Molecular cloning, characterization and antigenicity of *Babesia* sp. BQ1 (Lintan) (*Babesia* cf. *motasi*) apical membrane antigen-1 (AMA-1)

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SUMMARY

Apical membrane antigen-1 (AMA-1) has been described as a potential vaccine candidate in apicomplexan parasites. Here we characterize the *ama-1* gene. The full-length *ama-1* gene of *Babesia* sp. BQ1 (Lintan) (BLTAMA-1) is 1785 bp, which contains an open reading frame (ORF) encoding a 65-kDa protein of 594 amino acid residues; by definition, the 5′ UTR precedes the first methionine of the ORF. Phylogenetic analysis based on AMA-1 amino acid sequences clearly separated Piroplasmida from other Apicomplexa parasites. The *Babesia* sp. BQ1 (Lintan) AMA-1 sequence is most closely associated with that of *B. ovata* and *B. bigemina*, with high bootstrap value. A recombinant protein encoding a conserved region and containing ectodomains I and II of BLTAMA-1 was constructed. BLTrAMA-1-DI/DII proteins were tested for reactivity with sera from sheep infected by *Babesia* sp. BQ1 (Lintan). In Western-blot analysis, native *Babesia* sp. BQ1 (Lintan) AMA-1 proteins were recognized by antibodies raised in rabbits against BLTrAMA-1 *in vitro*. The results of this study are discussed in terms of gene characterization, taxonomy and antigenicity.

Key words: *Babesia* sp. BQ1 (Lintan), AMA-1, characterization, expression.

INTRODUCTION

Babesiosis is one of the most common tick-borne haemoparasitic diseases in wild and domesticated animals in tropical, subtropical and temperate regions worldwide. Babesiosis is caused by infection and subsequent intraerythrocytic multiplication of apicomplexan parasites of the genus *Babesia*. In China, *Babesia* cf. *motasi* is a widespread pathogen of small ruminants that is transmitted via the tick vectors *Haemaphysalis qinghaiensis* and *H. longicornis* (Guan *et al*. 2002, 2010*a*; Wang *et al*. 2013).

Vaccination is currently the best control strategy for preventing babesiosis, and the different live vaccines developed are able to efficiently control the disease. The first live *B. bovis* vaccine was produced in Australia using virulent *B. bovis* strains attenuated by multiple rapid passages and exhibited apparently reduced virulence (Callow *et al*. 1979; Bock *et al*. 1992; Pipano, 1995). An Australian chilled tick fever vaccine and three attenuated live vaccines using *B. bovis* strains have been produced, offering protection against *B. bovis* and *B. bigemina*. However, occasional reversion of live attenuated parasites to the virulent state has been observed by Australian researchers (Gohil *et al*. 2013).

The clinical symptoms of babesiosis appear when *Babesia* merozoites invade and replicate within host erythrocytes and reach a high parasitaemia level (Yokoyama *et al*. 2006). Initially, the merozoites attach to the host RBC surface in any orientation. After re-orientation, the parasites form tight junctions between the RBC surface and the apical region, and, subsequently, the invasion is initiated, followed by the internalization of the parasite within infected RBCs. RBC invasion is mediated by proteins located both on the merozoite surface coat and in organelles. Proteins involved in attachment include variable merozoite surface antigens, and proteins involved in invasion, such as associated membrane antigen 1 (AMA-1), thrombospondin-related adhesive protein, rhoptry-associated protein-1 (RAP-1) and spherical body proteins, are secreted by micronemes, rhoptries and dense granules (Lobo *et al*. 2012). These proteins involved in RBC invasion could be targets for developing recombinant or subunit vaccines to protect against babesiosis (Gohil *et al*. 2013).

After synthesis, the AMA-1 protein is stored in microneme organelles and is later transported to

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the parasite surface immediately prior to or during host-cell invasion (Healer *et al*. 2002). AMA-1 has been characterized in several *Babesia* species, including *B. bovis* (Gaffar *et al*. 2004), *B. bigemina* (Torina *et al*. 2010), *B. divergens* (Montero *et al*. 2009; Tonkin *et al*. 2013), *B. orientalis* (He *et al*. 2015) and *B. gibsoni* (Zhou *et al*. 2006). AMA-1, which is essential for host cell invasion, is a structurally conserved type I integral membrane protein with the following three characteristic structures: (i) an N-terminal, cysteine-rich ectodomain, (ii) a single transmembrane domain and (iii) a C-terminal cytoplasmic tail (Gaffar *et al*. 2004).

In the present study, the complete sequence of AMA-1 of *Babesia* sp. BQ1 (Lintan) was characterized and compared with homologous sequences of other apicomplexan parasites. In addition, recombinant expression of a conserved central region of BLTAMA-1, including domains I and II, were expressed *in vitro* and the immunoreactivity of these proteins were tested.

MATERIALS AND METHODS

Parasites

Babesia sp. (BQ1) Lintan was initially isolated from a sheep infested with adult *H. qinghaiensis* ticks from Lintan, Gansu Province in China (Guan *et al*. 2002).

gDNA and total RNA extraction

Genomic DNA was extracted from 300 μL of *Babesia* sp. BQ1 (Lintan) infected blood samples (parasitaemia about 5%) using a QIAamp DNA Blood Mini Kit (Gentra, USA), according to the manufacturer's instructions. The DNA samples were stored at −20 °C until further use.

