The observed high divergence of TIR sequences among Babesia and Theileria species was not surprising because no universal signature has been reported for TIR sequences of linear mt genomes from several unicellular flagellates: *Polytomella capuana*, *Po. parva*, and *Chlamydomonas reinhardtii* in the Reinhardtii clade of chlorophycean green algae (Smith and Lee 2008). Inverted complementary sequences at both ends of a linear mt genome are believed to play important roles in replication and stabilization (Nosek and Tomaska 2003). In TIR, small repeats with forward and reverse directions frequently occur, which are likely to form secondary structure. In general, sequence regions containing small repeats diverge rapidly compared with nonrepeat sequences. As a consequence, little sequence conservation is expected in TIR of a linear mt genome. An exception is *T. equi* TIR, in which sequence regions of *cox3* and two LSU rRNA gene fragments are relatively conserved, perhaps due to functional constraints.

In conclusion, our studies show the remarkable structural diversity in the mt genomes of Theileria species, in contrast to the highly conserved genome among Babesia species. The finding of a high structural divergence of the *T. equi* mt genome from other Babesia and Theileria species suggests the occurrence of lineage-specific evolution of mt genomes within the closely related Babesia and Theileria genera. Further investigations into mt genomes of other Theileria species and other related genera, such as those that belong to the Archaeopiroplasmids group, should provide insights into the evolutionary origin of a major structural divergence between Plasmodium (circular/concatemer genome) and Babesia/Theileria (linear genome) in the same phylum Apicomplexa.

Supplementary Material

Supplementary material, tables S1–S8, and figure S1 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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Short communication

Inhibitory effect of terpene nerolidol on the growth of *Babesia* parasites

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ABSTRACT

Nerolidol is a sesquiterpene present in the essential oils of many plants, approved by the U.S. FDA as a food flavoring agent. Nerolidol interferes with the isoprenoid biosynthetic pathway in the apicoplast of *P. falciparum*. In the present study, the *in vitro* growth of four *Babesia* species was significantly ($P<0.05$) inhibited in the presence of nerolidol (IC_{50}s values $= 21 \pm 1$, 29.6 ± 3, 26.9 ± 2, and 23.1 ± 1 μM for *B. bovis*, *B. bigemina*, *B. ovata*, and *B. caballi*, respectively). Parasites from treated cultures failed to grow in the subsequent viability test at a concentration of 50 μM. Nerolidol significantly ($P<0.05$) inhibited the growth of *B. microti* at the dosage of 10 and 100 mg/kg BW, while the inhibition was low compared with the high doses used. Therefore, nerolidol could not be used as a chemotherapeutic drug for babesiosis.

Babesia, a tick-born protozoan parasite, is one of the major pathogens that infect erythrocytes in a wide range of wild and economically valuable animals, such as cattle and horses. The clinical symptoms of babesiosis include malaise, fever, hemolytic anemia, jaundice, hemoglobinuria, and edema in the infected animals. *Babesia* parasites are prevalent worldwide, mainly in tropical and sub-tropical areas. Serious economic damage in the livestock industry has been caused by *Babesia* infections in such areas [1,2]. 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 [3,4]. Therefore, the development of new drugs that have a chemotherapeutic effect against babesiosis with high specificity to the parasites and low toxicity to the hosts has been desired.

The apicoplast was acquired by horizontal transfer (secondary endosymbiosis) from a eukaryotic alga [5] and has been identified in many apicomplexan parasites, including *Babesia* [6]. The apicoplast is essential for long term parasite viability and has been an attractive target for development of parasiticidal drug therapies as the biosynthetic pathways represented therein are of cyanobacterial origin and differ substantially from corresponding pathways in the mammalian host [7,8]. The apicoplast has synthetic pathways such as fatty acid biosynthesis and isoprenoid biosynthetic pathways. In apicomplexan parasites, synthesis of isopentenyl diphosphate (IPP), the universal isoprenoid precursor, has long been assumed to proceed exclusively via the acetate/mevalonate (MVA) ubiquitous pathway, a pathway that is absent from malaria parasites [9]. However, an alternative MVA-independent pathway for the formation of isopentenyl diphosphate (IPP) occurs in bacteria, algae and chloroplasts of higher plants [9]. The initial step of this MVA-independent pathway is the formation of 1-deoxy-D-xylulose4-phosphate (DOXP) by condensation of pyruvate and glyceraldehyde-3-phosphate, catalyzed by DOXP synthase. In the second step, DOXP reductoisomerase synthesizes 2-C-methyl-D-erythritol-4-phosphate (with 2-C-methyl-D-erythrose-4-phosphate as an intermediate), in a single step, by intramolecular rearrangement followed by a reduction process [9]. IPP is the basic five-carbon building block (C_5) that forms the next member of the series. Two C_5 units condense to form geranyl pyrophosphate (C_{10}), which condenses with another molecule of IPP to form farnesyl pyrophosphate (FPP) (C_{15}). FPP is incorporated in the formation of prenylated proteins, ubiquinones (Coenzyme Q), and dolichols [9]. Isoprenoid biosynthesis in malaria parasites operates by a different mechanism than that in humans; therefore, could be a promising target for drug therapy [9]. While in most eukaryotic cells, the isoprenoid biosynthesis is achieved through a mevalonate dependent pathway which generates essential metabolic products in addition to cholesterol and ergosterol, such as the dolichols, which are present in all membranes in variable amounts and, in a modified phosphorylated form, are required for the asparagine-linked glycosylation of proteins. The pathway also generates the isoprene side chains attached to the benzoquinone ring of ubiquinone, prenyl groups transferred to prenylated proteins, and prenylated transfer RNAs [9]. *Leishmania* species cannot synthesize cholesterol de novo but are able to produce ergosterol through the mevalonate pathway [10]. In addition to ergosterol, other products of the mevalonate pathway have been identified in *Leishmania*: coenzyme Q9 (CoQ9) was detected as the predominant species of ubiquinone in promastigotes and amastigotes of *L. amazonensis*, whereas CoQ7 and CoQ8 were also identified in promastigotes [11]; prenylated proteins were observed in *Leishmania mexicana*

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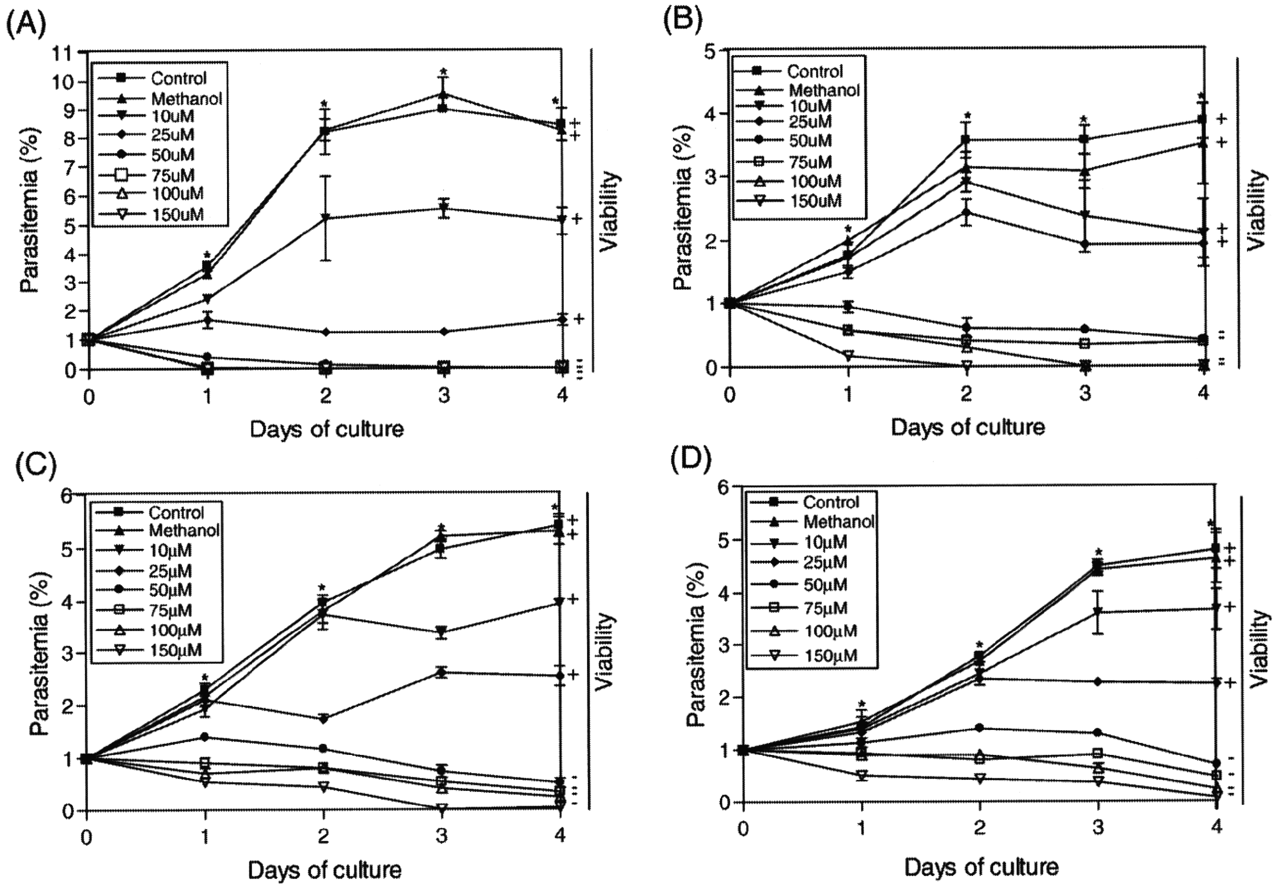

Fig. 1. Inhibitory effect of different concentrations of nerolidol on the *in vitro* growth of *B. bovis* (panel A), *B. bigemina* (panel B), *B. ovata* (panel C), and *B. caballi* (panel D). Each value represents the mean ± standard deviation in triplicate. These curves represent the mean of three experiments carried out in triplicate. *, statistically significant differences (student's *t* test, $P<0.05$) between 10 μM (panels A and B) and 25 μM (panels C and D) treated cultures and the control. Parasite viability was monitored in subcultures without nerolidol for 10 days (±, viable cells and −, dead cells).

after incorporation of labeled mevalonate, and phosphorylated dolichol has been detected as a sugar donor for glycosylation of proteins in *L. mexicana* [12]. The isoprenoid biosynthetic pathway is the only active pathway in *B. bovis* apicoplast, and is similar to that of *P. falciparum* [13]. Therefore, the blockade of the mevalonate independent pathway in the apicoplast could potentially have serious effects for *Babesia* parasites.

