Identification of Immunodominant Antigens in *Babesia microti* Using Phage Display cDNA Technology

A thesis submitted by

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Abstract

Babesiosis is a life-threatening infection of the red blood cells (RBC) caused by the parasite *Babesia microti,* an intraerythrocytic protozoan parasite. Babesia parasites are related to plasmodium, the causative agent of malaria. Babesia infection in erythrocytes causes babesiosis that often mimics clinical features of malaria. Babesia is transmitted by Ixodes ticks with little or no transmission occurring from ticks feeding on infected humans, thus making humans the dead-end hosts. Nevertheless, human-to-human transmissions can occur via blood transfusions. Babesia parasites are endemic to the Northeastern and upper Midwestern regions of the United States resulting in over 2,000 patients per year based on data reported in 2019. The purpose of this study was to identify immunodominant antigens in *Babesia microti* to develop a comprehensive diagnostic assay. Babesiosis is often difficult to diagnose, and infected blood can be lethal if transfused into immunocompromised patients. Therefore, the identification of novel *Babesia microti* antigens that can accurately detect antibodies against babesiosis is of high clinical significance. To identify such antigens, we used a phage display cDNA library constructed from *Babesia microti* to screen for novel antigens using plasma from humans afflicted with babesiosis. Previously, our laboratory identified *Babesia microti* antigens BmSA1 and BMN1-20 and showed that recombinant segments of these antigens can directly bind to human RBCs. I used these recombinant antigens as a positive control to develop a novel screening strategy. A novel plaque lift assay was developed in combination with Western blotting to identify unique segments of parasite antigens detected by the antibodies present in infected human plasma samples. Among other cDNA clones detected by the antibodies of the infected human plasma, the most promising antigen was BM4-12, which would be useful for the development of a

comprehensive diagnostic test against B*abesia microti*. The BM4-12 could also be included for the development of a viable vaccine against babesiosis in humans and other vertebrates.

Dedication

This paper is dedicated in loving memory of my late sister Osegbe Loveth Omorodion, and above all, God Almighty.

Acknowledgments

I would like to express my sincere gratitude to my thesis advisor, Dr. Athar Chishti, for making this work possible and for his time and availability. Also, for his encouragement at every stage of my research work and writing, I will forever be grateful. I would like to give a special thanks to Dr. Toshihiko Hanada for his support and guidance during my experiments, for taking the time to coach me, and for making sure that I understood every step of my research work. I acknowledge and give my warmest thanks to our lab manager, Donna-Marie Mironchuk, for the role she played in the completion of my thesis work, as well as her help in organizing things around the lab. I would like to say a big thank you to every past and present member of the lab who, one way or the other, supported this journey, and for making me understand what success feels like when there is teamwork and a friendly working environment.

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Last but not the least, I would like to thank my family, my parents Mr. Thomas Omorodion, and Mrs. Osaro Omorodion for all their care and support throughout this phase of my life. I am forever indebted to them.

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List of Abbreviations

AMP: Ampicillin
BSA: bovine serum albumin
cDNA: Complementary DNA
DNA: deoxynucleic acid
E. coli: *Escherichia coli*
HRP: Horse Radish Peroxidase
LB: Luria Broth
OD: Optical density
PBS: Phosphate buffered saline
PBST: Phosphate buffered saline with tween 20
PCR: Polymerase chain reaction
PFU: Plaques forming units
RBC: Red blood cell
TMB: 2,3',5,5' tetramethylbenzidine
TMHMM: Tied Mixture Hidden Markov Model

Chapter 1 : Introduction

Babesia microti is typically the cause of human babesiosis. It is prevalent in the Northeastern and upper Midwestern areas of the United States and less so in other parts of the world. This infectious disease is caused by an intraerythrocytic protozoan parasite of the red blood cell (RBC), transmitted by the deer tick, Ixodes scapularis (Vannier et al., 2015). Also, the parasite is often transmitted through blood transfusions, perinatally, and during organ transplantation.

The babesia parasite was first described by Hungarian pathologist, Victor Babes. Earliest reported cases of babesia infection were misrepresented as plasmodium infection (Liu et al., 2017). The first causative babesia species identified as *B. microti* was from a patient from Nantucket Island in Massachusetts. Other cases were reported around the New England regions and spread to other parts of the USA. Patients infected with *B. microti* can show symptoms similar to malaria infection. Babesiosis is caused by a parasite protozoan of the genus babesia, which closely resembles the *plasmodium* parasite, the disease-causing agent of malaria. Both parasites infect red blood cells in their life cycle, with the pathology occurring during the erythrocytic stages (Al-Nazal et al., 2021).

Babesiosis is usually more severe in the elderly, even in otherwise healthy patients. Although seroprevalence studies show that children are equally exposed to this disease, most pediatric cases were recorded in neonates that acquired the babesiosis infection from blood transfusion product or transplacental transmission. There have been over 170 transfusion-transmitted infections reported cases, with *B. microti* being the most common. Generally, infected blood can be lethal if transfused into immunocompromised patients (Bloch et al., 2021).

The severity of the babesia infection varies to different degrees. Individuals with weakened immune systems, those who previously had a splenectomy or an organ transplant, HIV patients, children, and the elderly are more susceptible to babesiosis with severe symptoms that can lead to death (Liu et al., 2017). While most people infected with *Babesia microti* are usually asymptomatic, some patients develop flu-like symptoms e.g., chills, sweats, headache, fever, loss of appetite, fatigue, or nausea. *B. microti* can lead to the destruction of RBCs resulting in hemolytic anemia. In severe cases of the disease, there can be the development of blood clots, fluctuating blood pressure, and/or organ failures. These severe symptoms may take months to appear after pathogen exposure (Vannier et al., 2015).

