

# Cloning and Expression of a 48-Kilodalton *Babesia caballi* Merozoite Rhoptry Protein and Potential Use of the Recombinant Antigen in an Enzyme-Linked Immunosorbent Assay

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**A cDNA expression library prepared from *Babesia caballi* merozoite mRNA was screened with a monoclonal antibody BC11D against the rhoptry protein of *B. caballi* merozoite. A cDNA encoding a 48-kDa protein of *B. caballi* was cloned and designated BC48. The complete nucleotide sequence of the BC48 gene had 1,828 bp and was shown to contain no intron. Southern blotting analysis indicated that the BC48 gene contained more than two copies in the *B. caballi* genome. Computer analysis suggested that this sequence contained an open reading frame of 1,374 bp with a coding capacity of approximately 52 kDa. The recombinant protein expressed by the vaccinia virus vector in horse cells had an apparent molecular mass of 48 kDa, which was the same as that of the native *B. caballi* 48-kDa protein. Moreover, recombinant proteins expressed by the pGEX4T expression vector in *Escherichia coli* as glutathione *S*-transferase fusion proteins were used for antigen in an enzyme-linked immunosorbent assay (ELISA). The ELISA was able to differentiate very clearly between *B. caballi*-infected horse sera and *B. equi*-infected horse sera or noninfected normal horse sera. These results suggest that this simple and highly sensitive test might be applicable to the detection of *B. caballi*-infected horses in the field.**

*Babesia caballi* is a tick-borne protozoan parasite which causes fever, anemia, jaundice, and edema in horses and, in some cases, the death of the infected animals (5, 9, 19, 22). The disease leads to great economic losses in the horse industry. The complement fixation test has been used as the standard test for the detection of antibodies against *Babesia* infection in horses since 1969 (9). However, it has been reported that, because of its low sensitivity and specificity, the complement fixation test fails to discriminate accurately between negative and carrier animals (27). Moreover, a large quantity of antigens are required to carry out this test. Since the parasitemia of *B. caballi* is usually very low in horses, it is very difficult to prepare the antigen from *B. caballi*-infected erythrocytes. By using the lysates of *B. caballi*-infected erythrocytes, the enzyme-linked immunosorbent assay (ELISA) causes extensive cross-reaction between *B. caballi* and *B. equi* (3, 28). Moreover, the serum from *B. equi*-infected horses sometimes shows a cross-reaction to *B. caballi*-infected erythrocytes by Western blotting (11). Therefore, the development of a high-quality system is required for the diagnosis of *B. caballi* infection.

Monoclonal antibody (MAb) BC11D was produced against a 48-kDa protein of *B. caballi*, and this MAb seemed to bind the rhoptry of merozoite observed by confocal laser microscopy (12). The 48-kDa protein has been shown to be an immunodominant protein (2-4, 11) and might be an important antigen for diagnosis (3, 4, 11, 12). Rhoptry proteins were reported to be involved in the invasion of erythrocytes (21) and

might be suitable candidates for incorporation in vaccines against *B. caballi* merozoites. The aim of this study was to examine ultrastructural localization of the protein recognized by MAb BC11D, to sequence and express the 48-kDa rhoptry protein of *B. caballi* by using a pGEX4T expression vector in *Escherichia coli*, and to apply the expressed protein for the development of a highly specific and sensitive diagnostic ELISA. A possible use of the gene encoding the 48-kDa protein and its product for the development of a vaccine is also discussed.

## MATERIALS AND METHODS

**Parasite.** *B. caballi* (U.S. Department of Agriculture [USDA] strain) was grown in horse erythrocytes in continuous microaerophilous stationary-phase cultures as described by Avarzed et al. (1).

**MAB and immunoelectron microscopy.** MAb BC11D against *B. caballi* merozoite was used because the confocal laser microscopic study has suggested that the location of protein recognized by MAb BC11D was within the rhoptry (12). Immunoelectron microscopy was done to examine the precise localization of epitope recognized by MAb BC11D as described before (26). Briefly, *B. caballi*-infected erythrocytes were collected when parasitemia was ca. 10% and were then washed three times in phosphate-buffered saline (PBS). Infected erythrocytes were then fixed with periodate-lysine-paraformaldehyde in phosphate buffer for 1 h. After three rinses with PBS, infected erythrocytes were dehydrated in a graded ethanol series (30 to 100%) and embedded in LR White resin (London Resin, Basingstoke, United Kingdom). Ultrathin sections mounted on nickel grids were blocked for 30 min in PBS containing 1% bovine serum albumin and then incubated with MAb BC11D for 12 h at room temperature. The ultrathin section were rinsed with PBS and then incubated with goat anti-mouse immunoglobulin G (IgG) + IgM conjugated with 10-nm gold particles (BioCell, Cardiff, United Kingdom) for 2 h at room temperature. After another rinse with PBS, the immunolabeled sections were fixed with 1% osmium tetroxide and then stained with a mixture of uranyl acetate and methyl cellulose. They were examined with a Hitachi-7500 (Hitachi, Tokyo, Japan) electron microscope at 80 kV. The specificity of immunohistochemical staining was confirmed by replacing MAb with normal mouse IgG.