Nerolidol is a sesquiterpene present in essential oils of several plants, approved by the U.S. Food and Drug Administration as a food flavoring agent, it has been tested as a skin penetration enhancer for the transdermal delivery of therapeutic drugs [14]. In *Leishmania amazonensis*, nerolidol inhibited the isoprenoid biosynthesis, as shown by reduced incorporation of mevalonic acid (MVA) or acetic acid precursors into dolichol, ergosterol, and ubiquinone, in treated promastigotes due to the blockage of an early step in the mevalonate pathway [14], while in *P. falciparum*, nerolidol interferes with the isoprenoid biosynthetic pathway of the apicoplast leading to the interference with the biosynthesis of the dolichols, with the isoprenic chain of ubiquinones, and with protein isoprenylation of the parasites [15]. Nerolidol have leishmanicidal [14], and antimalarial [15,16] activities. The aim of this study was to evaluate the inhibitory effect of nerolidol on the growth of *Babesia* parasites.

B. bovis Texas strain, *B. bigemina* Argentina strain, *B. ovata* Miyake strain, and *B. caballi* U.S. Department of Agriculture strain were used in this study. Parasites were grown in a micro-aerophilous stationary phase culture system using bovine (for bovine *Babesia*) or equine (for *B. caballi*) erythrocytes (RBC) and sera as described previously [17,18]. GIT medium alone was used to test the effect of nerolidol without serum on *B. bovis* and *B. caballi* [19]. The overlaying medium was replaced daily with a fresh medium, and the infected RBC were passaged every 3 days [20]. Nerolidol (a mixture of *cis-* and *trans*-nerolidol) was purchased from Sigma-Aldrich (St. Louis, MO). A 20 mM stock solution prepared in methanol was used for *in vitro* experiments.

The *in vitro* growth inhibition assay for nerolidol followed the methods previously described [17,21] with some modifications. Parasite-infected RBC were diluted with uninfected RBC to obtain an RBC pack with 1% parasitemia. Twenty microliters of RBC with 1% parasitemia was dispensed to a 96-well microtiter plate together with 200 μl of the culture medium containing the indicated concentration of nerolidol (10, 25, 50, 75, 100, and 150 μM), and then incubated at 37 °C in a humified multi-gas water-jacketed incubator. For the control, cultures without the drug and another containing only methanol similar to the highest concentration

Table 1
The IC_{50} values of nerolidol for *Babesia* parasites and mammalian cells.

Organism	IC_{50} (μM)[a]
B. bovis	21 ± 1
B. bigemina	29.6 ± 3
B. ovata	26.9 ± 2
B. caballi	23.1 ± 1
J774.A1 macrophages[b]	125.69 ± 14.40
Human foreskin fibroblasts[b]	134.94 ± 32.19

[a] IC_{50} values expressed as nerolidol concentration are in micromolar 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 3 separate experiments.

[b] The IC_{50} values for mammalian cells reported by Arruda et al. [14].

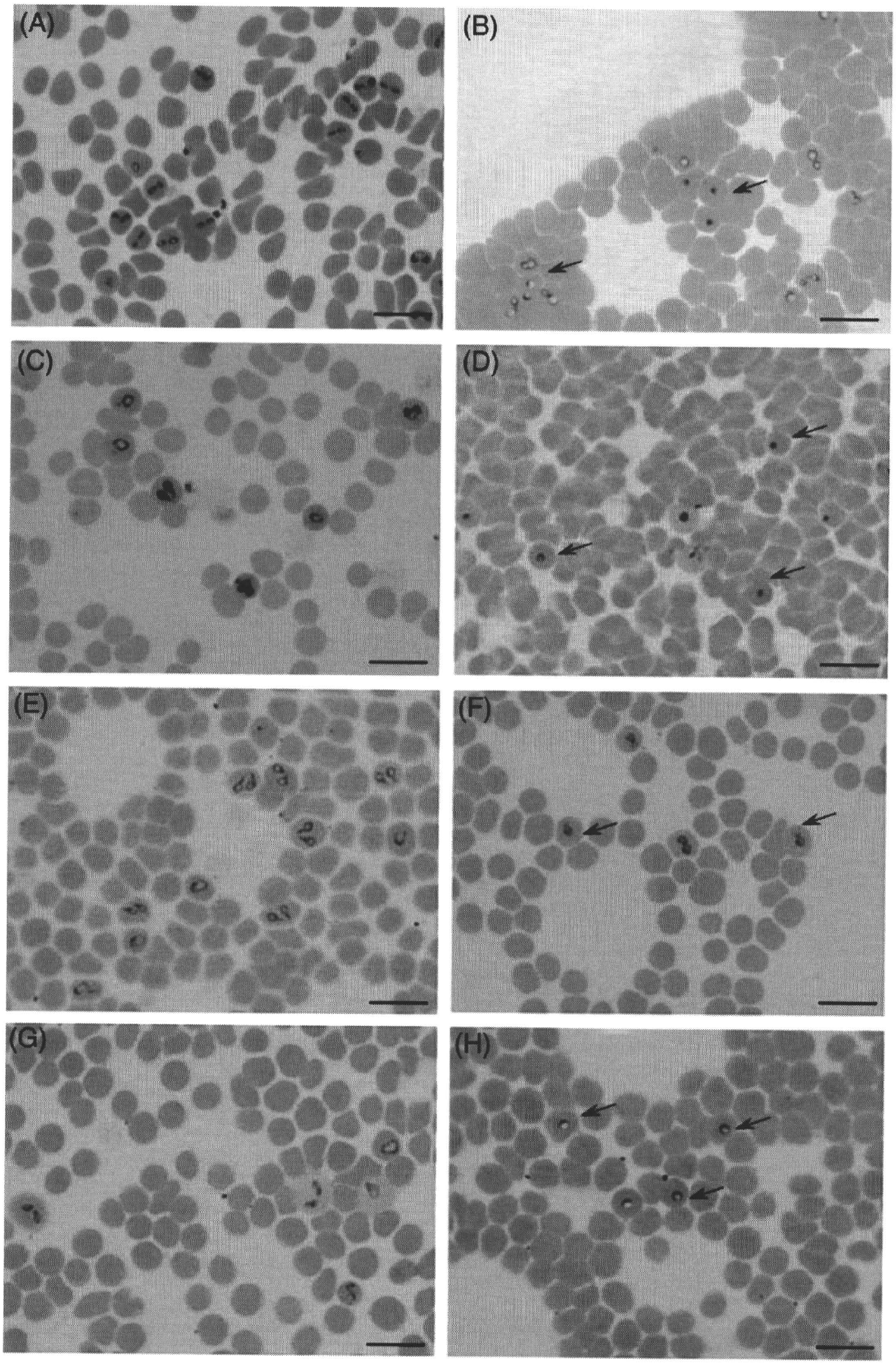

Fig. 2. Light micrographs of nerolidol-treated *Babesia* parasites in an *in vitro* culture. Micrographs were taken on day 3 of the experiments for 10 μ M treated cultures. *B. bovis*: control (panel A), and treated (panel B). *B. bigemina*: control (panel C), and treated (panel D). *B. ovata*: control (panel E), and treated (panel F). *B. caballi*: control (panel G), and treated (panel H). The drug-treated cultures showed a higher number of degenerated and swollen parasites than the control cultures. Scale bars = 10 μm.

used in drug were prepared. The experiments were carried out in triplicate for each drug concentration for 3 separate trails for a period of four days. The culture medium was replaced daily with 200 μl of a fresh medium containing the appropriate concentration of the drug. Parasitemia was monitored daily by counting the parasitized RBC to approximately 1000 RBC in Giemsia-stained thin blood smears. After the fourth day of treatment, 6 μl of each of the control and nerolidol-treated (at the various concentrations) RBC was mixed with 14 μl of

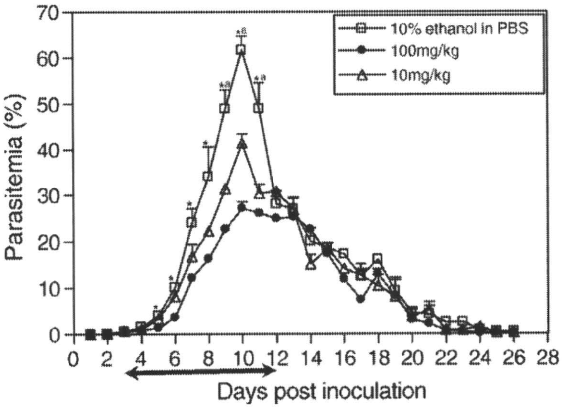

Fig. 3. Inhibitory effect of nerolidol (10 and 100 mg/kg) on the *in vivo* growth of *B. microti* for observations of 5 mice in each group. Each value represents the mean ± standard deviation of two separate experiments.* and ^{a}, statistically significant differences ($P<0.05$) between 100 mg/kg and 10 mg/kg treated groups, and the control group, respectively. The double head arrow (↔) indicates the nerolidol intraperitoneal injection period.

parasite-free RBC and suspended in fresh growth medium without nerolidol supplementation. The plates were incubated for the next 10 days. The culture medium was replaced daily, and parasite recrudescence was determined by light microscopy to evaluate the parasite viability [20]. The values of a 50% inhibitory concentration (IC_{50}) of nerolidol against all parasites were calculat/ed based on parasitemia observed at 3 days after drug treatment by interpolation after curve fitting technique.

The *in vivo* growth inhibition assay for nerolidol was carried out two times according to the method previously described [14,20] with some modifications. Fifteen 8-week-old female BALB/c mice (CLEA Japan, Tokyo, Japan) were divided into 3 groups each contain five mice and intraperitoneally inoculated with 1×10^6 Munich strain *B. microti*-infected RBC that was maintained by passage in the blood of BALB/c mice [22]. Nerolidol was administered at a dose rate of 10 and 100 mg/kg after dissolving in 0.3 ml 10% ethanol in 0.01 M phosphate buffer (pH 7.4) for the first and second groups respectively. The third group was only administered 0.3 ml 10% ethanol in 0.01 M phosphate buffer (pH 7.4) as a control. When the infected mice showed about 1% parasitemia, all groups underwent daily intraperitoneal injections from days 3 to 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 promulgated by the National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Hokkaido, Japan.

JMP statistical software (version 5.1., SAS institute Inc., USA) was used to compare means of parasitemia percentage in the *in vitro* and *in vivo* studies using the independent Student's *t* test and considered to be significantly different when $P<0.05$.

