Annals of Clinical Investigation

Research Article
Published: 31 Jul, 2019

Identification, Characterization, and Protective Effect of the Apical Membrane Antigen-1 (AMA-1) Homolog from the Argentina Strain of *Babesia bigemina*

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Abstract

The *Babesia bigemina* Apical Membrane Antigen-1 gene (*BbigAMA-1*) was cloned from a cDNA of the Argentina strain by Polymerase Chain Reaction (PCR) and sequenced. The ORF of the gene was 1,788 bp and the predicted polypeptide was 595 amino acids long with a calculated molecular weight of 65.67 KDa. In addition to the N-terminal ectoplasmic region, *BbigAMA-1* has a signal peptide of 30 amino acids, a transmembrane segment, and a cytoplasmic domain. Phylogenetic analysis of the amino acid sequence of *BbigAMA-1* showed that *B. bigemina* was most closely related to *B. bovis* and *B. gibsoni*. Recombinant truncated protein (rtBbigAMA-1) was expressed in *E. coli* as a soluble GST-fusion protein with a molecular mass of 50.3 kDa. The serum raised in mice against rtBbigAMA-1 detected the native protein in *B. bigemina*, *B. bovis*, and *B. microti* lysates and reacted with *B. bigemina*, *B. bovis*, and *B. microti* merozoites in the IFAT. Mice vaccinated with rtBbigAMA-1 showed lower parasitemia against the challenge infection with *B. microti* than GST-vaccinated and non-vaccinated controls. These results added a new member of *Babesia* AMA-1 and demonstrated its protective effects in an experimental model of rodent babesiosis.

Keywords: *B. bigemina*; Argentina strain; Apical membrane antigen-1; Expression; Characterization; *In vitro* inhibition; *In vivo*; Protective effect; *B. microti*

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Received Date: *08 Jun 2019*
Accepted Date: *23 Jul 2019*
Published Date: *31 Jul 2019*

Citation:
AbouLaila M, Terkawi M, Menshawy S, Yokoyama N, Igarashi I. Identification, Characterization, and Protective Effect of the Apical Membrane Antigen-1 (AMA-1) Homolog from the Argentina Strain of Babesia bigemina. Ann Clin Investig. 2019; 1(1): 1001.

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Introduction

Babesia bigemina is a parasite of blood cells. Tick is the vector transmits the infection *via* blood feeding. The infection resulted in the losses in livestock production. Infected animals demonstrated anemia, fever, and hemoglobinuria [1]. There is no available vaccine for controlling the infection. The live vaccine has the hazard of disseminating infection or propagation of viral diseases. Therefore, we should concentrate on recombinant proteins as vaccine candidates. Proteins involved in merozoite invasion of erythrocytes might be important candidates for vaccine development [2].

Apical Membrane Antigen-1 (AMA-1) is a surface protein with high conservation among apicomplexan parasites [3]. AMA-1, a microneme protein, is stored in the micronemes directly after production and is conveyed to the parasite surface just earlier to, or through, host-cell invasion. The architect of AMA-1 consists of three regions, an N-terminal ectoplasmic region, a single transmembrane segment, and a small cytoplasmic domain. The ectoplasmic segment of AMA-1 has three domains based on AMA-1's crystal structure [4,5] and disulfide bonds [6]. The exact role of AMA-1 is not recognized, but there are pieces of evidence on its important effect in the invasion of host-cell [7]. AMA-1 is regarded as a leading vaccine candidate and is used to develop a vaccine for malaria [8-10]. The AMA-1 gene was identified from Italian strains [11]. AMA-1 was also used for molecular [12] and serological [13] diagnosis of *Babesia* infections. Homologues of AMA-1 from the apicomplexan parasites *Toxoplasma gondii* (TgAMA-1) [14,15], *Neospora caninum* [16], *B. bovis* (BbAMA-1) [17,18], *B. gibsoni* [19], *B. microti* [20], and *B. orientalis* [21] have been characterized, while *B. bigemina* was only expressed in the Italian [13] and Turkish [22] strains. In this study, we identified, expressed, and characterized the gene of AMA-1 homolog from the Argentina strain of *B. bigemina* and evaluated its protective effects.

