An apical membrane antigen-1 homologue from *Babesia gibsoni* and its antibody response in the infected dogs

Jinlin Zhou,[1,2] Jun Yang,[1] Guohong Zhang,[1] Yoshifumi Nishikawa,[1] Kozo Fujisaki,[1] and Xuenan Xuan[1*]

1. National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan
2. Shanghai Institute of Animal Parasitology, Chinese Academy of Agricultural Sciences, 3 Lane 345, Shilong Road, Shanghai 200232, China

*****Corresponding author:
Dr. Xuenan Xuan,
National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan.
Tel.: 0081-155-49-5648
Fax: 0081-155-49-5643
E-mail: gen@obihiro.ac.jp

Abbreviations:

AMA-1, apical membrane antigen-1; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay

Abstract

A cDNA encoding the apical membrane antigen-1 (AMA-1) homologue was obtained by immunoscreening a cDNA expression library prepared from *Babesia gibsoni* merozoite mRNA. The complete nucleotide sequence of the gene was 2,062 bp. Computer analysis suggested that sequence contains an open reading frame of 1,794 bp with a coding capacity of approximately 66 kDa. Based on the homology analysis, this putative protein was designated *B. gibsoni* AMA-1 (BgAMA-1). The BgAMA-1 gene was expressed in *Escherichia coli* BL21 strain, and used as the antigen in the Western blotting and enzyme-linked immunosorbent assay (ELISA). These results indicated that BgAMA-1 was recognized as an immunodominant antigen by the host immune system and that it induced a strong antibody response only in chronic *B. gibsoni* infection in dogs, however, the antibody response could not detect in the early infection stage (within 15 days). This phenomenon might be explained by the limited stimulation with the low-abundance protein in the early infection. This result showed the BgAMA-1 was a new member of AMA-1 family and its immune response characteristic in canine *B. gibsoni* infection.

Key words:

Babesia gibsoni; AMA-1; antibody response

Babesia gibsoni is a tick-transmitted apicomplexan protozoan. It is a causative agent of canine babesiosis and is found throughout many regions of Asia, Africa, Europe, and the Americas [1,2]. Understanding the basic molecular mechanism of the asexual intraerythrocytic cycle, particularly the process of merozoite invasion of erythrocytes, may accelerate the development of an effective control strategy. Host cell invasion by apicomplexan parasites is a complex, multistep process [3,4], a number of proteins have been implicated in invision, but in most cases their precise functions remain unknown.

Apical membrane antigen-1 (AMA-1) is a malarial surface protein found in all characterized *Plasmodium* species and highly conserved among apicomplexan parasites [5]. AMA-1 is stored in the microneme organelles immediately after synthesis and is transported to the parasite surface just prior to, or during, host-cell invasion. It comprises an N-terminal ectoplasmic region, a single transmembrane segment and a small cytoplasmic domain. The ectoplasmic region of AMA-1 has been divided into three domain based on the disulfide bonds [6] and the recent crystal structure [7]. The precise function of AMA-1 is not known, but many lines of evidence point to a direct role in host cell invasion. AMA-1 is regarded as a leading vaccine candidate and currently being developed as a malaria

vaccine [8,9]. Homologues of AMA-1 from the apicomplexan parasites *Toxoplasma gondii* (TgAMA-1) [10,11] and *B. bovis* (BbAMA-1) [12] have been identified and sequenced. In this study, we report a gene encoding AMA-1 homologue from canine *B. gibsoni*, and the antibody response to AMA-1 homologue in the experimentally infected dogs.

Construction of a cDNA expression library from *B. gibsoni* merozoites and immunoscreening using the infected dog sera were performed as described previously [13]. The cDNA encoding the AMA-1 homologue was cloned, and the putative protein was designed as BgAMA-1 (*B. gibsoni* AMA-1 homologue). The complete nucleotide of the BgAMA-1 gene was 2,062 bp. Started from methionine at position 74, a single open reading frame (ORF) of 1,794 bp nucleotides was presented. The ORF encodes a polypeptide of 598 amino acid residues with a size of 66-kDa as calculated by computer. Blast analysis deduced that the amino acid sequences have a significant homology with the AMA-1 from other apicomplexan protozoan, especially the AMA-1 from reported *B. bovis* AMA-1 homologue (BbAMA-1). The BgAMA-1 showed 48% homology with BbAMA-1 (Fig. 1). Reminiscent of the previously described AMA-1 proteins, the hydrophobic N-terminal 32 amino acids of BgAMA-1 were predicted to form a signal peptide (SignalP 3.0), whereas the hydrophobic stretch from Ile-514 to Trp-

536 is likely to form a transmembrane region, leaving a 62-amino-acid cytoplasmic C terminus (TMHMM 2.0). Many of the invariant residues and conservative differences in AMA-1 are also present in BgAMA-1, three domains as predicted from the pattern of cystine-bridge formation were shown in Fig. 1. However, as the BbAMA-1, the cystine bridge corresponding to cys434-cys451 in PvAMA-1 is absent and the pair of CXC motifs characterizing the cystine knot structure is thus not present in BgAMA-1 [5,12].

