THE ROLE OF APICAL MEMBRANE ANTIGEN-1 IN ERYTHROCYTE INVASION BY THE ZOONOTIC APICOMPLEXAN *BABESIA MICROTI*

A Dissertation

by

ISSA BARADJI

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

DOCTOR OF PHILOSOPHY

August 2008

Major Subject: Veterinary Microbiology

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	James E. Womack
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ABSTRACT

The Role of Apical Membrane Antigen-1 in Erythrocyte Invasion

by the Zoonotic Apicomplexan *Babesia microti*. (August 2008)

Issa Baradji, B.S., Institut Polytechnique Rurale de Katibougou, Mali;

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Babesia microti is a tickborne hemoprotozoan parasite that causes the disease babesiosis in humans. *Babesia microti* Apical Membrane Antigen-1 (AMA-1) is a micronemal protein suspected to play a role in erythrocyte invasion. To investigate interaction between AMA-1 and the host cell, the ectodomain region of the *B. microti ama-1* gene was cloned into an expression vector, expressed as a histidine-tagged fusion protein, and used to probe red blood cell membrane proteins in far Western blot assays.

The *B. microti ama-1* ectodomain, which excludes the signal peptide and the transmembrane region of the open reading frame, was amplified from a cloned gene sequence. The AMA-1 ectodomain is a membrane bound polypeptide that extends into the extracellular space and is most likely to interact or initiate interaction with the host red blood cell surface receptor(s). The amplicon was ligated into a protein expression vector to produce a 58.1 kDa recombinant His-tagged fusion protein, which was confirmed by Western blot analysis.

The recombinant *B. microti* AMA-1 fusion protein was enriched on nickel affinity columns and then used to probe mouse, human and horse red blood cell membrane proteins in far Western blot assays. *Babesia microti* AMA-1 consistently reacted strongly with a protein migrating at 49 kDa. A similar reaction occurred between the *B. microti* AMA-1 and horse red blood cell membrane proteins, suggesting that similar interacting proteins of this size are shared by red blood cells from the three species.

The *B. microti* AMA-1 may bind to red blood cell membrane sialic-acid groups, as shown for other *Babesia* spp. This may explain the signal at the 49 kDa position observed between *B. microti* AMA-1 and red blood cell membrane proteins from three different species. Further studies may determine if the binding epitopes of the red blood cell binding partner at this position vary and contribute to the specificity of each parasite AMA-1 for their respective host cells.

DEDICATION

This dissertation is dedicated to my daughter Aissata Koute Baradji who died at a very young age. I still think of her as it was yesterday. Her image will always be with me.

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NOMENCLATURE

AMA-1	Apical Membrane Antigen –1
BmAMA-1	*Babesia microti* Apical Membrane Antigen-1
B. microti ama-1 gene	*Babesia microti ama-1* gene
BmAMA-1 protein	*Babesia microti* AMA-1 protein
ORF	Open Reading Frame
RAP-1	Rhoptry Associated Protein-1
TRAP	Thrombospondin Anonymous Protein
IPTG	Isopropyl β-D-1-thiogalactopyranoside
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel
Ni-NTA	Nickel-Nitrilotriacetic Acid

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CHAPTER I

INTRODUCTION

Babesia microti is a blood parasite that is transmitted by the tick *Ixodes scapularis* and is the most common cause of human babesiosis in the United States (Perry et al., 1977; Dammin et al., 1981; Piesman and Spielman, 1982). There is no reliable diagnostic test available today to detect infection prior to the clinical manifestations of the disease, there are no safe and effective drugs for treatment, and there is no vaccine to prevent transmission (Homer et al., 2000; Krause, 2003; Gelfand and Vannier, 2005).

Proteins from the apical complex of apicomplexan parasites have been shown to mediate invasion of the host cell by these parasites (Dubremetz et al., 1998; Hodder et al., 2001; Yokoyama et al., 2006). These proteins are released from organelles of the apical complex and have been shown to play a critical role in parasite entry, internalization, and multiplication in the host cell.

Apical membrane antigen-1 (AMA-1) is one of these proteins and is secreted from the micronemes. The characteristic features of AMA-1, such as a short transmembrane domain near the C-terminus, a predicted short cytoplasmic C-terminal sequence, and an extracellular domain containing 14 cysteine residues, are typically conserved in the amino acid sequence of the protein for all of the Apicomplexans studied

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This dissertation follows the formatting style of the Journal of Parasitology.

to date. This conservation indicates that this ligand plays an essential role in parasite functions (Triglia et al., 2000; Yokoyama et al., 2006). Antibodies to AMA-1 have been shown in *Plasmodium falciparum* and in *Babesia bovis* to inhibit host erythrocytes invasion by these parasites (Hooder et al., 2001; Mosqueda et al., 2002; Gaur et al., 2004; Silvie et al., 2004).

Host cell receptors involved in interacting with AMA-1 have also been documented (Pasvol et al., 1982; Perkins, 1984; Zintl et al., 2002; Gaffar et al., 2003; Lobo, 2005). Glycophorin receptors on host erythrocytes have been shown to interact with the merozoites during invasion (Gaur et al., 2004). For instance, *B. bovis* merozoites were shown to invade human, ovine, equine, porcine, and caprine erythrocytes by a sialic acid-dependent mechanism (Gaffar et al., 2003). In addition, *Babesia divergens* and *P. falciparum* were shown to use common receptors, glycophorins A and B, to invade the human red blood cell (Pasvol et al., 1982; Lobo, 2005). Treatment of bovine and non-bovine erythrocytes with neuraminidase decreased their susceptibility to invasion by *B. divergens*, which implicates sialic-acid as an important erythrocyte receptor for babesial invasion (Zintl et al., 2002).

Babesia microti AMA-1, as demonstrated in other apicomplexans, is thought to play a role in erythrocyte invasion by this parasite. In this study, interaction of the BmAMA-1 ectodomain ligand with the host erythrocytes is explored using bioactivity assays, which include far Western blot analysis, immunoflurescent sntibody test, and immunocapture assays.

The objective of this study was to construct an expression plasmid containing the *B. microti ama-1* ectodomain in order to produce recombinant protein that was used in downstream applications. Specific primers designed from the ectodomain portion of *B. microti ama-1* were used to amplify this portion from the full *B. microti ama-1* gene sequence (Fig. 1) by the polymerase chain reaction. The amplicon was purified and then ligated into an expression plasmid. The plasmid was sequenced to confirm the presence of the *B. microti ama-1* ectodomain gene insert, as well as to confirm its orientation, and to determine if the sequence was in frame with the histidine tag. The choice of the ectodomain was important because this portion of the membrane bound polypeptide presumably extends into the extracellular space and likely interacts or initiates interaction with the host erythrocyte surface receptors.

CHAPTER II

LITERATURE REVIEW

Babesiosis is caused by hemoprotozoan parasites of the genus *Babesia,* which belong to the phylum Apicomplexa. Apicomplexans are obligate intracellular parasites that cause important parasitic diseases of humans and animals including malaria, babesiosis, coccidiosis, and toxoplasmosis (Mahoney, 1977; Homer et al., 2000). More than 100 *Babesia* species have been reported, but only a few have been identified as causing human infections (Purnell, 1981; Brocklesby, 1979; Gorenflot, 1998; Zintl et al., 2003; Holman, Spencer, Droleskey et al., 2005; Holman, Spencer, Telford III, et al., 2005). However, these parasites infect a wide variety of wild and domestic animals, and together are responsible for enormous economic losses to the livestock industry worldwide (McCosker, 1981; Kuttler, 1988; Taboada and Merchant, 1991).

Babesia are tick-transmitted intraerythrocytic protozoan parasites (Herwaldt et al., 1996; Kjemtrup. and Conrad, 2000; Beattie et al., 2002). Transmission does occur through blood transfusion, organ transplant, or via vertical transmission from mother to newborn (Herwaldt et al., 1997; Kjemtrup and Conrad, 2000). Babesiosis is caused by any one of many *Babesia* species, which infect a wide variety of vertebrate hosts, including domestic and wild animals, as well as man (Mahoney, 1977; McCosker, 1981; Taboada.and Merchant, 1991; Telford et al., 1993).

In the United States, human babesiosis is considered an emerging infectious disease (Kjemtrup and Conrad, 2000; Krause, 2002). *Babesia microti* is the agent most

frequently identified as causing human babesiosis, which occurs mainly in the Northeastern and Midwestern regions of the U.S. (Gray et al., 2002). Although infections are often subclinical, severe disease is seen in immunosuppressed individuals (Homer et al., 2000; Kjemtrup and Conrad, 2000; Krause, 2002). Human babesiosis caused by *B. microti* first was described from sites along the Northeastern United States, and later from Minnesota and Wisconsin (Perry et al., 1977; Dammin et al., 1981; Piesman and Spielman, 1982). *Babesia microti* is transmitted by the deer tick, *Ixodes scapularis* (Kjemtrup and Conrad, 2000; Krause, 2002). Its most common vertebrate reservoir is the white-footed mouse (*Peromyscus leucopus*) (Borggraefe et al., 2006; Gray et al., 2002). Humans enter the cycle when bitten by infected ticks or by transfer of infected blood to an immunocompromised individual. The *Babesia*-infected tick introduces sporozoites into the human host during a blood meal. The sporozoites enter erythrocytes and undergo asexual replication. The parasites at this stage are referred to as piroplasms due to their pear-shaped appearance when within the infected host cells (Suarez et al., 1991; Kjemtrup and Conrad, 2000).

Multiplication of the blood stage parasites is responsible for the clinical manifestations of the disease (Gorenflot et al., 1998; Kjemptrup and Conrad, 2000). Humans are dead-end hosts and there is little or no subsequent transmission from ticks feeding on infected persons. But, human-to-human transmission is well documented and occurs through blood transfusions or organ transplantations (Herwaldt et al., 1996; Beattie et al., 2002).

Recently, worldwide interest in human babesiosis has increased as a result of human cases caused by new species distinct from *B. microti* and *B. divergens* European isolate (Herwaldt et al., 1996; Beattie et al., 2002; Kjemtrup et al., 2002; Holman, Spencer, Telford III et al., 2005; Holman, 2006). In Europe, human babesiosis is rare but often severe. On the basis of morphology and antigen reactivity, most cases have been attributed to the cattle pathogen *B. divergens*. However, some of these cases may have been due to a closely related organism called the European Strain (Herwaldt et al., 2003).

Symptoms in human babesiosis range from a silent infection to severe malaria-like disease which often results in the death of the infected host. Various determinants such as age, immune status of the infected host and coinfection with other pathogens appear to play a role in the severity of the disease manifestations (Brocklesby, 1979; Mahoney, 1979; Beattie et al., 2002; Krause, 2002; Krause, 2003).

Infections caused by *B. microti* in Europe may be underdiagnosed. *Babesia microti* has been detected in *I. ricinus*, the tick that transmits *B. divergens* to cattle. This tick has been collected in various regions in Switzerland (Gray et al.,2002; Foppa et al., 2002) and in Poland as well (Karbowiak, 2004). *Ixodes ricinus* also is an efficient vector for *B. microti*, which indicates that incidence of this parasite in Europe may be underestimated (Duh et al., 2005; Meer-Scherrer et al., 2004). Serum from residents in Germany and Switzerland react to *B. microti* antigen (Hunfeld et al., 2002; Foppa et al., 2002) indicating active or resolved infection with *B. microti*. In spite of all these

findings, the incrimination of *B. microti* in human illness in Europe is fairly recent (Duh et al., 2001; Foppa et al., 2002; Gray et al., 2002; Duh et al., 2005).

DISEASE SYMPTOMS AND DIAGNOSIS

Babesiosis is characterized by fever, anemia, icterus, and hemoglobinuria (Brocklesby, 1979; Krause, 2003). These symptoms are mainly due to the continuous cyclic asexual multiplication of the parasites in the host erythrocytes and the rupturing of the latter to invade new erythrocytes (Krause 2003). The severity of babesiosis is associated with this asexual reproduction, which leads to high parasitaemia, erythrolysis, anemia, and malaise in the infected animals (Taboada and Merchant, 1991; Herwaldt et al., 1996; Beattie et al., 2002; Krause, 2003).

The diagnosis of babesiosis is based on the above cited symptoms and the observation of the parasites in stained blood smears (Fig. 1) (Kjemtrup and Conrad, 2000; Krause, 2003). Parasite presence also can be confirmed by using diagnositc DNA probes to target parasite antigen in blood of infected hosts. The indirect fluorescent

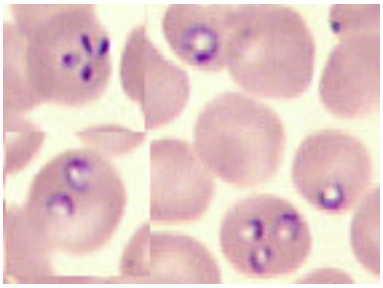


Figure 1. *Babesia microti* in mouse blood.

antibody test (IFAT) mainly is used for diagnosis of *B. microti* infection and has been specifically recommended for detecting the presence of IgG antibodies to *B. microti* (Taboada and Merchant, 1991; Brasseur et al., 1998). Additional tests include the enzyme-linked immunosorbent assay (ELISA), and Polymerase Chain Reaction (PCR) (Homer et al., 2000; Krause, 2003; Gelfand and Vannier, 2005). Antibodies to *Babesia* can be detected by IFAT and ELISA prior to the identification of the parasites in stained blood smears (Telford et al., 1993; Krause, 2003), but these assays cannot distinguish between active and resolved infections. PCR, a sensitive DNA detection test, detects early infection of the parasite before the development of detectable antibody, but does not provide information on the level of parasitemia (Homer et al., 2000; Krause, 2003; Gelfand and Vannier, 2005). However, real-time PCR allows detection of the increase in the amount of DNA as it is amplified and results using this assay correlated well with microscopic results (Swan et al., 2005).

CONTROL OF BABESIOSIS

In the United States, control of zoonotic babesial infections includes the use of acaricides, destruction of the tick habitat, and removal of animal reservoirs (Kuttler, 1988; Kjemtrup and Conrad, 2000; Krause 2002). Such measures are not used in Europe because of the low incidence of the infection. But, measures should be taken to avoid exposure to areas where zoonotic babesiosis is endemic, such as simple avoidance to habitat modification, use of tick repellents before entering a tick-infested area, thorough examination of skin after exposure, daily self-examination for persons engaged in

outdoor activities in endemic areas and examination of pets for ticks (Dewaal, 2000; Kjemtrup and Conrad, 2000; Krause, 2003).

Early treatment of babesial infections in humans, with well-recognized antimalarial drugs such as chloroquine, quinine, mefloquine and pyrimethamine, and pentamidine are not successful (Raoult et al., 1987; Brasseur et al., 1996; Denes et al., 1999; Krause, 2003). Although chloroquine therapy improves symptoms in some patients infected with *B. microti*, the parasitemias appear to be maintained (Krause, 2003). Berenil (diminazene aceturate), an antitrypanosomal compound known to be effective in veterinary cases, failed to cure a patient with a severe *B. divergens* infection (Raoult et al., 1987; Brasseur et al., 1996; Brasseur et al., 1998). In general, drug treatment of human babesiosis has failed because of the difficulty in clearing the parasite, and also the toxicity of the therapeutic procedures is problematic (Raoult et al., 1987; Brasseur et al., 1996). Today, the recommended treatment of human babesiosis consists of a massive blood exchange transfusion followed by administration of intravenous clindamycin and oral quinine together (Brasseur et al., 1996; Denes et al., 1999; Krause, 2003). The efficiency of clindamycin and quinine in clearing the parasite has been shown by *in vitro* experiments, yet *in vivo* experiments have the risk of increased parasitemia, consequent hemolysis, and renal damage (Brasseur et al., 1996; Brasseur et al,. 1998). Atovaquone is registered for use in humans and has been shown to clear *B. divergens* in human erythrocytes *in vitro* (Brasseur et al., 1998; Krause, 2003). The drug was reported to be effective *in vivo* when used daily at low doses in a

gerbil model (Krause, 2003) and has been suggested for prevention in immunosuppressed individuals when exposed to human babesiosis (Krause, 2003).

HOST CELL INVASION PROCESS IN APICOMPLEXANS

Studies have shown that interactions between merozoites and host erythrocytes during the asexual growth cycle of *Babesia* are mediated by adhesive interactions of several parasite ligands and target receptors on host eryhtrocytes (Dubremetz et al.,1998; Zint et al., 2002; Yokoyama et al., 2006). During the invasion process, several surface-coating molecules of merozoites might be involved in the initial attachment and entry of the parasite of the erythrocytes. Many proteins secreted from apical organelles (rhoptry, micronemes, and dense granules) have been identified as playing a role in erythrocyte penetration or internalization (Dubremetz et al., 1998). Studies have been carried out to identify interacting molecules in merozoite invasion of erythrocytes (Igarashi et al., 1988; Gaffar et al., 2003; Yokoyama et al., 2006). The detailed molecular interactions between merozoites and receptors on host erythrocytes remain incompletely understood (Dvorak et al., 1975; Jack and Ward, 1981; Dubremetz et al., 1998). This lack of detailed knowledge of interacting molecules has hindered the development of a good vaccine and therapeutic drugs for babesial infections (Thomas et al., 1984; Yokoyama et al., 2006).

The erythrocytes invasion process of *B. microti,* like many apicomplexans is thought to mimic that of *Plasmodium falciparum* and is mediated by an apical complex located at the anterior end of the parasite (Dvorak et al., 1975; Mota et al., 2002; Gaur et al., 2004). Secreted proteins from the apical complex (rhoptries and micronemes, dense granules) are thought to play a central role in the sequence of events leading to cell

invasion by apicomplexan parasites (Taylor et al., 1990; Gaffar et al., 2004; Yokoyama et al. 2006).

HOST CELL RECEPTORS INVOLVED IN INVASION BY APICOMPLEXANS

Many studies have documented the nature of the erythrocyte receptors involved in invasion by the Apicomplexan parasites (Gaffar et al., 2004; Yokoyama et al. 2006). Sialic acid residues, protease-sensitive proteins, or sulphated glycosaminoglycans located on the surface of the erythrocytes have been identified as the host erythrocytes receptors for babesial invasion (Igarashi et al., 1988; Dubremetz et al.,1998; Gaffar et al., 2003; Yokoyama et al., 2006). Sialic acid residues on bovine erythrocytes have been implicated to function as the receptors for *B. bovis*, *B. bigemina*, and *B. divergens* **(**Zintl et al., 2002; Gaffar et al., 2003). Pretreatment of the bovine erythrocytes with neuraminidase significantly decreases invasion by the parasite. In addition, *B. bovis* and *B. divergens* invade human and other animal erythrocytes in a sialic acid-dependent mechanism (Zintl et al., 2002).

PARASITE LIGANDS INVOLVED IN INVASION OF HOST CELL

Members of the apicomplexa infect different hosts and cell types, but they have similar host cell invasion processes. Unlike *Plasmodium* parasites, which require hepatocytes invasion and multiplication prior to the erythrocytic stage, sporozoites of *Babesia* parasites can directly invade the host erythrocytes and undergo an asexual growth cycle in the merozoite stage (Gelfand and Vannier**,** 2005). Since Rhoptry Associated Proteins 1 (RAP-1), *Babesia bovis* Apical membrane Antigen 1 (BbAMA-1), and *Babesia bovis* Thrombospondin Anonymous Protein (BbTRAP) are all detectable

in the supernatant of a *B. bovis* culture, these proteins seem to function as secretory proteins during erythrocyte invasion by merozoites (Gaffar et al., 2004).

Although many proteins are known to play an important role in invasion, their functional characteristics remain unclear. A complete understanding of the molecular interactions involved in the invasion process is crucial in the design of therapeutics and vaccines to counter these diseases (Gaur et al., 2004).

