Cloning of a Novel *Babesia equi* Gene Encoding a 158-Kilodalton Protein Useful for Serological Diagnosis

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In this study, we characterized a *Babesia equi* Bet158 gene obtained by immunoscreening a *B. equi* cDNA expression phage library with *B. equi*-infected horse serum. The Bet158 gene consists of an open reading frame of 3,510 nucleotides. The recombinant Bet158 gene product was produced in *Escherichia coli* and used for the immunization of mice. In Western blot analysis, mouse immune serum against the Bet158 gene product recognized 75- and 158-kDa proteins from the lysate of *B. equi*-infected erythrocytes. In an indirect fluorescent-antibody test with the mouse immune serum, the Bet158 antigen appeared in the cytoplasm of Maltese cross-forming parasites (which consist of four merozoites) and was located mainly in the extracellular merozoite body. When the recombinant Bet158 gene product was used in an enzyme-linked immunosorbent assay as a serological antigen, it was found to react to *B. equi*-infected horse sera, indicating that the Bet158 gene product is useful as a serologically diagnostic antigen for *B. equi* infection.

Babesiosis, a well-recognized disease of veterinary importance in horses, cattle, and dogs, is gaining attention as an emerging zoonotic disease (15). It has been found in a wide variety of mammals but is perhaps most prevalent in rodents, canivores, and cattle (25). Two species of *Babesia* parasites, *Babesia equi* and *Babesia caballi*, infect equids (22). Acute equine babesiosis is characterized by fever, anemia, icterus, hepatosplenomegaly, lethargy, and in some cases death (4, 5, 22), leading to great economic losses in the horse industry (14). The infected horses often remain carriers of the parasites for a long period and are known to act as sources for subsequent infections for other horses via tick vectors (11). Therefore, the development of a high-quality system for the serological diagnosis of babesial infection is necessary. In Japan, no clinical cases of equine babesiosis have been reported up to now (12), but there has been a long-term increase in the number of horses imported from foreign countries, including those from areas where equine babesiosis is endemic. The existence of two tick vectors, *Dermacentor reticulatus* and *Rhipicephalus sanguineus*, has also been reported in Japan (28). These conditions indicate that Japan is facing the risk of the introduction of infected or carrier horses.

Recently, we reported an enzyme-linked immunosorbent assay (ELISA) that is specific for the detection of equine anti-*B. equi* antibodies by using a recombinant Be82/236–381 gene product as the antigen (9, 10). The serodiagnostic ELISA could clearly distinguish the *B. equi*-infected horse sera from noninfected or *B. caballi*-infected horse sera (9, 10). However, in order to analyze all sera infected with various types of field strains, further study was necessary to search for other serological antigens applicable in epidemiological surveys. These studies might lead to a more practical usage of ELISA worldwide.

In this study, we identified a novel Bet158 gene by immunoscreening a cDNA library with *B. equi*-infected horse serum and characterized the gene product immunologically in *B. equi*-infected erythrocytes. Subsequently, the recombinant gene product was subjected to ELISA and evaluated for its serologically diagnostic utility against *B. equi* infection.

MATERIALS AND METHODS

**Parasites.** U.S. Department of Agriculture strains of *B. equi* and *B. caballi*, which had been kindly provided previously by the Equine Research Institute of the Japan Racing Association, were grown in equine erythrocytes in vitro as described by Avarzed et al. (1, 2). The parasite development was monitored by microscopic observation of Giemsa-stained thin smears.

**Immunoscreening of a *B. equi* cDNA library and DNA sequencing.** The immunoscreening and DNA sequencing were performed as described previously (9). Open reading frame (ORF) and protein homology searches were performed using the Mac Vector program (Oxford Molecular Ltd., Oxford, United Kingdom) and the National Center for Biotechnology Information database, respectively.

