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CLONING AND EXPRESSION OF AN ANTIGEN OF *BABESIA GIBSONI* IN *ESCHERICHIA COLI* AND ITS USE FOR THE IMMUNODIAGNOSIS OF CANINE BABESIOSIS

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ABSTRACT

A cDNA expression library prepared from *Babesia gibsoni* (*B. gibsoni*) merozoite mRNA was screened with a *B. gibsoni*-infected dog serum. A cDNA clone encoding 30 kDa protein was cloned and designated P30 gene. The complete nucleotide sequence of the P30 gene had 792 bp. Computer analysis suggested that the sequence of the P30 gene contained an open reading frame of 600 bp with a coding capacity of approximately 23 kDa. The native P30 protein of *B. gibsoni* with molecular mass of 30 kDa was identified by Westem blotting using anti-recombinant P30 mouse serum. The recombinant P30 protein expressed in *Escherichia coli* was used for antigen in an enzyme-linked immunosorbent assay (ELISA). The ELISA was able to detect the *B. gibsoni* infection and did not show any cross reactivity to *B. canis*-infected dog sera and normal dog sera. Furthermore, the antibody response against the recombinant P30 was maintained in dog experimentally infected with *B. gibsoni* even after the dog became the chronic stage of infection. These results demonstrated that the recombinant P30 might be a useful diagnostic reagent for detection of antibodies to *B. gibsoni in dogs.*

Keywords: *Babesia gibsoni*; *Babesia canis*; cDNA library; ELISA; P30 gene

INTRODUCTION

Babesia gibsoni (*B. gibsoni*) is a tick-borne hemoprotozoan parasite, which causes piroplasmosis in dogs. The disease is characterized by remittent fever, progressive anemia, haemoglobinuria, marked splenomegaly and hepatomegaly, and sometimes causes death. *B. gibsoni* infection is endemic in many regions of Asia, Africa, Europe and America (casapulla et al. 1998., Wozniak et al. 1997; Yamane et al. 1993a). This disease frequently occurs in the companion animals, and it becomes clinically a serious

problem (Adachi et al. 1993; Farwell et al. 1982).

The diagnosis of canine babesiosis is routinely performed by microscopic examination of Giemsa-stained thin blood smear film or indirect fluorescent antibody test (IFAT) (Yamane et al. 1993b). However, the detection of intraerythrocytic *Babesia* organisms by the microscopic examination is sometimes very difficult, because some dogs show clinical symptoms without appearance of intraerythrocytic *Babesia* organisms in the peripheral blood. On the other hand, IFAT has a high sensitivity to detect the antibodies to *B. gibsoni*. However, IFAT has a problem in the specificity, because sometimes *B. gibsoni*-infected dog serum show cross-reactivity to the other closely related *Babesia* parasites. Therefore, there is a great need for the development of highly sensitive and specific diagnostic method.

In the present study, we describe the cloning and expression of a 30 kDa antigen gene of *B. gibsoni* and evaluated its diagnostic potential use in enzyme-linked immunosorbent assay (ELISA) for diagnostic purpose. Our data indicate that the ELISA, with the recombinant P30 expressed in *Escherichia coli* (*E. coli*), might be useful tool for diagnosing canine *B. gibsoni* infection.

MATERIALS AND METHODS

Parasite. *B. gibsoni* isolated from a hunting dog in the Hyogo prefecture, Japan, designated NRCPD strain (Ishimine et al. 1978; Fukumoto et al. 2001) was experimentally infected and maintained in splenctomized Beagles or canine red blood cell-substituted severecombined immune deficiency (SCID) mice as described earlier (Fukumoto et al. 2000). *B. gibsoni*-infected dog erythrocytes were collected from the experimentally infected dog at peak parasitemia (14%) and stored at -80^0C.

Dogs. One year old Beagles were used. The dogs were confirmed to be free of natural *B. gibsoni* infection by detection of specific antibody prior to use the experiments.