It is well known in malaria that the plasmodium invasion process begins by reversible attachment, followed by reorientation of the merozoite, which brings the apical end of the parasite in contact with the plasma membrane of the erythrocyte (Dvorak et al., 1975; Jack and Ward, 1981; Mota and Rodriguez, 2002; Gaur et al., 2004). A tight junction is formed through which the parasite invades the red blood cell. From the first attachment until completion of the invasion process the parasite secretes proteins from apical organelles into the merozoite membrane and into the environment. The process is completed when the parasite is inside the erythrocytes (Dvorak et al., 1975; Jack and Ward, 1981; Gaur et al., 2004). The role of these proteins in the entry, internalization and multiplication of the parasites in the host cell yet needs to be determined (Jack and Ward, 1981; Dubremetz et al., 1998; Gaffar et al., 2004; Yokoyama et al., 2006). Proteins released from *Babesia* spp. apical end are often soluble and secreted by the parasite during host erythrocyte invasion (Perkins, 1984; Dubremetz et al., 1998; Yokoyama et al., 2006).

The roles played by parasites ligands Apical Membrane Antigen-1 (AMA-1), Rhoptries Associated proteins (RAP), and Thrombospondin-Related Anonymous Protein

(TRAP) during host cell invasion have been the focus of many scientists (Collins et al., 1994; Anders et al; 1998; Dubremetz et al., 1998; Triglia et al., 2000; Adrian et al., 2000; Soren et al., 2000; Hodder et al., 2001; Mosqueda et al., 2002; Gaur et al., 2004; Silvie et al., 2004; Yokoyama et al., 2006). Ligands from apicomplexan parasites are speculated to mediate the host cell invasion by these parasites. The role of these ligands in host cell invasion is still not well understood in spite of all the data collected so far supporting this view (Hodder et al., 2001; Triglia et al., 2000; Yokoyama et al., 2006).

AMA-1 is a micronemal protein secreted to the surface of merozoites of *Plasmodium* species and *Toxoplasma gondii* tachyzoites in order to fulfill an essential but uncharacterized function in host cell invasion (Triglia et al., 2000; Mota and Rodriguez, 2002; Gaur et al., 2004; Yokoyama et al., 2006). Although the biological function of AMA-1 is unknown, the subcellular localization, stage-specific expression, and secretion during host cell invasion suggest that it is involved in merozoite invasion (Triglia et al., 2000; Yokoyama et al., 2006). An AMA-1 homologue is present in all *Plasmodium* species studied to date and in *T. gondii*, which supports the suggestion that this protein is involved in an essential function (Triglia et al., 2000; Hodder et al., 2001; Silvie et al., 2004). That it functions in erythrocyte invasion is demonstrated by the ability of anti-AMA-1 IgG to inhibit parasite invasion (Collins et al., 1994; Anders et al., 1998; Adrian et al., 2000; Silvie et al., 2004). Also, a strong correlation exists between protection and *Plasmodium* AMA-1 (Thomas et al., 1984; Collins et al., 1994). AMA-1 homologs have been found in many apicomplexans parasites and the characteristic features of this parasite ligand, such as a transmembrane domain near the C terminus, a

predicted short cytoplasmic C-terminal sequence, and an extracellular domain containing 14 cysteine residues, are typically conserved in the amino acid sequence (Adrian et al., 2000; Gaffar et al., 2004; Gaur et al., 2004; Zhou et al., 2006). *Babesia bovis* AMA-1, like the *Plasmodium* spp. AMA-1, is implicated in erythrocyte invasion by the parasite. This is evidenced by the inhibition of invasion by parasites into bovine erythrocytes using anti-AMA-1 peptide specific antibodies to *P. falciparum* AMA-1 (Gaffar et al., 2004; Yokoyama et al., 2006). The *B. bovis* AMA-1, with molecular weight of 82 kDa, was localized to the apical half of merozoites with peptide specific antibodies (Gaffar et al., 2004).

Protective immunity is induced in squirrel monkeys by recombinant AMA-1 from *Plasmodium fragile* (Collins et al., 1994), whereas mice are protected against infection by *Plasmodium chabaudi* after immunization with recombinant AMA-1 (Anders et al., 1998). The existence of AMA-1 as homologs in different parasite genera is suggestive of its functional relevance (Taylor et al., 1990; Suarez et al., 1991). The specificity of the protective antibody to AMA-1 has been documented in *P. falciparum* (Hodder et al., 2001). Anti-AMA-1 antibodies from human and rabbit strongly inhibit merozoite invasion from homologous and heterologous strains of *P. falciparum* (Hodder et al., 2001). In addition, the inhibitory effects of the antibodies are within ectodomain of the ligands and are directed to the conserved and strain-specific epitopes (Hodder et al., 2001).

Rhoptries are complex organelles that contain numerous proteins, many of which are immunogenic (Dalrymple et al., 1993; Dubermetz et al., 1998; Gaur et al., 2004).

Rhoptry-associated proteins 1 (RAP-1) from *B. bovis* and *Babesia bigemina* play a role in invasion of these parasites (Suarez et al., 1991; Dubremetz et al., 1998; Yokoyama et al., 2002). The best-characterized rhoptry protein in *B. bigemina* and *B. bovis* is the 58- to 60-kDa rhoptry-associated protein 1 (RAP-1), which is detected on the merozoite surface in both species (Suarez et al., 1991; Suarez et al., 1994). RAP-1 is encoded by two genes in *B. bovis* and multiple polymorphic genes in *B. bigemina* (Suarez et al., 1991; Yokoyama et al., 2002). *Babesia bovis* RAP-1 has an apical localization and is clearly detectable as a rhoptry component by immuno-electron microscopy (Dalrymple et al., 1993; Suarez et al., 1991). RAP-1-encoding genes also have been identified in *Babesia canis*, *Babesia ovis*, and *Babesia divergens* (Suarez et al., 1991). Members of the RAP family have retained four conserved cysteine residues and considerable sequence homology. Furthermore, several conserved oligopeptide motifs are shared by the different RAP-1 proteins (Dalrymple et al., 1993; Suarez et al., 1991). Despite polymorphism in nucleotide sequences located within the RAP-1 open reading frame in *B. bovis* and *B. bigemina*, defined B-cell surface epitopes and the molecular size are conserved (Suarez et al., 1994). Conserved residues in amino-acid sequences in rhoptry proteins within and across genera indicate the importance of the role played by this parasite ligand in invasion (Taylor et al., 1990; Suarez et al., 1994).

The ability of rhoptry-associated proteins to induce partial protective immunity against parasite challenge has been documented for several apicomplexan parasites (Mosqueda et al., 2002; Yokoyama et al., 2002). Protection was demonstrated with

Plasmodium sp. by using rhoptry protein RAP-1 (Anders et al., 1998; Mosqueda et al., 2002; Gaffar et al., 2004).

An erythrocyte-binding assay with the lysate of *B. bovis* merozoites proved the binding ability of RAP-1 to bovine erythrocytes (Yokoyama et al., 2002). Moreover, anti-RAP-1-specific antibodies prevented the interaction of RAP-1 with bovine erythrocytes and these antibodies significantly inhibited the proliferation of *B. bovis* in *in vitro* culture (Yokoyama et al., 2002). These findings indicate that the RAP-1 plays a significant role in erythrocyte cell invasion by merozoites (Yokoyama et al., 2002; Gaffar et al., 2004).

Thrombospondin-related anonymous protein (TRAP) is another miconemal protein found in *Babesia* that has analogy to their most closely related counterparts in *P. falciparum* (Scarselli et al., 1993; Sharma et al., 1996; Soren et al., 2000). The *B. bovis* TRAP has only been found during the asexual erythrocytic stage and is located at the apical complex. A 75-kDa protein is secreted in the extracellular milieu, which is recognized by TRAP antisera (Gaffar et al., 2004). In addition, specific antibodies to *B. bovis* TRAP inhibit bovine erythrocyte invasion by the merozoites.

Plasmodium falciparum TRAP is detected in sporozoites rather than in merozoites (Gaffar et al., 2004; Yokoyama et al., 2006). TRAP homologues in *Plasmodium* spp. are identified as parasite ligands which interact with the host cell (Muller et al., 1993; Sultan et al., 1997). They share several adhesive domains that are not conserved. These findings indicate that TRAP is directly involved in both

recognition and invasion of host cells only in *Plasmodium* spp. and not in other apicomplexans.

From the data above aimed at elucidating the role played by apicomplexan apical ligands in host cell invasion, AMA-1 appears to play a critical role in this process. RAP-1 proteins have high sequence homology and conserved oligopeptide motifs, but RAP-1 is encoded by two genes in *B. bovis* and multiple polymorphic genes in *B. bigemina* (Suarez et al., 1991; Yokoyama et al., 2002) indicating a lack of strict conservation. In addition, RAP-1 proteins are able to produce only a partial immunity during invasion by merozoites (Yokoyama et al., 2006), which suggests that this ligand plays a minor role in the process of invasion.

TRAP involvement in host cell invasion needs further confirmation. TRAP has been implicated in gliding motility of *Plasmodium* and *Toxoplasma* (Muller et al., 1993; Sharma et al., 1996; Soren et al., 2000; Yokoyama et al., 2006). TRAP in malaria has been detected in sporozoites rather than in merozoites, whereas in *B. bovis* TRAP has only been found during the asexual erythrocytic stage, which indicates lack of conservation of this ligand between parasitic stages in apicomplexans. In addition, antibodies against TRAP do not inhibit invasion of erythrocytes by *P. falciparum* (Soren et al., 2000). On the other hand, fragments of monoclonal IgG to a merozoite apical antigen inhibited *P. knowlesi* invasion of erythrocytes, immunization of mice with recombinant AMA-1 protected mice against infection with *P. chabaudi*, and protective immunity was induced in squirrel monkeys immunized with recombinant AMA-1 antigen of *P. fragile* (Thomas et al., 1984; Collins et al., 1994; Anders et al., 1998).

Evidence of strain specific protective immunity by anti-AMA-1 antibodies has been described in *Plasmodium* spp. (Anders et al., 1998). Cross protection has been demonstrated using *P. falciparum* AMA-1 anti-peptides antibodies to inhibit bovine erythrocyte invasion by *B. bovis* (Urquiza et al., 2000; Gaffar et al., 2004; Yokoyama et al., 2006). Complementation of *P. falciparum* AMA-1 with AMA-1 from *P. chabaudi* allows the human parasite *P. falciparum* to efficiently invade mouse cells (Triglia et al., 2000). These ligands therefore play a critical function in cell invasion by *Plasmodia* species. The remarkable conservation of the AMA-1 molecule within and acrross genera and its protective role make it an excellent candidate for developing efficient therapeutics and vaccine for apicomplexan pathogens (Waters et al., 1991; Denise et al., 1996; Hodder et al., 2001).

CHAPTER III

EXPRESSION PLASMID CONSTRUCT

INTRODUCTION

The objective of the line of experiments in this chapter was to prepare an expression plasmid construct with the *B. microti* ectodomain sequence. The BmAMA-1 ectodomain sequence was amplified from the *B. microti ama-1*gene then cloned into the pET TOPO 101D expression vector, to provide a histidine-tagged fusion protein for affinity column capture. The plasmid construct carrying the correct sequence of the BmAMA-1 ectodomain in frame with the His-tag and the ATG initiation codon with no internal stop codon was selected for expression.

MATERIALS AND METHODS

Amplification of the *Babesia microti ama-1* ectodomain

The *B. microti ama-1* ectodomain was amplified using primers pETSigBm F (5'-CAC CATGAACG CAAGACCC GC-3') and pETTmBm R (5'-GCCATARAAAGT-CAATGGTGTTTGGTTCAATGTCTCACT-3') (Fig. 2) designed from the full sequence of the *B. microti* AMA-1 gene according to manufacturer's instructions (Champion pET Directional Expression kit; Invitrogen, Carlsbad, CA). The forward strand primer incorporated a 4-base overhang (5'CACC) directly preceding the ATG start codon. The *B. microti ama-1* ectodomain was amplified using a high fidelity polymerase according to manufacturer's instructions (Phusion High-Fidelity PCR Kit, New England Biolabs,

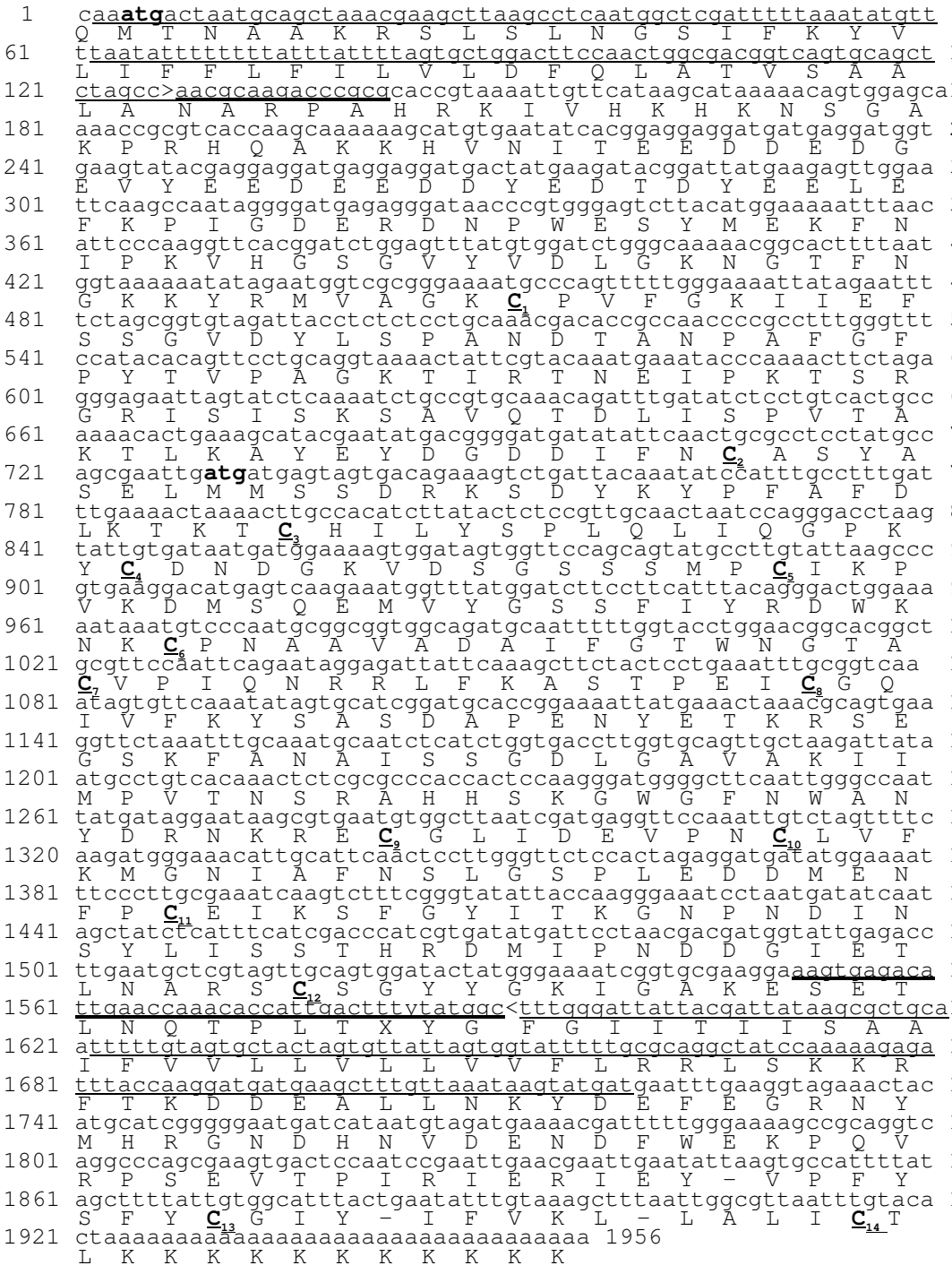


Figure 2. *Babesia microti* AMA-1 full gene sequence with predicted amino acid residues. The ectodomain is shown by < following the signal peptide (underlined) and > preceding the transmembrane region (underlined). Primer sequences (bold underline) and conserved cysteines $C_{1\text{-}14}$ are shown.

Beverly, MA) in a 12.5 µl total reaction volume. The cycling protocol included a hot start at 98 C for 30 sec, followed by 25 cycles of 98 C for 10 sec, 67 C for 10 sec, and 72 C for 1.5 min with a final extension at 72 C for 5 min. Amplified products were separated by 1% agarose gel electrophoresis, stained with ethidium bromide (CLP, San Diego, CA) and viewed by UV transillumination. The size of the amplicon was determined by comparison to a 100 bp DNA size ladder (Invitrogen, Carlsbad, CA).

Purification of the amplified *B. microti ama-1* ectodomain

The *B. microti ama-1* ectodomain amplicon was purified using QIAGEN Quick Purification Kit (QIAGEN, Valencia, CA). Briefly, 5 volumes of Buffer PB1 were added to the sample and mixed by stirring with a pipette. The mixture was applied to the column and the flow-through collected by centrifugation in a 2 ml collection tube and discarded. The column was washed with 750 µl of Buffer PE and the flow-through was collected by centrifugation and discarded. The residual wash buffer was removed by a second centrifugation and discarded. The column was placed into a new 1.5 µl microcentrifuge tube then 30 µl of deionized water was added to the column and the *B. microti ama-1* ectodomain amplicon was eluted by centrifugation. One microliter of the purified DNA was electrophoresed through and visualized on an ethidium bromide stained 1% agarose gel by ultraviolet transillumination. The DNA concentration was determined by spectrophotometry (NanoDrop ND-1000 Spectrophotometer, NanoDrop Technologies, Inc., Wilmington, DE).

Ligation and bacterial transformation

The purified *B. microti ama-1* ectodomain obtained above was ligated into the pET101/D-TOPO® C-terminal V5, 6xHis expression vector according to the protocol outlined in the Champion pET Directional TOPO Expression Kit (Invitrogen, Carlsbad, CA). A total volume of 6 µl of ligation reaction was prepared containing 8 ng of the purified PCR product and 1 µl of the pET101/D-TOPO vector. The reaction was mixed and incubated at room temperature for 5 min, then transferred to ice prior to transformation of TOP10 Competent *E. coli* cells.

TOP10 Competent *E. coli* cells were transformed with the ligation product according to manufacturer's instructions (Champion pET Directional TOPO Expression Kit, Invitrogen, Carlsbad, CA). Briefly, the cells were thawed on ice and 3 µl of ligation mixture from above was added and mixed gently with a pipette tip. The transformation mixture was incubated on ice for 20 min, then heat shocked for 30 sec at 42 C. Then, 250 µl of room temperature SOC (2% Tryptone, 0.5%Yeast Extract, 10 mm NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mm $MgSO_4$, 20 mM glucose) was added and the culture incubated on a shaker at 37 C at 200 rpm for 1 hr. Two LB agar plates containing 50 µg/ml carbenicillin were inoculated with 100 or 200 µl of culture and incubated overnight at 37 C.

Selection of clones

Colony PCR using pETTBm R and pETSigBm F primers was performed to detect clones carrying the BmAMA-1 ectodomain insert from among 24 colonies. Briefly, a total volume of 286 µl of master mix was made using 260 µl of 2X QIAGEN

PCR Buffer and 10.4 µl (104 pmol) each of the forward and reverse primers, and 5.2 µl of ultrapure water. A portion of each of the 24 selected colonies was transferred into 9 µl of ultrapure water and 11 µl of master mix was added to each tube. Two additional reactions with BmAMA-1 pDNA and water served as positive and negative control respectively. The cycling protocol included an initial denaturation for 2 min at 96 C, followed by 30 cycles of 96 C for 30 sec, 55 C for 30 sec and 72 C for 2 min, and a final extension at 72 for 10 min (PCR Express thermocycler, Hybaid). The resulting amplicons were analyzed on an ethidium bromide stained 1% agarose gel alongside a 100 bp DNA ladder (Invitrogen, Carlsbad, CA) and viewed by UV transillumination.

