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Original article

Immunomics analysis of *Babesia microti* protein markers by high-throughput screening assay

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ABSTRACT

Babesia microti is a protozoan considered to be a major etiological agent of emerging human babesiosis. It imposes an increasing public-health threat and can be overlooked because of low parasitemia or mixed infection with other pathogens. More sensitive and specific antigens are needed to improve the diagnosis of babesiosis. To screen the immune diagnostic antigens of *B. microti*, 204 sequences from homologue proteins between *B. microti* and *B. bovis* genome sequences in PiroplasmaDB were selected. The high throughput cloned and expressed *B. microti* proteins were screened with the sera from the BALB/c mice infected by *B. microti* using protein arrays. Ten (5.9%, 10/169) highly immunoreactive proteins were identified, and most (80%, 8/10) of these highly immunoreactive proteins had not been characterized before, making them potentially useful as candidate antigens for the development of diagnostic tools for babesiosis.

1. Introduction

Babesia microti is a tick-borne intraerythrocytic protozoan, considered to be a major etiological agent of emerging human babesiosis and responsible for the majority of cases of transfusion-transmitted babesiosis in the United States (Moritz et al., 2016; Vannier and Krause, 2015). Human babesiosis, a malaria-like disease, poses an increasing public health threat in the People's Republic (P.R.) of China (Vannier and Krause, 2012). The clinical manifestation in patients with *Babesia* infection varies substantially from asymptomatic presentation to severe and occasionally fatal infections. *B. microti* and *B. microti*–like organisms have been reported to cause illness in Japan, the P.R. China, and other Asia-Pacific regions (Zhou et al., 2014a, 2014b). In China, *B. microti* parasites have been found in local ticks, wild animals, and humans. Therefore it was regarded as an emerging public health threat (Zhou et al., 2013, 2014a, 2014b). The diagnosis of parasitological diseases has a major disadvantage due to its low sensitivity in cases of low density of parasites or co-infection cases. Human babesiosis may have previously been overlooked because of low parasitemia or mixed infection with other pathogens (Zhou et al., 2015). Additionally, parasitological detection is normally labor-intensive and time-consuming, and large-scale surveillance of disease transmission are strongly dependent on sensitive and accurate diagnostic methods (Xu et al., 2014). The development of more sensitive and specific antigens is needed for the rapid diagnosis of babesiosis for the disease control program.

The development of the genome, transcriptome, proteomics, and metabolomic information of parasites as well as the research technology of large-scale high-throughput sequencing and screening has laid a solid foundation for studies on the process of parasite gene regulation, expression, and interaction with the host. Genome data of *B. microti* has been released in recent years (Cornillot et al., 2012). Transcriptome data can provide extensive information on expressed genes, and it is helpful to screen the molecular surface of merozoites, but the current transcriptome data of *B. microti* are insufficient. The apical organelles of the *Babesia* merozoites are associated with its invasion of host cells and the immunity activities between the host and *Babesia* spp. These may participate in the adhesion between the merozoites and the erythrocyte membrane, related molecular diagnosis, vaccine and drug target candidates (Lobo et al., 2012; Tonkin et al., 2011). However, the existing studies on relevant candidates of *B. microti* merozoites are limited.

The protein array, as an important proteomics technology, is one of the high-throughput screening systems with the advantages of high throughput as well as fast and parallel detection (Claessens et al., 2011; Steenkeste et al., 2009; Winzeler, 2008). In combination with high-

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throughput gene cloning and protein expression, a parallel analysis of thousands of protein samples, such as the antigens and antibodies, the interaction between ligand and the receptor can be performed and has the advantages of high sensitivity and accuracy (Dharia et al., 2010). This technology has already been applied in *Toxoplasma*, *Plasmodium*, and *Schistosoma* during the related studies (Bahl et al., 2010; Chen et al., 2010, 2014, 2015; Fan et al., 2013; Lu et al., 2014).

In the current study, based on the bioinformatics, the native database of *B. microti* was composed with 204 sequences. The In-Fusion clone, wheat germ cell-free (WGCF) protein synthesis system and high-throughput proteomics assay were applied to screen some candidates for *B. microti* infection.

