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Original article

Characterisation of a *Babesia orientalis* apical membrane antigen, and comparison of its orthologues among selected apicomplexans

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ABSTRACT

In the present study, we identified and characterised the complete coding sequence of *Babesia orientalis* apical membrane antigen 1 (designated *Bo-ama1*); it is 1803 bp in length and encodes a polypeptide of 601 amino acids (aa). The *Bo-ama-1* gene product (*Bo*-AMA1) is predicted to be 67 kDa in size and contains a signal peptide. Mature Bo-AMA1 is predicted to have one transmembrane region and a short cytoplasmic tail (C-terminal domain). The extracellular part of *Bo*-AMA1 has three functional domains (DI, DII and DIII) with 14 conserved cysteine residues. A *Bo*-AMA1 fragment containing all three of these domains (designated *Bo*-AMA1-DI/II/III) was cloned into the plasmid vector pET-28a and expressed as a recombinant (His-fusion) protein of 53 kDa. Antibodies in the serum from a *B. orientalis*-infected water buffalo specifically recognised this protein in immunoblotting analysis. Rabbit antibodies raised against the recombinant protein were able to detect native *Bo*-AMA1 (67 kDa) from erythrocytes of *B. orientalis*-infected water buffalo. *Bo*-AMA1 is a new member of the AMA1 family and might be a good antigen for the specific detection of antibodies produced in *B. orientalis* infected cattle. This protein is likely to play critical roles during host cell adherence and invasion by *B. orientalis*, as the AMA1s reported in other organisms such as *Plasmodium falciparum* and *Toxoplasma gondii*. Further research is required to explore the biological functions of this protein and to determine whether its immunisation can induce protective effects in water buffalo against *B. orientalis* infection.

Introduction

Vector-borne apicomplexan parasites, including *Plasmodium*, *Theileria* and *Babesia*, invade and replicate within host cells to cause diseases. The process of host-cell invasion is best characterised in *Plasmodium falciparum* and consists of five main phases: (1) *initial attachment*: extracellular merozoites reversibly attach to erythrocytes; (2) *re-orientation*: merozoites re-orient themselves to bring the apical organelles close to erythrocytes; (3) *tight junction formation*: the parasites bind closely to erythrocytesurface from the apical end and form a tight junction with the erythrocyte; (4) *actual invasion*: merozoites penetrate the erythrocyte membrane and form a parasitophorous vacuole as they migrate into erythrocytes (Dubremetz et al., 1998; Soldati et al., 2001); (5) *internalisation*: parasites finish the invasion process and are completely internalised into erythrocytes (Carruthers and Boothroyd, 2007).

Tight junction formation is a crucial step for successful invasion (Carruthers and Boothroyd, 2007), in which the apical membrane antigen 1 (AMA1) forms a complex with rhoptry neck proteins (RONs) (Collins and Blackman, 2011). For instance, in *P. falciparum*, the rhoptry neck protein *Pf*-RON2 interacts with AMA1 and the other two rhoptry neck proteins (*Pf*-RON4 and *Pf*-RON5) to perform this function (Alexander et al., 2006; Cao et al., 2009; Richard et al., 2010; Straub et al., 2009). This AMA1-RONs complex is required for the parasite to maintain a close association with the erythrocyte, to allow the secretion of rhoptry contents into this cell and to enable efficient invasion (Tyler et al., 2011).

Many aspects of tight junction formation have been studied, particularly in *P. falciparum* (Alexander et al., 2006; Harvey et al.,

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2014; Yap et al., 2014), and there has been some progress in understanding the role of this phase of invasion in related parasites, such as *Babesia bovis* (Asada et al., 2012). However, very little is known in most other *Babesia* species, including *B. orientalis*. This latter species is a parasite of bovids and, to date, has been described only in China (He et al., 2011). This species appears to be of economic importance, but has mild pathogenicity (Liu et al., 2005; Mital et al., 2005). Although culturing *B. orientalis in vitro* is a major challenge, we have become interested in undestanding how this parasite invades erythrocytes and why it is less pathogenic in water buffalo than, for example, *B. bovis* and *B. bigemina* in cattle. In the first step, we focused here on the characterisation of the *B. orientalis* orthologue of *P. falciparum* AMA1 and studied its relationship with homologues from a range of apicomplexan parasites.

Methods

Experimental infection

The Wuhan strain of *B. orientalis* was previously isolated from water buffalo in Hubei province of China and preserved in liquid nitrogen in the State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, China (Liu et al., 1997). Two *Bubalus bubalis* (water buffalo; 2 years of age) used for parasite infection were confirmed to be *B. orientalis*-free by microscopic examination (Liu et al., 2005) and real-time PCR testing (He et al., 2011). These buffaloes were splenectomised 2 weeks before experimental *B. orientalis* infection. Each buffalo was subcutaneously injected with 4 ml of water buffalo blood containing *B. orientalis* (Wuhan-strain; 1% parasitaemia) (He et al., 2009). Blood samples were collected every day to monitor parasitaemia until it reached 3%. Experimental animals were housed, fed and given clean drinking water in accordance with the regulations of the Standing Committee of Hubei People's Congress, PR China. All protocols were approved by the Laboratory Animal Research Centre of Hubei province, and the ethical committee of Huazhong Agricultural University (permit number 4200696657).

