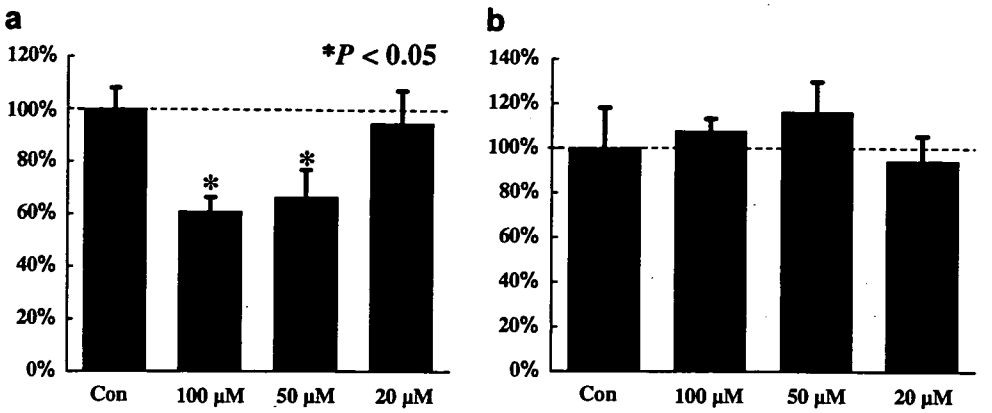

Fig. 2. Effects of E64d (a) and ALLN (b) on the erythrocyte invasion of *B. bovis*. Relative values are expressed as the percentage of the parasitemia in the culture with E64d or ALLN to that in the DMSO medium control (Con: 100%) in an *in vitro* invasion test. Each value represents the mean ± SD in three wells for each concentration of chemicals in three separate experiments. The asterisks indicate significant differences ($^*P < 0.05$) on the values between the chemical- and DMSO control-treated cultures analyzed using an independent Student's *t*-test.

As a result, the erythrocyte invasion activity of the parasite was significantly inhibited in the presence of 100 and 50 μM E64d ($P = 0.010$ and 0.045, respectively) (Fig. 2a). In contrast, none of the concentrations (100, 50, and 20 μM) of ALLN showed any significant inhibitory effects relative to the value of the control (Fig. 2b). Although the other two inhibitors (E64 and leupeptin) were also tested on the invasion and following replication tests, no inhibitory effects on these activities were noted (data not shown). This finding suggests the presence of an anonymous but functional babesial cysteine protease(s) that appears to be essential for the parasite's invasion to host RBCs. In contrast, the lack of effect of ALLN might be explained by the lack of activity of this inhibitor over the particular cysteine protease(s). In *P. falciparum*, it is known that a parasite's cysteine protease, falcipain 1, plays a specific role in host cell invasion and is also inhibited by E64d (Greenbaum et al., 2002; Dahl and Rosenthal, 2005). Our data will be useful for elucidating the molecular mechanism of the erythrocyte invasion of *Babesia* parasites in future.

Finally, the effects of E64d and ALLN on the intraerythrocytic replication of *B. bovis* were also evaluated using the high-voltage pulsing method described previously (Okubo et al., 2006b). After high-voltage pulsing, free parasites were incubated with normal RBCs at 37 °C. When the parasites were incubated for 30 min, almost all parasites invaded the RBCs and were observed to form rings on the stained smears (data not shown, Okubo et al., 2006b). At that time, the infected RBCs were washed with the GIT medium and then incubated with the indicated concentrations (20, 50, and 100 μM) of E64d or ALLN or with the same final concentration of DMSO for the control, at 37 °C for 5 h. After the incubation, Giemsa-stained smears were prepared, and the replication activity was calculated as the ratio of the number of divided parasite-containing RBCs to the entire population of infected RBCs (100%), among which more than 200 of the infected RBCs were monitored. The divided parasite-containing RBCs are infected RBCs with more than two replicated parasites before their egression (data not shown).