**Construction and immunoscreening of the cDNA expression library.** Total RNA was prepared from an in vitro culture of *B. caballi*-infected horse eryth-

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TABLE 1. Primers used for gene sequencing

Primer	Sequence (5'-3') <sup>a</sup>	Corresponding position on cDNA
<b>Group I</b>		
F1	<i>ACGAATTC</i> CCACAACAGCCGTGTT	17–32
R3	<i>AGAATTC</i> GTAAAGCGTGGCCATG	533–548
<b>Group II</b>		
F2	<i>ACGAATTC</i> AAGAACGTGACTCGCG	471–486
R4	<i>ACGAATTC</i> TGGTGTGCGTTGACAC	960–975
<b>Group III</b>		
F3	<i>ACGAATTC</i> GGTGAACAGGGTGTTTC	881–896
R2	<i>ACGAATTC</i> CTAGAGTGCAACCGAG	1757–1772

<sup>a</sup> BC48 sequences representing restriction enzyme sites are shown in italics.

rocytes (erythrocyte volume, 10 ml; parasitemia, ca. 10%) by acid guanidinium thiocyanate-phenol-chloroform extraction, and then polyadenylated RNA was purified by Oligotex-dT 30 (Takara, Tokyo, Japan). The cDNA was synthesized by using a Zap-cDNA synthesis kit, ligated to a  $\lambda$  Zap II phage expression vector, and packaged by using the Gigapack III packaging system (Stratagene, La Jolla, Calif.). The cDNA library ( $2.5 \times 10^5$  PFU) was screened with MAb BC11D recognizing 48-kDa antigen. MAb BC11D was incubated 1 h at room temperature with nitrocellulose sheets (Schleicher & Schuell, Keene N.H.) containing the phage plaques. Positive plaques were visualized by using alkaline phosphate-conjugated goat anti-mouse IgG (Stratagene) with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrates (Stratagene). Positive plaques were rescreened until 100% plaque purification was achieved. The cloned insert in the plaque-purified  $\lambda$  phage was subcloned into pBluescript SK(+) by using the in vivo excision capabilities of  $\lambda$  ZAP II (23).

**cDNA sequencing.** Restriction enzyme-generated fragments for sequencing were subcloned into pBluescript SK(+) vectors (Stratagene). An insert cDNA designated BC48 was sequenced by the dideoxy chain-termination method by using M13 reverse and universal primers (Perkin-Elmer, Foster, Calif.). Sequence analysis was performed with the computer program GeneWorks (IntelliGenetics, Mountain View, Calif.).

**Isolation of the BC48 genomic clone.** As shown in Table 1, three sets of oligonucleotide primers derived from BC48 were used. The nucleotide sequences of each primer, including an *Eco*RI restriction enzyme site and their corresponding positions on cDNA, are indicated in Table 1. The amplified products were inserted into the *Eco*RI site of pBluescript SK(+) and sequenced with M13 reverse and universal primers as described above.

**Northern and Southern blotting analyses.** Formaldehyde-denatured total RNA (10  $\mu$ g) was fractionated on 1.2% formaldehyde-agarose gel, transferred to a nylon membrane (Hybond-N; Amersham-Buchler, Braunschweig, Germany), and hybridized with a <sup>32</sup>P-labeled probe derived from the BC48 cDNA by using the random primer DNA synthesis method in the presence of [<sup>32</sup>P]dCTP (Amersham-Buchler) (8). Prehybridization and hybridization were performed overnight at 42°C. Membranes were washed three times with 0.1 $\times$  SSC (0.3 M NaCl plus 0.03 M trisodium citrate; pH 7.0) containing 0.1% sodium dodecyl sulfate (SDS) at 42°C for 15 min. Bands hybridizing to the probe were detected by standard techniques. *B. equi* merozoite RNA and horse leukocyte RNA were used as controls. For Southern blotting analysis, total DNA was extracted from *B. caballi* merozoites by the standard method (20). Restriction enzyme-digested *B. caballi* genomic DNA was run on a 0.7% agarose gel, and the DNA was transferred onto a nylon membrane as described earlier (13). Horse leukocyte DNA was used as a control. The membrane was processed and probed exactly as for Northern blotting analysis.