Total RNA was extracted by lysing *Babesia* sp. BQ1 (Lintan)-infected RBCs using a standard TRIzol reagent protocol (Life Technologies), followed by chloroform extraction, precipitation with isopropyl alcohol and ethanol and DNase I treatment (Amplification Grade; Life Technologies, Invitrogen, Carlsbad, CA, USA).

Analysis of the complete mRNA transcript (cDNA clone) encoding AMA-1

The amino acid sequences of AMA-1 from *B. bovis, B. bigemina, B. divergens, B. orientalis* and *B. gibsoni* (GenBank accession numbers: ACM44018, ADP02976, ACC96234, AHW45797 and ACY07255, respectively) were aligned. A set of degenerate primers (BLTF: 5′-ACM AAR TAY AGG TAY CCH and BLTR: GWA RTA VGT DCC ACA RCT-3′) corresponding to amino acid sequences TKYRYP and SCGTYY was used to amplify partial *Babesia* sp. (BQ1) Lintan *ama-1* sequences.

Several forward and reverse gene-specific primers were designed based on the partial sequences obtained and synthesized to amplify the full-length cDNA using 5′ and 3′ RACE. The primers (BLTama-1fullF: 5′-ATG CAG TGC ATA GTG AGA AAG T-3′ and BLTama-1fullR: 5′-TCA ATT GAT CTT GGT TAG GTG G-3′) were designed from obtained partial 5′ and 3′ putative sequences to amplify the entire open reading frame (ORF) of the *Babesia* sp. BQ1 (Lintan) *ama-1* gene. The gene fragment encoding the BLTAMA-1 extracellular region of BLTAMA-1-DI/DII corresponding to amino acids G^{116} to E^{428} was PCR amplified using specific primers (BLTrAMA1-F: 5′-GCC *TCT AGA* GGT GGT AAG CAT TAC CGC ATG-3′ and BLTrAMA1-R: 5′-GCC *GGA TCC* TTC CAG CGG GGA TCC AAG A-3′) modified to include *Xba* I and *BamH* I sites (underlined).

Amplification of an 807 bp fragment of the *ama-1* gene was achieved using degenerate primers followed by cloning into the pGEM-T easy vector (Promega, USA) and sequencing. Orthologous sequences from *B. ovata* (GenBank accession number KT312795), *B. bigemina* (GenBank accession number HM54372), *B. bovis* (GenBank accession number FJ588027), *B. orientalis* (GenBank accession number KJ196379), *B. divergens* (GenBank accession number EU486539) and *B. gibsoni* (GenBank accession number FJ800574) were identified by BLAST analysis.

Rapid amplification of 5′- and 3′-RACE-Ready cDNA from total parasite RNA was obtained using SMARTer® RACE 5′/3′ Kit (Clontech Laboratories, USA), according to the manufacturer's instructions. The PCR products were purified and cloned into the pGEM-T easy vector (Promega, USA), followed by sequencing. The ORF was determined using ORF Finder (www.ncbi.nlm.nih.gov/gorf). The full-length gene was further amplified and identified with a pair of specific primers, using cDNA and gDNA as templates.

Bioinformatic analysis

The sequences obtained in the present study were identified using BLASTn and PSI-BLAST [non-redundant (NR) protein database] programs. A multiple sequence alignment was performed using Clustal W 2·0·12: Multiple alignment. The per cent identity value among Chinese isolates was calculated after Clustal W alignment using DNAStar (Version 4.01, Madison, WI, USA) The phylogenetic analysis was conducted with MEGA 7·0·18 software (Kumar *et al*. 2016). The presence of potential transmembrane helices and signal peptides in the BLTAMA1 protein were predicted using the TMHMM Server v. 2·0 and the SignalIP 4·1 server (http://www.expasy.org/resources).

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Recombinant expression, purification of BLTrAMA-1-DI/DII and production of anti- BLTrAMA-1-DI/DII polyclonal antibodies

The extracellular region of BLT-AMA1 containing DI/II, was amplified and cloned into the pUC57 vector. Plasmid with the verified sequence was selected and digested using *Xba* I and *BamH* I restriction enzymes and cloned into the pET-30a expression vector (Genscript) according to the manufacturer's instructions. The recombinant plasmid pET-30a-AMA1-DI/DII, containing 939 bp fragment, was confirmed by sequencing.

Recombinant protein pET-30a-AMA-1-DI/DII was expressed in BL21 (DE3) cells in the presence of kanamycin (50 μg mL^{-1}) by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 37 °C for 4 h or 15 °C overnight. The bacterial culture was evaluated by SDS–PAGE and Western-blot analyses. One litre of bacterial culture was further induced with 0·5 mM IPTG using stored strain at 15 °C overnight. The bacterial cultures were harvested by centrifugation (8000 rpm, 10 min) and lysed by ultrasonication in binding buffer (50 mM Tris–HCl, 150 mM NaCl, 8 M urea; pH 8·0) containing PMSF (phenylmethylsulfonyl fluoride). The recombinant protein was purified as inclusion bodies; the pellet was re-suspended in solubilization buffer [20 mM Tris–HCl (pH 8·0), 50 mM NaCl and 1 mM DTT (dithiothreitol), 8 M urea] containing increasing concentrations of imidazole (20, 50 and 500 mM). The samples were centrifuged and collected. Recombinant protein expression was subsequently analysed using SDS–PAGE and Western-blot analyses. The purity of the recombinant protein was more than 85% according to SDS–PAGE gel scanning analysis.