Materials and Methods

Parasites and experimental animals

The Argentina strain of *B. bigemina* [23] was cultured using purified bovine RBCs in medium M199 (Sigma-Aldrich, Tokyo, Japan) supplemented with 40% normal bovine serum, 60 U of penicillin G per ml, 60 µg of streptomycin per ml, and 0.15 µg of Amphotericin B per ml (all three drugs were obtained from Sigma-Aldrich, Tokyo, Japan) [24]. The Munich strain of *B. microti* was sustained by passage in the blood of BALB/c mice [24] BALB/c (CLEA, Japan) mice was used for infection and immunization experiments. All animal experiments described in this study were conducted in accordance with the Guiding Principles for the Care and Use of Research Animals Promulgated by Obihiro University of Agriculture and Veterinary Medicine.

RNA and DNA extraction

Total RNA was extracted using Trizol® (Molecular Research Center Inc., USA). RNA was treated with DNase I (Invitrogen, USA). About 5 µg of the total RNA were used as a template for cDNA production. The Genomic DNA was extracted using a QiAmp DNA Blood Mini kit (QIAGEN, USA) and used as a template for PCR. The PCR primers used for the amplification of the entire *B. bigemina AMA-1* (*Bbig AMA-1*) gene were designed based on the sequence of the *Babesia bigemina* genome sequence at the Sanger Institute website (http://www.sanger.ac.uk/cgi- bin/blast/submitblast/b_bigemina) [25]. Using this tool, it was possible to compare the genes of the related organisms: *B. bovis* (*AAS58045*) and *B. gibsoni* (*ABD04040*) with the *B. bigemina* genome. In this manner, a region corresponding to the *AMA-1* gene of 1,788 bp was recognized in *B. bigemina*. This sequence was submitted to the ORF Finder software (available at http://www.ncbi.nlm.nih.gov/projects/gorf/) and ORF Finder (available at http://www.bioinformatics.org/sms/orf_find.html) to confirm that the sequence was a complete ORF. The nucleotide sequence was submitted to the Blast x server (available at http://blast.ncbi.nlm.nih.gov/Blast.cgi), and we found identities of 37.9% to 51.7% with other *AMA-1* genes. The primers F and R were designed for this sequence to identify the *BbigAMA-1* from the Argentina strain (Table 1).

RTPCR and PCR

The first-strand cDNA was synthesized using a Superscript® first-strand synthesis kit (Invitrogen, USA) using oligo (dT) to prime first-strand synthesis according to the manufacturer instructions. Approximately 200 ng of genomic DNA or cDNA in reaction mixes was used in PCR according to the supplied protocol (Taq Gold DNA polymerase, Applied Biosystems, Foster City, CA, USA). *BbigAMA-1* F and R primers were used to amplify the gene (Table 1). The PCR reaction includes 10 min at 95°C for activation of Taq polymerase, 35 cycles (denaturation for 1 min at 95°C, annealing at 64°C for 1 min, and extension at 72°C for 2 min), and a final extension at 72°C for 5 min in a Gene Amp PCR system 9,700 (Applied Biosystems). The PCR products were purified after 2% agarose gel electrophoresis and then cloned into a pCRII cloning vector using a TA Cloning Kit (Invitrogen, Carlsbad, CA, USA). The nucleotide sequences of inserts were determined using a Big Dye Terminator Kit (Applied Biosystems Japan, Ltd.) with an automated DNA sequence (ABI PRISM 3,100 genetic analyzer, Applied Biosystems Japan, Ltd.). *BbigAMA-1* primers F, F1, F2, R, R1, and R2 were used in the sequence analysis. The comparison and analysis of the nucleotide and the amino acid sequences and calculation of the molecular weight were performed using the Genetyx 7 package (Software Development Co., Ltd., Tokyo, Japan). In addition to *BbigAMA-1*, complete *AMA-1* amino acid sequences from the GenBank database for *B. bovis* (*AAS58045*), *B. gibsoni* (*ABD04040*), *Theileria annulata* (*XM_950337*), *Theileria parva* (*XM_761078*), *P. falciparum* (*DQ174774*), *P knowlesi* (*AF298218*), *Neospora caninum* (*BAF45372*), and *Toxoplasma gondii* (*AF010264*) were used in the phylogenetic analysis. The phylogenetic tree was inferred using Clustal X 1.8 and N-J plot. The identities between *BbigAMA-1* and other *AMA-1* nucleotides and amino acids were calculated using EMBOSS needle (available at http://emboss.bioinformatics.nl/cgi-bin/emboss/needle).

