A member of the HSP90 family from ovine *Babesia* in China: molecular characterization, phylogenetic analysis and antigenicity

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SUMMARY

Heat shock protein 90 (HSP90) is a key component of the molecular chaperone complex essential for activating many signalling proteins involved in the development and progression of pathogenic cellular transformation. A *Hsp90* gene (*BQHsp90*) was cloned and characterized from *Babesia* sp. BQ1 (Lintan), an ovine *Babesia* isolate belonging to *Babesia motasi*-like group, by screening a cDNA expression library and performing rapid amplification of cDNA ends. The full-length cDNA of *BQHsp90* is 2399 bp with an open reading frame of 2154 bp encoding a predicted 83 kDa polypeptide with 717 amino acid residues. It shows significant homology and similar structural characteristics to *Hsp90* of other apicomplex organisms. Phylogenetic analysis, based on the HSP90 amino acid sequences, showed that the *Babesia* genus is clearly separated from other apicomplexa genera. Five Chinese ovine *Babesia* isolates were divided into 2 phylogenetic clusters, namely *Babesia* sp. Xinjiang (previously designated a new species) cluster and *B. motasi*-like cluster which could be further divided into 2 subclusters (*Babesia* sp. BQ1 (Lintan)/*Babesia* sp. Tianzhu and *Babesia* sp. BQ1 (Ningxian)/*Babesia* sp. Hebei). Finally, the antigenicity of rBQHSP90 protein from prokaryotic expression was also evaluated using western blot and enzyme-linked immunosorbent assay (ELISA).

Key words: *Babesia motasi*, *Babesia* sp. BQ1 (Lintan), HSP90, phylogenetic analysis, antigenicity.

INTRODUCTION

Heat shock proteins (HSPs) are some of the phylogenetically conserved and ubiquitously expressed protein families in bacteria, mammals and plants. These proteins play essential roles in stress tolerance and the folding, activation and assemblage of many proteins. According to their homology, function and size, they can be divided into different families, e.g. HSP110, HSP90, HSP70, HSP60, HSP40 and small HSP (Buchanan, 2000; Gullo and Teoh, 2004). The molecular chaperone HSP90 is important in the folding and functioning of many proteins involved in cell survival, especially those participating in cell cycle regulation and signal transduction (Pearl and Prodromou, 2006). Due to its broad functions, it is highly abundant in both stressed and non-stressed cells, and constitutes 2·8% of the total cellular protein (Brandau *et al*. 1995). It has also been implicated as molecular marker for identifying and differentiating parasite species and genotypes, such as *Cryptosporidium* and *Ditylenchus* (Feng *et al*. 2009; Vovlas *et al*. 2015). Furthermore its contributions to the immune response have led to encouraging studies of its use as an antigen or adjuvant in vaccine, especially for cancer and get the promising results (Tosti *et al*. 2009; Reitsma and Combest, 2012; Crane *et al*. 2013).

However, little is known about the *Hsp90* gene of piroplasms. Gerhards *et al*. (1994) showed that an 87 kDa HSP90 protein was expressed by *Theileria parva* during both sporozoite and schizont but not in the piroplasm phases, although the corresponding transcript was detected. Unlike that of other microbial pathogens, *T. parva* and *Theileria annulata*-induced IκB kinase activity does not require functional HSP90 in the schizont stage (Hermann and Dobbelaere, 2006). Ruef *et al*. (2000) based on the phylogenetic analysis of *HSP70* and *Hsp90* genes, showed that *Babesia microti* was paraphyly with *Babesia bovis* and

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Theileria, which supports *B. microti* should be a basal group to *Babesia* and *Theileria* rather than *Theileria*. Khan *et al.* (2014) revealed that there were 2 HSP90 proteins in *Babesia orientalis*, BoHSP90-A and BoHSP90-B. Sera from buffalo infected by *B. orientalis* react with recombinant protein BoHSP90-A and BoHSP90-B. To date, nothing has been reported about HSP90 from ovine *Babesia* species.

Ovine babesiosis is one of the most important tick-borne hemoparasite diseases and is responsible for economic losses for small ruminant production. This disease is mainly due to *B. bovis* and *Babesia motasi*. In China, ovine babesiosis has been reported since 1980s (Chen, 1982; Zhao *et al.* 1986) and is a common disease in the north of China. Several geographic strains have been collected in our institute, such as *Babesia* sp. BQ1 (Lintan), *Babesia* sp. BQ1 (Ningxian), *Babesia* sp. Tianzhu, *Babesia* sp. Madang, *Babesia* sp. Hebei and *Babesia* sp. Xinjiang (Yin *et al.* 1997; Guan *et al.* 2001, 2002; Bai *et al.* 2002). Phylogenetic analysis of these strains based on the 18S RNA gene or ribosomal DNA internal transcribed spacer (ITS) sequences suggest that they could be separated into 2 clusters, *Babesia* sp. Xinjiang and *B. motasi*-like clusters (Liu *et al.* 2007; Niu *et al.* 2009). In the present study, a full-length *Hsp90* cDNA was cloned and characterized from *Babesia* sp. BQ1 (Lintan), and designed as *BQHsp90*. Five Chinese ovine *Babesia* isolates together with *T. annulata, T. parva, B. bovis* and other apicomplexa parasite species were subjected to phylogenetic analysis based on the HSP90 amino acid sequences. Furthermore, the antigenicity of recombinant BQHSP90 (rBQHSP90) expressed in the prokaryotic system was evaluated by western blot and enzyme-linked immunosorbent assay (ELISA) to investigate the potentiality as diagnostic antigen.

MATERIALS AND METHODS

Parasites and sera

A clonal line (G7) of *Babesia* sp. BQ1 (Lintan) was grown *in vitro* in sheep erythrocytes as described by Guan *et al.* (2010*b*) and infected sheep blood was cryopreserved in liquid nitrogen in the Vectors and Vector-borne Diseases (VVBD) Laboratory, LVRI, China.