As shown in Fig. 3, supplements with 100 and 50 μM E64d had stronger inhibitory effects on the parasite's replication than the control culture ($P = 0.0019$ and 0.018, respectively) (Fig. 3a). Moreover, 100, 50, and 20 μM

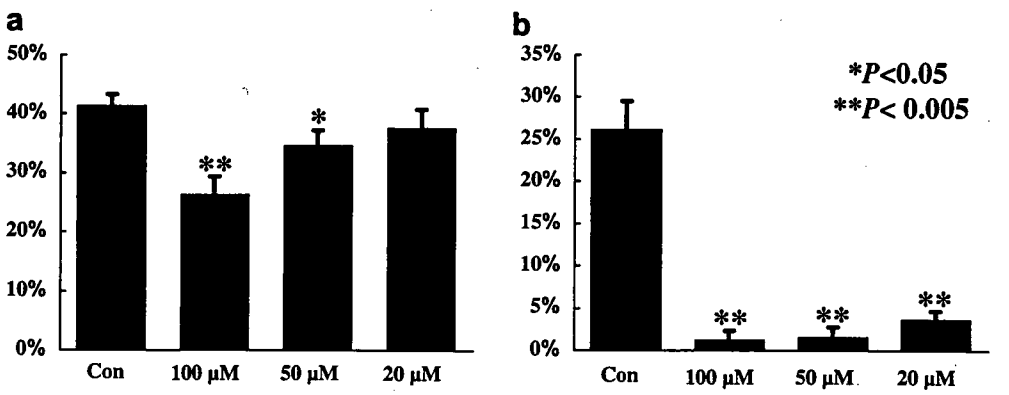

Fig. 3. Effects of E64d (a) and ALLN (b) on the intraerythrocytic replication of *B. bovis*. Relative values are expressed as the rates of divided parasite-containing RBCs to all infected RBCs. Each value represents the mean ± SD in three wells for each concentration of chemicals in three separate experiments. The asterisks indicate significant differences ($^*P < 0.05$, $^{**}P < 0.005$) on the values between the chemical- and DMSO control-treated cultures analyzed using an independent Student's *t*-test.

ALLN had more significant inhibitory effects on the replication activity ($P = 0.00026$, 0.00029, and 0.00038, respectively) (Fig. 3b) than E64d. In *P. falciparum*, the parasite's cysteine proteases have been reported to play a vital role in host hemoglobin degradation (Sijwali and Rosenthal, 2004). Both E64d and ALLN were reported to block the intracellular processing of two parasite's cysteine proteases, falcipain-2 and -3 (Dahl and Rosenthal, 2005), which function to degrade the host hemoglobin in the intracellular stage (Sijwali et al., 2001; Shenai et al., 2000). Accordingly, it is also possible that the anonymous babesial cysteine protease could digest the host hemoglobin for their metabolism even if the phenomenon has not been observed in *Babesia* parasites.

Consequently, our data strongly suggested the presence of cysteine protease(s) derived from *B. bovis*, in which the protease(s) would play important roles in the erythrocyte invasion and/or replication processes of *B. bovis*. However, since there is no report about the identification of *B. bovis* cysteine protease(s) related to their asexual growth or even the homologous proteins, the exact role of babesial cysteine protease is still not clear. Progress of the genome project is expected to lead to the elucidation of the *B. bovis* cysteine protease. Further research will be necessary to elucidate the molecular mechanism of the asexual growth cycle of *Babesia* parasites as well as for developing novel anti-babesial drugs in the future.

Acknowledgments

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Cyclin-dependent kinase inhibitors block erythrocyte invasion and intraerythrocytic development of *Babesia bovis in vitro*

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SUMMARY

Cyclin-dependent kinases (CDKs) are essential for the regulation of the eukaryotic cell cycle. A number of chemicals, which selectively inhibit the CDK activities, have been synthesized for the development of anti-cancer drugs. This report describes the inhibitory effect of purine derivatives known to be CDK inhibitors on the asexual growth of *Babesia bovis*. The 4 compounds, roscovitine, purvalanol A, CGP74514A, and CDK2 Inhibitor II, showed significantly suppressive effects on the *in vitro* growth *of B. bovis*. Three (roscovitine, purvalanol A, and CDK2 Inhibitor II) showed an inhibitory effect on the early stages of intraerythrocytic development of *B. bovis*. CGP74514A (CDK1-specific inhibitor) blocked the erythrocyte invasion by merozoites. Our data suggest the chemotherapeutic potential of the CDK inhibitors for babesiosis, and the target molecules of the compounds would participate in the process of successful erythrocyte invasion or intraerythrocytic development of *B. bovis*.