Expansion and plasmid DNA extraction of selected colonies

Eight clones were selected based on the presence of the appropriate sized amplicon determined above. The clones were expanded in 10 ml LB broth containing 50 µg/ml carbenicillin at 37 C and 200 rpm overnight. Plasmid DNA was prepared according to the manufacturer's instructions (QIAGEN Plasmid DNA miniprep kit, QIAGEN, Valencia, CA). Briefly, the cultures were centrifuged to pellet the *E. coli* cells at 1800 *g* for 30 min, the supernatant was removed from each, and the pellets were resuspended in 500 µl buffer P1. Lysis buffer P2 (500 µl) was added to the suspension, which was then mixed by gently inverting the tube 6 times. Then, 700 µl buffer N3 neutralization buffer was added and the suspension mixed immediately and thoroughly by inverting the tube 6 times. The tubes were centrifuged at 13,000 *g* for 10 min in a microcentrifuge. The supernatant was recovered and applied to the QIAprep Spin Column. The column was centrifuged for 60 sec and the flow-through was discarded.

The column was washed with 500 µl of binding Buffer B, then the column was centrifuged for 60 sec and the flow-through discarded. Then, 750 µl of wash buffer PE was added to the column then centrifuged for 60 sec. The flow-through was discarded then the column was centrifuged for an additional 60 sec to remove residual wash buffer. The column was placed in a new 1.5 ml microcentrifuge tube. To elute the plasmid DNA, 30 µl of nuclease free water was added to the center of each column, allowed to stand for 1 min, then centrifuged for 1 min to collect the flow-through. The collected plasmid DNA was size-analyzed on ethidium bromide stained 1% agarose and the DNA concentration determined by spectrophotometry (NanoDrop ND-1000 Spectrophotometer, NanoDrop Technologies, Inc., Wilmington, DE). The presence of the BmAMA-1 ectodomain insert in the plasmid DNA was reconfirmed by PCR using primers pETTBm R and pETSigBm F. BmAMA-1 ectodomain inserts in 8 confirmed clones were sequenced (Davis Sequencing, Davis, CA) using T7 and T7 Reverse primers. The insert nucleotide sequence, orientation, and frame relative to the vector His-tag were analysed by Sequencher^{TM} 4.2 (Gene Codes Corporation, Ann Arbor, MI).

RESULTS

Amplification of the *B. microti ama-1* ectodomain produced an amplicon of approximately 1500 base pairs (Fig. 2). After purification, the DNA concentration of the amplicon was 212.68 ng/µl.

Cloning the *B. microti ama-1* ectodomain amplicon into the pET101/D-TOPO vector yielded 16 colonies with inserts out of 24 tested (Fig. 3). Of these, eight were selected for expansion and plasmid DNA preparation. The purified plasmids were

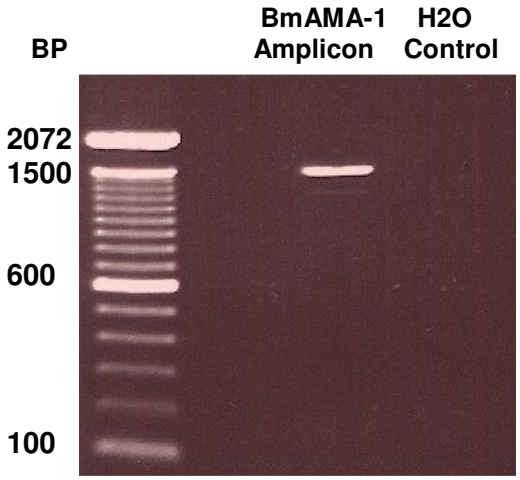


Figure 3. *Babesia microti* AMA-1 ectodomain amplicon. Ethidium bromide-stained 1% agarose gel. Lane 1: 100 bp DNA ladder, 3: *B. microti* AMA-1 ectodomain 1563 bp, 4: water control.

confirmed to contain the insert by PCR (Fig. 4). Upon sequencing, Clone 7 possessed the full nucleotide sequence (1563 bp) of the BmAMA-1 ectodomain in frame with the vector histidine tag and with no internal stop codons (Fig. 5). The ectodomain spans 1563 bp with an additional 96 bp for the vector, which includes 18 bp for the 6 His-tag residues. The DNA concentration of the BmAMA-1 Clone 7 plasmid was determined to be 212.68 ng/µl. This clone was used for recombinant protein expression.

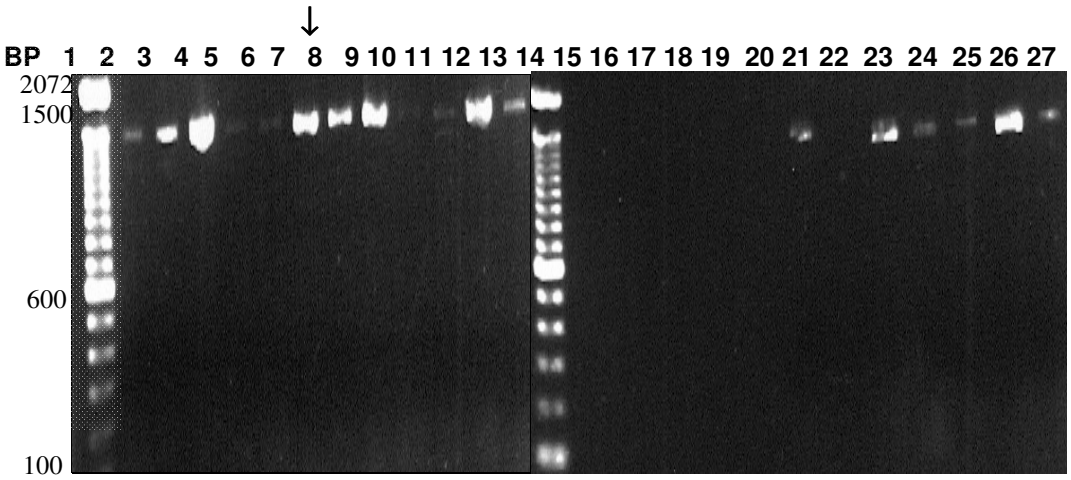


Figure 4. Colony PCR amplicons. *Babesia microti ama-1* ectodomain. 1% agarose gel stained with ethidium bromide. Lanes 1 and 14, 100 bp DNA Size marker; Lanes 2-12 and 16-27, tested colonies; Lane 13, *B. microti ama-1* DNA control; Lane 15, water control. Clone 7 is indicated by an arrow.

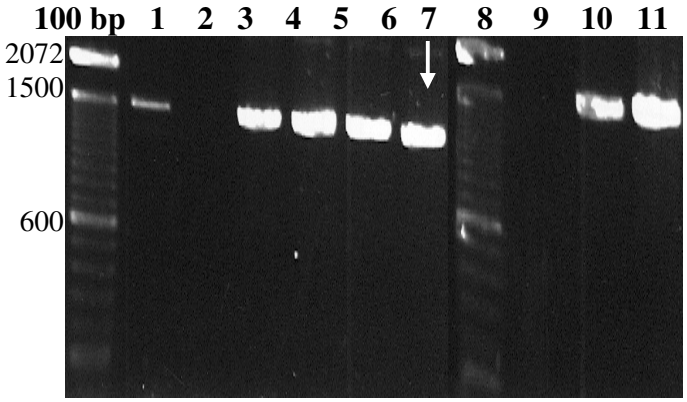


Figure 5. Plasmid constructs with *B. microti ama-1* ectodomain. Electrophoresed in 1% agarose gel Lanes 1 and 8, 100 bp DNA size marker (100 to 2072 bp). Lanes 2-7 and 10, cloned plasmid DNA. Lane 9 negative control. Clone 7 is indicated by an arrow.

CHAPTER IV

BABESIA MICROTI AMA-1 RECOMBINANT PROTEIN

INTRODUCTION

The objective of this line of experiments was to express the *B. microti* AMA-1 (BmAMA-1) ectodomain recombinant protein in a prokaryotic expression system. The expressed recombinant protein was enriched for use in downstream applications to determine its interactions with erythrocytes membrane proteins.

MATERIALS AND METHODS

Recombinant BmAMA-1 ectodomain protein expression in BL21 Star™ (DE3) One Shot® *E. coli*

BL21 Star™(DE3) One Shot® *E. coli* were transformed with BmAMA-1/pET Clone 7 according to manufacturer's recommendations (Invitrogen, Carlsbad, CA). Briefly, the DNA (8 ng in 2 µl) was mixed gently into BL21 Star™(DE3) One Shot® cell suspension and then the mixture was incubated on ice for 30 min. The cells were heat-shocked for 30 sec at 42 C without shaking and the tube immediately transferred to ice. After addition of 250 µl of room temperature SOC, the tube was incubated horizontally at 37 C for 30 min with shaking at 200 rpm.

Recombinant protein pilot expression in BL21 Star™ (DE3) One Shot® *E. coli*

The entire transformation reaction was added to 10 ml of LB broth containing carbenicillin (50 µg/ml) and the culture was grown overnight at 37 C with shaking at 200 rpm. The overnight culture (500 µl) was used to inoculate 10 ml of LB broth

containing carbenicillin and the cultures were grown for 2 hr at 37 C with shaking at 200 rpm. The OD_{600} was checked and was about 0.65 (midlog phase). The cultures were divided into two 5 ml cultures, with addition of IPTG to a final concentration of 1.0 mM to one of the cultures. For 0 hr time point samples, a 500 µl aliquot was collected from each culture, centrifuged at 13,000 g for 30 sec, the supernatant aspirated and discarded, and pellets frozen at –20 C for later analysis. The cultures were incubated at 37 C with shaking at 200 rpm and monitored over time for 6 hr with samples taken each hour as above.

The collected samples were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Briefly, each pellet was resuspended in SDS-PAGE sample buffer according to manufacturer's recommendations for the expression system used (Invitrogen), boiled for 5 min, then centrifuged briefly. The samples (10 µl each) were loaded on a vertical 12% polyacrylamide gel containing 10% SDS (12% SDS-PAGE) (Laemmli et al., 1970) alongside a molecular weight marker (Low Range Molecular Weight Marker; BIO-RAD, Hercules, CA). Electrophoresis was done in Mini PROTEAN II dual slab cell (BIO-RAD). The gel was run for 1 hr at 180 V. The gel was stained with Coomassie blue, a digital image acquired using AlphaEaseFc (Alpha Innotech, Berkeley, CA), and the expression profile was analyzed.

Large-scale recombinant protein expression in BL21 Star™ (DE3) One Shot® *E. coli*

One Erlenmeyer flask containing 100 ml of LB broth with carbenicillin (50 µg/ml) was inoculated with 2 ml of an overnight 10 ml culture obtained from a BL21 Star™(DE3) One Shot® *E. coli* transformation as above. The 100 ml culture was grown

for 2 hr at 37 C with shaking (225 rpm) to an OD_{600} of 0.5, then split into two 50 ml cultures. IPTG was added to a final concentration of 1.0 mM to one of the cultures and both cultures were incubated for 6 hr at 37 C with shaking at 200 rpm. Samples from induced and non-induced cultures were collected, processed, and analyzed as described above.

Nickel affinity column enrichment of recombinant protein- Ni-NTA Spin Column

The BmAMA-1 ectodomain recombinant proteins from BL21 Star™(DE3) One Shot® *E. coli* were purified by nickel affinity for the fusion protein histidine tag from the 50 ml-culture pellets collected above. Purification was done under denaturing conditions according to the manufacturer's protocols for purification (QIAGEN, Valencia, CA). Buffers B, C and E were prepared as follows: Buffer B: 8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-Cl, pH 8.0; Buffer C: 8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-Cl, pH 6.3; and Buffer E: 8 M urea, 0.1 M NaH_2PO4, 0.01 M Tris·Cl, pH 4.5. Pellets were thawed for 15 min and resuspended in 1 ml Buffer B to lyse the bacteria. The suspension was then incubated with agitation for 1 hr at room temperature. To pellet the cellular debris, the lysate was centrifuged at 10,000 *g* for 30 min at room temperature. The supernatant was collected and 20 µl of cleared supernatant was saved for SDS-PAGE analysis. The Ni-NTA Spin Column (QIAGEN) was equilibrated with 600 µl Buffer B, centrifuged for 2 min at 700 *g*, and 600 µl of cleared lysate supernatant was loaded onto the pre-equilibrated Ni-NTA Spin Column. After centrifugation for 2 min at 700 *g*, the flow-through was collected and saved for subsequent analysis by SDS-PAGE. The Ni-NTA Spin Column was washed 2 times with 600 µl denaturing wash buffer by

centrifugation at 700 *g* for 2 min, and the wash flow-through saved for analysis by SDS-PAGE. Bound His-tagged proteins were eluted 2 times with 200 µl Buffer E by centrifugation at 700 *g* for 2 min. Protein concentrations in both elutions were determined by spectrophotometry at wavelength 280 (Nanodrop ND-1000 Spectrophotometer, Nanodrop Technologies, Inc., Wilmington, DE).

SDS-PAGE sample buffer was added to each sample collected above and mixed. The samples were boiled for 5 min, centrifuged briefly, and 10 µl each were loaded into lanes of a 12% SDS-PAGE gel alongside a molecular weight marker (Low range molecular weight marker BIO-RAD, Hercules, CA) and electrophoresed at 180 V for 1 hr. The gel was stained with Coomassie blue overnight and destained with a solution of 45% H2O, 45% methanol and 10% Glacial Acetic Acid. A digital image was acquired using AlphaEaseFc (Alpha Innotech, Berkeley, CA), and the expression profile was analyzed.

Nickel affinity column enrichment of recombinant protein using QIAExpress FAST START

Denaturing lysis buffer (pH 8.0), denaturing wash buffer (pH 6.3), and denaturing elution buffer (pH 4.5) were prepared according to manufacturer's instructions (QIAExpress FAST START Purification KIT, QIAGEN, Valencia, CA) and stored at 4 C until use. Five pellets from 50-ml of 6 hr induced cultures were removed from frozen storage at -80 C and 20 µl of protease inhibitors cocktail (SIGMA Aldrich, St. Louis, MO) was stirred into each pellet thawed at room temperature. The pellets were refrozen for 15 min at –80 C, then thawed again by stirring gently at room temperature.

Two ml of denaturing lysis buffer prepared above containing protease inhibitors were added to each pellet and the pellets were resuspended by stirring gently. The suspensions were incubated at room temperature for 60 min and swirled to mix gently every 15 min. To pellet the cellular debris, the resulting bacterial cell lysates were transferred to 1.5 ml screw cap conical microtubes and centrifuged at 10,000 *g* at room temperature for 30 min. The supernatants, presumed to contain the recombinant proteins, were pooled from all tubes, and a 10-µl aliquot of the supernatant was removed for SDS-PAGE analysis. An equal volume of 2X SDS-PAGE sample buffer was added to the sample and the sample was stored frozen at -20 C until analysis.

The resin in a Fast Start Column (QIAGEN) was gently resuspended by inverting the column 3 times. The seal at the outlet of the column was broken and the screw cap opened to allow the storage buffer to drain out. Pooled supernatant (10.2 ml) was applied to the column and the flow-through fraction was collected. A 10-µl aliquot was removed and treated as above for SDS-PAGE analysis. The column was washed 2 times with 4 ml of denaturing wash buffer with protease inhibitors. Both wash fractions were collected and samples collected for later analysis as above. Two applications of 1 ml denaturing elution buffer were collected as flow-through and samples of each prepared for later analysis as described above. Protein concentrations in both elutions were determined by spectrophotometry at wavelength 280 (Nanodrop1000) and the samples were analyzed by SDS-PAGE as described above.

Western blot analysis of enriched BmAMA-1 ectodomain fusion protein

Each sample was adjusted to 10 µl by addition of ultrapure water and the appropriate amount of 2X Laemmli buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue and 0.125 M Tris HCl, pH approx. 6.8), boiled for 5 min and loaded into a lane of a 12% SDS-PAGE. The gel was electrophoresed at 180V for 1 hr to separate the proteins, which were then transferred overnight to either nitrocellulose (NC) or PVD (BIO-RAD, Hercules, CA) membranes at 30 volts (30 V, 40 mA-90 mA) followed by 2-hr transfer at 100 volts (150 m-240 mA) using a continuous cooling system. The membrane was removed from the transfer cassette and incubated in 10 ml blocking buffer composed of Tris Buffered Saline (TBS) with 5% nonfat dry milk for 1 hr with gentle agitation on a rocker platform. Then, the membrane was washed 2 times with 20 ml of TBS containing 0.5% Tween 20 (TBST) with gentle agitation for 5 min each. The membrane was then incubated in 10 ml mouse anti-His (C-term) monoclonal antibody (Invitrogen, Carlsbad, CA) diluted 1:5000 in blocking buffer with gentle agitation overnight at 4 C. The membrane was washed 2 times in 20 ml TBST 5 min each with gentle shaking, then incubated in 10 ml of alkaline phosphatase conjugated goat anti-mouse antibody (ImmunoPure Goat Anti-Mouse IgG+IgM (H+L) (min x BvHnHs Sr Prot), Pierce, Rockford, IL) diluted 1:10,000 in blocking buffer with gentle agitation for 1 hr at room temperature. The membrane was washed 2 times with 20 ml TBST containing 0.5% Tween 20 for 5 min each with gentle shaking. Then, the membrane was incubated in 10 ml 5-bromo-4-chloro-3-indolyl-

phosphate/Nitroblue Tetrazolium substrate (Sigma Fast BICP/NBT, Sigma, St Louis, MO).

Recombinant BmAMA-1 ectodomain fusion protein expression in BL21 Codon Plus *E. coli*

Recombinant BmAMA-1 ectodomain protein was expressed in BL21 Codon Plus (DE3)-RIPL Competent Cells according to the manufacturer's instructions (Stratagene, La Jolla, CA). A 100 µl aliquot of BL21 Codon Plus (DE3)-RIPL chemically competent *E. coli* cells was mixed with 2 µl of 1:10 dilution of XL10-Gold β-mercaptoethanol in 14-ml BD Falcon polypropylene round bottom tube on ice. The tube content was swirled to gently mix every 2 min for 10 min. BmAMA-1/pET Clone l7 (10 ng) was added to the cells and swirled gently to mix. The transformation was incubated on ice for 30 min, and then heat pulsed in a 42 C water bath for 20 sec. The reaction was incubated on ice for 2 min and 0.9 ml of preheated (42 C) SOC medium was added to the reaction and incubated at 37 C for 1 hr with shaking at 225 rpm. After incubation, 50 and 200 µl of transformed cells were used to inoculate LB agar plates containing carbenicillin.

Identification of transformed colonies

Colony PCR was performed on 10 colonies to determine if the BmAMA-1 ectodomain insert was present. PCR master mix was prepared using 100 µl of 2X QIAGEN PCR buffer (QIAGEN, Valencia, CA), 4 µl of BmAMA-1 specific primers pETSigBm F and pETTmBm R (40 pmol each) and 2 µl of ultrapure water. Ten colonies were selected and a portion of each added to 9 µl aliquots of water. Eleven µl of the master mix was then added to each tube, and the tubes placed in a PCR Express

thermocycler (Franklin, MA, U.S.A) along with a negative control. Amplification was done with an initial denaturation at 94 C for 2 min, then 30 cycles of 94 C for 30 sec, 50 C for 30 sec and 72 C for 2 min with a final extension at 72 C for 10 min.

