**NOTE** Parasitology

**Protein Analysis of Babesia caballi Merozoites by Two-Dimensional Polyacrylamide Gel Electrophoresis and Western Blotting**

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**ABSTRACT.** Babesia caballi merozoites were prepared by combining two improved methods of cultivation and purification of merozoites using Percoll-centrifugation, and the protein compositions of merozoites were analyzed by two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting. The relative molecular masses of the major proteins and protein masses separated by electrophoresis were >94, 80–70, 50–45, 34–30, 30–28 and 18 kDa. By Western blotting, twelve proteins or protein groups were recognized by pooled sera from two horses experimentally infected with B. caballi. Among twelve proteins, five new proteins (54, 30–26, 24, and two 18 kDa) were identified, and the 48 kDa protein was revealed to consist of 2 components in the B. caballi merozoite. One protein (54 kDa) of B. caballi was also recognized by the pooled sera from two horses experimentally infected with B. equi.—**KEY WORDS:** Babesia caballi, 2D-PAGE, Western blotting.

*Babesia caballi,* one of the major hemoprotozoan parasites that cause equine babesiosis, is widely distributed throughout tropical and subtropical areas of the world. In horses, this parasite causes fever, anemia, jaundice, and edema, and death in some cases [14, 25]. The disease causes significant economical losses in the horse industry [13, 15]. Various serological techniques, such as the complement fixation test (CFT), the indirect fluorescent antibody test (IFAT), and the enzyme-linked immunosorbent assay (ELISA), are used to detect antibodies against B. caballi and B. equi in host animal sera. The main disadvantages of these tests are their lower sensitivity and specificity [12, 27]. Therefore, the identification and characterization of the proteins of B. caballi and B. equi is necessary for the improvement of the serodiagnosis of equine *Babesia* infections.

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), a powerful method for resolving and characterizing proteins in complex biological materials [3, 4], has been used for the analysis of the proteins of malarial parasites [16, 26] and bovine Theileria and Babesia species [22–24]. A lysate of B. equi-infected erythrocytes was analyzed by 2D-PAGE and Western blotting [1, 10]. For B. caballi, several proteins have been identified using crude protein prepared from infected erythrocytes [9]. However, the proteins of purified B. caballi merozoites have not been examined by 2D-PAGE, to our knowledge, due to the low parasitemia of infected horses, which rarely exceeds 1%, and the difficulty of preparing pure merozoites without contamination of host components.

Recently, we have developed improved methods for the cultivation of B. caballi [6] and the purification of B. caballi merozoite by two-step centrifugations using the Percoll-gradient solution system [17]. These methods make it possible to analyze purified merozoites without contamination of host components. In the present study, we analyzed the protein compositions of B. caballi merozoites in more detail by 2D-PAGE and by subsequent Western blotting with pooled sera from horses experimentally infected with either B. caballi or B. equi.

*B. caballi* (USDA strain) was grown in equine erythrocytes in continuous microaerophilous stationary phase cultures as described by Avarzed et al. [6]. B. caballi-infected blood, with a parasitemia of approximately 60–90% with Percoll gradient [7], was lysed with an ammonium chloride solution. Then, merozoites were enriched by density gradient centrifugation on a Percoll-gradient [17]. These merozoites were stored at -80°C until use. As a control, erythrocyte membranes obtained from an uninfected horse were purified by the same method of hemolysis [19].

One-dimensional polyacrylamide gel electrophoresis (1D-PAGE) was performed using a 12% (w/v) polyacrylamide gel according to the method described by Laemmli [18]. Samples were boiled for 5 min in a 1D-PAGE sample buffer [18]. After electrophoresis, gels were stained with Coomassie brilliant blue. The samples for 2D-PAGE were prepared as follows: B. caballi merozoites after purification were sonicated in a phosphate-buffered saline (PBS) with a mixture of protease inhibitor (Complete™, Boehringer Mannheim, Germany) on ice. These sonicated-samples were solubilized in a solubilization buffer composed of 9 M urea, 4% (v/v) Nonidet P-40, 2% 2-mercaptoethanol, and 2% (v/v) carrier ampholites (pH 3.0–10.0; BIO-RAD, California, USA).
After insoluble components were removed by centrifugation at 15,000 g for 10 min at 4°C, the supernatant was used as the loading sample. Non-equilibrium pH gradient gel electrophoresis [21] was used as first step. One hundred µg of protein for each sample was electrophoresed for 2 hr at 400 V. A 12.0% polyacrylamide gel was used as the second dimension. After electrophoresis, the gels were stained using a silver-stain kit (Dai-ichi Pure Chemical Co., Tokyo, Japan).

After separation by 2D-PAGE, the merozoite proteins were electrophoretically transferred to polyvinylidene difluoride sheets (Immobilon transfer membranes; Millipore, U.S.A.) according to the Western blotting technique of Avarzed et al. [5]. The sheets were blocked with PBS containing 3% skim milk for 1 hr at 37°C. Membranes were then washed three times with PBS and incubated with the horse sera for 1 hr at 37°C. Bound antibody was detected by incubation with peroxidase-conjugated goat anti-horse IgG (1: 1,000) (Cappel Research Products, Durham, N.C., U.S.A.) in 3% skim milk-PBS for 1hr at 37°C. After being washed, the strips were treated with a freshly prepared substrate solution containing 5.3 mM 3,3'-diaminobenzidine, tetrahydrochloride (Wako, Japan) and 0.01% (v/v) hydrogen peroxide. The pooled sera taken from 2 horses experimentally infected with either B. caballi (USDA strain) or B. equi (USDA strain) and from 2 non-infected horses were used. All these sera were obtained from the Equine Research Institute, The Japan Racing Association, Tochigi, Japan.