Key words: *Babesia bovis*, cyclin-dependent kinase, asexual growth.

INTRODUCTION

Bovine babesiosis is a well-recognized, tick-borne protozoan disease of cattle in most tropical and subtropical as well as some temperate regions of the world, often resulting in great economic losses (Kuttler, 1988; Bock *et al.* 2004). *Babesia bovis*, one of the major causative agents of bovine babesiosis, induces severe clinical symptoms, such as fever, anaemia, haemoglobinuria, and hypotensive shock syndrome, with the occasional death (Bock *et al.* 2004; Vial and Gorenflot, 2006). *Babesia* sporozoites directly invade host erythrocytes after the animal has been bitten by an infected tick. The intraerythrocytic parasites multiply by forming 2–4 merozoites to establish a seemingly perpetual cycle of asexual reproduction (Vial and Gorenflot, 2006). The pathogenicity of *B. bovis* in the host animals is related to the erythrocytic stage of the parasite (Wright and Goodger, 1988; Bock *et al.* 2004). A better understanding of the biological processes involved in the growth cycle at the erythrocytic stage is required in order to develop effective therapeutic tools against bovine babesiosis.

Cell cycle progression is essential for the survival of any organism, and cyclin-dependent kinases (CDKs), in particular, are responsible for the control of cell cycle progression in cooperation with other regulatory molecules, called cyclins (Morgan, 1995). The CDK/cyclin activity is frequently up-regulated in human cancer cells, which has stimulated the interest in the chemical inhibition of these enzymes (Senderowicz and Sausville, 2000). After purine-derivative compounds were discovered to be inhibitors of CDK1, a large variety of chemical compounds have been extensively investigated as CDK inhibitors (Chang *et al.* 1999; Knockaert *et al.* 2002). Several CDK inhibitors suppress tumor growth although the specificity for CDK activities of these inhibitors in treated cells is still controversial (Knockaert *et al.* 2002; Becker *et al.* 2004).

The role of CDKs in protozoan parasites remains unclear. In *Plasmodium falciparum*, a protozoan parasite that belongs to the same phylum Apicomplexa as *Babesia* spp., the asexual growth was significantly suppressed by the addition of chemical inhibitors for the CDK/cyclin activity into the *in vitro* culture (Harmse *et al.* 2001). This report suggested that several CDK inhibitors have chemotherapeutic potential for malaria. The homologous genes coding for CDKs in *P. falciparum* have been identified. Experiments using recombinant enzyme systems showed that CDK inhibitors significantly inhibited the enzymatic activities of these CDK homologues (Geyer *et al.* 2005). These facts suggest that CDKs play an essential role in the asexual growth of *P. falciparum*. However, the

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effective doses of the CDK inhibitors against the parasite's growth were different from those obtained in an *in vitro* assay using purified higher eukaryotic CDK/cyclin complex (Harmse *et al.* 2001). Further, it has been reported that purvalanol B, one of the representative CDK inhibitors, interacts with casein kinase rather than CDKs as an intracellular target in protozoan parasites such as *Plasmodium*, *Toxoplasma*, *Trypanosoma* and *Leishmania* (Knockaert *et al.* 2000). These reports suggest that target molecules and biological mechanisms of CDK inhibitors in protozoan parasites might differ from those observed in higher eukaryotic cells. These differences are encouraging with regard to the identification of a parasite-specific inhibitor.