2. Materials and methods

2.1. Genes/open reading frames (ORFs) selection

To screen the immune diagnostic antigens of *B. microti,* homologue proteins between *B. microti* EST and *B. bovis* genome sequences (http://piroplasmadb.org/piro/) were selected by high blasting identification. High blast identity transcriptions between *B. microti* EST results and *B. bovis* genome sequences were researched first. Then, we set up sampling data for 500 sequences in length distribution of blast results. Finally, an optimal span with most of the sequences' size between 500 bp and 1500 bp was selected because as the reliability of producing desired polymerase chain reaction (PCR) products decreases as the length of the genomic DNA fragment increases (Chen et al., 2010, 2014; Lu et al., 2014). The antibody epitopes of the sequences were predicted online by Immune Epitope Database (IEDB) analysis resources (http://tools.immuneepitope.org/bcell/). The latest SignalP 4.1 server was used to predict the presence and location of signal peptide cleavage sites in amino acid sequences from different organisms. The method incorporates a prediction of cleavage sites and a signal peptide/non-signal peptide prediction based on a combination of several artificial neural networks (Petersen et al., 2011). The trans-membrane region and topology structure of the proteins were predicted by TMHMM server v.2.0 (http://www.cbs.dtu.dk/services/TMHMM/) and Phobius tools (http://phobius.cbr.su.se/).

2.2. Design of PCR primers

In-Fusion cloning allows for the joining of a vector and insert, as long as they share 15 bases of homology at each end. Therefore, In-Fusion PCR primers must be designed in such a way that they generate PCR products containing ends that are homologous to those of the vectors. The design pattern is also described in the previously reported study (Chen et al., 2010, 2014).

2.3. Preparation of linearized vectors

The pEU-E01-His-TEVMCS-N2 (pEU, Cell Free Sciences, Matsuyama, Japan) vector was used for In-Fusion cloning. The vector was first linearized by double digestion and purified. The quality of the digestion products was determined by 1% agarose gel electrophoresis, and the concentration of linearized vectors was measured using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technology, Rockland, DE).

2.4. PCR amplification of sequences

Plasmid DNA and ds cDNA of *B. microti* (*B. microti* ATCC PRA-99™) were used for PCR amplification of target genes. Each gene was amplified in a 25-μl PCR reaction containing 12.5 μl of Taq Platinum PCR MasterMix (Tiangen, Beijing, China), 0.5 μM each of the sense and antisense primers, 1 μl of plasmid DNA (20 ng/μl). The selected unique genes were amplified in a 96-well format. Some of the unamplified targets were amplified in a 20-μl PCR reaction containing 0.4 U Phusion High-Fidelity DNA Polymerase (Finnzymes, Espoo, Finland). The quality of each gene product was measured using 1% agarose gel electrophoresis and stained in a thidium bromide solution. The PCR products were visualized on an ultraviolet (UV) transilluminator, and the images were scanned with an imaging system (Gel Doc XR+, Bio-Rad, Hercules, CA).

2.5. In-Fusion cloning of PCR products

The In-Fusion cloning system enables vector and insert DNA sequences to be seamlessly joined in a ligation-independent reaction. This property of the In-Fusion process has been exploited in the design of vectors for the expression of proteins with precisely engineered His-tags (Berrow et al., 2009; Sleight and Sauro, 2013).

High-quality PCR products achieved on an agarose gel as a single, dense band of DNA, were treated with the Cloning Enhancer (Clontech). The cloning enhancer-treated PCR producer (2 μl) and 50 ng of the linearized pEU-His vector were mixed with In-Fusion Enzyme (Clontech) in the PCR tubes and incubated for 15 min at 37 °C, followed by 15 min at 50 °C. Solutions were then placed on ice. All reactions were diluted 1:5 with TE buffer (10 mM Tris·HCl pH 8.0, 1 mM EDTA) and then 5 μl diluted solutions was used to transform DH5α-competent cells (Tiangen, Beijing, China). Transformants were selected and screened by PCR amplification. Positive colonies were cultured, sequenced, and analyzed using the DNASTAR analysis software (DNASTAR Inc., Madison, WI).

