Babesia microti RECOMBINANT DNA VACCINE AS A MODEL FOR *Babesia bovis* PREVENTION

A Thesis

by

JULIETTE E. CARROLL

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2009

Major Subject: Veterinary Parasitology

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ABSTRACT

Babesia microti Recombinant DNA Vaccine as a Model for *Babesia bovis* Prevention.

(December 2009)

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Babesiosis is caused by a genus of tick-transmitted apicomplexan parasites with considerable economic, medical, and veterinary impact. Bovine babesiosis is an important impediment to livestock production throughout the world. Limited options are available for control of this widespread protozoal disease. This study evaluated the protective effect of DNA vaccines incorporating *Babesia* cysteine proteases and Apical Membrane Antigen-1 separately and in combination. The Helios Gene Gun System was used to vaccinate BALB/c mice with plasmid DNA constructs encoding different *B. microti* proteins (pBmCP1, pBmAMA1 or a combination of pBmCP1 and pBmAMA1). An analysis of the parasitemia post-challenge supports the hypothesis that pBmCP1 and pBmAMA1 induce protective effects against the progression of the parasite. However, the combination of the two constructs given simultaneously has no marked effect on parasite progression. Furthermore, the data obtained from the packed cell volumes of the mice indicates that only BmCP1 is able to reduce this effect of clinical disease with any level of significance. *Babesia bovis* constructs containing Cysteine Protease-2 and Apical Membrane Antigen-1 were created and sequence

verified for use in future vaccination studies. The results seen using the mouse model of Babesiosis may provide applicable information for the design of vaccines against other *Babesia* spp., particularly for *B. bovis.*

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1. INTRODUCTION

Members of the genus *Babesia* are tick-vectored intraerythrocytic parasites of the phylum Apicomplexa, order Piroplasmida (Levine, 1971; Allsopp et al., 1994). *Babesia* species infect a wide variety of mammalian hosts including humans. Currently, there are over 100 recognized species of *Babesia* throughout the globe, five of which infect cattle: *Babesia bovis, Babesia bigemina, Babesia divergens, Babesia major* and *Babesia ovata* (Levine, 1985; Krause, 2002; Liu et al., 2008).

The first *Babesia* sp. was described in the late 19th century by Hungarian scientist Victor Babes (Reviewed by Hunfeld et al., 2008). Babes discovered microorganisms in the erythrocytes of Romanian cattle suffering from hemoglobinuria, this pathogen would later be named *Babesia bovis*. Following its initial discovery, Smith and Kilborne were able to identify ticks as the source of infection for *Babesia* spp. This was a significant advance in both veterinary and human medicine, as it was the first time an arthropod was identified as the vector for disease (Smith and Kilborne, 1893).

Toward the end of the nineteenth century ranchers throughout the world began to experience devastating losses among their cattle. Cattle throughout the Americas, Australia, and Africa were becoming infected by what was discovered to be bovine babesiosis, commonly referred to as “Tick Fever” (Bock et al., 2004).

This thesis follows the style of Veterinary Parasitology.

Babesia bovis and *Babesia bigemina* became the most commonly recognized disease agents of bovine babesiosis in cattle located in tropical and subtropical regions of the world (Brown and Palmer, 1999).

Babesia bovis and *B. bigemina* share similar biological characteristics - both are transmitted by the same tick vector and infect the erythrocytes of cattle. However, the clinical disease caused by these two parasites is remarkably different (Wright et al., 1988; Criado-Fornelia et al., 2004). Generally, *B. bovis* is considered to be the more pathogenic of the two organisms, resulting in higher mortality rates among susceptible cattle (Brown and Palmer, 1999; Wright et al., 1988; Callow, 1984). Both forms of babesiosis are characterized by high fever, anemia, ataxia, anorexia, haemoglobinurea and hypotensive shock. In contrast to *B. bigemina* infections, *B. bovis* infections are commonly associated with severe central nervous system (CNS) signs. The damage done to the CNS is linked to sequestration of infected erythrocytes in the capillary bed of neural endothelial cells (Wright et al., 1988; O'Connor et al., 1999; Brown et al., 2006a). This rare phenomenon also makes *B. bovis* infections difficult to detect because of the low numbers of parasites circulating in peripheral blood (Fahrimal et al., 1992).

Accumulation and adherence of parasitized erythrocytes to organ vasculature is notably the most important aspect of *B. bovis* infections, however, the severe virulence of *B. bovis* has also been attributed to the overproduction of the pro-inflammatory cytokines associated with protective immunity resulting in a partially immune-mediated response (Brown et al., 2006b). Together these two factors make *B. bovis* an

economically devastating parasite with the potential to cause significant production losses among cattle producers.

In North America parasitism by cattle fever ticks is associated with two species of the family Ixodidae, *Boophilus annulatus* and *Boophilus microplus* (Bock et al., 2004; Estrada-Peña and Venzal, 2006). It should be noted that a recent change has been made with regards to the naming of these two species of ticks, transferring five species in the genus *Boophilus* into the genus *Rhipicephalus* (Horak et al., 2003). To avoid confusion this paper will retain the name *Boophilus* as a subgenus of *Rhipicephalus* (Murell and Barker, 2003).

Populations of *Boophilus* ticks were linked to a widespread occurrence of bovine babesiosis throughout the southern United States shortly after Smith and Kilborne published their detailed findings that identified ticks as the vector of this devastating pathogen. Epidemiologists believe these ticks were introduced into North America on cattle and horses transported to the New World by Spanish colonialists (George et al., 2002).

1.1 Life cycle and development of *Babesia bovis* in the tick vector

Babesia bovis parasites are transmitted by larval ticks of the subgenus *Boophilus* (Mahoney and Mirre, 1979). Adult female *Boophilus* ticks acquire *B. bovis* while feeding on the erythrocytes of infected cattle. After the tick has fed to repletion, *Babesia* gametocyte development occurs in the tick gut. Gametogenesis leads to zygote formation followed by progression into the kinete stage (Mackenstedt et al., 1995). The

motile kinete enters the hemolymph via penetration of the intestinal epithelium and begins to invade the tissues of the female tick, the most notable tissue of invasion being the ovaries (Howell et al., 2007). From the ovaries, kinetes are transmitted to the tick offspring through the egg. Because *Boophilus* ticks are one-host ticks, meaning larval, nymphal, and adult development occur on the same host animal, transovarial transmission ensures that the parasite will be spread to the next tick generation (Mahoney and Mirre 1979; Howell et al., 2007).

After an adult female tick has mated and taken a blood meal she drops off the host and deposits infected eggs in the environment. Larvae hatch from the eggs approximately 21 days after ovipositing and have the ability to survive for long periods of time without feeding (Needham and Teel, 1991; Corson et al., 2004). In larvae *Babesia* sporozoites migrate to and develop within the salivary glands (Mehlhorn and Schein, 1984; Sauer et al., 1995). Eventually larvae attach to the bovine host then development continues to the adult stage. *Babesia bovis* sporozoites are only transmitted to cattle by larval ticks; the tick cannot retain the infection following its first molt.

Tick saliva contains various anticoagulatory, anti-inflammatory and immunosuppressive components that increase the efficiency of sporozoite transmission to the host (Bowman et al., 1997). Infective sporozoites penetrate the host erythrocytes where they become trophozoites divide into merozoites. Merozoites exit the infected erythrocyte, enter the bloodstream and invade new erythrocytes in which they undergo asexual division (Bock et al., 2004).

The merozoite stage has characteristic features not found in trophozoites. These features include anterior secretory organelles known as rhoptries that are critical for cell invasion and an actomyosin motor driven inner membrane complex that is thought to propel the parasite into new cells (Lew et al., 2002; Yokoyama et al., 2002). Apicomplexans utilize rhoptry proteins secreted from organelles located at the apical end of merozoites for host cell invasion. Extracellular merozoites attach to the host erythrocyte, reorient to bring the apical organelles close to the attachment interface, and discharge rhoptry proteins onto the cell membrane (Yokoyama et al., 2002).

Erythrocyte invasion is accomplished by a novel form of locomotion called gliding motility. Although they do not have cilia or flagella, *Babesia* merozoites are motile. They have the ability to glide on solid substrates, such as the host cell surfaces (Menard, 2001). Gliding motility is thought to be driven by an actomyosin-based system associated with the inner membrane complex found beneath the parasite plasma membrane (Lew et al., 2002; Zhou et al., 2006). This theory is supported by studies that show the inability of *Toxoplasma* and *Plasmodium* parasites to glide and invade host cells in the presence of actomyosin inhibitors (Dobrowolski and Sibley, 1996; Pinder, 1998). Lew and others (2002) inhibited *B. bovis* parasite invasion in vitro using myosin and actin-binding drugs. This was the first reported case of actomyosin-based participation in erythrocyte invasion by *B. bovis* parasites.

1.2 Tick eradication efforts

One of most economically destructive outbreaks of bovine babesiosis in the United States occurred in 1868 when apparently healthy cattle from Texas were relocated to stockyards in northern states. As a result, 15,000 head of native cattle from Illinois and Indiana were said to have contracted *Babesia* and died from the subsequent infections (Dolman, 1969; Pelzel, 2005).

Despite being heavily infested with ticks, Texas cattle often remained clinically normal. It was later discovered that cattle driven from Texas had previously acquired immunity to the disease due to frequent vector exposure as calves. Calves less than 6 months of age possess a strong resistance against *B. bovis* diesase (Goff et al., 2001; Brown et al., 2006a). Exposure to the parasite during this period induces a long-lasting carrier state and concomitant immunity that prevents disease typically seen in susceptible adults upon initial infection with *B. bovis*. Cattle located in the northern states had not been previously exposed to the parasite as calves, making them highly susceptible to disease. The United States Congress estimated that the direct and indirect economic loss associated with bovine babesiosis during this period to be $130.5 million (approximately $3 billion today) (Dolman, 1969; APHIS, 2002).

Soon after this outbreak it was discovered that as infested Texas cattle advanced north, female *Boophilus* ticks would detach and distribute *Babesia* infected eggs (Bram and Gray 1983; APHIS, 2002; Pelzel, 2005). This prompted northern stockmen and ranchers to ban Texas cattle from export into their states. The U.S. Secretary of Agriculture placed strict quarantines on Texas cattle during summer months to prevent

the spread of the vector. Cattlemen quickly felt the crippling financial effects of the quarantines and turned to the Texas legislature. In 1893, the Livestock Sanitary Commission (now the Texas Animal Health Commission) was established to prevent the spread of tick fever.

In 1906, as part of the Cattle Fever Tick Eradication Program (CFTEP), state-federal quarantine and eradication programs were established to eliminate cattle fever ticks from the United States. Laws were instated that required cattle to be systematically immersed in acaricide solution in concrete dipping vats. Cattle in endemic areas were dipped before being transported to any tick-free zone, especially northern markets (Graham and Hourrigan., 1977; Pelzel, 2005).

By 1943 the two species of fever ticks, *Bo*. *annulatus* and *Bo*. *microplus*, were successfully eradicated from the United States, with the exception of a permanent quarantine zone between Texas and Mexico. Currently, tick riders employed by the U.S. Department of Agriculture's (USDA) Animal and Plant Health Inspection Service (APHIS) are responsible for maintaining the permanent quarantine zone, which extends 852-square miles from Del Rio, TX to Brownsville, TX and encompasses eight South Texas counties (Hillman, 2008a). The goal of an APHIS tick inspector is to prevent the introduction of new ticks from Mexico (APHIS, 2002; Hillman, 2008a). This is accomplished by horseback patrol and systematic quarantines along the buffer zone.

Tick inspectors capture stray Mexican cattle that have entered the country illegally or native cattle that may have crossed into Mexico and returned. Surveillance and control rely on a process called “scratching” for ticks. This requires the official to

inspect the skin of the animal with his fingers, feeling for ticks (Bram and Grey, 1983; Pelzel, 2005). If cattle are found to be infested with *Boophilus* ticks they are quarantined and dipped in 0.3% - 0.25% Coumaphos, an organophosphate acaricide, every 10 to 14 days (EPA, 1996, Miller et al., 2005). Once cattle are re-inspected and declared tick-free they may be moved out of quarantine. The Texas Animal Health Commission complements the USDA control efforts through tick inspection of livestock outside the quarantine zone and trace-out work at Texas livestock markets.

Recent literature indicates that *Boophilus* tick populations in Mexico have developed resistance to organophosphate acaricides, the only approved chemicals for use in the cattle dipping vats in the U.S. (Jamroz et al., 2000; Li et al., 2003). Rodriguez-Vivas and others (2006) reported that 83.7% of *Bo. microplus* ticks obtained from cattle in Yucatan, Mexico were confirmed resistant to organophosphates as well as other classes of acaricides. Evidence obtained by the USDA indicates that the resistant strains of ticks from Mexico are spreading into southern Texas and jeopardizing the tick eradication program (Jamroz, 2000; Miller et al., 2005; Davey, 2006).

In August 2004, ticks obtained from cattle in Starr County, TX were examined for resistance to several types of acaricides including Coumaphos using the Larval Packet DD Test (FAO, 1971). Engorged females were collected from native cattle and held in growth chambers until they produced eggs. Once egg production was complete, larvae were allowed to develop. On day 14 of larval development a discriminating dose test was performed with various concentrations of coumaphos, permethrin and amitraz. Resistance was detected by exposing larvae to an acaricide concentration expected to kill

99% of susceptible ticks, then increasing the dose to an amount twice that expected to kill 99% of susceptible ticks.

The results from these bioassays revealed that the acaricide resistance levels of ticks collected from Texas cattle were similar to those found in ticks from Mexican cattle (Miller et al., 2005). This is the first published report of organophosphate-resistant ticks in the U.S. (George et al., 2002, Davey et al, 2006).

According to the 2007 Veterinary, Medical and Urban Entomology Annual Report, an additional 1000 square miles extending over five Texas counties has been placed under temporary quarantine due to the presence of *Boophilus* ticks (Everett, 2008). Re-establishment of the tick from Mexico appears to be related to the movement of white-tailed deer and exotic ungulates along the Texas-Mexico border (George, 1989; Bram et al., 2002). *Boophilus microplus* in all life stages have been recovered from wild deer, demonstrating that deer may serve as an alternative host for these ticks (Kistner and Hayes, 1970). Treatment options for wildlife are limited to a bait system, which applies a pyrethroid to the animal, and ivermectin treated corn, which must be discontinued at least 60 days prior to hunting season (Hillman, 2008b). However, acarcide resistance may limit the effectiveness of these treatment options.