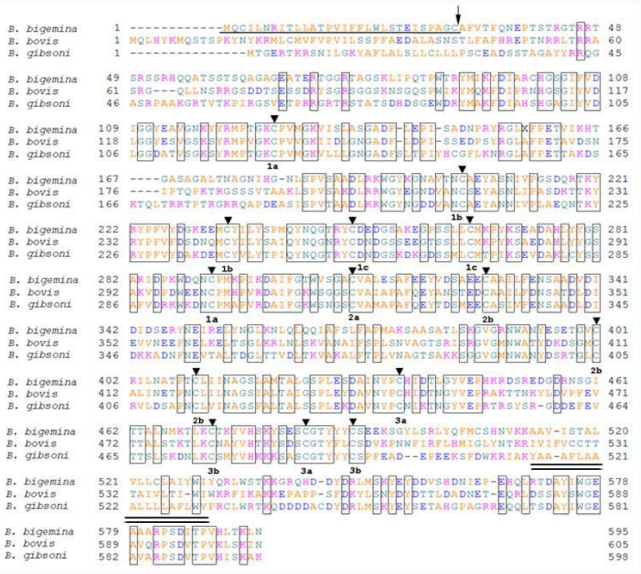

Figure 1: Multiple-sequence alignment of apical membrane antigen-1 (*AMA-1*) proteins of *B. bigemina*, *B. bovis*, and *B. gibsoni*. Identical residues are framed, and cysteine residues that form putative disulfide bonds in *B. bigemina AMA-1* are indicated by arrow heads and domain (1, 2, 3) and bond (a, b, c) designations. The signal peptide sequence of *BgAMA-1* is underlined and indicated by an arrow, and the transmembrane region is indicated by a double line. The nucleotide sequence accession numbers for *B. bigemina, B. bovis,* and *B. gibsoni* are *AB481200*, *AAS58045*, and *ABD04040*, respectively.

Cloning and expression of the recombinant protein

The tBbigmAMA-1 lacking the signal peptide (signal 3, Denmark) and transmembrane (TMHMM Server, Denmark) was amplified from cDNA of the Argentina strain of *B. bigemina* using the primers forward (Fe1) ACGAATTCGCCTTCGTGACCTTCCAGAA and reverse (Re1) AACTCGAGCCTTCTTCACGTTGTGCGCG (Table 1) containing EcoRI and XhoI sites, respectively. Then, the amplified products were cloned into the pGEX-4T-1 vector (Amersham Pharmacia Biotech, Madison, CA, USA) but the level of the expressed protein was very low after urea purification. Therefore, we tried shorter segment with higher solubility. The expressions primers forward (Ftre2) 5´-GCGAATTCAAGGTCATCAGCCTTGCGAGCG-3´ and reverse (Rtre2) 5´- GCTCGAGTTCATTGTACCTCTCCGAGTC-3´ were used to amplify 657 bp of the *AMA-1* gene (Table 1). The PCR reaction includes 10 min at 95°C for activation of Taq polymerase, 35 cycles (denaturation for 1 min at 95°C, annealing at 66°C for 1 min, and extension at 72°C for 1 min), and a final extension at 72°C for 5 min in a Gene Amp PCR system 9,700 (Applied Biosystems). The PCR products were inserted into the pGEX-4T-1 vector (Amersham Pharmacia Biotech, Madison, CA, USA) using the EcoRI and XhoI sites and expressed in the *Escherichia coli* BL21 strain. The recombinant proteins were then purified using glutathione Sepharose

Table 1: Sequence of primers used in this study

Primer	Sequence (5´–3´)
F	GTGCAGTGCATATTGAATAGGATTACGC
R	CCTCAGTTGAGCTTAGTCAGGTGTACTGGA
F1	GCGAGCGGTGCGGACTTCCTCGAACCG
R1	GCGGCTGATACTGGCGACAAATTACCG
F2	GAACTGGGCGAACTACGAGTCCGCAACG
R2	CGCCCAGTTCATACCGACACCCTTGGAC
Fe1	ACGAATTCGCCTTCGTGACCTTCCAGAA
Re1	AACTCGAGCCTTCTTCACGTTGTGCGCG
Fte2	GCGAATTCAAGGTCATCAGCCTTGCGAGCG
Rte2	GCTCGAGTTCATTGTACCTCTCCGAGTC

