Expression of sheep pathogen *Babesia* sp. Xinjiang rhoptry-associated protein 1 and evaluation of its diagnostic potential by enzyme-linked immunosorbent assay

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SUMMARY

Ovine babesiosis is one of the most important tick-borne haemoparasitic diseases of small ruminants. The ovine parasite *Babesia* sp. Xinjiang is widespread in China. In this study, recombinant full-length XJrRAP-1aα2 (rhoptry-associated protein 1aα2) and C-terminal XJrRAP-1aα2 CT of *Babesia* sp. Xinjiang were expressed and used to evaluate their diagnostic potential for *Babesia* sp. Xinjiang infections by indirect enzyme-linked immunosorbent assay (ELISA). Purified XJrRAP-1aα2 was tested for reactivity with sera from animals experimentally infected with *Babesia* sp. Xinjiang and other haemoparasites using Western blotting and ELISA. The results showed no cross-reactivities between XJrRAP-1aα2 CT and sera from animals infected by other pathogens. High level of antibodies against RAP-1a usually lasted 10 weeks post-infection (wpi). A total of 3690 serum samples from small ruminants in 23 provinces located in 59 different regions of China were tested by ELISA. The results indicated that the average positive rate was 30·43%, and the infections were found in all of the investigated provinces. This is the first report on the expression and potential use of a recombinant XJrRAP-1aα2 CT antigen for the development of serological assays for the diagnosis of ovine babesiosis, caused by *Babesia* sp. Xinjiang.

Key words: *Babesia* sp. Xinjiang, rhoptry-associated protein, expression, serodiagnosis.

INTRODUCTION

Babesiosis is a tick-borne haemoparasitic disease affecting a wide range of domestic and wild animals and is caused by the genus *Babesia*, which comprises many species of parasites that infect a large range of erythrocytes and various vertebrate hosts (Vannier and Krause, 2009). Many *Babesia* species are highly pathogenic for animals. Ovine babesiosis is one of the most important tick-borne haemoparasitic diseases of small ruminants in tropical, subtropical and temperate regions. The economic losses in sheep and goat production due to babesiosis are significant in tropical and subtropical areas, where it has increasingly attracted more attention (Luo and Yin, 1997; Ahmed *et al*. 2002). Three *Babesia* species are known to infect small ruminants: *Babesia ovis*, *Babesia motasi*, and *Babesia crassa* (Uilenberg, 2006).

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In China, several *Babesia* isolates have been described to infect small ruminants, most of which belong to *B. motasi*-like species, which has the similar morphology characteristics and form a sister clade with *B. motasi* species based on phylogenetic trees of the 18S rRNA and internal transcribed spacer (ITS) gene sequences (Liu *et al*. 2007; Niu *et al*. 2009). The other isolate, *Babesia* sp. Xinjiang, was isolated from sheep experimentally infected with *Rhipicephalus sanguineus* and *Hyalomma anatolicum anatolicum* collected from pastures in China (Guan *et al*. 2001). The demonstrated vector of this *Babesia* species is *H. a. anatolicum*. The pathogenic and morphological characteristics of this species are totally different from the other *Babesia* species of small ruminants previously reported worldwide, including China. It is a large parasite with various morphological forms in infected erythrocytes: single and paired piriform (the most common shapes), ring form, three-leafed shaped, rod shaped, budding forms and oval forms (Guan *et al*. 2009).

Although the *Babesia* parasites are specifically parasitic on a large range of obligate host red blood cells, the pathological changes caused by *Babesia* species are similar to those observed in *Plasmodium*

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falciparum, and the mechanism of host cell entry is thought to be conserved (Besteiro *et al*. 2011). Parasites in the genus *Babesia* are transmitted when sporozoites are released into the vertebrate host with the saliva during tick feeding, and then the apicomplexan parasites could display a machinery to invade into host cells. Clinical symptoms of the disease might appear when the merozoites invade and replicate within host erythrocytes, and reach high parasitaemia (Yokoyama *et al*. 2006). The vaccine development targeted on the molecules of the extracellular merozoites during invasion process might be a significant strategy against babesiosis.

The Babesia rhoptry-associated protein-1 (RAP-1) protein was first described in *Babesia bigemina* (designated p58) (McElwain *et al*. 1987) and was then characterized in all examined *Babesia* species: *B. bovis* (Suarez *et al*. 1991), *B. divergens* (Rodriguez *et al*. 2014), *Babesia canis, B. ovis* (Dalrymple *et al*. 1993), *Babesia orientalis* (Yu *et al*. 2014), *Babesia gibsoni* (Zhou *et al*. 2007), *B. motasi*-like (Niu *et al*. 2013, 2014) and *Babesia* sp. Xinjiang (Niu *et al*. 2015). Seven different *rap-1* genes (five *rap-1aα* and two *rap-1aβ*) in *Babesia* sp. Xinjiang have been recently described, and at least three *rap-1aα* and two *rap-1aβ* were transcribed *in vitro* (Niu *et al*. 2015).