The estimated cost of eliminating new infestations along the recently expanded quarantine region is $13 million (Everett, 2008). In March 2008, the U.S. Department of Agriculture's Animal and Plant Health Inspection Service awarded the Cattle Fever Tick Eradication Program a $5.2 million dollar grant to be spent on the control of cattle fever tick outbreaks in the U.S. Although this amount is significantly less than requested it

will provide supplementary surveillance, training and treatments to postpone re-infestation for the time being (Everett, 2008).

Should bovine babesiosis re-emerge in the United States, the effects on the cattle industry will be that of a severe economic impact. Texas is the number one cattle producing state in the country, with an inventory of 13.8 million cattle and calves (NASS, 2007). For Texas cattle alone, direct and indirect losses associated with *B. bovis* have been estimated at $1.5 billion within the first year of re-emergence (Teel and Wagner, 2001).

At this time the only effective prevention of bovine babesiosis in the United States is the use of intensive tick control measures such as quarantine zones and scrupulous surveillance. These methods are extremely costly and labor intensive. Due to the development of acaricide-resistant tick populations and the increased number of wildlife carrying these ticks into tick-free areas, it is only a matter of time before alternative babesiosis prevention strategies must be employed.

1.3 Host immune response

Young cattle possess a unique spleen-dependent innate immunity to babesiosis that lasts until approximately 6 months of age. Animals infected within this timeframe exhibit a persistent yet milder form of the infection (Trueman and Blight, 1978; Mahoney et al., 1979). Contrarily, aged cattle develop severe clinical disease signs upon initial exposure to *Babesia* (Mahoney and Ross, 1972; Mahoney et al., 1973; Goff, et al.,

2001). The phenomenon of age-related immunity allows cattle infected as calves to transmit the parasite throughout their lifetime without the ill effects of the disease.

Originally the age-related innate immunity observed in older calves was attributed to the presence of protective antibodies transferred through colostrum of immune mothers (Mahoney 1967). Contrarily, Riek (1963) and Christensson (1987) reported the same form of immunity in calves from unexposed dams and in calves that did not receive colostrum at birth. It has been hypothesized that age-related immunity to *B. bovis* involves a soluble babesiacidal factor (Levy et al., 1982).

Adler and others (1995) identified nitric oxide (NO) as the first babesiacidal molecule to contribute to arrested growth of the parasite. Gale et al. (1998) was able to significantly reduce the mean parasitemia of *B. bovis* infected cattle by treating them with aminoguanidine, an inhibitor of the inducible form of nitric oxide synthase (iNOS). Goff et al. (2001) showed that the occurrence of age-related non-specific immunity in calves was achieved by the rapid induction of splenic interleukin-12 (IL-12) and interferon-γ (IFN-γ) followed by iNOS mRNA expression in the spleen. In the same study involving adult cattle, IL-12 and IFN-γ occurred later and no iNOS could be recovered. This suggests different mechanisms for age-related innate immunity in calves versus the acquired (contaminant) immunity seen in older cattle (Brown et al., 2006a).

Animals that have recovered from an acute *B. bovis* infection, either naturally or after treatment, are often referred to as "immunologically primed" (Brown and Palmer, 1999). These animals remain persistently infected and develop a protective immunity that prevents the onset of clinical signs upon re-exposure to the parasite. Because the

parasite involves an extracellular and intracellular stage, the mechanisms required to maintain immunity against *B. bovis* infections are thought to rely on both cell-mediated responses and humoral antibody responses (Brown and Palmer, 1999; Homer et al., 2000).

Studies involving persistently infected, immune cattle suggested that merozoite antigens produced by the parasite induce a strong IFN-γ response from memory T helper type 1 cells (Th1), providing prolonged immunity (Brown and Palmer, 1999). Macrophages activated in presence of the parasite produce cytokines such as IFN-α, IL-12 and IL-18 that promotes IFN-γ production by $CD4^+$ Th1 cells (Brown and Palmer, 1999; Shoda et al., 2001). The role of IFN-γ is two-fold, it is required to further stimulate production of babesiacidal molecules by macrophages and to enhance the opsonizing of immunoglobulin G (IgG) antibodies (Estes and Brown, 2002). During the re-introduction of the parasite to the blood of an immune animal, immunoglobulin G (IgG) antibodies block erythrocyte invasion of the parasite by binding and neutralizing freely circulating sporozoites before they can reach host erythrocytes (Homer et al., 2000).

In vitro studies using lymphocytes from *Babesia*-immune cattle demonstrate that bovine $CD4^+$ T cells produce IFN-γ in response to parasite antigen stimulation and that the development of acquired immunity against *B. bovis* in cattle is related to this specific cell-mediated response (Brown et al., 1993). Murine models of *Babesia microti* confirm the theory that $CD4^+$ T lymphocytes rather than $CD8^+$ T lymphocytes play an essential role in the elimination of the infections in mice.

As with *B. bovis*, acquired immunity to *B. microti* is thought to involve the activation of memory and effector $CD4^+$ T cells that secrete IFN-γ and assist with the production of protective antibodies (Igarashi et al., 1994). Mice with depleted $CD4^+$ T cell levels are more susceptible to infection with *B. microti* compared to mice with adequate $CD4^+$ T cell levels (Shimada et al., 1996; Igarashi et al., 1999). Similarly, in the closely related protozoan *Plasmodium,* Stephens and Langhorne (2007) demonstrated that $CD4^+$ T cells were essential for parasite clearance in malaria.

1.4 Vaccination strategies

An alternative approach to strategic tick control is becoming increasingly important with regards to *B. bovis* infections. Sole reliance on one form of prevention is unrealistic, while integrating the use of acaricides with an appropriate vaccine will prove to be a better choice for the control bovine babesiosis (de Waal and Combrink, 2006).

The first *Babesia* vaccine was developed through the use of a live attenuated strain of *B. bovis* (Callow and Mellors, 1966; Callow and Tammemacli, 1967; Callow, 1979). Blood of carrier animals was passaged into splenectomized calves resulting in reduced virulence of the parasite. Although this type of vaccine has been successfully used to control babesiosis in many parts of the world, it induces a carrier state, allowing the animal to serve as a reservoir for transmission. The live annulated vaccine has a number of other deficiencies including, a short shelf life of 5-7 days at 5ºC, potential for reversion to virulence, and the possibility of co-transmission of other infectious agents such as viruses and haemoprotozoan parasites (Wright and Riddles, 1989). Vaccines

derived from killed *B. bovis* strains have also been employed (Mahoney et al., 1981) however, they were unsuccessful because of the unsatisfactory level of immunity produced (Timms et al., 1983).

Passive transfer studies show that antibodies play an important role in the prevention of bovine babesiosis (Mahoney, 1967). Antibodies bind to critical epitopes found on the surface of the merozoites and block the processes required for erythrocyte invasion and/or parasite replication (Heins et al., 1995, Brown et al., 2006b). Knowledge of this type of antibody production has driven vaccine development towards the use of functionally relevant merozoite antigens (Heins et al., 1995).

To overcome the difficulties seen with the production of live *Babesia* vaccines a variety of soluble parasite exoantigens has been isolated from *B. bovis* as potential vaccine candidates (Sibinovic et al., 1967; Goodger et al., 1987; Montenegro-James et al., 1987). Exoantigens are shed by the parasite during merozoite invasion of erythrocytes and can be obtained through the blood or plasma of *Babesia* infected animals or the supernatant of in vitro *Babesia* cultures (James, 1989). The use of parasite exoantigens appears to induce an immune response that decreases clinical disease manifestations through the rapid development of protective antibodies (Schetters et al., 2001). Early use of single strain exoantigen vaccines produced protection against the antigen-derived strain but failed to provide protection against different strains upon challenge (Wright et al., 1983; Goodger et al., 1987; Schetters et al., 1995).

Schetters et al. (2001) obtained promising vaccine results against canine babesiosis by using exoantigens obtained from a combination of culture-derived *Babesia*

canis canis and *Babesia canis rossi*. This study concluded that dogs immunized with a bivalent vaccine had an effective antibody response and reduced clinical signs upon challenge from both forms (Schetters et al., 2001).

Although studies indicate that exoantigens do confer some protection against *Babesia,* a specific individual exoantigen has yet to be isolated. Additionally, soluble parasite exoantigens require the use of cultured parasites or infected animals, making them difficult to obtain in large quantities. These drawbacks have resulted in a shift towards an alternate type of subunit vaccine. The use of recombinant proteins from cloned DNA has come to the forefront of *Babesia* vaccine research because of their stability and high reproducibility.

Proteins produced from micronemes, rhoptries, and dense granules at the apical end of the parasites are thought to play a major role in invasion of erythorocytes and establishment of infection by apicomplexan parasites (Dubremetz et al., 1998). Recognition of the host cell is initiated by receptor-ligand interactions, while entry into the host erythrocyte is initiated by contact between the apex of the parasite and the surface of the cell (Yokoyama et al., 2006). After contact with the red blood cells, the parasite will orient itself to allow the apical membrane to enter the cell first. A tight junction is then formed, permitting the parasite to invade the red blood cell and asexually reproduce. After division, the parasite exits the erythrocyte and travels through the blood where it can enter a new cell and undergo another round of division (Dubremetz et al., 1998).

Protective immunity against babesiosis may be directed against one or more surface antigens associated with the merozoite. Antibodies in sera obtained from cattle recovered from *B. bovis* and *B. bigemina* infections have been used to identify numerous surface-labelled merozoite proteins (McElwain et al., 1988; Suarez et al., 1991; Jasmer et al., 1992; Wilkowsky et al., 2003). In *B. bovis*, the most well-documented surface proteins are those which belong to the family of variable merozoite surface antigens (VMSA) including 60-kDa rhoptry-associated protein 1 (RAP-1), 42-kDa merozoite surface antigen-1 (MSA-1), and 44-kDa MSA-2c. RAP-1 antigen, a member of a polymorphic gene family, have undoubtedly been the most intensely studied of all the VMSAs.

Conservation of B-cell and T-cell epitopes among various *B. bovis* strains initiated interest in RAP-1 as vaccine candidate (Brown et al., 1996). *Babesia bovis* RAP-1 is encoded by two identical RAP-1 genes and possesses a sequence homologous to that of RAP-1 found in other *Babesia* spp. (Suarez et al., 1998; Dalrymple et al., 1993). Using a lysate of merozoites, Yokoyama and others (2002) showed binding of RAP-1 to bovine erythrocytes in an erythrocyte-binding assay. In addition to the proof of RAP-1 binding to the erythrocyte in that assay, anti-RAP-1 antibodies were successful in hindering the binding of the parasite to the host erythrocyte in vitro. However, Norimine et al. (2003) found no evidence of protective immunity using a RAP-1 recombinant vaccine in cattle, even though the vaccine initiated strong cell-mediated and humoral responses against RAP-1.

MSA-1 showed promise as a vaccine because of its ability to neutralize merozoite infectivity in vitro, suggesting its importance in merozoite invasion (Hines et al., 1992). As seen with RAP-1, recombinant MSA-1 immunization also failed to confer protection against challenge with virulent *B. bovis* (Hines et al., 1995). MSA-2c has been identified as being highly conserved among different *B. bovis* strains and serves as a possible vaccine candidate. Like MSA-1, bovine antibodies to recombinant MSA-2c neutralized the invasion of erythrocytes by *B. bovis* (Wilkowsky et al., 2003). In vivo vaccine trials have yet to be completed.

1.5 Apical Membrane Antigen – 1 as a vaccine target

Because of the availability of genomic sequences of *B. bovis,* recombinat vaccine candidates can be identified based on their genetic identity with homologous proteins of other apicomplexan parasites (Brayton et al., 2007). Apical Membrane Antigen-1 (AMA-1) is a highly conserved micronemal surface protein found among members of the phylum Apicomplexa, including *B. bovis*, *Toxoplasma gondii* and all species of *Plasmodium* examined to date (Waters et al., 1990; Donahue et al., 2000; Gaffar et al., 2004). In *Plasmodium*, AMA-1 is stored in the microneme organelles and expressed during merozoite formation in maturing schizonts where it is transported to the rhoptries prior to erythrocyte invasion. While the specific function of AMA-1 is not yet known, it is thought to be directly responsible for reorientation of the apical end or junctional contact of the parasite with the host cell, which is dependent on binding proteins for completion of the parasite (Kocken et al., 2000; Gaffar et al., 2004; Mitchell et al.,

2004). Because of its pivotal role in erythrocyte invasion, AMA-1 is a leading malaria vaccine candidate (Girard, et al., 2007; Nair, 2002).

While the overall amino acid similarity between *Plasmodium* AMA-1 and *B. bovis* AMA-1 is low (18%), characteristic features such as an N-terminal ectoplasmic region, a cysteine-rich ectodomain, a single transmembrane domain, and a C-terminal cytoplasmic tail are found in both proteins (Chesne-Seck et al., 2005). The important structural similarities between *B. bovis* AMA-1 and *Plasmodium* AMA-1 suggest that much of the research done with *Plasmodium* may be applied to *B. bovis* (Gaffar et al., 2004; Chesne-Seck et al., 2005).

Mice and non-human primates immunized with the ectodomain of *Plasmodium* AMA-1 are reported to have an increased protective immunity against challenge from blood-stage parasites (Anders et al., 1998; Hodder et al., 2001; Stowers et al., 2002). Evidence of this AMA-1 initiated immune response suggests that the protein could be a successful candidate for inclusion in a *Babesia* vaccine. Gaffer et al. (2004) identified *B. bovis* AMA-1 as a low-abundance 82-kDa protein that is synthesized into a 69-kDa protein upon invasion of erythrocytes in vitro. Additionally, antiserum made against *B. bovis* AMA-1 prevented parasite invasion of host erythrocytes in vitro.

As in the case with soluble parasite exoantigens, considerable antigenic polymorphism could limit the effectiveness of a recombinant AMA-1 vaccine when challenged under field conditions. Immunization with the highly polymorphic *Plasmodium falciparum* AMA-1 induces antibodies that block the growth of the homologous parasite but are less efficient in blocking the growth of heterologous

P. falciparum (Kennedy, 2002; Duan et al., 2008). To date, no variation has been shown in AMA-1 within *Babesia* spp. Studies in our lab comparing AMA-1 from four different *B. microti* strains have shown no difference among the amino acid sequences (unpublished results).

1.6 Cysteine proteases as a vaccine target

Cysteine proteases are known for their importance in propagation and proliferation of protozoan parasites (McKerrow, 1993; Holman et al., 2002; Sajid and McKerrow, 2002). Meirelles et al. (1992) demonstrated that cysteine protease-specific inhibitors prevent host cell invasion and arrest intracellular development of *Trypanosoma* sp. in vitro. Engel et al. (1998) went on to report the first successful treatment of Chagas' disease in an animal model using inhibitors designed to inactivate cruzain, the major cysteine protease of *T. cruzi*, the causative agent of Chagas' disease.

