., Gehring, R., Havel, J.A., Hollis, L.C., Olson, K.C., Apley, M.D., 2009a. Plasma pharmacokinetics of oral chlortetracycline in group fed, ruminating, Holstein steers in a feedlot setting. *J Vet Pharm Ther* 9999.

Reinbold, J.B., Coetzee, J.F., Hollis, L.C., Nickell, J.S., Riegel, C., Christopher-Huff, J., Ganta, R.R., 2009b. Comparison of iatrogenic *Anaplasma marginale* transmission by needle and needle-free injection techniques. *Am J Vet Res* (In press: AJVR-09-07-0279).

Reinbold, J.B., Coetzee, J.F., Hollis, L.C., Nickell, J.S., Riegel, C., Olson, K.C., Ganta, R.R., 2009c. Establishment of the *in vivo* pharmacokinetic-pharmacodynamic relationship between chlortetracycline and anaplasmosis carrier clearance using three oral treatment regimens *Vet Microbiol* Submitted.

Renshaw, H.W., Magonigle, R.A., Eckblad, W.P., Frank, F.W., 1976. Immunity to bovine anaplasmosis after elimination of the carrier status with oxytetracycline hydrochloride. *Proc Ann Meet U S Anim Health Assoc*, 79-88.

Richey, E.J., 1991. *Bovine anaplasmosis. In: The 24th Annual Convention of Proceedings of American Association of Bovine Practices*, Orlando, Florida, pp. 3-11.

Richey, E.J., Brock, W.E., Kliewer, I.O., Jones, E.W., 1977. Low levels of chlortetracycline for anaplasmosis. *Am J Vet Res* 38, 171-172.

Rikihisa, Y., 1991. The tribe *Ehrlichieae* and ehrlichial diseases. *Clin Microbiol Rev* 4, 286-308.

Rodgers, S.J., Welsh, R.D., Stebbins, M.E., 1994. Seroprevalence of bovine anaplasmosis in Oklahoma from 1977 to 1991. *J Vet Diagn Invest* 6, 200-206.

Rodriguez-Vivas, R.I., Mata-Mendez, Y., Perez-Gutierrez, E., Wagner, G., 2004. The effect of management factors on the seroprevalence of *Anaplasma marginale* in *Bos indicus* cattle in the Mexican tropics. *Trop Anim Health Prod* 36, 135-143.

Rogers, R.J., Shiels, I.A., 1979. Epidemiology and control of anaplasmosis in Australia. *J S Afr Vet Assoc* 50, 363-366.

Schuntner, C.A., Leatch, G., 1988. Radioimmunoassay for *Anaplasma marginale* antibodies in cattle. *Am J Vet Res* 49, 504-507.

Scoles, G.A., Broce, A.B., Lysyk, T.J., Palmer, G.H., 2005. Relative efficiency of biological transmission of *Anaplasma marginale* (*Rickettsiales: Anaplasmataceae*) by *Dermacentor andersoni* (*Acari: Ixodidae*) compared with mechanical transmission by *Stomoxys calcitrans* (*Diptera: Muscidae*). *J Med Entomol* 42, 668-675.

Scoles, G.A., Miller, J.A., Foil, L.D., 2008. Comparison of the efficiency of biological transmission of *Anaplasma marginale* (*Rickettsiales: Anaplasmataceae*) by *Dermacentor andersoni* Stiles (*Acari: Ixodidae*) with mechanical transmission by the horse fly, *Tabanus fuscicostatus* Hine (*Diptera: Muscidae*). *J Med Entomol* 45, 109-114.

Shkap, V., Bin, H., Ungar-Waron, H., Pipano, E., 1990. An enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies to *Anaplasma centrale* and *Anaplasma marginale*. *Vet Microbiol* 25, 45-53.

Sirigireddy, K.R., Ganta, R.R., 2005. Multiplex detection of *Ehrlichia* and *Anaplasma* species pathogens in peripheral blood by real-time reverse transcriptase-polymerase chain reaction. *J Mol Diagn* 7, 308-316.

Smith, R.D., Woolf, A., Hungerford, L.L., Sundberg, J.P., 1982. Serologic evidence of *Anaplasma marginale* infection in Illinois white-tailed deer. *J Am Vet Med Assoc* 181, 1254-1256.

Stevens, A.M., Shoemaker, N.B., Li, L.Y., Salyers, A.A., 1993. Tetracycline regulation of genes on *Bacteroides* conjugative transposons. *J Bacteriol* 175, 6134-6141.

Stiller, D., Coan, M.E., 1995. Recent developments in elucidating tick vector relationships for anaplasmosis and equine piroplasmosis. *Vet Parasitol* 57, 97-108.

Strik, N.I., Alleman, A.R., Barbet, A.F., Sorenson, H.L., Wamsley, H.L., Gaschen, F.P., Luckschander, N., Wong, S., Chu, F., Foley, J.E., Bjoersdorff, A., Stuen, S., Knowles, D.P., 2007. Characterization of *Anaplasma phagocytophilum* major surface protein 5 and the extent of its cross-reactivity with *A. marginale*. *Clin Vaccine Immunol* 14, 262-268.

Sweat, J.M., Abdy, M., Weniger, B.G., Harrington, R., Coyle, B., Abuknesha, R.A., Gibbs, E.P., 2000. Safety testing of needle free, jet injection devices to detect contamination with blood and other tissue fluids. *Ann N Y Acad Sci* 916, 681-682.

Tebele, N., McGuire, T.C., Palmer, G.H., 1991. Induction of protective immunity by using *Anaplasma marginale* initial body membranes. *Infect Immun* 59, 3199-3204.

Torioni de Echaide, S., Knowles, D.P., McGuire, T.C., Palmer, G.H., Suarez, C.E., McElwain, T.F., 1998. Detection of cattle naturally infected with *Anaplasma marginale* in a region of endemicity by nested PCR and a competitive enzyme-linked immunosorbent assay using recombinant major surface protein 5. *J Clin Microbiol* 36, 777-782.

Twiehaus, M.J., 1962. Control of anaplasmosis by feeding an antibiotic (Aureomycin). *Proc 4th Natl Anaplasmosis Conf*, 48-49.

Uilenberg, G., 1995. International collaborative research: significance of tick-borne hemoparasitic diseases to world animal health. *Vet Parasitol* 57, 19-41.

Valdez, R.A., McGuire, T.C., Brown, W.C., Davis, W.C., Jordan, J.M., Knowles, D.P., 2002. Selective in vivo depletion of CD4(+) T lymphocytes with anti-CD4 monoclonal antibody during acute infection of calves with *Anaplasma marginale*. *Clin Diagn Lab Immunol* 9, 417-424.

Van Donkersgoed, J., Linfield, T.F., Bridges, M., Raths, D., Dargatz, D., Wagner, B., Block, J., Knoop, D., Walton, T.E., 2006. Prevalence of antibodies to serotypes of Bluetongue virus and *Anaplasma marginale* in Montana feeder cattle: 2002-2003. *Can Vet J* 47, 692-694.

VMRD-2. Product information sheet: Anaplasma antibody test kit, cELISA (www.vmrd.com/docs/tk/Anaplasma/Anaplasma_Flyer_050113.pdf) (Veterinary Medical Research & Development (VMRD), Inc.).

VMRD. Anaplasma antibody test kit, cELISA. (Veterinary Medical Research & Development (VMRD), Inc.), pp. Assay instructions for catalog numbers: 282-282 and 282-285. USDA Product Code 5002.5020 For veterinary use only.

Vredevoe, L. Background information on the biology of ticks (http://entomology.ucdavis.edu/faculty/rbkimsey/tickbio.html).

Wanner, M., Walker, W., Sutter, H.M., Riond, J.L., Broz, J., 1991. Influence of dietary citric acid and calcium on the bioavailability of orally administered chlortetracycline in piglets. *Zentralbl Veterinarmed A* 38, 755-762.

Wilson, A.J., Parker, R., Trueman, K.F., 1980. Susceptibility of *Bos indicus* crossbred and *Bos taurus* cattle to *Anaplasma marginale* infection. *Trop Anim Health Prod* 12, 90-94.

Winkler, G.C., Brown, G.M., Lutz, H., 1987. Detection of antibodies to *Anaplasma marginale* by an improved enzyme-linked immunosorbent assay with sodium dodecyl sulfate-disrupted antigen. *J Clin Microbiol* 25, 633-636.

Zarlenga, D.S., Higgins, J., 2001. PCR as a diagnostic and quantitative technique in veterinary parasitology. *Vet Parasitol* 101, 215-230.

Zaugg, J.L., 1985. Bovine anaplasmosis: transplacental transmission as it relates to stage of gestation. *Am J Vet Res* 46, 570-572.

Zaugg, J.L., Kuttler, K.L., 1984. Bovine anaplasmosis: in utero transmission and the immunologic significance of ingested colostral antibodies. *Am J Vet Res* 45, 440-443.

CHAPTER 2 - Duplex detection of *Anaplasma marginale* and *Anaplasma phagocytophilum* in a bovine peripheral blood sample by real-time reverse transcriptase-polymerase chain reaction

Submitted for publication in the Journal of Clinical Microbiology (December 2009)

Introduction

Many species of the genus *Anaplasma* cause bovine anaplasmosis. The Office International des Epizooties (OIE) Animal Health Code categorizes anaplasmosis as a notifiable disease due to socioeconomic impact and international trade restrictions (OIE, 2009). However, the significance of anaplasmosis is frequently underestimated due to seasonal outbreaks and stability in endemic areas. Anaplasmosis, caused by *Anaplasma marginale*, is one of the most prevalent tick-transmitted, rickettsial diseases of cattle worldwide (Kocan et al., 2003). Vaccination with *A. centrale* is a common practice used to reduce disease morbidity in cattle subsequently infected with *A. marginale* (Molad et al., 2006). Infection with one *Anaplasma* spp. does not confer immunity from infection with other *Anaplasma* spp. Co-infection with two or more *Anaplasma* spp. occur in cattle due to ubiquitous disease susceptibility and animal husbandry practices such as vaccination with *A. centrale*.

Although *Anaplasma marginale* causes significant economic and health impacts in infected cattle worldwide, *A. phagocytophilum* also causes self-limiting, economically significant disease in cattle within 9 days post-infection (Hoar et al., 2008; Pusterla and Braun, 1997; Pusterla et al., 1999). Cattle that survive acute disease become subclinical carriers of *Anaplasma* spp. and serve as reservoirs of infection for naïve cattle in spite of vaccination practices and treatment (Coetzee et al., 2006; Coetzee et al., 2005; Kocan et al., 2003). In the absence of effective treatment strategies, vaccine availability, and problematic vector control, anaplasmosis control strategies are primarily focused on disease identification and prevention (Reinbold et al., 2009a).

Disease prevention strategies are centered on reliable diagnostic testing strategies for accurately and precisely identifying infected cattle. The subinoculation of whole blood into splenectomized cattle has served as the gold standard for anaplasmosis disease classification

caused by *A. marginale.* Currently, one of the most common diagnostic techniques used in commercial lab settings today, cELISA, relies upon the identification of bovine anti-major surface protein 5 (MSP-5) antibodies that recognize the MSP5 protein epitope of *A. marginale* (Knowles et al., 1996). Due to the establishment of a carrier state post-infection, the cELISA is regarded as a reliable screening test for identifying *A. marginale*-infected cattle. However, cross-reactivity among *Anaplasma* spp. has been reported when using cELISA to classify cattle infected with *A. centrale* and *A. phagocytophilum* (Bradway et al., 2001; Dreher et al., 2005; Strik et al., 2007). Additionally, the lag time between infection and anti-MSP5 antibody response may allow for the misclassification of cattle peracutely-infected with anaplasmosis (Coetzee et al., 2007; Reinbold et al., 2009a).

The selection of an appropriate target for the accurate and precise diagnosis of disease is critical for the development of a robust diagnostic method. Due to their role in the translation of genetic code, ribosomes, as well as ribosomal RNA, are present in high copy numbers when compared to a single copy of DNA. The extensive conservation of the primary and secondary structures of rRNA implies an ancient origin of these macromolecules (Gutell et al., 1985). The 16S rRNA gene segment is a common structure of bacterial rRNA genes of the genus *Anaplasma* (Rurangirwa et al., 2002). The 16S rRNA gene sequence has been shown to be identical among isolates of *A. marginale* (Dame et al., 1992; Dumler et al., 2001). However, sequence analysis has shown there are highly conserved and specific regions of the 16S rRNA gene segment of the family *Anaplasmataceae* (Sirigireddy and Ganta, 2005).

In this study, methods are described for the development of simplex real-time qRT-PCR assays for identifying 16S rRNA gene sequences of A. marginale and A. phagocytophilum and a duplex real-time qRT-PCR assay for the simultaneous detection 16S rRNA gene sequences of A. marginale and A. phagocytophilum in plasma-free bovine peripheral blood samples. The duplex real-time qRT-PCR assay and a cELISA were also used to screen field samples from cattle originating from anaplasmosis endemic herds. Finally, the results of the duplex assay were used to evaluate the cELISA as an anaplasmosis diagnostic tool.

Materials and Methods

This study was approved by the Kansas State University (KSU) Institutional Animal Care and Use Committee (protocol #2517) and Institutional Biosafety Committee (protocol #524).

36

Primer and probe design for the RT-PCR assays

The 16S rRNA gene sequences for *A marginale* and *A. phagocytophilum* were previously downloaded from the GenBank nucleotide sequence database and aligned using the University of Wisconsin Genetic Computer Group program, Pileup and Pretty (Sirigireddy and Ganta, 2005). Genera-specific regions were identified for the design of PCR primers. Species-specific regions were used to design TaqMan probes in real-time pathogen detection. TaqMan probes were designed with specific fluorescent reporter dyes and quencher molecules to facilitate the duplex assay (Table 1).

A. marginale and A. phagocytophilum positive control plasmids

A whole blood sample collected from a cow infected with the Florida isolate of *A. marginale* was previously stored at -80°C in a 50% glycerol solution at a ratio of 2 parts blood:1part glycerol. Genomic DNA was isolated from 300 µL of this sample using a kit (Puregene DNA purification kit, Gentra Systems; Minneapolis, MN) as described by the manufacturer's recommendations. The isolated DNA pellet was re-hydrated with 100 µL of the kit-supplied DNA hydration solution and stored at -80°C.

Genomic DNA of *A. marginale* was used as the template to amplify a 0.48-kb 16S rRNA gene segment. The PCR was performed with 200 ng of genomic DNA using the AmpliTaqPCR reagent kit (Applied Biosystems; Foster City, CA). Thermal cycles were defined by an initial denaturation cycle for 3 minutes at 94°C, 45 cycles of 94°C for 30 seconds, 52°C for 30 seconds, and 72°C for 3 minutes. The PCR product was resolved on a 1% agarose gel in 1X Tris-acetate EDTA buffer (40mmol/L Tris-acetate, 1mmol/L EDTA, pH 8.0) containing 0.1 µg/mL of ethidium bromide and visualized under UV light (Sambrook and Russell, 2001a). The amplicon was ligated into chemically competent *Eschirichia coli* using a cloning kit (TOPO TA Cloning-TOP10, pCR 2.1-TOPO version U, Invitrogen Corp.; Carlsbad, CA). The *E. coli* were streaked on ampicillin-impregnated (100µg/mL) LB media plates with 20 µL of kanamycin (50 µg/mL) applied to the plate surface. Transformants containing the *A. marginale* recombinant plasmid were isolated and propagated anaerobically at 37°C in an LB media solution containing kanamycin (50 µg/mL). Glycerol stock (Sambrook and Russell, 2001b) preserved transformants of an *A.* phagocytophilum positive control plasmid were used in the development of this method as previously described (Sirigireddy and Ganta, 2005). Similarly, transformants of the *A.*

phagocytophilum plasmid were re-established by growth in the LB media solution. A boiling preparation method (Sambrook and Russell, 2001a) was used to extract plasmid DNA from the transformants. Plasmid DNA was linearized with a restriction enzyme digest using *SpeI* and *BamHI* for *A. marginale* and *A. phagocytophilum*, respectively (Sambrook and Russell, 2001a).The sites for these restriction enzymes are located at the 3' end of the insert in the multiple-cloning site region of the plasmids. Each restriction enzyme was chosen due to the absence of a recognition sequence within the inserts. This allowed the plasmids to linearize downstream to the inserts and facilitated synthesis of *in vitro* transcripts with a T7 polymerase. The *A. marginale* plasmid insert was verified by sequencing with a thermo-sequencing reaction kit according to the manufacturer's recommendations (USB Corp., Cleveland, OH).

In vitro transcripts

In vitro transcripts were prepared for use in the quantitative RT-PCR (qRT-PCR) assay development according to the following procedures. Linearized inserts of *A. marginale* and *A. phagocytophilum* were purified with a phenol-chloroform extraction technique (Sambrook and Russell, 2001b). Two micrograms of each purified linear insert was used to generate recombinant transcripts with a T7 polymerase kit as recommended by the manufacturer (MEGAscript kit, Ambion, Inc.; Austin, TX). The recombinant *in vitro* transcripts were purified free of plasmid DNA by treating with DNAseI and using an RNA purification kit (MEGAclear kit, Ambion, Inc.; Austin, TX).

Real-Time qRT-PCR

TaqMan-based real-time amplification (Huang et al., 2005; Manoj et al., 2004) was performed using a Smart Cycler II system (Cepheid; Sunnyvale, CA). Simplex and duplex real time quantitative qRT-PCR assays were developed using previously designed forward and reverse primers (Sirigireddy and Ganta, 2005) and TaqMan probes designed as a part of this study (Table 1). A commercially available RT-PCR assay kit (SuperScript III Reverse Transcriptase, Invitrogen Corporation; Carlsbad, CA) was used for development of the simplex and duplex assays. Ten-fold serial dilutions made from known quantities of the *in vitro* transcripts (ranging from 1 billion to one molecule) were analyzed in triplicate to optimize these assays for species specificity and duplex pathogen detection. The temperature cycles used for the qRT-PCR assay were: an initial cDNA generation cycle at 48°C for 30 minutes, 3 minutes at

94°C followed by 45 cycles of 94°C for 15 seconds, 50°C for 30 seconds, and 60°C for 60 seconds. The RT-PCR product formation was monitored in real-time by measuring the emitted fluorescence associated with exponential growth of the PCR product during the log-linear phase. A reaction was qualified as positive for the presence of a template when seven fluorescent units for the emission channel of the fluorescent probe were detected. The PCR cycle at which fluorescence occurs, which was template concentration-dependent in the reaction, was regarded as the cycle threshold (Ct) value. Linear regression will be used to correlate the reported Ct value to the number of 16S rRNA template molecules in the 25 µL reaction.

Evaluation of blood samples from anaplasmosis endemic herds

Field sample collection was arranged during phone consultation with producers seeking recommendations for the control of anaplasmosis. The collection of samples from anaplasmosis endemic herds resulted in the screening of 237 cattle from northeastern and southeastern Kansas during September and October of 2008. These samples were collected from adult cows and bulls of *Bos taurus* influence. Samples were collected in no-additive evacuated tubes and evacuated tubes containing EDTA during 2008. Typically, the samples were collected on-site, transported on ice, and processed within 48 hours. However, some samples were shipped overnight on ice packs and processed within 48 hours of receipt.

Anaplasmosis cELISA

Serum was collected from no-additive evacuated tubes and analyzed by cELISA. A commercially available cELISA (Anaplasma Antibody Test Kit, cELISA, VMRD Inc.; Pullman, WA) was used in accordance with the method described by the OIE and recommended by the manufacturer (OIE, 2009; VMRD). The optical density (OD) of each well was measured by use of an ELISA plate reader at a wavelength of 620 nm. The percent inhibition (% I) of each sample was calculated by use of the following equation:

% inhibition = 100 – [(sample OD X 100) /mean OD of negative control sample]

A binary reporting format was used to report cELISA results. Reports with a % inhibition of < 30% were recorded as negative (0), whereas reports that were > 30% were recorded as positive (1) (OIE, 2009; Strik et al., 2007; VMRD).

RNA extraction

Samples collected in evacuated tubes containing EDTA were centrifuged at 2,750 X g at 4º C for five minutes. Plasma was removed by a single use pipette while ensuring the buffy coat and red blood cell fraction were not disturbed. The plasma-free sample was vortexed to mix the cell fractions. Extraction of RNA from the homogenous, plasma-free sample occurred according to the manufacturer's recommendations of a commercially available product (TRI Reagent, Sigma-Aldrich; Saint Louis, MO). The RNA pellet was resolubilized with 50 µL of nuclease free water. Samples from a known *A. marginale* carrier and a naïve cow were extracted and analyzed simultaneously for monitoring qRT-PCR assay performance and quality of the RNA extraction technique. Samples were stored in a -80°C freezer until analysis by qRT-PCR. Extracted samples were not subjected to a DNAse treatment; however, contamination was assessed by comparing qRT-PCR and qPCR assay results by replacing reverse-transcriptase (SuperScript III Reverse Transcriptase, Invitrogen Corporation; Carlsbad, CA) with taq polymerase (Platinum Taq DNA Polymerase, Invitrogen Corporation; Carlsbad, CA) in the optimized *A. marginale* simplex assay.

Ratio of 16S rRNA to 16S DNA of A. marginale

The number of 16S rRNA molecules present in *Anaplasma* spp. is unknown. This provides uncertainty for determining the minimum detection sensitivity of the assay in a 250 µL plasma-free sample of bovine peripheral blood. A comparison was made by extracting RNA and DNA from a 200 µL sample of ten plasma-free blood samples preserved in 50% glycerol as described previously. The extraction of RNA will proceed as described above. The extraction of DNA will occur using a kit (Wizard SV Genomic DNA Purification System, Promega Corporation; Madison, WI) according to the manufacturer's instructions and a protocol modification for blood products (Promega, 2002). The extracted DNA and RNA were re-hydrated with 500 µL of nuclease-free water.

Stability of RNA in plasma-free blood samples stored in 50% glycerol

The stability of RNA from whole blood samples stored in glycerol is unknown. Ten samples were processed on the day of sampling with the RNA extraction method described above and the optimized *A. marginale* simplex assay. Aliquots of these samples were stored in

50% glycerol at -80°C (as previously described) for an extended time period. Samples were thawed and underwent a similar extraction and analysis as the fresh samples. A percent difference change in frozen versus fresh RNA recovery was made following correction of the total RNA count of the frozen sample due to dilution during storage.

Non-equivalent molar ratios

Nonequivalent molar ratios of RNA may be encountered during analysis. This may preclude the duplex assay from detecting both pathogens during concurrent infection due to template competition for reaction components. The optimized duplex assay was used to compare detection of 100,000 molecules of *A. marginale in vitro* transcripts to *A. phagocytophilum in vitro* transcripts ranging from 100,000 to 1,000 molecules in triplicate.

Data analysis

Statistical analysis was conducted utilizing a one-way analysis of variance (ANOVA) procedure (Microsoft Excel 2007, Microsoft Corporation; Redmond, WA) (Martinez et al., 2006). This statistical procedure analyzed variance for a quantitative dependent variable by a single independent variable to test the null hypothesis that several means are equal. An alpha level of < 0.05 was designated *a priori* for the determination of statistical significance. ANOVA was used to determine statistically significant differences between the ratio of 16S DNA copies contaminating 16S rRNA-extracted samples versus genomic DNA-extracted samples and the percent difference of RNA molecules recovered from fresh samples versus samples preserved in 50% glycerol.

Diagnostic test results were also converted to a binary format (0 = negative, 1 = positive). Sensitivity and specificity with 95% confidence intervals were calculated for cELISA using RT-PCR assay results as the determinant of disease status. Additionally, one- and two-way receiver operator characteristic (R.O.C.) curves were constructed to illustrate the optimal negative cut-off values for the cELISA based upon RT-PCR assay results (Le, 2003). This was accomplished by evaluating cELISA % inhibition results in 10% increments beginning and ending at 0 and 90% inhibition, respectively.

Agreement between diagnostic results was assessed by calculating a κ statistic at 95% confidence (Le, 2003). Results were compared using a software program (WinEpiscope 2.0, CLIVE; Edinburgh UK) in a 2 X 2 contingency table to calculate κ with the following equations:

$$EP = [(a+b)/n \cdot (a+c)/n] + [(c+d)/n \cdot (b+d)/n]$$

$$\kappa = [(a+d)/n - EP]\ (1\text{-}EP)$$

where EP is the expected proportion of equal outcomes according to chance; (a+d)/n – EP is the observed proportion of equal outcomes beyond chance; and 1-EP is the maximal proportion of agreement not due to chance. The κ statistic measures the agreement between tests on a scale from 0 to 1(Le, 2003).

Results

The target selection of these molecular diagnostic methods was based upon the expected high, intracellular copy number of 16S rRNA. Real-time qRT-PCR methods were developed for the simplex detection of *A. marginale* and *A. phagocytophilum* and a duplex assay for the detection of both species in a 250 μL plasma-free, bovine peripheral blood sample. In 25 μL reactions assembled with template from ten-fold serial dilutions of *in vitro* transcripts, the linear dynamic range of all assays ranged from 100 to 1 billion molecules (Figure 1). The number of reagent molecules used in the 25 μL RT-PCR assay mixtures is provided in Table 2. Linear regression was used to correlate the reported Ct value to the number of 16S rRNA template molecules in the 25 μL reaction (Table 3). Additionally, the correlation coefficient (R_2) of each regression equation and efficiency of the RT-PCR assays were reported (Ginzinger, 2002).

The species-specific probes accurately and precisely identified the respective template molecules without cross-reactivity during assay development. The duplex assay did not identify the *in vitro* transcript of *A. phagocytophilum* when the non-equivalent molar ratio exceeded a 10-fold difference. When the molar ratios were set equal for analysis, the results reported a mean ratio (95% CI) of 1.53 (0.25,2.83):1. At the maximum allowance for detecting non-equivalent ratios (10-fold), the mean ratio was 12.94 (9.96,15.93):1.

The mean ratio of *A. marginale* 16S rRNA to 16S DNA determined in ten field samples from anaplasmosis-endemic herds was 117.9 (100.7, 135.2):1. Therefore, the minimum detection sensitivity would be equal to the minimum infective unit of one *A. marginale* bacterium in 250 μL of plasma-free bovine peripheral blood. Due to RNA samples not being DNAse-treated prior to analysis, an experiment was conducted to assess the level of 16S DNA contamination. The mean ratio of 16S rRNA to 16S DNA of *A. marginale* following extraction of RNA from field samples was 1,252 (641.4,1863.8):1. This ratio and the data gathered during non-equivalent

molar ratio experiments did not demonstrate the need for DNAse treatment of extracted RNA. Reverse-transcriptase was substituted with taq polymerase for comparison of the mean ratio of the qPCR results of 16S DNA-contaminated RNA samples to extracted genomic 16S DNA samples. The mean ratio was 1.28 (0,2.85):1. Additionally, the number of 16S DNA molecules was not significantly different between the extraction methods (P = 0.34). The stability of 16S rRNA in ten plasma-free blood samples stored in 50% glycerol for 311 days was compared to the original samples processed on the day of collection. The mean percent difference change between fresh and frozen was 32.1% (19.8,44.5); however, this difference was not statistically significant (P = 0.27).

The duplex qRT-PCR assay determined the prevalence of *A. marginale* to be 37.6% in field samples; however, *A. phagocytophilum* was not detected (Table 4). The prevalence determined by the cELISA was 26.1%. The *A. phagocytophilum* simplex qRT-PCR assay was performed on a total of fourteen samples that were cELISA+/RT-PCR- (4) and randomly selected samples (10). Likewise, *A. phagocytophilum* was not detected in these fourteen samples. The sensitivity and specificity of the cELISA for detecting *A. marginale* was evaluated by the disease status of these cattle determined by the duplex RT-PCR assay. The cumulative sensitivity and specificity of the cELISA was 65.2% (55.3,75.1) and 97.3% (94.7,99.9), respectively. The cumulative agreement between the cELISA and duplex qRT-PCR assay was 0.655 (0.542,0.788).

One- and two-way R.O.C. curves were constructed to evaluate the % inhibition of cELISA negative cut-off values for the detection of *A. marginale* based upon RT-PCR assay results (Figures 2a and 2b). A one-way R.O.C. curve determined the optimal negative cut-off value to be 20% inhibition. For a 20% inhibition negative cut-off value, the sensitivity and specificity of the cELISA was 73 and 91.2%, respectively. A 15.3% inhibition was determined to be the optimal negative cut-off value by

intersection of the plots for sensitivity and specificity in a two-way R.O.C. curve. For a 15.3% inhibition negative cut-off value, the sensitivity and specificity of the cELISA was 74.2 and 81.2%, respectively. The negative cut-off values recommended by the R.O.C. curve analyses enhanced the sensitivity of the cELISA by 7.8-9% while adversely affecting the specificity by 6.1-16.1%.

Discussion

Real-time RT-PCR methods that combine reverse-transcriptase, PCR chemistry and fluorescent probe detection of amplified product have greatly enhanced the ability to diagnose disease. This study has described the development of real-time qRT-PCR assays for the detection of *A. marginale* and *A. phagocytophilum* alone or in combination. Duplex methods have been described for the simultaneous detection of *A. marginale-A. centrale* and *A. phagocytophilum-Borrelia burgdorferi* (Courtney et al., 2004; Decaro et al., 2008). To our knowledge, this is the first study to describe a duplex method for the diagnosis of *A. marginale* and *A. phagocytophilum* from the same sample.

The selection of an appropriate target for the accurate and precise diagnosis of disease was critical for the development of these anaplasmosis diagnostic methods. The selection of the 16S rRNA gene segments enhanced the analytical sensitivity of the assay due to the high ratio of 16S rRNA:16S DNA. Furthermore, the extraction of RNA from a plasma-free blood sample ensured the maximum number of cells in a 250 μL sample were available for analysis. These real-time assays combined the sensitivity and specificity of conventional methods without the risk of environment contamination with amplified product. Furthermore, the analysis of 48 samples can occur in less than 6 hours from the start of RNA extraction to analysis completion by the real-time system.

Numerous real-time assays have been developed for the detection of *A. marginale*. However, key differences exist among these assays and the method described here. Simplex assays that target the DNA gene sequences encoding for a major surface protein (MSP1b) and groEL of *A. marginale* have been described (Carelli et al., 2007; Decaro et al., 2008). The high analytical sensitivity of both methods provided for the detection of 10 DNA copies or 10 infective *A. marginale* bacteria; however, our qRT-PCR methods were able to detect as few as 100 copies of 16S rRNA that is equivalent to the minimum infective unit of a single *A. marginale* bacterium. Similarity was observed in selection of the fluoresecent probe (FAM) and quencher molecule (BHQ) during TaqMan probe development for the assay detecting groEL (Decaro et al., 2008) and our assay.

The detection of *A. phagocytophilum* by multiplex real-time methods has recently been limited to the diagnosis of disease in humans and canines (Courtney et al., 2004; Sirigireddy and

44

Ganta, 2005). However, a simplex assay was described in cattle for detecting *A. phagocytophilum* (Pusterla et al., 1999). Similarities exist due to the use TaqMan probes in the development of the assays; nevertheless, the assay described in 1999 by Pusterla et al. differs by the improved analytical sensitivity of the assay by detecting as few as ten 16S rRNA molecules. The significance of this enhanced sensitivity is unknown due to the current lack of information regarding the ratio of 16S rRNA:16S DNA of *A. phagocytophilum*.

The correct classification of disease status is important for the collection of epidemiologic information and improvement of free-trade policy between endemic and non-endemic countries. The development of a real-time method for the detection of *A. marginale* and *A. phagocytophilum* was influenced by the insufficient specificity of the cELISA due to cross-reactivity among closely related *Anaplasma* spp. (Bradway et al., 2001; Dreher et al., 2005; Strik et al., 2007). Furthermore, *A. centrale* was not selected due to vaccination with this spp. not occuring in the United States. Our method described the capability of detecting as few as one hundred 16S rRNA molecules of these samples in the same reaction tube. Due to these *Anaplasma* spp. infecting different types of blood cells, the ability to only detect non-equivalent molar concentrations up to 10-fold may be problematic (Courtney et al., 2004; Sirigireddy and Ganta, 2005). However, this could not be accurately assessed during this study. The level of detection of non-equivalent molar concentrations may have been due to competition for reaction components. This may be a result of unequal TaqMan probe concentrations and the enhanced processivity of the *A. marginale* probe due to its shorter length by 7 bases. However, a simplex assay for the detection of *A. phagocytophilum* has been described and applied in this study for the screening of samples whose duplex results did not agree with that of the cELISA (Sirigireddy and Ganta, 2005).