In the present study, the gene encoding full-length and C-terminal truncated RAP-1aα2 in *Babesia* sp. Xinjiang was cloned and expressed in *Escherichia coli* and evaluated for its potential use as a diagnostic and vaccine candidate. The presence of antibodies directed against native and recombinant RAP-1a during the course of infection was detected using Western blotting and enzyme-linked immunosorbent assay (ELISA). The identification and localization of native RAP-1aα2 in *Babesia* sp. Xinjiang erythrocytes were detected by Western blotting and indirect fluorescent antibody test (IFAT) and its serodiagnostic performance as an antigen was evaluated by ELISA.

MATERIALS AND METHODS

Parasites

Biologically cloned *Babesia* sp. Xinjiang lines were derived from *in vitro* culture by limiting dilution, as described previously (Malandrin *et al*. 2009), and cryopreserved at the vector and vector-borne disease (VVBD) laboratory of Lanzhou Veterinary Research Institute (LVRI) (CAAS Lanzhou, China).

Sera

Sera collected from two sheep (Nos. 3201 and 026), which were infected by *Babesia* sp. Xinjiang were used to evaluate antibody kinetics. Sera collected from sheep Nos. 3216, 08026, 08040 and 08020, which were infected with *Babesia* sp. BQ1 (Lintan), *Babesia* sp. BQ1 (Ningxian), *Babesia* sp. Tianzhu and *Babesia* sp. Hebei, respectively, as well as the sera collected from sheep experimentally infected with *Theileria luwenshuni, Theileria uilenbergi* and *Anaplasma ovis*, or bovine *B. bovis* and *B. bigemina*, were used as controls to evaluate cross-reactivity.

The sera samples ($n = 110$) were selected from ten sheep, 3–12 weeks post-infection (wpi), experimentally infected with *Babesia* sp. Xinjiang and used as positive samples to evaluate the sensitivity of the ELISA and a mixture of these sera was used as the positive control serum (Guan *et al*. 2012*b*).

Sera from 197 lambs, purchased from the *Babesia*-free region in Jingtai County (Gansu Province of China) from 2005 to 2010 were collected. The genomic DNA samples corresponded to these serum samples were tested by different methods and has never found an animal positive. A mixture of these sera was used as the negative control serum to evaluate the specificity of the ELISA (Guan *et al*. 2012*b*).

A total of 3690 serum samples were randomly collected from clinically healthy sheep from 59 different locations in 23 Chinese provinces between 2010 and 2015. All of the blood samples were collected in tubes and transported to the laboratory on ice, where the serum was separated and stored at −20 °C until further use.

Recombinant protein expression and purification of the XJrRAP-1aα2

Genomic *Babesia* sp. Xinjiang DNA encoding full-length and the C-terminal truncated version of XJrRAP-1aα2 protein were amplified using the modified primers based on the RAP-1aα2 gene sequence (GenBank accession number: KF811194), rRAP-1aα-full-F: 5′-CGCATATGGGTTCGTC ACTATCAGAATGT-3′, or rRAP-1aα-CT-F: 5′-CGCATATGATCGCCATTCCAACAAAAGA-3′ and rRAP-1aα-R: 5′-GCCAAGCTTTTCTTGA GATACCTCATCCT-3′ to include NdeI and HindIII (New England BioLabs, USA) enzyme restriction sites (underlined) on the 5′ end and subcloned into the pET-30a vector. The resulting plasmids (pET-30a – XJRAP-1aα2 and pET-30a – XJRAP-1aα2 CT) were confirmed by enzyme restriction digestion and sequencing, and subsequently transformed into the *E. coli* BL21 (DE3 strain) according to the manufacturer's instructions for protein expression.

The bacterial cultures containing His6-fusion proteins of rXJRAP-1aα2 and rXJRAP-1aα2/CT were harvested after induction with 1 mM isopropy-β-D-thiogalactoside (IPTG) for15 °C for 16 h and

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lysed by ultrasonication in binding buffer (50 mM Tris, 6 M guanidine–HCl, pH 8·0) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and purified from the *E. coli*. The target protein was then eluted using urea followed by buffer containing increasing concentrations of imidazole (20, 50 and 250 mM). The recombinant proteins were then assessed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–10% PAGE). Recombinant protein expression was then analysed by Western blotting.

Preparation of anti-XJrRAP-1aα2 CT-specific rabbit immune serum

Purified XJrRAP-1aα2 CT was used to raise antibodies in New Zealand Rabbits (2 kg each). The sera were collected from each rabbit before the first immunization as a negative control. The rabbits were subcutaneously injected with 400 μg of purified rXJRAP-1aα2/CT protein emulsified in Freund's complete adjuvant (FCA; Sigma). Booster injections with the same amount of protein in Freund's incomplete adjuvant (FIA; Sigma) were administered on days 15, 20 and 28. The sera from the immunized rabbits were then collected 15 days after the last injection and purified and stored at −20 °C until further use.

Immunoblot analysis

The recombinant proteins (XJrRAP-1aα2 and XJrRAP-1aα2 CT) were separated on SDS–18% PAGE and then transferred to nitrocellulose (NC) membranes. The membrane was cut into 0·25 cm strips and incubated in blocking solution [5% skimmed milk powder in Tris-buffered saline (pH 7·6) with 0·1% Tween-20 (TBST)], for overnight at 4 °C on a shaker. After three washes, the NC strips were incubated for 1 h with each tested serum diluted at 1:100 in TBST. After three washes with TBST, the strips were incubated for 2 h 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). The reactions were developed using the 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) liquid substrate system (B1911-100ML, Sigma).