There have been few reports on the protein analysis of B. caballi-infected erythrocytes by 1D- or 2D-PAGE and Western blotting [8, 9, 11, 13]. The characterization of Babesia-specific proteins is generally impaired by the contamination of the erythrocyte membrane. We have solved this problem by combining two improved methods of cultivation [6] and purification of merozoites [17]. The patterns of B. caballi merozoite proteins recognized by 1D-PAGE are shown in Fig. 1. Several major bands, whose molecular masses ranged from >96 to 15 kDa, were recognized in B. caballi proteins by 1D-PAGE (Fig. 1, lane 1). The most strongly stained bands were 50 and 48 kDa proteins. Compared with the pattern of infected erythrocyte-membrane proteins (Fig. 1, lane 2), no protein bands attributed to contamination with the erythrocyte membrane were detected in the electrophoresis patterns of the merozoite sample.

The general patterns of merozoite proteins separated by 2D-PAGE are shown in Fig. 2A. More than 90 clearly visible protein spots and protein masses were observed in purified B. caballi merozoites, and these protein compositions could be identified in more detail by 2D-PAGE than 1D-PAGE. For example, a protein with a molecular mass of 48 kDa, which was observed as single band by 1D-PAGE, was shown to consist of 2 components (spots 3 and 4) by 2D-PAGE. Two thirds of these 90 proteins with relative molecular masses of >94, 80–70, 50–45, 34–30, 30–28, and 18 kDa were localized in acidic and neutral areas of the gel. By 2D-PAGE, no contamination of the erythrocyte membrane was detected in purified B. caballi merozoites either (Fig. 2B).

By means of Western blotting analysis, twelve proteins were recognized by the pooled sera from horses experimentally infected with B. caballi. These were proteins with molecular masses of 54 (spot 1), 50 (spot 2), 48 (2 dots, spots 3 and 4), 35 (spot 5), 33 (spot 6), 31 (spot 7), 30 (spot 8), 30–26 (spot 9), 24 (spot 10), and 18 (2 dots, spots 11 and 12) kDa (Fig. 3A), corresponding to the protein spots of 2D-PAGE indicated by arrowheads in Fig. 2A. Two components (2 dots, spots 3 and 4) of a 48 kDa protein were also identified by immune sera, and spot 4 appeared as a horizontal line extending from the neutral to basic area of the gel. No reaction was observed in the pooled sera from non-infected horses by the analysis (data not shown). We could identify five new proteins of immunodominant protein of B. caballi merozoite, with molecular masses of 54 (spot 1), 30–26 (spot 9), 24 (spot 10), and 18 (2 dots, spots 11 and 12) kDa, which were recognized by sera from horses experimentally infected with B. caballi and had not been reported before. Since it is very important and urgent to seek suitable diagnostic proteins for the development of new...
Serological test, further studies on these newly identified proteins by 2D-PAGE are required for their possible use as diagnostic proteins to improve or develop the serological test for equine babesiosis.

The 50 and 48 kDa proteins were suggested as target proteins for the *B. caballi*-serological test based on crude proteins [8, 9, 11]. We also detected the 50 and 48 kDa proteins in purified merozoites. Furthermore, we found that the 48 kDa protein was composed of 2 immunodominant components, spot 3 and spot 4 as a horizontal band, as reported by Böse [8]. Although the 48 kDa protein was suggested to localize in the apical complex of the parasite [8], the precise localization of 48 kDa protein must be examined by immunoelectron microscopy in the future. Several other proteins (the 35, 33, 31, and 30 kDa proteins) were also identified. These proteins have similar molecular masses to the 34 and 30 kDa proteins reported as strain-specific proteins [9]. Further study is needed to determine whether these proteins are also strain-specific proteins.

*B. caballi* (USDA strain) major proteins recognized by immune sera had apparent molecular masses of 141, 112, 70, 50, 48, 34, and 30 kDa by 1D-PAGE and Western blotting [9, 11]. However, no proteins with high molecular masses of 141, 112, and 70 kDa were observed in *B. caballi* merozoites by Western blotting in the present study. The lack of high-molecular-mass proteins might be explained by the difference in parasite preparation. Since the 141 kDa protein was present on the surface of infected erythrocytes by IFAT [8], high-molecular-mass proteins associated with the erythrocyte membrane could not be identified in purified merozoites of *B. caballi* in the present study and it was also confirmed that merozoite proteins did not contain common proteins with non-infected erythrocyte membrane.

It is interesting to note that a 54 kDa protein of *B. caballi*
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REFERENCES


Fig. 3. Demonstration of *B. caballi* proteins. A lysate of purified *B. caballi* merozoites was separated by two-dimensional polyacrylamide gel electrophoresis and blotted onto PVDF membranes. Membranes shown were reacted with: pooled sera from horses experimentally infected with *B. caballi* (A); *B. equi* sera (B). Immunodominant protein spots were indicated by arrowheads (numbers 1–13). The gels were orientated with the acidic end to the left and the basic end to the right. Molecular mass markers are indicated on the left in kilodalton (kDa).

was also recognized by Western blotting using the pooled sera from horses experimentally infected with *B. equi* (Fig. 3B, spot 13). This common 54 kDa protein of *B. caballi* may share a homologous molecule with *B. equi* which was suggested as *Theileria equi* [2, 20], but amino acid sequence or glycosylation may be partially different between two species. Possibly, this protein may be structurally and functionally conserved among species in piroplasmida, as seen in the major piroplasm proteins of the genus *Theileria* [24]. For further analysis of the 54 kDa common protein, determination of amino-acid sequence of the common protein in the other *Babesia* sp. and *Theileria* sp., cloning of these gene encoding the protein and its DNA sequencing, and comparison of homologue of these gene will be required.