The simplex and duplex assays could serve as effective diagnostic tools for the epidemiologic study of *A. phagocytophilum*. Even though anaplasmosis caused by A. *phagocytophilum* has been identified as an economically important disease (Hoar et al., 2008; Pusterla and Braun, 1997; Pusterla et al., 1997), the prevalence of this pathogen in cattle in the United States is unknown (Hoar et al., 2008). Furthermore, the zoonotic potential of *A. phagocytophilum*, also known as human granulocytic ehrlichiosis, lends cattle to the role of sentinel animals for monitoring the spread of this anaplasmosis disease form from endemic to non-endemic regions.

45

Cattle that recover from acute anaplasmosis caused by *A. marginale* commonly develop persistent infections characterized by a cyclic rickettsemia of 5 week intervals (Eriks et al., 1989). The ability to quantify the genetic template by regression analysis of the Ct value reported by the real-time simplex and duplex assays can further supplement the study of cyclic rickettsemias. This is further substantiated by the findings of our study of the mean ratio of 16S rRNA:16S DNA being 117.9 (100.7,135.2):1. Therefore, the number of *A. marginale* would be equitable to one bacterium per 118 molecules of 16S rRNA detected. Furthermore, there was not a significant difference in 16S DNA contamination of 16S rRNA-extracted samples compared to genomic 16S DNA-extracted samples (P = 0.34). This would allow for the monitoring of 16S rRNA:16S DNA in the same 16S rRNA-extracted sample by substituting reverse-transcriptase with taq polymerase in the optimized reaction mixtures as described (Table 2).

The evaluation of the cELISA as a diagnostic tool by the results of the duplex qRT-PCR assay may be controversial because this qRT-PCR is not a gold standard test. However, our group has previously demonstrated 100% sensitivity and specificity of the qRT-PCR method described here in cattle with experimental *A. marginale* infections (Reinbold et al., 2009a) and carrier cattle chemosterilized with tetracycline antimicrobials (Reinbold et al., 2009b). In these studies A. marginale infection status was confirmed using the gold standard splenectomized calves. Furthermore, the approach to validation described in this study was similar to the interpretation of in-house data by a nested PCR that was submitted to the United States Department of Agriculture in support of licensure of the cELISA by Veterinary Medical Research & Development, Inc. (Torioni de Echaide et al., 1998; VMRD-2). In these studies, the sensitivity and specificity of the cELISA was reported at 95% and 98%, respectively. However, the OIE does not recommend the use of this nested PCR due to the potential for non-specific amplification (OIE, 2009). Non-specific amplification is not a disadvantage of this qRT-PCR assay.

It is noteworthy that the sensitivity and specificity of the cELISA when evaluated by the duplex real-time qRT-PCR assay was 65.2% (55.3, 75.1) and 97.3% (94.7, 99.9), respectively. A similar number of cattle were screened in our study and the cELISA licensure study at 237 and 235 cows, respectively. The difference in disease prevalence between our study and the licensure study was 37.6 and 64.3%, respectively. The measure of agreement (κ) calculated in the licensure study was 0.91, whereas κ was only 0.655 (0.542, 0.788) in our study. Furthermore, the

licensure study used a negative cut-off value set at 28% inhibition. In order to achieve similar sensitivity results, the % inhibition used as the negative cut-off value in our study would have needed to be set at 40%. When referring to the two-way R.O.C. curve constructed in Figure 2b, 100% sensitivity was achieved at a % inhibition of 40%. However, the specificity at this % inhibition was approximately 60%. The one-and two-way R.O.C. curves constructed from the results of this study (Figures 2a and 2b) determined the optimal negative cut-off values to be set at 20% inhibition and 15.3% inhibition, respectively.

In conclusion, a highly sensitive and specific duplex real-time qRT-PCR assay was developed for the detection of as few as 100 copies of 16S rRNA molecules of *A. marginale* and *A. phagocytophilum* in the same reaction. The ability of this assay to correctly identify the disease status of cattle is critical for the development of anaplasmosis disease control programs. Furthermore, the correct classification of anaplasmosis disease status prior to export may prove to be an important tool for improving free-trade of cattle between endemic and non-endemic countries and regions. Finally, this novel, duplex assay may improve epidemiologic studies of anaplasmosis in cattle populations of unknown disease status.

Figures and Tables

Figure 2.1 Target detection sensitivity and linearity with RNA concentration for the simplex and duplex assays. Serial 10-fold dilutions of *in vitro* transcripts were made from positive control plasmids to optimize the qRT-PCR assays. The average Ct values from three independent experiments were plotted against the log number of rRNA molecules for optimized simplex assays detecting (a) *Anaplasma marginale* and (b) *Anaplasma phagocytophilum*. Equivalent molar concentrations of *in vitro* transcripts were used to optimize a real-time duplex qRT-PCR assay (c) from the average Ct values of three independent experiments plotted against the log number of *A. marginale* (white squares) and *A. phagocytophilum* (white diamonds) RNA molecules. The fluorescent emission from serial dilution templates for *A. marginale* and *A. phagocytophilum* are shown in the inset graphs of (a) and (b), respectively.

b

c

Y-axis: Ct value

X-axis: Log_{10} number of molecules
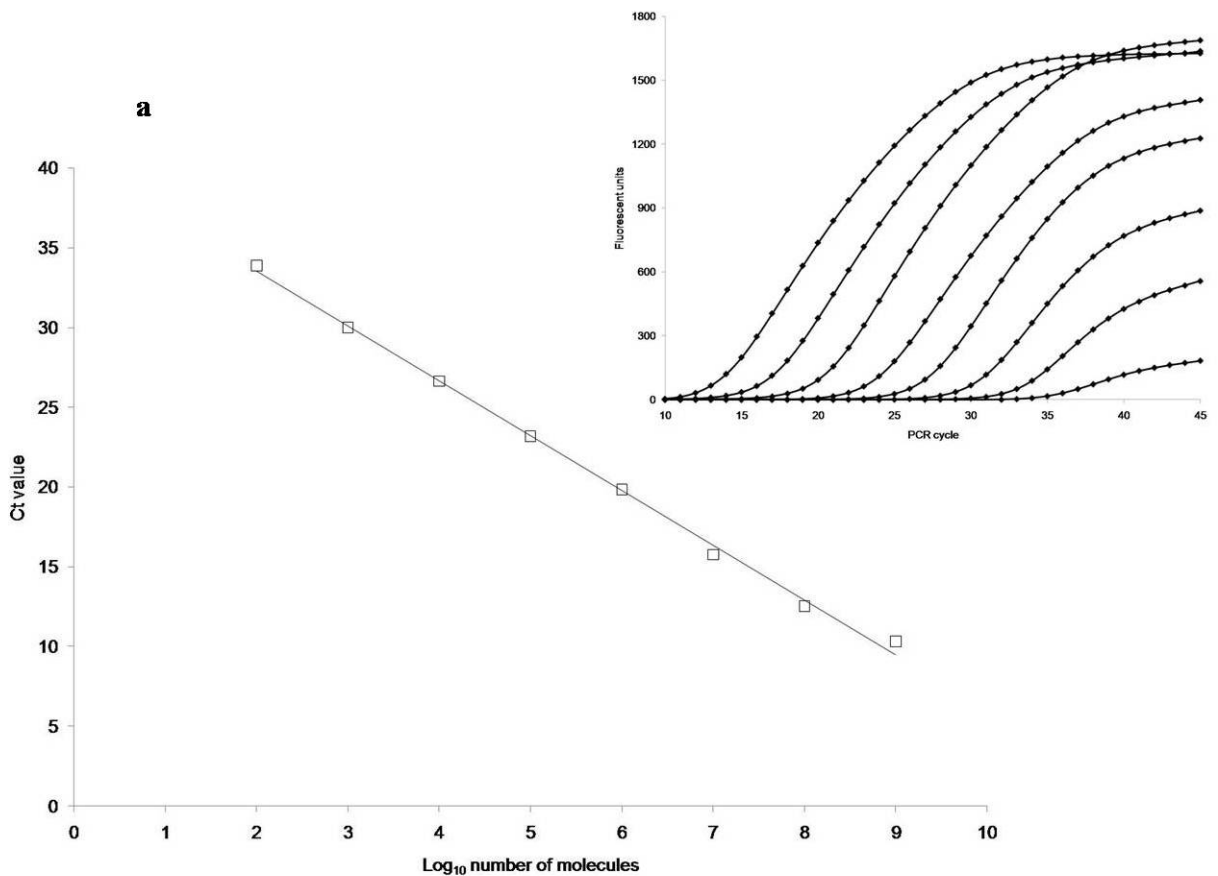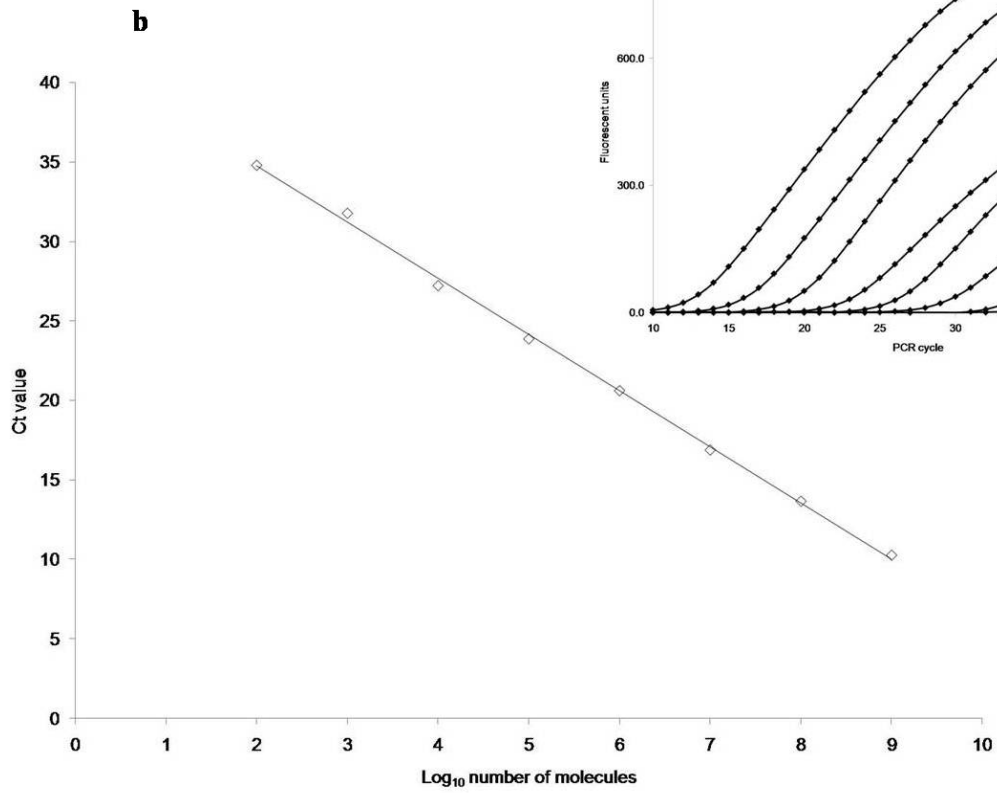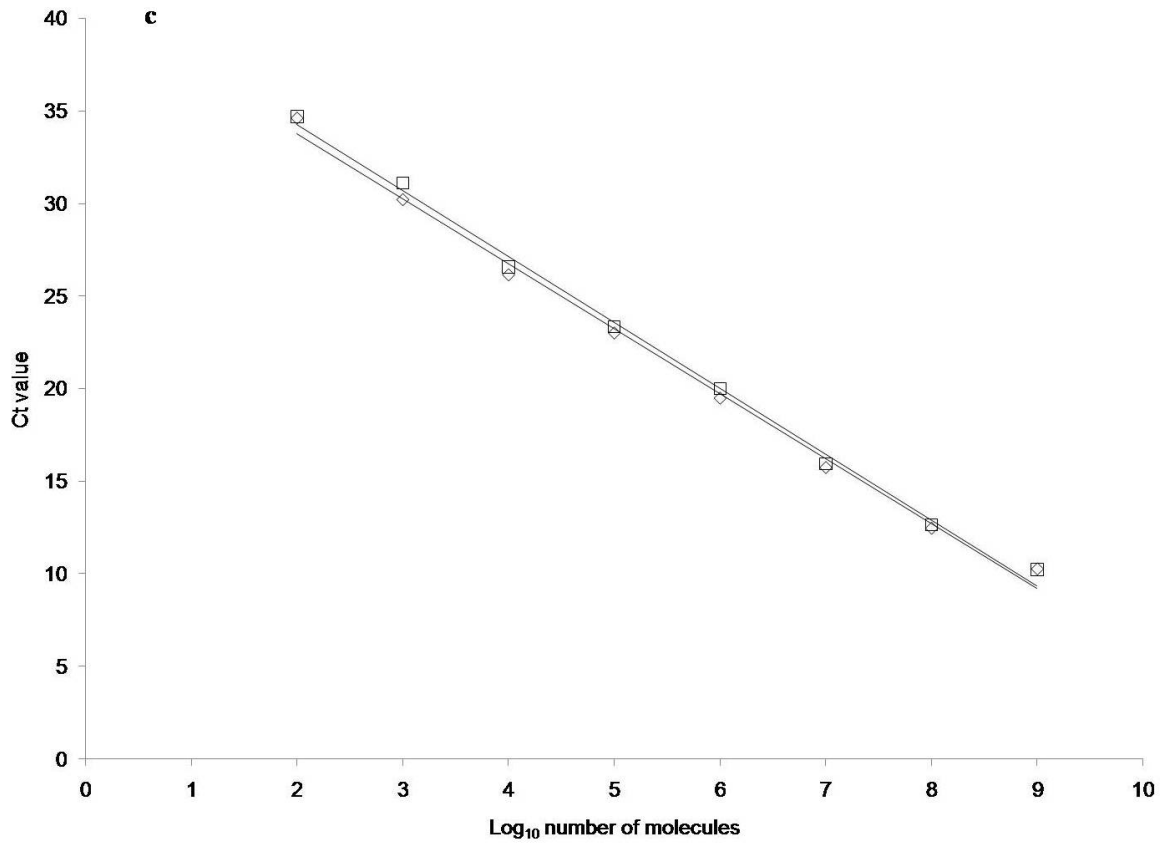
Figure 2.2 Determination of the optimal negative cut-off value of the cELISA through the construction of R.O.C. curves. (a) A one-way R.O.C. curve was constructed by plotting the true positive rate (sensitivity) by the true negative rate (1-specificity). The corresponding % inhibition is recorded adjacent to each data point. The recommended negative cut-off value for the one-way R.O.C. is represented as the data point located near the upper left portion of the graph (20%) (b) A two-way R.O.C. curve was constructed by plotting the sensitivity (black squares) and specificity (black diamonds) of the cELISA by the corresponding % inhibition. The recommended negative cut-off value for the two-way R.O.C. is represented by the intersection of the sensitivity and specificity plots (15.3%).
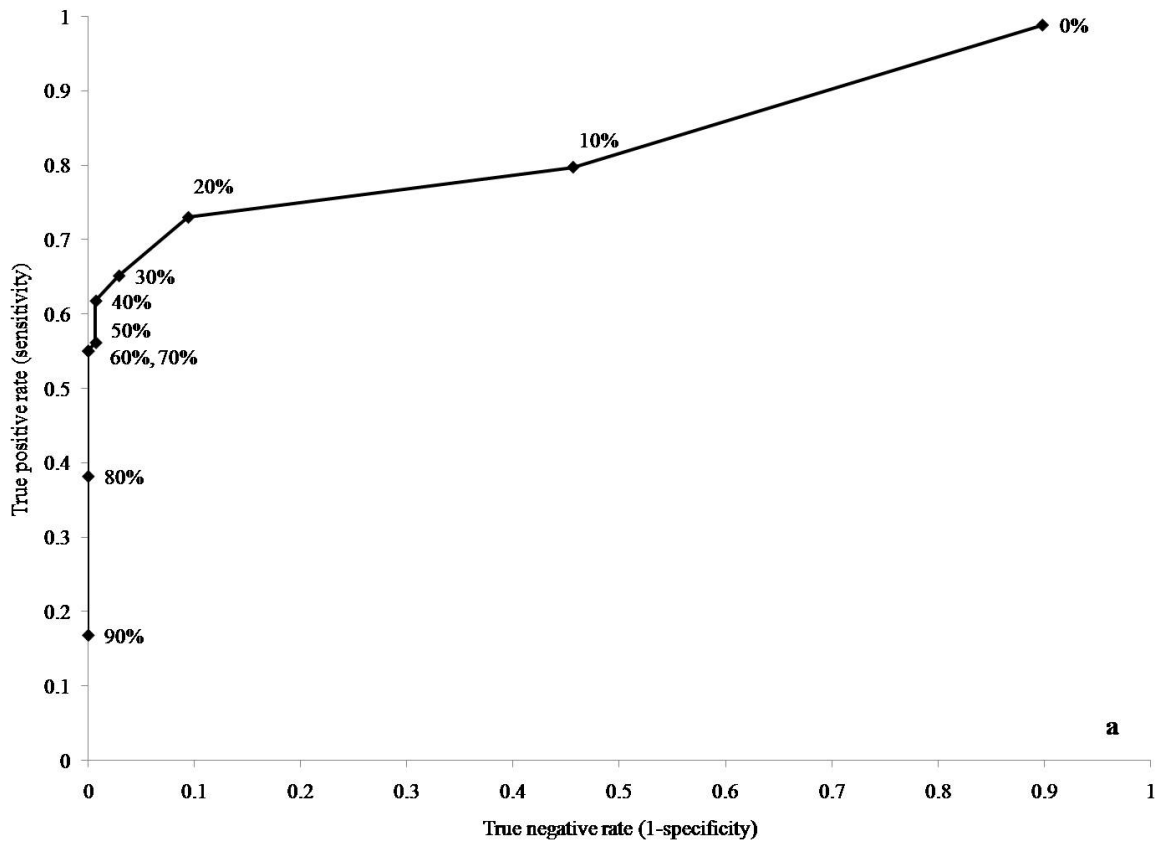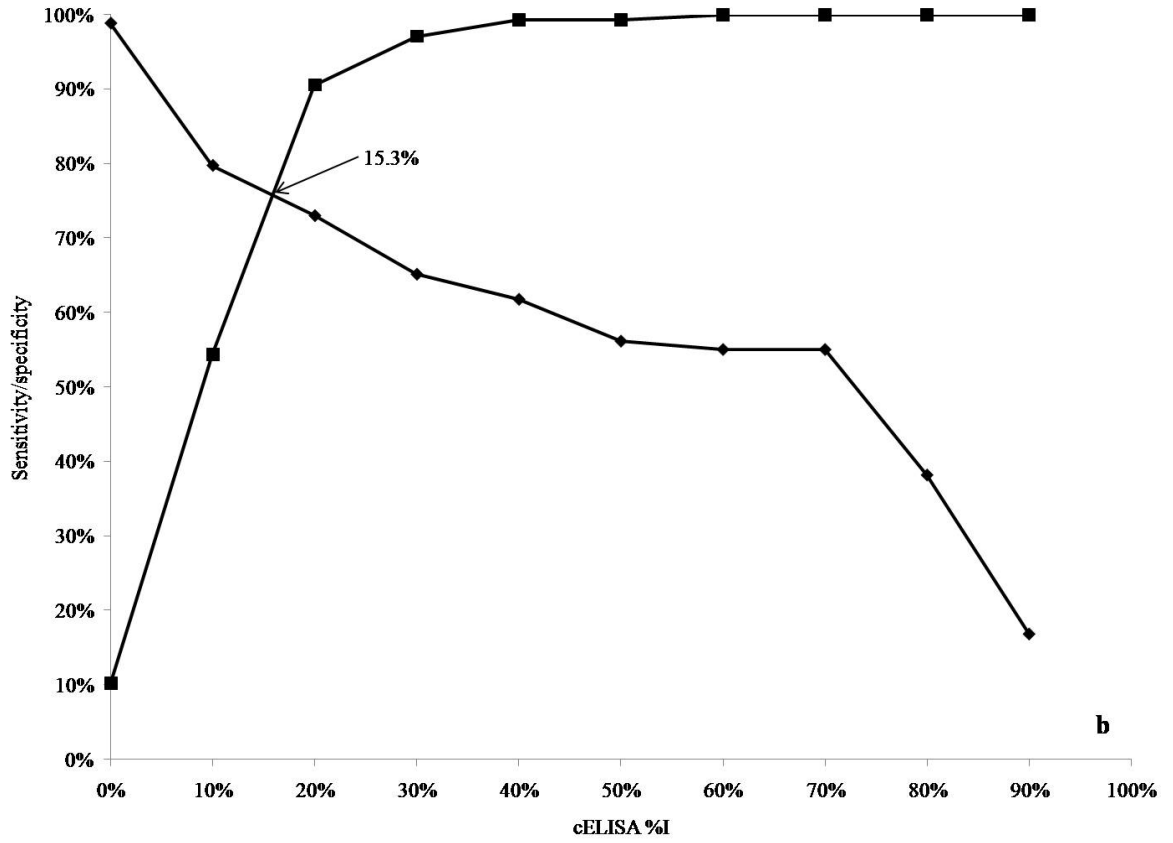
Table 2.1 TaqMan primers and probes used in the development of the simplex and duplex molecular assays.

TaqMan Primers/Probes[†]	Base Sequence	Length	Tm (°C)
Anaplasma forward primer	5'- CTCAGAACGAACGCTGG -3'	17	53.3
Anaplasma reverse primer	5'- CATTTCTAGTGGCTATCCC -3'	19	49.7
A. marginale probe	5'- /56-FAM/CGCAGCTTGCTGCGTGTATGGT/3BHQ_1/ -3'[*]	22	62.8
A. phagocytophilum probe	5'- /5TexRd-XN/TTGCTATAAAGAATAATTAGTGGCAGACG/3BHQ_2/ -3'[*]	29	55.4

[†]Primers and probes were synthesized by Integrated DNA Technologies Inc.; Coralville, IA. [*]Base sequences are recorded with reporter and quencher molecules. Tm = melting temperature in 50mM NaCl.

Table 2.2 RT-PCR assay reaction components for each of the simplex and duplex assays.

| Component | Simplex RT-PCR assay | | Duplex RT-PCR assay |
	A. marginale	*A. phagocytophilum*	
MgSO$_4$	187.5 nmol	187.5 nmol	187.5 nmol
each dNTP	5 pmol	5 pmol	5 pmol
Anaplasma forward primer	10 pmol	10 pmol	10 pmol
Anaplasma reverse primer	10 pmol	10 pmol	10 pmol
A. marginale probe	11.3 pmol	—	11.3 pmol
A. phagocytophilum probe	—	9.4 pmol	9.4 pmol

Each 25 µL RT-PCR reaction mixture consisted of 23 µL of master mix from a kit (SuperScript III Platinum One-step Quantitative RT-PCR System, Invitrogen Corporation; Carlsbad, CA), 8U of a ribonuclease inhibitor (Recombinant RNAsin, Promega Corporation; Madison, WI), and 2 µL of template. DNA contamination of RNA samples was assessed by replacing reverse transcriptase (Superscript III Reverse Transcriptase) with 2U of taq polymerase (Platinum Taq DNA Polymerase, Invitrogen Corporation; Carlsbad, CA).

Table 2.3 RT-PCR assay optimization criteria results and regression equations for the quantification of template in a 25 µL reaction.

Assay	Regression equation	Correlation Coefficient (R^2)	Efficiency (%)
Simplex - *Anaplasma marginale*	y = -3.4324x + 40.38	0.9973	95.59
Simplex - *Anaplasma phagocytophilum*	y = -3.532x + 41.815	0.9986	91.92
Diplex			
A. marginale	y = -3.5014x + 40.744	0.9950	93.02
A. phagocytophilum	y = -3.5571x + 41.392	0.9965	91.04

For the regression equation, y is the reported Ct value and x is the number of template molecules.
Efficiency = -1+10(-1/slope)

Table 2.4 Comparison of diagnostic method results during the screening of field samples for *Anaplasma marginale* infection.

Location: Northeast Kansas	RT-PCR +	RT-PCR -		Location: Southeast Kansas	RT-PCR +	RT-PCR -		Cumulative	RT-PCR +	RT-PCR -
cELISA +	15	1		cELISA +	43	3		cELISA +	58	4
cELISA -	11	37		cELISA -	20	107		cELISA -	31	144

cELISA sensitivity: 57.7% (38.7,76.7)   cELISA sensitivity: 68.3% (56.8,79.7)   cELISA sensitivity: 65.2% (55.3,75.1)

cELISA specificity: 97.4% (92.3,100)   cELISA specificity: 97% (93.7,100)   cELISA specificity: 97.3% (94.7,99.9)

Agreement (κ): 0.586 (0.356,0.816)   Agreement (κ): 0.695 (0.55,0.841)   Agreement (κ): 0.655 (0.542,0.788)

A κ result where $0 < κ < 0.4$, $0.4 < κ < 0.75$, and $κ > 0.75$ indicates poor, good, and excellent reproducibility, respectively.

References

Bradway, D.S., Torioni de Echaide, S., Knowles, D.P., Hennager, S.G., McElwain, T.F., 2001, Sensitivity and specificity of the complement fixation test for detection of cattle persistently infected with *Anaplasma marginale*. *J Vet Diagn Invest* 13, 79-81.

Carelli, G., Decaro, N., Lorusso, A., Elia, G., Lorusso, E., Mari, V., Ceci, L., Buonavoglia, C., 2007, Detection and quantification of *Anaplasma marginale* DNA in blood samples of cattle by real-time PCR. *Vet Microbiol* 124, 107-114.

Coetzee, J.F., Apley, M.D., Kocan, K.M., 2006, Comparison of the efficacy of enrofloxacin, imidocarb, and oxytetracycline for clearance of persistent *Anaplasma marginale* infections in cattle. *Vet Ther* 7, 347-360.

Coetzee, J.F., Apley, M.D., Kocan, K.M., Rurangirwa, F.R., Van Donkersgoed, J., 2005, Comparison of three oxytetracycline regimens for the treatment of persistent *Anaplasma marginale* infections in beef cattle. *Vet Parasitol* 127, 61-73.

Coetzee, J.F., Schmidt, P.L., Apley, M.D., Reinbold, J.B., Kocan, K.M., 2007, Comparison of the complement fixation test and competitive ELISA for serodiagnosis of *Anaplasma marginale* infection in experimentally infected steers. *Am J Vet Res* 68, 872-878.

Courtney, J.W., Kostelnik, L.M., Zeidner, N.S., Massung, R.F., 2004, Multiplex real-time PCR for detection of *Anaplasma phagocytophilum* and *Borrelia burgdorferi*. *J Clin Microbiol* 42, 3164-3168.

Dame, J.B., Mahan, S.M., Yowell, C.A., 1992, Phylogenetic relationship of *Cowdria ruminantium*, agent of heartwater, to *Anaplasma marginale* and other members of the order *Rickettsiales* determined on the basis of 16S rRNA sequence. *Int J Syst Bacteriol* 42, 270-274.

Decaro, N., Carelli, G., Lorusso, E., Lucente, M.S., Greco, G., Lorusso, A., Radogna, A., Ceci, L., Buonavoglia, C., 2008, Duplex real-time polymerase chain reaction for simultaneous detection and quantification of *Anaplasma marginale* and *Anaplasma centrale*. *J Vet Diagn Invest* 20, 606-611.

Dreher, U.M., de la Fuente, J., Hofmann-Lehmann, R., Meli, M.L., Pusterla, N., Kocan, K.M., Woldehiwet, Z., Braun, U., Regula, G., Staerk, K.D., Lutz, H., 2005, Serologic cross-

reactivity between *Anaplasma marginale* and *Anaplasma phagocytophilum*. *Clin Diagn Lab Immunol* 12, 1177-1183.

Dumler, J.S., Barbet, A.F., Bekker, C.P., Dasch, G.A., Palmer, G.H., Ray, S.C., Rikihisa, Y., Rurangirwa, F.R., 2001, Reorganization of genera in the families *Rickettsiaceae* and *Anaplasmataceae* in the order *Rickettsiales*: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and designation of *Ehrlichia equi* and 'HGE agent' as subjective synonyms of *Ehrlichia phagocytophila*. *Int J Syst Evol Microbiol* 51, 2145-2165.

Eriks, I.S., Palmer, G.H., McGuire, T.C., Allred, D.R., Barbet, A.F., 1989, Detection and quantitation of *Anaplasma marginale* in carrier cattle by using a nucleic acid probe. *J Clin Microbiol* 27, 279-284.

Ginzinger, D.G., 2002, Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream. *Exp Hematol* 30, 503-512.

Gutell, R.R., Weiser, B., Woese, C.R., Noller, H.F., 1985, Comparative anatomy of 16-S-like ribosomal RNA. *Prog Nucleic Acid Res Mol Biol* 32, 155-216.

Hoar, B.R., Nieto, N.C., Rhodes, D.M., Foley, J.E., 2008, Evaluation of sequential coinfection with *Anaplasma phagocytophilum* and *Anaplasma marginale* in cattle. *Am J Vet Res* 69, 1171-1178.

Huang, Y., Babiuk, L.A., van Drunen Littel-van den Hurk, S., 2005, Immunization with a bovine herpesvirus 1 glycoprotein B DNA vaccine induces cytotoxic T-lymphocyte responses in mice and cattle. *J Gen Virol* 86, 887-898.

Knowles, D., Torioni de Echaide, S., Palmer, G., McGuire, T., Stiller, D., McElwain, T., 1996, Antibody against an *Anaplasma marginale* MSP5 epitope common to tick and erythrocyte stages identifies persistently infected cattle. *J Clin Microbiol* 34, 2225-2230.

Kocan, K.M., de la Fuente, J., Guglielmone, A.A., Melendez, R.D., 2003, Antigens and alternatives for control of *Anaplasma marginale* infection in cattle. *Clin Microbiol Rev* 16, 698-712.

Le, C.T., 2003, *Introductory biostatistics*, 1st Edition. John Wiley and Sons, Inc., Hoboken, NJ.

Manoj, S., Griebel, P.J., Babiuk, L.A., van Drunen Littel-van den Hurk, S., 2004, Modulation of immune responses to bovine herpesvirus-1 in cattle by immunization with a DNA vaccine encoding glycoprotein D as a fusion protein with bovine CD154. *Immunology* 112, 328-338.

Martinez, M.N., Kawalek, J.C., Howard, K.D., Ward, J.L., Marroum, P., Marnane, W., Bensley, D., Pelsor, F.R., Hoag, S., Tatavarti, A.S., Xie, L., Fahmy, R., 2006, Comparison of bovine in vivo bioavailability of two sulfamethazine oral boluses exhibiting different *in vitro* dissolution profiles. *J Vet Pharmacol Ther* 29, 459-467.

Molad, T., Mazuz, M.L., Fleiderovitz, L., Fish, L., Savitsky, I., Krigel, Y., Leibovitz, B., Molloy, J., Jongejan, F., Shkap, V., 2006, Molecular and serological detection of *A. centrale-* and *A. marginale*-infected cattle grazing within an endemic area. *Vet Microbiol* 113, 55-62.

OIE 2009. Office International des Épizooties, Manual of standards for diagnostic tests and vaccines for terrestrial animals. Chapter 2.4.1 17th ed. 2008. Accessed July 13, 2009. http://www.oie.int/eng/normes/mmanual/2008/pdf/2.04.01_BOVINE_ANAPLASMOSIS.pdf

Promega 2002. Promega Application Notes: Genomic Purification from blood (10351-AN-PU, Part #AN101), Corporation, P., ed. (Madison, WI, Promega Corporation), p. Protocol Modification.