Plasmodium falciparum contains several cysteine proteases with various functions including hydrolysis of hemoglobin, erythrocyte rupture and erythrocyte invasion (Rosenthal, 2004). Hemoglobin digestion is the best characterized function of *Plasmodium* cysteine proteases and is thought to provide amino acids for parasite protein synthesis, allow space in the cell for the growing parasite and maintain the osmotic stability of parasites (McKerrow, 1993; Lew et al., 2003). Incubating cultured malaria parasites with cysteine protease inhibitors blocked the ability of the parasite to process hemoglobin therefore reducing its proliferation (Rosenthal, 1995).

In order to complete the erythrocytic cycle of *Plasmodium,* the parasite must rupture the infected erythrocyte and release merozoites. *Plasmodium* cysteine protease inhibitors inhibit the rupture of the erythrocytes preventing the continuation of the asexual cycle (Wickham et al., 2003). Additionally, Greenbaum et al. (2002) demonstrated the role of cysteine proteases in *Plasmodiu*m erythrocyte invasion by using inhibitors to block invasion of erythrocytes by merozoites.

Holman et al. (2002) identified the gene *cys-1,* which codes for a cathepsin L-like cysteine protease found in *Babesia equi*. In that study membrane permeable cysteine protease inhibitor E64d was shown to inhibit parasite propagation of *B. equi* in vitro, suggesting a target for vaccine development. Similar results were reported by Okubo et al. (2007) in which E64d significantly inhibited erythrocyte invasion activity of *B. bovis* in vitro. Although the exact role of cysteine protease in *Babesia* infections is not clear, data strongly suggest it plays an important role in erythrocyte invasion and/or replication of *Babesia.*

1.7 Study design

Because of the critical role AMA-1 plays in host cell invasion and the effect of cysteine protease on parasite proliferation, they have been selected as target genes for inclusion in a recombinant vaccine against *B. bovis* infection. Our first objective was to generate and verify plasmid constructs containing *B. bovis* AMA-1 and cysteine protease genes. Once it was established that these constructs could be obtained in vitro, a vaccine

utilizing *Babesia microti* AMA-1 and *B. microti* cysteine protease-1 (CP-1) constructs was created to test the efficacy of these targets as a model for the *B. bovis* vaccine.

The main focus of current malaria vaccines is the use of DNA technology. Multivalent DNA vaccines incorporate the cell-mediated and humoral roles of the immune system, both of which are required for optimal protective effects. Genes coding for different antigenic regions of malaria proteins have been successful in invoking strong immune responses against the parasite (Jones et al, 2001; Ivory and Chadee, 2004; Wang et al., 2004; Coban et al., 2004). Antigenic variation and the complex nature of the parasite life cycle allow for varied responses to natural infection. The use of a multivalent vaccine consisting of two antigens presents a greater number of protective epitopes and a stronger cell-mediated response. Studies using a multivalent vaccine against leishmaniasis proved that the combination of three antigens is more effective in stimulating $CD4^+$ Th1 cells and reducing parasite numbers than that of a single antigen (Mendez et al., 2001; Rafati et al., 2001). By targeting different parasite systems it is anticipated that we will be able to prevent invasion and proliferation of the parasite in the host, inhibiting the onset of clinical signs.

For this study, the eukaryotic expression vector, pCMV (Gene Therapy Systems, CA), will be used to generate recombinant plasmid DNA constructs. This vector has been modified to incorporate a CD5 secretory signal and a polypeptide protein tag (FLAG-tag) to the genes of interest, and the resultant vector is designated pCMVCD5flag (McIlhinney, 2004; Edwards and Aruffo, 1993). Addition of a FLAG-

tag allows fusion proteins to be purified by affinity chromatography and for evaluating protein expression using anti-Flag monoclonal antibodies.

Plasmid DNA is an ideal vaccine vector because it is internalized by antigen presenting cells and can induce antigen presentation by major histocompatibility complex class II for T-cell response. The unmethylated CpG dinucleotide motifs found in the plasmid DNA backbone readily induce Th1 cytokine expression and increase the antigen-specific production of IFN-γ. This increased IFN-γ production enhances the development of cell-mediated responses against the immunizing antigens (Klinman et al., 1999). The pCMVCD5flag vector contains cytomegalovirus (CMV) promoter that drives gene expression and is widely used for high-level protein expression in mammalian cells (Boshart et al., 1985; Foecking and Hofstetter, 1986; Nott et al., 2003).

The route of pDNA delivery plays an important role in the immunogenicity and efficacy of the vaccine. The skin is an ideal target for DNA immunization because of the large numbers of resident cutaneous antigen presenting cells, such as Langerhans cells and dermal dendritic cells. Intradermal gene vaccination induces antigen specific Th1 cells secreting high levels of IFN-γ and stimulating the production of IgG2a isotype antibody (Grunathan and Klinman, 2000). The use of a biolistic device (e.g. a gene gun) to propel plasmid DNA-coated gold particles into the epidermis has been reported to be more effective at eliciting a protective immune response than intramuscular or intraperitoneal delivery methods (Yoshida et al., 2000; Mohamed et al., 2003). Upon challenge, a single gene gun vaccination using 2 μg of *Toxoplasma gondii* HSP70 gene resulted in a significant reduction in the number of *T. gondii* organisms compared with

50 μg of *T. gondii* HSP70 given by intramuscular or intraperitoneal injection (Mohamed et al., 2003). Biolistic delivery of DNA to the skin using the gene gun resulted in the direct transfection of dendritic cells and subsequent migration to draining lymph nodes (Akbari et al., 1999; Larregina and Falo, 2000; Larregina et al., 2001).

The vaccine designed in this study will utilize biolistic delivery of pDNA encoding *B. microti* AMA-1 and CP-1 via the Helios Gene Gun System (Bio-Rad). Gold particles will be coated with plasmid DNA, and the coated particles will then be accelerated into the epidermis of BALB/c mice using a compressed helium driven gene gun.

Anesthetized mice will receive a series of 3 injections at 2-week intervals. Each injection will contain 2 μg of pDNA encoding for AMA-1 and/or CP-1, or control plasmid DNA. It is anticipated that the AMA-1 and CP-1 protein will be expressed by antigen presenting cells, stimulating a strong $CD4^+$ IFN- γ effector/memory T-cell response directed towards the inhibition of AMA-1 and CP-1 when challenged by infection with *B. microti* parasites. By targeting AMA-1 and CP-1, we hope to prevent parasite invasion of the host erythrocytes and arrest parasite proliferation. By disrupting the parasite, we can reduce parasitemia levels. If successful, this study will serve as a proof of concept for *B. bovis* vaccination and open the door to a new vaccine against bovine babesiosis.

1.8 Hypothesis

Vaccination with *Babesia* derived Apical Membrane Antigen-1 and Cysteine Protease-1 will elicit a protective immune response against challenge infection. The null hypothesis being that there is no significant reduction of parsitemia between the vaccinated mice and the control mice.

1.9 Objectives

The objectives for this study are to:

1. Generate and sequence verify plasmid constructs containing *B. bovis* AMA-1 ectodomain and full-length cysteine protease.

2. Generate and sequence verify plasmid constructs expressing *B. microti* Apical Membrane Antigen-1 and Cysteine Protease-1 to be used as vaccine in a mouse model.

3. Validate the use of Apical Membrane Antigen-1 and Cysteine Proteases as vaccine candidates for *B. bovis* using *B. microti* constructs in a mouse model.

2. MATERIALS AND METHODS

2.1 DNA extraction from *Babesia bovis*

DNA was obtained from a cryo-preserved Mexican-isolate of in vitro cultivated *Babesia bovis* using the FlexiGene DNA Extraction method (Qiagen). The DNA concentration was adjusted to 100 ng/ul based on quantification using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies).

2.2 Amplification of full-length *Babesia bovis* Cysteine Protease-2

Oligonucleotide primers for polymerase chain reaction (PCR) were designed based on the *B. bovis* (T2Bo Strain) cysteine protease-2 gene sequence (GenBank accession no. XP_001610695). The primers used for amplification of the full gene (1335 bp) were CP B.bov Forward (5′-ATG GGA ATA CCG GCT GCT GC-3′) and CP B.bov Reverse (5′-TTA ATA TGG GAC ATA ACC GTA AGA AAG AAC GC-3′) with an optimal annealing temperature of 63ºC (the table on p.31). To avoid nucleotide misincorporations during by PCR amplification, Phusion High-Fidelity DNA Polymerase was used (Finnzymes). The amplification profile for the PCR reaction was initial denaturation at 98ºC for 30 s, followed by 30 cycles of denaturation at 98ºC for 10 s, annealing at 63ºC for 10 s and extension at 72ºC for 2 min with a final extension at 72ºC for 10 min followed by hold at 4ºC (Hybaid PCR Express Thermocycler). The product was checked by electrophoresis on an ethidium bromide stained 1% agarose gel alongside a 100 bp DNA marker (Invitrogen).

Amplification with Phusion High-Fidelity DNA Polymerase does not result in the 3′ adenine overhangs required to ligate into the pCR-TOPO2.1 vector (Invitrogen); therefore, incubation of the PCR product with 2X Taq PCR Master Mix was required for addition of 3′ adenine overhangs. The Phusion High-Fidelity PCR amplicon was purified using the QIAquick PCR Purification method (Qiagen). The purified DNA was quantified on the NanoDrop ND-1000 Spectrophotometer at a concentration of 106 ng/µl. The purified PCR product (10 µl) was incubated in 10 µl of 2X Taq PCR Master Mix (Qiagen) at 72ºC for 10 min.

2.3 Cloning *Babesia bovis* Cysteine Protease-2 into pCR-TOPO2.1 and Champion pET 101 Directional TOPO vectors

A cloning reaction was prepared using 2 µl (53 ng/µl) of purified *B. bovis* cysteine protease PCR amplicon, 1 µl of salt solution, 2.0 µl of sterile water and 1 µl of pCR-TOPO2.1 vector according to the manufacturer's instructions (Invitrogen). The reaction was gently mixed and allowed to incubate at room temperature for 30 min. Following the initial incubation, 2 µl of the cloning reaction was added to One Shot TOP10 Competent *Escherichia coli* cells (Invitrogen) and incubated on ice for 30 min. Following the 30 min incubation on ice, the cells were heat-shocked at 42ºC for 30 s and supplemented with 250 µl of S.O.C medium (0.5% Yeast extract, 2.0% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 20 mM MgSO4, 20 mM glucose). The transformation mixture was shaken horizontally (200 rpm) at 37ºC for 1 h (Queue Orbital Shaker, Queue Systems, Inc) and spread onto pre-warmed LB agar plates (Luria

Bertoni Agar, Sigma-Aldrich) containing Kanamycin (final concentration 50 µg/ml)(Sigma-Aldrich) and X-Gal (5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside, 40 mg/ml, Fisher Scientific). The plates were then incubated at 37ºC overnight.

Transformants were analyzed by colony PCR to check for proper *B. bovis* cysteine protease insert. A master mix was made consisting of 300 µl of 2X Taq PCR Master Mix, 276 µl of sterile water, 12 µl of 1 pmol M13 Forward (-20) (5′-GTA AAA GCG CGG CCA G-3′) and 12 µl of 1 pmol M13 Reverse (5′-CAG GAA ACA GCT ATG AC-3′). A portion of each transformant colony was added to 20 µl of master mix in a 0.2 ml PCR tube. One tube containing 20 µl of master mix served as a negative control. The cycling program used was initial denaturation at 94ºC for 5 min, followed by 30 cycles of denaturation at 94ºC for 10 s, annealing at 50ºC for 1 min and extension at 72ºC for 2 min. A final extension at 72ºC for 10 min was followed by a 4ºC hold. The product was checked by electrophoresis on an ethidium bromide stained, 1% agarose gel alongside a 100 bp DNA Marker. Positive clones containing the desired *B. bovis* cysteine protease insert were expanded into 10 ml LB broth cultures containing Kanamycin and grown for 16 h with shaking (200 rpm).

The broth culture was centrifuged (1800 rpm for 20 min) to pellet the bacteria, and the supernatant discarded. *Babesia bovis* cysteine protease pDNA was column-purified from the pellet using the QIAprep Spin Miniprep method (Qiagen) and quantified using the NanoDrop ND-1000 Spectrophotometer. The purified pDNA clones were sequenced using M13 Forward (-20) and M13 Reverse primers to obtain the full nucleotide sequence of the cysteine protease insert (Davis Sequencing, Inc. Davis, CA).

The sequencing reactions were performed using Applied Biosystems Big Dye Terminator V3.0 sequencing chemistry and the resulting sequences analyzed using Sequencher 3.0 software (Gene Codes Corporation). BLAST searches were performed on the nucleotide sequences to confirm the correct amino acid sequence (National Center for Biotechnology Information, National Institutes of Health (http://www.ncbi.nlm.nih.gob/BLAST/) (Altschul et al., 1990).

The insert DNA from pCR-TOPO2.1 Clone 5 was identified as a 99% amino acid match for the *B. bovis* (T2Bo Strain) cysteine protease-2 gene sequence (GenBank accession no. XP_001610695) and subsequently used as the template DNA for ligation of the gene insert into the Champion pET 101 Directional TOPO vector (Invitrogen, Carlsbad, CA). A PCR reaction using Phusion High-Fidelity DNA Polymerase was performed with BbovCP(pET) forward primer (5′-CAC CAT GGA AAT ACC GGC TGC T-3′) and BbovCP(pET) reverse primer (5′-GAC ATA ACC GTA AGA AAG AAC GCC ACA T-3′) (the table on p.31). The amplification profile for the PCR reaction was initial denaturation at 98ºC for 30 s, followed by 30 cycles of denaturation at 98ºC for 10 s, annealing at 62ºC for 10 s and extension at 72ºC for 2 min with a final extension at 72ºC for 10 min followed by hold at 4ºC. The product was checked by electrophoresis on an ethidium bromide stained 1% agarose gel alongside a 100 bp DNA marker. Using the same method described with pCR-TOPO2.1 vector**,** the resulting PCR products were ligated into Champion pET 101 Directional TOPO vector and One Shot TOP10 Competent *E. coli c*ells were transformed. Upon removal of the transformation reaction from the shaker, it was spread onto pre-warmed LB agar plates containing

Carbenicillin at a final concentration of 50 µg/ml(Research Products International Corporation) and incubated at 37ºC overnight.

