Research Article

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Preparation of monoclonal antibodies against Bc48 and development of a rapid detection assay for infection with *Babesia caballi* in China

Panju Wang^{1,*}, Jingjing Song^{1,*}, Ruiqi Song¹, Mengyuan Zhang¹, Lijiang Wu¹, Fangxin Li¹, Yan Yan², Jiyong Zhou², Bayin Chahan¹, Min Liao²

¹Parasitology Laboratory, Veterinary College, Xinjiang Agricultural University, Urumqi, Xinjiang, P. R. China;

²Key Laboratory of Animal Virology of Ministry of Agriculture, Zhejiang University, Hangzhou, P. R. China

* These authors contributed on the work equally

Abstract: *Babesia caballi* (Nuttall, 1910) is one of the causative agents of equine piroplasmiasis which causes economic losses to horse industry in China. There is an urgent need for rapid detection method for *B. caballi* infection in Xinjiang Province, China. To prepare monoclonal antibodies (mAbs) against Bc48 gene of *B. caballi* (Xinjiang local strains) and establish colloidal gold-immunochromatographic (ICT) assay for diagnosis of the disease, recombinant Bc48 was expressed and purified from *Escherichia coli*. With purified Bc48 as immunogen in mice, three hybridoma cells named 11F4, 1H2 and 7F4 secreting mAbs against Bc48 of *B. caballi* were obtained, which showed strong reaction with recombinant Bc48 and Bc48 gene transfected cells. Furthermore, colloidal gold labelled ICT assay based on purified Bc48 recombinant antigen and its mAb was developed. The ICT assay showed high sensitivity and specificity and no cross-reaction with *Theileria equi* (Laveran, 1901). Total of 56 horse serum samples collected from Xinjiang were tested by ICT and compared with the detection by commercial ELISA kit. The results showed that 32 out of 56 serum samples were positive by ICT and 33 were positive by ELISA. ICT assay had high coincidence (98%) to the reference ELISA kit. mAbs and ICT developed in this study could be provided as an efficient diagnosis tool for infection with *B. caballi* in horse in Xinjiang area.

Keywords: Rhoptry protein of *B. caballi* (Bc48), hybridoma cells, colloidal gold-immunochromatographic test

Equine piroplasmiasis (EP) is a tick-borne blood protozoan disease caused by *Babesia caballi* (Nuttall, 1910) and *Theileria equi* (Laveran, 1901), which leads to anemia, edema, fever and jaundice in horse (Holbrook 1969). Equine piroplasmiasis has a serious impact on horse international trade (Friedhoff et al. 1990) and brought huge economic losses to horse industry (Kuttler and Kuttler 1988).

Several methods have been developed to detect *B. caballi*. Complement fixation test (CFT) is a commonly used method for the detection of equine infections with *B. caballi* (see Frerichs et al. 1969), but low sensitivity limited its wider application in the field. Occasionally, indirect immunofluorescence assay (IFA) is also used (Ogunremi et al. 2008), but this method is time-consuming and requires technical expertise. The enzyme-linked immunosorbent assay (ELISA) has been used to detect the antibody against equine *B. caballi*, but due to cross-reactivity with the antibody against *T. equi* (see Weiland 1986, Bose and Peymann 1994), this method is not used routinely.

Bc48 is rhoptry protein of merozoites of *B. caballi* with a molecular mass of 48kDa, which was previously evaluated as a promising antigen for the serological detection of antibody to *B. caballi* (see Ikadai et al. 1999a). Monoclonal antibody (mAb) raised against the rhoptry protein showed inhibition of the growth of the parasite (Ikadai et al. 1999b). Competitive ELISA for detection of *B. caballi* has been developed using mAb raised against Bc48 (Brining et al. 1997). These findings indicate that Bc48 and its mAbs might be good candidates for serological diagnosis of infection with *B. caballi*.

Infection of horses with *B. caballi* is common in many countries. In Xinjiang province of China, 18–30% of total equine population was found to be seropositive against *B. caballi* when tested using commercial ELISA kit (Zhu et al. 2016). Considering the vast geographical area of Xinjiang province where horses are widely distributed in different peasant households, there is a need for development of detection assay for infection with *B. caballi* which can be applied in the field conveniently.