The *in vitro* growth was significantly inhibited ($P<0.05$) at 10 µM (for *B. bovis*, and *B. bigemina*) (Fig. 1A, B), and at 25 µM (for *B. ovata*, and *B. caballi*) (Fig. 1C, D). The parasites' growth was completely suppressed at 50 µM (for *B. bigemina*), and at 75 µM (for *B. bovis*, *B. ovata*, and *B. caballi*). The calculated IC_{50}s values of nerolidol on the third day of culture for the growth of *B. bovis*, *B. bigemina*, *B. ovata*, and *B. caballi* were 21 ± 1, 29.6 ± 3, 26.9 ± 2, and 23.1 ± 1 µM, respectively (Table 1). *B. bovis is* more susceptible to nerolidol compared to other species of *Babesia*. Complete clearance was observed on the third (for *B. bovis*, *B. bigemina*, and *B. ovata*), and the fourth (for *B. caballi*) days of drug treatment. Subsequent cultivation of the parasites without the drug for a 10-day period showed no regrowth of the four species at 50 µM (Fig. 1) as shown by light microscopy. The serum has no effect on the inhibition of nerolidol to *Babesia* species as indicated from the use of GIT medium without serum for *B. bovis* and *B. caballi* (data not shown). The addition of only methanol to the culture had no influence on the growth. The effective doses of nerolidol for the growth inhibition of *Babesia* parasites were lower than the effective doses of heparin [20], and in nearly similar level to those of other drugs that had been tested as babesicidal drugs [21,23–27]. While they have medium level between those reported for *P. falciparum* [15] and *L. amazonensis* [14]. The average IC_{50} values of nerolidol for *Babesia* parasites were low compared with nerolidol IC_{50} values for mammalian cells (125.69 ± 14.40 µM; J774.A1 macrophages and 134.94 ± 32.19 µM; human foreskin fibroblast) [14]. The light microscope study showed that the drug affected the morphology of the parasite in treated cultures of *B. bovis* (Fig. 2B), *B. bigemina* (Fig. 2D), *B. ovata* (Fig. 2F), and *B. caballi* (Fig. 2H). The drug-treated cultures showed high number of degenerated and swollen parasites without cytoplasm compared to the control cultures. Based on light microscopic observations of the changes in the host cell shape, size, and color, nerolidol was non-toxic to erythrocytes.

The effect of nerolidol on the course of *B. microti* in mice was revealed. In the treated groups the level of parasitemia increased significantly (Student's *t* test, $P<0.05$) more slowly than the control, achieving peak parasitemia of 27 and 41.3% in the presence of 100 mg/kg and 10 mg/kg on day 10 post inoculation. In contrast, in the control group, the peak parasitemia was 58.3% (10% ethanol in PBS) on day 10 post inoculation (Fig. 3). The difference in growth inhibition (Student's *t* test, $P<0.05$) between 10 and 100 mg/kg and the control groups was significant on days 9–11 and days 5–11 PI, respectively. There was a significant difference between the 2 used doses of nerolidol ($P<0.05$) from days 5 to 11 PI, but the difference in the growth inhibition between the two doses was low (Fig. 2). The overall inhibition was moderate relative to the high doses used, while the inhibitory effect on *L. amazonensis* was low [14], this may be due to the differences in the life cycle, and pathogenicity of the two parasites. Toxic effects were not observed on mice after intraperitoneal administration of nerolidol; this is in good agreement with what was reported by Arruda et al. [14], where the mice injected with 100 mg/kg nerolidol for 12 days did not show any signs of toxicity. The half life of nerolidol may be very short; therefore, this may lead to its degradation and consequently low effect and no toxic signs even at high doses on the host.

The presence of an active isoprenoid pathway for the biosynthesis of dolichol of 11 and 12 isoprenic units was reported in *L. amazonensis* promastigotes [14] and in *P. falciparum* [28]. Dolichols composed of 11 to 13 isoprene units had been previously characterized in *Trypanosoma brucei* [29], and side chain of the 8 and 9 isoprenic units attached to benzoquinone rings of ubiquinones in *P. falciparum* [30]. While in the mammalian cells, the isoprenic chains of dolichols and ubiquinones comprise 20 to 22 and 10 isoprenic units, respectively [31]. In *Babesia* parasites, the presence of an active isoprenoid pathway was reported [13], while the structure of the dolichol and the side chain attached to the benzoquinone rings of ubiquinones are not known and need further studies.

In *Leishmania amazonensis*, nerolidol inhibited the isoprenoid biosynthesis, as shown by inhibiting incorporation of mevalonic acid (MVA) or acetic acid precursors into dolichol, ergosterol, ubiquinone, and by inhibiting the synthesis of geraniol, farnesol, and the putative hexaprenol intermediate in treated promastigotes due to the blockage of an early step in the mevalonate pathway [14], while in *P. falciparum*, nerolidol interferes with the isoprenoid biosynthetic pathway of the apicoplast leading to the interference with the biosynthesis of the dolichols, with the isoprenic chain of ubiquinones, and with protein isoprenylation of the parasites [15]. *B. bovis* has active isoprenoid pathway which is similar to that of *P. falciparum* [13]; therefore, the mechanism of inhibition for *Babesia* parasites may be similar to that of *P. falciparum* which requires further studies.

In conclusion, the results of the present study showed that nerolidol effectively inhibited the *in vitro* growth of *Babesia* parasites, while its *in vivo* effect on *B. microti* was weak in spite of the high doses used. Therefore, nerolidol could not be used for the treatment of babesiosis.

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Seroprevalence of *Babesia* infections of dairy cows in northern Thailand

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ABSTRACT

The present study was conducted to demonstrate the epidemiological distribution of bovine babesiosis in the northern regions of Thailand. A total of 700 serum samples of dairy cows in the northern provinces (Chiang Rai, Chiang Mai, Lumpang, and Mae Hong Sorn) were tested for antibodies against *Babesia bovis* and *B. bigemina*. Species-specific enzyme-linked (rRAP-1/CTs) were performed. According to the results, 517 (73.8%) and 484 (69.1%) were positive for *B. bovis* and *B. bigemina*, respectively. In addition, 370 (52.9%) were positive for mixed infections by both ELISAs. On the other hand, all samples were also examined by the indirect fluorescent-antibody test (IFAT) with *B. bovis*- and *B. bigemina*-infected blood smears. According to the IFAT, 482 (68.8%) and 531 (75.8%) were positive for these infections, respectively. The overall concordances between the ELISA and IFAT techniques were 93.6% and 90.7% for *B. bovis* and *B. bigemina* infections, respectively. These results indicated that *babesia* infections are widespread in the northern parts of Thailand. To our knowledge, this is the first report describing the epidemiology of *Babesia* infections using rRAP-1/CT-based ELISAs in these areas.

1. Introduction

Bovine babesiosis is known as one of the most economically important diseases in tropical and subtropical regions (McCosker, 1981). This tick-borne disease is mainly caused by two intraerythrocytic protozoan parasites, *Babesia bovis* and *B. bigemina*. The clinical signs induced by these parasites are characterized by fever, anemia, and hemoglobinuria in the infected cattle (de Vos and Potgieter, 1994). However, the disease caused by *B. bovis* is more severe than that by *B. bigemina* (Ristic, 1981). Acute infections are usually diagnosed by a microscopic examination of blood smears, whereas subclinical infections have been identified serologically (Weiland and Reiter, 1988). Therefore, differential diagnosis between *B. bovis* and *B. bigemina* infections will lead to a better understanding of their epidemiology, and the species-specific distribution in the field will provide useful information to establish a control program of these diseases (de Vos and Potgieter, 1994).

Thailand is a developing agricultural country located in Southeast Asia. In this country, livestock development, particularly for dairy cows, has been hampered by persistently low production of milk and meat due to many infectious pathogens, including *Babesia*. In 1990, 428 cattle sera of 12 provinces in Thailand were tested for the antibodies of bovine babesiosis by the IFAT (Nishikawa et al., 1990). The prevalence of antibodies to *B. bovis* and *B. bigemina* was 74.5% (Nishikawa et al., 1990); however, there are only a few reports of bovine babesiosis in Thailand. Therefore, further reliable information is needed to reduce losses to animal owners due to these diseases.

The objective of the present study is to determine the prevalence of *Babesia* infections among dairy cows in northern provinces of Thailand using the IFAT and

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Table 1
Comparison of ELISA and IFAT in the detection of anti-*B.bovis* or *B. bigemina*-specific antibodies[a].

Provinces	Total	*B. bovis*				*B. bigemina*			
		ELISA		IFAT		ELISA		IFAT	
		+	–	+	–	+	–	+	–
Chiang Rai	392	284(72.4)	108(27.6)	269(68.6)	123(31.4)	257(65.5)	135(34.5)	283(72.1)	109(27.9)
Chiang Mai	150	126(84)	24(16)	111(74)	39(26)	107(71.3)	43(28.7)	123(82)	27(18)
Lumpang	100	91(91)	9(9)	89(89)	11(11)	85(85)	15(15)	95(95)	5(5)
Mae HongSorn	58	16(27.5)	42(72.5)	13(22.4)	45(77.6)	35(60.3)	23(39.7)	30(51.7)	28(48.3)
Total	700	517(73.8)	183(26.2)	482(68.8)	218(31.2)	484(69.1)	216(30.9)	531(75.9)	169(24.1)

[a] Values in parenthesis are in percentage.

the enzyme-linked immunosorbent assay (ELISA) with recombinant rhoptry-associated protein-1 (RAP-1) of each parasite (Boonchit et al., 2004, 2006).

2. Materials and methods

2.1. Preparation of recombinant antigens

The rRAP-1/CT, which consisted of a C-terminal portion of *B. bovis* and *B. bigemina* RAP-1, were prepared as previously described (Boonchit et al., 2004, 2006). Briefly, a gene encoding the *B. bovis* and *B. bigemina* RAP-1/CTs (C-terminal portion of the RAP-1) were expressed as an rRAP-1/CT fused with glutathione S-transferase (GST) in *E. coli*. Recombinant RAP-1/CT in an elution buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl, and 2 mM EDTA) was used without thrombin protease, and rRAP-1/CT was obtained as a GST-fused protein according to the manufacturer's instructions (Amersham Pharmacia Biotech, Little Chalfont, UK). GST was used as control antigen in ELISA.

2.2. ELISA and IFAT

The purified proteins were subjected to species-specific diagnostic ELISAs (Boonchit et al., 2004, 2006). By optimization with 30 non-infected bovine sera, cutoffs for the OD at 415 nm in *B. bovis* and *B. bigemina* ELISAs were determined to be 0.07 and 0.096, respectively. The IFAT was also performed as previously described (Boonchit et al., 2004, 2006).

2.3. Sera

For the present investigation, 700 bovine sera were collected from 55 small dairy farms located in Chiang Rai (392), Chiang Mai (150), Lumpang (100), and Mae Hong Sorn provinces (58) in northern Thailand and stored at −20 °C until used.

2.4. Statistical analysis

The results of the ELISA were compared with those of the IFAT, and the percentages of overall concordance were calculated (percent concordance (%) = number of concordances between the ELISA and the standard IFAT reference × 100/number of tested samples) (Kim et al., 2007). The prevalence of *B. bovis* and *B. bigemina* according to the ELISA in each province was statistically analyzed employing Pearson's chi-square test $P < 0.05$ as the values representing a significant difference. The chi-square test was applied to compare the rates of seropositivity among age groups, study sites, and gender. The statistical significance in this study was defined as $P < 0.05$.