The ORF without the signal peptide sequence of BgAMA-1 gene was subcloned into pGEX-4T-3 and expressed as a glutathione *S*-transferase (GST)-fusion protein in the *E. coli* BL21 (DE3) strain. The molecular mass of the recombinant protein was estimated to be 88-kDa as expected, considering with 26-kDa the GST tag. The dog serum 60 days post-infection with *B. gibsoni* reacted with recombinant BgAMA-1, but the normal dog serum and the dog serum 15 days post-infection did not recognize the recombinant protein by Western blotting (Fig. 2).

To determine the antibody response of BgAMA-1 in the dogs infected with *B. gibsoni*, the ELISA was used to detect the immunoglobulin G level against recombinant BgAMA-1 in the experimentally infected dogs. Individual wells of a microtiter plate (Nunc) were coated with the purified

recombinant GST-BgAMA-1 protein (0.1 μg/well) or the control GST protein (0.1 μg/well) in an antigen coating buffer (0.05M carbonate-bicarbonate buffer, pH 9.6) and then used as an antigen for ELISA. The ELISA was performed as described previously [13]. Absorbance was shown as the distance between the GST-BgAMA-1 protein and the control GST protein. The canine sera used for ELISA were as follows: 10 sera from healthy dogs, 8 sera from dogs within 15 days post-infection with *B. gibsoni*, 7 sera from dogs more than 30 days post-infection with *B. gibsoni*, and the sequential serum samples from a dog experimentally infected with *B. gibsoni*. As shown in Fig. 3A, all 7 sera from dogs more than 30 days post-infection with *B. gibsoni* were positive (optical density >0.2), whereas the serum samples from uninfected dogs and sera from dogs within 15 days post-infection were negative (optical density < 0.1). A dog experimentally infected with *B. gibsoni* developed a significant level of antibody response to BgAMA-1 from 18 days post-infection. The antibody titer was maintained until 207 days post-infection, even when the dog became chronically infected with a significantly low level of parasitemia (Fig. 3B). These results indicated that BgAMA-1 was recognized as an immunodominant antigen by the host immune system and that it induced a strong antibody response only in chronic *B. gibsoni* infections in dogs.

Our results showed the second member of AMA-1 in *Babesia* parasites, the highly conserved sequences character between BgAMA-1 and BbAMA-1 suggests they may share a common biological function. Homologues of AMA-1 from the apicomplexan parasites *T. gondii* (TgAMA-1) and *B. bovis* (BbAMA-1) show the same pattern of cysteine residues in domains I and II of *Plasmodium*, but domain III in both TgAMA-1 and BbAMA-1 shows significant sequence differences to *Plasmodium* AMA-1, most notably in the cysteine residues [5]. Domain I and II belong to the PAN module superfamily, suggesting that they may function in adhesion to protein or carbohydrate receptors [7]. The differences in domain III may reflect the proximity of this part of AMA-1 to the parasite surface and its consequent interaction with other surface proteins in the different apicomplexan taxa, or perhaps interaction with different host-cell receptors [5]. Domain III of BbAMA-1 has only four cysteine residues but their alignment shows that they should form two disulphide bridges [12]. Our cloned BgAMA-1 also showed the same character with BbAMA-1, which indicated the more similar functions among species. Like other micronemal proteins, AMA-1 has a short cytoplasmic tail, which may be involved in binding to other intracellular proteins that may govern the timing of secretion, which is not synchronous for different micronemal proteins [14]. Alternatively, it may be

involved in transmitting a signal upon contact of the ectodomain with a host cell ligand, or it may be involved in linkage to cytoskeletal structures like those recently described for members of the TRAP family of micronemal proteins [15].