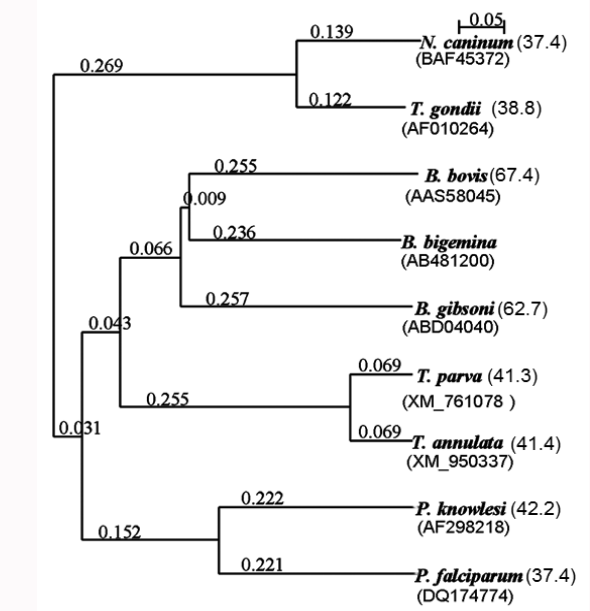

Figure 2: A phylogenetic tree based on a comparison of predicted amino acid sequences of *AMA-1* of *B. bigemina* with the corresponding amino acid sequences from other apicomplexan protozoan parasites. GenBank accession number for each parasite is placed in parentheses. Scale bar, 0.05.

4B beads (Amersham Biosciences, Piscataway, NJ, USA). Endotoxins removed from the recombinant protein (Detoxi-Gel™ Endotoxin Removing Gel; Pierce Biotechnology, Rockford, IL, USA). SDS-PAGE was used to determine the purity of recombinant proteins. The BCA protein assay kit was used to measure the quantity (Pierce Biotechnology).

Production of anti-rtBbigAMA-1 antiserum

Eight-week-old BALB/c mice (n=5) were immunized i.p. with 100 µg of the purified protein emulsified in 100 µL of Freund's complete adjuvant (Sigma, St. Louis, MO, USA). Three boosters were given i.p. using 50 µg of the same antigen emulsified in Freund's incomplete adjuvant (Sigma) at 14-day intervals. Sera were collected 2 weeks after the last booster and checked for specific antibodies by an indirect Immunofluorescence Assay (IFA).

SDS-PAGE and western blotting

Proteins in the extracts were size-separated under reducing conditions by electrophoresis in 12% SDS-PAGE and electroblotted onto a nitrocellulose membrane (Immobilon™ P; Millipore, Billerica, TN, USA). The Western blotting was performed as described previously [26]. The membranes were blocked for 1h with PBS containing 0.05% Tween 20 and 5% skim milk before incubation with specific anti-AMA1 mice serum diluted to 1:500 in 5% skim milk in PBST for 1h at room temperature. The membrane was incubated with Horseradish Peroxidase (HRP)-conjugated mice anti-IgG (H+L) (BETHYL, Montgomery, AL, USA) for 1 h. The reactions were developed using Diaminobenzidine (DAB). Western blotting was also conducted on the lysates from infected erythrocytes of *B. bigemina*, to detect the native protein in the parasite lysates. Non-infected cow erythrocytes were used as a control. Western blotting was also conducted using *B. bigemina* naturally infected field cow serum and HRP-conjugated bovine anti-IgG (H+L) (BETHYL, Montgomery, AL, USA).

IFAT and confocal laser microscopic observation

B. bigemina-parasitized erythrocytes were coated on IFAT slides, dried and fixed in absolute acetone for 10 min. For confocal microscopic observation, thin blood smears of cultured *B. bigemina*-, *B. bovis*-, and *B. microti*-infected erythrocytes were fixed in 1:1 absolute acetone: methanol at -20°C for 30 min. Immunofluorescence assays were carried out as described previously [26]. Briefly, antiserum raised against the rtBbigAMA-1 was applied as the first antibody (1:200) on the fixed smears and incubated at 37°C for 1h in a moist chamber. After washing with PBS Tween 20 (PBST) three times, Alexa-Fluor® 488-conjugated goat anti-mouse Immunoglobulin G (IgG) (Molecular Probes, Dallas, Texas, USA) was applied as a secondary antibody (1:200) and then incubated at 37°C for 30 min. The slides were washed three times with PBST and incubated with 2.5 µg/mL Propidium Iodide (PI) (Molecular Probes, Carlsbad, CA, USA) containing 50 µg/mL RNase (Qiagen, Hilden, Germany) at 37°C for 10 min. After washing with PBS twice, the glass slides were mounted by adding 10 µL of a 50% glycerol-PBS (v/v) solution and covered with a glass coverslip. The slides were examined using a confocal laser scanning microscope (TCS NT; Leica, Mannheim, Germany).