Positive sera of *Babesia* sp. BQ1 (Lintan) (16 sera from 2 sheep), *Babesia* sp. BQ1 (Ningxian) (11 sera, 1 sheep), *Babesia* sp. Tianzhu (10 sera, 1 sheep), *Babesia* sp. Hebei (9 sera, 1 sheep), *Babesia* sp. Xinjiang (2 sera, 2 sheep), *Theileria luwenshuni* (3 sera, 1 sheep) and *Theileria uilenbergi* (1 serum, 1 sheep) collected from infected sheep, were provided by VVBD. Sera from the 9 sheep of pre-infection were considered as the negative sera (Guan *et al.* 2012*b*). And sera collected from 3 sheep (numbers 2007, 3216 and 3533) (Guan *et al.* 2010*a*) during 84 days post-infection (dpi) with *Babesia* sp. BQ1 (Lintan) were used to evaluate antibody kinetics. All sera were pre-absorbed against lysates of *Escherichia coli* BL21 as previously described by Guan *et al.* (2012*a*).

Construction and immunoscreening of cDNA expression library

Purification of merozoites, construction and immunoscreening of *Babesia* sp. BQ1 (Lintan) cDNA expression library refer to the previous description (Guan *et al.* 2012*a*). Briefly, merozoites were purified from the *in vitro* culture when parasitemia reached 8–10%. The purified mRNA was used to construct cDNA library (NOVAGEN, USA). The *Babesia* sp. BQ1 (Lintan) merozoite cDNA expression library was immunoscreened using immune sera collected from sheep infected with *Babesia* sp. BQ1 (Lintan) in 6th week post-infection. Ninety-three positive plaques were revealed by primary screening of the library on plates. Phage plugs were removed from the plates according to the sites of positive signal on the membrane and subjected to re-screening until all the signals on the membranes were positive. Pure phage stock was converted to plasmid by using the *in vivo* auto-subcloning capabilities of the loxP-cre system of λ screen vector in host strain BM25·8. Recombinant plasmid isolated from BM25·8 was transformed into the host strain JM109 and sent to TaKaRa Company (China) for sequencing. Sequence analysis was done using the Lasergene software package for Windows (DNASTAR, Madison, WI) and the National Center for Biotechnology Information (NCBI) database. The nucleotide sequence of an Expressed Sequence Tag (EST) fragment showed high homology with the *Hsp90* gene of *B. bovis* based on the BLASTn in GenBank and was designated as *BQHsp90*.

Amplification of the full-length cDNA of BQHsp90

5′ end of *BQHsp90* was amplified using SMART™ rapid amplification of cDNA ends (RACE) cDNA amplification kit (Clontech Laboratories, USA) from cDNA of *Babesia* sp. BQ1 (Lintan) with Gene specific primer 1 (5′- ACGCTCAGTCCAC CTCCTCCATCTT -3′) designed from 3′ end of *BQHsp90* EST fragment according to the manufacturer's instructions (Guan *et al.* 2012*a*). Amplified polymerase chain reaction (PCR) fragments were routinely cloned into pGEM-T easy vector (Promega, USA) and nucleotide sequences determined by the TakaRa Company (China). The full-length cDNA sequence of *BQHsp90* was assembled using the Lasergene software package for Windows (DNASTAR, Madison, WI) and the open reading frame (ORF) was determined using ORF Finder (www.ncbi.nlm.nih.gov/gorf).

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Table 1. Information about the apicomplexa species included in this study

Species	Strain	Accession number of nucleotide	Accession number of protein
Babesia sp.	BQ1 (Lintan)	GQ397856[a], GQ443608[b]	ACV04849[a], ACV71146[b]
Babesia sp.	BQ1 (Ningxian)	GQ443604	ACV71142
Babesia sp.	Hebei	GQ443605	ACV71143
Babesia sp.	Tianzhu	GQ443606	ACV71144
Babesia sp.	Xinjiang	GQ443607	ACV71145
Babesia bovis	MEX	AF136649	AAF61428
Theileria annulata	Ankara	XM_947380	XP_952473
Theileria parva		M57386	AAA30132
Toxoplasma gondii		AY344115	AAQ24837
Eimeria acervulina		AY459430	AAS18319
Eimeria tenella	PAPt38	AF042329	AAB97088
Cryptosporidium parvum	Iowa II	XM_626924	XP_626924
Cryptosporidium muris	RN66	XM_002142364	XP_002142400
Plasmodium vivax	SaI-1	XM_001613401	XP_001613451
Plasmodium falciparum	7	Z29667	CAA82765

[a] Derived from cDNA.
[b] Derived from gDNA.

Characterization of BQHSP90

Multiple sequence alignment was performed on the deduced BQHSP90 amino acid sequence and those of *B. bovis* (AAF61428), *T. annulata* (XP_952473), *T. parva* (AAA30132) and *Plasmodium falciparum* (CAA82765) using MEGA 4. The putative signal peptide was predicted using SignalP (www.cbi.dtu.dk/services/SignalP). The molecular mass (Mw) and theoretical isolectric point (pI) were calculated on line (www.expasy.org/tools.pi_tool.html). Motif scan and transmembrane topology prediction were done using MyHits (http://myhits.isb-sib.ch/cgi-bin/motif_scan). The structure prediction of BQHSP90 was performed using the I-TASSER online services (http://zhanglab.ccmb.med.umich.edu/services/) and SWISS-MODEL (http://www.expasy.org/swissmod/SWISS-MODEL.html).