**Expression and purification of the recombinant Bet158 gene product in *Escherichia coli*.** Two oligonucleotide primers, 5′-aagctgaaAAATGAGTTACGCA CGCAGA-3′ and 5′-agcgccggctTTAACCATTGCTAGA-3′ (lowercase letters form Sall and NotI restriction site linkers, respectively), were used to amplify the Be158 gene from the cDNA clone by PCR (17). The amplified DNA was digested with Sall and NotI and then ligated into the Sall and NotI sites of a pgEX-4T E. coli expression plasmid vector (Amer sham Pharmacia Biotech, Little Chalfont, Buckinghamshire, United Kingdom). The resulting plasmid, designated pGEX/Be158, was used to transform the *E. coli* BL21 strain (Strat- age, La Jolla, Calif.) and express the recombinant Bet158 gene product fused with glutathione S-transferase (GST), designated GST/Be158 protein, by standard techniques (21). The GST/Be158 protein was purified from the soluble fraction with glutathione-Sepharose 4B (Amer sham Pharmacia Biotech), as described previously (9, 14, 23).

**Preparation of mouse anti-Bet158 protein immune serum.** Six-week-old female ddY mice (CLEA, Tokyo, Japan), which are often used for obtaining the specific immune serum in Japan, were intraperitoneally immunized with 0.2 ml of the purified GST/Be158 protein (0.1 mg/ml) emulsified with the same volume of...
Of diaminobenzidine/ml and 0.03% H2O2 to develop the color. An indirect
with PBS, the membranes were exposed to a substrate solution containing 0.5 mg
antibody (1:2,000; ICN Biochemicals, Aurora, Ohio) for 1 h. After three washes
horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG)
times with phosphate-buffered saline (PBS). The membrane was incubated with
mouse anti-Be158 protein immune serum (1:100) for 1 h and then washed three

The proteins were electrophoretically transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, Mass.). The blots were incubated with mouse anti-Be158 protein immune serum (1:100) for 1 h and then washed three times with phosphate-buffered saline (PBS). The membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) antibody (1:2,000; ICN Biochemicals, Aurora, Ohio) for 1 h. After three washes with PBS, the membranes were exposed to a substrate solution containing 0.5 mg of diaminobenzidine/ml and 0.03% H2O2 to develop the color. An indirect-fluorescent-antibody test was performed as described previously (27). In brief, smears of B. equi or B. caballi-infected test erythrocytes were prepared on slides and fixed in methanol at 3 min at 20°C. The mouse anti-Be158 protein immune serum (1:100) was applied as the first antibody on the fixed smear and incubated for 30 min at 37°C. After three washes with PBS, an Alexa Fluor 488 goat serum (1:100) was applied as the secondary antibody and incubated for 30 min at 37°C. The slides were washed three times with PBS, incubated with 25 μg of propidium iodide per ml (Molecular Probes) and 50 μg of RNase A (Roche, Basel, Switzerland) per ml for 10 min at 37°C, and then mounted in 50% glycerol–PBS with a coverslip. The slides were observed with a confocal laser scanning microscope (TCS NT; Leica, Heidelberg, Germany) (original magnification, ×4,000).

RESULTS AND DISCUSSION

Cloning of the Be158 gene. A cDNA clone was isolated from a B. equi cDNA expression plasmid library by immunoscreening with B. equi-infected horse serum. The cDNA had a total of 3,942 nucleotides (GenBank accession number AB159602) and showed an ORF of 3,510 nucleotides, which was designated the Be158 gene. The ORF encodes a polypeptide of 1,169 amino acid residues with a putative size of 134.2 kDa, as shown in Fig. 1. The amino acid sequence showed a broad glutamic acid-rich region from positions 34 to 908 and also contained an apical membrane antigen 1 (AMA-1) signature of Plasmodium falciparum (19) from positions 894 to 918. The AMA-1 is located in the microneme of Plasmodium merozoite and is anticipated to be a vaccine candidate to prevent merozoite invasion into host erythrocytes (8). In the homology search using the National Center for Biotechnology Information database, the Be158 amino acid sequence showed high similarity to the P. falciparum liver stage antigen (LSA-1; 28%) (GenBank accession number AE014834-50) (7), the p200 antigen located in the merozoite cytoplasm of Babesia bigemina (P200; 27%) (GenBank accession number AF142406) (24), and the P. falciparum erythrocyte-binding protein (MEAB) (26%) (GenBank accession number AY042084-2) (3). The LSA-1 plays an important role in hepatic cell invasion of sporozoites as well as erythrocyte invasion of merozoites (6, 20). The MEAB is an erythrocyte-binding protein located in the rhoptries and on the surface of mature merozoites; it is expressed at the beginning of schizogony (3, 18). P200 was previously identified as a diagnostic antigen for the serological detection of B. bigemina infection and also has a glutamic acid-rich region, as does the Be158 protein (23). Taken together, these findings indicate that the Be158 gene product might be a novel candidate for a vaccine molecule as well as a diagnostic antigen for B. equi infection.
Immunological characterization of native Be158 antigen.

