Examination of the impact of bovine anaplasmosis on dairy and beef operations, and evaluation of control with antimicrobial chemosterilization or vaccination with an ear-implant delivery platform

by

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B.S., University of Missouri, 2012
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AN ABSTRACT OF A DISSERTATION

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DOCTOR OF PHILOSOPHY

Department of Anatomy and Physiology
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Abstract

Bovine anaplasmosis is a mild to severe hemolytic disease caused by the intraerythrocytic pathogen *Anaplasma marginale* (*Am*). Transmission of bacteria occurs biologically via ticks and mechanically via blood-contaminated fomites and biting flies. Following inoculation, cattle hosts exhibit persistent bacteremia and can serve as reservoirs for subsequent infection. Bovine anaplasmosis can cause fever and lethargy but the most notable feature is anemia due to phagocytosis of parasitized erythrocytes. Total costs of disease to U.S. cattle producers are difficult to estimate but are thought to amount to several hundred million dollars per annum. Treatment and control have historically been predicated on the administration of the antimicrobials oxytetracycline and chlortetracycline, respectively. In the U.S., disease prevention is complicated by the lack of efficacy data for the conditionally approved vaccine.

In this dissertation, examples of the impact of bovine anaplasmosis on the domestic cattle population are explored. Two case reports are presented that describe the impact of disease outbreaks in areas considered both endemically stable and unstable. Two production systems and geographic regions were examined: a dairy in the state of Iowa and a beef cattle operation in Florida. Serology was performed in both cases using commercially available enzyme-linked immunosorbent linked assay (ELISA) kits. Outcome measures included seroprevalence of anti-*Am* antibodies, milk production, abortions, and mortality. In Iowa, seroprevalence of anti-*Am* antibodies was shown to have an inverse relationship with milk production. In Florida, seroprevalence of anti-*Am* antibodies was shown to be higher among open herds containing animals introduced from a different region (Texas). Likewise, open herds were shown to experience higher rates of mortality and abortion than herds containing exclusively Florida cattle. Statewide seroprevalence for Florida was also estimated by testing the blood of animals

destined for slaughter. Seroprevalence among herds experiencing the disease outbreak was found to be higher than the statewide average.

This dissertation also explores the efficacy of currently approved tetracycline-based treatment and control protocols. An experiment was conducted to study the impact of injectable oxytetracycline (OTC) and oral chlortetracycline (CTC) on *Am* bacteremia over time. Persistently infected cattle were treated with OTC, CTC, or no drug, and bacteremia was calculated using quantitative polymerase chain reaction (qPCR). Bacteremia was found to significantly but transiently drop among animals treated with OTC. Among those treated with CTC, no significant drop in bacteremia was noted. These findings are important as they show that neither drug, despite being approved by the FDA for treatment (OTC) and control (CTC) of bovine anaplasmosis, appears capable of bacterial clearance from persistently infected cattle.

As vaccine options for bovine anaplasmosis are limited, practical implications of vaccine delivery via a subcutaneous implant are also explored. A series of pilot studies were conducted to test the capability of a subcutaneous implant to deliver vaccine over an extended period of time. For these pilots, a gonadotropin-releasing hormone (GnRH) antigen was used. To test the versatility of the implant, GnRH was bound to different carrier proteins (ovalbumin, OVA; keyhole limpet hemocyanin, KLH) and packaged with different adjuvants (Quil-A®, DEAE-Dextran). Outcome measures were related to the immunological impact of a GnRH antigen and included scrotal and testicular changes, levels of anti-GnRH antibodies, testosterone concentration, and degree of spermatogenesis. The implant was shown to be capable of stimulating humoral immunity to GnRH as determined by ELISA. The implant was also shown to affect spermatogenesis when gonads were examined histologically. Although significant changes in testosterone were not noted, the implant technology is promising.

To apply the implantable vaccine concept to bovine anaplasmosis, a study was conducted to examine the ability of the implant to confer protection to heterologous disease challenge over a long period of time (20+ months). For this experiment, the major surface protein 1a (MSP1a), an adhesion conserved among many *Am* strains, was used as an antigen and both Quil-A® and DEAE-D were used as adjuvants. Outcome measures included bacteremia, body temperature, and packed cell volume (PCV) as an indicator of anemia. Animals that were provided vaccine implants with both adjuvants were shown to have reduced symptom severity after being challenged with *Am*. This is a promising outcome for the implant methodology and warrants further exploration.

Bovine anaplasmosis remains a challenge to profitable cattle production in the U.S. and abroad. As tetracycline antimicrobials may not reliably clear infection, further research towards refining strategies of disease prevention is warranted. A vaccine that not only limits disease severity, but also entirely prevents infection with *Am* would be ideal. Novel implantable platforms for vaccine delivery are an attractive option, as they may be able to confer protection over an extended period of time with a single dose. Towards this end, field studies of an implantable polyvalent vaccine tailored to deliver highly-conserved *Am* surface epitopes are needed.

Novel vaccine strategies for bovine anaplasmosis

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Table of Contents

List of Figures

List of Tables

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Dedication

Dedicated to my friends who never forgot about me and to my family that never gave up on me. Also dedicated to the most unwittingly noble of beasts: those members of the genus *Bos*.

Preface

My interest in bovine anaplasmosis began at an early age. Each year, animals on the family cattle farms would succumb to this illness, subsequent to periods of suffering of inconsistent duration. Mortality was usually concentrated in the early weeks of autumn, and animals were sometimes found moribund when feeding the first hay of the year. I remember veterinarians describing their blood as "watery" and administering oxytetracycline. Recovery wasn't guaranteed but at least treatment made *us* feel better about the whole thing. Growing up, anaplasmosis didn't receive a lot of consideration relative to other herd health issues. Pinkeye, for example, was a more overtly debilitating and often heartbreaking disease. In retrospect, "anaplas" was viewed, simply, as an unavoidable cost of doing cattle business in the Ozarks.

I was reintroduced to the disease by Drs. Hans Coetzee and Katie Reif at Kansas State University's College of Veterinary Medicine. Their expertise in the treatment and prevention of bovine anaplasmosis is impressive. Their ongoing efforts to remedy this often underestimated illness is to be lauded. I count myself fortunate to have wetted my feet (albeit in the shallow end) in the fount of rickettsial knowledge under their tutelage. With their help and the help of many others, I have examined facets bovine anaplasmosis ranging from herd effects and transmission to antimicrobial clearance and vaccine technology. It has been a formative experience and I am forever grateful.

Chapter 1 - Literature Review

Introduction

Bovine anaplasmosis, caused by the intracellular hemoparasite *Anaplasma marginale (Am)*, was first described by Swiss veterinarian Sir Arnold Theiler in South African cattle in 1910. Colloquially referred to as "Gall-Sickness" due to jaundice observed during the course of disease, anaplasmosis was noted to cause "marginal points" along the periphery of infected erythrocytes (Theiler, 1910a). Although likely observed and misdiagnosed earlier as cattle fever (Smith and Kilbourne, 1893), anaplasmosis was first correctly described in the U.S. by P.B. Darlington in the vicinity of Chanute, KS (Darlington, 1926). As a practicing veterinarian, Darlington noted a progressive and persistent anemia among dairy cattle in his care. Subsequent expert investigation of blood smears confirmed the disease as bovine anaplasmosis (Ackert, 1956). Today, over half of Kansas cattle herds have been shown to include at least one animal with bovine anaplasmosis (Spare et al., 2020).

Early efforts to better understand transmission and pathogenesis were summarized in a report by Dykstra et al. (1948). In this report, the tick *Dermacentor andersoni* and two genera of mosquitos, *Psorophora* and *Aedes* were incriminated as vectors of *Am*. It was also postulated that calves may acquire permanent, asymptomatic infection, and could be parasitized by experimental inoculation (Splitter, 1950). It is now known that disease features acute and persistent phases, following an incubation period of 7-60 days (Kocan et al., 2003). This incubation period is variable and likely depends on infectious dose (Gale et al., 1996). It is the asymptomatic status of persistently infected animals, paired with vectored bacterial transmission via infected fomites and arthropod vectors, that makes control of bovine anaplasmosis an ongoing problem for the

beef and dairy cattle industries. In addition, there is currently no fully approved vaccine to prevent infection, and no antimicrobial approved to clear *Am* bacteria.

Over a century after the disease was first described, there remains an apparent privation of peer-reviewed literature examining the production impact of both acute and persistent bovine anaplasmosis. This is true for production systems raising cattle for both beef and dairy products. There also remains no treatment approved to eliminate bacteria from infected cattle or vaccine to prevent *Am* infection. To answer questions relating to the production impacts of bovine anaplasmosis, available treatments, and current vaccinology, a systematic review of literature was conducted in October 2021. The online resources PubMed, Google Scholar, and AGRICOLA were used. Search terms included bovine anaplasmosis; seroprevalence; and then state or country. Search terms also included bovine anaplasmosis; vaccines; treatments. Conditions and procedures used in the search included antimicrobials, tetracyclines, oxytetracycline, chlortetracycline, imidocarb dipropionate, dairy cattle, Holsteins, abortions, anemia, bacteremia, epitopes, major surface proteins, and implants. Studies were limited to those published or translated to English via browser software or third-party publication.

Etiology and Clinical Signs

Following infection, *Am* invades erythrocytes via endocytosis and undergoes cyclic replication, removal of infected blood cells by the reticuloendothelial system, and subsequent reinvasion of erythrocytes within the ruminant (Aubry and Geale, 2011). Previous work has suggested an ability of *Am* to infect certain endothelial cells as well as erythrocytes. This has been demonstrated both *in vivo* (Carreño et al., 2007) and after in vitro challenge (Munderloh et al., 2004). These endothelial cells may serve as early reservoirs for bacteria following the

attachment of mechanical or biological vectors. During initial infection, bacterial replication is prolific, with the number of infected erythrocytes doubling every 24 hours (Richey and Palmer, 1990). During the acute phase of infection, bacteremia peaks, and the number of infected erythrocytes may be as high as 10^9 cells per ml of whole blood (Palmer et al., 1999). Clinical disease appears to manifest once at least ~15% of the erythrocytes have been parasitized (Radostits et al., 2007). The clinical signs of acute bovine anaplasmosis include anemia and icterus, resulting from profound phagocytosis of infected erythrocytes. Other signs may include fever, weight loss, abortion, lethargy, and death (Ristic, 1977). Young cattle appear to be less susceptible to acute disease (Jones et al., 1968; Roby et al., 1978). The specific mechanism of resistance warrants further investigation but may be related to the relatively high number of gamma-delta T lymphocytes present in the peripheral blood and spleens of calves (Valdez et al., 2020). These lymphocytes have shown an ability to lyse cells infected with *Theileria parva*, another tick-born intracellular pathogen of cattle (Daubenberger et al., 1999). Infected animals remain infected, often asymptomatically, regardless of age. As such, humoral immune response to *Am* has been shown to last for many years after the acute disease phase (Knowles et al., 1996). The resulting paradox of bovine anaplasmosis is that persistently infected cattle exhibit immunity to homologous reinfection concomitant to original infection. The potential thus exists for asymptomatic "carrier" animals to serve as reservoirs for bacteria to be transmitted to naïve hosts (Kocan et al., 2000).

Cyclic replication of *Am*, even in immunocompetent hosts, is a demonstrated feature of the persistent phase of disease (Kieser et al., 1990). Compared to the acute phase, bacteremia is lower during persistent disease, and has been shown to fluctuate by several orders of magnitude. Eriks et al. (1993) reported bacteremia ranging from $<10^4$ to 10^7 infected erythrocytes per ml

whole blood at approximately 5-week intervals during persistent infection. One mechanism by which bacteria persist appears to be related to immune subversion via antigenic variation through continuous elaboration of new cell surface structures (Barbet, 2009). The rapid deletion of antigen-specific CD4+ T lymphocytes also represents a strategy of immune evasion and persistence for *Am* (Han et al., 2008). Recombination of gene families that encode for major surface proteins (MSPs) has been shown to generate diversity in cell surface epitopes. This phenomenon has been suggested among several MSPs including MSP1 (Viseshakul et al., 2000), MSP2 (French et al., 1998) and MSP3 (Alleman et al., 1997). These changes in surface immunogens have implications not only for immune subversion, but also for vaccine design.

Production loss

It is worth noting the economic detriment that bovine anaplasmosis represents to producers during the course of disease. Losses can be difficult to estimate and can vary by breed, sex, age, and physiological status of the affected animal (Rodriguez et al., 2009). Material losses to producers may take the form of deaths (Henker et al., 2020; Hornok et al., 2012), abortions (Kirkbride, 1993; Correa et al., 1978), decreased milk yield (Machado et al., 2015; Howden et al., 2010), and costs associated with disease treatment and prevention (Alderink and Dietrich, 1983). Recent studies examining disease costs to producers are lacking in the published literature. Current data detailing the true economic impact of disease are desperately needed to inform future research directions.

Transmission

In general, bovine anaplasmosis may be considered to exhibit three modes of transmission: mechanical, biological, and transplacental.

Mechanical

Mechanical transmission can occur via biting insects or by blood-contaminated fomites such as ear tagging devices or needles (Sonenshine, 1991). Interestingly, it appears that early research did not routinely implicate mechanical transmission of *Am*. This began to change when Darlington (1926) reported the disease among cattle found to be free of ticks (Lotze et al., 1956). Since then members of the genus *Tabanus* biting insect family have been shown to experimentally infect splenectomized calves with as few as ten bites (Hawkins et al., 1982), though they appear much less efficient at transmitting *Am* than ticks such as *Dermacentor* species (Scoles et al., 2008). Data collected on stable flies of the genus *Stomoxys* (Bautista et al., 2018) have also implicated these insects in *Am* transmission. Lice (*Haematopinus*) have also been found to harbor *Am* genetic material (Hofmann-Lehmann et al., 2004) but it should be noted that this does not necessarily mean they are capable of transmission. Previous work has also suggested that blood-contaminated fomites can also contribute to iatrogenic spread of bovine anaplasmosis. For example, surveys conducted by Atif et al. (2013) and Rodriguez-Vivas et al. (2004) found that reuse of needles was a major risk factor for *Am* seroprevalence. Reinbold et al. (2010) demonstrated that iatrogenic transmission of *Am* can occur 60% of the time when needles are reused between acutely infected and naïve animals. These findings have clinical relevance to U.S. bovine practitioners as a nationwide survey of large animal veterinarians

indicated that most (69%) routinely reused hypodermic needles between cattle (Anderson and Silviera, 2008).

Biological

In general, biological transmission in the context of bovine anaplasmosis refers to transmission via ticks. Early work by Theiler (1910b) demonstrated the capacity for *Am* transmission via ticks (genus *Boophilus*). Since then, approximately 20 tick species worldwide have been incriminated as biological vectors (Kocan et al., 2010) including members of the genera *Dermacentor* (Boynton et al., 1936), *Hyalomma* (Potgeiter, 1979), *Ixodes* (Helm, 1924), *Rhipicephalus* (Brumpt, 1931), and *Argas* (Howell et al., 1943), although there have been some inconsistencies among these experiments (Kocan et al., 2004). It should be noted that not all *Am* strains have been proven transmissible by ticks. For instance, previous work has shed doubt on the ability of strains from Illinois (Smith et al., 1986), and Florida (Wickwire et al., 1987) to transmit by *Dermacentor variabilis* and *andersoni*, respectively. Tick transmission may depend on the ability of MSPs such as MSP1a to act as adhesions between *Am* and tick cells. It has been suggested that this ability is related to the presence of MSP glycosylation and is likely strain-dependent (Garcia-Garcia et al., 2004). However, biological transmission of many strains of *Am* via ticks is important as it has been shown to be significantly more efficient than mechanical transmission via biting flies due, in part, to the potential for *Am* replication within tick cells (Scoles et al., 2005). The *Am* bacteria can be harbored in tick midgut and salivary glands, making them potential reservoirs of infection for extended periods and among multiple cattle (Kocan et al., 1992a). Transmission by ticks can occur both intrastadially (i.e. within one life stage, in male ticks) as described in *Dermacentor* (Kocan et al., 1992b) and transstadially (i.e. between two life

stages) as described in *Boophilus* (Samish et al., 1993). The potential for ticks to ingest *Am* organisms when feeding has also been shown to be very high. Work conducted by Eriks et al. (1993) suggested the likelihood of acquisition of at least one *Am* bacterium by *Dermacentor andersoni* to be 95-100% during acute disease and 27-84% during the persistent phase.

Transplacental

Transplacental (vertical) transmission of *Am* can occur when infected erythrocytes move across the placenta in utero from an infected dam to her offspring. Data suggest that this can occur regardless of whether the dam experiences acute disease during pregnancy, though transmission does not appear to be universal. One study confirmed transplacental transmission of *Anaplasma* organisms (both *A. marginale* and *A. centrale*, as determined by rapid card agglutination) in 15.6% of calves (Potgieter and Van Rensburg, 1987). Interestingly, a much more recent study found a nearly identical (15% -20%) transplacental transmission rate as measured by ELISA and PCR (da Silva et al., 2016). Another investigation of transplacental transmission, with a focus on persistently infected cows, showed that 10.5% of calves were born seropositive for *Am* as measured using ELISA and indirect immunofluorescence (Grau et al., 2013). The effect of maternal antibodies on this finding was not investigated. Notably, none of the dams in the 2013 study experienced acute disease during gestation. Transplacental transmission has been demonstrated when dams are infected naturally (Costa et al., 2016) and when experimentally inoculated during late pregnancy (Swift and Paumer, 1976). Data suggest that transplacental transmission of *Am* may play a significant role in disease epidemiology, particularly in areas where vectors and contaminated fomites are uncommon or absent.

Seroprevalence

From a disease management perspective, an understanding of bovine anaplasmosis seroprevalence is valuable. There exist certain geographic regions around the world where disease is considered endemic (i.e. regularly found among susceptible hosts) and seroprevalence relatively high. In other regions, (Canada, for example) seroprevalence is lower and disease may not be routinely diagnosed. The distinction between endemic and non-endemic areas is particularly relevant when it comes to movement of cattle from one region to another. Cattle movement has been suggested (by Aubry and Geale, 2011) as a driver for the different genotypes of *Am* recorded within herds in endemic areas (Palmer et al., 2004, 2001). Importantly, outbreaks of anaplasmosis can occur through transfer of infected cattle to nonendemic areas (Kocan et al., 2000).

Seroprevalence of bovine anaplasmosis is variable and dependent on geographic region. In general, it is considered endemic in tropical and subtropical areas of the globe (~40ºN-32ºS Aubry and Geale, 2011). In the Western Hemisphere, endemicity is applied to areas of Mexico, the Caribbean, as well as Central and South America with the exception of the Andes, presumably due to lack of arthropod vectors (Guglielmone, 1995). Extensive seroprevalence testing using a variety of methods (complement fixation, blood smears, card agglutination assays, ELISA, PCR, and immunofluorescence assays) has revealed that seroprevalence varies widely not just across the Americas, but also within the U.S (Table 1). Length of the vector season may be a contributing factor to higher seroprevalence in warm regions, but it should be noted that *Am* has been found in drier, cooler, climates as well. Though bovine anaplasmosis has been considered endemic to the southeastern and midwestern regions of the continental U.S. (McCallon, 1973), it has been reported in every contiguous state (Coetzee et al., 2010). This is

likely due, in part, to cattle movement from one region to another (Kocan et al., 2003). In the contiguous U.S., estimates of seroprevalence have ranged from low in the north (1.93% in Montana, Van Donkersgoed et al., 2004) to higher in the Midwest (7.1 – 10.7% in Illinois, Hungerford and Smith, 1997), and even higher in the South (29.02% in Mississippi, Okafor et al., 2019a). However, even within a geographic region (e.g. the South), variability has been demonstrated in seroprevalence. For example, apparent seroprevalence was estimated at 4.44% in Georgia, (Okafor et al., 2019b) 15.91% in Texas (Okafor et al, 2018a), and 10.78% in Kentucky (Okafor et al., 2018b). Likewise, previous work suggests that freedom from bovine anaplasmosis should not be assumed for cattle raised in northern U.S. states. In Eastern Oregon, for instance, seroprevalence has been shown to vary from 26% - 71% using card (Peterson et al., 1977) and nested PCR (de Echaide et al., 1998) assays, though these studies focused on single herds. Lack of routine serological testing ahead of interstate cattle movement is one reason why endemic stability for disease should not be assumed based exclusively on geographic area.

In the U.S., there is an absence of universal seroprevalence data by state. There is also a lack of data detailing differential effects of disease on beef and dairy cattle production. This information would help inform decisions pertaining to cattle transport and screening. Considering potential logistical and financial constraints of this research, studies might focus first on states with significant cattle populations. It would also be beneficial to compare seroprevalence and impact of bovine anaplasmosis on cattle in states historically thought to be in either endemic or non-endemic areas. An example of a state in a region considered non-endemic for bovine anaplasmosis, and with a large cattle population (3.7 million, USDA NASS 2021) is Iowa. Conversely, an example of a state in a region considered endemic for bovine anaplasmosis, and with a large cattle population (1.7 million, USDA NASS 2021) is Florida. Studies that focus

on endemic and non-endemic states with large cattle industries stand to generate data with the highest applicability to the U.S. cattle industry.

Iowa

Bovine anaplasmosis in the U.S. state of Iowa has been described, although perhaps not to the extent that it has been in other states. As Iowa is not generally considered to be enzootic for bovine anaplasmosis (Dewell, 2010), cases of disease outbreaks and studies of seroprevalence are relatively deficient in the published literature. One early case report described several weak, lethargic animals near Aplington, IA that were eventually screened and confirmed positive for anaplasmosis using a card agglutination assay. In this case, animals died despite treatment with oxytetracycline, and the owner elected to disperse the remaining herd (n = 33) to slaughter (Porter and Greve, 1975). Recent investigations in Iowa are lacking, but one small survey estimated seroprevalence to be 2.3 – 2.8 % across several herds tested in the 1980s (Brinkman and Kersting, 1990). A more recent study which examined seroprevalence in 659 western Iowa feedlot cattle found a higher apparent seroprevalence via ELISA (30% inhibition) of 15.17% (Coetzee et al., 2010). Importantly, animals tested in the 2010 feedlot study originated from 31 consignors located within Iowa. Although seroprevalence appears to have increased over the 20 years between studies, further work is needed to accurately depict disease spread and seroprevalence changes in Iowa over time.

Florida

Bovine anaplasmosis in considered enzootic to the U.S. state of Florida (Richey, 1991) and the state is referenced in some of the earliest *Am* experiments performed in the U.S.

