by the ELISA with rP200/CT and for the detection of *B. bigemina* parasites in microscopy and DNA by PCR. As shown in Table 1, none of the animals were positive for *Babesia* parasites by microscopic examination of Giemsa stained blood smears. On the other hand, 66 (61%) and 67 (62%) of the tested samples were positive by the ELISA and PCR, respectively. In addition, 42 (38.9 %) samples were positive by both the ELISA and PCR, while 25 (23.1%) PCR positive samples were ELISA negative and 22 (20.3 %) PCR negative samples were ELISA positive (Table 1). Sequence analysis of all positive field PCR samples showed a 100% identity with p200 (data not shown).

Discussion

In the search for suitable antigens to detect *B. bigemina* infection, attention has been focused on the identification of immunodominant antigens recognized by sera from animals infected with distant geographical isolates and from both acute and chronically infected animals (Boonchit et al. 2006). *B. bigemina* 200 kDa protein (P200), one of such conserved and immudominant antigens, was previously identified as a candidate diagnostic antigen for serological assaying in the detection of *B. bigemina* antibodies in infected cattle (Tebele et al. 2000).

B. bigemina p200 is present in merozoite cytoplasm, suggesting that the antigen may be a structural protein involved in forming filament structures within the cytoskeleton. In addition, P200 is immunogenic and is conserved among different strains of *B. bigemina*. In this study, a truncated fragment of the 200 kDa (rP200/CT) protein was expressed in *E. coli* for the serological diagnosis of *B. bigemina* infection. To examine whether antibodies against rP200/CT were elicited during natural infection with *B. bigemina*, recombinant rP200/CT was examined for its reactivity with known positive sera collected from infected cattle of endemic areas. As shown in Fig. 2, sera from these animals showed reactivity with the recombinant protein on Western blotting. Furthermore, in a comparative ELISA with RAP-1/CT (Boonchit et al. 2006), rp200/CT protein was recognized by *B. bigemina*-infected serum samples. However, the OD values of *B. bigemina*-infected bovine sera in the rP200/CT ELISA were slightly lower than those in the rRAP-1/CT ELISA, probably indicating that some immunodominant region capable of inducing a stronger humoral immune response could have been removed as a result of the truncation. Although the sensitivity of rP200/CT was low, as compared to RAP-1, it proved to be highly conserved and specific for the detection of antibodies against *B. bigemina* infection across different geographical regions.

In the current study, none of the cattle examined had detectable *Babesia* parasites by microscopy; however, some positive detection was obtained by ELISA and PCR methods, respectively (Table 1). The mechanisms involved in parasitemia variations in carrier infections are not well understood. However, it has been observed that parasite densities in *Babesia*-carrier animals seem to fluctuate with time and periodically fall below the detection level of microscopy and PCR (Aguilar-Delfin et al. 2001, 2003; Brown et al. 2006; de Vos and Bock 2000). The proportions of seropositive samples from *B. bigemina* naturally infected cattle compared with the PCR results (67%) are indicative of high sensitivity of rp200/CT antigen. In addition, the rp200/CT-ELISA detected antibodies against *B. bigemina* in samples that were PCR negative (Table 1). Either this could imply low level of parasite DNA below detection limit of the PCR or the persistence of the parasite antibodies for a period even after the living pathogen has been eliminated from the host. In fact, microscopy and PCR-based detection methods are less likely to detect low parasite densities because of a higher probability that no infected erythrocytes are present in the minute blood volume used. In this respect, this ELISA could be useful in screening cattle, which are exposed to *B. bigemina* infection by monitoring their antibody levels. Such a strategy is normally vital in prevention of new infections rather than in treatment of clinical infection, which require specific methods of parasite identification. On the other hand, some 20 seronegative samples tested positive for parasite DNA by PCR suggesting that the samples could have been from cattle with early parasitic infection characterized by undetectable antibody titers. Alternatively, this could have been due to the relatively higher sensitivity of PCR compared with the rp200-ELISA. The rp200/CT-ELISA detected higher proportion of positive samples than

Table 1 Comparison of microscopy, PCR, and ELISA results

Number of Samples	Microscopy	ELISA	PCR	ELISA-PCR[a]	ELISA-PCR[b]	PCR-ELISA[c]
108	0	66 (61%)	67 (62%)	42 (38.8%)	25 (23.1)	22 (20%)

[a] Samples positive with both GST-rP200/CT-ELISA and PCR

[b] ELISA-positive field samples but negative according to the PCR

[c] PCR-positive field samples but negative according to the ELISA

PCR in samples collected from apparently healthy cattle (Table 1). From epidemiological point of view, screening for subclinical or latent infections is important because such cases could serve as potential source of re-infection (Stegeman et al. 2003); moreover, cases of relapses of infection are possible. Therefore, rp200/CT-ELISA could be useful in screening surveys and in identification of infected cattle that appear apparently healthy while PCR could be useful especially in cases where microscopic findings are inconclusive. Nevertheless, the ELISA and PCR each has its own advantages and disadvantages depending on the intended use. Alternatively, a repetition or a combination of different diagnostic tests might provide a more accurate picture of the true prevalence of infection.

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FULL PAPER *Parasitology*

Epidemiological Survey of *Theileria orientalis* Infection in Grazing Cattle in the Eastern Part of Hokkaido, Japan

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ABSTRACT. *Theileria orientalis* is one of the benign species of *Theileria* that is widely distributed in Japan and is sometimes responsible for serious economic losses in the livestock industry. In the present study, we surveyed the current status of *T. orientalis* infection in grazing cattle in the eastern areas of Hokkaido (Taiki, Otofuke, Shintoku, and Shin-Hidaka districts) using molecular methods, as well as traditional methods, of diagnosis. The genes encoding the major piroplasm surface protein (MPSP) and p23 of *T. orientalis* were identified using highly detectable polymerase chain reaction (PCR). Results of the MPSP-PCR assay indicated that grazing cattle in these districts, after about 1.5 months pasturage, showed high rates of infection, ranging from 10.0–64.8%. Although the main MPSP and p23 genotypes detected were the Ikeda- or Chitose-types, an MPSP gene closely relating to that found in Okinawa prefecture, and a p23 gene closely relating to the Australian (Warwick) Buffeli-type gene, were found in the cattle in Shintoku and Shin-Hidaka districts. The present survey indicated that there were at least five types of *T. orientalis* classified by their MPSP genes in Hokkaido, Japan, and that *T. orientalis* infection rates are still high in this region.

KEY WORDS: cattle, Hokkaido, MPSP, p23, *Theileria orientalis*.

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Bovine theileriosis in Japan is caused by *Theileria orientalis* which is a tick-transmitted, intraerythrocytic protozoan belonging to the phylum Apicomplexa [23]. In Hokkaido, the northern island of Japan, most cattle are pastured from spring to autumn to reduce rearing costs, but this is associated with a high risk of contracting theileriosis. *T. orientalis* is a member of the relatively benign Theileria group (*Theileria sergenti/buffeli/orientalis*). It shows a lower pathogenicity in cattle than *T. parva* and *T. annulata*, but causes symptoms including fever, anemia, and anorexia [7]. However, the livestock industry in Japan still suffers enormous economic losses due to this disease [9, 22], because no commercially effective medicines or vaccines are currently available for its control in Japan. Application of flumethrin pour-on to the grazing cattle is the main method used to control the parasite, by reducing the number of vector ticks during the pasturage season [20]. Shimizu *et al.* [20] reported that the mortality and morbidity in grazing cattle in Japan were less than 0.1% and about 2.5%, respectively. Although the tick densities in Hokkaido are lower than in other areas [26], many farmers still suffer from the consequences of bovine theileriosis and related losses in livestock production.

The purpose of the present study was to investigate the current status of bovine theileriosis in Hokkaido and to discover the significance of this disease in grazing cattle. The genes encoding the major piroplasm surface protein (MPSP) and p23 of *T. orientalis* were subjected to epidemiological molecular analysis. The MPSP and p23 genes are expressed in the intraerythrocytic stage of the parasite, called piroplasm, and are conserved to some degree among field isolates of *T. orientalis* [6, 8, 17]. *T. orientalis* populations have recently been found to consist of seven types, based on a series of all the registered MPSP gene sequences [10, 27]. Another gene, which encodes a 23-kDa piroplasm membrane protein, was also reported to show genetic diversity [18, 28]. We therefore collected a total of 501 blood samples from 230 grazing cattle in the eastern areas of Hokkaido, Japan (Taiki, Otofuke, Shintoku, and Shin-Hidaka districts) in 2007 and 2008. We surveyed the current status of *T. orientalis* infection in the cattle using molecular methods, including newly designed PCR primers and DNA sequencing analyses, together with classical methods of diagnosis.

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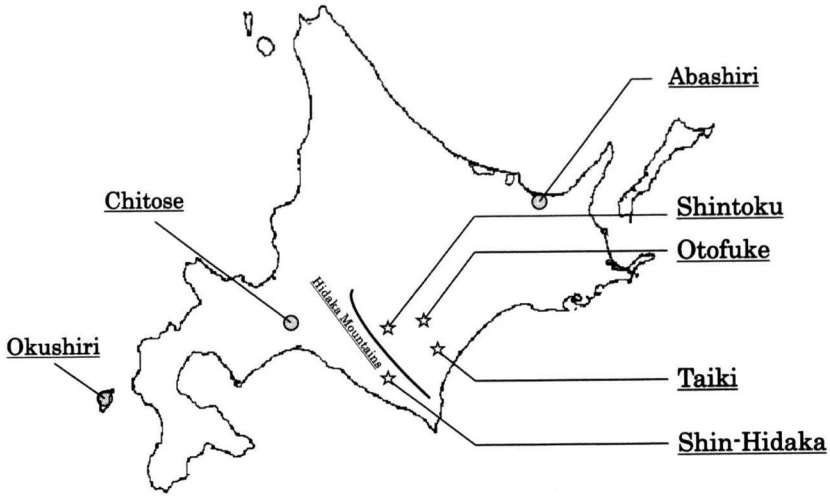

Fig. 1. Geographical map of Hokkaido, Japan. Stars indicate the locations of the four districts (Otofuke, Shintoku, Taiki, and Shin-Hidaka) where the blood samples were collected. Filled circles show the locations (Abashiri, Chitose, and Okushiri districts) where previous epidemiological surveys of *T. orientalis* have been carried out [10, 11, 14].