In vitro growth inhibition assay for *B. bigemina*

The *in vitro* growth inhibition assay for *B. bigemina* was adopted as previously described [24,27] with some modifications. Anti-AMA-1 and anti-GST mice sera were added to the culture at 4% of the total volume. Cultures were incubated at 37°C in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2. For a period of four days, the culture medium was replaced every day with 100 µl of fresh medium containing the sera. Parasitemia was monitored based on approximately 1,000 erythrocytes in a Giemsa-stained thin erythrocyte smear. Negative control composed of M199 enriched with perimmune serum was used.

Invasion inhibition assay

B. bigemina infected erythrocytes with 5% parasitemia were used to obtain free merozoites as previously described [28]. The erythrocytes were raptured using Gene *PulsarII* (Bio-Rad, US) at 1.5 Kv, 400 Ω, and 25 µØ. Free-merozoites were washed with PBS by centrifugation to remove hemoglobin remnants. Merozoites were incubated with anti-AMA-1 mice serum at 4% of the final volume of the culture for 10 min at 37°C [18] and then incubated with bovine erythrocytes at 10% in a 96-well plate at 37°C. Smears were made at 1, 6, and 24 h. Invasion inhibition for the anti-AMA-1 serum was compared with anti-GST serum control and calculated as a percentage of control preimmune serum.

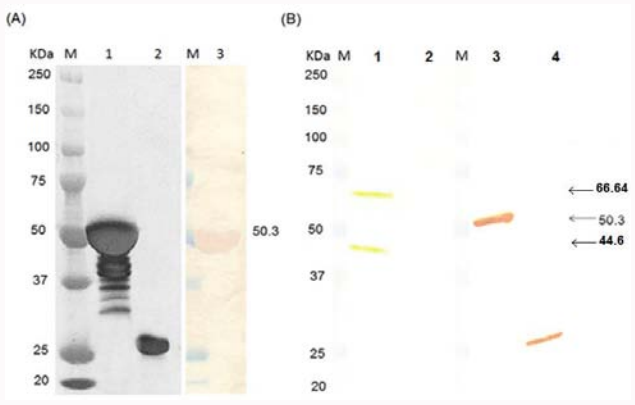

Figure 3: Sodium dodecyl sulfate poly acrylamide gel electrophoresis and Western blot of *AMA-1*. (A) SDS PAGE of the rtBbigAMA-1 (lane1) and GST (lane 2) and Western blot with infected serum (lane 3). (B) Western blots of the anti-rtBbigAMA-1 reacted with native protein (lane 1), with the rtBbigAMA-1 (lane 2) and GST (lane 3) but not with bovine erythrocytes (lane 2). M is a protein marker.

Vaccination and challenge infection

A total of 18 female BALB/c (6-week-old) mice were divided into three groups (n=6). One hundred micrograms of purified rtBbigAMA-1 emulsified in 100 µL of Freund's complete adjuvant (Sigma) was administered i.p. followed by three additional boosters (50 µg) with Freund's incomplete adjuvant (Sigma) i.p. at 14-day intervals. This group was designated as a test group. Control mice received either GST protein or no immunization. Two weeks after the final boosting, mice were challenged i.p. with 1×10^7 *B. microti*-infected erythrocytes. Parasitemia was observed every day for 23 days by examination of Giemsa-stained blood smears.

Statistical analysis

JMP statistical software (SAS Institute Inc., USA) was used in the analysis of data. One-way Analysis of Variance (ANOVA) followed by independent Student's t-test was used to analyze the differences in the percentage of parasitemia in the *in vitro* growth inhibition assay, invasion inhibition assay, and among groups of vaccinated mice in challenge experiment. A P value of <0.05 and <0.01 were considered statistically significant for the *in vivo* and *in vitro* studies, respectively. The effect of vaccination on the progress of parasitemia among groups of mice was evaluated by repeated measures analysis of variance (Manova). A P value of <0.001 was considered statistically significant.