**Expression of the BC48 gene in *E. coli*.** The insert BC48 gene in pBluescript SK(+) vectors was subcloned into the pGEX4T plasmid (Pharmacia, Uppsala, Sweden) of *E. coli* expression vector after digestion with *Eco*RI and *Xho*I. The resulting plasmid pGEX/BC48 was checked for accurate insertion by restriction enzyme analyses. The pGEX/BC48 was used to transform *E. coli* (BL21 strain; Stratagene) by standard techniques (20). The recombinant protein was expressed as glutathione *S*-transferase (GST) fusion protein, designated GST-BC48 protein. The GST-BC48 protein was purified with glutathione-Sepharose 4B beads (Pharmacia) (24) after lysis of the collected bacteria by sonication in PBS containing 1% Triton X-100.

**Production of anti-GST-BC48 serum.** Antiserum against the GST-BC48 protein was produced in mice. Recombinant bacteria were washed three times in PBS, lysed in PBS by sonication, and used as crude antigen. Seven-week-old female BALB/c mice were injected both intraperitoneally and subcutaneously with crude antigen suspended in 0.2 ml of PBS (8 mg/ml) emulsified with 0.2 ml of complete Freund's adjuvant (Difco, Detroit Mich.). At 2-week intervals, three additional stimulations with the same amount of crude antigen emulsified with

0.2 ml of incomplete Freund's adjuvant (Difco) were given. Sera from immunized mice were collected 10 days after the last immunization.

**Construction of the recombinant vaccinia virus.** Insert BC48 in pBluescript SK(+) vectors was subcloned after digestion with *Eco*RI and *Xho*I, blunt ended with a Klenow fragment of DNA polymerase, and then ligated into the *Sa*I site of pAK8 plasmid (30) of the vaccinia virus transfer vector. The accurate insertion of the resulting plasmid pAK8/BC48 was checked by restriction enzyme analyses. RK13 cells infected with vaccinia virus LC16mO (mO) strain were transfected with the recombinant transfer vector pAK8/BC48 by using a Lipofectin reagent (Gibco, Grand Island, N.Y.). Thymidine kinase negative (TK<sup>-</sup>) viruses were isolated by plaque assay on 143TK<sup>-</sup> cells in the presence of 5-bromo-2'-deoxyuridine at a concentration of 50  $\mu$ g/ml. TK<sup>-</sup> virus plaques were picked up and purified three times, and the recombinant vaccinia virus (rVV) carrying the BC48 gene (vvBC48) was obtained. E. Derm cells from the dermis of horses were infected with vvBC48 at 10 PFU per cell. After incubation for 48 h, cell lysates obtained by sonication in PBS containing 1% Triton X-100 were collected as recombinant protein and designated ED-BC48 protein.

**Western blotting analysis.** ED-BC48 and GST-BC48 proteins were analyzed by Western blotting as described previously (12).

**ELISA.** Ninety-six-well microtiteration plates (Nunc-Immuno Plate; Nunc, Roskilde, Denmark) were coated overnight at 4°C with 50  $\mu$ l (0.1  $\mu$ g/ $\mu$ l) of purified GST-BC48 protein or GST protein as the control. These proteins were diluted in a 0.05 M carbonate-bicarbonate buffer (pH 9.6). To reduce the non-specific binding, plates were blocked for 1 h at 37°C with PBS containing 3% skim milk. The microtiter plates were then incubated with individual horse serum diluted 1:80 in PBS containing 3% skim milk for 1 h at 37°C. After six washes times with PBS containing 0.05% Tween 20, the peroxidase-conjugated goat anti-horse IgG (Cappel, Durham, N.C.) antibody diluted 1:4,000 in PBS containing 3% skim milk was added to each well in 50  $\mu$ l and incubated for 1 h at 37°C. The plates were washed as described above, and then substrate solution (0.1 M citric acid, 0.2 M sodium phosphate, 0.003% H<sub>2</sub>O<sub>2</sub>, and 0.3 mg/ml 2,2'-azide-bis[3-ethylbenzthiazoline-6-sulfonic acid]; Sigma, St. Louis, Mo.) was added to each well in 100- $\mu$ l aliquots. The absorbance at 415 nm was read after 1 h of incubation at room temperature by using an ELISA reader (Corona Microplate Reader MTP-120; Corona, Tokyo, Japan).