Pusterla, N., Braun, U., 1997, Clinical findings in cows after experimental infection with *Ehrlichia phagocytophila*. *Zentralbl Veterinarmed A* 44, 385-390.

Pusterla, N., Huder, J., Wolfensberger, C., Braun, U., Lutz, H., 1997, Laboratory findings in cows after experimental infection with *Ehrlichia phagocytophila*. *Clin Diagn Lab Immunol* 4, 643-647.

Pusterla, N., Huder, J.B., Leutenegger, C.M., Braun, U., Madigan, J.E., Lutz, H., 1999, Quantitative real-time PCR for detection of members of the *Ehrlichia phagocytophila* genogroup in host animals and *Ixodes ricinus* ticks. *J Clin Microbiol* 37, 1329-1331.

Reinbold, J.B., Coetzee, J.F., Hollis, L.C., Nickell, J.S., Riegel, C., Christopher-Huff, J., Ganta, R.R., 2009a, Comparison of iatrogenic *Anaplasma marginale* transmission by needle and needle-free injection techniques. *Am J Vet Res* (In press: AJVR-09-07-0279).

Reinbold, J.B., Coetzee, J.F., Hollis, L.C., Nickell, J.S., Riegel, C., Olson, K.C., Ganta, R.R., 2009b, Establishment of the *in vivo* pharmacokinetic-pharmacodynamic relationship between chlortetracycline and anaplasmosis carrier clearance using three oral treatment regimens. *Vet Microbiol* Submitted.

Rogers, R.J., Shiels, I.A., 1979, Epidemiology and control of anaplasmosis in Australia. *J S Afr Vet Assoc* 50, 363-366.

Rurangirwa, F.R., Brayton, K.A., McGuire, T.C., Knowles, D.P., Palmer, G.H., 2002, Conservation of the unique rickettsial rRNA gene arrangement in *Anaplasma*. *Int J Syst Evol Microbiol* 52, 1405-1409.

Sambrook, J., Russell, D.W., 2001a, *Molecular Cloning: A Laboratory Manual*, Vol 1, 3rd Edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sambrook, J., Russell, D.W., 2001b, *Molecular Cloning: A Laboratory Manual*

Vol 2, 3rd Edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sirigireddy, K.R., Ganta, R.R., 2005, Multiplex detection of *Ehrlichia* and *Anaplasma* species pathogens in peripheral blood by real-time reverse transcriptase-polymerase chain reaction. *J Mol Diagn* 7, 308-316.

Strik, N.I., Alleman, A.R., Barbet, A.F., Sorenson, H.L., Wamsley, H.L., Gaschen, F.P., Luckschander, N., Wong, S., Chu, F., Foley, J.E., Bjoersdorff, A., Stuen, S., Knowles, D.P., 2007, Characterization of *Anaplasma phagocytophilum* major surface protein 5 and the extent of its cross-reactivity with *A. marginale*. *Clin Vaccine Immunol* 14, 262-268.

Torioni de Echaide, S., Knowles, D.P., McGuire, T.C., Palmer, G.H., Suarez, C.E., McElwain, T.F., 1998, Detection of cattle naturally infected with *Anaplasma marginale* in a region of endemicity by nested PCR and a competitive enzyme-linked immunosorbent assay using recombinant major surface protein 5. *J Clin Microbiol* 36, 777-782.

VMRD-2. Product information sheet: Anaplasma antibody test kit, cELISA (www.vmrd.com/docs/tk/Anaplasma/Anaplasma_Flyer_050113.pdf) (Veterinary Medical Research & Development (VMRD), Inc.).

VMRD. Anaplasma antibody test kit, cELISA. (Veterinary Medical Research & Development (VMRD), Inc.), pp. Assay instructions for catalog numbers: 282-282 and 282-285. USDA Product Code 5002.5020 For veterinary

CHAPTER 3 - Comparison of iatrogenic *Anaplasma marginale* transmission by needle and needle-free injection techniques

Introduction

Anaplasmosis, caused by *Anaplasma marginale*, is one of the most prevalent tick-transmitted, rickettsial diseases of cattle worldwide (Dumler et al., 2001; Kocan et al., 2003; Uilenberg, 1995). The Office International des Epizooties (OIE) Animal Health Code categorizes anaplasmosis as a notifiable disease due to socioeconomic impact and international trade restrictions (OIE, 2009). However, the significance of anaplasmosis is frequently underestimated compared with other diseases due to seasonal outbreaks and stability in endemic areas (Rogers and Shiels, 1979). Clinical disease symptoms in acutely-infected, adult cattle include, but are not limited to, anemia, fever, icterus, lethargy, and death (Radostits et al., 2000). Moreover, abortion, high mortality, reduced milk production, extensive treatment costs, and weight loss are key economic considerations of this disease. In 2003, anaplasmosis was estimated to cost the United States cattle industry over $300 million per year (Kocan et al., 2003).

Historically, vaccination has been used to modulate disease severity. In some countries, naïve cattle are inoculated intravenously with bovine blood infected with *Anaplasma centrale* for reducing disease morbidity prior to infection with *A. marginale* (Kocan et al., 2003). However, this strategy is not used or recommended in the United States due to the potential for transfer of other blood-borne pathogens and production of erythrocytic isoantibodies (Dennis et al., 1970). A killed vaccine was previously available for use in cattle in the United States, but is currently available under special permission only (Luther, 1999).

Cattle infected with anaplasmosis following natural infection and vaccination with live *Anaplasma spp.* remain lifelong carriers despite tetracycline therapy (Coetzee et al., 2006; Coetzee et al., 2005; Lincoln et al., 1982; Rogers and Shiels, 1979; Wilson et al., 1979). Carriers

are responsible for horizontal, iatrogenic, and vertical transmission of anaplasmosis to naïve cattle by providing a reservoir of infective blood for biological, mechanical, and *in utero* infection (Eriks et al., 1989; Futse et al., 2003; Norton et al., 1983; Potgieter and van Rensburg, 1987; Reeves and Swift, 1977; Zaugg, 1985; Zaugg and Kuttler, 1984).

Appropriately-timed application of insecticides is recommended for reducing biological transmission by haematophagous arthropods (De Wall, 2000; Peter et al., 2005; Rodriguez-Vivas et al., 2004). Due to the lack of significant success with treatment strategies, vaccine availability, and problematic vector control, anaplasmosis control strategies should primarily concentrate on established methods for disease prevention.

Anaplasmosis transmission has been documented during routine animal husbandry practices (Reeves and Swift, 1977; Rodriguez-Vivas et al., 2004). Disease prevention strategies associated with vaccination have been considered impractical, uneconomical or are potentially deleterious to the success of the procedure (Andrews and Lamport, 1985; Makoschey and Beer, 2004). In a survey of fifty-five large animal veterinary practices, 32% did not exchange hypodermic needles between each cow (Anderson and Silviera, 2008).

Needle-free injection, which uses a pneumatic-powered system to deliver vaccines, was used by the United States military to vaccinate recruits following World War II. Needle-free injection techniques have proven to be efficacious for the delivery of vaccines in cattle (Hollis et al., 2005; Huang et al., 2005; Manoj et al., 2004). However, research has suggested the potential for blood product transfer during the use of needle-free injectors for consecutive injections (Sweat et al., 2000). The purposes of this study are to compare iatrogenic transmission of *A. marginale* during simulated vaccination between needle-free and conventional needle injection techniques and diagnostic method performance of light microscopy, competitive ELISA (cELISA), and an *A. marginale*-specific Reverse transcriptase-polymerase chain reaction (RT-PCR) assay.

Materials and Methods

Animals

This study was approved by the Kansas State University (KSU) Institutional Animal Care and Use Committee (protocol #2517) and Institutional Biosafety Committee (protocol #524).

Twenty-six preconditioned, clinically healthy Holstein steers were purchased from the KSU Dairy Teaching and Research Center in Manhattan, KS. The mean age $\pm$ standard deviation was 172.2 $\pm$ 27.5 days at the time of study. All steers were prescreened for anti-*A. marginale* antibodies with a commercially available cELISA (Anaplasma Antibody Test Kit, cELISA, VMRD Inc.; Pullman, WA), as well as a novel RT-PCR for detecting a 16S ribosomal RNA subunit (16S rRNA) of *A. marginale* (Coetzee et al., 2007; OIE, 2009; Torioni de Echaide et al., 1998; VMRD). Pre-study negative disease status was determined though interpretation of cELISA and RT-PCR assay results in series (Dohoo I et al., 2007).

Housing and husbandry

Steers were blocked by body weight and randomized to one of three treatment groups: (1) needle injection (ND, n=10), (2) needle-free injection (NF, n=10), and (3) a non-injected control group (CONT, n=5). Steers were individually housed in a biolevel-2 (BL-2) safety facility at the KSU Animal Research Facility. An insecticide (Ultra Boss, Schering-Plough; Summit, NJ) was applied upon entry into the BL-2 facility as recommended by the manufacturer's label. A total mixed ration (TMR) diet was fed at 2.5% of bodyweight (as fed) divided into twice daily feedings. Monensin (80 g/ton) was the only antimicrobial included in the TMR. Water was supplied *ad libitum*. When handling was necessary, steers were individually restrained with a rope halter. Steers did not receive any antimicrobials that would potentially interfere with *A. marginale* disease transmission beginning 30 days prior to study initiation and continuing throughout the remainder of the study; furthermore, no procedure was permitted that was not outlined in the experimental design.

Splenectomy protocol and procedure

One steer was splenectomized and used to propagate a Virginia isolate of *A. marginale*. A hand-assisted, laparoscopic procedure was used to splenectomize this steer. Briefly, the steer was placed in right lateral recumbency after sedation was achieved by an intramuscular injection of xylazine (0.1 mg/kg), ketamine (0.1 mg/kg), and butorphanol (0.05 mg/kg). Following a local infusion of lidocaine hydrochloride (2%), a six centimeter paracostal incision was made centered over the costochondral arch and approximately three centimeters caudal to the thirteenth rib. A hand was placed through this hand-assist incision and used to bluntly dissect away the connective tissue between the spleen and the rumen until the splenic hilus was isolated. A

laparoscopic stapler, functioning as a ligating, dividing and stapling device (LDS), was placed through the left flank via a 1.5 cm incision. The intra-abdominal hand was used to guide the LDS unit around the vascular pedicle to apply two staples around the hilus. The spleen was then divided free from the rumen. The spleen was removed via the hand-assist incision. All incisions were closed by a routine, three layer closure. Post-operative analgesia was provided with an intravenous injection of flunixin meglumine (1 mg/kg). Procaine penicillin G (10,000 IU/kg) was administered intramuscularly for three days peri-operatively. Skin sutures were removed fourteen days after surgery.

Inoculation and monitoring of splenectomized steer

A tick transmissible Virginia isolate of *A. marginale* was collected from an infected cow and provided by Dr. Katherine Kocan, Oklahoma State University; Stillwater, OK. This fully characterized isolate (1978) was secured by Dr. Kocan from the United States Department of Agriculture Animal Parasitology Institute, Beltsville, MD (de la Fuente et al., 2003). The blood sample was treated with a heparin anticoagulant and prepared for overnight shipment on ice to KSU.

Five milliliters of heparinized whole blood sample was used for subinoculation of the splenectomized steer (referred to hereafter as SPS) intravenously on the eighth day postoperatively. The SPS was monitored daily for clinical signs of anaplasmosis, including anorexia, lethargy, and fever. Whole blood samples were collected and immediately prepared for percent parasitized erythrocyte count (PPE) and packed cell volume (PCV) determination.

Blood film preparation and PPE determination

Blood films were stained with an automated unit (Hema-Tek, Ames Company; Elkhart, IN) using a Modified Wright stain (Kutaish, 1982; Teerasaksilp et al., 2005). The number of parasitized erythrocytes was determined using a Miller reticle$_d$ (Riley et al., 2001). The Miller reticle (Miller reticle, Klarmann Rulings, Inc.; Litchfield, NH) is a large square inset with an additional square that is one-tenth the size of the large square. All parasitized erythrocytes in the large square were counted; however, only the erythrocytes present in the smaller square were counted. The number of parasitized erythrocytes and non-parasitized erythrocytes were recorded separately. A total of 1,000 erythrocytes were counted. The PPE was calculated by the following

equation which was modified from an equation for measuring the percent of reticulocytes under similar conditions (Riley et al., 2001):

PPE (%) = [(total parasitized erythrocytes in the larger square) ÷ (total erythrocytes in the smaller square × 9)] x 100

Measurement of PCV

The PCV was determined by partially filling no-additive capillary tubes (Hemato-Clad Plain, Drummond Scientific Company; Broomall, PA) with blood followed by centrifugation at12,600 X g for ten minutes (Coetzee et al., 2007). The PCV was then determined by measuring (Critocaps Micro-Hematocrit Capillary Tube Reader, McCormick Scientific; Saint Louis, MO) the height of the red blood cell portion in comparison to the total column height.

Serum collection and cELISA

A commercial cELISA (Anaplasma Antibody Test Kit, cELISA, VMRD Inc.; Pullman, WA) was used in accordance with the method described by the OIE and recommended by the manufacturer (OIE, 2009; VMRD). The optical density (OD) of each well was measured by use of an ELISA plate reader at a wavelength of 620 nm. The % inhibition (% I) of each sample was calculated by use of the following equation:

% inhibition = 100 – [(sample OD X 100) /mean OD of negative control sample]

Reports with an inhibition of < 30% were recorded as negative results, whereas reports that were > 30% were recorded as positive (OIE, 2009; Strik et al., 2007; VMRD).

RNA extraction and RT-PCR assay

Extraction of RNA from a plasma-free whole blood sample occurred according to the manufacturer's recommendations of a commercially available product (TRI Reagent, Sigma-Aldrich; Saint Louis, MO) with the following procedure specifications. Briefly, plasma was separated by centrifuging vacutainer tubes at 2,750 X g at 4° C for five minutes. Plasma was removed by a single use pipette while ensuring not to remove the buffy coat and red blood cell fraction. Two hundred microliters of plasma-free blood was transferred to a microcentrifuge tube. One milliliter of a monophase solution of guanidine thiocyanate and phenol was added to lyse the blood cells followed by vigorous vortexing. The solution then stood for ten minutes. Two hundred microliters of chloroform was added followed by vortexing vigorously for fifteen

seconds. The solution then stood for ten minutes prior to centrifuging at 12,000 x g for fifteen minutes at 4°C. The colorless, upper, aqueous phase of the solution was transferred to a new tube. Five hundred microliters of 2-propanol was added to each tube and mixed by vortexing briefly. The solution then stood for 10 minutes prior to centrifuging at 12,000 x g for ten minutes at 4°C. The supernatant was poured off and the pellet washed with 1mL of 75% ethanol. Next, the sample was vortexed briefly prior to centrifuging at 12,000 x g for five minutes at 4°C. The supernatant was poured off and allow to air dry. The RNA pellet was resolubilized with 50 μL of nuclease free water. Samples were stored in a -80°C freezer until analysis by RT-PCR.

A simplex, real-time qRT-PCR assay was developed for the identification of 16S rRNA of *A. marginale* using previously designed forward and reverse primers and a TaqMan probe designed as a part of this study (Sirigireddy and Ganta, 2005). The forward and reverse primer sequences (Integrated DNA Technologies Inc.; Coralville, IA) were 5′-CTCAGAACGAACGCTGG-3′ and 5′-CATTTCTAGTGGCTATCCC-3′, respectively. The TaqMan probe sequence (Integrated DNA Technologies Inc.; Coralville, IA) was 5'-/56-FAM/CGCAGCTTGCTGCGTGTATGGT/3BHQ_1/-3'. A commercially available, 23 μL RT-PCR assay mixture (SuperScript III Reverse Transcriptase, Invitrogen Corporation; Carlsbad, CA) included 10 pmol of each of the TaqMan forward and reverse primers; 5 pmol of each dNTP; 187.5 nmol of $MgSO_4$; 11.3 pmol of *A. marginale* probe; and 8U of ribonuclease inhibitor (Recombinant RNAsin, Promega Corporation; Madison, WI). Two microliters of template were added for a 25 μL final volume. The temperature cycles used for the RT-PCR assay were: an initial complementary DNA generation cycle at 48°C for 30 minutes, 3 minutes at 94°C followed by 45 cycles of 94°C for 15 seconds, 50°C for 30 seconds, and 60°C for 60 seconds.

An *in vitro* transcript of *A. marginale* plasmid DNA was prepared for use in the RT-PCR assay development. Three micrograms of plasmid DNA were digested with the restriction enzyme *SpeI*. *SpeI*-digested plasmid DNA was used to generate recombinant transcripts with a T7 RNA polymerase as outlined in the kit protocol (T7 MEGAscript High-Yield Transcription Kit, Ambion Inc.; Austin, TX). Ten-fold serial dilutions (ranging from 1 billion to one molecule) of the *in vitro* transcript were analyzed in triplicate to optimize the assay in a commercially available real-time quantitative polymerase chain reaction (qPCR) system (SmartCylcer II, Cepheid; Sunnyvale, CA). The RT-PCR product formation was monitored in real-time by measuring the emitted fluorescence in the extension phase of the PCR cycles within the real-time

PCR system. A reaction was qualified as positive for the presence of a template when it detects seven fluorescent units for the emission channel of the fluorescent probe. The PCR cycle at which fluorescence occurs, which is dependent on the concentration of the template in the reaction, was regarded as the cycle threshold (Ct) value. In 25 μL reactions assembled with template from the ten-fold serial dilutions, the standard curve of the assay ranged from 100 to 1 billion molecules (Figure 3.1). Linear regression was used to quantify the number of 16S rRNA template molecules in the 25 μL reaction based upon the corresponding Ct value with the following equation:

$$y = -3.4324x + 40.38$$

where y is the reported Ct value and x is the number of template molecules. The correlation coefficient (R^2) for the regression equation was 0.9973. The efficiency of the RT-PCR assay was 95.6% (Ginzinger, 2002). Samples from a known *A. marginale* carrier and a naïve cow were extracted and analyzed simultaneously for monitoring assay performance and quality of the rRNA extraction technique.

Injection system preparation and simulated vaccination

The needle-free injection system (Pulse 250 Needle-free Injection System, Felton International, Lenexa, KS) was primed with a 1L bag of sterile saline solution (0.9% NaCl, B. Braun Medical Inc.; Irvine, CA) according to the manufacturers recommendations. The pneumatic pressure was metered to 80 psi for delivery of 2 mL of saline intramuscularly in cattle weighing < 227.3 kg. Similarly, a multi-dose syringe (Ideal Instruments Pro-Shot 50cc Pistol Grip Syringe, Durvet Inc. Animal Health Products; Blue Springs, MO) was prepared with a 50 mL aliquot of saline acquired from the 1L bag previously described. The syringe was fitted with a single use, 1.7 X 25 mm hypodermic needle (Beckton Dickinson and Company; Franklin Lakes, NJ) and preset to deliver a 2 mL injection.

The transmission study initiated when a PPE of 2.0% developed in the SPS. For the provision of preemptive analgesia due to receiving multiple injections, the SPS was premedicated with an intravenous injection of flunixin meglumine (1 mg/kg) fifteen minutes prior to restraint. A halter was used to extend and secure the head of the SPS for provision of lateral exposure of the neck. Ten, naïve steers previously randomized to the ND treatment group were individually restrained adjacent to the SPS. A sham vaccination with 2 mL of sterile saline

(0.9% NaCl) was delivered intramuscularly into the left lateral cervical muscle of the SPS with the hypodermic needle fitted to the multi-dose syringe. The same needle and syringe were then utilized within sixty seconds to vaccinate a naïve steer from the ND group in the ipsilateral muscles of the neck as the SPS. This two step procedure was repeated until ten naïve steers in the ND treatment group were sham vaccinated ipsilaterally by needle injection. Similarly, the contralateral (left) cervical muscles of the SPS were injected with the needle-free injection system for the ten naïve calves in the NF treatment group. Briefly, the tip of the needle-free injection system apparatus was placed against the hide of the SPS. The apparatus was agitated in a circular motion to clear hair from between the apparatus and the epidermis. Manual, downward pressure was used to engage and ready the apparatus for injection. Finally, the injection trigger was depressed for release of the saline injection. This process was immediately repeated on a NF steer. This multi-stage procedure was repeated until ten naïve steers in the NF treatment group were sham vaccinated ipsilaterally by needle-free injection. Five calves in the CONT treatment group served as non-injected, sentinel steers. No injection site was disinfected nor was any injection apparatus exchanged or disinfected during either procedure. One operator performed the injections for each system used.

Steers were monitored daily for signs of illness. Disease status was monitored semi-weekly throughout a 61 day study period by light microscopy, cELISA, and RT-PCR as previously described here within.

Agreement and Statistical analysis

Data were entered into a software package (Microsoft Excel 2007, Microsoft Corporation; Redmond, WA) for subsequent calculations and manipulation. Geometric mean and geometric coefficient of variation (Geo CV%) were calculated for data acquired from responses recorded from diagnostic test results. Diagnostic test results were also converted to a binary format (0 = negative, 1 = positive). Sensitivity and specificity with 95% confidence intervals were calculated for light microscopy, cELISA, and RT-PCR for each time point (n = 25).

Agreement between diagnostic results of each semi-weekly sampling was assessed by calculating a Kappa statistic (κ) statistic (Le, 2003). Results were compared using a software program (WinEpiscope 2.0, CLIVE; Edinburgh UK) in a 2 X 2 contingency table to calculate κ with the following equations:

$$EP = [(a+b)/n \cdot (a+c)/n] + [(c+d)/n \cdot (b+d)/n]$$

$$\kappa = [(a+d)/n - EP] \ (1\text{-}EP)$$

where EP is the expected proportion of equal outcomes according to chance; $(a+d)/n - EP$ is the observed proportion of equal outcomes beyond chance; and 1-EP is the maximal proportion of agreement not due to chance. The κ statistic measures the agreement between tests on a scale from 0 to 1. A calculated κ result, where $0 < \kappa < 0.4$, indicates poor reproducibility. Good and excellent reproducibility is indicated when $0.4 < \kappa < 0.75$ and $\kappa > 0.75$, respectively. When κ could not be determined because of a lack of concordant results in 2 or more 2 X 2 cells, an overall proportion of agreement was calculated by dividing the sum of concordant test results by the number of samples tested (Le, 2003).

The association between disease outcome and the three diagnostic testing regimens was analyzed by generalized linear mixed models and generalized estimating equations (PROC Glimmix and PROC Genmod, SAS version 9.1, SAS Institute, Inc.; Cary, NC). Proper methods were employed to take into account the lack of independence among repeated observations of the same animal over time. A semi-parametric survival analysis (Stata v10.1; Stata Corp LP, College Station, TX) was performed to take into account the time variable of when each respective diagnostic test first detected *A. marginale*. A Kaplan-Meier survival analysis (non-parametric) (Stata v10.1; Stata Corp LP, College Station, TX) was performed on the raw data depicting the amount of time elapsed post-injection prior to positive outcomes for each of the three testing regimens. An alpha level of 0.05 was observed throughout the study for evaluating statistically significant differences.

Results

Prior to enrollment in the study, all steers were confirmed negative for anaplasmosis with the cELISA and an *A. marginale*-specific RT-PCR assay. No adverse reaction due to surgery was observed in the SPS. The SPS was subinoculated with 5 mL of a heparinized whole blood sample that had a parasitemia of 8.1% and PCV of 30%; however, rRNA molecule count was not determined in the inoculum used due to heparinization of the collected sample.

The time elapsed from the subinoculation of the SPS to the development of a PPE equal to 2% was thirty-four days. The PPE, PCV and number of 16S rRNA molecules were 2%, 23% and 8.9×10^9 upon study initiation, respectively. No adverse reactions were observed due to

injection method used. Currently, there are no indications suggesting the use of flunixin meglumine neither predisposes cattle to clotting disorders nor were any such complications noted after administration. The SPS was euthanized forty-five days after subinoculation due to anorexia, lethargy, and pyrexia; however, no other steer was removed from the study.

Upon study completion, 60% of the steers in the ND treatment group were positive for the bovine anaplasmosis pathogen, *A. marginale*, based upon the results of cELISA, light microscopy and RT-PCR. The order of injection of steers infected in the ND treatment group was first, second, fourth, sixth, seventh, and tenth. All steers in the NF and CONT groups remained negative for anaplasmosis throughout the study. A significant difference in the number of steers infected with *A. marginale* occurred between ND and NF treatments ($P < 0.05$) as well as ND and CONT ($P < 0.05$).

The predicted model-adjusted probability of becoming infected when comparing ND to NF and ND to CONT was 0.6 ± 0.16 and 0.65 ± 0.18, respectively. Since all cattle in the NF and CONT treatment groups remained negative to *A. marginale*, the model-adjusted probabilities of testing positive for *A. marginale* was zero. The odds of becoming infected with *A. marginale* due to ND treatment were 44.6 (19.5,101.8) times ($P < 0.05$) more likely than NF and CONT.

The RT-PCR assay identified 120 molecules of 16S rRNA (Table 3.1) in one infected steer as early as 9 days post-injection (DPI) (Figure 3.2). The sensitivity of the RT-PCR assay was consistent in identifying steers infected with *A. marginale* in successive samplings. The peak sensitivity (100%) of the RT-PCR assay occurred at 20 DPI and sustained through 61 DPI. The peak number of 16S rRNA molecules recovered from 250 µL of plasma-free, whole blood (1.6 X 10^8) occurred at 41 DPI. Positive assay results did not occur for the CONT and NF groups with the RT-PCR assay.

The cELISA identified one positive steer at 13 DPI (Figure 3.2); however, sensitivity of cELISA was observed to be inconsistent during the four successive samplings. No infected steer was identified by the cELISA at 16 DPI. The steer identified at 20 DPI was not the infected steer identified at 13 DPI. The cELISA did not demonstrate consistent sensitivity until 34 DPI. The peak sensitivity (100%) of the cELISA occurred at 41 DPI and sustained through 61 DPI. This coincided with the peak number of 16S rRNA molecules identified by the RT-PCR assay and a decreasing PCV (Table 3.1).

The PPE determination through examination of stained blood smears by light microscopy identified one infected steer as early as day 16; however, this steer was not consistently identified until day 34. Interestingly, the peak sensitivity of light microscopy (41 DPI) coincided with the peak sensitivity of cELISA, peak in rRNA molecules identified with RT-PCR, and the decline in PCV (Table 3.1). Furthermore, the odds of testing positive for *A. marginale* infection using light microscopy are only 0.5 times (P < 0.05) as likely as cELISA.

A comparison was prepared to illustrate the variability of diagnostic method performance and agreement during the peracute, acute, and chronic stages of infection (Table 3.2). Sensitivity was inadequate among all methods during the peracute phase post-injection. However, RT-PCR, cELISA and light microscopy detected an infected steer on days 9, 13 and 16 post-injection. Peak sensitivity was recorded on days 20, 41, and 41 post-injection for RT-PCR, cELISA and light microscopy, respectively. Diagnostic sensitivity was sustained for the cELISA and RT-PCR; however, sensitivity of light microscopy decreased to zero by day 57 post-injection. RT-PCR was the only diagnostic method that maintained 100% specificity throughout the study. Poor specificity was demonstrated by the cELISA due to eight false positive assay results for steers in the CONT and NF groups on days two (1), thirteen (2), sixteen (1), twenty-three (3), and thirty (1) DPI. The % inhibition recorded for each of these false positive results was between 30 and 40%. The odds of an *A. marginale* infected steer testing positive with the RT-PCR assay was 1.34 (P = 0.07) times more likely than with cELISA. Agreement was determined between the cELISA and RT-PCR (2 test) as well as the result of all three diagnostic methods (3 test). However, a proportion of concordance (0.76) was calculated on 0, 2 and 6 DPI due to a lack of concordant results in 2 or more cells in the 2X2 contingency table. The sensitivity and specificity for each of these test days were 0% and 100%, respectively. Kappa was calculated for the remainder of results occurring on 9 through 61 DPI. Perfect agreement occurred on day 41 when making a 2 and 3 test comparison; however, an exceptional level of agreement was only sustained through the remainder of the study when comparing the RT-PCR and cELISA. At 61 DPI, the 2 and 3 test agreement were 0.75 (0.53,0.97) and 1 (0.72,1.28), respectively.

The imprecision of diagnostic test results reported for the six steers infected with *A. marginale* in the ND group was calculated as the Geo CV% of the geometric mean (Table 3.1). The Geo CV% for RT-PCR at peak sensitivity (20 DPI) was greater than 15% (17%). However, this estimate did not exceed 22% throughout the study once infected steers were detected. The

Geo CV% for light microscopy, cELISA and RT-PCR at 41 DPI, which corresponds to the greatest three test agreement (1), were 68%, 43.2%, and 1.8%, respectively. The lowest estimates occurred at 48 DPI, 61 DPI and 44 DPI for light microscopy, cELISA, and RT-PCR responses, respectively.

Results from the semi-parametric survival analysis were expressed as hazard ratios. Hazard ratios were interpreted similarly as odds ratios, assumed to be proportional over time and represented the effect of a unit change in the predictor on the frequency of the outcome (Le, 2003). The hazard ratio of cattle testing positive to *A. marginale* in the ND group by RT-PCR when compared to cELISA and light microscopy was 1.15 (P = 0.06) and 1.58 (P < 0.05), respectively. Likewise, the hazard ratio of cattle testing positive to *A. marginale* in the ND group by cELISA when compared to RT-PCR and light microscopy was 0.74 (P=0.06) and 1.86 (P<0.05), respectively. Conversely, light microscopy was shown to be the least likely to find positive animals across the study period as the hazard ratio of cattle testing positive to *A. marginale* in comparison to RT-PCR and cELISA was 0.74 (P<0.05) and 0.81 (P<0.05), respectively. A Kaplan-Meier survival analysis was performed on raw data comparing the three diagnostic regimens (Figure 3.3). At 0 DPI, the risk of infection is 1 for all steers exposed. However, this risk reduced over time as the *A. marginale* infected steers were identified.

Additionally, the semi-parametric survival analysis was also used to test the significance of the injection sequence among the ND treatment group. Once the needle was contaminated post-exposure to the SPS, the steers that subsequently tested positive for *A. marginale* was the first, second, fourth, sixth, seventh, and tenth animals in the ND group. The hazard ratio for the sequence of injection (0.96; P>0.05) indicated the hazard of becoming positive to *A. marginale* was the same across all animals in the ND treatment group. Therefore, the sequence of injection was not associated with testing positive to *A. marginale*.

To further validate the results of the diagnostic methods, fifteen naïve steers from the NF and CONT groups were intravenously inoculated with five milliliters of whole blood from one of each of the six steers iatrogenically infected with the Virginia isolate of *A. marginale*. All fifteen steers became infected with *A. marginale* due to intravenous inoculation as determined by interpretation of cELISA, light microscopy and RT-PCR results in series. The four remaining steers were not challenged due to subsequent enrollment in a separate study.

Discussion

Anaplasmosis presents many problems to the cattle industry due to complications with disease control, eradication and treatment. When vaccinating cattle of unknown disease status, hygienic animal husbandry techniques are highly recommended. Although repetitive needle use among cattle is not suggested, this practice still occurs today. It may be argued that a cattle population with an endemic and stable disease prevalence may be advantageous due to minimizing clinical disease in adult cattle. However, this approach does not permit the comingling of cattle of unknown disease status. Another disadvantage would be the transmission of other blood-borne pathogens of cattle. If anaplasmosis disease prevalence is allowed to increase, culling practices may be amplified as well as trade restrictions intensified between endemic and non-endemic countries due to the current use of unreliable diagnostic methods.

This study was designed to vigorously challenge the utility of needle-free injection for the control of anaplasmosis transmission among cattle during vaccination. Needle-free injection was validated as a tool for controlling horizontal transmission of *A. marginale*. Additionally, a novel RT-PCR assay was evaluated for detecting *A. marginale* in bovine peripheral blood samples. This data set is clinically relevant due to the potential spread of *A. marginale* to naive cattle during routine animal husbandry practices as well as identifying the deficiencies in the sensitivity and specificity of currently available diagnostic methods. To our knowledge, this is the first report to evaluate needle-free injection techniques for the control of iatrogenic transmission of anaplasmosis as well as determine the performance of first, second and third generation diagnostic methods at sequential time points following a single exposure to *A. marginale* in cattle.