3. Results

According to ELISA, the prevalence of *B. bovis* and *B. bigemina* infection in Thailand was 73.8% (517/700) and 69.1% (484/700), respectively (Table 1). The percentages of *B. bovis*-positive sera in the four provinces, i.e., Chiang Rai, Chiang Mai, Lumpang, and Mae Hong Sorn, were 72.4% (284/392), 84% (126/150), 91% (91/100), and 27.5% (16/58), respectively. On the other hand, *B. bigemina*-positive sera represented 65.5% (257/392), 71.3% (107/150), 85% (85/100), and 60.3% (35/58), respectively. Mixed infections of *B. bovis* and *B. bigemina* were also determined to be 48.7% (191/392), 60.7% (91/150), 77% (77/100), and 18.9% (11/58), respectively. The rate of *B. bovis*-infected animals was significantly higher in Lumpang than in Chiang Rai ($P < 0.01$); it was also significantly higher in Chiang Mai than in Chiang Rai ($P < 0.01$) (Fig. 1A and C). In contrast, the rate of *B. bovis*-infected animals was significantly lower in Mae Hong Sorn than in the other three provinces ($P < 0.01$). These results indicated that the rate of *B. bovis*-infected animals is highly different in the four provinces. On the other hand, the rate of *B. bigemina*-infected animals was significantly higher in Lumpang than in Chiang Rai ($P < 0.01$), Chiang Mai ($P < 0.05$), and Mae Hong Sorn ($P < 0.01$) (Fig. 1B and D). The results of the ELISA were compared with those of the IFAT to evaluate the sensitivity and specificity of the ELISA for the detection of the specific antibodies to each parasite in Thailand. According to the IFAT, the overall prevalence for *B. bovis* and *B. bigemina* infections was determined to be 68.8% (482/700) and 75.9% (531/700), respectively (Table 1). The *B. bovis*-positive rates of four provinces were 68.6% (269/392), 74% (111/150), 89% (89/100), and 22.4% (13/58) in Chiang Rai, Chiang Mai, Lumpang, and Mae Hong Sorn, respectively. Moreover, *B. bigemina*-positive rates showed 72.1% (283/392), 82% (123/150), 95% (95/100), and 51.7% (30/58) in Chiang Rai, Chiang Mai, Lumpang, and Mae Hong Sorn, respectively. In addition, the prevalence of *B. bovis* and *B. bigemina* infections varied in different age groups, ranging from 68.4% to 93.8% and 45.5% to 88.2%, respectively (Table 2).

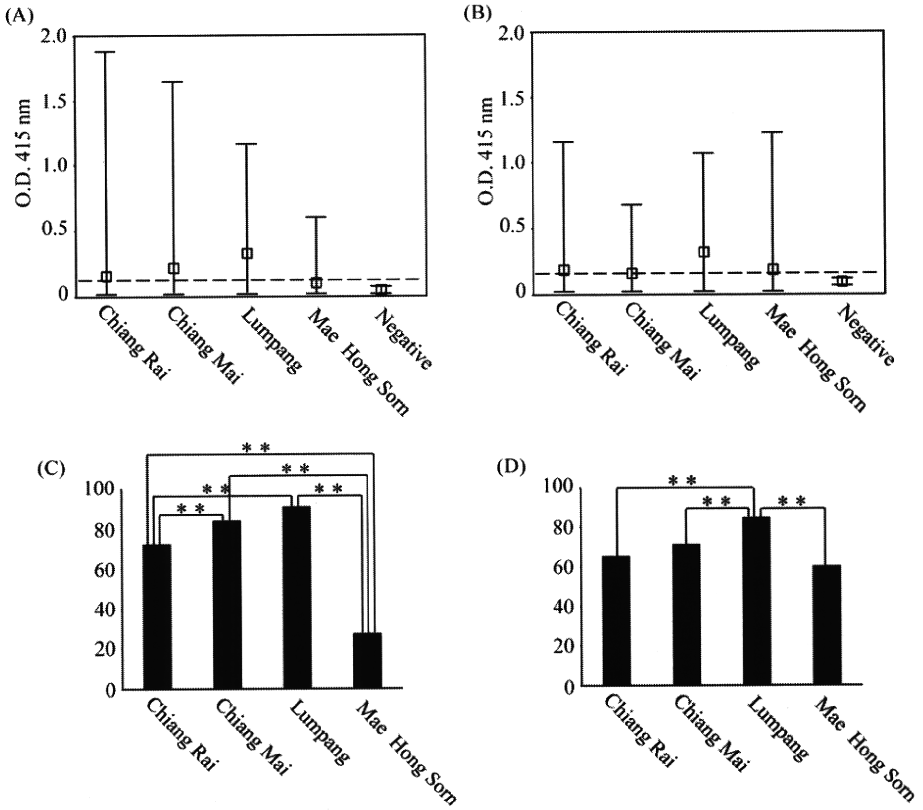

Fig. 1. ELISA O.D. values distribution of *B. bovis* (A) and *B. bigemina* (B) seroprevalence and sero-positive prevalence of *B. bovis* (C) and *B. bigemina* (D) in four different regions in Thailand. Maximum and minimum values (bars) and median values (boxes) from the ELISA are shown in panels A and B. The cutoff line was defined as the mean value plus the threefold standard deviation of the optical density (OD) obtained from 30 uninfected serum samples. These represent the OD at 415 nm of *B. bovis*-infected sera and *B. bigemina*-infected sera; Chiang Rai ($n = 392$), Chiang Mai ($n = 150$), Lumpang ($n = 100$), and Mae Hong Sorn ($n = 58$). The asterisks indicate significant differences in each region ($^{**}P < 0.01$).

4. Discussion

The overall concordances of the ELISA and the IFAT were determined to be 93.6% and 90.7% for *B. bovis* and *B. bigemina* infections, respectively, when the results of the IFAT were used as the reference standard. The prevalence of *B. bovis*-positive animals in the ELISA was higher than that in the IFAT; in contrast, the rate of *B. bigemina*-positive animals in the ELISA was lower than that in the IFAT. This contradiction could be explained by a report in which *B. bigemina*-infected sera had cross-reactivity against *B. bovis*-infected sera (Morzaria et al., 1992).

Resistance to babesiosis is influenced by several factors, including the age of the animal (Ristic, 1981). Therefore, we analyzed the relationship between the prevalence of *B. bovis* and *B. bigemina* and the age of the examined cattle. However, there were no statistically significant differences between the age group and the infection rates,

Table 2
Seroprevalence of *B. bovis* and *B. bigemina* in different age groups of dairy cows[a].

Provinces	Age (year)	No. of animals	No. of positives (seroprevalence (%))	
			B. bovis	*B. bigemina*
ChiangRai	1	0	0	0
	1-5	212	145(68.4)	141(66.5)
	>5	180	134(74.4)	116(64.4)
Chiang Mai	1	11	10(90.9)	5(45.5)
	1-5	96	81(84.4)	71(74)
	>5	43	36(83.7)	33(76.7)
Lumpang	1	7	6(85.7)	6(85.7)
	1-5	76	64(84.2)	67(88.2)
	>5	17	15(93.8)	13(76.5)

[a] Values in parenthesis are in percentage.

except for a group of *B. bigemina*-infected cattle in Chiang Mai. In Chiang Mai, a significant difference was obtained in the infection rate between <1-year and 1–5-year groups ($P < 0.05$). Calves can receive anti-*Babesia* antibodies via the colostrum immediately after birth from immune mothers, which is considered to be a challenge during the first month of life (James, 1988). Most cattle develop lasting immunity after recovering; while this immunity is not absolute, it may last for life, even in the absence of re-infection (de Vos and Potgieter, 1994). Further epidemiological surveys on antibodies would give some interesting information on the control of both bovine babesiosis.

We believe that the epidemiological data obtained in the present study will contribute to establish a plan and strategy to control or diminish bovine babesiosis in the northern regions of Thailand. The prevalence of bovine babesiosis in Thailand suggests that an endemic stability might be present in the studied areas. In addition, our data will be beneficial for provincial veterinarians to understand current epidemiological status of the infections and will help Thai farmers reduce losses from this disease.

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Development and evaluation of two nested PCR assays for the detection of *Babesia bovis* from cattle blood

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ABSTRACT

We developed and evaluated two nested polymerase chain reaction (nPCR) assays for the diagnosis of *Babesia bovis* infection in cattle based on two membrane protein genes from *B. bovis*, BBOV_IV005650 (BV5650) and BBO_IV008970 (BV8970). The specificities and sensitivities of the tests were compared with *B. bovis* Rhoptery associated protein 1 gene (RAP-1) nPCR. The specificity of the tests was 100% for *B. bovis* DNA. The sensitivities of nPCR to *B. bovis* from the *in vitro* cultured parasites were as low as 10^{-8}%, 10^{-6}%, and 10^{-7}% parasitemia for BV5650, BV8970, and RAP-1 nPCR, respectively. The nPCR detected as little as 1 fg genomic DNA per test for BV5650 and 100 fg per test for both BV8970 and RAP-1 genes. For field applications, the sensitivity was evaluated to a total of 165 field samples from Ghana, Mongolia, Brazil and Japan. The nPCR assay of BV5650 was the most sensitive for the detection of *B. bovis* from the field samples. The BV5650 nPCR assay provides a good diagnostic tool for laboratory diagnostic assessment of *B. bovis* infection in cattle worldwide.

1. Introduction

Babesia bovis is a tick-transmitted protozoan parasite of cattle. The disease is considered one of the most important tick-borne diseases of cattle worldwide, and of the 1.2×10^9 cattle in the world, over 500 million of these cattle are potentially at risk of having bovine babesiosis (McCosker, 1981). The clinical signs of *B. bovis* infection are fever, hemoglobinuria, acute anemia, and cerebral or nervous signs (Homer et al., 2000). Animals that survive *B. bovis* infection generally become low-level carriers of the parasite and serve as a reservoir for transmission (Mahoney, 1969).

Routine clinical diagnosis for babesiosis is usually based on the microscopic detection of parasites from collected blood smears. The detection had been considered to be the "gold standard" for the diagnosis of babesiosis (Böse et al., 1995). However, the technique is relatively laborious when large numbers of blood smear samples must be simultaneously quantified. Furthermore, it is extremely difficult to detect parasites in blood smears during low parasitemia as in the case of carrier animals (Almeria et al., 2001). Alternative techniques have been developed for the laboratory diagnosis of babesiosis. For example, many serological diagnostic tests have been developed for the detection of specific antibodies to bovine *Babesia* parasites, such as the complement fixation test (CFT), the indirect hemaglutination (IHA) test, the latex agglutination test (LAT), the indirect fluorescent antibody test (IFAT), the enzyme-linked immunosorbent assay (ELISA) (Weiland and Reiter, 1988; Boonchit et al., 2006; Goff et al., 2006), and immunochromatographic test (ICT) (Kim et al., 2008). However, the antibodies cannot always be detected in long-term carriers despite the presence of the parasite, and the lack of discrimination between previous exposure and current infections (Wagner et al., 1992). Fur-

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thermore, cross-reactivity of the antibodies against other *Babesia* species has limited the specificity of serological tests (Papadopoulos et al., 1996; Passos et al., 1998).