**Sera.** The following horse sera were used for ELISA: (i) 14 sera from noninfected horses; (ii) 4 sera from horses experimentally infected with *B. equi*; and (iii) 6 sera from horses experimentally infected with *B. caballi*. All sera were obtained from the Equine Research Institute, The Japan Racing Association, Tochigi, Japan. They were kept at -80°C until use.

**Nucleotide sequence accession number.** The sequence of the BC48 gene of *B. caballi* (USDA strain) has been submitted to the GenBank database under accession no. AB017700.

## RESULTS

**Ultrastructural localization of the 48-kDa protein.** Immunomicroscopic studies were undertaken to determine the intracellular localization of the 48-kDa protein in *B. caballi* merozoites. Immunogold labeling showed specific binding of MAb BC11D to the rhoptries in *B. caballi* merozoites (Fig. 1). Gold particles were observed only in rhoptries of *B. caballi* merozoites, but not in the nucleus, spherical bodies, micronemes, or merozoite cytoplasm. Infected erythrocytes incubated with control mouse IgG did not have any particles bound to them (data not shown). These results have confirmed previous results with confocal laser microscopic observations, suggesting that MAb BC11D specifically bound to rhoptries of *B. caballi* merozoites (12).

**Cloning and sequencing of the BC48 cDNA clone.** A cDNA clone containing a 1,828-bp insert was isolated after a screening with MAb BC11D. The cDNA sequence of the insert is shown in Fig. 2. Starting with methionine at position 45, a single open reading frame (ORF) of 1,374 nucleotides was present. Computer-aided searching of the GenBank database revealed that this ORF contained 189 bp of the *B. caballi* rhoptry protein gene previously reported by Dalrymple et al. (7) (GenBank accession number U46551) (Fig. 2).

The ORF encodes a polypeptide of 458 amino acid residues, with a size of 52 kDa as calculated by computer. Two conserved sequences with tandemly repeated 27- and 8-residue periodicities, starting as F(X)N(Y)EIR, occur five and four times from residues 292 to 458, respectively (Fig. 3). The 27-

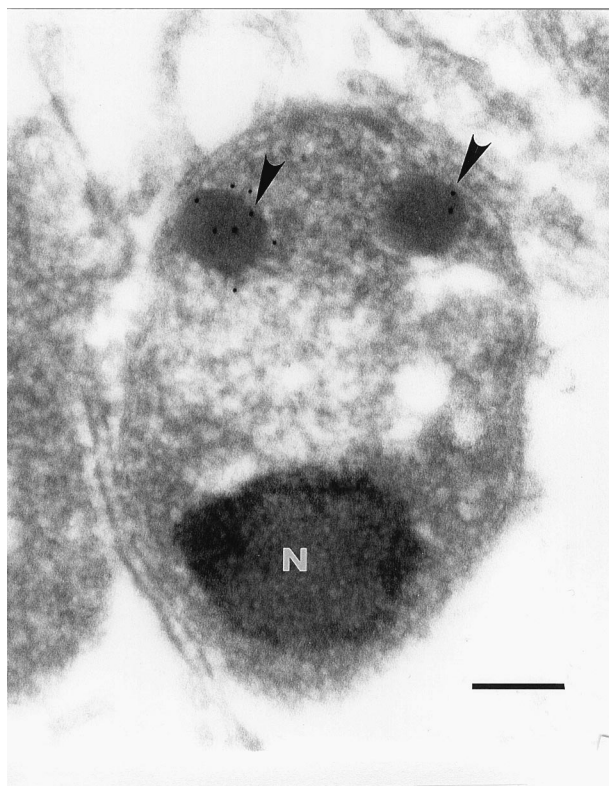


FIG. 1. Localization of the 48-kDa protein to rophtries of *B. caballi*. Sections of periodate-lysine-paraformaldehyde-fixed parasites were reacted with MAb BC11D and then reacted with a 10-nm-gold-conjugated second antibody and examined by electron microscopy. Gold particles (arrowheads) were observed in two rophtries located in the anterior area of merozoites. N, nucleus. Bar, 0.2  $\mu$ m.

residue sequence was almost identical, and the 8-residue sequence KIGQGTVD was exactly preserved.

**Characterization of the BC48 gene.** A cDNA clone BC48 was hybridized to the total RNA isolated from *B. caballi* merozoites but not to horse leukocyte RNA or *B. equi* RNA by Northern blotting. Two mRNA species of 2.6 and 2.0 kb were identified (Fig. 4).