Needle transmission of anaplasmosis from a known carrier to susceptible cattle has been documented (Reeves and Swift, 1977). In that report, only 1 out of 5 animals was infected. Furthermore, the authors described the ability to visually detect blood contamination on the needle between injections. In this study, 6 out of 10 animals were infected. Blood contamination on the single use needle was only visible prior to the injection of the last naive calf in the ND injection group. Otherwise, the needle appeared to be safe to use for multiple injections. Due to the random pattern of transmission and lack of statistical significance associated with injection sequence, the repetitive use of a needle among cattle of unknown disease status should be regarded as unacceptable. Furthermore, the fact that 60% of steers exposed to an *A. marginale*

contaminated needle became infected following an intramuscular injection leads to the hypothesis that this route of infection may be very common in current production systems.

The transmission of blood components during needle-free injection techniques has been documented (Sweat et al., 2000). This is apparently negligible when concerned with anaplasmosis disease transmission under the conditions of this study. The vaccination of the SPS prior to the injection of each steer in the NF group robustly challenged the potential for iatrogenic transmission of a Virginia isolate of *A. marginale* with needle-free injection. However, the utility of needle-free injection is unknown under situations where anaplasmosis carriers may have a PPE greater than 2%. Similar or worse conditions are reasonably unlikely to be encountered under field conditions. However, it may be necessary to account for the temporal association of previous vaccinations, disease prevalence and the timing of vaccination in regard to the seasonal distribution of clinical cases when applying needle-free injection techniques. Furthermore, it should be noted that disease resistance among breeds has not been shown to exist (Bock et al., 1999; Jonsson et al., 2008; Wilson et al., 1980).

The use of needle-free injection in production settings will aid in the reduction of biohazard waste, alleviate operator injury due to accidental needle puncture and eliminate the possibility of needle contamination due to vaccination in consumable meat products while maintaining processing rates that are necessary for minimizing handling and stress in cattle. Even though needle-free injection is superior to needle injection for preventing iatrogenic transmission of *A. marginale*, it is recommended that due care be observed to avoid the unwarranted vaccination of cattle that might occur as a result of improper cleansing of previously used vaccine products from the injection system (Makoschey and Beer, 2004). Further studies are necessary to fully evaluate the utility of needle-free injection for the control of other blood-borne diseases of cattle.

Upon study completion, the prevalence of anaplasmosis was 24%. It may be argued that the evaluation of sensitivity and specificity among the diagnostic methods included in this study be interpreted with caution due to the small study population and low number of infected steers; however, the accurate and precise diagnosis of anaplasmosis has historically been problematic due to diagnostic methods that lack adequate sensitivity and specificity (Bradway et al., 2001; Coetzee et al., 2007; Dreher et al., 2005; Gonzalez et al., 1978; Strik et al., 2007). Therefore, it is important to describe the inequality of these diagnostic methods.

First generation diagnostic methods rely on the growth or visualization of the organism of interest. These methods have limited sensitivity and lack adequate specificity to differentiate between morphologically similar pathogens, normal structures, and stain artifacts. During this study, light microscopy was proven to be unreliable due to false negative results. This was due to the low level of circulating rickettsemias encountered. Even though all steers infected with *A. marginale* were accurately classified during the study, light microscopy only demonstrated 100% diagnostic sensitivity at 40 DPI.

Second generation methods, which rely on the identification of cell components, metabolic products, and detection of antigenic components, are currently the most commonly used techniques for disease classification in clinical medicine and research. One disadvantage of these methods is the potential for cross-reactivity among coexisting diseases. Due to similarity among MSP5 surface proteins, cross-reactivity when using cELISA has been reported among *A. marginale, Anaplasma centrale* and *Anaplasma phagocytophilum* (Dreher et al., 2005; Strik et al., 2007). The cELISA demonstrated improved and sustainable sensitivity over light microscopy. However, cELISA did not demonstrate 100% sensitivity until 41 DPI in the ND group. The disadvantage of this technique is the cut-off value used to classify disease status. A cut-off of > 30% inhibition was used in this study to classify a steer as disease positive. This level led to multiple false positive results in the NF and CONT groups. However, the sensitivity of this assay at the earlier time points would have been compromised if a 40% inhibition had been used.

Third generation methods utilize nucleic acid-based techniques for the classification of disease status. These methods offer superior sensitivity and specificity over first and second generation methods. This study is not the first to develop or use a nucleic acid-based technique for the diagnosis of *A. marginale* in bovine peripheral blood samples (Carelli et al., 2007; Decaro et al., 2008; Eriks et al., 1989; Figueroa et al., 1998; Ge et al., 1997; Ge et al., 1995; Goff et al., 1990; Hoar et al., 2008; Molad et al., 2006; Torioni de Echaide et al., 1998). However, it is the first of its kind to use a real-time, quantitative RT-PCR method to identify 16S rRNA of *A. marginale*. The advantages of this assay are the enhanced sensitivity of identifying rRNA targets that are present in higher quantities than a single copy of DNA per organism; the ability to quantify the genetic template; the need for polyacrylamide gel electrophoresis is no longer required; and this assay could serve as a substitute for the subinoculation of splenectomized

cattle with blood from cattle of unknown disease status. The major disadvantage is the cost of the reagents and equipment as well as the necessity of modern elements that may not be readily accessible.

The RT-PCR assay demonstrated 100% sensitivity by 20 DPI. This is a marked improvement over light microscopy and cELISA for diagnosis in the prepatent period; however, false negative assay results occurred due to the inability of RT-PCR to detect disease prior to 20 DPI in 250 µL of plasma-free blood samples. Due to the performance of the diagnostic methods used during this study, the authors would recommend repeated sampling of cattle of unknown disease status on a 3 week and 6 week interval when using the RT-PCR assay and cELISA, respectively. However, light microscopy would not be recommended for determining disease status in unknown cattle.

Anaplasmosis is a complex and challenging disease for stakeholders in the cattle industry, foreign policy and research arenas. Due to the lack of significant success with treatment strategies, vaccine availability, and problematic vector control, anaplasmosis control strategies should primarily concentrate on established methods for disease prevention. This data set is clinically relevant due to the potential spread of *A. marginale* to naive cattle during routine animal husbandry practices as well as illustrating the deficiencies in the sensitivity and specificity of currently available diagnostic methods. Our results identified needle-free injection as a superior method for controlling the iatrogenic transmission of anaplasmosis. Furthermore, a novel, *A. marginale*-specific RT-PCR assay has the potential to significantly impact the future of disease classification prior to local, interstate, or international movement of cattle between endemic and non-endemic countries.

Figures and Tables

Figure 3.1 RT-PCR assay detection sensitivity and linearity with RNA concentration. Serial 10-fold dilutions of an *in vitro* transcript made from *Anaplasma marginale* plasmid DNA. The average Ct values from three independent experiments were plotted against the log number of rRNA molecules (a). The $\log_{10}$ 2 and $\log_{10}$ 9 correspond to 100 and 1 billion molecules of 16S rRNA, respectively. The correlation coefficient (R^2) and equation of the line are 0.9973 and y = -3.4324 + 40.38, respectively. The fluorescent emission from serial dilution templates is shown (b).
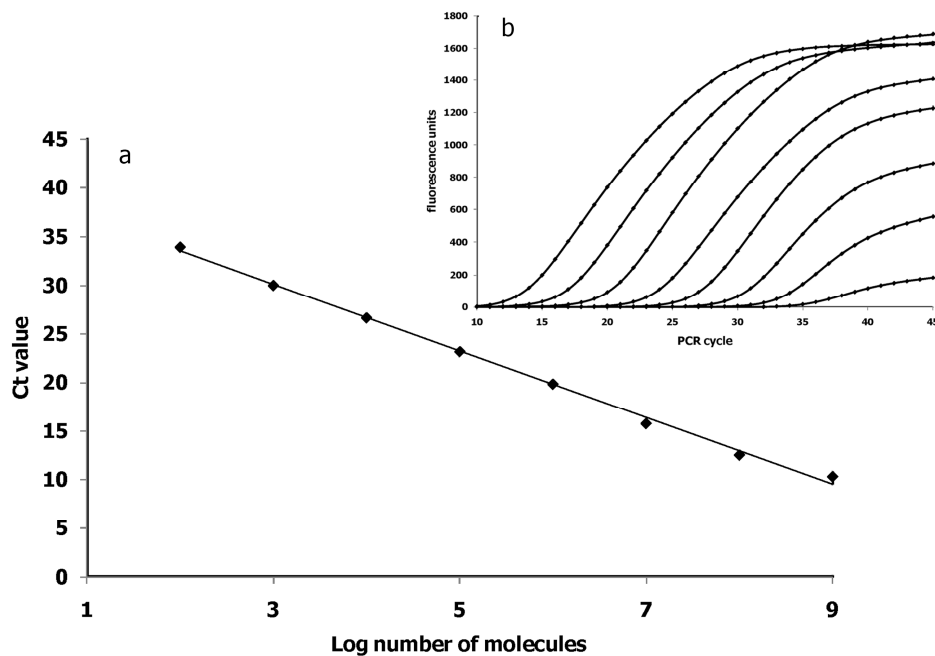
Figure 3.2 Diagnostic method Se for RT-PCR (white boxes), cELISA (black diamonds), and light microscopy (white triangles) for six steers iatrogenically infected with a Virginia isolate of *Anaplasma marginale* **in the ND injection group.**
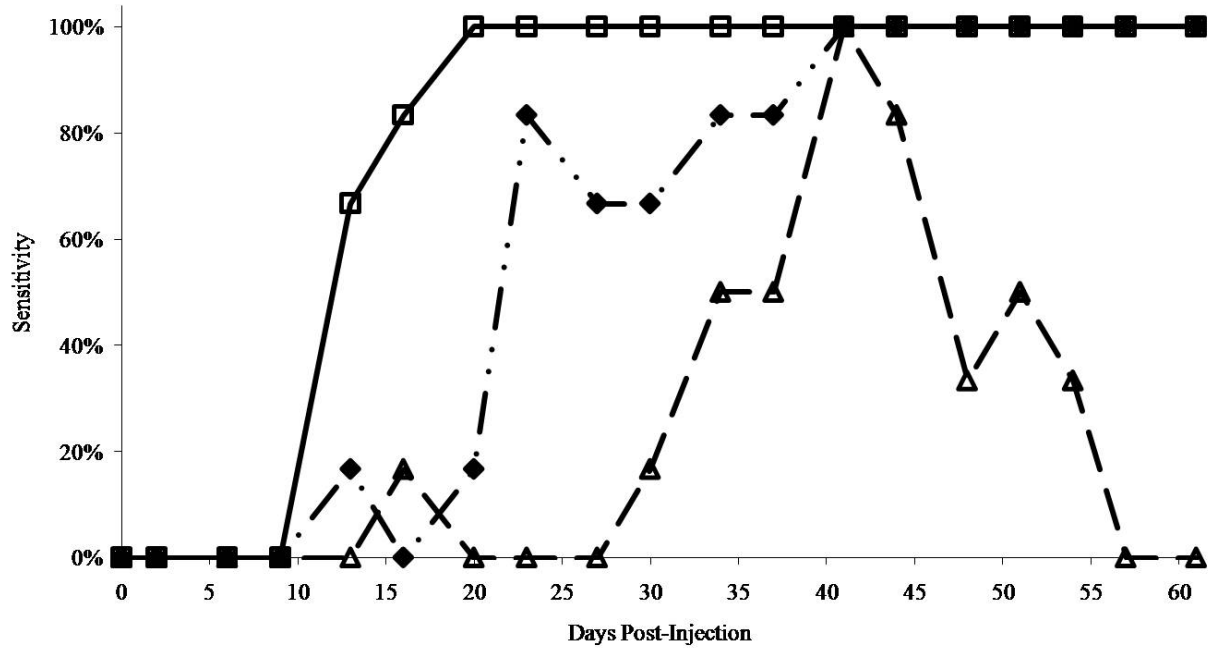
Figure 3.3 A Kaplan-Meier survival estimate derived from the results of six steers iatrogenically infected with a Virginia isolate of *Anaplasma marginale* in the ND injection group for the RT-PCR assay (bold line), cELISA (dashed line) and light microscopy (shaded line).
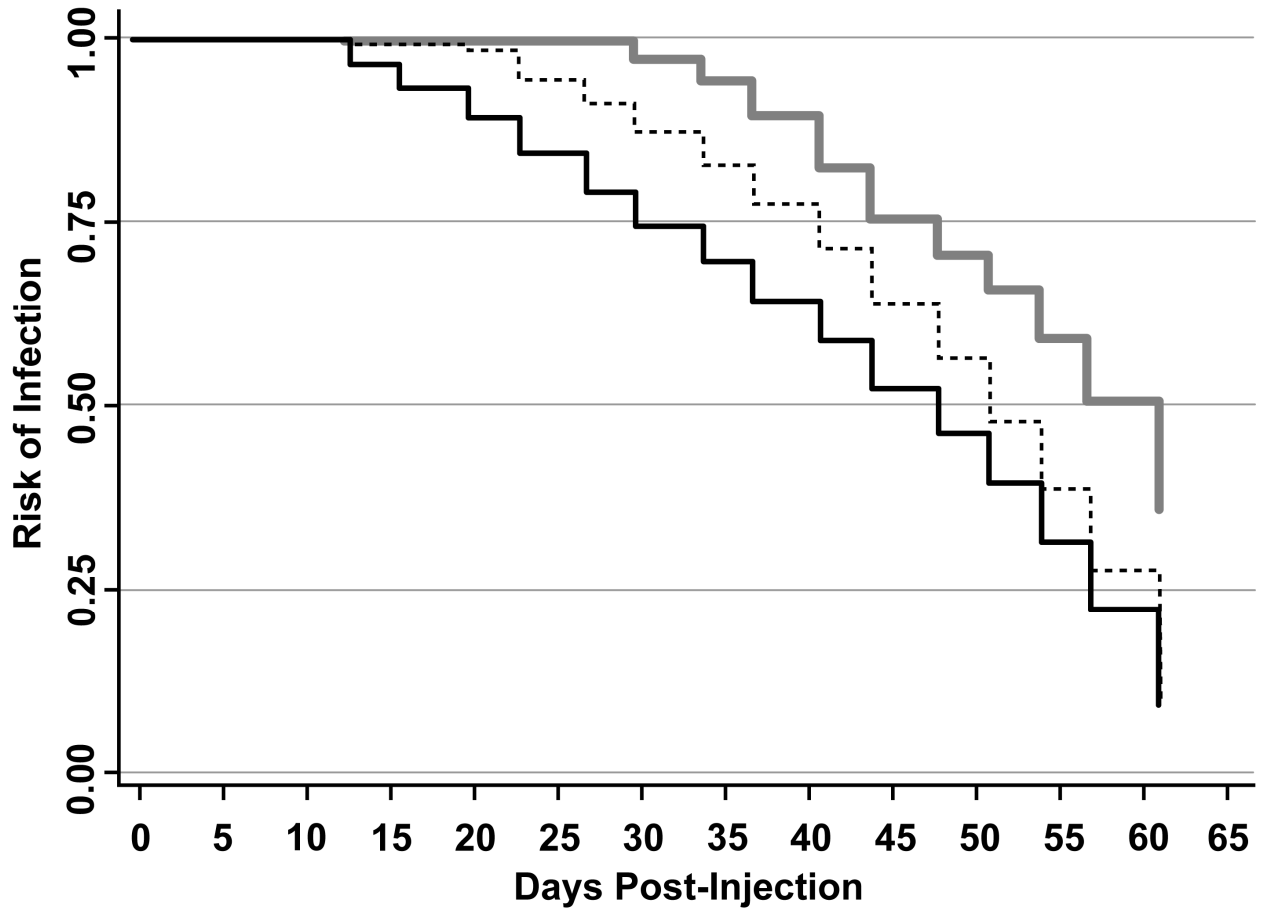
Table 3.1 Geometric mean and Geo CV% for each diagnostic test result response and PCV derived from the analysis of six steers iatrogenically infected with a Virginia isolate of *Anaplasma marginale* in the ND injection group (n = 6).

DPI	Light microscopy[▲]	cELISA[▲]	RT-PCR[*]	PCV
0	0% (--)^0	12.87% (25.9)^6	0 (--)^0	34.29% (5.4)^6
2	0% (--)^0	15.04% (30.9)^6	0 (--)^0	35.36% (9.69)^6
6	0% (--)^0	3.39% (122.4)^6	0 (--)^0	31.78% (6.1)^6
9	0% (--)^0	10.3% (75.6)^6	1.2×10^2 (--)^1	31.94% (6.84)^6
13	0% (--)^0	13.31% (77.0)^6	1.8×10^3 (8.9)^4	31.3% (5.3)^6
17	0% (--)^0	14.96% (36.5)^6	1.9×10^3 (13.5)^5	30.11% (6.84)^6
20	0% (--)^0	21.29% (70.8)^6	4.2×10^4 (17.0)^6	32.14% (4.61)^6
23	0% (--)^0	40.18% (53.5)^6	4.8×10^5 (19.0)^6	29.98% (4.17)^6
27	0% (--)^0	27.1% (243.4)^6	5.7×10^6 (21.8)^6	30.63% (5.3)^6
30	0.1% (--)^1	44.84% (94.2)^6	3.3×10^7 (20.4)^1	30.41% (8.6)^6
34	0.42% (119.1)^3	45.31% (170.2)^6	8.7×10^7 (10.1)^1	30.44% (6.8)^6
37	0.75% (96.3)^3	61.43% (73.1)^6	1.4×10^8 (2.7)^6	28.41% (8.6)^6
41	0.55% (68.0)^6	69.34% (43.2)^6	1.6×10^8 (1.8)^6	25.92% (14.8)^6
44	0.24% (219.9)^5	85.51% (18.9)^6	1.4×10^8 (1.2)^6	25.6% (8.0)^6
48	0.14% (52.1)^2	88.55% (10.7)^6	1.0×10^8 (4.8)^6	27.06% (9.9)^6
51	0.1% (--)^1	79.57% (8.4)^6	8.4×10^7 (10.9)^6	27.37% (10.9)^6
54	0.1% (--)^1	79.57% (11.3)^6	5.0×10^7 (15.5)^6	28.53% (10.9)^6
57	0% (--)^0	82.59% (9.7^6	2.9×10^7 (15.6)^6	29.03% (0.7)^6
61	0% (--)^0	84.65% (12.1)^6	1.9×10^7 (17.4)^6	30.26% (7.3)^6

The numbered superscripts indicate the total number of non-zero values included in the calculation of the geometric mean and Geo CV%. [▲]Data were divided by 100 prior to calculating the geometric mean and geometric coefficient of variation; however, the geometric mean was again multipled by 100% for reporting. [*]The number of 16S rRNA molecules recovered from 250 µL of plasma-free, whole blood.

Table 3.2 Diagnostic method performance as determined by the calculated sensitivity and specificity with 95% confidence intervals for each diagnostic method used during the 61 day study (n=25).

DPI [†]	Light microscopy		cELISA		RT-PCR		Agreement	
	Se (%)	Sp (%)	Se (%)	Sp (%)	Se (%)	Sp (%)	2 test (κ)	3 test (κ)
9	0 (--,--)±	100 (--,--)±	0 (--,--)±	100 (--,--)±	16.7 (0,46.5)	100 (100,100)	0.12 (0,0.25)	0.08 (0,0.17)
13	0 (--,--)±	100 (--,--)±	16.7 (0, 46.9)	89.5 (75.7, 100)	66.7 (28.9,100)	100 (100,100)	0.43 (0.16,0.69)	0.31 (0.11,0.50)
16	16.7 (0 ,46.5)	100 (100,100)	0 (--,--)±	94.7 (84.7,100)	83.3 (53.5,100)	100 (100,100)	0.47 (0.22,0.73)	0.4 (0.20,0.59)
20	0 (--,--)±	100 (--,--)±	16.7 (0,46.5)	100 (100,100)	100 (100,100)	100 (100,100)	0.68 (0.42,0.94)	0.49 (0.30,0.69)
23	0 (--,--)±	94.7 (84.7,100)	83.3 (53.5,100)	84.2 (67.8,100)	100 (100,100)	100 (100,100)	0.79 (0.52,1.1)	0.57 (0.35,0.80)
27	0 (--,--)±	94.7 (84.7,100)	66.7 (28.9,100)	100 (100,100)	100 (100,100)	100 (100,100)	0.88 (0.61,1.16)	0.62 (0.40,0.84)
30	16.7 (0,46.5)	94.7 (84.7,100)	66.7 (28.9,100)	94.7 (84.7,100)	100 (100,100)	100 (100,100)	0.83 (0.55,1.11)	0.64 (0.42,0.86)

34	50 (10,90)	94.7 (84.7,100)	83.3 (53.5,100)	100 (100,100)	100 (100,100)	100 (100,100)	0.94 (0.67,1.22)	0.81 (0.58,1.03)
37	50 (10,90)	94.7 (84.7,100)	83.3 (53.5,100)	100 (100,100)	100 (100,100)	100 (100,100)	0.94 (0.67,1.22)	0.81 (0.58,1.03)
41	100 (100,100)	100 (100,100)	100 (100,100)	100 (100,100)	100 (100,100)	100 (100,100)	1 (0.72,1.28)	1 (0.77,1.23)
44	83.3 (53.5,100)	100 (100,100)	100 (100,100)	100 (100,100)	100 (100,100)	100 (100,100)	1 (0.72,1.28)	0.96 (0.74,1.19)
48	33.3 (0,71.1)	100 (100,100)	100 (100,100)	100 (100,100)	100 (100,100)	100 (100,100)	1 (0.72,1.28)	0.84 (0.62,1.07)
51	50 (10,90)	100 (100,100)	100 (100,100)	100 (100,100)	100 (100,100)	100 (100,100)	1 (0.72,1.28)	0.88 (0.66,1.11)
54	33.3 (0,71.1)	100 (100,100)	100 (100,100)	100 (100,100)	100 (100,100)	100 (100,100)	1 (0.72,1.28)	0.84 (0.69,1.07)
57	0 (--,--)[±]	100 (--,--)[±]	100 (100,100)	100 (100,100)	100 (100,100)	100 (100,100)	1 (0.72,1.28)	0.75 (0.53,0.97)
61	0 (--,--)[±]	100 (--,--)[±]	100 (100,100)	100 (100,100)	100 (100, 100)	100 (100,100)	1 (0.72,1.28)	0.75 (0.53,0.97)

Se= sensitivity. Sp= specificity. [†]Values for 0, 2, and 6 DPI were omitted from the table due to a lack of concordant results in 2 or more cells in the 2X2 contingency table. For each of these time points, the calculated proportion of concordance was 0.76 with a Se and Sp of 0 and 100%, respectively. [‡]The 95% confidence interval was not calculated due to a lack of concordant results in 2 or more cells in

the 2X2 contingency table. The 2 test (κ) is a comparison of agreement between the cELISA and RT-PCR assays exclusively.

References

Uilenberg G. International collaborative research: significance of tick-borne hemoparasitic diseases to world animal health. *Vet Parasitol* 1995;57:19-41.

Dumler JS, Barbet AF, Bekker CP, et al. Reorganization of genera in the families *Rickettsiaceae* and *Anaplasmataceae* in the order *Rickettsiales*: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and designation of *Ehrlichia equi* and 'HGE agent' as subjective synonyms of *Ehrlichia phagocytophila*. *Int J Syst Evol Microbiol* 2001;51:2145-2165.

Kocan KM, de la Fuente J, Guglielmone AA, et al. Antigens and alternatives for control of *Anaplasma marginale* infection in cattle. *Clin Microbiol Rev* 2003;16:698-712.

OIE. Office International des Épizooties, Manual of standards for diagnostic tests and vaccines for terrestrial animals. Chapter 2.4.1 17th ed. 2008. Accessed July 13, 2009. http://www.oie.int/eng/normes/mmanual/2008/pdf/2.04.01_BOVINE_ANAPLASMOSIS.pdf 2009.

Rogers RJ, Shiels IA. Epidemiology and control of anaplasmosis in Australia. *J S Afr Vet Assoc* 1979;50:363-366.

Radostits OM, Gay CC, Blood DC, et al. *Veterinary Medicine: A textbook of the diseases of cattle, sheep, pigs, goats and horses*. 9th ed. St. Louis: W. B. Saunders Company Ltd, 2000.

Dennis RA, O'Hara PJ, Young MF, et al. Neonatal immunohemolytic anemia and icterus of calves. *J Am Vet Med Assoc* 1970;156:1861-1869.

Luther DG. http://anaplasmosisvaccine.com, 1999.

Lincoln SD, Eckblad WP, Magonigle RA. Bovine anaplasmosis: clinical, hematologic, and serologic manifestations in cows given a long-acting oxytetracycline formulation in the prepatent period. *Am J Vet Res* 1982;43:1360-1362.

Coetzee JF, Apley MD, Kocan KM. Comparison of the efficacy of enrofloxacin, imidocarb, and oxytetracycline for clearance of persistent *Anaplasma marginale* infections in cattle. *Vet Ther* 2006;7:347-360.

Wilson AJ, Parker R, Parker M, et al. Chemotherapy of acute bovine anaplasmosis. *Aust Vet J* 1979;55:71-73.

Coetzee JF, Apley MD, Kocan KM, et al. Comparison of three oxytetracycline regimens for the treatment of persistent Anaplasma marginale infections in beef cattle. *Vet Parasitol* 2005;127:61-73.

Zaugg JL, Kuttler KL. Bovine anaplasmosis: in utero transmission and the immunologic significance of ingested colostral antibodies. *Am J Vet Res* 1984;45:440-443.

Zaugg JL. Bovine anaplasmosis: transplacental transmission as it relates to stage of gestation. *Am J Vet Res* 1985;46:570-572.

Futse JE, Ueti MW, Knowles DP, Jr., et al. Transmission of Anaplasma marginale by *Boophilus microplus*: retention of vector competence in the absence of vector-pathogen interaction. *J Clin Microbiol* 2003;41:3829-3834.

Reeves JD, Swift BL. Iatrogenic transmission of *Anaplasma marginale* in beef cattle. *Vet Med Small Anim Clin* 1977;72:911-914.

Eriks IS, Palmer GH, McGuire TC, et al. Detection and quantitation of *Anaplasma marginale* in carrier cattle by using a nucleic acid probe. *J Clin Microbiol* 1989;27:279-284.

Potgieter F, van Rensburg L. The persistence of colostral *Anaplasma* antibodies and incidence of in utero transmission of *Anaplasma* infections in calves under laboratory conditions. O*nderstepoort J Vet Res* 1987;54:557-560.

Norton JH, Parker RJ, Forbes-Faulkner JC. Neonatal anaplasmosis in a calf. *Aust Vet J* 1983;60:348.

Peter RJ, Van den Bossche P, Penzhorn BL, et al. Tick, fly, and mosquito control--lessons from the past, solutions for the future. *Vet Parasitol* 2005;132:205-215.

De Wall DT. Anaplasmosis control and diagnosis in South Africa. *Ann N Y Acad Sci* 2000;916:474-50 483.

Rodriguez-Vivas RI, Mata-Mendez Y, Perez-Gutierrez E, et al. The effect of management factors on the seroprevalence of *Anaplasma marginale* in *Bos indicus* cattle in the Mexican tropics. *Trop Anim Health Prod* 2004;36:135-143.

Andrews AH, Lamport A. A practical method of reducing spread of disease by hypodermic needles. *Vet Rec* 1985;116:185-186.

Makoschey B, Beer M. Assessment of the risk of transmission of vaccine viruses by using insufficiently cleaned injection devices. *Vet Rec* 2004;155:563-564.

Anderson DE, Silviera F. Survey of Large Animal Veterinarians' Biosecurity Practices. *Proceedings 41st Annual Convention AABP in Charlotte, NC* 2008:244.

Hollis LC, Smith JF, Johnson BJ, et al. A comparison of serological responses when modified-live infectious bovine rhinotracheitis virus vaccine and *Mannheimia haemolytica* bacterin-toxoid are administered with Needle-free versus conventional needle-based injection in yearling feedlot steers. *Bovine Practitioner* 2005;39:106-109.

Huang Y, Babiuk LA, van Drunen Littel-van den Hurk S. Immunization with a bovine herpesvirus 1 glycoprotein B DNA vaccine induces cytotoxic T-lymphocyte responses in mice and cattle. *J Gen Virol* 2005;86:887-898.

Manoj S, Griebel PJ, Babiuk LA, et al. Modulation of immune responses to bovine herpesvirus-1 in cattle by immunization with a DNA vaccine encoding glycoprotein D as a fusion protein with bovine CD154. *Immunology* 2004;112:328-338.

Sweat JM, Abdy M, Weniger BG, et al. Safety testing of needle free, jet injection devices to detect contamination with blood and other tissue fluids. *Ann N Y Acad Sci* 2000;916:681-682.

Torioni de Echaide S, Knowles DP, McGuire TC, et al. Detection of cattle naturally infected with *Anaplasma marginale* in a region of endemicity by nested PCR and a competitive enzyme-linked immunosorbent assay using recombinant major surface protein 5. *J Clin Microbiol* 1998;36:777-782.

VMRD. Anaplasma antibody test kit, cELISA. . Version 060505 ed: Veterinary Medical Research & Development; Assay instructions for catalog numbers: 282-282 and 282-285. USDA Product Code 77 5002.5020 For veterinary use only.

Coetzee JF, Schmidt PL, Apley MD, et al. Comparison of the complement fixation test and competitive ELISA for serodiagnosis of *Anaplasma marginale* infection in experimentally infected steers. *Am J Vet Res* 2007;68:872-878.

Dohoo I, Martin W, Stryhn H. *Veterinary Epidemiologic Research*. Charlottetown, PrinceEdward Island: AVC Inc., 2007.

de la Fuente J, Blouin EF, Kocan KM. Infection exclusion of the rickettsial pathogen *Anaplasma marginale* in the tick vector *Dermacentor variabilis*. *Clin Diagn Lab Immunol* 2003;10:182-184.

Teerasaksilp S, Wiwanitkit V, Lekngam P. Comparative study of blood cell staining with wright giemsa stain, field stain, and a new modified stain. *Lab Hematol* 2005;11:76-78.

Kutaish N. Automated staining of bone marrow and peripheral blood by a modified Wright's technique. *Am J Clin Pathol* 1982;77:319-320.

Riley RS, Ben-Ezra JM, Goel R, et al. Reticulocytes and reticulocyte enumeration. *J Clin Lab Anal* 2001;15:267-294.

Strik NI, Alleman AR, Barbet AF, et al. Characterization of *Anaplasma phagocytophilum* major surface protein 5 and the extent of its cross-reactivity with *Anaplasma* marginale. *Clin Vaccine Immunol* 2007;14:262-268.

Sirigireddy KR, Ganta RR. Multiplex detection of *Ehrlichia* and *Anaplasma* species pathogens in peripheral blood by real-time reverse transcriptase-polymerase chain reaction. *J Mol Diagn* 2005;7:308-316.

Ginzinger DG. Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream. *Exp Hematol* 2002;30:503-512.

Le CT. *Introductory biostatistics*. 1st ed. Hoboken, NJ: John Wiley and Sons, Inc., 2003.

Wilson AJ, Parker R, Trueman KF. Susceptibility of *Bos indicus* crossbred and *Bos taurus* cattle to *Anaplasma marginale* infection. *Trop Anim Health Prod* 1980;12:90-94.

Bock RE, Kingston TG, De Vos AJ. Effect of breed of cattle on innate resistance to infection with *Anaplasma marginale* transmitted by *Boophilus microplus*. *Aust Vet J* 1999;77:748-751.

Jonsson NN, Bock RE, Jorgensen WK. Productivity and health effects of anaplasmosis and babesiosis on *Bos indicus* cattle and their crosses, and the effects of differing intensity of tick control in Australia. *Vet Parasitol* 2008;155:1-9.