There are different methods currently used for diagnosing babesiosis. Babesiosis can be diagnosed simply through a microscopic examination of the blood sample to visualize the presence of parasites in the RBCs. However, this test is carried out in a specialized laboratory. Also, babesiosis can be suspected by the healthcare provider with possible symptoms or tick bite exposure. The disease is diagnosed by a thin blood smear of a patient with babesia-infected RBCs or Babesia DNA amplification using polymerase chain reaction (PCR) (Kumar et al., 2021). The testing of blood smears must be carried out at the early stage of illness because of low parasitemia, common in asymptomatic patients, which can hide the signs and symptoms of the infection with no clinical manifestation for a long period. However, the PCR test is more sensitive than blood smear-based diagnosis in *B. microti* infection. In addition, there is another diagnostic test termed indirect immunofluorescence assay (IFA). The IFA is the most common serological test based on the detection of antibodies against babesia antigens (Vannier et al., 2015).

Despite the existing advanced diagnostic approaches for babesiosis, there are still substantial limitations. To prevent the *B. microti* infection in the blood supply chain as well as in humans, there is a need to identify the immunodominant antigens in *Babesia microti* using methods such as the phage display cDNA screening technology to develop a sensitive, reliable, and simple diagnostic test. In malaria, serological testing has been successful to detect dominant parasite antigens.

In our study, we used a phage display cDNA library made from *B. microti*. This cDNA library was screened using human plasma to identify immunodominant antigens of *B. microti*. Human plasma collected from patients infected with babesiosis was used for the screening of our *B. microti* cDNA library through several rounds of biopanning steps to identify a positive hit. The DNA sequencing of the positive clones was carried out. Among the several known babesia antigens identified by this screening strategy, we identified several overlapping clones encoding a novel antigen known as BM4-12. This novel phage clone belongs to the BM4 family, including the BM4-12. The BM4-12 has been previously identified as a surface antigen consisting of two types of repeat regions. One region possesses short degenerate hexapeptide repeats with 78% identity to each other (Homer et al., 2003).

The *B. microti* genome has four chromosomes with about 6.5 megabase pairs encoding around 3,500 predicted genes (polypeptides) (Cornillot et al (2012)). *B. microti* genome is about 28% the size of the *Plasmodium falciparum* genome, and it is the smallest of all sequenced apicomplexan parasites (Puri et al., 2021) (Cornillot et al., 2013). DNA sequencing of phage clones has helped to identify novel *B. microti* antigens suitable for diagnostic purposes. Originally, we aimed to identify the secreted antigens from *B. microti* for diagnostic testing as well as detect antibodies against dominant immunoreactive antigens expressed on the surface of the parasite.

To achieve this aim, a phage display cDNA library was constructed in our laboratory by Dr. Toshihiko Hanada and screened by human plasma from patients with babesiosis. From this screen, we have identified a novel segment of BM4-12 antigen as a potential diagnostic marker as well as a putative vaccine candidate against babesiosis.

Chapter 2 : Materials and Methods

2.1 Construction of phage display cDNA library of *Babesia microti*

The *B. microti* phage display cDNA library was constructed in our laboratory by Dr. Toshihiko Hanada by utilizing Novagen's OrientExpress random prime cDNA system and T7Select 10-3b phage display system protocol. The cDNA library was constructed from mRNA isolated from *B. microti* infected mouse RBCs. The Novagen T7Select phage display system takes advantage of the properties of bacteriophage T7. Two primers were used in creating the phage clones: T7SelectUP Primer and T7Down primers were used alongside EcoRI and HindIII arms. Following Novagen's protocol, cDNA libraries were created in the T7Select10-3b vector of EcoRI and HindIII arms with the use of OrientExpress random primer cDNA Synthesis kits. This strategy enabled the estimation of the cDNA insert sizes as well as the total number of phage clones encoding the cDNA inserts. The cDNA phage clones insert size ranged from 300bp to 1000bp and the total number of the cDNA clone plaques forming units (pfu) per unit volume (ml) in the library was approximately $3x10^9$ pfu/ml.

2.2 Plaque Lift Assay

For the plaque assay, the host strain (*E. coli* BLT5403) was inoculated in a 2 ml of M9LB medium supplemented with 70 μg/ml ampicillin, and incubated overnight by shaking at 37°C. The BLT5403 host cells were stored at 4°C for further characterization. From the freshly cultured bacteria, 100 ul was added into a 2 ml of M9LB medium with ampicillin (LB Amp) in a culture tube. Thereafter, the M9LB medium was placed in an incubator and the optical density value at 600 nm (OD600) was measured at intervals till it reached approximately 1.0 OD600. Also, a sufficient

amount of top agarose was melted to provide 3 ml for each plate to be used. The molten agarose was kept in a 45°C water bath to prewarm until needed. A series of dilutions of the phage samples were prepared using Luria Broth (LB) medium as diluent. Normally, the general dilution range for recombinant phage is 10^3 to 10^6. But we increased the dilution range to about 10^1 to 10^7.

A preliminary 1:100 dilution was prepared by adding 10 µl of phage sample to 990 µl of LB medium (10^2 dilution). Serial dilution was further made by adding 100 ul of 1:100 dilution to 900 ul medium (10^3 dilution), 100µl of the 10^3 dilution to 900 µl medium (10^4 dilution), and this process further continued till the right amount of dilution was needed. 250 µl of host bacterial cells was pipetted into each prepared series of 4 ml sterile tubes, starting with the highest dilution. 100 µl of phage dilution was added to each tube, and 3 ml of top agarose was added to the tubes and vortexed.

The content was poured onto a prewarmed (37°C) LB agar plate containing 70 µg/ml ampicillin. The plate was immediately swirled gently to evenly spread the agarose, and the plate was allowed to sit unmoved for 15 to 20 minutes until the top agarose solidified. Plates were then turned over inside the incubator for 3-4 hours at 37°C, after which the plaques were counted, and the phage titer was calculated for further experiments.

2.3 Plaque lift and Western blotting assays for screening of babesia antigens

Phage extract was made from a human reticulocyte cDNA library constructed by Dr. Toshi Hanada. This was done by infecting the cDNA library inside a 200 mL of host bacterial culture (BLT5403). The bacteria culture was centrifuged after lysis for 10 minutes at 6,000 rpm to remove any cell debris. The supernatant was used in treating a new nitrocellulose membrane and five pieces of nitrocellulose membrane

were incubated with the phage extract for 30 minutes. Thereafter, the membrane was removed and incubated in the blocking buffer (1% gelatin, 3% BSA in a 2X PBST) for 60 minutes. The patient's plasma was diluted in the blocking buffer, the nitrocellulose membrane was incubated for 20 minutes each at room temperature. In the end, the pre-absorbed antibody supernatant was stored at a 4°C in the presence of sodium azide for subsequent cDNA phage screening.