(Sanders, 1933). An isolate unique to the state was first isolated in 1955 and Florida organisms have been described as having an unusual, "tailless" (i.e. lacking cellular appendages) morphology (McGuire et al., 1984) with the genotype MSP5-GenBank M3392. The Florida isolate of *Am* has been the basis for experimental vaccine attempts spanning several decades (Oberle et al., 2021, Palmer et al., 1989) and has demonstrated a lack of transmission via the tick *Dermacentor andersoni* (Wickwire et al., 1987). Despite the seemingly rich history of scientific inquiry into bovine anaplasmosis in Florida, there is a paucity of published literature describing seroprevalence and economic effects. More research is needed to better understand the impact of bovine anaplasmosis on the Florida cattle industry.

Treatment

Since its description, treatment of bovine anaplasmosis has been attempted via a remarkably large and diverse number of chemotherapies. In the years following Theiler's early experiments, over 80 unique compounds were tested, including anti-malarials, antimony derivatives, dyes, and arsenicals, all with mixed results (Miller, 1956). More recently, some of the more experimentally efficacious drugs include dithiosemicarbazone (Todorovic et al., 1975) diminazene aceturate, and buparvaquone (Akhter et al., 2010). In the U.S., however, approved treatments are currently limited to oxytetracycline, chlortetracycline, and enrofloxacin (as of 2020).

Tetracyclines

After many failed attempts at treating bovine anaplasmosis in the early 20th century, it was eventually demonstrated that certain antimicrobials (i.e. chlortetracycline and

oxytetracycline) were able to reduce bacteremia to such an extent that blood from infected animals failed to infect naïve cattle (Splitter and Miller, 1953). These drugs were also found to reduce mortality and speed recovery when applied to clinical cases (Lotze et al., 1956). Early experiments went so far as to suggest the ability of tetracyclines to entirely clear bacteria (Joyner, 1973). More recent trials, however, have shown that while tetracyclines appear capable of reducing bacteremia and limiting clinical effects of disease, they have not been reliably shown to eliminate persistent infections (Aubry and Geale, 2011). Until recently in the U.S., oxytetracycline and chlortetracycline were the only compounds approved for parenteral and oral treatment of bovine anaplasmosis, respectively. Neither drug is approved for elimination of persistent bovine anaplasmosis nor prevention of *A. marginale* infection.

Enrofloxacin

Relatively recently, a new approval was granted to the fluroquinolone enrofloxacin (Baytril ® Elanco US, Greenfield, IN) for treatment of clinical bovine anaplasmosis in the U.S. Specifically, it is conditionally approved for use in replacement dairy heifers under 20 months of age and beef cattle (excluding veal calves and bulls for breeding) by veterinary prescription. Previous work has explored the therapeutic benefits of dosing enrofloxacin to cattle with acute anaplasmosis and suggests efficacy in production settings. Coetzee and Apley (2006) showed that enrofloxacin, when subcutaneously administered at 12.5 mg/kg bodyweight, was capable of reducing parasitemia (as quantified by the percent of parasitized erythrocytes) and anemia in splenectomized calves. In this study, enrofloxacin was administered twice at a 48-hour interval and reported treatment effect against several *Am* isolates. Another study showed that a lower dose (7.5 mg/kg) administered either once or three times at 72-hour intervals also reduced

parasitemia and anemia (Facury-Filo et al., 2012). Efficacy data are encouraging, but enrofloxacin is not approved for elimination of persistent bovine anaplasmosis nor prevention of *A. marginale* infection.

Total Bacterial Clearance

A common theme that is demonstrated throughout the literature is that of variable results when measuring the ability of a drug to entirely clear infection (i.e. chemosterilization). For example, imidocarb dipropionate has been reported both as incapable (Adams and Todorovic, 1974) and capable (Atif et al., 2012) of bacterial clearance, both alone and in conjunction with tetracyclines or enrofloxacin (Shaukat et al., 2019). Conversely, enrofloxacin has been reported unable to clear bacteria in both experimentally (Coetzee and Apley, 2006) and naturally infected animals (Alberton et al., 2015). Tetracyclines also have a lengthy history of inconclusive results when it comes to chemosterilization. Parenteral oxytetracycline has been shown to be both successful (Rogers and Dunster, 1984) and unsuccessful (Wallace et al., 2007) at clearing bacteria. Likewise, oral chlortetracycline has demonstrated both an ability (Franklin et al., 1965) and inability (Reinbold et al., 2009) to chemosterilize animals with bovine anaplasmosis. One possibility for conflicting results is the different methods used to verify bacterial clearance (e.g. card agglutination assays versus PCR). Conflicting reports also suggest that differences in antimicrobial susceptibility among *Am* isolates may exist. The amount of drug and dosing frequency used across studies is also not consistent. More studies are needed to determine the ability of drug therapies to clear infection entirely, as well as generate efficacy data among known strains of *Am*.

Vaccination

Live

Early live vaccine attempts were also pioneered by Sir Arnold Theiler, who experimented with protecting animals from *Am* infection by inoculating them with the less pathogenic *Anaplasma centrale* (Theiler, 1912). This strategy is used in several countries but has questionable efficacy when it comes to cross-protection across geographically disparate isolates as shown in South American (Brizuela et al., 1998) and African (Turton et al., 1998) studies. Importantly, evidence suggests that while vaccination with *A. centrale* can offer some protection against clinical symptoms during acute disease it has not been proven capable of outright prevention of *Am* infection (Shkap et al., 2002). Practical drawbacks also exist for live vaccines such as cold chain requirements, short shelf lives, purification costs, and potential for transmission of concurrent pathogens (Suarez and Noh, 2011).

Killed

Killed (inactivated) vaccines have been available in the U.S. since the 1960s (Kocan et al., 2003). Currently, a killed vaccine is provisionally approved by the USDA and is available in 28 states. However, this product lacks any robust efficacy data support its use. Early killed vaccines relied on purified and lyophilized antigen derived from infected erythrocytes, adjuvanted with mineral oil, and administered at intervals varying between 2 and 6 weeks with a yearly booster (Brock et al., 1965). Early field trials suggested that calves could be predisposed to hemolytic anemia after nursing dams vaccinated with killed vaccine (Dennis et al., 1970). Later experiments improved killed vaccine manufacture by greatly limiting host cell antigen contamination (Hart et al., 1981). Compared to live options, killed vaccines offer the advantages

of a low risk of contamination with concurrent pathogens, relatively inexpensive storage requirements, and low risk of inoculation reactivity or infectivity (McCorkle-Shirley et al., 1985). However, efficacy results on killed vaccines are generally mixed. Killed vaccines have demonstrated some ability to minimize disease severity when based on multiple isolates and tested against a single isolate (Rodriguez et al., 2000). When efficacy was tested against both homologous and heterologous challenges, Montenegro-James et al. (1991) reported solid protection among cattle administered killed vaccine. Conversely, Kuttler et al. (1984) reported little to no cross-protection against heterologous disease challenge.

Subunit

Subunit vaccines are a relatively novel approach to *Am* control. As molecular technologies improve, so does the potential for describing, isolating, and synthesizing protective *Am* antigens (Musoke et al., 1996). The potential for vaccines to be based on recombinant peptide subunits, such as those in MSPs associated with the *Am* cell surface, has been examined at length in the literature (Palmer and McElwain, 1995). Several MSPs of *Am* have been identified including MSP1, MSP2, MSP3, MSP4 and MSP5, and all have an ability to stimulate large antibody responses (Palmer and McGuire, 1984) making them attractive vaccine candidates. For example, previous work examining the value of antigenic MSP2, a protein thought to play a role in immune evasion, showed a strong humoral immune response (Noh et al., 2010). That immune response, however, did not appear to correlate with protection among challenged calves. Another MSP of particular interest to vaccinologists has been MSP1 (a heterodimer comprised of the MSP1a and MSP1b polypeptide subunits) due to the role of MSP1a as an adhesion used by *Am* organisms when invading erythrocytes (McGarey et al.,

1994). Previous work has also shown conservation of MSP1a within isolates (unlike MSPs 1b, 2, and 3) and it does not appear to undergo major sequence variation in persistently infected cattle (Bowie et al., 2002). Camacho-Nuez et al. (2000) reported encouraging results after vaccinating calves with native MSP1a as an antigen. Calves in this study demonstrated less severe acute disease, although they were not protected from *Am* infection.

Future work is needed to elucidate, synthesize, and test potentially protective *Am* antigens. In the current era of -omics science there is ample room for advancement in subunit vaccinology, with implications for many pathogens including bovine anaplasmosis. An ideal bovine anaplasmosis vaccine would not only limit disease severity, but would also induce wholly protective immunity against initial infection. Current vaccines have not been proven to achieve this goal, and more work is needed to characterize highly-conserved immunogenic epitopes for use as future antigens. In a perfect vaccine, these epitopes would be cross-protective and stable.

From an animal management standpoint, bovine anaplasmosis vaccines that require boosters are not ideal. The labor associated with catching cattle multiple times for revaccination could be reduced with long-acting vaccine(s) and/or vaccine delivery systems. A perfect vaccine would confer protection from infection after a single dose. Future work should focus on the refinement of technologies designed to prolong the immune response in animals. This area of inquiry will benefit from collaborations among microbiologists, immunologists, veterinary scientists, material scientists, and clinicians. The final product could be a biocompatible platform capable of delivering a variety of vaccines including one to protect against bovine anaplasmosis.

Conclusions

Despite being a long-characterized disease with global implications for livestock production, bovine anaplasmosis remains a challenge for cattle producers and veterinarians. Over a century after discovery, there is still no reliable vaccination strategy to prevent infection. There are no antimicrobials approved or demonstrated as consistently capable of eliminating infection in sick cattle. There are no drug or vaccine strategies that can be relied upon to prevent transmission from infected to naïve animals. Seroprevalence data are growing but still lacking, as are studies examining prevalence of specific *Am* isolates. Animals are not routinely tested for presence of *Am* prior to transport, which likely contributes to increased strain diversity in areas with large numbers of introduced cattle. Importantly, current data that describe the economic impact of disease are sparse.

Without high-quality and up-to-date economic data that show the financial impact of bovine anaplasmosis, it will remain difficult to incentivize its study. Economic data that exist are out of date and may not accurately depict the current costs associated with both acute and persistent bovine anaplasmosis. As infection can result in weight loss, decreased milk yield, abortions, morbidity, and mortality, the financial impact on cattle producers is likely considerable. Future work is urgently needed to accurately quantify disease costs incurred by cattle producers. Once a commercial case can be made for increased research into bovine anaplasmosis, future research should focus on therapies that can clear infection and vaccines that can prevent infection outright.

Tables

State	N	Low Prevalence (%)	High prevalence (%)	Test[a]	Reference
Alabama	17,755	7.90	12.50	cELISA	Whitlock et al., 2014
Arkansas	1,848	16.60	16.60	cELISA	Whitlock et al., 2014
Georgia	293	4.44	4.44	cELISA	Okafor et al., 2019b
Georgia	237	4.64	4.64	cELISA	Whitlock et al., 2014
Illinois	4,994	7.10	10.70	MCAT	Hungerford and Smith, 1997
Iowa	490	2.30	2.80		Brinkman and Kersting, 1990
Iowa	659	15.17	15.17	cELISA	Coetzee et al., 2010
Kentucky	232	10.78	11.58	cELISA	Okafor et al., 2019b
Kentucky	233	13.40	13.40	cELISA	Whitlock et al., 2014
Louisiana	11,175	4.27	5.64	CT	Hugh-Jones et al., 1988
Louisiana	11,085	3.80	11.20	IFA	Morely and Hugh-Jones, 1989
Mississippi	5,389	29.02	29.02	cELISA	Okafor et al., 2019a
Mississippi	402	27.60	32.48	cELISA	Whitlock et al., 2014
Missouri	54	35.18	35.18	cELISA	Whitlock et al., 2014
Montana	5,608	1.93	1.93	cELISA	Van Donkersgoed et al., 2004
North Carolina	24	10.90	10.90	cELISA	Whitlock et al., 2014
Oklahoma	20,155	4.70	17.60	CF	Rodgers et al., 1994
Oregon*	235	64.26	64.26	nPCR	de Echaide et al., 1998
Oregon*	124	26.00	63.00	CT	Peterson et al., 1977
South Carolina	467	5.10	5.10	cELISA	Whitlock et al., 2014
Tennessee	10,550	53.00	56.00	cELISA	Whitlock et al., 2014
Texas	15,675	15.91	15.91	cELISA	Okafor et al., 2018a
Texas	1,835	15.02	15.02	cELISA	Whitlock et al., 2014
Texas	20,866	14.4	14.40	cELISA	Whitlock et al., 2014
Virginia	41	2.44	2.44	cELISA	Whitlock et al., 2014

*Denotes a study that examined a single herd in that state

[a]cELISA, Competitive Enzyme-Linked Immunosorbent Assay; CF, Complement fixation; nPCR, nested Polymerase Chain Reaction; IFA, immunofluorescent Assay; MCAT, Modified Card Agglutination Test; CT, Card Test

Table 1. Studies examining seroprevalence of *Anaplasma marginale* among cattle are summarized by U.S. state

Approach	Vaccine Isolate	Challenge Isolate	Study Type	N	Outcome	Success (%)*	Reference
Am Outer Membrane	Florida	Florida	Experimental	3	2/3 PCR negative for *Am*	67	Brown et al., 1998
Am Mutant	St. Maries	St. Maries	Experimental	4	4/4 Blood smear negative for *Am*	100	Hammac et al., 2013
Am Initial Bodies	Mexico Morelos Yucatan	Mexico	Experimental	5	Partial protection for 5/5	100	Espinoza et al., 2006
	Mexico Morelos Yucatan	Veracruz	Experimental	5	Partial protection for 5/5	100	
	Morelos Veracruz Yucatan	Mexico	Experimental	5	Partial protection for 5/5	100	
Live, low virulence *Am*	Yucatan	Morelos	Experimental	44	Partial protection for 20/44	45	Camarillo et al., 2008
		Chiapas		10	Partial protection for 10/10	100	
		Aguascalientes		24	Partial protection for 23/24	96	
		Playa Vicente	Field	26	Partial protection for 26/26	100	
Am MSP1 subunit	Florida	Florida	Experimental	5	2/5 Blood smear negative for *Am*	40	Palmer et al., 1989
		Washington-O		5	5/5 Blood smear negative for *Am*	100	
Subdominant OMPs: AM:202, 368, 854, 936, 1041, 1091	Kansas Australia Mexico Puerto Rico Virginia	St. Maries	Experimental	5	No protection for 5/5	0	Ducken et al., 2015
Recombinant subdominant OMPs: AM:202, 368, 854, 936, 1041, 1091	Kansas Australia Mexico Puerto Rico Virginia	St. Maries	Experimental	5	Partial protection for 5/5	100	
Crosslinked Surface Proteome	St. Maries	St. Maries	Experimental	15	Partial protection for 15/15	100	Noh et al., 2008
Live *A centrale*	Australia	Zimbabwe	Experimental	37	Partial protection for 13/37	35	Turton et al., 1998
	South Africa			32	Partial protection for 5/32	16	
Killed *Am*	Anaplaz ®	Colombia	Field	5	No protection for 5/5	0	Zaraza and Kuttler, 1971
Live, attenuated *Am*	Iowa			10	Partial protection for 8/10	80	
Am Initial Body Membranes	Zimbabwe	Zimbabwe	Experimental	5	3/5 Blood smear negative for *Am*	60	Tebele et al., 1991
	Florida			5	4/5 Blood smear negative for *Am*	80	

*Success denotes an animal that exhibited one or more of the following when compared to controls: significantly reduced or absent anemia, parasitemia, fever, or need for antimicrobial intervention as reported by the author(s)

Table 2. Studies examining different vaccine approaches to prevent bovine anaplasmosis are summarized

Chapter 2 - Assessment of within-herd seroprevalence of Anaplasma marginale antibodies and associated decreased milk production in an Iowa dairy herd

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ABSTRACT

Objective: Bovine anaplasmosis, caused by the hemobacteria *Anaplasma marginale* (***Am***) is the most prevalent tick-transmitted disease of cattle worldwide and is associated with significant production losses in cattle. The objective of this survey was to investigate the within-herd seroprevalence of antibodies to *Am* and the relationship between disease status and milk production after anaplasmosis outbreak in a northern Iowa dairy herd.

Materials and Methods: In 2010, anaplasmosis was diagnosed in an Iowa dairy herd comprised of 680 lactating Holstein cows. Samples for serological testing by cELISA were gathered from 799 animals throughout 2011 in 24 separate accessions. Information on milk production, obtained from the Dairy Herd Improvement Association (**DHIA**), was gathered from 2010 to

2013. Monthly DHIA milk production was then statistically compared to 2011 anaplasmosis serostatus.

***Results and Discussion*:** Analysis of cELISA data found that 38% of the animals tested positive for bovine anaplasmosis. DHIA milk data showed seropositive cows produced significantly less milk during 2012 (P = 0.0041) and 2013 (P = 0.0351) than did seronegative animals. This resulted in a mean (± SEM) difference of 1,677 ± 579 kg and 2,175 ± 1,022 kg of milk during 2012 and 2013, respectively.

***Implications and Applications**:* Cows found to be seropositive for *Am* antibodies produced significantly less milk in subsequent lactations than seronegative cows. Therefore, subclinical anaplasmosis may represent a potential loss of income for dairy producers. Results also suggest that animals should not be assumed free of infection based on geographic location.

Key words: Anaplasmosis, dairy cattle, serological study, anaplasma seroprevalence

INTRODUCTION

Bovine anaplasmosis, caused by the intraerythrocytic rickettsial hemobacteria, *Anaplasma marginale (**Am**)*, is the most prevalent tick-transmitted disease of cattle and is associated with significant economic loss to producers in the United States (Uilenberg, 1995; Kocan et al. 2010). Ticks are recognized as biological vectors for *Am* (Dikmans, 1950). Mechanical vectors such as horseflies (Baldacchino et al. 2014) and blood-contaminated fomites (Reinbold et al. 2010a) have been implicated in disease spread. *Am* can also be transplacentally transmitted from a persistently infected cow to the calf during pregnancy (Zaugg, 1985).

Prepatency ranges from 7 to 60 days, and averages 28 days (Kocan et al., 2003). Symptoms of anaplasmosis during acute infection include anemia, icterus, fever, weight loss, abortion, lethargy, inappetence, and can be fatal. Mature (Ristic, 1977), high-producing dairy (Ristic 1968), and periparturient animals (da Silva and de Fonseca, 2014) appear to be at greater risk. Symptoms may appear to resolve in surviving animals but low, mostly undetectable, levels of infection exist in recovered cattle (Coetzee et al. 2005). Asymptomatic animals can serve as local reservoirs for disease transmission (Swift and Thomas, 1983). Previous work has estimated that, when introduced to a naïve herd, anaplasmosis can result in a 3.6% reduction in calf crop, a 30% increase in cull rate and a 3% mortality rate in infected adult cattle (Alderink and Dietrich 1983).

This report describes analysis of blood collected from an Iowa dairy herd that experienced a significant decrease in milk production in 2008 followed by an increase in mortality in 2009. Bovine anaplasmosis is not generally regarded as endemic to Iowa, although seroprevalence in feedlots has been shown to range from 5.00% - 15.17% (Coetzee et al., 2010). A study in the adjacent state of Illinois demonstrated an overall *Am* seroprevalence of 2.5% among surveyed dairy cattle (Hungerford and Smith, 1997) which was lower than that measured among beef breeds (5 - 10%). In Louisiana, seroprevalence was higher at 4.3% among surveyed dairy cattle (Hugh-Jones et al., 1988). However, recent reports detailing the seroprevalence of anaplasmosis in US dairy herds and the effect of the disease on milk production remains deficient in the published literature.

MATERIALS AND METHODS

Background

In late 2010, a dairy located in Northern Iowa was diagnosed as being infected with bovine anaplasmosis. Prior to this diagnosis, herd mortality data indicate annual death loss among all cows at the site increased from 124 (16.7%) and 121 (16.0%) in 2006 and 2007, respectively, to 182 (27.5%) cows in 2008. Death loss peaked in 2009 with 229 deaths (32.9%), and then declined. A 2014 National Animal Health Monitoring System survey (**NAHMS**, USDA, 2014) reported that average annual cow mortality was 5.6% across US dairy herds. This period of elevated death loss coincided with decreased milk production from January 2008 to January 2009. The first diagnosis of anaplasmosis was made in October 2010. The dairy herd was open, implying that animals were purchased and introduced without pre-purchase testing or quarantine. Records documenting the origin of new animal purchases were not kept, but a herd vaccination program was in place. Both calves and adults received standard cattle immunizations which included vaccines for bovine viral diarrhea virus (**BVDV**). Records indicate that calves received vaccinations against BVDV infection at 1 week of age, 6-8 weeks of age, and at 10-12 months of age. Dry cows were vaccinated against BVDV at 60 days pre-calving. Lactating cows were vaccinated against BVDV at between 35 and 41 days in milk (**DIM**). All lactating cows received oxytocin injections twice daily at the time of milking. Syringes and needles were reused among animals receiving oxytocin, and visible blood contamination was observed in the syringes and oxytocin bottles during a farm visit.

Mortality data were obtained from records archived in on-site dairy management software (Dairycomp 305, Valley Agricultural Software, Tulare, CA). Milking and DIM records

were obtained from Dairy Herd Improvement Association (**DHIA**) archives. Unlike animal health data, DHIA milk production data are collected once a month by an independent observer. Changes in DHIA milk production data and death loss over time were examined to establish the progression of the outbreak. Veterinary billing records were also examined as part of the herd health review.

Beginning in 2011, anaplasmosis infection status (positive or negative for *Am* antibodies) was determined using a commercially available competitive, enzyme-linked, immunosorbent assay (**cELISA**; catalog No: 283-2, VMRD, Pullman, WA) designed to provide results which will give guidance about the presence of *Anaplasma* infection in cattle. This test kit was approved by the United States Department of Agriculture and the test was conducted in an American Association of Veterinary Laboratory Diagnosticians accredited facility at Iowa State University. As per the manufacturer (VMRD), the test kit has a diagnostic sensitivity of 100% and a specificity of 99.7%, with a cutoff point of 30% inhibition. It has been reported that this test kit can be cross-reactive to *Anaplasma phagocytophilum* (Dreher et al., 2005) and a genotype of *Erlichia* (Al-Adhami et al., 2011). However, as there has never been a reported case of naturally occurring *A. phagocytophilum* infection in US cattle (Hairgrove et al., 2015), and only one reported instance of naturally occurring Erlichial infection among cattle in Canada (Gajadhar et al., 2010), the VMRD kit was deemed appropriate for the survey.

