REFINEMENT OF AN ESTABLISHED LARGE-ANIMAL MODEL TO UNDERSTAND THE TICK-PATHOGEN-HOST INTERFACE

A Dissertation
presented to
the Faculty of the Graduate School
at the University of Missouri-Columbia

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
in Microbiology

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July 2021

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Dedication

To my wife, family, friends, and our pets, I could not have done this without you and your continuous and loving support throughout these past few years. I am reminded through all of you that life is more than work.

ACKNOWLEDGEMENTS

I would like to first thank my wife, parents, and other family (blood or otherwise) that helped me through this journey to reach this point. The path of graduate school was not always a smooth and seamless one and I could not have done it without your continued love and support. I would like to thank my parents, my Aunt Mary and Uncle Joe, and Dr. Abdalla Aldras who encouraged me to further pursue this career pathway to become a Ph.D.; without them none of this would have been possible.

I would like to further thank Nicole for providing me with support throughout these countless hours of work. For encouraging me and helping me to get through the bad days. Thank you for all your patience during the time we were apart during this graduate schooling time. Twice in-fact (Texas and Iowa) I can say I was happy to be there with you in spirit. We have only just begun our journey and I am glad we get to spend it together.

I would like to specifically thank my friends and family: Josh, Kelsie, Nelvia, Bill, Alexis, Ashley, and Christa for being my pillars of support during these trying times. Especially the Connaway folks who were always around to provide good advice, support, wisdom, and much needed guidance through my experiments and otherwise.

Further, I would like to thank my committee members for all your advice. I appreciate all of the input and wisdom you have provided to me over the years.

Finally, I would like to thank Dr. Stich. I know I joined your lab under unique conditions, and I truly appreciate that. I want to say that you were an excellent mentor and scientist and provided me excellent room to grow. I could not have made it to where I am without you and the projects that we worked on together. I have enjoyed our time working together and look forward to opportunities to work with you in the future. I feel that the phrase you liked to say has really hit home, "Steel sharpened steel."

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REFINEMENT OF AN ESTABLISHED LARGE-ANIMAL MODEL TO UNDERSTAND THE TICK-PATHOGEN-HOST INTERFACE

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ABSTRACT

Ticks are globally distributed vectors of important pathogens of human and animal health. Since the discovery of tick resistance in 1918, the field has sought continuously for the development of effective biologic control of ticks and tick-borne pathogens. A goal of this dissertation was the refinement of a large-animal model system to study species indigenous to the United States. Further, the research described in this dissertation sought to target various aspects of the tick-pathogen-host interface. The research in chapter 2 detailed our work in high-quality sequencing of the non-tick-transmissible *Anaplasma marginale* Illinois strain. We identified several candidate genes and genomic elements associated with the tick-transmission of *A. marginale*. The research in chapter 3 sought to adapt and refine a large-animal model system to evaluate host resistance to *Dermacentor andersoni* ticks. Several proteins isolated from cattle with reduced tick performance were identified in calves immunized with tick salivary gland tissues. The proteins identified here are important for future experiments to determine their posited roles in reduction of tick feeding. Finally, the research in chapter 4 built on this work and suggested that immunization with native salivary gland (NSG) and native midgut (NMG) interfered with *A. marginale* transmission by *D. andersoni*. Further, NSG immunization also had the most consistent and greatest impact on

D. andersoni acquisition of *A. marginale*. The antigens responsible for this protection remain undetermined. The results in this dissertation demonstrate that there are several facets of the tick-pathogen-host life cycle that can be targeted for mitigation of tick-borne disease.

CHAPTER 1

Introduction

Approximately 1.8 million organisms are currently named and documented, and in the class Insecta alone, 1 million organisms exist. Thus, insects comprise approximately 56% of all species documented (1, 2). Human beings have interacted with arthropods, including insects, in positive ways through crop pollination to food sources; however, arthropods have also shaped human history through plagues, pathogen transmission, famine, crop destruction and so on (3–5). The way in which human history has been shaped by arthropods is incalculable, and one of the arguably most important factors is their ability to impact human and animal health as vectors of etiological agents of disease. Ticks are second only to mosquitoes as vectors of etiological agents of human disease and transmit a greater variety of pathogens than any other arthropod group (6, 7). Ticks are of the phylum Arthropoda, class Arachnida, order Parasitiformes and are important vectors of etiological agents of disease (8).

Tick biology and classification

Ticks are ectoparasites that require a blood meal for progression throughout their life cycle and are often important vectors of pathogens that they typically transmit during feeding (9). Ticks can be split into three families: the Argasidae, Nuttalliellidae and Ixodidae (8). The Argasidae is a family of soft ticks which is comprised of 5 genera containing 186 species (8). Due to the rapid nature of

Argasid feeding and their ability to hide, they are generally ignored; however, they are competent vectors of important human pathogens (10).

The Argasidae are mainly nidicolous, which means they prefer to live in burrows, cracks, holes or other small nesting or breeding areas (10). Soft tick feeding often goes unnoticed due to the nature of their feeding and behavior. The feeding period for soft ticks is significantly shorter than hard ticks, at 15-60 minutes depending upon the stage. The life cycle of soft ticks also varies in which there are 4-7 nymphal stages, allowing for one nymph to blood feed many times before molting to adult stages. Soft ticks may lay multiple egg clusters throughout their life span of up to 25 years. An important physiological distinction from hard ticks is that Argasidae lack a dorsal scutum, thus the common name soft tick, and argasid mouth parts originate ventrally so they are often only visible ventrally (10).

The family Nuttalliellidae consists of one valid species and it has been argued that the only current species, *Nuttalliella namaqua*, may be moved into one of the other tick families. However, phylogenetic analysis of the 18S nuclear and 16S mitochondrial rRNA genes has indicated that this species is more closely related to ancestral tick lineages, differentiating it from all other known living tick species as a living linkage between the other two tick families (11).

The family Ixodidae, hard ticks, is found on every continent, including Antarctica, and is arguably the most economically and medically important family of ticks due to their vector potential (12). Ixodidae includes 12 genera which are comprised a minimum of 720 species. Ixodidae is further defined by different phyletic groups, the prostriate and metastriate ticks. The Prostriata consists of the

genus *Ixodes* and all other genera belong to the Metastriata (13). Some prostriate ticks will mate on and off the host, before or after feeding. The majority of prostriate ticks will not hunt, they will instead exhibit questing behavior, where their first pair of legs are held outstretched in order to attach to a host (14). Metastriate ticks will often hunt or ambush their hosts and require blood meals prior to mating.

The focus of the research in this dissertation will mainly be on the genus *Dermacentor*, and specifically the tick *Dermacentor andersoni* or the Rocky mountain wood tick. *D. andersoni* is indigenous to the United States and a documented vector of *Anaplasma marginale*, *Francisella tularenesis* and *Rickettsia rickettsii* (15). In an NIH Bulletin in 1938 (16), Dr. Robert Allen Cooley described *D. andersoni* as a "veritable pandora's box of disease-producing agents." *D. andersoni* was chosen for the future studies outlined in this dissertation because it is indigenous in the U.S. and has a well-studied vectoring capacity for *A. marginale* (17–22).

Life cycle

The life cycle for Ixodidae consists of egg, larva, nymph and adult stages. Each motile stage is dependent upon a blood meal to reach the subsequent stage and adult females require a blood meal for egg production. An adult female typically lays between 2,000 to 10,000 eggs, depending upon the species (4). The eggs hatch and a larva that is approximately 1/10th the size of a nymph will emerge. Nymphs are typically the size of a pinhead and both these immature stages are often thought to feed on smaller animals, while adult feeding will typically occur on large animals (23). However, it is possible to see all three stages on one animal.

The presence of three stages on one animal is especially common among one-host ticks (24).

There are three types of ticks, if sorting by feeding preference and molting behavior: one-host, two-host and three-host ticks (24). The host number refers to the number of vertebrate hosts required for the tick to complete its life cycle. *D. andersoni* is a three-host tick and thus each stage will fall off the host after engorgement. The molted nymph or adult can reattach to the same host or a different host, making control of this type of tick significantly more difficult than a one-host tick. A good example of a one-host tick is *Rhipicephalus microplus*, the "cattle tick". *R. microplus* will have all three stages found on the same animal. *R. microplus* and *R. annulatus* are competent vectors of *Babesia bovis* and *Babesia bigemina* and as such were targeted for elimination from the United States with acaricide dips (25). These one-host ticks have been successfully extirpated from the U.S. using acaricides (25). Three-host ticks require control of all species the ticks may feed upon, which can be substantially more difficult. Two-host ticks involve the larva and nymph feeding on the same host before falling off to find a new host as an adult. Important examples of two-host ticks include *Hyalomma marginatum* and *Hyalomma rufipes* (26).

Mouthparts

The tick mouthparts are specifically designed for long-term attachment and blood feeding on hosts. Among the 20 times that blood feeding has evolved independently during arthropod evolution, tick feeding remains unique in that no other arthropod remains attached for blood feeding as long as ticks (27, 28). The

mouthparts of a tick consist of a basis capitulum, paired palps, unpaired hypostome and paired chelicerae. Ixodid ticks will cut into host skin with the paired chelicerae. The hypostome will hold the tick to the host with recurved teeth and contains an extended channel in the middle for blood sucking and saliva secretion. During the initial two days of feeding, ticks will secrete cement compounds to adhere themselves to the feeding lesion for the duration of the feeding process (28).

Midgut

The midgut is the primary organ involved in storage and digestion of the blood meal and is branched throughout the tick body. During feeding, the midgut will be engorged with blood and a female tick will expand up to 100 times her original weight to obtain the protein necessary for egg laying. Males will not expand to the same degree as they have a full-sized dorsal scutum preventing large imbibement (4). The midgut epithelium layer in ixodid ticks consists of a monolayer of large degenerating digestive cells. These cells will produce hemolysins that are secreted into the blood meal to lyse red blood cells (RBCs). Lysed RBCs are taken into midgut epithelial cells through receptor-mediated endocytosis. The intracellular vesicle will fuse with a lysosome, creating a heterolysosome, where intracellular digestion occurs (28).

The midgut is the primary mediator of tick-pathogen interactions. Ticks have an innate immune response, but to the best of our knowledge they lack an adaptive immune response (29). The host blood meal, when lysed, provides anti-microbial hemocidins and complement factors that play a posited role in response to

bacterial pathogens. The midgut cells themselves express defensins and lysozymes that protect against bacterial infection (29).

Hemolymph

Ixodid ticks, like other arthropods, have an open circulatory system in which a heart pumps hemolymph into arteries leading to hemocoel spaces. The hemolymph directly interacts with organs allowing for transport of nutrients (28). Oxygen is not transported through the hemolymph but is acquired through spiracles on the tick's body surface and transported through tracheal tubes. The hemolymph is important in the immune response to invading organisms, as it is considered the necessary medium pathogens must pass through to reach the salivary glands. Circulating hemocytes, located in the hemolymph, might be targeted for infection and eventual migration to the salivary glands (30). The hemocytes are divided into multiple cell types – granulocytes, plasmatocytes, prohemocytes, spherulocytes and oenocytoids (31). Granulocytes and plasmatocytes are primarily involved in phagocytosis. Prohemocytes, spherulocytes and oenocytoids do not have a defined role in the cellular immune response. Two other cellular mechanisms of response involve encapsulation and nodulation to surround organisms and isolate them. Humoral responses include lectins, complement-like proteins, lysozymes, hemolymph clotting and defensins (29).

Salivary glands

The tick salivary glands are involved in many processes from secretion of saliva to water balance and osmoregulation. The salivary glands are a grape-like

cluster situated near the front of the tick and close to the mouthparts (32). During periods off of hosts, ticks secrete a hygroscopic solution that absorbs water from the atmosphere before it is re-imbibed by the tick (33). When tick feeding begins, the salivary glands will undergo hypertrophy and expand type II and type III acini (32, 34). The secretion of saliva and uptake of blood occur at alternate intervals via the hypostome (35). The saliva contains many bioactive molecules that range from anti-inflammatory to immunosuppressive (35). These molecules evolved with the tick to assist with feeding; however, pathogens often are adapted to take advantage of these molecules to infect vertebrate hosts.

The tick salivary gland is comprised of 3 types of acini, the 4th type is only found in male ticks.

1. Type I Acini. The type I acini are found in all stages of the tick life cycle and function primarily in water balance (36). No secretory granules are found in type I acini. However, they are believed to play a role in initial tick attachment through cement compound secretion (36).

2. Type II Acini. The type II acini are comprised of six cell types that function in secretion (a, b, c1-4) (32). During tick feeding, cell morphology changes, but cell number does not. Type a cells will release secretory granules, while types b and c increase in size and number of secretory granules (36). Type b secretory granules are hypothesized to have a role in immunoregulation.

3. Type III Acini. The type III acini contain three types of cells (d, e and f) and the structure is similar to type II acini (36). Cells d and e are thought to be involved in secretion of cement protein during tick attachment. Type f are agranular

but increase in size and granularity during tick feeding. A majority of the bioactive molecules involved in tick feeding are believed to come from type II and type III acini (36). Alveoli, or several cells clustered together in acini, are found in large numbers in the type III acini and the size increase during tick feeding suggests that most of the excreted fluid passes through the type III acini (32).

4. Type IV Acini. The type IV acini are only found in male ticks and are comprised of only one cell type, g (32). During feeding, it fills with secretion granules; however, the function is not fully understood. It has been suggested that it is involved in copulation to lubricate the spermatophore and prevent adhesion to the male mouthparts or integument (32).

Female Ixodid salivary glands increase rapidly during feeding, approximately 25-fold, in size and protein concentration without increasing the number of cells (37). Within four days after detachment post-feeding, the salivary glands begin to degenerate in female ticks. Male ticks can feed multiple times; thus, this process is different. Copulation with males may assist with female salivary gland degeneration, by stimulating the release of 20-hydroxyecdysone, an ecdysteroid, into the hemocoel which controls the process of female salivary gland degeneration (38–40).

Tick-borne diseases

Ticks are capable of harming human and animal health through exsanguination, toxicosis, paralysis, dermatosis and subsequent secondary infection. However, the most important effect ticks have in the human and veterinary medical world is their ability to be competent vectors of important

pathogens. According to the CDC, of the 650,000 vector-borne disease cases reported in the United States between 2004-2016, approximately 75% of these cases were tick-borne (41). Secondly, the number of cases of tick-borne diseases doubled during that period. Thirdly, tick-borne disease cases are expected to rise, as tick ranges and human interactions with ticks are posited to increase due to climate change (42). Finally, with a globally connected world, the risk of introduction of invasive species is an ever-present threat and, in-fact, just recently the Asian long-horned tick, *Haemaphysalis longicornis*, has been introduced to the United States (43). This tick is a competent vector of severe fever with thrombocytopenia syndrome virus (SFTSV) and *Rickettsia japonica*. It is unknown if *H. longicornis* is a competent vector for pathogens already found endemic to the U.S (43). In the United States, there are multiple important tick-borne diseases of human and animal health, summarized below (Tables 1, 2).

Tick-borne pathogens

Ticks are amongst the most important blood feeding arthropods worldwide in their impact on human and animal health (4, 7). Ticks also transmit a greater number of species and variety of protozoans, bacteria, viruses and nematodes than any other arthropod vector (7, 44, 45). The life cycle of ticks, especially that of three-host ticks, allows them to be efficient vectors of many pathogens throughout their life cycle. Larval and nymphal ticks can easily become infected on reservoir hosts and spread pathogens to other vertebrates throughout the multiple feedings required for progression to adulthood. The typical infection life cycle for a tick-borne pathogen is thought to involve a very similar route, in which a naïve tick

Disease name	Etiological agent	Vector
Lyme disease	*Borrelia burgdorferi*	*Ixodes scapularis* and *Ixodes pacificus*
Hard tick relapsing fever	*Borrelia miyamotoi*	*Ixodes* spp.
Human anaplasmosis	*Anaplasma phagocytophilum*	*Ixodes* spp.
Bourbon virus disease	Bourbon virus	Potentially *A. americanum*
Heartland virus disease	Heartland virus	Potentially *A. americanum*
Tularemia	*Francisella tularensis*	*Dermacentor* spp. and *Amblyomma americanum*
Powassan disease	Powassan virus	*I. scapularis* and *I. cookei*
Human babesiosis	*Babesia microti*	*Ixodes* spp.
Human monocytic ehrlichiosis	*Ehrlichia chaffeensis*	*A. americanum*
Human granulocytic ehrlichiosis	*Ehrlichia ewingii*	*A. americanum*
Rocky mountain spotted fever	*Rickettsia rickettsia* and *R. parkeri*	*Dermacentor* spp.

Table 1. An abridged list of tick-borne diseases and pathogens of human medical importance transmitted by ticks in the United States.

Disease name	Etiological agent	Vector
Lyme disease	*Borrelia burgdorferi*	*Ixodes scapularis* and *Ixodes pacificus*
Bovine anaplasmosis	*Anaplasma marginale*	*Dermacentor* and *Rhipicephalus* spp.
Cytauxzoonosis	*Cytauxzoon felis*	*D. variabilis* and *Amblyomma americanum*
American canine hepatozoonosis	*Hepatozoon americanum*	*Amblyomma maculatum*
Canine anaplasmosis	*A. phagocytophilum* and *A. platys*	*R. sanguineus*
Canine babesiosis	*Babesia canis*, *Babesia vogeli* and *Babesia gibsoni*	*R. sanguineus*
Canine granulocytic ehrlichiosis	*Ehrlichia ewingii*	*R. sanguineus* and *A. americanum*
Canine monocytic ehrlichiosis	*Ehrlichia canis* and *Ehrlichia chaffeensis*	*R. sanguineus* and *A. americanum*

Table 2. An abbreviated list of tick-borne diseases and pathogens of veterinary medical importance transmitted by ticks in the United States.

feeds on an infected host and ingests infected blood into the midgut (46).

The midgut is subsequently infected, the pathogen replicates, then leaves the midgut and enters the hemocoel, the open circulatory system. The pathogen will then infect multiple tick tissues depending upon the type of organism, but usually a tick-borne pathogen moves through the salivary glands and is secreted into vertebrate hosts (35). Thus, the life cycle is completed until another tick feeds on this newly infected vertebrate host. Generally, the invertebrate host range for tick-borne pathogens is limited, while the vertebrate range can be quite broad. A good example of this phenomenon is *Borrelia burgdorferi*, the causative agent of Lyme disease. *B. burgdorferi* is primarily vectored by two tick species in the United States – *Ixodes scapularis* and *Ixodes pacificus* (47, 48). However, the *B. burgdorferi* vertebrate range is large and it is found in birds, lizards, small and large mammals and humans (49–51).

There are three routes for ticks to become infected: intrastadial, transstadial (also known as interstadial) and transovarial. Transstadial infection occurs when a naïve, immature stage of tick feeds on an infected host and then is infected in the subsequent molting stage (52). Intrastadial transmission is primarily a method of infection of male adults in which a male adult tick is infected during feeding and proceeds to feed on multiple different naïve hosts, thus spreading the pathogen.

Transovarial transmission occurs when a pathogen infects a tick ova and thus is passaged to offspring. Some notable examples of transovarial transmission include multiple *Babesia* species, tick-borne encephalitis virus (TBEV) and the Spotted Fever Group Rickettsiae, especially *Rickettsia rickettsii*, (53–57). In

laboratory conditions, it has been reported that *Anaplasma phagocytophilum* is transovarially passaged in *Dermacentor albipictus* ticks, this work has not been shown in its primary vector, *Ixodes* spp. ticks (58–60). It is thought that transovarial transmission in the *Anaplasmataceae* is unlikely, and that tick transmission occurs primarily with transstadial or intrastadial transmission of pathogens (52).

Ticks have evolved with vertebrate hosts for millions of years. It is theorized that ticks evolved as early as 400 million years ago (mya), although it also has been suggested that they evolved in the late Cretaceous Period, approximately 120 mya (9, 61). Ticks evolved to become highly adapted to vertebrate hosts, which is demonstrated through tick saliva and its multiple bioactive molecules. The saliva contains a myriad of bioactive molecules that function as immunosuppressants, immunomodulators, vasodilators, anti-coagulation factors and anti-itch molecules (35, 62). These salivary components have evolved to help ticks avoid host detection and encourage efficient tick feeding. Pathogens have evolved closely with ticks, for example *B. microti* was found in a 15-40 mya tick preserved in amber (63). *B. burgdorferi* is known to take advantage of tick saliva secretions, such as Salp15, which inhibits deposition of complement complexes on the cell membrane (64, 65). This kind of adaption in which pathogens take advantage of the saliva components to aid in infection occurs in many pathogen species (66–68).

Tick-borne pathogens were thought to not harm their vector host as harm to the vector would negatively impact fitness; however, it has been shown that infection with *R. rickettsii* reduces tick survival (69). *A. phagocytophilum* infection

may encourage survival through the induction of genes involved in the production of antifreeze glycoprotein (70). Unfortunately, the research on the topic of tick survival due to pathogen infection is limited. It does seem likely, though, that most pathogens do not negatively impact tick survival. If this were the case, tick-borne pathogen transmission would not occur with such prevalence globally.

Anaplasma

In the order *Rickettsiales*, family *Anaplasmataceae* lies five genera: *Ehrlichia*, *Anaplasma*, *Wolbachia*, *Aegyptianella* and *Neorickettsia* (71). The family *Anaplasmataceae* was expanded in 2001 and resulted in a myriad of changes, including adding *Anaplasma phagocytophilum*, *Anaplasma platys* and *Anaplasma bovis,* which were all previously classified as *Ehrlichia*, to the genus *Anaplasma* (72).

In Europe, in 1780, tick fever was reported in goats, cattle and sheep. The etiological agent at the root cause of this fever condition was unknown but symptoms corresponded well to what is now known as granulocytic anaplasmosis. Specifically, this bacterium in question was posited to be *A. phagocytophilum* – which could make this the first description of anaplasmosis (73, 74). Further, in 1893 Smith and Kilborne reported an inclusion in calf erythrocytes as *Babesia bigemina* (75). Only in 1910 did Sir Arnold Theiler define this ‘inclusion’ as the bacteria we know today as *A. marginale* (76).

The genus *Anaplasma* includes 9 species; however, there are 7 more proposed species (77). Some of the most notable species of *Anaplasma* that cause

significant impacts to human and animal health include *A. marginale*, *A. phagocytophilum* and *Anaplasma centrale*.

Anaplasmal pathogens of humans

Three species of *Anaplasma* infect humans: *A. phagocytophilum*, *Anaplasma platys*, *Anaplasma ovis* and *Anaplasma capra* (77–81). The most well-known of these species is *A. phagocytophilum*, which was first described in a modern report in Scotland (1932) in which a herd of sheep were reported to have tick-borne fever (TBF) (82). Several reports of TBF were documented in cattle and sheep throughout Europe (83–85). TBF was first documented in horses in 1969 (86). It was only in 1994 that a documented human case of *A. phagocytophilum*, at the time *E. phagocytophila*, finally helped lead to the realization in 2001 that all of these cases were caused by the same organism with distinct variants that are capable of infecting many mammalian species (87–90). *A. phagocytophilum* in humans is also identified in the literature by the name human granulocytic anaplasmosis (HGA). In the United States it is primarily vectored by *I. scapularis.*

A. ovis, *A. platys* and *A. capra* were reported to infect humans in 2007, 2014 and 2015, respectively (78, 79). The diagnosis of these three pathogens in humans is a new and as such there are relatively few published papers discussing the impact of these three pathogens. The number of reported human cases available for these three pathogens is also exceptionally small, thus the impact and range of these pathogens is unknown. It seems unlikely that they are as widespread as *A. phagocytophilum*. Due to the recent diagnosis and identification of some of

these *Anaplasma* species that infect humans, it seems possible that the future may yield new, currently unknown, species that may infect humans.

Anaplasmal pathogens of animals

Most species in the genus *Anaplasma* infect animals, and this list includes *A. ovis*, *A. bovis*, *A. phagocytophilum*, *A. centrale*, *A. marginale*, *A. platys*, and *A. odocoilei* (77). All species of *Anaplasma* target ruminants primarily, except for *A. platys* which targets camels and dogs. There are varying degrees of pathogenicity, for example *A. ovis* and *A. centrale* are generally thought to cause mild disease (91). *Anaplasma* spp. have a tropism for the erythrocyte; however, there are some exceptions: *A. phagocytophilum* – granulocytes (neutrophils, primarily), *A. bovis* – monocytes, *A. platys* and *A. odocoilei* – platelets, finally *A. capra* – unknown (77).

Anaplasma marginale

Anaplasma marginale is a gram-negative obligate intracellular pathogen of ruminant erythrocytes (92). *A. marginale* is named due to the location of the bacteria in the erythrocyte. It is often found on the margin of erythrocytes, often seen as a dot marking the edge. This dot is referred to as an marginal body which contains the replicating bacteria (93, 94). The exact molecular mechanisms for exocytosis of the initial bodies remain a mystery, since bovine anaplasmosis is classified as lacking hemoglobinuria and hemoglobinemia, indicating no or limited cell lysis (17).

Arguably, the most important problem presented by *A. marginale* is the economic impact on the cattle industry. Bovine anaplasmosis due to *A. marginale* has been estimated to cost $300 million in the U.S., $800 million USD in Latin

America and $30.5 million USD in Australia (17, 95). This problem is further enhanced by the worldwide presence of *A. marginale*, especially in tropical and subtropical climates.

Transmission

A. marginale can be spread through contact with infected blood through blood-contaminated fomites (17). However, the primary mode of spread is biologically through select ixodid ticks of the tribe Rhipicephalinae or mechanically by biting flies, respectively (17, 96, 97). Multiple reports have consistently shown lack of transmission by *Amblyomma* spp. and there is conflicting evidence for *Ixodes* spp. transmission (97). Multiple studies have reported on the conditions of intrastadial, transstadial and transovarial transmission of *A. marginale* to susceptible animals, summarized in Table 3.

Adult male ticks will intermittently feed several times and can exchange between hosts to allow transfer of pathogens, this process is termed intrastadial transmission (52). Experimental research has documented that intrastadial transmission of *Anaplasmataceae* species does occur (52, 98–100). Intrastadial transmission is reported to have an important epidemiological role in tick transmission of rickettsial pathogens (101).

The minimum number of reported ticks necessary for *A. marginale* transmission was reported by Rozeboom *et al.* in 1940, in which one tick was capable of transmitting *A. marginale* to a susceptible animal (102). This male *D. andersoni* was removed from an infected animal, held for one day, subsequently fed on a naïve host for five days and the host developed anaplasmosis as identified

Reference	Ticks used	Transtadial/intrastadial	Number of Ticks	Results
Rees (103)	*D.v. D.a.*	Transstadial	Not listed	+
Rees (104)	*D.a.*	Transstadial	Multiple with lowest at 50 pairs	+
Stich *et al.* (105)	*D.v.*	Transstadial	100 nymphs; 150 pairs of adults	Intrastadial +/ - Transovarial
Boynton *et al.* (106)	*D.a., D. o., D. albipictus*	Unknown	Not listed	+
Rozeboom *et al.* (102)	*D.a.*	Unknown	1 male or 4 larvae clutches on 3 cows	+ Adult male/ - Transovarial
Anthony & Roby (107)	*D.v.*	Unknown	2 males	+
Bram & Roby (108)	*D. albipictus*	Transovarial	10 egg clutches	-
Bram (109)	*D.a.*	Transstadial	Variable (lowest 25)	+
Howarth & Hokama (110)	*D.o.*	Transstadial & Transovarial	25 pairs	+ Transstadial/ - Transovarial
Kocan *et al.* (20)	*D.v., D.a.*	Transstadial	600 adults/cow	+
Kocan *et al.* (111)	*D.a.*	Transstadial	25 pairs	+
Wickwire *et al.* (112)	*D.a.*	Transstadial	25 pairs	+
Samish *et al.* (113)	*B. annulatus*	Both	100 and 220 nymphs; 100 & 300 adults	+
Eriks *et al.* (114)	*D.a.*	Both	Up to 50 pairs	+
Aguirre *et al.* (115)	*B.microplus*	Transstadial	10, 20, 40 & 80 nymphs	+
Kocan *et al.* (116)	*D.a.*	Transstadial	50 ticks total	+
Scoles *et al.* (96)	*D.a.*	Intrastadial	3 ticks and 30 ticks male only	+
Ueti *et al.* (117)	*D.a.*	Intrastadial	10 and 35 ticks male only	+
Scoles *et al.* (118)	*D.a., B. microplus, B. annulatus*	Intrastadial	120 *B.m.*, 66 *B.a.*, 59 *D.a.* total	+
Lankester *et al.* (119)	*D.v., D.a.*	Intrastadial	90 *D.v., D.a.* as low as 3 up to 48	+
Zivkovic *et al.* (120)	*D. reticulatus*	Intrastadial	30 adults total	+

Table 3. Reported cases of transstadial, transovarial and intrastadial transmission of *Anaplasma marginale* by *Rhipicephalus* and *Dermacentor* spp.

Abbreviations include *D.a.* for *Dermacentor andersoni*; *D.v.* for *Dermacentor variabilis*; *D.o.* for *Dermacentor occidentalis*; *D.* for *Dermacentor*; and *B.* for *Boophilus*.

by blood smear. Rozeboom *et al.* did not list the conditions in which the cattle were raised, which is a limitation of this study as it is unknown if these cattle were exposed to mechanical infection by biting flies outdoors. In 2005, Scoles *et al*. infected multiple adult male ticks, maintained under laboratory conditions for 24 hours, and subsequently fed infected ticks on susceptible cattle. A minimum number of three ticks was capable of transmitting the infection to susceptible animals for intrastadial transmission (96).

For transstadial transmission of *A. marginale* the minimum number of reported ticks was 16 females and 20 males; however, this study placed 50 pairs of ticks on bulls at once in a sack around the scrotum but only recovered 16 females and 20 males between 8 to 11 days after attachment (104). The limitation of this study is that it is unknown how long the 50 pairs fed before they died, thereby causing the lower recovery numbers. It is known that after 6 days of feeding, *A. marginale* can be transmitted by the tick to the vertebrate host (111). Therefore, it is conceivable these ticks attached for less than six days and died, thus not allowing for the conclusion that 16 females and 20 males is the minimum number necessary for transstadial transmission. Multiple reports have shown that 25 pairs of ticks transstadially infected is sufficient to transmit *A. marginale* to hosts (109, 110, 112). The information in the literature suggests that 25 pairs is commonly used. However, we have been successful with a lower number of transstadially infected adults for transmission of *A. marginale* to naïve cattle.

Transovarial transmission involves passage of a pathogen adult female ticks to progeny. This type of transmission has not been reported in *A. marginale*

and multiple reports in Table 3 suggest that *A. marginale*, is not transmitted through transovarial passage (108, 110).

Electron micrographs of infected blood from the midgut of fed ticks reveals that *A. marginale* bundles actin filaments, presumably from bovine red blood cells (121). These actin bundles are referred to as inclusion appendages and their role is not fully understood, but it is hypothesized that actin-based motility is utilized to allow for increased contact with the midgut epithelium which leads to infection of the tick (122, 123). A protein called the *A. marginale* appendage associated protein (AAAP) is found to be associated with the inclusion appendage, which may play a role in actin bundling, but the exact function of this protein is unknown (124). The molecular mechanisms involved with *A. marginale* infection and replication in the tick midgut are poorly understood; however, research has shown that the midgut acts as a distinct barrier for infection for the non-tick-transmissible Florida strain (118, 125). Comparative genomics and transcriptomics were used in an attempt to identify the mechanisms involved in *A. marginale* tick infection through the study of the Florida strain, which yielded 30 genes or promoters that were potentially involved (126).

After infection of the midgut, *A. marginale* begins replication in a parasitophorous vacuole, initially in the reticulate (vegetative) form. Afterwards, the organism changes to a dense form which appears to survive outside host cells (93). This form leaves the midgut epithelium through the basal surface and enters the hemocoel to migrate towards the salivary glands (127). The salivary glands are subsequently infected; however, the mechanism of entry has not been reported.

A. marginale will replicate in the salivary glands and can be secreted into the saliva on or after six days of tick feeding (111, 128).

Clinical disease

A. marginale infection is the primary cause of bovine anaplasmosis. The clinical signs of anaplasmosis include fever, anemia, icterus, loss of appetite, weakness, dehydration, difficulty breathing and abortion in pregnant animals (129). Younger animals are generally more resistant to clinical disease, with those older than 2 years of age at a higher risk of death (93). Disease is characterized by a lack of hemoglobinemia and hemoglobinuria; this is due to phagocytosis of infected erythrocytes by reticuloendothelial cells. The animals that survive are chronically infected carriers with cyclic levels of bacteremia, termed rickettsemia, between 10^2 to 10^6 organisms per mL of blood (130). Despite a persistent infection, these animals often remain subclinical, immunocompetent and protected from acute anaplasmosis upon subsequent exposure to *A. marginale* (131).

Immunology

Cattle that are not treated with antibiotics for *A. marginale* infection are classically referred to as carrier animals, as the host is typically unable to clear the infection and becomes a chronic carrier (132). Further, premunition is posited to play a role in cattle infected with *A. marginale*, in which an animal is infected with *A. marginale* and in subsequent infections the clinical symptoms are reduced or absent due to prior exposure the pathogenic organism (133). The erythrocyte, which lacks MHC presentation (134), is the target of *A. marginale* infection; thus, external antigen must be taken up and presented by antigen presenting cells for a

response against the bacteria (135). Typically, a response to *A. marginale* is classified by a robust antibody response, IgG1 and IgG2 against Major surface protein 2 (Msp2), with other major surface proteins inducing some antibody response (136). However, antibody responses are typically not protective against *A. marginale* infection. Passive transfer from either infected or immunized animals does not confer protection to naïve animals (137). The *A. marginale* Msp2 and Msp3 will create variants of themselves through nonreciprocal homologous recombination, theoretically allowing for immune evasion (138, 139). When an antibody response is generated to Msp2 and Msp3, the variants are cleared, and then a subsequent generation of new variants appears to which the antibodies do not respond, as if in a complex game of cat and mouse (140). Msp2 is flanked by conserved C and N terminal regions with a hypervariable region (HVR) in the middle (141). The variation encoded in the *msp2* locus comes from partial or whole hypervariable regions from *msp2* pseudogenes that are recombined into the HVR of the *msp2* operon, creating a new variant (139). The number of pseudogenes varies per strain, but there are multiple pseudogenes allowing for many different variants to be produced. It has been reported that this variation leads to loss of protective antibody responses (139).

A. marginale replicates to high levels in the host, resulting in cyclic levels between $10^{2.5}$ to 10^{7} *A. marginale* infected erythrocytes per milliliter of blood (96). The high bacterial load is believed to play a role in induction of a T-cell exhaustion state (142), which may be responsible, in part, for persistent *A. marginale* infections. In one study, cattle were immunized with gel-purified Msp2 to monitor

T-cell responses during acute phase anaplasmosis. Peripheral blood mononuclear cells (PBMCs) collected before or following challenge, were challenged with whole Msp2, Msp2-derived peptides, phytohemagglutinin (PHA), *Clostridium* vaccine control, IL-12 and IL-18 positive controls (143). These challenged PBMCs were used for IFN-γ specific ELISPOTs. The PHA, IL-12, IL-18 and clostridium vaccine controls remained elevated before, during and after peak rickettsemia; however, the responses to Msp2 were completely ablated during peak rickettsemia and afterwards. It was reported that antigenic variance of the HVR would not explain the lack of response, as there was no response to conserved flanking regions either. A similar response was observed with peptide immunization of an Msp1a construct known to elicit an MHC II response (144). This immunization was against a single T-cell and B-cell epitope, allowing for tracking of antigen specific $CD4^+$ T-cells using MHC Class II tetramer staining. A rapid loss of antigen specific $CD4^+$ T-cells was observed in immunized animals while response to the *Clostridium* vaccine remained stable. Not all cells were cleared, though, as some tetramer positive cells were found in the spleen and liver but did not respond to antigen challenge. The above results indicate that *A. marginale* does not induce a general T-cell suppressive response.

Lastly, a study in 2014 with cattle immunized against outer membrane proteins saw a similar T-cell exhaustion phenotype (142). The authors subsequently treated animals with tetracycline and monitored T-cell responses by assaying IFN-γ and TNF-α, and it was observed that these responses were partially restored upon tetracycline treatment. The above studies suggest that *A.*

marginale induces a T-cell exhaustive state combined with major surface protein variation to maintain a chronic infection in carrier animals.