Gonzalez EF, Long RF, Todorovic RA. Comparisons of the complement-fixation, indirect fluorescent antibody, and card agglutination tests for the diagnosis of bovine anaplasmosis. *Am J Vet Res* 1978;39:1538-1541.

Bradway DS, Torioni de Echaide S, Knowles DP, et al. Sensitivity and specificity of the complement fixation test for detection of cattle persistently infected with *Anaplasma marginale*. *J Vet Diagn Invest* 2001;13:79-81.

Dreher UM, de la Fuente J, Hofmann-Lehmann R, et al. Serologic cross-reactivity between *Anaplasma marginale* and *Anaplasma phagocytophilum*. *Clin Diagn Lab Immunol* 2005;12:1177-1183.

Carelli G, Decaro N, Lorusso A, et al. Detection and quantification of *Anaplasma marginale* DNA in blood samples of cattle by real-time PCR. *Vet Microbiol* 2007;124:107-114.

Decaro N, Carelli G, Lorusso E, et al. Duplex real-time polymerase chain reaction for simultaneous detection and quantification of *Anaplasma marginale* and *Anaplasma centrale*. *J Vet Diagn Invest* 2008;20:606-611.

Figueroa JV, Alvarez JA, Ramos JA, et al. Bovine babesiosis and anaplasmosis follow-up on cattle relocated in an endemic area for hemoparasitic diseases. *Ann N Y Acad Sci* 1998;849:1-10.

Ge NL, Kocan KM, Ewing SA, et al. Use of a nonradioactive DNA probe for detection of *Anaplasma marginale* infection in field cattle: comparison with complement fixation serology and microscopic examination. *J Vet Diagn Invest* 1997;9:39-43.

Ge NL, Kocan KM, Murphy GL, et al. Detection of *Anaplasma marginale* DNA in bovine erythrocytes by slot-blot and in situ hybridization with a PCR-mediated digoxigenin-labeled DNA probe. *J Vet Diagn Invest* 1995;7:465-472.

Goff WL, Stiller D, Roeder RA, et al. Comparison of a DNA probe, complement-fixation and indirect immunofluorescence tests for diagnosing *Anaplasma marginale* in suspected carrier cattle. *Vet Microbiol* 1990;24:381-390.

Hoar BR, Nieto NC, Rhodes DM, et al. Evaluation of sequential coinfection with *Anaplasma phagocytophilum* and *Anaplasma marginale* in cattle. *Am J Vet Res* 2008;69:1171-1178.

Molad T, Mazuz ML, Fleiderovitz L, et al. Molecular and serological detection of *Anaplasma centrale*- and *Anaplasma marginale*-infected cattle grazing within an endemic area. *Vet Microbiol* 2006;113:55-62.

Appendix A: Copyright permission letter from the American Veterinary Medical Association

AVMA — American Veterinary Medical Association

Diane A Fagen,
Librarian
1931 N. Meacham Rd.
Schaumburg, IL
60173-4360

phone 847.925.8070
fax 847.925.0329
dfagen@avma.org
www.avma.org

15 December 2009

James B. Reinbold DVM
111B Mosier Hall
Department of Diagnostic Medicine/Pathobiology
College of Veterinary Medicine
Kansas State University
Manhattan, KS 66506

Dear Dr. Reinbold:

Your manuscript, titled "Comparison of iatrogenic *Anaplasma marginale* transmission by needle and needle-free injection techniques" has been provisionally accepted for publication in the *American Journal of Veterinary Research (AJVR)*. At this time, the manuscript has not been assigned a publication date.
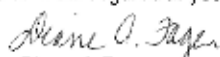
We grant permission to include the most recent revision of this article in your doctoral dissertation, provided:

1) the article is noted as "provisionally accepted for publication in the *American Journal of Veterinary Research (AJVR)*";

2) the article is embargoed from electronic access for a period of twelve (12) months after the publication date of the *AJVR* issue in which it appears.

The permission for immediate use includes publication in print, as well as authorization to University Microfilms / Bell & Howell / Xerox / Proquest to reproduce and distribute single print copies for scholarly use upon demand.

Please note that electronic release of this manuscript may be judged to be prior publication, thus compromising publication in *AJVR*.

Congratulations on your achievement. Best regards in your future work.

Diane A Fagen
Permissions Coordinator

CHAPTER 4 - Plasma pharmacokinetics of oral chlortetracycline in group fed, Holstein steers in a feedlot setting

Introduction

Chlortetracycline HCl (CTC) has served a pivotal role in profitable livestock production since its discovery in 1945. CTC is one of the many broad-spectrum tetracycline antibiotics widely used in veterinary medicine. This antibiotic was first approved for use in feed on June 23, 1954, for improving feed efficiency, growth promotion, and the treatment of CTC-sensitive pathogens (NADA 065-440, Freedom of Information Summary http://www.fda.gov/cvm/FOI/552.htm; NAHMS (Swine 2006), 2006; NAHMS (Feedlot 1999), 1999). Today, the label indications, based on multiple dosing regimens, are for increased rate of weight gain, improved feed efficiency, control of bacterial pneumonia associated with shipping fever complex caused by susceptible *Pasteurella spp.*, control of active infection of anaplasmosis caused by susceptible *Anaplasma marginale*, reduction of liver condemnation because of liver abscessation, and treatment of bacterial enteritis caused by susceptible *Escherichia coli* (Feed Additive Compendium, 2008). The 1999 NAHMS feedlot study reported that 51.9% of all feedlots surveyed administer CTC as a health or production management tool to 18.2% of cattle fed. Operations with capacities of 1000–7999 head, as well as those feeding cattle weighing <318.2 kg, tend to administer CTC to cattle more often than operations feeding >8000 head and cattle weighing >318.2 kg. On average, CTC is fed 8.6 and 7.7 days to cattle weighing <318.2 and >318.2 kg, respectively.

Pharmacokinetic parameters for orally administered CTC are available for preruminating calves, poultry, and swine (Bradley et al., 1982; Luthman & Jacobsson, 1983; Pollet et al., 1983; Dyer, 1988; Kilroy et al., 1990; Wanner et al., 1991; Nielsen & Gyrd-Hansen, 1996). However,

similar information for ruminating cattle is scarce in peer-reviewed literature. As a result of the controversial use of CTC for improved feed efficiency and growth in bovine diets at levels that have been erroneously termed 'subtherapeutic', this antimicrobial has been implicated as a potential variable for the promotion of antimicrobial resistance.

The Clinical and Laboratory Standards Institute (CLSI) has set the susceptible minimum inhibitory concentration (MIC) breakpoint of tetracycline antibiotics (200 mg∕mL of injectable oxytetracycline product) at 2 µg∕mL when treating bovine bacterial infection caused by *Mannheimia haemolytica, Pasteurella multocida, and Histophilus somni* (CLSI 2008); yet, it is unknown if currently approved dosages and treatment regimens applied in modern production systems achieve this recommended concentration in bovine plasma. To evaluate these breakpoints in relation to in-feed CTC regimens, a study was conducted to determine the pharmacokinetic parameters of CTC in ruminating cattle under group fed conditions in a feedlot setting.

Materials and Methods

This study was approved by the Kansas State University (KSU) Institutional Animal Care and Use Committee (IACUC protocol #2517).

Experimental cattle and animal husbandry

A total of 18 preconditioned, clinically healthy Holstein steers were purchased from the KSU dairy farm in Manhattan, KS, USA. The steers were housed in three pens in a dry lot located at the KSU Juniatta Beef Cattle research facility. One month prior to the start of the experiment, the steers were acclimated to a total mixed ration diet consisting of the following ingredients listed in descending quantity; wheat middling pellets, liquid molasses, cracked corn, loose cottonseed hulls, extender pellets, monensin (80 g∕ton), limestone, salt, corn gluten meal pellets, vitamin A, zinc sulfate, and dried distiller's grains. The diet was fed at 2.5% of body weight (as fed) divided into twice daily feedings. Water was supplied *ad libitum*. When handling was necessary, the steers were individually restrained with a head gate and a rope halter. This study was conducted concurrently with an *Anaplasma marginale* chemosterilization study.

Oral chlortetracycline preparation

A commercial chlortetracycline product (Aureomycin 50 Granular®; Alpharma Animal Health, Bridgewater, NJ, USA) was purchased for the formulation of a CTC topdress prepared by the KSU feed mill. A final concentration of 591.0; 1477.3; and 2954.4 mg of CTC per kg of ground corn carrier was used for the 4.4, 11, and 22 mg∕kg dosages administered during the study, respectively. These respective drug concentrations ensured that a similar total quantity of topdress was fed (kg) regardless of the dosage administered.

Treatment groups and administration

Steers were randomized by initial body weight and assigned to a 4.4, 11, or 22 mg∕kg per os, multi-dose CTC treatment (n = 6∕ treatment). Steers were collectively 314 ± 29.9 days old and weighed 312.4 ± 47.1 kg, 309.5 ± 43.6 kg, and 303.5 ± 47.2 kg, for the 4.4, 11, and 22 mg∕kg treatment groups, respectively. Twelve linear feet (3.7 m) of feed bunk space (0.61 m∕ head) was used per treatment group. The daily dietary ration and CTC dosages were determined using the mean weight of the treatment group, which was taken bi-weekly. Daily dosage and ration were divided equally and administered twice daily for 80 days (160 total doses∕group). Residues from the previous treatment were noted in a daily log sheet; however, observed residues were not removed or weighed. If plasma collection coincided with scheduled treatment, the plasma samples were collected prior to treatment administration. The feed bunk was inspected prior to each treatment.

Plasma collection

Plasma samples were collected throughout the 83-day study period; at 12-hour intervals on days 0–7, twice weekly on days 8–77, and every 4hours on days 80–83, according to the following standard procedures. Blood samples were collected via jugular venipuncture into a vacutainer tube containing lithium heparin (BD Vacutainer, Franklin Lakes, NJ, USA). Samples were stored on ice until plasma separation, which was performed by centrifuging the vacutainer tubes containing the plasma at 2750 g at 4°C for 5 minute. Plasma was separated and stored at -80 °C until analysis. Samples were analyzed <11 months after collection. A freezer stability evaluation was not performed.

Sample analysis

A novel solid phase extraction (SPE) and ultrahigh performance liquid chromatography-mass spectroscopy∕mass spectroscopy (UPLC-MS∕MS) method was developed for the analysis of CTC in bovine plasma. Frozen samples were thawed at room temperature with 0.15 mL of plasma transferred to a new tube. Fifty microliters of doxycycline solution (3200 ng∕mL of doxycycline), functioning as a structurally related internal standard, was added to each sample. Ten microliters of phosphoric acid was added followed by vortexing for 20 sec. Next, 150 µL of de-ionized water (D_i) was added and vortexed for 20 sec. The analyte and internal standard were isolated from bovine plasma by SPE using 10 mg, 1 mL Waters HLB cartridges (Waters OASIS Extraction Products; Waters Corporation; Milford, MA, USA). The cartridges were first conditioned with 0.5 mL of >99% methanol, then with 0.5 mL of D_i. Then, samples were transferred to the cartridges and allowed to elute freely. The cartridges were washed with 0.5 mL of 5:95 > 99% methanol: D_i (v:v) and allowed to elute freely. Then, the cartridges were dried under high vacuum for 10 minute. The elutant was separated by adding 0.3 mL of methanol (>99% purity) to each cartridge and collected into a new collection tube. Finally, 150 µL of the elutant was transferred to a UPLC-MS∕MS vial. Electrospray ionization and MS-MS analysis were carried out using a Waters Acquity TQD Tandem Quadrupole UPLC-MS∕MS system (Waters Corporation). While operating in positive ion mode, chromatographic separation was achieved using a 1.7-µm, 1.0 X 50 mm C18 analytical column (Waters Acquity UPLC BEH; Waters Corporation). A gradient elution from 95% of 0.1% trifluoroacetic acid: 5% acetonitrile to 5% of 0.1% trifluoroacetic acid: 95% acetonitrile was utilized in the analysis. Identification and quantitation were established on the following transitions: m∕z 479–154 for CTC and m∕z 445–321 for doxycycline. A 2-minute run time and a 3-minute cycle were used in the method. Quality controls of known concentrations were analyzed during sample analysis to monitor method performance. The method was proven to be accurate and precise across a linear dynamic range of 50–1000 ng∕mL. The limit of detection and limit of quantitation (LOQ) were 20 and 50 ng∕mL, respectively. The precision and accuracy for 36 quality controls instrumented over eighteen analytical runs were found to be $\leq$9% and 98.5%, respectively. Re-analysis of individual samples occurred if the coefficient of variation (CV %) for the known concentration of the internal standard was >15%.

Pharmacokinetic analysis

Noncompartmental pharmacokinetic analysis (NCA) using a commercial software package (WinNonlin 5.2, Pharsight Academic License; Pharsight Corporation, Mountain View, CA, USA) was performed by treatment group on individual steer data derived from data subsequent to the final treatment offered. The variables determined were the partial area under the curve (AUC) calculated from the time of the final dose to the next regularly scheduled dose ($AUC_{final-next}$) using the linear trapezoidal rule, clearance (Cl/F) calculated using $AUC_{final-next}$, apparent volume of distribution during the elimination phase (V_z/F), elimination rate constant (k_z), and elimination half-life ($t_{1/2}\lambda_z$). The maximum drug concentration (C_{max}) achieved at steady-state and time to C_{max} (T_{max}) was determined from the data after the time of the final dose. This method for determining C_{max} is equitable to the peak concentration achieved with these dosing regimens and method of drug administration. Data for drug concentration–time curves were also fit to a one-compartment open model with first order absorption and elimination by nonlinear-mixed effects (nonlinear mixed effects modeling, NLMEM) modeling with a commercially available software program (WinNonMix; PharSight, Mountain View, CA, USA). No covariates were used in model development. The choice of the model was based on minimizing values for Akaike's information criterion, Schwarz's Bayesian Criterion, and ML Log Likelihood; reduced variability of the parameter estimates; evaluation of agreement for predicted vs. observed plasma CTC concentrations; and uniformity of residual plots. The model estimated two primary parameters; apparent volume of distribution (mL/kg) and a rate constant (h^{-1}) for both absorption and elimination. Because of the inability to determine absolute bioavailability (F) for orally administered treatments, the parameters for the apparent volume of distribution and apparent clearance are reported as V/F and Cl/F, respectively, for NCA and NLMEM. The following secondary parameter estimates were generated during compartmental analysis; dose-normalized AUC (AUC/D), elimination half-life ($t_{1/2}$), maximum concentration (C_{max}), and time of maximum concentration (T_{max}). The residual variability was described by a proportional error structure model:

$$y = f + f\varepsilon \tag{1}$$

where y and f are the observed and predicted plasma drug concentrations, respectively. ε represents the randomly distributed terms with a mean (0) and variance (σ). Samples with

concentrations below the LOQ were not included in either pharmacokinetic analytical method. The geometric CV% for parameters derived by NCA was calculated according to the following equation:

$$\text{Geo CV\%} = \exp^{(\ln(\text{geometric standard deviation}))^2} \cdot 100\% \qquad (2)$$

The calculation of CV% for NLMEM is different because of the standard error of the mean being used in place of the standard deviation of the mean.

Statistical analysis

Statistical analysis was conducted by using a one-way analysis of variance (ANOVA) procedure (Microsoft Excel 2007; Microsoft Corporation; Redmond, WA, USA) (Martinez et al., 2006). This statistical procedure analyzes variance for a quantitative dependent variable by a single independent variable to test the null hypothesis that several means are equal. Treatment group was designated as the independent variable. An alpha level of ≤ 0.05 was designated a priori for the determination of statistical significance. ANOVA was used to determine statistically significant differences between treatment groups for the pharmacokinetic parameters reported by NCA and NLMEM; furthermore, dose linearity was determined by comparing the $\text{AUC}_{\text{final–next}}/\text{D}$ and the dose-normalized peak drug concentration ($\text{C}_{\text{max}}/\text{D}$) among treatment groups determined by NCA.

Results

Steers in the CTC treatment groups were not significantly different with respect to body weight at the initiation of the study ($P = 0.943$). Additionally, no significant difference in body weight ($P = 0.93$) was detected at the completion of the study. Oral administration of all treatments was well-tolerated. No steers were removed from the study. Feed bunk spatial allocation of 0.61 m/head (24 inches/head) was sufficiently adequate to allow each steer equal opportunity to consume the daily ration and treatment during the study.

The original (CV %) estimates for the NCA-derived parameters suggest that the data were not normally distributed (data not shown). This is because of a high level of inter-individual variability observed. Therefore, the geometric mean and Geo CV% for pharmacokinetic

parameter data derived by NCA are reported in Table 1. Statistically significant differences by treatment were not detected among the parameters reported by NCA. Dose linearity was confirmed with ANOVA by a comparison of the $AUC_{final-next}/D$ (P = 0.91) and C_{max}/D (P = 0.73) parameter estimates.

A one-compartment model, where the absorption rate constant was equal to the elimination rate constant, was fit to the data using NLMEM. This modeling method was selected as it weights each data point by the inverse of the variance of each data point when modeling sparse and rich data sets (Proost & Eleveld, 2006). This weighting technique made it possible to fit a model to highly variable data. Model goodness of fit was not improved by not setting the absorption rate constant equal to the elimination rate constant (data not shown). To simplify the model, the absorption rate constant was set equal to the elimination rate constant. Goodness of fit scatter plots for the model are presented in Figs 4.1 & 4.2. Figure 4.1 demonstrates the relationship between the observed and the model-predicted concentrations for CTC. The distribution of the observed data around the model-predicted concentrations suggests that the final model fits the observed data; however, a systematic overestimation of lower peak concentrations was observed in the scatter plot of weighted residuals vs. predicted plasma drug concentration in Figure 4.2.

Primary and secondary pharmacokinetic parameter estimates with calculations for standard error and CV are listed in Table 2. When considering CV as a measurement of precision, the primary parameters, V/F and K, were estimated with high precision at 3.125% and 4.184%, respectively; furthermore, these estimates are more precise based on the CV estimates reported for NCA. The secondary parameter estimates for AUC/D, Cl/F, $t_{1/2}$, and T_{max} were similarly precise at 2.207%, 2.035%, 2.022%, and 2.023%, respectively; however, the estimate for C_{max} was less precise at 18.32%. The overall imprecision of the NLMEM estimates tended to be less than comparably transformed Geo CV% estimates derived by NCA.

Semi-logarithmic scatter plots of plasma drug concentration–time curves were graphed for each of the CTC treatment groups with an overlay of the NLMEM model-predicted concentration estimates for the population ±2 SD (Figs 4.3–4.5). Oral administration of CTC in group fed, ruminating steers resulted in high inter- and intra-individual variability in observed plasma drug concentrations in all treatments; however, the model-predicted normal distribution of drug concentrations included the vast majority of the observed data points. This indicates that

a one-compartment open model with first order absorption and elimination, where the absorption rate constant was equal to the elimination rate constant, is an adequate model for calculating dosage and treatment regimens that achieve target plasma concentrations for ruminating steers fed in a feedlot setting. Because of dose-linearity and the reliability of the model, dose adjustments can be prepared for targeted plasma drug concentrations within the range of dosages studied.

Discussion

Because of the widespread use of CTC in ruminating cattle, it is essential that these pharmacokinetic parameters be reported in the literature. The dosage regimens selected were necessary for study design fulfillment of a concurrent *Anaplasma marginale* chemosterilization study. The daily ration and treatment were offered in a similar manner to feedlot situations; however, feed bunk spatial allocations are approximately 0.23 m∕head in typical commercial settings as opposed to 0.61 m∕head in this study (Montgomery et al., 2008). This change in practice was implemented to ensure equal access for all steers to the daily ration and CTC provided in the treatment.

The distribution of the ration and treatment was similar along the length of the feed bunk. It was assumed that steers would have similar observed plasma drug concentrations; however, high inter- and intra-individual variability was observed in plasma drug concentrations, as demonstrated in Figs. 4.3–4.5, and dose-related parameter estimates ($AUC_{final–next}$, CL/F, C_{max}, and V_z/F) in Table 1. Partitioning of the treatment may have occurred during ration consumption because of the treatment being offered as a topdress. This could lead to individual steers having the opportunity to consume more drug as compared with that by other steers. It may be possible that a threshold variability in treatment intake existed for time spent at the feed bunk to allow the steers that remained longer at the feed bunk to be exposed to more drug; however, this was not a measured covariate during the study. Additionally, the rumen may be a drug depot where variable amounts of CTC are presented to the small intestine for absorption based on hydration of rumen contents and physical fill. Twice-daily feed bunk evaluations were recorded in regard to complete or incomplete consumption of the ration and treatment. For plasma drug concentrations corresponding to incomplete consumption of the treatment, no

recognizable pattern was observed for a decrease or subsequent increase in plasma drug concentration. This is in part a result of the majority of occurrences happening during the twice-weekly sampling period, infrequency of the occurrence, and minimal quantity remaining; however, the length of the elimination half-life and the potential for the rumen to serve as a drug depot may have played an additional significant role in suppressing a recognizable pattern.

Divalent cations, as well as the addition of citric acid, have been shown to decrease and increase the absorption of CTC, respectively (Bradley et al., 1982; Luthman & Jacobsson, 1985). Citric acid was not an ingredient in the ration; however, divalent cations, specifically represented as calcium in limestone, were present in the diet. The static addition of calcium was necessary for homeostasis of normal metabolic processes and physiologic growth (Ross et al., 1994; Montgomery et al., 2004). The potential phenomenon of decreased absorption, caused by the binding of CTC to divalent cations, was assumed to be homogenous among all steers in this study.

The fed status and dietary feed ingredients may play a significant role in altering plasma drug concentration. In swine studies, fed pigs had lower bioavailability and mean plasma drug concentrations than fasted pigs (Kilroy et al., 1990; Nielsen & Gyrd-Hansen, 1996). Plasma drug concentrations are decreased in milk-fed calves as opposed to increased concentrations in calves fed citric acid in milk-replacer (Bradley et al., 1982; Luthman & Jacobsson, 1985). Furthermore, similar results were demonstrated when swine were fed calcium and citric acid (Wanner et al., 1991). Further studies are necessary to establish a relationship between plasma drug concentrations with fed or fasted status and feed ingredients.

Noncompartmental analysis was used to determine individual pharmacokinetic parameters of each treatment group. NLMEM was used to characterize the experimental data with a one-compartment open model with first order absorption and elimination. Although the data set was relatively rich at some periods during the study, the data were highly variable. Fitting a compartmental model using standard nonlinear regression methods was not successful because the algorithms were unable to converge as a result of the high, nonsystematic variability of the data. NLMEM was able to fit a model to the data and provide more precise parameter estimates as the method analyzes all the pharmacokinetic profiles at once and the data are weighted by their variance. As a result of the parameters not being skewed by a small number of outliers, NLMEM is known to give superior parameters estimated for data that is not normally or

log-normally distributed (Tam et al., 2003). Parameters estimated by other methods of nonlinear regression can be easily skewed by a small number of outliers because of each data point influencing the output equally. Furthermore, analyzing all data points and parameters at one time will increase the number of independent data points (degrees of freedom) that can be used to determine the central tendency and dispersion of the parameter estimates while accounting for the variance in the system. This also enhances the robustness of estimates with NLMEM methods over conventional nonlinear regression methods.

An overestimation of the lower plasma drug concentrations by NLMEM is demonstrated in Fig. 4.2. A more complex model may have characterized lower concentrations more accurately; however, a simpler model was chosen for the advantage of accurately estimating the higher concentrations that are likely to possess more clinically relevant information when compared with a more complex model that may accurately estimate lower concentrations. Pharmacokinetic parameters determined by NCA and NLMEM in this study were compared with other data derived from the literature. Two-week-old, conventionally fed (starter concentration at 2% body weight plus ad libitum alfalfa hay) Holstein calves were administered a single, 22 mg/kg dose of CTC by ruminal intubation (Bradley et al., 1982). Parameters derived for the conventionally fed calves are $AUC_{0\text{-LOQ}}$ (7.5 hr·µg/mL), $t_{1/2\,\lambda z}$ (17.75 h), Cl/F (1.3 L/kg/hr), and V_z/F (40.9 L/kg). The geometric mean for the 22 mg/kg treatment group derived by NCA are $AUC_{final\text{-}next}$ (0.99 hr·µg/mL), $t_{1/2\,\lambda z}$ (37.2 h), Cl/F (11.1 L/kg/h), and V_z/F (13.5 L/kg). Mean pharmacokinetic parameters determined by NLMEM are AUC/D (0.29 h·µg/mL), $t_{1/2}$ (16.2 hr), C_{max} (4.5 ng/mL), Cl/F (1.8 L/kg/h), and V/F 40.9 (L/kg). The dissimilarity of NCA-derived parameters is likely caused by significant differences in anatomy, metabolism, physiology, method of administration, and compartmental vs. noncompartmental analysis between the studies. Metabolic differences would be attributable to total body water and fat, as well as decreased bone density, of the 14-dayold calves compared with that of the 314-day-old steers in this study. Although the pyloric groove is likely to be open, conventionally fed, 14-day-old calves do not have a fully functional rumen. This incongruence in anatomy and physiology would affect the absorption of CTC because of differences in presentation of the drug to the small intestine. The similarities between parameters derived by NLMEM and that for the conventionally fed calves are remarkable. One possible explanation is these parameters were estimated by a one-compartment model for both studies. This may account for the shorter half-life compared with

NCA, as $t_{1/2}\lambda$ is calculated from the terminal phase of the time-concentration curve that is not considered in the one-compartment model.

An intravenous study was not performed in conjunction with the current study. The median F for the conventionally fed calves in the Bradley et al. study was calculated at 4.5% (±2.2%). In swine, F has been calculated at 17.88% (±5.3%) and 11% (±5%) (Kilroy et al., 1990; Nielsen & Gyrd-Hansen, 1996). Although the conventionally fed calves may not have a fully functional rumen, there appears to be a remarkable difference in oral F for CTC when comparing monogastric and ruminant species. Future studies are necessary to determine the absolute bioavailability of CTC in ruminating cattle.

Currently, CLSI has determined a concentration of 2 µg/mL for tetracycline antibiotics (200 mg/mL injectable oxytetracycline products) as a susceptible MIC for the treatment of bacterial infection caused by *M. haemolytica, P. multocida, or H. somnii*. This CLSI breakpoint is much higher than the observed plasma drug concentrations achieved with oral CTC in this study. In addition to different pharmacokinetic profiles, pharmacodynamic targets for extended duration antimicrobial regimens have not been established; therefore, susceptibility testing results should be interpreted with caution for in-feed CTC. The preferred susceptibility testing procedure would be an extended-range, dilution plate system that determines susceptibility as low as 0.5 µg/mL. It is also important to recognize that the inhibition of the pathogen growth curve may occur below the MIC which inhibits visible growth in the laboratory. Basing all interpretations of potential disease prevention or treatment activity on plasma drug concentration vs. in-vitro MIC values may lead to an underestimation of the utility of an antibiotic treatment regime in a production setting. The use of CTC at sub-MIC concentrations has been shown to be effective in reducing the incidence and effects of bovine respiratory disease (BRD) (Nanduri et al., 2005). This study demonstrated that a concentration of 0.25 µg/mL in vitro reduced the development of clinical lesions from BRD through inhibition of *Mannheimia haemolytica* leukotoxin A expression. Under the conditions of this study, the 11 and 22 mg/kg/day treatment groups reached this level that would be necessary for inhibition of *Mannheimia haemolytica* leukotoxin A expression in vivo. The dose-linearity of this drug across treatments suggests that higher plasma drug concentrations may be achieved by feeding higher dosages of CTC to ruminating steers. The data derived from this research could be used, with other in vivo studies, to support a new animal drug application (NADA) to the Food and Drug Administration Center

for Veterinary Medicine (FDA CVM) for the labeled use of inhibiting *M. haemolytica* leukotoxin A expression in ruminating cattle.

Current literature remains deficient in information regarding oral bioavailability in ruminating cattle. Until a sufficient body of scientific knowledge for a complete pharmacokinetic and pharmacodynamic profile of CTC is formed, the use of this antibiotic as a tool in profitable cattle production will continue to be a target of special interest groups for the removal of the FDA-approved, labeled use of CTC in animal feed. The NLMEM compartmental model could be a useful tool to simulate plasma drug concentrations for different dosage and treatment regime scenarios as well as the projected variability of the population. Recently, a population pharmacokinetic (PPK) study was established as a successful tool for gathering pharmacokinetic information in large populations of cattle ($\geq$100 head) (Fu et al., 2008). In addition to that study, the information gathered in this study, as well as the use of NLMEM techniques typical of PPK studies, could provide the framework for PPK research of CTC in group fed, ruminating cattle. Future studies of this kind are necessary to bridge the gap between experimentally derived data and confirmatory studies applied under normal conditions (Sheiner, 1997).