Results

Cloning and phylogenetic analysis

B. bigemina Argentina strain was used to obtain genomic and cDNA for PCR with primers *BbigAMA-1* F and R to amplify the *AMA-1* gene. The sequences had an identical length from both templates and of the estimated size according to primers' design (not shown). The nucleotide sequencing analysis presented a 1988-bp long gene (gene bank accession number: *AB481200*) and encoded a 595-amino acid peptide (gene bank accession number: *BAH22706*) with a computer-calculated molecular weight of 66.64 KDa (Figure 1). The protein has a signal peptide of 30 amino acids (Signal 3). The nucleotide sequence had 98.2% identity with the sequence reported in the *B. bigemina* genome sequence at Sanger Institute. The nucleotide sequence had 98% to 99% identity with other isolates of AMA-1 (Gene bank accession: JN572799, JN572796, HM543728, JN572800, JN572792, and GQ257738). The nucleotide sequence was most similar to the cDNA sequence of the *B. gibsoni AMA-1* gene, with an identity of 51.7%, compared to 46.6, 45.4, 44.1, 40.9, 38.3, and 37.9 for AMA-1 genes of *B. bovis*, *N. caninum*, *P. knowlesi*, *T. parva*, *P. falciparum*, and *T. gondii* and *T. annulata*, respectively. The amplified product has amino acid identities of 99.2% with the Australian isolate and 99.3% to 99.5% with other isolates of *B. bigemina AMA-1* (Gene bank accessions: AET36930, AET36927, ADP02975, AET36931, AET36926, and ACT46060). The protein sequence was compared with the sequences of *AMA-1* of *B. gibsoni*, *B. bovis*, *T. parva*, *T. annulata*, *P. knowlesi*, *P. falciparum*, *T. gondii*, and *N. caninum* with identities of 67.4, 62.7, 41.3, 41.4, 42.2, 37.4, 38.8, and 37.4%, respectively (Figure 2). Phylogenetic tree for *BbigAMA-1* showed that *B. bigemina* is most strictly correlated to *B. gibsoni* and *B. bovis* (Figure 2) and has a closer genetic relationship with *T. parva*, *T. annulata, P. knowlesi,* and *P. falciparum* compared to *N. caninum* and *T. gondii.*

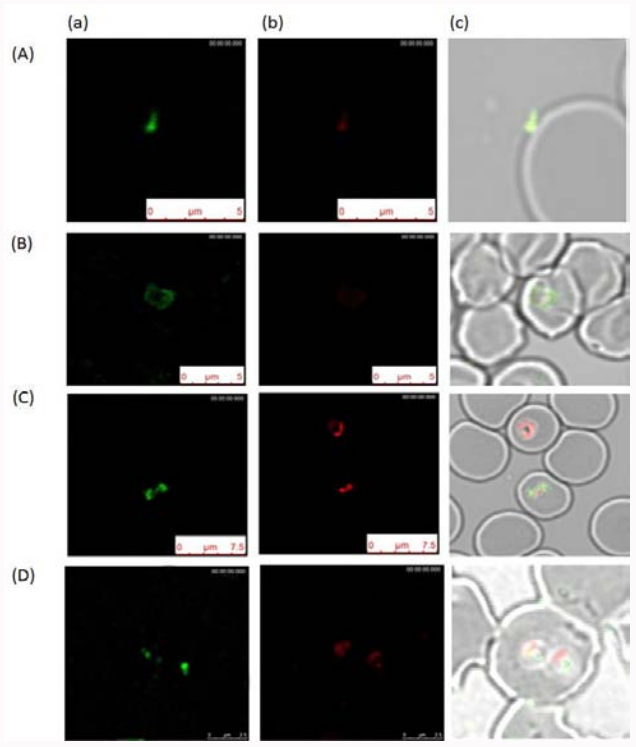

Figure 4: Indirect immunofluorescent assay of anti-rtBbigAMA-1 mouse serum. The antiserum reacted with *Babesia bigemina* extracellular (panel A) or intracellular (panel B), *Babesia bovis* (panel C), and *Babesia microti* (panel D) merozoites. (a) Green florescent of anti-rtBbigAMA-1, (b) Red florescent is propodeum iodide, and (c) Merge.