A probe derived from cDNA clone BC48 was strongly hybridized to *B. caballi* DNA fragments but not to horse leukocyte DNA in Southern blotting. As shown in Fig. 5, genomic DNA was digested by restriction enzymes *Hind*III, *Sac*I, *Eco*RV, *Sal*I, *Kpn*I, *Dra*II, *Pvu*II, *Acc*I, *Sty*I, and *Pst*I. The cDNA sequence, which did not contain any *Hind*III, *Sac*I, and *Eco*RV sites, contained only a single *Sal*I, *Kpn*I, *Dra*II, and *Pvu*II site and two *Acc*I, *Sty*I, and *Pst*I sites. However, the results showed the size of the fragments and the presence of more than two bands in lanes 4, 5, 6, and 7 and more than three bands in lanes 8, 9, and 10. These results indicate that BC48 contained more than two copies in the *B. caballi* genome.

*B. caballi* genomic DNA was amplified by PCR techniques by using three sets of primers, namely, groups I, II, and III (Table 1). The resulting DNA fragments were ca. 500, 500, and 900 bp, respectively. These amplified DNAs were molecularly cloned into each plasmid vector. The plasmids containing the gene from each representative group were isolated and subjected to DNA sequence analysis. The DNA sequences were found to coincide with BC48 (data not shown), which demonstrates that the BC48 gene contains no introns.

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GTGCCCTGGCCGTCGCCACACAGCCGTTTCCATCATGGCTCCCGAGGACTCTGTGGGGCAGGTGACTAAGACCTTATGGTGCCA 90
      M A P S D S V G D V T K T L L A A S
GCGAAAGTGGACTCAGCTGCCAATGCCATATATGATCAACAGTGAATGACGCGATTACTTGTCCGGCTGTGTGACAACCTGCCCGAGC 180
      E S V D S A A N A Y M I N S D M S D Y L S A V S D N F A E R
GCATTTCAGTCAGGTCCTAAGGGGAGTAAGTCAAGTCTCCGTTAGCGCATACATGAGTCCGCTAAACAGGACTGCCTGACTC 270
      I C S Q V P K G S N C S A S V S A Y M S R C A K Q D C L T L
TCCAAAGTCTTAAGTACCCTCTGAGGCTAAGTACCAACCGCTGACCCCTCTGACCCCTACCAGTTGGAGGCCGATTATACCTTCA 360
      Q S L K Y P L E A K Y Q P L T L P D P Y Q L E A A F I L F K
AGGAGAGTGAAGTAAATCCGGCCCAATAGCACTGAGAGGCGCTCTGGATCGTTTTCAGAGGGGCAAGAACCACAGTACTTCCAGCACT 450
      E S D A N P A N S T E K R F W M R F R R G K N H S Y F H D L
TAGTCTTCAATCTGTGGAGAGAGCTGACTCGCGAGCGGGATGCTACTGACATTGAGAACTTTCGCTCCAGGACTCTGATACGGCCA 540
      V F N L L E K N V T R D A D A T D I E N F A S R Y L Y M A T
CGCTTACTACAAGACGTACACGAATGTTGATGAGTTCGGTGTAGCTTCTTAAACAAGTGTCTTCACTACTGGTGTTCGGCTGGT 630
      L Y Y K T Y T N V D E F G A S F F N K L S F T T G L F G W G
GCATCAAGAGGCACTAAGCAGATTATTCCGCTCAACCTGACCCCTGACATCGGGACAGAACAGCGTCAGTCCGCTCAGCACATTA 720
      I K R A L K Q I I R S N L P L D I G T E H S V S R L Q H I T
CGAGCAGTACAAGATTACATGGATACGAGATTCTGCACTGCCAAGTTTGGGAAGGTTTCTCCCTTATGAGTGCAGAGGGCTGC 810
      S S Y K D Y M D T Q I P A L P K F A K R F S L M V V Q R L L
TGGCCCGTGGCTGGTACGTGACACCCCGTGGTATAAGAAAGTGGTACATGAAGCTGAAGAATTATGGTGAACAGGGTGTTCATTC 900
      A T V A G Y V D T P W Y K K W Y M K L N F M V N R V F I P
CTACAAGAAGTCTTCAATAAGAAATTCGTGAGCCTAGTAAGGCATTAAGAAAGGTTCAACCCAGCACCAAGGATTTATTCGAGA 990
      T K K F F N K E I R E P S K A L K E K V S T D T K D L F E N
ACAAATGGGGAGGACTGTGACTTCTCAATAGGAAATTCGTGACCCCTAGTAAGGCATTAAGAAAGGTTCAACAGCACGCCA 1080
      K I G Q G T V D F F N K E I R D P S K A L K E K V S N D A K
AGGATTTATCGAAGCAAAATGGGAGGGTACTGTGGACTTCAATAAGCAAAATTCGTGACCCCTAGTAAGGCATTAAGAAAGG 1170
      D L F E N K I G Q G T V D F I N N E I R D P S K A L I R K V
TGTCAACGGGGCCGAGGATTTATTTCGAGAACAATGGGCGAGGACTGTGGACTTCAATAAGCAAAATTCGTGACCCCTAGTAAGG 1260
      S T G A E D L F E N K I G Q G T V D F I N N E I R D P S K A
CATTAATAAGAAAGTGTACACCGAGGCGATGATTTATTTCGAGAACAATGGGCGAGGACTGTGGACTTCAATAAGCAAAATTC 1350
      L I R K V Y T E A D D L F E N K I G Q G T V D F I N K E I R
GTGACCCCTAGTAAGGCATTAATAAGAAAGTGTCTACCGAGGCGGATAATTTATGGAGAATAGGTTGGGAAGCCCTGAGGAAGCAC 1440
      D P S K A L I R K V S T E A D N L L E K
GCAAGGGCAACGTTAGTGCACCGGGGAATCTGAGGAAATTCGGCTGTGGTGAATCTTGGAACTCGCAACGAAATGAAGACCCAGG 1530
AGTCAATGAATCGAGAGGTCTTCTACGAACTCCCTCTGAGGAGTCCGAGGAAGTCCGGCTGCTATGTTATTACGAGCCCAACC 1620
TGGAGAGGCGAGGAGATCGCATTGCCTGCTGAAGAAGCAGCTCAGAGTTCGAGGAACCTCCGCAACTACTAAGCCTCTCTGAT 1710
CACCTTGACGTCATCGCACTGCTCGGAGAATATAAACGCAATTCGCTCGGTTGCACTCTAGTTGTTAAACATGCACAATTAATGTTAT 1800
AGTTGTTTTGAAAAAAAAAAAAAAAAA 1828
    