Transformants were analyzed by colony PCR to check for proper cysteine protease insert. A master mix of consisting of 400 µl of 2X Taq PCR Master Mix, 368 µl of sterile water, 16 µl of 1 pmol T7 Forward (5′-TAA TAC GAC TCA CTA TAG GG-3′) and 16µl of 1 pmol T7 Reverse (5′-TAG TTA TTG CTC AGC GGT GG-3′). Plasmid purification was done as described with the pCR-TOPO2.1 vector and quantified using the NanoDrop ND-1000 Spectrophotometer. Six clones containing the proper cysteine protease insert were sequenced using T7 Forward and T7 Reverse primers to obtain the full nucleotide sequence (Davis Sequencing, Inc. Davis, CA). The sequencing reactions were performed using Applied Biosystems Big Dye Terminator V3.0 sequencing chemistry and BLAST searches (NCBI) performed on the obtained nucleotide sequences to identify the correct gene sequence in the proper orientation.

2.4 Amplification of *Babesia bovis* Apical Membrane Antigen-1 (AMA-1) ectodomain

Primers were designed based on the *B. bovis* Apical Membrane Antigen-1 (T2Bo strain) (GenBank accession no. XM_001610993). Amplification of *B. bovis* AMA-1 ectodomain (1737 bp) was carried out using Platinum Taq DNA High Fidelity Polymerase with forward primer BbovisAMAF2 (5′-GGC CAA CCA ATT CAC GC-3′) reverse primer BbovisR1 (5′-CAA TTG ATT AAC AAG CGA CCA CG-3′) (Table 1). The amplification profile for the PCR reaction was initial denaturation at 96ºC for 2 min,

followed by 30 cycles of denaturation at 96ºC for 10 s, annealing at 62ºC for 10 s and extension at 68ºC for 2 min with a final extension at 68ºC for 10 min followed by hold at 4ºC. The product was checked by electrophoresis on an ethidium bromide stained 1% agarose gel, alongside a 100 bp DNA marker.

2.5 Cloning *Babesia bovis* AMA-1 ectodomain into pCR-TOPO2.1 and Champion pET 101 Directional TOPO vectors

Babesia bovis AMA-1 was cloned into the pCR-TOPO2.1 vector as described above for the *B. bovis* cysteine protease gene. Plasmid DNA from pCR-TOPO2.1 Clone 4 was identified as a 99% amino acid match for *B. bovis* Apical Membrane Antigen-1 (T2Bo strain) (GenBank accession no. XM_001610993) and subsequently used as the template DNA for ligation of the *B.bovis* AMA-1 ectodomain into the Champion pET 101 Directional TOPO vector. A PCR reaction using Phusion High-Fidelity DNA Polymerase was performed with forward primer BbovAMASigPF (5′-CAC CAT GTC CAA CTC CAC ACT CTT C-3′) reverse primer BbovAMAmreP (5′-GAT ACG CTT TGT GTT GTA GAG TCC G-3′) and an internal primer BbovMRE (5′-GAT ACG CTT TGT GTT GTA GAG TCC-3′) (Table 1). The amplification profile for the PCR reaction was initial denaturation at 98ºC for 30 s, followed by 30 cycles of denaturation at 98ºC for 10 s, annealing at 59ºC for 10 s and extension at 72ºC for 1.5 min with a final extension at 72ºC for 10 min, followed by hold at 4ºC. The product was checked by electrophoresis on an ethidium bromide stained 1% agarose gel alongside a 100 bp DNA

marker. Cloning of the *B. bovis* AMA-1 into Champion pET 101 Directional TOPO was also performed as described above for *B. bovis* cysteine protease.

Table 1

***Babesia bovis* Full Length Cysteine Protease and Apical Membrane Antigen-1 Ectodomain PCR Oligonucleotide Primers**

PCR Primer	Sequence	Annealing Temp.
CP B.bov Foward	5′-TGGGAATACCGGCTGCTGC-3′	63ºC
CP B.bov Reverse	5′- TATGGGACATAACCGTAAGAAAGAACGC-3′	63ºC
BbovCP (pET) Forward	5′-CACCATGGAAATACCGGCTGCT-3′	62ºC
BbovCP (pET) Reverse	5′-GACATAACCGTAAGAAAGAACGCCACAT-3′	62ºC
BbovisAMAF2	5′-GGCCAACCAATTCACGC-3′	62ºC
BbovisR1	5′-CAATTGATTAACAAGCGACCACG-3′	62ºC
BbovAMASigPF	5′-CACCATGTCCAACTCCACACTCTTC-3′	59ºC
BbovAMAmreP	5′-GATACGCTTTGTGTTGTAGAGTCCG-3′	59ºC
BbovMRE	5′-GATACGCTTTGTGTTGTAGAGTCC-3′	59ºC

2.6 Amplification and cloning of *Babesia microti* Cysteine Protease-1 (CP-1) hydrophilic domain into pCR-TOPO2.1 and pCMVCD5flag vectors

The hydrophilic domain of *B. microti* CP-1 (1113 bp) was amplified using Phusion High-Fidelity PCR from a previously cloned *B. microti* cysteine protease (CP-1) gene (Mahmoud et al., 2002) with primers carrying restriction sites for BamH1. The forward primer used was BmCPBAMH1FWD (5′-ATA GGA TCC TAT GGA GAC

TGA GGC TTC GAG AC-3′) and the reverse primer was BmCPBAMH1REV (5′-ATA GGA TCC TTA CAA CGG CAT TAA ACC G-3′). The amplification profile for the PCR reaction was initial denaturation at 93ºC for 3 min, followed by 35 cycles of denaturation at 93ºC for 30 s, annealing at 50ºC for 1 min and extension at 72ºC for 1.2 min with a final extension at 72ºC for 10 min followed by hold at 4ºC. The PCR product was electrophoresed through a 0.8% agarose gel (Sigma-Aldrich) in 1X TAE buffer (0.04M Tris acetate; 0.001 M EDTA) at 50 volts for 1 hour. The appropriate sized amplicon was excised from the gel using a sterile scalpel and the amplicon purified from the gel slice using the QIAquick Gel Extraction method (Qiagen).

A pCR-TOPO2.1 clone confirmed as carrying the *B. microti* CP-1 fragment was restriction digested with BamH1 (Fermentas), gel purified and the resulting fragment ligated into the respective sites of the BamH1-digested and tritrated pCMVCD5flag vector using T4 DNA ligase (Invitrogen, Carlsbad, CA). One Shot TOP10 Competent *E. coli* cells were transformed with the resulting BmCP-1 plasmids in the same manner as described above for *B. bovis*. The transformed cells were plated out onto LB agar plates containing Carbenicillin and incubated overnight at 37°C. The BmCP-1 pDNA was column-purified using the QIAprep Spin Miniprep method and quantified using the NanoDrop ND-1000 Spectrophotometer. After purification the BmCP-1 plasmids were sequenced to identify which clones contained the correct gene sequence and orientation. Once the correct clone, designated pBmCP-1, was identified, a large quantity (~10mg) of BmCP-1 endotoxin-free plasmid DNA was obtained using the Qiagen EndoFree Plasmid Giga method (Qiagen).

2.7 Amplification and cloning of *Babesia microti* Apical Membrane Antigen-1 (AMA-1) ectodomain into pCR-TOPO2.1 and pCMVCD5flag vectors

The *B. microti* AMA-1 ectodomain (1440 bp) was previously amplified and cloned into the pCMVCD5flag vector using the same general methodology as described above for *B. microti* CP-1 and the resultant recombinant construct, designated pBmAMA-1, was available for this study. The insert was sequence verified at the start of this study.

2.8 Validation of gene expression by pBmAMA-1 and pBmCP-1 constructs

Human embryonic kidney (HEK) Freestyle 293-A cells (Invitrogen) were transfected with the pBmAMA-1 or pBmCP-1 plasmid constructs to validate gene expression. For transfection, 2 µg plasmid DNA, 2 µl Turbofect transfection reagent (Fermentas) and 100 µl Opti-MEM 1 (GIBCO Laboratories) were mixed and incubated at room temperature for 15 min and pipetted into 6-well plate containing a monolayer of Freestyle 293-A cells. The 6-well plate was then incubated for 48 h at 37ºC in a humidified 8% CO_2 in air atmosphere.

Gene expression by the pBmAMA-1 and pBmCP-1 constructs was confirmed by immunocytometric analysis of transfected HEK 293-A cells by detecting the FLAG tag. The transfected HEK 293-A monolayers were methanol–fixed (-20°C, 5 min) and incubated with a 1/1000 dilution of a mouse anti-FLAG M2-alkaline phosphatase conjugate (Sigma-Aldrich) in blocking buffer [1X phosphate-buffered saline (PBS) with 5% fetal bovine serum (FBS)]. Following five washes in blocking buffer, the buffer was removed and the alkaline phosphatase activity detected using Fast Red TR/Naphthol AS-

MX substrate (SIGMA FAST; Sigma-Aldrich). To achieve this, one tablet of 0.1 M TRIS buffer was added to 10 ml deionized water and vortexed. A Fast Red TR/Naphthol AS-MX tablet was dissolved in the 10 ml TRIS buffer and filtered through a 0.45 µ syringe filter. Approximately 0.1 to 0.2 ml of Fast Red TR/Naphthol AS-MX solution was used to cover the cells. The cells were stained overnight and the reaction stopped by washing the cells with deionized water. The stained cells were visualized at 400 X magnification and photographed using an Olympus 1X70 inverted phase contrast microscope (Olympus Optical).

2.9 Expression and purification of *Babesia microti* AMA-1 and CP-1 protein

The *B. microti* CP-1 and AMA-1 recombinant proteins were produced by transient expression using the FreeStyle 293 Expression System (Invitrogen). The 293-F cells were cultured in suspension to a density of $1.2x10^6$ cells per ml in the presence of FreeStyle 293 Expression Medium (Invitrogen). Lipid-DNA complexes were prepared by diluting 30 µg of pBmAMA-1 or pBmCP-1 constructs in 1 ml of Opti-MEM 1. Following a 5-min incubation, the DNA dilution was added to a 50 ml tube containing 45 µl of 293fectin (Invitrogen) diluted in 1 ml of Opti-MEM 1 and incubated at room temperature for 20 min. The DNA-293Fectin mixture was added to a 40 ml FreeStyle293 cell suspension in a 250 mL polycarbonate disposable sterile Erlenmeyer flask (Fisher Scientific) and the transfected suspension was further incubated for 48 h at 37°C on an orbital shaker platform rotating at 120-130 rpm.

The transfected 293-F cells were harvested by centrifugation in conical centrifuge tubes (2800 rpm for 10 min). The supernatant was decanted and saved. The cell pellets were resuspended in 300 µl of 1X protease inhibitor cocktail (Sigma-Aldrich) and lysed using three freeze/thaw cycles (alternately liquid nitrogen and a 37°C water bath). The lysed cell suspension was centrifuged at 2800 rpm for 15 min and the resulting supernatant combined with the saved supernatant and clarified through a 0.45 µm filter. Forty milliliters of lysate was purified through batch absorption using Anti-FLAG M2 Affinity Gel (Sigma-Aldrich). The protein was eluted from the affinity gel by acid elution using 1 ml aliquots of 0.1 M glycine HCL, pH 3.5 into vials containing 25 µl of 1 M Tris, pH 8.0. The elution fractions were combined and concentrated by centrifugation using a 30kD molecular cut-off membrane (Millipore Corporation). The concentration of *B. microti* CP-1 or AMA-1 protein was determined using the BCA protein assay (Pierce Biotechnology).

2.10 Validation of expressed protein using western blotting

The molecular weights of the expressed *B. microti* CP-1 and AMA-1 proteins were confirmed by western blotting using anti-FLAG mAb. The proteins were resolved on a 10% Bis-Tris NuPAGE gel using the XCell Surelock electrophoresed and transfer apparatus with NuPAGE MES 1X Running Buffer (Invitrogen) at 135 volts for 1 h. The electrophroesed proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore) using NuPAGE transfer buffer at 35 volts for 1 h (Invitrogen). The PVDF membrane was first placed in blocking buffer (5% non-fat milk powder in

Tris-buffered saline containing 0.2% Tween 20 (TBS-T)) for 1 h at room temperature, and subsequently incubated in a 1:1000 dilution of monoclonal ANTI-FLAG M2 Affinity Purified Antibody (Sigma-Aldrich) in blocking buffer for 1 h at room temperature. The PVDF membrane was washed in TBS-T to removed unbound antibody, and then incubated with Immun-Star AP substrate (Bio-Rad) for 5 min. The antibody–antigen complexes were visualized by exposure to subsequent development of BioMax chemiluminescence film (Kodak).

2.11 Vaccine preparation

For DNA immunization with the Helios Gene Gun System (Bio-Rad), each dose consisted of 0.5mg of gold spheres (1.6 μm) coated with plasmid DNA (2 μg of pBmAMA-1 or 2 μg of pBmCP-1, or 2 μg of each combined (4μg total) for the three experimental groups, respectively, resuspended in water). Gold spheres were suspended in 100μl of 0.05M spermidine in water. Following a 5 s sonication step the DNA was precipitated onto the gold by addition of 1 M $CaCl_2$. The DNA–gold complex was incubated for 10 min at room temperature, centrifuged for 15 s, supernatant removed, and the gold washed three times with 500 μl 100% ethanol. After the last wash the pellet was resuspended in 0.05 mg/ml polyvinylpyrrolidone (PVP) in ethanol. The DNA-coated gold was applied to the inner surface of Tefzel tubing (Helios Tubing Prep Station, Bio-Rad) by centrifugal force and a slight flow of nitrogen was used to evaporate the ethanol. The tubing was cut into cassettes, each containing a dose of

plasmid DNA. Cassettes containing the DNA-coated gold were loaded into the gene gun and the vaccine administered to the animals using compressed helium (Fig.1).

2.12 Vaccination regimen

Four groups of 5 BALB/c mice each were used for this vaccine trial (Table 1). The abdomen of each animal was shaved and wiped with 70% ethanol. The spacer of the Helios Gene Gun was held directly against the abdominal skin (Fig. 1). The gun was discharged at a helium pressure of 300 psi. Mice were injected 3 times at 2-week intervals. Group 1 received inoculations containing 2 µg of pBmAMA-1 and 2 µg of pBmCP-1 pDNA (total 4 µg pDNA/injection). Group 2 received 2 µg of pBmAMA-1. Group 3 received 2 µg of pBmCP-1. Group 4 served as the negative control and received 2 µg of the expression vector only (no gene inserts). During the first two series of inoculations, the mice were anesthetized to effect with isoflurane in oxygen. However, it became apparent that the act of anesthetizing the animals was more stressful than administering the vaccine without anesthetic. On the recommendation of the Comparative Medicine Program (Texas A&M University) a change was made to the protocol and the mice were not anesthetized for the third vaccination.