Address for correspondence: Bayin Chahan, Parasitology Laboratory, Veterinary College, Xinjiang Agricultural University, 311 Nongda East Road, Wulumuqi, Xinjiang, P. R. China. E-mail: bynch@hotmail.com
Min Liao, Key Laboratory of Animal Virology of Ministry of Agriculture, Zhejiang University, 866 Yuhangtang Road, Hangzhou, P. R. China. E-mail: liaomin4545@zju.edu.cn

Fig. 1. SDS-PAGE analysis of tBc48 recombinant protein in *Escherichia coli*. M – protein molecular weight marker; 1 – lysates from *E. coli* without induction; 2 – lysates from *E. coli* after induction; 3 – purified recombinant proteins.

In this study, we first prepared and characterised the monoclonal antibodies (mAbs) against Bc48 protein of *B. caballi* of the Xinjiang local strain, followed by the development of a colloidal gold-immunochromatographic assay for diagnosis.

MATERIALS AND METHODS

Recombinant *Escherichia coli*, clinical samples, mice and other materials

Recombinant *E. coli* carrying truncated *Bc48* (*tBc48*) gene of local strain of *Babesia caballi* cloned into pET-28a vector was prepared by our research group and stored in the Laboratory of Parasitology of Xinjiang Agricultural University (Wang et al. 2018). Horse bloods were collected from horses in Ili Prefecture, Xinjiang. Six-week-old Bagb albino C (laboratory bred strain of the house mouse) (BALB/c) female mice were purchased from the Experimental Animal Center of Zhejiang University. Reagents and materials related to colloidal gold-immunochromatographic test (ICT) were purchased from Shanghai JieYi company (JieYi, Shanghai, China).

Purification of recombinant truncated Bc48 and immunisation of mice

The recombinant *E. coli* carrying pET-28a-tBc48 plasmid was cultured in Luria-Betani medium at 37°C and then tBc48 protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Recombinant tBc48 was purified according to the method described previously (Loughran et al. 2006).

Three six-week-old female BALB/c mice were immunised with 100 µg of purified recombinant tBc48 emulsified with equal volume of Freund's complete adjuvant (Sigma, St. Louis, MO, USA) by subcutaneous injection. At two-week-interval additional two boost injections with the same dose of antigen emulsified with equal volume of Freund's incomplete adjuvant (Sigma, St. Louis, MO, USA) were administered to the mice. The titres of antibody against Bc48 in the sera of immunised mice were monitored by indirect ELISA with tBc48 as antigen. The mouse with highest antibody titre was given a booster immunisation with 150 µg recombinant protein by intraperitoneal injection. Then, the immunised mouse was subjected to prepare hybridoma cells 3 days post final booster.

Preparation of monoclonal antibody to tBc48

SP2/0 myeloma cells were cultured a week prior to the organ harvesting. Spleen of the immunised mice was removed and spleen cells were suspended in Roswell Memorial Institute (RPMI)-1640 medium (Gibco-BRL Life Technologies, Grand Island, NY, USA). Spleenocytes were fused with SP2/0 myeloma cells using 50% polyethylene glycol-4000 (PEG4000). The resulting hybridoma cells were cultured in RPMI-1640 medium containing HAT (hypoxanthine–aminopterin–thymidine) and HT (aminopterin–thymidine) (Sigma) for 10–14 days. The hybridoma cells secreting antibody against tBc48 were screened by ELISA and then subcloned 4–5 times by limiting dilution assay. Secreting antibody was finally confirmed by IFA of tBc48 transfected 293T cells.

Ascites fluid containing mAb was prepared by injecting 5×10^5 selected hybridoma cells into the abdominal cavity of each mouse. One week prior to the culture of ascitic fluid, mice were injected with sterile liquid paraffin. mAbs collected from ascitic fluid were purified using protein-G beads (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Immunoglobulin subtypes of the mAbs were identified with Southern Biotechnology Associates Clonotyping™ Horseradish peroxidase system (HRP) according to the manufacturer's protocols (Southern Biotechnology Associates, Birmingham, AL, USA).