Diagnosis

Diagnosis of *A. marginale* infection is achieved by a few methods – blood smear, complement fixation, competitive-ELISA (cELISA) or PCR. The first clinical signs of anemia are often important in diagnosis of anaplasmosis. A simple method for diagnosis is a blood smear, stained with Camco-Quik or Diff-Quik stains (17). The marginal bodies, which contain multiple bacteria, will be visible on the margins of the red blood cells.

Some of the older methods of diagnosis include complement fixation and card agglutination tests. These tests lack accuracy and better methods are available for determination (145, 146). The cELISA commercially available for *A. marginale* detection is one of the most cost-effective and rapid methods for diagnosis. The cELISA detects antibodies to Msp5 in host serum (147). According to VMRD, the manufacturer of the *Anaplasma* cELISA, the cELISA assay will react with *A. marginale*, *A. ovis* and *A. centrale*.

Finally, PCR is a rapid and highly specific method for identification of many pathogens. Both PCR and qPCR are used in multiple publications for the detection of *A. marginale* organisms. The University of Missouri Veterinary Medical Diagnostic Laboratory also provides services for qPCR via Sybr Green or conventional PCR. The common targets of PCR for detection include *msp1b*, *msp4*, *msp5* and *16S* rRNA genes (80, 148–150).

Treatment

During the acute phase of clinical anaplasmosis, it is critical to provide immediate treatment of animals with antibiotics of the tetracycline family while packed cell volume (PCV) remains above 15% (151). Animals are treated intramuscularly, typically one 20 mg/kg dose of long-acting tetracycline is sufficient (151). Blood transfusions can help restore PCV in response to *A. marginale*-induced anemia. Not all animals require treatment, animals that recover become subclinical carriers. Younger animals tend to be able to recover much more quickly (151). We have seen a wide range of variation in response to infection; some animals become extremely sick and require intervention, while some may not display mild clinical disease and PCV will not drop precipitously. However, these animals test positive by PCR.

Vaccinology

Vaccinology, or the study of vaccines, formally began in the 18th century with smallpox and variolation. There is some controversy as to who first practiced variolation. In 2003, a paper was published that suggested Benjamin Jesty in 1774 was the first to practice vaccination with cowpox against smallpox (152). Regardless, the work of Edward Jenner is immortalized, from his first vaccine in May 1796 with an inoculation of an 8-year-old boy using a lesion of cowpox from a milk maid, Sarah Nelms (153). Edward Jenner was a strong proponent of vaccination throughout his life and his work was essential in establishing vaccines and changing the field of medicine forever. Vaccines have come a long way since this first attempt, with over 58 vaccines against 26 pathogens licensed in the United

States, according to the CDC. As of this writing, multiple vaccines are available for use against SARS-CoV-2, which is the cause of the 2019-2021 global COVID-19 pandemic (154).

Crude whole tick tissue immunization

One of the earliest reports to document animal resistance to tick feeding was the initial work of Johnston and Bancroft in Queensland, 1918 (155), in which animals were reported to be more resistant to tick feeding based upon breed. This report provided evidence of a hereditary component of resistance to tick feeding. Trager in 1939 subsequently demonstrated that infestation and vaccination against ticks is possible and effective (156). In this study, multiple rabbits and guinea pigs were repeatedly fed on by *D. variabilis* larvae and nymphs, resulting in the reduction of the number of larvae that engorge on challenge infestations. Furthermore, guinea pigs were immunized with an extract of homogenized larvae and a subsequent challenge infestation yielded very poor tick survival. Research since 1939 has documented similar phenomena of reduced tick performance post-immunization of hosts with crude tick extracts (157–161). Willadsen suggested that a benefit to immunization is that hosts are now exposed to "concealed" antigens, *i.e.* those that the hosts are not exposed to during tick feeding due to the physiologic characteristics of the tick (162). In contrast, Barriga *et al.* suggested that tick infestation may result in competition between irrelevant and protective antigens, because in their study it was observed that there was an inverse relationship between antibodies and tick resistance (163). Allergic responses to ticks are well documented (164–167). Thus, to the possibility that crude lysate

vaccination may induce hypersensitivity, it is ideal to identify purified target antigens for vaccination.

Midgut immunization

The salivary gland and midgut organs are the two organs that directly interface with the host immune response due to their functions. The salivary gland will secrete saliva directly into the host and the midgut will be engorged with blood containing antibodies and other immune effectors. Several studies have validated the approach of using midgut for immunization (168–170).

One of the earliest studies to document midgut immunization was done by Allen and Humphreys in 1979, in which guinea pigs and cattle were immunized with partially engorged *D. andersoni* midgut and reproductive tissues (171). Ticks that were recovered had decreased performance as indicated by lower engorgement weights, increased mortality, less egg laying, lower egg cluster weights and lower yield of larvae. Guinea pigs immunized with antigen prep II, containing all other organs, resulted in no engorged ticks recovered. Suggesting that other tick tissues may provide a more important target. In 1980, Ackerman *et al.*, immunized rats with *D. variabilis* midgut or whole tick antigen preparations (172). Tick performance was notably lower in midgut immunized animals, while those immunized with whole tick preparations did not have the same effect. The performance was in stark contrast to the work by Allen and Humphreys; this phenomenon is potentially explained by differences in the tick species used and the host species challenged. Alternatively, whole tick lysate may support Barriga's hypothesis that irrelevant antigens may compete with protective antigens.

Immunization with solely midgut tissue has been shown to have a more pronounced impact on tick fecundity. Sahibi *et al.* in 1997 first reported that immunization of cattle with “intestinal,” or midgut extract of *Hyalomma marginatum* subsp. *marginatum*, has the most pronounced impact on tick fertility (173). Further work by Jittapalapong *et al*. in multiple studies from 2000 and 2004 found a similar phenotype of reduced tick fecundity performance parameters of ticks fed on midgut immunized hosts (168, 169). The first study was with *R. sanguineus* and dogs and the second focused on *R.* (*Boophilus*) *microplus* and cattle. These studies validated the approach of midgut immunization with different *Rhipicephalus* species ticks and different vertebrate hosts. The above work also demonstrated a unique form of protection: targeting the midgut can affect tick fecundity.

Salivary gland immunization

Multiple studies with different vertebrate hosts and tick species have confirmed that immunization with tick salivary glands negatively impacts tick performance (173–176). In contrast to midgut immunization, salivary gland extract immunization has been demonstrated to consistently target tick feeding performance. In 1997, Sahibi *et al*. reported that ticks that fed on salivary gland extract immunized cattle had the lowest feeding performance parameters (173). Jittapalapong *et al*. confirmed this work in 2000 and 2004 with the same studies as previously mentioned (168, 169). Immunization with tick salivary gland extract provides a unique target because the secreted proteins in the saliva are expressed at significantly higher quantities in salivary gland extract. Thus, immunization with salivary gland extract allows the vertebrate host to produce a strong response to

all the proteins they would normally encounter in tick saliva and to concealed antigens that remain associated with the tick acini.

Anti-tick vaccines

Mechanisms of protection

The mechanism of protection against ticks may likely be dependent upon the type of tissue used for vaccination. The midgut is exposed directly to host blood, which is concentrated in females consequently also concentrating the immune effectors. Complement, antibodies and host cells directly interact with the midgut. Administration of midgut extract to pigs and mice isolated from *Ornithodoros erraticus* led to tick resistance in pigs and mice (177). Mice were decomplemented using cobra venom anticomplementary protein (CVF), which completely abolished protection. The authors hypothesized that the luminal surface midgut proteins were targeted by the complement cascade. Host antibodies have been found inside the tick hemolymph (178, 179). It is unknown if complement can cross the tick midgut barrier; however, if complement enters the hemolymph then a potential means of protection involves the complement cascade with antibodies specific to concealed tick antigens.

Vaccination of cattle with whole *R.* (*Boophilus*) *microplus* resulted in damage of tick midgut, allowing for leukocyte entry into the hemolymph (180). Other research by the same authors suggested complement is necessary for initial damage to the tick midgut epithelium, permitting leukocyte entry (181). The authors confirmed with histopathology that leukocytes damaged tick muscle, Malpighian tubules and reproductive organs. Control and naturally acquired resistant animals

did not present with this damage (180). This work provides another mechanism of host resistance to tick feeding through complement, antibody and leukocyte damage to tick tissues.

At the tick feeding site, basophils are recruited in response to repeated infestation (182, 183). In guinea pigs, ablation of basophils and subsequent lower recruitment of eosinophils in sensitized animals removed resistance to tick feeding (184). A more recent study found that acquired tick resistance in mice to *Haemaphysalis longicornis*, is dependent upon histamine and that adoptive transfer of basophils and mast cells from histamine-sufficient mice to mice lacking basophils or mast cells revealed that only histamine produced from basophils was essential (185). According to histopathology studies, basophils accumulated around tick mouth parts while mast cells were dispersed and found in the dermis, which is more distant from the tick mouth parts. Histopathology of feeding lesion sites from salivary gland-immunized rabbits demonstrated that salivary gland immunization induced strong inflammatory infiltrate, local edema, epithelial stratification, and vascular dilation as compared to controls (186). Vaccination against a tick cement protein (64TRP) protected mice against fatal tick-borne encephalitis virus (TBEV) infection from tick feeding, leading the authors to suggest that this was mediated by a strong cutaneous inflammatory response at the tick feeding site and that this was assisted by $CD8^+$ responses (187). Inflammatory responses through multiple cell types at the tick feeding site provide another mechanism of tick resistance and protection against tick-borne pathogens.

Transfer of sera from resistant animals will confer tick resistance (156). Sera from hyperimmune guinea pigs, due to repeated infestation, transferred intraperitoneally to naïve guinea pigs, negatively impacted larval engorgement. Passive transfer of serum from rabbits infested with *I. ricinus*, cattle with *B. microplus*, guinea pigs with *D. andersoni*, and separately with *I. holocyclus*, passively transferred acquired tick resistance (188–191). Further, damage to tick salivary gland acini can be found in histopathology studies of *R.* (*Boophilus*) *microplus* fed on cattle immunized with tick salivary gland extract (192). These studies indicated that antibodies may provide a mechanism of protection against ticks.

There are multiple mechanisms detailed that have been shown to influence ticks and tick feeding. It is conceivable that these mechanisms will work in tandem. The mechanism of protection may also depend upon the type of antigen used; however, whole tissue lysate may prove efficacious against multiple tissue types due to conserved housekeeping proteins expressed between different tissues. In summary, the mechanism of protection is likely dependent upon the type of antigen used and may provide protection against the ticks and tick-borne pathogens.

Commercially available vaccines

The first commercially available anti-tick vaccine was Tickgard, which was based upon the midgut *R.* (*Boophilus*) *microplus* glycoprotein, Bm86 (193). A re-cloned Bm86 from *R. microplus*, Bm95, is the basis for the only commercial vaccine currently available, Gavac (193–195). Gavac provides protection against ticks by reducing the number of females engorging, female engorgement weight

and reproductive potential for subsequent generations (196, 197). A study in Cuba with 588,573 Gavac-vaccinated dairy calves found an 87% reduction in the use of acaricides and a significant reduction in the number of clinical babesiosis cases (198). The authors suggested that this reduction in babesiosis was mediated by a significant reduction in tick offspring, thus affecting the endemic status of *Babesia* in the region. IgG specific to Bm86 mediated damage to gut epithelium in combination with the complement cascade (199–201).

Recombinant vaccine targets

Many recombinant targets have been identified using reverse vaccinology against multiple different tick species and these targets have been demonstrated to reduce tick feeding and reproduction (202). However, none of these vaccines are commercially available. Immunization with crude tick salivary gland extract reduced the numbers of ticks that were PCR-positive for *Babesia* and numbers of cattle with clinical babesiosis (203). Multiple tick recombinant proteins reduce tick-pathogen infection (204). For example, it was reported that vaccination of cattle with subolesin reduced *R. microplus* survival and infection by *B. bigemina* or *A. marginale* (205, 206). Mice immunized with salivary gland protein, Salp15, had lower levels of infection with *B. burgdorferi* (207). This work demonstrated that vaccination of vertebrate hosts against the tick can negatively impact tick survival, feeding and tick-borne pathogens.

Research problem

Tick-borne transmission of *A. marginale*

A. marginale has been studied extensively since the initial work of Sir Arnold Theiler in 1910. However, there is a great deal to learn about the molecular determinants of infection. Other pathogens, such as *B. burgdorferi* and *A. phagocytophilum*, have been studied to determine their molecular determinants of infection of ticks and vertebrate hosts (208–210). *A. marginale* infection and replication in the midgut, migration into the hemolymph and infection and replication in the salivary glands, has been documented and mentioned previously in this dissertation (19, 127, 211, 212). Bundled host cell actin filaments, termed inclusion appendages, have been identified with the *A. marginale* appendage-associated protein (AAAP) in infected erythrocytes (122–124). These inclusion appendages have been hypothesized to be involved in actin-based motility of *A. marginale*. Multiple *A. marginale* strains that are not tick-transmissible by *D. andersoni* have been documented (18, 112, 117). However, the molecular mechanisms involved in lack of tick infection or transmission in these strains remain unknown. One study described in this dissertation sought to build upon the research with other non-tick-transmissible strains as we sought to identify sequences associated with strains that were not experimentally transmitted by ticks. Thus, we sequenced the non-tick-transmissible Illinois strain of *A. marginale* with Pacific Biosciences single molecule, real time (SMRT) sequencing technology for bioinformatic comparison between other non-tick-transmissible and tick-transmissible strains. The research described herein narrowed the list of potential candidates associated with the non-tick-transmissibility phenotype. In addition, this

work provided a high-quality genome sequence of another non-tick-transmissible strain for future research to understand *A. marginale* genomics. The molecular determinants of tick competency may be applicable to other related tick-borne pathogens as well.

Forward vaccinology approach for tick vaccines

Forward vaccinology or conventional vaccinology is an approach in which a vaccine is developed without using targeted sequences, typically using whole cell, live-attenuated, killed organisms, lysed organisms or whole tissues for vaccine candidate antigen discovery. This type of immune protection allows for the host immune response to determine the preferentially targeted antigens (213). If a whole cell, live-attenuated, killed or inactivated vaccine is used and the entire target organism is to be used for immunization, then no further antigen identification is necessary. However, antigens can be individually isolated and identified using proteomic tools such as western blotting and mass spectrometry. Typically, the next step involves recombinant protein expression and confirmation of protection through immunization. These proteins are then paired with appropriate adjuvants and will pass through clinical trials to determine efficacy in humans or field trials for efficacy in animals (214). Most of the vaccines available in the 20th century were determined using forward vaccinology approaches. However, the current COVID-19 vaccines were largely developed using reverse vaccinology (215).

Reverse vaccinology is relatively new approach developed through genomics and bioinformatics (213, 216). In a reverse vaccinology approach, the

genome sequence is used with computer analysis to determine antigen targets. Animals are subsequently immunized, and the protective antigens are used for molecular analysis against different strains to determine efficacy. Vaccine candidates that are efficacious in animal models will proceed to clinical trials or field studies.

In this dissertation, the vertebrate immune response is used to target tick molecules. While reverse vaccinology can yield important targets, forward vaccinology studies host-parasite interactions based upon millions of years of evolution. The study in chapter 3 sought to determine utility of a large-animal model system for immunization against ticks indigenous to the United States, *D. andersoni*. The objectives of this study were to measure protection in cattle against tick feeding and to identify proteins associated with reduced tick performance. A subsequent study built on this research to target the tick to intervene with tick-borne pathogen transmission or acquisition. The overall objective of the latter study was to measure the effects of different tick midgut or salivary gland preparations on tick transmission or acquisition of *A. marginale*. These investigations examining the vertebrate response to tick tissues are expected to aid the field in the development of vaccines for the control of tick-borne diseases.

The research in this dissertation focused on different aspects of the tick-pathogen life cycle; from the molecular determinants of tick transmission of *A. marginale*, to vaccination to inhibit tick feeding, to affect tick-borne pathogen transmission or acquisition. These afore mentioned topics in the research problem section will be discussed in detail in the remaining chapters of this dissertation.

CHAPTER 2

A long read and high-quality genome sequence of a phenotypically novel *Anaplasma marginale* strain

Abstract

Background: *Anaplasma marginale*, is the primary etiologic agent of bovine anaplasmosis. *A. marginale* is biologically transmitted by ticks of the genera *Rhipicephalus* and *Dermacentor*. However, the molecular determinants pertinent to tick transmission of this pathogen remain undefined. Identification of the molecular determinants of vector competency is expected to lead to useful tools for vaccines such as to control biological transmission of this tick-borne pathogen. Several strains of *A. marginale* have been reported to be non-tick-transmissible. Thus, next generation sequencing was used to compare the phenotypically novel non-tick-transmissible Illinois strain of *A. marginale* with genomes of other strains with different tick-transmission phenotypes. This methodology allowed for high-massively parallel sequencing of the Illinois strain, providing a sequence with high depth of coverage that is useful for extensive sequence analysis.

Results: Next generation single molecule, real-time (SMRT) sequencing of the Illinois strain genome was conducted. Manual curation of the genome resulted in identification of a 14.5-kb inversion, uniquely present in the non-tick-transmissible Florida and Illinois strains and not identified in the tick-transmissible St. Maries strain. Further, 12 genes with high variability in the Illinois strain were identified as

compared to tick-transmissible *A. marginale* strains. Two frameshifted genes with premature stop codons were found to be unique to the Illinois strain. Further, comparison of several putative adhesins or tick-transmissibility associated genes found variation between them and tick-transmissible strains. Previous studies have reported 30 genetic loci identified as segregating with the transmissibility phenotype in the Florida strain genome. Screening of these 30 genetic elements for non-synonymous single nucleotide polymorphisms (SNPs) left 10 possible candidates associated with non-tick-transmissibility phenotypes of both the Florida and Illinois strains.

Conclusions: Next generation SMRT sequencing produced a high quality and deep coverage sequence that enabled prediction of several candidates associated with the non-tick-transmissibility phenotype. This approach identified multiple genomic elements and genes in the Illinois strain that are potentially involved in the adaption of *A. marginale* to its invertebrate host.

Keywords: *Anaplasma marginale*, anaplasmosis, single nucleotide polymorphisms (SNPs), ticks, biologic transmission, genome, whole genome sequencing (wgs)

Introduction

Bovine anaplasmosis is one of the most economically important vector-borne diseases of cattle worldwide (217). Clinical signs of bovine anaplasmosis primarily result from anemia, and economic losses result from preventive treatment and other veterinary services, weight loss, abortion, testicular degeneration, loss of libido, reduced milk production and death (218–221). *Anaplasma marginale,* the

primary etiologic agent of bovine anaplasmosis, is biologically transmitted by *Rhipicephalus* and *Dermacentor* spp. ticks (105), mechanically transmitted on the mouthparts of biting flies (96), or iatrogenically transmitted with blood-contaminated fomites (222–224). In nature, biologic transmission by ixodid ticks can spread *A. marginale* acquired from subclinical bovine carriers to susceptible vertebrate hosts (20), while mechanical transmission by hematophagous arthropods is thought to spread *A. marginale* acquired from hosts with acute or recrudescent infections (96). Notably, certain *A. marginale* strains have not been biologically transmitted by tick vectors under experimental conditions (18, 112, 117).

Biologic transmission of *A. marginale* is a complex interaction among the tick vector, the vertebrate host and the pathogen, which begins with transfer of the pathogen from ingested blood (*i.e.,* the blood meal) of an infected bovine host to the tick midgut epithelium (21, 225). From the infectious blood meal, *A. marginale* infects the tick midgut epithelium (127), multiplies in colonies within parasitophorous vacuoles and eventually disseminates to tick hemolymph (212, 226) and salivary glands (227) before transmission to a subsequent vertebrate host via tick saliva (117, 228, 229).

Previous reports have ascribed single traits to *A. marginale* infection of ticks. Kocan *et al.* observed anaplasmal inclusion appendages, also known as tails, in association with *A. marginale* in the blood meal within the tick midgut lumen, as well as directly juxtaposed to individual organisms next to apical membranes of tick midgut epithelial cells (123). Noting that such appendages were

not seen in erythrocytes infected with the non-tick-transmissible Florida strain (22), these observations led to the hypothesis that inclusion appendages were associated with infection of the tick vector. However, the presence of inclusion appendages was not indicative of tick transmissibility of the “tailed” *A. marginale* Illinois strain (18, 112), indicating that inclusion appendages were not the only factor associated with infection of tick vectors (122). Further, MSP1a and OmpA have been reported adhesins involved in infection of tick cells (230, 231). Among the strains tested to date, the *A. marginale* Illinois strain expressed the highest level of anaplasmal appendage-associated protein (AAAP) relative to the tailed tick-transmissible Virginia strain, with the lowest levels of AAAP expressed in the tailless Florida strain of *A. marginale* (124). Thus, comparison of the Illinois and Florida *A. marginale* strains could be instructive to protein expression mechanisms of this pathogen in addition to infectivity to its invertebrate host.

The Illinois strain of *A. marginale* is novel in that it possesses intraerythrocytic inclusion appendages, but it is not transmissible by *Dermacentor andersoni* or *Dermacentor variabilis* ticks. The objective of this study was to obtain a high-quality genome sequence of the tailed, non-tick-transmissible *A. marginale* Illinois strain with the Pacific Biosciences (PacBio) sequencing platform (232), in order to further define factors associated with non-tick-transmissible *A. marginale* strains.

Materials and Methods

Genome sequences

Roche 454 sequenced genome sequences were available for the Puerto Rico (233), Virginia (112), Gypsy Plains (234, 235), Oklahoma (236) and South Idaho (19, 237) strains of *A. marginale*, which reportedly were transmissible by *D. andersoni* or *Rhipicephalus microplus* ticks, while genomes of the Mississippi (117) and Dawn (238) strains were representative of those non-tick-transmissible by *D. andersoni* or *R. microplus.* BAC-based cloning whole genomes were available for the reportedly *D. andersoni* transmissible St. Maries strain (239) and non-*Dermacentor*-transmissible Florida strain (112).

DNA Isolation

DNA was isolated from blood prepared from a steer that was experimentally infected with *A. marginale* Illinois strain (18, 112, 124). The blood was stored at -80°C until a Roche High Pure Viral Kit (Roche, Indianapolis, IN) was used to isolate the genomic DNA, following the manufacturer's instructions.

Strain confirmation

A forward primer (GGTCGTAGGATTGCAGAGGA) and reverse primer (CAAGGGTCGCAATAGCAGTC) were purchased from IDT (Newark, NJ) and used to amplify *aaap* (GenBank accession number: AY514452.1). Primers were at a concentration of 0.5 μM in a standard 1X PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 0.02 U/μL of Platinum Taq (Invitrogen, Carlsbad, CA). 45 cycles with 94°C for 30 sec, 60°C for 30 sec and

72°C for 1 minute, and a final extension at 72°C for 7 minutes. Amplified PCR products were detected on a 1% agarose gel containing ethidium bromide. DNA was gel purified from the target band (960 bp) using the EZ Gel Extraction Kit (EZ BioResearch, Saint Louis, MO). Dideoxy sequencing of the target amplicon was conducted on both strands at the MU DNA Core Facility.

Depth of coverage and genome assembly

The *A. marginale* Illinois genome was sequenced at the National Center Genome Resources (NCGR; Santa Fe, NM). One PacBio Long-Read (CLR) 10-kb library insert was prepared for PacBio single molecular, real-time (SMRT) sequencing with one SMRT cell. This genome was sequenced to an average depth of 383x. The reads were assembled using hierarchical genome assembly process (HGAP) version 2.0 (232). A sequence alignment map (SAM) file was made using the PacBio Basic Local Alignment with Successive Refinement (BLASR) program. This SAM file was converted to a binary alignment map (BAM) file with samtools (240). A tab-delimited file was made with samtools from the BAM file. This file was used to calculate depth of coverage and was plotted against base pair position using SigmaPlot (Systat Software, San Jose, CA). The *de novo* Illinois strain sequence was compared to the high-quality St. Maries reference strain and rearranged in Artemis to start at the same position as the St. Maries genome (241).

Primers were designed, flanking a putative 1.7-kb gap in the Illinois genome, with forward primer (CGAAGTCTCCCTTGAGGACGCTTT) and reverse primer (GTACGATAATTGACATAGCTATAT) and these primers were purchased from IDT. Primers were at a concentration of 0.5 μM in a standard 1X PCR buffer

(20 mM Tris-HCl pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 0.02 U/µL of Platinum Taq (Invitrogen, Carlsbad, CA). 45 cycles with 94°C for 30 sec, 50°C for 30 sec and 72°C for 1 minute, and a final extension at 72°C for 7 minutes. Amplified PCR products were detected on a 0.75% agarose gel containing ethidium bromide. DNA was gel purified from the target band (2.2 kb) using the EZ Gel Extraction Kit (EZ BioResearch).

Primer walking was performed with the forward primers (ILAm_1144163-1144186_S CTTCTGACTTGCATTGTAGTGTTC, ILAm_1144450-1144469_S CCGAAGTACCAAGGGTTTTT, ILAm_1145029-1145052_S AGAGGAAATGGAAGAAGCCCTCTG) and reverse primers (ILAm_1145454-1145435_A GCGAATAGTATAGGGATTAG, ILAm_1145381-1145358_A ACTCATGGTAAAGTTATTCGTGGA, ILAm_1144594-1144570_A CTCTATAGGGTCCATCACCAACTCG). Dideoxy sequencing of the target amplicon was conducted on both strands at the MU DNA Core Facility. Incorporation of the missing 1.7 kb assembled the *de novo* full-length *A. marginale* Illinois strain genome sequence.

Annotation

The Prokaryotic Genomes Automatic Annotation Pipeline (Prokka) version 1.9 was used to annotate the assembled genome (242). Open reading frames (ORFs) were checked manually, using Artemis Comparison Tool (ACT) for frameshifts, truncations and general variations as compared to the St. Maries reference genome (NC_004842.2) (243). To ensure that annotations were correct, the National Center for Biotechnology Information (NCBI) basic local alignment

tool (BLASTx) was used to search protein databases with deduced *A. marginale* Illinois protein sequences (244). Annotations were changed when strong correlations (*i.e.,* E-values ≤ -30) to conserved protein domains were indicated (245). Such highly conserved domains were used to search the Universal Protein Resource (UniProt.org) to find consensus among gene names (246). Gene names were changed where a consensus existed. Frameshift mutations and in-frame stop codons in ORFs were checked by PCR and subsequent dideoxy sequencing; if frameshifts were found erroneous, they were corrected based upon these amplicon sequence analyses. Intra-species genomic variations were also detected using ACT. The EMBL-EBI InterPro software suite was used to identify predicted and conserved domains of protein sequences in FASTA format (247).

Visualization of the genome

The *A. marginale* Illinois strain genome sequence was loaded into DNAPlotter, a part of the Artemis suite, to create a circular genome map (248). The tracks indicating pseudogenes, coding sequence (CDS) regions and other notable features of the genome were visualized. Clusters of orthologous genes (COG) functional analysis were predicted using eggNOG 5.0 (249, 250).

Phylogenetic analysis

Different *A. marginale* strains and their accession numbers were St. Maries (NC_004842.2), Florida (NC_012026.1), Puerto Rico (NZ_ABOQ00000000.1), Virginia (NZ_ABOR00000000.1), Mississippi (NZ_ABOP00000000.1), South Idaho (NZ_AFMY00000000.1), Okeechobee (NZ_AFMV00000000.1), Oklahoma

(NZ_AFMX00000000.1), Gypsy Plains (NC_022784.1) and Dawn (NC_022760.1). Multiple sequence whole genome alignment was performed using Mugsy (release 2.3) and subsequent trimming of poorly aligned regions was done with trimAl (v1.4) (251, 252). The MAF file was converted to a FASTA file format using Galaxy (253). The phylogenetic tree was constructed using MEGA (v10) with 100 bootstraps using the generalized time reversible (GTR) model on a maximum likelihood method (254).

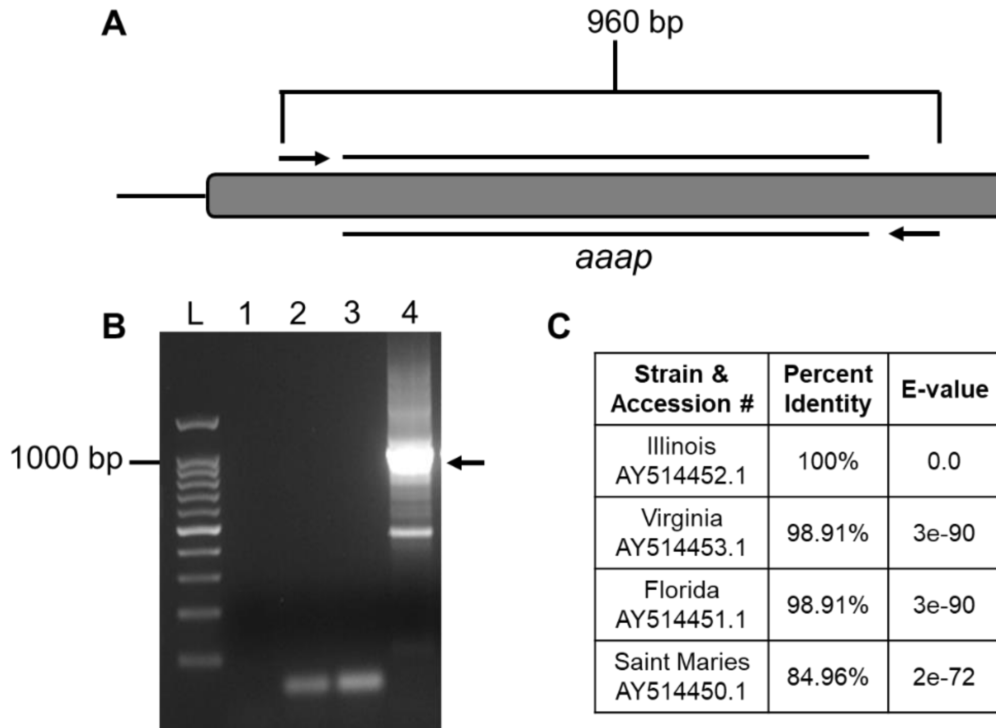
Contigs genome assembly

For previously submitted *A. marginale* genomes with multiple contigs, whole-genome shotgun contigs (WGS) were placed into scaffold_builder and contigs were re-ordered against the St. Maries strain reference genome, with default settings (255). These scaffolds had regions of contamination, which were removed.

Results

Confirmation of *A. marginale* Illinois strain identity

The identity of the previously reported *A. marginale* Illinois strain was confirmed from infected bovine blood stored at -80°C, to ensure sequencing of the Illinois strain genome. The polymorphic *aaap* ORF was used to confirm the Illinois strain. Amplicons were bidirectionally sequenced in two independent experiments. Sense and antisense strand sequences from both experiments overlapped for a total of 675 bp, which was 100% identical to the previously reported Illinois strain of *A. marginale* (Fig. 1).



Strain & Accession #	Percent Identity	E-value
Illinois AY514452.1	100%	0.0
Virginia AY514453.1	98.91%	3e-90
Florida AY514451.1	98.91%	3e-90
Saint Maries AY514450.1	84.96%	2e-72

Figure 1. Strategy for confirmation of the Illinois *A. marginale* strain from infected bovine blood.

A. Localization of primers (arrows) on the *aaap* sequence (AY514452.1) with an expected amplicon of 960 bp. Line indicates regions of sequencing for strain confirmation. **B.** Gel electrophoresis of *aaap* using primers described in A. Lanes are indicated as follows; 100 bp GoldBio ladder (L); non-template control #1 (1); non-template control #2 (2); non-template control #3 (3); PCR of purified DNA from Illinois strain infected blood (4), respectively. Target amplicon used from dideoxy sequencing indicated by arrow. **C.** BLASTn results with percent identity and E-score of *aaap* dideoxy DNA sequence searched against *Anaplasma marginale*.

Assembly of the *A. marginale* Illinois genome sequence

The presence of paralogs and other repetitive sequences can make assembly of *A. marginale* genomes problematic (256). The relatively long read reported for the PacBio RSII sequencer was expected to mitigate this challenge (257). Based on the small size of the *A. marginale* genome, coverage of >30X is expected to provide accuracy of 99.999% (258). Thus, PacBio sequencing of the Illinois genome was conducted to assemble a high-quality *A. marginale* Illinois genome sequence. Reads were aligned to build a *de novo* assembly of the *A. marginale* Illinois genome sequence. A binary alignment map (BAM) file was produced to evaluate depth of coverage of the genome sequence. Prokka annotation of the genome was performed, and the assembly was manually compared to the *A. marginale* St. Maries strain genome as a reference. Total reads, read size, depth of coverage, genome size, GC content, CDS content, pseudogene and functional pseudogene contents and functional predictions of CDS regions in the genome were compared to those of other high-quality genomes (*i.e.* Florida and St. Maries strains). The Illinois strain genome assembled to a single contig. Functional pseudogenes include truncated copies of *msp2* and *msp3* genes that recombine into the active site of the full-length gene to create variation of the gene. The PacBio reads of the Illinois strain genome totaled 532,104 with an average read size of 2,639 bp. The depth of coverage for any single nucleotide averaged 383x (Fig. 2). *De novo* assembly of the genome with HGAP resulted in a single contig. The *A. marginale* Illinois strain assembly was not aligned to the same start position as the St. Maries strain reference genome.

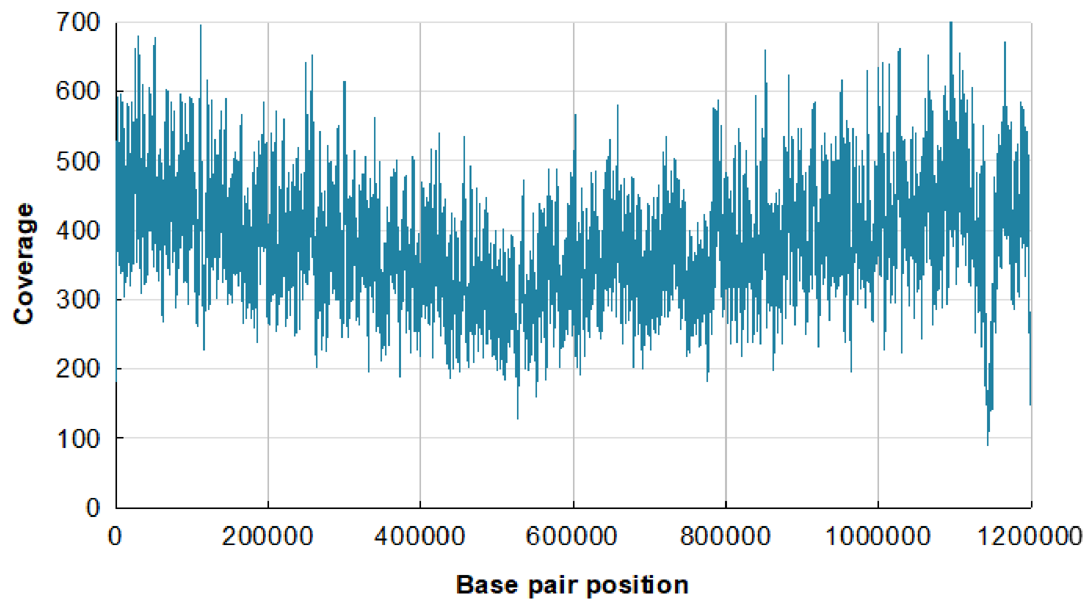


Figure 2. Coverage depth analysis of reads mapped to the *de novo* contig of the Illinois *A. marginale* strain genome.

Average coverage depth was plotted on the y axis as compared to the base pair position on the x axis.

Position 1 of the assembled *A. marginale* Illinois strain genome aligned to position 1,145,467 of the St. Maries strain reference genome. While position 1 in the St. Maries genome aligned to position 52,364 on the linearized Illinois genome. Rearrangement of the Illinois circular sequence aligned the Illinois and St. Maries genomes to have the same start position.

Further comparison of the Illinois and St. Maries genomes with the Artemis Comparison Tool (ACT) revealed a 1.7-kb gap in the Illinois sequence, which was initially located on the ends of the linearly assembled Illinois contig. Subsequent PCR analysis confirmed that this sequence was missing from the assembly (Fig. 3). To complete the sequence assembly, primer walking with dideoxy sequencing was conducted for bidirectional sequencing of the missing nucleotides. Initial annotation of the genome was conducted with Prokka, followed by, manual curation to confirm the annotations. The Illinois strain genome was comprised of 1,197,953 bp encoding 961 CDS regions, a coding percentage of 85.6% and GC content of 49.81% (Fig. 4; Table 4). The genome contained 21 pseudogenes. COG functional prediction of the Illinois genome ORFs, using eggNOG-mapper, revealed that 11% of the CDS regions had unknown functions (Fig. 5).