2.6. Collection serum of the different infection stages from BALB/c mice infected by B. microti

The clinical characteristics of babesiosis in the majority of babesiosis include mild to moderate malaria-like symptoms. To establish the experimental animal model of *B. microti* (strain of ATCC PRA-99™, the same one used in preparing Plasmid DNA and ds cDNA of *B. microti*) in different infection stages, 8 BALB/c mice were infected with *B. microti* by intraperitoneal injections. Before intraperitoneal injection, blood from the mouse (red blood cell infection rate is approximately 60%) was taken from the eyelids, anticoagulated with ethylenediaminetetraacetic acid (EDTA), mixed with sterile 0.9% physiological saline in a ratio of 1:2, and infected by 100 μl per BALB/c mouse via intraperitoneal injection (Lu et al., 2012). Sera from 7 different infection points were collected: sera from 7 days, 14 days, 21 days, 28 days, 2 months, 4 months, and 5 months, respectively. The normal serum was collected before infection and treated as the negative control. In different infection stages, mice were anesthetized using 50% isoflurane, and blood collection (approx. 800 μl) was performed via retroorbital bleeding. The blood was collected in microcentrifuge tubes and left at room temperature for 1 h. The infection rate was monitored by blood smears on each sera collection point. The erythrocyte infection rate of BALB/c mice peaked at Day 7 (82.4%). After that, the parasites in peripheral blood began to decrease, D12 erythrocyte infection rate fell below 20%, and on D20 erythrocyte infection rate dropped to 2%. The low parasitemia persisted for about one month and developed to the inapparent infection later. Other blood samples were spun down at 4 °C, 10,000 × g for 10 min. The sera were then separated from the coagulated blood, transferred into new 1.5-ml microcentrifuge tubes, and stored at −80 °C until use. All animal experimental protocols followed National Institution of Parasitic Diseases (NIPD) of China guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of the NIPD of China.

2.7. Protein expression and Western blots

Cell-free protein synthesis systems can synthesize proteins with high speed and accuracy, but the yields are low due to their instability over

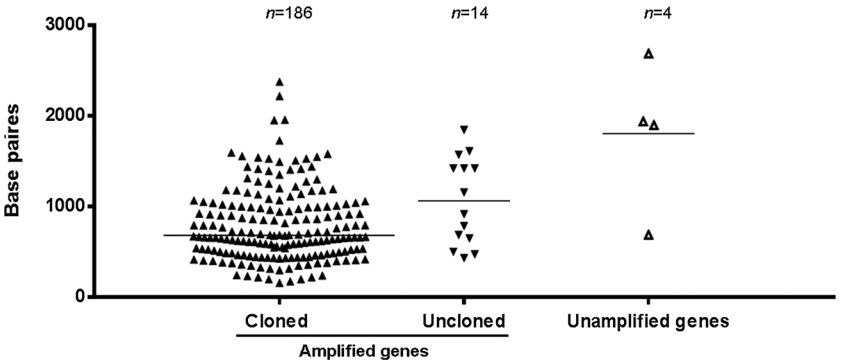

Fig. 1. Size distribution of *B. microti* genes selected for PCR amplification and In-Fusion cloning. Both size distribution of amplified and unamplified ($p = 0.004 < 0.05$), and cloned and uncloned genes ($p = 0.02 < 0.05$) were significantly different.

time (Endo and Sawasaki, 2003). By improving this cell-free system (Endo and Sawasaki, 2004), the application of wheat germ cell-free (WGCF) system-based protocol for the discovery of malaria vaccine candidates was reported in 2008 (Tsuboi et al., 2008). Proteins of *B. microti* were expressed by a 226-μl WGCF system using the bilayer translation reaction method described previously (Chen et al., 2014). The total fraction of recombinant proteins was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. The separated proteins were transferred to 0.45-μm polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA). After blocking with 5% skim milk in tris-buffered saline with Tween (TBS/T), Penta-His antibody (QIAGEN) and secondary horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (IgG, Pierce) were used to detect His-tagged recombinant proteins. The immunoblots were incubated with diaminobenzidine (DAB). The results were documented by a ScanJet 5300C scanner (Hewlett-Packard). Crude protein samples were stored in aliquots at −80 °C until further use.

