Morphological, Serological and Antigenic Characteristics, and Protein Profile of Newly Isolated Japanese Bovine Babesia Parasite with Particular Reference to Those of B. ovata

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ABSTRACT. An intraerythrocytic large protozoan, tentatively designated Babesia sp.1, was recently isolated from cattle in Hokkaido Prefecture, Japan. This parasite closely resembled B. ovata in shape of piroplasms, but was distinguishable by other morphological, immunological, and biochemical characters. The paired pyriform piroplasm of B. sp.1 was larger than that of B. ovata. The results from serological and antigenic examination by enzyme-linked immunosorbent assay (ELISA) and Western blot analysis showed that there were cross- but distinguishable-reaction between B. sp.1 and B. ovata. Protein profiles of both Babesia parasites piroplasms analyzed by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) were apparently different from each other. Several major proteins revealed by 2D-PAGE and the immunodominant proteins resolved by Western blot analysis (40 kDa for B. sp.1 and 29 kDa for B. ovata) were unique to each parasite. The results of the present study indicate the possibility that B. sp.1 is a species different from B. ovata.—KEY WORDS: Babesia, ELISA, 2D-PAGE, Western blot.


The genus Babesia is composed of unicellular eukaryotic organisms which are intraerythrocytic, tick-transmitted parasites of man and animals [16]. There are at least seven Babesia species described in cattle [13]. B. ovata has been the only species of Babesia that has been identified as the causative agent of bovine babesiosis in Japan, except for the Okinawa islands [18].

In 1993, we detected large intraerythrocytic parasites, tentatively designated Babesia sp.1, in blood from cattle in Hokkaido Prefecture, Japan. It was not easy to distinguish morphologically of this parasite from B. ovata by light microscopy.

Since Babesia parasites show pleomorphic tendencies, it is difficult to characterize them by morphology alone, so those in domestic animals have been characterized by pathogenicity, epidemiology, morphology, serological property, and vector tick species [13, 15]. The complement fixation test and indirect fluorescent antibody test were used to analyze the serological and antigenic properties [4].

Biosynthetically radio-labeled proteins of in vitro cultivable Babesia species have been analyzed by gel-electrophoresis with and without immunoprecipitation [11, 17]. While, antigens and structural proteins of non-labeled B. equi piroplasms have been analyzed by gel-electrophoresis followed by Western blot analysis or silver staining [1]. Recently, species of tick-borne and intraerythrocytic protozoan parasites were discussed on the basis of their antigenic and genotypic properties obtained by enzyme-linked immunosorbent assay (ELISA) and Western blot analysis [12], structural protein profiles [23], and DNA sequence [8].

In this report, newly isolated B. sp.1 was characterized and compared to B. ovata (Miyake stock [18]) for identification.

MATERIALS AND METHODS

Parasite stocks and infection of cattle: B. sp.1 isolated in 1993 from Japanese Brown in Oshima area of Hokkaido Prefecture, Japan and B. ovata (Miyake stock [18]) isolated in 1967 from grazing cattle in Tokyo, Japan were used within 15th passage after isolation. Holstein-Friesian calves 4–6 months old, which had been splenectomized before infection, were infected with each parasite stock by blood inoculation.

Antisera: Anti-B. sp.1 and anti-B. ovata sera were obtained from the calves experimentally infected with each parasite stock after parasitaemia had progressed markedly and stored at −20°C until used. Anti-B. bigemina and anti-B. bovis sera which had been used for positive reference sera in the previous studies [5, 19], were used in this study.

Purification of piroplasms: Blood was collected from the calves experimentally infected with each parasite stock after the level of parasitaemia showed >7% for B. sp.1, and >15% for B. ovata. Piroplasms were purified from erythrocytes by nitrogen cavitation [22]. No contaminants were observed by light microscopy. The purified piroplasms were divided into aliquots and stored at −80°C until used.

Measurement of piroplasms: The paired pyriform piroplasms of B. sp.1 and B. ovata in blood smears stained with Giemsa were measured in length and width with a realtime image analyzer (LUZEX F; Nikon, Tokyo, Japan).