MATERIALS AND METHODS

Collection of blood samples: The cattle examined in the present study were grazed on 4 public farms in the eastern areas of Hokkaido (Taiki, Otofuke, Shintoku, and Shin-Hidaka districts) (Fig. 1), from May to July in 2007 and 2008. In the Taiki district, Holstein cattle aged 12–20 months were firstly allowed to graze from 4th June, 2007, and 89 or 91 blood samples were collected 4 times (on 29th May, 25th June, 9th July, and 23rd July, 2007) from the cattle. These samples were collected in order to investigate the changes in *T. orientalis* infection throughout the grazing period. Thirty-six and 83 blood samples were collected from cattle in the Shintoku (firstly grazing cattle aged approximately 9 months) and Shin-Hidaka districts (cattle of various ages with various grazing histories) on 17th July and 25th July, 2007, respectively. The other 20 samples were collected from cattle in the Otofuke district (cattle of various ages, including 4 firstly grazing cattle) on 14th July, 2008. The animals sampled included Japanese Black, Angus, Hereford, Japanese Short-horn, and Holstein cattle (Table 1). The sampling was performed at approximately 1.5 months after the start of pasturage. Thus, a total of 501 blood samples were collected from 230 grazing cattle in these districts.

Diagnoses of parasitemia and anemia: Approximately two m*l* of blood were collected from the tail veins of cattle and added to ethylenediaminetetraacetic acid, to a final concentration of 0.15–0.22%. Thin blood smears were made and then stained with Giemsa solution, for classical microscopic diagnosis. Parasitemia was observed in *T. orientalis*-infected red blood cells (RBC) by microscopy. The blood smear was classified based on the followed method, referred to as the Ishihara's method in the Japanese literature: –; no detection, +; 1 parasite in 10 fields (<0.05%), ++; >1 parasite in 10 fields (0.05–0.5%), +++; more than 1 parasite in 1 field (0.5–5%), ++++; more than 10 parasites in 1 field (> 5%). Additionally, the RBC count, hematocrit (HCT) value, and hemoglobin (Hb) concentration of respective blood samples were determined using a Celltac α (Nihon Koden, Tokyo, Japan), to detect anemia. Anemia was diagnosed if the RBC, HCT, or Hb values were $<5 \times 10^6$ cells/μl, 24%, or 8 g/d*l*, respectively [5].

DNA extraction and plasma preparation: Genomic DNA was extracted from 100 μl of blood sample using a QIAamp® DNA Blood Mini Kit (Qiagen, Hilden, Germany), and 200 μl of the DNA solutions (approximately 6 $\mu g/\mu l$) were obtained and stored at –30°C. Blood plasma fractions were obtained by centrifugation at 700 × g for 15 min at 4°C, and then stored at –30°C, for subsequent analysis by enzyme-linked immunosorbent assay (ELISA).

PCR: A pair of universal primers (857 base pairs (bp)) [8, 24], and three kinds of type-specific primer pairs for the Ikeda strain (826 bp), Chitose strain (831 bp), and *T. buffeli* Warwick stock (825 bp) [10, 13, 27] were used for the PCR identification of *T. orientalis*. All the primer pairs were targeted to the MPSP (p32/33/34) gene of *T. orientalis*, and were able to amplify the indicated sizes of DNA fragments by PCR.

In the present study, we designed two new kinds of primer pairs universal for *T. orientalis*, by aligning all of the registered sequences of MPSP and p23 genes listed in Figs. 2 and 3. Based on the designation, the first "MPSP" primers, MPSP-F (5'-CTTTGCCTAGGATACTTCCT-3') and MPSP-R (5'-ACGGCAAGTGGTGAGAACT-3'), amplified a 776-bp DNA fragment from the MPSP gene of *T. ori-*

entalis, while the second "p23" primers, p23-F (5'-GTACACACCTTGAAATCTGGC-3') and p23-R (5'-CAAGAGAGGCAACAACAACGA-3'), amplified a 601-bp DNA fragment from the p23 gene. These primer pairs targeted completely conserved regions in these database sequences, and the PCR analyses were expected to be adopted for the surveillance of *T. orientalis* infections.

These diagnostic PCR analyses were performed using 2 μl of stored DNA template extracted from the blood sample, mixed with 18 μl of reaction buffer, consisting of 0.2 μl of Ex Taq DNA polymerase (Takara, Tokyo, Japan), 0.2 μl of 10 μM each primer, 10 μl of 2 × Ampdirect plus (PCR buffer: Shimadzu Biotech., Kyoto, Japan), and 7.4 μl of double distilled water (DDW). These reactions were performed using an iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, U.S.A.) for 35 cycles. After an initial denaturation step for 10 min at 94°C, all the cycles consisted of denaturation for 1 min at 94°C, annealing for 1 min at 58°C, and extension for 1 min at 72°C, followed by an additional 4 min at 72°C. The amplified PCR products were subjected to 1.5% agarose gel electrophoresis, stained with ethidium bromide (Sigma-Aldrich, St. Louis, MO, U.S.A.), and then visualized under an ultraviolet light.

ELISA: ELISA was performed as described previously [21] with some modifications to check the presence of *T. orientalis*-specific immunoglobulin G (IgG) in the collected blood plasma fractions. Crude parasite antigens including a mixture of Ikeda- and Chitose-type piroplasms kindly provided by Kaketsuken (The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan) were used. In brief, each well of a micro-titer plate (Nalge Nunc, Roskilde, Denmark) was coated with 1 μg/m*l* of the antigen in 100 μl of 0.05 M carbonate-bicarbonate buffer (pH 9.6), and then incubated overnight at 4°C. Samples were washed five times with 0.05% Tween-20 in phosphate-buffered saline (PBS-T) between all steps. The whole well was then blocked with 200 μl of 4% skimmed milk in PBS-T for 1 hr at 37°C. Plasma samples diluted to 1:400 with 1% skimmed milk in PBS-T were added as the first antibodies in 100 μl, and incubated at 37°C for 1 hr. Horseradish peroxidase-conjugated rabbit serum anti-bovine IgG (Bethyl Laboratories Inc., Montgomery, TX, U.S.A.) was diluted to 1:10,000 with 1% skimmed milk in PBS-T, and 100 μl of the second antibody was added to each well. After incubation at 37°C for 1 hr, 100 μl of SureBlue Reserve TMB Microwell Peroxidase Substrate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, U.S.A.) were added, and the mixture was incubated at room temperature for 20 min. After adding an equal volume of 1N HCl to each well to stop the reaction, the optical density (OD) was measured at a wavelength of 450–630 nm, using an MTP-120 ELISA reader (Corona Electric, Ibaraki, Japan). The sample was considered to be positive for *T. orientalis* infection when the OD value was > 0.5.

DNA sequencing and phylogenetic analysis: DNA sequencing analysis of PCR fragments was carried out to examine the diversities of the MPSP and p23 genes derived from the blood samples. At least six blood samples were randomly selected from the diagnostic PCR-positive samples from each of the Taiki, Shintoku, and Shin-Hidaka farms. The DNA fragments were amplified by a further PCR step for subsequent DNA sequencing. PCR was performed using 2 μl of the selected DNA template mixed with 18 μl of reaction buffer, consisting of 0.2 μl of Expand HiFi Plus Enzyme Blend (Roche Applied Science, Basel, Switzerland), 0.2 μl of 10 μM of each primer, 4 μl of 5 × Expand HiFi Plus Reaction Buffer with 7.5 mM $MgCl_2$, 0.4 μl of 10 mM PCR Grade Nucleotide Mix (Roche Applied Science), and 13 μl of DDW. All of the PCR cycles consisted of an initial denaturation step for 2 min at 94°C , followed by 35 cycles for 1 min at 94°C, annealing for 30 sec at 58°C, and extension for 1 min at 72°C, followed by an additional 7 min at 72°C. The amplified PCR products were cloned into a pCR2.1 vector, according to the manufacturer's instructions for the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, U.S.A.). DNA sequencing of the inserts was performed by a DNA sequencing service (Sigma-Aldrich, Tokyo, Japan). The CLUSTAL X program (University College Dublin, Dublin, Ireland) was used to align the obtained sequences, and a neighbor joining method using an NJplot program [16] constructed 2 phylogenetic trees based on the MPSP and p23 genes. The accuracy of the branches of the phylogenetic trees was confirmed by a bootstrap method [1]. The representative sequences obtained in the present study were registered in the GenBank database (National Center for Biotechnology Information, National Institutes of Health), as indicated in Figs. 2 and 3.

RESULTS

A study of grazing cattle in Taiki district: In Taiki district (Fig. 1), grazing was started on 4th June in 2007. On 29th May, before the cattle were put out to graze, only 2 out of 89 cattle (2.2%) were positive for *T. orientalis* infection, based on microscopic observation, sero-diagnostic ELISA, and PCR detections of the MPSP and p23 genes, as shown in Tables 1 and 2. However, the number of *T. orientalis*-positive cattle had increased dramatically, to 48.4% and 49.5%, based on PCR detections of the MPSP and p23 genes, respectively, by 25th June, 3 weeks after being put out to pasture. By 23rd July, more than half of the cattle (64.8% by MPSP-PCR and ELISA) were infected with *T. orientalis*. Importantly, the percentage of cattle with anemia increased from 0.0% (29th May) to 22.0% (23rd July) in a group of MPSP PCR-positive cattle (Table 1).

The newly designed MPSP and p23 primers used for PCR analyses could detect the parasite in a greater number of blood samples than the classical universal primers could, as shown in Table 2. PCR-based typing for the Ikeda, Chitose, and Buffeli stocks showed that at least the individuals of *T. orientalis* carrying the Ikeda-type (type 2 in Fig. 2) and Chitose-type MPSP genes (type 1) were distributed in the farm in Taiki district, and approximately half of the infected cattle exhibited a mixed infection with the Ikeda- and Chitose-

Table 1. Classical diagnoses and disease occurrence of *T. orientalis*-positive cattle grazing in the east part of Hokkaido

Districts	Species	Date (total cattle)	Number of positive cattle (%)		
			Microscopic test[a)]	ELISA[a)]	Anemia[b)]
Taiki	Holstein	5/29 (89)	2 (2.2)	2 (2.2)	0 (0.0)
		6/25 (91)	13 (14.3)	14 (15.4)	3 (6.8)
		7/9 (91)	40 (44.0)	37 (40.7)	5 (8.6)
		7/23 (91)	47 (51.6)	59 (64.8)	13 (22.0)
Otofuke	Japanese Black/Angus	7/14 (20)	5 (25.0)	1 (5.0)	0 (0.0)
Shintoku	Japanese Black/Angus	7/17 (36)	8 (22.2)	4 (11.1)	0 (0.0)
Shin-Hidaka	Hereford/Japanese Shorthorn/Holstein	7/25 (83)	57 (68.7)	39 (47.0)	16 (32.6)

a) Number (%) of cattle showing positive reactions (of total cattle).
b) Number (%) of cattle showing clinical anemia in a group of MPSP PCR-positive cattle as shown in Table 1.