2.8. Protein expression analyzed by protein array

OPEpoxy glass slides (75 × 25 mm) were purchased and used for protein arrays (CapitalBio, Beijing, China). The protein assays were screened by sera from different infection stages. Each slide has 8 blocks, applying different sera from different stages: normal sera from BALB/c mice and sera from BALB/c mice of 7 days, 14 days, 21 day, 28 days, 2 months, 4 months, and 5 months post-infection. Each block with 10 columns × 10 row wells, 2 continuous wells in 1 row were set as the duplication for 1 protein. Each block has 50 (5 × 10) proteins to be detected; the last 8 wells in each block were set as positive controls (His-tagged *Bm*SA1), the blank controls by phosphate-buffered saline (PBS), and the negative controls by WGF (wheat germ lysate with blank plasmid vector and without plasmid vector).

One nanoliter of each crude *B. microti* protein solution was spotted to each well of the array and incubated for 2 h at 37 °C. In addition, the array contained an area spotted with purified His-tagged *Bm*SA1, wheat germ lysate with blank plasmid vector, without any plasmid vector, and PBS, which were regarded as 1 positive and 2 negative controls and 1 blank control, respectively. The array was first blocked with 5% bovine serum albumin (BSA) in PBS containing 0.1% Tween 20 (PBS-T) for 1 h at 37 °C and incubated with Penta-His antibody (1 μl, 10 ng/μl) in PBS-T for 1 h at 37 °C. Antibodies were visualized with Alexa Fluor 546 goat anti-mouse IgG (10 ng/μl, Introgen) in PBS-T for 1 h at 37 °C and fluorescence intensities of array spots quantified by the fixed circle method were scanned using ScanArray Express software version 4.0 (PerkinElmer). The setting of the slide, the block, and the well incubated with expressed protein were analyzed by protein arrays.

2.9. Data analysis

A two-tailed unpaired Student *t*-test was used to compare the PCR amplification of genes and In-Fusion cloning, version 5.0 (GraphPad, San Diego, CA). Statistical differences of $p < 0.05$ were considered significant.

3. Results

3.1. PCR amplification of sequences and In-Fusion Cloning

Polymerases with proofreading characteristics were used for PCR amplification to increase the quality of the PCR products because the non-specific PCR products or primer dimmers might hinder the efficiency of cloning. From a total of 204 *B. microti* genes selected for PCR amplification, 200 genes were amplified successfully with the amplified rate 98.0% (200/204). The amplified genes have an average size of 812 bp (95% CI, 754–870 bp), which had statistical difference with 4 unamplified genes with an average size of 1802 bp (95% CI, 486–3117 bp, independent samples *t*-test, two-tailed, $p = 0.004 < 0.05$) (Fig. 1).

Four colonies per transformation were selected at random for screening by colony PCR to evaluate the efficiency of In-Fusion cloning. Of 200 genes screened, 186 were successfully cloned with the cloning rate of 93.0% (186/200). The successfully colonized sequences had an average size of 793 bp (95% CI, 734–851 bp) and 14 genes were unsuccessfully cloned with an average size of 1061 bp (95% CI, 782–1340 bp). Statistical analyses indicated that the cloned genes were significantly shorter than the uncloned genes in length (independent samples *t*-test, two-tailed, $p = 0.019 < 0.05$) (Fig. 1).

3.2. High-throughput expression of B. microti proteins

One hundred sixty-nine proteins expressed by the WGCF system were detected by Western blots. The rate of proteins detected by Western blots was 90.9% (169/186). A partial listing of the Western blots results is attached in Fig. 2. Of the 169 proteins detected by Western blots, 22 proteins were determined to contain the repeated sequences, and 54 proteins, analyzed by online software SignalP4.1, were determined to contain secreted proteins. The rates of amplified genes and In-Fusion cloning are 98.0% and 93.0%, respectively. The results were consistent with the reports by previous studies for high-throughput construction of expression vectors of *Plasmodium falciparum*, *Plasmodium vivax,* and *Schistosoma* carried out in our group (Table 1).