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FIG. 2. Complete sequence, including the 5'- and 3'-untranslated regions, of BC48. The amino acid sequence translated from the long ORF is depicted. The sequence of 189 bp of the *B. caballi* rophtry protein previously reported by Dalrymple et al. (GenBank accession number U46551) is underlined.

**Western blotting analysis of the recombinant protein.** E. Derm cells infected with vvBC48 were analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting to determine whether the ED-BC48 protein was expressed. A single band of ED-BC48 protein was observed in the cell lysate, and the molecular mass of the ED-BC48 protein was demonstrated to be the same as that of the native *B. caballi* 48-kDa protein by Western blotting (Fig. 6a), which proves that the ORF in the BC48 gene was the complete length. Moreover, the antibodies against GST-BC48 protein from mice recognized only the 48-kDa native protein as MAb BC11D (Fig. 6b). These results indicate that this 48-kDa protein did not contain the

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FFNKEIREPSKALKEKVSVDTKDLFENKIGQGTVD 326
FFNKEIRDPSKALKEKVSNDKDLFENKIGQGTVD 361
FINNEIRDPSKALIRKVSSTGAEDLLENKIGQGTVD 396
FINNEIRDPSKALIRKVYTEADDLFENKIGQGTVD 431
FINKEIRDPSKALIRKVSSTEADNLLK 458
    
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FIG. 3. Deduced amino acid sequence of the BC48 coding region from residues 292 to 458. Highly conserved residues are denoted by underlining.

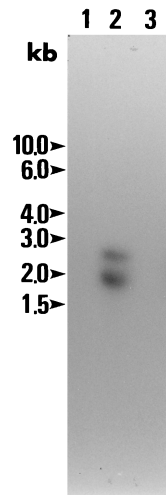


FIG. 4. Northern blotting hybridization of the BC48 of *B. caballi*. Tracks contained 10 µg of total RNA prepared from *B. equi* derived from infected blood (lane 1), *B. caballi*-infected blood (lane 2), and horse leukocytes (lane 3) and were hybridized with the <sup>32</sup>P-labeled BC48 cDNA. Molecular size markers are shown on the left (in kilobases).

epitope of other constituent proteins in *B. caballi* and was unique among these proteins.