Serology

Blood samples were obtained from 799 of the Iowa dairy cows throughout 2011 in 24 separate accessions. Blood samples were submitted by one of two licensed veterinarians who had professional relationships with the dairy owner. Samples were analyzed for *Am* antibodies using

cELISA, which assumed a cutoff of 30% inhibition for a sample to be considered positive for antibodies. Although serology data became available throughout 2011, animals remained intermingled regardless of serostatus and were managed under the same production conditions. The number of animals tested was not entirely comprehensive as cows were tested at different time points and animals were bought and sold during the sampling process. Documents relating to the case indicate that sampling days occurred approximately biweekly and timing was based on the discretion and availability of a licensed veterinarian familiar with the operation. Sampling occurred, at least in some cases, when cattle were being caught and tested for other diseases (e.g. brucellosis). Each animal contributed a single data point to the analysis.

Statistical Analysis

Microsoft Excel® (Excel for Mac Version 16.38, Microsoft Corporation, Redmond, Washington) was used for data compilation and descriptive statistics. To examine the association between anaplasmosis serological status and milk production, data were analyzed in JMP® (SAS Institute, Inc., Cary, NC, USA) using a Mixed Effects statistical model. Animal nested in anaplasmosis serological status (positive or negative) were designated as a random effect with anaplasmosis status, year and their interaction designated as Fixed Effects in the model. To account for differences in DIM, milking frequency, season of calving and cow age, mature equivalent (**ME**) lactation data calculated and reported by DHIA was used for comparing milk production between positive and negative cows. Statistical significance was designated *a priori* as $P < 0.05$.

RESULTS AND DISCUSSION

Diagnostic Testing

An investigation into the prevalence of *Am* in the 799 cows sampled from an Iowa dairy was conducted in 2011. The cELISA results from this survey indicated 38% of the animals were positive for anaplasmosis. This number does not take into account the animals dying from the disease before the diagnosis of anaplasmosis in late 2010. These results are consistent with what is typically encountered in herds experiencing an outbreak of anaplasmosis since approximately two-thirds of the herd would be considered susceptible to the disease while one-third of the herd is infected. As infected cattle serve as reservoirs of infection, bacteria can be vectored to local naïve cattle primary through contaminated equipment or arthropods. This endemic instability favors disease outbreaks as occurred at the Iowa dairy.

Needle Transmission

Outbreaks of anaplasmosis can occur due to a combination of different factors. These include a lack of a disease control program, a high ratio of susceptible cattle relative to cows persistently infected in a herd, and the prevalence of vector/fomite transmission (Gill, 1994). The ratio of susceptible to unsusceptible cattle in this case was variable, but testing revealed that the majority of animals in this herd were naïve. Contributing to observed seroprevalence was the lack of a quarantine or testing process for newly purchased animals before being introduced to the herd.

Natural vectors have been implicated in the spread of *Am* and include members of the genera *Tabanus* (Ewing, 1981), *Psorophora* (Ristic, 1968), *Stomoxys* (Baldacchino et al., 2013),

and *Dermacentor* (Kocan et al., 1981). Species of *Tabanus* (Sutton and Millspaugh, 1950), *Stomoxys* (Raun and Casey, 1956), *Psorophora* (Dunphy et al., 2014) and *Dermacentor* (Lingren et al., 2005) are known to exist in Iowa and could have contributed to transmission at the dairy. In this case, however, it is apparent that a lack of comprehensive biosecurity measures was likely a significant contributor to *Am* spread from seropositive to seronegative cows.

Blood-contaminated fomites, such as needles, dehorning saws, nose tongs, tattooing instruments, ear tagging devices and castration instruments have also been shown to contribute to disease transmission (Kocan et al., 2003). Needles as a potential vector appear particularly likely as it was discovered that needles were shared among cattle when oxytocin was injected for milk ejection. One study that considered iatrogenic transmission of *Am* concluded that, when exposed to contaminated needles, naïve steers exhibited disease 60% of the time following single exposure (Reinbold et al., 2010a). As *Am* positive animals were comingled with those that were *Am* negative, the reuse of needles in the milking parlor likely served as a point of disease transmission to naïve cows.

Milk production analysis

Milk production data were obtained from DHIA records archived in on-site dairy management software (Dairycomp 305). Review revealed that before January 2008, herd milk production peaked at 665,863 kg in May 2007, with a nadir of 529,760 kg in October 2006. After January 1, 2008, milk production peaked approximately 90,718 kg lower than in 2007, with trough production reduced by approximately 136,078 kg to 403,194 kg in February 2010. Rolling herd average milk production decreased by 880 kg/cow from 2008 to 2010. This represented an 8% decrease in milk production.

A statistical analysis of the ME milk production per cow by anaplasmosis status over 3 years revealed evidence of serological status ($P = 0.005$) and a year-by-serological status interaction ($P = 0.0297$). Specifically, cows that tested positive for anaplasmosis on the cELISA test in 2011 produced on average 1,389 kg less per year than cows that tested negative. This difference over time from 2011-2013 is presented in Figure 1. The difference in milk production between positive and negative cows was not significant in 2011 ($P > 0.05$) but was highly significant in 2012 ($P = 0.0041$) and 2013 ($P = 0.0351$). In 2012, *Am* positive cows produced, on average, 10.4% less milk on a ME basis than those that were *Am* negative. In 2013 positive cows averaged 15.3% less milk than negative animals. Ordered differences between positive and negative cows are shown in Table 3.

Though there is a paucity of literature on the subject of milk production during an anaplasmosis outbreak, a few reports have supported these observations. Pazinato et al. (2016) reported that cows identified as seropositive for *Am* produced, on average, 3 liters of milk less per day than identically managed cows found to be seronegative. Research conducted on a dairy in an area considered endemic for bovine anaplasmosis showed that low producing dairy cattle (i.e. < 1,500 kg milk per annum) were 3.9 times more likely to be seropositive for *Am* than high-producing animals (i.e. > 3,000 kg milk per annum). In the same study, primiparous dairy cattle were found to have an 88% greater chance of being seropositive than multiparous herd mates (Silva et al., 2013). Unspecified decrease in milk production were reported in Turkish dairy cows subsequently tested and found to be seropositive for *Am* (Aktas and Ozubek, 2017, Birdane et al., 2006). McDowell et al., (1964) reported an average loss in lactation yield of 26% in milk and 31% in milkfat during the clinical phase of disease. These results are consistent with our findings of a decrease of 10.4% to 15.3% when comparing seronegative to seropositive cows. However,

our survey focused on the long-term impact of positive serological status as opposed to the effect of clinical disease.

One potential limitation to the survey is the unknown impact of bovine leukosis virus (**BLV**) on milk production in this case. Evidence suggested that at least some animals at the Iowa dairy had developed lymphoma as a result of BLV infection. Previous studies have shown that that BLV-positive cows produce from 3% (Ott et al., 2003) to 11% (D'Angelino et al., 1998) less milk than did BLV-negative animals. However, it is noteworthy that cows in these reports were not screened for *Am*. It is possible that the decrease in production resulted from a combination of factors (i.e. animals could have been positive for both *Am* and BLV). As the BLV status of animals subject to this survey was unknown, this relationship could not be considered.

Cattle were routinely vaccinated as part of the herd management program on the dairy, and animals did not exhibit signs of infection with BVDV during the period described. However, it is possible that latent BVDV could predispose cattle to developing clinical anaplasmosis. A report on Hungarian dairy cattle demonstrated that if susceptible animals are infected with both *Am* and BVDV at roughly the same time, the immunosuppressive effect of BVDV will support the progression of *Am* infection to the point of clinical disease (Szabara et al., 2016). As animals were not routinely screened for BVDV, the potential for simultaneous infection cannot be ruled out completely.

APPLICATIONS

This survey details the apparent decrease in production among dairy cows with anaplasmosis. First, data suggest that cows found to be seropositive for *Am* antibodies tend to produce less milk on a ME basis. This is an important finding as it demonstrates the need for further study of the effects of *Am* in dairy settings. In addition, the survey indicates that freedom from bovine anaplasmosis cannot be assumed for an entire geographic region (i.e. northern Iowa). Similarly, and regardless of location, the survey suggests that freedom from *Am* infection should not be assumed for open herds without rigorous diagnostic testing. The importance of herd biosecurity is not dependent on the site of the production system. In this case, failure to quarantine new livestock purchases and the reuse of hypodermic needles for routine treatments among animals serve as examples of poor biosecurity. By managing risks associated with new introductions into the herd and reducing conditions that may favor iatrogenic transmission of disease, production and herd health at this facility could have been preserved.

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FIGURES

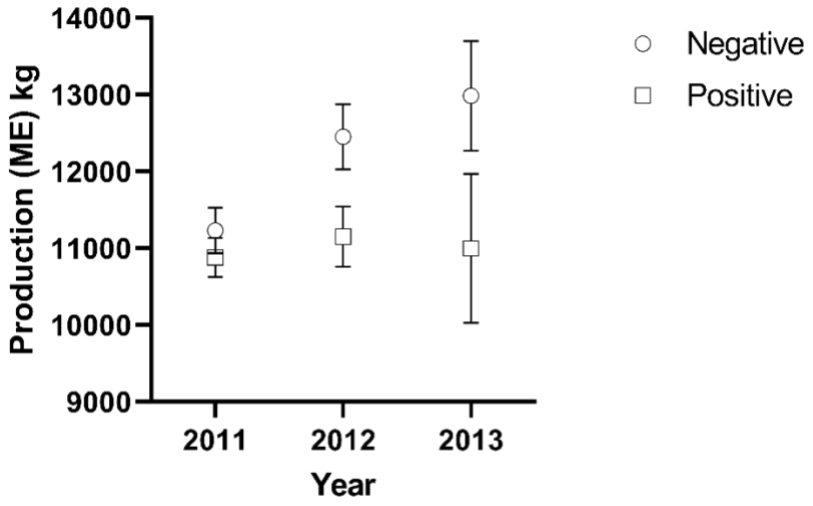

Figure 1 DHIA recorded mature equivalent (ME) milk production by anaplasmosis infection status

TABLES

Ordered Difference						
Level 1	Level 2	Difference (kg)	Std Err Dif	Lower CL	Upper CL	p-Value
Negative, 2013	Positive, 2013	2,174.85	1021.71	154.14	4195.56	0.0351
Negative, 2012	Positive, 2012	1677.35	579.07	537.53	2817.17	0.0041
Negative, 2011	Positive, 2011	315.87	397.02	-465.88	1097.62	0.427

Table 3 Ordered differences in milk production between anaplasmosis positive and negative status

Chapter 3 - Assessment of statewide and within-herd seroprevalence of Anaplasma marginale antibodies in 12 Bos taurus – Bos indicus cow herds and the association with sporadic outbreaks of bovine anaplasmosis in Florida

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ABSTRACT

Objective: To assess within-herd seroprevalence of *Anaplasma marginale* (*Am*) antibodies across 12 Florida beef cattle herds and compare to statewide seroprevalence.

Materials and Methods: Twelve surveyed herds ranged in size from 160 to 456 adult *Bos taurus - Bos indicus* cattle. Statewide *Am* seroprevalence relied on blood samples obtained at slaughter from 201 Florida cattle. Screening relied on competitive ELISA.

Results and Discussion: Prior to serology, an outbreak of anaplasmosis resulted in increased mortality (up to 17.8%) and abortions in several herds. Up to 29.2% of cows aborted late in gestation in 2 herds that included many cattle introduced from Texas. Among 1,085 cattle tested in the 12 herds, seroprevalence of *Am* varied from 2.6% to 85%, with an overall seropositive rate of 50.3%. Cattle in open herds were 6.23 (95% CI: 4.26-9.17) times more likely to experience mortality, and 3.10 (95% CI: 2.39-3.98) times more likely to abort than animals in closed herds. Average mortality (12%) and abortion (16.3%) among open herds were significantly ($P < .05$) higher than mortality (1.9%) and abortion (5.3%) among closed herds. Overall seropositivity among affected herds was higher than the apparent statewide seroprevalence of 20.32%.

Implications and Applications: This survey provides estimates of seropositivity among Florida cattle and reports the absence of uniform herd immunity in an area considered endemic for bovine anaplasmosis. These data highlight unrestricted cattle movement and environmental conditions that favor vector-borne disease transmission as risk factors for disease outbreaks even in regions that are considered endemic for bovine anaplasmosis.

Key Words: Endemic disease, vector-borne, beef cattle, serological study

INTRODUCTION

Anaplasmosis, caused by the hemobacteria *Anaplasma marginale (Am)* is the most prevalent tick-transmitted disease of cattle worldwide and causes economic loss to cattle

producers in the US (Uilenberg, 1995; Kocan et al. 2003). When introduced to a naïve herd, anaplasmosis can result in a 3.6% reduced calf crop, a 30% increased cull rate, and a 3% mortality rate in clinically infected adult cattle (Alderink and Dietrich 1983). Transmission of *Am* occurs through biological vectors such as ticks (Dikmans, 1950), mechanical vectors such as horseflies (Baldacchino et al. 2014; Foil, 1989), mosquitos (Potgieter et al., 1981), and stable flies (Ewing, 1981), blood-contaminated fomites such as needles (Reinbold et al. 2010a), or transplacentally from cow to fetus (Zaugg, 1985). Weight loss from disease progression averages 86 kg (Alderink and Dietrich 1983) with adult (>2 yrs) cattle being more susceptible to severe clinical disease and death (Kocan et al. 2003). The prepatent period of anaplasmosis varies with infectious dose, but likely ranges from 7 to 90 days, with an average of 28 days. Infected erythrocytes are phagocytosed, and resulting anemia and icterus generally occur without hemoglobinemia or hemoglobinuria. Clinical symptoms include fever, weight loss, abortion, lethargy, icterus, and death, particularly in mature cattle (Ristic, 1977). Symptoms diminish in survivors, but low levels of infection are maintained in recovered cattle (Coetzee et al. 2005). These carrier animals are considered immune to reinfection but serve as reservoirs for disease transmission (Swift and Thomas, 1983).

Florida and the Southeastern US are considered endemic for anaplasmosis (Stiles, 1942). While data are available for other states, recent reports describing seroprevalence of anaplasmosis in Florida are deficient in the published literature. The objective of this report is to describe serological findings and risk factors associated with an anaplasmosis outbreak in an area considered endemically stable for the disease. This report compares results of a state-wide serological survey for *Am* antibodies conducted at a slaughter facility with a focused seroprevalence analysis of blood collected from 12 beef cattle herds. Results provide ranchers

and veterinarians knowledge for improved control of bovine anaplasmosis in Florida and other areas *Am* is considered endemic.

MATERIALS AND METHODS

Herd Background and Sampling

Bos taurus - *Bos indicus* cross cattle were housed in 12 separately managed herds totaling 3,118 adult (> 3 years of age) cows (Table 4). The sample population was larger than the average Florida beef herd of 795 animals (Hodges et al., 2019). The operations encompassed over 33,000 acres in Osceola County, a county with the third-largest cattle inventory in the state with approximately 60,000 head. These pastures spanned approximately 16 km from the northwest to southeast corners of the property. Several, though not all, herds shared fence lines. Animals were in good condition (BCS of +/- 5) and were managed similarly. Individual herds enrolled in the survey consisted of 160 to 456 cows. Herd history noted the introduction of approximately 1,100 cows of unknown anaplasmosis infection status from Texas into several herds starting in 2011. Herds where Texas cattle were commingled with those from Florida were designated as open herds (Herds B, C, D, F, G) for data analysis. Open herds were comprised of approximately 1,450 animals.

It was reported that several of the 12 surveyed herds experienced sudden disease challenge from anaplasmosis beginning in October 2014. This was approximately three months after all animals on the ranch were checked for pregnancy and vaccinated against clostridial and reproductive infections. Comprehensive (i.e., all 12 herds) pregnancy records indicate that animals affected by the outbreak included late-gestation and recently post parturient cows. The

most impacted herds consisted of a group of 309 cows (*Herd C*) and another group of 321 (*Herd F*). Over a period of 6 weeks, mortality rates in *Herd C* and *Herd F* reached 17.8% (Table 4). Adjacent and nearby herds also suffered mortality, but at a much lower extent (from 0.4% to 11.3%) than did *Herd C* and *Herd F*. Not all animals found dead were subject to postmortem testing for presence of *Am* due to rapid decomposition and predation by wildlife and carrion-eating birds. The decision of whether to test a dead animal for *Am* was made by a veterinarian on-site and was influenced by the estimated elapsed time since death. Animals found to have recently died were subject to blood harvest and testing, while those that died some time (i.e., several days) before discovery were not tested for *Am*. Postmortem blood testing relied on either a competitive enzyme-linked immunosorbent assay (cELISA) or a quantitative polymerase chain reaction to determine *Am* positivity. Not every animal subject to blood testing was also subject to comprehensive necropsy. Some dead animals were suited only to singular organ biopsy (e.g., liver, lung, spleen) depending on the integrity of remains. All animals that were tested at post-mortem during this period of the outbreak were found to be positive for *Am*.

A comprehensive investigation of the prevalence of *Am* in the 12 different herds in Florida was conducted in April 2015. Blood samples were collected via convenience sampling when animals were worked as part of routine management practices. Every cow was checked for pregnancy at this time. Approximately every other cow that was caught was sampled for purposes of determining *Am* antibody presence. This survey relied on the VMRD cELISA testing of blood samples collected from 1,085 individual animals (cat No: 283-2, Veterinary Medical Research and Development (VMRD), Pullman WA). It has been reported that this test kit can be cross-reactive to *Anaplasma phagocytophilum* (Dreher et al., 2005) and a genotype of *Ehrlichia* (Al-Adhami et al., 2011). However, as there has never been a reported case of naturally

occurring *A. phagocytophilum* infection in US cattle (Hairgrove et al., 2015), and only one reported instance of naturally occurring Ehrlichial infection among cattle in Canada (Gajadhar et al., 2010), the VMRD kit was deemed appropriate for the survey. The owner of the cattle did not choose to genotype *Am* during this survey because routine gene sequencing for *Am* is not widely available at veterinary diagnostic laboratories and financial constraints precluded development of these assays specific for this production system.

Environmental History

Cattle in the 12 sampled herds were housed on approximately 33,000 acres of Central Florida grassland dominated by native species and grass of the genus *Hemarthria*. Cattle had unrestricted access to natural water sources in the forms of ponds and ditches. Climatic records for this area of Florida revealed that a period of below average rainfall occurred in August 2014 followed by a period of rainfall that was higher than the seasonal average during September 2014. Specifically, climatic data indicated that the area where anaplasmosis outbreaks occurred likely experienced more than double the expected rainfall for the month of September (Figure 2). This amounted to 150 mm above the rainfall expected for the month, or a 110% increase over the September average (usclimatedata.com). It was noted that sections of pasture had been flooded and were still drying prior to and during the outbreak of illness.

Screening at Slaughter

In order to compare seroprevalence of animals subject to local outbreak with statewide seroprevalence, a screening survey was conducted of beef animals subject to slaughter. Slaughter survey of Florida beef cows for *Am* was performed as previously described (Okafor, Collins et

al. 2018a). Descriptively, based on a population of 982,790 beef cows (NASS 2014), an estimated seroprevalence of 10% and a confidence level of 95%, 138 beef cows were required to estimate the seroprevalence of *Am* in FL beef cows. This sample size was calculated using the Epi Info™ Version 7.0 software (Centers for Disease Control and Prevention, Atlanta, GA, USA). A slaughterhouse (FPL Food; located in Augusta, Georgia) that slaughtered a significant portion of beef cattle from FL was purposively selected for collection of specimens. Between August to December 2014, blood specimens were collected from cull beef cows presented at FPL Food slaughterhouse. Specimens were collected only from cows with a USDA-approved backtag identification beginning with the prefix "58", indicating Florida as the state of last origin; with the first mature incisors erupted, indicating the cow was at least 18 months of age; a phenotype consistent with beef cattle. On specimen collection dates, blood specimens were collected from all beef cows that met the above criteria. During exsanguination, blood was collected (~8 ml) from each cow in a blood collection tube (BD Vacutainer Serum Separator; 8.5 mL). All blood specimens were transported in ice-pack containers and tested with cELISA, using the VMRD Anaplasma Antibody Test Kit. Blood was tested for presence of *Am* antibodies at the Veterinary Diagnostic Laboratory at Iowa State University. In estimating the true seroprevalence of *Am*, a previously described (Coetzee et al. 2007, Aubry and Geale 2011) sensitivity of 95% and specificity of 98% for the VMRD cELISA was used with a cutoff point of 30% inhibition. In accordance with commercial testing guidelines, all specimens having a ≥30% inhibition were reported as serologically positive.

Statistical Analysis

For purposes of live herd screening, Microsoft Excel® (Excel for Mac Version 16.38, Microsoft Corporation, Redmond, Washington) was used for both descriptive and inferential statistics, including Chi squared tests. Chi squared tests were used to determine significant differences in mortality and abortion rates among open and closed herds. Chi squared tests were also used to detect differences in mortality and the number of open cows in herds with a greater than 1:1 ratio of *Am* seronegative: seropositive animals and those with less than a 1:1 ratio of seronegative: seropositive animals. Descriptive outcome measures included percent mortality by herd, percent of open cows by herd, serology status, and odds ratios. Statistical significance was set *a priori* at $p \leq 0.05$. Odds ratios were calculated using JMP® (SAS Institute, Inc., Cary, NC, USA) to quantify the strength of relationships between ratios of serostatus and herd type (i.e. open or closed) with rates of abortion and mortality. Statewide true seroprevalence estimates were calculated as described previously (Reiczigel et al., 2010; Rogan and Gladen, 1978). Wilson's confidence intervals were calculated on the assumption that sensitivity and specificity were known exactly as described previously (Reiczigel et al., 2010). Cattle population data for each county in Florida were obtained from the 2012 census of the National Agricultural Statistical Service (USDA NASS, 2014).

RESULTS AND DISCUSSION

Diagnostic Testing of 12 Herds

The group of 1,085 animals accounted for approximately 35% of the total number of animals in the surveyed herds. On a herd basis, prevalence of *Am* antibodies ranged from 2.6% to 85%, with an overall positive rate of 50% (Figure 3). Based on the overall positive rate of 50%, half of the cattle among the herds surveyed showed no serological evidence of being exposed to anaplasmosis. Cattle in open herds were 1.63 (95% confidence interval: 1.45-1.83) times more likely to have antibodies to *Am* (indicating prior exposure) than cattle from a herd with no herd introductions (Figure 3). This can also be expressed as an odds ratio of 2.8 (95% CI 2.18-3.60):1 in favor of open herds having *Am* antibodies. Further, average mortality rates (12%) among herds that included introduced Texas animals were significantly ($P < .05$) higher than average mortality rates (1.9%) among herds containing no animal introductions (closed herds).