AMA-1 is a low-abundance type I integral membrane protein in *P. falciparum* [16]. Despite its low abundance, AMA-1 is a highly immunogenic protein. Most individuals exposed to malaria develop anti-AMA-1 antibodies after relatively few exposures [17]. It has been calculated that 1% of the total immunoglobulin G in Papua New Guineans living with endemic malaria is against AMA-1 [18]. Our results also demonstrated the BgAMA-1 was a highly immunogenic protein in chronic infected dogs, but the antibody response could not detect in the early infection stage (within 15 days). This phenomenon could be explained by the limited stimulation with the low-abundance protein in the early infection. As mentioned above, the AMA-1 homologues are low-abundance protein not only in *Plasmodium*, but also in *B. bovis* [12]. The BgAMA-1 may also be a low-abundance protein in *B. gibsoni* merozoites, because the anti-rBgAMA-1 serum could not detect the native protein in lyses of infected dog erythrocyte by Western blotting in our experiment (data not shown). This result showed the BgAMA-1 was not suitable as a diagnostic antigen in canine *B. gibsoni* infection. The biological

function and vaccine effect of BgAMA-1 against *B. gibsoni* infection will be our further works.

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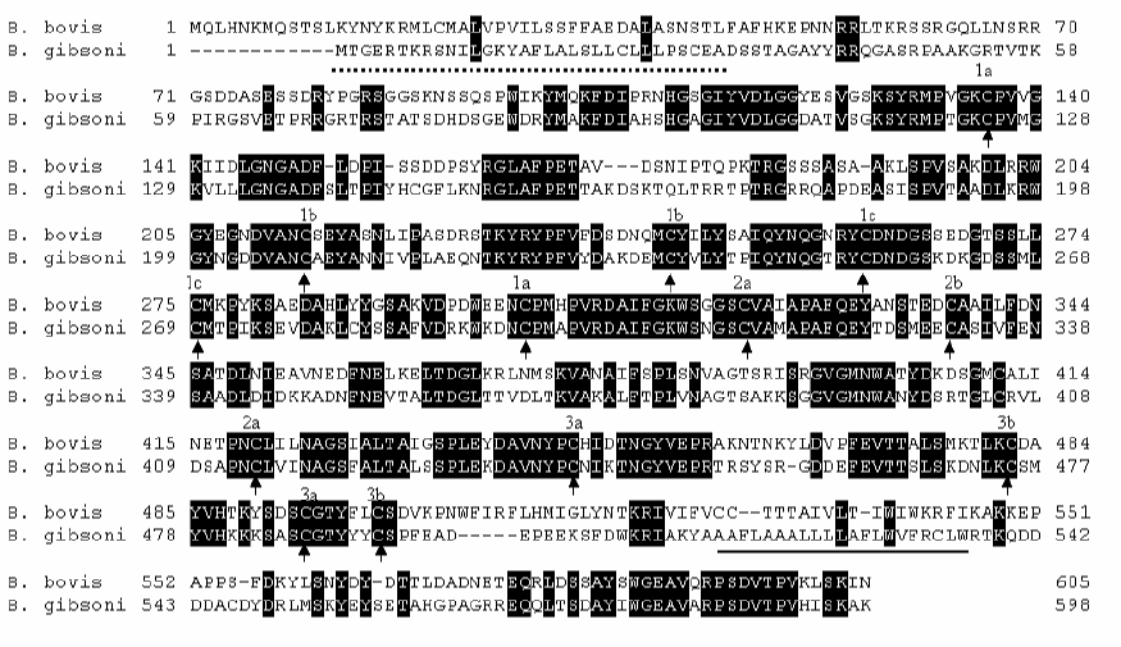
Figures legends:

Fig. 1. Alignment of putative amino acid of *B. gibsoni* AMA-1 (BgAMA-1) with *B. bovis* AMA-1 (BbAMA-1). Identical residues are black shading, cysteine residues that putative form disulfide bonds in *B. bovis* AMA-1 are indicated by arrow, domain (1, 2, 3) and bond (a, b, c) designations. The signal peptide sequence of BgAMA-1 is indicated by dotted line, and the transmembrane region is indicated by solid line.

Fig. 2. Western blot analysis of recombinant BgAMA-1 reacted with the infected dog serum. Recombinant BgAMA-1was electrophoresed on a 12% plolyacrylamide gel and blotted onto a PVDF membrane. The membrane was incubated with a dog serum 60 days post-infection with *B. gibsoni* (lane 2), a dog serum 15 days post-infection with *B. gibsoni* (lane 3) and a normal dog serum. Lane 1, molecular weight markers.

Fig. 3. Antibody detection by enzyme-linked immunosorbent assay (ELISA). A, values from ELISA with recombinant BgAMA-1 and experimentally *B. gibsoni* infected dog sera. Lane 1, seven sera from dogs more than 30 days post-infection; lane 2, ten sera from healthy dogs; lane 3, eight sera from dogs more than 30 days post-infection. $OD_{415nm,}$ Optical density at 415 nm. B, Antibody titer to recombinant BgAMA-1 in a dog experimentally infected with *B. gibsoni* by ELISA. The antibody titer is indicated by optical density at 415 nm. The parasitemia is indicated by percentage.