Enzyme-linked immunosorbent assay: ELISA was performed in a slightly modified procedure compared with the previous one [22]. In brief, the purified piroplasms were suspended in phosphate-buffered saline (PBS; pH 7.2) and mixed with an equal volume of a 4% (v/v) Triton X-100 in PBS. Without sonication, the mixed sample was
left for 3 hr at 4°C. and then centrifuged at 15,000 g for 10 min at 4°C. The supernatant was used as ELISA antigen. Optimal dilutions of reagents were obtained by checkerboard titrations. The antigens prepared from B. sp.1 and B. ovata piroplasm suspensions were diluted to 1:4,000 and 1:3,000, respectively. The sera collected from the calves that had been experimentally infected with Babesia parasites were used at dilution of 1:200 as primary serum. The horseradish peroxidase (HRP)-conjugated rabbit IgG fraction to bovine IgG (Organon Teknika, Durham, U.K.) was used as secondary antibody. Azinobenzolene (0.5 mg/ml) in 0.1 M sodium/citric buffer (pH 4.0) containing 0.03% hydrogen peroxidase was used as a substrate. Enzyme reaction was stopped with an equal volume of 1% sodium dodecyl sulfate (SDS) and optical density was measured.

Western blot: The purified piroplasm was sonicated in SDS-sample buffer [14] on ice. Sonicated sample was heated at 100°C for 5 min, and immediately after that chilled on ice. After removing insoluble components by centrifugation at 15,000 g for 5 min at 4°C, the supernatant was loaded onto a 12.5% (W/V) polyacrylamide gel. One-dimensional SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli [14]. Samples were equally loaded with proteins equivalent to 3 mg of the piroplasms per well.

The piroplasm proteins separated by SDS-PAGE were electrophoretically transferred onto polyvinylidene difluoride sheets (Immobilon-P transfer membranes; Millipore, Massachusetts, U.S.A.). After electrophoresis, the gel was equilibrated with 50 mM Tris-HCl (pH 7.4), 20% glycerol, and semi-dry electrophoretic blotting was performed at constant current using carbonate transfer buffer [7].

Blocking of sheets and detection of antigenic proteins of the piroplasm were performed as described previously [24]. The sera collected from the calves that had been experimentally infected with B. sp.1 and B. ovata at dilutions of 1:800 and 1:100 respectively, were used as the primary antisera. The HRP-conjugated rabbit IgG fraction to bovine IgG was used as the secondary antibody.

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE): The purified piroplasms were solubilized in the solubilization buffer [12]. After insoluble components were removed by centrifugation at 15,000 g for 5 min at room temperature, the supernatant was used as the loading sample. 2D-PAGE was performed as previously described [12]. Non-equilibrium pH gradient gel electrophoresis (NEPHGE) [20] was used for the first dimension, and SDS-PAGE with a 12.5% (W/V) polyacrylamide gel was used for the second dimension. Samples were equally loaded with proteins equivalent to 3 mg of the piroplasms per column.

After separation by 2D-PAGE, the piroplasm proteins in the gel were detected by silver staining (Silver staining kit; Dai-ichi Pure Chemical, Tokyo, Japan).

RESULTS

Morphology: In blood smears stained with Giemsa, B. sp.1 piroplasms showed a variety of forms; paired pyriform, pyriform, round, drop-like, budlike, oval and so on and no clear difference in shape compared to B. ovata (Fig. 1). The paired pyriform piroplasm of B. sp.1 was significantly larger than that of B. ovata (Table 1).

Enzyme-linked immunosorbent assay: In comparative ELISA between antigens of B. sp.1 and B. ovata, standardized ELISA values of heterologous combinations

Fig. 1. Piroplasms of Babesia parasite (B. sp.1) stained with Giemsa. Bar=5 μm
Several bands of *B. sp.1* piroplasm proteins were detected with anti-*B. sp.1* serum, while few bands of *B. ovata* were detected with anti-*B. sp.1* serum (Fig. 2, lanes a, b). The most intensively reactive piroplasm protein with anti-*B. sp.1* serum in *B. sp.1* and *B. ovata* was 40 kDa (Fig. 2, lane a, band 1) and 34 kDa (Fig. 2, lane b, band 2), respectively.