Phylogenetic analysis of the *A. marginale* Illinois strain genome

Several *A. marginale* strains were reportedly non-tick-transmissible by ticks classified in the ixodid tribe Rhipicephalinae (112, 117, 238). It was hypothesized that the non-tick-transmissible Illinois strain may more closely align with one of the other non-tick-transmissible *A. marginale* strains, and the relatedness between Illinois and other *A. marginale* strains could direct strategies to identify factors

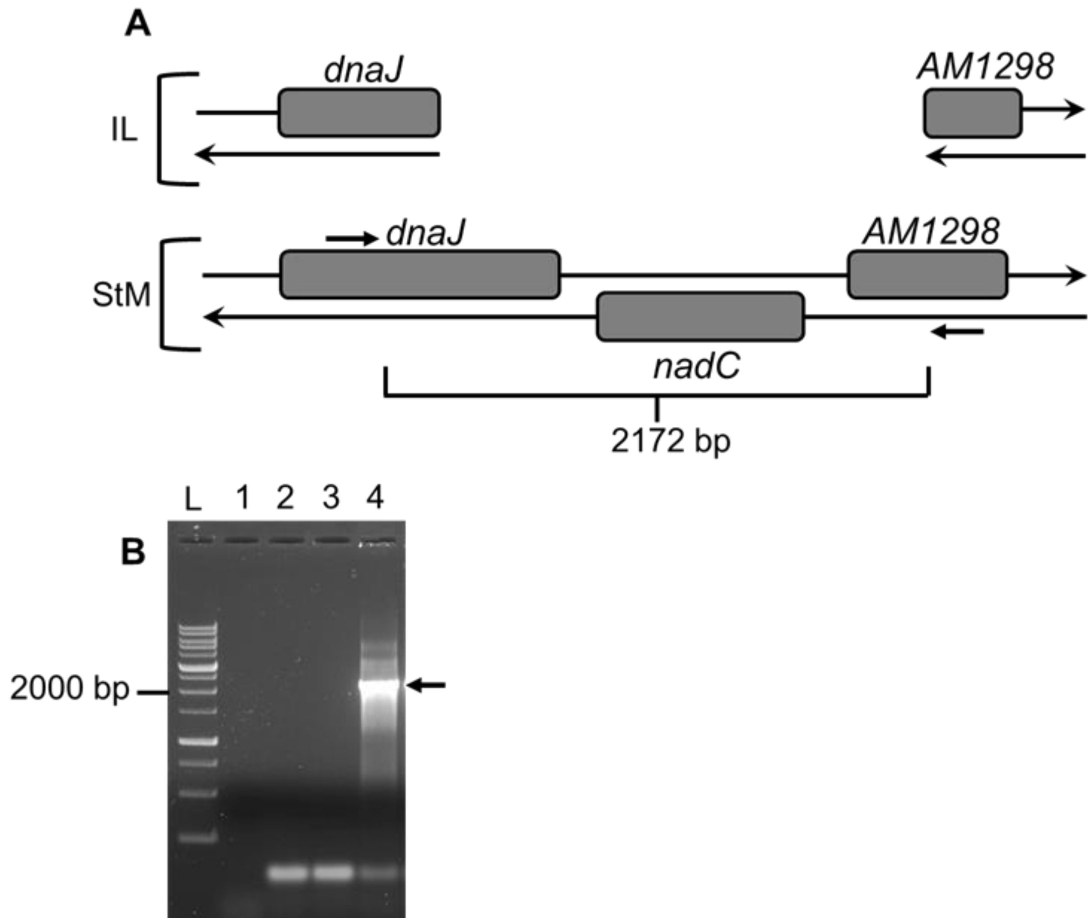


Figure 3. Sequencing and completion of the Illinois *A. marginale* strain genome.

A. Localization of primers (arrowheads) on the St. Maries reference genome for sequencing of the 1.7-kb gap sequence with an expected amplicon of 2,172 bp. The top illustration indicates the Illinois genome with truncated genes *dnaJ* and *AM1298*. The bottom illustration is the reference St. Maries annotated genome. **B.** Gel electrophoresis of the amplicon with the primers described in A. Lanes are indicated as follows; 1 kb GoldBio ladder (L); non-template control #1 (1); non-template control #2 (2); non-template control #3 (3); PCR of purified DNA from Illinois strain infected blood (4), respectively. Target amplicon used from dideoxy sequencing indicated by arrow. Abbreviations include IL – Illinois and StM – St. Maries.

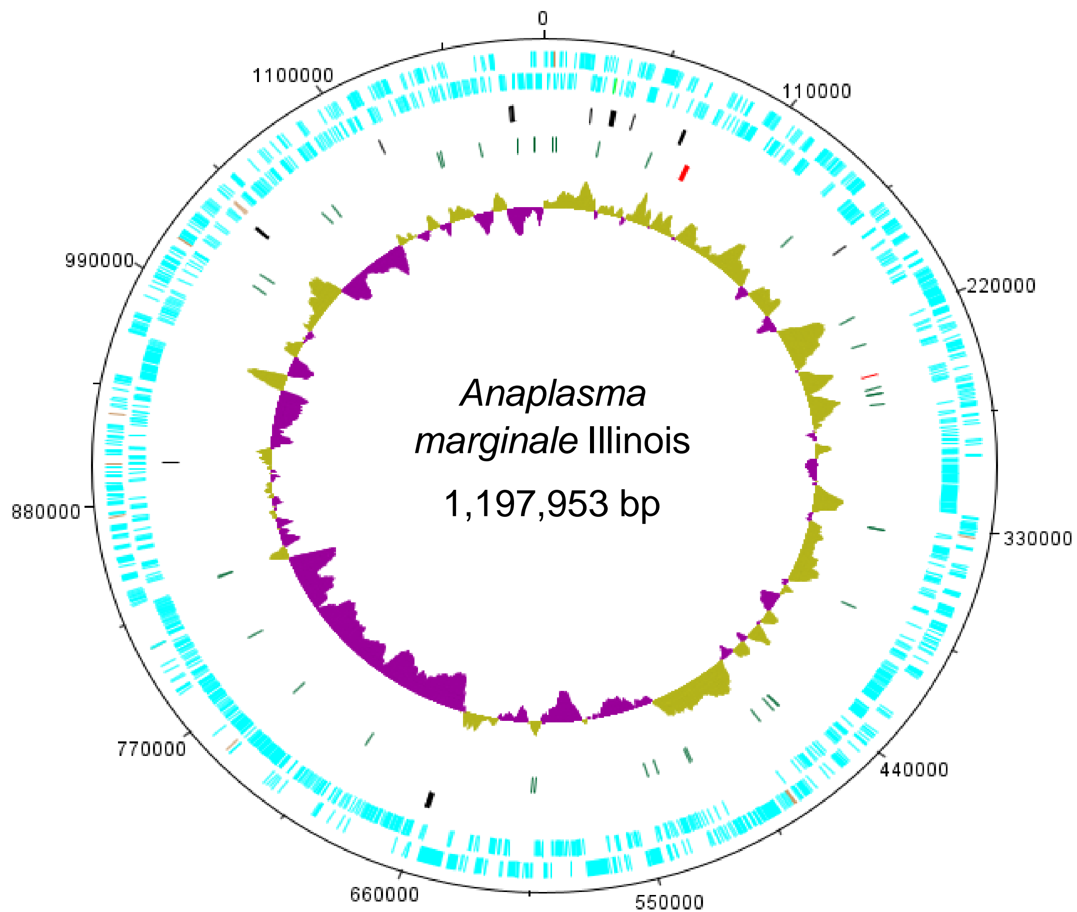


Figure 4. Circular representation of the Illinois *A. marginale* strain genome.

The genome is circularized with markings indicating the size of the genome in base pairs. The first outer and second tracks indicate coding sequences on forward and reverse strands and the black markings on the third track indicate pseudogenes. The fourth track contain red markings for rRNAs, green for tRNAs, and ncRNAs in orange. The innermost track indicates guanine-cytosine (GC) skew with purple for negative skew and olive for positive skew. The circular genome image was made in DNAplotter.

	A. marginale Illinois	*A. marginale* Florida	*A. marginale* St. Maries
Total Bases	1,197,953	1,202,435	1,197,687
CDS Count	961	942	949
tRNAs	37	37	37
ncRNAs	3	3	3
rRNAs	3	3	3
tmRNA	1	1	1
Pseudogenes	21	19	20
Functional pseudogenes	15	15	14
Coding %	85.6	85.7	85.4
GC %	49.81	49.77	49.9

Table 4. General genome statistics of the Illinois *A. marginale* strain genome.

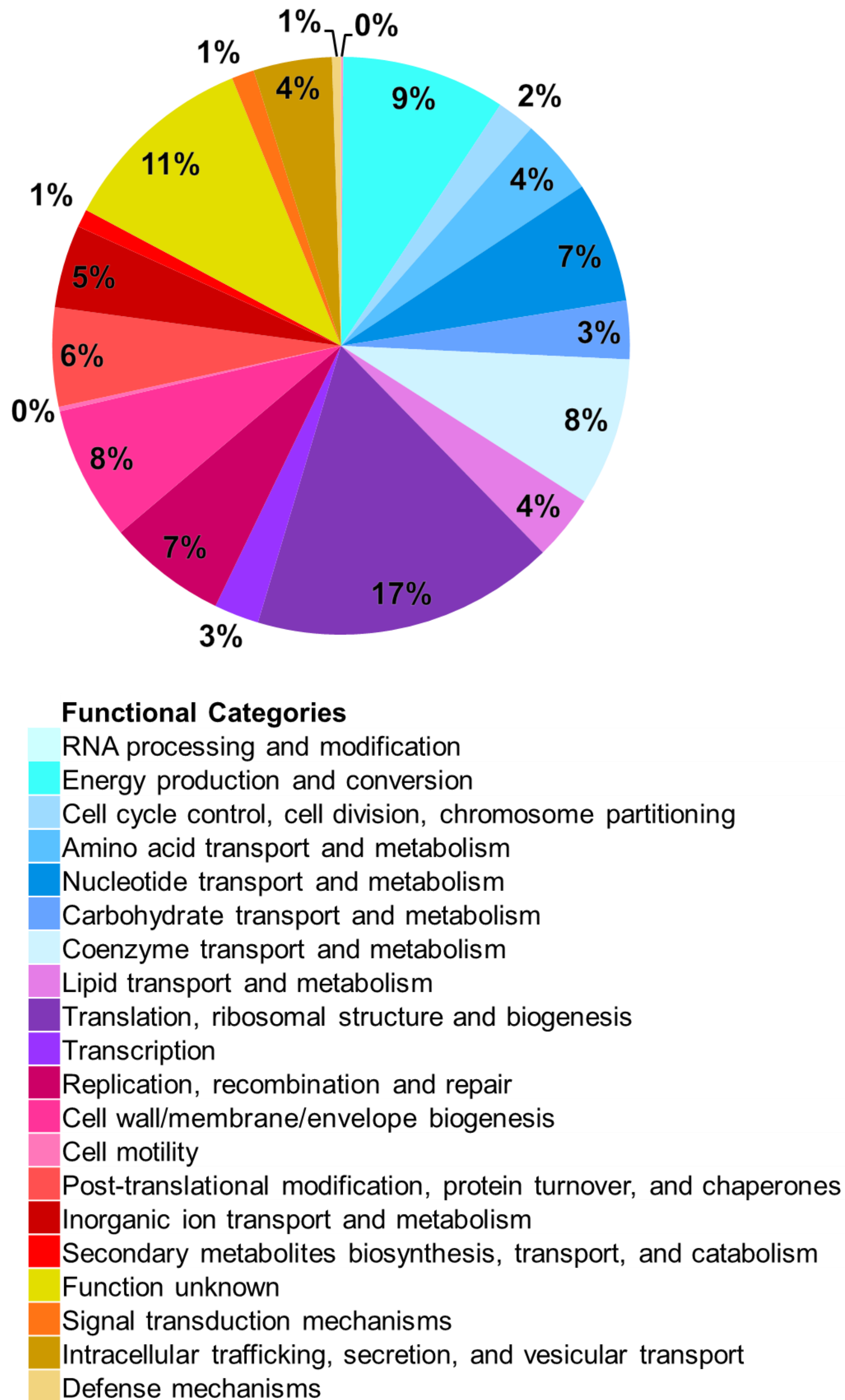


Figure 5. Clusters of orthologous groups (COG) classification of the CDS encoded in the Illinois *A. marginale* strain genome.

Color coding is matched to the legend provided with predicted function. Cell motility and defense mechanisms have less than 1% of the CDS align to their predicted function group.

associated with adaption to tick vectors. Interestingly, the Illinois strain genome aligned most closely to the tick-transmissible St. Maries strain genome (Fig. 6), and the non-tick-transmissible Florida, Mississippi and Dawn strains were also all more similar to tick-transmissible strains than to each other or to the Illinois strain genome.

Illinois strain MSP1a

Adherence of *Escherichia coli* expressing rMSP1a to IDE8 tick cells was previously reported (125) and predictions of tick infectivity were reported based upon MSP1a sequence analysis (231). MSP1a contains tandem peptide repeats and these repeats are reported to be predictive of MSP1a-based adherence to these tick cells. Specifically, it was reported that adhesion to tick cells was abrogated if the 20th amino acid residue of the tandem repeat is glycine instead of glutamic or aspartic acid. It was also reported that other amino acid substitutions elsewhere in this repeat region had no impact on adhesion to tick cells. It was unknown if the Illinois strain MSP1a sequence is consistent with non-adherence to tick cells. Thus, to test whether the Illinois strain MSP1a was consistent with the MSP1a-based paradigm of adherence to tick cells, the Illinois *msp1α* sequence was located and the deduced protein sequence compared to previously published sequences. Amino acid residues present at 20th positions of tandem repeats, the neutralization sensitive epitope and putative secretion domain were compared to those of previously reported *A. marginale* strains. The Illinois MSP1a most closely aligned with what was reported as non-adherent to tick cells, based upon having four of its seven MSP1a tandem repeats containing glycine residues at the 20th

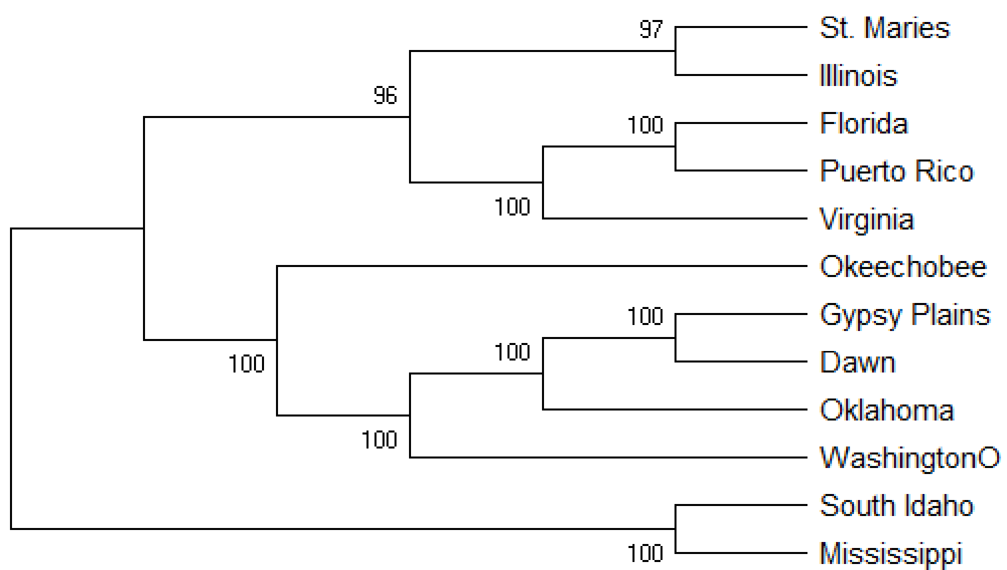


Figure 6. Phylogenetic analysis of 12 sequenced strains of *A. marginale* based upon whole genome alignments.

The tree shown is a maximum-likelihood distance tree using 100 bootstraps. The tree was constructed using MegaX using a maximum likelihood method with a general time reversible model.

amino acid position (Table 5). The neutralization sensitive epitope, QASTSS (amino acids 21-26), were conserved in all 7 tandem repeats of the Illinois strain. The putative secretion and anchorage domain, localized within 10 residues after after the final tandem repeat, was 100% identical to the tick-transmissible and tick cell adherent Oklahoma strain (231).

Another outer-membrane protein and posited adhesin

Anaplasma phagocytophilum outer membrane protein A (OmpA) is another putative adhesin. (259), and a more recent report suggested that *A. marginale* OmpA serves as an adhesin and invasin for mammalian endothelial (RF/6A) and acarine embryonic (ISE6) cells (230). Residues 50-67 are reportedly involved in binding to tick cells. To assess if the Illinois strain OmpA has mutations compared to other strains, the *A. marginale* Illinois strain OmpA amino acid sequence was compared against those of other reported strains. A single nucleotide change at position 373 (residue 125) in the Illinois strain *ompA* resulted in a non-synonymous change, alanine to serine, which was not found in any tick-transmissible strain.

Sequences associated with AAAP expression levels

Among the strains tested to date, the *A. marginale* Illinois strain was found to express the highest level of *A. marginale* appendage-associated protein (AAAP) compared to the tailed tick-transmissible Virginia strain and the tailless non-tick-transmissible Florida strain of *A. marginale* (124). The *aaap* locus is variable between strains and has reported plasticity (260); in the *aaap* gene cluster there are the *aaap* and *alp* (AAAP-like protein) genes. It is unknown why the Illinois strain expresses a higher level of AAAP than the Florida strain, so analysis of the *aaap*

MSP1a Repeats	Tandem Repeats #	Repeats with G at 20th amino acid
Florida	8	0
Illinois	7	4
Mississippi	5	0
Oklahoma	3	2
Puerto Rico	2	0
St. Maries	3	0
Virginia	2	0

Table 5. MSP1a tandem repeat numbers and 20th amino acid designations from several tick-transmissible and non-tick-transmissible strains.

region was expected to allow for better understanding of *aaap* expression mechanisms.

To understand the *aaap* loci in the two strains, Clustal Omega (ClustalO) multiple sequence alignments of the 5' intergenic region for *aaap* and the intergenic region upstream of the *aaap* and *alp* gene cluster were analyzed (Fig. 7) and previously reported *A. marginale* -10 promoter sequences (TACACT, TATCCT, TACCTT) were searched for in the *aaap* gene cluster (261, 262). The promoters reported in these papers were not found in the *aaap* gene cluster region in the intergenic regions. The canonical *E. coli* -10 promoter (TATAAT) was found upstream of the entire intergenic region; however, the sequence identity between the Illinois and Florida strains was 100% identical in this region. The Florida strain *aaap* and 5' intergenic region was duplicated and 100% identical (Fig. 7, Florida panel). Initial comparison of the Florida and St. Maries strains *aaap*, the upstream intergenic region and the *alp* ORF to RNA-seq data (accession number: SRP014580) indicated that RNA-seq reads mapped to the *aaap* and *alp* genes. However, they did not map to the full intergenic region between these two genes in the Florida strain (Fig. 7, intergenic region 1), and in the St. Maries strain RNA-seq reads mapped to almost the full length of the intergenic region. The Florida strain RNA-seq reads suggested *aaap* is not part of a polycistronic mRNA with the promoter in this intergenic region; however, the St. Maries strain alignment suggested otherwise, as such both the intergenic region upstream of *aaap* and upstream of the *aaap* gene cluster were observed. Others have reported that the *aaap* region is unlikely to be polycistronically transcribed due to breaks in the

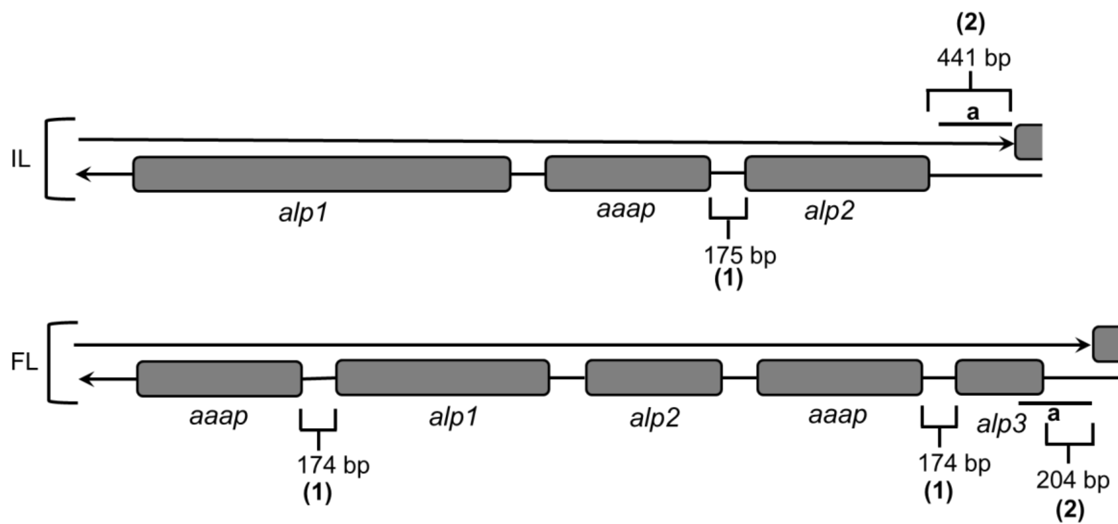


Figure 7. Alignment of the *A. marginale* Illinois and Florida strains *aaap* gene cluster and intergenic regions.

The *alp* and *aaap* genes are labeled between the two strains. (1) The intergenic region immediately upstream of *aaap*. (2) The intergenic region upstream of the *aaap* gene cluster. Abbreviations include IL – Illinois and FL – Florida.

coverage of RNA-seq reads (260). Alignment of the intergenic region immediately upstream of *aaap* in the Illinois and Florida strains indicated that the Florida and Illinois sequences were 100% identical except for one homopolymeric G tract at the 5' end of the intergenic region. Illinois contains 10 Gs whereas Florida contains 9 Gs (Fig. 7, intergenic region 1).

The intergenic region upstream of the entire gene cluster in Illinois is 441 bp long; however, the Florida strain intergenic region is 204 bp with the *alp3* gene starting at base pair 204 (Fig. 7, intergenic region 2). Direct comparison of the low-AAAP expression Florida strain and high-AAAP expression Illinois strain intergenic regions showed that the Illinois and Florida sequences are nearly 100% identical with only two base pairs different between the two strains until base pair 352 (Fig. 7, intergenic region 2a), at which point the sequences begin to deviate heavily with little to no sequence identity. Further, comparison of the entire *aaap* gene cluster found three PolyG tracts of seven more base pairs in intergenic regions for Illinois and four in the Florida strain. One of the PolyG tracts in Illinois and two in Florida are in the 5' intergenic region immediately upstream of *aaap* (Fig. 7, intergenic region 1). The duplicated *aaap* and intergenic region in the Florida strain results in this extra PolyG tract. No rho-independent terminators were found in the Illinois *aaap* gene cluster using ARNold rho-independent terminator program (263). The lack of these rho-independent terminators suggests that the *aaap* and *alp* sequences are polycistronic.

Sequences uniquely shared between the Florida and Illinois strains

SNPs

There have been several advances since the first *A. marginale* St. Maries genome was published (264), allowing new approaches to understand *A. marginale* adaptions to tick vectors. Previously, comparative genomics and transcriptomics associated based on the Florida strain genome resulted in identification of 30 candidate genes and promoters associated with non-infectivity to ticks (126). Assuming the same mechanism is responsible for non-infectivity of the Florida and Illinois strains to ticks, these sequences served as a starting point to further eliminate Illinois strain sequences which were more similar to tick-transmissible strains. Candidate genes associated with the non-tick-transmissibility phenotype from Pierlé *et al*. (2012) were aligned with the same genes from other *A. marginale* strains. The genes were loaded into ClustalO and were aligned using default settings (265). The alignment file was manually searched to identify SNPs that resulted in non-synonymous amino acid changes found in the Illinois *A. marginale* strain, but not found in a transmissible strain, these ORFs with SNPs were retained as candidate sequences. Those sequences identical to transmissible strain sequences were eliminated from candidacy. Two ORFs contained non-synonymous mutations in the same position for both the Florida and Illinois strains, AnPIll_01015 and AnPIll_03145 (Table 6). Eight other ORF or promoter regions contained mutations found in the Illinois strain, which were not found at the same position as in Florida; however, these mutations were also not found in any tick-transmissible strain, a total of 10 candidates were

Product	Strains	Gene Annotation/Position	Domains
Hypothetical protein, unknown function	IL, FL, MS, Dawn	AnPIII_02090, Pos. 489022-496713	Tm, non-cyto
Promoter and gene: Sigma 54 modulation protein	IL, FL	AnPIII_02455, Pos. 1039540-1040757	Ribosome hibernation promoting factor
Hypothetical protein, unknown function	IL, FL, MS, Dawn	AnPIII_01015, Pos. 223155-226574	None identified
Hypothetical protein, unknown function	IL, FL, MS, Dawn	AnPIII_01015, Pos. 223155-226574	None identified
Promoter and gene: Hypothetical protein, unknown function	IL, FL, MS, Dawn	AnPIII_04045, Pos. Complement (940934-944863)	Non-cyto, signal peptide
Hypothetical protein, unknown function	IL, FL, MS	AnPIII_01370, Pos. Complement (298803-303416)	None identified
Hypothetical protein, unknown function	IL, FL, MS, Dawn	AnPIII_01395, Pos. Complement (317992-321033)	Tm, cyto, non-cyto
Hypothetical protein, unknown function	IL, FL, MS, Dawn	AnPIII_02730, Pos. 644744-655207	None identified
Hypothetical protein, unknown function	IL, FL, MS, Dawn	AnPIII_02665, Pos. 626291-629323	Tm, cyto
Hypothetical protein, unknown function	IL, FL, MS, Dawn	AnPIII_03145, Pos. 755655-759734	Tm, cyto, non-cyto
Hypothetical protein, unknown function	IL, FL	AnPIII_01332, Pos. 289055-289345	Tm, cyto, non-cyto

Table 6. SNPs shared between four non-tick-transmissible strains of *A. marginale* from the 30 candidate proteins associated with the non-transmissibility phenotype of the Florida *A. marginale* strain.

Abbreviations include: Tm – transmembrane, Cyto – cytoplasmic, Non-cyto – non-cytoplasmic, FL – Florida, IL – Illinois and MS – Mississippi.

identified with the above-described approach. Two of these candidates contained nucleotide changes found both in the promoter region and in the ORF. One candidate was annotated as a Sigma 54 modulation protein. The second candidate was a hypothetical protein (AnPIII_04045). The eight remaining candidates encoded hypothetical proteins with unannotated functions. InterPro prediction of all the hypothetical proteins revealed that five of these proteins contained putative transmembrane domains, suggesting they were likely membrane-bound or secreted proteins. Comparison of the 10 candidates shared between the Illinois and Florida strains indicated seven and eight of these genes were shared with the non-tick-transmissible Dawn and Mississippi strains, respectively (Table 6).

A DNA inversion that is found in the Illinois and Florida strains

Manual annotation of the genome revealed multiple unique elements in the Illinois genome as compared to St. Maries. A 14.5-kb inverted region, previously reported as 30-kb and unique to the Florida strain (256), was also present in the Illinois strain (Fig. 8A,B). This sequence is not inverted in the tick-transmissible St. Maries strain. Comparison of this sequence in 454 sequenced tick-transmissible strain genomes was unable to identify if this sequence is inverted in these strains. In the Illinois and Florida strains, *virB2* (AnPIII_00180) and *virB3* (AnPIII_00250) flank this inverted sequence. Both genes are 393 bp long and encoded 100% identical sequences. The genes within the inversion, from 5’ to 3’ ends, included pseudogene *msp3* #1, *proP_1*, *rpsP*, *glmU*, *kduD*, *pheS*, *rplT*, *rpmI*, hypothetical protein AnPIII_00225, *rho_1* and pseudogene *msp2* #2. There were no amino acid changes unique to only the Florida and Illinois strains as compared to the genes

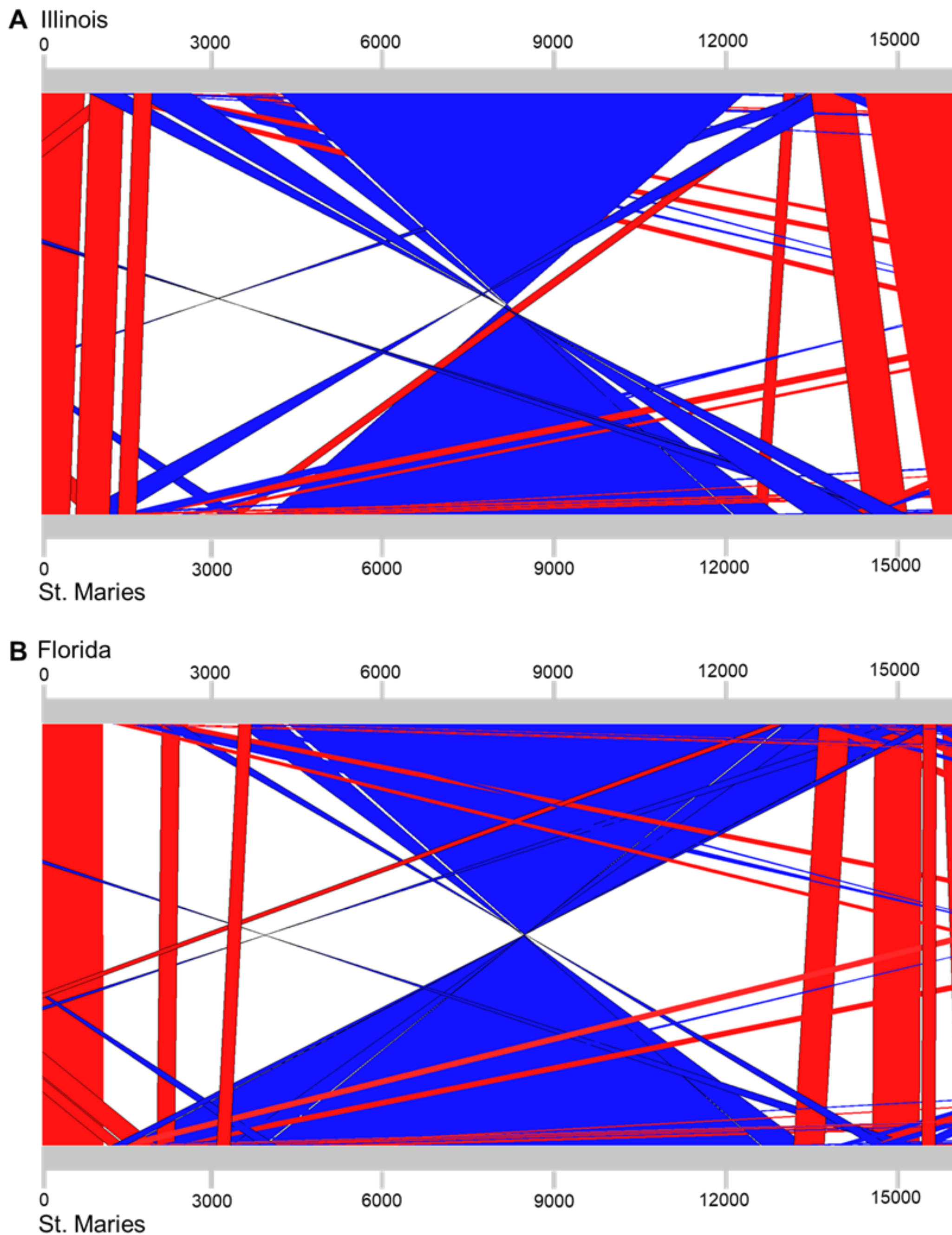


Figure 8. An inversion not found in the tick-transmissible St. Maries strain, but conserved in the non-tick-transmissible Florida and Illinois strains.

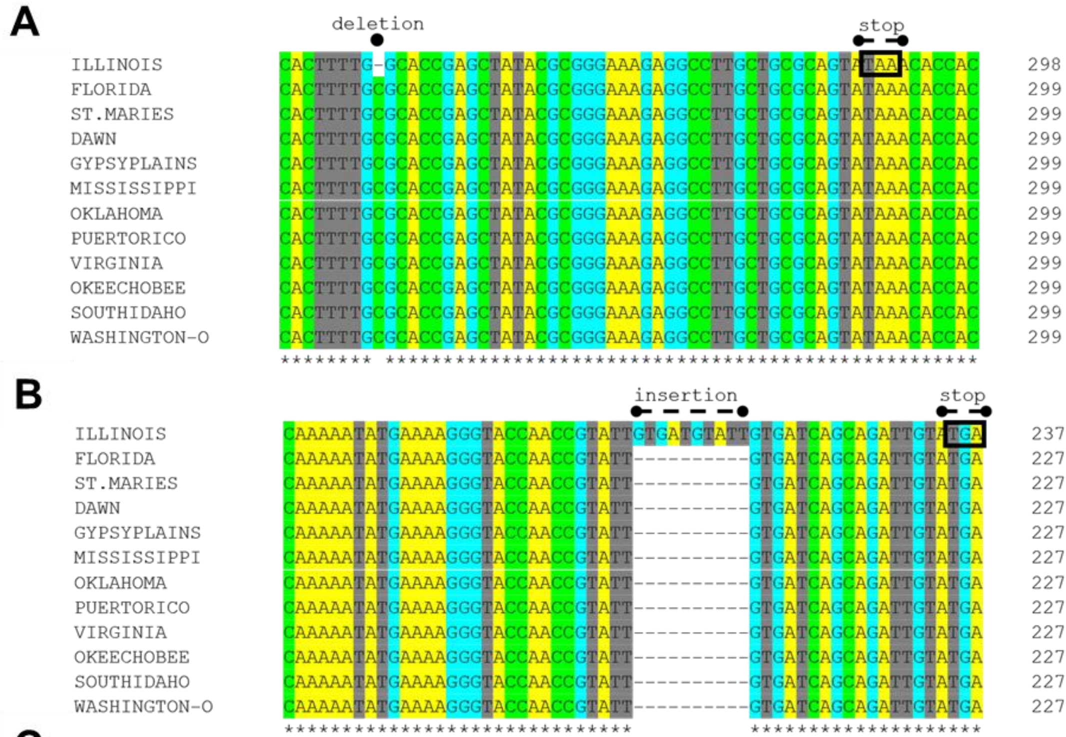
Alignments were made in Artemis comparison tool. **A.** Alignment of the Illinois (top) and St. Maries strains (bottom). **B.** Alignment of the Florida (top) and St. Maries strains (bottom). Red lines indicate regions with high percent sequence identity, blue indicates sequences that are flipped between the two strains with high percent sequence identity and white spaces indicate regions of low sequence identity between the two strains.

within this inversion fragment in tick-transmissible strains.

Sequences unique to the Illinois strain genome

Frameshifts

It is conceivable that the Illinois and Florida strains are non-tick-transmissible for several different reasons. Several frameshifts were found in the Illinois strain genome during manual annotation. In gene frameshifts are potentially indicative of candidate sequences associated with *A. marginale* adaptions to tick vectors. Thus, amplicon sequence analyses were used to confirm the authenticity of frameshifts in Illinois strain sequences encoding truncated proteins that were observed during the manual annotation process. Two Illinois genome unique indels, insertion or deletion of base pairs, resulted in premature stop codons, truncating the proteins (Fig. 9). The first ORF, annotated as a xenobiotic response element (XRE) transcriptional regulator, contained a 10 bp insertion 323 bp upstream of the orthologous stop codon. To the best of our knowledge, function of the *A. marginale* XRE transcriptional regulator has not been reported. A second ORF, encoding a hypothetical protein (AnPIll_04873) at position 1,120,828-1,121,135, contained a deletion changing the final 20 amino acids and creating an early stop codon 18 bp upstream of the stop codon in the St. Maries homolog. Analysis with BLASTn (266), did not reveal an orthologous sequence outside of the genus *Anaplasma*, and InterPro and BLASTx did not reveal any conserved proteins or domains for this hypothetical protein.



Product	Indels	Gene Annotation/Position	SNPs Effect
Hypothetical protein, unknown function	1	AnPIII_04873, Pos. 1120828-1121135	Frameshift resulting in internal stop codon
XRE Family Transcriptional Regulator	10	AnPIII_04405, Pos. 1025958-1026524	Frameshift resulting in internal stop codon

Figure 9. Frameshift mutations resulting in premature stop codons.