Construction and immunoscreening of cDNA expression library. Total RNA was prepared from *B. gibsoni*-infected dog erythrocytes (erythrocyte volume, 10 ml., parasitemia, 14%) by acid guanidium thiocyanate-phenol-chloroform extraction methods (Chomczynski et al. 1987), and then polyadenylated RNA was purified by Oligotex-dT 30 (Takara, Japan). The CDNA was synthesized by using a Zap-cDNA synthesis kit, ligated to aλ Zap I phage expression vector and packaged by using Gigapack packaging system. The cDNA library (1 x 10^5 PFU) was screened with a *B. gibsoni*-infected dog serum. The antiserum was incubated for 1 hr at room temperature with nitrocellulose membranes containing the phage plaques. Positive plaques were visualized by using alkaline phosphate-conjugated goat anti-dog IgG antibody with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrates. Positive plaques were rescreened until 100% positive plaques were obtained. The cloned insert in the plaque-purified A phage was subcloned into pBluescript SK(+) by using in vivo excision capabilities of λ ZAP II (Shortetal. 1988).

cDNA sequencing. Restriction enzyme-generated fragments for sequencing were subcloned into pBluescript SK(+) vectors. Nucleotide sequencing was performed on both strands with double-stranded

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CACATATTATTCATCTAGAATTACGGATTCATAATTTAAATATATTAAAT  50
TCCGTAACCATGGAATGCTGCTCAAGGGATACCCGCCTCTTGGATGAGGA  100
          M  E  C  C  S  R  D  T  R  L  L  D  E  E
AGAAGAAACTCAAGAGACCGAGGTCCGTACCGTCGGTACAGTTGCCGACA  150
  E  E  T  Q  E  T  E  V  R  T  V  G  T  V  A  D
GGTCCAGGGCCCAGGAAATCAGCCAGAACGTAGAAAGACAATGGGTCGCC  200
 R  S  R  A  Q  E  I  S  Q  N  V  E  R  Q  W  V  A
GTTACCACCTACCAACCAGTTGACACCGTTACCAAGACCATTGAGGTCCC  250
 V  T  T  Y  Q  P  V  D  T  V  T  K  T  I  E  V  P
CGTTGTTAAGACTGTTGAGAGAATTGTTCACAAGCCAGTCATCCAGGAGC  300
  V  V  K  T  V  E  R  I  V  H  K  P  V  I  Q  E
GTGTTATTCAGGTTCCCCGTGAGGTACCTCAGGTTGTAGAGAAGGTCGTT  350
 R  V  I  Q  V  P  R  E  V  P  Q  V  V  E  K  V  V
GAGATCCCTGACGTCAAATTCGTCGAGAAGATCATCGAAGTACCTCAGGT  400
  E  I  P  D  V  K  F  V  E  K  I  I  E  V  P  Q  V
GCAGTACCGTAACAAGTTGGTTCCTAAAGTGGAGGTCGTGGAGCGCATTG  450
  Q  Y  R  N  K  L  V  P  K  V  E  V  V  E  R  I
TCGAGAAGCCTCAGATTATTGAACAGTGGACAGAGCGCAAGGTTGAGGTT  500
 V  E  K  P  Q  I  I  E  Q  W  T  E  R  K  V  E  V
CCTCAAATCAAGGAAGTCGTACGCTACAAGGAGATTGACGAGACTGAGGA  550
  P  Q  I  K  E  V  V  R  Y  K  E  I  D  E  T  E  E
AATCATTCGCTACTACCCCAAGGGACATGGAAACATTGACTGGGATAAGG  600
  I  I  R  Y  Y  P  K  G  H  G  N  I  D  W  D  K
AATGTGAGAAGGCACACATCATTATTCCACATGAGGGCCAGGAGGAAAAG  650
 E  C  E  K  A  H  I  I  I  P  H  E  G  Q  E  E  K
GCTGAGTAATAAACAGGTTATGGGCGCCAATAAGGCTTATTAAACCTCTC  700
  A  E  *
TGCAGGTTGATTCTGTCGTATAAATCAACACTAAAAATTTTGATGATATG  750
ATTCAATATTATGCCGACAAGAAAAAAAAAAAAAAAAAAAAA          792
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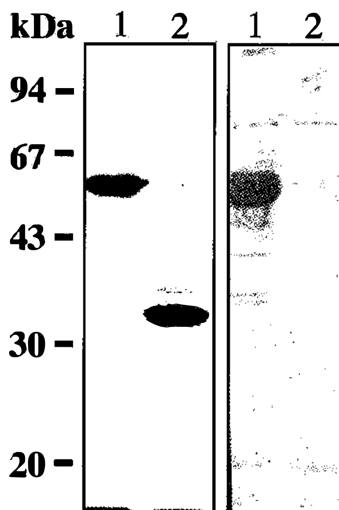
FIG. 1. The nucleotide sequence of the coding region of the P30 cDNA and its flanking sequences. The predicted amino acid is indicated below each codon