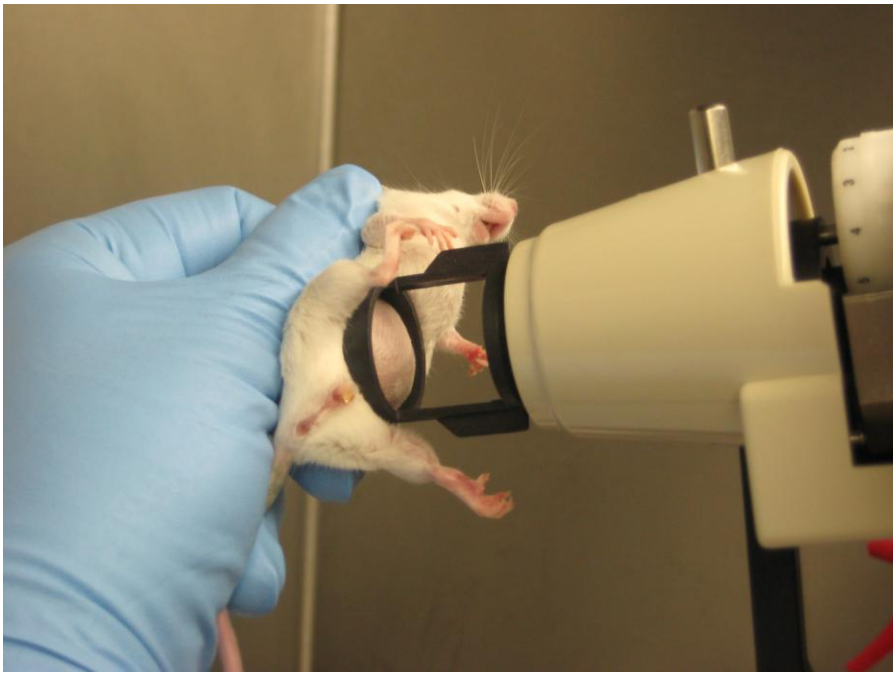

Fig. 1. Gene transfer into the abdominal skin of BALB/c mice using Helios Gene Gun.

2.13 Analysis of infection following challenge with *Babesia microti*

One month after the last gene gun vaccination was administered, the mice were challenged by intraperitoneal administration of 10^7 *B. microti*-infected red blood cells. The challenge dose was obtained from two infected C.B-17/IcrHsd-Prkdc-scid mice at a combined parasitemia of 65%, adjusted with Dulbecco's phosphate buffered saline (pH 7.4) to a concentration of 10^7 *B. microti*-infected red blood cells in 0.3 ml. The animals were monitored daily by obtaining blood smears from the tip of the tail, which were methanol-fixed and Giemsa-stained for microscopic examination at 1000X under oil immersion. Percent parasitemias were measured by counting the number of *B. microti*-infected erythrocytes in 1000 erythrocytes. The mean PCV of each group was

determined before challenge and every 5 days following challenge. Blood was collected from the tail tip in heparinized microhematocrit capillary tubes (Fisherbrand) and centrifuged (IEG MB Centrifuge); the PCV was then measured and recorded. The plasma was collected from the microhematocrit tubes and stored at –80º C for future serologic analysis (not in the scope of the current study). After the parasitemia cleared, as determined by three consecutive days without parasite observation in blood smears, the mice were humanely euthanized. Euthanasia was effected by carbon dioxide asphyxiation followed by cervical dislocation.

2.14 Statistical analyses

A repeated data analysis was used to compare parasitemias and PCVs of vaccinated animal to unvaccinated animals. P-values ≤ 0.05 are considered statistically significant. All data analyses were performed using SPSS version 12.0 (SPSS, Inc., 2001, Chicago IL).

3. RESULTS

3.1 *Babesia bovis* constructs

The *B. bovis* Cysteine Protease-2 full open reading frame was successfully amplified (Fig. 2), cloned into pCR-TOPO2.1 and subcloned into the Champion pET Directional TOPO expression vector. Figure 3 shows that the clone obtained was a 99% amino acid match to the *B. bovis* Cysteine Protease-2 sequence in the genome project (http://www.ncbi.nlm.nih.gob/BLAST/). The AMA-1 ectodomain gene region was successfully amplified (Fig. 4) and cloned into the pCR-TOPO2.1 and Champion pET 101 Directional TOPO vectors. The clone obtained was a 99% amino acid match to the *B. bovis* AMA-1 sequence in the genome project (Fig. 5). The constructs were stored at –80ºC for future applications.

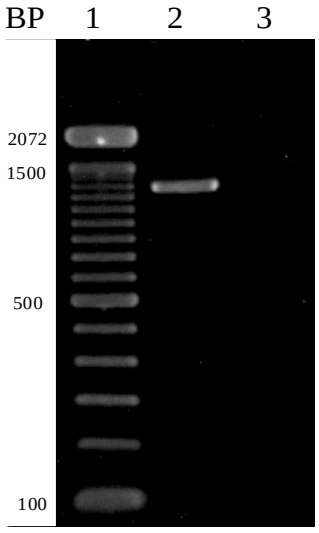

Fig. 2 . **PCR-amplified *Babesia bovis* Cysteine Protease gene product.** Amplification of *Babesia bovis* cysteine protease (2), negative control (3), alongside 100 base pair marker (Invitrogen) (1).

```
BbCP1           MEIPAAASDLSNLDDHYVRSDDEVRDTTLIGRSRRCCVGKKTMWIVLLGTAILTAAITSG
BbCP1Clone      MEIPAAASDLSNLDDHYVRSDDEVRDTTLIGRSRRCCVGKKTMWIVLLGTAILTAAITSG
                ************************************************************

BbCP1           IILLVTSLSGSKAKPSGGVKHIGKFDGLNRADCHVSPETFAELSSMAHLGEINVSDPAEI
BbCP1Clone      IILLVTSLSGSKAKPSGGVKHIGKFDGLNRADCHVSPETFAELSSMAHLGEINVSDPAEI
                ************************************************************

BbCP1           VKYMDFTRMAKKFDRKYDTVAERHTAFLNFRRNHDIVKSHEHNKAATYTKDLNHFFDKDI
BbCP1Clone      VKYMDFTRMAKKFDRKYDTVAERHTAFLNFRRNHDIVKSHEHNKAATYTKDLNHFFDKDI
                ************************************************************

BbCP1           KAVAAKLLHKIDVYNESNISVTPTDTTATKENQPIYATLKNYSVSAGYPPIGSKVNFEDI
BbCP1Clone      KAVAAKLLHKIDVYNESNISVTPTDTTATKENQPIYATLKNYSVSAGYPPIGSKVNFEDI
                ************************************************************

BbCP1           DWRRADAVTPVKDQGMCGSCWAFAAVGSVESLLKRQKTDVRLSEQELVSCQLGNQGCNGG
BbCP1Clone      DWRRADAVTPVKDQGMCGSCWAFAAVGSVESLLKRQKTDVRLSEQELVSCQLGNQGCNGG
                ************************************************************

BbCP1           YSDYALNYIKFNGIHRSEE-PYLAADGKCVAHDGTKYYIKGYHAAKGRSVANQLLVMGPT
BbCP1Clone      YSDYALNYIKFNGIHRSEEWPYLAADGKCVAHDGTKYYIKGYHAAKGRSVANQLLVMGPT
                ****************** ****************************************

BbCP1           VVYIAVSEDLMHYSGGVFNGECSDSELNHAVLLVGEGYDSALKKRYWLLKNSWGTSWGED
BbCP1Clone      VVYIAVSEDLMHYSGGVFNGECSDSELNHAVLLVGEGYDSTLKKRYWLLKNSWGTSWGED
                ****************************************** ******************

BbCP1           GYFRLERTNTPTDKCGVLSYGYV
BbCP1Clone      GYFRLERTNTPTDKCGVLSYGYV
                ***********************
```

Fig. 3. Alignment of predicted amino acid sequence of *Babesia bovis* Cysteine Protease gene insert (pCR-TOPO2.1) with the *B. bovis* T2Bo Strain Cysteine Protease-2 (GenBank accession no. XP_001610695). Amino acids identical in both sequences are indicated by stars (⋆).

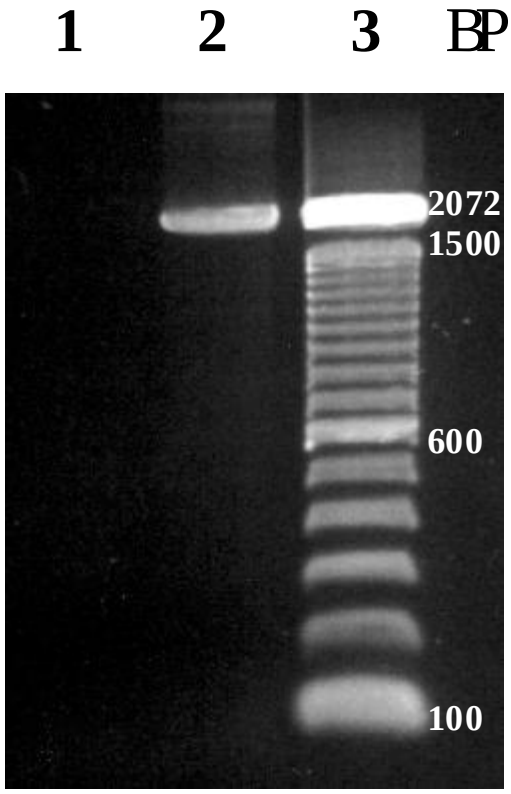

Fig. 4. PCR-amplified *Babesia bovis* AMA-1 gene product. Amplification of *B. bovis* AMA-1 (2), negative control (1), alongside 100 base pair DNA marker (Invitrogen) (3).

```
BbAMA1          MQSTSPKYNYKRMLCMVFVPVILSSFFAEDALASNSTLFAFHREPTNRRLTRRASRGQLL
BbAMA1Clone     MQSTSPKYNYKRMLCMVFVPVILSSFFAEDALASNSTLFAFHREPTNRRLTRRASRGQLL
                ************************************************************

BbAMA1          NSRRGSDDTSESSDRYSGRSGGSKNSGQSPWIKYMQKFDIPRNHGSGIYVDLGGYESVGS
BbAMA1Clone     NSRRGSDDTSESSDRYSGRSGGSKNSGQSPWIKYMQKFDIPRNHGSGIYVDLGGYESVGS
                ************************************************************

BbAMA1          KSYRMPVGKCPVVGKIIDLGNGADFLDPISSEDPSYRGLAFPETAVDSNIPTQPKTRGSS
BbAMA1Clone     KSYRMPVGKCPVVGKIIDLGNGADFLDPISSEDPSYRGLAFPETAVDSNIPTQPKTRGSS
                ************************************************************

BbAMA1          SVTAAKLSPVSAKDLRRWGYEGNDVANCSEYASNLIPASDKTTKYRYPFVFDSDNQMCYI
BbAMA1Clone     SVTAAKLSPVSAKDLRRWGCEGNDVANCSEYASNLIPASDKTTKYRYPFVFDSDNQMCYI
                ******************* ****************************************

BbAMA1          LYSAIQYNQGNRYCDNDGSSEEGTSSLLCMKPYKSAEDAHLYYGSAKVDPDWEENCPMHP
BbAMA1Clone     LYSAIQYNQGNRYCGNDGSSEEGTSSLLCMKPYKSAEDAHLYYGSAKVDPDWEENCPMHP
                ************** ****************************************

BbAMA1          VRDAIFGKWSGGSCVAIAPAFQEYANSTEDCAAILFDNSATDLDIEVVNEEFNELKELTS
BbAMA1Clone     VRDAIFGKWSGGSCVAIAPAFQEYANSTEDCAAILFDNSATDLDIEVVNEEFNELKELTS
                ************************************************************

BbAMA1          GLKRLNLSKVANAIFSPLSNVAGTSRISRGVGMNWATYDKDSGMCALINETPNCLILNAG
BbAMA1Clone     GLKRLNLSKVANAIFSPLSNVAGTSRISRGVGMNWATYDKDSGMCALINETPNCLILNAG
                ************************************************************

BbAMA1          SIALTAIGSPLEYDAVNYPCHIDTNGYVEPRAKTTNKYLDVPFEVTTALSTKTLKCNAYV
BbAMA1Clone     SIALTAIGSPLEYDAVNYPCHIDTNGYVEPRAKTTNKYLDVPFEVTTALSTKTLKCNAYV
                ************************************************************

BbAMA1          HTKYSDSCGTYFLCSDVKPNWFIRFLHMIGLYNTKRIVIFVCCTTTAIVLTIWIWKRFIK
BbAMA1Clone     HTKYSDSCGTYFLCSDVKPNWFIRFLHMIGLYNTKRIVIFVCCTTTAIVLTIWIWKRFIK
                ************************************************************

BbAMA1          AKKEPAPPSFDKYLSNYDYDTTLDADNETEQRLDSSAYSWGEAVQRPSDVTPVKLSKIN
BbAMA1Clone     AKKEPAPPSFDKYLSNYDYDTTLDADNETEQRLDSSAYSWGEAVQRPSDVTPVKLSKIN
                ************************************************************
```

Fig. 5. Alignment of predicted amino acid sequence of *Babesia bovis* AMA-1 gene insert (pCR-TOPO2.1) with the *B. bovis* T2Bo strain AMA-1 (GenBank accession no. XM_001610993). Amino acids identical in both sequences are indicated by stars (⋆).

3.2 BmCP-1 and BmAMA-1 plasmid constructs

The *B. microti* Cysteine Protease-1 hydrophilic region was successfully amplified (Fig. 6), cloned into pCR-TOPO 2.1 and subcloned into the pCMVCD5flag expression vector. Figure 7 shows that the clone obtained was a 100% amino acid match to the predicted *B. microti* CP-1 sequence previous identified in our lab (Mahmound et al., 2002). The AMA-1 ectodomain gene region was previously amplified and cloned into pCR-TOPO 2.1 and subcloned into the pCMVCD5flag expression vector. The clone was sequenced verified to confirm a 100% amino acid match to the *B. microti* AMA-1 sequence previously identified in our lab (P.J. Holman, unpublished results) (Fig. 8).