3.3. High-throughput analysis of B. microti protein by protein arrays

A total of 169 proteins were screened by sera collected from 8 different stages: normal BALB/c mice sera, sera of 7 days, 14 days, 21

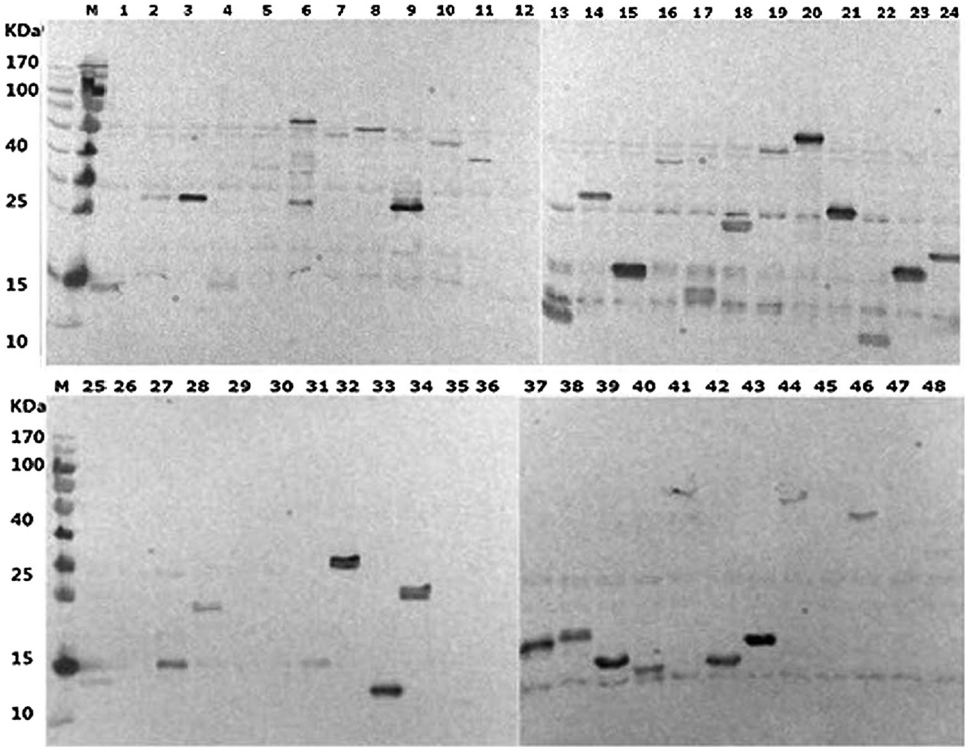

Fig. 2. Analysis of the level of expressed *B. microti* recombinant proteins by Western blots (partial) M: Protein marker; 1–48: The recombinant proteins.

Table 1
Efficiency of PCR amplification and cloning of parasite genes.

	PCR (%)	In-fusion cloning (%)	Expressed proteins (%)
B. microti	98.0% (200/204)	93.0% (186/200)	90.8% (169/186)
P. vivax [a]	90.8% (99/109)	92.9% (92/99)	93.4% (86/92)
P. falciparum [b]	79.2% (152/192)	90.8% (138/152)	85.5% (118/138)
S. japonicum [c]	97.0% (194/200)	99.0% (192/194)	98.4% (189/192)

[a, b, c] The results of PCR amplification and cloning of parasite genes were published in the previous studies (Bahl et al., 2010; Chen et al., 2014, 2015).

days, 28 days, 2 months, 4 months and 5 months post infection mice, respectively. Totally, there are 10 proteins screened to be highly immunoreactive proteins to sera collected in different infective stages. There is 1 secreted protein having signal peptides. This secreted protein has higher immunreaction from the sera collected on 7 days, 14 days, and 21 days and lasted until 5 months post-infection. There are 3 proteins with higher immunoreactive from the sera collected on 14 days, 4 beginning from sera of 21 days post-infection, and the last 2 having higher immunreactive from sera of 28 days post-infection. These 10 proteins have higher immunoreactivity with sera sustaining to 5 months. The screening results were shown in Fig. 3A & B.