Three New Zealand rabbits (2·3 kg each) were subcutaneously injected with 500 μg purified BLTrAMA-1/DI/DII protein with Freund's complete adjuvant (FCA, Saint Louis, Missouri, USA, Sigma). Booster injections containing the same amount of protein in Freund's incomplete adjuvant (FIA, Saint Louis, Missouri, USA, Sigma) were administered on days 15, 20 and 28. Sera were collected from the immunized rabbits at 15 days after the last injection, purified (Protein A-affinity Purified, Genscript, China) and stored at −20 °C until further use. For the negative control, sera were collected from each rabbit prior to the first injections.

Immunoblotting analysis

Two micrograms per lane of BLTrAMA-1-DI/DII or 5 μg per lane of native soluble antigens from *Babesia* sp. BQ1 (Lintan) merozoites (BQMA, Guan *et al*. 2010*b*) were separated by SDS–PAGE (12%) gel and subsequently transferred to nitrocellulose membranes (BioRad) at 24 V and 50 W for 35 min. The membranes were then cut into 3-mm strips and blocked in 5% (w/v) skimmed milk in Tris-buffered saline (pH 7·6) with 0·1% Tween-20 (TBST) overnight at 4 °C on a shaker. The strips were probed with serum (diluted at 1:100 in TBST), obtained as described by Guan *et al*. (2010*b*), from sheep (No. 3216, 3 weeks post-infection) experimentally infected with *Babesia* sp. BQ1 (Lintan) (Guan *et al*. 2010*b*), with serum from a rabbit immunized with the BLTrAMA-1 protein, or serum from a *Babesia*-free sheep or rabbit prior to immunization (negative control) for 1 h. After washing three times with TBST, the strips were incubated with secondary antibodies (monoclonal anti-goat/sheep IgG-alkaline phosphatase conjugate, Sigma, A8062, dilution: 1:5000 or polyclonal anti-rabbit IgG–alkaline phosphatase conjugate, Sigma, A9919, dilution: 1:5000) for 2 h. Positive blots were developed using the 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium liquid substrate system (B1911-100ML, Sigma).

RESULTS

Cloning and sequencing of the ama-1 gene of Babesia sp. BQ1 (Lintan)

The full-length *ama-1* gene from *Babesia* sp. BQ1 (Lintan) cDNA was amplified using 5′ and 3′ RACE. The sequence obtained is 2066 bp and contains a predicted ORF of 1785 bp, a 208 bp 5′ UTR and a 72 bp 3′ UTR following a 30 bp poly (A) tail. The full-length *ama-1* gene was also amplified from *Babesia* sp. BQ1 (Lintan) gDNA. Comparison of the sequence with the cDNA indicated no introns in the *ama-1* gene. The sequence has been deposited in GenBank under accession number KX570629.

AMA-1 amino acid sequences from different parasite species were compared, as shown in Table 1. The results indicated that the AMA-1 protein of *Babesia* sp. BQ1 (Lintan) has significant identity with AMA-1 of other *Babesia* parasites, i.e. a maximum per cent identity of 84·4% with *B. ovata* and 77·9% identity with *B. bigemina*, 54·1% with *B. divergens*, 53·5% with *B. orientalis*, 52·1% with *B. bovis* and 50·3% with *B. gibsoni*. In addition, *Babesia* sp. BQ1 (Lintan) AMA-1 shows 21·3–42·1% identity with AMA-1 proteins of *Theileria* species and other apicomplexan parasites.

Characterization and sequence comparison of the AMA-1 protein

Sequence analysis using the SignalP 4·1 program predicts a 23-aa signal sequence (MQCIVR KLSLLAMPVVIAGMLSAE) in the BLTAMA-1 protein. The program TMHMM version 1.0 predicts an extracellular region (ectodomain) from M^{1} to K^{511}, a single hydrophobic C-terminal transmembrane helix from A^{512} to N^{531}, and a small cytoplasmic tail from R^{532} to N^{594} (Fig. 1).

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Table 1. Per cent identity of AMA-1 amino acid sequences between *Babesia* sp. BQ1 (Lintan) and other 16 apicomplexan parasites deduced after CLUSTAL W alignment