Figures and Tables

Figure 4.1 Scatter plot of observed plasma drug concentration vs. predicted plasma drug concentration predicted by nonlinear mixed effects modeling for chlortetracycline hydrochloride in ruminating Holstein steers (n = 18).
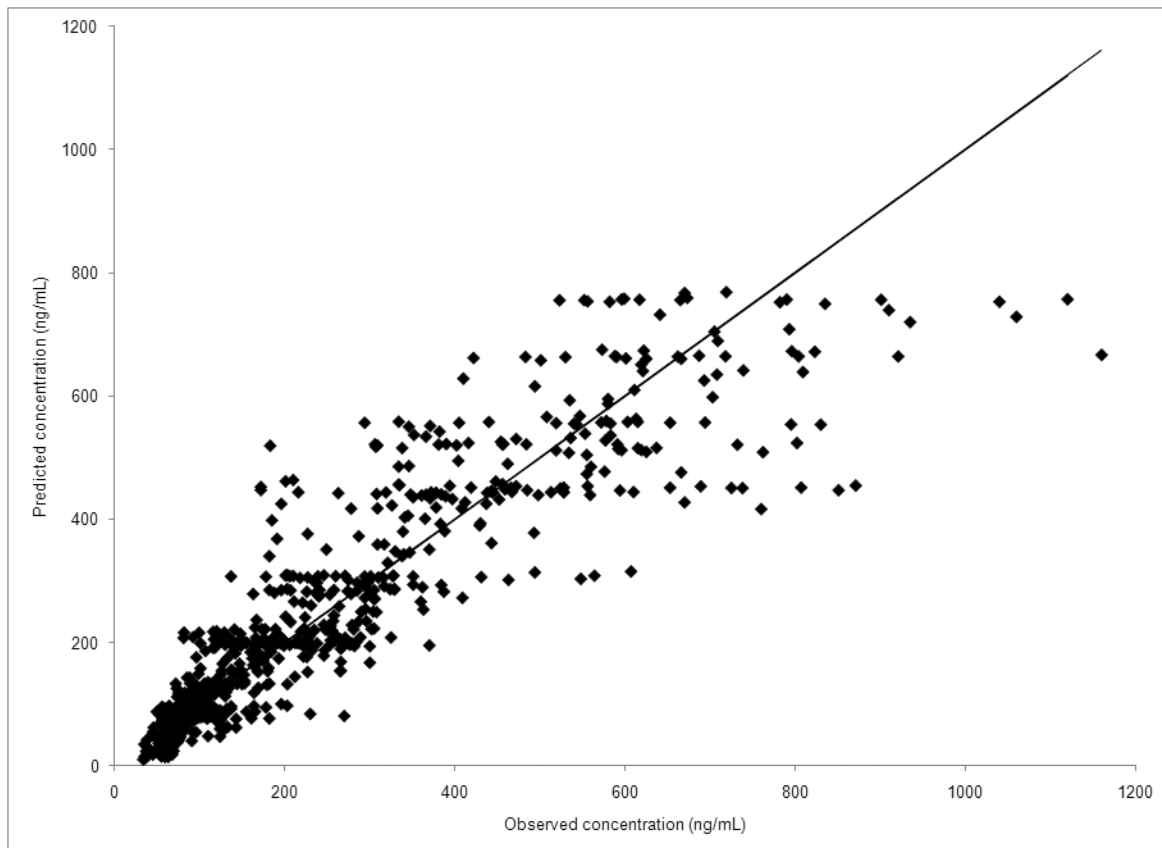
Figure 4.2 Scatter plot of weighted residuals versus predicted plasma drug concentration by NLMEM for chlortetracycline hydrochloride in ruminating, Holstein steers (n=18).
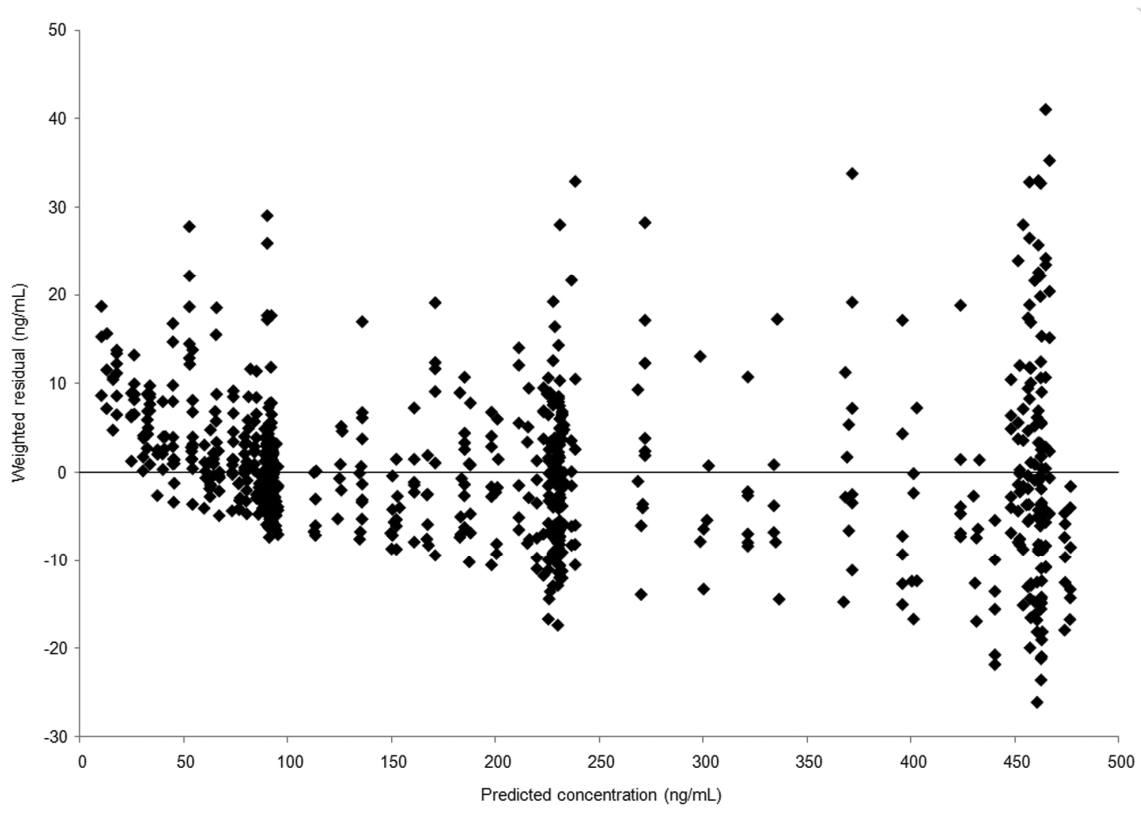
Figure 4.3 Plasma drug concentration of chlortetracycline hydrochloride administered at 4.4 mg/kg/day *p.o.* to ruminating, Holstein steers (n=6) with NLMEM model-predicted drug concentration for the population (+/- 2 SD).
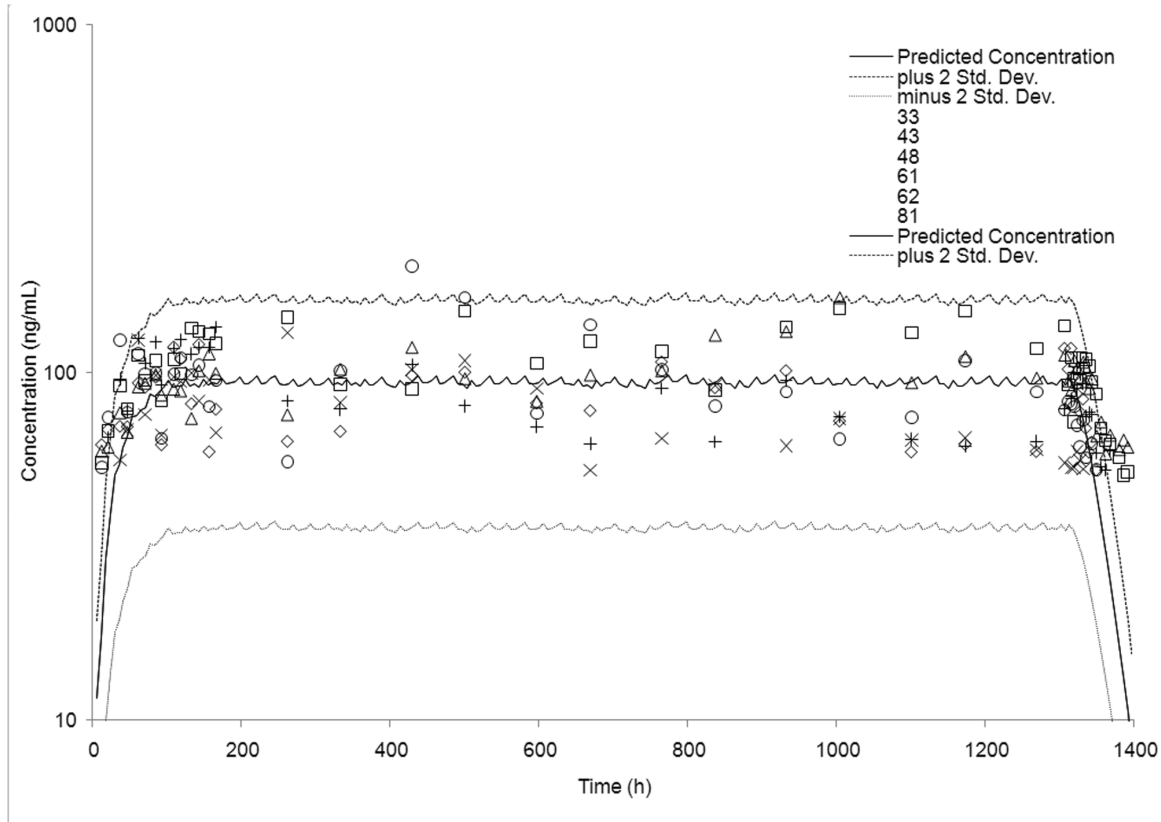
Figure 4.4 Plasma drug concentration of chlortetracycline hydrochloride administered at 11 mg⁄kg/day *p.o.* to ruminating, Holstein steers (n=6) with NLMEM model-predicted drug concentration for the population (+/- 2 SD).
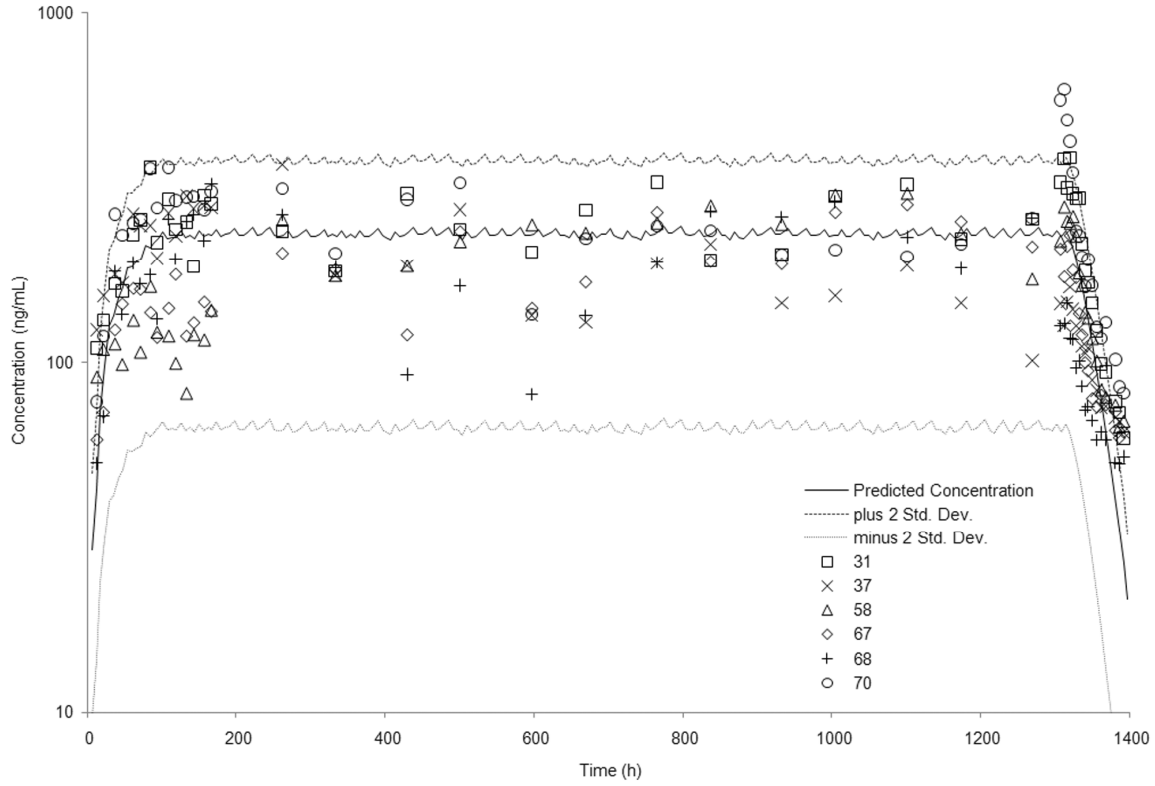
Figure 4.5 Plasma drug concentration of chlortetracycline hydrochloride administered at 22 mg/kg/day *p.o.* to ruminating, Holstein steers (n=6) with NLMEM model-predicted drug concentration for the population (+/- 2 SD).
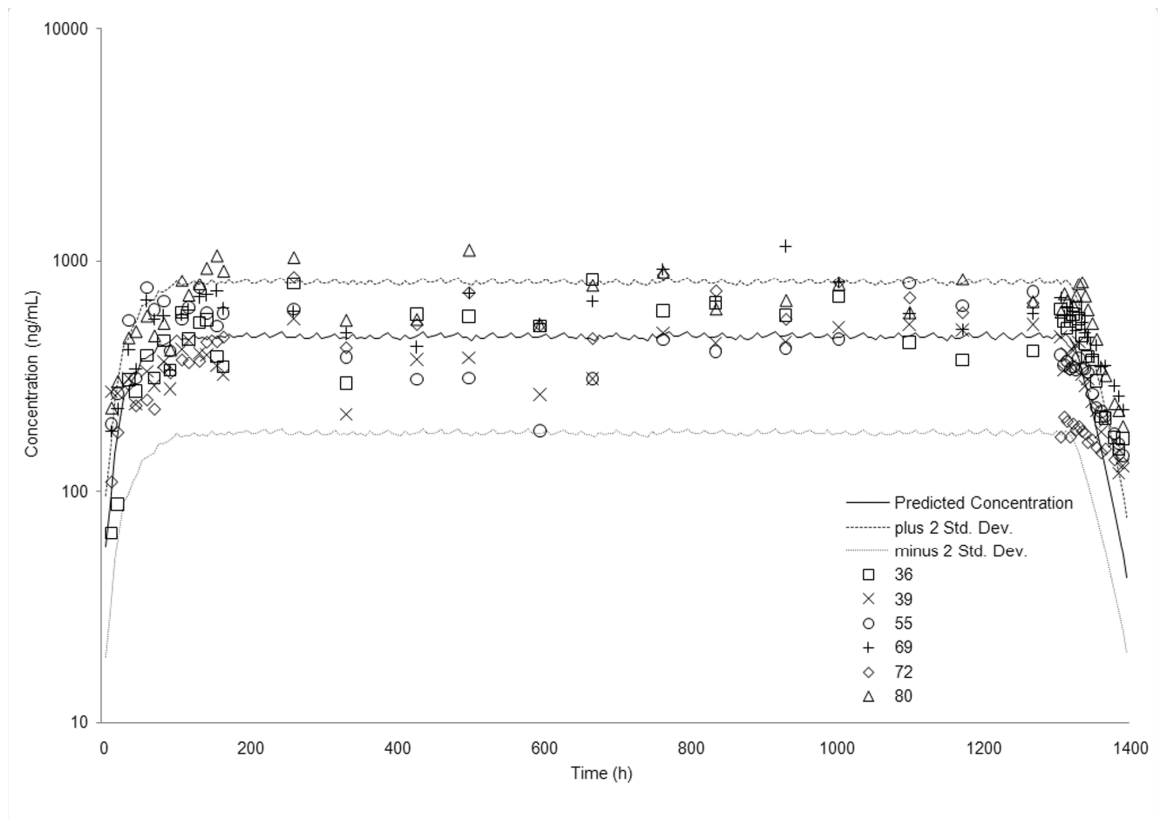
Table 4.1 Geometric mean and coefficient of variation (Geo CV%) of pharmacokinetic parameters derived by NCA for chlortetracycline hydrochloride administered *p.o.*

Parameter	Units	4.4 mg/kg (CV%)$^{\pm}$	11 mg/kg (CV%)$^{\pm}$	22 mg/kg (CV%)$^{\pm}$	p-value
$AUC_{\%Extrap}$	%	29.7 (20.5)	17.8 (26.5)	19.8 (46.7)	0.06
$AUC_{final\text{-}next}$	h·µg/mL	0.20 (9.5)	0.52 (29.9)	0.99 (35.5)	--
$AUC_{final\text{-}next}/D^{\dagger}$	h·µg/mL	0.046 (--)	0.047 (--)	0.045 (--)	0.91
Cl/F	L/kg /h	10.9 (32.0)	10.6 (43.4)	11.1 (59.1)	0.51
C_{max}^{*}	ng/mL	97.7 (9.5)	267.8 (25.4)	485.9 (26.8)	0.77
λ_z	1/h	0.0197 (0.4)	0.0195 (0.2)	0.0186 (13.2)	0.99
$t_{\frac{1}{2}} \lambda_z$	h	35.2 (--)	35.5 (--)	37.2 (--)	--
T_{max}	h	38.4 (0.002)	42.1 (0.00)	41.5 (0.008)	0.73
V_z/F	L/kg	12.8 (5.8)	15.2 (12.8)	13.5 (37.2)	0.77

$\pm$ n= 6; $\dagger$ Reported estimate is the dose-normalized partial AUC calculated from the time of the final dose to the next scheduled dose ($AUC_{final\text{-}next}/D$); *Reported estimate for C_{max} is derived from steady state concentrations.

Table 4.2 Pharmacokinetic parameter estimates derived by NLMEM for chlortetracycline hydrochloride administered *p.o.*

Parameter	Estimate$^{\pm}$	Standard Error	CV (%)
Primary			
V/F (L/kg)	40.92	1.28	3.125
K (h^{-1})	0.0478	0.002	4.184
Secondary			
AUC/D† (h·µg/mL)	0.29	0.0064	2.207
Cl/F (L/kg/h)	1.8	36.914	2.035
t$_{1/2}$ (h)	16.174	0.327	2.022
C$_{max}$/D (ng/mL)	4.502	0.044	18.32
T$_{max}$ (h)	23.334	0.472	2.023

Interindivdual variability	CV (%)
V/F (L/kg)	8.68%
K (h^{-1})	16.87%

$\pm$ n= 18; $\dagger$ Reported estimate is the dose-normalized AUC (AUC/D).

References

2008 Feed Additive Compendium. Chlortetracycline. *Feedstuffs*, 46, 227, 262.

Bradley, B.D., Allen, E.H., Showalter, D.H. & Colaianne, J.J. (1982). Comparative pharmacokinetics of chlortetracycline in milk-fed versus conventionally fed calves. *J Vet Pharmacol Ther*, 5(4), 267-278.

Clinical and Laboratory Standards Institute (2008). Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; Approved standard - Third edition. CLSI document M31-A3. Table 2. 28(8), 65-72.

Dyer, D.C. (1988). Pharmacokinetics of chlortetracycline in the turkey: evaluation of biliary secretion. *Am J Vet Res*, 49(1), 36-37.

Fu, L.X., Jiang, Z.G., Ding, H.Z. & Liu, Y.H. (2008). Population pharmacokinetics of enrofloxacin and its active metabolite ciprofloxacin in ill cows. *J Vet Pharmacol Ther*, 31(3), 240-245.

Kilroy, C.R., Hall, W.F., Bane, D.P., Bevill, R.F. & Koritz, G.D. (1990). Chlortetracycline in swine--bioavailability and pharmacokinetics in fasted and fed pigs. *J Vet Pharmacol Ther*, 13(1), 49-58.

Luthman, J. & Jacobsson, S.O. (1983). The availability of tetracycline in calves. *Nordisk veterinarmedicin*, 35, 292-299.

Luthman, J. & Jacobsson, S.O. (1985). The effect of citric acid on the availability of tetracyclines in calves. *Nord Vet Med*, 37(1), 22-26.

Martinez, M.N., Kawalek, J.C., Howard, K.D., Ward, J.L., Marroum, P., Marnane, W., Bensley, D., Pelsor, F.R., Hoag, S., Tatavarti, A.S., Xie, L. & Fahmy, R. (2006). Comparison of bovine in vivo bioavailability of two sulfamethazine oral boluses exhibiting different in vitro dissolution profiles. *J Vet Pharmacol Ther*, 29(6), 459-467.

Montgomery, J.L., Galyean, M.L., Horst, R.L., Morrow, K.J., Jr., Blanton, J.R., Jr., Wester, D.B. & Miller, M.F. (2004). Supplemental vitamin D3 concentration and biological type of beef steers. I. Feedlot performance and carcass traits. *J Anim Sci*, 82(7), 2050-2058.

Montgomery, J.L., Krehbiel, C.R., Cranston, J.J., Yates, D.A., Hutcheson, J.P., Nichols, W.T., Streeter, M.N., Swingle, R.S. & Montgomery, T.H. (2008). Effects of dietary zilpaterol

hydrochloride on feedlot performance and carcass characteristics of beef steers fed with and without monensin and tylosin. *J Anim Sci*.

NADA (065-440). Freedom of Information Summary http://www.fda.gov/cvm/FOI/552.htm.

NAHMS (Swine 2006). Naitonal Animal Health Monitoring Service Swine Report Part II: Reference of swine health and health management practices in the United States.

NAHMS (Feedlot 1999). *National Animal Health Monitoring System Feedlot Report Part III: Health Management and Biosecurity in US Feedlots*. In National Animal Health Monitoring System Feedlot Report Part III: Health Management and Biosecurity in US Feedlots p 15.

Nanduri, B., Lawrence, M.L., Vanguri, S. & Burgess, S.C. (2005). Proteomic analysis using an unfinished bacterial genome: the effects of subminimum inhibitory concentrations of antibiotics on Mannheimia haemolytica virulence factor expression. *Proteomics*, 5(18), 4852-4863.

Nielsen, P. & Gyrd-Hansen, N. (1996). Bioavailability of oxytetracycline, tetracycline and chlortetracycline after oral administration to fed and fasted pigs. *J Vet Pharmacol Ther*, 19(4), 305-311.

Pollet, R.A., Glatz, C.E., Dyer, D.C. & Barnes, H.J. (1983). Pharmacokinetics of chlortetracycline potentiation with citric acid in the chicken. *Am J Vet Res*, 44(9), 1718-1721.

Proost, J.H. & Eleveld, D.J. (2006). Performance of an iterative two-stage bayesian technique for population pharmacokinetic analysis of rich data sets. *Pharm Res*, 23(12), 2748-2759.

Ross, J.G., Spears, J.W. & Garlich, J.D. (1994). Dietary electrolyte balance effects on performance and metabolic characteristics in growing steers. *J Anim Sci*, 72(7), 1842-1848.

Sheiner, L.B. (1997). Learning versus confirming in clinical drug development. *Clin Pharmacol Ther*, 61(3), 275-291.

Tam, V.H., Preston, S.L. & Drusano, G.L. (2003). Comparative pharmacokinetic analysis by standard two-stage method versus nonparametric population modeling. *Pharmacotherapy*, 23(12), 1545-1549.

Wanner, M., Walker, W., Sutter, H.M., Riond, J.L. & Broz, J. (1991). Influence of dietary citric acid and calcium on the bioavailability of orally administered chlortetracycline in piglets. *Zentralbl Veterinarmed A*, 38(10), 755-762.

CHAPTER 5 - Establishment of the *in vivo* pharmacokinetic relationship between chlortetracycline and anaplasmosis carrier clearance using three oral treatment regimens

Submitted to *Veterinary Microbiology* (September 2009).

Introduction

Anaplasmosis, caused by *Anaplasma marginale*, is one of the most prevalent tick-transmitted, rickettsial diseases of cattle worldwide (Kocan et al., 2003). The Office International des Epizooties (OIE) Terrestrial Animal Health Code categorizes anaplasmosis as a notifiable disease due to socioeconomic impact and international trade restrictions (OIE, 2009). However, the significance of anaplasmosis is frequently underestimated due to seasonal outbreaks and stability in endemic areas (Brock et al., 1957). Breed differences are reported for peak change in packed cell volume, parasitemia level, and tick susceptibility (Bock et al., 1999; Jonsson et al., 2008; Wilson et al., 1980). However, all cattle are susceptible to infection with *A. marginale*.

Cattle infected with anaplasmosis following natural infection and vaccination with live *Anaplasma* spp. remain lifelong carriers (Kocan et al., 2003). Carriers are responsible for horizontal, iatrogenic, and vertical transmission of anaplasmosis to naïve cattle by providing a reservoir of infective blood for biological, mechanical, and *in utero* infection (Futse et al., 2003; Norton et al., 1983; Reinbold et al., 2009b).

Anaplasmosis chemosterilization regimens using chlortetracycline hydrochloride (CTC) have been assessed using antigen/antibody-mediated second generation diagnostic methods, such as capillary agglutination and complement fixation, and subinoculation of splenectomized calves (Table 5.1) (Brock et al., 1959; Franklin et al., 1966; Franklin et al., 1967; Franklin et al., 1965; Richey et al., 1977; Twiehaus, 1962). This is problematic due to deficiencies in sensitivity and specificity of second generation methods (Coetzee et al., 2007; Goff et al., 1990; Gonzalez et al., 1978) as well as animal welfare concerns associated with the use of splenectomized cattle. Furthermore, the time from commencement of therapy to chemosterilization is currently

unknown. CTC is only labeled for the control of active infection of anaplasmosis caused by *A. marginale* susceptible to CTC in cattle in the United States (2009 Feed Additive Compendium).

The Clinical and Laboratory Standards Institute (CLSI) has set the susceptible minimum inhibitory concentration (MIC) breakpoint of tetracycline antibiotics (200 mg/mL injectable oxytetracycline product) at 2 µg/mL when treating bovine bacterial infection caused by *Mannheimia haemolytica, Pasteurella multocida,* and *Histophilus somni* (CLSI, 2008); yet, a similar pharmacokinetic relationship does not exist for the treatment of bacterial infection caused by *A. marginale.* The purpose of this study was to identify a practical and efficacious CTC chemosterilization regimen and diagnostic testing strategy to determine therapeutic success. The specific objectives were to (1) evaluate *A. marginale* chemosterilization regimens using oral chlortetracycline antibiotics at different dosages; (2) establish the pharmacokinetic relationship between chlortetracycline and chemosterilization and determine the time of chemosterilization; and (3) determine the susceptibility of chemosterilized steers to re-infection with the original Virginia isolate.

Materials and Methods

Twenty-five preconditioned, Holstein steers were enrolled in this study under Kansas State University (KSU) Institutional Animal Care and Use Committee protocol #2517 and KSU Institutional Biosafety Committee protocol #524. Six of these steers were iatrogenically infected with a Virginia isolate of *A. marginale* by a contaminated, 1.7 X 25 mm hypodermic needle in a previous iatrogenic transmission study (Reinbold et al., 2009b). Nineteen steers were confirmed negative for anaplasmosis by a commercially available cELISA and an *A. marginale*-specific RT-PCR assay. In order to assemble a group of 21 steers chronically infected with *A. marginale,* fifteen naïve steers were randomly assigned to receive a 5 mL intravenous inoculation of whole blood from an iatrogenically infected steer 48 days prior to study initiation. The whole blood inoculum samples were collected into separate evacuated tubes containing K2EDTA from each iatrogenically infected steer prior to subinoculation. The four remaining naïve steers were splenectomized 36 days prior to the study to serve as disease transmission sentinels during treatment.

Steers were randomized by body weight and assigned to a (1) 4.4 mg/kg/day (LD; n=6); 2) 11 mg/kg/day (MD; n=6); 3) 22 mg/kg/day (HD; n=6) CTC treatment groups; or 4) placebo

treatment group (CONTROL; n=7). The LD, MD and HD treatment groups each consisted of five infected steers and one splenectomized steer. The CONTROL group consisted of six infected steers and one splenectomized steer. Steers were collectively 314 ± 29.9 days old and weighed 312.4 ± 47.1, 309.5 ± 43.6, 303.5 ± 47.2 and 320.8 ± 29 kg for the LD, MD, HD and CONTROL treatment groups, respectively. Four dry lot pens located at the KSU Juniatta Beef Cattle research facility accommodated the treatment groups.

A commercial CTC product (Aureomycin 50 Granular; Alpharma Animal Health, Bridgewater, NJ, USA) was purchased for the formulation of a CTC top dress prepared by the KSU feed mill. A final concentration of 591.0; 1477.3; and 2954.4 mg of CTC per kg of ground corn carrier was used for the LD, MD, and HD dosages administered, respectively. These respective drug concentrations ensured that a similar total quantity of top dress was fed (kg) regardless of the dosage administered. The CONTROL group received the ground corn carrier as a placebo based upon the average weight of top dress fed to the CTC-treated groups.

Steers were acclimated to a total mixed ration diet over a one month period pre-study. During the study, this diet was rationed twice daily at 1.25% (as fed) of the average pen weight. The daily dietary ration and CTC dosages were determined using the mean weight of the treatment group, which was taken bi-weekly. Daily dosage and ration were divided equally and administered twice daily for 80 days (160 total doses∕group). The daily ration and top dress treatment were distributed evenly along 3.7 m of a concrete feed bunk (0.61 m∕head). The feed bunk was inspected prior to each treatment. Residues from the previous treatment were noted in a daily log sheet; however, observed residues were not removed or weighed. Water was supplied *ad libitum*. When handling was necessary for the collection of venous blood samples, the steers were individually restrained with a head gate and rope halter. If sample collection coincided with scheduled treatment, samples were collected prior to treatment administration.

Sample collection and analysis

cELISA—Blood was collected from the jugular vein with evacuated tubes containing no additive. Serum was removed and analyzed for antibody against *A. marginale* by a commercially available cELISA in accordance with the method described by the OIE and recommended by the manufacturer (OIE, 2009; VMRD). The optical density of each sample was measured by an ELISA plate reader at a wavelength of 620 nm. The optical density was used to calculate a

percent inhibition (% inhibition). Samples were considered negative for anaplasmosis if the % inhibition was <30%. All samples with a % inhibition >30% were considered positive (Coetzee et al., 2007; OIE, 2009; Strik et al., 2007; VMRD).

Real time RT-PCR assay—Two hundred and fifty microliters of plasma-free whole blood sample was removed from blood samples collected from the jugular vein with evacuated tubes containing K_2EDTA. The plasma-free whole blood sample was used to extract RNA using a commercially available product according to manufacturer recommendations (TRI Reagent, Sigma-Aldrich; Saint Louis, MO). The RNA sample was rehydrated with 50 μL of nuclease–free water. An *A. marginale*-specific real-time RT-PCR assay was used to detect and quantify a highly conserved and specific region of 16S ribosomal RNA subunit (16S rRNA) as previously described (Reinbold et al., 2009b). The RT-PCR was optimized over a linear, dynamic range with one hundred to one billion molecules of 16S rRNA template that correspond to cycle threshold (Ct) values from 10 to 35, respectively. Linear regression was used to quantify the number of 16S rRNA template molecules in the 25 μL reaction based upon the corresponding Ct value with the following equation:

$$y = -3.4324x + 40.38 \text{ (1)}$$

where y is the reported Ct value and x is the number of template molecules. The correlation coefficient (R^2) for the regression equation was 0.9973. Samples from a known *A. marginale* carrier and a naïve cow were extracted and analyzed simultaneously for monitoring assay performance and quality of the RNA extraction technique.

Light microscopic examination of stained blood smears—Blood films were prepared from whole blood collected from the jugular vein in evacuated tubes containing K2EDTA. Blood films were stained with an automated unit (Hema-Tek, Ames Company; Elkhart, IN) using a Modified Wright stain. A total of 1,000 erythrocytes were counted in each sample.

Plasma drug concentration analysis—Plasma drug concentrations were determined from whole blood samples collected from the jugular vein with evacuated tubes containing lithium heparin. Plasma was subjected to solid phase extraction and analysis with an ultrahigh performance liquid chromatography-mass spectroscopy∕mass spectroscopy method as previously described (Reinbold et al., 2009a). The limit of quantitation of the method was 50 ng/mL. Plasma drug concentrations > 50 ng/mL were reported and used for analysis.

114

Evaluation of chemosterilization

A heparinized, 10 mL whole blood sample was collected from each steer in the LD, MD and HD treatment groups. Heparinized whole blood samples were pooled within each treatment group to compose a 50 mL final volume. Fifty milliliters of a whole blood sample was used to intravenously subinoculate the splenectomized calf assigned to the respective treatment group during the 80 day study. The disease status of the splenectomized steers was evaluated weekly by cELISA, light microscopic examination of stained blood smears and RT-PCR assay.

Determining susceptibility to re-infection

After chemosterilization was achieved, steers confirmed negative for *A. marginale* by subinoculation of splenectomized calves were monitored until the reported % inhibition of the cELISA declined below 40%. Five steers were exposed to re-infection with a frozen stabilate prepared from the same Virginia isolate of *A. marginale*. This stabilate was prepared from a splenectomized steer used to propagate the isolate *in vivo* for the previous iatrogenic transmission study (Reinbold et al., 2009b). The stabilate was prepared 280 days prior to the time of exposure from a heparinized, whole blood sample with a 2% parasitemia. The parasitemia at the time of collection was 2.0%. Four milliliters of stabilate was used to intravenously inoculate five chemosterilized steers. The disease status of the chemosterilized steers was evaluated by cELISA, light microscopic examination of stained blood smears and RT-PCR assay.

Chemosterilization of CONTROL group

Upon validation of chemosterilization results for the LD, MD, and HD treatment groups, chemosterilization was assessed in the CONTROL treatment group with a single, subcutaneous injection of a long-acting oxytetracycline (Tetradure 300, Merial Limited, Duluth, GA) at 20 mg/kg followed by 30 days of treatment with CTC at 4.4 mg/kg/day (Figure 5.4). The CTC treatment preparation and administration was similar to the LD treatment group. Samples were collected on days 0, 10, 17, 24, 31 and 38 for analysis by cELISA and RT-PCR. Plasma drug concentrations were not determined during this treatment.

Statistical analysis

Data were entered into a software package (Microsoft Excel 2007, Microsoft Corporation; Redmond, WA) for subsequent calculations and manipulation. Geometric mean and

CV% were calculated for data acquired from responses recorded from diagnostic assay results. Diagnostic assay results were converted to a binary format (0 = negative, 1 = positive). Sensitivity and specificity with 95% confidence intervals were calculated for cELISA and RT-PCR for each time point. Results were compared using a software program (WinEpiscope 2.0, CLIVE; Edinburgh UK) in a 2 X 2 contingency table. Agreement between diagnostic results of each semi-weekly sampling was assessed by calculating a κ statistic (Le, 2003). The κ statistic measures agreement on a scale from 0 to 1.