Outbreaks of anaplasmosis occur due to a high ratio of susceptible cattle relative to cows that are persistently infected carriers in a herd as well as environmental or management conditions that favor transmission of infected blood from sick to healthy animals (Gill, 1994). The variable seroprevalence results from this survey could be attributed to both factors. Herd histories suggest that approximately 1,100 cows of unknown anaplasmosis infection status were purchased from Texas in 2011. These cows were commingled with native Florida cows in 7 herds including *Herd C* and *Herd F*. Seroprevalence of *Am* averaged 31.2% among closed herds, and 65.6% among open herds (Figure 3). These data suggest that the introduction of cattle that were potentially infected with bovine anaplasmosis were introduced into previously naïve, open, herds in Florida. This hypothesis is supported by the observation that, relative to the open herds,

the closed herds generally had lower seroprevalence suggesting that the Florida herds were not endemically stable at the time the anaplasmosis infection was introduced.

Conversely, naïve cattle from Texas may have been exposed to *Am* infection in the Florida herds resulting in seroconversion prior to blood testing. It is noteworthy that the likelihood of cattle from Texas being naïve is dependent on the region from which the animals came. In a survey of Texas cattle, Hairgrove et al. (2015) demonstrated a spatial trend for intrastate seropositivity. Specifically, depending on the area surveyed, apparent seroprevalence for anaplasmosis in Texas ranged from 5% to approximately 40%. As documents relating to this case do not indicate the specific region within Texas from which animals originated, and animals were not tested upon arrival in Florida, it remains unclear if the infection originated from the Texas cattle, or the outbreak was associated with infection of the Texas cattle after comingling with the Florida cattle.

Local environmental conditions may also have contributed to the transmission of anaplasmosis. An examination of climatic records taken from the area reveals that in September 2014 the ranch experienced more than double the expected rainfall (Figure 2; usclimatedata.com). It has previously been reported that increased rainfall is a risk factor for seroprevalence of bovine anaplasmosis (Oliveira et al., 2011). The elapsed time from the period of high rainfall in September 2014 to the onset of the anaplasmosis outbreak at the end of October 2014 corresponds with the reported prepatent period of anaplasmosis (7 to 90 days) considered typical following vector borne transmission of infection to naïve cattle (Potgieter and Stoltsz, 2004). Therefore, local weather records support the hypothesis that the anaplasmosis outbreaks were clustered in certain herds due to climatic and ecological conditions that promoted

vector-borne disease transmission in herds containing roughly equal proportions of susceptible and infected cattle.

Pregnancy and Mortality

A survey of the number of open cows (likely indicating abortion) was also conducted beginning in April 2015. A positive association between the herds that experienced an increase in mortality and the herds that had the largest number of open cows 5 months later was observed (Figure 6). Analysis of the ratio of positive and negative cows by herd showed a tendency for herds that included similar numbers of naïve and prior exposed cattle to experience higher mortality (Figure 4). In this case, *Herd C* and *Herd F* exhibited seronegative to seropositive ratios of 1:1.16 and 1:1.28, respectively, in addition to highest mortality. Analysis revealed that average mortality rates (2.2%) among herds with higher than a 1:1 ratio of seronegative: seropositive animals were significantly ($P < .05$) lower than average mortality rates (8.5%) among herds with lower than a 1:1 ratio of seronegative: seropositive animals (Figure 4). Likewise, abortion rates (5.7%) among herds with higher than a 1:1 ratio of seronegative: seropositive animals were significantly ($P < .05$) lower than average abortion rates (12.0%) among herds with higher to a 1:1 ratio of seronegative: seropositive animals (Figure 6).

A similar trend was discovered when comparing the ratio of seronegative to seropositive cattle to the percentage of open cattle in April 2015 (Figure 6). This showed that *Herd C* and *Herd F* exhibited the highest percentage of open cows in April (18% and 29.2%, respectively). Herds that included introduced cattle were 3.10 (95% CI: 2.39-3.98) times more likely to abort than animals in closed herds. Furthermore, average abortion rates (16.3%) among open herds were significantly ($P < .05$) higher than average abortion rates (5.3%) in closed herds.

Data gathered from all herds provide support for the reported outbreak of anaplasmosis causing an increase in pregnancy loss. As periparturient immunosuppression around calving is a documented risk factor for development of anaplasmosis (da Silva and de Fonseca, 2014), the large proportion of animals that had recently calved or were calving during the disease challenge may have been at higher risk for illness and death. Since the incident in October 2014 was not associated with mortality in every bred animal impacted by disease, those animals that survived infection were at greater risk for pregnancy loss (Fowler and Swift, 1975, Ristic, 1977, Correa et al., 1978, Henker et al., 2020). It is likely that surviving cattle aborted causing an associated increase in open cows in the herds with highest mortality (Figure 5, Figure 6).

Slaughter Screening

In the active *Am* beef cow screening, 201 beef cows originating from 7 (5.97%) of the 67 counties in FL were sampled (Table 5). Of the 201 beef cows, 42 were positive for *Am*. Hence, the overall observed apparent seroprevalence (those tested animals that are rightly or wrongly found seropositive) of *Am* in FL was 20.90% (95% CI: 15.85 — 35.50%) while the estimated true seroprevalence (those animals that are actually seropositive) was 20.32% (95% CI: 14.63 — 26.97%). However, the county level apparent seroprevalence ranged from 0 to 30% (Table 5). This county information corresponds to the stockyard where the animal received its backtag identification and may not necessarily correspond to the county of residence before sale and subsequent slaughter.

Anaplasmosis is considered endemic in the Southeastern United States and the disease has been known to exist in Florida for more than 70 years (Stiles, 1942). However, recent surveys have revealed variances in estimated true seroprevalence rates. These surveys have been

conducted in several Southeastern states, including Mississippi (estimated true seroprevalence of 21.62% to 47.90%, Okafor et al., 2019a), Georgia (2.62%, Okafor et al., 2019b), Texas (12.35% to 12.78%, Okafor et al., 2018a), and Kentucky (9.44% to 10.3%, Okafor et al., 2018b). This survey is the first to estimate *Am* seroprevalence for the state of Florida (20.32%). Variation from one state (e.g. Texas) to another (e.g. Florida) could have been a contributing factor in disease transmission. An investigation of the prevalence of *Am* in the 12 different herds found that prevalence of the disease ranged from 2.6% to 85%, with an overall positive rate of 50% (Figure 3). This wide range in seroprevalence suggests the endemic stability of bovine anaplasmosis in Florida should not be presumed. Specifically, the high ratio of anaplasmosis negative to anaplasmosis positive cattle indicated that a substantial number of animals were susceptible to infection, potentially resulting in clinical illness, and death (Figure 4).

APPLICATIONS

This survey presents several novel findings for livestock producers and veterinarians. Specifically, these data suggest that an assumption of endemic stability should not be made for an entire geographic region and that clusters of naïve cattle may exist within an endemic area. Furthermore, data suggest that herds exhibiting an approximate 1:1 ratio of naïve to previously exposed animals may be at higher risk of experiencing anaplasmosis outbreaks, resulting in increased mortality and abortions. In herds with comparable ratios of naïve to exposed cattle, findings support the institution of biosecurity and vector-control measures to limit disease transmission. Finally, management decisions should be focused on mitigating biosecurity risks

associated with new introductions into the herd and environmental conditions that may favor natural vector proliferation.

ACKNOWLEDGEMENTS

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FIGURES

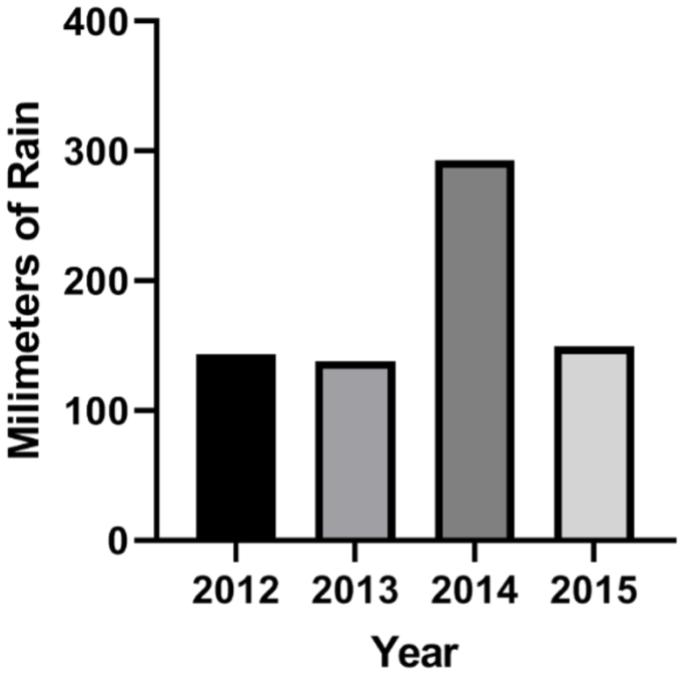

Figure 2 Central Florida September rainfall averages (mm) following arrival of Texas Cattle in 2011. Bartow is approximately 85 km from the location of the surveyed cattle.

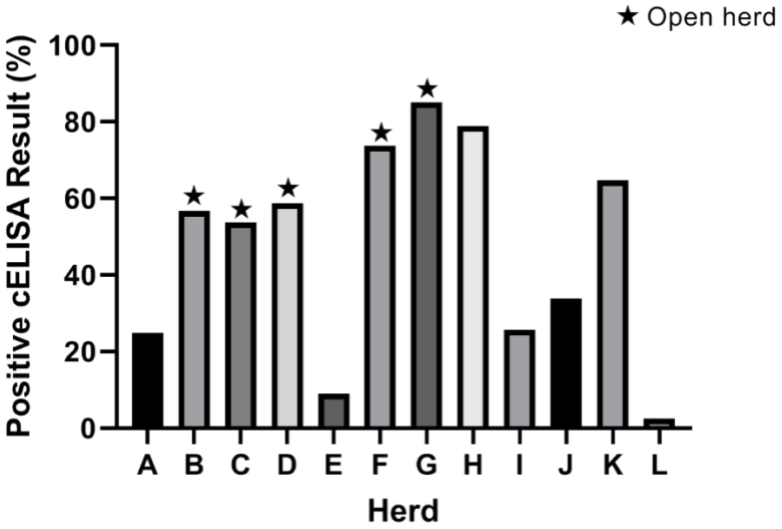

Figure 3 Results of a survey of seroprevalence of Anaplasma marginale. Data are separated by herd. The cELISA assay has a cut-off value of 30% inhibition. Open herds that included Texas cattle are shown

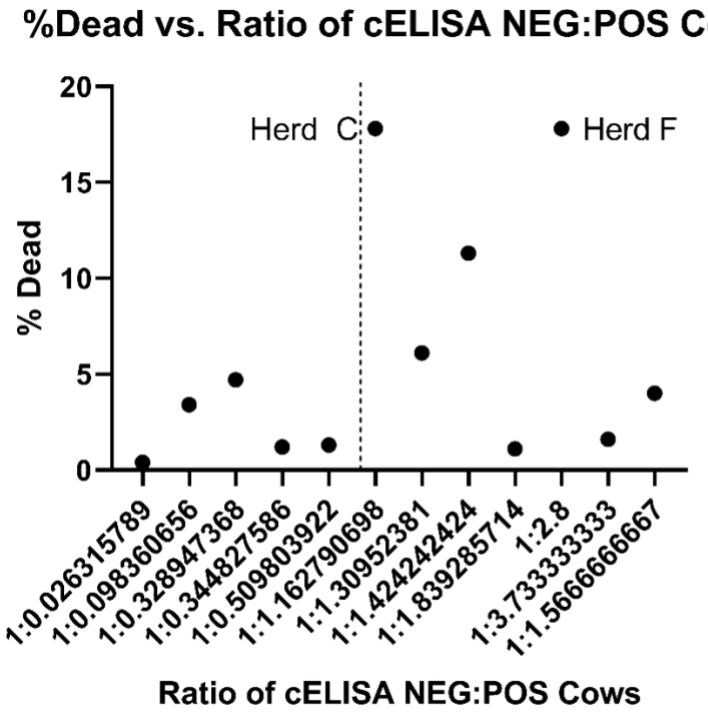

Figure 4 Herd mortality (%) is plotted by the ratio of cattle found to be seronegative to those found to be seropositive for Anaplasma marginale. Data are separated by herd. Herd C and Herd F are shown. A vertical dashed line denotes a 1:1 ratio

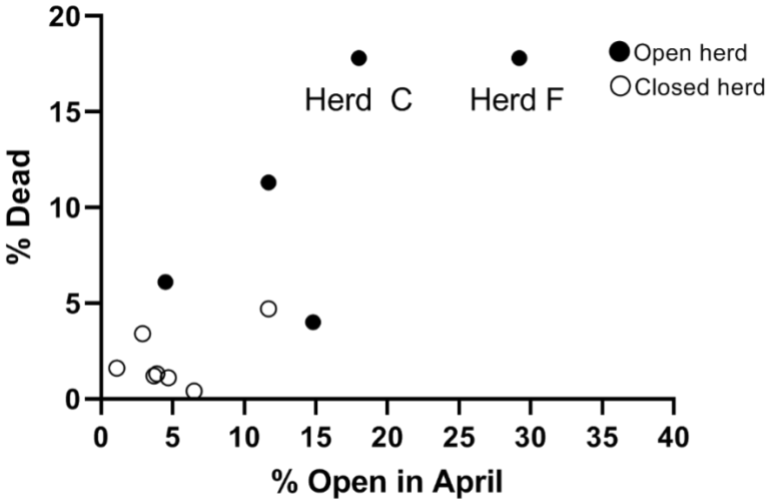

Figure 5 Incorporating all herds on the property, a comparison between the percent mortality during the 2014 outbreak and percent of open cows in April 2015 shows the impact on fertility. Herd C and Herd F are shown

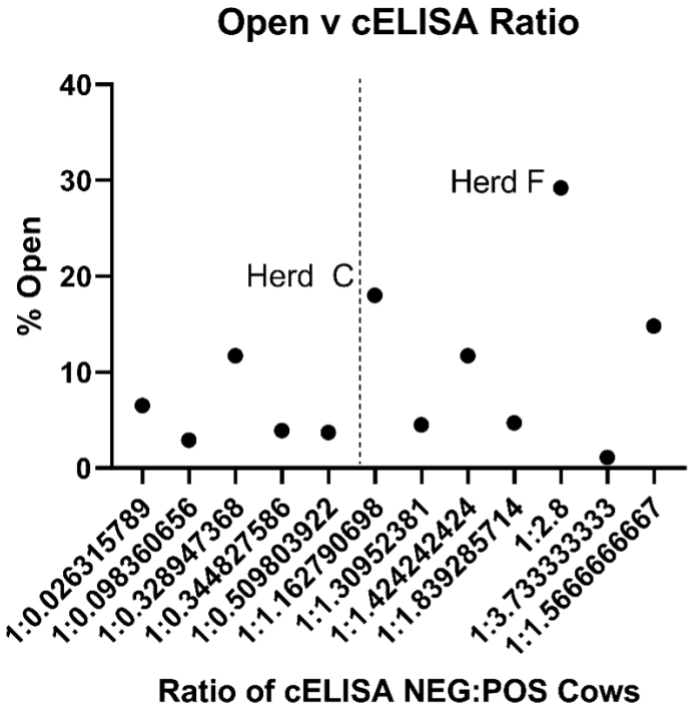

Figure 6 Open cows (%) are plotted by the ratio of cattle found to be seronegative to those found to be seropositive for Anaplasma marginale. Data are separated by herd. Herd C and Herd F are shown. A vertical dashed line denotes a 1:1 ratio

TABLES

					Anaplasmosis Prevalence Data	
Herd	Number of cattle	% Dead	% Open in April	Open or Closed Herd	Ratio of anaplasmosis seronegative vs. seropositive cattle	Percent of Herd seropositive for bovine Anaplasmosis
A	257	4.7	11.7	Closed	1:0.3	24.75
B	246	6.1	4.5	Open	1:1.3	56.70
C	309	17.8	18	Open	1: 1.2	53.76
D	319	11.3	11.7	Open	1:1.4	58.75
E	208	3.4	2.9	Closed	1:0.1	8.96
F	321	17.8	29.2	Open	1:2.8	73.68
G	251	4	14.8	Open	1:5.7	85.00
H	185	1.6	1.1	Closed	1:3.7	78.87
I	160	1.3	3.9	Closed	1:0.3	25.64
J	163	1.2	3.7	Closed	1:0.5	33.77
K	456	1.1	4.7	Closed	1:1.8	64.78
L	243	0.4	6.5	Closed	1:0.02	2.56

Table 4 Summary of the demographic data from 12 Central Florida beef herds included in this survey. Open herds indicate herds that allowed comingling of cattle from other geographic locations

County	**Total beef cattle population**	**Number of beef farms**	**Number of beef cows screened for Anaplasmosis by cELISA (no. Positive)**	**Apparent prevalence for Anaplasmosis by cELISA (95% CI)**	**Estimated true prevalence for Anaplasmosis by cELISA (95% CI)**
Columbia	24,407	483	10 (3)	30 (10.78 — 60.32)	30.11 (7.23 — 54.45)
Desoto	36,820	516	80 (23)	28.75 (19.99 — 39.46)	29.76 (19.18 — 40.81)
Okeechobee	88.046	518	39 (10)	25.64 (14.57 — 41.08)	25.42 (12.52 — 43.24)
Madison	N/A	337	4 (1)	25 (4.56 — 69.94)	24.73 (0.01 — 78.64)
Jackson	24,039	448	39 (4)	10.26 (4.06 — 23.58)	8.88 (1.70 — 23.74)
Sumter	N/A	905	10 (1)	10 (1.79 — 40.42)	8.60 (0.02 — 45.86)
Polk	66,158	1,082	16 (0)	<0 (0 — 19.36)	<0 (0 — 20.25)
Unknown	N/A	N/A	3 (0)	<0 (0 — 56.15)	<0 (0 — 65.76)
State Total	**982,790**	**18,433**	**201 (42)**	**20.90 (15.85 — 27.04)**	**20.32 (14.63 — 26.97)**

Table 5 Apparent and estimated true seroprevalence of bovine anaplasmosis in Florida counties estimated with slaughter survey between August to December 2014

Chapter 4 - Failure to eliminate persistent Anaplasma marginale infection from cattle using labeled doses of chlortetracycline and oxytetracycline antimicrobials

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ABSTRACT

Bovine anaplasmosis, caused by the intracellular rickettsial pathogen Anaplasma marginale, is the most prevalent tick-transmitted disease of cattle worldwide. In the U.S., tetracycline antimicrobials are commonly used to treat and control anaplasmosis. Oxytetracycline, administered by injection, is indicated for treatment of clinical anaplasmosis in beef and dairy cattle and calves. Chlortetracycline, administered orally, is indicated for control of active anaplasmosis infection in beef and nonlactating dairy cattle. Tetracyclines have been demonstrated to be effective for treating active anaplasmosis, but their ability to eliminate A. marginale at currently approved therapeutic doses or dosing regimens remains unclear. In the absence of approved dosing regimens for A. marginale clearance, a study was conducted to determine the effect of approved oxy-tetracycline and chlortetracycline indications on A. marginale bacteremia. Fifteen animals with persistent anaplasmosis were enrolled and divided among 3 treatment groups. Group 1 (n=6) received oral chlortetracycline (1.1 mg/kg bodyweight) administered via hand-fed medicated feed for 60 consecutive days. Group 2 (n=6) received injectable oxytetracycline administered subcutaneously at 19.8 mg/kg bodyweight 3 times in 3-week intervals. Group 3 (n=3) served as an un-treated control. After 60 days, bacteremia failed to permanently decrease in response to treatment. This result indicates that clearance of A. marginale is unlikely to be reliably achieved using currently approved tetracycline-based regimens to manage anaplasmosis.

Key Words: anaplasmosis; antibiotic; bacteremia; beef; Holstein; management; persistent infection; subclinical; tetracycline.

INTRODUCTION

Bovine anaplasmosis is caused by the intracellular rickettsial hemobacteria Ana-plasma marginale (Am) and is the most prevalent tick-transmitted disease of cattle worldwide (Uilenberg et al., 1995). Clinical signs include anemia, fever, weight loss, abortion, and death (Ristic, 1977). Disease can be divided into acute and persistent phases. During the acute phase of bovine anaplasmosis, bacteremia peaks, and the number of infected erythrocytes may be as high at 109 per mL of whole blood (Palmer et al., 1999). Clinical signs of acute disease have been shown to appear once at least ~15% of an animal's erythrocytes have been parasitized (Radosits et al., 2007). When compared to the acute phase, bacteremia is markedly lower during persistent bovine anaplasmosis. It has been reported that cyclic bacteremia can range from <104 to 107 parasitized erythrocytes per mL whole blood during persistent infection (Eriks et al., 1993). Anaplasmosis represents a significant obstacle for profitable beef production in the United States (U.S.), and losses associated with death, abortion, treatment, and control cost hundreds of millions of dollars per annum (Kocan et al., 2003; Alderink and Dietrich, 1983). Seroprevalence is variable and depends on geographic region (Kocan et al., 2003). Among U.S. beef cattle, seroprevalence has been shown to range from 4.44% in Georgia (Okafor et al., 2019a) to 28.99% in Mississippi (Okafore et al., 2019b). Importantly, low levels of bacteremia are maintained in surviving cattle (Kieser et al., 1990). Persistent, cyclical, Am bacteremia contributes to concomitant immunity among Am "carrier" cattle and overall disease endemic stability. Once infected, cattle often remain Am carriers for the duration of their life, thus chronic anaplasmosis is the most common disease state among infected cattle (Kocan et al., 2010). Carrier animals represent a challenge to disease control as they can serve as asymptomatic reservoirs for vectored transmission of Am to naïve cattle (Swift and Thomas, 1983). The importance of anaplasmosis to

the U.S. cattle industry is supported by its status as a national priority under the USDA ARS 2022-2027 National Program 103 Animal Health Action Plan in Component 3: Endemic Bacterial Diseases (USDA, 2021).

In the absence of a fully USDA approved vaccine, anaplasmosis control has been predicated on the administration of tetracycline antimicrobials. The antimicrobial action of tetracyclines is generally bacteriostatic and is associated with reversible binding to the 30S ribosomal subunit in susceptible bacteria (Merck, 2021). Tetracyclines are widely used in both human and veterinary medicine, and various studies have suggested that antimicrobial resistance has emerged partially as a result of selective pressure exerted by expansive use in animals (Holmes et al., 2016). From 2009 to 2016, tetracyclines accounted for 70% by weight of all medically important antibiotics sold or distributed in the U.S. for use in food-producing animals (FDA, 2021a). Oxytetracycline (OTC) and chlortetracycline (CTC) are indicated for treatment (FDA, 2021b) and control (FDA, 2021c) of anaplasmosis, respectively. Currently, there is no antimicrobial approved for elimination or prevention of Am.