Characterization of rtBbigAMA1

BbigAMA1 (1,450 bp positions 91 to 1,541) lacking the signal peptide (signal 3, Denmark) and transmembrane (TMHMM Server, Denmark) was cloned using expression primers Fe1 and Re1 (Table 1) in *PGEX4T1* vector using EcoRI and XhoI enzyme sites and expressed as 76 KDa GST fusion protein, but the levels of purified protein using urea were very small to continue the experiments. Therefore, we used a truncated AMA1 of 657 bp (nucleotide positions 394 to 1,050) and cloned in *PGEX4T1* vector using EcoRI and XhoI enzyme sites. The rtBbigAMA1 was expressed in *E. coli* as a soluble GST-fusion protein with a molecular mass of 50.3 kDa (Figure 3A, lane 1). The rtBbigAMA1 reacted with serum naturally infected with *B. bigemina* in Western Blotting (Figure 3A, lane 3), suggesting the

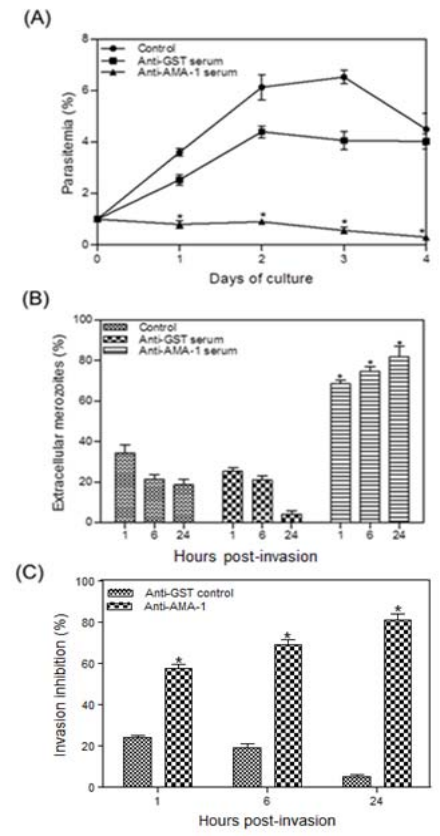

Figure 5: Growth and invasion inhibition effects of antirtBbigAmA-1. (A) *In vitro* growth inhibition of anti-rtBbigAMA-1 and anti-GST on *Babesia bigemina* for 4 days. (B) Extracellular merozoites of *Babesia bigemina* in *in vitro* invasion inhibition by anti-rtBbigAMA-1 or anti-GST at 1, 6, and 24 hours. (C) Invasion inhibition percent of *Babesia bigemina* merozoites after incubation with anti-rtBbigAMA-1 or anti-GST at 1, 6, and 24 h. *- Asterisks indicate significant inhibition (P<0.01).

maintenance of antigenicity of *E. coli*-expressed rtBbigAMA-1. To characterize the *BbigAMA-1*, antibodies were raised to the protein in mice. In Western blot analysis, the anti-rtBbigAMA-1 serum reacted specifically with protein bands of about 66.64-kDa in *B. bigemina* merozoite lysates (Figure 3B, lane 1) which represent the unsecreted form of *AMA-1*. It also reacted with a smaller band of 44.6 KDa. The mice serum reacted positively with the recombinant protein at 50.3 KDa. Finally, the antiserum raised against rtBbigAMA1 reacted positively with merozoites of *B. bigemina* extracellular (Figure 4A) or intracellular (Figure 4B), *B. bovis* (Figure 4C), *and B. microti* (Figure 4D) in IFAT. The green fluorescence of the *BbigAMA1* was observed by IFAT in extracellular and intracellular stages of *B. bigemina* merozoites.

In vitro growth inhibition and invasion inhibition assays

B. bigemina growth was significantly inhibited by anti-rtAMA-1 mouse serum (P<0.01). Parasites in the treated cultures had a parasitemia of 0.56% on day 3 while control cultures treated with anti-GST serum and normal bovine serum had 4% and 6.5%, respectively (Figure 5A). The effect of anti-rtAMA-1 mice serum on merozoite invasion was evaluated. Extracellular merozoites were significantly higher (P<0.01) with anti-rtAMA-1 mice serum than the anti-GST and the control sera (Figure 5B). The invasion inhibition was 57.8%, 67.8%, and 81.2% at 1, 6, and 24 hours post-invasion, respectively (Figure 5C).