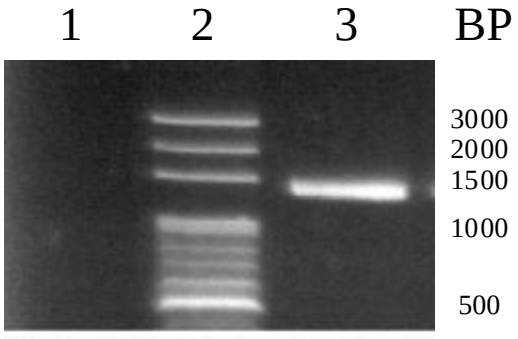

Fig. 6. PCR-amplified *Babesia microti* CP-1 gene product. Amplification of *B. microti* CP-1 (3), negative control (1), 1 Kb marker (New England Bio Labs) (2).

```
B.microti CP-1   ETEASRQVKARLIDDMLSGQSLYNCYPYDQQRYHVGKPAQKPESVGEFIVRTLTEHGYTI
BmCP-1 clone     ETEASRQVKARLIDDMLSGQSLYNCYPYDQQRYHVGKPAQKPESVGEFIVRTLTEHGYTI
                 ************************************************************

B.microti CP-1   DPDLEAKIYKEFNIFMAKFGKIYFTPKEKGDKYINFRKSYEIVMAHNNNKNVSYKMALGQ
BmCP-1 clone     DPDLEAKIYKEFNIFMAKFGKIYFTPKEKGDKYINFRKSYEIVMAHNNNKNVSYKMALGQ
                 ************************************************************

B.microti CP-1   FSDKSPEEFENSVLNPMMSNEHYVNAIKSGRFNLFRPDPRQEGIPEQFIWDHKFLGPVLN
BmCP-1 clone     FSDKSPEEFENSVLNPMMSNEHYVNAIKSGRFNLFRPDPRQEGIPEQFIWDHKFLGPVLN
                 ************************************************************

B.microti CP-1   QGACGSCWAFATAGAVQSLFNIVNNSKLVLSPQELVDCTINANGCKGGNPIYAFNYVRDH
BmCP-1 clone     QGACGSCWAFATAGAVQSLFNIVNNSKLVLSPQELVDCTINANGCKGGNPIYAFNYVRDH
                 ************************************************************

B.microti CP-1   GLCTLNDYPYVGFQQKCSSSSCKHKIPIKNKMLVTSGFDIALAQGSPMVVGIDANGPFQH
BmCP-1 clone     GLCTLNDYPYVGFQQKCSSSSCKHKIPIKNKMLVTSGFDIALAQGSPMVVGIDANGPFQH
                 ************************************************************

B.microti CP-1   YSHGIFEAPCTPGTSNHAVLLVGYGVDKETGKKYWVIKNSWGPDWGEKGYARILRSDDGN
BmCP-1 clone     YSHGIFEAPCTPGTSNHAVLLVGYGVDKETGKKYWVIKNSWGPDWGEKGYARILRSDDGN
                 ************************************************************

B.microti CP-1   GADCNLTKFGL
BmCP-1 clone     GADCNLTKFGL
                 ***********
```

Fig. 7. Alignment of cloned *Babesia microti* Cysteine Protease-1 predicted amino acid sequences. The amino acid sequence of the hydrophilic region from the previously identified *B. microti* Cysteine Protease-1 (*B. microti* CP-1) gene is aligned with the obtained sequence of the region cloned into pCMVCD5flag (BmCP-1 clone). Amino acids identical in both sequences are indicated by stars (⋆).

```
B.microtiAMA-1   ALANARPAHRKIVHKHKNSGAKPRHQAKKHVNITEEDDEDGEVYEEDEEDDYEDTDYEEL
BmAMA-1 clone    ALANARPAHRKIVHKHKNSGAKPRHQAKKHVNITEEDDEDGEVYEEDEEDDYEDTDYEEL
                 ************************************************************

B.microtiAMA-1   EFKPIGDERDNPWESYMEKFNIPKVHGSGVYVDLGKNGTFNGKKYRMVAGKCPVFGKIIE
BmAMA-1 clone    EFKPIGDERDNPWESYMEKFNIPKVHGSGVYVDLGKNGTFNGKKYRMVAGKCPVFGKIIE
                 ************************************************************

B.microtiAMA-1   FSSGVDYLSPANDTANPAFGFPYTVPAGKTIRTNEIPKTSRGRISISKSAVQTDLISPVT
BmAMA-1 clone    FSSGVDYLSPANDTANPAFGFPYTVPAGKTIRTNEIPKTSRGRISISKSAVQTDLISPVT
                 ************************************************************

B.microtiAMA-1   AKTLKAYEYDGDDIFNCASYASELMMSSDRKSDYKYPFAFDLKTKTCHILYSPLQLIQGP
BmAMA-1 clone    AKTLKAYEYDGDDIFNCASYASELMMSSDRKSDYKYPFAFDLKTKTCHILYSPLQLIQGP
                 ************************************************************

B.microtiAMA-1   KYCDNDGKVDSGSSSMPCIKPVKDMSQEMVYGSSFIYRDWKNKCPNAAVADAIFGTWNGT
BmAMA-1 clone    KYCDNDGKVDSGSSSMPCIKPVKDMSQEMVYGSSFIYRDWKNKCPNAAVADAIFGTWNGT
                 ************************************************************

B.microtiAMA-1   ACVPIQNRRLFKASTPEICGQIVFKYSASDAPENYETKRSEGSKFANAISSGDLGAVAKI
BmAMA-1 clone    ACVPIQNRRLFKASTPEICGQIVFKYSASDAPENYETKRSEGSKFANAISSGDLGAVAKI
                 ************************************************************

B.microtiAMA-1   IMPVTNSRAHHSKGWGFNWANYDRNKRECGLIDEVPNCLVFKMGNIAFNSLGSPLEDDME
BmAMA-1 clone    IMPVTNSRAHHSKGWGFNWANYDRNKRECGLIDEVPNCLVFKMGNIAFNSLGSPLEDDME
                 ************************************************************

B.microtiAMA-1   NFPCEIKSFGYITKGNPNDINSYLISSTHRDMIPNDDGIETLNARSCSGYYGKIGAKESE
BmAMA-1 clone    NFPCEIKSFGYITKGNPNDINSYLISSTHRDMIPNDDGIETLNARSCSGYYGKIGAKESE
                 ************************************************************
```

Fig. 8. Alignment of cloned *Babesia microti* Apical Membrane Antigen-1 predicted amino acid sequences. The amino acid sequence of the ectodomain from the previously identified *B. microti* Apical Membrane Antigen-1 (*B. microti* AMA-1) gene is aligned with the obtained sequence of the region cloned into pCMVCD5flag (BmAMA-1 clone). Amino acids identical in both sequences are indicated by stars (⋆).

3.3 Confirmation of in vitro BmCP-1 and BmAMA-1 protein expression using immunohistochemistry

To confirm the expression of the flag-tagged BmAMA-1 and BmCP-1 proteins, HEK 293A cells were transfected and expression verified through immunohistochemistry detecting the presence of the FLAG marker peptide (Fig. 9).

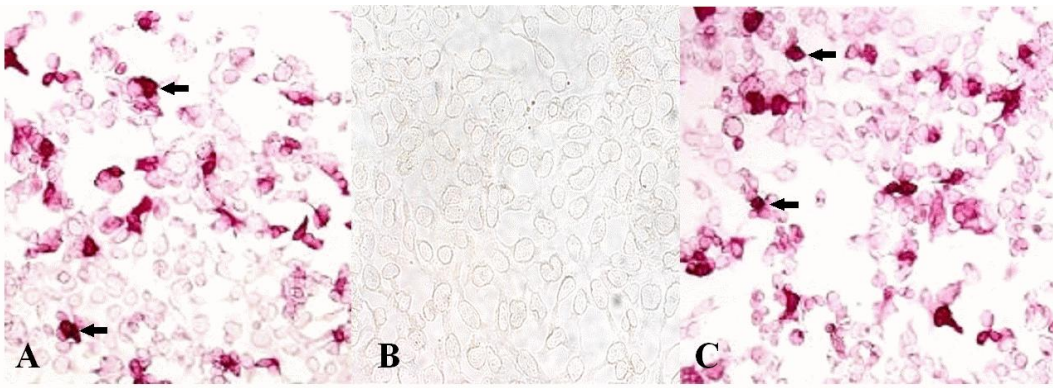

Fig. 9. Expression of *Babesia microti* FLAG-tagged CP-1 and AMA-1 proteins in HEK 293-A cells. Probed with ANTI-FLAG M2 Monoclonal Antibody alkaline phosphatase conjugate. (A) Cells expressing CP-1 (arrows), (B) control cells, (C) cells expressing AMA-1 (arrows) (400X).

3.4 Identification of *Babesia microti* AMA-1 and CP-1 proteins

The open reading frames of the *B. microti* AMA-1 and CP-1 genes in the expression vector pCMVCD5flag were expressed and affinity purified. As shown in Fig. 10, a strong specific 54 kDa band was obtained from lysate of HEK293F cells transfected with the BmAMA-1 construct and a 48 kDa band was obtained from the lysate of cells transfected with the BmCP-1 construct. The molecular size of both proteins is consistent with the expected size from the predicted amino acid sequence.

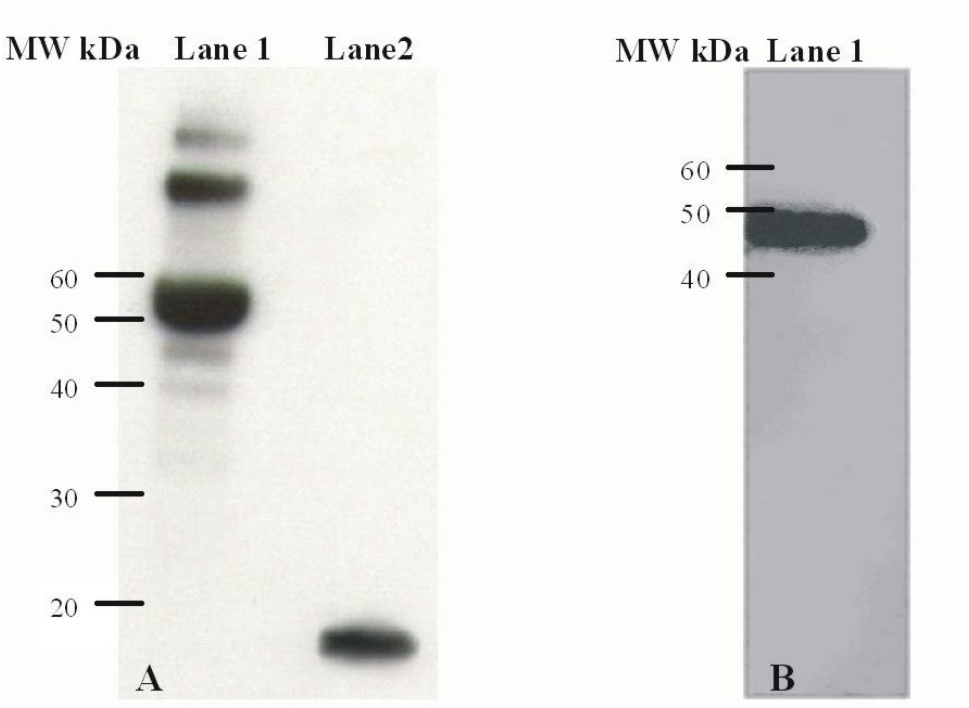

Fig. 10. Western blot analysis of ANTI-FLAG M2 Affinity purified *Babesia microti* AMA-1 and CP-1 expressed proteins. (A) FLAG-tagged *B. microti* AMA-1 protein migrating at approximately 54 kDA (Lane 1). FLAG-tagged Interleukin-4 positive control (Lane 2). (B). FLAG-tagged *B. microti* CP-1 protein migrating at approximately 48 kDa.

3.5 Analysis of parasitemia

In order to examine the protective effects of the *B. microti* AMA-1 and CP-1 DNA vaccines, mice were challenged with 10^7 *B. microti*-infected erythrocytes passaged from infected mice. By day 2 post-challenge, all mice developed a measurable parasitemia lasting until day 18 for the BmCP1 and BmAMA1 groups and until day 21 for the BmCP1/BmAMA1 and the plasmid control groups. The mean daily percent parasitemia values for each group (Fig. 11) have the highest mean peak parasitemia, 20.04%, in the plasmid control group on day 9 and the lowest mean peak parasitemia,

7.42%, in the BmCP1 group on day 8. Figure 12 shows parasitemia values for each individual over time, color-coded according to vaccine group.

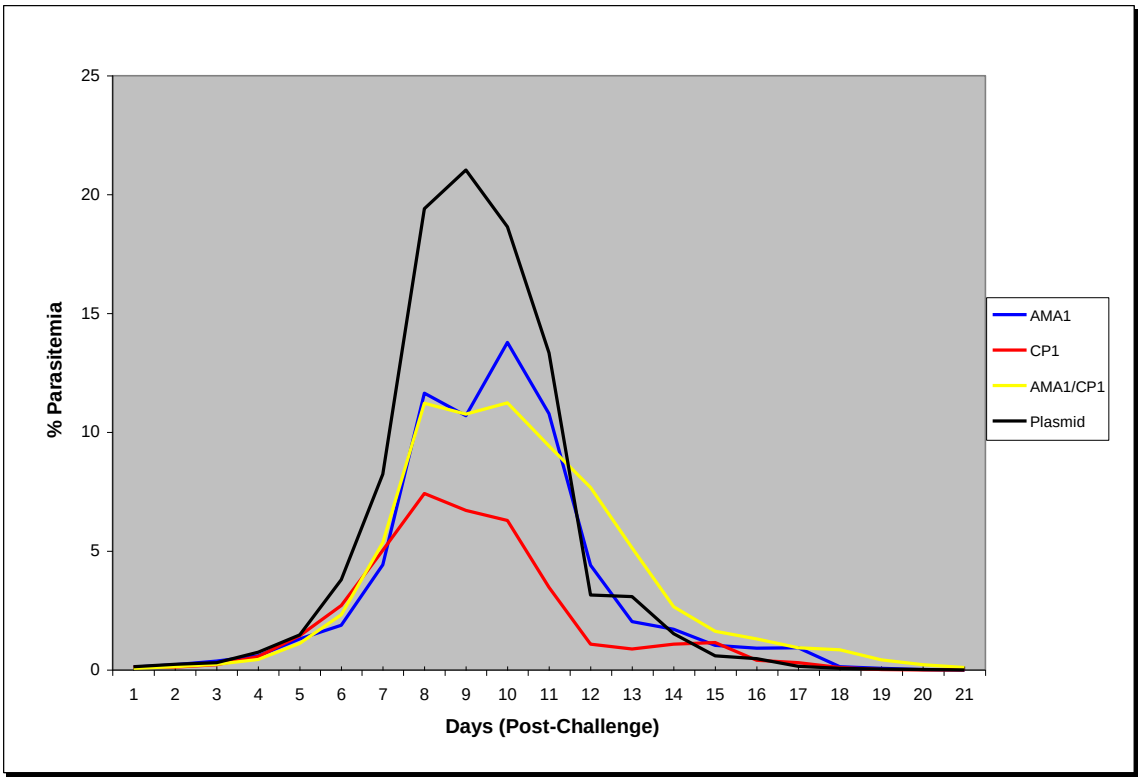

Fig. 11. Mean group percent parasitemias.