Per cent identity																				
Divergence		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**		
	1		51·2	49·7	48·9	29·7	70·7	61·1	52·1	25·9	22·0	25·7	25·1	26·9	25·7	36·4	41·8	24·5	1	*B. bovis* ACM44018
	2	76·8		54·5	51·4	28·4	52·7	91·4	77·9	26·0	20·5	26·7	27·5	28·2	27·4	35·9	41·6	24·1	2	*B. bigemina* ADP02976
	3	80·7	68·7		55·6	28·4	52·7	60·7	54·1	25·8	21·3	24·7	25·6	27·2	25·8	35·8	41·2	22·8	3	*B. divergens* ACC96234
	4	82·9	76·3	66·1		31·2	51·6	58·4	50·3	27·7	21·6	26·8	26·1	30·3	28·1	35·9	43·2	25·9	4	*B. gibsoni* ACY07255
	5	161·8	170·3	170·5	152·5		29·7	37·7	28·8	25·7	25·0	24·7	25·5	26·5	26·9	28·0	32·9	21·9	5	*B. microti* AGJ72906
	6	37·2	72·9	73·0	75·6	161·6		60·5	53·5	25·9	21·8	26·7	26·0	27·3	27·3	35·5	42·0	24·1	6	*B. orientalis* AHW45797
	7	54·4	9·2	55·2	59·9	120·7	55·5		84·4	35·9	25·9	28·4	31·2	32·2	31·0	47·5	48·3	29·9	7	*B. ovata* AKR16110
	8	74·4	26·2	69·7	79·2	167·6	71·1	17·5		28·3	21·3	26·9	27·7	29·1	27·6	37·0	42·1	25·5	8	*Babesia* sp. BQ1 Lintan
	9	190·5	189·5	191·3	175·5	192·1	190·5	128·5	171·0		27·3	24·3	24·6	26·9	25·6	24·9	28·5	42·7	9	*E. maxima* CBL80633
	10	223·0	239·0	231·0	227·0	195·0	226·0	190·4	230·0	179·0		24·2	25·0	25·4	26·5	21·6	26·4	26·7	10	*N. caninum* XP_003886059
	11	191·9	183·8	198·0	183·1	198·0	183·9	170·4	181·9	200·0	202·0		47·8	53·8	53·4	26·4	27·2	21·8	11	*P. berghei* AAC47192
	12	197·2	177·4	192·8	188·7	193·9	189·2	152·6	175·7	199·0	195·0	85·8		57·8	58·0	25·3	28·8	22·0	12	*P. falciparum* XP_001348015
	13	181·8	171·7	179·5	158·3	185·1	178·9	146·8	166·1	181·6	194·7	70·3	61·1		85·4	24·8	30·5	22·2	13	*P. knowlesi* XP_002259339
	14	192·4	178·3	191·6	173·1	181·6	178·5	153·8	176·5	193·2	185·5	71·1	60·7	16·3		26·0	29·9	22·1	14	*P. vivax* AAC16731
	15	126·2	128·3	128·7	128·3	173·6	130·2	86·8	123·5	195·0	228·0	186·0	195·6	196·0	189·6		48·1	24·6	15	*Th. annulata* CAI73460
	16	104·9	105·6	106·9	99·9	142·9	104·2	84·5	103·9	169·7	186·1	179·4	168·1	157·0	160·5	85·1		25·4	16	*Th. equi* XP_004833099
	17	200·0	203·0	215·0	190·0	223·0	203·0	160·8	193·9	101·8	183·6	224·0	222·0	220·0	222·0	198·0	194·9		17	*To. gondii* AAB65410
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17		