Injectable OTC is an FDA-approved treatment, by or on the order of a licensed veterinarian, for anaplasmosis caused by Am (FDA, 2021b). Injectable OTC can be formulated in a sterile solution that contains 200 mg of OTC per mL of product. In this form, it can be delivered intramuscularly or intravenously at a dose of 1.1 mg/kg bodyweight (BW) per day for treatment of anaplasmosis. Where retreatment of anaplasmosis is impractical, a single injection of OTC can be intramuscularly or subcutaneously provided at 19.8 mg/kg BW. Presently, no OTC product available in the U.S. has a specific anaplasmosis label indication. Therefore, use of these products to treat anaplasmosis, though common, are off-label.

Oral CTC is approved by the FDA for controlling active anaplasmosis caused by susceptible strains of Am infecting beef and nonlactating dairy cattle (FDA, 2021c). CTC-medicated feed products can be hand-fed or provided free-choice within a drug-specific approved free-choice medicated feed formulation. The hand-fed FDA-approved dose of 1.1 mg CTC per kg BW is used to control active infection caused by Am susceptible to CTC. This regimen is indicated for beef cattle over 318 kg and requires a 48 hour withdraw period before animals are slaughtered. In addition, CTC can be fed to beef and nonlactating dairy cattle in free-choice feeds such as feed blocks or salt-mineral mixes as an aid in the control of active infection of anaplasmosis caused by Am susceptible to CTC. These free-choice feeds and mixes are formulated to provide a range of 1.1 and 4.4 mg CTC/kg BW. When formulated this way, CTC has no required withdrawal period. As of January 1, 2017, use of a CTC-medicated feed product (provided hand-fed or free choice) requires a veterinary client patient relationship and a veterinary feed directive from a licensed veterinarian.

Currently no OTC injectable or CTC-medicated feed products are approved or labeled for elimination of persistent Am infections. Identification of a robust and reliable antimicrobial-based Am elimination regimen is highly sought by producers and veterinarians seeking to not only reduce severity and duration of active anaplasmosis but also to resolve persistent infection among carrier animals. Elimination of persistent infection may not be appropriate for all animals. However, a reliable Am clearance protocol would be useful to cattle producers and veterinarians that want to clear infection from valuable stock for production or export purposes that require anaplasmosis-free cattle (e.g. embryo transfer cows, breeding stock), or other producers that are willing to maintain stringent biosecurity measures to prevent anaplasmosis introduction into their herd. Previous experiments have suggested that Am carrier clearance with tetracycline drugs

range from successful (Swift and Thomas, 1983; Roby et al., 1978), to unsuccessful (Coetzee et al., 2005). Swift and Thomas (1983) and Roby et al. (1978) reported that OTC can eliminate the carrier state of anaplasmosis, but currently no OTC product has a specific label indication or FDA approval for this use. Similarly, previous studies have demonstrated that oral CTC antimicrobials are effective in controlling acute infection, but not for clearance of bacteria at approved doses (Stewart et al., 1979; Kuttler and Simpson, 1978). The challenge of clearance may be further complicated by Am strain diversity. At least 43 strains of Am are known to circulate in the U.S. (Catanese et al., 2016), and treatment efficacy is likely strain-dependent (Coetzee et al., 2006). In addition to potential variable susceptibility among Am strains, differences in drug dosing regimens (approved or experimental) can make direct comparisons of results among previous studies challenging (Turse et al., 2014; Reinbold et al., 2010b). Finally, standardized methods of determining clearance are not present across the published literature (e.g. PCR, serum agglutination and complement fixation assays, xenodiagnoses in splenectomized steers). Thus, rigorous studies are needed to evaluate and confirm the efficacy of antimicrobial treatment protocols, ideally using already approved drugs and drug dosages, to reliably clear Am infection caused by diverse Am strains.

Towards understanding the potential for tetracycline antimicrobials to eliminate Am infection, we conducted a study to evaluate Am bacterial level changes in persistently infected carrier cattle administered currently available OTC and CTC products. Specifically, the objective was to evaluate the ability of repeated oral CTC and injectable OTC administration to continually lower Am bacteremia to the point of clearance. Persistently infected steers were treated with oral CTC, delivered in feed each day for 60 consecutive days or injectable OTC, delivered subcutaneously 3 times, once every 3 weeks, and their Am bacterial levels or status

(infected versus uninfected) were compared to untreated controls and each other. While both tetracycline products share a similar mechanism of action, OTC was hypothesized to have a greater likelihood to eliminate Am infection due to greater drug dosages and dose timing, the latter expected to interrupt the normal cyclical bacteremia by reducing opportunity for emergence of new antigenic variants. Data gathered from this study will help inform dosing regimens and responsible anti-microbial stewardship when elimination of Am infection is desired by producers.

MATERIALS AND METHODS

This study was conducted under approved Institutional Animal Care and Use Committee protocol #3959 on file in the University Research Compliance Office at Kansas State University, Manhattan, Kansas.

Animals

A cohort of 15 Holstein steers, aged approximately 30 months and weighing 807.9 +/- 57.1 kg (mean +/- SD), were enrolled in the study. All steers were confirmed persistently infected with Am by PCR prior to study enrollment. Steers had been experimentally infected with a field isolate of Am (Msp1a genotype M-F-F, sourced from a naturally infected Am carrier cow in Oklahoma in 2018) approximately 120 days prior to onset of this clearance study (Curtis et al., 2020). As a requirement for enrollment, cattle had to be beyond the established withdrawal periods of any previously administered antimicrobial. Although not expected, all animals were monitored daily for signs of clinical anaplasmosis such as: anorexia (>24 h), pale mucus membranes, depression (>24 h), and/or increased respiratory rate (>60 breaths per min). Steers

were co-housed in isolated dry lot pens away from study unrelated cattle; fed a standard, balanced ration; and provided water ad libitum. To reduce arthropod vector transmission risk, steers were regularly treated with a permethrin-containing pour-on product (Ultra Boss®, Merck Animal Health, Kenilworth, NJ) per label instructions. At study conclusion, animals were humanely slaughtered after all drug withdrawal periods had been satisfied.

Experimental Design

Steers were blocked by weight and randomly allocated into 1 of 3 treatment groups using the RAND function in a spreadsheet program (Microsoft Excel, Richmond, WA). Study start was day 0, the first day of treatment administration. Group 1 steers (n=6) were co-housed in the same pen and hand-fed CTC-medicated feed (Mid Kansas Cooperative Association, Moundridge, KS; CTC product: Pennchlor 50, Pharmgate Animal Health, Wilmington, NC) mixed to provide 1.1 mg CTC/kg BW daily for 60 consecutive days. Feed containing CTC was manufactured once and was maintained in an outdoor bulk feed bin for the study duration. Group 2 steers (n=6) were administered OTC (300 mg/mL, Noromycin 300 LA, Norbrook, Newry, UK) subcutaneously at 19.8 mg/kg once every 3 weeks for 6 weeks (at study day 0, 21, and 42). Finally, Group 3 steers (n=3) received no antimicrobial treatment. Steers in Group 2 and 3 were co-housed in the same pen and received an unmedicated version of the same feed ration as Group 1. On a weekly basis (± 1 day if inclement weather), beginning 1 week prior to treatment and continuing for 10 weeks, blood samples were collected to evaluate bacteremia (Am/mL blood), OTC plasma concentration (parts per billion, ppb), and CTC plasma concentration (ppb). To collect blood samples and administer OTC to Group 2 steers, steers were led into and safely restrained in a cattle chute. Venipuncture utilized jugular or coccygeal veins. At each blood sampling time point,

approximately 20 mL of blood was collected into a combination of evacuated tubes containing EDTA (for evaluation of Am bacteremia) or lithium heparin (for evaluation of OTC or CTC plasma concentration). Depending on availability of personnel and handling equipment, Group 1 steers were normally sampled after CTC feeding. Steers were maintained until all drug withdrawal times were met.

Quantification of A. marginale Bacteremia

To determine Am bacteremia (Am/mL blood), a quantitative PCR assay (qPCR) targeting a portion of the single copy Am Msp5 gene was used (Hammac et al., 2013). First, genomic DNA was extracted from 100 µL whole blood using the Quick DNA Miniprep Kit (Zymo Research, Irvine, CA) according to manufacturer instructions. The resulting genomic DNA was eluted in 35 µL of DNA elution buffer. The PCR mixture was set up in 20 µL reaction volumes and included: 0.2 µM of each primer (Am msp5 F: ATA CCT GCC TTT CCC ATT GAT GAG GTA CAT and Am msp5 R: AGG CGA AGA AGC AGA CAT AAA GAG CGT), 10 µL of SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA), nuclease free water, and 2 µL gDNA. Reaction cycling was performed using a CFX Connect Real-Time PCR System (Bio-Rad) with the following cycling parameters: 1 cycle of 98°C for 2 min; followed by 40 cycles at 98°C for 5 sec, 60°C for 5 sec and 74°C for 15 sec; and a final melt curve cycle of 65-95°C with increasing 0.5°C temperature steps at 10 sec/step. Real-time qPCR data was visualized and analyzed using CFX Maestro Software v1.1 (Bio-Rad).

CTC and OTC Quantification

For the analysis of CTC, OTC was used as an internal standard. Similarly, for the analysis of OTC, CTC was used as an internal standard. CTC hydrochloride and OTC hydrochloride and phosphoric acid were sourced from Fisher Scientific (Thermo Fisher, Hampton, NH) and stored at 4°C until use. All LC-MS grade solvents and phosphoric acid (85%) were sourced from Fisher Scientific. Ultrapure water (18 Ω) was obtained from an in-house Millipore UV-R system. Cleanup was performed using HLB Prime µElution plate, 3 mg sorbent per well from Waters Co. (Milford, MA).

On the day of analysis, standards working solutions were prepared fresh from a stock solution of OTC at 100 µg/mL in methanol (free base). The following concentrations were prepared in aqueous phosphoric acid 4%: 1; 2.5; 5; 10; 25; 50; 100; 250 ppb. A solution of CTC (internal standard) at 50 ppb in aqueous phosphoric acid 4% was also prepared. Conversely, standards working solutions were prepared fresh from a stock solution of CTC at 100 ppb. The concentrations used for CTC were the same as OTC. A solution of OTC (internal standard) at 50 ppb in aqueous phosphoric acid 4% was prepared as well. Quality controls (QCs) for analysis of OTC were prepared in untreated bovine serum at the following OTC concentrations: 4.75, 47.5, and 95 ppb. For the analysis of CTC, QCs were prepared in untreated bovine serum at the following CTC concentrations: 7, 70 and 210 ppb.

Calibration standards, controls, samples and QCs were prepared in a 48-well mixing plate. Calibration standards were prepared by mixing 100 µL of untreated serum with 100 µL of each standard. Negative controls were prepared by adding 100 µL of untreated serum to 200 µL of aqueous phosphoric acid 4%. Samples and QCs (100 µL) were mixed with 100 µL of aqueous phosphoric acid 4%. To each solution (except negative control) was added 100 µL of internal

standard at 50 ppb. The plate was covered and shaken gently at 300 rpm on a platform for 10 min. The content of each well (300 µL) was loaded on the SPE µElution plate using a nitrogen processor to push the fluid through the sorbent. After washing with 300 µL of water-methanol (95:5), the CTC was eluted with 50 µL of acetonitrile-methanol (90:10) in a collection plate. To each well was added 50 µL of aqueous 0.2% formic acid. The collection plate was covered with a cap-mat and shaken gently with a vortex mixer before analysis.

An ultra-high pressure liquid chromatography system (ULPC) Acquity H system combined with a XEVO TQ-S triple mass spectrometer (Waters Co.) was used for analysis. The chromatographic separation was performed using the UPLC column Waters Acquity HSS T3 50 x 2.1 mm, 1.8 µm. The mobile phase consisted in a gradient of water with 0.1% formic acid (A) and acetonitrile (B) as follows: 0 min: 98% A; 1.5 min: 0% A; 2.0 min: 2.01 min: 98% A; 2.5 min: 98% A. The total run time was 2.5 min. The flow rate was set at 0.5 mL/min, the column temperature at 55°C and the autosampler compartment at 8°C. The injection volume was 5 µL.

The acquisition was done by electrospray ionization in positive mode. The operating parameters for the mass spectrometer were as follows: the capillary voltage was 3.0 kV, source and desolvation temperatures were 150°C and 600°C, respectively and the cone energy was set t 25 V. Nitrogen was used as the desolvation and cone gas flows of 1,000 L/h and 150 L/h, respectively. Helium was used as the collision gas at a flow rate of 0.15 mL/min. Data acquisition and analysis were done using Waters MassLynx (Waters Co.) and TargetLynx (Waters Co.) software, respectively. The detection of OTC and CTC was performed using multiple reaction monitoring.

The lower limit of quantitation (LLOQ) was determined according to the FDA guidelines for the bioanalytical Method Validation Guidance for Industry (FDA, 2018) with a signal over

noise ratio > 5, precision ≤ 20% and accuracy between 40 and 120%. The LLOQ for CTC and OTC was determined to be 2.5 ppb (2.5 parts per billion, ppb) to 250 ng/mL (250 ppb). Linear regression was used with a weighing factor of 1/x. The calibration curve was linear from 2.5 ppb and accepted if the correlation coefficient was at least 0.99. The intra-day and inter-day precisions were <15% and the accuracies for both CTC and OTC ranged from 80 to 100%.

Statistical Analyses

Statistical analyses evaluated the relationships between CTC and OTC concentrations (ppb) with bacteremia (Am/mL blood) over time. Bacteremia was log transformed prior to analysis. The outcome variables of bacteremia and CTC or OTC concentration were analyzed using a repeated measures test in SigmaPlot (SPSS Statistics, Chicago, IL). Linear regressions were performed using JMP (SAS Institute, Cary, NC) to examine relationships between drug concentrations and bacteremia. For all outcomes, statistical significance was set a priori at $P < 0.05$.

RESULTS

Effect of CTC treatment on A. marginale bacteremia

The ability of oral CTC to reduce Am bacteremia to the point of clearance was evaluated in Group 1 persistently infected steers. Summary statistics of Am bacteremia changes in CTC-treated steers and untreated steers are presented in Table 6. Group 1 animals treated daily with oral CTC, at 1.1 mg/kg BW, did not experience significantly decreased bacterial loads over the treatment period compared to mean starting Am bacteremia ($P = 0.9980$) (Figure 7). In addition, mean Am bacteremia (1.77×106 copies/mL $\pm$ 2.64×105 copies/mL) among Group 1 steers did

not significantly differ from mean Am bacteremia (2.31 × 106 copies/mL ± 4.78 × 105 copies/mL) among Group 3 steers (untreated control) (P = 0.1834) during the study. Likewise, untreated control animals in Group 3 maintained persistent bacteremia that did not significantly differ over the study period (P = 0.3920).

Oral CTC treatment resulted in plasma CTC concentrations ranging between <2.5 and 84.6 ppb, with an average of 29.3 ppb ± 2.6 ppb. Drug concentrations are summarized in Table 7. Plasma CTC concentrations peaked (mean 64.1 ppb ± 21.0 ppb) 13 days after beginning treatment (Figure 7) before steadily declining for the remainder of the study. Linear regression indicated a poor (R2 = 0.0348, P = 0.1064) relationship between CTC concentration and bacteremia (Figure 9).

Effect of OTC treatment on A. marginale bacteremia

The ability of injectable OTC to reduce Am bacteremia to the point of clearance was evaluated in Group 2 persistently infected steers. Summary statistics of Am bacteremia changes in OTC-treated steers is presented in Table 6. Injectable OTC administered once every 3 weeks at study days 0, 21, and 42 at 19.8 mg/kg BW, elicited a significant but transient reduction of Am bacteremia (Figure 8). OTC suppression of Am bacterial load was evident by each subsequent post-OTC treatment evaluation time point (~1 week later) and continued to decrease through at least another week after which infection rebounded to near pre-treatment levels. The mean infection nadir observed post-OTC treatment was 5.25 × 105 copies/mL ± 3.10 × 105 copies/mL. Compared to Group 1 (CTC), Group 2 steers exhibited significantly lower Am bacteremia at study day 13 (P = 0.0168), 34 (P = 0.0103), 48 (P = 0.0094), and 55 (P = 0.0172). Compared to Group 3 (untreated control) steers, Group 2 steers exhibited significantly lower Am bacteremia at

study day 7 (P = 0.0296), 27 (P = 0.0088), 34 (P = 0.0002), 48 (P = 0.0011), and 55 (P = 0.0087). Compared to baseline, Group 2 steers exhibited significantly lowered Am bacteremia at study day 7 (P = 0.0007), 13 (P = 0.0006), 27 (P = 0.0064), 34 (P = 0.0003), 48 (P = 0.0022), and 55 (P = 0.0005). However, these drops were transient, and by the third week post each OTC treatment, mean bacteremia in Group 2 had returned to or exceeded baseline bacteremia or time-matched bacteremia levels in the untreated steers (e.g. at study day 69, Group 2 mean bacteremia was greater than mean bacteremia of untreated steers).

Treatment resulted in plasma OTC concentrations of between 9.3 and 420 ppb, with an average of 124 ppb ± 13.4 ppb over the study period (day 7 to 70; day 7 is the first OTC plasma concentration evaluation time point post-initial treatment and day 70 is 29 days post-final OTC treatment). Drug concentration data are summarized in Table 7. Plasma OTC concentrations peaked the week following each treatment (Figure 8) before declining. Peak plasma OTC concentrations averaged 247 ppb ± 12.17 ppb. The relationship between drug concentrations and log transformed bacteremia over time is illustrated in Figure 10. A linear relationship (R2 = 0.2033, P = 0.0001) between OTC concentration and bacteremia was noted, suggesting that as OTC concentration increases, bacteremia tends to decrease.

DISCUSSION

This study investigated the ability of FDA-approved, commercially-available tetracycline products to reduce Am bacteremia to the point of infection clearance in persistently infected steers. Groups of steers were either provided CTC daily at 1.1 mg/kg BW for 60 days, injected with OTC at 19.8 mg/kg BW 3 times at 3-week intervals, or received no treatment. Outcome measures included bacteremia, OTC concentration, and CTC concentration over time. Compared

to pre-treatment Am bacteremia levels and untreated controls, the OTC treatment regimen significantly but transiently lowered Am bacteremia but the CTC treatment regimen had no significant effect on Am bacteremia. By the end of the study, bacteremia had rebounded to near pre-treatment levels in both treatment groups and were similar to untreated control steer bacteremia levels. Currently, no antimicrobial drugs or products are approved for elimination of Am infection; and, use of the tetracycline products investigated in this study for Am clearance were for experimental purposes only.

In this study, Noromycin 300 LA, a commercially-available injectable OTC product, failed to achieve Am bacterial clearance in steers with persistent anaplasmosis. It should be noted there is no specific FDA approval for the Noromycin 300 LA OTC formulation to be used in the context of bovine anaplasmosis. However, Noromycin 300 LA does include a label indication for use against disease caused by a wide range of susceptible gram-negative bacteria. Further, the dose of 19.8 mg/kg BW is approved by the FDA for less concentrated OTC products (e.g. 200 mg/mL) where retreatment with injectable OTC is impractical (FDA, 2021b). Given the average weight of Group 2 steers (793 kg) use of Noromycin 300 LA required an average treatment volume of 52 mL instead of an average treatment volume of 79 mL had a 200 mg/mL OTC product been used. Further, the reduced volume required for Noromycin 300 LA reduced the total number of injections needed per treatment (6 versus 8 when using 10 mL/injection site as per manufacturer product administration directions). As this study was conducted in an experimental setting and limiting the number of injections was preferable in the interest of animal welfare, Noromycin 300 LA was used. Administration of injectable OTC resulted in reduced bacteremia (~26-fold reduction), with the greatest reduction observed 7-14 days post-treatment administration. Despite leading to a reduction in bacterial load, likely in part facilitated

by the drug as well as the animal's own immune response, Am infection was not cleared, rebounding to pre-treatment levels 7-14 days post treatment bacteremia nadirs.

The OTC results in the present study are contradictory to previous work in which clearance was reportedly achieved using OTC dosing regimens ranging from 11 to 22 mg/kg BW given at intervals ranging from daily to weekly for between 5 and 14 days (Magonigle and Newby, 1982; Kuttler et al., 1980; Roby and Simpson, 1978). Other studies have achieved clearance through OTC injections at 20 mg/kg BW following 3-4 administrations at 3-day intervals (Özlem, 1988; Swift and Thomas, 1983). It is possible that differences in methodology among previous experiments or the infecting Am strain contributed to different outcomes in this study versus previous studies. For example, Magonigle and Newby (1982) and Roby et al. (1978) confirmed carrier clearance by subinoculating splenectomized blood harvested from OTC treated Am carrier cattle at least 83 days after carrier cattle were treated. Özlem et al. (1988) confirmed carrier clearance by harvesting blood from OTC treated Am carrier cattle and observing a lack of organisms in stained blood smears. Although subinoculation of blood into a splenectomized calf is a robust way to investigate clearance, the available methodologies at that time to monitor infection (e.g. blood smears) have low sensitivity. Conversely, the present study relied on qPCR to quantify infection (direct visualization of Am-infected red blood cells on a thin blood smear are rare and not reliably detected during persistent Am infection). It is also notable that previous studies investigating possible Am clearance protocols often used different Am strains, some of which may be more-or-less relevant when extrapolating which tetracycline-based Am elimination protocols may work best for contemporary Am strains. For example, previous work tested stains originating in Florida (Kuttler et al., 1984) and Oklahoma (Goff et al., 1990), and another (Özlem, 1988) did not specify. Our results agree with a more recent study

that reported clearance failure in naturally infected cattle using 2 doses of long-acting injectable OTC at 20 mg/kg (Goff et al., 1990). Likewise, Coetzee et al. (2005) reported clearance failure after injecting persistently infected steers with either 1 dose of OTC at 30 mg/kg, 2 doses of OTC at 30 mg/kg 5 days apart, or 5 doses at 22 mg/kg daily for 5 days. Data from the present study support that injectable OTC may be appropriate for reducing Am bacteremia to limit disease severity during acute anaplasmosis while the animal mounts an effective immune response but should not be considered reliable to achieve total Am clearance.