In the case of *B. bovis*, the effect of CDK inhibitors on the parasite's growth is unknown. Recently, an EST sequence database of *B. bovis* has been published (de Vries *et al.* 2006). In this database, the 3 kinds of CDK gene homologues (annotated as cdc2 (CDK1)-like kinase or cyclin-dependent serine/threonine protein kinase) and a cyclin gene homologue (annotated as cyclin-putative) can be observed. Therefore, the CDKs potentially act as key molecular switches for the cell cycle progression of *B. bovis*. In this study, we evaluated the inhibitory effects of selected chemical compounds, referred to as CDK inhibitors, on the asexual growth of *B. bovis*.

MATERIALS AND METHODS

Chemical compounds

Five purine derivatives, olomoucine, roscovitine, purvalanol A, CGP74514A, and CDK2 Inhibitor II, were purchased from Calbiochem-Novabiochem Co. (Darmstadt, Germany). They have high permeability to cells and selective inhibitory activities against purified eukaryotic CDKs through the competitive binding to the ATP-binding pocket of CDKs (Vesely *et al.* 1994; Meijer *et al.* 1997; Gray *et al.* 1998; Davis *et al.* 2001; Dai *et al.* 2002). Spectra and 50% inhibitory concentration (IC_{50}) against the purified CDKs of each compound, as evaluated in previous research, are summarized in Table 1.

Table 1. Compounds used in this study

Chemicals	Spectra[a]	IC_{50} (μM)[a]
Olomoucine	CDK1/Cyclin B	7
	CDK2/Cyclin A	7
	CDK2/Cyclin E	7
	CDK5/p35	3
Roscovitine	CDK1/Cyclin B	0·65
	CDK2/Cyclin A	0·7
	CDK2/Cyclin E	0·7
	CDK5/p35	0·2
Purvalanol A	CDK1/Cyclin B	0·004
	CDK2/Cyclin A	0·07
	CDK2/Cyclin E	0·035
	CDK5/p35	0·075
CGP74514A	CDK1/Cyclin B	0·025
CDK Inhibitor II	CDK2/Cyclin A	0·06
	CDK2/Cyclin E	0·06

[a] These data are available in a CALBIOCHEM® online catalogue (www.calbiochem.com).

In vitro *growth inhibition assay*

The Texas strain of *B. bovis* was maintained in purified bovine erythrocytes (RBC) with a serum-free GIT medium (Wako Pure Chemical Industrial, Ltd, Osaka, Japan) as described previously (Bork *et al.* 2005). The *in vitro* growth inhibition assay followed the method previously described by Bork *et al.* (Bork *et al.* 2003, 2004) with some modifications. Parasitized RBC were mixed with uninfected RBC to obtain RBC packs with 1% parasitaemia, and GIT medium containing an appropriate concentration of each compound was added into each of 3 wells. Dimethyl sulfoxide (DMSO), which was used as a solvent for each drug, was added at the same final concentration into control wells. All cultures were incubated at 37 °C in a humidified multi-gas incubator. Every 24 h for a period of 3 days, the medium was replaced with a fresh medium containing the appropriate compound. Thin blood smears were periodically prepared, stained with a Giemsa's solution, and then the number of infected RBC was counted out of a total of 1000 RBC for the calculation of parasitaemia. Counting RBC was performed without any information about the samples to eliminate any bias. The IC_{50} values of each compound were determined by interpolation after curve-fitting using a Prism® program (GraphPad Software, Inc., San Diego, CA, USA) based on the parasitaemia observed at 72 h after the treatment.

RBC invasion assay

The RBC invasion assay was performed according to the procedure described previously (Franssen *et al.* 2003; Okubo *et al.* 2006). PBS (pH 8·0) containing 1 mM $CaCl_2$ (PBS/Ca^{2+}) was selected as a culture maintenance buffer instead of GIT medium to reduce the influence of medium components on the drug activity. After *B. bovis*-infected RBC were suspended in an equal volume of a PBS/Ca^{2+}, the mixture of 400 μl was subjected to 5 intermittent (10 sec, 0 °C) high-voltage pulses (1·5 kV, 400 Ω, 25 μF) in a Gene Pulser II (Bio-Rad Laboratories, Hercules, CA, USA) with a 0·2 cm pulser cuvette (Bio-Rad Laboratories) to purify the intraerythrocytic parasites from infected RBC artificially. After washing with the PBS/Ca^{2+}, the parasites were distributed into a 10% RBC suspension in PBS/Ca^{2+} with an appropriate concentration of each compound. After incubation at 37 °C for 1 h, the