A. Hypothetical protein with a premature truncation. **B.** XRE family transcriptional regulator with a premature truncation. **C.** Table of truncated proteins described in A. and B. (*) indicate regions of complete homology. Gaps in the sequence are indicated by dashes (-) and strain names are listed to the left of the genes. Deletions, insertions, and early stop codons are marked.

Candidate factors associated with the non-tick-transmissibility phenotype of the Illinois strain

Whole genome comparison of the Illinois and St. Maries strains with ACT revealed several CDS regions with variation, greater than single nucleotide polymorphisms, between the two strains, suggesting additional candidates if the non-tick-transmissible phenotype of the Illinois strain is not shared with the Florida strain. Some of these highly variant genes in the Illinois strain were also found in tick-transmissible and non-tick-transmissible strains. Such genes are potential additional candidates associated with the non-tick-transmissibility of the Illinois strain. Each ORF with variability from the corresponding St. Maries homolog was compared to available *Anaplasma marginale* sequences with known tick-transmission phenotypes. If the variable sequence was found in a tick-transmissible strain, then the sequence was removed from candidacy. Variable Illinois sequences with no match to tick-transmissible strains were considered candidates. Of the 20 genes found by whole genome comparison with St. Maries strain homologs, 12 candidate genes did not have a matching variable sequence in any tick-transmissible strain (Table 7).

Discussion

The purpose of this study was to identify candidate genes associated with non-tick-transmissibility of the Illinois strain of *A. marginale*. The Illinois strain genome was similar in size, GC content, coding percentage, pseudogene and functional pseudogene content as the Florida and St. Maries strain genomes.

Category	Gene Loci	Product	Explanation for candidacy
Variable Sequence	AnPIII_01340, Pos. 289876-290769	Hypothetical protein	Variable 3' end not consistent with tick-transmissible strains
	AnPIII_02595, Pos. 606939-607940	Hypothetical protein	Variable sequence, not consistent with tick-transmissible strains
	AnPIII_02605, Pos. 609464-610999	Hypothetical protein	Variable gene from the St. Maries strain, insufficient sequence coverage in tick-transmissible strains
	AnPIII_02955, Pos. 702290-703687	Hypothetical protein	Variable gene from the St. Maries strain, insufficient sequence coverage in tick-transmissible strains
	AnPIII_03745, Pos. 877039-878538	Hypothetical protein	Variable gene from the St. Maries strain, insufficient sequence coverage in tick-transmissible strains
	AnPIII_04055, Pos. 945067-949149	Hypothetical protein	Variable sequence from the St. Maries strain and several tick-transmissible strains, insufficient sequence coverage in the Gypsy Plains and Virginia strains
Insertion	AnPIII_01380, Pos. 303937-308271	Hypothetical protein	Unique insertion of 207 bp near the 3' end as compared to the St. Maries strain. Most closely aligns to the Oklahoma strain.
	AnPIII_01415, Pos. 326120-326518	Hypothetical protein	Insertion of a gene with 20% sequence coverage in tick-transmissible strains, partial sequence coverage found in *A. centrale*
	AnPIII_02430, Pos. 564613-564789	Translation initiation factor IF-2 (infB_2)	Unique insertion of a truncated 176 bp fragment of *infB*
	AnPIII_02670, Pos. 629376-629642	Hypothetical protein	Insertion of 63 bp as compared to the St. Maries strain. Insertion of variable base pair lengths as compared to tick-transmissible strains.
	AnPIII_03837, Pos. 898651-899193	Outer membrane protein 15 (Omp15)	Insertion of 87 bp compared to the St. Maries strain. Longer coding sequence than Oklahoma and Gypsy Plains strains. Insufficient sequence coverage in some tick-transmissible strains
Deletion	AnPIII_02625, Pos. 615120-615554	Hypothetical protein	Truncated gene 957 bp shorter than the St. Maries strain, insufficient sequence coverage in tick-transmissible strains

Table 7. Candidate genes with high variation associated with the non-tick-transmissibility phenotype of the Illinois strain of *A. marginale*.

Several proteins have been posited to play a role in tick infection or transmission. Comparison of these proteins confirmed that the Illinois strain *msp1α* most closely aligned to what was reported as non-tick-transmissible. In addition, the Illinois strain *ompA* contained a single nucleotide change that resulted in a non-synonymous amino acid change not found in any tick-transmissible strain. Several variations in intergenic regions in the *aaap* gene cluster were found, especially in homopolymer G tracts. This study found 27 candidate loci that are associated with the non-tick-transmissibility phenotype of the Illinois strain. 10 of these candidates are shared with the non-tick transmissible Florida strain.

Genome comparison of the Illinois, Florida and St. Maries strains revealed minor variation in terms of coding sequences. However, 961 CDS were identified in the Illinois strain as compared to 949 in the St. Maries strain with only two unique inserted genes and one truncated version of a gene in the Illinois strain as compared to the St. Maries strain (Table 4). The remainder of the CDS number variation is attributable to different annotation software. COG functional prediction of the Illinois strain CDS found that 11% of genes were identified as “function unknown”, in contrast to *Anaplasma ovis* with 26% of all predicted CDS regions having unknown function (91). Further, whole-genome phylogenetic analysis of available *A. marginale* genomes was consistent with previous alignments, with the addition of the Illinois strain genome in this study (234). During manual annotation of the Illinois strain genome, it was observed that it contained a 14.5-kb inversion (Fig. 8A) flanked by *virB2_2* and *virB2_3* genes. This inversion was also present in the Florida strain genome (Fig. 8B). It was posited that the genes in this inversion

or the inversion itself may play a role in non-tick-transmissibility phenotype between these two strains. The genes in Illinois that flank this inversion were 100% identical; however, in Florida and St. Maries strains, these genes were highly similar but not 100% identical. Comparative analysis of the genes encoded within the inversion as compared to other transmissible strains did not reveal anything remarkable as they were highly conserved with identical gene content. It has been reported in *Salmonella spp.* and other bacteria that inversions can aid in control of gene expression (267, 268). Hence, it is conceivable that inversion of this large fragment could be a method for controlling expression of these genes. In *E.coli* there is a strong preference towards lower numbers of genes encoded in a single operon (269). There were 13 genes encoded in the inversion, including pseudogenes and the flanking *virB2*; the data from *E. coli* would suggest that there are multiple promoters in this sequence. Thus, if the inversion controls expression it would likely affect those genes closest to the inversion start sites, which include *msp2* pseudogene, *msp3* pseudogene, a proline/betaine transporter (*proP*) and transcription termination factor Rho (*rho_1*). It seems unlikely that protein expression of the internal genes encoding the essential 30S and 50S subunits would be changed, as this would significantly affect or abrogate translation and thus make these strains self-limiting. Further, several putative rho-independent terminators are found in 3’ intergenic regions of genes encoded in the inversion (data not shown). The combination of the putative rho-independent terminators and several genes encoded on different strands in the inversion suggest that there

are multiple operons and not one long polycistronic mRNA encoding all the genes found in the inversion.

Research by McGarey *et al*. in 1994 reported that Msp1a and Msp1b are adhesins that bind to *A. marginale* initial bodies and may have a role in infection of erythrocytes (94, 270). Further *in vitro* studies on these two putative adhesins found that Msp1a has binding potential for both tick cells and bovine erythrocytes, while Msp1b only bound bovine erythrocytes (125). This led to the hypothesis that Msp1a tandem repeats are major players in tick transmission of *A. marginale* (231). This phenotype appears to be affected by the identity of the 20th amino acid residue in the tandem repeat region of Msp1a. In this study we report that the Illinois strain Msp1a contains 7 tandem repeats with 4 containing the posited non-tick adhering 20th amino acid, a glycine residue. The other repeats contain acidic residues associated with adherence to tick cells (Table 5). In addition, the neutralization sensitive epitope, QASTSS, and putative secretion domain of the Illinois Msp1a were 100% identical to the tick-transmissible Oklahoma strain. These results suggest that Msp1a alone is not the only determinant for the non-tick-transmissibility phenotype of Illinois because of the agreement of the sequence identity with a known tick-transmissible *A. marginale* strain. The other putative adhesin, OmpA, contained a single mutation of alanine to serine change (230). The domain of residues reported to be important for *A. marginale* infection of tick cells were reportedly far upstream from the location of this residue (230).

Comparison of the 5’ intergenic region between the low AAAP-expression Florida strain and high AAAP-expression Illinois strain found that the intergenic

region was 100% identical except for 1 base region in a PolyG tract at the 5' end of the intergenic region. The Illinois strain contained 10 guanine nucleotides and Florida contained 9 guanine nucleotides. It has been reported in *Neisseria meningitidis* that changes to a homopolymer G tract between the -35 and -10 affects expression of the protein PorA (271). Further, homopolymeric tracts in other species have been reported to affect expression in promoter regions as well (272, 273). If the *aaap* homopolymeric G tract, located in the intergenic region, is between the -35 and the -10 sigma recognition motifs then it is possible that this single indel could dictate the different AAAP expression levels seen between the strains by changing the face of the helix. Comparison of the 441 bp long intergenic region upstream of the entire *alp* and *aaap* cluster found that the Florida and Illinois strain sequences were virtually identical, except for 1 bp, for the first 352 bp, after which point the sequences deviated. This divergence in the final 89 bp of the upstream intergenic region flanking the *aaap* gene cluster may encode regulator or promoter sequences responsible for transcription or translation of this cluster. The Virginia strain *aaap* region is reportedly polymorphic (260), and comparison of the two different intergenic regions flanking the *aaap* gene cluster found variation between the two strains that was not consistent with the Illinois or Florida strains.

The premise underlying results shown in Table 6 is that Illinois strain is non-tick-transmissible for the same reason as the Florida strain. Pierlé *et al.* conducted comparative genomics and transcriptomics on the Florida strain, with several tick-transmissible strains, and identified 30 potential candidates associated with the

Florida strain's inability to be transmitted by ticks (126). SNPs resulting in non-synonymous residue changes shared uniquely between Florida and Illinois were considered candidates, and 20 of the 30 non-tick transmissibility gene or promoter candidates were eliminated. Two of the remaining 10 candidates had non-synonymous mutations found in the exact same position between the Illinois and the Florida strains. The remaining eight contained mutations found only in the Illinois strain, which could have potentially interrupted function or expression. The high degree of identity between the *A. marginale* strains, with different tick-transmission phenotypes, allowed for identification of interruptions in genes uniquely found in the Illinois strain. Two frameshifts resulted in a premature stop codon, truncating the protein, and plausibly affecting protein function (Fig. 9c). The first gene, a posited XRE transcriptional regulator, contained a helix-turn-helix (HTH) domain according to the NCBI conserved domain database and InterPro. BLASTn analysis showed this HTH domain has conservation with species outside of *Anaplasmataceae* including many organisms in the Bacteria domain. To the best of our knowledge, the binding sites in *A. marginale* and potential co-regulators are unknown. Interruption of a transcriptional regulator will most likely affect protein expression and result in uncontrolled regulation of target genes. Thus, it is possible that the *A. marginale* XRE transcriptional regulator affects expression involved with infection of the invertebrate host and/or expression of AAAP. The other frameshifted gene encoded a hypothetical protein with unknown function, which was interrupted near the 3' end, resulting in an early termination. Future research

is warranted to determine the function of this hypothetical protein and to delineate the regulon governed by the XRE transcriptional regulator.

Genomic comparisons of the *A. marginale* Illinois and St. Maries strains revealed multiple genes in the Illinois strain with high sequence variation (Table 7). One hypothetical protein, AnPIll_01415, was only found with 20% coverage in other *A. marginale* genomes. When compared to all *Anaplasmataceae*, this same sequence was found with 91% coverage and 97.54% identity to *Anaplasma centrale*, indicating that this gene is uniquely encoded in the Illinois strain and in *A. centrale*, but not in any other *A. marginale* genome. The annotated *A. centrale* protein is 87 amino acids longer than the Illinois strain protein. This poses an interesting question as to why this gene might be restricted to the *A. marginale* Illinois strain and *A. centrale*. Further study is warranted to ascertain the biologic function(s) of this hypothetical protein. A truncated duplication of the *infB* gene (*infB_2*), was also observed, but the presence of the full-length *infB* gene suggests that the truncated sequence is not as likely to impact the Illinois strain phenotype. Another gene, *omp15*, was identified in Illinois. The Illinois strain *omp15* is variable from St. Maries, but limited sequence coverage in 454-dervied sequences from tick-transmissible strains prevents determination of the sequence.

Future studies with whole genome sequencing with long read methods will be a logical next step to identify if these candidates are found in other tick-transmissible strains. Published *A. marginale* genomes often have gaps in membrane protein encoding loci assemblies and in homopolymer regions, 3 or more repeated bases in a row, and 454 technology is known to have issues with

homopolymeric tracts due to light intensity variance (274). SMRT sequencing has a noted reduction in error bias due to multiple reads at the same location allowing for consensus building (275). In addition, multiple *A. marginale* outer membrane proteins are known for having large repeat sequences. In this report, SMRT sequencing was used to overcome the limitation of Illumina and 454 sequencing and provide sequence coverage of these repeat-prone genes. As such, SMRT sequencing of other tick-transmissible and non-tick transmissible strains could be a valid approach for comparison of additional high-quality genome sequences.

This paper has identified several genes/protein candidates associated with the non-tick-transmissibility of the Illinois strain. The high-quality sequence of another non-tick-transmissible strain of *A. marginale* will further assist in understanding the function and identification of *A. marginale* genes. The candidates identified in this paper are expected to be important to guide future studies of the *A. marginale* tick-transmission phenotype, and these results justify further work to generate additional high-quality genomes with the same approach. High-depth PacBio sequencing of the repeat and homopolymeric tract prone *A. marginale* Illinois genome permitted the identification of several candidates that may be associated with *A. marginale* infection and transmission by ticks. Functional studies will be necessary to delineate the role of these candidates.

Acknowledgements

This research was supported by USDA NIFA PROJ NO: MO.W-2016-09373 and the University of Missouri Department of Veterinary Pathobiology.

CHAPTER 3

Protein antigens associated with seroreactivity of cattle producing reduced tick performance

Abstract

Dermacentor andersoni, the Rocky Mountain wood tick, is indigenous to the United States and is an important vector of multiple tick-borne pathogens. The tick midgut (MG) and salivary gland (SG) are the two main organs that directly interact with the vertebrate host. Importantly, immunization of vertebrate hosts with extracts of these organs has been shown to reduce tick performance after feeding on immunized animals compared to control animals. Here we adapted a large-animal model system to *D. andersoni* for immunization against the tick host using MG and SG. Immunization of cattle with SG resulted in a greater reduction in tick performance of ticks fed on those animals than baseline or midgut immunized calves. Immunization of calves induced significant seroconversion as demonstrated by increasing titers throughout the immunization schedule and during tick feeding. Two-dimensional western blots developed with serum from SG immunized animals reacted with 44 candidate protein spots associated with reduced tick performance. Protein spots that reacted with SG immune sera were isolated and processed for liquid chromatography with tandem mass spectrometry, this process identified 258 proteins. A bioinformatic elimination strategy found eight proteins that only reads in *D. andersoni* SG transcriptomic datasets. Collectively,

these results indicate that the *D. andersoni* SG has the potential to yield antigenic targets that significantly reduce tick feeding and fecundity performance.

Introduction

Ticks infest every class of land-dwelling vertebrate, are found on every continent including Antarctica and transmit a greater variety of pathogens than any other arthropod group (4, 276). Tick populations and tick-borne pathogens are increasing globally, with the number of tick-borne infections in the United States doubling from 2004 to 2016 (277, 278). Historically, the best method for control of vectors and vector--borne pathogens is through environmental control by controlling the vector. Acaricides are one of the most commonly employed methods of tick control in livestock; however, acaricides unfortunately result in contamination of the environment, contamination of food products from animals and promote acaricide resistance (279). Biological control of the vector has been reported with success marked by limited performance of ticks during feeding but research towards an effective vaccine has been slow (168, 169, 171, 173, 194, 280, 281). Gavac is the only internationally licensed commercial vaccine against ticks and is based off a concealed midgut antigen, Bm86. An unfortunate limitation with Bm86 is that there is limited success with more divergent tick species not closely related to the species this glycoprotein was isolated from, *Rhipicephalus microplus* (199, 282, 283). Due to the current nature of control of ticks, primarily to environmental control and acaricides, a method of immunological control of the vector is desirable as it limits the need for acaricides.

Several studies have shown that repeated infestation and immunization with tick tissues induce resistance to tick feeding. Some of the earliest research regarding the hereditary component of tick resistance in cattle was documented in 1918 by Johnston and Bancroft (155). Trager was the first to report that repeated infestation induces tick resistance (156). Other studies have shown that repeated infestation of animals by various species of ticks also induces vertebrate resistance to tick feeding (163, 284, 285). In addition to repeated infestation, immunization of hosts with tick tissues has been reported to reduce tick feeding and fecundity performance parameters and this resistance is passively transferable (161, 168, 169, 175, 188–190). Investigations that have used immunization of vertebrate hosts with tick tissues have resulted in significant success in reducing tick performance. This phenomenon is repeatable with cattle (160, 161, 169, 181).

Dermacentor andersoni, the Rocky Mountain wood tick, is indigenous to North America and an is important vector of multiple pathogens including *Anaplasma marginale*, *Francisella tularensis, Coxiella burnetii* and *Rickettsia rickettsii* (286). It is unknown if immunization against *D. andersoni* midgut (MG) and salivary gland (SG) from different time points during tick feeding induces similar effects seen with other tick species. The purpose of this study is to adapt the research of previous immunizations of vertebrate hosts with tick tissues to a model system in the United States and identify antigens associated with reduced tick performance parameters. Calves in this study were immunized with *D. andersoni* denatured SG proteins or native MG proteins as previously described

(168, 169). Performance of ticks that fed on immunized cattle were recorded. Seroconversion was measured in calves with ELISAs and two-dimensional western blots. Immunization with *D. andersoni* SG had the greatest impact on tick performance. Thus, proteins uniquely recognized by sera from calves immunized with tick SG were identified with liquid chromatography with tandem mass spectrometry (LC-MS/MS) and searched bioinformatically with tBLASTn to identify proteins present in *D. andersoni* SG transcriptomic data. This study demonstrated that immunization with *D. andersoni* tick tissues is capable of significantly reducing tick performance.

Materials and Methods

Ticks

D. andersoni adults were obtained from the Oklahoma State University Medical Entomology Laboratory. Ticks were stored at 25°C, >90% relative humidity, and provided a photoperiod of 12 h:12 h (L:D).

Antigen preparation

Adult, 3-5 day fed, male and female *D. andersoni* were bisected along their dorsal surface. *D. andersoni* SG and MG organs were removed, dissected free of other tissues, and placed in sterile 1X PBS, pH 7.4, at 4°C. SG were denatured overnight in 1% (w/v) SDS, 5% (v/v) β-mercaptoethanol at 56°C followed by boiling for 5 minutes. The solution was cooled to room temperature, transferred to a 12,000-14,000 molecular weight cut off dialysis tube (Spectra/Por7, Denver, CO), immersed in 1 L of PBS, and maintained at 4°C on a magnetic stirrer overnight

(PBS was changed every 4-6 h). Protein concentrations were estimated using the Bradford method. The mixture (0.5 mL, 2 mg protein/mL) was filtered and mixed with 0.5 mL of complete or incomplete Freund's adjuvant H37Ra (Fisher Scientific, Pittsburg, PA) immediately prior to immunization of the hosts.

Tick MG was also removed and stored in 1X PBS at 4°C. Organs were disrupted with a tissue homogenizer followed by sonication for 15 seconds (model 300 sonic dismembrator, Fisher Scientific) set at 35 and 50% output power, a total of 10 times. MG tissues were dialyzed in PBS at 4°C overnight and centrifuged at 16,000 *xg* for 30 minutes at 4°C. MG samples were processed through a 0.45-μm filter (Millipore, Bedford, MA). The protein concentration of the extract was estimated using the Bradford method. 0.5 mL of MG mixture (2 mg of protein/mL) was mixed thoroughly by sonication with 0.5 mL of complete or incomplete Freund's adjuvant H37Ra immediately prior to immunization of hosts.

Immunization

Four Holstein steers of 3-6 months old were used in this experiment. Calves were housed in an enclosed building at the University of Missouri Middlebush farm. Calves were maintained according to the University of Missouri OAR, ACUC protocol #8981. Four calves, two per group, were immunized intradermally at 10 sites along the dorsal surface with 1 mg of the SG or MG extracts plus Freund's complete adjuvant. The second and third immunizations were given with Freund's incomplete 2 weeks after the first and second immunizations, respectively.

ELISA

Sera from calves immunized with native or denatured *D. andersoni* SG or MG were tested with an indirect ELISA against *D. andersoni* MG or SG antigens (Ag). 96-well high binding ELISA microplates (Greiner Bio-One, Monroe, NC) were coated overnight at 4°C with 75 µL of protein antigen at a concentration of 1.0 µg/mL in 0.1 M carbonate coating buffer (pH 9.6). Plates were washed with 0.5% (v/v) Tween-20 PBS (PBS-T) five times and then wells were blocked for thirty minutes with PBS-T. Plates were further blocked for 2 h with 100 µL 3% (w/v) BSA in PBS. After blocking, 60 µL of primary antibody was added at a concentration of 1/100 and diluted 1/10 until a final dilution of $1x10^8$ was achieved and samples were incubated for 3 h at 37°C. Plates were washed five times with PBS-T and 50 µL of rabbit anti-bovine IgG (Invitrogen, Carlsbad, CA), diluted 1:10,000 in PBS-T, was added to each well for 15 minutes at room temperature. 50 µL of 3,3,5,5-tetramethylbenzidine (TMB) solution (1 mM TMB, 0.0665 M citric acid, 0.0306 M sodium citrate dihydrate and 0.01068% (v/v) H_2O_2) was added to each well and incubated for 15 minutes. The reaction was stopped with 50 µL of 2 M H_2SO_4 and optical densities were measured at 450 nm. Negative controls included samples without 1° antibody (Ab) and 2° Ab alone without 1° Ab to ensure the colorimetric reaction was due to Ag-Ab complex formation.

Tick performance parameters

After 14 days post-second boost, calves were challenged with 25 pairs of adult male and female *D. andersoni*. Calves were checked daily for ticks, and

recovered (detached) ticks were counted, weighed, and maintained in tick humidity chambers until oviposition was complete. Egg clusters were weighed and maintained until hatching occurred. The following biological tick performance parameters were measured during each infestation: feeding period, mortality, tick weight and egg clutch weight. These performance parameters were used to calculate feeding and fertility efficiency indices as previous described (163, 168, 169). Engorged female weight was measured immediately after detachment. Egg clutch masses were determined after oviposition was complete. The engorgement period was assumed to be the time that elapsed from tick attachment to tick removal at partial or full engorgement. The feeding efficiency index was calculated by dividing the weight of each engorged female by her feeding period. The fecundity efficiency index was calculated by dividing the weight of each egg mass by the weight of each respective female.

One-dimensional gel electrophoresis

Unfed tick SG and MG tissues were isolated as previously described from *D. andersoni* and stored in lysis solution (8 M urea, 2 M thiourea, 2% (w/v) CHAPS or 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate) at 4°C. Tissues were homogenized using a dounce homogenizer. Long-term storage of proteins was at -20°C. For electrophoresis, equal volumes of male and female MG or SG tissues were mixed with 1X PBS and 1X loading solution (10% (v/v) glycerol, 60 mM Tris/HCl pH 6.8, 2% (w/v) SDS, 0.01% (w/v) bromophenol blue, 1.25% (v/v) β-mercaptoethanol) and then heated to 90-100°C for 10 minutes. The mixture was loaded onto 12% SDS-PAGE gels in a Mini-PROTEAN Tetra Cell (BioRad,

Hercules, CA) with running buffer (192 mM glycine, 3.45 mM SDS, 25 mM Tris-base pH 8.3) and separated at 125 V constant for 1 h or until the dye front proceeded to reach the bottom of the gel. After gels were completely separated, they were then transferred to PVDF membrane at 350 mA constant in the Mini Trans-Blot Electrophoretic Transfer Cell (BioRad) for 1 h and 10 minutes following the manufacturer's instructions. After transfer, gels were fixed with 25% (v/v) isopropanol and 10% (v/v) acetic acid for 15 minutes and subsequently rinsed with water three times at 10 minutes each. Finally, gels were stained overnight with PageBlue (Thermo Scientific, Waltham, MA) and then destained in water overnight and images were taken.

Two-dimensional gel electrophoresis

SG and MG tissues were isolated and homogenized as previously described. Protein concentrations were calculated using the Bradford method and 20 µg of male and 20 µg of female tissue were rehydrated overnight on a 7 cm 3-11NL immobilized pH gradient (IPG) Strips (GE, Pittsburg, PA) in rehydration solution (8 M urea, 2 M thiourea, 2% (w/v) CHAPS, 0.5% (v/v) ZOOM carrier ampholytes pH 3-10, 0.002% (w/v) bromophenol blue) according to the manufacturer's instructions. Separation of proteins in the first dimension was performed in the ZOOM IPGRunner System (Invitrogen) at 200 V for 20 minutes, 450 V for 15 minutes, 750 V for 15 minutes, and a final step of 2000 V for 1 h and 30 minutes at 4°C. IPG strips were removed from cassettes and transferred to a 12% SDS-PAGE gel and separated in the second dimension following the same

protocol as for one-dimensional gel electrophoresis. Gels were transferred to membranes or stained with PageBlue.

Western blotting

MG and SG antigenic proteins were detected using primary immune sera from immunized cattle. Membranes were incubated while rocking for 1 h with immune sera diluted 1:400 in 1X TBS 0.1% Tween-20 (TBS-T). Afterwards, the membranes were washed 3X with TBS-T for 5 minutes each, secondary antibody rabbit anti-bovine IgG (Invitrogen) diluted at 1:10,000 in TBS-T was incubated with membranes for 1 h at room temperature (RT). Membranes were subsequently washed 3X with TBS-T for 5 minutes each, developed in a diaminobenzidine (DAB) solution (0.8 mg/mL DAB, 100 mM Tris-HCl pH 7.5, 0.4 mg/mL $NiCl_2$, 0.009% H_2O_2) for 40 minutes. Staining was halted with water.

Sample preparation, trypsin digestion, peptide enrichment

Protein spots were excised from a Coomassie stained gel. Proteins were destained, trypsin digested (Promega, Madison, WI), and subjected to C18 tip purification according to the manufacturer’s instruction (Pierce, Appleton, WI). Sample containing peptides were lyophilized, resuspended in 10 µL of 5% (v/v) acetonitrile, 0.1% (v/v) formic acid and transferred to an autosampler vial.

Mass spectrometry

Peptides were analyzed by mass spectrometry as follows: a 1 µL injection was made directly onto a 20 cm long x 75 µm inner diameter pulled-needle analytical column packed with ethylene bridged hybrid-C18 (BEH-C18) (Waters,

Milford, MA), 1.7 μm reversed phase resin. Peptides were separated and eluted from the analytical column with a gradient of acetonitrile at 300 nL/minute. The Bruker nanoElute system was attached to a Bruker (Billerica, MA) timsTOF-PRO mass spectrometer via a Bruker CaptiveSpray source. Liquid chromatography (LC) gradient conditions: Initial conditions were 3%B (A: 0.1% formic acid in water, B: 99.9% acetonitrile, 0.1% formic acid), followed by 7.5 minutes gradient to 17%B. Then 17-25%B over 7.5 minutes, 25-37%B over 5 minutes, 37-80%B over 2 minutes, hold at 80%B for 5 minutes, ramp back to (1 minute) and hold at (2 minutes) initial conditions. Total run time was 30 minutes. MS data were collected in a positive-ion data-dependent parallel accumulation-serial fragmentation (PASEF) mode (1) over an m/z range of 100 to 1700, the samples were run on 2/26/2021 with the last calibration date of 2/22/2021. PASEF and trapped ion mobility spectrometry (TIMS) were set to "on". One MS and ten PASEF frames were acquired per cycle of 1.27 sec (~1 MS and 120 MS/MS). Target MS intensity for MS was set at 20,000 counts/sec with a minimum threshold of 250 counts/s. A charge-state-based rolling collision energy table was used from 76-123% of 42.0 eV. An active exclusion/reconsider precursor method with release after 0.4 minutes was used. If the precursor (within mass width error of 0.015 m/z) was >4X signal intensity in subsequent scans, a second MSMS spectrum was collected. Isolation width was set to 2 m/z (<700 m/z) or 3 (800-1500 m/z). Mass spectrometry was performed at the University of Missouri Gehrke Proteomics Center.

Database searches (protein identification)

The trapped ion mobility spectrometry time of flight (timsTOF) data were converted to mascot generic format (MGF) files using the "shotgun-pasef" script in the Bruker Compass Hystar acquisition software and submitted to the Proteome Discoverer (Sequest HT) search engine for protein identifications. The protein database (Genbank accession number: GCA_013339745.1) for *Dermacentor silvarum* (26,821 sequences) was downloaded and used for the search. An automated decoy database search was conducted in which all sequences are reversed and added to the search to generate a false discovery rate (FDR) of 1% for protein/peptide matches. Data were searched with trypsin as enzyme, two missed cleavages allowed; carbamidomethyl cysteine as a fixed modification; oxidized methionine, deamidation of N/Q, as variable modifications; 20 ppm mass tolerance on precursor ions, 0.1 Da on-fragment ions. Search results files were filtered for "high" confidence (1% FDR for protein matches).

Bioinformatic analysis of mass spectrometry data

Proteins were accessed from the GenBank accession number provided with the mass spectrometry data set. Protein sequences were subsequently put into the tBLASTn engine (244) and searched against sequence read archives (SRA) databases for *D. andersoni* SG transcriptomic studies including SRX540759, SRX174799 and SRX174800 (245, 287). SRA accession numbers for *D. andersoni* MG were SRX608533, SRX540759, SRX540760 and SRX540761.

Statistical analysis

Performance parameter data collected during tick feeding were compared between pre-immune control and treatment groups (SG and MG) using a t-test or its nonparametric equivalent, where necessary. ELISA statistical analysis was conducted using a two-way analysis of variance (ANOVA) with a Holm-Sidak post-hoc. All analyses were performed using SigmaPlot (Systat Software, San Jose, CA). A $P \leq 0.05$ was considered statistically significant.

Results

Immunization of calves with *D. andersoni* tissues

Repeated infestation and immunization with tick tissues is known to induce antibodies (175, 288), and antibodies are posited to play a role in tick resistance (188, 189). To evaluate the utility of this approach for the bovine-*D. andersoni*-model system, immunization with similarly prepared *D. andersoni* MG and SG was used to quantify seroconversion. Two groups of calves (n = 2 per group) were immunized with native MG or denatured SG antigen preparations. Trial 1 included calves 2249 (MG immunized) and 2335 (SG immunized) and trial 2 calves were 2324 (MG immunized) and 2468 (SG immunized). Titers to *D. andersoni* MG or SG were measured using indirect ELISAs.

Antibody titers and tick tissue cross-reactivity were compared between calves. All animals exhibited increasing titers during timepoints measured throughout the immunization schedule and during tick feeding (Fig. 10). Serum from calf 2249, MG immunized (Fig. 10A), first had a detectable titer on day 9 post-immunization. The titer for calf 2249, to both tissue types, increased throughout

the immunization period and further increased significantly on the measured timepoint at the beginning of tick feeding, day 42. Serum from calf 2324, MG immunized (Fig. 10B), showed a higher cross-reactive titer to SG than MG for all measured timepoints. In addition, titers for calf 2324 displayed increasing titers to both tissues throughout the immunization schedule and it increased significantly at day 42. Calf 2335, SG immunized (Fig. 10C), showed a rapid increase in titers to both tissue types throughout immunization. Titers significantly increased after the secondary boost at several measured timepoints including day 28 and during early tick feeding at day 42. Finally, calf 2468, SG immunized (Fig. 10D), titers increased throughout the course of immunization to both tissue types and significantly at day 28 at which point it remained elevated throughout tick feeding.

Comparative performance of female *D. andersoni* fed on normal calves or after feeding on midgut or salivary gland immunized calves

Previous research with several tick species has shown that a significant reduction in tick feeding or fecundity performance parameters occurs when ticks are fed on animals that were immunized with SG or MG tissues, respectively (151, 152, 156, 185). Thus, it was hypothesized that tick performance would be reduced after feeding on immunized calves as compared to pre-immune controls. A reduction in tick performance after feeding on *D. andersoni* immunized calves would demonstrate that *D. andersoni* is a suitable model for tick tissue immunization. A random block design was chosen with the pre-immune tick performance, baseline feeding, measured prior to tick feeding. This approach allowed for elimination of variation between cattle by feeding ticks prior to

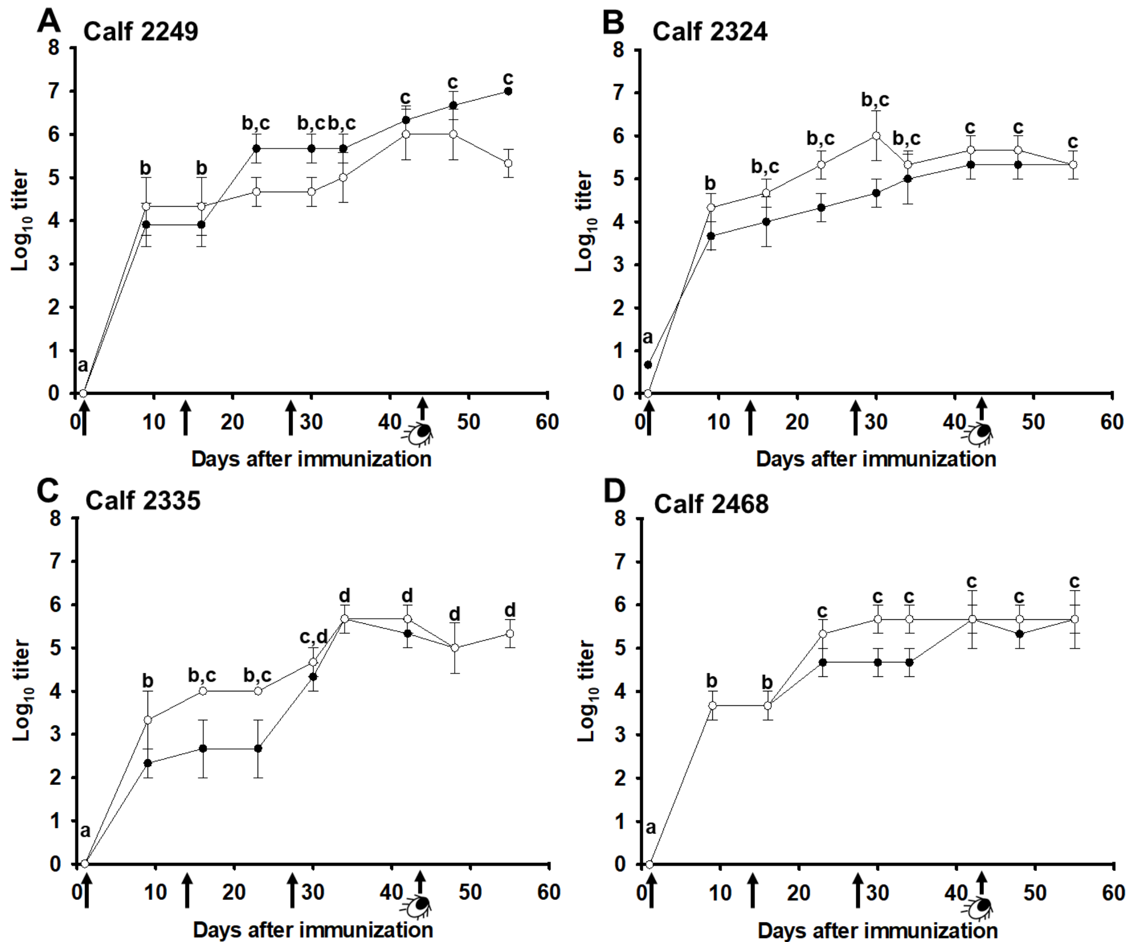


Figure 10. Antibody responses of calves immunized with *D. andersoni* native midgut or denatured salivary gland homogenates.