Fig. 1


B. bovis 1 MQLHNKMQSTSLKYNYKRMLCMALVPVILSSFFAEDAIASNSTLFAFHKEPNNRRLTKRSSRGQLLNSRR 70
B. gibsoni 1 ----------MTGERTKRSNILGKYAFLALSLLCLLLPSCEADSSTAGAYYRRQGASRPAAKGRTVTK 58
1a
B. bovis 71 GSDDASESSDRYPGRSGGSKNSSQSPWIKYMQKFDIPRNHGSGIYVDLGGYESVGSKSYRMPVGKCPVVG 140
B. gibsoni 59 PIRGSVETPRRGRTRSTATSDHDSGEWDRYMAKFDIAHSHGAGIYVDLGGDATVSGKSYRMPTGKCPVMG 128
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B. gibsoni 129 KVLLLGNGADFSLTPIYHCGFLKNRGLAFPETTAKDSKTQLTRRTPTRGRRQAPDEASISPVTAADLKRW 198
1b 1b 1c
B. bovis 205 GYEGNDVANCSEYASNLIPASDRSTKYRYPFVFDSDNQMCYILYSAIQYNQGNRYCDNDGSSEDGTSSLL 274
B. gibsoni 199 GYNGDDVANCAEYANNIVPLAEQNTKYRYPFVYDAKDEMCYVLYTEIQYNQGTRYCDNDGSKDKGDSSML 268
1c 1a 2a 2b
B. bovis 275 CMKPYKSAEDAHLYYGSAKVDPDWEENCPMHPVRDAIFGKWSGGSCVAIAPAFQEYANSTEDCAAILFDN 344
B. gibsoni 269 CMTPIKSEVDAKLCYSSAFVDRKWKDNCPMAPVRDAIFGKWSNGSCVAMAPAFQEYTDSMEECASIVEEN 338
B. bovis 345 SATDLNIEAVNEDPNELKELTDGLKRLNMSKVANAIPSPLSNVAGTSRISRGVGMNWATYLKDSGMCALI 414
B. gibsoni 339 SAADLDIDKKADNPNEVTALTDGLTTVDLTKVAKALPTPLVNAGTSAKKSGGVGMNWANYLSRTGLCRVL 408
2a 3a 3b
B. bovis 415 NETPNCLILNAGSPALTAIGSPLEYDAVNYPCHIDTNGYVEPRAKNTNKYLDVPFEVTTALSMKTLKGDA 484
B. gibsoni 409 DSAPNCLVINAGSPALTALSSPLEKDAVNYPCNIKTNGYVEPRTRSYSR-GDDEFEVTTSLSKDNLKGSM 477
3a 3b
B. bovis 485 YVHTKYSDSCGTYTLCSDVKPNWFIRFLHMIGLYNTKRIVIFVCC--TTTAIVLT-IWIWKRFIKAKKEP 551
B. gibsoni 478 YVHKKKSASCGTYYYCSPFEAD-----EPEEKSFDWKRIAKYAAAFLAAALLLIAFLWVFRCLWRTKQDD 542
B. bovis 552 APPS-FDKYLSNYDM-DTTLDADNETEQRLDSSAYSWGEAVQRPSDVTPVKLSKIN 605
B. gibsoni 543 DDACDYDRLMSKYDYSETAHGPAGRREQQLTSDAYIWGEAVARPSDVTPVHISKAK 598

