

PCR Detection of *Babesia ovata* from Cattle Reared in Japan and Clinical Significance of Coinfection with *Theileria orientalis*

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We describe here the clinical significance of coinfection with *Theileria orientalis* and *Babesia ovata* in cattle. Anemia status in a herd of dairy cattle in Japan was investigated in relation to infection with these parasites. Our findings indicate that while *B. ovata* infection might not be the primary cause of anemia in the cattle, it may contribute to the clinical development of anemia in animals coinfecting with both *B. ovata* and *T. orientalis*.

The benign group of *Theileria* parasites (*Theileria sergenti*, *Theileria buffeli*, and *Theileria orientalis*) and *Babesia ovata* constitute the only bovine piroplasms that are known to be endemic in Japan (1, 7, 13). *T. orientalis* is not considered to be highly pathogenic; however, clinical signs of anemia have sometimes been observed in cattle affected by this parasite (5, 8). In contrast, previous studies of splenectomized calves indicate that only immunocompromised animals exhibit anemia-related clinical signs when they are infected with *B. ovata* (3). Both *T. orientalis* and *B. ovata* are transmitted to the bovine host by *Haemaphysalis longicornis*, which is an ixodid tick vector (6). Therefore, mixed infections with the two parasites are expected to be very common in areas of endemicity (3). Although several researchers have focused on the clinical consequences of infection with each of these parasites alone (3, 5), the pathobiology of mixed infection with *T. orientalis* and *B. ovata* has not been evaluated. Infections with *T. orientalis* and *B. ovata* can be diagnosed by examination of Giemsa-stained thin blood smears under a light microscope. However, the lack of sensitivity and difficulty of species differentiation have limited the use of this technique (2). Therefore, PCR methods are employed because of their greater sensitivity and specificity (11). Although several PCR assays have been developed for diagnosis of *T. orientalis* infection (8, 11), microscopy remains the only available technique for detecting *B. ovata*.

In the current study, we developed a novel PCR assay for detection of *B. ovata* in DNA samples extracted from infected bovine blood. A set of forward (5'-GATACGAGGCTGTCCGTAGC-3') and reverse (5'-AGTATAGGTGAGCATCAGTG-3') primers was designed to amplify a 504-bp fragment of the *B. ovata* Apical membrane antigen 1 (AMA-1) gene (GenBank accession number AB634843). One microliter of template DNA was added to 25 μ l of a reaction mixture containing 2.5 μ l of 10 \times PCR buffer (Applied Biosystems, Branchburg, NJ), 200 μ M (each) deoxynucleoside triphosphates (dNTP) (Applied Biosystems), 0.8 μ M (each) primer, 1 U *Taq* polymerase (Applied Biosystems), and double-distilled water. PCRs were conducted using a Veriti thermal cycler (Applied Biosystems). Optimized cycle conditions were used for the PCR: briefly, 95°C for 5 min, followed by 45 cycles each of which consisted of a denaturing step at 95°C for 30 s, an annealing step at 56°C for 1 min, and an extension step at 72°C for 1 min. After a final elongation step at 72°C for 10 min, agarose gel elec-

trophoresis and ethidium bromide staining were followed by visualization of amplicons under UV light. The AMA-1 PCR amplified a 504-bp product from *B. ovata* DNA; no amplicons were produced using DNA derived from 11 other bovine blood pathogens (Fig. 1A). Furthermore, the assay was sensitive enough to detect 100 fg genomic DNA from *B. ovata* (Miyake strain) *in vitro* culture (Fig. 1B).

Blood samples were collected from 94 randomly selected dairy cattle from a dairy farm within the Shin-Hidaka district in Japan on 19 June 2009. Microscopic examination of Giemsa-stained thin blood smears indicated that 29 (30.8%) and 3 (3.2%) of the animals were positive for infection with *T. orientalis* and *B. ovata*, respectively. However, when DNA from the blood samples was screened using a previously described PCR method based on the gene encoding the major piroplasm surface protein (MPSP-PCR) (8) and our newly developed AMA-1 PCR assay, 47 (50%) and 23 (24.5%) of the animals were found to be infected with *T. orientalis* and *B. ovata*, respectively (Table 1). All of the blood samples that were microscopy positive were also found to be positive using the two PCR assays (i.e., MPSP-PCR for *T. orientalis* and AMA-1 PCR for *B. ovata*). Sequencing analysis confirmed that all of the *B. ovata*-specific amplicons had nucleotide sequences identical to those of the *B. ovata* AMA-1 gene (GenBank accession number AB634843). The degree of anemia in the animals was determined by red blood cell (RBC) counts, hemoglobin (Hb) concentrations, and hematocrits (HCT), using a Celltac (Nihon Kohden, Tokyo, Japan) automated hematological analyzer (8). An animal was considered to be anemic if the RBC, Hb, or HCT value was less than 5×10^6 RBCs/ μ l, 8 g/dl, or 24%, respectively (4).