The plates were stored in a 4°C refrigerator for 30 minutes, thus making the plaque lifts feasible by reducing the tendency of the top agarose to stick to the nitrocellulose membrane during plaque lifting. The density of the two large Petri dishes (15cm) used was about 40,000 to 20,000 plaques per plate. The plaques lift was carried out by the use of a nitrocellulose membrane, this was done by marking the membrane with a sterile needle and then placed on the surface of the top agarose inside the 15 cm dish. Minutes later, when the membrane was recovered from the top agarose, it was lifted at one end with forceps and immersed into a container with phosphate-buffered saline with tween 20 (PBST 1X) solution for further experiment.

Western blotting was carried out on the nitrocellulose membrane after the plaque lifting procedures. For the primary antibody, *B. microti* infected human plasma (obtained from a commercial source) was used. To reduce the nonspecific reactivity of the primary antibody, the human plasma was pretreated with unrelated phage extract (T7 phage cDNA library of human reticulocytes RBCs).

The nitrocellulose membrane with the plaques was incubated with a blocking buffer containing 1% gelatin, and 3% BSA in a 2X PBST for 30 minutes. Thereafter, human plasma was diluted in the previously used blocking buffer using a 1:500 dilution, membrane treated with diluted plasma and covered with a plastic wrap was incubated at room temperature for 1 hour.

After the primary antibody treatment, the nitrocellulose membrane was washed with PBST buffer 5 times every 10 minutes (50 minutes). The secondary antibody, anti-human IgG conjugated with HRP (Horse Radish Peroxidase), treatment was carried out using a 1:4000 dilution with a PBST also for 1 hour. Finally, the membrane was washed five times with PBST every 10 minutes. Super Signal (PIERCE) was used for chemiluminescent imaging. The treated membrane was immersed for less than two minutes in Super Signal solution and placed in plastic wrap. Thereafter, the membrane was wrapped in plastic wrap and placed in an X-Ray cassette. The imaging was obtained using the X-Ray film developer in the darkroom.

2.4 Phage clone selection through cDNA library screening

The image on the film was aligned with the petri dish containing agarose gel and phage clones. The regions with the positive plaques were identified and picked with a micropipette disposable tip into the Eppendorf tube containing 100 µl EDTA phage extraction buffer and incubated for 10 minutes at 70°C. After the first screen using large plates, plaques were picked and stored in the phage extraction buffer to keep the phage alive. These phage extracts were plated for the second screening of plaque lift. After the second screening, plaques were picked and stored in the same extraction buffer. After the third and final screen, confirmed single positive plaques were collected in EDTA buffer for PCR.

2.5 PCR Amplification of plaques DNA

The inserts of the phage clones were amplified by PCR using the T7SelectUP and T7SelectDOWN primer pair. The EconoTaq was used for PCR. The conditions for PCR were optimized with the initial denaturation at 95°C for five minutes, 35 cycle amplification of 94°C for 30 seconds, 55°C for one minute, and 72°C for one

minute. Finally, with an extension at 72°C for six minutes, the PCR products were analyzed by agarose gel electrophoresis. This step was carried out by first making agar gel using one gram of agarose LE, 2 ml of 50X TAE, and 98 ml of deionized water. The gel was placed in the microwave to dissolve and then cool a little, 2.5ml of ethidium bromide was added, mixed, and poured into the gel tray. A running buffer containing 5 ml of 50X TAE and 250 ml of deionized water was added to the buffer tank. When the agar gel was solidified, it was placed inside the buffer, 1.0 µL of loading buffer was mixed with the 5 µL of PCR samples individually and was added into the sample wells. One kb DNA ladder was used for insert size estimation. The electrophoresis was performed for 15 minutes at 100 volts. Imaging was carried out using the Bio-Rad Gel Doc system.

2.6 DNA Sequencing

The PCR products were purified using an E.Z.N.A cycle pure kit. DNA concentration was measured using Nanodrop. DNA sequencing was carried out at Tufts University Core Facility using T7SelectUP and T7SelectDOWN primers.

2.7 Detection of antigen-antibody interaction by ELISA

The BM4-12 phage insert was cloned in a pET32b plasmid for recombinant protein expression. The expressed recombinant protein was purified using Ni-agarose affinity chromatography. This protein was used for the ELISA. Cloning, expression, and purification of the recombinant protein were accomplished with the help of Dr. Toshi Hanada. The ELISA experiments were carried out in our laboratory by Dr. Christopher Schwake and Nicholas DaRosa. 31 patients infected with *B. microti* sera samples were used to assess the efficacy of the BM4-12 antigens for the detection of antigen-antibody interaction. The negative controls were from two uninfected

subjects as well as four patients infected with malaria to verify the antigen's specificity to babesiosis. 96 well plates were coated with 50 µL of 0.5 µg/ml of the previously identified antigen phage lysate in PBS and kept overnight in a 4°C. Plates were then blocked with a blocking buffer containing 3% BSA, 1% gelatin, and PBST for one hour, followed by Western blotting as described above.

In a two-step process, ELISA was performed to determine how effective is the antibody-antigen interaction. 100 µL of 3,3'5,5'-tetramethylbenzidine (TMB) in buffer was added for 12 minutes in each well. The antibody-antigen interaction was monitored by the development of blue color and quenched by the addition of 50 µL of 1 M HCL. The resulting yellow color in the ELISA plate wells was measured by optical density at 450 nm using a VersaMax plate reader.

Chapter 3 : Results

3.1 Characterization of random primed phage display cDNA library

Plasma samples from humans infected with *Babesia microti* were obtained from multiple collaborators. Multiple plasma samples were used for screening. Plasma samples from infected patients are expected to contain antibodies that can recognize immunodominant parasite proteins that may neutralize the pathogen. Hence, we used these plasma samples to identify the *B. microti* antigen(s) expressed on the phage surface recognized by the antibodies. The *B. microti* random primed phage display cDNA library was screened by the plaque lift and Western blotting assays.