Fig.2

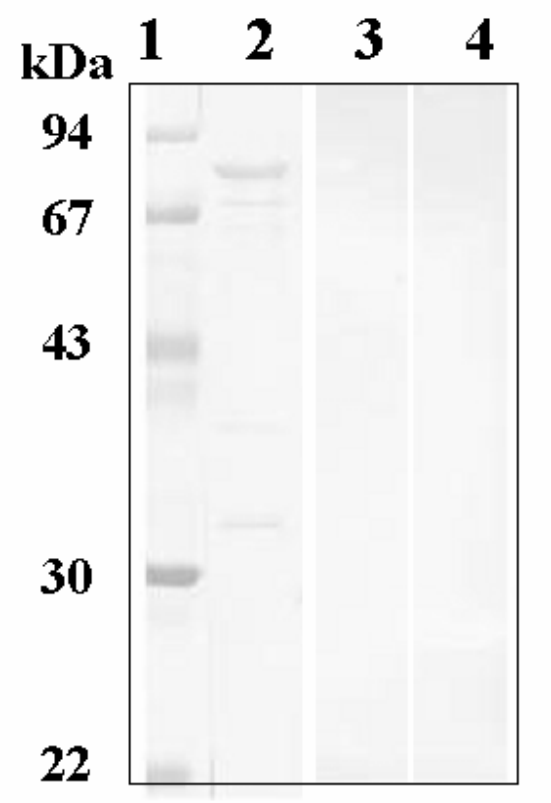
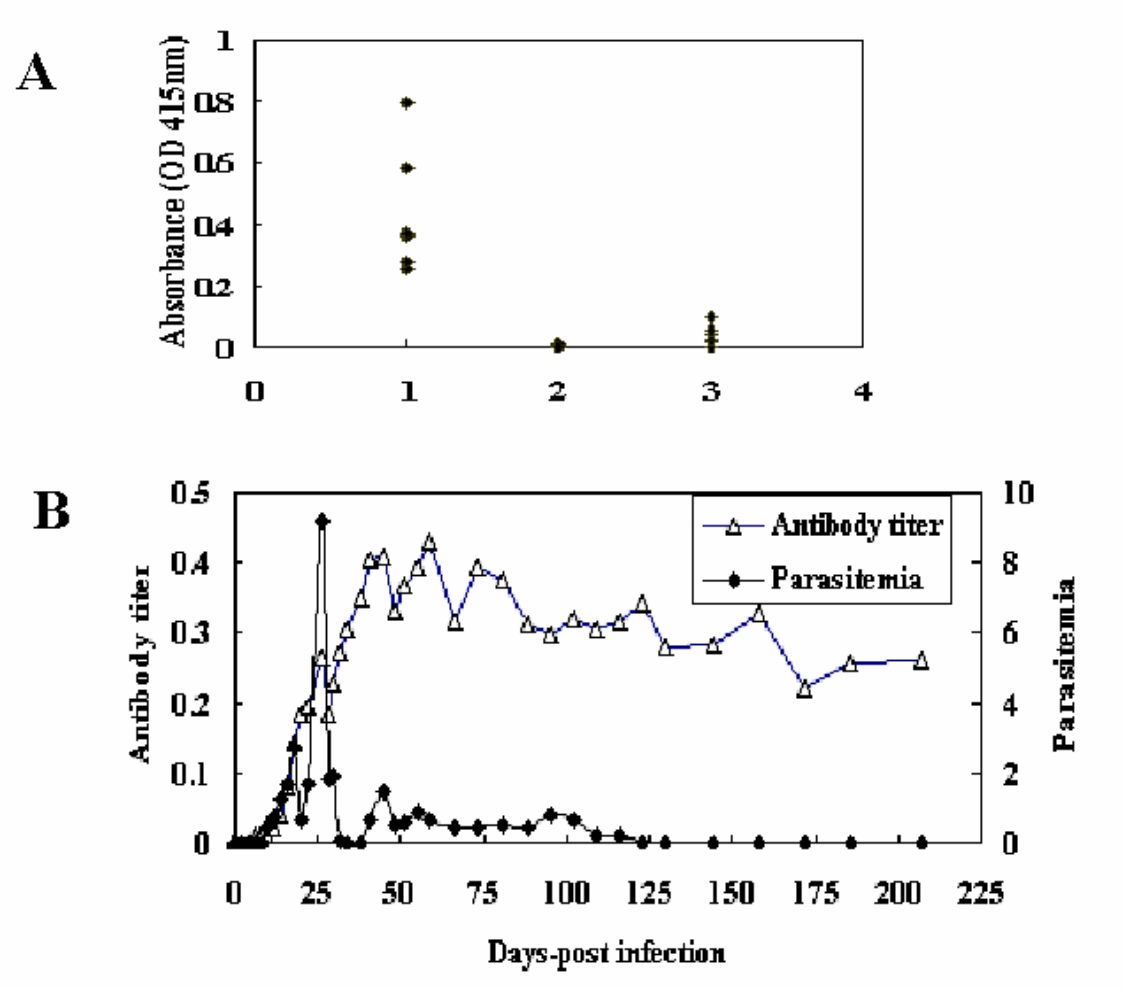


Fig. 3


A
Absorbance (OD 415nm)
1
0.8
0.6
0.4
0.2
0
0
1
2
3
4
B
Antibody titer
Parasitemia
0.5
0.4
0.3
0.2
0.1
0
10
8
6
4
2
0
0
25
50
75
100
125
150
175
200
225
Days-post infection