Among these 10 highly immunoreactive proteins, 8 proteins were uncharacterized proteins, and 2 proteins were the reported seroactive antigens of *B. microti*: seroactive antigen BMN 1–3 and BMN 1–3-related protein (Lodes et al., 2000). The features, including the molecular weight (MW), isoelectric point (pI), signal peptides (SP), transmembrane domains (TMD,) and protein domains of 10 screened proteins of *B. microti* were analyzed (Table 2). One protein screened to have high immunoreactivity to sera from mice 7 days post-infection contained secreted peptides analyzed by SignalP4.1 software (http://www.cbs.dtu.dk/services/SignalP/). One protein contained the transmembrane domains analyzed by SMART (http://smart.embl-heidelberg.de/).

4. Discussion

The completion of genome, transcriptome, and proteome of *Babesia*, and these new approaches, such as high-throughput screening, have the potential to identify more new vaccine target molecules that may induce greater efficiency than the current candidate *Babesia* antigens (Cornillot et al., 2012; Ellis et al., 2003; Gull, 2000; Gutierrez, 2000). However, annotation and validation of more than 10,000 *Babesia* genes and proteins are difficult, and there is an urgent need to discover new antigens or vaccine candidates using innovative screening approaches. Reverse vaccinology and high-throughput genome-based screens are 2 interrelated techniques that hold much promises in this regard (Claessens et al., 2011; Steenkeste et al., 2009; Winzeler, 2008). Thus, the establishment of high-throughput cloning and protein expression system would be extremely helpful.

The In-Fusion cloning method, which is based on the In-Fusion enzyme, could be used in combination with any 15-bp homology region to enable ligation-independent cloning of PCR products to any linearized vector. We used In-Fusion cloning methods to construct a total of 186 vectors from 200 PCR products of *B. microti* by colony PCR screening of 4 randomly selected colonies. It had an overall cloning efficiency of 93.0% (186/200), which is almost equal to the rate of cloning of *P. vivax* genes conducted previously (e.g., 92.9%). The results by other studies for high-throughput construction of expression vectors of *P. falciparum* (e.g., 97% PCR product to expression clone efficiency) and construction of expression vectors of *Schistosoma mansoni* and *S. japonicum* (e.g., 100%) using the homologous recombination in *Escherichia coli*. The reasons for some PCR products not cloning by the In-Fusion cloning method are not clear, although the average size of uncloned genes is much higher than cloned genes both for genes from *B. microti, S. japonicum,* and *P. vivax*.

E. coli-based expression systems have been widely used for the expression of parasite proteins. However, it is difficult to efficiently produce many proteins due to the complexity of the parasite genome (Tsuboi et al., 2008). In contrast, it was reported 95% (89/94) of the genes of *P. vivax* (Chen et al., 2010) and 98.4% (189/192) of genes of *S.*