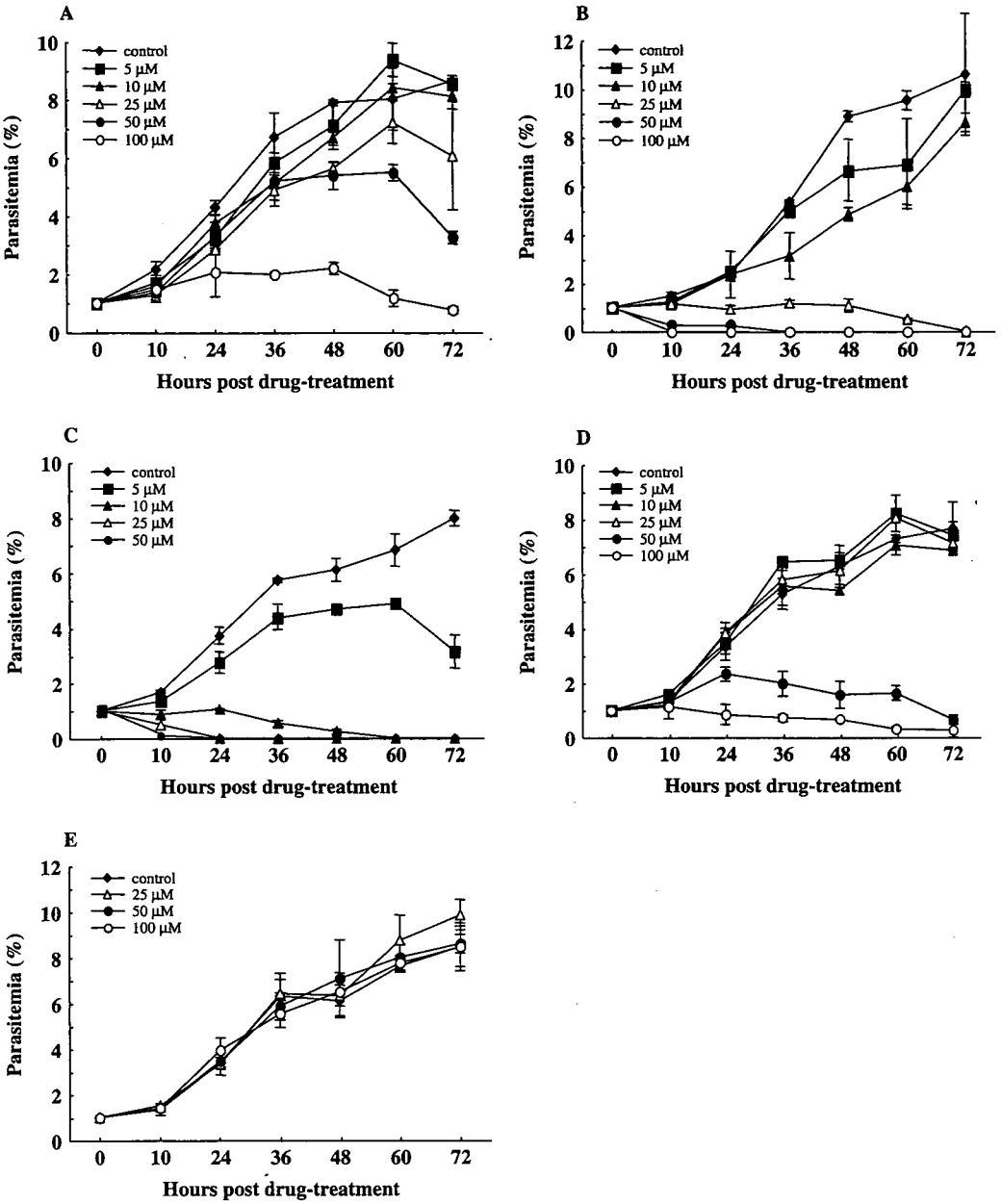

Fig. 1. *In vitro* growth curve of *Babesia bovis* in the presence of different concentrations of roscovitine (A), purvalanol A (B), CGP74514A (C), CDK2 Inhibitor II (D) and olomoucine (E) based on the dynamics of parasitaemia. Each value represents the mean ± S.D. in triplicate. These curves represent the results of 1 representative experiment out of similar experiments repeated twice.