Polymerase chain reaction (PCR) assays for the diagnostic detection of *Babesia* parasites have the potential to provide rapid results with high specificity and sensitivity, in particular nested PCR assay (Fahrimal et al., 1992; Smeenk et al., 2000; Almeria et al., 2001; Gayo et al., 2003; Oliveira-Sequeira et al., 2005; Costa-Júnior et al., 2006; Martins et al., 2008). These PCR assays have various advantages over the microscopic and serological diagnostic test. For example, PCR diagnosis is possible in animals as young as 1 month of age, and the data obtained by PCR assays refer to the current prevalence, in contrast to the data obtained by serological assays (Wagner et al., 1992). In addition, the sensitivity of these assays for detecting bovine babesiosis has been shown to be higher than that of microscopic detection methods (Fahrimal et al., 1992; Figueroa et al., 1993; Almeria et al., 2001).

Completion of the genome sequence of *B. bovis* resulted in a large number of candidate genes for designing new PCR methods (Brayton et al., 2007). The search for new genes is required for the diagnosis of *B. bovis* infection. We chose two membrane protein genes, BBOV_ IV005650 (BV5650) and BBOV_ IV008970 (BV8970), from *B. bovis* genome sequence database to be used in the nested PCR assay. These two genes are unique for *B. bovis* and have no homologues either in *B. bigemina* genome sequence database or in the other apicomplexan parasites (Brayton et al., 2007). Unlike other apical complex proteins (e.g., Rhoptry-Associated Protein 1), which are conserved among *Babesia* species, the genes encoding BBOV_ IV005650 (BV5650) and BBOV_ IV008970 (BV8970) have not been detected in *B. bigemina* genome sequence (www.sanger.ac.uk). Therefore, the use of these genes for the diagnosis of *B. bovis* infection may increase the specificity, and sensitivity of the PCR. The aim of this study was to evaluate the diagnostic efficiency of the nested PCR assays based on these two membrane protein genes for diagnosis of *B. bovis* compared with the Rhoptry-Associated Protein 1 (RAP-1) nested PCR used in the previous studies.

2. Materials and methods

2.1. Parasites

The Texas strain of *B. bovis* (Hines et al., 1995) was maintained in purified bovine red blood cells (RBC) with a microaerophilic stationary-phase culture system (Bork et al., 2003a). Medium M199 (Sigma–Aldrich, Tokyo, Japan) was supplemented with 40% normal bovine serum to prepare the culture medium for the parasites.

2.2. DNA extraction

B. bovis-infected RBC were washed three times with cold phosphate-buffered saline (PBS) by centrifuging at 1000 × g for 5 min at 4 °C and resuspended in PBS. The infected RBC were serially diluted 10-fold with normal RBC to adjust the parasite concentrations from 2.7×10^{-2} (parasitemia: 0.0000000001%) to 2.7×10^{6} (parasitemia: 1%) infected RBC/200 μl of the total RBC. Also another concentration of 7×10^{7} (5.2%) infected RBC/200 μl of total RBC was prepared (as positive control). Then all the dilutions separately subjected to DNA extraction with a QIAamp DNA Blood Mini Kit (QIAGEN, Tokyo, Japan). The purified DNA samples were used as templates for the subsequent PCR method. Genomic DNA was extracted from cultured parasites with 5% parasitemia as mentioned above for measuring the sensitivity of the primers to genomic DNA (Alhassan et al., 2005). The DNA measured spectrophotometrically and diluted 10-fold from 200 ng/μl to 2 fg/μl. DNA from the field samples were extracted from blood-spotted filter papers (Abe and Konomi, 1998; da Silva et al., 2004). Briefly, the spotted filter papers were cut out with a 2-mm hole puncher (2.0-mm Harris Micro Punch; Whatman, Middlesex, UK). DNA samples were extracted from the cut portion containing the spotted blood using a QIAamp DNA Mini Kit (QIAGEN). As negative controls, distilled water, extracted DNA samples of normal bovine blood and other cultured parasites (*B. bigemina*, *Theileria parva*, *Trypanosoma conglonse*, and *Neospora caninum*) were prepared as described above.

2.3. PCR and nested PCR amplifications

PCR and nested PCR (nPCR) amplifications were carried out with the developed primers specific to *B. bovis*, BBOV_IV005650 (BV5650) and BBOV_IV008970 (BV8970) membrane protein genes, Gene Bank accession numbers of XM_001610444, and XM_001610769, respectively (Table 1). The published pairs of species-specific primers for the detection of *B. bovis* (Figueroa et al., 1993) were used as a control for the developed primers. PCR was performed in 25 μl of a mixture containing 0.5 μl of the extracted DNA template, 50 pmol of each primer, 200 μM of each dNTP, and 1.25 U of Taq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA) in a PCR buffer (Applied Biosystems). The reactions were performed at the following temperatures: initial denaturation at 95 °C for 5 min to activate the Taq Gold DNA polymerase, followed by 35 cycles (1 min of denaturation at 94 °C, 1 min of annealing (at 57.5 °C for BV897 and at 64.5 °C for BV565), 1 min of extension at 72 °C), and 10 min of final extension at 72 °C in a Gene Amp PCR system 9700 (Applied Biosystems). The amplified PCR products of 0.5 μl were used for the subsequent nPCR with a limited annealing temperature at 57 °C. The PCR and nPCR conditions for *B. bovis* RAP-1 follow the method previously described (Figueroa et al., 1993). Cross-contamination was prevented by using plugged tips, performing the PCR in a separate room from that used for DNA extraction. The PCR and nested PCR products were subjected to electrophoresis in 2% agarose gel and then visualized under an ultraviolet (UV) light after staining with ethidium bromide (Sigma-Aldrich).

2.4. DNA sequencing

Positive DNA products from the specificity test (PCR, and nPCR), and from field samples nPCR were purified after 2% agarose gel electrophoresis using QIAquick Gel Extraction Kit (Qiagen K.K., Tokyo, Japan) and then cloned into a

Table 1
Primers sets developed for *B. bovis* DNA amplification in the present study.

Target gene	Assay	Primers	Sequences (5′→3′)	Amplicons size
BV5650[a]	PCR	F	ccggaattccaaatggcaacaaaggttga	720
		R	ccgctcgagggagcagcgtattacttcctcacgt	
	nPCR	F1	cgaggatttggtagacctcatc	561
		R1	cgtaaaatgtgtacaactattt	
BV8970[b]	PCR	F	ccggaattcacggaagagccgctagatgta	590
		R	ccgctcgagtcataatcagcctcggtgaaagc	
	nPCR	F1	cgctccgtagatgtggtgtcc	420
		R1	actatcatcagagtcggaatca	

[a] BBOV_IV005650 gene.
[b] BBOV_IV008970 gene.

pCR2.1® cloning vector using The Original TA Cloning Kit (Invitrogen, Carlsbad, CA, USA). The nucleotide sequences of inserts were determined using a Big Dye Terminator Kit (Applied Biosystems Japan, Ltd.) with ABI PRISM 3100™ genetic analyzer (Applied Biosystems Japan, Ltd.). The Genetyx 7 package (Software Development Co., Ltd., Tokyo, Japan) was used to align the determined sequences.

2.5. Field samples

FTA cards were used to collect 40, 24, and 81 field blood samples from cattle living in Ghana, Mongolia, and Brazil, respectively. DNA samples were extracted from the FTA cards as described above. Moreover, 20 blood samples were collected from Japan (a region considered free of *B. bovis* infections) and the DNA was extracted with a QIAamp DNA Blood Mini Kit (QIAGEN, Tokyo, Japan).

3. Results

3.1. Specificities of PCR and nested PCR methods

The species-specific PCR primers for *B. bovis* specifically produced the positive amplicons of 720 bp for BV5650, 590 bp for BV8970, and 356 bp for RAP-1 genes of only *B. bovis* DNA. The nested primers targeted 561 bp for BV5650, 420 bp for BV8970, and 291 bp for RAP-1 genes only from *B. bovis* DNA. There was no amplification for DNA from *B. bigemina*, *Theileria parva*, *Trypanosoma conglonse*, and *Neospora caninum* that were used as negative controls. In order to confirm the nucleotide sequences of PCR and nested PCR products, the amplified DNA products were purified from the positive reactions and cloned into the vector. The determined sequences of all DNA fragments were 100% identical to the reported ones of BBOV_IV005650, BBOV_IV008970, and *B. bovis* RAP-1 genes (Gene Bank accession numbers: XM_001610444, XM_001610769 and AF027149, respectively) (data not shown).

3.2. Sensitivities of the PCR and nested PCR methods

3.2.1. Sensitivities to DNA extracted from diluted infected RBC

To evaluate the sensitivities of the PCR and nested PCR methods of the newly developed primers, *B. bovis* DNA samples were extracted from a 10-fold serial dilution of the culture and then subjected to the PCR and nested PCR methods. In addition, the detection limits of the PCR and nested PCR methods were compared to those of PCR and nPCR methods of RAP-1 gene. In the PCR methods (Fig. 1), positive band of 720 bp (Panel A, lane 10) was detected from the dilution of 0.00000001% parasitemia (2.7×10^{-2} infected RBC) for BV5650. Positive bands of 590 bp (Panel B, lane 4), and 356 bp (Panel C, lane 4) were detected from the dilution of 0.01% parasitemia (2.7×10^{4} infected RBC) for BV8970, and RAP-1 genes, respectively. Subsequently, in the nPCR methods (Fig. 2), specific band of 561 bp was observed from the dilution of 0.00000001% parasitemia (2.7×10^{-2} infected RBC) for BV5650 gene (Panel A, lane 10). Specific bands of 420 bp from the dilution of 0.000001% parasitemia (2.7×10^{0} infected RBC) for BV8970 gene (Panel B, lane 8), and of 291 bp from the dilution of 0.0000001% parasitemia (2.7×10^{-1} infected RBC) for RAP-1 gene (Panel C, lane 9) were detected, respectively.

3.2.2. Sensitivities to 10-fold diluted genomic DNA

We evaluated the sensitivities of the PCR and nested PCR methods of the newly developed primers for the genomic DNA of *B. bovis*. DNA was diluted 10-fold and then subjected to the PCR and nested PCR methods. The detection limit for the PCR was 10 fg genomic DNA per test for BV5650, while it detected 10 ng for both BV8970, and RAP-1 genes (data not shown). The nPCR (Fig. 3) detected as low as 1 fg genomic DNA per test for BV5650 (Panel A, lane 9), 100 fg per test for both BV8970 (Panel B, lane 7), and RAP-1 (Panel C, lane 7) genes.