Recombinant protein pilot expression in BL21 Codon Plus *E. coli*

Three single colonies were used to inoculate duplicate 1 ml aliquots of LB broth containing 50 µg/ml of chloramphenicol and 50 µg/ml carbenicillin. The transformed bacteria were grown overnight at 37 C, 220 rpm, then one set was used to inoculate three 20 ml LB broth cultures without antibiotics. The cultures were grown for 2 hr then induced with 1.0 mM IPTG. At time 0 hr, 20-µl samples of culture were collected for later SDS-PAGE analysis. The cultures were grown for 4 hr after induction and 20-µl samples were again collected for SDS-PAGE analysis. The remaining culture was centrifuged to pellet the *E. coli,* the supernatant was removed, and the pellet stored frozen at -80 C.

Large-scale expression in BL21 Codon Plus *E. coli*

The second set of 1-ml cultures from the duplicate sets above was used to inoculate 5 ml LB broths containing 50 µg/ml of chloramphenicol and 50 µg/ml carbenicillin, which were then incubated overnight at 37 C. Each 5 ml overnight culture was used to inoculate 100 ml LB broth with no selection antibiotic. The cultures were grown for 2 hr then induced with 1.0 mM IPTG and incubated for 4 hr at 37 C, 220 rpm. At time 0 hr, 20-µl samples of culture were collected for SDS-PAGE analysis. The cultures were grown for 4 hr after induction and 20-µl samples were again collected for SDS-PAGE analysis. Protein expression in samples collected before and 4 hr after

induction were analysed on SDS-PAGE stained with Coomassie blue (BIO-RAD, Hercules, CA).

Nickel affinity enrichment using NI-NTA Spin Column

Enrichment of the expressed recombinant BmAMA-1 fusion protein was done as described above. The samples were analyzed by SDS-PAGE stained with Coomassie blue and by Western blot analysis.

Nickel affinity column enrichment using QIAExpress FAST START

Native lysis buffer, wash buffer and elution buffer were prepared according to the manufacturer's instructions (QIAGEN, Valencia, CA). The pellets from 250 ml BL21 Codon Plus culture (Table 1) stored at –80 C were thawed on ice and resuspended in 10 ml of the prepared buffer. The suspension was incubated on ice for 30 min, mixing 3 times gently by swirling the contents. The lysate was centrifuged at 14,000 *g* for 30 min at 4 C to pellet the cellular debris. A 5 µl aliquot of the cell lysate supernatant was saved for SDS-PAGE analysis and the remainder was stored at –20 C for Western blot analysis.

The FAST START column protocol as given above was utilized except for the buffers which were designed for the native protein enrichment procedure. Aliquots were analyzed by SDS-PAGE as previously described.

Enrichment of expressed protein from BL21 Codon Plus *E. coli* was accomplished under denaturing conditions as described above. Samples were collected as previously described.

SDS-PAGE analysis of the enriched recombinant protein

Samples were analyzed by SDS-PAGE gel stained with Coomassie blue and by Western blot analysis. No band was observed in the elutions from native enrichment instead the protein was found in the cell lysate and the cleared lysate flow-through. Protein concentrations from denaturing enrichment were determined at wavelength 280 (Nanodrop1000).

Other recombinant protein enrichments using QIAexpress FAST START

BugBuster (Novagen, Madison, WI) protein extraction reagents were used to extract soluble protein from 100 ml culture pellet of transformed BL21 Codon Plus *E. coli*. Prechilled microfuge tubes at 4 C were used to process samples in this experiment. First the weight of the *E. coli* pellet collected from 100 ml transformed BL21 Codon Plus culture was determined. The pellet was resuspended in BugBuster lysis buffer (Novagen) at a rate of 1 g in 5 ml of buffer containing 10 µl/g lysonase and protease inhibitors. The lysate was incubated for 15 min at room temperature by slow rotation, then aliquoted in 2 ml snap cap tubes. The tubes were centrifuged at 16,000 g for 20 min at 4 C. Supernatants were collected and pooled then kept on ice. Protein concentration was determined on Nanodrop 1000, then the supernatant was loaded onto the FAST START column and the flow-through was collected. The column was washed 2 times with FAST START denaturing wash buffer containing protease inhibitors. The flow-through was collected. Bound BmAMA-1 was eluted with 1 ml denaturing elution buffer pH 4.

A pellet from 10 ml culture of transformed BL21Codon PLUS RIPL (Invitrogen, Carlsbad, CA) was used for enrichment using His-Select resin (Sigma). The pellet was frozen at –80 C then thawed on ice 3 times in the presence of protease inhibitor cocktail (Sigma, Aldrich, CA). The pellet was stirred to mix between cycles.

For enrichment under native conditions, the pellet was resuspended after the final cycle in 400 µl of native lysis buffer (FAST START) and mixed well. Ten µl of the suspension was removed for SDS-PAGE analysis. The suspension was incubated on ice for 30 min, mixed 3 times by swirling while incubating, then centrifuged at 14,000 g for 30 min at 4 C. Supernatant was collected in small snap cap tubes and the protein concentration was measured. Protease inhibitors were added, then the tubes were labeled and stored at –80 C.

For enrichment under denaturing conditions, the pellet was resuspended in 400 µl denaturing lysis buffer (FAST START) and mixed. Ten µl of the suspension was removed for SDS-PAGE analysis. The suspension was incubated 1 hr at room temperature with mixing by swirling 3 times during incubation. The suspension was centrifuged at 14,000 g for 30 min at room temperature. The supernatant was collected, protease inhibitors were added, and the supernatant was stored at –80 C.

Preparation of Sigma His–select nickel affinity gel (Sigma, Aldrich, CA)

Aseptically, the His-select affinity gel was resuspended by inverting several times. Aliquots of 25 µl resuspended His-select gels were placed in two 1.5 ml screw cap microfuge tubes on ice. Tubes were centrifuged 30 sec at 5000 g to pellet the gel.

The supernatant was discarded and the gel was washed 2 times with 200 µl of sterile distilled water.

Enrichment under native conditions

One of the 25 µl aliquots of washed His-select gel was equilibrated with 200 µl of native equilibration buffer pH 8.0 from FAST START Kit (QIAGEN). The tube was centrifuged for 30 sec at 5000 g and the native equilibration buffer was removed. One hundred µl of clarified BmAMA-1 recombinant fusion protein prepared using native lysis buffer (FAST START Kit, Sigma) was added and mixed gently for 1 min. The tube was centrifuged as indicated above to pellet the gel. Unbound fusion proteins were removed by centrifugation. The gel was washed 2 times by adding 500 µl of native wash buffer pH 8.0. The tube was centrifuged as above and the wash buffers were saved as a pool for SDS-PAGE analysis. Bound fusion protein was eluted 2 times with 50 µl of elution buffer (50 mM sodium phosphate, pH 8; 0.3 mM Sodium chloride, 250 mM imidazole). First the gel was mixed with 50 µl of the elution buffer by inverting gently for 1 min, then centrifuged for 30 Sec at 5000 g to collect the BmAMA-1 ectodomain fusion protein.

Enrichment under denatured conditions

Two hundred µl of denaturing lysis buffer (FAST START, QIAGEN) was added to a 25-µl aliquot of the washed His-select gel and centrifuged for 30 sec at 5000 g. The supernatant was removed then 100 µl of clarified BmAMA-1 recombinant fusion protein prepared using denaturing lysis buffer (FAST START Kit, QIAGEN) was added to the gel and mixed gently for 1 min. Tube was centrifuged for 30 sec at 5000 g to pellet gel

bound fusion protein and to remove unbound protein. The gel was washed 2 times by centrifugation by adding 500 µl of denaturing wash buffer, pH 6.3. Wash buffers were collected as a pool for SDS-PAGE analysis. Bound BmAMA-1 ectodomain fusion protein was eluted by adding 50 µl of denaturing elution buffer, pH 4.5 as above.

RESULTS

A summary of the expression trials and results is shown in Table 1. Overall, the best expression of recombinant BmAMA-1 fusioin protein was achieved using BL21 Star DE3 *E. coli* (Fig. 6). Nickel affinity capture using QIAExpress FAST START Kit method produced the best BmAMA-1 ectodomain recombinant protein (Figs. 7 and 8).

Enrichment of BmAMA-1 ectodomain recombinant protein from BL21 Star DE3 *E. coli* or BL21 Codon Plus under native conditions was unsuccessful (not shown). Nickel affinity capture of BmAMA-1 fusion protein using denaturing conditions resulted in enriched BmAMA-1 recombinant protein preparations with either the Spin Column or FAST START gravity flow (Figs. 7-9). The expected size for the recombinant BmAMA-1 ectodomain fusion protein was 58.1 kDa, but the elution showed multiple bands in SDS-PAGE stained with Coomassie blue and these were confirmed by Western blot analyses (Figs. 7-9). The multiple bands may have been due in part to lack of addition of protease inhibitors in the purification buffers in some of the assays (example in Fig. 8). With the FAST START enrichment, the recombinant BmAMA-1 ectodomain fusion protein band at 58 KDa was more prominent in Western blot analysis (Figs. 7 and 8), but when enriched by the Spin Column, the signal produced in all these bands appears to be the same whether assayed by Coomassie blue or by Western blot analysis (Fig. 9).

Table 1. Summary of BmAMA-1 ectodomain recombinant protein expression trials.

Date	Culture amount	*E. coli* BL21 strain	SDS-PAGE	Nickel column	Denaturing or Native	Resulting Bands(kDa)
10.6.07	50 ml	Star DE3	CB*	NI-NTA	Denaturing	
10.7.07	"	"	"	"	"	
10.8.07	"	"	"	"	"	33
10.8.07	"	"	"	"	"	58,53,43,33,23
10.11.07	"	"	"	"	"	58,53,43,33,23
10.12.07	"	"	"	"	"	58,53,43,33,23
10.12.07	"	"	"	"	"	58,54,43,37
10. 16.07	"	"	WB**	"	"	58
10.17.07	"	"	WB	"	"	58,54,43,37
10.18.07	250 ml	Codon Plus	CB	"	Native	
10.21.07	100 ml	Star DE3	"	FAST START	"	80,76,54,43,37,33
10.25.07	20 ml	Codon Plus	"	NI-NTA	Denaturing	58
10.26.07	"	Star DE3	"	"	"	
10..28.07	"	Codon Plus	"	"	"	58
10.31.07	"	"	"	"	"	
11.4. 07	"	"	"	"	"	
11.5.07	"	"	"	"	"	
11.6.07	"	"	"	FAST START	"	
11.9.07	50 ml	"	CB, WB	NI-NTA Spin	Denaturing	58
11.14.07	"	"	"	FAST START	"	58, 46,43
11.19.07	250 ml	"	"	"	"	58, 46,43,33,30
12.1.07	100 ml	"	CB	His select Sigma	Native	
12.5.07	"	"	"	BugBuster Novagen	"	
3.1.08	200 ml	Star DE3	"	His select Sigma	Denaturing	
3.3.08	"	"	"	BugBuster Novagen	Denaturing	
3.4.08	250 ml	"	"	FAST START	Denaturing	58,36,33,30
3.5.08	"	"	"	"	Denaturing	58, 49, 36

*CB, Coomassie blue
** WB, Western blot

The detection of these bands in Western blot assays probed with anti-His tag monocolonal antibody indicates that these bands are all histidine rich proteins or possibly breakdown products of the BmAMA-1 ectodomain fusion protein.

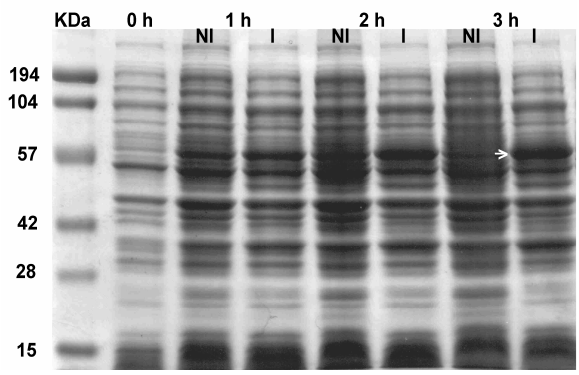


Figure 6. Timeline expression of recombinant BmAMA-1 ectodomain in BL21 STAR DE3 *E. coli*. Lane 1, molecular weight marker. Lanes 2 through 8, IPTG Non-induced (NI) and Induced (I) samples over time as indicated. Arrow, recombinant BmAMA-1 ectodomain.

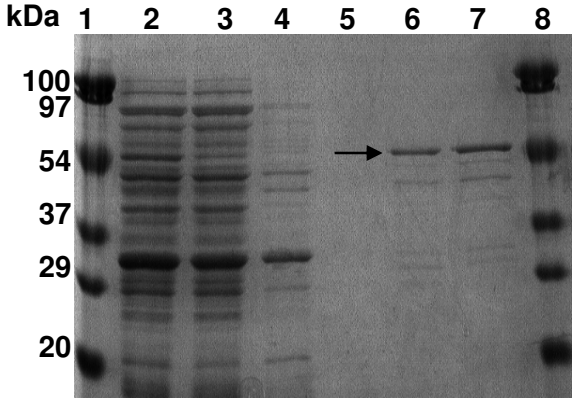


Figure 7. SDS-PAGE of enriched (FAST START) BmAMA-1 ectodomain fusion protein expressed in BL21 Codon Plus *E. coli*. Lane 2, *E. coli* lysate; Lane 3, cleared *E. coli* lysate flow-through after binding to the column. Lanes 4 and 5, first and second washes. Lanes 6 and 7, eluted BmAMA-1 ectodomain fusion protein.

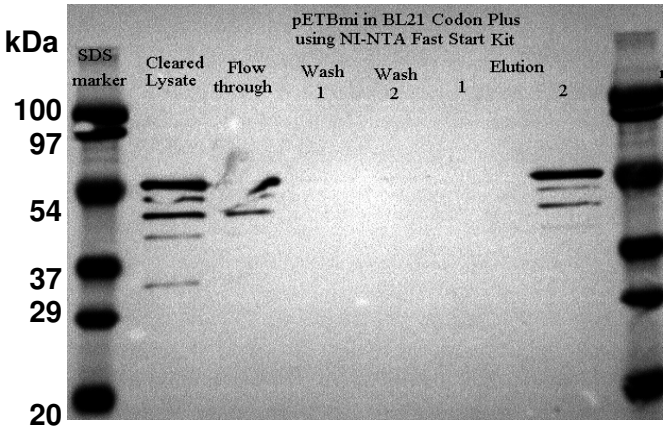


Figure 8. Western blot of His tag *B. microti* AMA-1 ectodomain fusion protein expressed in BL21 Codon Plus. Enriched by FAST START. Lanes 1 and 8, molecular weight marker. Lane 2, *E. coli* lysate cleared supernatant. Lane 3, supernatant flow-through. Lanes 4 and 5, first and second wash.Lanes 6 and 7, *B. microti* AMA-1 ectodomain fusion protein (58.1 kDa) elutions 1 and 2, respectively.

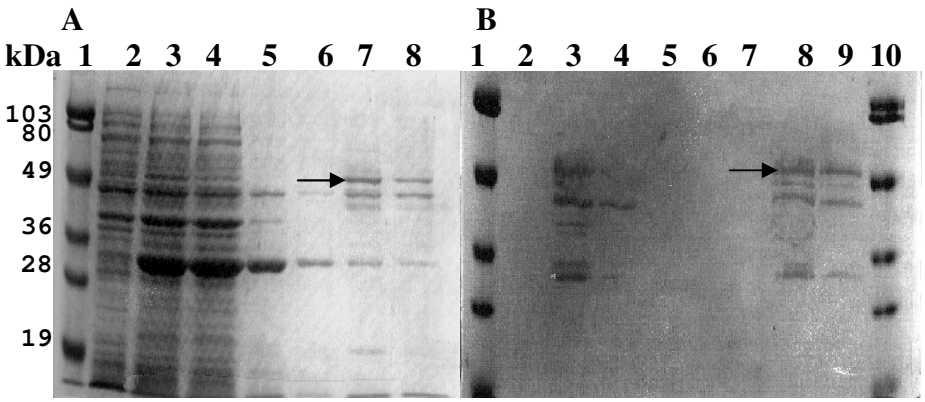


Figure 9. BmAMA-1 ectodomain fusion protein expressed in BL21 DE3. Ni-NTA spin column enrichment. **(A)** Coomassie blue stained 12% SDS-PAGE. Lane 1, molecular mass marker. Lane 2, *E. coli* lysate. Lanes 5 and 6, first and second washes. Lanes 7 and 8, first and second elutions of the fusion protein. **(B)** Western blot probed with anti-Histidine tag antibody. Lanes 1 and 10, molecular weight marker. Lane 3, cleared lysate. Lane 4, first flow-through. Lanes 5 and 6, first and second washes after binding. Lanes 8 and 9, first and second elutions of His-tagged recombinant protein (arrow, 58.1 kDa recombinant AMA-1).

Transformed BL21 Codon Plus E. coli were confirmed by colony PCR before expression. Of eight colonies tested, all possessed the *B. microti ama-1* insert (Fig. 10). Samples collected over 6 hours and analyzed according to the manufacturer's protocol showed very little increase in the intensity of the 58.1 kDa protein band over time, but the best expression was observed at 4 hr following IPTG induction (not shown). When larger samples (500 µl) were collected and the *E. coli* pellets were analyzed, marked differences in protein expression were observed between non induced and induced bacteria. The best recombinant expression was observed at 4 hr post IPTG induction (Figs. 11 and 12).

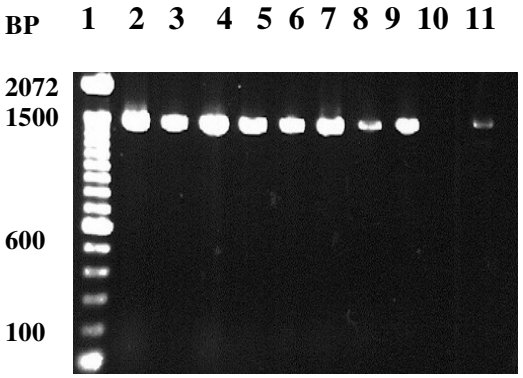


Figure 10. Colony PCR products. BL21 Codon Plus *E. coli* clones transformed by pET/ BmAMA-1 ectodomain construct. Lane 1, 100 bpDNA size marker. Lanes 2 through 9, BmAMA-1 ectodomain amplicons from individual colonies. Lane 11, BmAMA-1 DNA control.

Protein enrichment from larger samples under denaturing conditions using NI-NTA Spin Column produced low yield after purification of 50 ml pellet by NI-NTA Spin Column (not shown). This possibly due to the amount of cleared cell lysate supernatant processed on the Spin Column, which was 600 µl on the Spin Column

instead of 1 ml by FAST START. The elution for both was done in 50 µl denaturing elution buffer, resulting in less concentrated protein yield for the Spin Column.

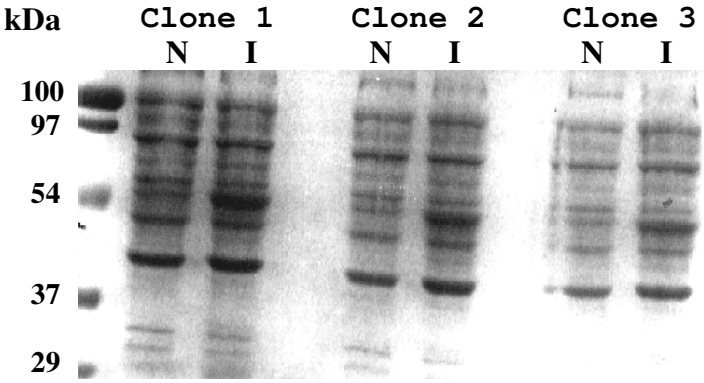


Figure 11. Expression of BmAMA-1 ectodomain in three colonies of BL 21 Codon Plus *E. coli*. Samples from induced and non-induced cultures collected 6 hr post IPTG induction. Clones 1, 2, 3 non- induced (NI) and induced (I) samples are indicated. Arrow, BmAMA-1 recombinant protein. Coomassie blue stained SDS-PAGE.