number of infected RBC was counted out of a total of 1000 RBC for calculation of parasitaemia. Percentage inhibition of RBC invasion by treatment with each of the compounds was determined by comparison of parasitaemia observed in the treated culture with that in the control culture. The ratio of extraerythrocytic merozoites to total number of merozoites was also calculated to determine invasion efficiency more precisely.

Parasite-replication assay

An inhibitory effect on the intraerythrocytic development of *B. bovis* was examined by a parasite-replication assay. Briefly, purified parasites were prepared from the infected RBC by high-voltage pulsing, as described above, and incubated with uninfected RBC. After incubation for 1 h to allow the parasites to invade the RBC, each compound was added into the culture. After an additional 5-h incubation, Giemsa-stained smears were made. The ratio of RBC containing degenerated parasites to the total number of infected RBC was calculated to evaluate intracellular killing effect by the compounds. The ratio of divided parasites to the total number of live parasites was also calculated to ascertain the effect of each drug on intraerythrocytic replication.

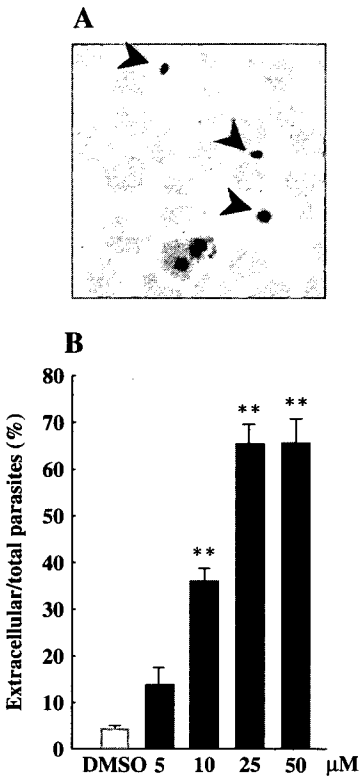

Fig. 2. Abnormal accumulation of extraerythrocytic merozoites (arrowheads) observed at 10 h post-treatment with 10–50 μM CGP74514A (A). The percentages of extraerythrocytic to the total number of merozoites in the cultures treated with CGP74514A (B). Each value represents the mean ± S.D. in triplicate.

RESULTS

Inhibitory effect of each compound on the in vitro *growth of* B. bovis

Treatment with roscovitine, purvalanol A, CGP74514A, and CDK2 Inhibitor II showed a reduction of the *in vitro* growth of *B. bovis* (Fig. 1A–D) with IC_{50} values (at 72 h) of 45·1, 11·0, 4·9 and 42·7 μM, respectively, whereas olomoucine did not show a clear reduction even at a 100 μM concentration (Fig. 1E). In the CGP74514A-treated culture only, an abnormal accumulation of extraerythrocytic merozoites was prominently observed at 10 h after addition of the compound (Fig. 2A). The percentage of extraerythrocytic merozoites in the total number of observed parasites reached about 70% when cultures were treated with a high concentration (25 or 50 μM) of the compound (Fig. 2B). The accumulation of extraerythrocytic merozoites suggested that this compound was affecting RBC invasion.

To confirm that the effects of the compounds were not due to their action on RBC, we performed culture experiments using RBC that had been pre-treated with the compounds. Briefly, RBC were incubated with a high concentration of each compound (100 μM for roscovitine and CDK2 Inhibitor II, 50 μM for purvalanol A and CGP74514A) at 37 °C for 3 h, washed with a normal GIT medium 3 times, and then used for standard parasite cultivation. It was found that there was normal growth of *B. bovis* in all cultures with pre-treated RBC, similar to the control (data not shown), indicating that these compounds directly act on the parasites, but not on the host RBC.