IFAT and confocal laser microscopic observation

The native *Babesia* sp. Xinjiang RAP-1 was identified in merozoites by IFAT using a procedure previously described by Moitra *et al*. (2015). *Babesia* sp. Xinjiang-infected red blood cells (iRBC) were washed using serum-free medium, and the iRBC membrane was stained using PKH26 (MINI26, Sigma-Aldrich) according to the manufacturer's protocol. The iRBCs were fixed in a buffer containing 4% paraformaldehyde and 10 mM piperazine-N, N-bis (2-ethanesulfonic acid) in PBS (PIPES) at pH 6·4 on coverslips for 30 min at room temperature. The coverslips were washed in PBS and then blocked for 1 h in 3% bovine serum albumin (BSA in PBS containing 0·25% Triton X-100). The coverslips were stained at 4 °C overnight with either pre-immune serum or purified anti-rXJRAP-1aα2/CT sera (1:100) diluted in blocking buffer without Triton X-100. The coverslips were washed three times in PBS and stained for 1 h with FITC-conjugated anti-rabbit IgG secondary antibody (Sigma; F0382; 1:80). The coverslips were then stained for 10 min in 1 μg mL^{-1} 4′, 6-diamidino-2-phenylindole (DAPI) (Sigma: D8417) and examined for reactivity using confocal microscopy.

ELISA

The optimum coating concentration of XJrRAP-1aα2 CT (100 μL at 2 μg mL^{-1}) in coating buffer (0·1 M carbonate/bicarbonate, pH 9·6) was distributed and adsorbed in 96-well flat-bottom microplates at 4 °C overnight. The plates were then blocked with 200 μL of 2% gelatin in PBST at 37 °C for 1 h. After washing, sera diluted with 1:100 were distributed in duplicate, and the plates were incubated for 1 h at 37 °C. After three washes, 100 μL of monoclonal anti-goat/sheep IgG-peroxidase (Sigma; A9452; dilution: 1:1000) was added to each well and incubated for 1 h at 37 °C. The reaction was detected with 100 μL of 1-Step™ Ultra TMB-ELISA (34028–250 mL; Thermo Scientific) for 15–25 min at room temperature and then halted by adding 100 μL of 2 M H_2SO_4. The plates were read at 450 nm with an automated ELISA plate reader (Bio-RAD Model 680 microplate reader, USA).

Statistical analysis

The 95% confidence intervals (95% CIs) for the overall prevalence values of *Babesia* sp. Xinjiang infection were calculated using IBM SPSS Statistics version 19.0.

RESULTS

Expression of recombinant XJRAP-1aα2 protein

The genes encoding XJrRAP-1aα2 (aa 22–447) and XJrRAP-1aα2 CT (aa 313–447) were successfully amplified by PCR. The pET-30a-XJRAP-1aα2 and pET-30a-XJRAP-1aα2 CT recombinant plasmids were identified by enzyme restriction analysis and subsequently confirmed by sequencing using specific primers; the insert sequences have also been verified (data not shown). The *E. coli* lysates

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containing recombinant protein were analysed by SDS–10% PAGE and Western blotting using anti-His6 serum (THETM His Tag Antibody, mAb, Mouse, GenScript, A00186). The results show that XJrRAP-1aα2 and XJrRAP-1aα2 CT were expressed as apparent approximately 52-kDa and 19-kDa proteins, respectively (Fig. 1).

Evaluation of the specificity of XJrRAP-1aα2 and XJrRAP-1aα2 CT via Western blotting

The reactivity of each positive serum sample from infected sheep with XJrRAP-1aα2 and XJrRAP-1aα2CT was analysed by Western blotting. Strong reactions with the expected size of XJrRAP-1aα2 CT (approximately 19 kDa) were observed with sera from *Babesia* sp. Xinjiang infected sheep (No. 3201). In contrast, the sera positive for other *Babesia*, *Theileria* and *Anaplasma* species giving a negative reaction with the RAP-1aα2 CT gave a positive reaction under identical conditions when tested with their corresponding antigens (data not shown) (Fig. 2). However, the cross-reactivity of positive serum from *B. motasi*-like and *T. uilenbergi* was observed with full length of XJrRAP-1aα2 (data not shown).

Identification of native XJRAP-1 proteins

The IFAT results show that native rhoptry proteins could be interacted with serum from a rabbit immunized by XJrRAP-1aα2 CT with *Babesia* sp. Xinjiang merozoites. Non-specific fluorescence signalling was detected using serum from pre-immunized rabbits (Fig. 3).

Cross-reactivity with other haemoparasites via ELISA

The sera collected from *Babesia* sp. Xinjiang and other haemoparasites-infected animals were used to evaluate cross-reactivity with the rRAP-1aα2 CT in the ELISA. The tests were repeated in triplicate, and the mean AbRs [AbR % = (Sample mean OD − Negative control mean OD)/(Positive control mean OD − Negative control mean OD) × 100] and S.D. were calculated with Excel 2007. The results indicate that no cross-reactivity was observed with sera against other *Babesia*, *Theileria* and *A. ovis* parasites (Fig. 4).