Enzyme-linked immunosorbent assay (ELISA)

Antibody titre of the sera of tBc48 immunised mice, supernatant of hybridoma cell culture and ascitic fluid were determined by ELISA. Briefly, 100 µl of the tBc48 protein (2 µg/ml in 50 mM sodium carbonate buffer, pH 9.6) were added into each well of a 96-well microtitre plate and then incubated overnight at 4°C. The plate was then washed three times with phosphate buffered saline with Tween-20 (PBST) (0.05% Tween-20 in PBS) and blocked with 100 µl/well of phosphate buffered saline (PBS) containing 5% skim milk and incubated at 37°C for 1 h. Plate was washed three times with PBST and then incubated with mouse sera or mAbs for 1 h at 37°C. After wash, the plate was incubated with 100 µl of HRP-conjugated goat anti-mouse IgG (KPL company, Gaithersburg, MD, USA) (1 : 5000) for another one hour at 37°C. The plate was then washed again and the colorimetric reaction was developed using hydrogen peroxide and o-phenylenediamine (OPD, Sigma) for 10 min at 37°C. The reaction was stopped with 2 mol/l H₂SO₄ and the optical density (OD) was recorded at 450 nm.

Table 1. Characterisation of the three monoclonal antibodies to Bc48 of *Babesia caballi* (Nuttall, 1910)

mAb	mAbs against Bc48 of <i>B. caballi</i>		
	11F4	1H2	7F4
Titres of supernatant	1 : 6,000	1 : 5,000	1 : 10,000
Titres of ascites	1 : 64,000	1 : 51,200	1 : 64,000
Subclass	IgG2a	IgG1	IgG2a
Type of light chain	Kappa	Kappa	Kappa

Table 2. Comparison of the immunochromatographic test with ELISA for detection of antibodies to *Babesia caballi* (Nuttall, 1910) in horses in Xinjiang

ELISA results	ICT test results		
	Positive	Negative	Total
Positive	32	1	33
Negative	0	23	23
Total	32	24	56

Indirect immunofluorescence assay (IFA)

To detect the reactivity of the mAbs, 293-T cells in 96-well plate were transfected with the tBc48 recombinant plasmid. After 48 h, the cells were fixed with 4% paraformaldehyde and then incubated with mAbs for 1 h at 37°C, followed by three washes with PBS. Subsequently, the cells were incubated with fluorescein isothiocyanate labelled goat anti-mouse IgG secondary antibody (KPL company, Gaithersburg, MD, USA) for 1 h at 37°C. The stained cells were rinsed five times with PBS and then visualised with a fluorescence microscope (Zeiss, Oberkochen, Germany).

Preparation of gold nanoparticle and conjugated antigen

Gold nanoparticle was prepared by deoxidisation of chloroauric acid with trisodium citrate. Briefly, 1 ml chloroauric acid (HAuCl₄) solution (Jieyi, Shanghai, China) was added into a boiling ddH₂O (around 100 ml) containing 10% (w/v) trisodium citrate. After the colour turned from gray to black and then to red, the solution was continuously boiled for another 8 min at 100°C. Solution was cooled to room temperature and 100 ml ddH₂O was added and stored in dark.

For preparation of gold nanoparticle conjugated antigen, 0.315 ml purified tBc48 protein (1.03 mg/ml) was added to 10 ml of above mentioned gold solution and pH was adjusted to 7.5 with 0.1M K₂CO₃ buffer. The solution was constantly stirred for 30 min and after that 1 ml 10% BSA was added to make the final concentration of 1%. After stirring for another 30 min, the solution was centrifuged at 12,000r/min for 15 min. Ninety percent of the supernatant was discarded and the remaining was used for resuspension of the pellets to get the gold conjugated tBc48 antigen.