In the present study, peak serum OTC values were much lower than those measured in some previous trials. For example, Luthman and Jacobsson (1982) found that injectable OTC peaked at between 1,500 and 4,000 OTC ppb in serum approximately 4 h after injection. One possible explanation for this discrepancy is the blood sampling schedule of the present study. In this case, blood was drawn at intervals much longer than the re-ported OTC half-life of 8 h (Luthman and Jacobsson, 1982). Similarly, Xia et al. (1983) reported peak plasma values of between 4,000 and 10,000 ppb 6-9 h after injection. Cattle in the present study were sampled 7, 14 and 21 days after each OTC administration, and the observed drug concentrations likely reflected that regimen. Sampling animals with closer temporal proximity to treatment would have likely revealed higher peak OTC concentrations.

In the present study, oral CTC failed to clear Am or reduce Am bacterial load from subclinical, persistently infected steers. The CTC dose of 1.1 mg/kg BW used in this study is approved by the FDA for control of active anaplasmosis. One potential reason for this result is the FDA-approved dosing regimen (1.1 mg/kg BW per day) is not high enough to result in clearance of Am infection. Previous work has demonstrated Am clearance with CTC feeding when cattle were fed between 4.4 to 22 mg/kg BW daily (Reinbold et al., 2010c). Higher daily

dosing in that study yielded higher mean CTC concentrations in plasma (85.3 – 518.9 ppb) than those measured in the present study (mean 29.3 ppb). Reinbold et al. (2010c) also gathered blood samples more frequently (sometimes as often as every 4 h) than the present study, likely contributed to differences in plasma CTC concentrations. However, at no time during the present study did more than 16.2 h, the oral CTC elimination half-life established for cattle (Reinbold et al., 2010c), elapse between CTC feeding and blood sampling. Higher drug concentrations, achieved by higher-than-approved CTC administration, may have contributed to greater bacteriostasis and subsequent Am clearance. In addition, the Am strain used in the present study differs from the Virginia isolate used by Reinbold et al (2010b). As with the OTC results, it is possible that genetic differences between isolates contributed to differences in susceptibility and overall results between studies.

A decline in plasma CTC concentration was noted in Group 1 steers during the course of their treatment regimen suggesting that there may be drug stability issues in the medicated feed. The CTC-medicated feed used in the present study was manufactured in a single batch (received 3 days prior to study start) which was used for the duration of the study. Similar to the unmedicated feed, the CTC-medicated feed was stored in a water-proof outdoor bin during the study, as is the case on many commercial cattle operations. During the study, temperatures were cold to moderate, ranging from -2.2 to 25.6°C, with 38.7 cm of precipitation (U.S. Climate Data, 2020). It is conceivable that diminishing steer plasma CTC concentration was due to loss of drug integrity over time, non-uniform initial feed ingredient mixing (less likely), or non-uniform drug concentration due to settling of feed in the bin (Figure 7). Because feed was not tested during the study, these possibilities are not able to be investigated.

Again, no CTC-medicated product is currently approved for prevention or elimination of Am infection in cattle. Oral CTC is approved for the control of active anaplasmosis. If disease control is interpreted as prevention of disease spread, oral CTC did not reduce Am bacteremia levels below untreated controls and therefore would be unlikely to reduce risk of disease spread (e.g. via arthropod vectors or iatrogenic transmission) based on the assumption that treated animals would have lower bacteremia levels. Because Am can replicate in vector-competent tick species, ticks can effectively acquire Am from cattle with high or low levels of bacteria to subsequently transmit to naïve cattle (Kocan et al., 2003). If 'control active anaplasmosis' is interpreted as prevention of clinical anaplasmosis, then it could be considered that CTC performed accordingly as no CTC-treated animal displayed any clinical signs of anaplasmosis; however, none of the untreated controls did either. Results presented here suggest that CTC, at current approved dosages, would be unlikely to eliminate Am infection or even reduce likelihood of transmission as Am bacteremia did not significantly vary from pre-treatment baseline or untreated controls during the 60 days of continuous treatment.

Despite the value it would have to the U.S. cattle industry, especially cow-calf and seed stock producers, a broadly effective, antimicrobial-based protocol to clear Am from carrier animals remains elusive. Presently, no OTC or CTC product or dosage have a label or approved indication for Am infection elimination from cattle. Data detailed here suggest that cattle producers and veterinarians should not anticipate or rely on labeled doses of OTC or CTC to eliminate Am in persistently infected cattle (nor are these products indicated for this purpose). Future efforts to identify a reliable Am infection elimination protocol could explore using current tetracycline products at different dosing frequencies or concentrations; or, explore the utility of other drug products as tetracyclines are no longer the only antimicrobials approved for the

treatment of bovine anaplasmosis in the U.S. As of 2020, the fluoroquinolone enrofloxacin has received conditional approval for the treatment of clinical anaplasmosis. Fluoroquinolone antimicrobials are generally bactericidal and exert their action through inhibition of topoisomerases (Merck, 2021). Like CTC and OTC, enrofloxacin is not labeled for total Am infection clearance but has been shown to be effective at limiting mortality and anemia during acute anaplasmosis (Shane et al., 2020). More research is needed to develop a robust and reliable antimicrobial-based protocol to eliminate persistent Am infection.

CONCLUSIONS

Long-term, persist infection by Am remains a challenging aspect of bovine anaplasmosis management around the world. Treatment regimens designed to eliminate infection during this phase of disease are needed, but previous attempts have yielded varying results. Data from the present study indicate that U.S. FDA-approved dosages of either CTC or OTC are unlikely to eliminate Am infection. Although specific regulations on use may differ, the results from this study are broadly informative to other countries that rely on tetracyclines to combat bovine anaplasmosis. Future work is needed to evaluate the ability of antimicrobials to eliminate Am bacteremia and resolve the carrier state to promote economic potential and manage disease spread.

FIGURES

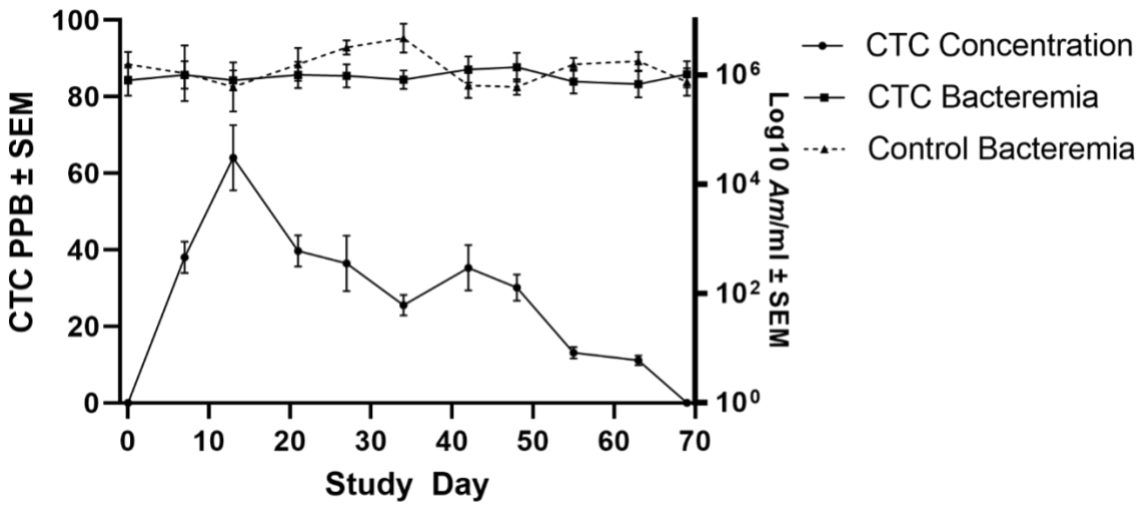

Figure 7. Changes in Am bacteremia in steers treated with CTC for 60 days. CTC concentration, in parts per billion (ppb), is plotted over time with bacteremia (Am/mL blood) for animals treated daily with 1.1 mg/kg bodyweight CTC for 60 days. Un-treated control steer mean Am bacteremias are included for comparison. CTC treatment was not found to have significant (P > 0.05) effect on reducing bacteremia.

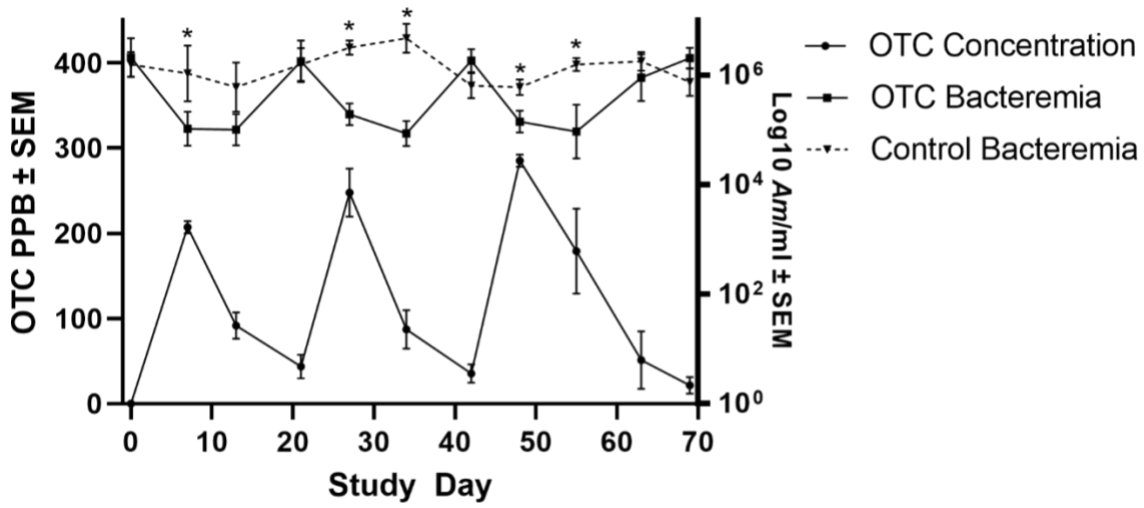

Figure 8. Changes in Am bacteremia in steers treated with multiple doses of OTC. OTC concentration, in parts per billion (ppb), is plotted over time with bacteremia (Am/mL blood) for animals administered 3 doses of OTC (19.8 mg/kg bodyweight) in 3-week intervals. Untreated control steer mean Am bacteremias are included for comparison. Asterisks denote statistically significant differences in bacteremia between treatment groups ($P < 0.05$).

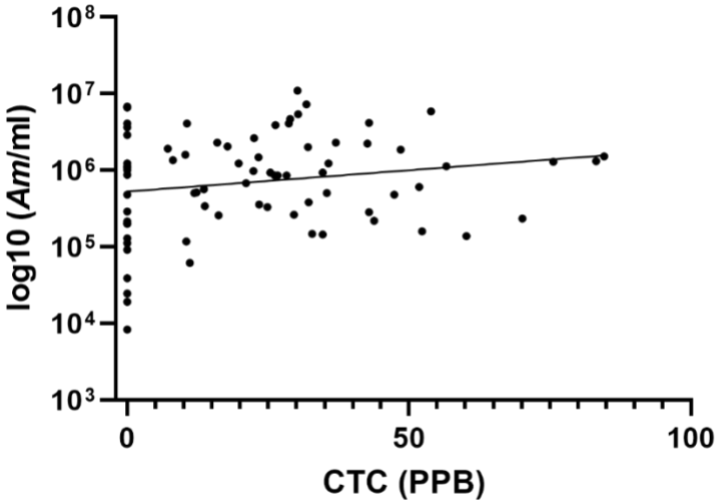

Figure 9. Relationship between Am bacteremia and drug concentration in steers treated with CTC for 60 days). Bacteremia (Am/mL blood) for animals treated with CTC is plotted by CTC concentration, in parts per billion (ppb). A straight line denotes linear regression (R^2=0.0348, P=0.1064)

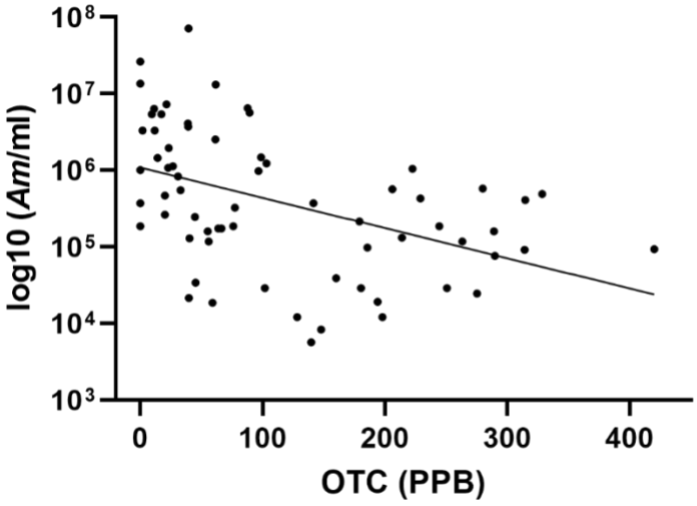

Figure 10. Relationship between Am bacteremia and drug concentration in steers treated with OTC. Bacteremia (Am/mL blood) for animals treated with OTC is plotted by OTC concentration, in parts per billion (ppb). A straight line denotes linear regression (R2 = 0.2033, P=0.0001)

TABLES

Day	Bacteremia (Log10 copy/mL)																	
	CTC						OTC						Untreated Control					
	N	Mean	Median	Min	Max	SD	N	Mean	Median	Min	Max	SD	N	Mean	Median	Min	Max	SD
0	6	5.90	5.87	5.05	6.83	0.68	6	6.32	6.26	5.27	7.42	0.86	3	6.19	6.04	5.90	6.64	0.39
7	6	5.96	5.97	5.20	6.67	0.62	6	5.02	5.06	4.08	6.02	0.75	3	6.03	5.69	5.37	7.02	0.88
13	6	5.90	6.12	5.14	6.27	0.44	6	5.00	4.92	4.27	6.17	0.70	3	5.78	6.19	4.87	6.27	0.79
21	6	5.96	5.88	5.16	6.86	0.59	6	6.25	6.45	4.53	7.12	0.93	3	6.19	6.28	5.64	6.66	0.52
27	6	5.98	5.96	5.37	6.73	0.51	6	5.28	5.42	4.46	5.75	0.50	3	6.50	6.38	6.36	6.76	0.23
34	6	5.91	6.01	5.41	6.42	0.41	6	4.93	5.24	4.08	5.39	0.57	3	6.67	6.76	6.18	7.08	0.46
42	6	6.10	6.12	5.17	6.77	0.59	6	6.26	6.41	5.42	6.81	0.51	3	5.81	5.80	5.43	6.21	0.39
48	6	6.14	6.10	5.45	7.04	0.63	6	5.15	5.14	4.39	5.76	0.50	3	5.78	5.91	5.50	5.94	0.25
55	6	5.88	5.92	5.07	6.61	0.54	6	4.97	4.72	3.75	6.75	1.19	3	6.19	6.25	5.96	6.37	0.21
63	6	5.83	5.92	4.79	6.36	0.58	6	5.95	5.63	5.07	7.85	1.05	3	6.25	6.14	6.02	6.59	0.30
69	6	6.01	5.97	5.30	6.82	0.60	6	6.31	6.31	5.67	6.80	0.46	3	5.87	6.00	5.38	6.22	0.44

Table 6 Summary of steer Am bacteremia values by study day and treatment group

Day	Drug Concentration (ppb)											
	CTC						OTC					
	N	Mean	Median	Min	Max	SD	N	Mean	Median	Min	Max	SD
0	6	0	0	0	0	0	6	0.28	0	0	1.70	0.69
7	6	38.00	38.80	25.40	52.30	10.14	6	207.10	205.95	185.50	228.90	17.18
13	6	64.05	67.90	32.20	84.60	20.95	6	91.58	82.50	58.90	160.10	38.28
21	6	39.67	35.05	31.80	56.60	10.09	6	43.77	33.30	14.20	103.10	34.39
27	6	36.43	29.30	22.40	70.10	17.92	6	247.50	247.55	141.40	328.50	69.26
34	6	25.58	24.75	16.20	35.70	6.66	6	87.12	65.50	39.60	179.10	55.43
42	6	35.28	30.75	17.80	53.90	14.48	6	35.37	27.05	11.60	87.60	27.23
48	6	30.15	28.25	21.10	42.90	8.39	6	285.30	284.35	263.30	314.60	17.36
55	6	13.10	12.10	10.30	19.80	3.65	6	179.00	143.65	89.40	420.00	122.83
63	6	11.10	11.50	7.20	16.00	3.17	6	51.18	48.10	32.80	77.20	16.71
69	6	0	0	0	0	0	6	21.45	21.35	9.30	39.10	10.91

Table 7 Summary of steer plasma drug concentration levels by study day and treatment group

Chapter 5 - Delivering an immunocastration vaccine via a novel subcutaneous implant

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ABSTRACT

Objective: To determine the feasibility of a single dose immunocastration vaccine implant in cattle. To examine subcutaneous implant retention and humoral immune response to gonadotropin releasing hormone (GnRH) antigen. To study the ability of the implant to limit testicular growth, spermatogenesis, and testosterone production.

Materials and Methods: A series of 5 pilot studies were conducted using 44 male Holsteins (*bos taurus*) to determine optimal vaccine composition and validate the ability of a stainless-steel subcutaneous implant to deliver vaccine. Outcome measures included duration of implant retention, scrotal dimensions and temperature, implant site temperature, anti-GnRH antibodies, and serum testosterone concentration.

Results and Discussion: Over the course of several studies, anti-GnRH antibodies were successfully stimulated by vaccine implants. No significant treatment effects on scrotal dimensions or testosterone were detected over time, but changes in spermatogenesis were detected across treatment groups. Results indicate that the single-dose implantable immunocastration vaccine elicits a humoral immune response and could impact spermatogenesis.

Implications and Applications: A single dose immunocastration vaccine would be beneficial to producers interested in reducing pain and increasing the ease with which cattle are managed. Additional research is needed to refine the platform and improve implant retention before it can be practically applied in production settings.

Key Words: Vaccine, castrate, dairy cattle, immunocastration

INTRODUCTION

Approximately 88% of male beef cattle are castrated in the United States (USDA-ARS-MWA, 2011). This translates to around 15 million procedures per year, making it one of the most common livestock management practices currently employed by the beef cattle industry (USDA Agricultural Statistics, 2009). Cattle are castrated for a variety of reasons that generally pertain to increased ease of management and improved carcass traits. Castrates exhibit reduced aggressiveness (Goodrich and Stricklin 1997), reduced mounting behavior and mounting-related injuries (Tarrant, 1981), and improved meat quality and market premium (Jones, 1995; Romans et al., 1994). In addition, sterilization that results from castration prevents unwanted breeding (Stafford and Mellor, 2005).

Methods of castration are varied, but all have side-effects and cause pain (Stafford and Mellor, 2005). The American Veterinary Medical Association (AVMA) Animal Welfare Division divides forms of castration among physical, chemical, and immunological methods (i.e. immunocastration). Physical castration involves surgical removal of testicles, application of a constricting elastic band at the base of the scrotum, and/or external clamping such as a Burdizzo clamp (Stilwell et al., 2008). Physical castration predominates in production settings (USDA-ARS-MWA) and is often performed in combination with other painful husbandry practices (Dinniss et al., 1997, Mellor et al., 2002, Becker et al., 2012). Chemical castration includes injection of sclerosing or toxic agents into the testicular parenchyma to cause irreparable damage and loss of function (AVMA) but may preserve androgenesis and associated behavior (Fordyce et al., 1989).

A less-painful alternative to traditional castration relies on immunization against reproductive hormones to control function (immunocastration). One target for vaccine

development has been gonadotropin releasing hormone (GnRH) for its upstream role in endocrine signaling cascades. Vaccination against GnRH conjugated to carrier proteins (i.e. hapten-carrier complexes) such as human serum albumin (Finnerty et al., 1998) and ovalbumin (Geary et al., 2006; Hoskinson et al., 1990) has been shown to provoke humoral immune responses in bulls and heifers. Early work in rams and bull calves relied on 4 doses of vaccine to immunize against GnRH and resulted in diminished testis size and weight as well as reduced plasma testosterone concentrations (Jeffcoate et al., 1982). Since 2007, a vaccine (Bopriva ®, Pfizer Animal Health) marketed specifically for use in cattle has been available in several markets outside the US. Janett et al. (2012) reported significant reduction in testosterone levels, testicular development, and physical activity in pubertal bulls treated with Bopriva. However, studies examining Bopriva and other vaccine cocktails have relied on multiple doses of vaccine in order to impair reproductive function in cattle (Janett et al., 2012; Aissat et al., 2002; Michael et al., 2001). A GnRH vaccine that replicates the benefits of surgical castration with a single dose would be useful to producers and clinicians interested in reducing pain during routine management. Thus, a series of studies were conducted in order to determine the ability of an implantable vaccine to stimulate humoral immunity, limit testicular development, and reduce testosterone production in bulls.

MATERIALS AND METHODS

All animal studies were conducted under an approved Institutional Animal Care and Use Committee (IACUC) protocol (IACUC #: 4394) on file at Kansas State University, Manhattan, Kansas.

Study Animals and treatments

Pilot 1

A total of 11 dairy bull calves (aged 6 months and with fully descended testicles) were randomly assigned to one of four treatment groups using the RAND function in a spreadsheet program (Microsoft Excel, Richmond, WA). Treatment groups are summarized in Table 8. Briefly, vaccine constructs consisted of 1 of 4 GnRH-based treatment iterations with varying amounts and types of adjuvants. Treatment iterations included GnRH complexed with ovalbumin (OVA) and keyhole limpet hemocyanin (KLH) in addition to adjuvants Quil-A ® (InvivoGen, San Diego CA; Implants 1 and 2) and/or DEAE-Dextran (DEAE-D; Sigma-Aldrich, St. Louis MO; Implants 3 and 4). Calves were sampled every 2 weeks for a total of 56 days. At the end of the study, calves were surgically castrated, and gonads were histologically evaluated. Major outcome measures for Pilot 1 were external scrotal/testicular changes measured over time as well as registration of spermatogenesis by histology following castration.

Pilot 2

A total of 12 male Holstein calves (aged 6 months) were implanted with empty stainless-steel implants (Implant 5) in order to determine the viability of stainless steel as a biocompatible delivery system. Calves were monitored for 42 days and implant site reactions were monitored along with attrition. The primary outcome measure for Pilot 2 was implant rejection/attrition over time.

Pilot 3

A total of 12 male Holstein calves (aged 10 months) were randomly allocated to 1 of 2 treatment groups. Group 1 (n = 6) received Implant 6 and Group 2 (n = 6) received Implant 7. Animals were monitored for 175 days and sampled every 14 days. Implant 6 contained GnRH-KLH with no adjuvant, whereas Implant 7 contained GnRH-OVA with DEAE-D and Quil-A®. Outcome measures included implant attrition and anti-GnRH antibody production as assessed by ELISA.