Calves were immunized with *D. andersoni* SG or MG every two weeks indicated by arrows. 25 pairs of adult *D. andersoni* were applied for challenge infestation as indicated by the tick illustration. **A.** Serum samples were measured from MG immunized calves 2249 (A) and 2324 (B) and SG immunized calves 2335 (C) and 2468 (D) with an ELISA using tick salivary glands (open circles) or midgut (closed circles). Superscripts a, b, c, d represent statistical differences based upon a two-way ANOVA with a Holm-Sidak post-hoc ($P \leq 0.05$).

tick feeding. This approach allowed for elimination of variation between cattle by feeding ticks prior to immunization. Ticks were applied to calves after immunization and fed to repletion and the parameters, including feeding period, feeding efficiency, female engorgement weight, egg cluster weight and fecundity efficiency were monitored. Tick performance parameters were compared between pre-immune and post-immune.

Tick feeding periods for all immunized calves increased in response to immunization, indicating ticks took longer to reach repletion. Adult *D. andersoni* fed on trial 1 calves 2249 (MG immunized) and 2335 (SG immunized) had a pre-immune feeding period of 9.36 and 9.08 days, respectively (Fig. 11). The feeding period increased to 13.61 ($P < 0.001$; 45% increase) and 12.08 days ($P < 0.001$; 40% increase) for post-immune feeding. For *D. andersoni* fed on trial 2 calves 2324 (MG immunized) and 2468 (SG immunized) pre-immune feeding period was 9.2 and 9.76 days, respectively. Feeding period lengthened to 11.63 ($P < 0.001$; 40% increase) and 15 days ($P < 0.001$; 53% increase) for ticks fed on immunized calves. There was no visible trend among the immunogen groups that would suggest one immunogen more significantly affects feeding period. *D. andersoni* adult female engorgement weight decreased in all immunized calves (Fig. 11).

The female engorgement weight after immunization was significantly lower in all immunized calves. Trial 1 pre-immune female engorgement weight for 2249 and 2335 calves was 592 and 610 mg, respectively. Female engorgement weight decreased to 461 ($P < 0.039$; 22% decrease) and 297 mg ($P < 0.001$; 51% decrease) after immunization.

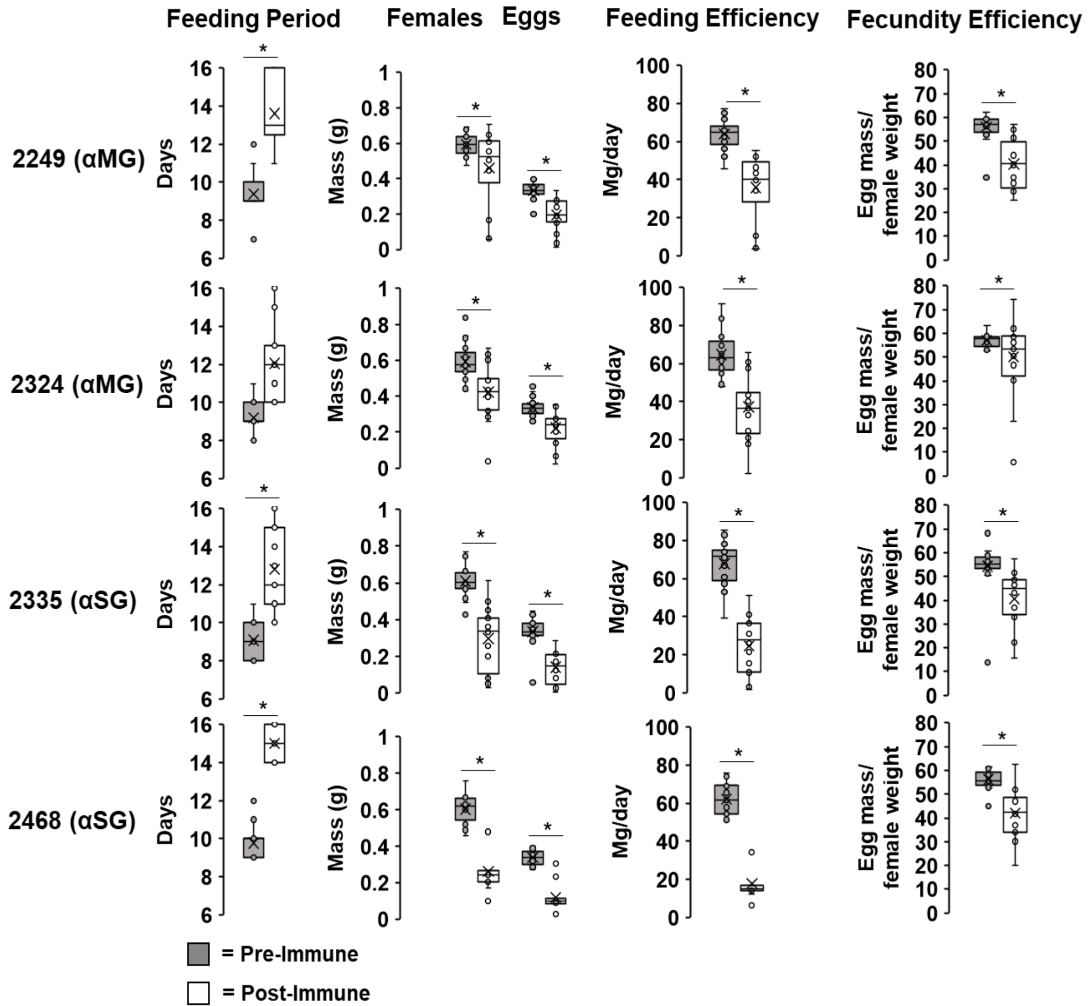


Figure 11. Performance of engorged female *D. andersoni* recovered from immunized and control calves.

Calves were immunized, as described in the text, with SG or MG tissues. Calves were subjected to tick feeding challenge with 25 female and 25 male ticks. Ticks were collected subsequently post-feeding and weighed. Performance parameters were measured, and these are defined in the methods. Boxes indicate the median, 1st and 3rd quartiles. Whiskers extend to the high and low values in the range of values. An asterisk indicates a significant difference at $P \leq 0.05$ based upon a t-test or its non-parametric equivalent.

Trial 2 calves 2324 and 2468 pre-immune female engorgement weights were 591 and 603 mg, respectively. After immunization, the female engorgement weight fell to 421 ($P < 0.001$; 28% decrease) and 262 mg ($P < 0.001$; 56% decrease). Female engorgement weight had a higher percent decrease in ticks fed upon SG immunized calves.

The *D. andersoni* egg cluster weight was significantly reduced after immunization in all groups (Fig. 11). Egg cluster weights for ticks fed on trial 1 calves 2249 and 2335 were 317 and 335 mg, respectively. After immunization egg cluster weight decreased to 194 ($P < 0.001$; 41% decrease) and 141 mg ($P < 0.001$; 57% decrease). Pre-immune egg cluster weights for ticks fed on trial 2 calves 2324 and 2468 were 336 mg for both calves. The trial 2 egg cluster weights decreased to 223 ($P < 0.001$; 34% decrease) and 118 mg ($P < 0.001$; 64% decrease) after immunization. While all immunizations had a significantly different egg cluster weight after immunization, SG immunization of calves had the greatest impact on tick egg cluster weight.

Feeding efficiency, female engorgement weight divided by feeding period for each female, was significantly reduced in all calves after immunization (Fig. 11). Trial 1 pre-immune feeding efficiency for 2249 and 2335 was 63 and 67 mg/day, respectively. The feeding efficiency decreased to 35 ($P < 0.001$; 43% decrease) and 24 mg/day ($P < 0.001$; 63% decrease) after immunization. In trial 2, pre-immune for calves 2324 and 2468 the feeding efficiency was 64 and 62 mg/day, respectively. The feeding efficiency decreased to 37 ($P < 0.001$; 42% decrease) and 17 mg/day ($P < 0.001$; 71% decrease) after immunization.

Fecundity efficiency, dividing the weight of the egg mass by the female engorgement weight for each female, was significantly reduced in all calves after immunization (Fig. 11). Trial 1 calves 2249 and 2335 pre-immune fecundity efficiency was 55 and 54, respectively. This decreased to 40 ($P < 0.001$ for both; 27 and 25% reduction for 2249 and 2335, respectively) for both calves after immunization. Trial 2 pre-immune fecundity efficiency for calves 2324 and 2468 was 57 and 56, respectively. This decreased to 50 ($P < 0.046$; 12% decrease) and 42 ($P < 0.001$; 25% decrease) after immunization. *D. andersoni* post-immunization performance parameters, excluding fecundity efficiency and feeding period, were more significantly decreased in SG immunized calf 2335 than MG immunized calves. However, the most significant reductions in all tick performance parameters, excluding fecundity efficiency, was recorded in SG immunized calf 2468.

Recognition of antigens

Antibodies are induced in response to immunization and titers increase during subsequent tick extract booster immunizations of calves. Antibodies produced by immunized calves are expected to have specific tick tissue targets that will be associated with reduced tick performance. Ticks fed on SG immunized calf 2468 displayed the lowest tick performance in all calves tested and it was hypothesized that western blots with sera from 2468 would have uniquely recognized antigens. Two-dimensional gel electrophoresis of MG and SG proteins yielded a sizable number of proteins found throughout the pH gradient (Fig. 12). Some of the proteins observed in the two tissues were apparently similar. Notably,

several strongly reactive proteins are seen in the low molecular weight and basic region of the SG protein gel and these are also detectable with the MG protein gel. Pre-immune sera were used as a negative control, and as expected, little to no recognition was observed (Fig. 13A-D). MG immune serum did not illicit strong protein spot patterns on SG tissues and had similar patterns between the two MG immunized calves (Fig. 14A,B). SG immune serum was highly reactive with protein spot patterns between 37-50 kDa and a pH greater than 6.0 on both antigen preparations (Fig. 14C,D). Several MG proteins cross-reacted with SG immune sera (Fig. 14C,D). Calf 2468 had the strongest western protein spot pattern with the highest cross-reactivity to MG tissues.

SG unique proteins

Cross-reactive proteins for this study are defined as proteins that react with another tick tissue other than the one used for immunization. Several cross-reactive proteins were recognized by MG or SG immunized calf sera. *D. andersoni* performance was most significantly reduced on SG immunized calves; therefore, SG unique proteins were targeted for LC-MS/MS. An elimination strategy was employed which included MG immunized calf serum incubated with SG tissues to identify cross-reactive proteins. In addition, further elimination involved SG immunized calf serum used with MG and SG tissues to identify cross-reactive and unique proteins, respectively. Comparison of the protein spot patterns between MG and SG immune calf sera yielded several proteins uniquely recognized in SG tissue by SG immunized calf sera. Post-immune calf 2335 serum recognized 19 unique western blot spots and 2468 post-immune serum recognized 25 unique

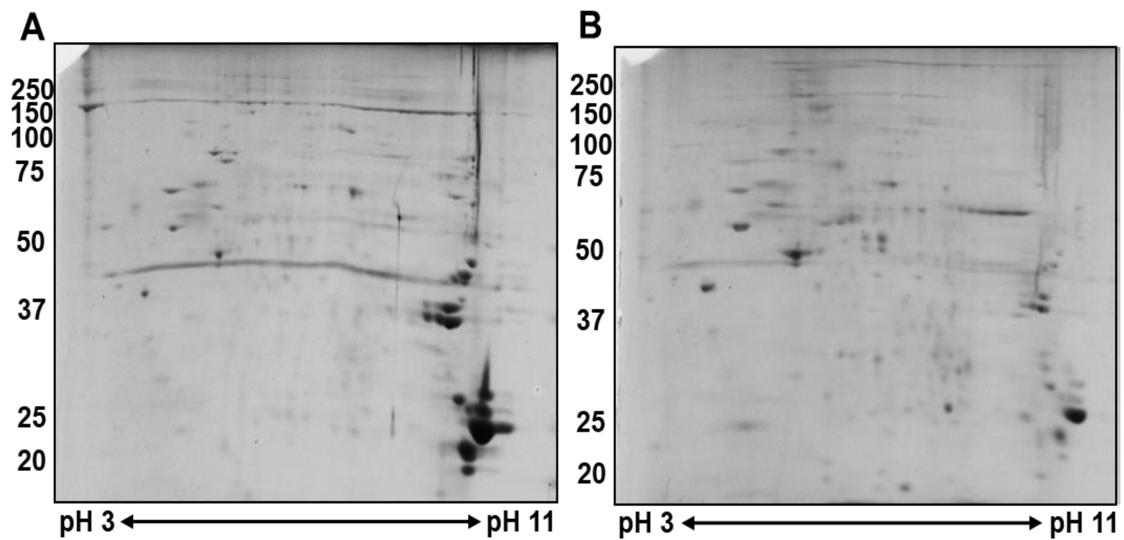


Figure 12. Coomassie stained *D. andersoni* salivary gland and midgut proteins.

D. andersoni SG (A) and MG (B) proteins were separated on two-dimensional gel electrophoresis, as described in the methods, and stained with PageBlue. The pH range for isoelectric focusing (IEF) is indicated at the bottom of each image. The molecular weight standard (kDa) is indicated on the left of each gel image.

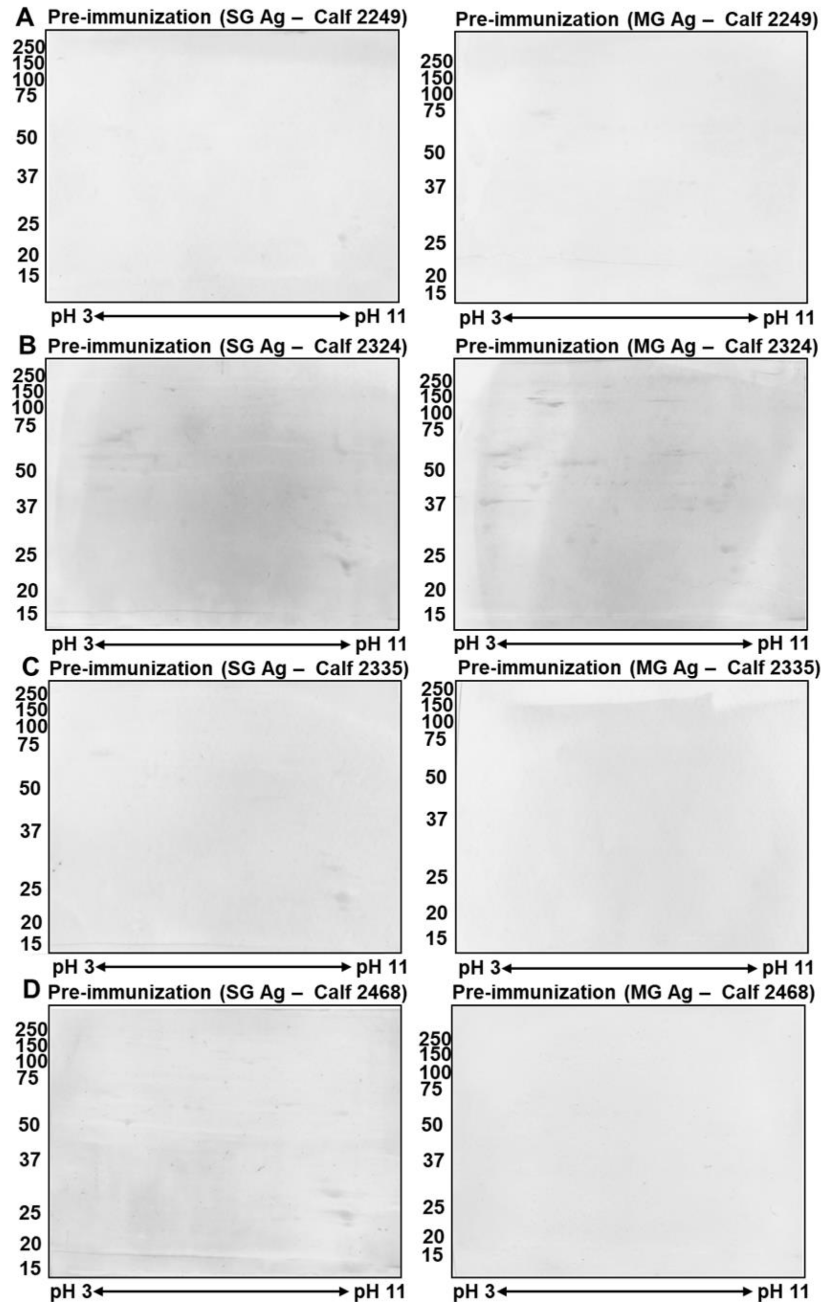


Figure 13. Pre-immune sera reactivity on midgut and salivary gland protein western blots.

D. andersoni SG (left column) and MG (right column) proteins were separated on two-dimensional gel electrophoresis and transferred to membranes. Immunoblot PVDF of transferred proteins was visualized with sera from immunized calves and DAB substrate as described in the methods. Pre-immune sera were incubated with membranes from MG immunized calves 2249 (A) and 2324 (B) and SG immunized calves 2335 (C) and 2468 (D). The pH range for IEF is indicated at the bottom of each image. The molecular weight standard (kDa) is indicated on the left. Abbreviations include Ag – antigen, SG – salivary gland, MG – midgut.

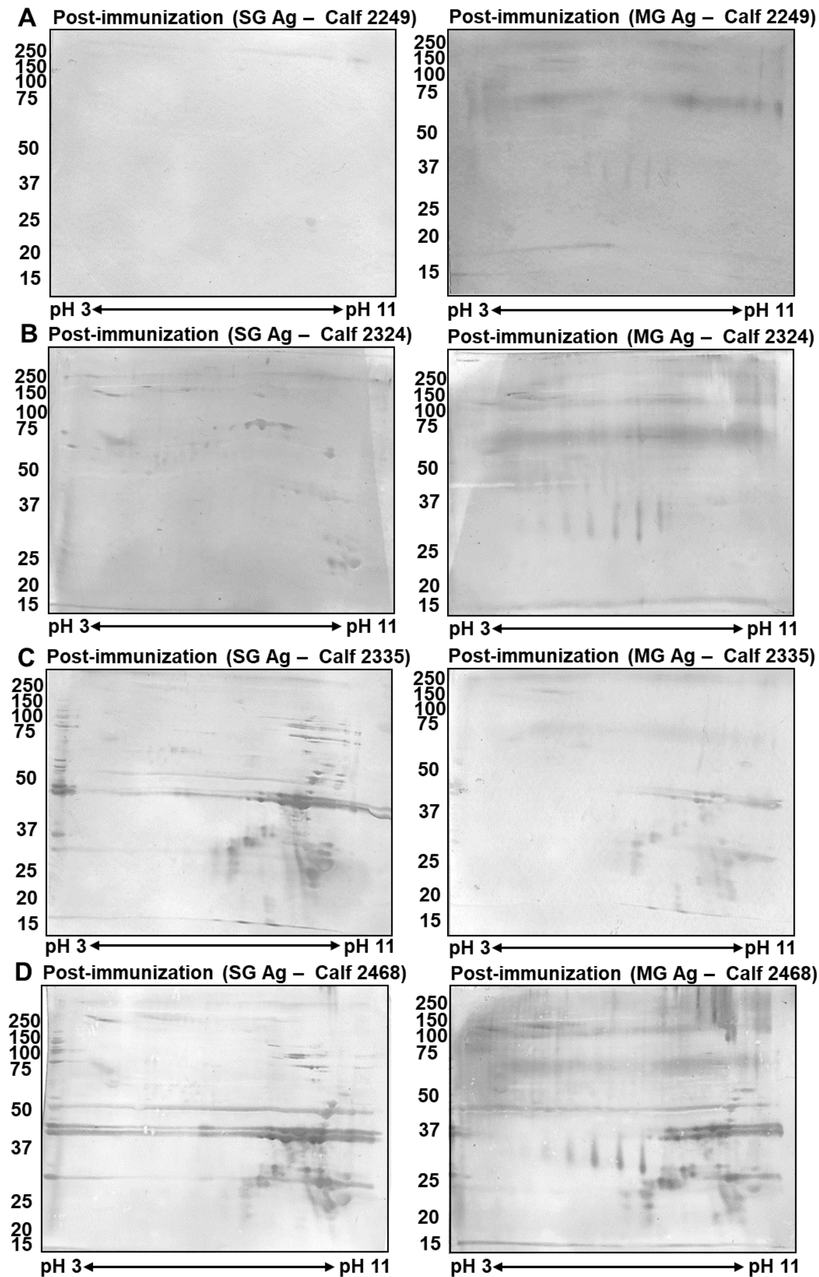


Figure 14. Recognition of *D. andersoni* midgut or salivary gland proteins that react to immune sera of immunized calves.

D. andersoni SG (left column) and MG (right column) proteins were separated on two-dimensional gel electrophoresis and transferred to membranes. Immunoblot PVDF of transferred proteins was visualized with sera from immunized calves and DAB substrate as described in the methods. Post-immune sera were incubated with membranes from MG immunized calves 2249 (A) and 2324 (B) and SG immunized calf sera from calves 2335 (C) and 2468 (D). The pH range for IEF is indicated at the bottom of each image. The molecular weight standard (kDa) is indicated on the left. Abbreviations include Ag – antigen, SG – salivary gland, MG – midgut.

spots (Fig. 15). Some spots are shared between the two SG immunized calves. Shared proteins include protein 1 (2335) and proteins 2, 3, 4 (2468), several proteins ranging from 7-13 (2335) and from 6-18 (2468), 17 (2335) and 21 (2468), 18 (2335) and 24 (2468) and 19 (2335) and 25 (2468) (Figure 15; Table 8).

LC-MS/MS and identification of SG unique proteins

Calves 2335 and 2468 have 19 and 25 SG unique protein spots recognized, respectively (Table 8). The identity of these proteins is unknown, and LC-MS/MS followed by a database search with a closely related *Dermacentor* genome was expected to yield potential candidates to identify the SG unique proteins. The *D. silvarum* proteome was chosen for LC-MS/MS database searching as no *D. andersoni* genome is currently available. Proteins were excised from SG protein two-dimensional gels and processed for LC-MS/MS. Database search of m/z against the *D. silvarum* proteome identified 258 proteins from the excised gel spots. Identified *D. silvarum* proteins were searched against *D. andersoni* transcriptomic data for MG and SG tissues. *D. silvarum* proteins that had strong read coverage and sequence identity to *D. andersoni* MG RNA transcriptomic data were removed from candidacy. *D. silvarum* proteins that limited read coverage and sequence identity to either *D. andersoni* MG or SG RNA transcriptomic data were unable to be eliminated and considered candidates. The *D. silvarum* proteins that only had strong coverage and sequence identity with *D. andersoni* SG RNA transcriptomic data were considered candidates.

Analysis of these proteins with clusters of orthologous groups (COG) functional prediction identified, large numbers of proteins involved in RNA

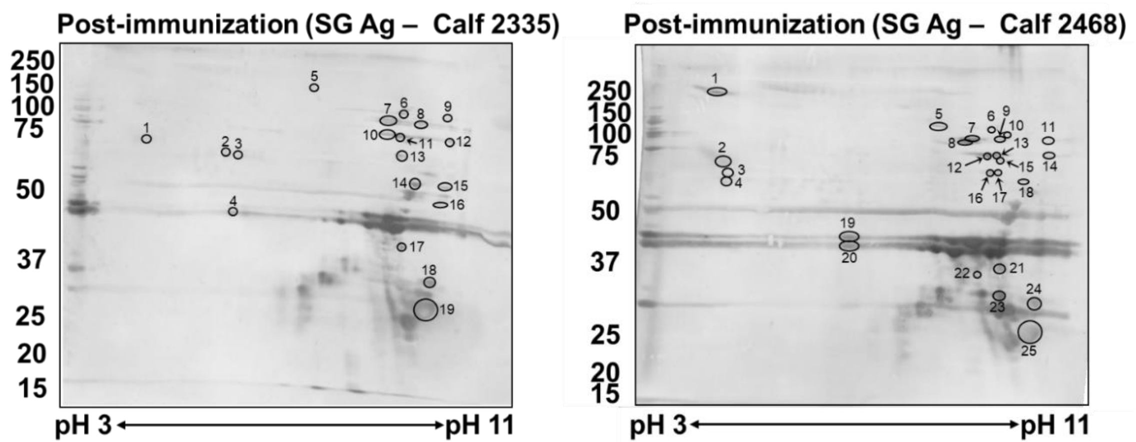


Figure 15. Salivary gland protein spots candidates.

SG proteins were separated on two-dimensional western blots as described in the methods. Immunoreactive protein spots that are not cross-reactive with MG proteins for calves 2335 and 2468 are shown. The pH range for IEF is indicated at the bottom of each image. The molecular weight standard (kDa) is indicated on the left. Abbreviations include Ag – antigen, SG – salivary gland, MG – midgut.

	Calf #2335		Calf #2468	
Spots	Size (kDa)	pI	Size (kDa)	pI
1	65	4.4	91	4.2
2	58	5.6	62	4.3
3	60	5.7	56	4.4
4	42	5.7	53	4.4
5	93	7.0	81	7.4
6	83	9.1	81	8.5
7	78	8.8	75	8.0
8	78	9.5	74	7.9
9	81	10.2	77	8.7
10	71	8.8	78	8.9
11	68	9.2	78	9.9
12	71	10.3	69	8.4
13	64	9.1	71	8.7
14	57	9.6	71	9.8
15	55	10.1	67	8.7
16	46	10.0	62	8.5
17	37	8.9	62	8.6
18	29	9.7	59	9.2
19	25	9.6	40	6.0
20			38	6.0
21			34	8.8
22			33	8.3
23			29	8.8
24			29	9.6
25			24	9.7

Table 8. Apparent molecular weight and isoelectric point (pI) of candidate SG proteins observed with two-dimensional western blots.

The isoelectric point was calculated following the manufacturer's instructions for pH distribution on a 3-11NL IPG strip. Protein kDa was calculated using a standard curve of the protein ladder migration pattern by measuring from the dye front to the protein band or protein spot location. All kDa numbers were rounded to the nearest whole number.

RNA processing and modification (25), post-translational modification, protein turnover and chaperones (36), translation, ribosomal structure and biogenesis (25) or were of an unknown function (23) (Fig. 16). Not all genes were assigned a COG functional prediction; 91% of the genes were assigned a predicted functional category. The large number of proteins identified from several individual spots and regions with LC-MS/MS data suggest that a single antigenically recognized spot contains several proteins. Comparison of the 258 proteins against RNA-seq data from *D. andersoni* MG or SG tissues revealed eight proteins with reads found in the SG RNA-seq data and no reads in MG RNA-seq data (Table 9). The proteins with limited sequence coverage or poor sequence alignment in *D. andersoni* SG or MG RNA-seq data were assigned the term "weak." 15 *D. silvarum* proteins had limited sequence coverage or identity with RNA-seq reads from both *D. andersoni* MG and SG tissues and an additional 14 *D. silvarum* proteins had no match to any *D. andersoni* RNA-seq reads. Finally, four *D. silvarum* proteins had limited sequence coverage to *D. andersoni* MG RNA-seq data and no sequence coverage to *D. andersoni* SG RNA-seq data. The proteins with weak or no reactivity in either tissue were unable to be eliminated as candidates. In summary, of the 258 proteins 41 protein candidates remain and these may be associated with reduced tick performance.

Discussion

The results of this investigation demonstrate that *D. andersoni* can be used to adapt a large-animal model system for immunization against ticks. Immunization with *D. andersoni* tick tissues induced seroconversion (Fig. 10) and significant

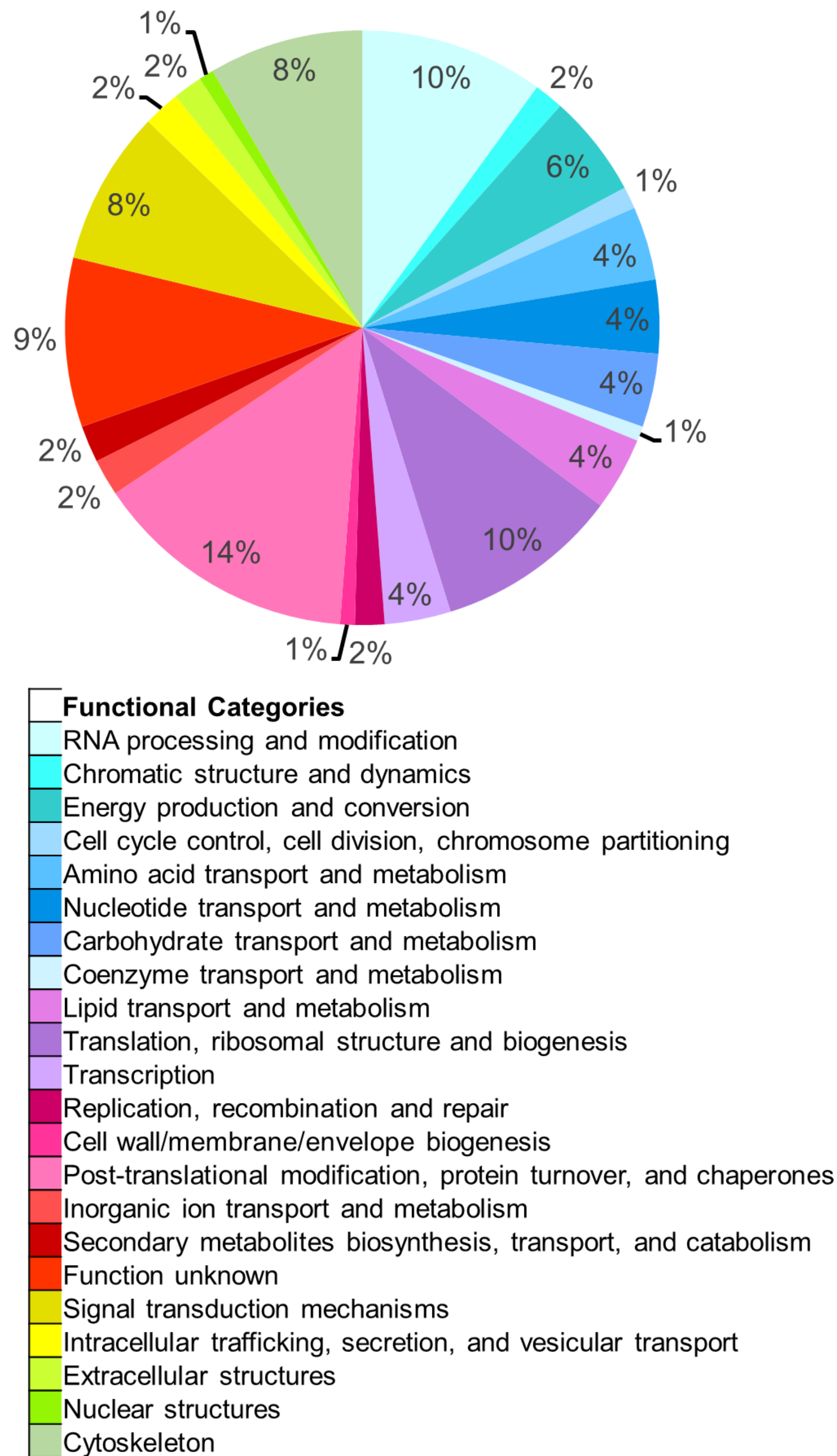


Figure 16. Clusters of orthologous groups (COG) functional prediction of candidate salivary gland proteins identified using LC-MS/MS.

Functional prediction of protein spots identified in LC-MS/MS from Table 8. Color coding is matched to the legend provided with predicted function.

Protein #	Accession #	Putative protein	*D. andersoni* RNA-Seq SG Reads	*D. andersoni* RNA-Seq MG Reads
1	XP_037573424.1	uncharacterized protein LOC119455948	Yes	No
2	XP_037582046.1	uncharacterized protein LOC119465317	Yes	No
3	XP_037554463.1	transcription elongation factor A protein 1-like	Yes	No
4	XP_037578958.1	neurofilament heavy polypeptide-like isoform X1	Yes	No
5	XP_037572424.1	extensin-2-like	Yes	No
6	XP_037580853.1	elastin-like isoform X1	Yes	No
7	XP_037578688.1	collagen alpha-1(I) chain-like isoform X1	Yes	No
8	XP_037565863.1	uncharacterized protein LOC119445661 isoform X2	Yes	No
9	XP_037557137.1	transcriptional activator protein Pur-beta-like isoform X1	Weak	Weak
10	XP_037581354.1	shematrin-like protein 2 isoform X1	Weak	Weak
11	XP_037582267.1	uncharacterized protein LOC119465860	Weak	Weak
12	XP_037582173.1	scaffold attachment factor B2-like	Weak	Weak
13	XP_037573921.1	limbic system-associated membrane protein-like isoform X1	Weak	Weak
14	XP_037573914.1	uncharacterized protein LOC119456293	Weak	Weak
15	XP_037581110.1	venom metalloproteinase antarease-like TtrivMP_A	Weak	Weak
16	XP_037572019.1	uncharacterized protein LOC119454084	Weak	Weak
17	XP_037572763.1	angiotensin-converting enzyme-like	Weak	Weak
18	XP_037559393.1	nucleolin-like isoform X1	Weak	Weak
19	XP_037571891.1	protein 5NUC-like	Weak	Weak
20	XP_037554372.1	probable protein phosphatase 2C 11	Weak	Weak
21	XP_037566502.1	RNA-binding protein cabeza-like isoform X1	Weak	Weak
22	XP_037569896.1	glycerol-3-phosphate dehydrogenase, mitochondrial-like	Weak	Weak
23	XP_037563896.1	myelin expression factor 2-like isoform X1	Weak	Weak
24	XP_037579803.1	cuticle protein 70, isoforms A and B-like	No, unable to eliminate	No
25	XP_037561755.1	extensin-1-like	No, unable to eliminate	No
26	XP_037565544.1	titin-like	No, unable to eliminate	No
27	XP_037556264.1	uncharacterized protein LOC119433189 isoform X1	No, unable to eliminate	No
28	XP_037559895.1	fibroin heavy chain-like isoform X1	No, unable to eliminate	No
29	XP_037579240.1	glycine-rich protein 5-like	No, unable to eliminate	No
30	XP_037561328.1	cuticle protein 16.8-like	No, unable to eliminate	No
31	XP_037575379.1	uncharacterized PE-PGRS family protein PE_PGRS54-like isoform X3	No, unable to eliminate	No
32	XP_037577602.1	glycine-rich cell wall structural protein-like	No, unable to eliminate	No
33	XP_037578464.1	uncharacterized protein LOC119461287	No, unable to eliminate	No
34	XP_037571299.1	mucin-12-like isoform X1	No, unable to eliminate	No
35	XP_037566106.1	cuticle protein 16.5-like	No, unable to eliminate	No
36	XP_037560630.1	collagen alpha-1(XVIII) chain-like	No, unable to eliminate	No
37	XP_037557971.1	synaptopodin-2-like	No, unable to eliminate	No
38	XP_037577792.1	LOW QUALITY PROTEIN: collagen alpha-1(VII) chain-like	No, unable to eliminate	Weak
39	XP_037575219.1	LOW QUALITY PROTEIN: serotransferrin-B-like	No, unable to eliminate	Weak
40	XP_037557449.1	toll-like receptor 3	No, unable to eliminate	Weak
41	XP_037578628.1	collagen alpha-1(IV) chain-like isoform X1	No, unable to eliminate	Weak

Table 9. Candidate proteins searched with RNA-seq data from *D. andersoni*.

Candidate LC-MS/MS proteins from *D. silvarum* were searched for with tBLASTn against *D. andersoni* RNA-seq reads from MG or SG transcriptomic data. Reads were listed if they aligned with the *D. silvarum* candidate. The term “weak” indicates *D. silvarum* proteins that had limited coverage or limited sequence identity with *D. andersoni* RNA-seq reads.

reductions in tick performance occurred post-immunization (Fig. 11). The greatest decrease in tick performance parameters occurred in SG immunized calves. All calves demonstrated little to no pre-immune western blot reactivity; however, significant reactivity was observed after immunization (Figs. 13, 14). Several protein spots, separated by pI and kDa, are uniquely found only in SG immunized calves (Fig. 15; Table 8). LC-MS/MS of the uniquely reactive SG spots identified 258 proteins which are noted to have several different predicted functions (Fig. 16). Some of these proteins are found in *D. andersoni* MG transcriptomes, suggesting they are not uniquely expressed in the tick SG. However, different isoforms or splicing variants could be uniquely isolated to the *D. andersoni* SG or MG.