The structure of the *BQHsp90* genomic sequence was confirmed by performing PCR analysis with *BQHsp90*-G primers (F10-34: AGTATCTACC CAGCGACATCTTTCT, R2200-2221: TCAGT CCACCTCCTCCATCTTA) designed from the sequence data of the *BQHsp90* cDNA clone with initial denaturation at 94 °C for 1 min, then 94 °C for 30 s; 55 °C for 30 s and 72 °C for 2 min, for 35 cycles, then at 72 °C for 10 min. Sequencing was performed as described above. Locations and exon–intron structures of the *BQHsp90* genomic sequences were predicted using the GENSCAN programme (http://genes.mit.edu/GENSCAN.html) and ClustalW.

Phylogenetic analysis

Hsp90 genomic DNA (gDNA) was amplified with primers UHSP90 (F436–460: CGGTGTCGGT TTCTACTCGGCTTAC, R2200–2224: CGCT CAGTCCACCTCCTCCATCTTA) designed on the conserved region of *Hsp90* from Chinese *Babesia* isolates infective for small ruminants, *Babesia* sp. BQ1 (Lintan), *Babesia* sp. BQ1 (Ningxian), *Babesia* sp. Hebei, *Babesia* sp. Tianzhu and *Babesia* sp. Xinjiang (at 94 °C for 1 min as initial denaturation, then 94 °C for 30 s; 63 °C for 30 s and 72 °C for 2 min, for 35 cycles, then at 72 °C for 10 min). The PCR products were processed as described above for sequencing by the TakaRa Company. The prediction of introns and splicing sequences, and the deduction of amino acid sequences were performed with GENSCAN (http://genes.mit.edu/GENSCAN.html) and the ClustalW programme in MEGA 4 software. Several HSP90 protein sequences of apicomplexa species that are important for humans and domestic animals were identified, i.e. *B. bovis, T. annulata, T. parva, Toxoplasma gondii, Eimeria acervulina, Eimeria tenella, Crytosporidium parvum, Cryptosporidium muris, Plasmodium vivax* and *P. falciparum* and downloaded from the Blast server (www.ncbi.nlm.nih.gov/Blast) (Table 1). Multiple sequence alignment and evaluation of the phylogenetic relationships were performed using the ClustalW programme in MEGA 4 and DNAStar.

Prokaryotic expression of the BQHsp90 gene

The *BQHsp90* containing the entire ORF was amplified from cDNA of *Babesia* sp. BQ1 (Lintan) using *BQHsp90* primers and the PCR product was ligated into pGEM-T-easy (pGEM-*BQHsp90*) for sequencing. The Champion™ pET Directional TOPO® Expression Kit (Invitrogen, USA) was used for the expression of BQHSP90. The ORF of *BQHsp90* was amplified from recombinant plasmid pGEM-*BQHsp90* with a pair of expression primers *BQHsp90*-TOPO: F68–88: CACCATGGCGACG GAGAGTCAGGAG, R2197–2221: TCAGTCCA CCTCCTCCATCTTAGGG) as follows: initial denaturation at 94 °C for 1 min, then 94 °C for 30 s; 65 °C for 30 s and 72 °C for 2 min, for 30 cycles,

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then at 72 °C for 10 min. The PCR product was gel-purified with Agarose Gel DNA Extraction Kit (TakaRa, China). The pET200/D-TOPO® cloning reaction was set up and recombinant plasmid was constructed into One Shot® TOP10 Chemically Competent *E. coli* for characterization following the user manual. After sequencing, the recombinant plasmid extracted from One Shot® TOP10 was transformed into BL21 Star™ (DE3) One Shot® Chemically Competent *E. coli* for expression. To induce recombinant BQHSP90 (rBQHSP90) expression, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, and expression was induced for 4 h at 37 °C. The rBQHSP90 was purified from supernatants of the lysates by Ni affinity chromatography according to the manufacturer's protocol (Invitrogen, USA).

Antigenicity and specificity analysis of rBQHSP90 by western blot and ELISA

The details could refer to previous description (Guan *et al.* 2012*a*). Briefly, for western blot, the purified rBQHSP90 proteins were electrophoresed and transferred to nitrocellulose (NC) membranes. The NC sheets were blocked with 10% skimmed milk powder in 0·1 M Tris-buffered saline (pH 7·6) and 0·1% Tween (TBST) overnight at 4 °C, and then incubated for 1 h with sera diluted at 1/20 in TBST. The sheets were incubated with monoclonal anti-goat/sheep IgG-alkaline phosphatase conjugates (Sigma) diluted at 1/1000 in TBST for 1 h. Positive signals were revealed using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) liquid substrate system (Sigma). For ELISA, microplates (Nunc) were coated with 2 μg mL^{-1} of rBQHSP90 in 0·1 M pH 9·6 carbonate buffer at 37 °C for 1 h and then at 4 °C overnight. The plates were blocked with 150 μL of 2% gelatin in carbonate buffer at 37 °C for 30 min. After drying the plate, blank (phoshphate-buffered saline containing 0.1% Tween 20, PBST) and sera (dilution of 1:20) were distributed in duplicate and the plates were incubated at 37 °C for 1 h. Peroxidase conjugate of monoclonal anti-goat/sheep IgG clone GT-34 (Sigma) diluted at 1:1000 was added to each well and the plates were again incubated at 37 °C for 1 h. 50 μL 3, 3′, 5, 5′ - Tetramethylbenzidine (TMB) (Sigma) were added to each well and incubated at room temperature for 15 min. The reaction was stopped by adding 50 μL of 0·1 M H_2SO_4 and the plates were then read at 450 nm with an ELISA automat (Bio-RAD, USA).