Pilot 4

A total of 8 male Holstein calves (aged 14 months) were administered Implant 8. Implant 8 contained GnRH-OVA and GnRH-KLH with both Quil-A® and DEAE-D. Animals were monitored for 56 days and sampled every 14 days. Outcome measures included implant attrition and anti-GnRH antibody production as assessed by ELISA.

Pilot 5/USDA1

A total of 12 male Holsteins (aged 3 months, with fully descended testicles) were enrolled in a study to examine two implantable vaccines (Implant 9 and Implant 10). Implant 9 contained 100 µg of GnRH antigen, whereas Implant 10 served as a negative control and contained only scrambled peptide (SP). The SP implants delivered the constituent amino acids of GnRH in a randomized sequence. Animals were monitored for 252 days and sampled every 14 days. Outcome measures included rectal temperature, body weight (BW), scrotal circumference,

estimated testicle volume, histological evaluation of spermatogenesis, temperature changes at the scrotum and implant site, and testosterone concentrations.

Blood Sampling and Analysis

During pilot studies that relied on blood draws, the sampling regimen was performed every 14 days. Beginning at day 0, immediately before vaccination, baseline blood was drawn from the jugular vein into evacuated tubes (Vacutainer, Becton Dickinson, Franklin Lakes, NJ) containing EDTA (1.8 mg/mL whole blood) or no anticoagulant. Approximately 10 ml of whole blood was drawn each time. Blood was centrifuged (IEC Centra CL2, Thermo Electron Corporation, Waltham, MA) at 1150 x g for 10 minutes, serum and plasma were drawn off, and samples were frozen in cryotubes at -27 ^{o}C until further analysis.

A commercially available double antibody RIA kit was used to detect total unconjugated testosterone (125 I RIA Kit, MP Biomedicals LLC, Solon, OH) per manufacturer's instructions. Testosterone was then measured via radioimmunoassay (RIA) using an automatic gamma counter (2470 Wizard 2, PerkinElmer, Waltham MA). Anti-GnRH antibodies were assessed at 1:10,000 dilution using enzyme-linked immunosorbent assays (ELISA) using EvenCoat Streptavidin Coated Plates (Cat# P004, R&D Systems, Minneapolis, MN). Optical density (OD) was measured using a SpectraMax i3 Multi-Mode Microplate Reader (Molecular Devices LLC, San Jose, CA).

Implantation

Prior to implantation, hair was removed from caudal aspects of the ears using livestock clippers (Powerpro Ultra, Oster, Milwaukee, WI) and skin was cleaned using chlorhexidine

surgical scrub (Chlorhexidine 4%, VetOne, Boise, ID) and gauze soaked in 70% isopropyl alcohol (Vedco, St Joseph, MO). Local nerve block was provided using injections of lidocaine hydrochloride (without epinephrine; Lidocaine 2%, VetOne, Boise, ID) approximately 5 minutes before implantation. Implants were placed in the subcutaneous space of the caudal aspect of the ear pinna. Incisions were approximately .5 cm long and were sealed using a single suture (000 silk, Arrow International, Morrisville, NC) and cyanoacrylate (Loctite Super Glue, Henkel North American Consumer Goods, Hartford, CT.

Vaccine Design

Stainless steel cylindrical implants measured approximately 5 mm x 41 mm and were delivered through a standard Compudose ® (Elanco Animal Health, Greenfield, IN, USA) needle using a proprietary applicator. Cylinders were packed with vaccine components and sealed at one end with a polyvinylidene fluoride (PVDF) membrane. Dry mixtures of all vaccine components were pressed in a custom-made mold at 0.5 tons-on-ram for 5 seconds, using a hydraulic press (International Crystal Laboratories Inc., Garfield, NJ). The implant was designed and formulated in a way similar to that previously described (Schaut et al. 2018). Depending on iteration, GnRH was complexed as a hapten with either OVA or KLH carrier proteins. Vaccine ingredients were arranged sequentially for controlled delivery of both antigen and adjuvant. Implant design was such that the priming component (primer) was presented first, then the boost component, then the vaccine platform for extended antigen release (VPEAR) adapted from Boggiatto et al. (2019).

Scrotal and Ear Surface Temperature

Temperature of the scrotal surface was assessed via infrared thermography using a digital camera capable of capturing thermographic images (TiX580 Thermal Imager, Fluke Corporation, Everett, WA). The thermographic camera was perpendicularly positioned approximately 45 cm away from the caudal aspect of the surface of the scrotum and image focus and quality was verified before saving to memory. Similarly, images of the implant site were captured by aiming the camera at the caudal aspect of the ear from approximately 20 cm away. Scrotal and ear surface temperatures were assessed via thermography every 14 days. Images were evaluated using Fluke Tools software (Smartview 4.3, Fluke Corporation, Everett, WA).

Scrotal Dimensions

Scrotal dimensions were assessed using a flexible scrotal tape (Reliabull, Lane Manufacturing Inc., Denver, CO) and digital calipers (Tool Shop 6" Stainless Steel Digital Caliper, Menards, Eau Claire, WI). To measure scrotal circumference, both testicles were manipulated by hand so that they rested at the lowest and most distal aspect of the scrotum. The scrotum was then held firmly with one hand while the scrotal tape was applied at the level judged to have the largest circumference. The tape was then drawn firmly against the circumference of the scrotum to provide a value as per the manufacturer's instructions. Volume of individual testicles was estimated by measuring external length, width, and depth of individual testes through the scrotal skin using digital calipers. Approximate volume was then calculated from caliper measurements using the prolate spheroid formula (Bailey et al., 1998).

Surgical Castration

In cases where animals were surgically castrated, scrotums were washed using 4% chlorhexidine (VetOne) and gauze soaked in 70% isopropyl alcohol (Vedco). Local anesthesia was induced by injecting 2% lidocaine hydrochloride (without epinephrine) into each spermatic cord (VetOne). Open orchidectomy was performed approximately five minutes after nerve block. Incisions were closed with purse string sutures (2 violet monofilament, Ethicon Inc, Somerville, NJ). Animals were monitored under close surveillance for 2 hours and then once daily for 14 days as per institutional requirements.

Testes Histology

In cases where animals were surgically castrated, testes were histologically evaluated to compare differences in spermatogenesis between treatment groups. Histologic slides were prepared, and analysis used 3 parenchymal slides and 1 epididymis from each testicle. Seminiferous tubular cross sections were selected at random (6 or 7 per slide) for each testicle. Scoring relied on methods previously described by Johnsen (1970) and adapted by Daigle et al. (2009) with higher numbers indicating greater degrees of spermatogenesis (Table 9). Scoring was conducted independently by the Veterinary Diagnostic Laboratory at the Iowa State University College of Veterinary Medicine and scorers were blinded to treatment.

Statistical Analysis

Statistical analyses evaluated the relationships between treatment and scrotal dimensions (in mm, g, or cc), antibody production, and testosterone over time. Treatment effect on spermatogenesis

among castrates was also examined. Scrotal dimensions, antibodies, and testosterone concentration over time were analyzed as repeated measures using JMP (SAS Institute, Cary, NC). Treatment effect on spermatogenesis, quantified as described by Daigle et al. (2009), was determined using t test. For all outcomes, statistical significance was set *a priori* at $P < 0.05$.

RESULTS AND DISCUSSION

Pilot 1

During the course of the study, 5 of 12 implants were rejected before day 28. Within 6 weeks, all implants had been rejected. At study conclusion, animals were surgically castrated and testes were examined. Post-castration histology scoring revealed significant ($P < 0.05$) differences in extent of spermatogenesis among treatment groups (Figure 11). Animals provided with Implant 3 exhibited significantly ($P < 0.0001$) less spermatogenesis than animals treated with Implants 1, 2, and 4. Animals treated with implant 2 exhibited significantly ($P < 0.0001$) less spermatogenesis than those treated with 1 and 4, but significantly ($P < 0.0001$) more than those with Implant 3. There was no significant difference ($P = 0.5186$) in spermatogenesis noted between animals treated with Implants 1 and 4. Scrotal measurements were also compared across treatment groups. There was no significant treatment by time effect on total estimated testicular volume ($P = 0.9387$), total estimated testicular mass ($P = 0.9387$), scrotal circumference ($P = .9934$), or percent change in circumference over baseline ($P = 0.0809$).

Histological results from Pilot 1 seem to support the use of the adjuvant DEAE-D in single-dose immunocastration vaccine design. Animals implanted with vaccine containing 100mg DEAE-D and no Quil-A® exhibited less spermatogenesis than animals implanted with only 20mg of DEAE-D. Similarly, animals implanted with 100mg DEAE-D exhibited less spermatogenesis than animals administered implants containing any amount of Quil-A®. Previous work has also indicated variable adjuvant effects on experimental GnRH vaccines. A study examining an oil-adjuvanted (Freund's complete with Freund's incomplete) GnRH-KLH vaccine (Adams and Adams, 1990) gave poor suppression of testosterone but appeared to reduce aggressive behavior (Price et al., 2003). Another experiment using GnRH-OVA in a water-oil

adjuvant containing *M. butyricum* was unsuccessful at reducing testosterone to castration levels but did appear to reduce testicular growth (Hernandez et al., 2005). It is possible that different outcomes could be related to differences in adjuvants or other vaccine components, and further work is needed to fully describe these differences in the context of single-dose GnRH vaccines. Additionally, implant rejection may have also limited duration and profundity of immune response.

Pilot 2

During the course of the study, implants were monitored for rejection. Of 12 calves that were administered Implant 5, only 2 lost their implants over the course of 42 days. One was rejected between day 7 and 14. The other was rejected between day 35 and 42. The retention rate of 10/12 was improved over the previous pilot study, indicating that rejection was related to implant contents as opposed to implant construction material. This is in agreement with previous work that has demonstrated biocompatibility of stainless steel in animal models (as reviewed by Syrett and Davis, 1979). As such, attrition during Pilot 1 was attributed to secondary infection. Future pilots incorporated .5 mg oxytetracycline in the implants to combat this.

Pilot 3

Anti-GnRH antibody responses were measured using ELISA. Implant attrition was also recorded. A total of 5/12 implants were rejected over 105 days. Over the course of the study, antibody response was significantly ($P < 0.0001$) greater for animals administered Implant 7 than for those administered Implant 6 (Figure 12). Additionally, there was a significant ($P < 0.0001$) treatment by time interaction. Antibody levels among animals administered Implant 7 were

significantly higher than animals administered Implant 6 at day 14 (P = 0.0001), 28 (P = 0.0001), and 91 (P = 0.0140). Interestingly, at day 161 antibody levels were significantly (P = 0.0461) higher among animals administered Implant 6 than animals administered Implant 7 (Figure 13).

Results suggest that the addition of at least some adjuvant is useful in stimulating a humoral anti-GnRH response. Implant 7 contained both DEAE-D and Quil-A® whereas Implant 6 contained neither. This result is in agreement with previous work including a recent experiment (Huenchullan et al., 2021) that demonstrated significantly increased anti-GnRH antibodies in response to a recombinant GnRH vaccine adjuvanted with chitosan. In Pilot 3, .5 mg of oxytetracycline was added to all implants in an attempt to halt rejections potentially caused by infection. However, implant attrition again could have impacted the quality of the immune response to vaccination during Pilot 3.

Pilot 4

Anti-GnRH antibody responses were measured using ELISA. Over the course of the study, antibody response was significantly different among treatment groups. Animals administered Implant 7 had significantly higher antibody levels when compared to animals administered Implant 6 (P < 0.0001) as well as those administered Implant 8 (P = 0.0134). Animals administered Implant 8 also had significantly (P < 0.0001) higher antibody levels than animals administered Implant 6. For antibodies, there was a significant (P = 0.0044) treatment by time interaction. When time-matched with animals in Pilot 3, antibody levels among animals administered Implant 7 were significantly higher than animals administered Implant 6 at days 14 (P < 0.0001) and 28 (P < 0.0001; Figure 14). Likewise, antibody levels among animals administered Implant 8 were significantly higher than animals administered Implant 6 at days 14

(P < 0.0001) and 28 (P = 0.0015). At day 28, animals administered Implant 7 displayed significantly (P = 0.0294) elevated antibody response when compared to animals administered Implant 8. Among animals implanted with Implant 6, antibody response did not significantly vary over time.

Results from Pilot 4 support the use of adjuvant(s) in the design of immunocastration vaccines. Antibody response to GnRH was elevated in adjuvanted implants when compared to Implant 6. Specifically, the use of DEAE-D is in agreement with previous work that has examined immunocastration vaccines in pigs (Improvac ®; McNamara, 2009) and cattle (Bopriva ®; Wicks et al., 2013; Walker, 2008). The use of the adjuvant Quil-A® has been documented as a component of immunocastration products aimed at controlling estrus in horses and deer (Equity ®; Campal-Espinosa et al., 2020). Results suggest that adjuvants are valuable components to an implantable immunocastration vaccine, but future work is needed to determine their impact on long-term implant retention.

Pilot 5/USDA1

Over the course of the study, there was a significant (P = 0.0051) antibody difference between treatment groups, with animals receiving Implant 9 exhibiting a greater antibody response than animals that received Implant 10 (Figure 15). However, there was no treatment by time interaction (P = 0.1002). There were no significant time by treatment interactions on raw scrotal circumference (P = 0.815), percent change over baseline (P = 0.8315), or estimated testicular volume (P = 0.3677). Likewise, there was no treatment effect over time on BW (P = 0.9977), average scrotal temperature (P = 0.6766), or average implant site temperature (P = 0.9137). Finally, there was no treatment by time effect on testosterone concentrations between groups (P

= 0.2574). At study conclusion, animals were surgically castrated and testes were examined. Post-castration histology scoring revealed no significant (P = 0.4917) differences in extent of spermatogenesis between treatment groups.

Testicular measurements did not suggest a treatment effect in this pilot. Previous work has shown inconsistent impacts of immunocastration on the size of gonads. One study using an experimental GnRH-KLH vaccine with Freund's complete and incomplete adjuvants noted a reduction in aggressive behavior, but no change to testicular weight in response to treatment (Huxsoll et al., 1998). Conversely, Cook et al. (2000) found that GnRH-vaccinated animals had significantly reduced scrotal circumference over time when compared to controls. In addition, BW did not appear to be affected by treatment during Pilot 5. This is in agreement with Amatayakul-Chantler et al. (2013) who found no impact on BW in response to treatment with Bopriva ®. Implant 9 appeared successful in stimulating anti-GnRH antibodies, but testosterone production remained unchanged. This is contrary to previous work that showed an inverse relationship between anti-GnRH antibody production and androgenesis (Finnerty et al., 1996). Ultimately implant attrition was likely a factor in Pilot 5, as 9/12 implants were rejected over the course of the trial. Predominant in the previous literature are examples of testosterone suppression via soluble vaccines (Theubet et al., 2010, Jago et al., 1997, Robertson et al., 1979) as opposed to implants. Soluble vaccines, despite requiring boost doses, are not subject to rejection from the animal. It is plausible that treatment effects would have been more robust if implants had been retained throughout the experiment.

APPLICATIONS

An immunocastration vaccine capable of delivering the benefits of surgical castration in a single dose remains an attractive prospect to producers and clinicians interested in reducing pain during routine animal husbandry. Results presented here may serve as the foundation on which future implantable vaccines are built. Future work is needed to determine the optimal cocktail of excipients needed to increase implant retention, reduce testosterone, and halt spermatogenesis. Future work should also examine the role that adaptive immunity plays in responding to vaccine delivered over an extended period of time via implant.

FIGURES

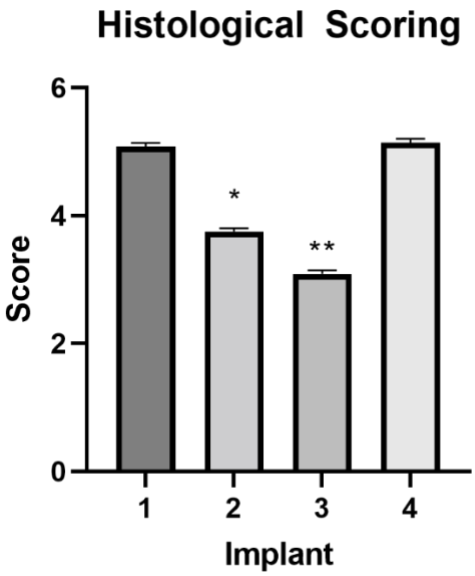

Figure 11 Differences in extent of spermatogenesis among treatment groups in Pilot 1.

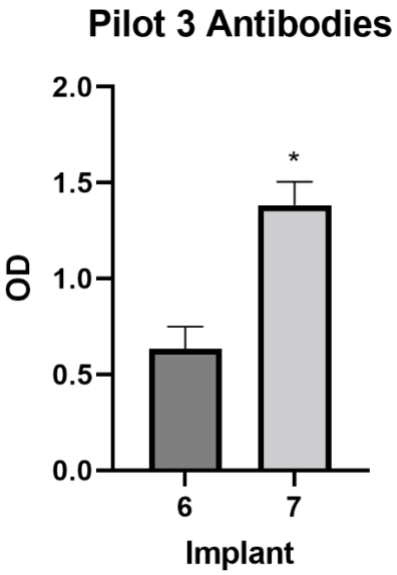

Figure 12 Differences in antibody production between treatment groups in Pilot 3.

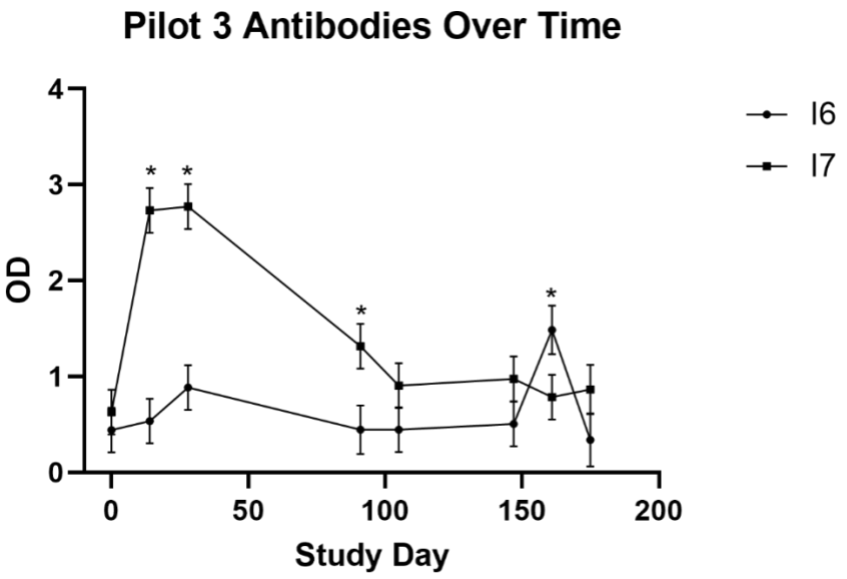

Figure 13 Antibody changes over time as measured during Pilot 3.

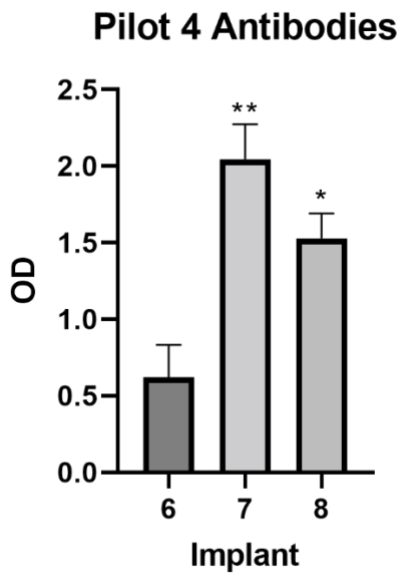

Figure 14 Differences in antibody production as measured over the first 28 days of Pilot 3 and 4

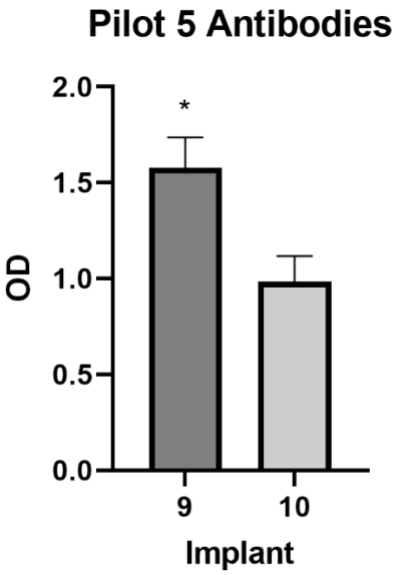

Figure 15 Differences in antibody production as measured during Pilot 5.

TABLES

Implant		PVP	DEAE-D	Quil-A	SP-	GnRH-	GnRH-	Total GnRH	Polyanhydride	OTC
	Primer	100	100			250		.1		
1	Boost		100	.5		250		.1	200	
	VPEAR		100	.5			2.6	1	140	
	Primer	100	100			250		.1		
2	Boost		20	1		250		.1	200	
	VPEAR		10	.5			2.6	1	140	
	Primer	100	100			250		.1		
3	Boost		100			250		.1	200	
	VPEAR		10				2.6	1	140	
	Primer	100	100			250		.1		
4	Boost		20			250		.1	200	
	VPEAR		10				2.6	1	140	
5	Empty									
	Primer									
6	Boost					250				.5
	VPEAR									
	Primer									
7	Boost									
	VPEAR		10	.5			2.6			.5
	Primer									
8	Boost					250				.5
	VPEAR		10	.5			2.6			.5
	Primer									
9	Boost			20		250		.1	100	
	VPEAR			10			2.6	1	140	
	Primer				.2					
10	Boost		10	.5	2.6					
	VPEAR									.5

Table 8 Summary of implant iterations across pilot studies.

Score	Description
1	No cells in tubular cross section
2	Sertoli cells only
3	Spermatogonia only
4	No spermatozoa, no spermatids, < 5 spermatocytes
5	No spermatozoa, no spermatids, many spermatocytes
6	No spermatozoa, < 5-10 spermatids
7	No spermatozoa, many spermatids
8	All stages present, < 5-10 spermatozoa
9	Many spermatozoa, germinal epithelium disorganized
10	Complete spermatogenesis

Table 9 A scoring system designed to quantify degree of spermatogenesis was used.

