Isolation of *Babesia gibsoni* Piroplasms from Infected Erythrocytes of Dogs

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**ABSTRACT.** To eliminate the influence of anti-dog erythrocyte membrane antibodies in *Babesia gibsoni*-infected dogs on the immunological investigations of parasites, an attempt was made to purify *B. gibsoni* piroplasms from infected erythrocytes by N2 cavitation and centrifugation in a Percoll discontinuous density gradient. Although electron microscopic examination revealed that the concentrated piroplasms visible as a band at the interface between 30% and 40% Percoll solutions were contaminated with a small amount of erythrocyte membrane debris, little contamination was detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western immunoblots. Hence, the isolated parasites may be usable for immunological examinations of parasites.—**KEY WORDS.** *B. gibsoni*, isolation.

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*Babesia gibsoni*, a causative agent of canine babesiosis, is a tick-transmitted hemoparasitic protozoan. Immunological investigations such as the antigenic identification and characterization of *B. gibsoni* components are now carried forward by many investigators. However, anti-dog erythrocyte membrane antibodies induced by *B. gibsoni* infection [1, 2] possibly obstruct the analysis of *B. gibsoni* components immunoreactive with sera in *B. gibsoni*-infected dogs, because the method for the separation of *B. gibsoni* piroplasms from erythrocyte membranes has not yet been established. So far, several methods have been devised for purifying piroplasms, as follows: those for isolation of parasites by hemolysis using N2 cavitation [10], osmotic pressure [4, 9] and hemolysin [11], and those for concentration of parasites by ultracentrifugation in a Percoll [4, 10, 11] and sucrose density gradient [8]. A rapid and simple method by a filtration apparatus [5], suitable for the preparation of a small amount of blood, has been also contrived. In spite of these efforts to improve the technique, slight contamination of purified parasites with erythrocyte membrane debris has been inevitable.

In the present study, we attempted to purify *B. gibsoni* piroplasms freed from infected erythrocytes so as to be applicable for immunological examinations.

*B. gibsoni* utilized in this study was originally isolated from a dog diagnosed as babesiosis at the Veterinary Teaching Hospital, Miyazaki University, Miyazaki, Japan.

Blood with 30% parasitemia was drawn from a dog, which was previously splenectomized and injected with 1×108 parasitized erythrocytes, and was subjected to dextran sedimentation [3]. Erythrocytes suspended in an equal volume of cold physiological saline (PS) were centrifuged twice at 700 G for 15 min at 4°C. Leukocytes were removed by a filtration apparatus, Immunoguard IG 200 (Terumo, Tokyo, Japan). The eluted solution was centrifuged at 700 G for 10 min. Packed erythrocytes were suspended in cold PS, and centrifuged at 700 G for 10 min. A 30% suspension of erythrocytes in cold phosphate-buffered saline (PBS), pH 7.4 were disrupted to set parasites free from host cells by the N2 cavitation method [10] at 70 kg/cm2 for 1 min. The hemolyzed solutions were stood still at room temperature for 20 min and centrifuged at 350 G for 15 min at 4°C. The supernatant containing many parasites was centrifuged at 6,000 G for 30 min at 4°C. The sediments were resuspended in PBS. The suspension was layered carefully on the top of a discontinuous density gradient consisting of 20%, 30% and 40% Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) and centrifuged at 2,500 G for 30 min. The resultant preparations visible as a band at the interface between 40% and 30% were washed and centrifuged twice with PBS at 4,000 G for 10 min.

Some parts of a pellet of the finally concentrated parasites were fixed for four hours in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), and rinsed and stored in 0.1 M cacodylate containing 5.1% sucrose at 4°C overnight. Small pieces of the samples were postfixed in 1% osmium tetroxide in cacodylate buffer at 4°C, dehydrated in ethanol and embedded in Quetol 812. Ultra-thin sections were cut by a Porter-Blum MT-1 ultramicrotome (Sovial, Connecticut, U.S.A.) and stained with uranyl acetate and lead citrate before examined by a Hitachi H-800 transmission electron microscope (Hitachi, Tokyo, Japan).

Erythrocyte membranes were prepared with blood from a healthy, two years old dog according to the method of Tomoda et al. [12]. The protein contents of purified dog erythrocyte membrane antigens and *B. gibsoni* piroplasm antigens were estimated by Lowry's method [5]. A 50 µl portion of the *B. gibsoni* piroplasm antigens (2 mg protein/ml) and the purified dog erythrocyte membrane antigens (2 mg protein/ml) were boiled in sodium dodecyl sulfate (SDS) sample buffer [6] and electrophoresed in a slab gel with a 4.5% stacking gel and a 10% resolving gel by the discontinuous buffer system [6] for 90 min. The gel was stained with Coomassie brilliant blue in a solution of 50% methanol and 10% acetic acid.