.FIG. 2. SDS-PAGE analysis and Western blots of the recombinant P30 expressed in *E. coli* by pGEMEX-2 vector. (A) SDS-PAGE analysis. (B) Westem blots using *B. gibsoni*-infected dog serum. Lanes 1, bacteriophage T7 gene 10-P30 fusion protein, lanes 2, bacteriophage T7 gene 10 protein. The positions of molecular mass standards are indicated on the left (in kilodaltons).

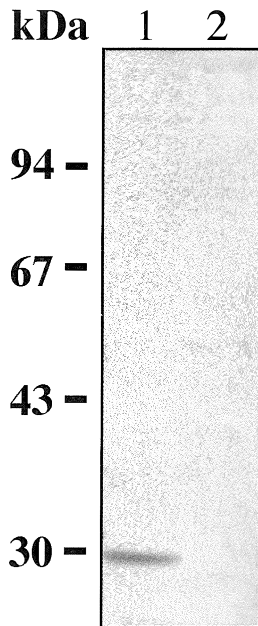


FIG. 3. Western blots of native P30 protein using mouse anti-gene 10-P30 serum. Lane 1, *B. gibsoni*-infected erythrocytes; lane 2, normal dog erythrocytes. The positions of molecular mass standards are indicated on the left (in kilodaltons).

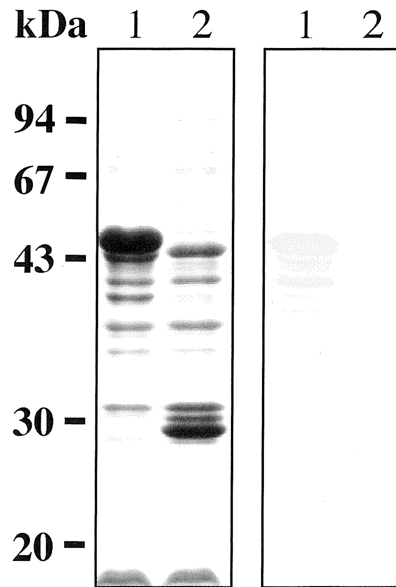


FIG. 4. SDS-PAGE analysis and Western blots of the recombinant P30 expressed in *E. coli* by PGEX-4T-3 vector. (A) SDS-PAGE analysis. (B) Western blots using *B. gibsoni*-infected dog serum. Lanes l, GST-P30 fusion protein; lanes 2, GST protein. The positions of molecular mass standards are indicated on the left (in kilodaltons).

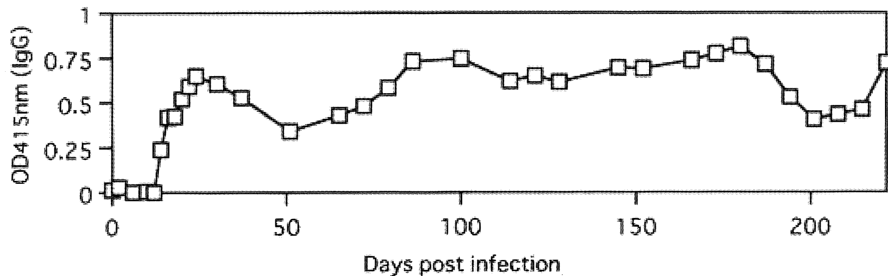


FIG. 5. Detection of antibody to recombinant P30 in a dog experimentally infected with *B. gibsoni* by ELISA

plasmid templates, using the Taq polymerase cycle sequencing method supplied by Applied Biosystems, and then analyzed with a model 377A ABI sequencer. Sequence data were analyzed by computer program MacVector Ver. 6.5.3.