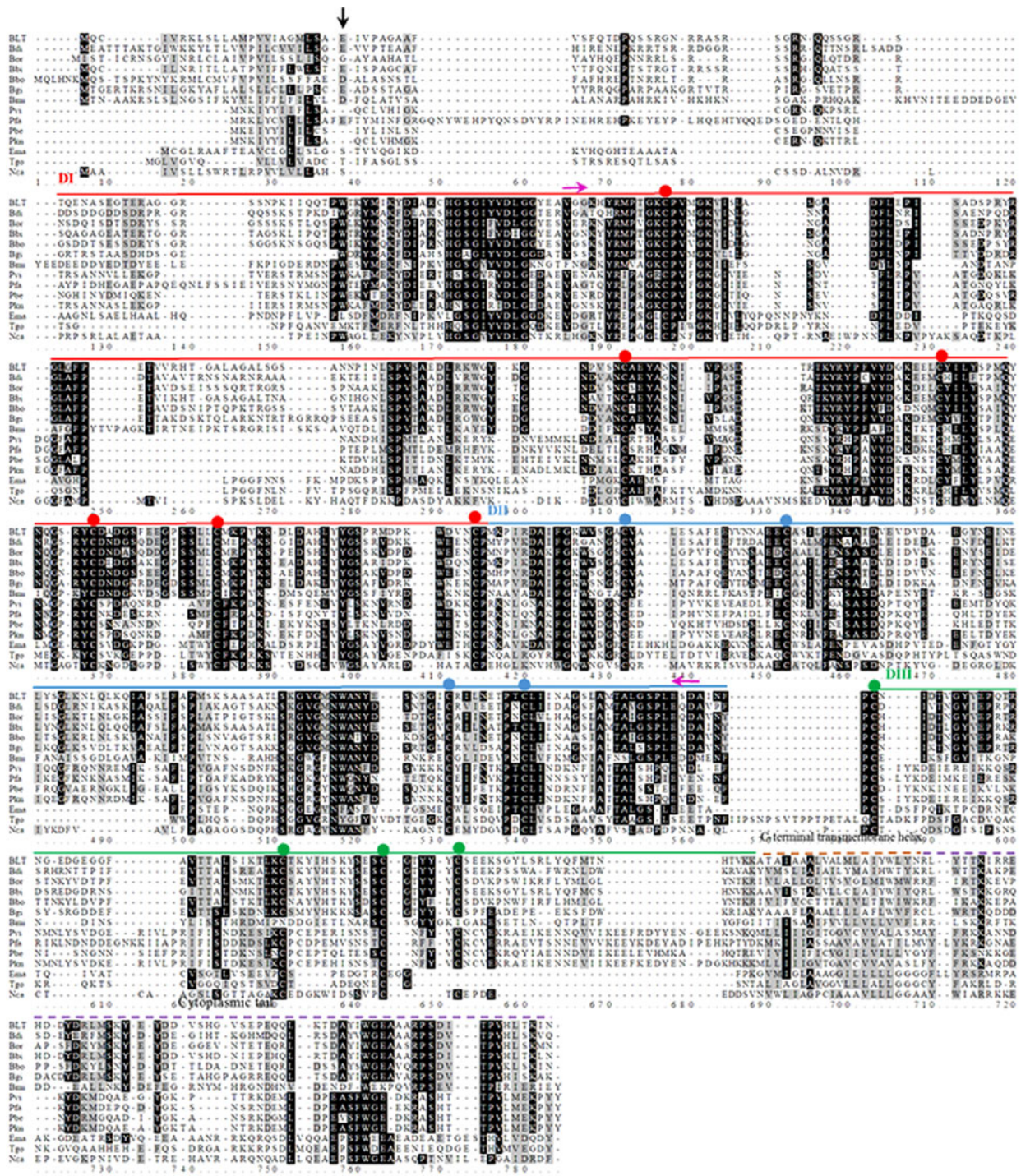

Fig. 1. Multiple alignment of AMA-1 amino acid sequences from *Babesia* sp. BQ1 (Lintan) (BLT), *B. divergence* (Bdi, GenBank accession number: ACC96234), *B. orientalis* (Bor, GenBank accession number: AHW45797), *B. bigemina* (Bbi, GenBank accession number: ADP02976), *B. bovis* (Bbo, GenBank accession number: ACM44018), *B. gibsoni* (Bgi, GenBank accession number: ACY07255), *B. microti* (Bmi, GenBank accession number: AGJ72906) *Plasmodium vivax* (Pvi, GenBank accession number: AAC16731), *P. falciparum* (Pfa, GenBank accession number: XP001348015), *P. berghei* (Pbe, GenBank accession number: AAC47192), *P. knowlesi* (Pkn, GenBank accession number: XP002259339), *Eimeria maxima* (Ema, GenBank accession number: CBL80633), *Toxoplasma gondii* (Tgo, GenBank accession number: AAB65410) and *Neospora caninum* (Nca, GenBank accession number: XP_003886059). The signal peptide cleavage site is indicated by a black arrow. Domains I (red), II (blue) and III (green) by the formation of disulphide bridges between conserved cysteine residues (indicated by solid circular) were marked. The transmembrane helices are marked by orange dotted line and a cytoplasmic tail is indicated by purple dotted line. The DI/DII region for recombinant protein expression was marked by pink arrows.

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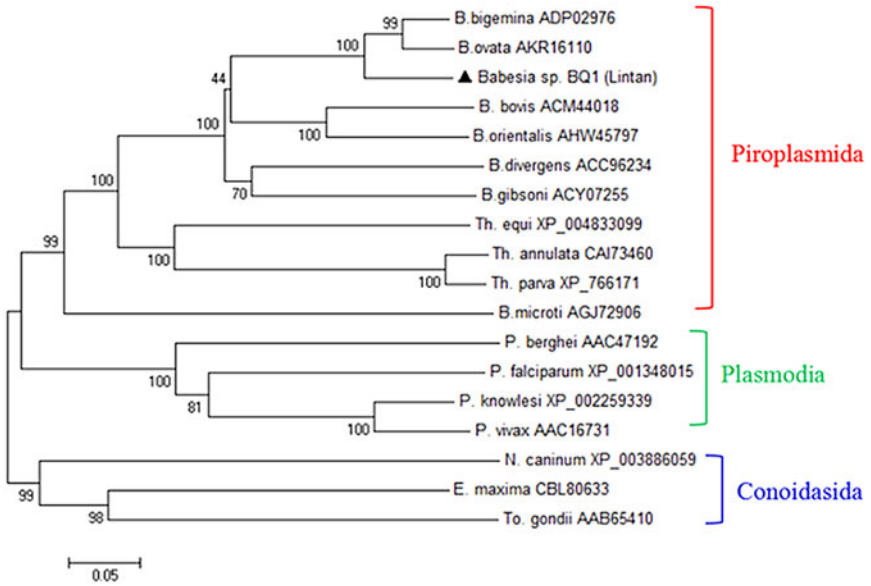

Fig. 2. Phylogenetic tree of the AMA-1 amino acid sequences of *Babesia* sp. BQ1 (Lintan) and other apicomplexan parasites. The accession numbers were showed after parasite species name and the species of Plasmodia and Conoidasida AMA-1 amino acid sequences were used as outgroups. The BLTAMA-1 sequence obtained in this study was indicated with bold triangle. The analysis involved 18 amino acid sequences. The tree was inferred using the Neighbour-Joining method of MEGA7·0·18; bootstrap values are shown at each branch point. Numbers above the branch demonstrate bootstrap support from 1000 replications. All sites of the alignment containing insertions–deletions, missing data were eliminated from the analysis (option 'complete deletion'). The optimal tree with the sum of branch length = 3·64528684. The evolutionary distances were computed using the *p*-distance method and are in the units of the number of amino acid differences per site. There were a total of 274 positions in the final dataset.