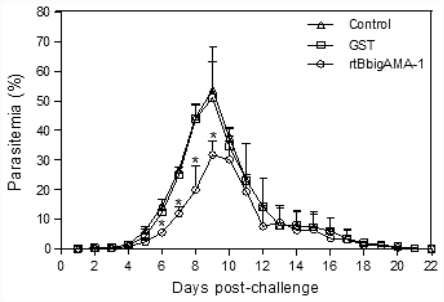

Figure 6: Infection course of *Babesia microti* in BALB/c mice over a period of 22 days. Following the immunization regime with rtBbigAMA-1 and GST, the mice were challenged with 1×10^7 *B. microti*. Each point represents the mean ± standard deviation. The means were statistically compared by one-way ANOVA followed by an independent Student's t-test and each asterisk indicates a significant difference (*P <0.05).

Vaccination and challenge infection

To test the efficacy of rtBbigAMA1 as a potential vaccine, all mice were vaccinated with rtBbigAMA1 and GST. Fifteen days next to last immunization, mice have been intraperitoneally infected with 1×10^7 *B. microti*-infected erythrocytes. Parasitemia was detected as early as day 1 post-challenge and reach the peak levels at day 9 post-challenge (Figure 5). BALB/c mice that received rtBbigAMA1 had significant reductions in progress (Manova, P>0.001) and peak levels (Student's t-test, P>0.05) of parasitemia from days 6 to 9 compared with the control mice. The inhibition ratio was 42.3% on day 9, the peak of parasitemia (Figure 6).

Discussion and Conclusion

Babesia has worldwide economic importance through the enormous losses it causes for the livestock industry. Life vaccines have the hazard of disseminating infections or the return to virulence. Therefore, subunit antigens are attractable for anti-*Babesia* vaccine production [29]. In this respect, *AMA-1* has arisen as a prospective invasion-blocking vaccine candidate. Therefore, we investigated the characterization of *AMA-1* from *B. bigemina*. The sequence of *BbigAMA-1* was found in the genome sequence database of *B. bigemina* through the blast search with homolog sequences of *B. bovis* and *B. gibsoni.* The sequence of *BbigAMA-1* from the Argentina strain was identified by sequencing. Phylogeny grouped *AMA-1* of *Plasmodium* spp. and other blood parasites outside the paraphyletic group containing *N. caninum* and *T. gondii*. Analysis of the protein indicated conservation (37.4% to 67.4%) with *AMA-1* from other apicomplexan parasites. The *AMA-1* of *Babesia*, *Theileria*, and *Plasmodium* were closely related; therefore, the function of *AMA-1* might be similar among these parasites.

For the immunological characterization of *BbigAMA-1*, the truncated gene was expressed with GST tag in *E. coli.* The rtBbigAMA-1 reacted with *B. bigemina* positive field serum in Western blot indicating antigenicity of the protein. Furthermore, Antiserum raised against the rtBbigAMA-1 reacted specifically with a 66.64 kDa native protein of *B. bigemina* which agreed with *B. bovis AMA-1* reported by Salama et al. [17] while not agreed with Gaffar et al. [18] who reported unsecreted protein of 82 KDa and the secreted protein of 69 KDa this might be due to the type of protein used as they used small peptides from the protein in contrast to Salama et al. [17]. Immunofluorescent staining of BbigAMA-1 was observed within the matrix of the merozoite of *B. bigemina*, *B. bovis*, and *B. microti* near to the apical end indicating the conservation of the protein among

Babesia species.

Antiserum against rtBbigAMA-1 prevented the growth and invasion of merozoites into host erythrocytes. These findings were similar to the action of other *Babesia* AMA-1 indicating its use as a vaccine candidate for babesiosis [17,18,20].

The vaccination efficacy of recombinant AMA-1 from *B. bigemina* was evaluated against a challenge infection with *B. microti* in BALB/c mice. The challenge with 1×10^7 infected erythrocytes resulted in obvious parasites in blood on day 2, afterward a rapidly ascending parasitemia that approached peak at day 9. Parasitemia of BALB/c mice that received rtBgAMA-1 showed significant reductions in parasitemia during the advancement and topmost levels compared with GST and non-vaccinated groups. Thus, the vaccination with rtBgAMA-1 offered cross-protection from the infection with *B. microti*. Further studies on cattle are advised to assess its protective effects. The moderate defense elicited by rtBgAMA-1 might advise its use as a vaccine nominee in a cocktail with other antigens against babesiosis.

In conclusion, the study started a characterization of a member of *AMA-1* of *B. bigemina* and demonstrated its importance for the invasion process and defending properties against *B. microti*. The promising and safe efficacies of the *AMA-1* make it interesting for further studies as a vaccine nominee against other babesiosis.

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