For the first round of screening, the density of the phage cDNA library was plated between the range of 20,000 to 40,000 plaques in two large plates of 12 cm petri dish. Western blotting was carried out and several positive plaques were detected from each plate as shown in Figures 1a, and 1b. The first plate showed eight positive plaques, and the second plate had 10 positive plaques. These positive plaques were picked for second screening and re-plated at a density of 2,000 to 5,000 plaques per plate. Western blotting was performed again and a total of 17 positive plaques were detected in the second screening.

Finally, for the third screen, positive plaques were isolated and replated at the density of 200 to 500 plaques per plate. From the third screen, 5 positive plaques were identified and isolated from each plate. PCR analysis was performed on various phage clones. A photograph of the screen along with the DNA ladder is shown (Figure 2), Multiple bands of isolated phage clones from the last biopanning were detected by agarose gel electrophoresis. The multiple bands of all the phage clones ranged from 500 bp to 800 bp (Figure 2).

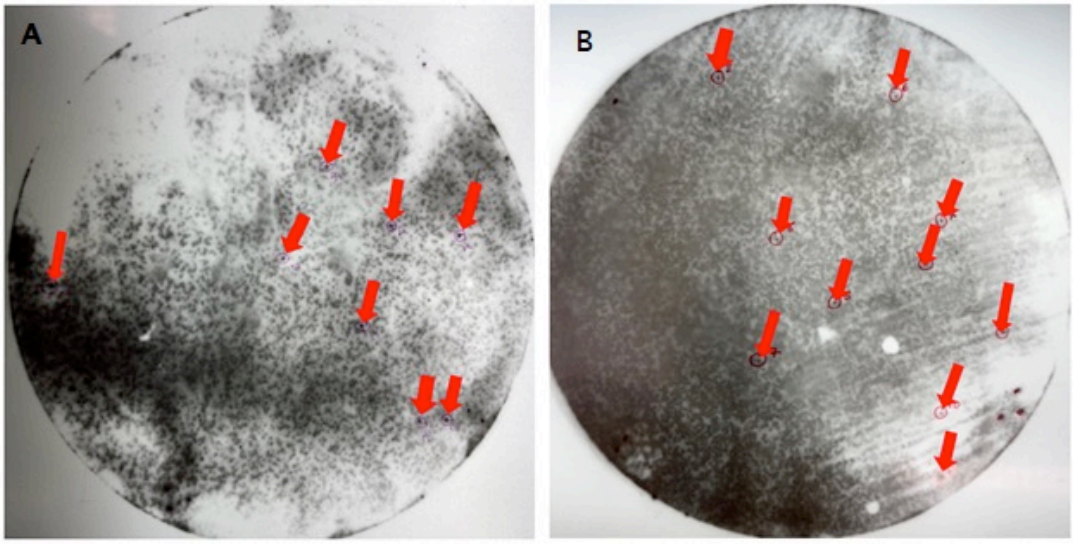

Figure 3.1: Plaque lift results showing positive hits of phage clones. Plate images show the density of positive plaques from our phage library that was plated between the range of 20,000 to 40,000 plaques per plate in each 6 cm petri dish.

3.2 Phage display screening identified BM4.12 as a binding protein

PCR samples of the two most prominent bands (Figure 2) were sent for DNA sequencing by the T7SelectUP and T7SelectDOWN primers. This procedure was carried out for all positive phage clones isolated from the third round of plating. National Center for Biotechnology Information (NCBI) database was used for BLAST nucleotides and protein sequence analysis. The ExPASy translation tool was used for the translation of nucleotide sequence to amino acid (AA). Among other antigens identified, the BM4.12 segment emerged as a novel antigen. Other phage clones included previously identified known genes such as the BMN1-20. We identified five independent BM4.12 clones and 3 independent BMN1-20 clones by these screens.

DNA sequencing performed at the Tufts University Core Facility identified the cloning restriction sites. The EcoRI and HindIII restriction sites in the cDNA library were used for cloning inserts in Novagen's T7Select phage Display System. From multiple DNA sequencing results, each clone showed slightly different size

inserts cloned at these restriction sites. The NCBI-BLAST analysis of all clones revealed an overlapping segment of BM4.12 (accession number AY170614) recognized by the human plasma from patients with babesiosis. Some phage clones differed by a single amino acid whereas others encoded polypeptides shorter by three amino acids (Figures 3 and 4).

Data in Figure 3 show the amino acid sequence of BM4.12 in red color that was identified as a possible epitope identified by the antibodies present in the human plasma.

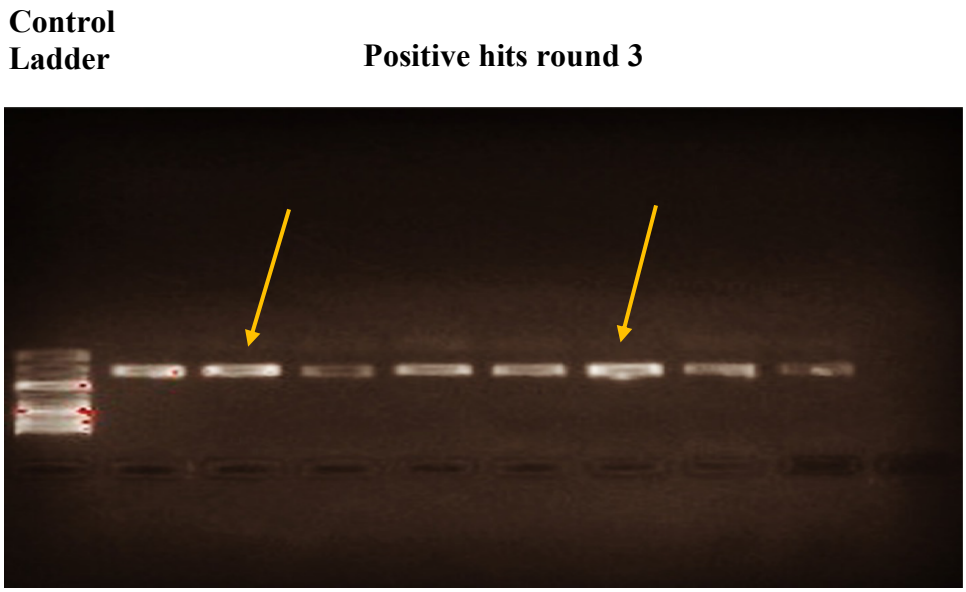

Figure 3.2:Agarose gel electrophoresis analysis of identified phage clones DNA. PCR amplified products of phage clones loaded on agarose gels showing respective DNA bands. The two most prominent bands were selected for further purification and characterization.