Table 2. PCR diagnoses of *T. orientalis*-positive cattle grazing in the east part of Hokkaido

Districts	Date (total cattle)	Number of positive cattle (%)					
		Universal[a)]	MPSP[a)]	p23[a)]	Ikeda[b)]	Chitose[c)]	I&C[d)]
Taiki	5/29 (89)	0 (0.0)	2 (2.2)	2 (2.2)	2 (100.0)	0 (0.0)	0 (0.0)
	6/25 (91)	13 (14.3)	44 (48.4)	45 (49.5)	35 (79.5)	7 (15.9)	7 (15.9)
	7/9 (91)	38 (41.8)	58 (63.7)	58 (63.7)	57 (98.3)	23 (36.7)	23 (36.7)
	7/23 (91)	44 (48.4)	59 (64.8)	58 (63.7)	57 (96.6)	32 (54.2)	32 (54.2)
Otofuke	7/14(20)	1 (5.0)	2 (10.0)	2 (10.0)	2 (10.0)	0 (0.0)	0 (0.0)
Shintoku	7/17 (36)	6 (16.7)	10 (27.8)	8 (22.2)	7 (70.0)	7 (70.0)	6 (60.0)
Shin-Hidaka	7/25 (83)	21(25.3)	49 (57.8)	51 (59.0)	41 (83.7)	31 (63.3)	26 (53.1)

a) Number (%) of cattle showing positive reactions (of total cattle).
b) Number (%) of cattle showing a PCR-positive reaction for Ikeda-type in a group of MPSP PCR-positive cattle.
c) Number (%) of cattle showing a PCR-positive reaction for Chitose-type in a group of MPSP PCR-positive cattle.
d) Number (%) of cattle showing both reactions for Ikeda-and Chitose-types in a group of MPSP PCR-positive cattle.

type parasites (54.2% in a group of MPSP PCR-positive cattle on 23rd July) (Table 2). The detection rate of the Ikeda-type gene (96.6% on 23rd July) was consistently higher than that of the Chitose-type gene (54.2% on 23rd July), while another gene of the *T. buffeli*-type (type 3) was not detected by the present PCR analysis.

Based on the results from Taiki on 25th June and 9th July, we divided the samples without microscopically detected parasites into 2 groups (Table 3): Group A had a negative reaction in the MPSP-PCR assay (–/–), while Group B had a positive reaction in the PCR assay (–/+). Parasitemia and anemia were assessed approximately 2 weeks after the initial division. In Group A, 25.6% of the cattle had become positive in both the microscopic and MPSP-PCR tests, but showed no anemia. On the other hand, 88.6% of the cattle in Group B showed positive reactions in both tests, and importantly, 22.6% and 58.1% of the positive cattle exhibited anemia and high levels of parasitemia (> +++), respectively (Table 3). These findings suggest that the MPSP-PCR method is able to detect infection 2 weeks earlier than traditional methods.

Geographical variation in infection of grazing cattle among Otofuke, Shintoku, and Shin-Hidaka districts: To examine whether the high rate of *T. orientalis* infection observed in Taiki district was also seen in the eastern areas of Hokkaido, blood samples were collected from grazing cattle on three different farms in Otofuke, Shintoku, and Shin-Hidaka districts (Fig. 1), approximately 1.5 months after they were put out to pasture. The results of MPSP-PCR analysis indicated high infection rates of 10.0%, 27.8%, and 57.8% on these farms, respectively, as shown in Table 2. The MPSP-PCR-positive cattle showed a high rate of anemia (32.6%) in the farm in Shin-Hidaka district, while anemia was not observed in the cattle on the Otofuke and Shintoku farms (Table 1). As in Taiki district, the newly developed MPSP- and p23-PCR analyses were more useful than the classical universal PCR analysis for detecting the parasite, and MPSP-PCR-positive cattle were infected with at least the Ikeda type or the Chitose type, or a mixture of these 2 types (Table 2).

Table 3. Infection status of the groups 2 weeks after initial division based on MPSP-PCR results

Initial division	Infection status after 2 weeks		
Parasitemia[a]/PCR[b]	Parasitemia[a]/PCR[b]	Numbers of cattle (%)	Anemia (%)[c]
Group A (n=39)[d] –/–	–/–	29 (74.4)	1 (3.4)
	+/+	5 (12.8)	0 (0.0)
	++/+	4 (10.3)	0 (0.0)
	+++/+	1 (2.6)	0 (0.0)
	++++/+	0 (0.0)	0 (0.0)
	subtotal	10 (25.6)	0 (0.0)
Total		39 (100.0)	1 (3.4)
Group B (n=35)[d] –/+	–/+	4 (11.4)	0 (0.0)
	+/+	3 (8.6)	0 (0.0)
	++/+	10 (28.6)	0 (0.0)
	+++/+	9 (25.7)	3 (33.3)
	++++/+	9 (25.7)	4 (44.4)
	subtotal	31(88.6)	7 (22.6)
Total		35 (100.0)	7 (20.0)

a) Parasitemia was determined by microscopic examination using the Ishihara method.
b) Judgment was based on MPSP-PCR, but p23-PCR showed similar results to those of MPSP-PCR.
c) Number (%) of cattle showing clinical anemia in the same group.
d) Samples were sorted from the data on microscopic test-negative cattle on the Taiki farm on 6/25 and 7/9 (Table 1).

Phylogenetic analyses of isolated MPSP and p23 genes: For DNA sequencing of the MPSP and p23 genes from field isolates, 24, 6, and 10 samples were randomly selected from the PCR-positive blood samples from the Taiki, Shintoku, and Shin-Hidaka farms, respectively. Forty PCR fragments derived from the MPSP and p23 genes were isolated, and the complete sequences were determined. Two phylogenetic trees were generated. Thirty-five and 34 samples of the MPSP and p23 genes, respectively, showed complete or high sequence identities with the registered sequences of Ikeda-type *T. orientalis* (D11046 and D84447, respectively), as shown in Figs. 2 and 3. In Taiki district, only Ikeda types were detected for both the MPSP and p23 genes. On the other hand, two MPSP and three p23 Chitose-type genes (D12689 and D844462, respectively) were obtained from three and one of the grazing cattle on the Shintoku and Shin-Hidaka farms, respectively (Figs. 2 and 3). Type-4 (Brisbane type) and type-5 MPSP genes (Cheju type) which were closely related to the types previously isolated in Okinawa prefecture, were found in one animal each from the Shin-Hidaka and Shintoku farms (Fig. 2). One Shin-Hidaka animal showed the rare type-4 MPSP gene, and the third Buffeli-type p23 gene (AB021223) which is readily distinguishable from the Ikeda- and Chitose-type p23 genes was also isolated (Fig. 3).

DISCUSSION

Based on the results from 25th June in Taiki district, 48.4% of grazing cattle were MPSP-PCR-positive (Table 2), indicating that about a half of the cattle became infected with *T. orientalis* during a brief 3-week span after being put out to pasture. After 1.5 months pasturage, we also observed high infection rate in all the other districts. Because suitable medicines and vaccines are currently unavailable, this infectious disease is becoming an increasingly serious problem for the livestock industry in Japan. The increase in *T. orientalis* infection is related to the seasonal activities of the vector ticks in the pastures [2]. Although Ixodid ticks are commonly found in the eastern part of Hokkaido [4], the presence of *Haemaphysalis longicornis*, a tick known to be a major vector of *T. orientalis*, has not been investigated [3]. Further studies are needed to identify the vector(s) capable of transmitting the infectious organism in these cold, eastern areas of Hokkaido.

In our study, 88.6% of the cattle that were initially microscopic test-negative but MPSP-PCR-positive became microscopic test-positive 2 weeks later (Table 3). This result demonstrates that a high incidence of *T. orientalis* infection can exist even when the piroplasms of *T. orientalis* cannot be detected by microscopic observation. It also indicates that the newly designed MPSP and p23 PCR methods provide useful means for detecting hidden infections before the appearance of symptoms in the cattle. The clinical application of these methods could help livestock producers to avoid economic damage caused by bovine theileriosis. In contrast, the detection rates of these PCR methods (10% and 57.8% in MPSP-PCR) for the blood samples obtained from the Otofuke and Shin-hidaka farms were lower than those of the microscopic test (25% and 68.7%, respectively) (Tables 1 and 2). Since there is a possibility of infection with unidentified new *T. orientalis* members, further investigations are necessary.

Two major types of MPSP genes categorized as the Chi-

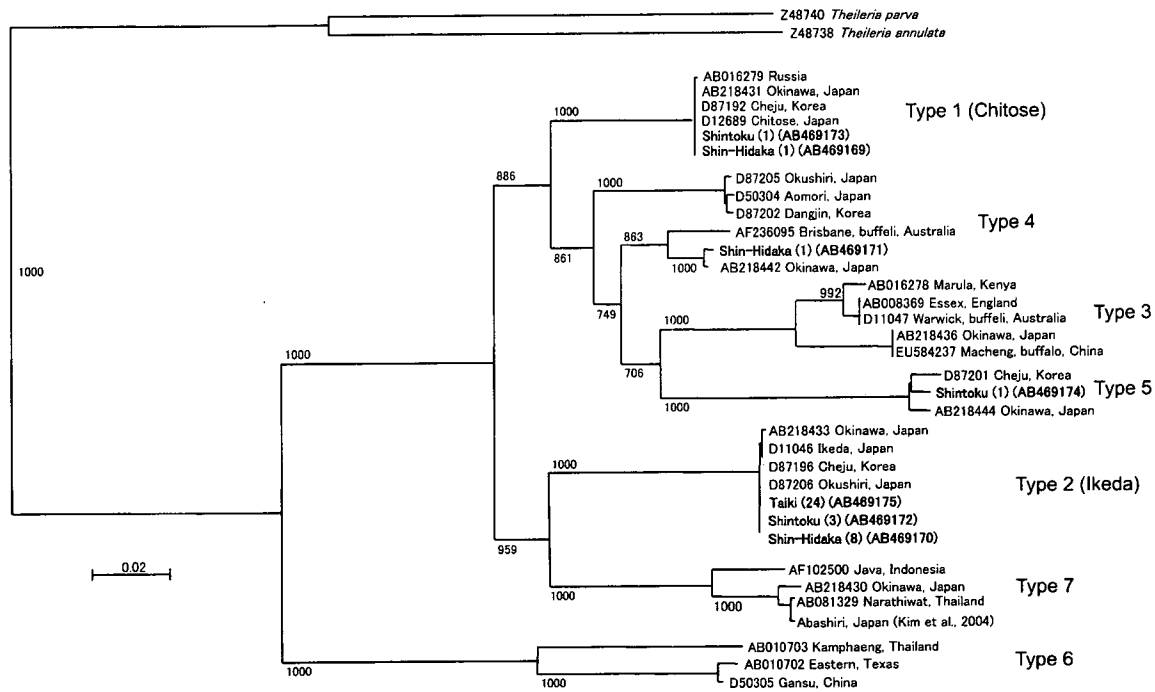

Fig. 2. Phylogenetic tree of the MPSP genes based on blood samples collected from Taiki, Shintoku, and Shin-Hidaka districts, together with previously registered sequences from the GenBank database. The sequences of the MPSP genes determined in the present study are expressed in bold-faced type, together with the number of collected samples. The representative sequences of isolated MPSP genes refer to the GenBank accession numbers, as indicated at the end of each branch. The numbers shown at the branch nodes indicate the bootstrap values. Different to the previous discrimination [10], a population of type-4 MPSP genes might be divided into two further groups due to the complexity of the branches.