Chapter 6 - Development of a subcutaneous ear implant to deliver an anaplasmosis vaccine to dairy steers

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ABSTRACT

Bovine anaplasmosis is the most prevalent tick-transmitted disease of cattle worldwide and a major obstacle to profitable beef production. Use of chlortetracycline-medicated feed to control active anaplasmosis infections during the vector season has raised concerns about the potential emergence of antimicrobial resistance in bacteria that may pose a risk to human health. Furthermore, the absence of effectiveness data for a commercially available, conditionally licensed anaplasmosis vaccine is a major impediment to implementing anaplasmosis control programs. The primary objective of this study was to develop a single-dose vaccine delivery platform to produce long-lasting protective immunity against anaplasmosis infections. Twelve Holstein steers, aged 11-12 weeks, were administered a novel 3-stage, single-dose vaccine against *Anaplasma marginale (Am)* major surface protein 1a. The vaccine consisted of a soluble vaccine administered subcutaneously (s.c.) for immune priming, a vaccine depot of a biodegradable polyanhydride rod with intermediate slow release of the vaccine for boosting immune response, and an immune-isolated vaccine platform for extended antigen release (VPEAR implant) deposited s.c. in the ear. Six calves were randomly assigned to two vaccine constructs (n=3) that featured rods and implants containing a combination of two different adjuvants, diethylaminoethyl (DEAE)-Dextran and Quil-A (Group A). The remaining 6 calves were randomly assigned to two vaccine constructs (n=3) that featured rods and implants containing the same adjuvant (either DEAE-Dextran or Quil A) (Group B). Twenty one months post-implantation, calves were challenged intravenously with *Am* stabilate and were monitored weekly for signs of fever, decreased packed cell volume (PCV) and bacteremia. Data were analyzed using a mixed effects model and chi-squared tests **(**SAS v9.04.01, SAS Institute, Cary, NC). Calves in Group A had higher PCV than calves in Group B ($P = 0.006$) at day 35 post-

infection. Calves in Group A were less likely to require antibiotic intervention compared with calves in Group B ($P = 0.014$). Results indicate that calves exhibited diminished clinical signs of anaplasmosis when antigen was delivered with a combination of adjuvants as opposed to a single adjuvant. This demonstrates the feasibility of providing long lasting protection against clinical bovine anaplasmosis infections using a subcutaneous ear implant vaccine construct.

Key Words: *Anaplasma marginale*, anaplasmosis, *Bos taurus*, cattle, implant, vaccine

INTRODUCTION

Anaplasmosis, caused by the rickettsial hemoparasite, *Anaplasma marginale (Am)*, is the most prevalent tick-transmitted disease of cattle worldwide and causes significant disease loss to beef producers in the United States (Uilenberg, 1995; Kocan et al. 2003). In the absence of an effective vaccine, control of anaplasmosis infection is predicated on the administration of low doses of in-feed chlortetracycline for several months (Reinbold et al. 2010c). As of January 2017, control of active anaplasmosis using in-feed chlortetracycline requires veterinary oversight in the form of a veterinary feed directive (VFD). As a result, federal law restricts this medicated feed to use by or on the order of a licensed veterinarian (FDA, 2019). The VFD places an additional regulatory burden on livestock producers and makes anaplasmosis control in extensive and smaller livestock operations especially challenging.

Vaccination strategies to control anaplasmosis are urgently needed to assist livestock producers in combating this disease. Major surface protein (MSP) 1a (MSP1a) is one of six MSP previously described on *Am* derived from bovine erythrocytes (Palmer et al. 2001) and is involved in immunity to *Am* infection in cattle (Palmer et al. 1987). Previous work has shown

that cattle vaccinated with erythrocyte-derived *Am* antigens demonstrated preferential recognition for MSP1a (Brown et al. 2001). The present study was conducted to determine the optimal delivery and adjuvant combination of *Am* MSP1a using a 3-stage vaccine administered as a single injection and long-term subcutaneous (s.c.) ear implant. The unique vaccine implant design allows for a sustained release of the target antigen with an immunoregulatory design to minimize tolerance and achieve long-term immunization in a single dose. We hypothesize that this device will mimic the life-long concomitant immunity associated with persistent *Am* infection after field exposure.

MATERIALS AND METHODS

All animal studies were conducted under an approved Institutional Animal Care and Use Committee (IACUC) protocol (IACUC #: 3959) on file at Kansas State University, Manhattan, Kansas.

Antigen

The peptide antigen R1OK was designed from an *Am* strain originally isolated from a cow in Oklahoma as previously described by Blouin et al. (2000) and characterized by de la Fuente et al. (2003a). The MSP1a genotype of this *Am* Oklahoma strain is K;S-C-H. The multiple antigenic peptide (MAP) for this vaccine was the R1OK peptide, NH2-ADGSSAGGQQQESSVSSQSDQASTSSQLG-COOH, derived from MSP1a tandem repeat K;S (de la Fuente et al. 2003a), which was synthesized as an 8-subunit MAP (Biosynthesis, Lewisville, TX) and shipped as a powder.

Priming Solubilization

To yield an immune-priming dose, 2 mg of R1OK-MAP was solubilized in 2 mL of MES buffer [0.1 M 2-(N-morpholino) ethanesulfonic acid, 0.9% sodium chloride, pH 4.7] and linked via 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride to 2 mg Imject Blue carrier protein (BP) (Thermo Fisher Scientific, Waltham, MA) in 1 mL of MES buffer following manufacturer's recommendations. The conjugate was washed through a polyethersulfone 3K molecular weight cutoff protein concentrator (Pierce, Thermo Fisher Scientific) three times in pH 7.42 phosphate-only buffer prepared according to manufacturer's instructions (Thermo Fisher Scientific). The soluble vaccine consisted of 300 µg R1OK-MAP in 1.0 mL pH 7.42 phosphate-only buffer mixed with 1.0 mL Montanide ISA 61 VG adjuvant (Seppic, Paris, France) for a final volume of 2 mL per injection.

Rod and implant design

The boosting dose relied on a bioerodible polyanhydride (PA) rod 15mm long and 4mm in diameter composed of 20% 1,8-bis(*p*-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) and 80% 1,6-bis(*p*-carboxyphenoxy) hexane (CPH) (20:80 CPTEG:CPH) (Schaut et al. 2018). The molecular weight and copolymer composition of the 20:80 CPTEG:CPH copolymer were 6.618 kDa and 23:77, respectively. The boosting dose rod consisted of 208 mg of PA, 100 µg of R1OK-MAP, and 100 mg of diethylaminoethyl-dextran (DEAE-Dextran) or 500 µg Quil-A (Sigma-Aldrich, Saint Louis, MO) as indicated. The VPEAR implant (Jones et al., 2016) for long-term release (up to 3 years) consisted of 140 mg of PA, 100 µg of R1OK-MAP and 100 mg of DEAE-Dextran or 500 µg Quil-A as indicated. Dry mixtures of all components were pressed in a custom-made mold at 0.5 tons-on-ram for 5 s, using a hydraulic press (International Crystal

Laboratories Inc., Garfield, NJ). The implant was designed and formulated as previously described (Schaut et al. 2018). All but three calves required implant replacement after initial rejection. Rejections were preceded by local inflammation and formation of an abscess around the implant sites. Implant replacements were of identical design to the original implant, except the amount of adjuvant was reduced to either 10 mg of DEAE-Dextran or 50 µg of Quil-A.

Study Animals

A cohort of 12 Holstein steers ranging from 11-12 weeks of age, weighing 102.1 ± 2.3 kg (mean ± SEM) was enrolled in the project. Calves were randomly assigned to vaccine treatment groups using the RAND function in a spreadsheet program (Microsoft Excel, Richmond, WA). Six calves were assigned to two vaccine constructs that featured rods and implants containing a combination of different adjuvants (DEAE-Dextran and Quil-A) (Group A). The remaining 6 calves were randomly assigned to two vaccine constructs that featured rods and implants containing the same adjuvant (either DEAE-Dextran or Quil A) (Group B). All calves received an initial soluble vaccine priming dose s.c. before implantation (See Table 10). As this was a proof-of-concept study conducted over 2 years and animal numbers were limited, we did not enroll a negative control group to evaluate the differences in composition of the vaccine constructs.

Animal vaccinations

The soluble vaccine was administered into the s.c. tissue on the right side of the neck. Immediately afterward, the rod was inserted into the s.c. tissue at the base of the right ear pinna through a six-gauge needle; the implant was then manually inserted through the same incision.

The incision was closed with a single suture. Reimplantation, when required, was done into the left (contralateral) ear, five weeks after the initial implant. All but three calves required reimplantation within the first 5 weeks of the study with devices containing a reduced adjuvant load. All implants were subsequently maintained for the duration of the study, suggesting that rejection may have been due to excessive activation of an immune response to the initial implant device. Reimplantation was not believed to impact total antigen delivery as the rate of release from the implants was engineered to remain consistent for the duration of the study regardless of whether the device was reimplanted.

Anaplasma marginale infection challenge

A cryopreserved field isolate of *Am*, with the MSP1a genotype M-F-F, was administered IV at 21 months after vaccination to infection challenge immunized animals. This represented day zero of the infection challenge phase of the study. Calves weighed a mean ± SEM of 632.7 kg ± 16.3 kg and were approximately 24 months of age at the time of challenge. The *Am* challenge isolate was obtained from a persistently-infected cow in Oklahoma in 2017. Briefly, to prepare the cryopreserved *Am* isolate, 60 mL of blood from the donor cow was collected into a blood bag containing heparin and sub-inoculated into a splenectomized calf to amplify *Am*. The resulting infected red blood cells were washed three times in phosphate buffered saline (PBS), resuspended 1:1 in a stabilate buffer (31.2% dimethylsulfoxide in 1X PBS) after the final wash, and stored in liquid nitrogen as described by Love (1972). Two milliliters of cryopreserved *Am* (M-F-F genotype) stabilate were intravenously inoculated into the jugular vein of each vaccinated steer. The target *Am* challenge dose was approximately 2×10^9 bacteria per inoculation.

Sampling

Beginning at day zero of the infection challenge component of the study, approximately 20 mL of whole blood was drawn from the jugular or coccygeal vein into evacuated tubes (Vacutainer, Becton Dickinson, Franklin Lakes, NJ) containing EDTA (1.8 mg/mL whole blood) or no anticoagulant once weekly post-infection to monitor development of anemia, bacteremia, and antibody response to *Am*. Anemia was evaluated by quantifying packed cell volume (PCV) from whole blood that was collected into EDTA tubes and centrifuged (Micro-Hematocrit Centrifuge CMH30, UNICO, Dayton, NJ). Development of bacteremia was monitored by PCR and microscopic examination of Wright-Giemsa-stained blood smears (HEMA-3, Fisher HealthCare, Pittsburgh, PA). Total genomic DNA was extracted from 100 µL of whole blood collected into EDTA tubes using the *Quick*-DNA Miniprep Kit (Zymo Research, Irvine, CA) according to manufacturer instructions, and DNA was eluted in 35 µL of DNA Elution Buffer. A quantitative, real-time PCR (qPCR) assay targeting a portion of the single-copy *Am* gene MSP5 was used to quantify *Am* infection levels in blood as previously described (Hammac et al. 2013). Quantitative specificity for this qPCR assay is 100 copies per template and qualitative sensitivity is 10 copies per template. Serum was centrifuged at 2,000 x g for 10 min at 20°C from whole blood collected into evacuated tubes containing no anticoagulant. Serum samples were submitted to the Iowa State University Veterinary Diagnostic Laboratory (Ames, IA) for *Am* serological screening using a commercial cELISA that detects host antibodies produced against *Am* MSP5 (Catalog No: 283-2, VMRD, Pullman, WA).

Animal Health

Anaplasmosis is potentially fatal, and animal health was monitored closely to determine need for antibiotic intervention. Animal PCV and rectal temperature were measured twice per week. Veterinary exams were performed if: temperatures measured greater than 39.2^{o}C or less than 36.7^{o}C, PCV was measured at less than 22%, respiration rate was measured at greater than 60 breaths per minute, inappetence was noted for more than 24 hours, or if severe depression was observed for more than 24 hours. Veterinary physical exams included verification of symptoms in addition to assessment of depressed mentation and icteric mucus membranes. Cattle were treated with a single label dose of 200 mg/mL oxytetracycline (Bio-Mycin 200, Boehringer Ingelheim Vetmedica Inc, Duluth, GA) at 20 mg/kg body weight if two or more of the preceding symptoms were displayed as determined by the attending veterinarian (EJR). If an animal required antibiotic intervention, then vaccination was deemed a failure.

Statistical Analysis

Outcome variables PCV, cELISA percent inhibition, bacteremia, and body temperature were analyzed using a generalized linear mixed model incorporating both fixed effects and random effects (PROC GLMMIX; SAS university edition v9.04.01, SAS Institute, Cary, NC). All of these responses best fit to log-normal models. Day post-infection, vaccine treatment group (each of four vaccine treatments tested), vaccine construct (Group A or B; depending on combination or single adjuvants), and their interactions were analyzed as fixed effects in the model with cattle nested in vaccine construct designated as a random effect. Where there was evidence for a vaccine construct by day post-infection interaction, simple effect comparisons of least squares means were conducted using Tukey-Kramer adjustment for multiple comparison. For all

outcomes, statistical significance was set *a priori* at $P < 0.05$. Two-tailed chi-squared tests, with and without Yate's corrections, were used to compare disease outcome between vaccine constructs (combination adjuvants vs. single adjuvant). A Fisher's exact test was also used for comparison between vaccine constructs.

RESULTS AND DISCUSSION

The effect of vaccine treatment group alone was examined but no differences were found when comparing bacteremia ($P = 0.136$) or body temperature ($P = 0.068$). Animals administered the combination adjuvant vaccine construct had higher PCV than those receiving the single adjuvant vaccine construct at day 35 post-infection ($P = 0.006$) (Figure 1). Bacteremia peak coincided with PCV nadir (Figure 1). It is noteworthy that a chi-squared test without Yate's correction revealed that animals vaccinated with combination adjuvants (vaccine construct A) were less likely to require antibiotic intervention compared with calves vaccinated with single adjuvants (vaccine construct B) ($P = 0.014$). A Fisher's exact test revealed a similar trend ($P = 0.061$), as did a chi-squared test with Yate's correction ($P = 0.066$). These results indicate that calves exhibited diminished clinical signs of anaplasmosis when vaccine antigen was delivered with a combination of adjuvants as opposed to a single adjuvant.

These results are in agreement with previous studies that indicated that immunization with native MSP1 (a heterodimer containing disulfide and noncovalently bonded polypeptides MSP1a and MSP1b) of the erythrocytic stage of *Am* conferred protection against pathogen challenge (Palmer et al. 1986). Likewise, Hope et al. (2004) demonstrated the value of using multiple adjuvants to confer immunity and decrease the need for antibiotic intervention in infected animals. This may be due to a broader repertoire of immune effector cells being stimulated by

multiple adjuvants. Previous work has suggested that Quil-A (a heterogenous fraction of saponin) induces activation of dendritic cells and leads to strong antibody and T cell responses (Maraskovsky et al., 2009). Though mode of action hasn't been studied in detail, DEAE-Dextran has shown antibody enhancing properties in anti-fertility vaccines (Vizcarra et al., 2012) and appears to stimulate antigen-specific antibodies and eosinophilia when used in helminth vaccines (Piedrafita et al., 2013).

Assuming vaccine efficacy is based on a reduced need for antimicrobial therapy and increased survival, our data indicate that the use of multiple adjuvants in the vaccine construct could limit disease severity. Though caution is necessary considering the small sample size, it is noteworthy that 100% (6/6) of the animals vaccinated using a single adjuvant (DEAE-Dextran or QuilA) required antibiotic intervention during the study, compared to only 33% (2/6) of animals vaccinated using combination adjuvants (DEAE-Dextran and QuilA).

Despite the documented negative impact of anaplasmosis in cattle herds (Alderink and Dietrich, 1982), there remains no effective means of disease prevention. Controlling the disease once endemic is challenging, as transmission of *Am* can be mediated through biological vectors such as ticks (Dikmans, 1950), mechanical vectors such as horseflies (Baldacchino et al. 2014), blood-contaminated fomites such as needles (Reinbold et al. 2010a), or transplacentally from cow to calf during gestation (Zaugg, 1985). Average weight loss associated with disease progression is reported to be 190 lbs. (Alderink and Dietrich, 1982) with adult (>2yrs of age) cattle being more susceptible to severe clinical disease and death (Kocan et al. 2003). Symptoms diminish in surviving animals, but recovered cattle maintain low, sometimes undetectable, levels of infection (Coetzee et al. 2005). These carrier animals subsequently serve as local reservoirs for disease transmission (Swift and Thomas, 1983). Previous work has estimated that, when

introduced to a naïve herd, anaplasmosis can result in a 3.6% reduction in calf crop, a 30% increase in cull rate, and a 3% mortality rate in clinically infected adult cattle (Alderink and Dietrich, 1982). In spite of these challenges, strategies to control anaplasmosis have not changed markedly in the last several decades (Kocan et al. 2003).

Though not available in the U.S., use of live vaccines containing attenuated or less pathogenic strains of *Am* or *A. centrale* for the control of clinical anaplasmosis is widespread in many parts of the world (Rogers et al. 1988). These vaccines are predicated on the principle of concomitant immunity, the paradoxical immune status in which resistance to reinfection coincides with the persistence of the original infection. Live vaccine-vaccinated cattle develop persistent infections which induce lifelong protective immunity in cattle such that revaccination is usually not required (Shkap et al. 2008). Although generally effective, use of live *Am* vaccines is not legal in the U.S. There are currently no USDA-approved vaccines for protection against *Am* infection or lowering disease severity. An experimental killed vaccine is available in 14 U.S. states, but no efficacy data for this vaccine are available (Aubry and Geale, 2011). Killed vaccines that contain purified *Am* organisms from erythrocytes are expensive to manufacture, may have the potential to induce isoimmune erthrolysis following repeated administration, have unknown efficacy against heterologous strains, and usually require annual revaccination (Kocan et al. 2003). Thus, there remains no vaccine universally accepted as safe and effective against bovine anaplasmosis (Hammac et al. 2013).

This study tested the efficacy of a set of s.c. vaccine implants to protect against the development of antibiotic-intervention-requiring clinical anaplasmosis. Work presented here agrees with previous studies using cell culture-derived *Am* antigens (Kocan et al. 2001). Six MSPs of *Am* have been identified on erythrocyte-derived organisms (Kocan et al. 2003). The

MSP1a is an *Am* adhesin for both bovine erythrocytes and tick cells (de La Fuente et al. 2003b). MSP1a has been explored as a vaccine target for *Am* because the individual tandem repeats contain B and T cell epitopes (Cabezas-Cruz et al. 2015). Cattle immunized with erythrocyte-derived *Am* have been shown to have a preferential antibody response to MSP1a (Brown et al. 2001). Immunization of cattle with MSP1a has also been shown to reduce infection of *Am* for the tick *Dermacentor variabilis* (de La Fuente et al. 2003c). The tandem repeats of the MSP1a subunit-based vaccines with *Am* MSP1a functional motifs have also been shown to induce a balanced humoral and cellular immune response in mice (Santos et al. 2013). It is possible that we would have observed a more robust protective response if we had used an *Am* strain that contained the K;S tandem repeat sequence, as the B and T cell epitopes differ between K;S, M and F tandem repeats (Catanese et al. 2016).

In the present study, our group demonstrated the feasibility of a subunit-based vaccine delivered in a single, subcutaneous ear implant 21 months prior to disease challenge. Future work may expand upon this observation by incorporating other conserved antigens, such as those previously identified (Riding et al. 2003) utilizing a similar vaccine delivery platform. Future work may also establish an optimal adjuvant concentration in order to achieve high immunogenicity without implant rejection.

FIGURES

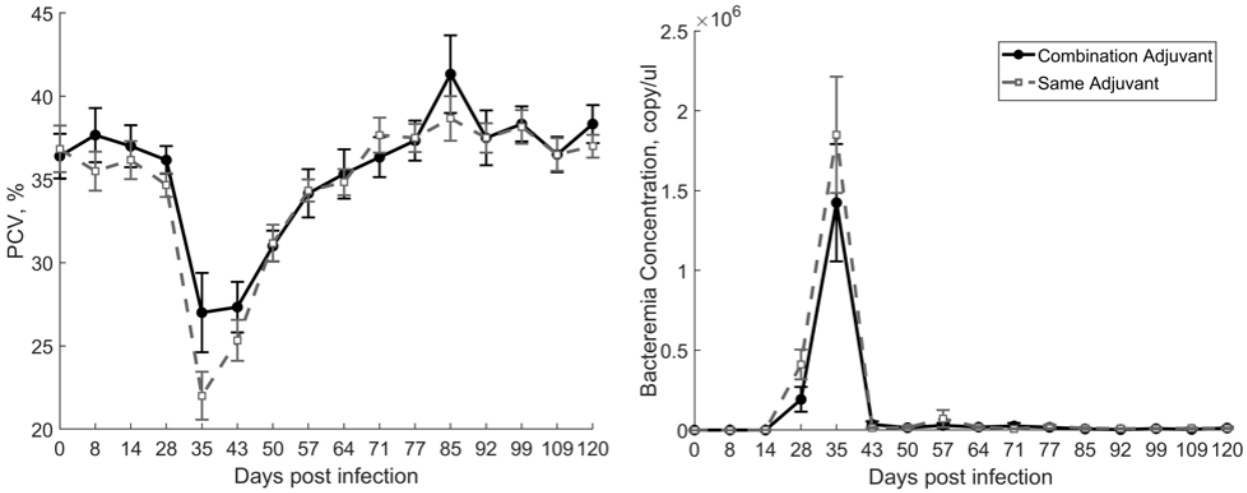

Figure 16. Mean (± SEM) bacteremia and packed cell volume (PCV) of vaccinated animals challenged with *Anaplasma marginale.* (A) PCV is shown over time and separated by vaccine construct. (B) Bacteremia is shown over time and separated by vaccine construct. PCV was significantly higher among animals within the combination adjuvant construct than those within the same adjuvant construct at day 35. * *P* = 0.006.

TABLES

Vaccine Treatment Group[a] (n = 3)	Vaccine Construct[b] (n = 6)	Priming Dose (300 ug R1OK-MAP-BP/ Montanide)	Boosting dose (rod) adjuvant	Implant adjuvant	Vaccine Outcome (Failure/ Treatment)
1	B	1 dose s.c.	DEAE-dextran	DEAE-dextran	3/3
2	B	1 dose s.c.	Quil A	Quil A	3/3
3	A	1 dose s.c.	Quil A	DEAE-dextran	1/3
4	A	1 dose s.c.	DEAE-dextran	Quil A	1/3

Table 10 Random assignment of calves to vaccine treatment groups

[a] Calves were randomly assigned to one of four vaccine treatment groups

[b] Calves were divided between two vaccine constructs (A or B) denoting single or combination adjuvants - diethylaminoethyl-Dextran (DEAE-Dextran) and Quil A

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