One hundred ninety kilodaltons of GST/Be158 gene product was expressed in E. coli and, after purification (data not shown), used for the immunization of mice to produce the anti-Be158 protein serum. In Western blot analysis, the immune serum against the GST/Be158 gene product recognized 75- and 158-kDa proteins from the lysate of B. equi-infected erythrocytes as well as the 58-kDa protein from the lysate of B. caballi-infected erythrocytes (Fig. 2A). No reaction with anti-GST protein immune serum was observed in these lysates (data not shown). These results suggested that the 158-kDa protein might be a precursor of the 75-kDa protein in B. equi and also that an antigenically similar antigen of the Be158 protein might exist in B. caballi. Further study would contribute to a broader understanding of the biological function of the Be158 protein in the asexual growth cycle.

Detection of anti-Be158 protein antibodies from B. equi-infected horse sera in ELISA. To evaluate the utility of GST/Be158 as a diagnostic antigen, the GST/Be158 antigen or control GST antigen was subjected to ELISA. None of the horse sera showed any reaction to the GST antigen (data not shown). The anti-Be158 protein antibodies were detected in all 13 B. equi-infected horse sera at an OD 415 of >0.4, whereas all 9 B. caballi-infected and all 25 uninfected serum samples had an OD415 of <0.4 (Fig. 3A). The ELISA using the GST/Be158 antigen was able to differentiate clearly between the sera of B. equi-infected horses (OD415, 1.06 ± 0.5 [mean ± standard deviation]) and those of either B. caballi-infected (OD415, 0.13 ± 0.12) or uninfected horses (OD415, 0.04 ± 0.04) at an OD415 of 0.4 (P < 0.05), which was considered the cutoff. Next, to confirm the sensitivity and specificity of the ELISA, we further examined the reactivity of sequential sera obtained from horses experimentally infected with B. equi or B. caballi that had been shown to have specific antibodies to B. equi and B.

FIG. 2. (A) Western blot analysis of the lysates of B. equi (lane a)- and B. caballi (lane b)-infected and noninfected (lane c) equine erythrocytes with the mouse anti-Be158 protein immune serum. The positions of the standard molecular mass markers are indicated on the left side of the panel. (B) Methanol-fixed smears of B. equi-infected erythrocytes (panels a to c) and B. caballi-infected erythrocyte (panel d) were incubated with the mouse anti-Be158 protein immune serum. All samples were observed with confocal laser scanning microscopy (magnification, ×2,964). The immune reaction (green) and nucleus (red) were visualized with an Alexa Fluor 488 goat anti-mouse IgG-conjugated secondary antibody and propidium iodide staining, respectively. Bar, 5 μm.


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For B. equi infection, several diagnostic ELISAs have been developed by using recombinant EMA-1, Be82/236–381, Be82, and EMA-2 gene products (9, 10, 16). The ELISA described here, which uses the GST/Be158 protein, also proved to have high specificity and sensitivity for the detection of B. equi-specific antibodies. In order to analyze all sera infected with various types of field strains, it is important to use ELISAs with various antigens. In conclusion, we have provided convincing data demonstrating the utility of a Be158 gene product specific for the serological detection of B. equi infection in horses.
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