tose and Ikeda types have been recognized from field isolates in Japan [13], and it has been also reported that these populations are distinguishable by MPSP allele-specific PCR methods [13, 15, 24]. The Chitose-type genotype is widely distributed [6, 12, 13], while the Ikeda-type parasite is commonly found in Korea and Japan but to a lesser extent in China [6], and not at all in Italy [19]. Most of field isolates consist of a mixture of 2–3 MPSP-type parasites, and single MPSP-type infections rarely occur [23]. In the present study, unique types of MPSP and p23 genes were found on the farms in Shintoku and Shin-Hidaka districts, in addition to the Chitose and Ikeda types. Type-3 (Buffeli-type), type-6 (Gansu-type), and type-7 (Abashiri-type) MPSP genes were not detected in the present study. The cow (an Angus) from the farm in Shintoku district that harbored *T. orientalis* with the type-5 MPSP gene was originally born on the farm, but its ancestors had been introduced from Miyazaki and Kagoshima prefectures, located in Kyushu Island, in the southern part of Japan. Type-4 and -5 MPSP genes have previously been found in Okinawa prefecture [27], and were also detected in the Kyushu area in our latest study (manuscript in preparation). In Shin-Hidaka district, the cow (a Hereford) with the type-4 MPSP and Buffeli-type p23 genes was also born on the study farm. The Buffeli-type p23 gene was derived from *T. buffeli* and has only previously been reported in the isolates from Australia [17]. The farm in Shin-Hidaka district had previously introduced cattle from Australia. This is the first report of the Buffeli-type p23 gene being isolated in Japan. Taken together with the results of a previous report concerning the isolation of a type-7 MPSP gene from Abashiri district (Fig. 1) [10], the present survey indicates that there are at least five types of *T. orientalis* (Types 1, 2, 4, 5, and 7) that can be categorized by their MPSP genes.

Terada *et al.* [25] reported that the indigenous Japanese Black breed of cattle was more resistant to *T. orientalis* infection than the exotic Holstein. Japanese Black cattle in Otofuke and Shintoku districts showed no sign of anemia, while the exotic Holsteins and Herefords in Taiki and Shin-Hidaka districts showed a high rate of anemia. Although differences in topography of the pastures, numbers and species of ticks, and age of the examined cattle among these districts make it difficult to evaluate the significance of the breed in the sensitivity to infection, it is possible that Japanese Black cattle are more resistant to *T. orientalis* than Holsteins and Herefords.

The present survey indicated that a population of *T. orientalis* carrying at least five types of MPSP genes and three types of p23 genes exists in Hokkaido. While the diversities of these immunogenic genes suggest that the control of *T.*

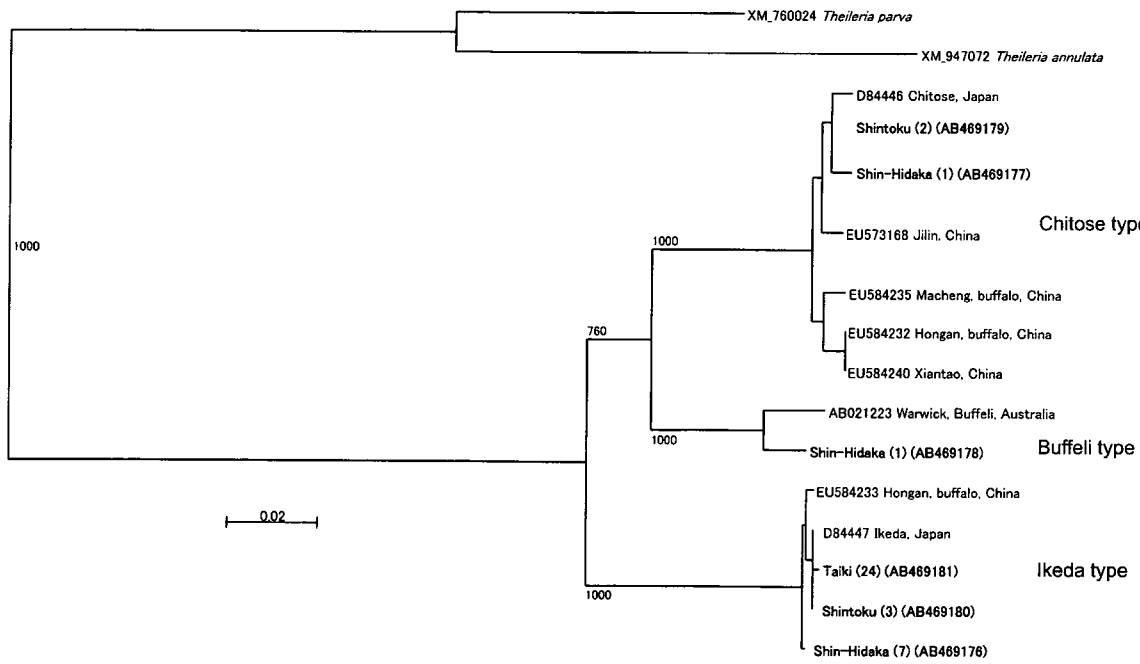

Fig. 3. Phylogenetic tree of the p23 genes of *T. orientalis* based on the blood samples from Taiki, Shintoku, and Shin-Hidaka districts, together with previously registered sequences from the GenBank database. The sequences of the p23 genes determined in the present study are expressed in bold-faced type, together with the number of collected samples. The representative sequences of isolated p23 genes refer to the GenBank accession numbers, as indicated at the end of each branch. The numbers shown at the branch nodes indicate the bootstrap values.

orientalis infection could be difficult, molecular epidemiological surveys are essential to provide the information needed for the successful developments of vaccines and diagnostic measures. The present study also demonstrated that *T. orientalis* infection is still a potentially serious problem in Hokkaido, Japan.

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Evaluation of the *in vitro* growth-inhibitory effect of epoxomicin on *Babesia* parasites

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ABSTRACT

Epoxomicin potently and irreversibly inhibits the catalytic activity of proteasomal subunits. Treatment of proliferating cells with epoxomicin results in cell death through accumulation of ubiquinated proteins. Thus, epoxomicin has been proposed as a potential anti-cancer drug. In the present study, the inhibitory effects of epoxomicin on the *in vitro* growth of bovine and equine *Babesia* parasites were evaluated. The inhibitory effect of epoxomicin on the *in vivo* growth of *Babesia microti* was also assessed. The *in vitro* growth of five *Babesia* species that were tested was significantly inhibited ($P < 0.05$) by nanomolar concentrations of epoxomicin (IC_{50} values = 21.4 ± 0.2, 4 ± 0.1, 39.5 ± 0.1, 9.7 ± 0.3, and 21.1 ± 0.1 nM for *Babesia bovis*, *Babesia bigemina*, *Babesia ovata*, *Babesia caballi*, and *Babesia equi*, respectively). Epoxomicin IC_{50} values for *Babesia* parasites were low when compared with diminazene aceturate and tetracycline hydrochloride. Combinations of epoxomicin with diminazene aceturate synergistically potentiated its inhibitory effects *in vitro* on *B. bovis*, *B. bigemina*, and *B. caballi*. In *B. microti*-infected mice, epoxomicin caused significant ($P < 0.05$) inhibition of the growth of *B. microti* at the non-toxic doses of 0.05 and 0.5 mg/kg BW relative to control groups. Therefore, epoxomicin might be used for treatment of babesiosis.

1. Introduction

Babesia, a protozoan parasite that is transmitted by ticks, is one of the major pathogens that infect erythrocytes in a wide range of wild as well as economically valuable animals, including cattle, sheep, and horses. *Babesia* parasites induce clinical symptoms such as malaise, fever, hemolytic anemia, jaundice, hemoglobinuria, and edema. The *Babesia* parasites, which are prevalent mainly in tropical and sub-tropical areas, cause serious economic damage in the livestock industries in these regions (Kuttler, 1988; Inci, 1997; Homer et al., 2000; Kjemtrup and Conrad, 2000; Ica et al., 2005, 2007). Thus, from the economic and public health perspectives, sustained research on babesiosis and the on-going search for chemotherapeutic drugs that are effective against this disease are important for the future development of treatment strategies. Due to intolerable toxic effects, combined with the emergences of resistant parasites, several babesiacidal drugs that widely have been used over many years have since proven ineffective (Bork et al., 2005b; Vial and Gorenflot, 2006). Therefore, the development of novel drugs that have low toxicity and exhibit chemotherapeutic effects specifically against *Babesia* parasites is highly desirable.

In eukaryotes, intracellular protein degradation is necessary for maintaining key cellular functions, and represents a process critical to the survival of the cells. Intracellular protein degradation is mediated predominantly through the ubiquitin pathway, which involves ligation of ubiquitin to proteins targeted for destruction (Varshavsky, 1997). Following ubiquitination targeted proteins recognized by the 26S proteasome, which is a multicatalytic protein complex composed of various proteases that cleaves proteins into short peptides. This is accomplished through the actions of three major

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proteolytic components that confer the chymotrypsin-like, trypsin-like, and peptidyl-glutamyl peptide-hydrolyzing (PGPH) catalytic activities (Hilt and Wolf, 1996). Thus, proteasome-mediated degradation plays a key role in intracellular protein processing (Ciechanover, 1998).