**Detection of anti-GST-BC48 antibodies in *B. caballi*-infected horses by ELISA.** The recombinant protein was expressed as GST-BC48 protein with an apparent molecular mass of approximately 75 kDa (data not shown). Ninety-six-well plates coated with the GST-BC48 protein or the GST control protein were used for ELISA. Although the sera of *B. caballi*-infected horses and those of *B. equi*- or noninfected horses were confirmed to be nonreactive to the GST control protein (data not

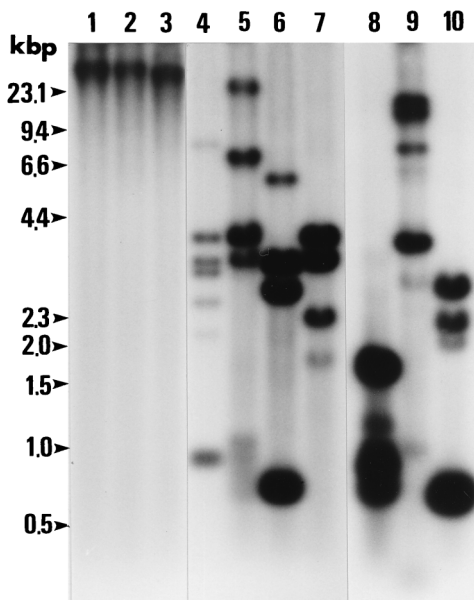


FIG. 5. Southern blotting hybridization of the BC48 of *B. caballi*. Genomic DNA (10 µg per lane) from *B. caballi* merozoites was digested with the indicated enzymes (lane 1, *Hind*III; lane 2, *Sac*I; lane 3, *Eco*RV; lane 4, *Sal*I; lane 5, *Kpn*I; lane 6, *Dra*II; lane 7, *Pvu*II; lane 8, *Acc*I; lane 9, *Sty*I; lane 10, *Pst*I) and hybridized with the <sup>32</sup>P-labeled BC48 cDNA. Molecular size markers are shown on the left (in kilobase pairs).

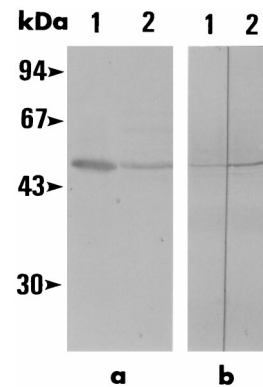


FIG. 6. Western blotting analysis. (a) Native *B. caballi* and ED-BC48 protein with MAb BC11D. The molecular mass of the ED-BC48 protein (lane 1) was the same molecular mass as that of the native *B. caballi* protein (lane 2). (b) Native *B. caballi* protein with antibodies against GST-BC48 protein from mice (lane 1) and MAb BC11D (lane 2). The molecular mass of the reacted pattern was recognized in the same position. The positions of molecular mass standards are indicated on the left (in kilodaltons).

shown), the test was able to distinguish (optical density = 0.2) between the sera of *B. caballi*-infected horses and those of *B. equi*- or noninfected horses (Fig. 7). The GST-BC48 protein did not show any cross-reaction with anti-*B. equi* horse sera or normal horse sera in ELISA.

**DISCUSSION**

In the present study, the cDNA gene encoding a 48-kDa rhoptry protein of *B. caballi* merozoite was cloned, and its complete nucleotide sequence was determined. The genes encoding the rhoptry protein have been cloned and sequenced from a number of *Babesia* spp. (6, 7, 16, 17, 25). Those Bv60/p58 family genes encoding the rhoptry protein were found to be conserved in all isolates of *B. bovis* (18) and *B. bigemina* (14, 15). In our previous study, we developed a MAb BC11D that

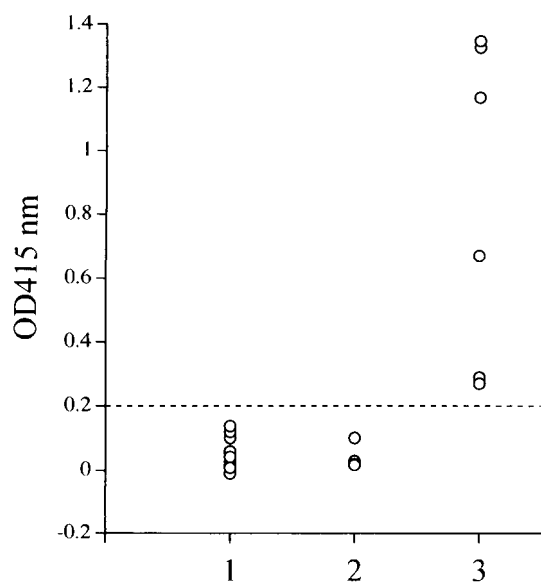


FIG. 7. ELISA analysis of GST-BC48 protein with experimentally infected horse sera. Lane 1, noninfected horse sera; lane 2, *B. equi*-infected horse sera; lane 3, *B. caballi*-infected horse sera.