3.3. Detection of B. bovis from field samples by PCR and nPCR methods

Field blood samples collected from cattle living in Ghana, Mongolia, and Brazil were surveyed using the PCR and nPCR methods in order to demonstrate the field utility of these methods as a diagnostic tool for epidemiological studies. Forty, 24, 20, and 81 field blood samples were collected from Ghana, Mongolia, Japan, and Brazil, respectively. The PCR of BV5650 detected *B. bovis* DNA in 70% (28/40), 54.2% (13/24), and 27.2% (22/81) of the samples from Ghana, Mongolia, and Brazil, respectively (Table 2). The PCR of *B. bovis* RAP-1 only detected *B. bovis* DNA in 2.5%

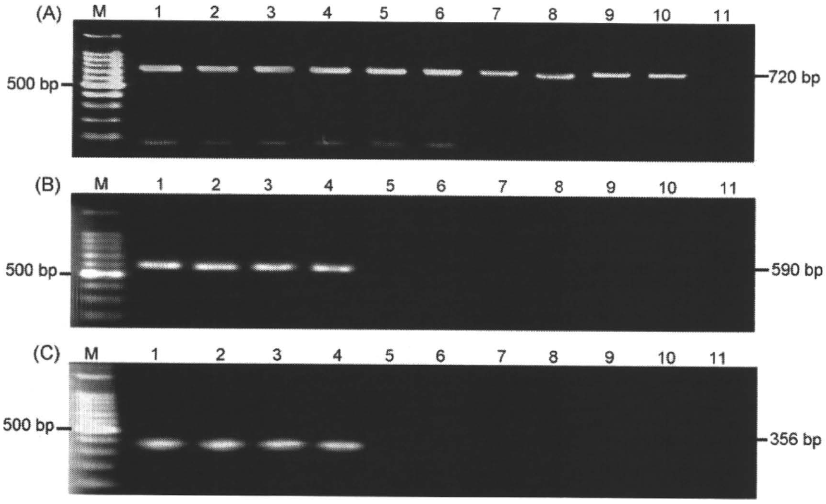

Fig. 1. Sensitivities of the PCR methods. PCR methods were carried out using the extracted DNAs from dilutions of infected RBC in the *in vitro* culture. BV5650 PCR (F and R) (Panel A), BV8970 PCR (F and R) (Panel B), and *B. bovis* RAP-1 (F and R) (Panel C) primers. In all panels, lane 1, 7 × 10^7; lanes 2–10, 2.7 × 10^6, 2.7 × 10^5, 2.7 × 10^4, 2.7 × 10^3, 2.7 × 10^2, 2.7 × 10^1, 2.7 × 10^0, 2.7 × 10^{-1}, and 2.7 × 10^{-2} infected RBC; and lane 11, bovine DNA. Lane M shows a 100-bp ladder size marker and the band of 500 bp is indicated on the left. The size of the positive bands is indicated on the right.

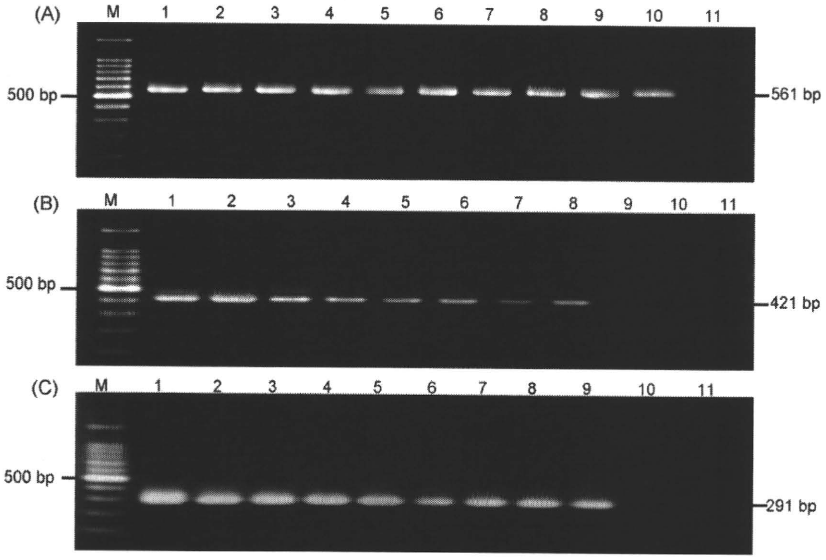

Fig. 2. Sensitivities of the nPCR methods. nPCR methods were carried out using the extracted DNAs from dilutions of infected RBC in the *in vitro* culture. BV5650 nPCR (F1 and R1) (Panel A), BV8970 nPCR (F1 and R1) (Panel B), and *bovis* RAP-1 nPCR (Panel C) primers. In all panels, lane 1, 7 × 10^7; lanes 2–10, 2.7 × 10^6, 2.7 × 10^5, 2.7 × 10^4, 2.7 × 10^3, 2.7 × 10^2, 2.7 × 10^1, 2.7 × 10^0, 2.7 × 10^{-1}, and 2.7 × 10^{-2} infected RBC; and lane 11, bovine DNA. Lane M shows a 100-bp ladder size marker and the band of 500 bp is indicated on the left. The size of the positive bands is indicated on the right.

Table 2

Comparison of the positive numbers among conventional PCR and nested PCR methods for *B. bovis* detection from field bovine blood samples collected from Ghana, Mongolia, Brazil, and Japan.

Country	Number	BV5650[a]		BV8970[b]		BVRAP-1[c]	
		Positive numbers (%)					
		nPCR	PCR	nPCR	PCR		
Ghana	40	28 (70)	33(82.5)	0 (0)	15(37.5)	0 (0)	10 (25)
Mongolia	24	13(54.2)	22(91.7)	0 (0)	2(8.33)	0 (0)	13(54.2)
Brazil	81	22(27.2)	52(64.2)	0 (0)	17 (21)	2(2.5)	31(38.2)
Japan	20	0	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

[a] BV5650 is BBOV_IV005650 gene.
[b] BV8970 is BBOV_IV008970 gene.
[c] *B. bovis* RAP-1 gene.

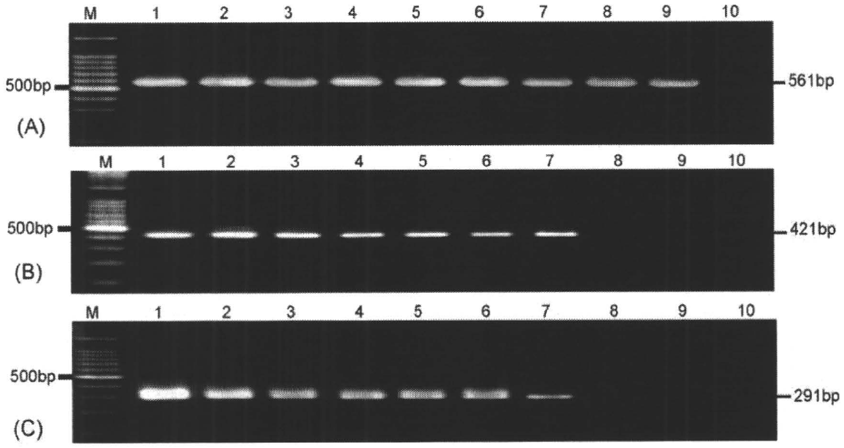

Fig. 3. Sensitivities of the nPCR methods. The nPCR methods were carried out using the 10-fold diluted genomic DNA extracted from the *in vitro* culture. BV5650 nPCR (F1 and R1) (Panel A), BV8970 nPCR (F1 and R1) (Panel B), and *bovis* RAP-1 nPCR (Panel C) primers. In all panels, lane 1, 100 ng; lane 9, 1 fg; and lane 10, bovine DNA. Lane M shows a 100-bp ladder size marker and the band of 500 bp is indicated on the left. The size of the positive bands is indicated on the right.

(2/81) of the samples from Brazil while the PCR of BV8970 could not detect *B. bovis* in any of the samples from the three countries (Table 2). Subsequently, the nPCR detection rates for *B. bovis* by BV5650 nPCR were 82.5% (33/40), 91.7% (22/24), and 64.2% (52/81) in Ghana, Mongolia, and Brazil, respectively (Table 2). BV8970 nPCR detected *B. bovis* in 37.5% (15/40), 8.33% (2/24), and 21% (17/81) of the samples from Ghana, Mongolia, and Brazil, respectively (Table 2). *B. bovis* RAP-1 nPCR detected *B. bovis* in 25% (10/40), 54.2% (13/24), and 38% (31/81) of the samples from Ghana, Mongolia, and Brazil, respectively. The BV5650, BV8970, and *B. bovis* RAP-1 PCR and nPCR could not detect *B. bovis* in any of the samples from Japan (Table 2). The sequences of the positive nested DNA fragments were almost 100% identical to the reported ones of BBOV_IV005650, BBOV_IV008970, and 99% identical to the *B. bovis* RAP-1 genes (Gene Bank accession numbers: XM_001610444, XM_001610769, and AF027149, respectively) (supplementary data).

4. Discussion

In this report, we described the successful development of two nested PCR methods for the detection of *B. bovis*. In the methods, sets of the designed primers specifically amplified the target DNAs derived from the respective BV5650 and BV8970 membrane protein genes. The specificity of the new primers was in agreement with that of *B. bovis* RAP-1 gene in PCR and nPCR methods.

The PCR and nPCR methods of BV5650 gene were more sensitive than the PCR and nPCR of BV8970, and *B. bovis* RAP-1 genes, respectively. They provide the detection of 10^{-8}% parasitemia (0.027 parasite) per test for DNA extracted from diluted infected RBC. The PCR and nPCR methods of BV5650 gene permitted the detection of 10 fg and 1 fg/test of the genomic DNA, respectively. The PCR and nPCR methods of BV5650 gene were more sensitive than the PCR and nPCR methods of BV8970, and *B. bovis* RAP-1 genes.

The detection rates for the field samples collected from Ghana, Mongolia, Japan, and Brazil were assessed. The PCR method of BV5650 gene had the highest positive numbers of the total samples tested. The nPCR method of the BV5650 gene also had the highest positive numbers of the total samples tested. The high number of positive samples detected by BV5650 nPCR were consistent with that were detected by spherical body protein 2 gene nPCR primers for the samples from Ghana (36/40), Mongolia (23/24), and Brazil (67/81) (AbouLaila et al., 2010). The high number of positive samples detected by BV5650 nPCR may be due to the presence of several gene copies in the genome. However, further studies are required to confirm this hypothesis. The sequence conservation among strains may also be a factor for these high positive numbers. While the *B. bovis* RAP-1 has two nearly identical copies (Norimine et al., 2002).

