Antigenic and Genetic Diversities of *Babesia ovata* in Persistently Infected Cattle

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ABSTRACT. Exploring the antigenic and genetic diversities of *Babesia ovata*, we obtained several field isolates from grazing cattle in the Okushiri island, Japan. Parasite isolation was greatly facilitated by using bovine red blood cell-substituted SCID mice (Bo-RBC-SCID mice), into which the blood samples of the cattle were inoculated. Isolates from different individuals within a herd of cattle were compared in immunoblot analysis with an anti-*B. ovata* serum and also in Southern blot analysis with a probe for the small subunit ribosomal RNA gene. In both analyses, the isolates exhibited banding patterns that were significantly different from each other. We were also able to obtain a series of parasite isolates from a single cow in different seasons of a nine months period, including winter when active vector ticks were not in the field environment. Different seasonal isolates showed different banding patterns in both immunoblot and Southern blot analyses. By contrast, these analyses detected little difference among the parasites that had been passed various times in Bo-RBC-SCID mice, where no specific immune responses should be generated. These results indicate that individual animals within a herd of cattle were infected with antigenically and genetically diversified populations of *B. ovata*, and that the parasites could persistently infect a single animal with dynamic change in their predominant subpopulations. — KEY WORDS: *Babesia ovata*, diversity, persistent infection.

Babesiosis, a tick-transmitted disease caused by *Babesia* parasites, still remains to be a major threat to livestock animals in tropical and subtropical regions of the world [10]. *Babesia bovis* and *B. bigemina* are the two major *Babesia* species that cause serious diseases among cattle. In Japan, however, these two *Babesia* species have been found only in Okinawa prefecture [12], and in the other part of the country, *B. ovata* is the sole bovine *Babesia* species reported so far. This parasite is known to be a relatively benign, large-type *Babesia* species transmitted by a vector tick *Haemaphysalis longicornis* [12], but details of its biology, pathology and epidemiology have remained to be studied.

Recently, we have reported on a *Babesia* parasite (previously referred to as *B. sp. 1 Oshima stock*) that was isolated from grazing cattle in Japan using bovine red blood cell-substituted SCID mice [19]. Analyses on the parasite proteins and genomic DNA [15, 16, 19] indicated that this parasite stock was quite different from the Miyake stock which had been used as the reference parasite of *B. ovata* [12]. However, since both parasite stocks showed substantial antigenic cross-reaction and were transmitted by the same vector tick, this newly isolated parasite was designated as *Babesia ovata oshimensis* n. var. [14]. An important implication from these studies was that there may be a great deal of diversity within *B. ovata*. Antigenic and genetic diversities may play a significant role in the perpetuation of *B. ovata* infections in the hosts, as those suggested in the other hemoparasitic protozoa [1–5, 7, 8].

In the present study, we investigated antigenic and genetic diversities of *B. ovata* which were isolated from grazing cattle in the Okushiri island of Hokkaido prefecture, Japan. Interestingly, significant diversity was seen not only among the parasites isolated from different individuals within a herd of cattle, but also among the isolates obtained from a single persistently infected animal.

MATERIALS AND METHODS

*Cattle*: A herd of Japanese Brown cattle grazing in the Okushiri island of Hokkaido prefecture, Japan, were examined in the present study. The place was known to be *B. ovata* endemic. Thirty-six heparinized blood samples, of which 24 were from cows and 12 from calves, were obtained in early June, 1995. The blood samples from the cows Nos. 26, 29 and 249, and from the calf No. 36, which contained relatively high numbers of *Babesia*-infected red blood cells (RBCs), were chosen for isolation of the parasites. In addition, blood samples were withdrawn from the cow No. 29 in July, September and December of 1995, and also in March of 1996.

*Isolation of *B. ovata*: The RBC samples of the cattle were washed three times with 0.85% NaCl, and 0.5 ml packed cell volume of RBCs were intravenously inoculated into a SCID mouse whose circulating RBCs had been substituted with bovine RBCs (Bo-RBC-SCID mouse). Two mice were used for each blood sample. The tail blood samples were collected from the mice every day, and microscopic examination was carried out with Giemsa-stained thin smeared blood films to detect *Babesia* parasites.
When the mice showed parasitemia levels between 10% and 40%, blood samples were collected from the mice by heart punctation under ether anesthesia. The infected RBCs were suspended in a cell freezing solution (Cell Banker, Nippon Zenyaku Co., Ltd.), dispensed in small aliquots, and stored in liquid nitrogen. When needed, the frozen parasites were propagated by intraperitoneal inoculation into Bo-RBC-SCID mice. For preparing the samples for immunoblot and Southern blot analyses, 10 to 20 Bo-RBC-SCID mice were infected, and the B. ova-infected RBCs were pooled and stored at -80°C.