RESULTS

Construction and immunoscreening of cDNA expression library

Analysis of the cDNA expression library of *Babesia* sp. BQ1 (Lintan) merozoites revealed sequences more than 200 bp in length involved in 10 EST with high homology to *B. bovis* genes. Except for 2 hypothetical proteins, these included gliding-associated protein 45 (GAP45), p200, Rab1b, histone H2A protein, cyclophilin, RNA recognition motif containing protein, membrane protein and HSP90 by sequence alignment. The clone 45, a 699 bp length containing a 94 bp poly (A) tail but no entire ORF was found. It showed 81% identity with the *Hsp90* gene of *B. bovis* (accession numbers: XM_001611504 and AF136649) when aligned by BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and was designated *BQHsp90*.

Amplification and characteristics of BQHsp90 full-length

A complete cDNA sequence of *BQHsp90* was obtained based on the 5′ RACE amplification. *BQHsp90* is a 2392 bp cDNA containing a 2154 bp ORF which encodes a predicted 717 amino acid residues protein with a theoretical pI of 5·0 and mass of 83 kDa. The sequence was deposited in GenBank under the accession number GQ397856. Multiple sequence alignment of the deduced BQHSP90 amino acid sequence with the HSP90 of *B. bovis, T. annulata, T. parva* and *P. falciparum* revealed identities of 92·0, 81·9, 81·7 and 70·6%, respectively.

No signal sequence was identified in the transcript using the SignalP software. MyHits analysis revealed that BQHSP90 contains a HSP90 family signature at position 27–36 and a tetratricopeptide repeat (TPR) binding site at the COOH-terminus. In addition, a nuclear localization signal (NLS) was also detected from 356 to 359 residues in BQHSP90. SMART and MyHits analysis revealed a conserved adenosine triphosphate (ATP) binding domain between positions 29 and 183, including a conserved GxxGxG motif from 121 to 126 residues (essential for ATP binding) and HATPase_c domain from 31 to 144 residues (essential for ATPase activity) (Fig. 1). And simple three-dimensional (3D) structure analysis using the crystal structure of yeast HSP90 (Protein Data Bank (PDB) code: 2cg9) as a template showed that BQHSP90 comprised 3 domains including N-terminal, middle domain and C-terminal domain, and the binding sites for ATP located in N-terminal. All these domains/subdomains, essential for client protein binding and ATP hydrolysis confirmed that BQHSP90 should belong to HSP90 family.

gDNA and cDNA of *BQHsp90* were amplified with *BQHsp90*-G primers and sequenced. Exon–intron structure analysis with GENSCAN revealed that the *BQHsp90* gDNA consisted of 2 introns and 3 exons, and the introns were 403 and 142 bp in length and located at sites 1423/1424 and 1924/1925, respectively. The predicted splice sites of the introns conformed to the guanine thymine - adenine guanine (GT-AG) rule (online Fig. S1).

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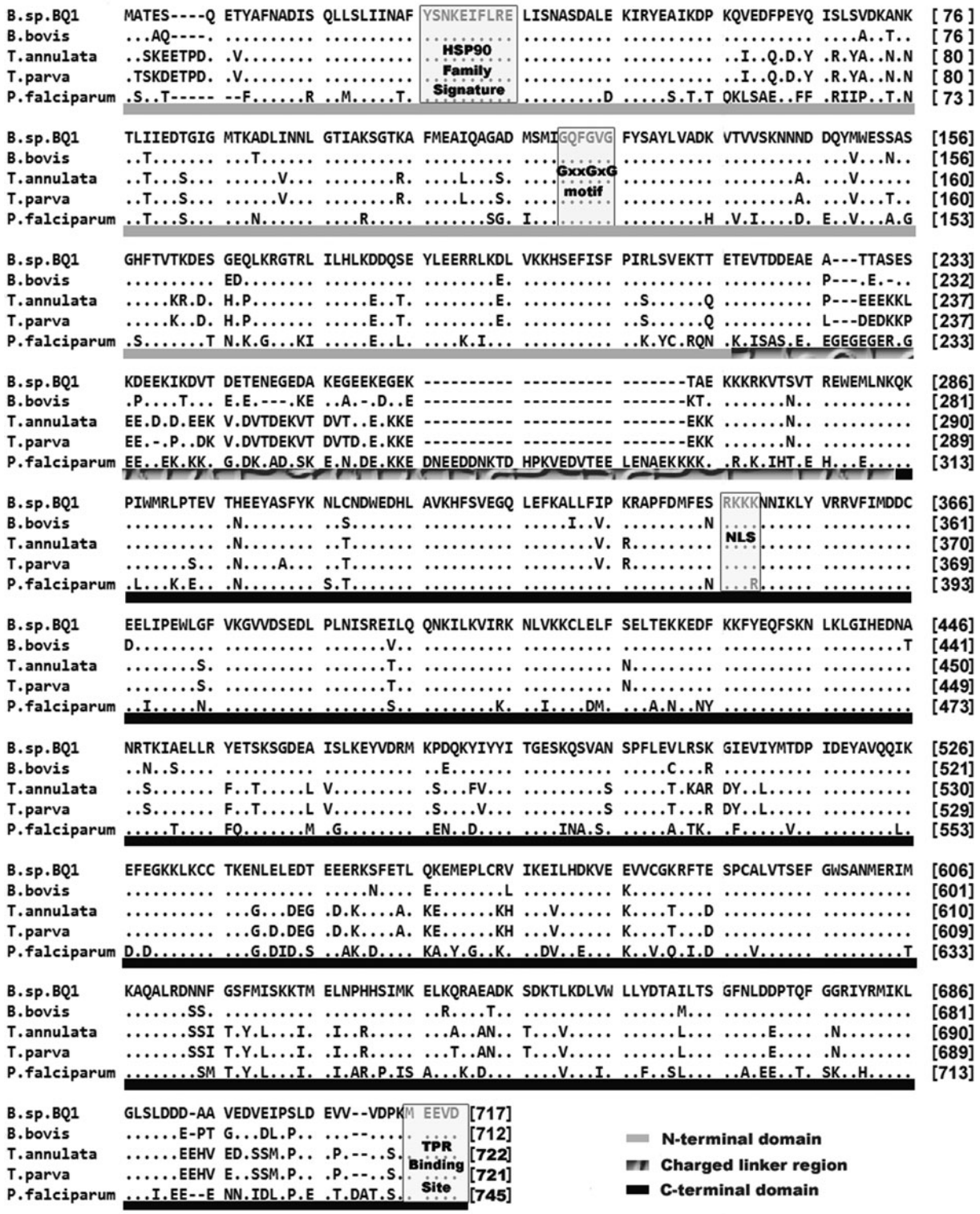