There are several reported methods describing the detection of *B. bovis*, but only some of them have been tested with random field samples: the nPCR (Figueroa et al., 1993; Almeria et al., 2001; Gayo et al., 2003; Oliveira et al., 2005; Costa-Júnior et al., 2006; Goff et al., 2006), the RLB (Gubbels et al., 1999; Brígido et al., 2004; Oura et al., 2004), the LAMP assay (Iseki et al., 2007), and recently the seminested hot-start PCR (Martins et al., 2008). The sensitivity of our nPCR is higher than reported sensitivities in previous studies using the nPCR of around 10^{-7}% parasitemia (Oliveira-Sequeira et al., 2005), 10^{-6}% parasitemia (Costa-Júnior et al., 2006), and 10^{-4}% parasitemia (Iseki et al., 2007).

The high positive numbers by our nPCR for *B. bovis* in the field samples from Brazil, and Ghana were consistent with that was detected by Oliveira-Sequeira et al. (2005), and the LAMP test by Iseki et al. (2007), respectively indicating high prevalence of babesiosis caused by *B. bovis* infection in both countries. To our knowledge, this is the first molecular detection of *B. bovis* from Mongolia. High detection rates of *B. bovis* by nPCR indicate that not only equine babesiosis (Boldbaatar et al., 2005), but also bovine babesiosis caused by *B. bovis* infection are prevalent in Mongolia.

In conclusion, we developed two nPCR-based methods for the detection of *B. bovis* from cattle blood. The BV5650 nPCR assay has higher levels of sensitivity than BV8970 nPCR; therefore, BV5650 nPCR method provides a good diagnostic tool for laboratory diagnostic assessment of *B. bovis* infection in cattle worldwide.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetpar.2010.04.011.

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Evaluation of a Loop-Mediated Isothermal Amplification Method as a Tool for Diagnosis of Infection by the Zoonotic Simian Malaria Parasite *Plasmodium knowlesi*▽

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Loop-mediated isothermal amplification (LAMP) is a novel method that rapidly amplifies target DNA with high specificity under isothermal conditions. It has been applied as a diagnostic tool for several infectious diseases, including viral, bacterial, and parasitic diseases. In the present study, we developed a LAMP method for the molecular diagnosis of *Plasmodium knowlesi* infection (*Pk*LAMP) and evaluated its sensitivity, specificity, and clinical applicability. We designed three sets of *Pk*LAMP primers for the species-specific β-tubulin gene. The primer sets for *Pk*LAMP specifically amplified the autologous DNA extracts of *P. knowlesi*, and the sensitivity of the test was 100-fold that of single-PCR assay. These results indicate that our *Pk*LAMP method can be used to efficiently distinguish between *P. knowlesi* and other malaria parasites. To evaluate the feasibility of using *in vivo* materials, comparisons of *Pk*LAMP and the conventional nested PCR (nPCR) method and microscopic examination were made with blood samples from two experimentally infected monkeys. These studies showed that *P. knowlesi* infection can be identified much earlier with *Pk*LAMP than with nPCR and microscopy. Moreover, the detection performance of *Pk*LAMP using whole blood as the template was identical to that of *Pk*LAMP when genomic DNA extracts were used. These results suggest that the *Pk*LAMP method is a promising tool for molecular diagnosis of *P. knowlesi* infection in areas of endemicity.

Naturally acquired human infections with a macaque malaria parasite, *Plasmodium knowlesi*, have now been referred to as the fifth human malaria (4, 17). In fact, recent studies have shown that naturally occurring *P. knowlesi* malaria cases are not rare and are widely distributed in Southeast Asia, particularly in forested areas inhabited by the natural macaque host and vectors such as the *Anopheles leucophyrus* group (4, 5, 16).

Until recently, numerous cases of *P. knowlesi* infections in humans may have been misdiagnosed as ordinary *Plasmodium malariae* malaria (4, 5, 16), since the morphological characteristics of the blood stages of *P. knowlesi* parasites are similar to those of *P. malariae*, and it can be easily misidentified as *P. malariae* on microscopic examination (16). Moreover, our recent study showed that some commercial rapid malaria diagnostic tests based on the detection of parasite lactate dehydrogenase enzyme (pLDH) are unable to distinguish between human malaria parasites and *P. knowlesi*, since certain antibodies to pLDH that were thought to be specific for *Plasmodium falciparum* and *Plasmodium vivax* also bind to *P. knowlesi* (9). Although the development of a PCR diagnostic method has been essential to solving these problems of misdiagnosis, PCR assays are not a simple method of detection and are not a viable option for routine diagnosis.

Loop-mediated isothermal amplification (LAMP) has been developed as a novel method to amplify DNA with high specificity and simplicity (13). It consists simply of incubating a mixture of the target gene, four or six different primers, *Bst* DNA polymerase, and substrates. The significant advantages of the LAMP method are (i) high amplification efficiency under isothermal conditions (63 to 65°C) and (ii) visual judgment based on the turbidity or fluorescence of the reaction mixture, which is kept in the reaction tube (10, 12). LAMP has thus emerged as a powerful tool to facilitate genetic testing for the rapid diagnosis of several infectious diseases, including viral, bacterial, and parasitic diseases (8, 11). Although the detection performances of LAMP for four human malaria parasites have been assessed in clinical and epidemiological settings, the LAMP method has not yet been evaluated for the diagnosis of *P. knowlesi* infection (3, 7, 14). In the present study, we developed a LAMP method for diagnosis of *P. knowlesi* infection (*Pk*LAMP) and evaluated its sensitivity, specificity, and clinical applicability using blood samples obtained from experimentally *P. knowlesi*-infected monkeys.

MATERIALS AND METHODS

Specific primers for *Pk*LAMP. The LAMP method requires a set of four specific primers: a forward inner primer (FIP), a backward inner primer (BIP), and two outer primers (F3 and B3), which recognize a total of six distinct nucleotide sequences (B1, B2, B3, F1, F2, and F3) on the target gene (10, 12, 13).

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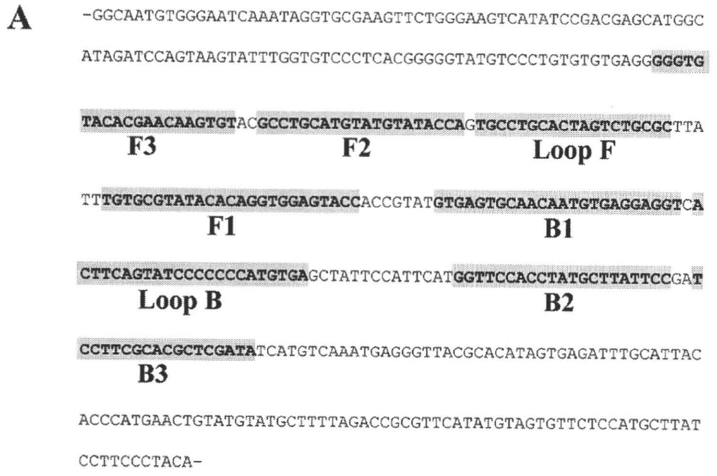

-GGCAATGTGGGAATCAAATAGGTGCGAAGTTCTGGGAAGTCATATCCGACGAGCATGGC

ATAGATCCAGTAAGTATTTGGTGTCCCTCACGGGGGTATGTCCCTGTGTGTGAGG**GGGTG**

TACACGAACAAGTGTAC**GCCTGCATGTATGTATACCA**G**TGCCTGCACTAGTCTGCGC**TTA

TT**TGTGCGTATACACAGGTGGAGTACC**ACCGTAT**GTGAGTGCAACAATGTGAGGAGGT**C**A**

CTTCAGTATCCCCCCCATGTGAGCTATTCCATTCAT**GGTTCCACCTATGCTTATTCC**GA**T**

CCTTCGCACGCTCGATATCATGTCAAATGAGGGTTACGCACATAGTGAGATTTGCATTAC

ACCCATGAACTGTATGTATGCTTTTAGACCGCGTTCATATGTAGTGTTCTCCATGCTTAT

CCTTCCCTACA-

B

Primer	Type	Sequence *
Knowlesi-F3	F3	5′-GGGTGTACACGAACAAGTGT-3′
Knowlesi-B3	B3c	5′-TATCGAGCGTGCGAAGGA-3′
Knowlesi-FIP	F1c-F2	5′-GGTACTCCACCTGTGTATACGCACA*GAATTC*CGCCTGCATGTATGTATACCAG-3′
Knowlesi-BIP	B1-B2c	5′-GTGAGTGCAACAATGTGAGGAGGT*GAATTC*CGGAATAAGCATAGGTGGAACC-3′
Knowlesi-FLP	Loop F	5′-GCGCAGACTAGTGCAGGCA-3′
Knowlesi-BLP	Loop B	5′-ACTTCAGTATCCCCCCCATGTGA-3′

*Underlining indicates a restriction enzyme site of *Eco R* I.

FIG. 1. Locations and sequences of LAMP targets and priming sites for the *P. knowlesi* β-tubulin gene. (A) Locations of priming sites of the *Pk*LAMP primer set in the reference sequence (GenBank accession number AY639984) are indicated by gray shading. (B) Primer sets used for amplification of the *P. knowlesi* β-tubulin gene in LAMP.

Since it has been demonstrated that additional loop primers increase the amplification efficiency, loop primers for each target gene were also synthesized. The specific primers for *P. knowlesi* were designed against species-specific β-tubulin gene sequences (GenBank accession number AY639984) (Fig. 1A). For easy confirmation of the amplified sequences, we modified the FIP and BIP by inserting a restriction enzyme (EcoRI) cleavage site between the F1 complementary sequence and F2 and between the B1 complementary sequence and B2, respectively, as shown in Fig. 1B.