Epoxomicin (α′,β′-epoxyketone) is a potent proteasome inhibitor. Epoxomicin covalently binds the LMP7, X, Z, and MECL1 catalytic β subunits of the proteasome, resulting in the inhibition of the chymotrypsin-like, trypsin-like, and PGPH catalytic activities of the proteasomal subunits. These inhibitory effects cause cell death by promoting the accumulation of ubiquitinated proteins within the cytoplasm (Meng et al., 1999). The inhibitory effect of epoxomicin upon growth of *Plasmodium* species was recently shown to occur through the blockade of two of three catalytically active proteasomal subunits (Mordmüller et al., 2006). In view of the potent inhibitory effect of epoxomicin upon in *Plasmodium* species, and considering the close biological similarities between *Plasmodium* and *Babesia* parasites, there is a strong incentive for studies that test the chemotherapeutic potential of epoxomicin in treating babesiosis. Thus, the aim of the present study was to evaluate the inhibitory effect of epoxomicin upon the *in vitro* growth of bovine and equine *Babesia* parasites; as well as its inhibitory effect on the *in vivo* growth of *Babesia microti*.

2. Materials and methods

2.1. Parasites

The Texan strain of *Babesia bovis*, the Argentine strain of *Babesia bigemina*, the Miyake strain of *Babesia ovata* and the U.S. Department of Agriculture strains of *Babesia* (*Theileria*) *equi* (Mehlhorn and Schein, 1998), *Babesia caballi*, and the Munich strain of *B. microti* were used in this study.

2.2. Culture conditions

Bovine and equine *Babesia* parasites used in this study were maintained in purified bovine or equine red blood cells (RBCs), using a microaerophilic stationary-phase culture system (Igarashi et al., 1994; Bork et al., 2004). Medium M199 (for bovine *Babesia* isolates and *B. equi*) and RPMI 1640 (for *B. caballi*) (both from Sigma–Aldrich, Tokyo, Japan) supplemented with 40% normal bovine serum (for bovine *Babesia* isolates) or normal equine serum (for equine *Babesia* isolates), 60 U/ml of penicillin G, 60 μg/ml of streptomycin, and 0.15 μg/ml of amphotericin B (all three drugs from Sigma–Aldrich) were prepared and used in the culture media. Additionally, 13.6 μg of hypoxanthine (ICN Biomedicals Inc., Aurora, OH) per ml was added to the *B. equi* culture as a vital supplement, while 229 mg/ml of *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid hemisodium salt (Sigma–Aldrich) was added to the bovine *Babesia* parasite cultures as a pH stabilizer (pH 7.2). Serum-free GIT medium (Wako Pure Chemical Industrial, Ltd., Osaka, Japan) also was used for culturing *B. bovis* and *B. caballi* to assess the growth-inhibitory effects of epoxomicin without serum (Bork et al., 2005a).

2.3. Chemical reagents

Epoxomicin (α′,β′-epoxyketone) was purchased from BIOMOL International, LP (Butler Pike, USA) and used as a test drug. A working stock solution of 1 mM epoxomicin dissolved in dimethyl sulfoxide (DMSO) (Wako Pure Chemical Industrial, Ltd., Osaka, Japan) was prepared and stored at −30 °C until required for use. Diminiazine aceturate (GANASEG) was purchased from (Ciba-Geigy Japan Limited, Tokyo, Japan) and used as a comparator drug. Stock solution of 10 mM was prepared in distilled water and stored at −30 °C until use. Tetracycline hydrochloride was purchased from Sigma–Aldrich (USA) and used as a comparator drug. A stock solution of 20 mM was prepared in distilled water and stored at −30 °C until use.

2.4. Mice

The Munich strain of *B. microti* was maintained by passage in the blood of BALB/c mice (Nishisaka et al., 2001). Forty female BALB/c mice (aged, 8 weeks) were purchased from CLEA Japan (Tokyo, Japan) and were used for the *in vivo* studies.

2.5. Mammalian cells

Vero cells (Estacia et al., 2002) were used for the cytotoxicity assay. The cells were maintained in a suspension culture consisting of Menimum Essential Medium (Sigma–Aldrich) supplemented with 5% heat-inactivated fetal bovine serum (Gibco, NY, USA) and 500 μl kanamicin (Sigma–Aldrich) and incubated at 37 °C in a 5% CO_2 atmosphere.

2.6. In vitro growth inhibition assay and drug combination test

The inhibitory effects of epoxomicin upon *Babesia* growth were tested using a modified version of an assay previously described by Bork et al. (Bork et al., 2003a, 2004; Matsuu et al., 2008). Parasite-infected RBCs were diluted with uninfected RBCs to obtain an RBC stock supply with 1% parasitemia. Twenty microliters of RBCs with 1% parasitemia was dispensed into a 96-well microtiter plate (Nunc, Roskilde, Denmark) with 200 μl of the culture medium containing the indicated concentration of epoxomicin (5, 10, 25, 50, and 100 nM), diminazene aceturate (5, 25, 50, 250, 1000 and 2000 nM), and tetracycline hydrochloride (5, 50, and 100 μM) and then incubated at 37 °C in a humidified multi-gas water-jacketed incubator. For experimental control, cultures without the drug and cultures containing only DMSO (0.04%, for epoxomicin), or distilled water (0.02%, for diminazene aceturate and 0.2%, for tetracycline hydrochloride) were prepared. Combination therapies of epoxomicin and diminazene aceturate were tested in the *in vitro* cultures of *B. bovis*, *B. bigemina*, and *B. caballi* as models for bovine and equine *Babesia* parasites. Epoxomicin/diminazene aceturate combinations (M1, M2, M3, and M4) were prepared as previously described (Bork et al., 2003c) with some modifications. The concentration of each drug used in the combination was

not destructive to the parasites. The concentrations (epoxo/dimin) applied simultaneously to the cultures were for *B. bovis* (5/75, 10/75, 20/75, and 20/333.3 nM), for *B. bigemina* (2/50, 4/50, 4/80, and 4/165 nM), and for *B. caballi* (2.5/1.75, 5/3.5, 5/7, and 10/7 nM). Three separate trials were performed, consisting of triplicate experiments for individual drug concentrations, over a period of 4 days. During the incubation period, the overlaying culture medium was replaced daily with 200 μl of fresh medium containing the indicated concentration of epoxomicin. Parasitemia was monitored daily by counting the parasitized RBCs to approximately 1000 RBCs in Giemsia-stained thin blood smears. The IC_{50} values (50% inhibitory concentration) for the three drugs upon growth of all parasites tested were calculated based on parasitemia observations recorded on day 3 in the *in vitro* cell culture system; using interpolation after curve fitting technique.

2.7. Viability test

After 4 days of treatment, 6 μl of each of the control and drug-treated (at the various indicated concentrations) RBCs were mixed with 14 μl of parasite-free RBCs and suspended in fresh growth medium without epoxomicin supplementation. The plates were incubated for the next 10 days. The culture medium was replaced daily, and parasite recrudescence was determined by light microscopy in order to assess the parasite viability (Bork et al., 2004).

2.8. In vivo growth inhibition assay

The *in vivo* growth inhibition assay for epoxomicin was performed twice in BALB/c mice, according to the method previously described (Bork et al., 2004; Meng et al., 1999; Yokoyama et al., 2003) with some modifications. Twenty 8-week-old female BALB/c mice were divided into four groups each contain five mice and intraperitoneally inoculated with 1×10^6 *B. microti*-infected RBCs. In the first and second groups, epoxomicin was administered at non-toxic dose rates of 0.05 and 0.5 mg/kg, respectively after dissolving in 0.5 ml phosphate-buffered saline (PBS). DMSO was administered to the third group in 0.5 ml PBS (0.02%). In the fourth group, 0.5 ml PBS only was administered as a placebo control. When the infected mice showed approximately 1% parasitemia, mice in all of the experimental groups were administered daily intraperitoneal injections from days 3–12 post-infection. The levels of parasitemia in all mice were monitored daily until 26 days post-infection by examination of stained thin blood smears prepared from venous tail blood. All animal experiments were conducted in accordance with the Standard Relating to the Care and Management of Experimental Animals set by the National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Hokkaido, Japan.

2.9. Cytotoxicity assay

Cytotoxicity of epoxomicin was evaluated against Vero cells using a colorimetric assay for lactate dehydrogenase (LDH) release (Chouby et al., 2007). Vero cells were seeded in triplicate in 96-well tissue culture plates at a density of 5×10^3 cells per well, and incubated at 37 °C in 5% humidified CO_2 atmosphere for 4 h. When the cells completely adhered to the plate following the incubation period, culture medium was replaced with 100 μl of fresh medium containing different concentrations of epoxomicin (dissolved in DMSO). As a negative control, cultures were used containing either fresh medium with DMSO or fresh medium only. As a positive control, cells were treated with 0.9% Triton X-100. The culture plates were then incubated for either 24, 48, or 72 h and 50 μl of the supernatant from each well of the assay plate was taken from the corresponding well of a flat-bottom 96-well plate. The colorimetric reactions for LDH assays were performed using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, USA), by following the instructions of the manufacturer. Optical densities at 490 nm were measured using a microplate reader (Corona Electric Co.). Three experimental trails were performed. Comparative analysis of LDH release was performed by setting LDH release in Triton X-100-treated cells at 100% (Chouby et al., 2007).

2.10. Statistical analysis

The differences in percentage of parasitemia for the *in vitro* cultures and drug combination test, among groups of the *in vivo* studies, and the cytotoxicity assay were analyzed with JMP statistical software (SAS Institute Inc., USA) using the independent Student's *t*-test (Bork et al., 2004). A P value of <0.05 was considered statistically significant for all the tests.