Bo-RBC-SCID mice: SCID mice (C.B-17 scid, CLEA), mixed sex of age between 10 and 16 weeks old, were used. The mice were housed in a vinyl film isolator at temperature between 22 and 25°C and were provided with γ-ray irradiated pellet diet and autoclaved tap water. All mice were splenectomized, and used for experiments after the surgical wounds have healed completely. Bo-RBC-SCID mice were prepared by giving periodic transfections of parasite-free bovine RBCs into SCID mice together with administration of anti-mouse RBC monoclonal antibody 2E11, according to the method described previously [13, 20].

Anti-B. ova serum: In order to produce polyclonal antibodies which are reactive to various B. ova isolates, Bo-RBC-SCID mice were infected with a mixture of all the B. ova isolates obtained in the present study, and the parasites propagated in these mice were used for immunization. Ten BALB/c mice (SLC Inc.) were intraperitoneally injected three times at daily intervals with 0.3 ml of 30% RBCs suspension containing approximately 3 × 10⁷ B. ova-infected RBCs. One month later, the mice were boosted with an equal amount of infected RBCs. Blood samples were withdrawn from all the mice and the sera were pooled. Prior to use for immunoblot analysis, the pooled anti-B. ova serum was absorbed with an excess amount of uninfected bovine RBC ghosts.

Immunoblot analysis: Frozen B. ova-infected RBCs were thawed and washed five times at 4°C in phosphate-buffered saline (PBS), pH 7.2, by centrifugation at 10,000 × g for 10 min. The resulting crude membrane pellets were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 7.5% acrylamide gels. Proteins were electropherically transferred from the gels to Fluorotrans membranes (Pall BioSupport) for 1 hr at 100 V. The membranes were incubated in PBS containing 0.5% casein for 2 hr at room temperature. Without being washed, the membranes were placed directly in a solution of mouse anti-B. ova serum diluted 1:200 in TBS (500 mM NaCl, 20 mM Tris-HCl, pH 7.5) containing 1% gelatin for 2 hr at room temperature. The membranes were washed three times in TBS containing 0.05% Tween 20, incubated for 1 hr at room temperature with alkaline phosphatase-conjugated goat anti-mouse IgG+A+M (Jackson ImmunoResearch) diluted 1:5,000 in TBS. The membranes were again washed four times in TBS containing 0.05% Tween 20, and antibodies bound to the membranes were detected by the BCIP/NBT Alkaline Phosphatase Substrates Kit IV (Vector Laboratories, Inc.).

Southern blot analysis: Frozen B. ova-infected RBC samples, containing more than 40% parasitemia, were thawed and washed three times at 4°C in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) by centrifugation at 10,000 × g for 10 min. Pellets were resuspended in TE containing 1% SDS and 0.1 mg/ml Proteinase K and incubated at 56°C for 4 hr. Genomic DNA samples were prepared by phenol-extraction followed by ethanol precipitation according to the standard procedure [18]. Approximately 0.5 µg of DNA digested with Sac I was separated in 1% agarose gels, and transferred to Hybrid-N membrane (Amersham Life Science) using a vacuum blotter. As a template to prepare a probe for Southern blot analysis, small subunit ribosomal RNA (SSUrRNA) sequence was amplified from the B. ova isolate of the cow No. 26 by polymerase chain reaction (PCR) [11], followed by cloning into pBluescript (Stratagene). Digoxigenin (DIG)-labeled SSUrRNA probe was produced by the DIG DNA Labeling Kit (Boehringer Mannheim) according to the protocol given by the manufacturer. Probe hybridization was carried out in a hybridization buffer consisting of 5x SSC (1x SSC = 0.15 M NaCl, 15 mM sodium citrate, pH 7.0), 2% Blocking Reagent (Boehringer Mannheim), 0.1% N-lauroylsarcosine, 7% SDS and 50% formamide for 16 hr at 42°C. The membranes were washed in 0.1x SSC containing 0.1% SDS at 68°C, and were incubated with 1:5,000 dilution of alkaline phosphatase-conjugated anti-DIG antibody (Boehringer Mannheim). DNA bands on the membranes that hybridized with the DIG probe were detected with a substrate solution containing 2-hydroxy-3-naphthoic acid-2′-phenylenamide phosphate (HNPP; Aishin Cosmo).