Expression of the P30 gene in *E. coli*. The insert P30 gene in pBluescript SK(+) vector was subcloned into the pGEMEX-2 and pGEX4T-3 plasmids of bacterial expression vectors after digestion with *Eco* R I and *Xho* I, respectively. The resulting plasmids pGEMEX-2 / P30 and pGEX-4T-3 / P30 were checked for accurate insertion by restriction enzyme analyses. The recombinant proteins were expressed as fusion protein of the bacteriophage T7 gene 10 protein (gene 10-P30) in *E. coli* JM 109 (DE3) strain or that of the glutathione S-transferase (GST) protein (GST-P30) in *E. coli* DH5 a strain according to the manufacturer's instructions.

Production of anti-gene10-P30 serum. Antiserum against gene 10-P30 protein was produced in mice. One hundred micrograms of the purified recombinant fusion protein in Freund's complete adjuvant was injected into 8 weeks old BALB/c mice intraperitioneally. The same antigen in Freund's incomplete adjuvant was injected intraperitonially into the mice on days 14 and 28. Sera from immunized mice were collected 14 days after last immunization.

IFAT. IFAT was performed as described previously (Fukumoto et al.2001).

Western blotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were carried out as described previously (Martin et al. 1971; Xuan et al. 1996).

ELISA. The insoluble GST-P30 protein was solubilized in urea according to the previous report (Iwata et al. 2000). In brief, the pellet after centrifugation of bacterial lysates was resolved in 8 M urea solution containing 50 mM Tris-HCl pH 8.0, I mM DL-dithiothretol and 1 mM EDTA, and incubated at room temperature for 1 hr. The solubilized supernatant was dialyzed against 4 M urea solution and then 2 M urea solution for 1 hr at 4 ^{0}C twice. Finally, after dialysis against the solution without urea 3 times at 4^{0}C for overnight, the refolded soluble GST-P30 was purified with Glutathione-Sepharose 4B beads according to the manufacture's instructions, and then used as an antigen for ELISA. The antigen diluted in antigen coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6) was dispensed into the wells of flat-bottomed 96 well microplates in 50 μl aliquots. After incubation at 4 ^{0}C for 24 hr, unadsorped antigen was discarded, and 100μl of blocking solution (phosphate-buffered saline (PBS) containing 3% skim milk) was added. After incubation at 37 ^{0}C for 1 hr, the blocking solution was discarded and 50 ul of test serum diluted in blocking solution added to each well. After incubation at 37^{0}C for 1 hr, the plate was washed 6 times with wash solution (PBS containing 0.05% Tween 20), and 50μl of horse radish peroxidase-conjugated goat anti-dog IgG antibody diluted in blocking solution was added to each well. After incubation at 37 ^{0}C for 1 hr, the plate was washed 6 times with wash solution, and then 100μl of substrate solution (0.1 M citric acid, 0.2 M sodium phosphate, 0.003% H_20_2, 0.5 mg / ml of 2, 2'-azino-di-[3-ethylbenzthiazoline solution]) was added to each well. The absorbance at 415 nm was read after 1 hr, and ELISA titer was expressed as the reciprocal

of the maximum dilution that showed an ELISA value equal to or over 0.1 which is the absorbance difference between GST-P30 antigen and control antigen (GST) wells.

Sera. Serum samples from a dog experimentally infected with *B. gibsoni* (NRCPD strain) or a dog experimentally infected with *B. canis*, and negative serum samples from normal dogs were used.

RESULTS

Cloning and sequencing of the P30 CDNA clones. A total of 20 positive clones were obtained from immunoscreening of *B. gibsoni* CDNA expression library (1 X 10^5 PFU) with dog antisera raised against *B. gibsoni.* The insert sizes of these clones ranged from 800 bp to 3,000 bp. phagemids were excised from the clones and partially sequenced using M13 and universal primers. The clone 9 containing 792 bp insert gene was chosen for further analysis. The clone of insert DNA that was digested with *Hind* II and subcloned into the *Hind* II site of pBluescript SK(+) vectors was completely sequenced using M13 and universal primers. The CDNA sequence of clone 9 is shown in Fig. 1. Stating with a methionine at position 58, a single open reading frame (ORF) of 600 bp nucleotides was present. The ORF encodes a polypeptide of 199 amino acid residues, with a size of 23 kDa as calculated by computer.