Clustal W alignment of multiple AMA-1 sequences, including *Babesia* sp. BQ1 (Lintan) and other *Babesia* species (*B. bigemina, B. bovis, B. divergens, B. orientalis, B. gibsoni* and *B. microti*), some plasmodia (*Plasmodium* species: *P. falciparum, P. vivax, P. berghei* and *P. knowlesi*), and some coccidia (*Toxoplasma gondii, Eimeria maxima* and *Neospora caninum*) revealed the characteristic features of AMA-1: three conserved extracellular domains (DI–DIII), containing 14 conserved cysteines, including six in DI, four in DII and four in DIII (Fig. 1).

Phylogenetic analysis

A phylogenetic tree, based on babesial AMA-1 sequences and homologues from other related apicomplexans deposited in GenBank, including 10 species of piroplasmida, four species of Plasmodia and three species of Conoidasida, was constructed using the Neighbour-Joining method (Fig. 2). The results showed that these apicomplexan parasites could be divided into two major clades, with high bootstrap values. One branch corresponds to two vertebrate blood-infecting clades (Piroplasmida and Plasmodia), and the other corresponds to Conoidasida species. Within Piroplasmida, *Babesia* sp. BQ1 (Lintan), with *B. bigemina* and *B. ovata* infecting for ruminants, form one clade; *B. bovis* and *B. orientalis*, which infect Bovidae animals, form another clade, with a highly significant bootstrap value. In contrast, *B. divergens* and *B. gibsoni*, which infect cattle and dogs, respectively, form another clade, with a lower bootstrap value that is over 50%. *Babesia microti* was found outside other *Babesia* species in a single cluster of Piroplasmida, with a high bootstrap value. All Piroplasmida sequences clustered, with a highly significant bootstrap value, into a clade different from Plasmodia and Conoidasida sequences, containing all analysed *Babesia* and *Theileria* species. These results indicate the validity of tree construction based on the sequence of AMA-1.

Cloning, expression and purification of the DI/DII region of BLTrAMA-1

The gene encoding the extracellular region of BLTAMA-1 was successfully cloned into the pET-30a expression vector and expressed in *Eschrichia coli* BL21 (DE3). The recombinant plasmid pET-30a-BLTAMA-1-DI/DII was identified by restriction analysis and subsequently confirmed by sequencing using specific primers. The expressed recombinant proteins were analysed by SDS–PAGE (Fig. 3A) and Western-blot (Fig. 3B) using anti-His6 serum (THETM His Tag Antibody, mAb, Mouse, GenScript, A00186). The predicted mass of the recombinant protein (His-BLTAMA-1-DI/DII) is ~35 kDa (Fig. 3).

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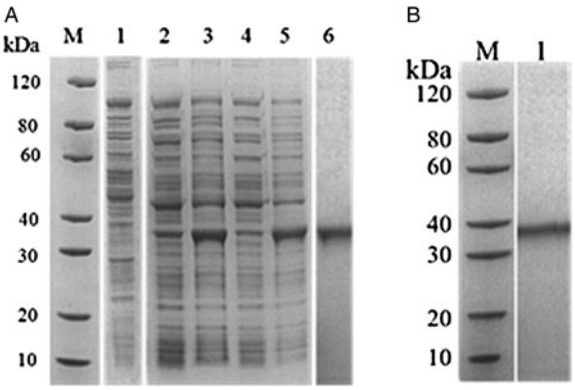

Fig. 3. Expression and purification of recombinant BLT-AMA-1-DI/DII protein. A: SDS–PAGE (Coomassie stained) analysis of recombinant protein produced in *E. coli*. M: molecular weight marker (Genscript, M00516). Lane 1, recombinant protein before induction; lane 2: supernatant of cell lysate of recombinant protein induced by 1 mM IPTG for 16 h at 15 °C; lane 3: cell lysate pellet of recombinant protein induced by 1 mM IPTG for 16 h at 15 °C after ultrasonication; lane 4: supernatant of cell lysate of recombinant protein induced by 1 mM IPTG for 4 h at 37 °C; lane 5: cell lysate pellet of recombinant protein induced by 1 mM IPTG for 4 h at 37 °C after ultrasonication; lane 6: purified recombinant BLT-AMA-1-DI/DII. B: Western-blot analysis. M: molecular weight marker. Lanes 1: Purified recombinant BLT-rAMA-1-DI/DII protein was detected by using monoclonal anti-His6 Tag antibody in mouse.

Antigenic characterization of BLTrAMA-1 and native BLTAMA-1 by immunoblot analysis

Sera from sheep (No. 3216) infected with *Babesia* sp. BQ1 (Lintan) and the sera from rabbits immunized with BLTrAMA-1-DI/DII were used to identify BLTrAMA-1 (~35 kDa) by Western-blot analysis, whereas the sera obtained from sheep or rabbits prior to infection did not react with BLTrAMA-1 (Fig. 4A). No reaction of the pET-30a vector control with positive control sera from sheep or rabbits was observed (Fig. 4B).