***Pk*LAMP procedures.** The *Pk*LAMP reaction was performed as described previously (10, 12, 13). Briefly, the reaction was performed with 25 μl of a mixture containing 1 μl of the extracted DNA template, 40 pmol each of the FIP and BIP, 5 pmol each of the F3 and B3 primers, 20 pmol each of the forward loop primer (FLP) and backward loop primer (BLP), and 1 μl of fluorescent detection reagent (Eiken Chemical Co., Ltd., Tokyo, Japan) with Loopamp DNA amplification kit (Eiken Chemical Co., Ltd., Tokyo, Japan). The *Pk*LAMP reaction was performed as described above with each of the specific primers. In a conventional heat block, the mixture was incubated at 66°C (temperatures of 47 to 72°C were also tested) for 60 min, and the reaction was then terminated by heating the mixture at 80°C for 5 min. For the initial validation study, *Pk*LAMP was confirmed with real-time monitoring of the increase of turbidity using a Loopamp real-time turbidimeter (LA-200; Teramecs, Kyoto, Japan). To confirm the amplified DNA products of each parasite, 1 μg/μl of the product was digested with the EcoRI at 37°C for 1 h. The nontreated and EcoRI-digested LAMP products were subjected to electrophoresis on a 2% agarose gel and then visualized under UV light after staining with ethidium bromide (Sigma). Digested LAMP DNA products were purified after 2% agarose gel electrophoresis and then cloned into a pCRII cloning vector using a TA cloning kit (Invitrogen, Carlsbad, CA). The nucleotide sequences of inserts were determined using a Big Dye Terminator kit (Applied Biosystems Japan, Ltd.) with an automated DNA sequencer (ABI Prism 3100 genetic analyzer; Applied Biosystems Japan, Ltd.). The Genetyx 7 package (Software Development Co., Ltd., Tokyo, Japan) was used to align the determined sequences. For the challenge infections, the amplified products in the reaction tube were directly detected with the naked eye using Loopamp fluorescent detection reagent (Eiken Chemical Co., Ltd.) according to the manufacturer's instructions.

Specificity of *Pk*LAMP primers. Specificity of the *Pk*LAMP primers was tested using genomic DNAs (gDNAs) of various *Plasmodium* species in a gel electrophoresis and fluorescent analysis. The gDNAs of *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale* were kindly provided by Takefumi Tsuboi of Ehime University of Japan. Blood samples infected with *P. inui*, *P. simiovale*, *P. fieldi*, *P. fragile*, *P. hylobati*, and *P. gonderi* were obtained from American Type Culture Collection (ATCC), and gDNAs of these parasites were extracted from frozen infected blood with a QIAamp DNA blood mini kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. *P. coatneyi*- and *P. cynomolgi*-infected blood samples were obtained from experimentally infected monkeys and were subjected to DNA extraction with the QIAamp DNA blood mini kit. These purified DNA samples were used as templates for the subsequent *Pk*LAMP and single-PCR assays. As a negative control, DNA extracted from normal monkey blood was prepared as described above.

Sensitivity tests for *Pk*LAMP and single PCR. For sensitivity testing, the *Pk*LAMP reaction was tested using 10-fold serial dilutions of plasmid DNA containing the target sequence by cloning from *P. knowlesi* H strain genomic DNA and compared against results of the single-PCR assay using F3 and B3 primers. PCR amplification was performed in 25 μl of a mixture containing 1 μl of the extracted DNA template, 50 pmol of each primer, 200 μM each deoxynucleoside triphosphate (dNTP), and 1.25 U of *Taq* Gold DNA polymerase (Applied Biosystems, Foster City, CA) in a PCR buffer (Applied Biosystems). The reaction was performed for 35 cycles under the following conditions: 10 min at 95°C to activate the *Taq* Gold DNA polymerase, 1 min of denaturation at 94°C, 1 min of annealing at 60°C, 1 min of extension at 72°C, and 10 min of final extension at 72°C in a Gene Amp PCR system 9700 (Applied Biosystems). The PCR products were subjected to agarose gel electrophoresis and then visualized as described above.

Evaluation of *Pk*LAMP using blood samples from infected monkeys. *Pk*LAMP was evaluated for fluorescence detection of *P. knowlesi* target DNA using blood samples obtained from experimentally *P. knowlesi*-infected monkeys. Two monkeys, J58 (male) and J64 (male), which were 3-year-old Japanese macaques (*Macaca fuscata*) weighing 4.2 kg and 4.7 kg, respectively, were used in this experiment. Both monkeys were second-generation offspring bred in captivity. The investigators adhered to the Guidelines for the Use of Experimental Animals authorized by the Japanese Association for Laboratory Animal Science. Monkey J58 was inoculated intravenously with 1×10^8 fresh *P. knowlesi* H strain (ATCC 30158) parasitized red blood cells (PRBCs) obtained from another infected Japanese macaque. Monkey J64 was inoculated intravenously with frozen *P. knowlesi* Hackeri strain (ATCC 30153)-infected blood obtained from the ATCC. After infection, Giemsa-stained thin blood films were prepared daily from peripheral blood obtained by ear prick, and parasitemia in the infected monkeys was monitored by microscopic examination. Heparinized blood samples for *Pk*LAMP assay were obtained daily from the infected monkeys during the course of infection. The infected blood samples were subjected to DNA extraction with a QIAamp DNA blood mini kit (Qiagen) as described above. The DNA extracts and whole blood samples were frozen at −80°C until use.

Comparison of *Pk*LAMP and nested PCR using DNA extracts and whole blood as template. We compared the sensitivities of *Pk*LAMP and conventional nested PCR (nPCR) assays using DNA extract of *P. knowlesi* and whole blood obtained from two infected monkeys during the course of infection. The nPCR assay, based on the *Plasmodium* DNA sequence of the small-subunit (SSU) rRNA gene, was performed according to a standard protocol as described previously (15). The nest 1 reaction was carried out in a 50-µl reaction mixture containing 2× PCR master mix (Ampli*Taq* Gold PCR master mix; Applied Biosystems), 250 nM each primer (rPLU1 and rPLU5) (15), and 2 µl of DNA template. The reaction mixture for nest 1 PCR amplification was placed in a thermal cycler (TP600; Takara Bio Inc., Shiga, Japan) at 95°C for 5 min for initial denaturation. This was followed by 40 cycles of 94°C for 30 s, 55°C for 60 s, and 72°C for 120 s for amplification and then 72°C for 10 min for final extension. Nest 2 PCR amplification was performed in a 20-µl reaction mixture containing 2× PCR master mix (Applied Biosystems), 250 nM each primer (Pmk8 and Pmkr9) (16), and 2 µl of the nest 1 PCR products used as DNA templates. the reaction mixture for nest 2 PCR amplification was placed in a thermal cycler (TP600) at 95°C for 5 min for initial denaturation. This was followed by 40 cycles of 94°C for 30 s, 60°C for 60 s, and 72°C for 60 s for amplification and then 72°C for 10 min for final extension. Nest 2 PCR products were electrophoresed separately on a 2% agarose gel and illuminated with UV light.

RESULTS

Specificity of *Pk*LAMP primers. The specificity of the *Pk*LAMP primers was investigated by using various *Plasmodium* gDNAs as templates for *Pk*LAMP. As shown in Fig. 2A, a typical ladder pattern was detected in *P. knowlesi* DNA (lane 1) but not in the DNAs of other *Plasmodium* species. Moreover, fluorescent detection was also specifically obtained in the reaction tube including gDNA of *P. knowlesi*, as shown in Fig. 2B. The sizes of the *Pk*LAMP fragments digested by EcoRI were identical to the predicted sizes for the parasite (data not shown). To evaluate the accuracy and robustness of the LAMP method, the *Pk*LAMP reaction was carried out in a water bath at 47 to 72°C separately. Positive ladder patterns were observed at 48 to 71°C and strongly at 56 to 70°C. These findings demonstrated that a set of species-specific primers was highly specific for the detection of the corresponding parasite in *Pk*LAMP. To confirm the nucleotide sequences of the LAMP products, the amplified and digested DNA products were purified from the positive controls and cloned into a vector. The determined sequences of the DNA fragments were completely identical to the reported ones (data not shown) (*P. knowlesi*, accession no. AY639984).

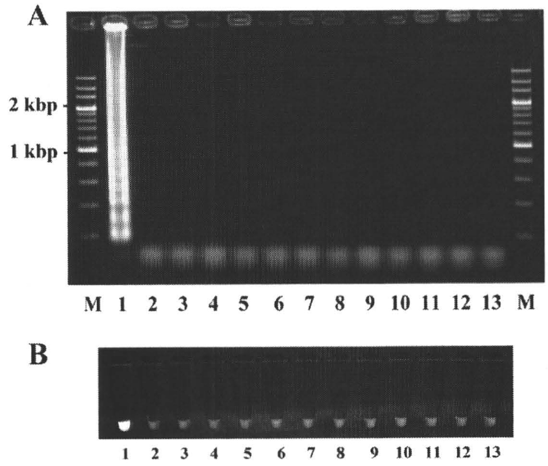

FIG. 2. Specificity of *Pk*LAMP for *P. knowlesi*. (A) Agarose gel electrophoresis of LAMP products from genomic DNAs of 13 *Plasmodium* spp. and ethidium bromide staining. (B) Visual detection of LAMP products under UV light using the Loopamp fluorescent detection reagent. Lanes M, 200-bp ladder size markers; lanes 1, *P. knowlesi*; lanes 2, *P. falciparum*; lanes 3, *P. malariae*; lanes 4, *P. vivax*; lanes 5, *P. ovale*; lanes 6, *P. coatneyi*; lanes 7, *P. cynomolgi*; lanes 8, *P. inui*; lanes 9, *P. simiovale*; lanes 10, *P. fieldi*; lanes 11, *P. fragile*; lanes 12, *P. gonderi*; lanes 13, *P. hylobati.*

Sensitivity of *Pk*LAMP reaction. To examine the sensitivity of *Pk*LAMP, three *Pk*LAMP detection methods were compared with conventional single PCR using two outer primers, F3 and B3, for the detection of *P. knowlesi* β-tubulin gene. As shown in Fig. 3A, amplification by real-time *Pk*LAMP was obtained in reaction tubes containing from 10^8 to 10^2 copies/µl of the DNA template in a 60-min reaction with a turbidity assay. On gel electrophoresis analysis, the amplified products also showed ladder-like patterns from 10^8 to 10^2 copies/µl (Fig. 3B). The amplified products in these positive reaction tubes were also visually detectable using the Loopamp fluorescent detection reagent, as shown in Fig. 3C. In contrast, the limit of detection for PCR using the F3 and B3 primers was 10^8 to 10^4 copies/µl (Fig. 3D). Therefore, it appeared that the sensitivity of the *Pk*LAMP, regardless of the detection method, was 100-fold higher than that of the single-PCR assay.

Evaluation of *Pk*LAMP and nPCR using DNA extracts and whole blood samples as templates. The course of infection of *Macaca* monkeys experimentally infected with *P. knowlesi* was monitored by *Pk*LAMP and nPCR for detecting parasite DNA (Table 1). Both monkeys infected with *P. knowlesi* developed a fulminating acute infection, and they finally became lethargic and severely withdrawn just before autopsy. In monkey J58 inoculated with fresh PRBCs of *P. knowlesi* strain H, the parasites in the peripheral blood were first detected by microscopy on day 1; parasite densities then increased to around 10% within 3 days after infection. *P. knowlesi* DNA could be detected by *Pk*LAMP as well as nPCR assay on all days during the course of infection (Table 1). In monkey J64 inoculated with frozen PRBCs of *P. knowlesi* strain Hackeri, the parasites were first detected by microscopy on day 6; parasite densities then increased sharply to around 58% within 9 days after