NOTES

Molecular Cloning of a Babesia caballi Gene Encoding the 134-Kilodalton Protein and Evaluation of Its Diagnostic Potential in an Enzyme-Linked Immunosorbent Assay

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A Babesia caballi gene encoding the 134-kDa (BC134) protein was immunoscreened with B. caballi-infected horse serum. An enzyme-linked immunosorbent assay (ELISA) using recombinant BC134 protein could effectively differentiate B. caballi-infected horse sera from Babesia equi-infected or noninfected control horse sera. These results suggest that the recombinant BC134 protein is a potential diagnostic antigen in the detection of B. caballi infection.

Babesia caballi, like Babesia equi, is a tick-borne protozoan parasite which causes fever, anemia, jaundice, and edema in the infected horses and sometimes results in death (3, 4, 17, 19). These equine babesioses are known to induce significant economic losses in the horse industry (3, 5). These parasites are usually detected in blood smears during the acute stage of infection but not easily in those of the recovered animals that remain carriers of the parasite (8). Clinical signs are not specific diagnostic measures for babesiosis, especially in asymptomatic or mixed infection in areas of endemicity (1). Therefore, serological distinction of the two infections is very important for choice of prophylactic treatment, epidemiological surveillance, and strategies of babesiosis control since B. caballi-infected animals become carriers for 1 to 3 years while horses infected with B. equi are lifelong carriers of this pathogen (5, 8). Additionally, in view of the fact that these Babesia parasites are currently absent in Japan but an increasing number of horses is being imported from countries where the infection is endemic, the development of a highly specific and sensitive diagnostic system for B. caballi is an urgent necessity for the enhancement of quarantine.

The enzyme-linked immunosorbent assay (ELISA) using the whole lysates of B. caballi-infected erythrocytes was found to cause an extensive cross-reaction between B. caballi- and B. equi-infected horse sera (2, 24). Competitive-inhibition ELISA using recombinant antigens was also developed for the detection of B. equi and B. caballi infections (11, 12). Recently, we succeeded in developing an ELISA system that could specifically detect anti-B. caballi equine antibodies by using a recombinant BC48 protein (10). The ELISA using the recombinant antigen could clearly distinguish the B. caballi-infected horse sera from noninfected or B. equi-infected horse sera (10). However, in order to cover all sera infected with various types of field strains, it is necessary to conduct further studies to search for other immunodominant antigens applicable to epidemiological surveillance. Such studies might lead to a more practical use of ELISA worldwide. In this study, we succeeded in obtaining a novel cDNA clone by immunoscreening a B. caballi cDNA expression library. On the basis of a nucleotide sequence that we determined, a recombinant antigen was designed, produced in an Escherichia coli expression system, and subjected to ELISA for the evaluation of its diagnostic utility for B. caballi infection.

A B. caballi (U.S. Department of Agriculture strain) cDNA expression phage library (2.5 × 10^8 PFU/ml) was immunoscreened with experimentally B. caballi-infected horse serum according to the previously reported method (10, 20). Nucleotide sequencing was performed as described previously (7). Open reading frame (ORF) and protein homology searches were performed using the MacVector program (Oxford Molecular, Ltd., Oxford, United Kingdom) and the National Center for Biotechnology Information database, respectively. Two oligonucleotide primers, 5’-caggatcTGTATGATATGGTTGCCTTAGTTGGGG-3’ and 5’-gataccGCGCCCTTCTTTCGCTGGG-3’ (lowercase letters show BamHI and EcoRI restriction site linkers, respectively), were designed on the basis of the nucleotide sequence of the BC134 gene (accession number AB095267 in GenBank, EMBL, and DDBJ databases), and 2,876 bp of a DNA fragment containing the BC134 ORF was amplified by PCR (16). The amplified DNA product was digested with BamHI and EcoRI, purified with the QIAquick gel extraction kit (Qiagen, Inc., Hilden, Germany), and then ligated into the BamHI and EcoRI sites of a pGEX-4T E. coli expression plasmid vector (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, United Kingdom). The resulting plas-
mid, designated pGEX/BC134, was used to produce a recombinant BC134 protein fused with glutathione S-transferase (GST) (designated the GST-BC134 protein) in the E. coli BL21 strain (Stratagene) according to standard techniques (18). Next, the protein was purified as described previously (10, 22). The purified GST-BC134 protein was resuspended in phosphate-buffered saline to a final concentration of 0.1 mg/ml and used as an immune antigen for the production of an anti-BC134 protein mouse immune serum or as a diagnostic antigen for an ELISA system, as described below. Seven-week-old BALB/c mice (CLEA Japan, Tokyo, Japan) were intraperitoneally immunized with 20 μg of the purified GST-BC134 protein emulsified with the same volume of complete Freund’s adjuvant (Difco, Detroit, Mich.). At 2-week intervals, the mice were stimulated five additional times with the same amount of purified antigen emulsified with Freund’s incomplete adjuvant (Difco). Sera were collected from the mice at 10 days after the last booster. The indirect fluorescent antibody test was performed as described previously (27). Purification of the B. caballi cells and B. equi (U.S. Department of Agriculture strain) cells was performed according to the previously reported method (13, 14). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis were performed as described previously (26). An ELISA was performed as described previously (10, 25). Seven sera of noninfected control horses, eight sera of experimentally B. caballi-infected horses, and eight sera of experimentally B. equi-infected horses were obtained from the Equine Research Institute of the Japan Racing Association.