Kinetics of anti-Babesia sp. Xinjiang antibodies in experimentally infected sheep via ELISA

The kinetics of antibody production against RAP-1a have been studied via ELISA using XJrRAP-1aα2 CT and sera from two infected intact sheep (Nos. 3201 and 026) collected before (0 weeks post-infection, WPI0) and after infection (from WPI1 to WPI12). A significant increase in antibodies produced against RAP-1a was observed after infection and with similar kinetics between each individual sheep. In general, antibodies were produced at 1 week post-inoculation and continued to increase through 3 weeks (WPI3), after which slight decreases were observed through 12 weeks (WPI4–12) (Fig. 5).

Evaluation of the specificity, sensitivity and positive threshold value of the ELISA in detecting XJrRAP-1aα2 CT

MedCalc statistical software was used to evaluate the sensitivity and specificity, as well as the positive threshold value of the ELISA, by testing 110 sera of *Babesia* sp. Xinjiang positive and 197 sera of *Babesia* sp. Xinjiang negative. The specific antibody mean rate (AbR %) was calculated for each serum sample. The results indicate that the positive threshold value was 29·10%, corresponding to 94·3% sensitivity (95% CI = 80·1–97·8) and 95·1% specificity (95% CI = 82·3–99·6). The numbers of false-positive and false-negative sera were 3 and 14, respectively (Fig. 6).

Identification of XJrRAP-1aα2 CT protein as a potential antigen for the serological epidemiology of Babesia sp. Xinjiang infection by ELISA

A total of 3690 field serum samples from 59 different regions of 23 Chinese provinces were detected by the ELISA to evaluate the prevalence of infection with *Babesia* sp. Xinjiang. The results of the ELISA for positive sample screenings are summarized in Table 1. Sera samples infected with *Babesia* sp. Xinjiang were examined in 57 prefectures of 23 surveyed provinces. The average positive rate was 30·43% (1123/3690). The sero-prevalence ranged from 0 to 73·58%. The significantly high positive rates exceeded 50% in eight of 59 surveyed prefectures, mainly from North China (Table 1).

DISCUSSION

According to molecular taxonomy and phylogenetic reconstruction based on the 18S rRNA gene, *Babesia* sp. Xinjiang was closely related to *Babesia* recently described from wild ruminants in South Africa and with *Babesia pecorum* isolated from red deer in Spain (Oosthuizen *et al.* 2009; Jouglin *et al.* 2014). Presently, a *Babesia* sp. Xinjiang-like parasite (which shared 99·5% identity with the original strain of *Babesia* sp. Xinjiang) was isolated using an *in vitro* culture system from one of 19 sheep blood samples collected from Dunhuang city, Western Gansu Province, China (Guan *et al.* 2012*a*). Moreover, a recent report of sero-epidemiological investigation by ELISA for the infections of small ruminants by

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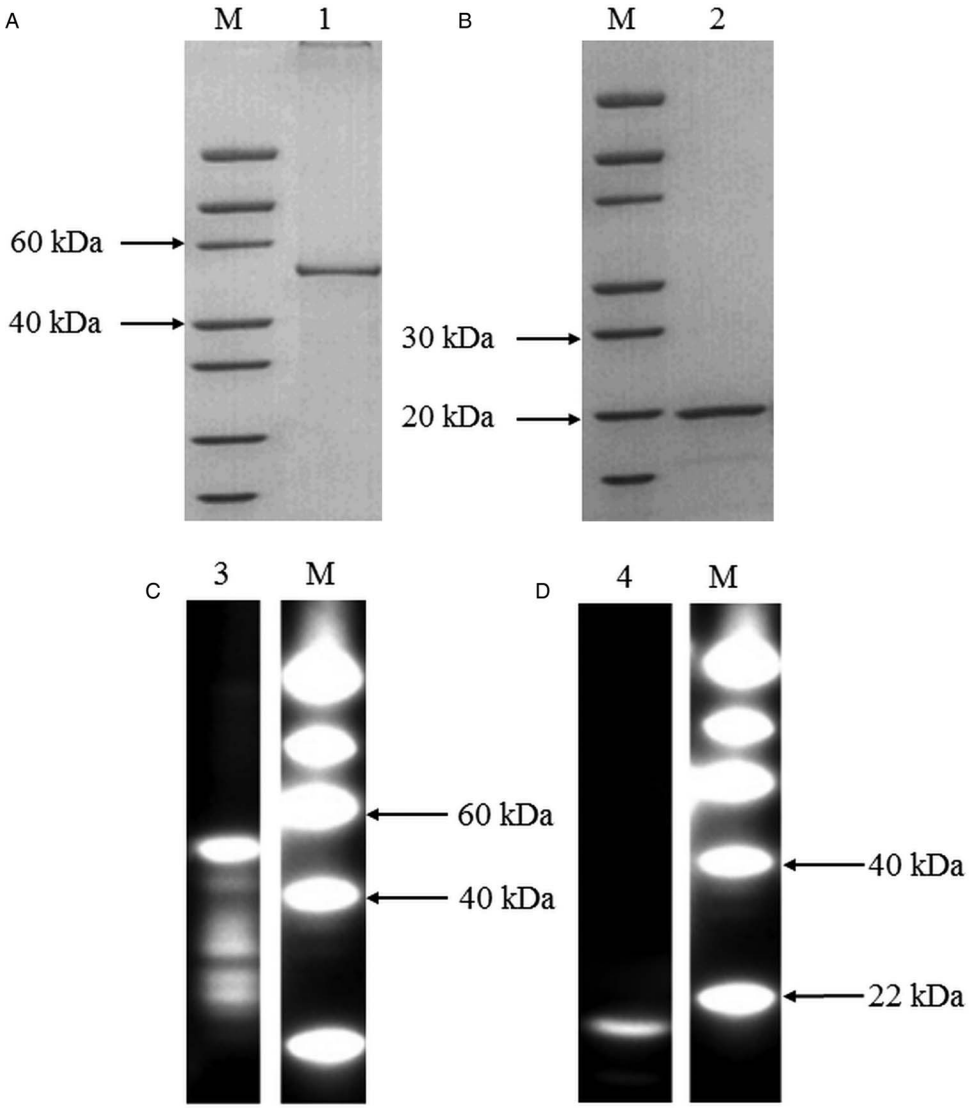