Passage of B. ova in Bo-RBC-SCID mice: The frozen stock of the July isolate from the cow No. 29 was thawed and inoculated into a Bo-RBC-SCID mouse. When parasitemia levels became more than 40%, the RBCs were harvested from the mouse. A small portion, which contained roughly 2 × 10⁷ parasitized RBCs, were passed to another newly prepared Bo-RBC-SCID mouse, and the rest portion was divided in half and processed to prepare samples for immunoblot and Southern blot analyses as described above. This process was repeated until the 10th passage.

RESULTS

B. ova infections in a herd of grazing cattle: We carried out microscopic examination of Giemsa-stained blood smears for 24 cows and 12 calves in a herd of cattle grazing on a B. ova endemic pasture. The results are shown in Table 1, revealing that 8 of the cows and 8 of the calves were infected with B. ova and all the cattle were infected with Theileria sergenti; another hemoparasite highly prevalent in Japanese cattle. Parasitemia by B. ova was much lower than that by T. sergenti. The blood samples from the cows Nos. 26, 29 and 249, and from the calf No. 36 contained relatively high numbers of Babesia-infected
We were able to isolate \textit{B. ovata} from the four blood samples using Bo-RBC-SCID mice. Although all the samples contained both \textit{T. sergenti} and \textit{B. ovata}, the former parasites were eliminated because of much faster growth of the latter in the mice. To investigate antigenic diversity among the parasite isolates, antibodies were raised in BALB/c mice with the mixture of the \textit{B. ovata} isolates obtained from the cow Nos. 26, 29 and 249 and from the calf No. 36. Figure 1 shows the results of immunoblot analysis with the anti-\textit{B. ovata} antibodies, which clearly demonstrated that the four isolates differed from each other.

**Table 1. Prevalence of \textit{Babesia ovata} and \textit{Theileria sergenti} infections among a herd of grazing cattle in the Okushiri island, Hokkaido, Japan**

<table>
<thead>
<tr>
<th>Parasitemia (%)</th>
<th>Cows</th>
<th>Calves</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Babesia ovata}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive(^a)</td>
<td>8/24</td>
<td>8/12</td>
</tr>
<tr>
<td>Negative(^b)</td>
<td>16/24</td>
<td>4/12</td>
</tr>
<tr>
<td>\textit{Theileria sergenti}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;4.0</td>
<td>0/24</td>
<td>6/12</td>
</tr>
<tr>
<td>0.40–3.99</td>
<td>4/24</td>
<td>3/12</td>
</tr>
<tr>
<td>0.04–0.39</td>
<td>20/24</td>
<td>3/12</td>
</tr>
<tr>
<td>Negative</td>
<td>0/24</td>
<td>0/12</td>
</tr>
</tbody>
</table>

\(^a\) Only a few parasites, less than 0.01%, were detected.\(^b\) Microscopically undetectable.

**Fig. 1. Immunoblot analysis of \textit{B. ovata} isolated from different individuals in a herd of cattle. Antigens were prepared with four \textit{B. ovata} isolates obtained from the cows Nos. 26, 29 and 249, and from the calf No. 36, and with uninfected bovine erythrocytes (RBC). Following SDS-PAGE and blotting onto a Fluorotrans membrane, parasite antigens were detected with a pooled anti-\textit{B. ovata} serum. The relative mobilities of the molecular mass standard proteins are shown on the left.**

**DISCUSSION**

In the present study, we have clearly demonstrated that significantly diversified \textit{B. ovata} infects cattle under the field conditions. Antigenically and genetically distinct parasites were isolated not only from different individuals within a herd of cattle, but also from a single animal. Although antigenic and genetic diversities were documented in various hemoprotozoa \cite{4, 7, 9}, most of them had been demonstrated with parasites isolated from geographically distant places. Whether or not (and how frequently) individual hosts are infected with mixed populations of diversified parasites is an important issue, because the
presence of multiple parasite clones in a vector’s blood meal is a prerequisite to cross-fertilization which will result in further genetic diversity. Recent studies employing new techniques, such as allele-specific PCR, make it increasingly clear that mixed infections of multiple, diversified parasite clones are common for many hemoprotozoan species [2, 5, 9].