By immunoscreening with B. caballi-infected horse serum, the nucleotide sequence of one cDNA clone that showed the strongest immunoreactivity was determined. Computer-aided analysis demonstrated that the cDNA insert had a total of 3,227 bp (accession number AB095267 in GenBank, EMBL, and DDBJ) and contained a single ORF, which was designated the BC134 gene (Fig. 1). The ORF from the initial codon...
(position 285) to the terminal codon (TAA) (position 3137) contained 2,853 nucleotides, corresponding to 950 amino acid residues with a predicted molecular mass of 104 kDa. The predicted protein had an isoelectric point (pI) of 5.62, which was suggestive of the acidic nature of the BC134 protein. Of the deduced amino acids of the BC134 gene, 28.6% were charged. The predicted protein has a typical signal peptide sequence at the N-terminal end (Fig. 1, underlined).

A recombinant BC134 protein (GST-BC134) with a molecular mass of approximately 160 kDa was expressed in the transformed E. coli with pGEX/BC134 and then purified using glutathione-Sepharose 4B beads, as shown in Fig. 2. The GST-BC134 protein was also recognized with the B. caballi-infected horse serum in Western blot analysis (data not shown). Since the GST-BC134 protein contains a 26-kDa GST tag, the molecular mass of the GST-BC134 protein was estimated at approximately 134 kDa after removal of the GST tag. The detected size of the GST-BC134 protein, however, was still larger than the size estimated by computer-aided analysis (about 104 kDa).

A mouse immune serum was prepared against the purified GST-BC134 protein. In Western blot analysis (Fig. 3), the anti-recombinant BC134 protein mouse serum recognized only 134 kDa of the native protein in the lysate of B. caballi-infected erythrocytes (lane a) and not in the lysates of B. equi-infected and noninfected erythrocytes (lanes b and c). Next, in the indirect fluorescent antibody test with mouse antiserum to the recombinant BC134 protein, positive reactions were observed in all of the developmental stages of B. caballi in erythrocytes as well as in extracellular parasites (Fig. 4). In the intraerythrocytic stage of merozoites (the ring and subsequent pear-shaped forms), the BC134 protein appeared as a dense accumulation in the parasite cytoplasm (Fig. 4a, b, and c), which later blanketed the membrane of ruptured erythrocytes (Fig. 4c), and retained a denser mass or accumulation in the cytoplasm of extracellular merozoites (Fig. 4b and c).

To evaluate the efficacy of the recombinant BC134 protein as a diagnostic antigen, the purified GST-BC134 antigen was subjected to diagnostic ELISA (Fig. 5). All of the sera used were confirmed to be nonreactive to the GST control antigen (data not shown). Positive reactions to the GST-BC134 antigen were detectable in six out of eight B. caballi-infected horse sera at optical densities at 415 nm (OD415) higher than 0.25 (lane 1). However, the antigen showed a cross-reaction or a nonspecific reaction to B. equi-infected horse or healthy horse sera with OD415 below 0.25 in the ELISA. These results demonstrate that the ELISA with GST-BC134 antigen is able to distinguish between the sera of B. caballi-infected horses and those of B. equi- or noninfected horses at OD415 of 0.25 and higher.

In the present study, a B. caballi-cDNA expression phage library was screened with serum from a horse experimentally infected with B. caballi, and a novel recombinant BC134 protein of B. caballi was identified as the most immunoreactive antigen. The deduced amino acid sequence of the BC134 gene has a signal peptide sequence at the N-terminal end, suggesting that it might function as a membrane-associated or secreted protein (21).
BC134 protein is a specifically Babesia bigemina (23), and the Be82 protein of the recombinant BC134 protein (6). Similar have disrupted the binding of sodium dodecyl sulfate to the dues and to the low pI of the predicted protein, which may attributed to the presence of highly charged amino acid resi-

The complete nucleotide sequence of the BC134 gene was identified, but the native BC134 protein, as well as the synthesized GST-BC134 protein, was found to be about 30 kDa larger than expected. This discrepancy in molecular masses may be sized GST-BC134 protein, was found to be about 30 kDa larger, but the native BC134 protein, as well as the synthe-

In the indirect fluorescent antibody test, the native BC134 protein was observed in the cytoplasm and/or membrane of the developmental stages of the intracellular parasites (the ring and subsequent pear-shaped forms) and free merozoites. The BC134 protein also seemed to have interacted with the cytoskeleton and/or the membrane of erythrocytes at the later phase of parasite development, which is associated with merozoite maturation and release and host cell rupture, similarly to the B. bovis rhoptry-associated protein 1 (27).

Although we already developed a diagnostic ELISA for B. caballi infection using a recombinant BC48 antigen (9, 10), we consider that further study is necessary to search for other immunodominant antigens applicable to diagnostic ELISA in order to cover all of the sera infected with various types of field strains. In the present study, an ELISA using a newly identified recombinant BC134 protein was developed and proved to be highly specific for the detection of B. caballi-specific equine antibodies at OD415 higher than 0.25. Although the diagnostic ELISA that we have developed detected B. caballi infection clearly, in the ELISA using the recombinant BC134 protein two B. caballi-infected serum samples tested negative. Although the number of serum samples was not enough to ex-

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