Expression of the P30 gene in *E. coli* by PGEMEX-2 vector. The p30 gene was ligated into the bacterial expression vector PGEMEX-2, and expressed as a fusion protein of bacteriophage T7 gene 10 protein in *E. coli.* As shown in Fig. 2A, the molecular weights of the gene 10 and gene 10 P30 fusion protein were estimated as 35 kDa and 61 kDa as expected, respectively. *B. gibsoni*-infected dog serum showed a strong reactivity to the 61 kDa band (Fig. 2B). Mice immunized with gene 10-P30 fusion protein induced specific antibodies against *B. gibsoni* by IFAT (data not shown).

Identification of native P30 protein. The lysates of *B. gibsoni*-infected dog erythrocytes were analyzed by Western blotting using mouse antisera against gene10-P30 protein. As shown in Fig. 3, a specific band of 30 kDa was detected in *B. gibsoni*-infected erythrocytes. In addition, the 30 kDa band was detected as a major antigen in Western blotting with *B. gibsoni*-infected dog serum (data not shown).

Expression of the P30 gene in *E. coli* by PGEX-4T-3 vector. The P30 gene was ligated into the bacterial expression vector PGEX-4T-3, and then P30 was expressed as a fusion protein of GST protein in *E. coli.* As shown in Fig. 4A, the molecular weights of the GST and gene GST-P30 fusion protein were estimated as 26 kDa and 52 kDa as expected. *B. gibsoni*-infected dog serum showed a strong reactivity to the 52 kDa band (Fig. 4B). The GST-P30 protein solubilized by urea and purified with GST 4B beads was used for further analysis. The solubilization and purification was confirmed by SDS-PAGE and Western blots (data not shown).

Antibody responses to the GST-P30 protein in a dog experimentally infected with *B. gibsoni.* To evaluate whether recombinant P30 expressed in *E. coli* can be a suitable antigen for diagnosis of *B. gibsoni* infection in dogs, the antibody response to the GST-P30 protein was measured by ELISA in a sequential dog

sera experimentally infected with *B. gibsoni*. As shown in Fig. 5, the antibody was detected by day 14 and the antibody response was maintained at high levels until 222 days post infection, the chronic stage of infection, which was characterized by recovering hematocrit rate and significantly low level of parasitemia (Fukumoto et al. 2001). In addition, *B. canis*-infected dog sera and normal dog sera did not show any cross-reactivity to the GST-P30 protein (data not shown).

DISCUSSION

In the present study' we described the cloning of a gene encoding 30 kDa antigen of *B. gibsoni*. A cDNA expression library of *B. gibsoni* merozoites was screened with serum from a dog experimentally infected with *B. gibsoni* in order to identify antigens that induce high antibody response. This led to the isolation of an antigen called P30, which exhibited good immunoreactivity with *B. gibsoni*-infected dog serum. The cDNA encoded a polypeptide of 199 amino acid residues, with a size of 23 kDa as calculated by computer. However, the native protein coded by the P30 gene with molecular mass of 30 kDa was detected in Western blotting with anti-gene 10-P30 serum. The size difference may be due to the difference of post-translational modification.

In Westem blotting analyses, *B. gibsoni*-infected dog sera showed a strong reactivity to the both gene-10-P30 protein and GST-P30 protein expressed in *E. coli*. These results suggested that both recombinant P30 proteins have a good antigenicity. Furthermore, ELISA with the GST-P30 protein could detect the antibodies to *B. gibsoni* from the early stage to chronic stage of infection. These results indicate that the recombinant GST-P30 protein has a good potential as an antigen for the sero-diagnosis such as ELISA. However, in this study, we tested only Japanese isolate (NRCPD strain) of *B. gibsoni*-infected dog sera. Therefore, further studies on ELISA with GST-P30 are necessary using a large numbers of *B. gibsoni*-infected dog sera from worldwide.

In the present study, mice immunized with recombinant P30 expressed in *E. coli* induced good antibody response against blood merozoites of *B. gibsoni*. Our further step will be to implement immunization trials in dogs to determine the potency of the recombinant P30 produced in *E. coli* as a potential subunit vaccine to control canine *B. gibsoni* infection.

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