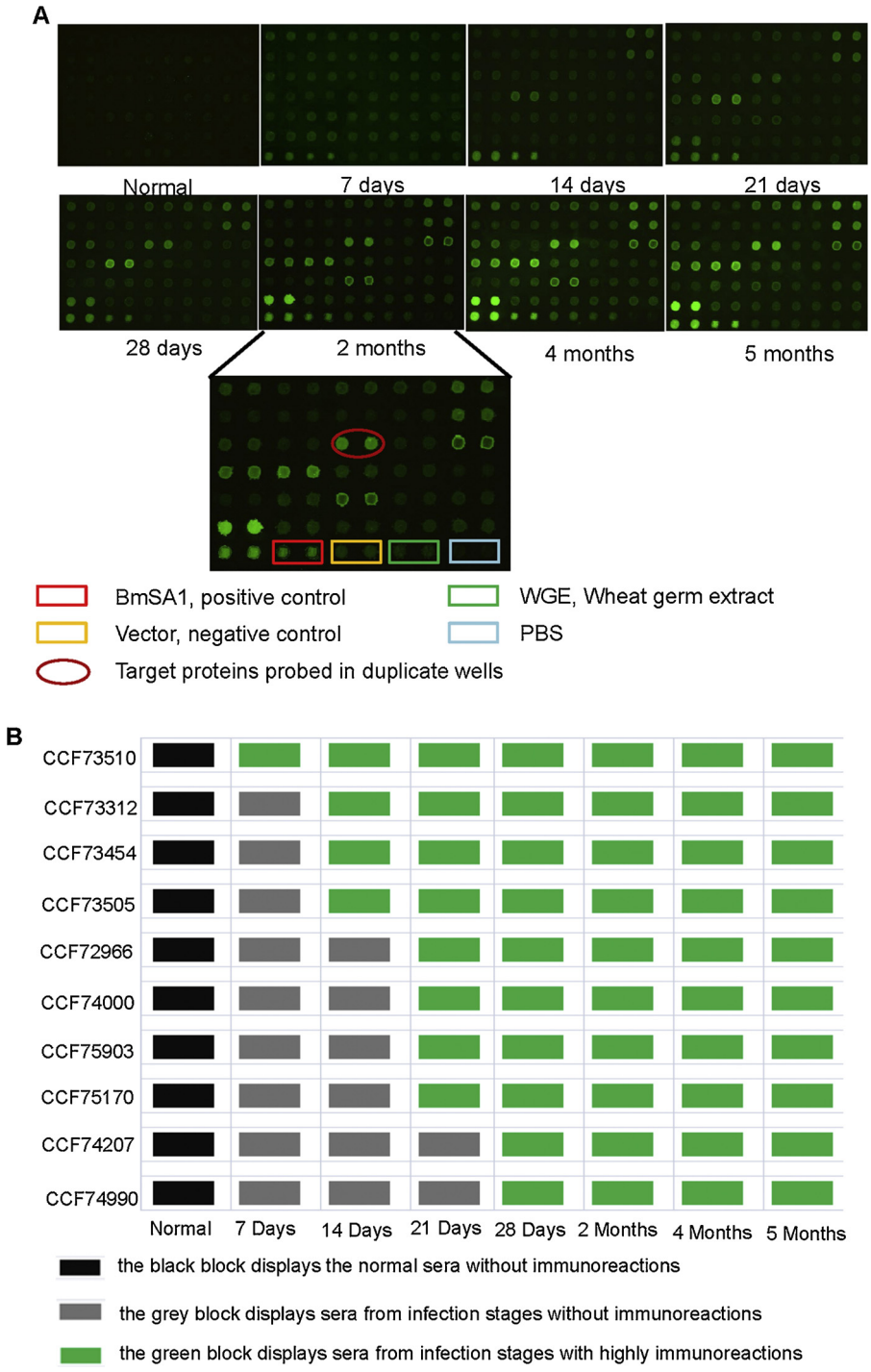

Fig. 3. The recombinant proteins detected by sera from infected BALB/c mice in different stages. A. Normal: the sera from the normal BALB/c mouse; the sera from the BALB/c mice infected after 7 days, 14 days, 21 days, 28 days, 2 months, 4 months, and 5 months. pEU-His vector, wheat germ extract (WGE), and PBS were used as negative controls, and BmSA1 was used as the positive control. **B**. The protein arrays were probed with mouse serum from 8 different infection stages, and a total of 10 proteins were detected.

japonicum (Chen et al., 2014) were expressed by the WGCF protein synthesis system. In the present, 169 of 204 genes (82.8%) have yielded protein products analysis by Western blots. Using protein assay, we developed a high-throughput method for the analysis of His-tag fusion protein expression.

The mortality rate associated with human babesiosis is estimated to be between 3 and 28%. Most severe cases occur in people over the age of 50 years or those who are asplenic, have cancer or human immunodeficiency virus (HIV), or who are on an immunosuppressive therapy (Vannier and Krause, 2012). Both host and parasite factors contribute to these symptoms, but the exact pathogenic mechanisms remain unknown (Krause et al., 2007). The majority of patients experience mild to moderate malaria-like symptoms; however, in severe cases, the disease may be associated with respiratory failure, multi-organ system dysfunction or coma (Krause et al., 2008; Leiby, 2011). Some of the babesiosis cases can even be co-infected with malaria, or latent infection in low parasitemia cases (Zhou et al., 2013, 2014a, 2014b). Based on the clinical characteristics of babesiosis, BALB/c mice

Table 2
Features of 10 proteins screened by protein assays with sera of different infection stages.