Fig. 1. Multiple sequence alignment of HSP90 proteins from *Babesia* sp. BQ1 (Lintan), *B. bovis, T. annulata, T. parva* and *P. falciparum*. The dots and short lines in the sequence represent identical residues and non-existent residues, respectively. Abbreviations: Hsp90, heat shock protein 90.

Phylogenetic analysis

Hsp90 gene fragments were amplified from all gDNA of *Babesia* sp. BQ1 (Lintan), *Babesia* sp. BQ1 (Ningxian), *Babesia* sp. Hebei, *Babesia* sp. Tianzhu and *Babesia* sp. Xinjiang. The sequence sizes were 2333, 2339, 2340, 2332 and 2166 bp, corresponding to the accession numbers GQ443608, GQ443604, GQ443605, GQ443606 and GQ443607, respectively. Exon–intron analyses of these genomic *Hsp90* with GENSCAN revealed that the variety of sequence lengths resulted in different sized introns (in GenBank database). Multiple sequence alignments

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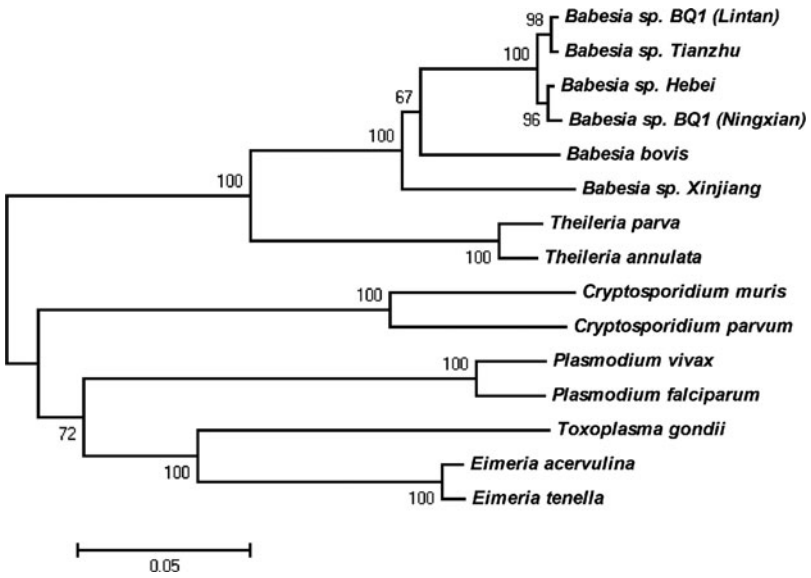

Fig. 2. Phylogenetic relationships between 5 Chinese *Babesia* isolates and 10 other apicomplexa species based on the amino acid dataset of *Hsp90* genes. The evolutionary history was inferred using the NJ method. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. There were a total of 564 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4. Abbreviations: NJ, neighbour-joining; Hsp90, heat shock protein 90.

based on the gDNA and deduced amino acid sequences of HSP90 in 5 Chinese ovine *Babesia* isolates revealed that the major varieties are present in the charged linker region (online Fig. S2). The identities of the 5 Chinese ovine *Babesia* isolates were determined on the basis of the nucleotide and amino acid sequences of HSP90. The minimum identities of nucleotide and amino acid sequences in 5 Chinese *Babesia* isolates were present in *Babesia* sp. Xinjiang (less than 80 and 90%). The identities of the amino acid and nucleotide sequences were more than 97·8 and 93·2%, respectively, between *Babesia* sp. BQ1 (Lintan), *Babesia* sp. BQ1 (Ningxian), *Babesia* sp. Hebei and *Babesia* sp. Tianzhu. In addition, the maximum identities were found for *Babesia* sp. BQ1 (Lintan) and *Babesia* sp. Tianzhu (99·3 and 99·4% for the amino acid and nucleotide sequence) and for *Babesia* sp. BQ1 (Ningxian) and *Babesia* sp. Hebei (99·5 and 98·8% for the amino acid and nucleotide sequence) (online Table S1). The bootstrap test of phylogeny for the apicomplexa HSP90 amino acid dataset generated identical tree topologies for neighbour-joining (NJ) analyses, using the ClustalW programme in software MEGA 4. The relationships were in agreement with the traditional taxonomic classification as all *Babesia, Theileria, Cryptosporidium, Eimeria, Toxoplasma* and *Plasmodium* were classified into separate branches. The *Babesia* group could be divided into 3 clades: *Babesia* sp. Xinjiang, *B. bovis* and the 4 Chinese *B. motasi*-like isolates. However, these 4 Chinese *B. motasi*-like isolates could be further divided into 2 subclades, one including *Babesia* sp. BQ1 (Lintan) and *Babesia* sp. Tianzhu and the other *Babesia* sp. BQ1 (Ningxian) and *Babesia* sp. Hebei (Fig. 2).