According to the method of Towbin et al. [13], *B. gibsoni* piroplasm antigens were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrophoretically to a nitrocellulose sheet. The nitrocellulose sheet was washed three times in 10 mM Tris-buffered saline (TBS), pH 7.4, and incubated in TBS containing 3% bovine serum albumin (BSA) for 1 hr at
37°C. Serum of a *B. gibsoni*-infected dog containing anti-dog erythrocyte membrane antibodies determined by enzyme-linked immunosorbent assay (ELISA) and Western immunoblots [1], and the same serum adsorbed with normal dog erythrocyte membranes, which included no detectable anti-dog erythrocyte membrane antibodies by ELISA and Western immunoblots, were diluted 1:100 in TBS containing 3% BSA. Each serum was incubated with the sheet for 30 min at room temperature. After washed twice for 10 min in TBS containing 0.05% Nonidet P-40 (NP-40) (Sigma, Saint Louis, U.S.A.), the sheet was mixed with peroxidase-conjugated rabbit anti-dog immunoglobulins (IgG fraction) (Heavy & light chain specific, Cappel, PA, U.S.A.) diluted 1:1,000 in TBS containing 3% BSA. After incubated at room temperature for 30 min, the sheet was washed three times for 30 min in TBS containing 0.05% NP-40. The sheet was developed in a solution of 0.025% 3,3’-diaminobenzidine tetrahydrochloride, 0.015% hydrogen peroxide and 0.05 M Tris-HCl (pH 7.6). Colorimetric reaction was terminated by thorough rinsing in deionized water.

By Percoll density-gradient centrifugation, a few bands were detected, among which the band at the interface between 30% and 40% Percoll solutions contained highly purified parasites.

Electron microscopic observation showed that the middle and bottom layers of the pellet had highly concentrated parasites though the upper layer was contaminated with a small amount of erythrocyte membrane debris. Most parasites in the pellet were intact in structure with the cell membrane, though some were partially vacuolated to various extents. A small number of parasites seemed to be ruptured. Parasites were contaminated with a small amount of erythrocyte membrane debris (Fig. 1).

As shown in Fig. 2, the SDS-PAGE protein profiles of the isolated parasites and dog erythrocyte membranes were almost distinct from each other. Most of major erythrocyte membrane components were not observed in the lane of isolated parasites.

In Western blotting analyses, the isolated parasites immunoblotted with *B. gibsoni*-infected dog serum with (lane A) and without (lane B) anti-dog erythrocyte membrane antibodies were similar to each other in the pattern and intensity of protein profiles (Fig. 3).

In the present study, an attempt was made to purify *B. gibsoni* piroplasms to such a degree that immunological investigations of parasites could be carried out.

Electron microscopic examination revealed that the isolated parasites were contaminated with a small amount of erythrocyte membrane debris. Figueroa et al. [4] reported similar results that isolated *B. bigemina* piroplasms were contaminated with a small amount of erythrocyte membrane debris. However, parasites were isolated from erythrocytes and concentrated applicable enough for SDS-PAGE and Western blotting analyses. Figueroa et al. [4] and Sugimoto et al. [11] concentrated parasites by ultracentrifugation at more than 10,000 G, while Shimizu et al. [10] did so at 2,700 G. In our present study, centrifugation at 2,500 G was more efficient than ultracentrifugation at more than 10,000 G (data not

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Fig. 1. Electron microphotographs of sections of the bottom layer in the pellet of the centrifuged *B. gibsoni* piroplasma. A: Well concentrated parasites. × 10,800. Though some are partially vacuolated or swollen, most parasites have the intact cell membrane. B: Showing sections of parasites of high magnification. × 41,700.
shown) to separate parasites from erythrocyte membranes.

Both B. gibsoni-infected dog sera with and without anti-dog erythrocyte membrane antibodies demonstrated the same immunoreactive protein profiles. Therefore, the isolated parasite antigens had no components immunoreactive with anti-dog erythrocyte membrane antibodies. This result indicates that no contamination of parasites with erythrocyte membranes was detected by Western blotting analysis, so no reduction was recognized in sensitivity and specificity in immunological studies on parasites.

In the present study, parasites were contaminated with a small amount of erythrocyte membrane debris, however, the contamination was only to a slight degree to eliminate the influence of anti-dog erythrocyte membrane antibodies in B. gibsoni-infected dogs on such immunological examinations as the antigenic identification and characterization of B. gibsoni components. Furthermore, Western blotting analyses revealed that the isolated parasites retained their antigenic characteristics. As no experiments were conducted regarding viability and infectivity of parasites, it remains to be made clear whether the parasites isolated by the present method is useful for biological and biochemical studies. However, leukocytes were sufficiently removed (evaluated by the electronic method) for the purified parasites to be used in molecular biological studies.

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