Parasite and merozoite antigen preparation

Babesia orientalis (Wuhan-strain) was collected from water buffalo and cultured as described previously (He et al., 2011). Merozoite antigen was prepared from *B. orientalis*-infected buffalo blood using a modified saponin lysis method (Conrad et al., 1987). In brief, 1 ml of *B. orientalis*-infected erythrocytes were washed 3 times with phosphate-buffered saline (PBS, pH 7.4) and subsequently suspended in 9 ml of RBC lysis buffer (TIANGEN, China), incubated at 37 °C for 5 min, and then centrifuged at 500 × *g* for 5 min. The supernatant was centrifuged again at 10,000 × *g* for 20 min and removed the subsequent supernatants. The pellet, which contained parasite antigens, was washed three times with PBS (pH 7.4). The merozoite antigen was enriched in pellets after centrifuged at 10,000 × *g* for 1 h, resuspended in 1 ml PBS (pH 7.4) and then stored at −20 °C until use.

DNA extraction

Blood from experimentally infected water buffalo was collected into EDTA tubes (BD Vacutainer, USA). Genomic DNA was extracted from 200 μl of *B. orientalis*-infected blood using the QIAamp DNA Blood Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. The DNA samples were used immediately or stored at −20 °C until use.

Immunoscreening of cDNA expression library

A cDNA library expressing *B. orientalis* antigens from phage was constructed using the SMART cDNA Library Construction Kit (Liu, CNKI, Parasitology Department; 2007). Briefly, total RNA was extracted from *B. orientalis* using the E.Z.N.ATM Blood RNA Kit, reverse transcribed using LD-PCR and used for cDNA Library construction using the SMART cDNA Library Construction Kit following the manufacturer's instructions. To screen for *B. orientalis* antigens, isolated phage plaques were transferred to a nitrocellulose membrane (Millipore, USA) and probed with serum (diluted 1:100 in 3% [w/v] bovine serum albumin in PBS) from *B. orientalis* infected water buffalo for 60 min at 37 °C. Primary antibodies were detected with horseradish peroxidase-conjugated goat anti-cattle IgG (1:2000 dilution) (Cappel, USA) and subsequent incubation with 3′,3′-diaminobenzidine tetrahydrochloride substrate (ZSGB-BIO kit) (He et al., 2009). Positive clones that could be recognised by anti-*B. orientalis* serum were isolated. Inserts in these positive clones were identified using Dye Terminator Cycle Sequencing reaction in ABI PRISM 377 DNA sequencer.

Cloning of Bo-*AMA1 and* Bo-*AMA1-DI/II/III*

The full-length *Bo-ama1* gene was amplified from genomic DNA of *B. orientalis* using the primers, AMA1-F: 5′-ATGATATCGACTATTTGCAGAAACTCCGG-3′ and AMA1-R: 5′-TCAGTTCATTTTTGAGAGGTGCACAG-3′, which were designed based on the *Bo*-AMA1 cDNA sequence. In addition, the extracellular domain (containing DI–DIII) of *Bo-ama1* was amplified from *B. orientalis* cDNA using primers E-AMA1-F: 5′-CCGGAATTCCCATGGTTGAAATACAT-3′ and E-AMA1-R: 5′-GCGTCGACGCGCTTTGTGTTGTAC-3′. The amplicons (1803 bp for full length *Bo*-AMA1 and 1281 bp for *Bo*-AMA1-DI/II/III) were cloned into the pMD19-T vector (TaKaRa Biotechnology, China) between *EcoR*I and *Sal*I sites. Positive clones were confirmed by Dye Terminator Cycle Sequencing reactions in ABI PRISM 377 DNA sequencer using vector originated primers M13 (-47) and M13 (-48) in separate reactions.

Bioinformatic analysis of Bo-*AMA1*

The amino acid sequence of *Bo*-AMA1 inferred from the cDNA sequence in the phage clone that identified *Bo*-AMA1 was aligned to AMA1 from other organisms that include *B. bovis* (XP001611043), *B. bigemina* (ADP02977), *B. gibsoni* (ABD04040), *P. berghei* (AAC47192), *P. falciparum* (XP001348015), *P. knowlesi* (XP002259339), *P. vivax* (AAC16731), *Neospora caninum* (BAF45372), *Toxoplasma gondii* (AAB65410) and *Eimeria maxima* (CBL80633) using the program MAFFT v.7 (Katoh and Frith, 2012; Katoh and Standley, 2013) and manually adjusted employing BioEdit v.7.1.11 (Hall, 1999). Phylogenetic analysis of the aligned sequences was conducted using the maximum likelihood (ML) algorithm in the program MEGA6 (Tamura et al., 2011). Evolutionary divergence was also calculated in the program MEGA6 using the Poisson correction model (Tamura et al., 2011; Zuckerkandl and Pauling, 1965). The transmembrane region and signal peptide in the *Bo*-AMA1 protein were predicted using the programs SOSUI and SOSUIsignal (http://expasy.org/tools/#topology). The antigen index was assessed using the program DNAstar Protean 7.5 (www.dnastar.com).

Expression and purification of Bo-*AMA1-DI/II/III in* Escherichia coli

The extracellular region of *Bo*-AMA1 containing DI/II/III, was cloned into the expression vector pET-28a (Addgene, USA) by using the primers E-AMA1-F and E-AMA1-R. The positive construct was