Assembly of the immunochromatography test strip

Before assembly of the immunochromatography test (ICT) strip, purified tBc48 with concentration of 0.5 mg/ml was jetted linearly on a nitrocellulose membrane as test line and purified mAb 11F4 with concentration of 6.33 mg/ml was jetted on the same membrane as control line. Gold colloid conjugated antigen was sprayed on a glass fibre and dried in a vacuum drying instrument. Finally, the NC membrane, conjugated pad, sample pad, and absorbent pad were assembled on an adhesive card and cut into 3-mm-wide strips. To test the ICT strip, 10 µl of serum was added to the sample pad of the strip, and incubated for 5–10 min to observe the results.

Sensitivity and specificity test of ICT strips

Specificity of the ICT strip was tested with sera of the horses infected with *B. caballi* and *Theileria equi* along with uninfected negative control. Sensitivity of the method was measured by making serial dilution of the positive serum by two fold and each dilution was applied to the strip.

Detection of clinical samples with the ICT strip

Test samples consisting of 56 field horse sera were detected by ICT strip and ELISA with a commercial kit (VMRD®, Inc, Pullman, WA).

RESULTS

Purification of recombinant tBc48 as immunogen

Recombinant protein was expressed and purified from *Escherichia coli* with expected molecular mark of 15 kDa (see Wang et al. 2018) (Fig. 1).

This purified protein was further used in ELISA, preparation of mAb to tBc48 and development of ICT for detection of antibody against *Babesia caballi*.

Production of mAbs to Bc48

After three immunisation with recombinant protein tBc48, the spleen cells of immunised mouse with the highest antibody titre (1 : 25,600) were fused with SP2/0 cells. After three subclonings, three hybridoma clones persistently secreted antibodies reacting with tBc48 protein as detected by both ELISA and IFA were obtained and named 11F4, 1H2 and 7F4, respectively. The ascites was developed in mice with selected hybridoma cells. As indicated by ELISA, the antibody titres of the hybridoma cell culture supernatants and ascetic fluid of 11F4, 1H2 and 7F4 were 1 : 6,000 and 1 : 64,000, 1 : 5,000 and 1 : 51,200, 1 : 10,000 and 1 : 64,000, respectively (Table 1). SDS-PAGE analysis showed that the purity of purified antibody to Bc48 was high with two clear heavy and light chain protein bands (Fig. 2A). Furthermore, mAbs subtypes were identified by the Rapid ELISA Mouse mAb Isotyping Kit (Southern Biotechnology Associates) and the results revealed that mAbs 11F4, 1H2 and 7F4 were IgG2a, IgG1 and IgG2a subtypes, respectively, with Kappa light chains (Fig. 2B).

Fig. 2. Characterisation of monoclonal antibodies to tBc48. **A** – SDS-PAGE analysis of purified mAbs; **B** – identification of subtypes of mAbs by ELISA. M – protein molecular weight marker.

Reactivity of the mAbs

The reactivity of mAb 11F4, 1H2 and 7F4 was confirmed by Western blot analysis with recombinant tBc48 and IFA in tBc48 transfected 293T cells. All three mAbs reacted clearly with purified recombinant tBc48 (Fig. 3A), and Bc48 transfected cells (Fig. 3B).

Assembly of the immunochromatographic (ICT) strips

In the ICT strip system, purified tBc48 (0.5 mg/ml), purified mAb 11F4 (6.33 mg/ml) were sprayed linearly on a nitrocellulose membrane, as test and control lines, respectively. The nitrocellulose membrane sprayed with tBc48 and purified mAb to IgG, as well as the conjugated pad, sample pad and absorbent pad, were assembled on an adhesive card and cut into 3-mm-wide strips (Fig. 4A, lane 1). Test of the ICT strip was performed with *B. caballi*-positive and negative serum. As shown on the strips, positive reaction was indicated by purplish red on both test line and control line (Fig. 4A, lane 3). In contrast, negative reaction exhibited no colour on the test line (Fig. 4A, lane 2).

Specificity and sensitivity of the immunochromatographic assay

In order to validate the specificity of the assay, we tested the *B. caballi*-infected serum and compared with the detection of *T. equi*-infected serum using the strips. As shown in Fig. 4B, the ICT strip could specifically detect *B. caballi*-infected serum, but showed no cross-reactivity with *T. equi*-infected serum (Fig. 4B). In contrast, the strip could detect the antibody in the serum after 16 fold dilution (Fig. 4C).