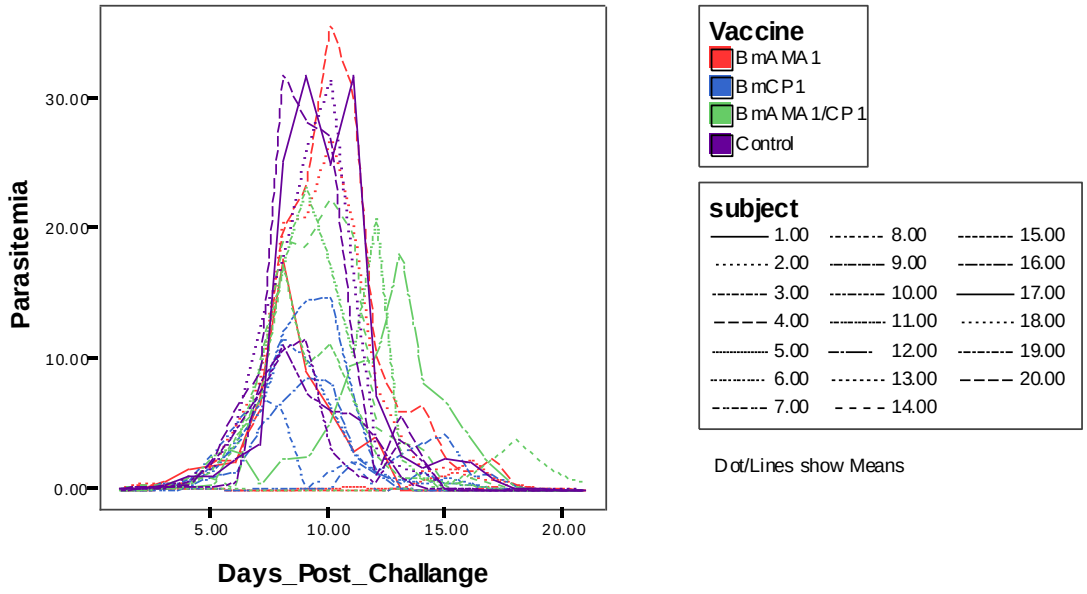

Fig. 12. Individual parasitemia for each subject.

A repeated data analysis was used to analyze the data obtained from the observed parasitemias. The model for this analysis was:

$Y_{ijk} = \mu + \tau_i + d_{ik} + \beta_j + (\tau\beta)_{ij} + e_{ijk}$ where i=1,2,3,4, j=1,..,21 and k=1,…,5 τ_i *i-th* treatment effect, β_j *j-th* time effect, $(\tau\beta)_{ij}$ treatment-time interaction effect, e_{ijk} is the error for every observation and considered independent and identically distributed (i.i.d), d_{ik} is the random effect of the subject (mouse) which is normally distributed with mean zero and variance decided from Akaike's information criterion (AIC) where d_{ik} is independent from e_{ijk}. After a check with the AIC, it was decided that the variance of the subject would be different in every time step. Therefore, there will be a diagonal covariance matrix of random effect for the subject in every time step. This choice can be

justified from Fig. 12 showing the individual parasitemias for each animal or from the table of estimated covariance (Table 2).

Table 2

Model Dimension for Covariance

Model analysis table for selection of the proper covariance matrix for the error variance.

Model Dimension [a]

		Number of Levels	Covariance Structure	Number of Parameters	Subject Variables	Number of subjects
Fixed Effects	Intercept	1		1		
	Vaccine	4		3		
	Days	21		20		
	Vaccine * Days	84		60		
Repeated Effects	Days	21	Diagonal	21	subject	20
Total		131		105		

[a] Dependent Variable: Parasitemia

Table 3

Type III Test of Fixed Effects: Parasitemia

Results of a fixed effect model based on the chosen diagonal covariance matrix (parasitemia).

Type III Test of Fixed Effects [a]

Source	Numerator df	Denominator df	F	Sig.
Intercept	1	82.554	197.271	.000
Vaccine	3	82.554	5.762	.001
Days	20	19.013	16.065	.000
Vaccine * Days	60	19.013	.907	.629

[a] Dependent Variable: Parasitemia

The analysis of variance table (Table 3) shows that there is no significant interaction between time and vaccine. The mean group parasitemias given in Fig. 11 indicate interaction; however, Table 3 proves that this is because of variability as opposed to real interactions. The main effect of the vaccines is statistically significant, meaning that statistically the vaccines react differently compared to each other and the control group. A least significant difference (LSD) test of all the possible vaccine comparisons is shown in Table 4.

Table 4

Pairwise Comparison: Parasitemia

Pairwise comparison of parasitemia for each experimental group.

Pairwise Comparison [b]

(I) Vaccine	(J) Vaccine	Mean Difference (I-J)	Std. Error	df	Sig. [a]	95% Confidence Interval for Difference [a]	
						Lower Bound	Upper Bound
BmAMA1	BmCP1	1.331*	.662	82.554	.047	.015	2.647
	BmAMA1/CP1	-.292	.662	82.554	.660	-1.608	1.024
	Control	-1.339*	.662	82.554	.037	-2.715	-.083
BmCP1	BmAMA1	-1.331*	.662	82.554	.047	-2.647	-.015
	BmAMA1/CP1	-1.624*	.662	82.554	.016	-2.940	-.308
	Control	-2.730*	.662	82.554	.000	-4.046	-1.415
BmAMA1/CP1	BmAMA1	.292	.662	82.554	.660	-1.024	1.608
	BmCP1	1.624*	.662	82.554	.016	.308	2.940
	Control	-1.107	.662	82.554	.098	-2.423	.209
Control	BmAMA1	1.339*	.662	82.554	.037	.083	2.715
	BmCP1	2.730*	.662	82.554	.000	1.415	4.046
	BmAMA1/CP1	1.107	.662	82.554	.098	-.209	2.423

Based on estimated marginal means

* The mean difference is significant at the .05 level.
[a] Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).
[b] Dependent Variable: Parasitemia

Based on the information in Table 4, it can be concluded that the mice vaccinated with BmCP1 developed a statistically lower parasitemia than any of the other groups, including the control group (P = .000). Mice vaccinated with BmAMA1 also developed a statistically lower parasitemia compared to the control group (P = .037); however,

based on the confidence intervals, it is obvious that the BmCP1 vaccine was more effective than AMA-1 at providing protection against *B. microti* challenge.

3.6 Analysis of packed cell volume

As with the analysis of parasitemia, a repeated data analysis model was used to analyze the PCV data obtained from the animals within each group (Fig. 13 and Fig. 14). From day 0 to day 15, the mean PCV of all the groups decreased. The substantial drop in PCV between days 5-10 correlates with the mean peak parasitemias for all groups, which occurred between days 7 and 12. This time frame correlates with previous published reports of *B. microti* infections (Hu et al., 1996). The group that had the least drop in PCV was the group that received BmCP1. The mean PCV of the BmCP1 group was considered significantly different from that of the control group ($P = .011$). The group that had the greatest drop in PCV was the control group. After the mice appeared to be clear of parasitemia, a final PCV was determined. The BmCP1 group and the BmAMA1 had the PCV returning to normal levels at a faster rate than all other groups (Fig. 13). The PCVs of the control group remained lower than all other groups (Fig. 13).

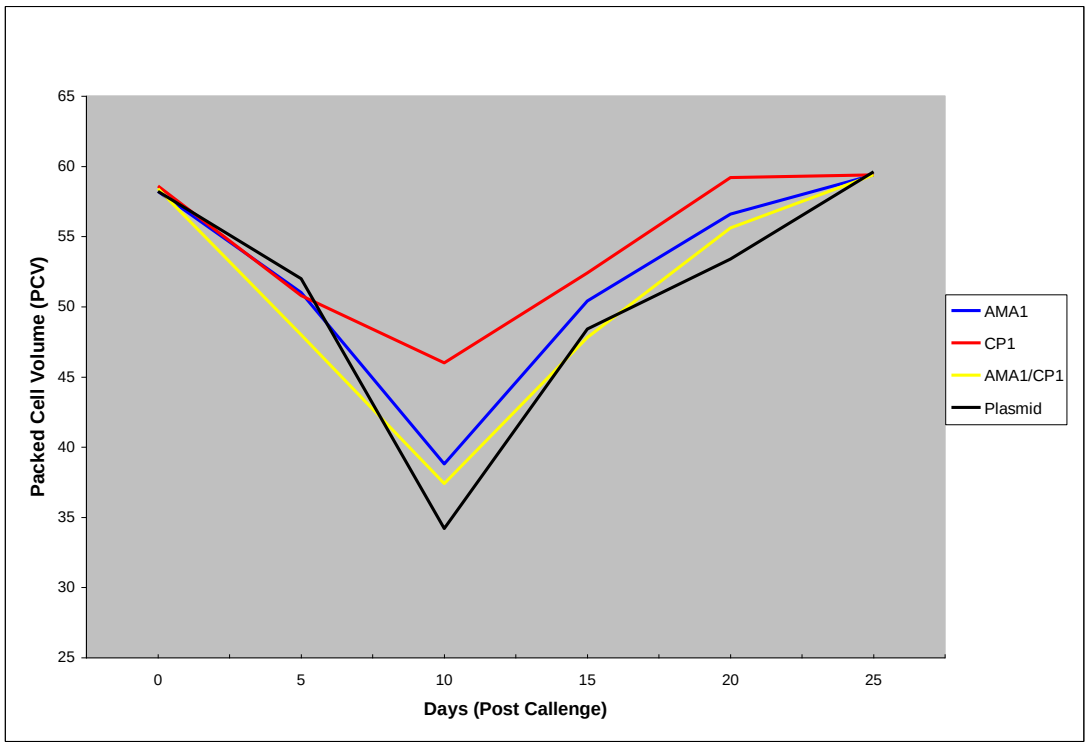

Fig. 13. Mean group packed cell volume (PCV).

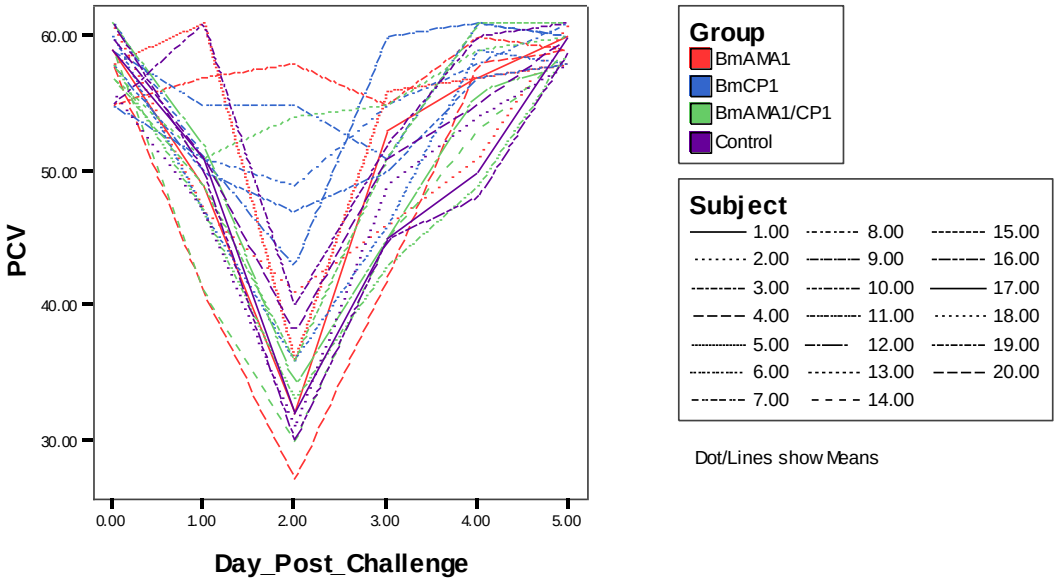

Fig. 14. Individual PCV for each subject.

The model used was: $Y_{ijk} = \mu + \tau_i + d_{ik} + \beta_j + (\tau\beta)_{ij} + e_{ijk}$ where i=1,2,3,4, j=1,…,6 and k=1,…,5 τ_i *i-th* treatment effect, β_j *j-th* time effect, $(\tau\beta)_{ij}$ treatment-time interaction effect, e_{ijk} is the error for every observation and is considered i.i.d., d_{ik} is the random effect of the subject (mouse) which is normally distributed with mean zero and variance decided from AIC criteria (shown in Table 5).

Table 5

Comparison of Different Statistical Models

Measure of the goodness of fit for different statistical models.

	AR1	CS	DIAG
-2 Restricted Log Likelihood	605.862	612.157	**560.454**
Akaike's Information Criterion (AIC)	605.862	612.157	**572.454**

From the above table, the smallest AIC is met when the assumption is made of diagonal covariance matrix within the subject error. The criteria chose the optimal covariance matrix in terms of best fit and over-parameterization. This choice can be justified from the variability seen in Fig. 14 or from the table of estimated covariance (Table 6).

Table 6

Type III Test of Fixed Effects: PCV

Results of a fixed effect model based on the chosen diagonal covariance matrix (packed cell volume).

Type III Test of Fixed Effects [a]

Source	Numerator df	Denominator df	F	Sig.
Intercept	1	49.230	12919.630	.000
Vaccine	3	49.230	3.005	.039
Days	5	20.764	44.057	.000
Group * Days	15	20.764	.918	.559

[a] Dependent Variable: PCV

From Table 7, BmCP1 and BmAMA1/CP1 as well as BmCP1 and the plasmid control are statistically different at a significance level of 0.05. Based on the available information, it cannot be concluded that BmCP1 is superior to BmAMA1 even though the P-Value is close to 0.1. More observations (a larger sample size), are needed to reject the null hypothesis that the vaccines are the equivalent to each other and the control.

Table 7

Pairwise Comparison: PCV

Comparison of PCV for each experimental group.