3.3 Identification of novel *Babesia microti* putative antigen BM4.12

BM4.12 sequence analysis was performed by the NCBI BLAST and ExPASy software to determine the ORF sequence of multiple sequences identified by the phage clones.

This analysis also mapped the precise amino acid number of each encoded polypeptide of BM4. 12. Importantly, the BM4.12 gene was identified multiple times from our phage screens. These findings show that our screening method correctly identified the *Babesia microti* antigens that were recognized by the antibodies present in the patient's plasma samples.

Interestingly, all phage clones mapped the same segment of the BM4.12 gene. Although these clones encoded the same segment of BM4.12, the clones differed by one to three amino acids suggesting that multiple independent clones were captured by our screen (Figure 4).

ORIGIN

MLRVKDASSTEATIRMFLRFNAFIKFLNEEKSRGDKSALNDEGLMRFISMTSGFIDDLELVLDELSKHSLLINNEGAKSMLSSLI
LSFRYINHIRNLINGIYLGLNNPSSSIGETAQETTEPSTPTPTPSTQTILKPKGSEIRGYIIKVDQTANLITFIDALIKELNVHIKQTTT
SSVVGTKETNGTTSGSPESNPGSTDSGSIQAEVAELLKKFATIASFDEKFTNLHINKPFADALIKRLNEIKAELSSNSGTPPKLPDI
SCLRLSEIVQKLNRLIKFNTSRLINKSFPELCKLFIKMPDVDSNKFMALDVDISNTLVNRRVRYSDGRFTIVSTGSNFRYTLAPTA
AGHDLSLFSQLPISMITVTSPQEQALTSCVSHGNEFSIVSTAGKTTYTTQSKLLSLFKLSAETLRDFNEARFALGNMTDSANKS
KALEVYKSTLTTMKSISVELEKIFGILKSTPNITFESVVSKYKLTGVNTVDTANADVINETMFDDLSKAISSYLYSLISIIFPEDIKGQ
GTSEGQQTSEGQQTSEGQQTSGDQDTSGGQDTNETIFSYLYSLISIIFPEDIKGQGTSAQLLEYRTQLASLSKIKSLRKKIKRRL

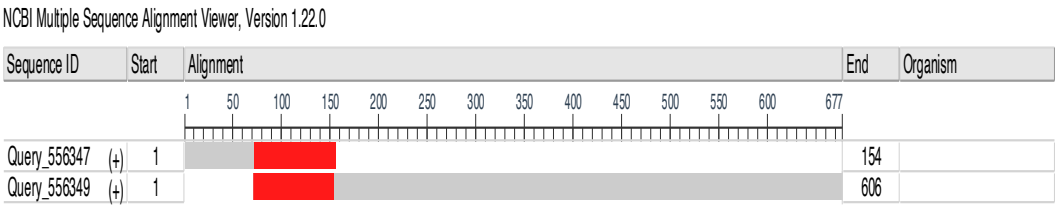

Figure 3.3: Analysis of complete BM4.12 amino acid sequence by FASTA. The complete amino acid sequence of BM4.12. The red region depicts the amino acid sequence alignment of the cDNA inserts encoded polypeptides identified in one of the selected clones. BM4.12 protein consists of 606 amino acids, the immunodominant region identified in our screen includes the first 78 amino acids.

Previously, BM4.12 has been identified as a putative B. *microti* antigen in a screen that also identified BMN1-9 and BMN1-20. BM4.12 was reported as a partial clone at that time (Luo et al., 2011). Furthermore, another group also identified BM4, which belongs to the family of BM4.12, along with BM2, BM9, and BM15. Although

the precise function of BM4 is currently not known, it is considered a secretory protein as detected in *Babesia microti* infected models (Liu et al., 2017).

The positive BM4.12 segment we have identified in our phage screens defines a unique immunodominant region located at the N-terminus of the full-length BM4.12 sequence (Figure 4). From the database search, another gene was identified termed RI N1-15, which is a close match to BM4.12. RI N1-15 is a large protein, and its function is not known. Our BM4.12 sequence aligns with the RI N1-15 clone with almost 100% identity. However, the RI N1-15 gene has been reported with multiple gene variations. These changes may reflect differences in various *B. microti* strains. Alternatively, the sequence variation may originate from early technical variations in the determination of parasite DNA sequences.

From sequence alignment statistics, our BM4.12 clone matched within the RI N1-15 nucleotide sequence at 4323-4729 and 1442-1576 amino acids. Moreover, another *Babesia microti* gene designated as N1-21b has been previously identified that resembles the RI N1-15 gene. This gene appears to be a subtype of the same protein with high sequence homology. The N1-21b protein has been shown to have an excellent diagnostic sensitivity to detect babesiosis (Liu et al., 2017).

Recent bioinformatics analysis suggests that BM4 protein may contain a signal peptide sequence. The SignalP4.1 software predicts that the mature BM4 protein does not contain the signal peptide sequence (Liu et al, 2017). Moreover, BM4 sequence analysis by Target P1.1 and TMHMM server v.2.0 indicates the absence of any transmembrane region in the protein. Prediction analysis by SMART showed the presence of low-complexity domains in BM4. Moreover, the IPTG-induced BM4 protein was localized in the inclusion bodies with a relative molecular mass (Mr) of about 30 kDa as assessed by SDS-PAGE (Luo et al., 2011) (Liu et al., 2017).