Application of ICT strips on detection of field serum samples and compared with the detection by commercial ELISA kit

A total of 56 serum samples collected from horses in Xinjiang, China were detected by the use of the ICT strips, and the results were compared with those of a commercial

ELISA kit. As shown in Table 2, out of the total 56 serum samples, 32 were found to be positive by ICT, and 33 were positive by ELISA (Table 2). The positive coincidence rate of the ICT was 97% (32/33), and the total coincidence rate was 98% (55/56), when the commercial ELISA was used as reference.

DISCUSSION

Bc48 of *Babesia caballi* is a conserved protein which plays important role for merozoite invasion into red blood cells of the host and acts as potent immunogen (Ikadai et al. 1999a, b). Bc48 has been evaluated as potential antigen for serodiagnosis of infection with *B. caballi* (see Bose et al. 1994).

In the present study, we expressed and purified the recombinant truncated protein Bc48 from *E. coli*, which showed good immunogenicity and immunoreactivity in our previous study (Wang et al. 2018). This purified tBc48 were then used as antigen for ELISA, mAb preparation and ICT development. Three mAbs against Bc48 were prepared. They showed high reactivity with the purified recombinant protein as well as the ability to detect *Bc48* gene in transfected cells by immunofluorescent assay. These mAbs can be used as efficient tool for diagnosis and functional study of Bc48 during parasite infection in horses.

We further used the purified recombinant tBc48 protein and the mAbs in ICT development. As indicated by the tests, the ICT showed high specificity and sensitivity (Fig. 4B, C) comparable to the commercially available ELISA kit (Table 2). Considering that horses in Xinjiang are raised in free-range, ICT could be more convenient for diagnosis of infection with *B. caballi* in the area. The antigen and mAb used in the development of ICT were prepared originally from the local strain circulating in Xinjiang area.

The sequence analysis showed that the amino acid sequence of tBc48 of the local *B. caballi* strain had 91.74% identity to the corresponding sequence of USDA strain

Fig. 3. Reactivity of monoclonal antibodies. **A** – Western-blot analysis; **B** – IFA analysis of MAbs with Bc48 transfected 293T cells. M – protein molecular weight marker.

Fig. 4. Immunochromatographic test. **A** – ICT strip assembly and test. 1 – pretest strip; 2 – negative reaction; 3 – positive reaction. **B** – specificity test. 1 – positive serum to *Babesia caballi*; 2 – negative serum to *B. caballi*; 3 – positive serum to *Theileria equi*; 4 – negative serum to *T. equi*. **C** – sensitivity test. Positive serum to *B. caballi* was diluted at 1 : 2, 1 : 4, 1 : 8, 1 : 16, 1 : 32, 1 : 64.

(GenBank sequence ID: BAA83725.1), 98.3% identity to that of the reference strain from Cuba (GenBank sequence ID: ASS33866.2), 96.6% to those of strains from India (GenBank sequence ID: AXQ59181.1) and Brazil

(GenBank sequence ID: AXY04137.1), around 91.7% to those of strains from Mongolia (GenBank sequence ID: BAL72551.1) and Egypt (GenBank sequence ID: ALL27276.1), and around 97.0% to those of strains from Thailand (GenBank sequence ID: AMR43788.1) suggesting that Bc48 is conserved among different strains but with some mutations. Therefore, the tBc48 is suitable for application in serological diagnosis of *B. caballi* infection especially in Xinjiang area.

Since there is still no vaccine available against *B. caballi* and equine piroplasmiasis, the level of antibody against *B. caballi* in horse sera could indicate the infection status of *B. caballi*. Our data show that ICT has potential use for diagnosis of *B. caballi* infection in equine population.

To conclude, three monoclonal antibodies to *B. caballi* were produced by hybridoma technology in the present study. Based on recombinant Bc48 and the mAbs, ICT for detection of antibody to *B. caballi* was developed. The ICT kit showed superior specificity and sensitivity compared to the commercial ELISA kit. ICT could be utilised as an efficient diagnostic tool for *B. caballi* infection in horses.

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