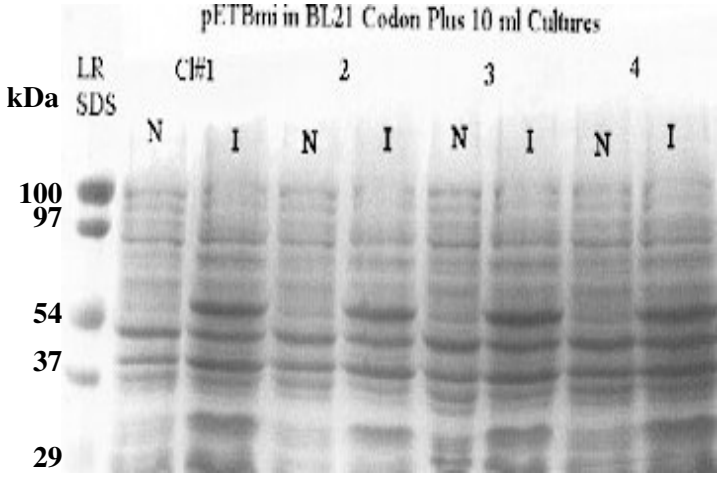


Figure 12. SDS-PAGE of expressed BmAMA-1 ectodomain fusion protein in Codon Plus *E. coli*. Cultures from 4 clones, 1, 2, 3, and 4, non-induced (N) and induced (I) samples from each 4 hours after induction. Molecular weight marker in first lane.

Purification of recombinant BmAMA-1 fusion protein from Codon Plus *E. coli* pellets from large-scale cultures using QIAExpress FAST START Kit under denaturing conditions produced prominent BmAMA-1 ectodomain protein bands along with weaker additional protein bands evident in Western blot analysis as mentioned above (Figs. 8 and 9). The multiple bands were observed might indicate that other histidine rich proteins bound to the nickel column were not removed by washing and were eluted along with BmAMA-1 ectodomain fusion protein. These eluted proteins could be contaminants or truncated breakdown products of BmAMA-1 fusion protein that carry the His-tag.

Expression was optimal at 3 hours post induction as evidenced by SDS-PAGE analysis of induced (I) and non induced (NI) samples over time (Fig. 6). All subsequent large scale expressions were grown for 3 hours following induction with 1 mM IPTG (Fig. 13). A clone from transformed BL21 STAR DE3 *E. coli* cell yielded a stronger BmAMA-1 ectodomain fusion protein band (58.1 kDa) as shown on SDS-PAGE stained with Coomassie blue (Fig. 14). Two FAST START nickel affinity capture procedures under denaturing conditions using pellets from 250 ml transformed BL21 STAR DE3 *E. coli* culture produced 0.28 and 0.29 mg/ml for elution 1 and 2, respectively (Fig. 14) and 0.24 and 0.83 mg/ml for elution 1 and 2, respectively (Fig.15).

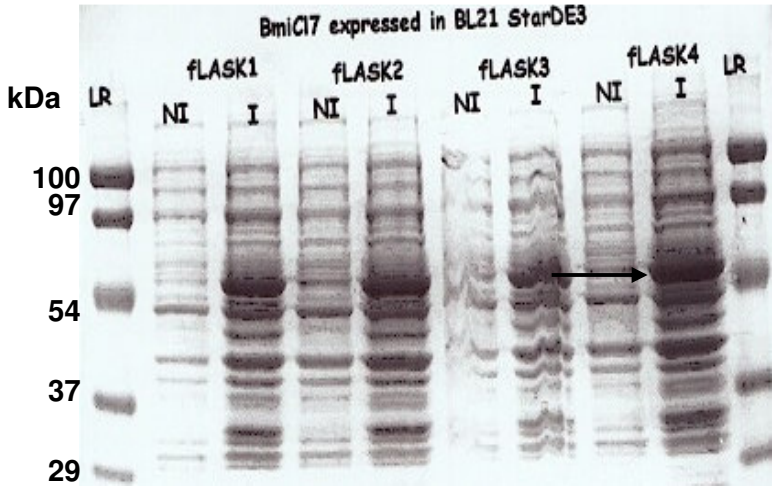


Figure 13. SDS-PAGE of expressed BmAMA-1 ectodomain fusion protein. Large scale cultures from 4. Lanes 1 and 8, molecular weight marker. Lanes 2-9, non-induced and induced samples from the same cultures as indicated. Coomassie blue stained.

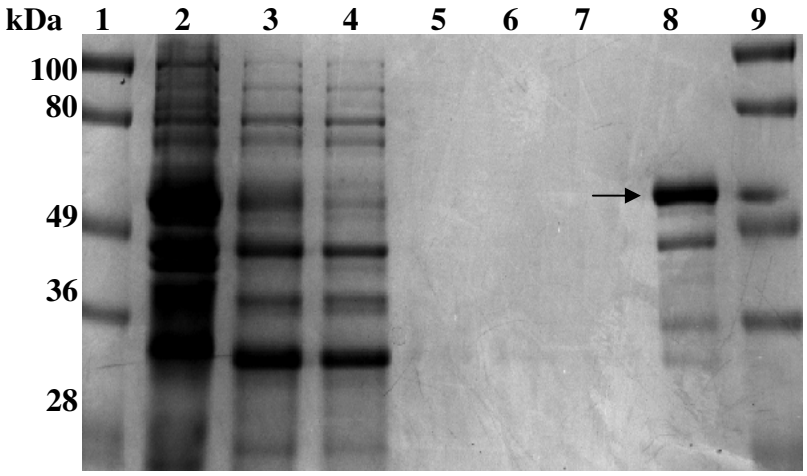


Figure 14. SDS-PAGE analysis of BmAMA-1 ectodomain enriched protein. Lanes 1 and 9, molecular weight marker. Lane 2, starting *E. coli* lysate. Lane 3, cleared lysate. Lane 4, flow-through after binding. Lanes 5 and 6, first and second washes. Lanes 7 and 8, eluted bound protein. Coomassie blue stained.

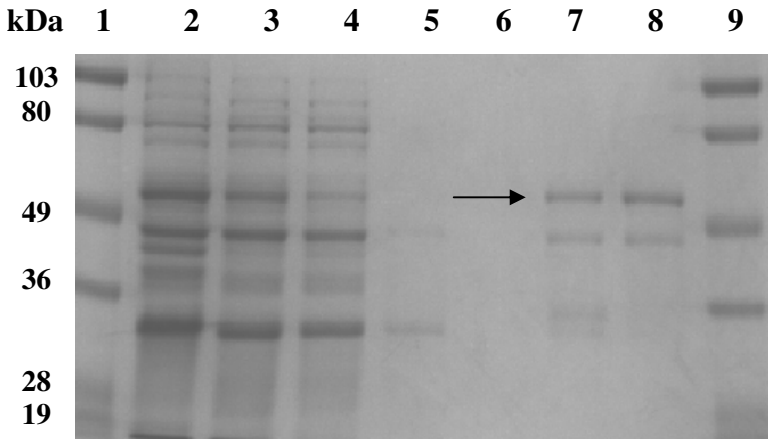


Figure 15. BmAMA-1 ectodomain enriched protein. SDS-PAGE. Lanes 1 and 9, molecular weight marker. Lane 2, *E. coli* lysate. Lane 3, cleared lysate. Lane 4, flow-through after binding. Lanes 5 and 6, first and second washes. Lanes 7 and 8, eluted bound protein. Coomassie blue.

CHAPTER V

PROTEIN-PROTEIN INTERACTIONS BETWEEN PARASITE AND HOST ERYTHROCYTE

INTRODUCTION

The aim of this chapter was to determine valid interactions between BmAMA-1 ectodomain protein and the receptors on the host erythrocyte. The role of many ligands (RAP, TRAP, AMA-1) in host cell invasion by apicomplexan has been studied. AMA-1 appears to be critical in this process as demonstrated by the ability of anti-AMA-1 to inhibit host cell invasion *in vitro* and *in vivo* as well. AMA-1 proteins when released by the parasite during cell invasion bind to the erythrocyte surface receptors thus enabling the parasite to invade the cell. Knowledge of a true interaction between BmAMA-1 ectodomain and the host cell will enable *in vitro* and *in vivo* studies to inhibit parasite invasion of the host cell. Our first objective was to determine that there is an interaction between the *B. microti* AMA-1 ligand and its putative receptors on the host erythrocyte. In some apicomplexans, such as *Plasmodium falciparum, Babesia divergens,* and *Babesia bovis,* these receptors have been shown to be the sialoglycoproteins of the RBCs, notably Glycophorin A and B, which are the most prominent. However, the composition of these residues that react to the parasite ligand (AMA-1) may be species specific. The analysis of interacting bands by tandem mass spectrometers can lead to structural information on the host cell receptors involved. In conclusion, determination of the interaction between BmAMA-1 ectodomain and analysis of the structure of the

cell binding partner structures are crucial in design of therapeutics and vaccines to counter babesiosis caused by *B. microti*. Here we used far Western blot analysis, immunocapture and immunofluorescent antibody test (IFAT) to demonstrate interactions between the *B. microti* AMA-1 and its interacting receptors on the host cell membrane.

MATERIALS AND METHODS

Red blood cell (RBC) membrane preparation

Mouse, human, and horse RBC membrane preparations were made according to the protocol below. Whole blood was collected in Alsever's solution, then transferred to prechilled microfuge tubes which were kept cold throughout the process. The blood was centrifuged at 300 *g* for 30 min at 10 C to pellet the cells. The buffy coat was removed, and the RBC pellet was washed 3 times by centrifugation in several volumes of cold Dulbecco's PBS, pH 7.2, with the buffy coat removed at each wash. The pellets were combined after the final wash. To each ml of packed RBC, 40 ml cold 5 mM Na_2HPO_4, pH 8.0 with protease inhibitor (Protease Inhibitor Cocktail, SIGMA Aldrich, St. Louis, MO) was added and the cells resuspended in this solution. The tubes were completely immersed in ice for 30 min, then the resulting RBC lysate was dispensed in 1.3 ml volumes into 1.5 ml conical snap-top microfuge tubes on ice. The lysates were centrifuged at 15,000 *g* for 10 min at 4 C and the supernatant removed and discarded. The resulting RBC membranes were washed 2 times in 5 volumes of 5 mM Na_2HPO_4, pH 8.0 containing protease inhibitors, then 2 times with PBS containing protease inhibitors by centrifugation at 15,000 *g* for 10 min at 4 C. After the last centrifugation, the supernatant was removed and the protein concentrations of the purified RBC

membrane pellets were determined by spectrophotometry (NanoDrop ND-1000 Spectrophotometer, NanoDrop Technologies, Inc., Wilmington, DE). The RBC membrane preparations were stored frozen at -80 C.

Far Western blot analysis

The far Western blot protocol is depicted in the schematic shown in Figure 16. For this assay, 60 μg of each RBC membrane preparation was loaded per lane on a vertical 12% polyacrylamide gel containing 10% SDS (Laemmli et al., 1970). Electrophoresis was done in Mini PROTEAN II dual slab cell (BIO-RAD, Hercules, CA). The gel was run for 1 hr at 180 V. After electrophoresis the separated proteins were electrophoretically transferred to a nitrocellulose membrane (Towbin et al., 1973). Transfer was done overnight in a Mini-Trans-Blot Cell (Bio-Rad) at 30 volts, then at 100 volts for 2 hr the next morning. The nitrocellulose membrane was cut into strips corresponding to protein lanes and one strip was stained with Ponceau Red stain (Sigma, Saint Louis, MO) to visualize the transferred protein bands. The strips were incubated in 10 ml blocking buffer composed of Tris Buffered Saline (TBS) with 5% nonfat dry milk and 0.05% Tween 20 for 2 hr with gentle agitation on a rocker platform, then washed 4 times with 300 ml PBS for 20 min each. The strips were incubated overnight at 4 C in purified BmAMA-1 ectodomain fusion protein diluted in blocking buffer at a concentration of 6 μg/ml. After blocking, control strips were washed once with PBS, 2 times with PBS containing 0.05% Tween 20, and once more with PBS for 20 min with agitation each wash and then stored overnight at 4 C. The next day, the strips incubated with BmAMA-1 ectodomain fusion protein were washed 4 times as described above.

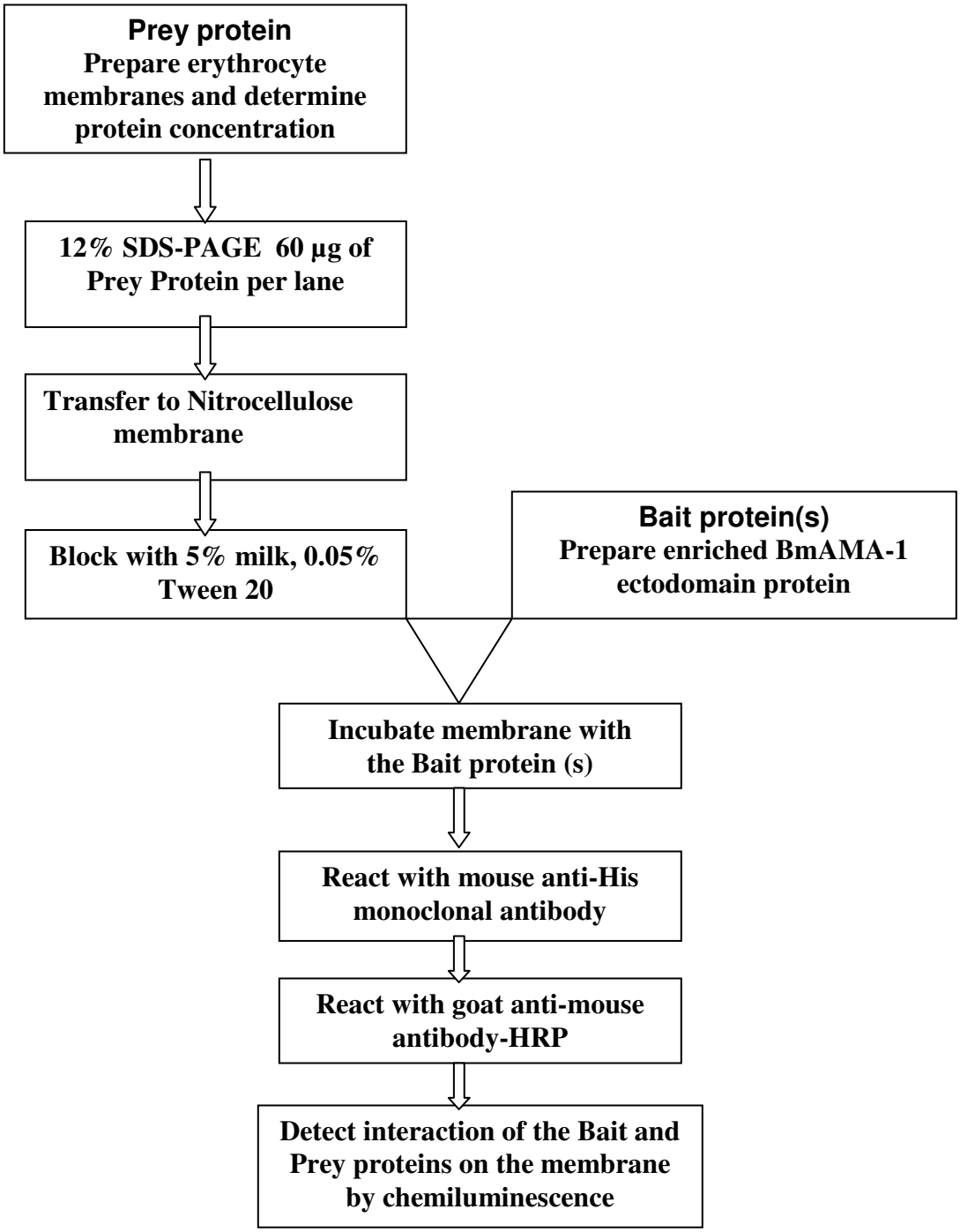


Figure 16. Schematic of the far Western blot assay protocol.

The strips and primary antibody (unreacted control strip) were incubated in mouse anti-His primary monoclonal antibody (Invitrogen, Carlsbad, CA) diluted 1:5000 for 1 hr at room temperature with slow rotation. After incubation, the strips were washed 4 times as above. The strips and the secondary antibody control (unreacted control strip), were incubated in 10 ml of goat anti-mouse Horse Radish Peroxidase conjugated (Pierce, Rockford, IL) diluted 1:10,000 in blocking buffer with gentle agitation for 1 hr at room temperature. All strips were washed as above, then incubated in Immobilon Substrate for 5 min according to the manufacturer's instructions (Millipore, Billerica, MA). Strips were blotted to drain off excess substrate then analyzed by X-ray radiography.

Immunoprecipitation

To prepare soluble RBC membrane proteins, 2 mg of human purified RBC membrane (above) was added to 1 ml solubilization buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% sodium deoxycholate) containing protease inhibitors and incubated on ice for 1 hr. After centrifugation at 13,000 *g* for 5 min at 4 C, the supernatant containing solubilized membrane proteins was recovered. The protein concentration was determined as above and 14 µg of soluble protein was analysed on an SDS-PAGE gel stained with Coomassie blue.

BmAMA-1 ectodomain recombinant protein was enriched from a 50 ml induced culture pellet of transformed BL21 Star DE3 *E. coli* (Table 1). The pellet was subjected to 3 freeze/thaw cycles in liquid nitrogen. Then, it was resuspended in 5 ml B-Per lysis buffer (Thermo Scientific, Rockford, IL) containing protease inhibitors and vortexed.

The bacterial lysate was incubated for 10 min at 4 C with shaking on a tube rotator at 20 rpm. The lysate was divided into 1 ml aliquots in screw-cap microfuge tubes, then centrifuged at 13,000 *g* for 20 min at 4 C. The supernatant containing the clarified BmAMA-1 ectodomain recombinant protein was transferred to screw-cap tubes and stored frozen until use. The pellets presumed to contain inclusion bodies as the fusion was not found in solution were also saved.

His-Select Nickel Affinity gel suspension (100 µl) (Sigma, Aldrich, CA) was dispensed into a 2-ml microfuge tube, centrifuged for 30 sec at 5,000 *g* to pellet the resin. The supernatant was discarded and the resin was washed 2 times by centrifugation with 3 volumes of sterile deionised water, then equilibrated by the addition of 500 µl of equilibration buffer (QIAExpress, QIAGEN, Valencia, CA) in which it was resuspended. The resin was centrifuged for 30 sec at 5000 *g*, then the supernatant was removed and discarded.

Purified BmAMA-1 recombinant protein 200 µl at 5.52 µg/µl prepared above was added to the equilibrated His-select nickel affinity gel resin and mixed on a tube rotator at 20 rpm for 1 hr at 4 C. After centrifugation at 5000 *g* for 30 sec, the fluid was removed by careful pipetting. The affinity gel was washed 2 times by centrifugation in 1 ml wash buffer (QIAExpress Fast Start Kit, QIAGEN). The washes were saved as a pool for SDS-PAGE analysis. The bound protein was eluted 2 times with 50 µl of elution buffer (QIAExpress Fast Start Kit, QIAGEN), with 5 min incubation with rotation at 20 rpm at 4 C in the elution buffer prior to centrifugation as above.

A volume of 200 µl (28 µg) of solubilized RBC proteins was mixed with the AMA-1 protein bound-resin from above and incubated overnight at 4 C with gentle rocking. After incubation the resin was allowed to settle, then the supernatant was removed. The resin was washed 3 times with 100 µl of RIPA buffer (50 mm Tris pH7.2, 150 mM NaCl, 5 mM EDTA, 0.1%SDS, 0.1% sodium deoxycholate, 1.0% Tween 20). The resin was allowed to settle at each wash and wash buffer removed by aspiration using a long pipette tip. Washes were saved as a pool for SDS-PAGE analysis. The resin-bound proteins were eluted with 50 µl of 100 mM glycine, pH 2.5 into tubes containing 10 µl 1.0 M TRIS, pH 7.5. The protein concentration was determined by spectrophotometry as above and stored at –80 C in 10 µl aliquots until use.