Pairwise Comparison [b]

(I) Vaccine	(J) Vaccine	Mean Difference (I-J)	Std. Error	df	Sig. [a]	95% Confidence Interval for Difference[a]	
						Lower Bound	Upper Bound
BmAMA1	BmCP1	-2.000	1.299	49.230	.130	-4.611	.611
	BmAMA1/CP1	1.300	1.299	49.230	.322	-1.311	3.911
	Control	1.433	1.299	49.230	.275	-1.178	4.044
BmCP1	BmAMA1	2.000	1.299	49.230	.130	-.611	4.611
	BmAMA1/CP1	3.300*	1.299	49.230	.014	.689	5.911
	Control	3.433*	1.299	49.230	.011	.822	6.044
BmAMA1/CP1	BmAMA1	-1.300	1.299	49.230	.322	-3.911	1.311
	BmCP1	-3.300*	1.299	49.230	.014	-5.911	-.689
	Control	.133	1.299	49.230	.919	-2.478	2.744
Control	BmAMA1	-1.433	1.299	49.230	.275	-4.044	1.178
	BmCP1	-3.433*	1.299	49.230	.011	-6.044	-.822
	BmAMA1/CP1	-.133	1.299	49.230	.919	-2.744	2.478

Based on estimated marginal means

* The mean difference is significant at the .05 level.
[a] Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments)
[b] Dependent Variable: PCV

4. DISCUSSION AND SUMMARY

Babesia bovis is an arthropod-transmitted apicomplexan pathogen infecting cattle in tropical and subtropical regions of the world. *Babesia bovis* infections cause significant morbidity and mortality resulting in devastating production and economic losses. Control of this parasite remains an important issue for cattle industries worldwide. Although *B. bovis* and the vector responsible for transmission were eradicated from the U.S. by 1943, reintroduction is a significant threat. Attenuated forms of the parasite have been used for effective control in endemic areas such as Australia; however, due to their drawbacks noted previously (Introduction, page 13), the development of recombinant vaccines is a more promising method for protection. The recently sequenced *B. bovis* genome (http://www.ncbi.nlm.nih.gob/BLAST/) allows the identification of a number of vaccine candidates, including Cysteine Protease-2 and Apical Membrane Antigen-1.

Cysteine proteases are critical to the pathogenicity of many parasites. Recombinant cysteine protease antigens have been used to stimulate a strong Th1 immune response, increase the rate of survival of infected animals, and inhibit parasites (Engle et al., 1998; McKerrow et al., 1999; Somanna et al., 2002; Rosenthal, 2004). Bart et al. (1997) reported that a cysteine protease-deficient mutant of *Leishmania mexicana* greatly reduced parasite infectivity to macrophages in vitro. The prophylactic potential of cysteine proteases has also been used in both cutaneous Leishmaniasis and visceral Leishmaniasis models of mice and dogs (Rafati et al., 2001; Rafati et al., 2005). Khoshgoo et al. (2008) reported that the parasite load of mice

vaccinated with *Leishmania infantum* Cysteine Proteinase Type III was significantly lower than control animals. Similar studies involving *Trypanosoma cruzi, Toxoplasma gondii* and *Plasmodium* spp. have given encouraging results in the use of cysteine proteases as vaccine targets (McKerrow et al., 1999; Teo et al., 2007). In the current study, to optimize exposure of specific T- cell receptors, the hydrophilic region of *B. microti* CP-1 was selected for expression in the pCMVCD5Flag vector.

AMA-1 is a microneme protein that is highly conserved among apicomplexan parasites including *Plasmodium* spp. *Toxoplasma gondii* and *Babesia* spp. (Chesne-Seck et al., 2005). Experimental evidence has shown that *Plasmodium* spp. AMA-1 is involved in merozoite invasion of erthrocytes, and is essential to the proliferation and survival of malaria (Triglia et al., 2000). *Plasmodium* AMA-1 is a highly immunogenic protein. The majority of individuals exposed to malaria develop anti-AMA-1 antibodies after only a few exposures (Riley et al., 2000). *Toxoplasma gondii* tachyzoites depleted of TgAMA1 are severely compromised in their ability to invade host cells, providing direct evidence that AMA-1 functions during host cell invasion (Mital et al., 2005). For this study, the ectodomain of *B. microti* AMA-1 was incorporated into the pCMVCD5Flag expression vector. The ectodomain is the area of the protein will be exposed on the parasite cell surface so it is the most likely region to encounter antibody, and therefore the best candidate for inclusion in the vector.

The choice of vaccine targets for this study was predicated on considerable evidence of the potential of both cysteine protease and AMA-1 to be effective vaccine components. Cysteine proteases have long been known for their involvement in

parasitic disease; however, they are also known for their involvement in tumor progression, arthritis, osteoporosis and virus replication (Selzer et al, 1999; Turk et al., 2004). Targeting cysteine proteases appears to be a promising strategy in the development of new chemotherapy for a number of diseases, including babesiosis. Immunization of animals with the ectodomain of AMA-1 induces protective immunity against challenge with asexual blood-stage malaria parasites in murine and simian models (Crewther et al., 1996; Stowers et al., 2002) and, thus, has recently been tested in a phase II clinical vaccine trial for *P. falciparum*-human malaria (Sagara et al., 2009).

To create an efficient detection and purification system based upon fusion polypeptides, a specific sequence encoding for the FLAG marker peptide (amino acid sequence, DYKDDDDK) is included at the C-terminus of the pCMV-2CD5Flag vector (Sigma-Aldrich). Because of its hydrophilic nature, the FLAG peptide is likely to be located on the surface of a fusion protein making it readily accessible to anti-FLAG antibodies (McIlhinney, 2004). Our lab has yet to obtain either *B. microti* CP-1 and AMA-1 antigen in substantial amounts, thus we have no specific antibody raised against these proteins. Adding a FLAG-tag to these recombinant proteins has allowed us to detect them using antibody against the FLAG sequence.

BALB/c mice are useful for immunology research involving protozoan parasites because they are highly susceptible to trypanosomiasis, toxoplasmosis, various forms of leishmaniasis and malaria as well as infections from *B. microti* and *Babesia rodhaini* (Shimada et al., 1996; Rafati et al., 2000; Narum et al., 2000; Boyle et al., 2007; Antoine-Moussiaux et al., 2008). BALB/c mice are an inbred strain and are therefore

isogenic (genetically identical). These mice are considered ideal for experimental work because they do not possess the genetic variability that often contributes to inconsistent data. *Babesia bovis* is host specific for bovines, thus cannot infect mice or other small animal laboratory models. Undoubtedly, differences will be seen between murine models of *B. microti* and bovine *B. bovis*. However, this shouldn't be considered as an obstacle to studying bovine babesiosis through mouse models because the advantage lies in the highly conserved genomes found among *Babesia* spp.

Subunit vaccines generally require the addition of an adjuvant to be effective (Singh and O'Hagan, 2002). Given their potent immunostimulatory capacity, bacterial substances are an excellent source of potential adjuvants (Klinman et al., 1999; Klinman et al., 2004). Bacterial cell wall peptidoglycans enhance the immune response without themselves being highly immunogenic. The demonstration that mycobacterial DNA had adjuvant activity led to the discovery that the adjuvant activity is correlated with a higher content of CpG motifs (Introduction, page 21) present in bacterial nucleic acids. DNA that contains CpG motifs is one of the most potent cellular adjuvants, increasing antigen-specific immune responses 5 to 500-fold (Klinman et al., 2004). CpG acts via activation of a Toll receptor pathway thereby promoting the production of T helper 1 (Th1) and pro-inflammatory cytokines (van Duin et al., 2006). Plasmid DNA, apart from encoding the antigen, can also be optimized to include adjuvants such as CpG. The expression vector used in this study incorporates a CpG motif as its adjuvant.

The use of a priming dose of DNA constructs followed by a recombinant protein boost has been reported to be effective in invoking T-cell production against

Plasmodium berghei in mice and *P. falciparum* in Rhesus monkeys (Schneider et al., 1999; Jones et al., 2001; Dunachie and Hill, 2003). However, this method of vaccination requires ample amounts of recombinant protein. Despite having high protein expression in an in vitro cell model prior to use in this study, sufficient amounts of the recombinant proteins from the BmCP1 and BmAMA1 plasmid constructs used in this study could not be obtained through affinity purification. Lysates of BmCP1 and BmAMA1 proteins expressed in HEK293F cells were sampled prior to loading on the purification column and samples of the agarose beads from the loaded purification column indicated the presence of the proteins in both the lysates and the columns by western blotting. However, western blotting also indicated that the protein was not successfully eluted in the expected amounts. There are various reasons that might explain why the proteins are not being purified with high yield: 1) The elution conditions (pH) are not sufficient to disrupt the binding to remove the protein from the column; 2) The proteins may fold in such a way that they are bound too tightly to be effectively eluted and 3) The ratio of resin to protein was not optimal.

Biolistic particle delivery systems such as the Helios Gene Gun (Bio-Rad) have been used successfully to deliver DNA vaccines to humans and experimental animals (Larregina, 2000; Cesco-Gaspere et al., 2008). By accelerating DNA-particle complexes into target tissue, DNA is effectively introduced without the pain and discomfort associated with needles and without the stress/danger of anesthetic. Studies indicate that DNA immunization using conventional needle methodologies is less successful than gene gun immunization in non-human primates and humans. As reported in HIV, DNA

immunization via needle injection is only effective in priming human immune responses when it is given in high (mg) DNA doses (Wang et al., 2008a). Gene gun technology enhances DNA vaccination because it requires only a small amount to elicit a protective immune response, usually less than 2 μg (Pertmer et al., 1995). DNA immunization by the gene gun-mediated delivery approach has been the only vaccination technique able to elicit protective levels of immunity in healthy, adult human volunteers without the use of another vaccine system, i.e. target protein with adjuvant, as a boost (Wang et al., 2008b).

In this study, the protective response to challenge *B. microti* infection that was induced by gene gun DNA vaccination by BmCP1, BmAMA1, or BmCP1 and BmAMA1 combined was compared based on parasitemia and clinical presentation upon challenge infection. The BmCP1 gene and the BmAMA1 gene vaccines induced enhanced protection against *B. microti* parasitemia compared to that of the plasmid control vaccine, P = 0.000 and 0.037, respectively. Although the BmCP1 group and the BmAMA1 group were both significantly superior to the control at protecting the mice against progression of the parasitemia, the BmCP1 vaccine induced greater protection than the BmAMA1 vaccine group (P = 0.047). BmCP1 vaccine was also significantly more effective than BmCP1/BmAMA1 vaccine (P = 0.016); however, there was no significant difference between BmAMA1 and the BmCP1/BmAMA1 vaccines (P = 0.660). This concludes that BmCP1, and to a lesser extent BmAMA1, are effective in decreasing the mean parasitemia of mice challenged with *B. microti.* However, the ability to reduce parasitemia is irrelevant if it fails to prevent clinical manifestations of

the disease. Evaluation of the packed cell volumes of the mice throughout the vaccine showed that only BmCP1 was able to reduce this effect with any level of significance (P = 0.011).

It was anticipated that the dual vaccine, BmCP1 and BmAMA1 combined, would provide the best protection against *B. microti* challenge. Optimal protective responses from Gene Gun delivery have been reported to be dependent on the delivery of a sufficient number of DNA-coated particles, as well as how much DNA coats the gold particles (Eisenbraun et al., 1993). It is possible that the combination vaccine containing a total of 4 μg/shot was too much DNA to properly precipitate onto the 0.5 mg of 1.6 μm diameter gold beads used. Future experiments might incorporate more beads or 0.5 mg of smaller beads to increase the surface volume, thereby allowing better binding of the DNA. In the future, optimization of DNA binding on the beads could be evaluated by in vitro transfection of cell lines to quantitate expression of the recombinant proteins. Using smaller beads also might facilitate their dispersal on the cartridges used to deliver the dose so that a more uniform dose is obtained. A wide range in response to challenge was seen in all of the vaccine groups, which is contrary to the expected results in an inbred mouse strain. This may have been in part a result of uneven dispersal of the DNA-gold complexes as they were coated in a long tube that was then cut into individual dose cartridges for use.

Based on the work of previous researchers and observations of infected BALB/c mice in this laboratory, animals were challenged with 10^7 infected erythrocytes (Iseki et al., 2007). However, because of the rapid onset of parasitemia it is suspected that

this dose may have been too high. The parasites used for challenge were collected from immume-deficient C.B-17/IcrHsd-Prkdc-scid mice. Although 10^7 infected erythrocytes were administered, the number of parasites per erythrocytes was not quantified. C.B-17/IcrHsd-Prkdc-scid mice lack lymphocytes therefore, the parasite replicates at a higher rate in the erythrocytes of these mice resulting in more parasites within a single erythrocyte compared to immunocompetent BALB/c mice. In future, the challenge dose should be determined based on parasite number as much as possible.

This study would be enhanced by the inclusion of assays to determine immune responses of the vaccinated mice and confirmation that cysteine protease and AMA-1 are expressed in vivo. Ideally, seroconversion and cytokine production in the recipients should be examined during the course of the trial. For this trial, recombinant antigen and specific antibody were not available in order to design assays to evaluate the serologic response to vaccination. The standard assay used to evaluate seroconversion is an ELISA assay. Cytokine quantification, particularly the production of IFN-γ, is best measured by an ELISPOT assay. Both the ELISA and ELISPOT assay require specific antigen in order to produce the necessary data. If an antigen was available and serology had been possible, the dosing of the vaccines could have been optimized. The vaccination protocol used in this study was based on successful Gene Gun administered vaccines against apicomplexan parasites (Ishii et al., 2006; Dautu et al., 2007). Ideally, titer levels should be determined by ELISA assays throughout the course of vaccination.

Recombinant protein expression in the recipients was not determined during this study. Larger groups of mice would need to be employed so that animals could be

euthanized at critical timepoints throughout the trial and evaluated for specific protein expression. This might be accomplished through western blotting (Dautu et al., 2007).

Typically, a larger sample size leads to increased precision when making statistical inferences. This study required daily data collection, and the sample size was limited based on cost and available manpower. If repeated, a large sample size should be utilized to ensure greater statistical significance.

While it is obvious that the pBmCP1 and pBmAMA1 pDNA vaccines were able to reduce the mean parasitemia of the vaccinated animals, it did not confer total protection. Ideally, the most successful vaccine would confer protection against the establishment of infection, rather than just reducing the number of parasites. The multiallelic *P. falciprum AMA1-C1* vaccine currently in Phase II clinical trials is an equal mixture of the correctly folded ectodomain portion of recombinant AMA-1 proteins (Sagara et al., 2009). The success seen with this vaccine suggests that recombinant proteins are most effective at conferring total protection against protozoan parasites. Future efforts should focus on obtaining the recombinant BmCP1 and BmAMA1 proteins in high yield for incorporation into a vaccination either alone or in conjunction with BmCP1 and BmAMA1 plasmids.

In conclusion, although a significant protective response was seen using *B. microti* CP-1 and AMA-1 plasmid constructs, vaccine optimization is necessary to confer total protection against the parasite. Secondly, these results indicate future application to a potential *B. bovis* vaccination. This laboratory was able to

obtain *B. bovis* Cysteine Protease-2 and Apical Membrane Antigen-1 for use towards the development of a bovine babesiosis vaccine.

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