Fig. 1. SDS–18% PAGE (panels A and B) and Western blot (panels C and D) analysis (ECL) of the expressed and purified rXJRAP-1aα2 and rXJRAP-1aα2 CT proteins with His6-tag expressed in *E. coli*. M: molecular weight marker. Lanes 1 and 2: SDS–18% PAGE analysis for rXJRAP-1aα2 and rXJRAP-1aα2 CT proteins; lanes 3 and 4: Western blot analysis for rXJRAP-1aα2 and rXJRAP-1aα2 CT proteins using monoclonal anti-His6 Tag antibody in mouse.

Babesia sp. Xinjiang has indicated that this *Babesia* species was widespread in 22 provinces of China (Guan *et al*. 2012*b*). These findings indicated that ovine babesiosis caused by *Babesia* sp. Xinjiang presented a tendency to expand in China.

The *N*-terminal regions of RAP-1, as well as their sequences, are more conserved at the species level among geographically distant isolates than the C-terminal region and several C-terminal sequences encode conserved or degenerate repetitive motifs in many *Babesia* species (Dalrymple *et al*. 1993; Hötzel *et al*. 1997; Terkawi *et al*. 2009; Bhoora *et al*. 2010). In *Babesia* sp. Xinjiang RAP-1, the sequence is identical among copies over the first 300 and the last 30 amino acids, with few differences in the sequences of the repeats of the C-terminus of the protein. The prediction of B-cell epitopes in all RAP-1a using software demonstrated the presence of B-cell epitopes primarily in the repeat region of the polymorphic C-terminus (Niu *et al*. 2015). In general, repetitive regions of a protein are thought to mediate important functions in parasite survival and immune evasion (Mendes *et al*. 2013). The variable C-terminal region among *Babesia* species could induce a high humoral response; therefore, it could be considered as a useful antigen for specific diagnostic and epidemiological investigation purposes. The C-terminal truncated region of the RAP-1a protein has been widely used as a specific diagnostic antigen to differentiate *Babesia* infection (Boonchit *et al*. 2002, 2006; Zhou *et al*. 2007).

In the present study, the XJrRAP-1aα2 proteins were subjected to Western blot analysis, and cross-reactivity was observed between full-length XJrRAP-1aα2 and serum samples infected with *B. motasi*-like and *T. uilenbergi* parasites. Previous

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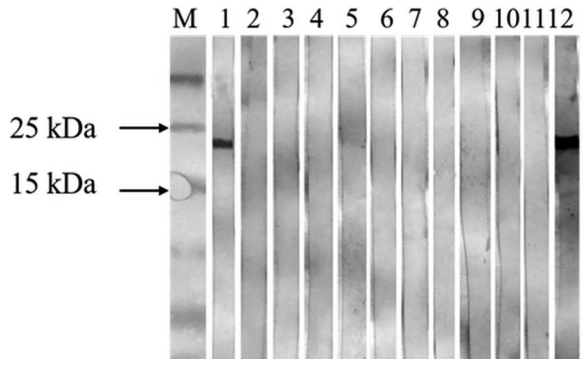

Fig. 2. Western blot analysis of the recombinant RAP-1aα2 CT in *Babesia* sp. Xinjiang. M: molecular weight marker. Lanes 1–10: sera positive for *Babesia* sp. Xinjiang, *Babesia* sp. BQ1 (Lintan), *Babesia* sp. Tianzhu, *Babesia* sp. BQ1 (Ningxian), *Babesia* sp. Hebei, *B. bigemina, B. bovis, T. luwenshun, T. uilenbergi* and *A. ovis*; lane 11: pool of negative sera; lane 12: positive control (anti-XJrRAP-1aα2 CT rabbit immune serum). The sera positive for other pathogens (lanes 2–10) giving a negative reaction with the RAP-1aα2 CT gave a positive reaction under identical conditions when tested with their corresponding antigens (data not shown).

studies in bovine *Babesia* species also indicated that the full-length recombinant RAP-1a from *B. bovis* could cross-react with *B. bigemina* due to the conserved features in the N-terminus of RAP-1 (Boonchit *et al.* 2002). By contrast, XJrRAP-1aα2 CT could be specifically recognized by the serum from *Babesia* sp. Xinjiang infection only by Western blot analysis. Recombinant RAP-1a constructed on the C-terminal region in *B. bovis* and *B. bigemina* has been used as a diagnostic antigen to develop an ELISA method and could improve the sensitivity and specificity of antibody detection (Terkawi *et al.* 2011).

