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

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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, carnivores, 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 Be158 gene by immunoscreening a cDNA library with *B. equi*-infected horse serum and characterized the gene product immunologically in *B. equi*-infected equine erythrocytes. Subsequently, the recombinant gene product was subjected to ELISA and evaluated for its serodiagnostically 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 expression phage 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 Be158 gene product in *Escherichia coli*.** Two oligonucleotide primers, 5′-aagtggaaATGAAGTTACGCA CGCAGA-3′ and 5′-aagtggagctTAAACATTGCTAGA-3′ (lowercase letters form SaII 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 SaII and NotI and then ligated into the SaII and NotI sites of a pGEX-4T expression plasmid vector (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, United Kingdom). The resulting plasmid, designated pGEX/Be158, was used to transform the *E. coli* BL21 strain (Stratagene, La Jolla, Calif.) and express the recombinant Be158 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 (Amersham Pharmacia Biotech), as described previously (9, 14, 23).

**Preparation of mouse anti-Be158 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...
Mouse anti-Be158 protein immune serum (1:100) for 1 h and then washed three times with PBS, the membranes were exposed to a substrate solution containing 0.5 mg of the antigen at 4°C overnight. 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 the unabsorbed antigen was discarded, the wells were blocked with 3% skim milk in PBS (blocking solution) at 37°C for 1 h. Then the blocking solution was washed three times with PBS, incubated with 25 μl of serum sample diluted in blocking solution (1:80) and 50 μl of the substrate [0.1 M citric acid, 0.2 M sodium phosphate, 0.003% H2O2, 0.3 mg of 2,2'-azino-di-(3-ethylbenzthiazoline sulfonate) per ml] was added per well. The optical density at 415 nm (OD415) was read after 1 h of incubation with an MTP-120 ELISA reader (Corona Electric, Ibaraki, Japan). The nucleotide sequence data reported in this paper are available in the GenBank, EMBL, and DDBJ databases under accession number AB159602.

RESULTS AND DISCUSSION

Cloning of the Be158 gene. A cDNA clone was isolated from a B. equi cDNA expression phage 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 P. falciparum (6, 20). The MAEBL is an erythrocyte-binding protein located in the rhoptries and on the merozoite cytoplasm of P. falciparum (6, 20). The MAEBL is an erythrocyte-binding protein located in the rhoptries and on the merozoite cytoplasm of P. falciparum (6, 20). The MAEBL is an erythrocyte-binding protein located in the rhoptries and on the merozoite cytoplasm of P. falciparum (6, 20). The MAEBL is an erythrocyte-binding protein located in the rhoptries and on the merozoite cytoplasm of P. falciparum (6, 20). The MAEBL is an erythrocyte-binding protein located in the rhoptries and on the merozoite cytoplasm of P. falciparum (6, 20). The MAEBL is an erythrocyte-binding protein located in the rhoptries and on the merozoite cytoplasm of P. falciparum (6, 20).

Serum samples. Ten serum samples from 25 uninfected horses, 13 from horses experimentally infected with B. equi, and 9 from horses experimentally infected with B. caballi were used for the ELISA. These horses were infected with both protozoan parasites by intravenous inoculation of infected erythrocytes or by infected ticks. All experimental horse sera were collected 30 days to 2 years after infection without significant hemolysis at the Equine Research Institute of the Japan Racing Association in Japan. Student’s t test was used to determine the significant difference of anti-B. equi titers in the three groups. A P value of <0.05 was considered a significant difference. Four additional sequential horse serum samples were collected on days 6, 12, 18, 25, 30, and 36 after the experimental infection with either B. equi (E3 and E4) or B. caballi (C3 and C4) to further examine the specificity and sensitivity of the ELISA using the GST/Be158 protein. All serum samples were kept at −80°C until use in the ELISA.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper are available in the GenBank, EMBL, and DDBJ databases under accession number AB159602.
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*. In an indirect fluorescent-antibody test with the immune serum (Fig. 2B), the Be158 antigen appeared in the cytoplasm of Maltese cross-forming parasites (Fig. 2B, panel a) and was mainly located in an extraerythrocytic merozoite body (Fig. 2B, panels b and c) in *B. equi*. However, the Be158 antigen was not detected in the ring stage of *B. equi* (Fig. 2B, upper middle part of panel a). The anti-Be158 protein immune serum was also found to react with the extraerythrocytic merozoites of *B. caballi* but did not recognize the intraerythrocytic parasites in the stages of the ring-shaped and subsequent pear-shaped forms (Fig. 2B, panel d). The control immune serum against GST was not reactive in any of the developmental stages of *B. equi* and *B. caballi* (data not shown). These results indicated that the Be158 protein of *B. equi* is expressed at a late stage of the asexual cycle of development and suggested the presence of an antigenically similar antigen 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 OD 415 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 (OD 415, 1.06 ± 0.5 [mean ± standard deviation]) and those of either *B. caballi*-infected (OD 415, 0.13 ± 0.12) or uninfected horses (OD 415, 0.04 ± 0.04) at an OD 415 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.
caballi by the complement fixation test (13, 26). As shown in Fig. 3B, sequential sera from the two B. equi-infected horses recognized the GST/Be158 antigen as early as days 12 and 18 after infection. On the other hand, sequential sera from two B. caballi-infected horses failed to recognize the antigen. These results demonstrated that the GST/Be158 antigen specifically recognizes the sera of B. equi-infected horses at an OD_{415} of 0.4 or higher in an ELISA and is useful for the detection of B. equi-infected horses.

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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