Inhibition of RBC invasion or intraerythrocytic development of B. bovis *by each compound*

To determine which step during the asexual growth cycle of *B. bovis* was influenced by the treatment with these compounds, we first performed an RBC invasion assay. In the control culture, 52·0 ± 6·8% (mean ± standard deviation, S.D.) of merozoites invaded RBC, reaching 1·8–2·8% absolute parasitaemia after incubation for 1 h (Fig. 3A). Treatment with 25 μM CGP74514A showed 52·9 ± 9·3% inhibition of RBC invasion compared to that of control ($P < 0·05$). A higher inhibitory effect (75·4 ± 5·4% compared to control, $P < 0·01$) was observed in the 50 μM CGP74514A-treated culture (Fig. 3A). Both concentrations of CGP74514A also induced a significant increase in the ratio of extraerythrocytic merozoites to total merozoites (Fig. 3B). Treatment with 10 μM CGP74514A showed 30·9 ± 2·7% inhibition which was not significant (Fig. 3A). A 15·5 ± 1·5% or 25·6 ± 8·6% (not significant) decrease of parasitized RBC was observed in the cultures treated with 100 μM roscovitine or 50 μM purvalanol A, respectively (Fig. 3A). However, a decreased number of parasitized RBC in these cultures was possibly due to a direct killing effect by these compounds because the number of extraerythrocytic merozoites was not increased (Fig. 3B). In contrast, invasion efficiency observed in cultures treated with CDK2 Inhibitor II was similar to that of control (Fig. 3A and B). These results could support the hypothesis that CGP74514A, especially in high concentration, affected RBC invasion by merozoites.

In general, *B. bovis* forms a ring-shape after the parasite's invasion into host RBC (Fig. 3C), which is then followed by an intraerythrocytic replication as it divides. However, the parasites treated with roscovitine, purvalanol A, and CDK2 Inhibitor II hardly ever formed the typical ring-shape within the infected RBC, but they did show the parasite's degeneration into a 'dot-shape' parasite (Fig. 3D). These results suggested that these compounds might affect the subsequent step of RBC invasion of *B. bovis*. Therefore, an inhibitory effect on the intraerythrocytic development of *B. bovis* was examined by a parasite-replication assay. The ratio of extraerythrocytic merozoites to total merozoites at 5 h after drug-treatment was the same as that immediately before drug-treatment. It indicated that mature merozoites had completed RBC invasion