With anti-*B. ovata* serum, several bands of both *B. sp.1* and *B. ovata* piroplasm proteins were detected, but each antigenic protein possessed a different molecular mass and intensity of reaction. The most intensively reactive piroplasm protein with anti-*B. ovata* serum in *B. sp.1* and *B. ovata* was 28 kDa (Fig. 2, lane c, band 3) and 29 kDa (Fig. 2, lane d, band 4), respectively.

**Protein analysis by 2D-PAGE:** To compare the protein profile, we initially performed isoelectric focusing (IEF) gel electrophoresis as the first dimension. Majority of major protein spots were compressed at the end of the IEF gels (data not shown). For better resolution of proteins, we performed NEPHGE as the first dimension.

Most of protein spots from *B. sp.1* and *B. ovata* piroplasms were present in the neutral to basic area of the gels, and there were fewer neutral protein spots than basic protein spots (Fig. 3).

Many proteins with a molecular mass >35 kDa of *B. sp.1* were in the basic area of the gel. In contrast, many proteins with a molecular mass <35 kDa of *B. ovata* were in the neutral to basic area of the gel as well as protein with a molecular mass >35 kDa in the basic area of the gel. Especially, spots 1, 2, and 3 of major piroplasm proteins were unique for *B. sp.1* (Fig. 3A), and spots 4 and 5 of major piroplasm proteins were unique for *B. ovata* (Fig. 3B). The comparisons between the purified piroplasms and bovine erythrocyte lysate on 2D-PAGE suggested that the piroplasm samples loaded on the gels were free of the erythrocyte membrane (data not shown).
B. sp.1 closely resembled B. ovata in the shape of piroplasms, however, there were statistically significant differences between these two species in the length, width, and ratio of length to width of paired pyriform organisms. Minami and Ishihara compared B. ovata with other well-known bovine Babesia species for identification [18]. In their report, it is one reason for B. ovata to be a new species that the length and the ratio of length to width of paired pyriform organisms are different from those of B. major or B. bigemina, although B. ovata closely resembles these two species in shape. So, it might be reasonable to regard B. sp.1 and B. ovata as morphologically different species.

Comparative ELISA between antigens of B. sp.1 and B. ovata using sera from cattle that had infected with either of these parasites revealed that B. sp.1 was possibly a different species from B. ovata. It must be noted that antigenic differences within the same species [6, 21] and species-cross-reactive epitopes [17] were reported in Babesia, however, the results may not negatively deny the speculation that B. sp.1 is a species distinct from B. ovata.

Detailed information about antigen can be obtained by immunoblotting rather than ELISA [2]. The most responsive proteins to homologous antiserum (Fig. 2, lane a, band 1 and lane d, band 4) had no reaction to heterologous antiserum (Fig. 2, lane c and lane b) in Western blot analysis. Other differences in the band patterns between B. sp.1 and B. ovata antigens were also observed against either of antiserum. Although a difference in the immunological response between the animals should be taken into account, these results also indicated that B. sp.1 was possibly a parasite different from B. ovata.

Sugimoto et al. [23] analyzed piroplasm proteins of three Theileria parasites by 2D-PAGE for classification. The results obtained from their study are consistent with the findings in transmission studies performed using several species of tick vectors [9]. In the present study, the major and unique structural proteins of B. sp.1 (Fig. 3A, spots 1, 2, and 3) and B. ovata (Fig. 3B, spots 4 and 5) were indicated by 2D-PAGE, and almost all the structural proteins of B. sp.1 showed different molecule from those of B. ovata. In contrast, Kahl et al. analyzed radio-labeled protein of three varieties of B. bovis by 2D-PAGE, and almost all of these structural proteins were conserved among them [11]. Therefore, the results from our study indicated probability that B. sp.1 and B. ovata are distinct from each other.

The morphological, serological and antigenic characteristics, and structural protein profile suggested the possibility that B. sp.1 is a distinct species, otherwise, B. ovata has wide diversity in features that allows B. sp.1 to be involved into this species. Further studies, inclusive of epidemiology, tick vector, and pathogenicity, should be carried out for obtaining conclusive identification of B. sp.1. In recent years, a nucleic acid probe has been described as a useful tool for identification of bovine Babesia [3, 10]. The development of nucleic acid probes is now in progress in order to confirm the present speculation.

REFERENCES


NEWLY ISOLATED JAPANESE BOVINE BABESIA 675