To the best of our knowledge, this is the first report demonstrating immunization of bovine hosts with *D. andersoni* tick SG and MG tissue homogenates separately. A previous investigation had shown that immunization against *D. andersoni* reduces tick performance (171). The limitations of that previous study were that the antigen preparation consisted of non-denatured antigen from only one day of feeding with pooling of multiple different tick tissues together including, MG and reproductive tissues, or all other internal organs of the tick. It has been reported that protein expression changes in the tick MG and SG during feeding (281, 289–291). Secondly, it is posited that competition between irrelevant antigens and protective antigens affects resistance (163). This information is pertinent because the research described in this current study sought to address these limitations by using proteins from specific tick tissues from

ticks fed for 3-5 days. Pooled tissues from several days of tick feeding used for calf immunization is expected to provide a more diverse range of antigens for recognition by the vertebrate immune response.

Repeated infestation of guinea pigs with *D. andersoni* resulted in reduced larvae engorgement after feeding upon immunized hosts and tick resistance is passively transferrable to naïve hosts (190, 292). The same phenomenon has been reported with *D. variabilis* fed on guinea pigs (156). *Rhipcephalus sanguineus* show diminished performance parameters after feeding on repeatedly infested dogs (168). Repeated infestation of cattle with *Hyalomma marginatum* subsp. *marginatum* induced antibodies to tick tissues and reduced tick performance (293). Further, repeated infestation induced an immune response in different breeds of cattle that reacts with two-dimensional western blots of tick antigens (288). Several investigations have sought to identify antigens responsible for the phenotype of reduced tick performance after repeated infestation, but without success (294–296). The only internationally available commercial anti-tick vaccine, Gavac, is based upon a concealed midgut glycoprotein, Bm86 (157, 194, 199). This anti-tick vaccine has been largely successful against the antigens source, *R. microplus* (197, 198), and the research that led to the identification of this protein demonstrates the utility of whole tick tissue immunizations to identify antigens. In addition, ticks and their hosts have evolved together for millions of years (63), making the likelihood of identifying an antigen that is secreted and capable of preventing tick feeding, unlikely. It seems probable that several secreted antigens and/or concealed antigens together would likely perform better

at providing a target for an anti-tick vaccine such as the concealed glycoprotein Bm86.

In contrast to previous research, this current study found that immunization with SG tissues affected both tick feeding and fecundity performance parameters (168, 169, 173). An explanation for the difference in fecundity performance parameters in this study may be due to the different tick species used in the previous studies as compared to *D. andersoni* used in this investigation. Further, the Holstein calves in this study were younger and a different breed than those previously described (169). The lower reduction of tick performance parameters with MG immunized calves suggests the antigens involved are unlikely to be due to cross-reactive antigens because the ticks that fed on MG immunized hosts did not perform as poorly. In general, the reduced tick performance in this study confirms the results of previous studies involving immunization of various vertebrate hosts with different species of ticks (160, 161, 168, 169, 175).

Antibody titers to MG or SG tissues increased throughout the immunization protocol and during tick feeding (Fig. 10). The two-dimensional westerns revealed many cross-reactive proteins; however, midgut immunized calf sera reactivity to midgut proteins was notably lower than protein reactivity with sera from salivary gland immunized calves (Fig. 14). The lower reactivity phenomenon is explainable by the denaturation step required for SDS-PAGE and two-dimensional gel electrophoresis. The calves in this study were immunized with native midgut and denatured salivary gland homogenates. The conformational epitopes that are recognized in native midgut immunized calves are likely damaged or destroyed on

two-dimensional westerns, while salivary gland immunized calf sera will bind to their recognized denatured and linear epitopes.

Although limited group sizes were used in this study, these limited group sizes were overcome by having each calf act as a negative control with pre-immune tick feeding. Unfortunately, but as expected, immunization did not result in complete mortality of ticks. Proteins were identified using two-dimensional gel electrophoresis which is a well-established system for protein separation for subsequent identification (288, 297–299). Further, the method of spot elimination reduces the candidate targets to those potentially associated with the reduced performance phenotype. This study sought to used *D. andersoni* as it is a prominent vector of *A. marginale* and is indigenous to the United States. Future studies can investigate if immunization has an effect on pathogen transmission from invertebrate vector to the bovine host (300).

In conclusion, this study reports that immunization against *D. andersoni* reduces tick performance and immunization with *D. andersoni* SG has a more significant impact on tick performance than MG immunization. LC-MS/MS of the unique SG proteins has yielded many candidate proteins associated with this phenotype. Bioinformatic analysis of these proteins found 41 candidate proteins that were associated with reduced tick feeding and fecundity performance parameters after feeding on immunized hosts. This research demonstrates adaption of the large-animal anti-tick vaccine model to a tick species indigenous to the United States. Future research with recombinant proteins of these SG unique proteins is warranted to determine immunoreactivity with *D. andersoni* SG

immunized calf sera. The immunoreactive proteins will be essential for future studies to determine which proteins are immunogenic and responsible for reduced tick feeding and fecundity performance parameters after feeding on immunized hosts.

Acknowledgements

This research was supported by USDA NIFA PROJ NO: MO.W-2016-09373 and the University of Missouri Department of Veterinary Pathobiology. The authors thank the University of Missouri Gehrke Proteomics core, C. Zorn, L. Dougherty, S. Scott and Dr. A. Al-Hosary for technical assistance.

CHAPTER 4

Immunization against *Dermacentor andersoni* to interfere with *Anaplasma marginale* transmission or acquisition

Abstract

Dermacentor andersoni, the Rocky Mountain wood tick, is indigenous to the United States and is an important vector of several tick-borne pathogens. Specifically, *D. andersoni* is a competent vector of *Anaplasma marginale*, a globally important pathogen of ruminants and a causative agent of bovine anaplasmosis. The tick midgut (MG) and salivary glands (SG) are the two main organs that directly interact with the vertebrate host immune system. Immunization of vertebrate hosts with these organs has been shown to negatively impact tick performance and lower tick transmission or acquisition of tick-borne pathogens. Here we developed a model system with *D. andersoni* for abrogation of tick-borne pathogen transmission or acquisition. Immunization of cattle with native (N) MG or SG prevented tick transmission of *A. marginale* to immunized animals while immunization with denatured (D) MG or SG did not prevent transmission. Further, NSG immunization had the most significant impact on tick performance and the greatest impact on *D. andersoni* acquisition of *A. marginale*. Collectively, these results indicate that the *D. andersoni* NSG have the potential to yield antigenic targets that will negatively impact tick feeding and abrogate tick transmission and acquisition of *A. marginale*.

Introduction

Ticks are important vectors of a myriad of different pathogens that impact human and animal health (7). Tick-borne pathogens in the rickettsial family *Anaplasmataceae* have been subject to increasing scrutiny and study from human and animal health perspectives. In addition, increasing research has sought to identify vaccines against ticks and tick-borne pathogens. Utilizing the vertebrate immune response to control the tick-borne pathogen life cycle by targeting the vector, is the approach employed in this study.

Vaccination against ticks was first demonstrated to be an effective method of inducing tick resistance to immunized animals in 1939 (156). Since then, multiple studies have used crude antigens or recombinant proteins to prevent tick feeding and/or limit tick-pathogen transmission (157, 168, 301, 302, 169, 170, 173, 181, 195, 203–205). Currently there are two general types of immunization strategies, a forward vaccinology approach targeting whole tick tissue homogenates or reverse vaccinology to target specific recombinant proteins. Reverse vaccinology has led a shift towards the use of expressed sequence tag (EST) libraries, whole genome sequencing and RNAi with *in vitro* systems to identify proteins of interest to negatively impact ticks and tick-borne pathogens (303–305). Despite these advances with reverse vaccinology, there is only one commercially available anti-tick vaccine based upon the glycoprotein Bm86. Further, the global distribution and large number of ticks makes it difficult to apply the bioinformatically chosen sequences as applicable to all globally distributed tick species (13). Previous research by our laboratory has demonstrated that

immunization of cattle with denatured tick salivary gland extracts from *Rhipicephalus* (*Boophilus*) *microplus* negatively impacts tick acquisition and transmission of the tick-borne pathogen, *Babesia bigemina* (203). Further research by our laboratory with *Dermacentor andersoni* demonstrated decreased tick fecundity and feeding performance after feeding upon denatured salivary gland (SG) immunized calves. It was theorized that immunization with specific and whole tick tissues exposes the host to concealed antigens that the host is never exposed to during natural tick feeding (162). Consequently, denaturation of the SG tissues is believed to enhance the immune responses against tick-borne pathogen transmission by exposing secretory granule proteins and concealed epitopes, thereby enhancing the immune responses by the vertebrate host.

To test this hypothesis and adapt this research to a model system with *A. marginale*, calves were immunized with four different *D. andersoni* tick tissue homogenate preparations including native salivary gland (NSG), native midgut (NMG), denatured salivary gland (DSG) or denatured midgut (DMG). Seroconversion of immunized calves was confirmed by ELISA, and *Anaplasma marginale* transmission by *D. andersoni* was monitored by tracking packed cell volume (PCV) and parasitemia. It was observed that NSG and NMG immunized calves were protected from *D. andersoni* mediated transmission of *A. marginale*. *D. andersoni* tick acquisition of *A. marginale* on immunized calves was tested with qPCR. Ticks that fed on NSG immunized calves consistently maintained the lowest number of ticks that acquired the pathogen and the lowest acquisition level of *A. marginale*.

Materials and Methods

Ticks

Dermacentor andersoni adults were obtained from the Oklahoma State University Medical Entomology Laboratory. Ticks were stored at 25°C, >90% relative humidity, and provided a photoperiod of 12 h:12 h (L:D).

Antigen preparation

Adult, 0-6 day fed, male and female *D. andersoni* were bisected along their dorsal surface. *D. andersoni* SG and MG organs were removed, dissected free of other tissues, and placed in sterile 1X phosphate buffer saline (PBS) with protease inhibitor mini tablets (Thermo Fisher Scientific, Waltham, MA) at 4°C. Tick SG and MG organs were homogenized using a dounce homogenizer (Corning Inc., Corning, NY). To denature tissues, samples were heated to 56°C overnight in 1% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol followed by boiling for 5 minutes. The solution was cooled to room temperature and excess SDS was removed with an SDS removal kit (Thermo Fisher Scientific) following the manufacturer's instructions. The protein concentration of the extract was measured by using the Bradford method. Tissues were mixed with Montanide ISA VG 61 (Seppic, Fairfield, NJ), a mineral-oil based adjuvant, in a 2:3 ratio of antigen to adjuvant following the manufacturer's instructions immediately prior to immunization of the hosts.

Native tick SG or MG were homogenized with a dounce homogenizer and protein concentration was determined with the Bradford method. Tissues were

mixed with Montanide ISA VG 61 in a 2:3 ratio of antigen to adjuvant following the manufacturer's instructions immediately prior to immunization of the hosts.

Animals

12 male or female Holstein or Holstein-Angus cross calves of 3 months to 1 year of age were used in this experiment. All calves were dehorned and castrated prior to use in the experiment. Calves were housed in an enclosed building at the University of Missouri Middlebush farm. Calves were maintained according to the University of Missouri OAR, ACUC protocol #8981.

Immunization

Calves were immunized intradermally at 10 sites along the dorsal surface with 1 mg of DSG, NSG, DMG or NMG extracts plus Montanide ISA VG 61. The second and third immunizations were given two weeks after the first and second immunizations, respectively. A final booster of 1 mg was provided one week prior to acquisition feeding.

A. marginale-infected *D. andersoni* challenge infestation

Calves were challenged with 10 pairs of *A. marginale*-infected and uninfected adullt *D. andersoni* ticks. Transmission of *A. marginale* to calves was monitored by blood smear and PCV change. Those calves that did not become symptomatic were continuously monitored by PCR, blood smear and PCV to determine if transmission occurred. The end of the prepatent period, the time from infection to diagnosis, has been reported to be 100 days (151). Thus, all calves were monitored for 100 days. To confirm susceptibility to infection after 100 days,

all calves were subsequently needle challenged intravenously with 2 mL of *A. marginale*-infected carrier calf blood and monitored for acute phase anaplasmosis.

D. andersoni **acquisition of** ***A. marginale***

Primers, as previously described, were used for detection of *A. marginale* targeting *msp5* (118). Briefly, primers used include forward primer (StM_194752-194775_S CTTATCGGCATGGTCGCCTAGTTT) and reverse primer (StM_194954-194931_A CTTCCGAAGTTGTAAGTGAGGGCA) and these primers were purchased from IDT (Newark, NJ). PowerUp SYBR Green (Thermo Fisher Scientific) master mix was used for qPCR. Cycling conditions were uracil N-glycosylase (UNG) activation at 50°C for 2 minutes, polymerase activation at 95° for 2 minutes, 50 cycles of melting at 95°C for 15 sec, annealing at 55°C for 30 sec, and extension at 72°C for 1 minute, followed by a dissociation stage. Amplified PCR products, with a target size of 202 bp, were detected on a 1% agarose gel containing ethidium bromide.

Animal monitoring

Calves were monitored daily for *A. marginale* infection in transmission and acquisition phases through examination of blood smears stained with Diff-Quick (Thermo Fisher Scientific). PCV was determined through hematacrit centrifugation of EDTA sampled blood and parasitemia (% parasitized erythrocytes) was monitored with stained blood smears and calculated by counting at a minimum 500 erythrocytes and dividing the number of infected erythrocytes by the total number

counted. PCR targeting *msp5* was used to confirm *A. marginale* infection of ticks and cattle, as previously described (117, 148).

ELISA

Sera from calves immunized with native or denatured *D. andersoni* SG or MG were tested with an indirect ELISA against *D. andersoni* MG or SG antigens (Ag). 96-well high binding ELISA microplates (Greiner Bio-One, Monroe, NC) were coated overnight at 4°C with 75 µL of protein antigen at a concentration of 1.0 µg/mL in 0.1 M carbonate coating buffer (pH 9.6). Plates were washed with 0.5% (v/v) Tween-20 PBS (PBS-T) five times and then wells were blocked for 30 minutes with PBS-T. Plates were further blocked for 2 h with 100 µL 3% (w/v) BSA in PBS. After blocking, 60 µL of primary antibody was added at a concentration of 1/100 and diluted 1/10 until a final dilution of $1x10^8$ was achieved and samples were incubated for 3 h at 37°C. Plates were washed five times with PBS-T and 50 µL of rabbit anti-bovine IgG (Invitrogen, Carlsbad, CA), diluted 1:10,000 in PBS-T, was added to each well for 15 minutes at room temperature. 50 µL of 3,3,5,5-tetramethylbenzidine (TMB) solution (1 mM TMB, 0.0665 M citric acid, 0.0306 M sodium citrate dihydrate and 0.01068% (v/v) H_2O_2) was added to each well and incubated for 15 minutes. The reaction was stopped with 50 µL of 2 M H_2SO_4 and optical densities were measured at 450 nm. Negative controls included samples without 1° antibody (Ab) and 2° Ab alone without 1° Ab to ensure the colorimetric reaction was due to Ag-Ab complex formation.

Tick performance parameters

For baseline infestation of cattle, calves were challenged with 20 pairs of adult uninfected *D. andersoni* in trial 1. In trials 2 and 3, calves were challenged with 25 pairs of adult *D. andersoni*. For baseline infestation, calves were checked daily for ticks, and recovered (detached) ticks were counted, weighed and isolated in tick humidity chambers. Transmission feeding using *D. andersoni* that were transstadially infected occurred 7 days post-second boost. Two trials of transmission feeding were conducted with calves being challenged with 10 pairs of *A. marginale*-infected *D. andersoni* and 10 pairs of uninfected *D. andersoni*. Two trials of acquisition feeding were conducted 7 days post-final boost. Trial 1 and trial 2 calves were challenged with 25 and 30 pairs of uninfected *D. andersoni*, respectively.

Calves were checked daily, recovered (detached) ticks were counted, weighed, and maintained in tick humidity chambers until oviposition was complete. Egg clusters were weighed and maintained until hatching occurred. The following biological tick performance parameters were measured during each infestation: feeding period, survival, tick weight and egg cluster weight. These performance parameters were used to calculate feeding and fertility efficiency indices as previously described (163, 168, 169). Engorged female weight was measured immediately after detachment. Egg masses were determined after oviposition was complete. The feeding period was assumed to be the time that elapsed from tick attachment to tick removal at partial or full engorgement. The feeding efficiency index was calculated by dividing the weight of each engorged female by her

feeding period. The fecundity efficiency index was calculated by dividing the weight of each egg mass by the weight of each respective female.

Statistical analysis

Performance parameter data collected during tick feeding was compared using a one-way analysis of variance (ANOVA). Pairwise comparison of the means was completed using a Tukey-Kramer post-hoc or its non-parametric equivalent where necessary. All analyses were performed using SigmaPlot (Systat Software, San Jose, CA). A $P \leq 0.05$ was considered statistically significant.

Results

Strategy for immunization of calves

Because immunization with tick homogenates affects tick performance and tick-pathogen acquisition and transmission (168, 169, 203), it was hypothesized that immunization of cattle with denatured *D. andersoni* tick tissue homogenates would negatively impact tick-borne pathogen transmission or acquisition. Baseline infestation was conducted as a negative control (Fig. 17), afterwards cattle were immunized with *D. andersoni* NSG, NMG, DSG and DMG tick homogenates with subsequent boosters every two weeks. Transmission feeding of *A. marginale*-infected ticks was one week post-final boost and after 100 days calves were needle challenged with *A. marginale* to confirm susceptibility to infection. Calves were immunized with a final boost of tick tissue homogenates after acute anaplasmosis had passed and naïve *D. andersoni* were acquisition fed upon calves to determine the infection rate and level of *A. marginale* in different immunized calves.

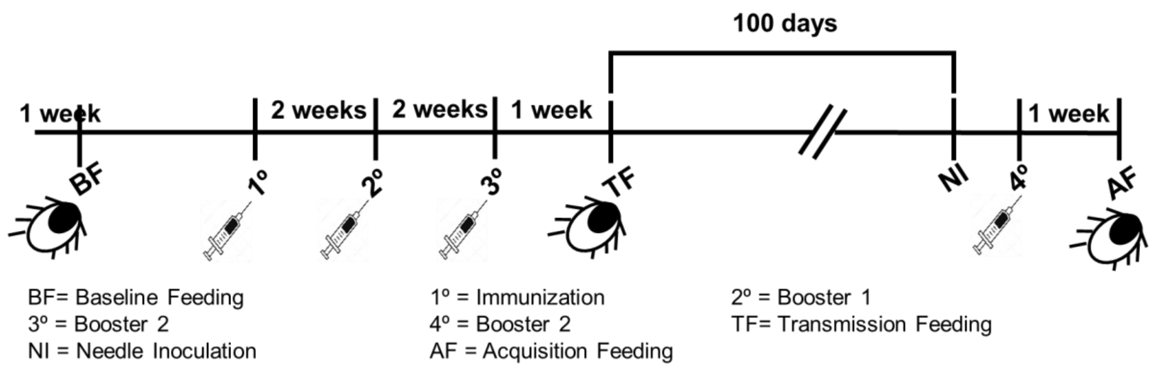


Figure 17. Immunization of cattle and tick feeding timeline.

Immunization of calves with *D. andersoni* tick tissues

Previously it was shown that immunization of animals with tick tissues induces seroconversion (306). Thus, it was hypothesized that *D. andersoni* tick tissue homogenate immunization will induce reactivity to the tissue used for immunization and cross-reactivity to other tick tissues. Three trials were performed in which one calf per each immunization group was inoculated with tick tissue homogenates. Three timepoints were measured for seroreactivity including pre-immune, prior to immunization, transmission feeding (TF) challenge and acquisition feeding (AF) challenge. Increasing titers in all calves indicated a strong seroconversion and cross-reactivity to both tick tissue types at TF or AF timepoints (Fig. 18). Some calves exhibited pre-immune titers as denoted by the blue bars. Calves 3342 and 3371 exhibited stronger pre-immune titers to both tissue types often near 1/1,000 or a Log_{10} of 3. The highest pre-immune titer was recorded in calf 3342 with a pre-immune Log_{10} titer of 4 (1/10,000) to SG antigen, which increased to 7.33 (1/21,544,346) during TF and AF timepoints (Fig 18A). At minimum, TF and AF titers increased 6700 times as compared to pre-immune.

Tick performance of female *D. andersoni* fed on pre-immune calves or calves immunized with denatured or native salivary gland or midgut tissues

Immunization with tick tissue homogenates has been reported to negatively impact tick performance (168, 175, 180). Also, variation between individual animals and breeds of cattle in response to tick feeding has been documented (155, 288). Thus, immunization with *D. andersoni* tick tissue preparations is expected to negatively impact tick feeding and fecundity performance parameters.

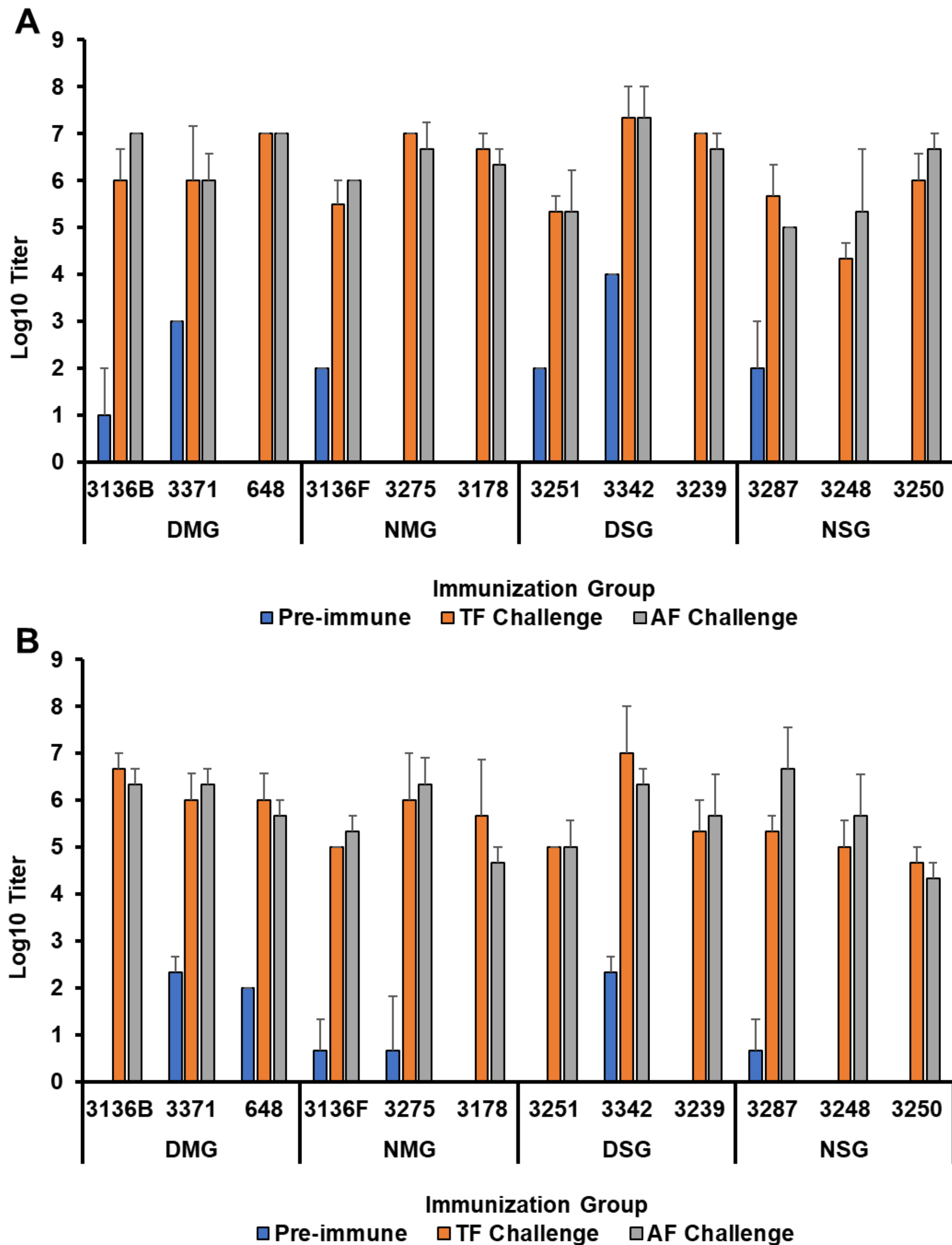


Figure 18. Titers of calves immunized and fed upon by *D. andersoni.*

Calves were immunized three times (intradermally) at two week intervals with denatured or native tick SG or MG. Serum samples were measured with an ELISA, as described in the methods, with different antigen preparations including salivary gland (A) or midgut (B). Sera were measured from three timepoints of pre-immune, transmission feeding (TF) challenge or acquisition feeding (AF) challenge. The research in this figure was conducted with the assistance of Samuel Shahzad.

A reduction in performance after feeding on immunized hosts would confirm the research of previous studies. To control for variation in tick performances between calves, tick baseline performance parameters were collected. After immunization, ticks were applied to the calves and fed to repletion in which parameters including feeding period, tick survival, feeding efficiency, female engorgement weight, egg cluster weight and fecundity efficiency were monitored and recorded. Performance parameters between baseline and post-immunization timepoints were compared to determine if immunization had an impact on tick feeding.

The feeding period for ticks fed on pre-immune calves was completed with all engorged females removed within 24 days, with an average removal period of 17 days (Fig. 19A). Pre-immune feeding period for ticks fed on DSG immunized calves was 17.61 days which decreased to 14.06 for TF ($P < 0.028$) and 12.14 days for AF ($P < 0.001$ vs. pre-immune). Pre-immune feeding period for ticks fed on NSG immunized calves was 16.32 days which decreased to 11.78 for TF ($P < 0.008$) and 13.72 days for AF. The reduced feeding period seen in DSG-immunized calves is not due to fewer numbers of ticks recovered as more ticks were recovered during TF challenge than baseline (Fig. 19B). Tick survival varied widely; however, there were no statistically significant differences between pre-immune and post-immunization in any immunogen group. There was a trend towards increased survival after immunization in DSG and NMG immunized calves. NSG is the only immunization group with a trend towards lower tick survival after immunization (Fig. 19B).

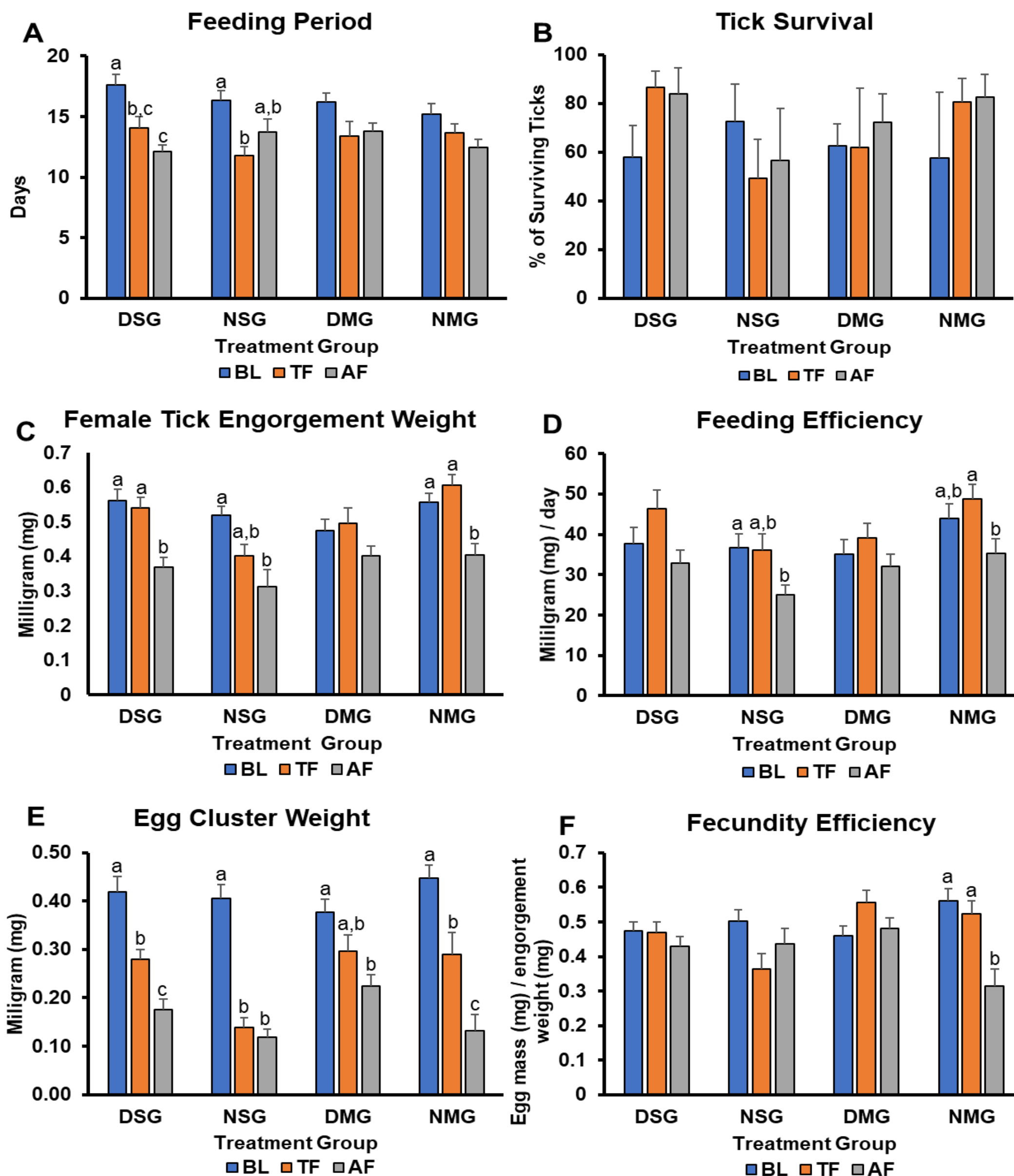


Figure 19. Performance parameters of engorged female *D. andersoni* recovered from immunized calves.

Calves were immunized with *D. andersoni* tissue homogenates and fed upon by *D. andersoni* as described in the methods. Performance parameters were measured from ticks fed during baseline (BL) feeding, transmission feeding (TF) or acquisition feeding (AF). The bars in each figure represent the mean (n = 3) ± standard error (SE) of ticks fed on immunized cattle with DSG, NSG, DMG or NMG. Parameters measured include feeding period (A), tick survival (B), engorgement weight (D), feeding efficiency (D), egg cluster weight (E) and fecundity efficiency (F) and are described in the methods. Female ticks < 0.1 g were removed from consideration due to partial engorgement. Superscripts a, b, c represent statistical differences based upon means and a one-way ANOVA and Tukey-Kramer post-hoc ($P \leq 0.05$) or its non-parametric equivalent. The research in this figure was conducted with the assistance of Samuel Shahzad.

Female engorgement weight was significantly lower at the AF timepoint for DSG, NSG and NMG immunized calves. There was not a statistically significant difference observed in ticks fed on DMG immunized calves (Fig. 19C). Female engorgement weight on DSG immunized calves for pre-immune feeding was 0.56 g which decreased to 0.53 during TF and to 0.368 g during AF ($P < 0.001$ vs. pre-immune). Ticks fed on NSG immunized calves had a pre-immune engorgement weight of 0.52 g which decreased to 0.402 during TF and to 0.312 g for AF ($P < 0.001$ vs. pre-immune). The ticks fed on NMG immunized calves increased in engorgement weight from 0.556 g during pre-immune to 0.607 g in TF. Engorgement weight decreased to 0.404 g during the AF period ($P < 0.003$ vs. pre-immune).

The feeding efficiency, milligrams engorged per day, was not significantly different in ticks fed on calves immunized with DSG or DMG. Only NSG or NMG immunized calves had significantly lower tick feeding efficiencies during the AF timepoint (Fig. 19D). Pre-immune tick feeding efficiency on NSG immunized calves was 37.80 mg/day. During TF feeding efficiency increased to 46.44 and decreased to 32.82 mg/day for AF ($P < 0.037$ vs. pre-immune). Ticks fed on NMG calves had a pre-immune feeding efficiency of 43.85 mg/day, at TF it increased to 48.69 and decreased to 35.31 mg/day for AF ($P < 0.044$ vs. pre-immune).

Egg cluster weight was significantly impacted in all immunizations for TF, except for DMG immunization, and AF timepoints (Fig. 19E). Pre-immune egg cluster weight for ticks fed on DSG immunized calves was 0.419 g, egg cluster weight decreased to 0.28 during TF ($P < 0.001$) and further decreased to 0.175 g

during AF (P < 0.001 vs. pre-immune). Tick egg cluster weight on NSG immunized calves for pre-immune fed ticks was 0.406 g, decreased to 0.139 g during TF (P < 0.001) and to 0.119 g during AF (P < 0.001 vs. pre-immune). Ticks fed on DMG immunized calves had an egg cluster weight average for pre-immune feeding of 0.377 g which decreased to 0.296 g during TF and to 0.224 g during AF (P < 0.001). Finally, egg cluster weights from pre-immune fed ticks on NMG immunized calves were 0.446 g which decreased to 0.29 g during TF (P < 0.001) and 0.132 g during AF (P < 0.001 vs. pre-immune).

The fecundity efficiency, egg mass divided by engorgement weight, was only significantly affected in ticks fed on calves immunized with NMG (Fig. 19F). Pre-immune fecundity efficiency for ticks that fed on NMG immunized calves displayed a pre-immune fecundity efficiency of 0.56 which decreased to 0.524 during TF and to 0.314 during AF (P < 0.002 vs. pre-immune).

D. andersoni **transmission of** ***A. marginale*** **to immunized calves**

DSG immunization of cattle decreased the number of cattle reportedly infected with *Babesia bigemina*, suggesting that DSG immunization negatively impacts tick transmission (203). Consequently, it was hypothesized that *A. marginale*-infected *D. andersoni* would have reduced *A. marginale*-transmission after feeding on DSG immunized calves. In the current study, several different tick tissue homogenates were used for calf immunization to determine which tick tissue homogenate would negatively impact *D. andersoni* transmission of *A. marginale*. *A. marginale*-infected *D. andersoni* adults were applied to immunized calves and

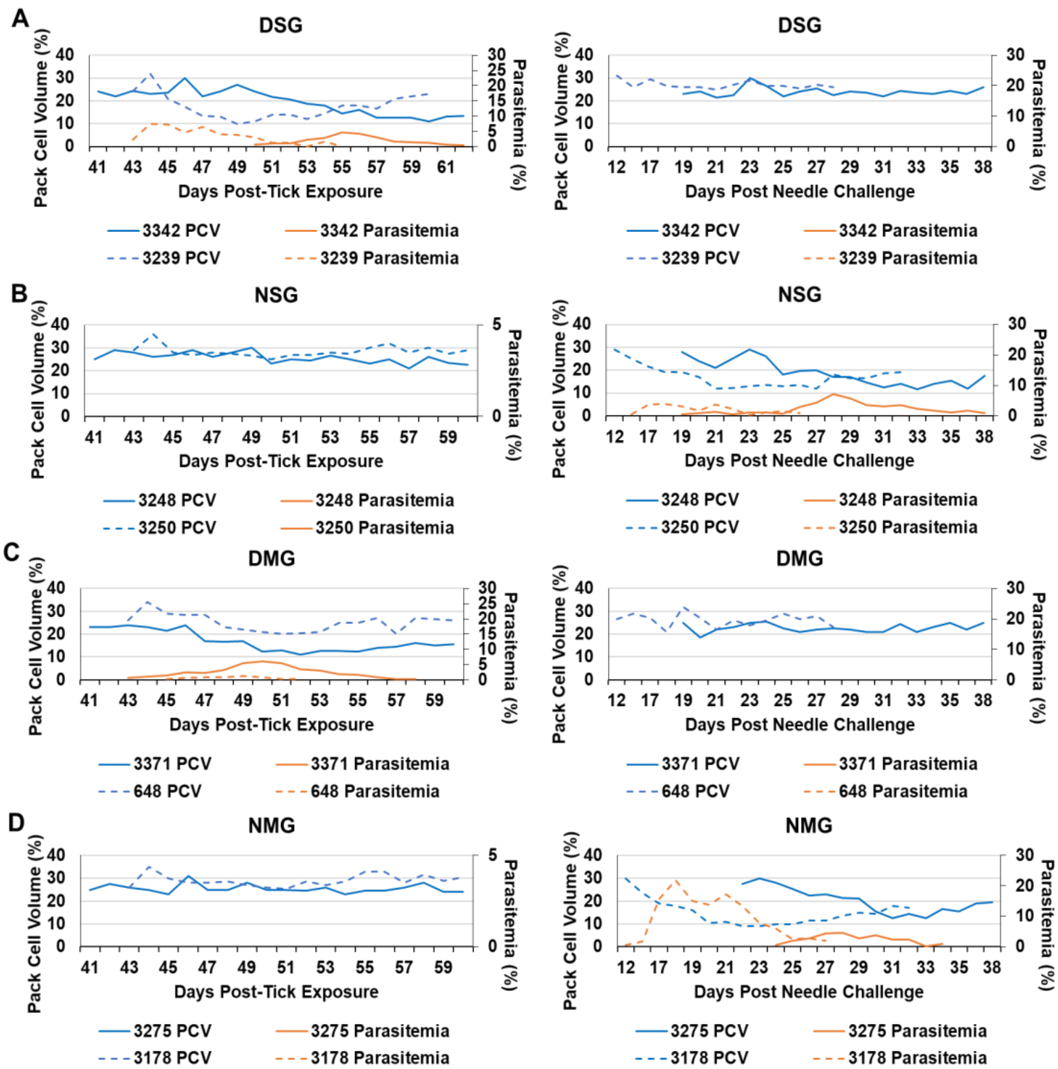


Figure 20. Parasitemia and packed cell volume of calves immunized with different antigen preparations after exposure to *A. marginale*-infected *D. andersoni*.