To identify native AMA-1 in *Babesia* sp. BQ1 (Lintan) merozoites, antibodies against recombinant BLTAMA-1 obtained from rabbits and sera obtained from sheep (No. 3216) infected with *Babesia* sp. BQ1 (Lintan) were examined for reactivity with native BQMA. By Western-blot, the native AMA-1 protein was detected at a size of approximately 65 kDa in the lysate of *Babesia* sp. BQ1 (Lintan)-infected sheep RBC s (Fig. 4C) but not in the lysate from uninfected sheep RBCs (data not shown). These results indicate that AMA-1 could induce antibody production in sheep infected by *Babesia* sp. BQ1 (Lintan).

DISCUSSION

In the present study, we report the cloning, recombinant expression, genetic and biological characterization of the *ama-1* gene from *Babesia* sp. BQ1 (Lintan). The results confirmed that AMA-1 in the sheep parasite *Babesia* sp. BQ1 (Lintan) resembled AMA-1 orthologs of other apicomplexan parasites sharing a similar domain organization. AMA-1 possesses an N-terminal signal sequence followed by an ectodomain region, a single transmembrane region, and a short cytoplasmic domain.

The BLTAMA-1 structure of the ectodomain region is divided into three domains (DI, DII and DIII) (Fig. 1). In a previous study, domains I and II, containing 10 conserved cysteine residues (six in D1 and four in DII) in all of apicomplexan AMA-1 proteins, were proposed to have an important function in adhesion to proteins or glycoprotein receptors (Pizarro *et al*. 2005). Domain III was relatively variable and located adjacent to the parasite surface, suggesting that this region might be under fewer selective constraints or that this variability is associated with immune evasion (Moitra *et al*. 2015). In addition, within highly variable domain III of the extracellular region, four conserved cysteines are observed in most *Babesia* spp., including *Babesia* sp. BQ1 (Lintan). Exception are that of *B. microti*, which contains only two cysteines and *Plasmodium* AMA-1, which contains six cysteines (Hodder *et al*. 1996). In general, the analysis of the protein structure of *Babesia* sp. BQ1 (Lintan) AMA-1 is consistent with the common features of apicomplexan parasites AMA-1, as described above.

The constructed phylogenetic tree based on all apicomplexan parasite AMA-1 amino acid sequences indicates that *Babesia* sp. BQ1 (Lintan) AMA-1 is closely related to that of *B. ovata* and *B. bigemina*. The per cent identity between *Babesia* sp. BQ1 (Lintan) and *B. ovata* (84·4%) and *B. bigemina* (77·9%) also reflects the evolutionary relationships of these parasites. Interestingly, these findings are consistent with previous phylogenetic analyses based on 18S rRNA, whereby *B. bigemina* and *B. motasi* (Lack *et al*. 2012) as well as *B. ovata, B. crassa* and *B. major* were grouped together (Schnittger *et al*. 2012), and on RAP-1, whereby *B. bigemina* and *Babesia* cf. *motasi* [*Babesia* sp. BQ1 (Lintan)] were grouped in the same clade (Niu *et al*. 2013). In general, the AMA-1 proteins of Piroplasmida are more closely related to those of Plasmodia than to those of Conoidasida. In addition, the phylogenetic analysis indicated that *B. microti* AMA-1 forms a separate branch from all other piroplasm species. This finding is consistent with phylogenetic analyses based on AMA-1 (Moitra *et al*. 2015) and the *eta* (η) subunit of the chaperonin-containing *t*-complex polypeptide 1 (CCT η) gene (Nakajima *et al*. 2009), suggesting that *B. microti* is distinct from all other *Babesia* species and represents a sister group to *Theileria* and *Babesia*.

In the present study, a recombinant protein based on the BLTAMA-1 conserved region containing

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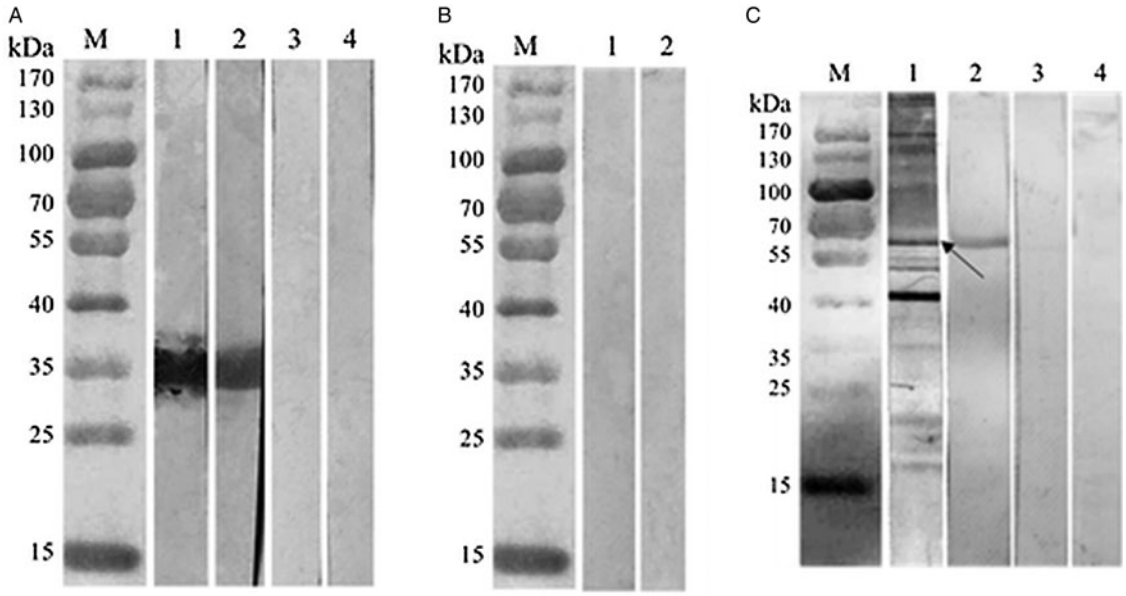