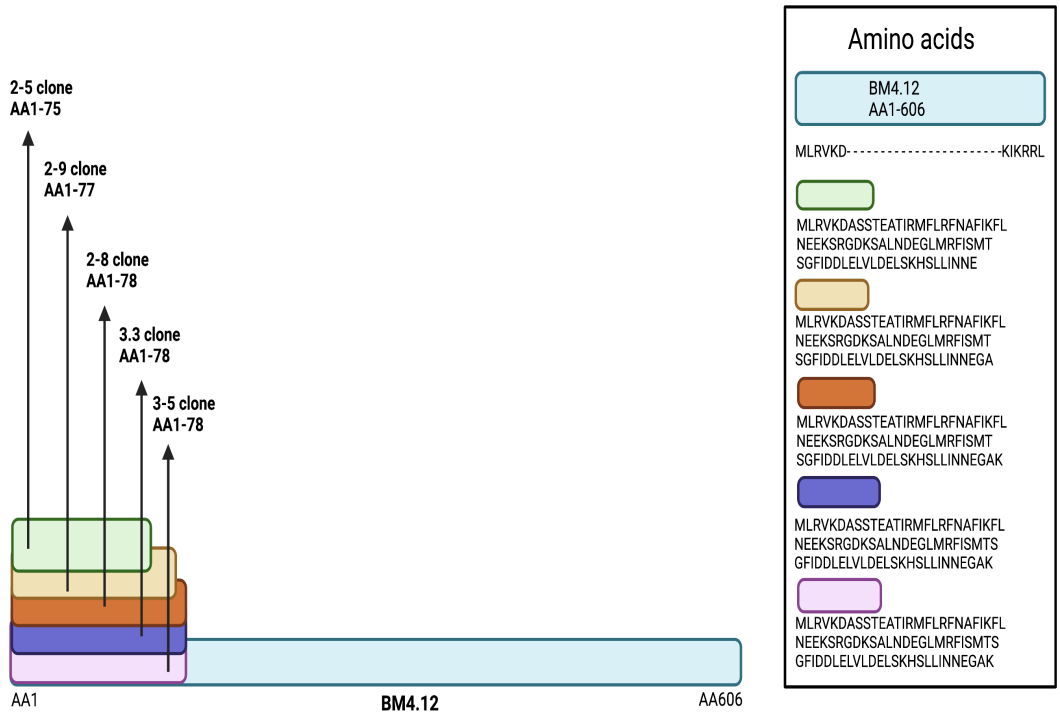

Figure 3.4: Primary structure of BM4.12. All phage clones encoded the same N-terminal region of the BM4.12 gene, with minor differences in their boundaries, respectively. Minor differences between the various phage clones suggest that our screens identified independent clones encoding the same region of BM4.12.

3.4 Depleted antibody plasma screening of phage display cDNA library

My screens identified two major antigens, BMN1-20 and BM4.12. The detection of BMN1-20 is consistent with the previous identification of this antigen in our laboratory. We reasoned that by removing BMN1-20 and BM4.12 reactive antibodies from the human plasma, it might be possible to identify relatively minor novel antigens. Therefore, we pretreated the human plasma with recombinant BMN1-20 and BM4.12 proteins. Specifically, acetone powders were prepared from *E. coli* culture expressing these recombinant proteins and added to the human plasma in excess. We confirmed that depleted human plasma did not recognize BMN1-20 plaques by the plaque lift assays (data not shown). The plasma serum depletion was performed by Dr. Toshihiko Hanada.

Since BmSA1 is a dominant antigen expressed by *Babesia microti*, we depleted human plasma of antibodies against BmSA1, BMN1-20, and BM4.12. The depleted plasma was used to screen the phage display cDNA library of *B. microti*. After several biopanning steps, as previously described in Chapter 3, several proteins were identified (Table 1). Importantly, these screens did not capture any dominant antigens thus validating the efficiency of the serum depletion strategy as outlined in this protocol. Although the biological significance of these low-abundance proteins remains to be investigated, it is noteworthy that our screens identified a *Babesia microti* protein of unknown function (XM_0127919) that is conserved in Plasmodium species (Table 1). Such target genes may be of interest as potential low expressor proteins that were likely missed by the presence of dominant epitopes by classical phage display cDNA screens.

Identified Proteins from the Depleted Serum	
Sequence ID	Proteins
XM_012792816.1	*Babesia microti* strain RI superoxide dismutase, Fe-Mn family partial mRNA
XM_021482810.1	*Babesia microti* strain RI transcription factor with AP2 domain(s), putative (ApiAP2) partial mRNA
XM_0127919	*Babesia microti* strain RI conserved Plasmodium protein, unknown function partial mRNA
XM_021482014.1	*Babesia microti* strain RI nucleoside-triphosphate pyrophosphatase partial mRNA
XM_021482364.1	*Babesia microti* strain RI nucleoside-triphosphate pyrophosphatase partial mRNA
XM_012793840	*Babesia microti* strain RI cytochrome c oxidase subunit II partial mRNA

Table 3.1: List of proteins identified by the depleted serum screen

3.5 Detection of antigen-antibody interactions by ELISA

The BM4.12 antigen (also designated as CTA-12) was identified by our phage display cDNA library screens. The rationale of this study was to test the utility of this antigen in the diagnosis of babesiosis. A statistical reactivity cut-off was determined at an optional density of 0.46 by using 5 negative controls, the H2 sample was an uninfected human donor, and M86, M87, M88, and M89 samples were obtained from individuals negative for babesiosis but positive for malaria (Figure 5). All negative controls displayed optical density below the determined cutoff. From patients' plasma samples, about 90% of samples detected positive for babesiosis. Of note, as shown in Figure 5, patient # 17-01 was splenectomized and therefore was not captured by the detection method. Patient #19-10 showing negative reactivity was treated with a monoclonal antibody drug (Rimuxan). Patient 20-2 showed negative reactivity despite being not splenectomized or treated with any other medication.