An attractive hypothesis for the presence of antigenically diversified parasites is that it may give some advantages to the parasites for their perpetuation in a herd of cattle and also for persistent infection within a host animal [5, 8]. In other words, the parasites may increase their antigenic diversity in order to evade the immunity of an individual animal and also of an animal herd. Indeed, significant differences were observed among parasite antigens detected in the immunoblot analysis with the isolates obtained from a persistently infected cow, but not with those grown in immunodeficient mice even after as many as 10 passages. The results strongly suggest that the predominant parasite subpopulations within an infected animal change dynamically in association with the host immune responses. A similar finding has recently been reported in cattle infected with *T. sergenti* [8].

Genetic diversity of *B. ovata* was evidenced by restriction-fragment-length polymorphism observed in Southern blot analysis with the SSUrRNA probe. A study on the genomic DNA of *B. bigemina* showed that the parasite has three rRNA gene units, each one of which encodes almost identical SSUrRNA sequence [17]. The presence of more than three bands in the Southern blot analysis, therefore, probably indicates the presence of genetically distinct parasite populations. Additional evidence for this was brought from our preliminary sequence analysis on the SSUrRNA genes of various *B. ovata* isolates, which revealed sequence differences at as much as 34 positions in 1685 nucleotides (unpublished data). Since SSUrRNA gene is a typical house-keeping gene whose product is unlikely to be the target of the host immune responses, the immune pressure may not be a sole driving force for expanding the parasite diversity.

Another important finding in the present study was that *B. ovata* persistently infected cattle, contributing to the presence of multiple parasite clones in a vector’s blood meal is a prerequisite to cross-fertilization which will result in further genetic diversity. Recent studies employing new techniques, such as allele-specific PCR, make it increasingly clear that mixed infections of multiple, diversified parasite clones are common for many hemoprotozoan species [2, 5, 9].

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parasite survival during winter season when active vector ticks were not in the field environment. Persistent infection of B. ovata has previously been demonstrated experimentally with splenectomized cattle [6], but whether or not it occurs under the field conditions was not clear, because after the initial attack, parasitemia in an spleen-intact animal decreases rapidly and usually becomes undetectable by microscopic examination. The Bo-RBC-SCID mice used in the present study allowed us to isolate B. ovata from blood samples containing microscopically undetectable levels of parasitemia. As previously reported [19], Bo-RBC-SCID mice were also useful to isolate B. ovata from cattle infected with both T. sergenti and B. ovata.

In an attempt to investigate the relationship between the host immune responses and the changes in predominant parasite subpopulations, the serum samples obtained from the cow No. 29 in different seasons were tested by immunoblot technique for anti-B. ovata antibodies (data not shown). However, all the samples exhibited only a weak reaction at 1:50 dilution, and did not show unequivocal differences against the parasite antigens of various seasonal isolates. It would be an interesting speculation that low antibody titers in the cow may be due to low levels of parasitemia. If this is the case, lowering parasitemia to a level unable to mount immune responses may be another factor, in addition to the presence of antigenically diverse parasite subpopulations, contributing to the persistent infection of B. ovata in cattle.

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Fig. 4. Southern blot analysis of different seasonal *B. ovata* isolates from a cow. Five Sac I-digested genomic DNA samples were prepared from the parasite stocks isolated from the cow No. 29 in June, July, September and December, and March in the following year. Lanes M and B contain Sac I-digested murine and bovine genomic DNAs, respectively. DNA fragments containing the SSUrRNA gene were detected with a DIG-labeled probe. The sizes of the λHind III fragments are indicated on the left.


Fig. 5. Immunoblot analysis of *B. ovata* passed in Bo-RBC-SCID mice. The parasite antigens of the 1st, 3rd, 5th, 7th, 9th and 10th passages were detected with a pooled anti-*B. ovata* serum. An uninfected bovine erythrocyte ghost sample was included as control (RBC). The relative mobilities of the molecular mass standard proteins are shown on the left.