Serological diagnostic testing based on the immunoreactive proteins generated from parasite genes is an essential tool used for the control of babesiosis (Ooka *et al.* 2012; Sevinc *et al.* 2015*a*). Recombinant antigens (merozoite antigen, RAP-1 and spherical body proteins (SBPs)) used in serological assays have been widely and effectively used for the diagnosis of bovine, equine and canine babesiosis (Boonchit *et al.* 2002; Huang *et al.* 2003; Zhou *et al.* 2007; Terkawi *et al.* 2011; Mandal *et al.* 2016). In the ovine *Babesia* parasite, a crude *B. bovis* antigen and a synthetic *B. bovis*-derived antigen (11C5) are described to detect *B. ovis* antibodies by the development of an ELISA (Duzgun *et al.* 1991), and few novel recombinant proteins have been identified as candidate diagnostic antigens: *B. ovis* secreted antigen 1 and 2 (BoSA1 and BoSA2) for *B. ovis* detection (Sevinc *et al.* 2015*a*, *b*) and BQP35 and heat shock protein 90 (HSP90) for *Babesia* sp. BQ1 (Lintan) (*B. motasi*-like species) detection (Guan *et al.* 2012*c*, 2015). Western blotting and an ELISA based on rBQP35 showed cross-reactivity between Lintan and Tianzhu isolates of *B. motasi*-like (Guan *et al.* 2012*c*), whereas rBQHSP90 could not differentiate among any ovine *Babesia* species (Guan *et al.* 2015). Until now, for *Babesia* sp. Xinjiang, only one report described that an ELISA

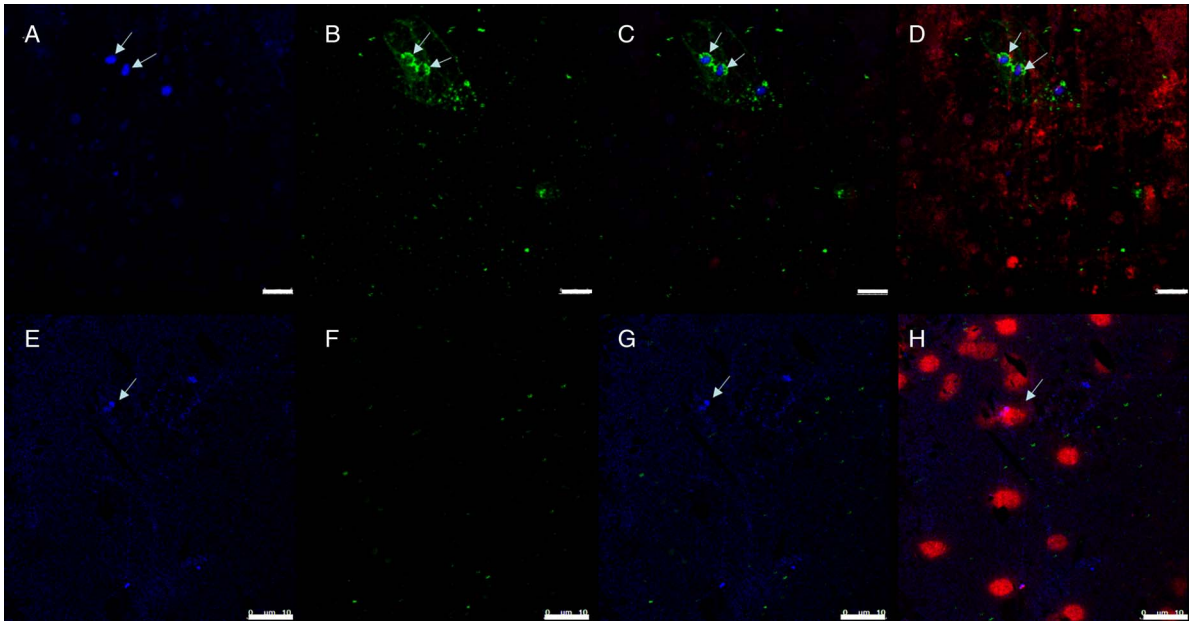

Fig. 3. Immunofluorescence microscopy analysis. *Babesia* sp. Xinjiang-iRBC were stained with the rabbit anti-XJrRAP-1aα2 CT antibody (A–D) and pre-immune serum (E–H). Nuclei were counterstained with DAPI (blue, A and E). Anti-XJrRAP-1aα2 CT antibody (green) reacted with native RAP-1 on merozoites (B and F). Overlaid image of fluorescent green reactivity and blue DAPI staining of nuclei (C and G). Merged images of fluorescent green reactivity, RBCs staining with PKH26 (red) and blue DAPI staining are shown in the right (D and H). Arrowheads showed the representative merozoites. Bar, 5 μM (A–D), 10 μM (E–H).