3. Results

3.1. In vitro inhibitory effect and drug combination test

The *in vitro* growth of *B. bovis* was significantly inhibited (Student's *t*-test, $P < 0.05$) by 10 nM epoxomicin treatment (Fig. 1A); while 5 nM epoxomicin treatments significantly inhibited the growth of *B. bigemina* (Fig. 1B), *B. ovata* (Fig. 1C), *B. caballi* (Fig. 3A), and *B. equi* (Fig. 3B). The *in vitro* growth of the five *Babesia* species was significantly inhibited (Student's *t*-test, $P < 0.05$) at 5 nM diminazene aceturate treatment, while tetracycline significantly inhibited (Student's *t*-test, $P < 0.05$) the growth at 5 μM (*B. caballi*) and 50 μM (*B. bovis*, *B. bigemina*, *B. ovata*, and *B. equi*). In the presence of 50 nM epoxomicin, growth of *B. bigemina* and *B. equi* was completely suppressed. An epoxomicin concentration of 100 nM was needed to completely suppress the growth of *B. bovis*, *B. ovata*, and *B. caballi*. Complete suppression of diminazene aceturate treated parasites was observed at a concentration of 2000 nM (Figs. 1 and 3A), while a concentration of 50 nM was required to suppress the growth of *B. caballi* (Fig. 3B). The concentration of 5 μM tetracycline completely suppressed *B. caballi* growth, while a concentration of 100 μM suppressed the other *Babesia* species (Figs. 1 and 3A). The epoxomicin IC_{50} values for growth inhibition of *B. bovis*, *B. bigemina*, *B. ovata*, *B. caballi*, and *B. equi* were 21.4 ± 0.2,

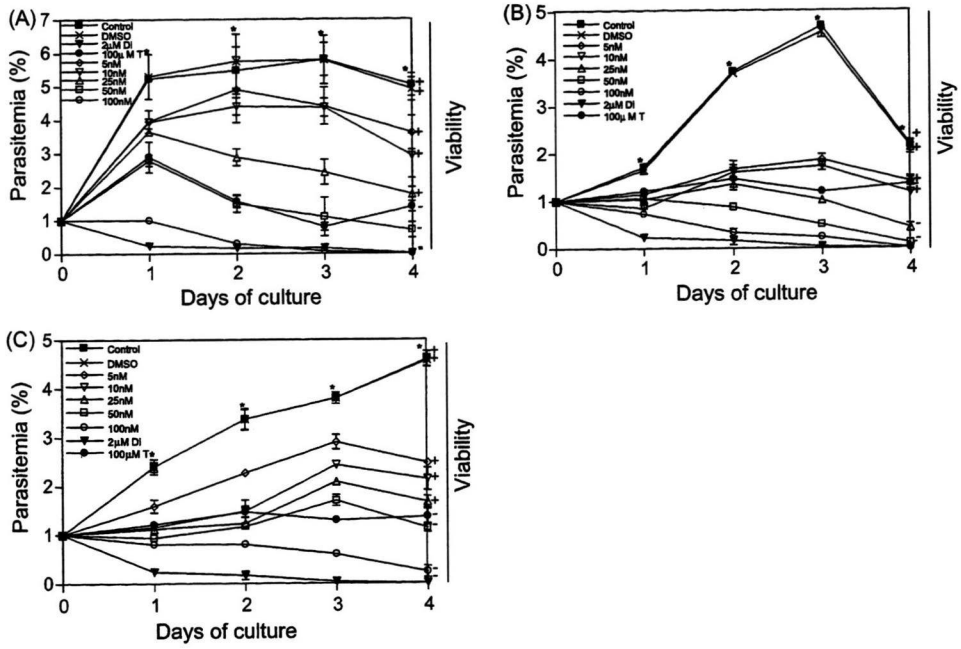

Fig. 1. Inhibitory effect of different concentrations of epoxomicin on the *in vitro* growth of *B. bovis* (A), *B. bigemina* (B), and *B. ovata* (C). Diminazene aceturate (Di) and tetracycline hydrochloride (T) were used as positive controls. Each value represents the mean ± standard deviation for experiments performed in triplicate. Curves represent the results of one representative experiment out of three separate replicates. *Statistically significant differences (Student's *t*-test, $P < 0.05$) between the drug-treated cultures and the control cultures.

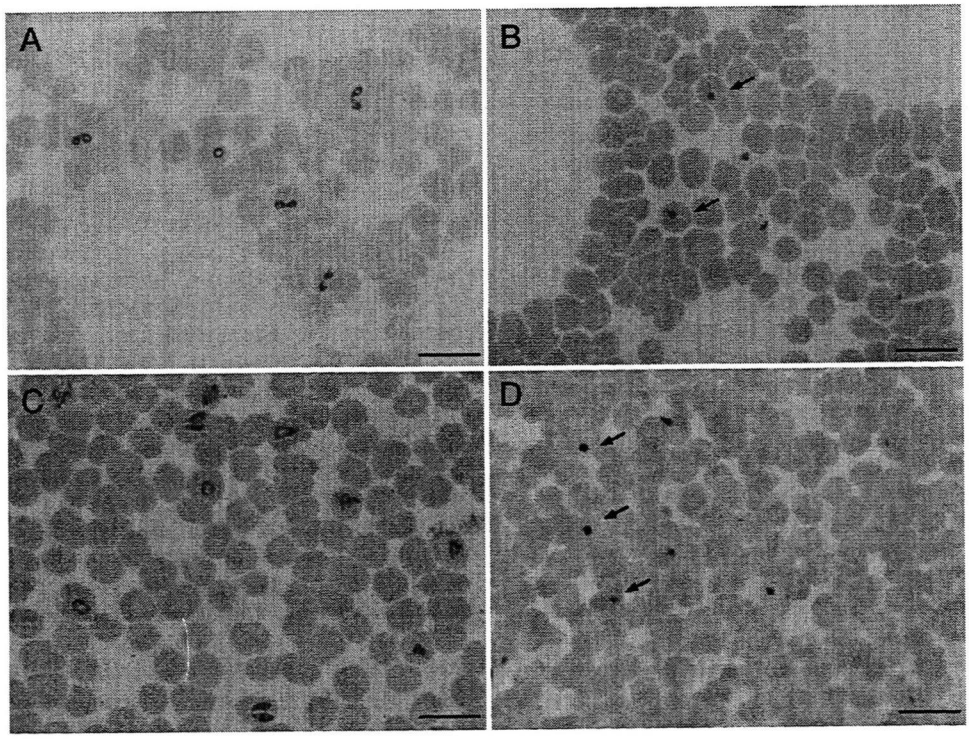

Fig. 2. Light micrographs of epoxomicin-treated bovine *Babesia* parasites in an *in vitro* culture. Micrographs were taken on day 3 of the experiment. *B. bovis*: (A) control and (B) 50 nM epoxomicin. *B. bigemina*: (C) control and (D) 25 nM epoxomicin. The drug-treated cultures showed a higher number of degenerated parasites than the control cultures. Scale bars = 10 μm.

Table 1
IC_{50} values of epoxomicin, diminazene aceturate, and tetracycline hydrochloride for growth inhibition of different *Babesia* species and proliferative cells.

Organism	IC_{50} (nM)[a]		IC_{50} (μM)[b]
	Epoxomicin	Diminazene aceturate	Tetracycline hydrochloride
B. bovis	21.4 ± 0.2	333.3 ± 20	44 ± 4
B. bigemina	4 ± 0.1	165 ± 7	41 ± 2
B. ovata	39.5 ± 0.1	186 ± 10	42 ± 3
B. caballi	9.6 ± 0.3	6.9 ± 0.3	4 ± 1
B. equi	21.1 ± 0.1	604 ± 30	62 ± 2
Bovine aortic Endothelial cells[c]	4 ± 1	ND	ND

ND was not determined.
[a] IC_{50} values expressed as drug concentration are in nanomolar of the growth medium and were determined on day 3 of *in vitro* culture using a curve fitting technique. IC_{50} values represent the mean and standard deviation of three separate experiments.
[b] IC_{50} values are in micromole of the growth medium.
[c] IC_{50} value of epoxomicin for the bovine aortic endothelial cells in the proliferation inhibition assay (Kim et al., 1999).

4 ± 0.1, 39.5 ± 0.1, 9.7 ± 0.3, and 21.1 ± 0.1 nM, respectively (Table 1). While the diminazene aceturate IC_{50} values for growth inhibition of *B. bovis*, *B. bigemina*, *B. ovata*, *B. caballi*, and *B. equi* were 333.3 ± 20, 165 ± 7, 186 ± 10, 6.9 ± 0.3, and 604 ± 30 nM, respectively (Table 1). Tetracycline IC_{50} values for growth inhibition of *B. bovis*, *B. bigemina*, *B. ovata*, *B. caballi*, and *B. equi* were 44 ± 4, 41 ± 2, 42 ± 3, 4 ± 1, and 62 ± 2 μM, respectively (Table 1). Compared to other species of *Babesia*, *B. bigemina* is highly susceptible to the growth-inhibitory effects of epoxomicin, while *B. caballi* is highly susceptible to diminazene aceturate and tetracycline hydrochloride. Subsequent viability tests showed that there was no re-growth of the parasites at the following epoxomicin concentrations: 25 nM, for *B. bigemina* and *B. caballi*, and 50 nM, for *B. bovis*, *B. ovata*, and *B. equi*. Using light microscopy, some re-growth of parasites was noted at lower epoxomicin concentrations. There was no re-growth of the diminazene aceturate treated parasites in the subsequent viability test at the concentration of 25 nM (*B. caballi*), and 1000 nM (*B. bovis*, *B. bigemina*, *B. ovata*, and *B. equi*). The parasites' re-growth was inhibited at the concentrations of 5 μM (*B. caballi* and *B. equi*), and 50 μM (*B. bovis*, *B. bigemina*, and *B. ovata*) tetracycline hydrochloride. Following the epoxomicin-treatment regimen, *Babesia* parasites were completely eradicated as early as day 3 (*B. bovis*, *B. caballi*, and *B. equi*) and day 4 (*B. bigemina* and *B. ovata*). Complete eradication of the five parasites from diminazene aceturate treated cultures was observed on day 4 of the treatment (Figs. 1 and 3), while tetracycline hydrochloride only eradicated *B. caballi* on day 4 of the treatment (Fig. 3B). The addition of only DMSO and distilled water to the cell culture system of epoxomicin, diminazene aceturate, and tetracycline hydrochloride did not influence parasitic growth. Serum-free GIT medium did not show any effect on either the IC_{50} values or the growth inhibition of epoxomicin for both of *B. bovis* and *B. caballi* relative to media supplemented with serum (data not shown). Epoxomicin also affected the morphology of *B. bovis* (Fig. 2B), *B. bigemina* (Fig. 2C), *B. caballi* (Fig. 4B), and *B. equi* (Fig. 4C) in the treated cultures. Epoxomicin-treated cultures showed a high number of degenerated parasites, which appeared dot shaped when compared to those in control cultures.

