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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