Fig. 4. Western-blot analysis for recombinant pET-30a-AMA1-DI/DII (A), control strain harboring pET-30a (B) and native BQMA (C) recognized by sheep serum against *Babesia* sp. BQ1 (Lintna) or rabbit serum anti-BLTrAMA-1-DI/DII. M: molecular weight marker; Lane 1: Positive serum from infected sheep (No. 3216) for *Babesia* sp. BQ1 (Lintan); Lane 2: Positive serum from immunized rabbits anti-BLTrAMA-1-DI/DII; Lane 3: Serum from pre-immunized sheep (No. 3216); Lane 4: Serum from before immunized rabbit. Native BLTAMA-1 in lane 1 was indicated by arrow.

domains I and II, was constructed and expressed. Sera produced from rabbits immunized with the BLTrAMA-1-DI/DII protein specifically recognized parasite-produced native AMA-1 protein, with the expected size of BLTAMA-1; this result indicates that *Babesia* sp. BQ1 (Lintan) merozoites expressed the AMA-1 protein. Sera (3 weeks post-infection) from *Babesia* sp. BQ1 (Lintan) experimentally infected sheep strongly reacted with BLTrAMA-1-DI/DII, suggesting that the antibody responses of AMA-1 are elicited early during sheep *Babesia* sp. BQ1 (Lintan) infection. However, kinetic studies of humoral responses against *B. divergens* AMA-1 indicated that antibody production against extracellular DI and DII of BdAMA-1 is weak and late, between more than 3 and 5 months post-infection during experimental infection of sheep by *B. divergens* (Moreau *et al*. 2015). Rabbit sera against the BLTrAMA-1-DI/DII protein did not react with uninfected sheep RBCs and the serum obtained prior to infection of sheep did not react with BLTrAMA-1 in Western-blot analyses. These finding suggest that the AMA-1 protein is present in *Babesia* sp. BQ1 (Lintan) and that BLTrAMA-1 is a potential diagnostic antigen for detecting *Babesia* sp. BQ1 (Lintan) and/or other isolates of *Babesia* cf. *motasi* in China, as AMA-1 is considered highly conserved among different isolates of the same species (Torina *et al*. 2010; Moreau *et al*. 2015).

Several studies of *Plasmodium* AMA-1 as a potential vaccine candidate have reported promising vaccine trials conducted in animal-model systems (Collins *et al*. 1994); in particular, recombinant Plasmodium AMA-1 containing only the ectodomain provided complete protection against challenge (Lal *et al*. 1996). In *B. bovis*, the use of specific antibodies directed against certain synthetic peptides (Gaffar *et al*. 2004) and conserved DI and DII regions of the AMA-1 protein inhibited parasite invasion, potentially serving as a vaccine candidate against *B. bovis* infection (Salama *et al*. 2013). In *B. divergens*, antibodies against DI and DII of AMA-1 have a potent inhibitory effect on merozoite invasion, decreasing invasion by ~50% (Montero *et al*. 2009). Because the structure of AMA-1 is conserved among apicomplexans, the data obtained in this study provide a basis for further investigation of the function of the AMA-1 protein in the *Babesia* cf. *motasi* group and suggest this protein's potential as a vaccine for controlling ovine babesiosis in China and even worldwide.

Further work is necessary to obtain the sequences of other sheep *Babesia* species (*B. motasi, B. ovis* and *Babesia* sp. Xinjiang) and to investigate polymorphism of AMA-1 sequences between isolates of the same species or different sheep-infection *Babesia* species. The kinetics of antibody production against *Babesia* sp. BQ1 (Lintan) during sheep infection and cross-reaction with other haemoparasites should to be further studied.

In conclusion, this study is the first to report the *ama-1* gene sequence in the sheep parasite *Babesia* sp. BQ1 (Lintan) (*Babesia* cf. *motasi* group) and show a sequence comparison of its orthologues from different apicomplexans. We cloned and expressed

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the BLTAMA-1-DI/DII region and detected the presence of antibodies directed against native and recombinant forms of AMA-1. Future studies should examine sequence polymorphisms of *ama-1* in different isolates of the *Babesia* cf. *motasi* group for the purpose of potential vaccine development, the process by which BLTAMA-1 mediates RBC invasion, the potential of BLTAMA-1 for disease diagnosis and development of a common vaccine against different isolates of *Babesia* cf. *motasi*.

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