Combination therapies of epoxomicin and diminazene aceturate were assessed in the *in vitro* cell cultures of *B. bovis*, *B. bigemina*, and *B. caballi* as models for bovine and equine *Babesia* parasites. Drug combination experiments were performed in order to evaluate the potential synergistic or antagonistic effects. Epoxomicin/diminazene aceturate combinations were prepared and applied simultaneously to the cultures of *B. bovis*, *B. bigemina*, and *B. caballi*. The simultaneous application of epoxomicin/diminazene aceturate significantly enhanced the killing efficacy in *B. bovis*, *B. bigemina*, and *B. caballi in vitro* cultures even at the combination M1 that consisted of 1/4 the effective doses of the two drugs (Table 2). Complete eradication of the parasites was observed on day 4 of treatment from the cultures of *B. caballi* (M1, M2, M3, and M4), *B. bigemina* (M2, M3, and M4), and *B. bovis* (M3, and M4). Subsequent viability tests showed that there was no re-growth of the three parasites at all the drug combinations used (Table 2).

3.2. *In vivo* effect of epoxomicin on *B. microti* infection

In the epoxomicin-treated groups, the levels of parasitemia increased at a significantly lower rate relative to the control groups (Student's *t*-test, $P < 0.05$). Peak parasitemia levels reached an average of 34.8 and 42.3% in the presence of 0.5 and 0.05 mg/kg epoxomicin 11 days

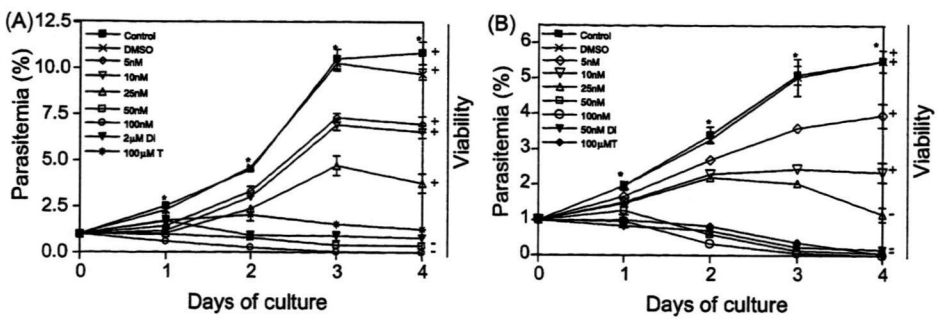

Fig. 3. Inhibitory effect of different concentrations of epoxomicin on the *in vitro* growth of *B. caballi* (A) and *B. equi* (B). Diminazene aceturate (Di) and tetracycline hydrochloride (T) were used as positive controls. Each value represents the mean ± standard deviation for experiments performed in triplicate. Curves represent the results of one representative experiment out of three separate replicates. *Statistically significant differences (Student's *t*-test, $P < 0.05$) between the drug-treated cultures and the control cultures.

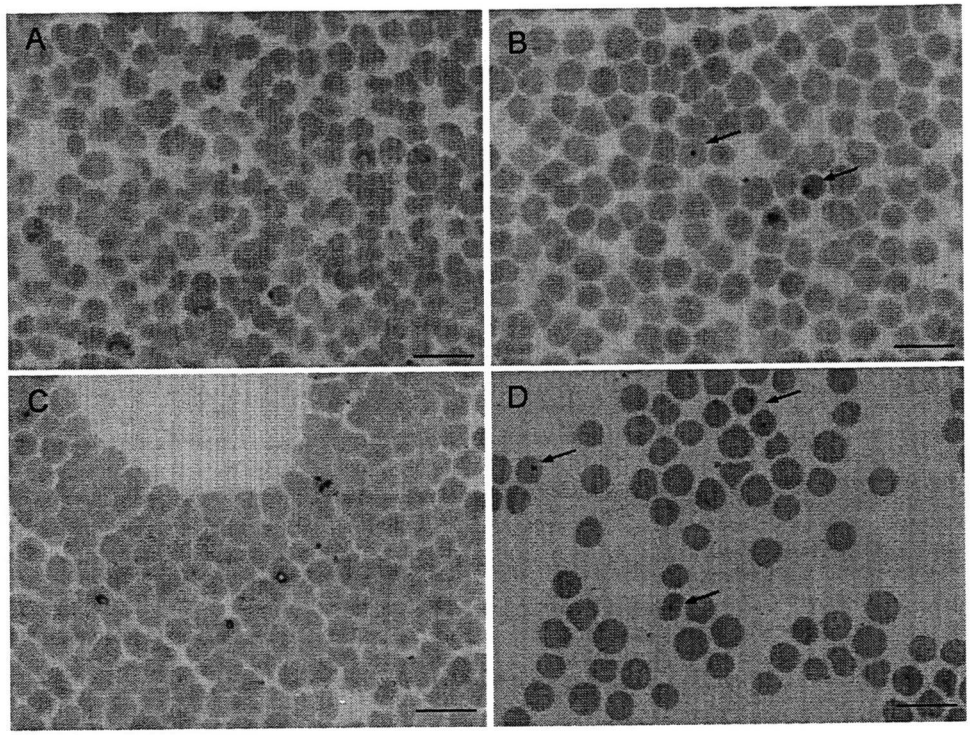

Fig. 4. Light micrographs of epoxomicin-treated equine *Babesia* parasites in an *in vitro* culture. Micrographs were taken on day 3 of the experiment. *B. caballi*: (A) control and (B) 50 nM epoxomicin. *B. equi*: (C) control and (D) 50 nM epoxomicin. The drug-treated cultures showed a higher number of degenerated parasites than the control cultures. Scale bars = 10 μm.

Table 2
Actual parasitemia (*P*%), and growth inhibition (*I*%) of combined applications of epoxomicin and diminazene aceturate in *B. bovis*, *B. bigemina*, and *B. caballi*.

Epoxomicin/diminazene (nM)[a]	*B. bovis*			*B. bigemina*			*B. caballi*		
	P%[†]	*I*%[§]	*V*[‡]	*P*%[†]	*I*%[§]	*V*[‡]	*P*%[†]	*I*%[§]	*V*[‡]
0	6.1 ± 0.7	0	+	6.9 ± 0.9	0	+	4.6 ± 0.8	0	+
0[b]	6.3 ± 0.8	0	+	7.1 ± 0.8	0	+	4.55 ± 0.9	0	+
M1	2.3 ± 0.05	63.5*	–	0.2 ± 0.05	97.6*	–	0.83 ± 0.2	81.8*	–
M2	1.2 ± 0.2	81*	–	0.1 ± 0.01	98.6*	–	0.53 ± 0.2	88.4*	–
M3	1 ± 0.1	84.1*	–	0.1 ± 0.01	98.6*	–	0.43 ± 0.1	90.5*	–
M4	0.03 ± 0.001	99.5*	–	0.03 ± 0.001	99.6*	–	0.03 ± 0.001	99.4*	–

[a] Epoxomicin/diminazene aceturate combinations for each parasite in nanomolar (M1, M2, M3, and M4) = *B. bovis* (5/75, 10/75, 20/75, and 20/333.3 nM), *B. bigemina* (2/50, 4/50, 4/80, and 4/165 nM), and *B. caballi* (2.5/1.75, 5/3.5, 5/7, and 10/7 nM), respectively.
[b] DMSO (0.0002%) + DDW (0.03%).
* Significant difference ($P < 0.05$) between the treated and control groups.
[†] *P*% is indicated by the average ± standard deviation at day 3 of culturing.
[‡] Viability after 10 days of subsequent drug free culturing; (+) viable, (–) not viable.
[§] *I*% was determined at day 3 of culturing compared to the control parasitemia.

after inoculation, respectively; relative to 66.4% (PBS) and 62.1% (DMSO) 10 days after inoculation in the control groups (Fig. 5). There was no significant difference in the inhibitory effect between the selected doses of the drug. No significant difference was found in parasitic growth for the PBS and DMSO control conditions.

3.3. Cytotoxicity assay

To study the toxicity of epoxomicin in mammalian cells, Vero cells were incubated with the same concentrations of epoxomicin used for experiments *in vitro* (5–100 nM). Cytotoxicity was assessed using LDH release assay. There was no significant difference (Student's *t*-test, $P < 0.05$) in LDH release between epoxomicin-treated cell cultures and the negative control cell cultures. LDH release from the maximum LDH release condition (which served as a positive control) significantly (Student's *t*-test, $P < 0.05$) differed from epoxomicin-treated cultures tested at 24, 48, and 72 h (Fig. 6).

4. Discussion

In the present study, the inhibitory effect of epoxomicin on the growth of bovine and equine *Babesia* was investigated *in vitro*. Exposure of cell cultures to high

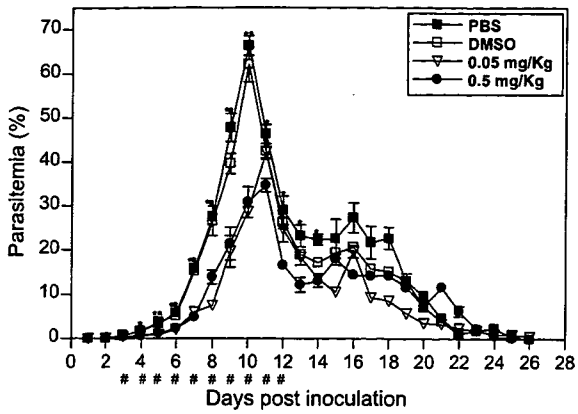

Fig. 5. Inhibitory effect of epoxomicin (0.05 and 0.5 mg/kg) on the *in vivo* growth of *B. microti*, based on observations taken from five mice per experimental group. Each value represents the mean ± standard deviation for two separate experiments. (*) and (#) statistically significant differences (Student's *t*-test, $P < 0.05$) between the 0.5 and 0.05 mg/kg treated groups and the control groups, respectively; #time of intraperitoneal epoxomicin inoculation or control reagent application (DMSO and PBS).

concentrations of epoxomicin completely suppressed the growth of all parasites tested in this study. Because treatment only with DMSO had no effect on parasitic growth, it is certain that this growth inhibition was due to the effects of epoxomicin. The effective dose for growth inhibition of *Babesia* parasites was similar to that observed in other studies using *Plasmodium falciparum* (Mordmüller et al., 2006; Kreidenweiss et al., 2008). Furthermore, the doses of epoxomicin that proved effective in this study were very low compared with other drugs that have been tested for the treatment of babesiosis (Igarashi et al., 1998; Nagai et al., 2003; Bork et al., 2003a,b,c, 2004, 2006; Nakamura et al., 2007; Okubo et al., 2007). The effective doses of epoxomicin for *Babesia* parasites were significantly low compared with those of diminazene aceturate and very low compared with tetracycline hydrochloride. The IC_{50} values of diminazene aceturate were nearly in similar range with that was reported for *B. gibsoni* (Matsuu et al., 2008), while very high compared to the IC_{50} value for *B. divergens* (Brasseur et al., 1998). The IC_{50} values of tetracycline hydrochloride are similar range to that was reported in a previous study (Nott et al., 1990). The principal aims of drug combinations are anchored in evaluation of combination compounds and also in delay of resistance phenomena. The combinations of epoxomicin with diminazene aceturate produced synergistic effects on the *in vitro* cultured parasites. Therefore, epoxomicin hold much promise for *in vivo* combined applications which require further studies.