The association between chemosterilization and diagnostic assay results were analyzed by generalized linear mixed models and generalized estimating equations (PROC Glimmix and PROC Genmod, SAS version 9.1, SAS Institute, Inc.; Cary, NC). Proper methods were employed, when necessary, to take into account the lack of independence among repeated observations of the same animal over time. A semi-parametric survival analysist was performed to analyze the variation when each respective diagnostic assay first detected *A. marginale* clearance. A non-parametric, Kaplan-Meier survival analysis (Stata v10.1; Stata Corp LP, College Station, TX) was performed on raw data depicting the time elapsed prior to RT-PCR negative outcomes for each of the antibiotic treatment regimens. An alpha level of 0.05 was observed throughout the study for evaluating statistically significant differences.

Results

The mean weight among treatment groups was not significantly different at the initiation (P = 0.943) and completion of treatment (P = 0.93). The initial RT-PCR assay results between iatrogenically infected steers and subinoculated steers were significantly different (P < 0.0001). However, the RT-PCR assay results were not significantly different after randomization (P = 0.16). There were no statistically significant differences in cELISA results prior to randomization (P = 0.86). One splenectomized steer was removed from the study due to death caused by post-surgical hemorrhage from an aneurysm of the splenic vein. This complication reduced the CONTROL group to six, *A. marginale* infected steers.

When evaluating the time of chemosterilization by the RT-PCR assay, the LD, MD and HD groups were chemosterilized following 46, 46 and 49 days of CTC treatment, respectively. Chlortetracycline treatment was significantly associated with RT-PCR assay results (P = 0.018). There was no significant difference when comparing LD to MD (P= 0.07) and MD to HD (P =

0.30); however, a significant difference was detected between LD and HD (P = 0.005). The estimated risk of chemosterilization based upon RT-PCR assay results for the LD, MD and HD treatment groups were 0.735 (0.726,0.744), 0.746 (0.737,0.755) and 0.753 (0.744,0.761), respectively. The cELISA did not confirm chemosterilization until 18, 54 and 18 days after the completion of the 80 day CTC treatment regime in the LD, MD, and HD treatment groups, respectively (Figure 5.2). CTC treatment was not significantly associated with cELISA results (P = 0.43). The estimated risk of chemosterilization based upon cELISA results for the LD, MD and HD treatment groups were 0.658 (0.636,0.679), 0.671 (0.650,0.692) and 0.676 (0.655,0.697) respectively.

Diagnostic method performance was calculated for the cELISA and RT-PCR assay throughout the 80 day study (Table 5.2). The cELISA demonstrated 100% sensitivity throughout the study. However, cELISA specificity was imprecise for days 14 through 80 of treatment. This imprecision was likely caused by the continued presence of anti-*A. marginale* antibodies following chemosterilization. Furthermore, the positive predictive value of the cELISA was reduced accordingly on days 14 through 80 during treatment. The specificity of the RT-PCR was 100% throughout the study. However, RT-PCR sensitivity was inaccurate during days 18 through 39 of treatment. This was likely caused by low parasitemia levels encountered as a result of treatment. Furthermore, the negative predictive value was reduced accordingly on days 18 through 39 of treatment.

Diagnostic method agreement (κ) was negatively influenced by persistent antibody levels and a reduction in parasitemia during treatment. Agreement was perfect on days 0 through 11 of treatment. However, agreement reduced accordingly until peak reduction was reported on days 49 through 53 of treatment. The agreement at the end of the 80 day treatment was 0.44.

Oral administration of all treatments was well tolerated throughout the study. Feed bunk spatial allocation of 0.61 m/head was adequate to allow each steer equal opportunity to consume the daily ration and treatment. However, a remarkable level of intra- and inter-individual variability was observed within treatment groups (Figure 5.3). The geometric mean (CV%) of plasma drug concentrations collected on days 4 through 53 of the study for the LD, MD, and HD treatment groups were 85.3 (28), 214.5 (32), and 518.9 (40) ng/mL for samples, respectively. This data suggests a concentration independent pharmacokinetic relationship between

117

chemosterilization and plasma drug concentration where time above the minimum inhibition concentration is required for chemosterilization.

The CONTROL group was not chemosterilized during the 80 day study. However, these steers were subsequently chemosterilized with a single, subcutaneous injection of a long-acting oxytetracycline followed by 30 days of treatment with CTC at 4.4 mg/kg/day (Figure 5.4). Plasma drug concentrations were not determined during this treatment.

Chemosterilization, as assessed by the cELISA and RT-PCR assays, was confirmed through subinoculation of splenectomized steers. Three splenectomized steers, subinoculated with a pooled blood samples collected from chemosterilized steers 50 days after the end of the 80 day study, were monitored for 6 weeks by cELISA, light microscopy, and RT-PCR. No change in disease status of the splenectomized steers was detected by these methods. Similarly, there was no change in disease status of a splenectomized steer subinoculated with blood collected from the CONTROL group 12 days after chemosterilization with oxytetracycline and CTC.

Results from the semi-parametric survival analysis were expressed as hazard ratios. Hazard ratios, interpreted similarly as odds ratios, were assumed to be proportional over time and represent the effect of a unit change in the predictor on the frequency of the outcome (Le, 2003). The hazard ratio of cattle chemosterilized by multi-modal treatment of the CONTROL group using oxytetracycline and CTC when compared with CTC alone in the LD, MD and HD groups were 5.67 (1.4, 23.2; $P < 0.016$), 5.4 (1.4, 21.5; $P = 0.016$) and 7.6 (1.7, 33.0; $P = 0.007$), respectively. A Kaplan-Meier survival analysis depicts the time elapsed prior to RT-PCR negative outcomes for each of the antibiotic treatment regimens (Figure 5.6).

Five steers previously infected in an iatrogenic transmission study were re-infected by a stabilate of the Virginia isolate of *A. marginale* used to infect these steers (Reinbold et al., 2009b). An immune response was detected by the cELISA 10 days after exposure in all steers. Re-infection was detected as early as 10 days, but by 24 days of exposure in all steers. A splenectomized steer subinoculated with a pooled blood sample collected from the re-infected steers was monitored by cELISA, light microscopy, and RT-PCR for change in disease status. Re-infection was confirmed by positive results in all methods within 21 days following subinoculation.

118

Discussion

This study has established multiple chemosterilization regimens using chlortetracycline for the clearance of persistent infection with *A. marginale*, as assessed with the Virginia isolate. The dose and length of treatment of these strategies differ from previous studies (Brock et al., 1959; Franklin et al., 1966; Franklin et al., 1967; Franklin et al., 1965; Richey et al., 1977; Twiehaus, 1962). Dosages were selected based upon a chlortetracycline pharmacokinetic study reporting dose linearization of plasma drug concentrations among the CTC dosages prescribed in this study (Reinbold et al., 2009a). Accordingly, these dosages were prescribed to determine if dose combined with a fixed duration of treatment affected the rate of chemosterilization.

These CTC treatment regimens resulted in plasma drug concentrations that successfully chemosterilized steers persistently infected with *A. marginale*. However, these plasma drug concentrations were less than the minimum inhibitory concentration recommended for the treatment of bacterial infection caused by *M. haemolytica, P. multocida,* or *H. somnii* (CLSI, 2008). Therefore, the success of an *A. marginale* chemosterilization strategy should not be based upon the aforementioned minimum inhibitory concentration. A key finding of this study is the pharmacokinetic relationship between plasma drug concentration and chemosterilization. This relationship illustrated that carrier clearance was not influenced by higher dosages of CTC; furthermore, 4.4 mg/kg was the minimum effective dose necessary to chemosterilize cattle infected with *A. marginale*. Indeed, a difference in RT-PCR assay results, which were used to determine the time of chemosterilization, was detected between the LD and HD treatment groups. However, the authors do not recommend the usage of higher doses when formulating a chemosterilization strategy due to all CTC-treated groups being chemosterilized between 46 and 49 days of treatment.

A clinically and statistically significant difference was observed when comparing CTC treatment alone to a regime using a combination of oral and injectable tetracycline antibiotics. This was likely due to the time of maximum concentration occurring earlier when a 300 mg/mL preparation of oxytetracycline (4.7 h) (Dowling and Clark, 2003) is administered than with the LD (38.4 h), MD (42.1 h) and HD (41.5 h) treatments (Reinbold et al., 2009a).

The absorption of tetracycline into the erythrocyte has been characterized as a simple diffusion process (DeLoach and Wagner, 1984). Once inside the erythrocyte, drug passes as a

cation through porin channels of the outer membrane of Gram negative bacteria into the periplasm, becomes an uncharged molecule to diffuse through the inner cytoplasmic membrane and reversibly binds to the 30S ribosome to inhibit protein synthesis (Chopra and Roberts, 2001). Tetracyclines are also known to interact with the 16S rRNA subunit (Moazed and Noller, 1987). However, this did not affect RT-PCR assay performance during the study.

Efflux and ribosomal protection proteins, as well as enzymatic inactivation, are mechanisms of resistance to counteract their efficacy during treatment. These mechanisms are driven by numerous resistance genes found in commensal and pathogenic bacteria today (Chopra and Roberts, 2001) The ability to define tetracycline resistance is difficult due to the frequent occurrence of mutants that are impermeable to drug uptake (Moazed and Noller, 1987). Furthermore, no apparent difference is distinguishable between resistance genes of the tetracycline family of antibiotics.

CTC is only labeled for the control of active infection of anaplasmosis caused by *A. marginale* susceptible to CTC in cattle in the United States (2009 Feed Additive Compendium). The continuous feeding of CTC to naïve cattle in high risk areas for anaplasmosis infection is advocated during the vector season (Brock et al., 1957). However, this practice may have the potential to inadvertently disrupt endemic stability by chemosterilizing infected cattle and select for or facilitate the distribution of resistance determinants in bacterial species present (Stevens et al., 1993). A significant change was reported in antimicrobial susceptibility of enteric bacteria from cattle fed chlortetracycline in three consecutive, five day pulse treatment regimens at 22 mg/kg (Platt et al., 2008). However, this change was only temporary as values returned to pre-exposure levels within 33 days. Due to length of treatment being critical to successful chemosterilization, it is unknown if this return to pre-exposure levels would be similar in situations where the duration of therapy is more extensive. The development and application of improved animal husbandry practices (Reinbold et al., 2009b), as well as establishment of an endemically stable herd (Figueroa et al., 1998), could considerably reduce the need for tetracycline antibiotics when managing anaplasmosis in cattle. However, the existence of an endemically stable herd does not permit the comingling of cattle of unknown disease status or reduce trade restrictions between endemic and non-endemic countries.

The host immune response to *A. marginale* infection depends upon the production of anti-parasitic and anti-erythrocytic antibodies inducing the erythrophagocytosis of parasitized

erythrocytes (Jatkar and Kreier, 1969). The mechanism by which anaplasmosis eludes the host immune response is unknown; however, the lack of conservation of gene sequences encoding major surface protein antigens of *Anaplasma* spp. has been the focus of research (de la Fuente et al., 2005). Evidence exists for a rapid decrease in antigen-specific T cells and immunologic memory following infection of cattle pre-immunized with the major surface protein 1a (Han et al., 2008). The chemosterilization process was not facilitated immunologically by a second exposure prior to and during treatment (Kuttler, 1983). Furthermore, our study and the findings by others (Magonigle and Newby, 1984; Renshaw et al., 1976) have illustrated a loss of immunologic memory through the loss of anti-parasitic antibody and re-infection of cattle previously chemosterilized.

The mechanism for the extensive duration of treatment necessary for chemosterilization success is unknown. The reversible binding of the 30S ribosome and lifespan of parasitized erythrocytes may be key contributors to this phenomenon. However, the concentration of drug achieved in parasitized erythrocytes may be inadequate for a bactericidal effect. It may be likely the inhibition of protein synthesis may prevent the infection of non-parasitized erythrocytes. However, parasitized erythrocytes must still be removed from circulation by erythrophagocytosis in the spleen. Ultimately, carrier clearance is influenced by an extensive drug absorption process, reversible binding of the tetracycline antibiotic to the 30S ribosome, an inadequate host immune response, and erythrophagocytosis of parasitized erythrocytes.

Previously recommended oral CTC chemosterilization regimens established with second generation diagnostic methods have inadequate sensitivity and specificity for accurately and precisely determining disease status (Coetzee et al., 2007; Goff et al., 1990; Gonzalez et al., 1978). Second generation methods most commonly used in the literature to determine anaplasmosis disease status in clinical medicine and research, such as capillary agglutination, complement fixation and cELISA, identify cell components, metabolic products, and detection of antigenic components. However, there is a body of evidence that suggests these methods are non-specific among related *Anaplasma spp.* (Bradway et al., 2001; Dreher et al., 2005; Richey et al., 1977; Strik et al., 2007). The results of this study illustrated the insufficient specificity of cELISA for identifying true negative cattle at the time of chemosterilization. The % inhibition (< 30%) used for the cELISA negative cut-off value in this study was previously set to optimize sensitivity (Coetzee et al., 2007; OIE, 2009; Strik et al., 2007; VMRD). However, the use of a

121

cut-off set at a greater % inhibition would do little to improve the specificity of this diagnostic method under the circumstances of this study. Indeed, the cELISA did eventually confirm a negative disease status. The time delay involved would make third generation diagnostic methods more attractive. However, the use of cELISA does offer a practical and cost effective way for veterinarians and producers to determine chemosterilization success.

A novel and quantitative third generation method, RT-PCR, was validated for determining chemosterilization in this study. The advantages of the RT-PCR assay were the ability to precisely identify the time of chemosterilization (specificity), enhanced sensitivity as a result of identifying high copy numbers of 16S rRNA versus a single cellular DNA copy (Sirigireddy and Ganta, 2005); and quantification of the 16S rRNA template. The disadvantages were the cost of the reagents and equipment as well as the necessity of modern elements.

The RT-PCR assay demonstrated improved sensitivity and specificity over cELISA. However, this is not the first study to compare second and third generation diagnostic methods during a chemosterilization strategy. A previous chemosterilization study reported sensitivity and specificity deficiencies of cELISA and a nested PCR (Coetzee et al., 2006). The validity of diagnostic results of this and previous chemosterilization studies were validated through the subinoculation of whole blood into splenectomized calves. However, the RT-PCR assay could be considered as a tool for the reduction of animal pain and suffering by serving as a reliable substitute to the subinoculation of splenectomized cattle.

Anaplasmosis is a complex and challenging disease for stakeholders in the cattle industry, foreign policy and research communities alike. Many of the current methods used for the diagnosis, treatment, eradication and control of bovine anaplasmosis present many problems to the cattle industry. A highly sensitive and specific RT-PCR assay was instrumental in characterizing the time of chemosterilization as well as establishing the pharmacokinetic relationship between plasma drug concentration and chemosterilization *in vivo*. Due to the widespread use of CTC for controlling and treating anaplasmosis in cattle, it is essential these effective plasma drug concentrations be reported in the literature to prevent imprudent use of this antibiotic. These treatment regimens offer an alternative strategy for minimizing animal handling and eliminate individual treatment. Furthermore, this study identifies a viable chemosterilization and testing strategy that could be considered as an alternative to culling valuable cattle infected with *A. marginale*. Ultimately, the findings of this study have the potential to significantly

impact local, interstate, and international movement of cattle between endemic and non-endemic regions.

Figures and Tables

Figure 5.1 Diagram of the study design and decision tree analysis used to determine disease status.
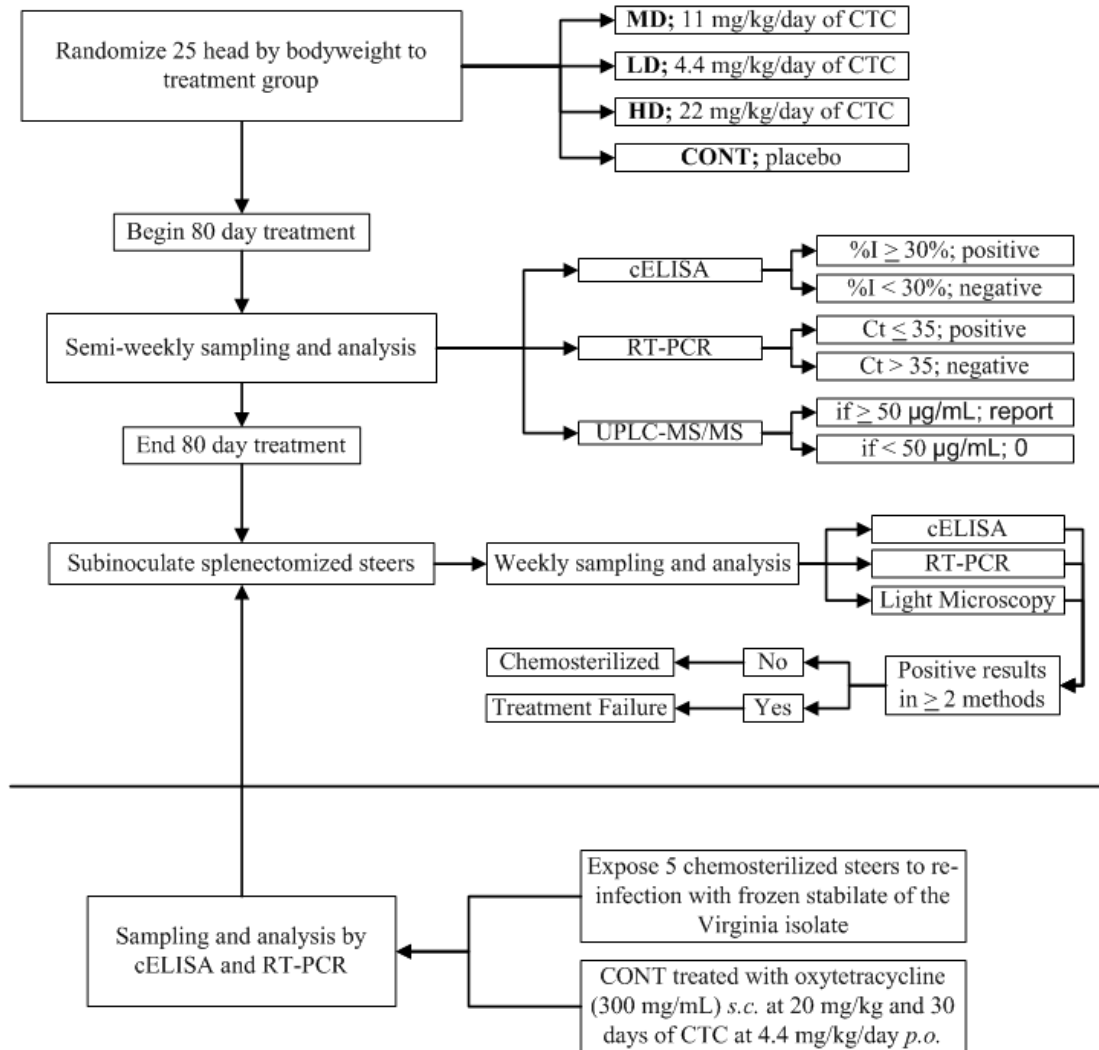
Figure 5.2 Comparison of the rate of chemosterilization detected by an *Anaplasma marginale* - specific RT-PCR assay following 80 days of treatment with placebo in the CONTROL group (open circles; n=6) and oral chlortetracycline in the LD (open diamonds; n=5), MD (open squares; n=5), and HD (open triangles; n=5) groups. Data points are represented as the inverse of the geometric mean of the Ct value reported during analysis. The geometric CV% is included as error bars for the CONTROL group.
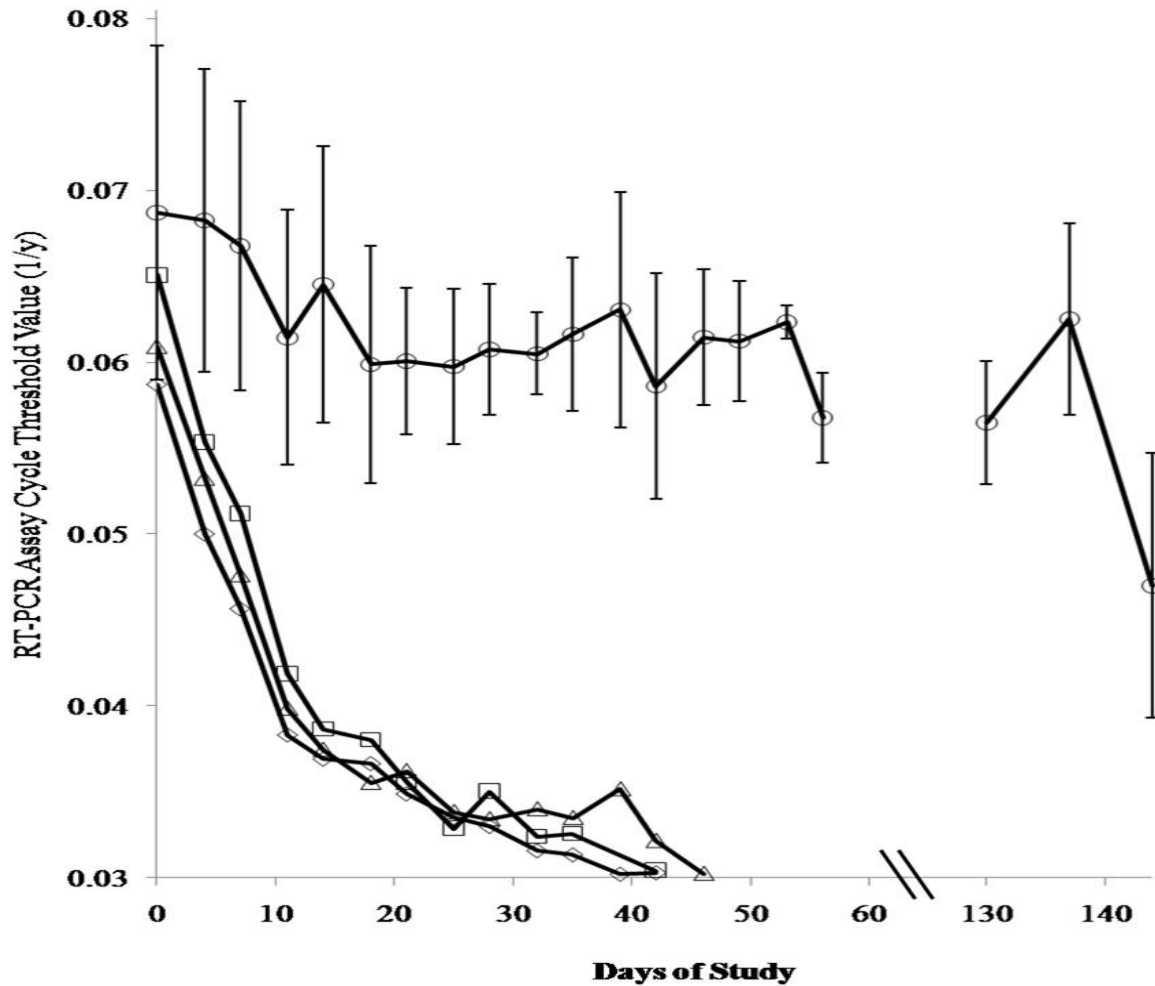
Figure 5.3 Comparison of the rate of antibody decline against *Anaplasma marginale*
detected by cELISA. The data series represented are the CONTROL (crosses; n=6), LD
(open diamonds; n=5), MD (open squares; n=5), and HD (open triangles; n=5) treatment
groups. Data points are represented as the geometric mean of the % inhibition reported
during analysis. The geometric CV% is included as error bars for the CONTROL group.
Commonly used cut-off points for disease status interpretation are represented as 30%
inhibition (- · · -) and 40% inhibition (- · -). The cumulative time to chemosterilization
detected by the RT-PCR assay is represented on the x-axis (closed circle). The end of
treatment is represented on the x-axis (closed square). Negative values on the x-axis refer to
the days of CTC treatment whereas positive values represent the days observed after the
end of treatment.
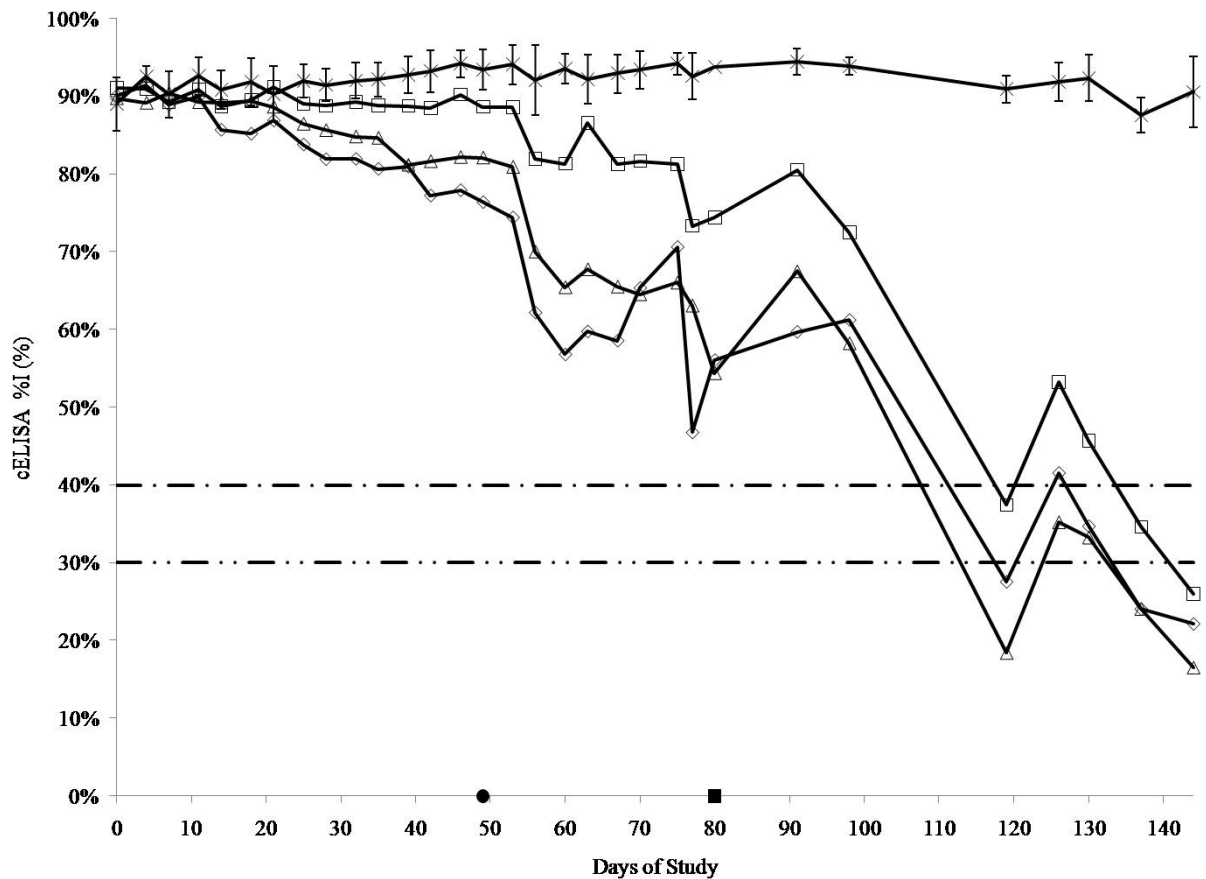
Figure 5.4 Comparison of plasma drug concentrations achieved with 4.4, 11, and 22 mg/kg/day of oral chlortetracycline in the LD (open diamonds; n=6), MD (open squares; n=6), and HD (open triangles; n=6) treatment groups, respectively. Data points are represented as the geometric mean and CV% (error bars) of plasma drug concentrations reported during analysis. The cumulative time to chemosterilization detected by the RT-PCR assay is represented (closed circle) on the x-axis. A geometric mean is included for plasma drug concentrations recorded on days 4 through 53 of treatment in the LD (85.3 ng/mL; -), MD (214.5 ng/mL; - · -), and HD (518.9 ng/mL; - · · -) treatment groups (n=90/treatment).
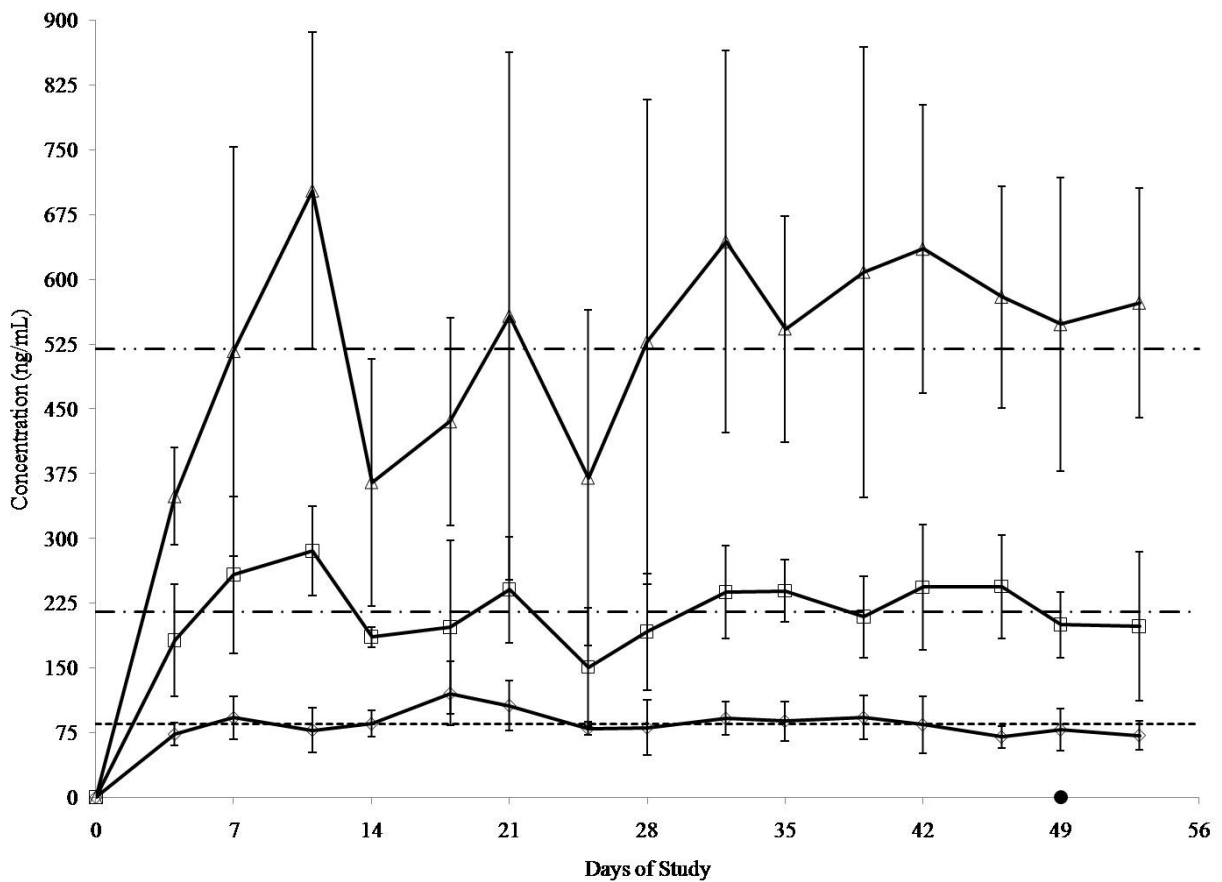
Figure 5.5 Comparison of the rate of chemosterilization detected by an *Anaplasma marginale* -specific RT-PCR (open diamonds) and antibody decline against *A. marginale* detected by cELISA (open triangles) in six steers treated with a subcutaneous injection of a long-acting oxytetracycline (300 mg/mL) at 20 mg/kg and 30 days of treatment with oral chlortetracycline at 4.4 mg/kg/day. The end of treatment with CTC treatment is indicated (closed circle) on the x-axis. Data points are represented as the geometric mean and CV% (error bars) of results reported during analysis.
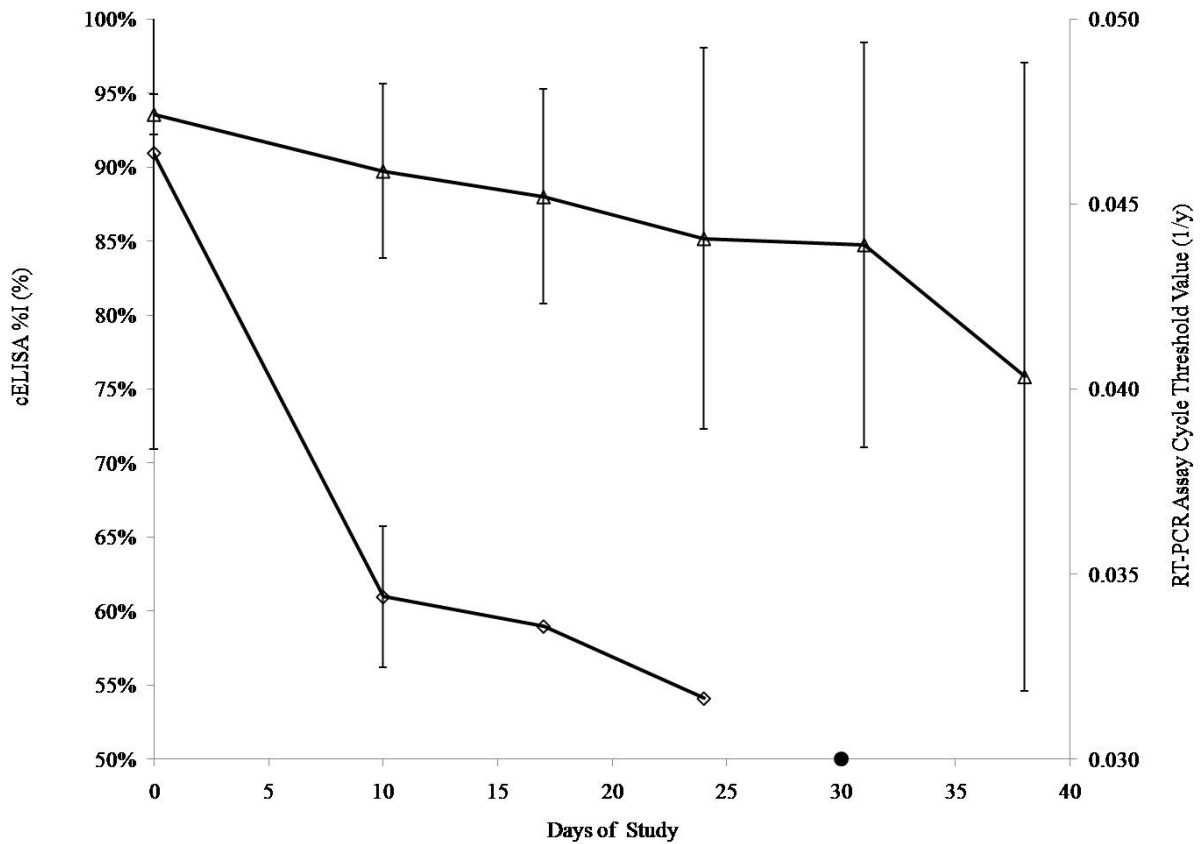
Figure 5.6 A Kaplan-Meier survival analysis derived from RT-PCR assay results for the LD (— —), MD (– – –), HD (⋯), and multi-modal treatment of the CONTROL group (—) for illustrating the probability of a positive RT-PCR assay result over time.
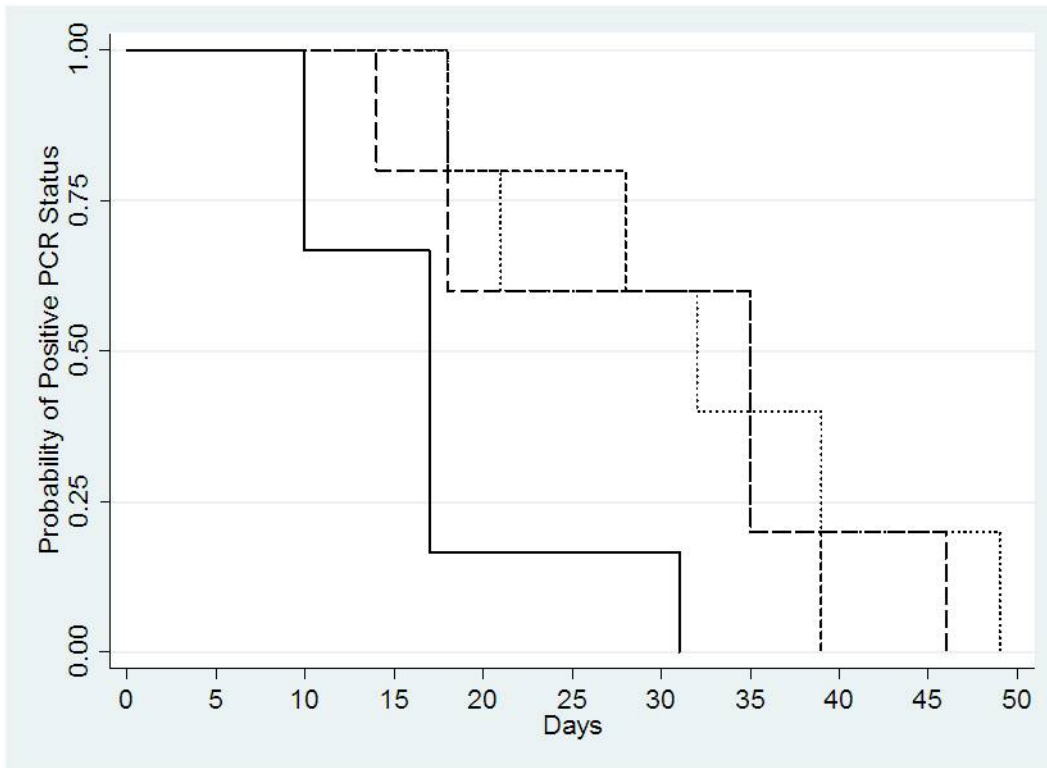
Table 5.1 Comparison of previously reported *Anaplasma marginale* chemosterilization strategies in cattle treated with chlortetracycline hydrochloride.