Calves were immunized with DSG (A), NSG (B), DMG (C) and NMG (D). Calves were then challenged with *A. marginale*-infected ticks (post-tick exposure, left) and monitored for PCV (left axis) changes and parasitemia (% parasitized erythrocytes, right axis) development during the acute phase. All calves were subsequently challenged with *A. marginale*-infected carrier calf blood (post-needle challenge, right) to confirm susceptibility to *A. marginale* infection. The research in this figure was conducted with the assistance of Samuel Shahzad.

fed to repletion and transmission of *A. marginale* to calves was monitored by blood smear, PCV change and PCR.

Calves immunized with DSG or DMG became infected with *A. marginale* after infected tick feeding and did not recrudesce after needle challenge (Fig. 20A,C). NSG or NMG immunized calves did not become infected in two trials (Fig. 20B,D). Needle challenge of NSG and NMG immunized calves resulted in all calves becoming infected, confirming calf susceptibility to infection. The parasitemia in response to challenge varied greatly between calves and within immunization groups. For example, needle challenged NMG immunized calf 3178 displayed a parasitemia of 17.3% and a minimum PCV of 10% while NMG immunized calf 3275 highest parasitemia was 6.1% with a minimum PCV of 12.5% (Fig. 20D). PCV for all calves, excluding 648, dropped to near 10% after infection with *A. marginale*. The parasitemia between immunization group varied considerably and did not appear to have a trend within a specific immunization group.

A. marginale acquisition by naïve _D. andersoni_ fed on _A. marginale_-infected, tick tissue immunized calves

The number of *R.* (*Boophilus*) *microplus* ticks which were positive for *B. bigemina* after feeding upon DSG-immunized calves was reported to be reduced, suggesting DSG negatively impacts tick acquisition of tick-borne pathogens (203). It was unknown if this same phenomenon would occur with *A. marginale* and *D. andersoni* tick tissue immunized calves. Therefore, this research sought to confirm if tick tissue immunization negatively impacts *D. andersoni* acquisition of a tick-

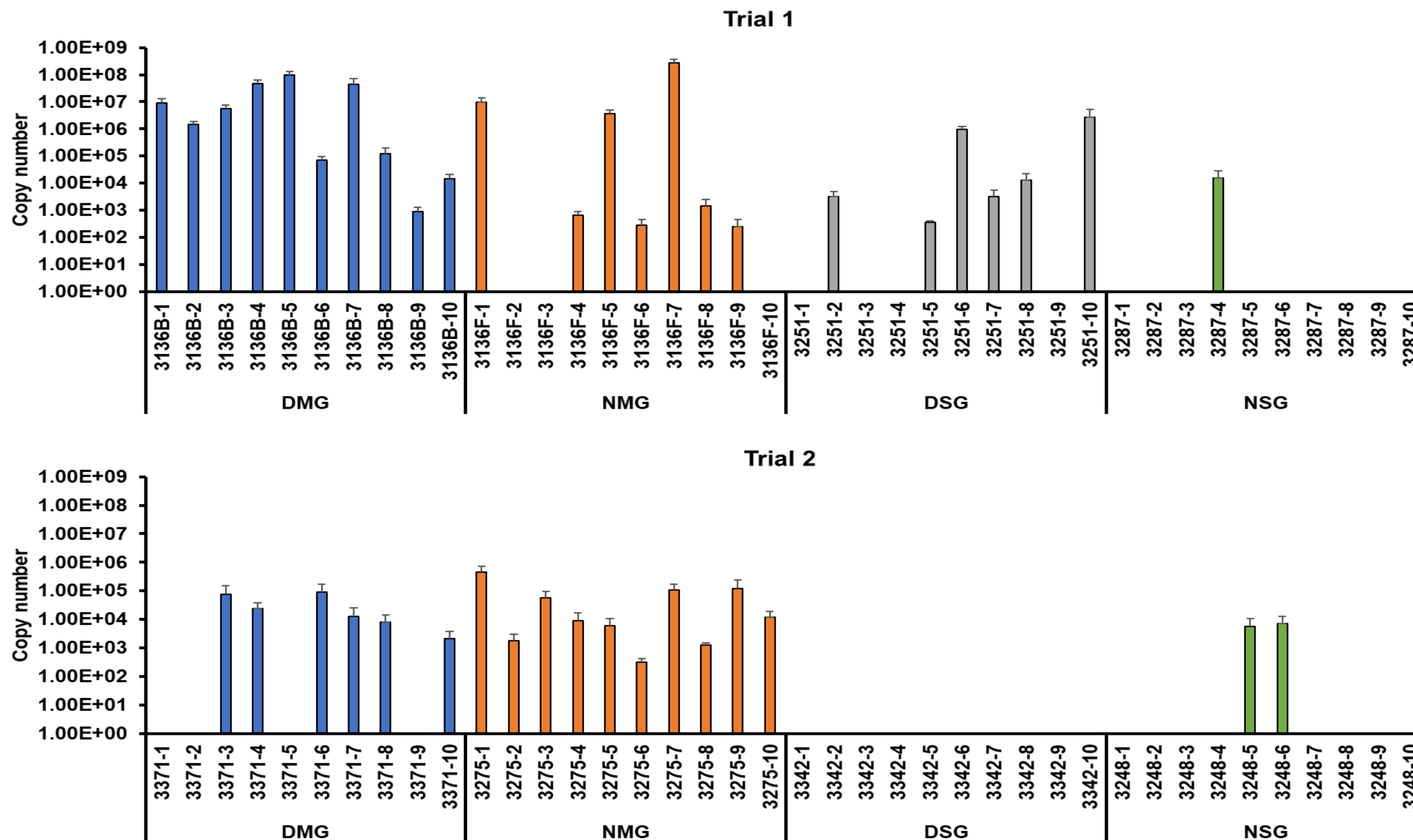


Figure 21. Acquisition of *A. marginale* in male *D. andersoni* fed on immunized calves.

A. marginale infection level per tick was quantified with qPCR from 10 adult *D. andersoni* males fed on infected, immunized calves. The bars in each panel represent the $\log_{10}$ (least-squared mean, n = 3) ± SE of ticks fed on cattle immunized with DSG, NSG, DMG or NMG. The Y-axis indicates copy number and the X-axis indicates calf numbers for Trial 1 (top) and Trial 2 (bottom). The research in this Figure was conducted with the assistance of Samuel Shahzad.

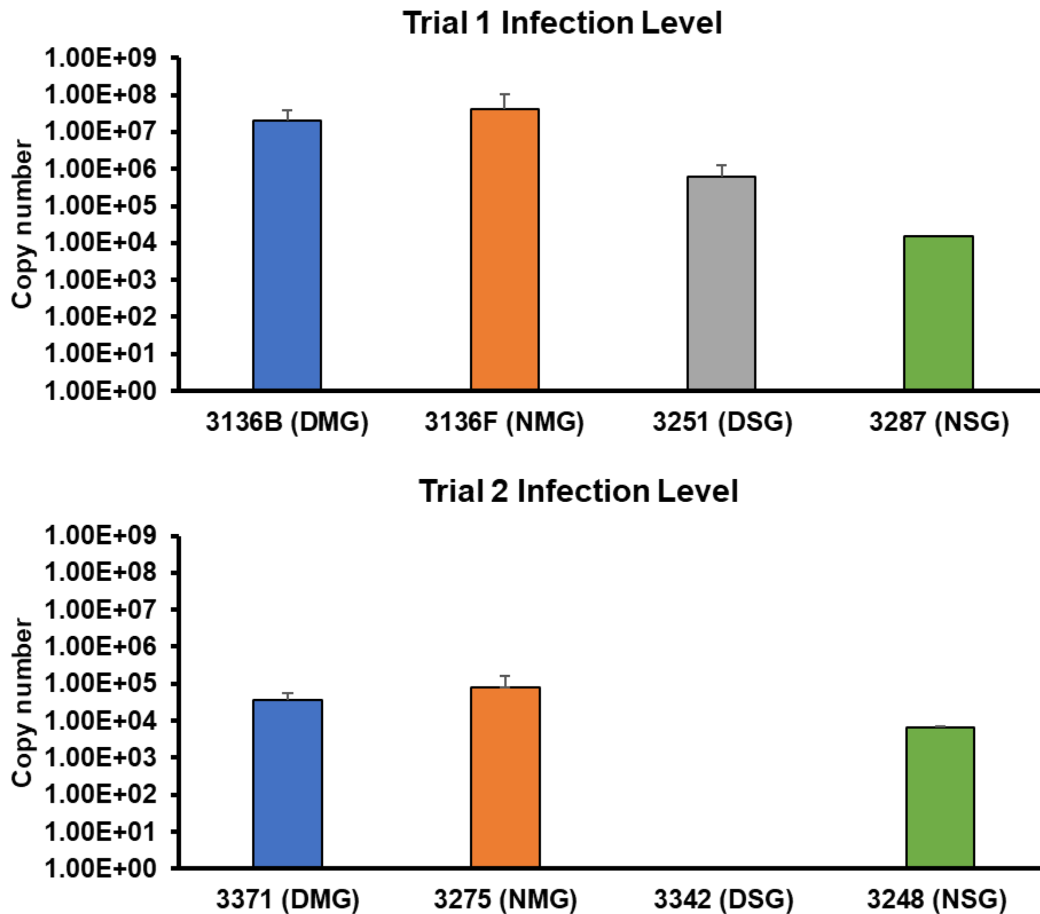


Figure 22. Infection level of *A. marginale* in male *D. andersoni* fed on immunized calves.

The average *A. marginale* infection level from 10 pairs of adult *D. andersoni* males fed on infected, immunized calves was quantified with qPCR. The bars in each panel represent the $\log_{10}$ (least-squared mean, n = 3) ± SE of ticks fed on cattle immunized with DSG, NSG, DMG or NMG. The Y-axis indicates copy number and the X-axis indicates calf numbers for Trial 1 (top) and Trial 2 (bottom). The research in this Figure was conducted with the assistance of Samuel Shahzad.

borne pathogen. Naïve adult *D. andersoni* were fed on *A. marginale*-infected, tick tissue immunized calves and fed to repletion and male *D. andersoni* were evaluated for *A. marginale* infection and level with qPCR. The infection rate and level were compared among NSG, DSG, NMG and DSG immunized calves. NSG immunized calves had one or two out of 10 tested ticks acquire *A. marginale* infection in trials 1 or 2, respectively (Fig. 21). In addition, the lowest *A. marginale* infection level was observed in ticks fed on NSG calves in both trials (Fig. 22). Ticks fed on DSG immunized calves followed with second lowest infection level with six and zero ticks acquiring infection in trials 1 or 2, respectively. NMG and DMG immunized calves had the greatest number of ticks acquire infection and the highest infection level per tick in both trials (Figs. 21, 22).

Discussion

The purpose of this study was to investigate if immunization with *D. andersoni* tick tissue homogenates negatively impact *D. andersoni* transmission or acquisition of the tick-borne pathogen *A. marginale*. Immunization with tick tissues, induced seroconversion for all calves (Fig. 18). In addition, antibodies were cross-reactive to MG or SG, regardless of the immunization tissue. Some calves had detectable pre-immune titers; however, the titer was a minimum of 6700 times higher in immunized calf sera than in pre-immune calf sera. NSG immunized calves had significantly lower tick feeding efficiency, female engorgement weight, or egg cluster weight than pre-immune and these parameters were the lowest recorded for all immunized calves (Fig. 19). Ticks fed on DSG immunized calves exhibited significantly reduced performance in feeding period, female

engorgement weight and egg cluster weight. Ticks fed on NMG immunized calves did have significant decreases in egg cluster weight and fecundity efficiency. *D. andersoni* transmission of *A. marginale* was abrogated in calves immunized with NMG or NSG (Fig. 20B,D). In addition, ticks fed on NSG immunized calves consistently had the fewest number of ticks acquire *A. marginale* and the lowest *A. marginale* acquisition level (Fig. 21,22).

It has been reported that immunization against tick tissues reduces tick performance after feeding on immunized hosts (156, 168, 169, 171, 173, 199). Our laboratories previous research with day 3-5 fed *D. andersoni* immunized calves found that DSG immunization had the most significant impact on tick feeding performance parameters, including feeding efficiency, feeding period and female engorgement weight (168, 169). Further, ticks fed on NMG immunized calves had significantly lower tick fecundity performance parameters, including egg cluster weight and fecundity efficiency. In this study, ticks that fed on SG immunized calves exhibited the lowest performance in several feeding and fecundity parameters including feeding efficiency, female engorgement weight and egg cluster weight (Fig. 19C,D,E). Additionally, in contrast to previous work, ticks fed on SG or MG immunized calves reached repletion faster and tick survival was not reduced. It is known that the protein expression profile changes during feeding (291, 307), suggesting that targets seen in this study could be variable compared to those in previous studies. Furthermore, Montanide ISA 61 VG, a mineral oil-based adjuvant, was used in this study as compared with the cited work that used

Freund's adjuvant, which is *mycobacteria*-based (168, 169). Different adjuvants may alter the immune response to tick homogenates.

In this study days 0-6 fed tick SG and MG homogenates were used for immunization of calves, as tick protein expression profiles change during feeding and because *A. marginale* is transmitted on or after six days of tick attachment (111, 289, 308, 309). During *A. marginale* tick challenge, only native tissue immunized calves did not become infected (Fig. 20). Needle challenge with *A. marginale* confirmed that native tissue immunized calves were susceptible to infection. Initially, we hypothesized that DSG immunization would have the greatest impact on tick transmission of *A. marginale* because of similar results with *B. bigemina* (169). This hypothesis, as stated, is rejected and we propose the adoption of an alternative hypothesis: that immunization of calves with *D. andersoni* NSG or NMG will abrogate *D. andersoni* transmission of *A. marginale*. Comparison of the denatured and native tissues with SDS-PAGE and Coomassie staining did not reveal significant changes in protein band profiles, suggesting denaturation is not degrading proteins (data not shown). It is likely that the conformation of the epitope is important in reducing *D. andersoni* transmission or acquisition of *A. marginale*. Ticks fed on NSG immunized calves had the lowest reported number of ticks acquire *A. marginale* in both trials. However, ticks fed on DSG immunized calves in trial 2 reported zero ticks acquiring *A. marginale*. Further research to test extra ticks collected from this calf is warranted to rule out technical error.

The antigens targeted by the host immune response are unknown. Antigens recognized by NSG immunized calf sera would be an important target, as NSG immunization negatively impacts tick performance, abrogates *A. marginale* transmission by ticks and negatively impacts *D. andersoni* acquisition of *A. marginale*. The mechanism associated with this described phenotype requires further analysis. However, it has been demonstrated that ticks that feed on SG immunized cattle present with damage to type II and type III acini as compared to controls (192). Consequently, histopathology of tick samples fed on immunized calves would likely yield pertinent information to the mechanism of protection in calves immunized with NSG and NMG.

In conclusion, NSG and DSG immunization of cattle reduced tick performance to the greatest degree in several fecundity and feeding performance parameters. Further, immunization with native tick tissue homogenates, either MG or SG, impacted *A. marginale* transmission by *D. andersoni*. Acquisition of *A. marginale* was negatively impacted in calves immunized with NSG and DSG. Determining the antigens associated with the reduced *D. andersoni* transmission or acquisition of *A. marginale* could provide the potential for new targets to use in vaccination of animals to limit tick-borne pathogen transmission and acquisition. Finally, the research in this study expands the body of knowledge that immunization with tick tissues can negatively impact the tick-pathogen life cycle.

Acknowledgements

This research was supported by USDA NIFA PROJ NO: MO.W-2016-09373 and the University of Missouri Department of Veterinary Pathobiology. The authors thank S. Shahzad for technical assistance.

CHAPTER 5

Molecular key for speciation of unknown tick samples

Abstract

Accurate identification of tick species is important in control of tick-borne pathogens. Morphological identification of ticks is dependent upon multiple factors and is confounded by sample quality and the tick stage. Previous reports in the literature have discussed the importance of molecular keys for determination of tick species identification. In this study, tick samples were collected from an elk herd in Missouri and were used for molecular identification with PCR targeting the 12S RNA gene and the internal transcribed spacer 2 (ITS-2). All ticks were identified morphologically and PCR of the 12S RNA gene was conducted on several tick samples. 12S RNA gene targeted PCR and dideoxy sequencing of *Dermacentor andersoni* positive controls demonstrated the utility of the 12S assay. In addition, PCR of unknown tick samples provided robust sequences for identification of tick samples. Those species identified morphologically matched the molecular identity in all but one tick. Collectively, the preliminary results from this study support the research demonstrating the utility of molecular identification of ticks with PCR targeting the 12S RNA gene.

Introduction

Ticks are important vectors of many pathogens worldwide and transmit a greater number of species and variety of protozoa, bacteria, viruses and nematodes than any other arthropod vector (7, 44, 45). Morphological identification of ticks is based upon dichotomous keys and often requires prior experience for accurate identification (310). In addition, damage to tick samples or immature stages often confound the process of identification. Some targets for molecular identification of ticks or phylogenetic studies of tick species involved targeting mitochondrial genes including 12S RNA gene and the cytochrome oxidase I (COI) (311, 312). Further targets include the ribosomal 16S RNA gene or the internal transcribed spacer 2 (ITS-2) located between 5.8S and the 28S (313–315). There is debate over which approach will provide the most accurate sequences for tick sample identification.

Cervus canadensis (elk) were extirpated from Missouri and only recently were reintroduced (316). Because elk are susceptible to tick-borne pathogens, and as such the Missouri Department of Conservation (MDC) monitors the reintroduced herd for ticks and tick-borne pathogens (317). The University of Missouri Veterinary Medical Diagnostic Laboratory (VMDL) received multiple tick samples from the MDC, collected from elk herds in the winter or spring of 2016 or 2017, respectively. Our laboratory morphologically identified tick samples and designed 12S RNA gene and ITS-2 targeted primers for molecular confirmation of species. Primers to the 12S RNA gene successfully amplified laboratory raised *Dermacentor andersoni* and sample DNA from ticks collected from elk, and

dideoxy sequences from these samples were uncontaminated allowing for species identification of samples. The research here confirmed that morphological identification and molecular identification coincided in all but one tested tick.

Materials and Methods

Primer selection

Sequences from ticks representative of each genus, excluding *Bothriocroton* which was unavailable, were found in GenBank and used for sequence alignment. WebPRANK, a progressive alignment software available through the European Bioinformatics Institute (EBI), was used to align sequences and primers (318). The 12S RNA gene GenBank accession numbers are as follows *Ixodes affinis* (U95878.1), *I. pacificus* (L43902.1), *I. ricinus* (NC_018369.2), *I. scapularis* (L43891.1), *I. simplex* (KM455965.1), *I. loricatus* (U95891.1), *Amblyomma americanum* (U95849.1), *A. cajennense* (KF614697.1), *A. maculatum* (U95854.1), *A. variegatum* (JF949801.1), *Haemaphysalis longicornis* (NC_037493.1), *Haemaphysalis leporispalustris* (U95873.1), *Hyalomma anatolicum* (KF583616.1), *H. rufipes* (U95875.1), *Rhipicephalus microplus* (U95867.1), *Dermacentor andersoni* (U95868.1), *D. variabilis* (U95869.1) and *Argas straitus* (KR907242.1).

ITS-2 GenBank accession numbers are as follows: *I. affinis* (JX982149.1), *I. pacificus* (L22279.1), *I. ricinus* (JF703110.1), *I. scapularis* (X63868.1), *I. simplex* (KY457499.1), *I. loricatus* (AF327343.1), *A. americanum* (AF291874.1), *A. cajennense* (AF469605.1), *A. maculatum* (MG076928.1), *A. variegatum* (HQ856803.1), *Haemaphysalis longicornis* (HQ005301.1), *Haemaphysalis*

leporispalustris (JQ868582.1), *H. anatolicum* (HQ005303.1), *H. rufipes* (KY457486.1), *R. microplus* (KY457506.1), *D. andersoni* (S83084.1), *D. variabilis* (S83088.1), *D. rhinocerinus* (KY457495.1) and *Argas straitus* (KY457493.1). 12S RNA gene and ITS-2 primers have been previously described (311, 313). A new reverse primer for both the ITS-2 and 12S RNA gene was selected in a region slightly upstream of the original reverse primer. Primer analysis was conducted in using the IDT (Newark, NJ) OligoAnalyzer to ensure that the primer design included low-self dimerization, low hetero-dimerization, low hairpin formation and to check that the melting temperature (Tm) was similar to the forward primer (319). Ticks were identified to genus or species level through the use of a dichotomous key (320).

12S RNA gene PCR

HotStart PCR 2X Master Mix (Lamda Biotech, Ballwin, MO) was used for PCR following the manufacturer's instructions. Briefly, each reaction of 25 µL contained 1X HotStart PCR Master Mix, 0.5 µM forward and reverse primers (pooled reverse primers each at 0.5 µM), and HPLC H_2O. Thermocycler conditions for the reaction were 95°C for 3 minutes, followed by 40 cycles at 94° for 30 seconds, 50°C for 30 seconds, and 72°C for 30 seconds with a final extension time at 72°C for 5 minutes.

Tick identification

Amplicons were excised from the gel and DNA was isolated using a gel extraction kit (EZBiosciences, Roseville, MN). Nucleotide sequences were

determined in both directions with the same forward and reverse primers used for the PCR assays. Samples were sent in 96-well plates to Eurofins Genomics (Eurofins, Louisville, KY) for dideoxy sequencing. Regions of similarity were assessed with the NCBI BLASTn program (245). DNA sequences with the highest score and lowest E-value to any species in correlation with high sequence identity were used to determine species identity as previously described (312).

Results

Primer design for molecular identification of tick species

The 12S RNA gene has been used in the field for identification of tick species and phylogeny in several studies (310, 311, 321, 322). A second target chosen for this study was the ITS-2 locus (313). Previously designed primers were compared to available ITS-2 and 12S RNA gene sequences (311, 313). The primers used in these studies were based upon information available in 1999 and 2001, respectively. A different reverse primer for both ITS-2 and 12S RNA gene was selected as these primers had a strong match to several tick species in every genus, excluding *Bothriocroton* which was unavailable, and did not have mismatch issues with the forward primer. Primers were 10-fold degenerate for 12S and 5-fold degenerate for ITS-2 to match most species (Figs. 23, 24; Table 10).

To demonstrate the utility of this assay, DNA was extracted from laboratory raised *D. andersoni* and used for 12S RNA gene targeted PCR. Dideoxy sequencing of the amplicons and subsequent searching with BLASTn revealed a high level of sequence agreement between *D. andersoni* laboratory raised amplicons and those sequences available in NCBI database (Fig. 25). Positive

Figure 23. Alignment of 12S RNA gene sequences of representative tick species aligned to matching degenerate primers.

Multiple 12S RNA gene sequences were selected from each genus of Ixodidae and one out group of *Argas* were aligned. Several primers were selected to have representation for all the sequences provided, for a total of 2 forward and 8 reverse degenerate primers. The alignment was created using webPRANK alignment software.

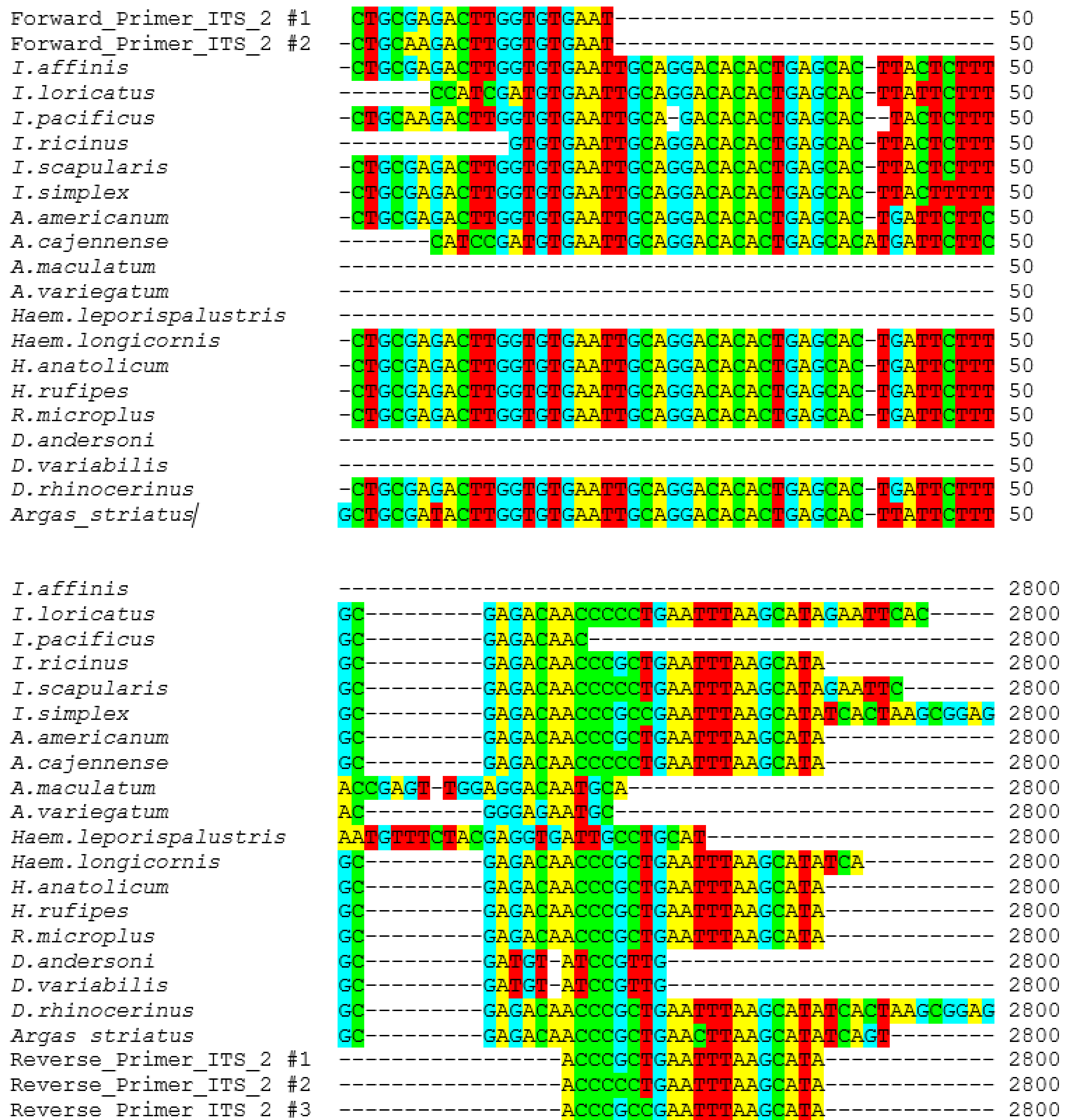


Figure 24. Alignment of ITS-2 sequences of representative tick species aligned to matching degenerate primers.

Multiple ITS-2 sequences were selected from each genus of Ixodidae and one out group of *Argas* were aligned. Multiple primers were selected to have representation for all the sequences provided, for a total of 2 forward and 3 reverse degenerate primers. The alignment was created using webPRANK software.

A

12S Primers	Sequence	Species recognized
Forward #1	AAA CTA GGA TTA GAT ACC CT	*I. affinis, I. pacificus, I. ricinus, I. scapularis, I. simplex, I. loricatus, A. americanum, A. cajennense, Haemaphysalis longicornis, Haemaphysalis leporispalustris, H. rufipes, R. microplus, D. andersoni, D. variabilis*
Forward #2	AAA CTA GGA TTA TAT ACC CT	*A. maculatum*
Reverse #1	CTA TGT TAC GAC TTA TCT TA	*A. maculatum, R. microplus, D. andersoni, D. variabilis*
Reverse #2	CTA TGT TAC GAC TTA TCT CA	*I. affinis, I. pacificus, I. scapularis, I. simplex*
Reverse #3	CTA TGT TAC GAC TTA TCT TT	*Haemaphysalis leporispalustris, A. americanum*
Reverse #4	CTT TGT TAC GAC TTA TCT CA	*I. ricinus*
Reverse #5	CTT TGT TAC GAC TTA TCT TT	*Haemaphysalis longicornis*
Reverse #6	CTA TGT TAC GAC TTA TCT CT	*I. loricatus*
Reverse #7	CTA TGT TAC GAC TTA GCT TA	*H. rufipes*
Reverse #8	CTA TGT TAC GAC TTA TCT TCA	*A. variegatum*

B

ITS-2 Primers	Sequence	Species recognized
Forward #1	ACC CGC TGA ATT TAA GCA TA	*I. affinis, I. scapularis, I. simplex, A. americanum, Haemaphysalis longicornis, H. anatolicum, H. rufipes, R. microplus, D. rhinocerinus*
Forward #2	ACC CCC TGA ATT TAA GCA TA	*I. pacificus*
Reverse #1	TAT GCT TAA ATT CAG CGG GT	*I. ricinus, A. americanum, H. anatolicum, H. rufipes, R. microplus, Haemaphysalis longicornis, D. rhinocerinus*
Reverse #2	TAT GCT TAA ATT CAG GGG GT	*I. scapularis, I. loricatus, A. cajennense*
Reverse #3	TAT GCT TAA ATT CGG CGG GT	*I. simplex*

Table 10. Primers for 12S RNA gene and ITS-2 for molecular identification of divergent tick species.

Degenerate primers were designed after mapping primers for the 12S RNA gene and ITS-2. **A.** The 12S RNA gene primers from left (5') to right (3'). **B.** ITS-2 gene primers from left (5') to right (3').

```
D.andersoni_U95868.1        AAACTAGGATTAGATACCCTATTATTTATGAAGCTAAACATTGTAAGTAAATGTTAATTA     60
D.andersoni_EU711297.1      ----------------------------------------------------GTTAATTA     8
D.andersoni_AF150040.1      --------------------ATTATTTATGAAGCTAAACATTGTAAGTAAATGTTAATTA     40
PositiveControl#1           ---------------------------------------------------------TTA     3
PositiveControl#2           ----------------------------------------------------GTTAATTA     8
PositiveControl#3           ----------------------------------------------------GTTAATTA     8
PositiveControl#4           ----------------------------------------------------GTTAATTA     8
                                                                                     ***

D.andersoni_U95868.1        T-AAAGCAAAAAATTATGGCGGTATCTTAAGCTTTTCAGAGGAATTTGCTCTATAATGGA     119
D.andersoni_EU711297.1      TGAAAGCAAAAAATTATGGCGGTATCTTAAGCTTTTCAGAGGAATTTGCTCTATAATGGA     68
D.andersoni_AF150040.1      TGAAAGCAAAAAATTATGGCGGTATCTTAAGCTTTTCAGAGGAATTTGCTCTATAATGGA     100
PositiveControl#1           TGAAAGCAAAAAATTATGGCGGTATCTTAAGCTTTTCAGAGGAATTTGCTCTATAATGGA     63
PositiveControl#2           TGAAAGCAAAAAATTATGGCGGTATCTTAAGCTTTTCAGAGGAATTTGCTCTATAATGGA     68
PositiveControl#3           TGAAAGCAAAAAATTATGGCGGTATCTTAAGCTTTTCAGAGGAATTTGCTCTATAATGGA     68
PositiveControl#4           TGAAAGCAAAAAATTATGGCGGTATCTTAAGCTTTTCAGAGGAATTTGCTCTATAATGGA     68
                            * **********************************************************

D.andersoni_U95868.1        TAAAACGCCTAAACCTTACTTTGACTTGTGA--GCAATTTGTATACCACTATTAAAATAA     177
D.andersoni_EU711297.1      TAAAACGCCTAAACCTTACTTTGACTAGTAAAATCAATTTGTATACCACTATTAAAATAA     128
D.andersoni_AF150040.1      TAAAACGCCTAAACCTTACTTTGACTAGTAAAATCAATTTGTATACCACTATTAAAATAA     160
PositiveControl#1           TAAAACGCCTAAACCTTACTTTGACTAGTAAAATCAATTTGTATACCACTATTAAAATAA     123
PositiveControl#2           TAAAACGCCTAAACCTTACTTTGACTAGTAAAATCAATTTGTATACCACTATTAAAATAA     128
PositiveControl#3           TAAAACGCCTAAACCTTACTTTGACTAGTAAAATCAATTTGTATACCACTATTAAAATAA     128
PositiveControl#4           TAAAACGCCTAAACCTTACTTTGACTAGTAAAATCAATTTGTATACCACTATTAAAATAA     128
                            ************************** ** *   **************************

D.andersoni_U95868.1        TAACATACAACTATTATTTCAATATATATAATATAAAAAATTAAGTCAAGGTGCAGTAAA     237
D.andersoni_EU711297.1      TAACATACAACTATTATTTCAATATAATTTATATAAAAAATTAAGTCAAGGTGCAGTAAA     188
D.andersoni_AF150040.1      TAACATACAACTATTATTTCAATATAATTTATATAAAAAATTAAGTCAAGGTGCAGTAAA     220
PositiveControl#1           TAACATACAACTATTATTTCAATATAATTTATATAAAAAATTAAGTCAAGGTGCAGTAAA     183
PositiveControl#2           TAACATACAACTATTATTTCAATATAATTTATATAAAAAATTAAGTCAAGGTGCAGTAAA     188
PositiveControl#3           TAACATACAACTATTATTTCAATATAATTTATATAAAAAATTAAGTCAAGGTGCAGTAAA     188
PositiveControl#4           TAACATACAACTATTATTTCAATATAATTTATATAAAAAATTAAGTCAAGGTGCAGTAAA     188
                            **************************  * ******************************

D.andersoni_U95868.1        AGTCAATGAATGAAGTGAATTACATTTCTTTTTAGAAATTAGAAAAATGAAAAGATAATT     297
D.andersoni_EU711297.1      AGTCAATGAATGAAGTGAATTACATTTCTTTTTAGAAAAG--AAAAATGAAAAGTAAATT     246
D.andersoni_AF150040.1      AGTCAATGAATGAAGTGAATTACATTTCTTTTTAGAAAAG--AAAAATGAAAAGTAAATT     278
PositiveControl#1           AGTCAATGAATGAAGTGAATTACATTTCTTTTTAGAAAAG--AAAAATGAAAAGTAAATT     241
PositiveControl#2           AGTCAATGAATGAAGTGAATTACATTTCTTTTTAGAAAAG--AAAAATGAAAAGTAAATT     246
PositiveControl#3           AGTCAATGAATGAAGTGAATTACATTTCTTTTTAGAAAAG--AAAAATGAAAAGTAAATT     246
PositiveControl#4           AGTCAATGAATGAAGTGAATTACATTTCTTTTTAGAAAAG--AAAAATGAAAAGTAAATT     246
                            **************************************    ************  ****

D.andersoni_U95868.1        AGGATTTGAAAGTAAAATTAAAATAAAATGTTAATTTGAATTAAGCTCTAAGATATGTAC     357
D.andersoni_EU711297.1      AGGATTTGAAAGTAAAATTAAAATAAAATGTTAATTTGAAT-------------------     287
D.andersoni_AF150040.1      AGGATTTGAAAGTAAAATTAAAATAAAATGTTAATTTGAATTAAGCTCTAAGATATGTAC     338
PositiveControl#1           AGGATTTGAAAGTAAAATTAAAATAAAATGTTAATTTGAAT-------------------     282
PositiveControl#2           AGGATTTGAAAGTAAAATTAAAATAAAATGTTAA--------------------------     280
PositiveControl#3           AGGATTTGAAAGTAAAATTAAAATAAAATGTTAATTTGAAT-------------------     287
PositiveControl#4           AGGATTTGAAAGTAAAATTAAAATAAAATGTTAATTTGAAT-------------------     287
```

Figure 25. Proof of concept for PCR with the 12S RNA gene using laboratory raised *D. andersoni* ticks.