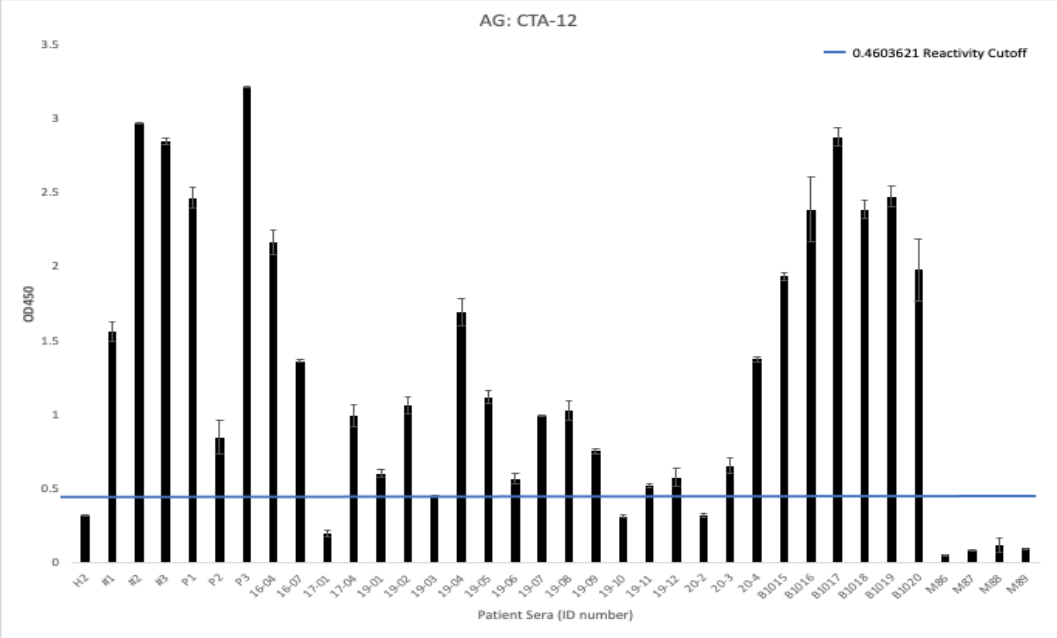

Figure 3.5: ELISA-based reactivity of CTA-12 by antibodies in patient's plasma. CTA-12 was screened to assess the efficacy of this antigen as a diagnostic method for babesiosis. Reactivity cutoff was set at 0.46 optical density. The diagnostic coverage of the patients' samples was 90% along with multiple negative controls.

There was a varying degree of optical density-based reactivity responses observed in patients. Patient #19-006 showed strong reactivity for BmSA1 short clone but low reactivity against CTA-12. In contrast, patient #P3 showed low reactivity for the BmSA1 short clone but was strongly positive against CTA-12 (Figure 5).

Finally, we pooled three antigens (BmSA1, BMN1-20, and BM4.12) to detect babesiosis. Pooled recombinant antigens were coated on the ELISA plate and examined for reactivity by patients' plasma samples. As shown in Figure 6, a statistical reactivity cutoff of 0.18 optical density was determined. The data revealed 100% detection of babesiosis in the patient's plasma samples. Patient #17-01 was splenectomized. The majority of the patient's plasma samples showed optical density readings between 1.5 and 3 indicating strong antigen-antibody-specific interactions under these conditions.

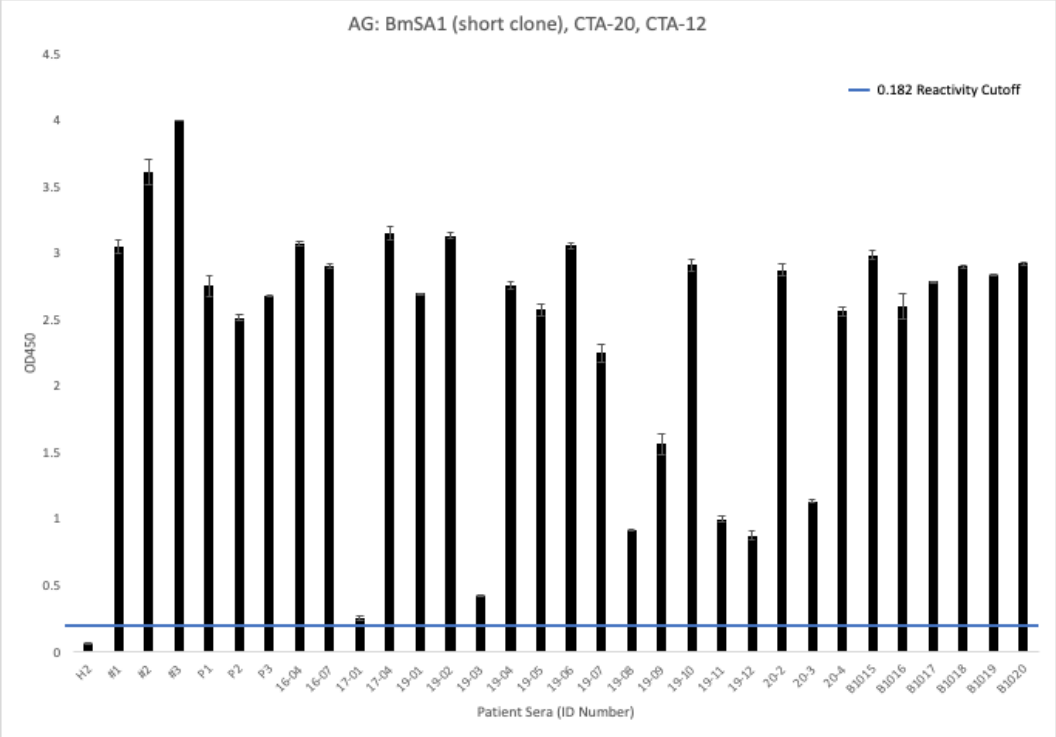

Figure 3.6: ELISA Screen of BmSA1 short clone, CTA-20, and CTA-12 with patients' plasma.

The optical density-based reactivity cutoff was set at 0.18. Data show 100% coverage for the detection of babesiosis in patients indicating that pooled three antigens provide a robust means of detecting babesiosis.