seemed to bind a 48-kDa rhoptry protein of *B. caballi* (12). The ultrastructural localization of a 48-kDa protein in the rhoptries of *B. caballi* merozoites was demonstrated by immunoelectron microscopic studies with MAb BC11D (Fig. 1). A cDNA expression library prepared from *B. caballi* merozoites was screened with MAb BC11D, and it was found that the complete DNA sequence encoding the rhoptry protein of *B. caballi* consisted of 1,828 bp. This DNA sequence contained 189 bp in *B. caballi*, as previously reported (7) with regions of highly conserved amino acid sequences in the Bv60/p58 in members of the genus *Babesia*. Therefore, the DNA sequence of *B. caballi* in the present study confirmed that the BC48 gene is a member of the Bv60/p58 family of the rhoptry protein in the genus *Babesia*. The 48-kDa protein was reported to be common to all strains of *B. caballi* obtained from different European countries and Brazil (2, 4), although it has not yet shown to be identical to the 48-kDa rhoptry protein. Taken together, these results may suggest that the BC48 gene encoding the rhoptry protein of *B. caballi* is an important gene for the conservation of the genus.

Two conserved sequences in the amino acids of the BC48 gene encoding the rhoptry protein of *B. caballi* with tandemly repeated 27- and 8-residue periodicities occurred five and four times from residues 292 to 458, respectively. The 27-residue sequence was almost identical, and the 8-residue sequence KIGQGTV D was exactly preserved. The polypeptide also contained a large region of two kinds of repeated amino acid sequences accounting for 36% of the total number of amino acids. Computer analysis indicated that the pattern present in this region has many T-cell epitopes. Suarez et al. (25) hypothesized that if the repeat regions of the amino acids were surface-exposed merozoite epitopes, the antibodies against the conserved and surface-exposed epitopes could block infectivity for host erythrocytes. Analyses of the mRNA from erythrocyte-stage parasites confirmed the transcription of mRNAs of two different sizes (2.6 and 2.0 kb). These results indicated that the BC48 gene is differentially expressed in the erythrocyte stage of the parasite. This BC48 gene was shown to be the product of more than two copies in the *B. caballi* genome by Southern blotting analysis, suggesting a multicopy gene. However, this protein expressed by the BC48 gene in *B. caballi* did not contain the epitope of other constituent proteins in *B. caballi*. Moreover, Western blotting indicated that it was unique among these proteins with antibodies against GST-BC48 protein from mice. The mechanisms for transcription and translation remain unclear at present because the origin of the rhoptries and the mechanism of their formation are still poorly understood. In addition, it is unclear whether proteins identified within the organelle are structural components of the organelle or whether they are inserted into the organelle post-translationally.

Proteins with molecular masses of 48- and 50-kDa were detected by Western blotting with anti-*B. caballi* antibody at an early stage of infection and in a wide range of horses in different countries (2, 4). Sera from all experimentally infected horses were still positive against 48- and 50-kDa proteins more than 1 year after infection. The 48- and 50-kDa proteins were also confirmed to be major antigens of *B. caballi* merozoites (4, 11). Therefore, these proteins were considered to be suitable antigens for use in immunodiagnostic tests for *B. caballi* infection. GST-BC48 protein expressed by *E. coli* was used for ELISA in the present study. The ELISA was able to differentiate very clearly between *B. caballi*-infected horse sera and *B. equi*-infected horse sera or noninfected normal horse sera. However, the number of horse sera was small, and further studies on ELISA with this recombinant protein are thus nec-

essary with a large number of horse sera to ascertain whether this simple and highly sensitive test is applicable to the detection of *B. caballi*-infected horses in the field.

Native and recombinant rhoptry proteins have been demonstrated to induce protective immune responses in cattle infection (15, 29). The ED-BC48 protein expressed by rVV has the same molecular mass as the native protein in the present study. Honda et al. (10) reported that the sporozoite surface antigen (p67) of *Theileria parva* expressed by rVV with rVV interleukin-2 of cattle enhanced protection against East Coast fever disease caused by *T. parva*, and that rVV expressing the p67 of *T. parva* with rVV interleukin-4 also enhanced anti-p67 antibody production. These results indicate the potential use of rVV as a delivery system in vaccination against protozoan infection. Further examination of the potency of vvBC48 as a potential subunit vaccine might provide interesting insights into how to control *B. caballi* infection in horses.

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