The samples collected during immunoprecipitation were evaluated by SDS-PAGE stained with Coomassie blue. The electrophoresed samples included human RBC membrane lysate (13.61 µg), human RBC solubilized protein (14 µg), purified recombinant BmAMA-1 protein (5.5 µg), solubilized protein from transformed *E. coli* (5.5 µg), presumed transformed *E. coli* inclusion bodies (4.66 µg), elution of presumed RBC protein-AMA-1 complex (7.3 µg), and a second elution of presumed RBC protein-AMA-1 complex (5.1 µg).

Immunofluorescent binding assay

BALB/C mouse blood collected in Alsever's solution was washed 2 times in 5 volumes of PBS containing 15 mM ethylenediaminetetraacetic acid (EDTA) followed by 4 washes in PBS, with removal of the buffy coat at each wash. Washed RBCs were tested for auto-immunofluorescence prior and to non-specific binding and fluorescence

of Protein G-FITG. Five µl of Protein G-FITC conjugate was added to 1 µl of washed RBCs and incubated for 10 min with gentle rocking. The suspension was washed to remove unbound Protein G-FITC, then washed 3 times with 100 µl of PBS by centrifugation at 3,500 *g* for 3 min. After the last wash, the supernatant was removed and the pellet resuspended in 5 µl PBS. A sample of the RBC was viewed by UV microscopy to check for non-specific binding of the Protein G-FITC conjugate to the RBCs.

Protein G binds to the FC portion of the leaving the binding site free (Sjobring et al., 1991). Mouse anti-His Moab will bind to the BmAMA-1 ectodomain fusion protein thus enabling the FC portion to interact with Protein G conjugated to FITC.

For the immunofluorescence assay, 25 µl of purified BmAMA-1 ectodomain fusion protein at a concentration of 6µg/ml was added to 5 µl of washed RBC and incubated under gentle mixing at 37 C for 1 and 2 hr. The RBC pellet was washed 3 times with 300 µl PBS and the final pellet was incubated in 25 µl of mouse anti-His monoclonal antibody (Invitrogen, Carlsbad, CA) (diluted 1:5,000) for 30 min with gentle mixing at 37 C. The RBC were washed 3 times by centrifugation with 300 µl of PBS per wash. The final pellet was incubated with 25 µl of Protein G-FITC (Fluorescein isothiocyanate) conjugate diluted 1:10 in PBS for 30 min at 37 C with gentle mixing. After washing the RBC 3 times with 300 µl PBS, a sample was viewed by UV microscopy. Controls included RBC incubated with mouse anti-His monoclonal antibody and Protein G-FITC conjugate and RBC incubated with Protein G-FITC conjugate only.

RESULTS

SDS-PAGE analysis of mouse, human, and horse RBC membrane preparations revealed by Coomassie blue staining showed 12 bands in the mouse (lane 2) of approximately 207, 114, 75, 64, 53, 49, 44, 35, 33, 30, 28, 6 kDa, whereas in the human there were 8 bands of approximately 207, 160, 114, 93, 53, 49, 44, 35 kDa. Horse RBC membrane protein preparations analyzed by SDS-PAGE and revealed by Coomassie blue produced 10 bands of approximately 207, 160, 137, 84, 56, 53, 49, 37, 28, 6 kDa (Fig. 17). Only bands of apparent molecular weights 207, 53 and 49 kDa were observed in RBC membrane preparations from all three species indicating possible conservation of these proteins.

The detection of signal by far Western blot analysis of mouse RBC membrane proteins probed with BmAMA-1 ectodomain fusion protein indicated binding of the ligand which was detected by X-ray radiography. Validity of the interaction in each experiment was confirmed by testing for nonspecific binding of the primary antibody and the secondary antibody-conjugate to the RBC membrane proteins. BmAMA-1 ectodomain fusion protein used in the interaction was included as a positive control. The results of the far Western blot analysis using mouse RBC membrane indicated interaction with the BmAMA-1 at approximate position 130 and 49 kDa (Fig.18). This interaction was observed in subsequent experiments indicating conserved binding of the BmAMA-1 ligands at this position to mouse RBC membrane proteins. The signal of this interaction was decreased when the RBC proteins were probed with a 2-fold decrease of the BmAMA-1 recombinant protein (Fig. 18).

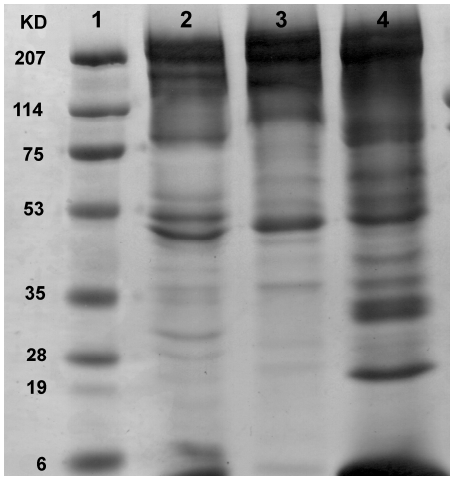


Figure 17. SDS-PAGE of horse, human and mouse RBC membrane protein preparations. Lane 1, molecular weight marker. Lane 2, horse RBC; lane 3, human RBC; and lane 4, mouse RBC. Coomassie blue stain.

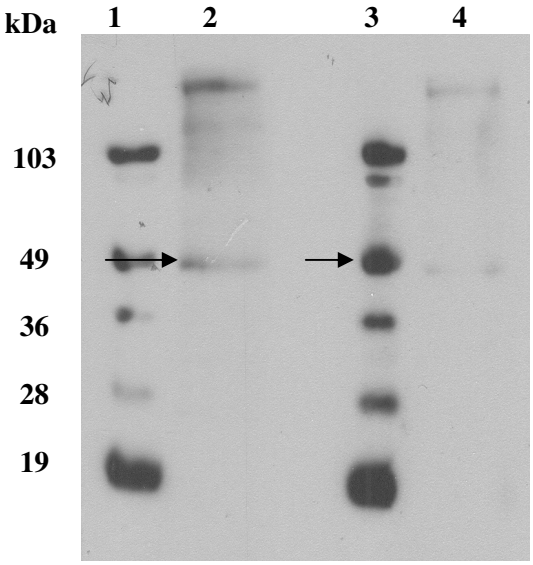


Figure 18. Far Western blot of mouse RBC membrane proteins. Lanes 1 and 3, molecular weight marker. Lane 2, probed with 60 µg BmAMA-1 ectodomain fusion protein. Lane 4, probed with 30 µg BmAMA-1 ectodomain fusion protein. Chemiluminescence.

In far Western blot analysis of mouse and human RBC membrane proteins probed with BmAMA-1 ectodomain fusion protein, binding of BmAMA-1 ectodomain fusion protein was detected at approximately 207, 114, and 49 kDa for mouse RBC and at 207 and 49 kDa for human RBC (Fig. 19). The reaction at 130 kDa observed above with mouse RBC membrane proteins was not seen.

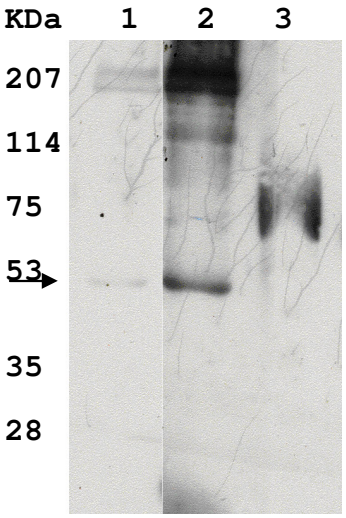


Figure 19. Far Western blot of mouse and human RBC membrane protein. Lane 1, human RBC. Lane 2, mouse RBC. Lane 3, BmAMA-1 ectodomain fusion protein. Probed with BmAMA-1 ectodomain fusion protein control. Chemiluminescence.

In far Western blot analysis comparing mouse, human, and horse RBC membrane proteins probed with BmAMA-1 ectodomain fusion protein (Fig. 20), BmAMA-1 binding to mouse RBC membrane proteins was detected at approximately 207, 56, 49, 37, 28 and 27 kDa, whereas binding to human RBC membrane protein was observed at 207, 114, 78, 56, 49 kDa. Thus BmAMA-1 fusion protein bound to RBC

membrane proteins was conserved at position 207, 56 and 49 kDa. The 49 kDa position was also conserved in the horse which indicates inter species conservation of the receptor at this position. However, the binding signal at 49 kDa was stronger in the

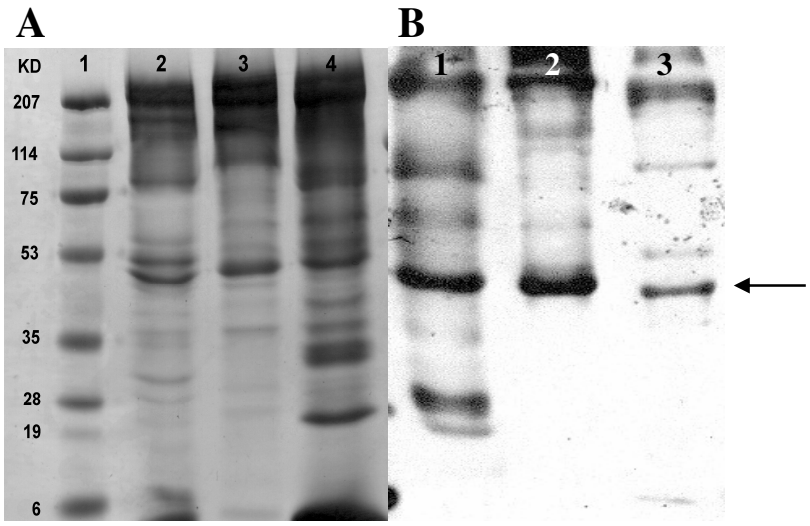


Figure 20. Comparison of horse, mouse and human RBC membrane proteins. **(A)** Coomassie blue stained SDS-PAGE and **(B)** far Western blot probed with BmAMA-1 ectodomain protein Lane 1, molecular weight marker. Lane 2, mouse RBC. Lane 3, human RBC. Lane 4, horse RBC. Arrow indicates binding between BmAMA-1 and 49 kDa RBC protein.

mouse and human RBC protein compared to the horse, possibly indicating more specific binding of BmAMA-1 ectodomain fusion to the mouse and human RBC membrane proteins. Subsequent far Western blots used more stringent washing to decrease the background observed in the previous experiments in analysis of binding of BmAMA-1 ectodomain fusion protein to mouse, human, or horse RBC membrane proteins. The BmAMA-1 binding to mouse RBC membrane proteins was detected at

approximately 75, 64, 49 kDa positions, whereas with human RBC proteins, the binding signal was detected at 205, 114, and 49 kDa (Fig. 21). Binding to the horse RBC membrane proteins was observed at approximately 53, 49 and 28 kDa (Fig. 22). Only binding to the protein at position 49 kDa appeared to beconserved in the 3 species. Under more stringent wash condition, the marked difference in signal intensity among the three species was no longer observed.

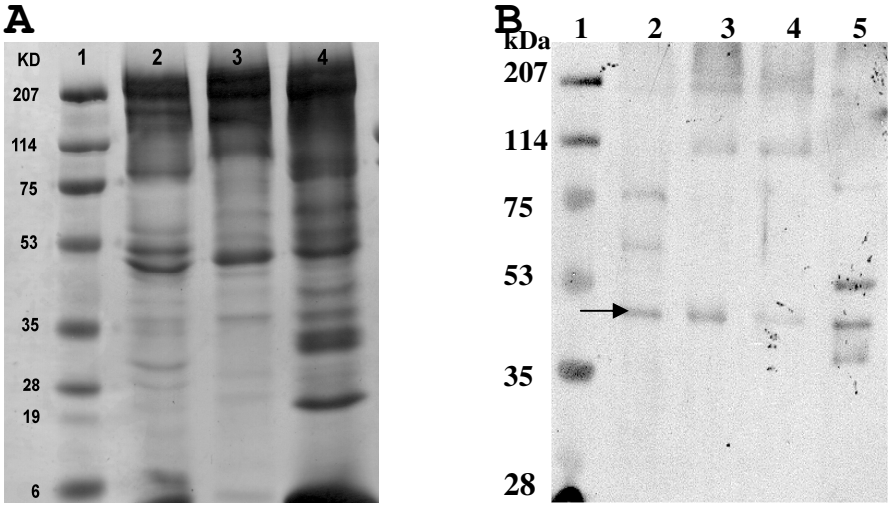


Figure 21. Far Western blot analysis. Mouse, horse and human RBC membrane protein probed with BmAMA-1 ectodomain protein. **(A)** Coomassie blue stained SDS-PAGE. Lane 1, molecular weight marker. Lanes 2, 3 and 4, mouse, horse, human and mouse RBC membrane protein, respectively. **(B**) Chemiluminescent far Western blot analysis. Lane 1, molecular weight marker. Lanes 2, 3 and 5 mouse, human and horse RBC membrane protein.

CHAPTER VI

DISCUSSION AND CONCLUSIONS

Babesia microti is a blood parasite that is transmitted by the tick *Ixodes scapularis* and is the most common cause of human babesiosis worldwide (Perry et al., 1977; Dammin et al., 1981; Foppa et al., 2002). The vast majority of cases occur in the United States (Dammin et al., 1981; Borggraefe et al., 2006). There are no reliable tests that can be used to screen blood and organ donors in order to prevent transmission. There is no vaccine for the disease and side effects following drug treatment make therapy problematic (Homer et al., 2000; Krause, 2003; Gelfand and Vannier, 2005).

Host cell invasion is a critical step in the pathogenesis of apicomplexan parasites including *Babesia* spp. (Mota et al., 2002; Krause, 2003; Lobo, 2005; Okamura et al., 2005). It is thought to involve a parasite ligand-host receptor interaction, which is supported by the following evidence. *In vitro* studies using *Babesia bovis* demonstrated binding ability of the merozoite RAP-1 to bovine erythrocyte lysate (Yokoyama et al., 2002), which suggests interaction between this parasite ligand and the bovine erythrocyte. Antibodies to RAP-1 protein on the other hand significantly inhibit the proliferation of *B. bovis* in *in vitro* culture (Yokoyama et al., 2002), which suggests that these antibodies blocked the ligand partner of the cell receptors needed for interaction and invasion.

Implication of apical membrane antigen 1 (AMA-1), another parasite ligand, in interaction with receptors on erythrocytes is evidenced by using antibodies to merozoite

AMA-1 to inhibit *Plasmodium knowlesi* invasion of erythrocytes *in vitro* (Thomas et al., 1984). This again shows that the ligand on the merozoites is blocked by these antibodies preventing its interaction with host cell to enable invasion. Evidence of the interaction between ligand and cell receptors was also demonstrated in *Toxoplasma gondii* by depleting the tachyzoites of their AMA-1, which severely compromised their ability to invade the host cells. This data again provides evidence that there is interaction between this ligand and the host cell receptors which precede invasion of the cells (Adrian et al., 2000; Mital et al., 2006). Also, complementation of the human malaria parasite *Plasmodium falciparum* AMA-1 with AMA-1 from the mouse parasite *Plasmodium chabaudi* allows *P. falciparum* to efficiently invade murine cells (Triglia et al., 2000), which also provides evidence that this ligand interacts with the host cell during invasion and that the interaction is species specific. Specific antibodies to *B. bovis* RAP-1 protein were used to inhibit sporozoite attachment to erythrocytes, indicating that these antibodies blocked the ligand that interacts with the host cell. Host cell invasion by *Eimeria tenella* sporozoites was completely inhibited by treating the sporozoites with proteases (Fuller and McDougald, 1990), which suggests that these proteases have hydrolyzed the proteins involved in interaction between the parasite and the erythrocytes.

The involvement of host cell receptors in parasite invasion has also been evidenced *in vitro*. First, pretreatment of the bovine erythrocytes with neuraminidase or trypsin, significantly decreases invasion by the parasite *B. bovis* and *Babesia divergens* (Zintl et al., 2002). This suggests that this treatment has removed the cell receptors that

could interact with the parasite to enable invasion. *Babesia equi* and *Babesia caballi* invasion of equine erythrocytes, on the other hand, was inhibited by treatment with neuraminidase, indicating again the involvement of cell surface receptors in invasion (Okamura et al., 2005). Erythrocytes deficient in glycophorin resist invasion by the malaria parasite *P. falciparum* (Pasvol et al., 1982), suggesting that the removal of this receptor from the host cell inhibits interaction with the parasite. Finally, surface proteins of *P. falciparum* merozoites bind to the erythrocyte receptor glycophorin and to products released by secretory organelles (Perkins, 1984), which indicates that *P. falciparum* merozoites interact with erythrocyte receptors and the proteins released from the apical organelles.

These data suggest parasite ligand-host cell receptor interactions occur in invasion by apicomplexans. This also suggests that there is basic step in invasion consisting of an interaction between a parasite stage ligand and its receptor, which is followed by the internalization of the parasite. The process of invasion is mediated by proteins released from the organelles of the apical complex, shared by all apicomplexans (Dubremetz et al., 1998; Yokoyama et al., 2006) and the receptors on host cells (Mitchell et al., 1986; Lobo et al., 1991; Jones et al., 1992; Zintl, et al., 2002; Yokoyama, et al., 2006). Interacting parasite ligand-host cell receptors may vary as a result of the stage of the parasite involved in invasion and host cell types infected.

The process of the entry of *Babesia* in host cell is likely similar to the entry described in other apicomplexan parasites, *Plasmodium, Toxoplasma, Eimeria,* and *Isospora*, because they are all mediated by an apical structure located in the apical

anterior end of these parasites. As indicated above, the type of cell infected may vary depending on the stage of the parasite involved in invasion. In *Eimeria* and *Isospora,* for instance, the oocyst excysts in the small intestine and the tachyzoites directly invade the cells lining the small intestine, which suggests that this stage of the parasite has the appropriate ligand to invade these cells. In *Babesia,* it is the sporozoite from the tick salivary glands that directly invades the erythrocytes (Jack and Ward, 1981; Igarashi et al., 1988; Krause, 2002), suggesting that this stage of the parasite has already developed the specific ligand to enter these cells. This is not seen in *Plasmodium,* where the sporozoite has to invade first the hepatocyte where it develops to merozoites which will in turn invade the erythrocytes.

In the most studied parasite, *Toxoplasma*, the asexual stage tachyzoites move over the cell surface by an unusual form of substrate-dependent gliding motility, which brings the apical end of the parasite in contact with the host cell membrane. Apical secretory organelles (micronemes and rhoptries) then sequentially discharge their contents (Dubremetz et al., 1998; Carruthers and Sibley, 1997) and a zone of tight interaction forms between the parasite and the host cell (Dubremetz et al., 1998; Grimwood and Smith, 1996). An invagination in the host cell plasma membrane develops at the point of entry and progressively deepens, ultimately surrounding the fully internalized parasite (Suss-Toby et al., 1996). Only the slowly replicating forms of the parasite, the bradyzoites, are found within the parasitophorous vacuoles. These vacuoles form cysts in tissue to protect the parasite from the host immune system (Hutchison et al., 1969; Dubey, 1977; Dubey et al., 1998). This type of invasion is

similar to that described in *Plasmodium* (Jack and Ward, 1981), but in malaria two types of host cells are involved. As stated above, the sporozoite stage does not invade the erythrocytes directly, but travels to the liver and passes through several hepatocytes before establishing in one hepatocyte, then develops to merozoites (Mota et al., 2002). Merozoites then invade the erythrocytes and begin the asexual reproductive cycle (Jack and Ward, 1981). This suggests that the *Plasmodium* sporozoites are not able to directly invade the erythrocytes as do *Babesia,* which suggests that when coming from the mosquito, this stage does not carry the proper ligand to infect erythrocytes. Perhaps the *Plasmodium* interacting ligand for the erythrocytes needs processing in sporozoites when developing to merozoites in the hepatocytes. This also suggests that the receptor on the erythrocyte may be different from that found on the hepatocyte, which are easily invaded by the sporozoites. The sporozoites of *Babesia*, on the other hand, invade the erythrocytes directly and do not use any intermediary host cell (Jack and Ward, 1981), which suggests that sporozoites from the tick stage have already developed the proper ligand for directly entering the erythrocyte. In addition to this difference, there is no pigment formation in *Babesia* merozoites establishment while this is observed in *Plasmodium,* which suggests different requirements for establishing in the host erythrocytes.