3.6 Contributors

Dr. Toshihiko Hanada constructed the *Babesia microti* phage display cDNA library. The ELISA experiments were carried out in our laboratory by Dr. Christopher Schwake and Nicholas DaRosa. The pooled antigen concept was first proposed by Dr. Chishti in consultation with Dr. Hanada.

Chapter 4 : Discussion

Phage display technology was first described by G.P. Smith et al. in 1985, and it was used for the expression of cloned antigens on the viral surface. This approach is now well established as *in vitro* selection technology used for the study of linear peptides, protein-protein, and protein DNA interactions that utilize bacteriophages encoding genetic information for specific peptides. The phage display technology can generate highly specific antibodies from comprehensive libraries and can identify molecules of defined size by taking into account the limitations of the immune system (Smith & Petrenko, 1997). To identify specific interactions mediated by the peptides displayed on bacteriophages, the phage display cDNA libraries are screened by defined antibodies and/or by various ligands serving as detection probes. Large protein libraries encoded by the cDNAs can be screened and amplified by *in vitro* selection methods for many basic and clinical applications (Kuhn et al., 2016).

The phage display bacteriophage approach used in this study utilized the T7 select system. We used the commercially available reagents from Novagen's T7Select system to generate multiple phage display cDNA libraries. The T7 bacteriophage is a double-stranded DNA phage, unlike other bacteriophages, the phage assembly occurs inside the host *E. coli* and cell lysis releases the matured phage particles. The T7 bacteriophages are relatively easy to grow fast and replicate faster than other bacteriophages. The plaques form within three hours at 37^{o}C (Lemon et al., 2019).

Babesiosis is reported as the most common transfusion-transmitted infection in the United State especially in the Northeastern region, having a seroprevalence of about 2.5% in the endemic regions of the population (Kumar et al., 2021). This tick-borne infection has become a major issue for blood banks and donation services due to the risk of infected healthy donors that are asymptomatic with the disease. The

babesia-infected blood when transfused to immunocompromised patients is an issue of major health concern. Recent studies suggest that donated blood units should be tested for babesiosis before transfusions. It is therefore critical to develop specific, sensitive, and easy-to-use diagnostic assays for babesiosis. The recent progress in this direction is demonstrated by the development of serologic diagnostic assays using a mixture of peptides chemically synthesized from the BMN1 family of babesia antigens (Homer et al., 2003). Such approaches have been used to prescreen the blood donors as well as continue to identify novel surface parasite antigens for novel screens (Elton et al., 2019).

In this study, we used human plasma samples from patients afflicted with babesiosis. We used both characterized plasma samples and blood donors from regions endemic to babesiosis. The rationale of the study was to identify novel antigens from *Babesia microti* using the phage display cDNA technology. I utilized the phage display cDNA library previously constructed in our laboratory from *Babesia microti*. This cDNA library was screened by multiple plasma samples from patients with babesiosis.

With the guidance of Dr. Toshihiko Hanada in our laboratory, I optimized a plaque lift assay for screening multiple plasma samples to identify novel *Babesia microti* antigens suitable for a diagnostic assay. My initial screens identified several antigens that are well established as immunodominant epitopes for babesiosis. These findings validated the feasibility of the plaque lift assay to identify novel antigens suitable for improved diagnostic assays. Upon multiple biopanning steps including PCR amplification of positive plaques and DNA sequencing, as outlined in the Results section, I identified BM4.12 as a putative antigen to detect babesiosis. The identification of multiple phage clones encoding BM4.12 suggested the existence of

independent clones in the phage display cDNA library. Minor variations in the BM4.12 indicates that the core immunodominant segment of the antigen is located within the N-terminus of the protein (Figure 4).

There is very little known about the function and properties of BM4.12. BM4.12 appears to be a novel antigen and has been previously identified as BM4/BM4.12. Moreover, BM4 is also identified as RI N1-15, which is closely matched with the BM4.12 antigen. RI N1-15 was previously recognized as a partial clone (Liu et al., 2017), but little else is known about its biological function and properties.

Several *Babesia microti* antigens have been identified in our laboratory as potential diagnostic candidates for babesiosis (unpublished data). The novelty of the phage display screening approach is the identification of defined linear peptide epitopes in the candidate antigens that can be readily developed as synthetic peptides and/or recombinant proteins. Some examples of candidate antigens include BmSA1 (also known as BMN1-9) and BMN1-20. The BMN1-20 antigen shows sequence identity to BMN1-17, which encodes a copper transport protein in *Babesia microti*. The precise function of this copper transporter is not known in the pathogenesis of babesiosis (Homer et al., 2003; Luo et al., 2011). Although both BmSA1 and BM4.12 appear to be secretory proteins, it is technically challenging to rigorously distinguish the surface and secretory proteins since the infected erythrocytes continually rupture *in vivo* as well as release microvesicles as part of the pathogenesis.

I also performed several phage display cDNA library screens using depleted plasma samples. The rationale of this experiment was to remove dominant antibodies in the human plasma and then use the depleted plasma to identify low abundance minor antigens as potential diagnostic epitopes. The plasma was depleted using

recombinant BmSA1, CTA-20, and CTA-12/BM4.12 antigens. The depletion of serum was validated by the absence of three immunodominant clones in the phage display screens. The depleted plasma identified several putative antigens (Table 1). Some of these antigens could be artifacts. However, putative hits such as a *Babesia microti* antigen of unknown function that is conserved in the Plasmodium genome should be evaluated as potential diagnostic antigens in future studies.

Finally, using recombinant BmSA1, CTA-20 and CTA-12, our ELISA screening has confirmed positive cases of babesiosis as well as identified new infections in the de-identified blood donors (Figures 5 and 6) (manuscript in preparation). If successful as a diagnostic approach for babesiosis, our triple antigen approach could be extended for the diagnosis of other blood-borne diseases such as malaria caused by *Plasmodium falciparum*.

In summary, I have identified BM4.12 as a potential secretory/surface antigen that could be useful for the development of a diagnostic assay for babesiosis caused by *Babesia microti*. Moreover, this antigen could be included as part of a potential multi-subunit vaccine against babesiosis.

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