Expression and antigenic analysis of rBQHSP90

An entire *BQHsp90* ORF was successfully inserted into the pET200/D-TOPO® vector and expressed in BL21 Star™ (DE3). The recombinant BQHSP90 protein (rBQHSP90) was expressed in 2 forms; soluble protein and inclusion bodies. The soluble rBQHSP90 protein was purified from supernatant of recombinant BL21 (Fig. 3A). Western blot analysis showed that *Babesia* sp. BQ1 (Lintan) infected sheep serum could specifically recognize the rBQHSP90. No reactions from rBQHSP90 were detected with negative sera, or from the pET200/D-TOPO® vector control with either positive or negative sera (Fig. 3B), which indicates BQHSP90 could induce antibodies production when animal was infected by *Babesia* sp. BQ1 (Lintan).

Specificity analysis of rBQHSP90 for Babesia sp. BQ1 (Lintan) infection

Specificity of rBQHSP90 for *Babesia* sp. BQ1 (Lintan) infection was evaluated *via* testing sera from individually infected sheep by *Babesia* sp. BQ1 (Lintan), *Babesia* sp. BQ1 (Ningxian), *Babesia* sp. Tianzhu, *Babesia* sp. Hebei, *Babesia* sp. Xinjiang, *T. luwenshuni, T. uilenbergi*, together with negative sera, using ELISA and western blot. The results indicated that rBQHSP90 could not specifically differentiate *Babesia* sp. BQ1 (Lintan) from

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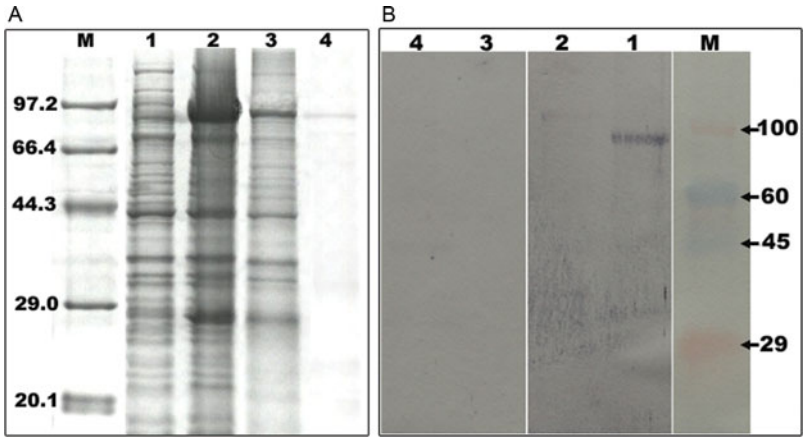

Fig. 3. Sodium dodecyl sulfate polyacrylamide gel electropheresis (SDS-PAGE) (A) and western blot (B) of rBQHSP90. (A) M is the marker of mass (kDa); 1–4 represent lysates of recombinant BL21 (DE3) before induction, lysates of recombinant BL21 (DE3) after induction (LRAI), soluble proteins in LRAI and purified rBQHSP90, respectively. (B) M is the protein marker (kDa). 1 and 3 are rBQHSP90 protein reacted each with positive serum from sheep infected by *Babesia* sp. BQ1 (Lintan) and negative serum; 2 and 4 are pET200/D/*lacZ* control reacted with positive serum from sheep infected by *Babesia* sp. BQ1 (Lintan) and negative serum, respectively. The right and left numbers represent the mass of each band in the protein marker.

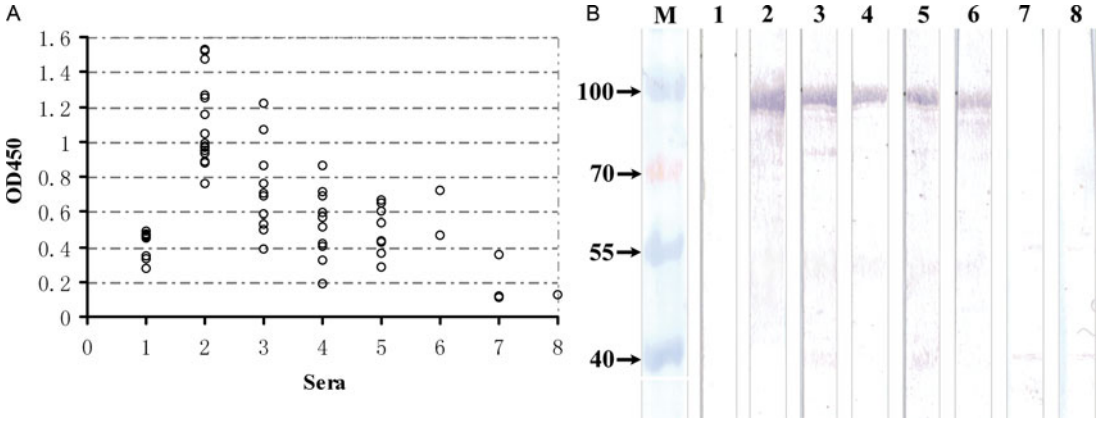

Fig. 4. Specificity analysis of rBQHSP90 for ovine piroplasms infection. (A) Level of BQHSP90 protein specific antibodies in sera from sheep of pre- and post-infection in ELISA. (B) Western blot analysis of rBQHSP90 specificity using sera from sheep of pre- and post-infection. M, standard molecular weight markers (kDa); 1, negative sera from sheep of pre-infection; 2–8, positive sera from sheep infected by *Babesia* sp. BQ1 (Lintan), *Babesia* sp. Tianzhu, *Babesia* sp. BQ1 (Ningxian), *Babesia* sp. Hebei, *Babesia* sp. Xinjiang, *T. luwenshuni* and *T. uilenbergi*, respectively.

other *Babesia* species/strains infection. However, rBQHSP90 had no reaction with positive sera of *T. luwenshuni* and *T. uilenbergi* (Fig. 4). Thus, BQHSP90 could be used to detect *Babesia* infection but not to distinguish different *Babesia* species infection. In addition, the antibody kinetics of BQHSP90 were evaluated using sera from 3 sheep during 84 dpi with *Babesia* sp. BQ1 (Lintan). BQHSP90 specific antibodies produced from 1st week post-infection, and peaked in 3rd–4th weeks post-infection. In 5th week, it returned to the level of 1st week and later, showed a fluctuation (Fig. 5).