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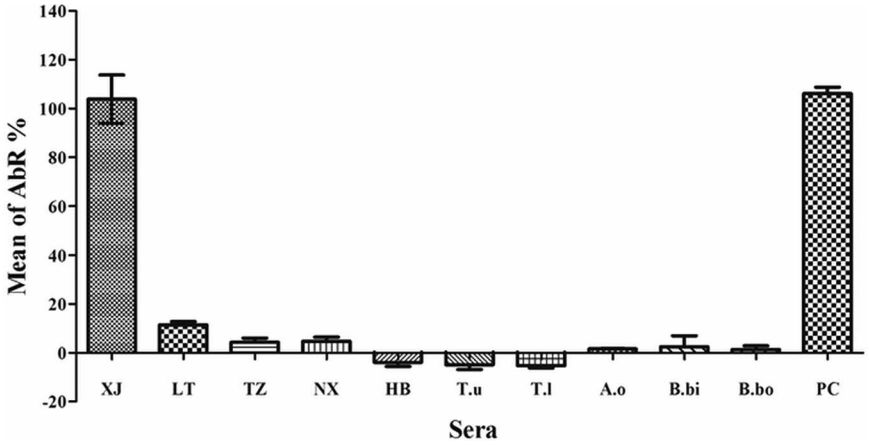

Fig. 4. Cross-reactivity of XJrRAP-1aα2 CT of *Babesia* sp. Xinjiang with the sera positive for *Babesia, Theileria* and *Anaplasma* infective to sheep or cattle in ELISA. XJ: *Babesia* sp. Xinjiang; LT: *Babesia* sp. BQ1 (Lintan); TZ: *Babesia* sp. Tianzhu; NX: *Babesia* sp. BQ1 (Ningxian); HB: *Babesia* sp. Hebei; T.u: *T. uilenbergi*; T.l: *T. luwenshuni*; A.o: *A. ovis*; B. bi: *B. bigemina* and B. bo: *B. bovis*; PC: positive control (anti-XJrRAP-1aα2 CT rabbit immune serum). AbR % = (Sample mean OD – Negative control mean OD)/(Positive control mean OD – Negative control mean OD) × 100).

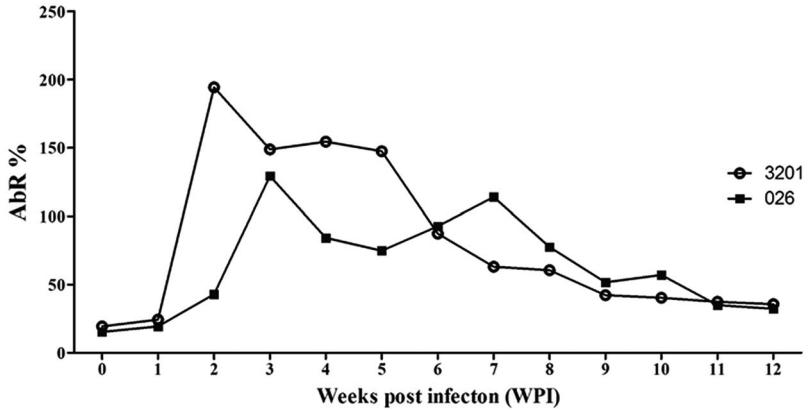

Fig. 5. Kinetics of anti-*Babesia* sp. Xinjiang antibodies in two sheep (Nos. 3201 and 026) infected experimentally. AbR % = (Sample mean OD – Negative control mean OD)/(Positive control mean OD – Negative control mean OD) × 100)

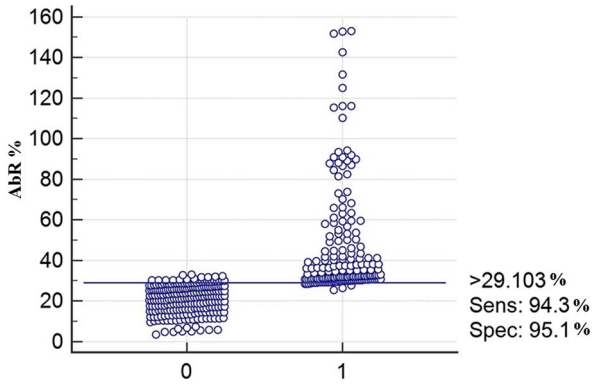

Fig. 6. Evaluation of specificity, sensitivity and positive threshold value of XJrRAP-1aα2 CT with ELISA using antibody mean rates (AbR %) of positive and negative sera. Spec: specificity, Sens: sensitivity. 0 = negative sera; 1 = positive sera.

was recently developed using soluble merozoite antigens (BXJMA) from the *in vitro* culture of *Babesia* sp. in Xinjiang, eight common proteins were recognized by antibodies, one of them was recognized by anti-*B. bovis*, -*B. bigemina* and -*Babesia* U sp. Kashi antibodies (Guan *et al.* 2012*b*). No report was focused on the production of the recombinant immunological antigens of *Babesia* sp. Xinjiang and their use as antigens in any serological test to detect ovine babesiosis, caused by this parasite. The result in this study suggest that rRAP-1aα2 CT could be replaced native crude parasite antigens, which require large parasite reproduction from the sacrifice of experimentally infected animals or the harvesting of cultures *in vitro* (Böse *et al.* 1995).