Study	Cattle	Dose (mg/kg)	Duration (days)	Method	Infection	Group Treatment	Test	Confirmed with subinoculation
1	26 Adults, intact	4.4	30	In ration	Natural	Y	CF	Yes[*]
2	60 Adults, intact	2.2	30	Range Cubes	Natural	Y	CF, CA	No
	60 Adults, intact	2.2	60	Range Cubes	Natural	Y	CF, CA	No
	60 Adults, intact	5.5	30	Range Cubes	Natural	Y	CF, CA	No
	60 Adults, intact	5.5	60	Range Cubes	Natural	Y	CF, CA	No
	60 Adults, intact	11	30	Range Cubes	Natural	Y	CF, CA	No
3	3 Adults, intact	11	60	In ration	Experimental	Y	CF	Yes
	7 Adults, intact	5.5	60	In ration	Experimental	Y	CF	Yes
	4 Adults, intact	3.3	60	In ration	Experimental	Y	CF	Yes
4	1 Calf, intact	2.2	41	In ration	Experimental	Y	CF, CA	No[#]
	8 Calf, intact	1.1	45	Hand fed	Experimental	N	CF, CA	No[#]

	1 Calf, splenectomized	1.1	90	Hand fed	Experimental	N	CF, CA	No[#]
5	10 Adults, intact	11	30	Hand-fed	6 Natural,4 Experimental	N	CF	No
	10 Adults, intact	11	60	Hand-fed	Natural	N	CF	Yes
6	10 Adults, intact	1.1	120	In ration	Natural	N	CF	Yes

Studies 1, 2, 3, 4, 5 and 6 were reported by Twiehaus 1962; Franklin et al., 1966; Brock et al., 1959; Franklin et al., 1967; Franklin et al., 1965; and Richey et al., 1977, respectively. CA = capillary agglutination; CF = complement fixation; [*] = only 1 head splenectomized; [#] = treated calves were splenectomized 75 days after treatment ended.

Table 5.2 Comparison of diagnostic method performance for the cELISA and RT-PCR assays as determined by the calculated sensitivity (%), specificity (%), positive predictive value (%), negative predictive value (%), and agreement (κ) during an 80 day *Anaplasma marginale* chemosterilization study (n=24).

| Day | cELISA | | | | RT-PCR | | | | |
	Se (%)	Sp (%)	PPV (%)	NPV (%)	Se (%)	Sp (%)	PPV (%)	NPV (%)	Agreement (κ)
0	*	*	*	*	*	*	*	*	1
4	*	*	*	*	*	*	*	*	1
7	*	*	*	*	*	*	*	*	1
11	*	*	*	*	*	*	*	*	1
14	*	75	95.2	*	*	*	*	*	0.92
18	*	60	90.5	*	84.2	*	*	62.5	0.7
21	*	60	90.5	*	84.2	*	*	62.5	0.7
25	*	60	90.5	*	94.7	*	*	83.3	0.8
28	*	42.9	81	*	82.4	*	*	70	0.64
32	*	37.5	76.2	*	87.5	*	*	80	0.66
35	*	30	66.7	*	91.7	*	*	92.3	0.66
39	*	25	57.1	*	66.7	*	*	75	0.46
42	*	25	57.1	*	*	*	*	*	0.63

46	*	17.6	33.3	*	*	*	*	*	0.46
49	*	16.7	28.6	*	*	*	*	*	0.41
53	*	16.7	28.6	*	*	*	*	*	0.41
56	*	22.2	30	*	*	*	*	*	0.44
60	*	22.2	30	*	*	*	*	*	0.44
63	*	22.2	30	*	*	*	*	*	0.44
67	*	22.2	30	*	*	*	*	*	0.44
70	*	22.2	30	*	*	*	*	*	0.44
75	*	16.7	28.6	*	*	*	*	*	0.42
77	*	22.2	30	*	*	*	*	*	0.44
80	*	22.2	30	*	*	*	*	*	0.44

n = 24; Se = Sensitivity; Sp = Specificity; PPV = Positive Predictive Value; NPV = Negative Predictive Value; * indicates the cell value is 100%. 0 < κ < 0.4 = poor reproducibility; 0.4 < κ < 0.75 = good reproducibility; and κ > 0.75 = excellent reproducibility.

References

2009 Feed Additive Compendium. Chlortetracycline. *Feedstuffs* 46, 223-229.

Bock, R.E., Kingston, T.G., De Vos, A.J., 1999. Effect of breed of cattle on innate resistance to infection with *Anaplasma marginale* transmitted by *Boophilus microplus*. *Aust Vet J* 77, 748-751.

Bradway, D.S., Torioni de Echaide, S., Knowles, D.P., Hennager, S.G., McElwain, T.F., 2001. Sensitivity and specificity of the complement fixation test for detection of cattle persistently infected with *Anaplasma marginale. J Vet Diagn Invest* 13, 79-81.

Brock, W.E., Pearson, C.C., Kliewer, I.O., 1959. Anaplasmosis control by test and subsequent treatment with chlortetracycline. *Proc US Livestock San Assoc* 62, 66-70.

Brock, W.E., Pearson, C.C., Staley, E.E., Kliewer, I.O., 1957. The prevention of anaplasmosis by feeding chlortetracycline. *J Am Vet Med Assoc* 130, 445-446.

Chopra, I., Roberts, M., 2001. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol Mol Biol Rev* 65, 232-260.

CLSI, 2008. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; Approved standard - Third edition. *CLSI document M31-A3*. Table 2. 28, 65-72.

Coetzee, J.F., Apley, M.D., Kocan, K.M., 2006. Comparison of the efficacy of enrofloxacin, imidocarb, and oxytetracycline for clearance of persistent *Anaplasma marginale* infections in cattle. *Vet Ther* 7, 347-360.

Coetzee, J.F., Schmidt, P.L., Apley, M.D., Reinbold, J.B., Kocan, K.M., 2007. Comparison of the complement fixation test and competitive ELISA for serodiagnosis of *Anaplasma marginale* infection in experimentally infected steers. *Am J Vet Res* 68, 872-878.

de la Fuente, J., Lew, A., Lutz, H., Meli, M.L., Hofmann-Lehmann, R., Shkap, V., Molad, T., Mangold, A.J., Almazan, C., Naranjo, V., Gortazar, C., Torina, A., Caracappa, S., Garcia-Perez, A.L., Barral, M., Oporto, B., Ceci, L., Carelli, G., Blouin, E.F., Kocan, K.M., 2005. Genetic diversity of anaplasma species major surface proteins and implications for anaplasmosis serodiagnosis and vaccine development. *Anim Health Res Rev* 6, 75-89.

DeLoach, J.R., Wagner, G.G., 1984. Pharmacokinetics of tetracycline encapsulated in bovine carrier erythrocytes. *Am J Vet Res* 45, 640-642.

Dowling, P.M., Clark, C. 2003. Pharmacokinetics comparison: Bio-mycin 200 and Tetradure (http://www.bi-vetmedica.com/BIMS/docs_ts/TB03-108.pdf?-session=BISITE:D8A83FAA118e216BBDvOg1456A5D), pp. 1-2.

Dreher, U.M., de la Fuente, J., Hofmann-Lehmann, R., Meli, M.L., Pusterla, N., Kocan, K.M., Woldehiwet, Z., Braun, U., Regula, G., Staerk, K.D., Lutz, H., 2005. Serologic cross-reactivity between *Anaplasma marginale* and *Anaplasma phagocytophilum*. *Clin Diagn Lab Immunol* 12, 1177-1183.

Figueroa, J.V., Alvarez, J.A., Ramos, J.A., Rojas, E.E., Santiago, C., Mosqueda, J.J., Vega, C.A., Buening, G.M., 1998. Bovine babesiosis and anaplasmosis follow-up on cattle relocated in an endemic area for hemoparasitic diseases. *Ann N Y Acad Sci* 849, 1-10.

Franklin, T.E., Cook, R.W., Anderson, D.J., 1966. Fedding chlortetracycline to range cattle to eliminate the carrier state of anaplasmosis. *Proc US Livestock San Assoc* 66, 85-90.

Franklin, T.E., Cook, R.W., Anderson, D.J., Kuttler, K.L., 1967. Medium and low level feeding of chlortetracycline with comparisons to anaplasmosis CF and CA tests. *Southwest Vet* 20, 101-104.

Franklin, T.E., Huff, J.W., Grumbles, L.C., 1965. Chlortetracycline for elimination of anaplasmosis in carrier cattle. *J Am Vet Med Assoc* 147, 353-356.

Futse, J.E., Ueti, M.W., Knowles, D.P., Jr., Palmer, G.H., 2003. Transmission of *Anaplasma marginale* by Boophilus microplus: retention of vector competence in the absence of vector-pathogen interaction. *J Clin Microbiol* 41, 3829-3834.

Goff, W.L., Stiller, D., Roeder, R.A., Johnson, L.W., Falk, D., Gorham, J.R., McGuire, T.C., 1990. Comparison of a DNA probe, complement-fixation and indirect immunofluorescence tests for diagnosing *Anaplasma marginale* in suspected carrier cattle. *Vet Microbiol* 24, 381-390.

Gonzalez, E.F., Long, R.F., Todorovic, R.A., 1978. Comparisons of the complement-fixation, indirect fluorescent antibody, and card agglutination tests for the diagnosis of bovine anaplasmosis. *Am J Vet Res* 39, 1538-1541.

Han, S., Norimine, J., Palmer, G.H., Mwangi, W., Lahmers, K.K., Brown, W.C., 2008. Rapid deletion of antigen-specific CD4+ T cells following infection represents a strategy of immune evasion and persistence for *Anaplasma marginale*. *J Immunol* 181, 7759-7769.

Jatkar, P.R., Kreier, J.P., 1969. Pathogenesis of anaemia in anaplasma infection II--Auto-antibody and anaemia. *Indian Vet J* 46, 560-566.

Jonsson, N.N., Bock, R.E., Jorgensen, W.K., 2008. Productivity and health effects of anaplasmosis and babesiosis on *Bos indicus* cattle and their crosses, and the effects of differing intensity of tick control in Australia. *Vet Parasitol* 155, 1-9.

Kocan, K.M., de la Fuente, J., Guglielmone, A.A., Melendez, R.D., 2003. Antigens and alternatives for control of *Anaplasma marginale* infection in cattle. *Clin Microbiol* Rev 16, 698-712.

Kuttler, K.L., 1983. Influence of a second anaplasma exposure on the success of treatment to eliminate anaplasma carrier infections in cattle. *Am J Vet Res* 44, 882-883.

Le, C.T., 2003. Introductory biostatistics, 1st Edition. John Wiley and Sons, Inc., Hoboken, NJ.

Magonigle, R.A., Newby, T.J., 1984, Response of cattle upon reexposure to *Anaplasma marginale* after elimination of chronic carrier infections. *Am J Vet Res* 45, 695-697.

Moazed, D., Noller, H.F., 1987. Interaction of antibiotics with functional sites in 16S ribosomal RNA. *Nature* 327, 389-394.

Norton, J.H., Parker, R.J., Forbes-Faulkner, J.C., 1983. Neonatal anaplasmosis in a calf. *Aust Vet J* 60, 348.

OIE 2009. Office International des Épizooties. Manual of standards for diagnostic tests and vaccines for terrestrial animals. Chapter 2.4.1 17th ed. 2008. Accessed July 13, 2009. http://www.oie.int/eng/normes/mmanual/2008/pdf/2.04.01_BOVINE_ANAPLASMOSIS.pdf

Platt, T.M., Loneragan, G.H., Scott, H.M., Norby, B., Thomson, D.U., Brown, M.S., Ives, S.E., Brashears, M.M., 2008. Antimicrobial susceptibility of enteric bacteria recovered from feedlot cattle administered chlortetracycline in feed. *Am J Vet Res* 69, 988-996.

Reinbold, J.B., Coetzee, J.F., Gehring, R., Havel, J.A., Hollis, L.C., Olson, K.C., Apley, M.D., 2009a. Plasma pharmacokinetics of oral chlortetracycline in group fed, ruminating, Holstein steers in a feedlot setting. *J Vet Pharm Ther* DOI: 10.1111/j.1365-2885.2009.1116.x.

Reinbold, J.B., Coetzee, J.F., Hollis, L.C., Nickell, J.S., Riegel, C., Christopher-Huff, J., Ganta, R.R., 2009b. Comparison of iatrogenic *Anaplasma marginale* transmission by needle and needle-free injection techniques. *Am J Vet Res* (Provisional Acceptance: AJVR-09-07-0279).

Renshaw, H.W., Magonigle, R.A., Eckblad, W.P., Frank, F.W., 1976. Immunity to bovine anaplasmosis after elimination of the carrier status with oxytetracycline hydrochloride. *Proc Ann Meet U S Anim Health Assoc*, 79-88.

Richey, E.J., Brock, W.E., Kliewer, I.O., Jones, E.W., 1977. Low levels of chlortetracycline for anaplasmosis. *Am J Vet Res* 38, 171-172.

Sirigireddy, K.R., Ganta, R.R., 2005. Multiplex detection of *Ehrlichia* and *Anaplasma* species pathogens in peripheral blood by real-time reverse transcriptase-polymerase chain reaction. *J Mol Diagn* 7, 308-316.

Stevens, A.M., Shoemaker, N.B., Li, L.Y., Salyers, A.A., 1993. Tetracycline regulation of genes on *Bacteroides* conjugative transposons. *J Bacteriol* 175, 6134-6141.

Strik, N.I., Alleman, A.R., Barbet, A.F., Sorenson, H.L., Wamsley, H.L., Gaschen, F.P., Luckschander, N., Wong, S., Chu, F., Foley, J.E., Bjoersdorff, A., Stuen, S., Knowles, D.P., 2007. Characterization of *Anaplasma phagocytophilum* major surface protein 5 and the extent of its cross-reactivity with *A. marginale*. *Clin Vaccine Immunol* 14, 262-268.

Twiehaus, M.J., 1962. Control of anaplasmosis by feeding an antibiotic (Aureomycin). *Proc 4th Natl Anaplasmosis Conf*, 48-49.

VMRD. Anaplasma antibody test kit, cELISA. (Veterinary Medical Research & Development), pp. Assay instructions for catalog numbers: 282-282 and 282-285. USDA Product Code 5002.5020 For veterinary use only. 25

CHAPTER 6 - Implications for further research

The qRT-PCR described in Chapter 2 has proven to be a robust diagnostic method for determining *A. marginale* disease status in cattle. Furthermore, the qRT-PCR was demonstrated as a replacement for the subinoculation of splenectomized cattle for the confirmation of study results. Additionally, a robust UPLC-MS/MS method has been described for monitoring CTC plasma drug concentrations (Chapter 4). From these novel diagnostic and quantification techniques developed in this dissertation, two novel studies will be modestly described in relation to future research implications.

Assessment of horizontal *A. marginale* transmission with a tick vector subsequent to feeding on a chlortetracycline-treated, carrier calf

Introduction:

Specific host vector relationships have previously been elucidated in ticks for the transmission of *A. marginale* in cattle (Kocan et al., 2000). Ticks have been demonstrated to be more efficient vectors of anaplasmosis than Tabanidae and Muscidae on the order of >240-fold and >300-fold, respectively (Scoles et al., 2005; Scoles et al.,2008). Additionally, the percentage of ticks infected during feeding is related to the circulating parasitemia (Eriks et al., 1989; Eriks et al., 1993). However, research has not been performed to determine the transmission rate of *A. marginale* by ticks subsequent to feeding on carrier cattle treated with CTC. The gathering of this data is imperative due to the chemoprophylactic use of CTC during the vector season (Brock et al., 1957).

Phase I:

Nine Holstein calves weighing an average of 200 kg will be purchased and screened for *A. marginale* infection with the qRT-PCR. One naive calf will be infected with the tick transmissible Virginia isolate of *A. marginale* as described in Chapter 3. A carrier state will be established and confirmed through monitoring of the parasitemia level with the qRT-PCR. Upon confirmation of the carrier state, Phase II will begin.

Phase II:

The carrier calf will be treated with the labeled dose of 350 mg of CTC per day as described in Table 1.3. Four days will elapse to ensure a steady-state plasma drug concentration has been achieved. Then, a total of 120 adult male, *Dermacentor variablis* ticks will be placed on the carrier calf and allowed to feed until self-detachment. Ticks will be stored for 1 week to allow multiplication of *A. marginale* in gut and salivary tissues. The parasitemia of the carrier calf will be monitored daily to assess the change in parasitemia levels prior to and during treatment with CTC. The CTC plasma drug concentration will be monitored with the UPLC-MS∕MS method (Chapter 4). A correlation (R^2) will be made between the change in parasitemia and CTC plasma drug concentration by regression analysis for the dose administered. Indeed, the limitation of this correlation will be an n=1. However, this will be a gathering of preliminary data for the monitoring of this scenario under field conditions.

Phase III:

Ten ticks will be transferred to each of the 8 naïve, non-medicated calves and allowed to feed until self-detachment to assess disease transmission success or failure. The remaining ticks (40) will be mounted, sectioned, and screened for infection by light microscopy. The prevalence of infection in these ticks will be used to estimate the prevalence of infection in the ticks feeding on the naïve calves. After 60 days, transmission success/failure will be determined through analysis of blood samples by the qRT-PCR.

Addendum to protocol:

If tick transmission is successful under the conditions of this study, then Phases I and II will be repeated in a separate group of calves and ticks. However, Phase III will be altered to include the treatment of the new group of 8 naïve calves with the dose and the conditions as described in Phase II for the carrier calf. This will assess the ability of infected ticks to transmit infection to calves treated with a labeled dosage of CTC.

Conclusion:

Valuable information will be gathered in regard to the utility of feeding CTC to an endemically unstable group of calves during the tick vector season for prevention of horizontal transmission.

Discovery of the mechanism of carrier clearance in cattle fed chlortetracycline during chemosterilization

Introduction:

The mode of action of tetracycline antibiotics was described in Chapter 1 for Gram negative bacteria (Chopra and Roberts, 2001). However, the mechanism of carrier clearance subsequent to the interaction of CTC with the 30S ribosome of *A. marginale* is unknown. It is imperative to understand this relationship in order to elucidate the future direction of anaplasmosis chemosterilization research efforts and the treatment of other pathogenic bacteria with tetracycline antibiotics.

Phase I:

Sixteen Holstein calves weighing an average of 200 kg will be purchased and screened for *A. marginale* infection with the qRT-PCR. Each naive calf will be infected with the tick transmissible Virginia isolate of *A. marginale* as described in Chapter 3. A carrier state will be established and confirmed through monitoring of the parasitemia level with the qRT-PCR. Upon confirmation of the carrier state, Phase II will begin.

Phase II:

Carrier calves will be randomly assigned to a (1) CTC treatment group (4.4 mg/kg of bodyweight/day for 45 days (n = 8) or (2) a placebo-treated, control group (n = 8). The parasitemia and plasma drug concentration of all calves will be monitored on a daily basis by the qRT-PCR (Chapter 2) and UPLC-MS∕MS methods (Chapter 4), respectively. The 16S rRNA: 16S DNA ratio will also be monitored in the same sample extracted for RNA analysis as described in Chapter 2. A flow cytometric method will be used at each time point to assess the viability of *A. marginale* bacteria as previously described in (Coetzee et al., 2006). Finally, a

protocol modification will be developed to monitor CTC concentration in the intra-erythrocytic compartment as compared to the vascular compartment.

Conclusion:

At the completion of the 45 day study, the purpose of this study is to assess the potential change in *A. marginale* intracellular metabolism relative to treatment administered. The rate and time of carrier clearance will be monitored with the qRT-PCR. A correlation (R^2) will be made between the change in metabolism according to treatment administered and the 16S rRNA: 16S DNA data. A correlation will also be made between the change in metabolism detected by the qRT-PCR and the viability of *A. marginale* bacteria assessed with the flow cytometric method. The plasma and intra-erythrocytic drug concentrations will be used to determine pharmacokinetic parameters during treatment. Furthermore, an attempt will be made to establish a relationship between plasma drug concentration, intra- erythrocytic drug concentration, *A. marginale* viability, and chemosterilization. If a relationship can be established, then it may be concluded that the cytotoxic effects of chlortetracycline contribute to the mechanism of carrier clearance. If no relationship can be established, then it may be concluded that an unknown mechanism contributes to the mechanism of carrier clearance.

Research conclusions and practical implications

The study in Chapter 2 described the development of real-time qRT-PCR assays for the detection of *A. marginale* and *A. phagocytophilum* alone or in combination. This is the first study to describe a duplex method for the diagnosis of *A. marginale* and *A. phagocytophilum* from the same sample. The selection of the16S rRNA gene segments enhanced the analytical sensitivity of the assay due to the high ratio of 16S rRNA:16S DNA. Furthermore, the extraction of RNA from a plasma-free blood sample ensured the maximum number of cells in a 250 µL sample were available for analysis. The correct classification of disease status is important for the collection of epidemiologic information, development of anaplasmosis disease control programs, and improvement of free-trade policy between endemic and non-endemic countries.

Anaplasmosis presents many problems to the cattle industry due to complications with disease control, eradication and treatment. When vaccinating cattle of unknown disease status, hygienic animal husbandry techniques are highly recommended. The study in Chapter 3 was designed to vigorously challenge the utility of needle-free injection for the control of anaplasmosis transmission among cattle during vaccination. Needle-free injection was validated as a tool for controlling iatrogenic transmission of *A. marginale*. Additionally, the *A marginale*-specific qRT-PCR assay was evaluated for detecting *A. marginale* in bovine peripheral blood samples. This data set is clinically relevant due to the potential spread of *A. marginale* to naive cattle during routine animal husbandry practices as well as identifying the deficiencies in the sensitivity and specificity of cELISA. This study is the first report to evaluate needle-free injection techniques for the control of iatrogenic transmission of anaplasmosis as well as determine the performance of first, second and third generation diagnostic methods at sequential time points following a single exposure to *A. marginale* in cattle.

Due to the widespread use of CTC in ruminating cattle, it is essential these pharmacokinetic parameters be reported (Chapter 4). Current literature remains deficient for oral bioavailability in ruminating cattle. Until a sufficient body of scientific knowledge for a complete pharmacokinetic and pharmacodynamic profile of CTC is formed, the use of this antibiotic as a tool in profitable cattle production will continue to be a target of special interest groups for the removal of the FDA-approved, labeled use of CTC in animal feed. Recently, a population pharmacokinetic (PPK) study was established as a successful tool for gathering pharmacokinetic information in large populations of cattle ($\geq$ 100 head) (Fu et al., 2008). In addition to that study, the information gathered in the present study, as well as the use of nonlinear mixed effects modeling techniques typical of PPK studies, could provide the framework for PPK research of CTC in group fed, ruminating cattle. Future studies of this kind are necessary to bridge the gap between experimentally-derived data and confirmatory studies applied under normal conditions (Sheiner, 1997).

The study in Chapter 5 established multiple chemosterilization regimens using CTC for the clearance of persistent infection with *A. marginale*, as assessed with the Virginia isolate. These treatment regimens offer an alternative strategy for minimizing animal handling and eliminates individual treatment. However, these plasma drug concentrations were less than the minimum inhibitory concentration recommended for the treatment of bacterial infection caused

by *M. haemolytica, P. multocida,* or *H. somnii* (CLSI, 2008). Therefore, the success of an *A. marginale* chemosterilization strategy should not be based upon the aforementioned minimum inhibitory concentration. A key finding of this study was the pharmacokinetic relationship evaluated among 3 doses of CTC in a fixed treatment regimen. Furthermore, this study identified a viable chemosterilization and testing strategy that could be considered as an alternative to culling valuable cattle infected with *A. marginale*. The qRT-PCR was instrumental in characterizing the time of chemosterilization as well as establishing the pharmacokinetic relationship between plasma drug concentration and chemosterilization *in vivo*.

References

Brock, W.E., Pearson, C.C., Staley, E.E., Kliewer, I.O., 1957, The prevention of anaplasmosis by feeding chlortetracycline. *J Am Vet Med Assoc* 130, 445-446.

Chopra, I., Roberts, M., 2001, Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol Mol Biol Rev* 65, 232-260 ; second page, table of contents.

CLSI, 2008. *Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; Approved standard - Third edition*. CLSI document M31-A3. Table 2. 28, 65-72.

Coetzee, J.F., Apley, M.D., Kocan, K.M., Jones, D.E., 2006, Flow cytometric evaluation of selected antimicrobial efficacy for clearance of *Anaplasma marginale* in short-term erythrocyte cultures. *J Vet Pharmacol Ther* 29, 173-183.

Eriks, I.S., Palmer, G.H., McGuire, T.C., Allred, D.R., Barbet, A.F., 1989, Detection and quantitation of *Anaplasma marginale* in carrier cattle by using a nucleic acid probe. *J Clin Microbiol* 27, 279-284.

Eriks, I.S., Stiller, D., Palmer, G.H., 1993, Impact of persistent *Anaplasma marginale* rickettsemia on tick infection and transmission. *J Clin Microbiol* 31, 2091-2096.

Fu, L.X., Jiang, Z.G., Ding, H.Z., Liu, Y.H., 2008. Population pharmacokinetics of enrofloxacin and its active metabolite ciprofloxacin in ill cows. *J Vet Pharmacol Ther* 31, 240-245.

Kocan, K.M., Blouin, E.F., Barbet, A.F., 2000, Anaplasmosis control. Past, present, and future. *Ann N Y Acad Sci* 916, 501-509.

Scoles, G.A., Broce, A.B., Lysyk, T.J., Palmer, G.H., 2005, Relative efficiency of biological transmission of *Anaplasma marginale* (*Rickettsiales: Anaplasmataceae*) by *Dermacentor andersoni* (*Acari: Ixodidae*) compared with mechanical transmission by *Stomoxys calcitrans* (*Diptera: Muscidae*). *J Med Entomol* 42, 668-675.

Scoles, G.A., Miller, J.A., Foil, L.D., 2008, Comparison of the efficiency of biological transmission of *Anaplasma marginale* (*Rickettsiales: Anaplasmataceae*) by *Dermacentor andersoni* Stiles (*Acari: Ixodidae*) with mechanical transmission by the horse fly, *Tabanus fuscicostatus* Hine (*Diptera: Muscidae*). J Med Entomol 45, 109-114.

Sheiner, L.B., 1997. Learning versus confirming in clinical drug development. *Clin Pharmacol Ther* 61, 275-291.