Animals were categorized in four groups according to their infection profiles (Table 1). All of the noninfected animals had normal values for all three hematological parameters (i.e., RBC

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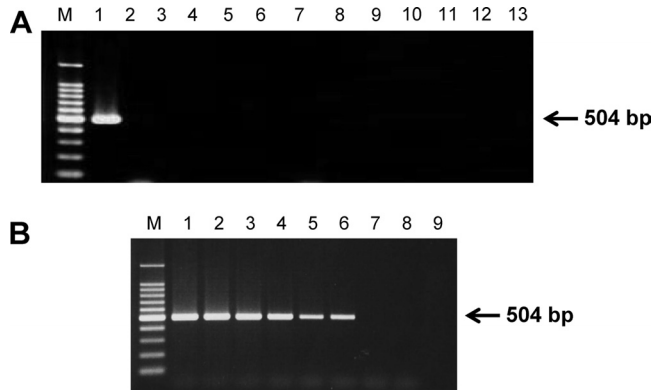


FIG 1 *Babesia ovata*-specific AMA-1 PCR assay development. A new PCR method was developed for the diagnostic detection of *B. ovata*, based on the *B. ovata* AMA-1 gene. (A) PCR specificity. Lanes 1 to 13 represent genomic DNA samples from *B. ovata*, *Babesia bigemina*, *Babesia bovis*, *Theileria annulata*, *T. orientalis*, *Trypanosoma brucei gambiense*, *Trypanosoma evansi*, *T. theileri*, *Anaplasma marginale*, *Anaplasma bovis*, *Anaplasma centrale*, *Anaplasma phagocytophilum*, and normal bovine blood, respectively. (B) PCR sensitivity. 10-fold serial dilutions of *B. ovata* genomic DNA extracted from *in vitro* culture. Lanes 1 to 8 represent 10 ng/ μ l, 1 ng/ μ l, 100 pg/ μ l, 10 pg/ μ l, 1 pg/ μ l, 100 fg/ μ l, 10 fg/ μ l, and 1 fg/ μ l, respectively. An uninfected bovine DNA sample was included as a negative control (lane 9). M, 100-bp DNA marker ladder (both panels).

count, Hb, and HCT); hence, other agents that could have contributed to the anemia among the animals could be ruled out. In addition, all the DNA samples were PCR screened for the presence of *Anaplasma* species as described previously (9, 12). Because only a single animal in the noninfected category was found to be infected with *Anaplasma bovis*, possible involvement of anaplasmosis as a cause of the anemia could be excluded. We found that the anemic animals were infected with *T. orientalis* alone or with *T. orientalis* and *B. ovata* (Table 1). Among the anemic cattle, all animals were positive for *T. orientalis* by microscopy, while *B. ovata* was detected on a blood smear from a single animal. In agreement with findings of a previous study (3), none of the animals that were infected only with *B. ovata* were anemic. The anemia rates among the coinfecting animals (42.9%) were significantly higher than those for animals infected with *T. orientalis* (18.2%) alone (Table 1). Among the anemic animals, three exhibited low values for all three hematological parameters; two of these animals were found to be coinfecting with both of the parasites (data not shown). The mean RBC count of the animals ($n = 33$) infected only with *T. orientalis* ($5.90 \times 10^6/\mu\text{l}$) was lower than that of the noninfected animals ($7.29 \times 10^6/\mu\text{l}$), while the RBC count

TABLE 1 Diagnostic results obtained for 94 field grazing cattle using *T. orientalis* MPSP-PCR and *B. ovata* AMA-1 PCR assays

Infection type ^a	No. (%) of positive animals	No. of anemic animals	Anemia rate (%)
Noninfected	38 (40.4)	0	0
<i>T. orientalis</i> ^b	33 (35.1)	6	18.2
<i>B. ovata</i> ^b	09 (9.6)	0	0
<i>T. orientalis</i> and <i>B. ovata</i>	14 (14.9)	6	42.9

^a Animals were categorized in 4 groups, which consisted of noninfected, *T. orientalis*-infected, *B. ovata*-infected, or coinfecting animals, based on the infection types.

^b Animals were infected with a single parasite species.

TABLE 2 Mean red blood cell counts and hemoglobin and hematocrit values for the different categories of animals

Infection type	Mean value \pm confidence interval		
	RBC count ($\times 10^6/\mu\text{l}$)	Hb concn (g/dl)	HCT value (%)
Noninfected	7.29 ± 0.29	11.6 ± 0.31	32.0 ± 0.91
<i>T. orientalis</i>	5.90 ± 0.35^a	11.2 ± 0.49	30.1 ± 1.39
<i>B. ovata</i>	7.24 ± 0.63	11.4 ± 0.44	31.7 ± 1.45
<i>T. orientalis</i> and <i>B. ovata</i>	5.64 ± 0.56^a	10.3 ± 0.88^a	27.8 ± 2.33^a

^a Statistically significant reduction in the mean value obtained compared to that for the noninfected animals ($P < 0.01$).

of the coinfecting animals ($n = 14$) ($5.64 \times 10^6/\mu\text{l}$) was similar to that obtained for those animals infected with *T. orientalis* alone (Table 2). In contrast, a significant reduction of the mean Hb concentration was observed only in the coinfecting animals (10.3 g/dl), compared with results for the noninfected animals (11.6 g/dl), whereas *T. orientalis* infection alone did not alter the mean Hb concentration (11.2 g/dl). A similar observation was also made for the mean HCT value, in which only the coinfecting animals (27.8%) showed a lower HCT value than the noninfected animals (32.0%) (Table 2).

Based on these findings, we suggest that *B. ovata* might be a risk factor for the induction of clinical anemia when animals are coinfecting with *T. orientalis*, although *B. ovata* is unlikely to be the sole agent causing anemia in the cattle population in regions of endemicity. In common with other *Babesia* parasites, *B. ovata* induces intravascular hemolysis, as was evident from the hemoglobinuria detected when splenectomized cattle were infected with the parasite (3). In contrast, the anemia induced by *T. orientalis* is thought to be due to erythrophagocytosis of red blood cells (10). Therefore, we assume that the combination of these two different mechanisms for anemia could have potentiated the serious development of clinical anemia among the animals that were coinfecting with *B. ovata* and *T. orientalis*. In this report, we have described the effects *T. orientalis* and *B. ovata* infections have on the anemia status of a cattle population. Further studies using a larger sample size are now necessary to confirm our findings. In addition, *in vivo* experiments to study the effects of coinfection with both parasite species in susceptible animals are a high priority.

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