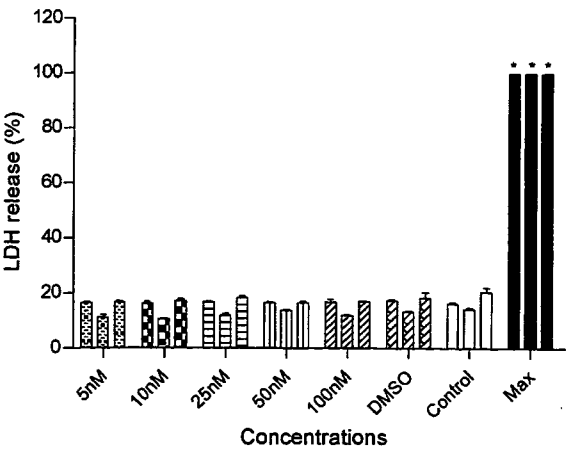

Fig. 6. Cytotoxicity of epoxomicin in Vero cells. Cells were exposed to different concentrations of epoxomicin (dissolved in DMSO) or medium with and without DMSO (as negative controls). As a positive control, cells were treated with 0.9% Triton X-100. The culture plate was then incubated at 37 °C for 4 h. Medium was replaced with fresh medium containing appropriate concentrations of epoxomicin and incubated for 24, 48, and 72 h. LDH release subsequently was evaluated. Data are presented as mean ± standard deviation for three separate experiments performed in triplicate. *Statistically significant differences (Student's *t*-test, $P < 0.05$) between the maximum LDH release positive control and LDH release from epoxomicin-treated cultures.

Bearing in mind epoxomicin exhibited a strong inhibitory effect on the growth of cultured parasites; this provided a strong incentive that led us to investigate its *in vivo* effects in mice infected with *B. microti*. These studies revealed a significant inhibitory effect of epoxomicin on the parasitic growth of *B. microti* in infected mice. The difference in growth inhibition between the control and the 0.05 and 0.5 mg/kg treated groups was evident on days 5–10 and 4–14 post-inoculation, respectively. Exposure to DMSO alone did not affect the growth of the parasites, meaning that the growth inhibition observed in this study was due to epoxomicin.

Mice treated with non-toxic doses of epoxomicin (0.05 and 0.5 mg/kg) did not show signs of toxicity and survived the entire experimental course. This is in good agreement with the results obtained before (Meng et al., 1999), where epoxomicin at a non-toxic dose of 0.58 mg/kg was injected intraperitoneally for 6 days and potently blocked *in vivo* inflammation in the murine ear edema assay. Furthermore, epoxomicin was found to be non-toxic to mice when it was injected subcutaneously at a dose rate of 5 mg/kg/day for 5 days (Garrett et al., 2003). These authors also demonstrated in mice that epoxomicin only increased the bone volume and the bone formation. Therefore, epoxomicin in these small doses is not toxic to the mice and might be used for treatment of babesiosis.

The IC_{50} values of epoxomicin for *Babesia* parasites are lower than epoxomicin concentrations needed for the inhibition of chymotrypsin-like activity (40–80 nM) except for *B. ovata* (39.5 ± 0.1), while are very low compared with the concentrations required for inhibition of trypsin-like activity (6–10 μM), and PGPH activity (25–75 μM) of the purified proteasome of bovine erythrocyte (Meng et al., 1999); furthermore, the erythrocytes that were pre-incubated with the concentration of 50 nM epoxomicin were not affected and permitted the growth of *P. falciparum* (Kreidenweiss et al., 2008). The IC_{50} values for *Babesia* parasites also are lower than a concentration of 100 nM at which the signs of toxicity started to be observed on the cultured mesencephalic dopamenergic neurons (Kikuchi et al., 2003). While the cytotoxicity assay did not show any toxic effects for the treated Vero cells at different concentrations up to 100 nM as indicated from LDH release. IC_{50} values of epoxomicin for *Babesia* parasites are higher than

an IC_{50} value of 4 ± 1 nM epoxomicin for bovine aortic endothelial cells in the proliferation inhibition assay (Kim et al., 1999). Although the cytotoxicity assay using LDH release and *in vivo* growth inhibition assays did not show any toxic effects for epoxomicin, the results of *in vitro* culture compared with the proliferation inhibition assay for aortic endothelial cells may have an evidence for the development of toxicity. Therefore, to avoid the risk of using epoxomicin alone at high dose it is advisable to use non-toxic dose in combination with another drug for *in vivo* therapy that requires further studies.

The plasmodial genome is known to encode molecules associated with proteasomal subunits. The proteasomal predecessor, ClpQ/hslV-orthologue, was identified as a target molecule of epoxomicin in plasmodia (Mordmüller et al., 2006). Genes homologous to proteasome-associated molecules are listed in the EST database for the *B. bovis* at the erythrocyte stage of its life cycle (deVries et al., 2006), which supports the hypothesis that the proteasome has a crucial role in the growth cycle of *Babesia* parasites.

In conclusion, the results of the present study showed that epoxomicin potently inhibited the *Babesia* parasites proteasome *in vitro* cell culture and *in vivo* for the first time. Furthermore, combinations with diminazene aceturate potentiated its inhibitory effect *in vitro* cell cultures. Epoxomicin has a moderate *in vivo* effect in the non-toxic doses, and further studies are required for its use as a part of combination therapy for babesiosis that may help to avoid the development of toxicity. Further studies that identify the molecule(s) targeted by epoxomicin in *Babesia* species should provide new insights relating to the precise role of the proteasome in controlling the growth cycle of *Babesia* parasites.

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Inhibitory effects of (-)-Epigallocatechin-3-gallate from green tea on the growth of *Babesia* parasites

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SUMMARY

(-)-Epigallocatechin-3-gallate (EGCG) is the major tea catechin and accounts for 50–80% of the total catechin in green tea. (-)-Epigallocatechin-3-gallate has antioxidant, anti-inflammatory, anti-microbial, anti-cancer, and anti-trypanocidal activities. This report describes the inhibitory effect of (-)-Epigallocatechin-3-gallate on the *in vitro* growth of bovine *Babesia* parasites and the *in vivo* growth of the mouse-adapted_rodent babesia *B. microti*. The *in vitro* growth of the *Babesia* species was significantly ($P<0{\cdot}05$) inhibited in the presence of micromolar concentrations of EGCG (IC_{50} values = 18 and 25 μM for *B. bovis*, and *B. bigemina*, respectively). The parasites showed no re-growth at 25 μM for *B. bovis* and *B. bigemina* in the subsequent viability test. The drug significantly ($P<0{\cdot}05$) inhibited the growth of *B. microti* at doses of 5 and 10 mg/kg body weight, and the parasites completely cleared on day 14 and 16 post-inoculation in the 5 and 10 mg/kg treated groups, respectively. These findings highlight the potentiality of (-)-Epigallocatechin-3-gallate as a chemotherapeutic drug for the treatment of babesiosis.

Key words: (-)-Epigallocatechin-3-gallate, *in vitro* growth, *in vivo* growth, *Babesia microti*.

INTRODUCTION

Babesia parasites are tick-transmitted protozoa that affect a wide variety of wild and domestic animals and are responsible for enormous economic losses to the livestock industry worldwide (Kuttler, 1988). Moreover, some species are major aetiologic agents of human babesiosis (Homer *et al.* 2000; Kjemtrup and Conrad, 2000). *Babesia microti* infects laboratory mice, causes a relatively mild but persistent disease, and has served as a useful experimental model for the analysis of animal and human babesiosis (Cox and Young, 1969). The analysis of the disease in this model has enabled a search for anti-babesial drugs (Wittner *et al.* 1996; Marley *et al.* 1997; Bork *et al.* 2004*a*). Several babesicidal drugs that have been in use for years have proven to be ineffective owing to problems related to their toxicity and the development of resistant parasites (Bork *et al.* 2005*a*; Vial and Gorenflot, 2006). Therefore, there is a need to develop new drugs that have a chemotherapeutic effect against babesiosis with high specificity to the parasites and low toxicity to the hosts.

Tea (*Camellia sinensis*) is one of the most popular beverages in the world, and its beneficial effects on health have attracted great attention (Yang and Landau, 2000). The most significant groups of tea components are polyphenols, especially the catechin group called flavonols. (–)-Epigallocatechin-3-gallate (EGCG) is the major tea catechin and EGCG accounts for 50–80% of the total catechin (Graham, 1992). EGCG have anti-tumorigenic (Chen and Zhang, 2007), anti-inflammatory (Lin and Lin, 1997), anti-oxidative (Fraga *et al.* 1987), anti-proliferative (Shammas *et al.* 2006), anti-bacterial (Mabe *et al.* 1999), and anti-viral (Fassina *et al.* 2002; Yamaguchi *et al.* 2002; Williamson *et al.* 2006) effects. EGCG also have anti-trypanocidal activity against *Trypanosoma cruzi* (Paveto *et al.* 2004). In this study we present the inhibitory effect of EGCG on *B. bovis* and *B. bigemina* in the *in vitro* cultures and on the *in vivo* growth of *B. microti*.

MATERIALS AND METHODS

Chemical compounds

(-) Epigallocatechin-3-gallate (EGCG) was purchased from Sigma-Aldrich (USA) and used as a test drug. A working stock solution of 1 mM in PBS was prepared and stored at −30 °C until use. Diminiazine aceturate (GANASEG) was purchased from (Ciba-Geigy Japan Lit., Tokyo, Japan). A working stock solution of 10 mM in distilled water was prepared and stored at −30 °C until use. Tetracycline hydrochloride was purchased from Sigma-Aldrich (USA). A working stock solution of 20 mM in distilled water was prepared and stored at −30 °C until use.

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