The difference between *Babesia* and *Toxoplasma* is that the tachyzoites reside within an intracellular vacuole and, following an unknown mechanism differentiates into encysted bradyzoites within host tissues (Dubey et al., 1998). These stages are not seen in *Babesia* where the sporozoites directly invade the host erythrocytes then reproduce by

asexual multiplication (Purnell, 1981; Taboada and Merchant, 1991; Homer et al., 2000). *Toxoplasma* and *Plasmodium* form a vacuole that is derived from the cell plasma membrane by an actin-myosin moving junction. But, the vacuole in *Plasmodium* does not form cyst as in *Toxoplasma,* and this stage is not found in *Babesia* (Jack and Ward, 1981; Muller et al., 1993; Sharma et al., 1996; Soren et al., 2000). Another parasite ligand, TRAP (Thrombospondin Anonymous Protein), is thought to interact with skeletal proteins in gliding motility in malaria sporozoites and in *Toxoplasma* tachyzoites. This mechanism has not yet been described in *Babesia* (Sultan et al., 1997; Dubremetz et al., 1998).

The roles played by proteins released from the apical complex of the parasite during erythrocyte invasion by some apicomplexan parasites such as *Plasmodium* and *Toxoplasma* have been investigated (Jack and Ward, 1981; Muller, 1993; Dalrymple et al., 1993; Collins et al., 1994; Denise et al., 1996; Adrian et al., 2000; Mota et al., 2002; Gaur et al., 2004). Proteins released from the rhoptries and micronemes have been shown to play a role in host cell invasion, but in most cases their precise functions remain incompletely understood (Dubremetz et al., 1998; Adrian et al., 2000; Gaur et al., 2004; Yokoyama et al., 2006). Apical membrane antigen-1 (AMA-1) is one of these proteins that is secreted from the organelle microneme and has been shown to be critical for invasion. The precise function of AMA-1 is not well elucidated, but its role in host cell invasion is well documented (Triglia et al., 2000; Mota and Rodriguez, 2002; Yokoyama et al., 2006). AMA-1 homologues have been found in all apicomplexans studied to date (Triglia et al., 2000; Hodder et al., 2001; Silvie et al., 2004) and

characteristics such as the N-terminal ectoplasmic region, single transmembrane segment and small cytoplasmic domains are conserved. The AMA-1 molecule has remarkable conservation within and across genera (Waters et al., 1991; Denise et al., 1996, Hodder et al., 2001). AMA-1 is stored in the microneme organelles after synthesis and is transported to the parasite surface just prior to, or during, host-cell invasion (Gaffar et al., 2004; Chesne-Seck et al., 2005).

AMA-1 has been shown in *P. falciparum* and in *B. bovis* to inhibit host erythrocyte invasion by these parasites (Anders et al., 1998; Gaffar et al., 2004). Furthermore, *T. gondii* tachyzoites depleted of TgAMA-1 were severely compromised in their ability to invade host cells, providing direct genetic evidence that AMA-1 functions during invasion (Adrian et al., 2000; Mital et al., 2006). Also the specificity of protective antibody against AMA-1 has been documented in *P. falciparum* and was found in ectodomain portion of the ligand and was directed to conserved and strain-specific epitopes (Hodder et al., 2001). The presence of conserved epitopes in this domain and the ability of antibodies to this domain to inhibit parasite invasion suggests that it plays an important role in the process of invasion.

Based on the above data, the ectodomain of *B. microti* AMA-1 was used in this study. The full cDNA of the *B. microti* AMA-1 was previously prepared in the Dr. P.J. Holman laboratory by reverse transcription of mRNA obtained from U.S. isolate Peabody strain (Ruebush and Hanson, 1979). The *B. microti* full AMA-1 sequence possesses characteristics in common with AMA-1 from other apicomplexans, including a transmembrane domain, short cytoplasmic C-terminal, extracellular domain and 14

conserved cysteine residues. The complete nucleotide sequence of the *B. microti ama-1* gene is 1956 base pairs. The sequence contains an open reading frame of 1944 base pairs with a coding capacity of approximately 73 kDa (648 amino-acids) (Protein Calculator v3.3). The hydrophobic N-terminal, 42 amino-acids of *B. microti* AMA-1 were predicted to form a signal peptide (Signal P 3.0), whereas the hydrophobic stretch of 111 amino-acids from Phe-1590-to Leu-1923 likely forms a transmembrane region, leaving a 11 amino-acids as a cytoplasmic C-terminus (TMHMM 2.0).

The ectodomain of the *B. microti* AMA-1 carrying 12 cysteine residues was used in this study instead of the entire *B. microti ama-1* gene because, as previously indicated, the specificity of the protective antibody to the AMA-1 is located in this portion, and is directed to conserved and strain-specific epitopes (Hodder et al., 2001). In addition, this portion of the membrane bound polypeptide presumably extends into the extracellular space and likely interacts or initiates interaction with the host erythrocyte surface receptors.

The 1474 base pair open reading frame of the *B. microti* AMA-1 ectodomain was amplified using specific primers designed from the 5´ and 3´ends excluding the signal peptide and the transmembrane region of *B. microti ama*-1 gene. These two domains were removed because the first attempts to express AMA-1 sequence carrying these regions were not successful. Generally, expression of recombinant proteins carrying hydrophobic regions, such as the signal peptide and the transmembrane domain, often have a toxic effect on the host cell which blocks expression. This is most likely due to the association of the protein with toxic products and the incorporation of these products

into vital membrane systems (Waeber et al., 1993). For the various known signal peptides, there is no consensus sequence in the primary structure, but they share common structural features consisting of three distinct regions: the basic region, the hydrophobic region, and the cleavage region (Goldstein et al., 1990). Signal peptide hydrophobicity appears to be more critical for the efficiency of early stages in protein expression and export (Goldstein et al., 1990). A hydropathic plot of *B. microti* AMA-1 amino acid sequence showed 3 hydrophobic domains in the transmembrane region (TMHMM 2.0). Based on the data above and the potential toxic effect of hydrophobic domains on expression, the sequences coding for the signal peptide and the transmembrane domain were removed from the *B. microti ama-1* gene sequence.

The ectodomain region was amplified from the full *B. microti ama-1*gene sequence using specific primers internal to the signal peptide and the transmembrane region. The ectodomain was ligated into the pET TOPO 101D vector, which produces a fusion protein with a V5 epitope and 6x His tag at the C-terminus. Vectors that place the His-tag at the C-terminus are recommended for open reading frames which may result in premature translation termination (Fry and Loeb, 1992) and to ensure that only full-length proteins are purified. The features of the pET TOPO 101D plasmid, such as T7*lac* promoter, IPTG-inducible expression of the gene in *E. coli,* C-terminal fusion tags for detection and purification of recombinant fusion, pBR322 origin for stringent replication and maintenance in *E. coli* of the *lacI* gene that reduces basal transcription from the T7*lac* promoter make this plasmid an excellent choice for this study. Use of pBR322 origin for stringent replication is important because high copy number (relaxed

plasmids) allows maximum gene expression, but the metabolic burden effects resulting from multiple plasmid copies could be detrimental to expression (Jones et al., 2000).

An advantage of the pET TOPO101 D was that the primer design allowed directional ligation of the *B. microti* ectodomain gene sequence in this vector. PCR products were generated using a high fidelity DNA polymerase (Phusion, New England BioLabs, Ipswich, MA). The choice of the polymerase is important because the specificity of the interaction between the ectodomain and the receptors on the host cell will depend on the correct sequence of the residues in each binding partner and may be linked to a single or few residiues in the ectodomain. This particular polymerase has a strong proofreading activity and a processivity-enhancing domain. Its error rate is approximately 50-fold lower than that of Taq polymerase (New England BioLabs). This polymerase amplifies with an extreme fidelity and possesses a 5´→3´ DNA polymerase activity and 3´→5´ exonuclease activity. Denaturation and annealing were carried out at elevated temperatures, thus increasing the specificity of the amplification. This characteristic makes this polymerase a good choice for this study. But, the pitfall in ligating the insert in this vector is that the percentage yield for suitable plasmid was very low. Only one clone out of seven (15%) was found with proper orientation with no internal stop codon. All the clones sequenced had the insert. However, internal stop codons were found in all except one probably due to mis-incorporation or deletion of nucleotide during gene amplification which resulted in translational frameshifting (Farabaugh, 1996). This may be due to polymerase inaccuracy during amplification which causes nucletotides to be incorporated or deleted from the sequence. In order to

correct this pitfall and increase our chance of getting more clones with correct sequence and no internal stop codons, a polymerase having a tight control of polymerase-catalyzed DNA extension with very low probability of base mis-incorporation should be used to amplify the *B. microti* ectodomain fusion protein. Isis DNA polymerase (MP Biomedicals, Solon, OH) shows this advantage with a rate of one base per 1600 molecules of 1.0 kb DNA per PCR cycle. This polymerase has also a very robust activity and requires less time for reaction set-up and optimization, which will decrease base deletion or mis-incorporation. This polymerase is recommended for PCR applications requiring very low probability of base mis-incorporation (MP Biomedicals, Solon, OH). Even though this polymerase has a lower proofreading capacity (error rate 40-fold lower than Taq) than the Phusion High Fidelity Polymerase, the features cited above make it a good choice for amplifying *B. microti* AMA-1 ectodomain fusion protein.

For expressing *B. microti* AMA-1 ectodomain fusion protein, BL21 *E. coli* strain (Invitrogen, Carlsbad, CA) was used. This strain was selected because of previous reported success in expressing *B. bovis* apical membrane antigen-1 (Gaffar et al., 2004). BL 21 *E. coli* lacks two key proteases *lon* protease and *OmpT,* which reduce degradation of heterologous proteins expressed in this strain (Invitrogen, Carlsbad, CA). *Babesia microti* AMA-1 ectodomain fusion proteins were obtained with molecular mass of 58.1 kDa for fusion protein as expected, with 4 kDa accounting for the vector sequence including the 6x His tag. However, the pitfall of using this strain is that expression was not reproducible using clones from the same batch of cultures which suggests clonal variation in ability to express the protein. This may be caused by clones losing the

expression plasmid or the plasmid losing the insert during the transformation or expression process. It may also be due to low level of expressed protein, which may not be detectable by our analysis methods. In order to prevent the loss of expression plasmid resulting from non-selective conditions and to increase expression levels, carbenicillin was used in selective media as suggested by the manufacturer. Carbenicillin is generally more stable than ampicillin, and studies have shown that using carbenicillin in place of ampicillin may help to increase expression levels by preventing loss of the pET TOPO® plasmid (Invitrogen, Carlsbad, CA). However, the use of carbenicillin in place of ampicillin did not correct this problem in our expression.

The low expression in this strain could also be attributed to stalling of expression, possibly due to depletion of specific tRNAs not found in many prokaryotic expression systems. This occurs especially when expressing proteins from AT or GC rich genomes of protozoan origin like *Babesia* spp. (Marshall et al., 1989; Flick et al., 2004; Kirsten et al., 2004). Analysis of the *B. microti* AMA-1 ectodomain sequence showed 58% AT (mbcf.dfci.Harvard.edu/docs/oligocalc.htm) which is low when compared to over 69 to 70% for *Plasmodium* spp. (Marshall et al., 1989). In order to correct potential codon bias in expressing the *B. microti* AMA-1 ectodomain we selected BL21 Codon Plus. This strain contains extra copies of specific tRNAs for ile, leu, pro and arg (Stratagene, La Jolla, CA). Availability of these tRNAs may allow high-level expression of many heterologous recombinant proteins not highly expressed in other *E. coli* strains. The BL21 codon Plus strain was successfully used to express malaria AMA-1 gene (Flick et al., 2004). Therefore, it was reasoned that using this strain may

correct potential codon bias that might stall the expression of our protein. The *B. microti* ectodomain possesses 7, 2, 4, 6 and 5 residues corresponding to the rare ile, leu, pro and arg tRNAS provided by this strain. Thus, the requirement for these particular tRNAs is relatively low in the *B. microti* AMA-1 ectodomain protein sequence for constituting a potential problem for expression. Another advantage in using this strain is that chloramphenicol is required in the medium to maintain plasmid stability in the cell in addition to the selective marker carbenicillin which also prevents the loss of the plasmid. Thus, plasmid stability in this strain is reinforced by the use of chloramphenicol in addition to the carbenicillin in the bacterial culture medium.

Babesia microti AMA-1 ectodomain was successfully expressed in the BL21 Codon Plus strain and the expression was reproducible. However, the concentration of protein yielded was less (0.29 ug/ul) than what was achieved in BL21 Star DE3 (0.83 ug/ul), suggesting that the low level or lack of expressed protein in certain clones of BL21 Star DE3 was not a result of stalling due to lack or depletion of certain tRNAs pool, but probably to other components in the expression process. For instance, growth inhibition due to toxic products released by the cells or cessation of plasmid-bearing cells after induction could well result in no yield or reduced yield. Changes in expression capacity of certain cells have been documented and were attributed to metabolic burden or toxicity caused by the overexpression of the target gene or to loss of the expression plasmid (Zhao and Wei, 2007). In our specific case, only the loss of the expression plasmid can be suspected using BL21 Star DE3 because the expression was done successfully using some clones. This suggests that the clone which did not express our

protein may have lost the plasmid when growing on agar plate or during cell growth in Luria Bertani (LB) medium.

Expressed recombinant BmAMA-1 fusion protein was analyzed by SDS-PAGE gel stained with Coomassie blue (BIO-RAD, Hercules, CA), then by Western blot analysis. Although Coomassie blue detects 50-fold less protein (30-100 ng) than silver stain (1 ng), we were able to detect our protein using this stain and monitor the timeline signal of our desired 58.1 kDa protein. However, silver stain should be used in future expression experiments to assess the efficiency of our purification process. This will help determined the number of non-specific bands (or contaminants) at the nanogram level that might not react in Western blot analysis.

Enrichment of the *B. mciroti* AMA-1 recombinant protein under native conditions was not successful as the protein was found in the lysate flow-through, which indicates that the protein did not bind to the nickel column, or did not carry the His tag to allow it to bind. This suggests that the protein extraction method did not result in protein that was in a form that could bind to the affinity column. In general, heterologous protein expression in *E. coli* most often leads to the protein being expressed in insoluble forms known as inclusion bodies. The tag of the protein likely is partially hidden in these forms (Bowden and Gergiou, 1990; Thomas and Baneyx, 1996; Chumpia et al., 2003). Under denaturing conditions, the tag will be exposed to allow binding to the Ni-NTA (Nickel-Nitrilotriacetic Acid) matrix (Thomas and Baneyx, 1996).

Based on the results outlined above, affinity enrichment was used under denaturing conditions, which produced fusion protein that was visualized using SDS-

PAGE stained with Coomassie blue and by Western blot analysis. Ni-NTA Spin Column (QIAGEN, Valencia, CA) was used for rapid screening and enrichment of the 6x His-tagged *B. microti* AMA-1 ectodomain proteins from 50-ml cultures. When eluted fusion proteins were analyzed on SDS-PAGE stained with Coomassie blue and in Western blot one to six bands between 58 kDa and 23 kDa were observed. The extra bands could be other histidine rich protein or possibly breakdown products of the *B. microti* AMA-1 ectodomain fusion protein. Since protease inhibitors were not added to these elutions, these bands, or some of these bands, could well be breakdown products of the fusion protein.

The failure of the native purification and the success in enrichment under denaturing conditions suggest that the His-tag on the fusion protein was not exposed under native conditions and that denaturing agents exposed the histidine residues allowing interaction with the nickel ions on the column.

The microspin nickel affinity resin used for rapid screening and enrichment of the expressed protein accommodates only 600 µl of cleared cell lysate derived from a 50-ml culture. The column is centrifuged immediately after loading the cleared supernatant containing the fusion protein, which does not give much time for the fusion protein histidine residues to interact with the nickel resin. This might explain the low yield after enrichment as revealed by low signal of bands following staining with Coomassie blue and in Western blot using anti-His antibody.

Downstream applications are focused specifically on epitope interactions between the fusion protein and the host cell receptors, and raising antibody against the

fusion protein to use in invasion inhibition and other assays. Enrichment of recombinant proteins under native conditions is generally preferred, but if proteins are to be used for antibody production there often is no need for native enrichment or renaturation (Wingfield et al., 1995; Von Specht et al., 2000). Downstream applications such as examining protein interactions and antibody production are epitope-based assays, therefore native enrichment is not critical for these interactions. However, proper controls should be set in each experiment to determine the validity of the results.

Enrichment of *B. microti* AMA-1 recombinant protein from large scale cultures was achieved using a gravity-driven nickel affinity column (FAST START, QIAGEN, Valencia, CA) under denaturing conditions. To increase the yield, the cell pellet was frozen and thawed three times in liquid nitrogen to free the recombinant protein. Protease inhibitors were added before the pellets were thawed for the first time to prevent degradation of the protein. SDS-PAGE and Western blot analysis of the subsequently enriched proteins revealed almost no recombinant protein in the clear lysate flow-through indicating binding of the target protein to the capture resin. Including freeze/thaw cycles increased the binding of the BmAMA-1 protein and, therefore, the efficiency of the enrichment. There were 3 to 4 bands between 58 kDa and 30 kDa observed on SDS-PAGE and Western blot following the enrichment by this method, with eluted protein concentrations from 0.28 µg/µl to 0.83 µg/µl for all enrichments using this method. The gravity-driven affinity column holds up to 10 ml of cleared cell lysate. Pellets from 250 ml-culture were used to increase the concentration of the eluted protein to get the best yield. This procedure gives more time for the His tag

to interact with the nickel ions that could lead to more affinity binding and more eluted fusion protein. These *B. microti* eluted fusion proteins were used in downstream applications (far Western blot, immunoprecipitation, and immunofluorescent binding assays).

To produce *B. microti* AMA-1 ectodomain fusion protein in high concentration for other applications using these methods, a large volume of the cleared cell lysate supernatant must be enriched because microgram amounts will be needed for antibody production and *in vivo* immunization assays, for example. Five to 10 liters of bacterial culture should be processed and enriched under denaturing conditions using bigger affinity purification columns. This large volume could yield 22 to 44 μg of *B. microti* fusion protein considering the lowest yield 0.28 μg/μl (in approximately 1 ml elution) under the conditions cited here.

Interaction of *B. microti* AMA-1 ectodomain with host red blood cell membrane proteins was investigated. Enriched recombinant *B. microti* AMA-1 showed bioactivity in far Western blot analysis using mouse, human, or horse RBC membrane protein. Interaction with a protein at position 49 kDa was observed when RBC membrane proteins from the mouse, human and horse were reacted with *B. microti* AMA-1 ectodomain protein. This band was consistent in all experiments. The specificity of the binding was confirmed by testing potential binding of the primary and secondary antibodies to the RBC membrane proteins.