DISCUSSION

A *Hsp90* gene from a *B. motasi*-like parasite was firstly cloned and characterized in the present study. The protein encoded by the *BQHsp90* gene is an 83 kDa member of the HSP90 family, sharing high homology with *B. bovis, T. annulata* and *T. parva* HSP90 around the N- and C-terminal domains. *In silico*, several HSP90 conserved signatures, such as the ATP-binding domain, HSP90 signature, TPR binding site, GXXGXG motif and NLS, were identified on BQHSP90 protein. Comparison of the gDNA and cDNA sequences of BQHSP90 showed that the *BQHsp90* gene contains 2 introns. This is in agreement with reports that all *Hsp90*s contain introns but that the number of introns differs from one to another (Girvitz *et al.* 2000; De Luca *et al.* 2009; Khan *et al.* 2014). The structural analysis of BQHSP90 also showed high similarity with the 3D structure of other HSP90 proteins, i.e. presence of N-terminal domain and COOH-terminal domain linked by a charged linker region. As the BQHSP90 protein exhibits several

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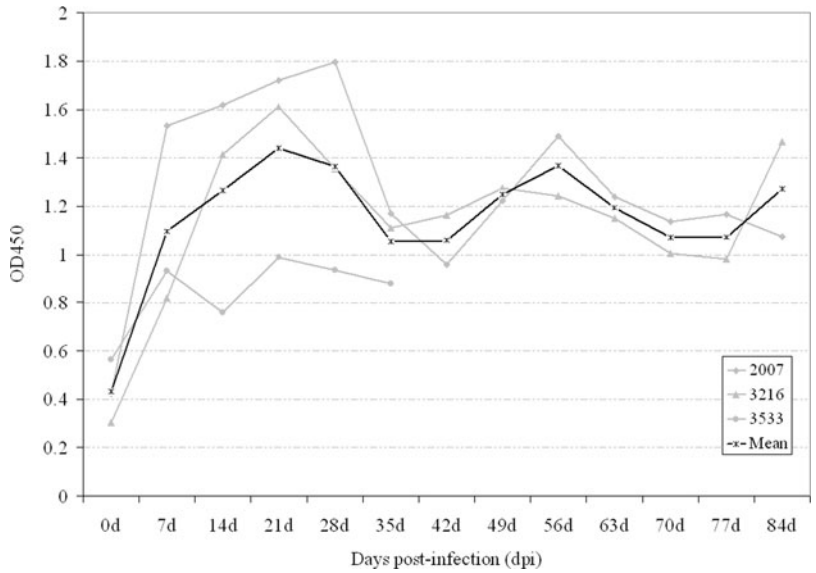

Fig. 5. Kinetics of humoral response against rBQHSP90 of 3 sheep experimentally infected by *Babesia* sp. BQ1 (Lintan).

structural characteristics common to the HSP90 family, it probably shares some important functions such as ATPase activity. For example, Kumar *et al.* (2007) and Zhang *et al.* (2008) used 3D structure analysis to show that conserved domains of HSP90 were involved in nucleotide binding, ATPase activity, co-chaperone binding and intersubunit interactions. It might therefore be possible to use inhibitors of BQHSP90 to develop therapeutic treatments against *Babesia* sp. BQ1 (Lintan) infection similar to these of toxoplasmosis and malaria previously described by Echeverria *et al.* (2005) and Kumar *et al.* (2003, 2007).

Although small-subunit (SSU) rRNA genes are commonly used to determine the molecular phylogenies of eukaryotes and prokaryotes and some new species were discovered based on SSU rRNA gene sequences (Inokuma *et al.* 2003; Oosthuizen *et al.* 2008), several authors (Philippe *et al.* 2000; Cavalier-Smith and Chao, 2003; Stechmann and Cavalier-Smith, 2003) have demonstrated that, due to the extremely variable rate and mode of rRNA evolution, unsound methods or artefactual grouping may also produce phylogenies if we just used SSU rRNA genes in phylogenetic analysis. Thus, together with conserved protein-coding genes, it makes phylogenetic analysis more sound and close to virtual evolutional relationship of organisms. For instance, Fukuda and Endoh (2008) used both the *Hsp90* and *β-tubulin* genes to determine the phylogeny of the dinoflagellate *Noctiluca scintillans* and proposed a possible evolutionary position between the diploid dinoflagellates and haploid core dinoflagellates. To date, 18S rRNA gene was the primarily molecular marker used to understand phylogenetic relationships of piroplasms (Gubbels *et al.* 2000; Ahmed *et al.* 2006). However, the phylogenetic analysis based on single gene marker cannot reflect the relationships of species clearly (Schnittger *et al.* 2012). Thus, more gene targets have been introduced for the phylogenetic analysis of piroplasms, including ITS, 28S rRNA, HSP70 and mitochondrial genes cytochrome b (cob), cytochrome c oxidase subunit I (cox I) and III (cox III) (Tian *et al.* 2013*a*, *b*; Yamasaki *et al.* 2007; Gou *et al.* 2013*a*, *b*).