According to the results of Western blotting and ELISA, the serum of *Babesia* sp. Xinjiang

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Table 1. Prevalence of *Babesia* sp. Xinjiang in field samples collected from 23 provinces by detected antibodies produced from rRAP-1aα2 CT with ELISA

Province	Prefecture	Number of sera	Number of positive sera	Positive rate (%) in different regions	Positive rate (%) in each province
Xinjiang	Yili	168	74	44·05	(231/443) 52·14
	Akesu	175	105	60·00	
	Habahe	100	52	52·00	
Inner Mongolia	Manzhouli	24	10	41·67	(107/345) 31·01
	Baotou	11	0	0	
	Chifeng	134	52	38·81	
	Ordos	176	45	25·57	
Gansu	Lintan	106	78	73·58	(139/350) 39·71
	Jiuquan	7	4	57·14	
	Tianzhu	81	11	13·58	
	Jiayuguan	81	8	9·88	
	Yongchang	75	38	50·67	
Qinghai	Haibei	180	58	32·22	(93/278) 33·45
	Qilianshan	98	35	35·71	
Ningxia	Wuzhong	81	21	25·93	(21/81) 25·93
Shaanxi	Yulin	74	22	29·73	(22/74) 29·73
Tibet	Lhasa	113	41	36·28	(41/113) 36·28
Jilin	Tonghua	14	2	14·29	(11/67) 16·42
	Yongji	26	8	30·77	
	Jiutai	27	1	3·70	
Liaoning	Fengcheng	28	20	71·43	(39/84) 46·43
	Anshan	27	4	14·81	
	Huanren	29	15	51·72	
Shanxi	Lvliang	50	9	18	(41/245) 16·73
	Datong	195	32	16·41	
Hebei	Baoding	170	27	15·88	(27/170) 15·88
Shandong	Dongying	90	13	14·44	(13/90) 14·44
Henan	Anyang	94	14	14·89	(14/94) 14·89
Anhui	Hefei	143	47	32·86	(47/143) 32·86
Sichuan	Panzhihua	31	4	12·90	(15/73) 20·55
	Hejiang	42	11	26·19	
Hubei	Suizhou	82	22	26·83	(22/82) 26·83
Chongqing	Jiangjin	30	12	40·00	(15/53) 28·30
	Wanzhou	23	3	13·04	
Hunan	Xinhuang	29	7	24·14	(18/55) 32·73
	Xiangtan	26	11	42·31	
Guizhou	Guiyang	95	19	20	(47/251) 18·73
	Dushan	30	4	13·33	
	Yuping	32	6	18·75	
	Ziyun	32	5	15·63	
	Qinglong	34	7	20·59	
	Rongjiang	28	6	21·43	
Zhejinag	Hangzhou	52	1	1·92	(15/117) 12·82
	Jingning	36	8	22·22	
	Qingtian	29	6	20·69	
Guangdong	Qingyuan	36	14	38·89	(16/73) 21·92
	Zhaoqing	37	2	5·41	
Guangxi	Guilin	45	15	33·33	(48/154) 31·17
	Pingxiang	18	2	11·11	
	Henglin	31	12	38·71	
	Jingxi	35	13	37·14	
	Tianyang	25	6	24	
Yunnan	Kunming	90	41	45·56	(81/255) 31·76
	Ruili	32	18	56·25	
	Menghai	12	1	8·33	
	Jinghong	20	2	10	
	Yanshan	34	11	32·35	
	Honghe	32	8	25	
	Fuyuan	35	0	0	
Total		3690	1123	30·43	(1123/3690) 30·43

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experimentally infected sheep interacted with rRAP-1aα2 CT, indicating that the antibody responses of RAP-1 may be elicited in a *Babesia* sp. Xinjiang infection. High levels of antibodies against RAP-1 could be attained for 2·5 months. Moreover, IFAT analysis showed that immunofluorescence signals were observed using rabbit antiserum raised against XJrRAP-1aα2 CT in *Babesia* sp. Xinjiang, which is indicated the expression of native rhoptry proteins in *Babesia* sp. Xinjiang merozoites. These data indicated that the RAP-1 proteins exist in *Babesia* sp. Xinjiang and suggested that XJrRAP-1aα2 CT could be a potential specific antigen for the detection of *Babesia* sp. Xinjiang antibodies.

Until now, there is no standard *Babesia* sp. Xinjiang ELISA assay based on specifically recombinant protein reported. In the present study, based on the calculated specificity, sensitivity and positive threshold value, the results of a sero-investigation indicated that *Babesia* sp. Xinjiang infections were widespread in China. The results similar to a recent report, and a positive rate as high as 68·13% was found from the Shandong Province (Guan *et al*. 2012*b*). The positive samples in our study were from the Shaanxi and Jilin Provinces, with 29·73 and 16·24% rates, respectively; however, no positive samples were found in these two provinces in a previous study (Guan *et al*. 2012*b*). The distribution of *Babesia* sp. Xinjiang infection in small ruminants shows a tendency to expand.

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