No.	Protein ID	MW	pI	SP[a]	TMD[b]	Protein Domain
1	CCF73510	24.08	5.39	yes	0	No
2	CCF74990	54.82	4.67	no	0	Herpes_BLLF1 domain
3	CCF73312	22.61	9.29	yes	0	No
4	CCF73454	34.83	8.28	no	0	GCC-2 GCC-3 domain
5	CCF75903	45.45	4.40	no	0	2A & PRK domain
6	CCF73505	32.84	9.29	no	1	S3 Ae domain
7	CCF72966	48.98	5.03	no	0	PTZ domain
8	CCF74000	17.55	5.83	no	0	No
9	CCF75170	30.67	4.68	yes	0	No
10	CCF74207	42.52	5.13	yes	0	No

[a] SP, Signal peptide, analyzed by SignalP4.1 (http://www.cbs.dtu.dk/services/SignalP/).
[b] TMD, transmembrane domain and protein domain analyzed by SMART (http://smart.embl-heidelberg.de/).

were infected with *B. microti* by intraperitoneal injections.

The blood smears were applied to detect the concentration of the parasites in the peripheral blood of the mice. The serum of different infection stages were collected from 7 days post-infection, then 14 days, 21 days, 28 days, 2 months, 4 months, and 5 months. These sera collected in developing stages of infections of *B. microti* partly imitated the different infection stages of *Babesia* in humans. Sera from 7 to 14 days post-infection displays the immunity and antibody of the host's peak parasitemia and fever stage. The later stages of 21 days, 28 days, 2 months, 4 months, and 5 months can repectively display the later recovery stages and even the latent infection stages.

In babesiosis cases, most symptomatic patients become ill 1 to 4 weeks after the bite of a *B. microti*–infected tick and 1 to 9 weeks after transfusion of contaminated blood products (Vannier and Krause, 2012). However, the antibodies detected by the available antigens are normally detected after the parasitemia. It has been reported that in the earliest stage of infection, before a detectable immunologic response, there is a "window period" (WP) in these babesiosis cases (Moritz et al., 2017). In this research, 10 stage-specific antigens were identified by protein assays and 1 protein was screened to have strong immunoreactions to the sera collected from 7 days, 14 days, and 21 days post-infection and until the sera of 5 months after infection was collected, if this candidate antigen has the same immunoreactions to human cases. Further evaluation is needed to determine the activities of this protein in human babesiosis cases. We would also evaluate 9 other candidates applied in surveillance populations in different infection stages of babesiosis.

In addition to the clinical infective cases, individuals with latent infections of *Babesia*, *Leishmania*, *Plasmodium*, *Trypanosoma*, and other protozoa taking residence in erythrocytes may experience asymptomatic infection, increasing the possibility of parasite transmission through the transfusion of contaminated blood products (Assennato et al., 2014; Fukutani et al., 2014). The omics studies on parasites, including genome, transcriptome, proteome and metabolomic studies have the main advantage of analyzing large amounts of data by high throughput technology and revealing the biological nature of parasites, pathogenic mechanism, and the relationship with the host. They have also provided a new basis to improve disease diagnosis, drug therapy, and vaccine target screening efficiency (Gutierrez, 2000).

5. Conclusions

From 169 proteins screened by protein assays, 10 (5.9%, 10/169) proteins were identified with highly immunoreactive. Most (80.0%, 8/10) of them were not characterized before.

Among 10 stage-specific antigens, 1 protein was screened to have strong immunoreactions from the sera collected from 7 days, and from 14 days until 5 months post-infection. This study could be expanded to evaluate the activities of this protein diagnosis of babesiosis cases and apply other candidates in surveillance of different infection stages of babesiosis populations.

Conflicts of interest

There are no conflicts of interest to declare.

Authors' contribution

XZ conceived the study, collected and analyzed the data, and drafted the manuscript. JLH, HMS, BX and JHC revised the manuscript and provided interpretation of the findings. JHC and XNZ conceived the project and provided technical support for data collection and analysis. All authors read and approved the final manuscript. Written consent to publish was obtained.

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