The signal of binding at 49 kDa observed with the horse RBC appears to be less than with mouse and human RBC membrane proteins, possibly indicating less

specificity of *B. microti* AMA-1 ectodomain fusion protein for the horse RBC. The relatively higher signal observed in mouse and human suggests more specific binding of the protein for the RBC proteins of these species, which are known hosts for this parasite. However, this difference in the binding signal was not consistently observed in subsequent experiments using interaction with the same erythrocyte membrane proteins, especially under more stringent wash conditions. The non-reproducibility of the results under different experimental conditions suggests that alternatives should be tested, such as using specific anti-BmAMA-1 antibody instead of anti-His antibody for detecting the interaction with the RBC membrane proteins. The current results suggest that the difference in the intensity of the interacting signal observed at 49 kDa may not be linked to the susceptibility of the host to this parasite, but more likely to the nature of the RBC receptor involved in interaction. It is also possible that the binding observed is not BmAMA-1 binding to the RBC protein, but rather one of the multiple bands found in the enriched protein. Using a specific antibody would determine if BmAMA-1 is binding to the 49 kDa protein. Therefore, further investigations are needed to confirm that the *B. microti* AMA-1 ectodomain fusion protein binds at this position. Once confirmed, then further investigation also is needed to assess the composition of the binding partner, the 49 kDa RBC membrane protein, as discussed below. .

Further work will require more BmAMA-1 recombinant protein of high quality and specific antibody to this protein. Large volumes of culture (4-6 liters to yield approximately 4-8 µg of protein considering a lowest yield of 0.28 µg/µl from 250-ml cultures) should be grown and processed to purify the *B. microti* AMA-1 ectodomain

fusion protein. The fusion protein will be enriched from the cleared supernatant using anti-V5 antibody (Southern et al., 1991) coupled to magnetic beads for more specific binding of the protein to the capture matrix. The capture antibody must react specifically with our *B. microti* AMA-1 ectodomain fusion protein, thus using antibody to the V5 epitope will minimize the non-specific capture of histidine-rich contaminants observed using His-tag affinity enrichment. Unbound proteins will be washed away then the fusion protein will be eluted in solution containing protease inhibitors. Samples will be processed by SDS-PAGE and protein bands will be revealed by silver staining to assess the level of enrichment and by Western blot analysis using both anti-His antibody and anti-V5 antibody. If there are extra bands observed in using silver staining and Western blot using His-tag this confirms the presence of histidine-rich proteins which are not breakdown products of the fusion proteins stated earlier. Therefore, affinity enrichment using nickel column may need to be followed by another methods such as V5 antibody or specific BmAMA-1 antibody affinity purification to obtain highly purified BmAMA-1 recombinat protein for use downstream applications. The quality of the protein purified by this method will be determined by SDS-PAGE stained with silver stain and by Western blot analysis, and the concentration determined. Antibodies to highly purified fusion protein will be raised in rabbits.

To show interaction between the 49 kDa RBC membrane protein and BmAMA-1, purified *B. microti* AMA-1 ectodomain fusion protein obtained above will be electrophoresed on SDS-PAGE as previously described, then transferred to nitrocellulose membrane. Membranes carrying the *B. microti* AMA-1 ectodomain fusion

protein will be incubated with erythrocyte membrane proteins (mouse, human and horse). Unbound membrane proteins will be washed away and the membranes probed with anti-V5 antibody tagged with horseradish peroxidase, or another enzyme, then reacted with the substrate to detect binding. There should be no reaction at the 49 kDa position, indicating that the erythrocyte membrane proteins bound to the protein at this position and prevented the AMA-1 antibody from binding. Controls will include reacting anti-V5 antibody directly with the fusion protein on the membrane.

Another experiment to confirm binding of *B. microti* AMA-1 ectodomain fusion protein at the 49 kDa band position will be done using far Western blot analysis. Erythrocyte membrane proteins from mouse, human, and horse will be separated by SDS-PAGE in two gels and transferred to nitrocellulose membranes for far Western blot assays. The first membrane will be probed with *B. microti* AMA-1 ectodomain fusion protein with the V5 epitope, whereas the second membrane will be probed with an irrelevant protein also carrying a V5 epitope. Binding of the two proteins at 49 kDa position will be determined by anti-V5 antibody tagged with horseradish peroxidase or with fluorescein for detection. There should be no interacting band at position 49 kDa on the membrane probed with the irrelevant protein, indicating that the irrelevant protein did not bind at this position, whereas the band at position 49 kDa should be detected on the membrane probed with BmAMA-1, showing that the *B. microti* AMA-1 ectodomain fusion protein bound to that RBC protein. Controls should include anti-V5 antibody that will be reacted with the erythrocyte membrane proteins and *B. microti* AMA-1

ectodomain fusion protein that will be reacted with the anti-V5 antibody. Results from this experiment will determine binding of *B. microti* AMA-1 ectodomain at this position.

The next experiment will be to elucidate the *B. microti* AMA-1 erythrocyte binding partner(s) by far Western blot analysis and mass spectrometry analysis of the interacting band at 49 kDa position. Erythrocytes membrane proteins from mouse, human and horse will be separated by SDS-PAGE in duplicate gels. One gel will be stained with silver stain and the other will be analyzed by far Western blotting to confirm binding of BmAMA-1 with the protein at 49 kDa. The RBC proteins that show binding with the BmAMA-1 protein will be further analyzed by mass spectrometry and Edmann amino acid sequencing. Analysis of the amino-acid composition of these bands in *B. microti* susceptible and in non-susceptible host erythrocytes may provide insight in the amino-acids residues involved in interaction.

The sialic-acid rich residues of the erythrocyte receptors (glycophorin) have been implicated as receptors for AMA-1 in many apicomplexans (Pasvol et al., 1982; Perkins et al., 1984; Heinz, 1978; Zintl et al., 2002; Gaffar et al., 2003; Gaur et al., 2004; Lobo, 2005; Okamura et al., 2005). Glycophorin implication as erythrocyte receptors in cell invasion by *B. equi* and *B. caballi* has been investigated (Murayma et al., 1981; Okamura et al., 2005). Dimers and monomers of glycophorin in terms of their molecular weights and sialic acid-rich nature were then removed by neuraminidase treatments. *In vitro* growth of both parasites was influenced by the removal of sialic acids from the surface of equine erythrocytes, suggesting that glycophorin interacts with the parasites to allow invasion by *B. caballi* and *B. equi. Babesia divergens* invasion of bovine

erythrocytes was also inhibited by neuraminidase treatment, suggesting that the sialic acid-rich residues (glycophorin) are the interacting partner of the parasite on the surface of the cells (Zintl et al., 2002). *Babesia bovis* merozoites were shown to invade human, ovine, porcine and caprine erythrocytes by sialic acid dependent mechanisms (Gaffar et al., 2003), indicating these parasites interact with these receptors to enable them to invade the cell.

Furthermore, *Plasmodium* spp. merozoites and products released by secretory organelles were shown to bind to glycophorin (Perkins, 1984). Erythrocytes deficient in glycophorin resisted invasion by *P. falciparum*, indicating that this parasite and its released products bind to this receptor and that removal of this receptor prevents invasion by this parasite. Finally, it has been shown that *Babesia* and *Plasmodium* use common receptors, glycophorin A and B, to invade host erythrocytes (Lobo, 2005), which suggests that the role of this receptor in cell invasion is shared by different genera in the phylum apicomplexan.

The findings described above indicate that current knowledge targets the sialic acid-rich residues of glycophorin as the erythrocyte receptor involved in invasion by both *Babesia* and *Plasmodium*. But, as yet the detailed knowledge of these residues and the structure of the ligand or part of the ligand that binds to them remain incompletely understood (Dubremetz et al., 1998; Gaur et al., 2004; Triglia et al., 2000; Yokoyama et al., 2006). For *B. microti* AMA-1 ectodomain fusion protein, further studies are needed to determine if the band at position 49 kDa is first sialic acid-rich (glycophorin) and then determine the structural composition of this band. Potentially, this will enable not just

identification of the receptor structures required for invasion by *B. microti*, but potentially could be used to identify potential susceptible hosts for this parasite by just comparing the amino-acid composition of their erythrocyte receptors to those of susceptible hosts (mouse, human).

Far Western blot analysis in the current study indicates binding of *B. microti* *A*MA-1 ectodomain fusion protein to horse erythrocyte membrane proteins as well as to mouse and human, suggesting that the interacting receptors on these erythrocytes may share similar structures. It is possible that these receptors are the sialic acid-rich residues present on the erythrocytes of these three species, as demonstrated for other apicomplexans, since erythrocytes from all species possess sialic acid-rich reidues (glycophorin) (Pasvol, 1982; Perkins, 1984; Zintl et al., 2002; Okamura et al., 2005). However, other residues like those suspected in sialic acid independent pathway might be involved (Lobo et al., 1991; Yokoyama et al., 2006)**.** Additional experiments will be needed to further confirm the involvement of these receptors in invasion by *B. microti*.

The role played by glycophorin as a receptor in invasion by *B. microti* may be investigated using *in vitro* assays and *in vivo* assays. *In vitro* assays might include enzyme treatment of erythrocytes, blocking the putative receptor with anti-glycophorin antibody, and far Western blot analysis testing binding of BmAMA-1 to membrane proteins from normal erthryrocytes and from glycophorin deficient erythrocytes. *In vivo* assays might use infection studies of transgenic mice or hamsters deficient in glycophorin compared to normal controls.

Babesia microti AMA-1 binding to mouse, human and horse erythrocytes treated by neuraminidase could be evaluated based on the discussion above (Pasvol et al., 1982; Zintl et al., 2002; Lobo et al., 2005). Briefly, erythrocytes from mouse, human, and horse will be washed with PBS then incubated in neuraminidase solution. Incubated erythrocytes will be washed in PBS containing non ionic detergents, then erythrocyte membrane proteins will be prepared as previously described. Far Western blot assays will be done as previously described. Treated and non-treated erythrocyte membrane proteins will be separated by SDS-PAGE, transferred to nitrocellulose, then probed with *B.microti* AMA-1 ectodomain fusion protein. Anti-V5 antibody labeled with an enzyme or with fluorescein will be used to detect binding. Non-treated erythrocyte membrane proteins will be used as controls. Controls will also include probing the RBC proteins with anti-V5 antibody alone. Expected results will be no signal between the treated RBC protein and BmAMA-1, which would suggest that glycophorin is involved in the interaction between *B. microti* AMA-1 and the host erythrocyte. Signal may be observed if treatment with neuraminidase is not complete, meaning all sialic residues were not removed during treatment. Signal may also occur if glycophorin is not the erythrocyte receptor for BmAMA-1. Using more concentration of neuraminidase or longer incubation in neuraminidase followed by stringent washing could be used to test for binding to residual sialic acid.

Anti-glycophorin antibody that binds to the erythrocyte membrane proteins to prevent binding of *B. microti* AMA-1 ectodomain fusion protein might be used in a far Western assay. Electrotransferred erythrocyte membrane proteins will be incubated with

anti-glycophorin antibody then reacted with recombinant BmAMA-1 fusion protein. Controls will include erythrocyte membrane proteins probed with *B. microti* AMA-1 ectodomain fusion protein as a positive control, and the V5 antibody that will bind directly to the erythrocyte membrane proteins. Absence or very low signal at position 49 kDa in the reaction compared to the positive control signal, would suggest that anti-glycophorin antibody prevented binding of *B.microti* AMA-1 ectodomain fusion protein. This might indicate that glycophorin is an interacting partner for *B. microti*.

Offspring of transgenic mice or hamsters produced by knocking out a gene that codes for glycophorin or by disrupting this gene could be assessed for infectivity by *B. microti.* The following generation of mice or hamsters deficient in glycophorin on their erythrocytes would be challenged by inoculating them with *B. microti* infected blood. Controls would include normal animals challenged with the same dose as the transgenics. The parasitemias would be monitored daily to determine infection. The level of parasitemias in test animals when compared to the controls would indicate that glycophorin acts or does not act as a receptor in invasion by *B. microti*.

Future studies will be focused on elucidating the structure of the interacting partner of *B. microti* AMA-1 and the 49 kDa mouse, human and horse erythrocyte membrane proteins. For erythrocyte receptor involvement in invasion, sialic acid-dependent and sialic acid-independent interactions (Mitchell et al., 1986; Lobo et al., 1991; Lobo, 2005) must be investigated to determine the impact of each in the invasion process by this parasite. This will indicate if in interaction is restricted to glycophorin or if other interacting partners are involved.

Ideally, in order to investigate the role played by transmembrane proteins (sialic independent pathway) erythrocytes will be treated with neuraminidsae and then trypsin respectively and assayed for invasion *in vitro*. However, at present there is no *in vitro* culture system for *B. microti*. Further reduction of invasion by these two treatments when compared to treatment by neuraminidase only would suggest that the receptor role is not restricted to glycophorin only and that an alternative pathway may be involved.

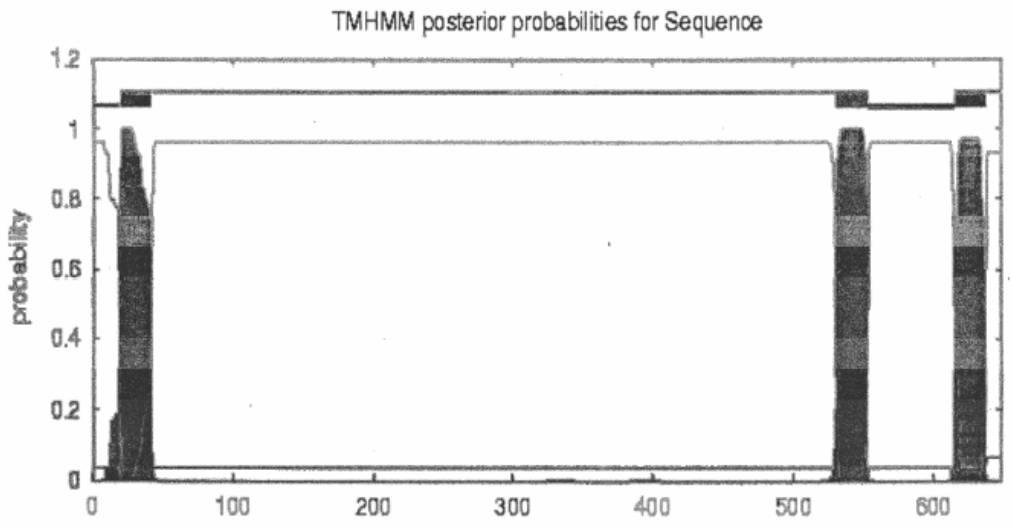
Studies should also include additional *in vivo* invasion inhibition assays. These might include passive transfer of anti-*B. microti* AMA-1 antibodies to mice or hamsters as described in infection studies for other apicomplexans. Then, mice or hamsters would be challenged with *B. microti* infected blood (Thomas et al., 1984). Controls would include mice and hamsters that receive no anti-*B. microti* AMA-1 antibodies. Parasitemia in test and control animals would be monitored daily and compared on a plot. Low, static, or no parasitemia would indicate protection conferred by anti- *B. microti* AMA-1 antibodies.

Protection from infection after inoculating mice or hamsters with the recombinant *B. microti* AMA-1 ectodomain fusion protein might be assayed as described in similar experiments for *Plasmodium* spp. (Deans et al, 1984; Collins et al., 1994; Anders et al 2005). The V5 epitope and the His-tag would be removed from the recombinant protein before immunization. Test mice or hamsters will be immunized with *B. microti* AMA-1 ectodomain protein in Freund's Adjuvant (complete, incomplete). The resulting antibody level would be be measured by Enzyme Linked Immunosorbant assay (ELISA) until it reaches a plateau. Controls would include mice or

hamsters that receive only the adjuvant. The animals would be challenged with the same dose of *B. microti* infected blood after the antibody plateaus. Parasitemias in test animals and in controls after challenge would be evaluated daily and analysed on a plot. If the immunized animals do not develop a parasitemia or if they develop a low parasitemia compared to the controls, this would indicate that immunization with *B. microti* AMA-1 ectodomain protein inhibits erythrocyte invasion *in vivo*, which would suggests a protective role by this portion of the *B. microti* AMA-1 ligand. Studies may also include *in vitro* cross invasion inhibition assays and cross protection studies using *B. divergens*.

Finally, an important inference in the interaction between *B. microti* AMA-1 ectodomain fusion protein and the receptors on the erythrocytes membrane could be the transmembrane domain of the *B. microti* AMA-1. This domain could have a profound effect on the stability of interaction.Therefore, a hydropathic plot of *B. microti* AMA-1 was constructed using TMHMM 2.0 (Fig. 22). Analysis of this plot showed three hydrophobic domains represented by residues 20-42, 531-553 and 616-638 and four hydrophilic domain residues 1-19, 43-530, 554-6145 and 639-649.

The hydrophobic domain of the protein resides only in the lipid bilayer core of the membrane, while hydrophilic domains protrude into the watery environment inside and outside the cell. The lipid environment provided by the transmembrane domain could affect the stability of interaction as a result of signal transduction through hydrophobic or hydrophilic interactions between the erythrocytes and this domain. The impact of this domain on the stability of the interaction might be investigated by expressing the *B. microti* AMA-1 protein with this domain and probing RBC membrane




```
# Sequence Length: 649
# Sequence Number of predicted TMHs:  3
# Sequence Exp number of AAs in TMHs: 66.61247
# Sequence Exp number, first 60 AAs:  22.50695
# Sequence Total prob of N-in:        0.96281
# Sequence POSSIBLE N-term signal sequence
Sequence        TMHMM2.0        inside       1    19
Sequence        TMHMM2.0        TMhelix     20    42
Sequence        TMHMM2.0        outside     43   530
Sequence        TMHMM2.0        TMhelix    531   553
Sequence        TMHMM2.0        inside     554   615
Sequence        TMHMM2.0        TMhelix    616   638
Sequence        TMHMM2.0        outside    639   649
```

Figure 22. Hydropathic plot of B. microti AMA-1. TMHMM 2.0 (CBS; Denmark). Len=649, ExpAA=66.61, First 60= 22.51, PredHel=3.

proteins in far Western blot assays. Because of its hydrophobic nature, other expression systems such as yeast or mammalian cells may be better used to express this domain.

In summary, invasion of host cells by apicomplexans is likely a multi-step process which involves proteins released from the rhoptries and the micronemes and possibly two types of receptors (sialic-acids and proteins) on the host cells. The role played by both the parasite ligands and the receptors on the host cells are not yet well understood. However, data obtained so far target three ligand proteins, RAP, AMA-1

and TRAP, from the parasite apical complex, which appear to be involved in invasion. However, the involvement of these ligands during invasion may vary depending on the parasite stage and cell type. Among these ligands, based on the information to date, AMA-1 appears to be the most critical in invasion.

Cell surface receptor partners involved in invasion have also been investigated, especially the receptors on erythrocytes. Data obtained so far suggest that glycophorin is the main interacting receptor partner on these cells. However, an alternative pathway of invasion has been described (Lobo et al., 2003) called the sialic-independent pathway. Further investigation is needed to study the roles played by these two pathways in *Babesia* invasion of the host erythrocyte and to determine the structures of the receptors and the structures of the ligands involved in the host-parasite interaction.

In conclusion, this study provides a new approach in investigating the role played by *B.microti* AMA-1 in erythrocytes invasion by this parasite. A specifc domain (ectodomain) of this ligand (AMA-1) was used to investigate this role. Results obtained suggest that there is interaction between *B. microti* AMA-1 and a 49 kDa host red blod cell protein and suggest that an erythrocyte membrane protein of 49 kDa is likely to be involved in invasion by *B. microti*. However, further investigation is needed. Studies aimed at elucidating the structure of the host cell receptor(s) at this position and the structure of the interacting parasite ligands should also be investigated to bring more insight to the invasion process of *B. microti*. Information from such studies will pave the way to developing better therapeutics and vaccines to counter the disease caused by this parasite.

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