Sequencing was performed on PCR amplicons from adult *D. andersoni* using 12S RNA gene primers. The alignment was created using Clustal Omega alignment software. Positive control sequences (#s 2-4) start at base pair 53 in the 12S alignment from Figure 23.

control sequences matched 2 of the 3 available *D. andersoni* 12S sequences.

Morphological identification of ticks collected from *C. canadensis* in Missouri

Ticks were collected from elk in the winter or spring of 2016 or 2017, respectively. Tick samples were identified using a dichotomous key to the genus, or preferably species level if possible. The results yielded a substantial number of *A. americanum* ticks, 60, followed by *I. scapularis*, 19, *D. albipictus*, 12, and a smaller number of the other species, including some that were unable to be identified to species level or identified to any group (Fig. 26).

PCR of the 12S RNA gene of ticks collected from *C. canadensis* in Missouri

116 ticks and 6 positive control *D. andersoni* were amplified with primers against the 12S RNA gene (Fig. 27). A clean and robust amplicon at approximately 400 bp was produced in almost all tick samples collected. The PCR resulted in some limited background with a small amplicon sometimes occurring at 200 bp; however, gel excision and dideoxy sequencing of the target was without contamination. Preliminary identification of ticks to the species level was achieved with NCBI BLASTn results as determined by the highest score and lowest E-value (Fig. 28). Only one morphologically identified tick, *I. minor*, was determined to be *I. scapularis* with 100% identity and 99% coverage and an E-score of 0.0. Multiple tick species were identified and matched the morphological identification (Fig. 28). As expected,- dideoxy sequencing of samples and subsequent BLASTn analysis revealed that most of the samples matched *A. americanum*, followed by *I. scapularis* and *D. albipictus*.

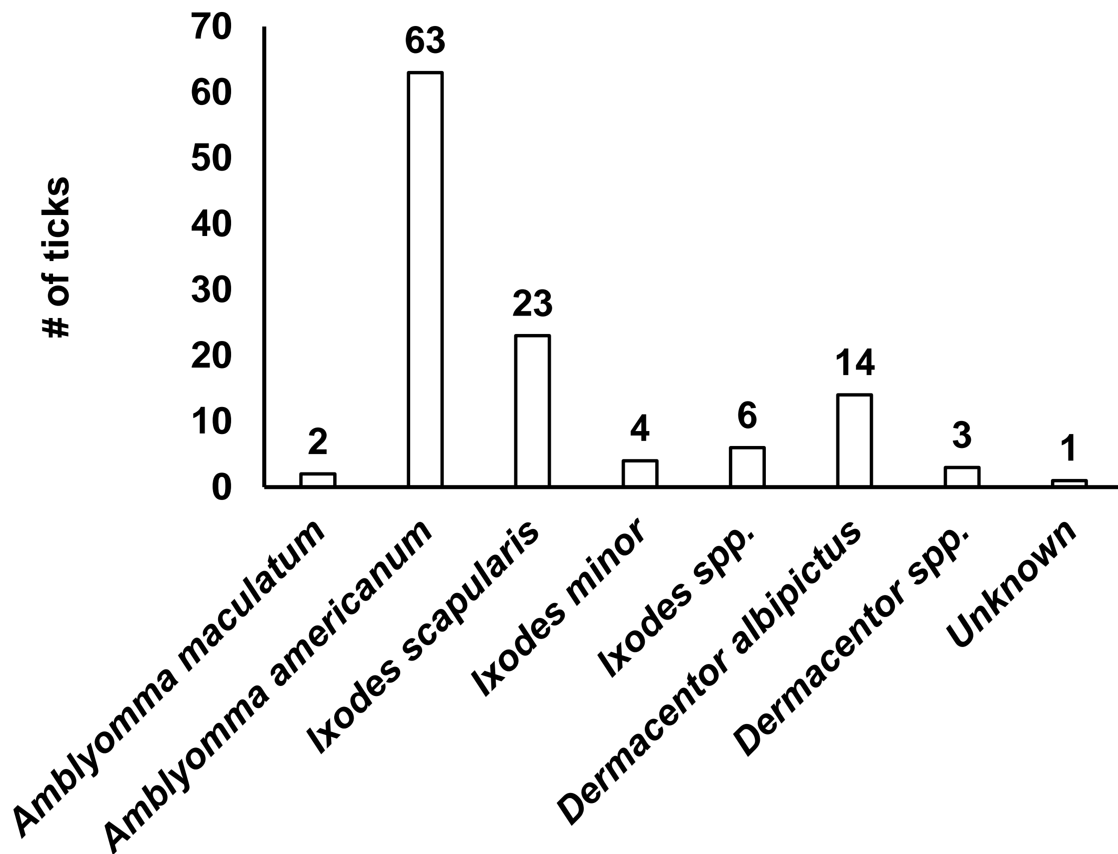


Figure 26. Morphological identification of ticks collected from *C. canadensis* reintroduced to Missouri.

Morphologic identification of tick species was evaluated based upon a dichotomous key. Data labels indicate number of ticks in each group. The research in this figure was conducted by D. Thompson and Dr. R. Stich.

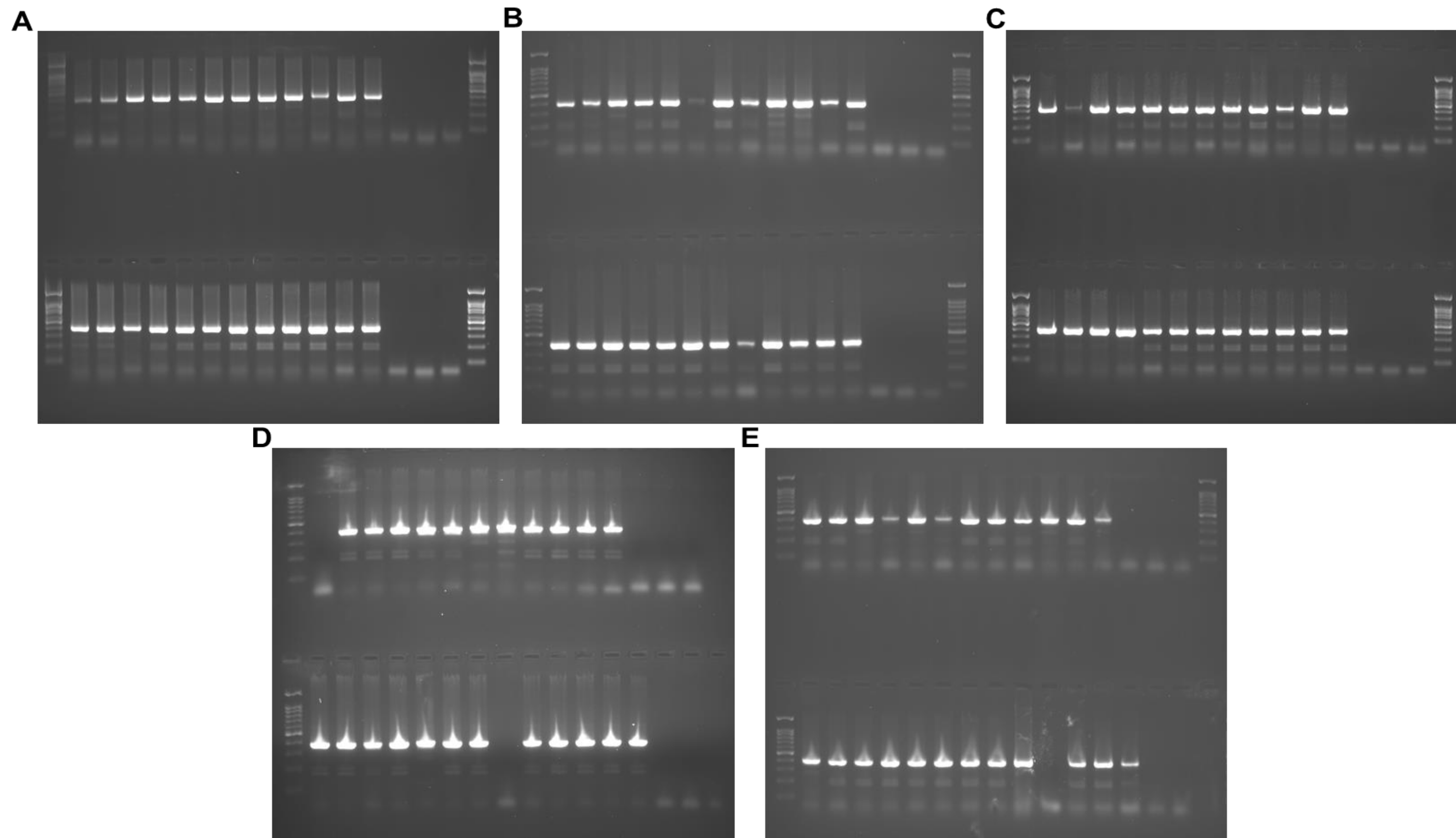


Figure 27. 12S RNA gene targeted PCR of 116 tick samples collected from elk.

The amount of DNA used as template was 1/10th the final volume per reaction of isolated whole tick DNA. A. Tick samples 1-24. B. samples 25-48. C. samples 49-72. D. samples 73-97. E. samples 98-121. Gel images are split into two, top gel with the top 12 samples followed by 3 non-template controls (NTCs). Bottom, samples 13-24 followed by 3 non-template controls (NTCs). PCR products were separated on a 1.0% agarose gel stained with ethidium bromide with a target amplicon of approximately 400 bp. A Goldbio 1kb ladder was used. Ticks 59-64 are positive control *D. andersoni* colony raised ticks.

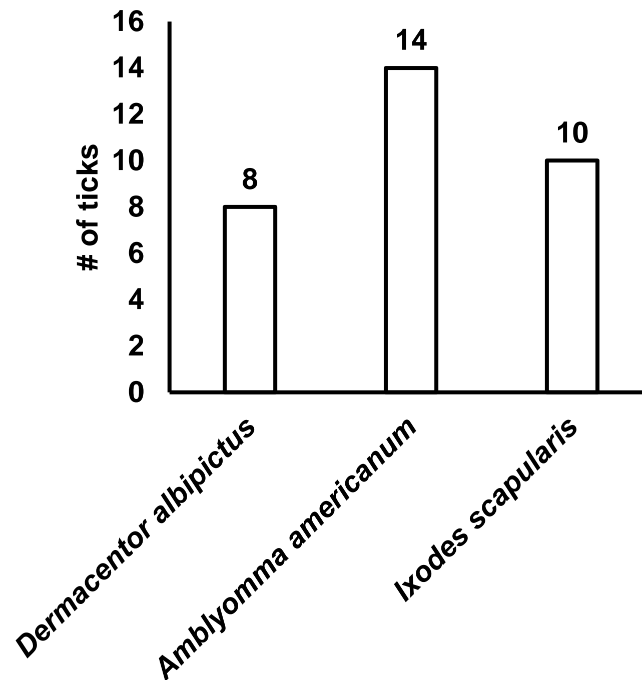


Figure 28. 12S PCR based identification of tick samples.

Amplicons from 12S targeted PCR of tick samples were sequenced with dideoxy sequencing. Further, BLASTn analysis of tick sequences against the family Ixodidae identified several ticks to the species level as determined by the highest score and lowest E-value. Identification is based upon sequencing of 1 strand. Data labels indicate number of ticks in each group.

Discussion

The purpose of this study was to create a molecular key for identification of tick species. Morphological identification of ticks is laborious and often requires substantial experience in tick identification to correctly identify samples to the species level. In this study, 12S primers were based upon primers in a 2001 phylogeny study of the genera *Rhipicephalus* and *Boophilus* (311). A second set of primers was chosen based upon a study that utilized a molecular key for identification of *Ixodes* spp. targeting the ITS-2 gene. The forward primers used in our work matched previously reported primers; however, the reverse primers varied from the original source due to strong identity with all sequences upstream of the original primer (Figs. 23, 24; Table 10). (313). Alignment of the 12S RNA gene and the ITS-2 from multiple tick samples of each genus revealed high conservation of the loci for the primer sequences (Figs. 23, 24). The ITS-2 aligned sequences displayed greater inter-species sequence variation than the 12S RNA aligned sequences. Morphological identification of ticks collected from elk and provided to our laboratory from the VMDL at the University of Missouri revealed large numbers of *A. americanum*, followed by *I. scapularis* and *D. albipictus* (Fig. 26). 12S RNA gene targeted PCR was successful in identification of positive control *D. andersoni* ticks. Finally, BLASTn analysis of 12S RNA gene sequences of tick samples matched, in all but one case, the morphological identification of ticks matched the molecular identification (Fig. 28).

Several studies have demonstrated the utility of molecular identification of tick species by targeting the cytochrome c oxidase I (COI), 16S RNA, ITS-2 or 12S

RNA genes or loci (312, 323–327). A study directly compared the four described molecular targets, COI, 16S, ITS-2 and 12S RNA genes or loci, and found no statistically significant difference in successful tick species identification between the four tested DNA targets (312). Our study evaluated and confirmed the use of molecular identification of tick species in the United States. The preliminary results in this study support the utility of the 12S RNA gene assay, and the alternative reverse primer described in this study.

Further work is necessary for ITS-2 confirmation of species identified with the 12S RNA gene. In addition, molecular identification of tick samples from Figure 26 that were not identified to the species level will be necessary. Further, preliminary primer design using the consensus sequence for ITS-2 and the 12S RNA gene, has revealed another primer target that will be useful for testing in the future (data not shown). In conclusion, the preliminary data in this study agree with previous studies that the 12S RNA gene is a strong target for DNA based identification of unknown tick samples.

Acknowledgements

This research was supported by USDA NIFA PROJ NO: MO.W-2016-09373 and the University of Missouri Department of Veterinary Pathobiology. The authors thank S. Shahzad and H. Smith for technical assistance.

CHAPTER 6

Conclusions

Chapter 2

Tick transmission of *Anaplasma marginale*

The precise molecular mechanisms involved in pathogen infection of ticks are not fully understood. Identification of these mechanisms is expected to provide targets in the pathogen life cycle to help control the spread of tick-borne diseases. Similarly, the mechanisms identified in pathogen infection of and transmission by ticks may provide insight into the control of other tick-borne pathogens, thus allowing for control strategies that are broader than a single targeted pathogenic organism. Several studies have outlined the molecular mechanisms involved in infection of ticks by *Anaplasma phagocytophilum*. One study reported that *A. phagocytophilum* regulates host cell porin and subsequent cytochrome c release to prevent apoptosis in infected tick cells (209). Secondly, *A. phagocytophilum* appears to appropriate *I. scapularis* antifreeze glycoprotein (IAFGP), which inhibits bacterial biofilms, altering the midgut microbiome (208, 328), and it is thought that this may enhance *A. phagocytophilum* infection of the tick. These two examples suggest mechanisms employed by pathogens to aid in infection of tick midgut tissues. It is not fully understood how *A. phagocytophilum* reaches the midgut epithelium. It is possible that *A. phagocytophilum* enters the midgut cells during receptor-mediated pinocytosis of lysed RBCs by tick midgut epithelial cells.

Another mechanism employed by a related pathogen, *Rickettsia parkeri*, is actin motility in the vertebrate and tick host. Ticks exposed to mutant *R. parkeri* without RickA, an Arp2/3 complex activator leading to actin polymerization, had lower levels of infection than wild type *R. parkeri* in most tick tissues, excluding the ovaries in early infection (329). Further, as the infection progressed mutant *R. parkeri* persisted in organs, while the wild type *R. parkeri* was eliminated from the ovaries. This suggests that actin-based motility is important in early and rapid dissemination in ticks but may not be important in the overall dissemination of the pathogen in the tick. The described molecular mechanisms employed by *A. phagocytophilum* and *R. parkeri* are likely to be conserved in the prototypical species of the rickettsial family *Anaplasmataceae*, *Anaplasma marginale*.

As previously stated, multiple stages of the *A. marginale* infection and replication life cycle in the tick have been documented with electron microscopy, immunofluorescence assay (IFA) or PCR, while the molecular mechanisms associated with these stages are poorly understood. The focus of chapter 2 was the identification of genomic elements, specifically genes and promoters, associated with *A. marginale* adaptions to acarine hosts. The non-tick-transmissible *A. marginale* Illinois strain was first reported in 1986 (18). The Illinois strain was not transmitted by two *Dermacentor* spp. ticks fed on naïve calves (18, 112). Percutaneous infection of *D. andersoni* did not result in successful transmission of the Illinois strain and transmission electron microscopy (TEM) did not reveal any colonies in the midgut of the Illinois exposed ticks (112). This work suggests that the Illinois strain of *A. marginale* is incapable of infecting the tick.

Secondly, it is unknown if the Illinois strain can be adapted to infect tick species or strains other than those which were tested. Thus, future experiments will be necessary to tease out the alternatives described above. Experiments involving feeding different tick species indigenous to the state of Illinois, where the Illinois strain was isolated from, including *Rhipicephalus sanguineus*, *Dermacentor albipictus*, *Ixodes scapularis* and *Amblyomma americanum*, would be instructive to determine if other local tick species or strains could be competent vectors of the Illinois strain. Additionally, it will be useful to pair *A. marginale*-targeted PCR of salivary glands and midguts of the above tick species and *D. andersoni* to determine the stage in the Illinois strain life cycle that is not adapted to use *Dermacentor* spp. as its invertebrate host.

Putative candidates factors associated with tick-transmission of *A. marginale*

To the best of our knowledge, chapter 2 describes the first sequence of the *A. marginale* Illinois strain. The sequencing was done with Pacific Biosciences (PacBio) next generation single molecule, real time (SMRT) sequencing, which contrasts with the majority of the *A. marginale* genomes currently available in the NCBI database that were sequenced with Roche 454 sequencing technology. SMRT sequencing allowed for a more complete sequence, with sequence coverage that spans the numerous repetitive regions found in *A. marginale* genomes. The experiments and bioinformatic analyses described in chapter 2 yielded 27 candidates associated with the non-tick-transmissibility phenotype of the Illinois strain, 10 of which may also contribute to the non-tick-transmissibility of

the Florida strain. Additionally, a large 14.5-kb inversion was found to be unique to the Florida and Illinois strains. One candidate, *aaap*, is associated with the inclusion appendage and has several variations in the intergenic regions surrounding the *aaap* gene cluster that are potentially involved in different expression levels between the Florida and Illinois strains (124). Finally, two of the candidate genes responsible for the Illinois strain phenotype are posited adhesins and another candidate gene is associated with the inclusion appendage actin filament.

One candidate is a sequence similar to a xenobiotic response element (XRE). The XRE transcriptional regulator is predicted to be a transcriptional regulator, based upon conserved motifs for a helix-turn-helix domain which could be capable of binding DNA (330). This candidate was interesting to find as a putative XRE transcription regulator that could be associated with tick transmission of *A. marginale*. For example, the transcriptional regulator may regulate expression of *aaap*, resulting in the noted difference in AAAP expression between the Florida and Illinois strains. Further, this XRE transcriptional regulator may affect other genes that are important in tick-transmission of *A. marginale*. One approach to determine the function of the XRE regulator could be chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq). This technique was used successfully with eukaryote and prokaryote systems to find binding sites for several proteins, including NF-κB, cyclic AMP-responsive element-binding protein (CREB), *Escherichia coli* leucine-responsive protein (Lrp), *E. coli* RNA polymerase and *E. coli* cyclic AMP-receptor protein (CRP) (331–335).

In theory, the technique is straightforward, but a significant limitation involves requirements for certain proteins to bind to DNA (336). For example, CRP will not bind to DNA unless it is bound to cyclic-AMP. To the best of my knowledge, the conditions for the XRE transcriptional regulator to bind to DNA have not been reported to date. An alternative is to express recombinant XRE transcriptional regulator that is tagged (*e.g.*, polyhistidine-tagged), from a non-frameshifted gene, and to mix this recombinant fusion protein with homogenate of cell-culture raised *A. marginale* because the cell-culture homogenate may provide molecules necessary for the XRE transcriptional regulator to bind to DNA. It is also possible that the XRE transcriptional regulator may bind to DNA without requiring another bound molecule.

Whole genome sequencing of additional tick-transmissible strains

A major limitation of the research described in chapter 2 is that several genes were not eliminated from association with adaption to tick vectors due to the limited coverage of sequences from genomes of other *A. marginale* strains. A previous study with the *A. marginale* Florida strain described elimination of candidate genes using targeted PCR and amplicon sequence analyses of transmissible strains, EMΦ and 6DE (126). Whole-genome sequencing with long-read SMRT technology of the known tick-transmissible strains would further aid in elimination of candidates. In 2019, Pacific Biosciences has released a new version of the sequel RS II, which can generate eight times the data as compared to the instrument used in chapter 2. Hybrid library assemblies with Illumina and Pacific Biosciences sequencing technologies would be expected to further enhance the

sequence quality. Due to the significant decrease in price for DNA sequencing, future studies can use this approach with additional tick-transmissible and non-tick-transmissible strains to narrow the list of candidates (337, 338).

Transformation of *A. marginale*

There are at least two reports of transformation of *A. marginale,* both using the *Himar1* transposon system (339, 340). The *Himar1* system only requires a transposase recognizing a TA dinucleotide and as such it is not targeted to specific genes. Thus, the *Himar1* system would likely prove difficult for mutagenesis studies of the chapter 2 candidate genes associated with tick-transmission of *A. marginale*. In addition, transformation of *Anaplasmataceae* is expected to be relatively challenging because these organisms are restricted to intracellular parasitophorous vacuoles. These organisms must be purified from the cell culture milieu, and any manipulation cannot interfere with their ability to reinfect new host cells (339). Further, it has been reported that organisms in *Anaplasmataceae*, unlike *Rickettsiaceae*, do not maintain plasmids (341). More recent work has reported genetic disruption and restoration of target genes in a closely related intracellular pathogen, *Ehrlichia chaffeensis* (342). In the future, this work is expected to be translatable to *A. marginale*. The current research and candidates described in chapter 2 assist with the body of knowledge of *A. marginale* tick-pathogen interactions, because future studies are warranted to identify functionality and binding partners for these candidates.

Chapter 3

Ticks are important vectors of pathogens worldwide and in the U.S. alone are responsible for 75% of all reported vector-borne infections (41). *Dermacentor andersoni* and *Dermacentor variabilis* are important vectors of multiple pathogens including *Anaplasma* spp., *Francisella tularensis*, *Coxiella burnettii* and *Rickettsia rickettsii* (286, 343).

Research on vertebrate resistance to tick feeding began in 1918 with Johnson and Bancroft (155). In 1939, Trager revolutionized the field by demonstrating that immunization against ticks and passive transfer of resistance is possible (156). Since this seminal work, attention has turned to the phenomenon of tick resistance due to repeated infestation or immunization against ticks with crude tick tissues or recombinant tick proteins (163, 169, 181, 187, 189, 207, 283, 292, 344). Despite these efforts, the only internationally available commercial vaccine is based upon the glycoprotein, Bm86, which was isolated from *Rhipicephalus microplus* over 30 years ago (181, 199). The research discussed in chapter 3 sought to adapt an economically relevant animal model for tick immunization to a tick indigenous to the United States, *D. andersoni*, a well-documented vector of *A. marginale* (17, 96, 100, 109, 345, 346).

Performance of *D. andersoni* after feeding on immunized hosts

Immunization with *D. andersoni* tick tissues led to seroconversion of these animals with an associated reduction in tick feeding and fecundity. Immunization with salivary glands had the greatest reduction on both tick feeding and fecundity. This was in contrast to the previous reports that denatured salivary gland

immunization reduced feeding performance while native midgut immunization reduced tick fecundity performance (168, 169, 173). For previous studies, was posited that denaturation exposes the salivary gland molecules more efficiently for presentation to the vertebrate host immune response. The tissue types, and methods used to prepare these immunizations were identical to previous studies from Jittapalapong *et al.* (168, 169), and it seems possible that the differences in tick species and host ages may explain minor differences in resultant tick performance (168, 169). Further, previous studies were done with ticks in the genera *Hyalomma* and *Rhipicephalus*. *D. andersoni* may express different proteins and thus the immunogenic targets recognized by the host may vary, resulting in different readouts of protection. Additionally, our study used aged 3-6 months old male Holstein steers for tick feeding, while the previous studies used adult dairy cows or adult dogs. Age is known to shift immune responses (347), and it is plausible that the young age of these calves and the tick species tested could contribute to the different tick performances seen between studies. Additionally, one study by Jittapalapong *et al.* reported different tick performance in *R. sanguineus* fed on dogs (168). A different host species may also contribute to different tick performance after feeding on immunized hosts.

Mechanism associated with reduced tick performance

The potential mechanisms responsible for the reduction of tick performance after immunization are quite intriguing. Briefly, research by Kemp *et al.* suggested that antibodies and the complement cascade mediate damage to the midgut epithelium for ticks fed on vaccinated animals (181). A potential explanation is that

the reduction in fecundity due to midgut immunization is a result of damage to the midgut tissues, either by directly killing the tick or through prevention of hemoglobin processing due to a damaged midgut epithelium. A second posited mechanism is that midgut epithelium damage allows entry of humoral and cellular immune components into the hemocoel, resulting in subsequent damage of tick tissues including those involved in reproduction.

Histopathology of *R.* (*Boophilus*) *microplus* ticks after feeding on denatured salivary gland-immunized cattle showed increased vacuolization, shrinkage of membranes, irregular cell shape, incomplete cell boundaries and distended endoplasmic reticulum, which indicated damage to type II and type III acini as compared to controls (192). Further, histopathology of ticks that fed on *R.* (*Boophilus*) *microplus* immunized cattle found sloughing off of midgut digestive cells which allowed entry of leukocytes, mainly neutrophils, basophils and eosinophils, into the hemocoel (180). Several studies have reported vertebrate antibodies in the hemolymph of ticks (178, 179). Thus, the damage to these salivary glands may be explained by protective vertebrate immune effectors which enter the tick hemocoel and these effectors damage tick tissues after ticks have fed on immunized hosts. The tick salivary glands are not known to directly interface with host tissues, therefore, host immune components are expected to first passage through the midgut. Damage to the salivary glands could interfere with feeding, negatively impact water homeostasis and release proteases that may damage internal structures.

The tick salivary glands contain many secretory granules that are used to secrete bioactive saliva into the host. Immunization with tick salivary gland homogenates likely results in exposure to proteins found in the saliva, but at significantly higher levels. Thus, immunization against the salivary glands may impair feeding by not only targeting the internal structures of the tick, but by also interfering with the feeding lesion. Antigen-specific IgE and mast cells are thought to play a role in acquired resistance to ticks (348). Studies have indicated that after repeated infestation with *Haemaphysalis longicornis* larvae, host basophils, but not mast cells, will be the mediators of tick resistance at the tick feeding site (182, 185). Basophils were found to be recruited to the feeding lesion during the second infestation and surrounded the tick mouth parts and localized release of histamine was essential to tick resistance. Additionally, a study with *A. americanum* feeding on sensitized rabbits revealed that, at the tick feeding site, basophils increased from 1 to 11% (percent of total cells) during second infestations, mast cells remained constant and eosinophils increased from 7 to 33% (349). Other studies have reported that eosinophils and basophils work together for acquired tick resistance (191). The above studies suggest that resistance to ticks, resulting from immunization or repeated infestation, is probably a cooperative effect between inhibition at the feeding site and damage to the tick. For future studies, it would be ideal to isolate tick feeding sites and perform histopathology of these sites to determine cell types recruited to the feeding site as compared to controls and baseline feeding. Further, experiments involving *in vitro* feeding of ticks on

immunized calf blood or sera, paired with electron microscopy or histopathology of ticks, would assist in determining the mechanism of reduced tick performance.

Identification of proteins associated with reduced tick performance

Much of the research in chapter 3 was focused on the isolation of proteins reactive with host immune sera associated with reduced tick performance. Immunization of calves had the greatest negative impact on tick performance parameters. Thus, an elimination strategy using sera from midgut-immunized animals on salivary gland tissues was employed to eliminate cross-reactive proteins. Additionally, we developed anti-salivary gland sera on midgut antigens to further eliminate cross-reactive proteins. This research identified 258 LC-MS/MS candidate proteins from the 44 two-dimensional western protein spots uniquely reactive to salivary gland proteins.

Bioinformatic analysis of these proteins helped to narrow the list of proteins to those with RNA-seq reads not found in *D. andersoni* MG transcriptomic datasets. The hypothesis underlying this approach was that proteins uniquely reactive to protective anti-sera were not expressed in the midgut. However, there are drawbacks to two-dimensional gel electrophoresis and the bioinformatic approach described in chapter 3. First, it is conceivable that the tick performance on immunized calves were quantitatively different due to a higher expression of protective antigens in the salivary glands. Second, elimination of proteins based upon expression in midgut *D. andersoni* RNA-seq data is not complete. Several proteins in the *D. silvarum* genome, the reference genome for the LC-MS/MS data, did not have reads map from *D. andersoni* RNA-seq data of salivary gland or

midgut tissues. It is possible that the limited reads are a technical limitation of the transcriptomic data in which there is not enough read coverage of transcripts for accurate determination of presence or absence of transcripts in *D. andersoni* midgut or salivary gland tissues. Further, a publicly available *D. andersoni* genome is not available. If a *D. andersoni* genome was available, it is possible more candidates would be identified with m/z search of the LC-MS/MS data.

An alternative strategy to identify antigens would be immunoprecipitation. This strategy would involve coating magnetic beads with antibodies from midgut-immune sera, then placing these beads with salivary gland proteins to allow for antigen-antibody binding. Removal of these beads is expected to eliminate cross-reactive proteins. Subsequently, magnetic beads incubated with salivary gland-immune sera would be employed on denatured depleted salivary gland proteins and elution of these proteins for LC-MS/MS is expected to yield candidate denatured proteins. The outcome of this experiment, in theory, would be salivary gland proteins only recognized by salivary gland-immune sera. Unfortunately, this strategy does not address the hypothesis that the same protein, at different expression levels, is responsible for the unique reduction in tick performance in salivary gland immunized calves. To address this, sera from salivary gland-or midgut-immune calves would need to be incubated with their cognate antigen and LC-MS/MS identification of these proteins would reveal proteins that both sera recognize together. The proteins shared between the two preparations would be candidates associated with the stated hypothesis.

Finally, the data collected from chapter 3 indicated that the bovine-*Dermacentor* model system can be adapted to investigate immune intervention with tick vectors. The culmination of this research is the basis for chapter 4 and intervention with acquisition or transmission with the tick-borne pathogen *A. marginale*.

Chapter 4

In chapter 4, we sought to interfere with *A. marginale* transmission or acquisition after immunization with tick tissue homogenates. These antigens would be the basis for future research to help reach the long-term goal of mitigating tick-borne diseases. Briefly, chapter 4 involved immunization of calves with four *D. andersoni* tissue homogenates of native (N) or denatured (D) salivary gland (SG) or midgut (MG). These calves were challenged with *A. marginale*-infected adult *D. andersoni* ticks. After all calves were infected with *A. marginale*, they were then acquisition fed upon by uninfected *D. andersoni* and tick acquisition of *A. marginale* was measured. NSG-and NMG-immunized calves were resistant to *D. andersoni* transmission of *A. marginale* but were still susceptible to infection upon challenge with carrier blood. Further, *D. andersoni* fed on NSG-immunized calves had the lowest consistent acquisition rates and *A. marginale* infection levels.

Native protein identification

Because immunization with denatured tissues did not interfere with tick transmission of *A. marginale*, these data suggest that conformational epitopes are important for reduction in transmission of *A. marginale* between ticks and cattle. Two-dimensional gel electrophoresis and SDS-PAGE employed in chapter 3

required denaturation of the tissues, potentially destroying the putatively protective conformational epitopes. As such, a method to identify native antigens is necessary. There are several methods available including Native PAGE or Blue Native PAGE, phage display or immunoprecipitation. Native PAGE involves separation of proteins on a polyacrylamide gel by their negative charge in an alkaline buffer separating them on size and charge while in a native state. In Blue Native PAGE, a negative charge is added to proteins by the addition of Coomassie dye allowing for separation of proteins in a native state. A limitation with these approaches will be the two-dimensional separation of these proteins as there are limited protocols that describe two-dimensional separation of proteins while conserving native structure (350–352). This work could potentially involve significant effort to discover and optimize a protocol for native two-dimensional gel electrophoresis.

Phage display relies on genetically modified bacteriophages that express small random peptides on their coat protein (353). These bacteriophages could be used for biopanning to isolate specific epitopes that are bound by immune sera. NSG immunization of calves had the greatest impact on tick performance, *D. andersoni* transmission of *A. marginale* and naïve *D. andersoni* acquisition of *A. marginale*. As such, it would be prudent to perform phage display biopanning with NSG immunized calf sera. In addition, biopanning separately with DSG-or DMG-immune sera would be a useful negative selection tool to eliminate denatured cross-reactive epitopes that are presumably not protective.

This technique has a few limitations. The small amino acid sequences identified may have no known match to any currently available protein in the *Dermacentor silvarum* genome. Secondly, bias with over incorporation of G in the base pairs encoding the displayed peptides reduces the randomness of randomly generated phage peptide libraries, this can limit sequence coverage (354–356).

Another method involves possibly full length proteins expressed through a cDNA library. The expression library can be screened to identify proteins that react with sera from immunized calves that were not infected by *A. marginale* during infected tick challenge.

Finally, immunoprecipitation, in theory, appears to be the most straightforward application for identification of antigens. This protocol would be the similar to the protocol suggested above for chapter 3. Briefly, an ideal first step would be elimination of antigens recognized from DSG and DMG. Magnetic beads bound with anti-DSG and anti-DMG immunoglobulins can be used to eliminate cross-reactive antigens in NSG. This elimination strategy would be expected to yield only antigens recognized by potentially protective anti-NSG immune sera. A further important consideration is that both NMG and NSG immunization of calves prevented *A. marginale* transmission. Thus, antigen(s) responsible for blocking transmission may be shared between both tissue types used for immunization. These tissues will need to be thoroughly evaluated and compared for antigens recognized by calves immunized with both native tissue homogenates. A limitation with immunoprecipitation is that the amount of antigen isolated from immunoprecipitation can be low or the antibody response to the antigen is limited

resulting in poor yield after immunoprecipitation. This could result in higher false negatives as the protective antigens may unidentifiable due to technical limitations. The affinity of the antibodies to the tick targets is unknown and with a polyclonal response that has different affinities for the target, it is possible that the protein may not be immunoprecipitated from the solution in high enough quantities for identification. The above-described techniques will be appropriate going forward to identify the native antigens associated with the abrogation of tick transmission and acquisition of *A. marginale*.

Mechanism of abrogation of tick transmission of *A. marginale*

The mechanisms responsible for reduction in tick transmission or acquisition described in chapter 4 are unknown. The mechanism may involve antibodies or host immune cells damaging the tick thus killing it or preventing feeding. Electron microscopy of tick MG, SG and reproductive tissues would be useful to understand the damage to the tick after feeding on immunized hosts, if there is any. It is anticipated that damage to tick SG would be seen as previously described in *R. microplus* feeding on DSG-immunized calves (192). A mechanism that may prevent tick acquisition or transmission involves increased inflammation and clearance of tick saliva and pathogens at the tick feeding lesion following immunization. Histopathology of tick feeding lesions on NSG and NMG immunized animals would help to reveal the immune cells at the lesion site as compared to control tick feeding lesions. It is possible that the reduction in tick transmission is a result of reduced tick feeding and salivary secretions. However, an alternative is that immunization with SG induces antibodies to tick proteins that are necessary

for *A. marginale* infection of the host. For example, Dai *et al.* demonstrated that Salp15, an *I. scapularis* protein, coats *Borrelia burgdorferi* and antibodies raised to Salp15 significantly abrogate transmission but do not affect tick feeding (207).

Conclusion

The research described in this dissertation focused on different aspects of the tick-pathogen life cycle. The primary focus of the research discussed in this dissertation was on the transmission of *A. marginale* between cattle and *D. andersoni* ticks. Future objectives include identification of the tick antigens targeted by bovine immune effectors responsible for protection against transmission and reduction of tick acquisition of *A. marginale*. Identification of the *A. marginale* genomic loci associated with tick transmission may assist in providing new targets to intervene with tick acquisition of *A. marginale*. While the research discussed in this dissertation helps to fill the knowledge gaps of tick-borne pathogen transmission, a great deal of work is still necessary before sustainable control of tick-borne disease is fully possible.

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VITA

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