In this study, we used the *Hsp90* gene to carry out a phylogenetic analysis within the apicomplexa species and to clarify the phylogeny of Chinese ovine *Babesia* isolates. Multiple sequence alignment of the deduced BQHSP90 amino acid sequence showed that BQHSP90 shared more than 70% identity with the HSP90 of other apicomplexa. The *Babesia, Theileria, Eimeria, Cryptosporidium, Plasmodium* and *Toxoplasma* genera were apparent as clearly separate clusters as in traditional taxonomy. In China, several geographical strains of ovine large *Babesia* have been isolated from field-collected blood or ticks in the past 2 decades. Liu *et al.* (2007) subjected these Chinese ovine *Babesia* isolates to a phylogenetic analysis based on the 18S rRNA gene sequences, and showed that 7 Chinese isolates could be separated into 2 clusters, *Babesia* sp. Xinjiang and *B. motasi*-like (*Babesia* sp. BQ1 (Lintan), *Babesia* sp. BQ1 (Ningxian), *Babesia* sp. Tianzhu and *Babesia* sp. Hebei together with European *B. motasi*) clusters. Phylogenetic analyses, based on ribosomal DNA ITS sequences, suggest that all these strains, except *Babesia* sp. Xinjiang, should be considered as *B. motasi* (Niu *et al.* 2009). In our phylogeny analysis, based on the amino acid sequences of HSP90, the results were similar to those of analyses based on ribosomal genes, 18S rRNA and ITS genes in that *Babesia* sp. Xinjiang, *B. bovis* and 4 Chinese *B. motasi*-like isolates were separated into 3 distinct clusters on the phylogenetic tree. This provides further evidence that *Babesia* sp. Xinjiang appears to be a new *Babesia* species

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infective for small ruminants. Interestingly, the 4 Chinese *B. motasi*-like isolates could be further divided into 2 subclades, one containing *Babesia* sp. BQ1 (Lintan) and *Babesia* sp. Tianzhu and the other *Babesia* sp. BQ1 (Ningxian) and *Babesia* sp. Hebei. Multiple sequence alignment indicated that *Babesia* sp. BQ1 (Lintan) and *Babesia* sp. Tianzhu were more closely related, as were *Babesia* sp. BQ1 (Ningxian) and *Babesia* sp. Hebei. Uilenberg (2006) provided a detailed description of the taxonomic state of *B. motasi*, declaring that it could be separated into at least 2 species or subspecies based on the differences in pathogenicity (low virulence in northern Europe and high virulence in southern Europe and the Mediterranean basin), infectivity to sheep and goats, serology and even morphology. The taxonomy of the Chinese *B. motasi*-like isolates, with one group of low virulence (*Babesia* sp. BQ1 (Lintan) subclade) (Guan *et al.* 2002) and another of high virulence (*Babesia* sp. BQ1 (Ningxian) subclade) (Bai *et al.* 2002), seems to align with this viewpoint. In addition, Guan *et al.* (2010*a*) showed that a soluble merozoite antigen of *Babesia* sp. BQ1 (Lintan) from *in vitro* culture cross-reacted with sera from *Babesia* sp. Tianzhu infected sheep, but not with those from *Babesia* sp. BQ1 (Ningxian) and *Babesia* sp. Hebei. Nevertheless, before these Chinese ovine *B. motasi*-like isolates can be classified as 2 species or subspecies, further evidence from biological studies of the tick vector, virulence or antigenicity will be required.

HSP90-specific antibodies have been used as a diagnostic marker of disease progression in some tumours and psychiatric disorders (Shen *et al.* 2006; McCarthy *et al.* 2008). They are also found in patients with various autoimmune disorders such as rheumatoid arthritis, ankylosing spondylitis, systemic lupus erythematosus and inflammatory bowel diseases. HSP90 has rarely been considered for use in the diagnosis of diseases caused by pathogen invasion, due to the presence of anti-HSP90 antibodies in the normal IgG repertoire and cross-reaction between antibodies and HSP90 from different organisms (Pashov *et al.* 2002; Virdi *et al.* 2009). Nevertheless, the level of anti-HSP90 specific antibodies significantly increases when hosts are infected by pathogens. De Andrade *et al.* (1992) showed that recombinant HSP90 from *Leishmania donovani donovani* did not cross-react with sera from *Trypanosoma cruzi* and *T. gondii* patients in either western blot or ELISA. HSP90 from *Trichinella spiralis* was also specific and showed no reaction with irrelevant immune rat sera by western blot (Martinez *et al.* 2001). In the present study, no reaction was detected between recombinant BQHSP90 and sera recovered from sheep of pre-infection and infected by *T. luwenshuni* and *T. uilenbergi* either in western blot or ELISA. Sera positive for *Babesia* sp. BQ1 (Lintan) had strong reactions with rBQHSP90, following by *Babesia* sp. Tianzhu, *Babesia* sp. BQ1 (Ningxian), *Babesia* sp. Hebei and *Babesia* sp. Xinjiang. Thus, it had cross-reaction with sera from other Chinese ovine *Babesia* isolates and cannot be used to differentiate infection of these *Babesia* parasites infections. However, rBQHSP90 can be used to distinguish the infection of ovine *Babesia* from those of *Theileria*. Antibody kinetics of BQHSP90 in 3 sheep during 84 dpi with *Babesia* sp. BQ1 (Lintan) revealed that specific antibody levels of BQHSP90 drastically increased from 1st week post-infection, and peaked in 3rd–4th weeks post-infection. In 5th week, it returned and later, showed a fluctuation, which suggests rBQHSP90 may be a potential sero-diagnostic antigen to detect early infection of ovine *Babesia*.

In summary, there is a member of HSP90 family in ovine *Babesia* and it has similar characteristics and predictive function involved in chaperone protein, which suggests possibly it could be used as adjuvant in vaccine during the control babesiosis. Recombinant protein rBQHSP90 can specifically recognize antibodies of *Babesia* HSP90 post-infection. It indicates that the molecule may have potential as an antigen for detecting parasites of *Babesia* genus infection but not differentiating species infection. Finally, HSP90 can be considered as molecular marker for clarifying phylogenic relationships of piroplasms.

SUPPLEMENTARY MATERIAL

To view supplementary material for this article, please visit http://dx.doi.org/10·1017/S0031182015000797.

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