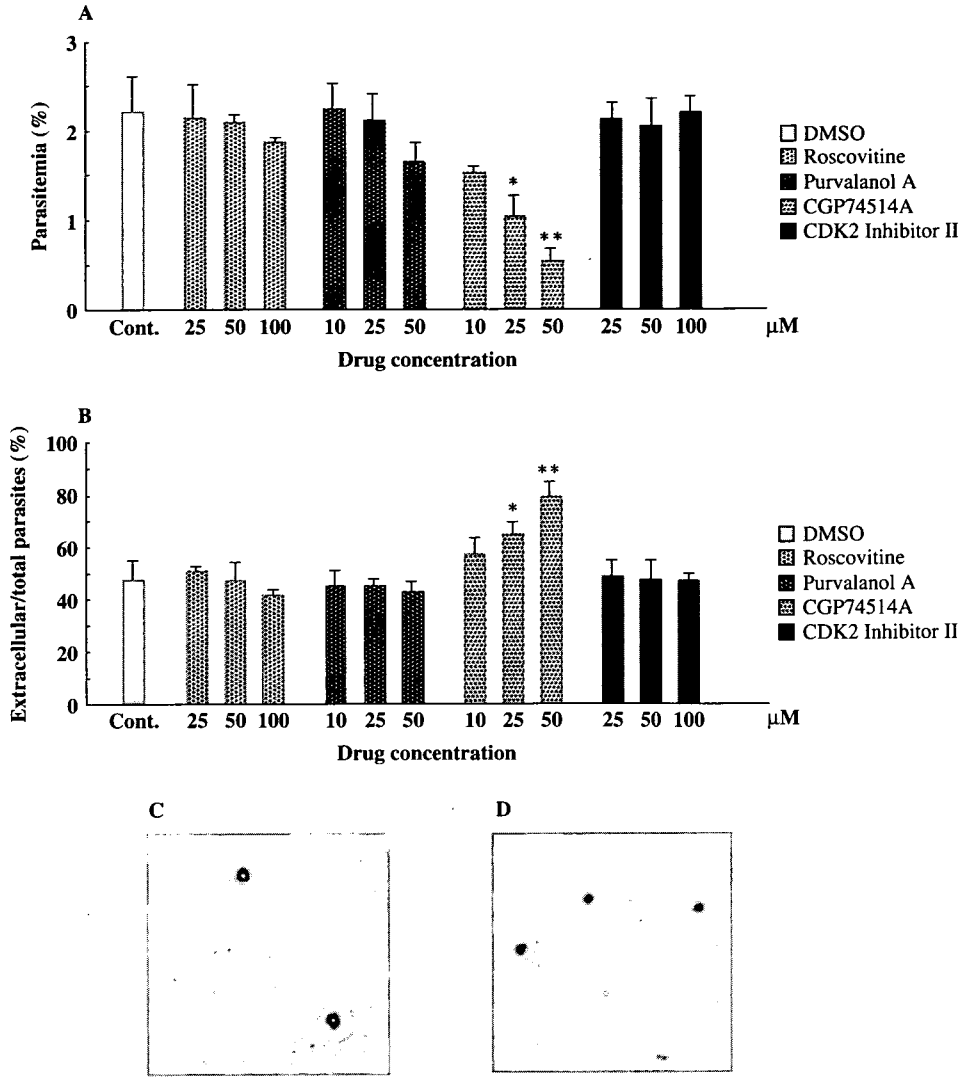

Fig. 3. Effects of the inhibitory compounds on the invasion of RBC by *Babesia bovis* (A). The percentages of extraerythrocytic to the total number of merozoites in the cultures treated with each of the compounds (B). Asterisks indicate significant differences (Student's *t*-test; * $P<0{\cdot}05$, ** $P<0{\cdot}01$) between the drug-treated and control cultures. Each value represents the mean ± S.D. in triplicate. Ring-shaped parasites observed in a control culture immediately after the RBC invasion (C). Dot-shaped parasites observed in a 25 μM Purvalanol A-treated culture (D).

within 1 h pre-incubation of the culture, whereas immature merozoites liberated by a high-voltage pulse had remained outside the RBC. Therefore, an influence on RBC invasion by the treatment with each of the compounds could be ruled out in this assay. In the control culture, degenerated parasites were seen at the ratio of $13{\cdot}5 \pm 0{\cdot}9$% to total parasites, and $23{\cdot}5 \pm 1{\cdot}3$% of total live parasites developed into dividing-forms (Fig. 4A and B). An increased number of degenerated parasites was observed in a dose-dependent manner by treatment with roscovitine, purvalanol A and CDK2 Inhibitor II (Fig. 4A). The appearance of dividing-form parasites was significantly ($P<0{\cdot}05$) decreased in 25 and 50 μM roscovitine-, 25 and 50 μM purvalanol A-, and 100 μM CDK2 Inhibitor II-treated cultures (Fig. 4B). These results suggested that the early stages of intraerythrocytic development of *B. bovis* were affected by treatment with these compounds. A concentration less than 25 μM of CGP74514A did not affect the viability of the intraerythrocytic parasites or the replication capacity (Fig. 4A and B). However, the addition of 50 μM CGP74514A to the culture caused intracellular killing of the parasites (Fig. 4A). A suppression of *in vitro* growth observed in 50 μM CGP74514A-treated culture could be promoted by this killing effect.

DISCUSSION

In this study, 4 different CDK inhibitors, roscovitine, purvalanol A, CGP74514A, and CDK2 Inhibitor II, significantly suppressed the *in vitro* growth of *B. bovis*, suggesting the chemotherapeutic potential of CDK inhibitors for bovine babesiosis. Olomoucine did not show any inhibitory effect on

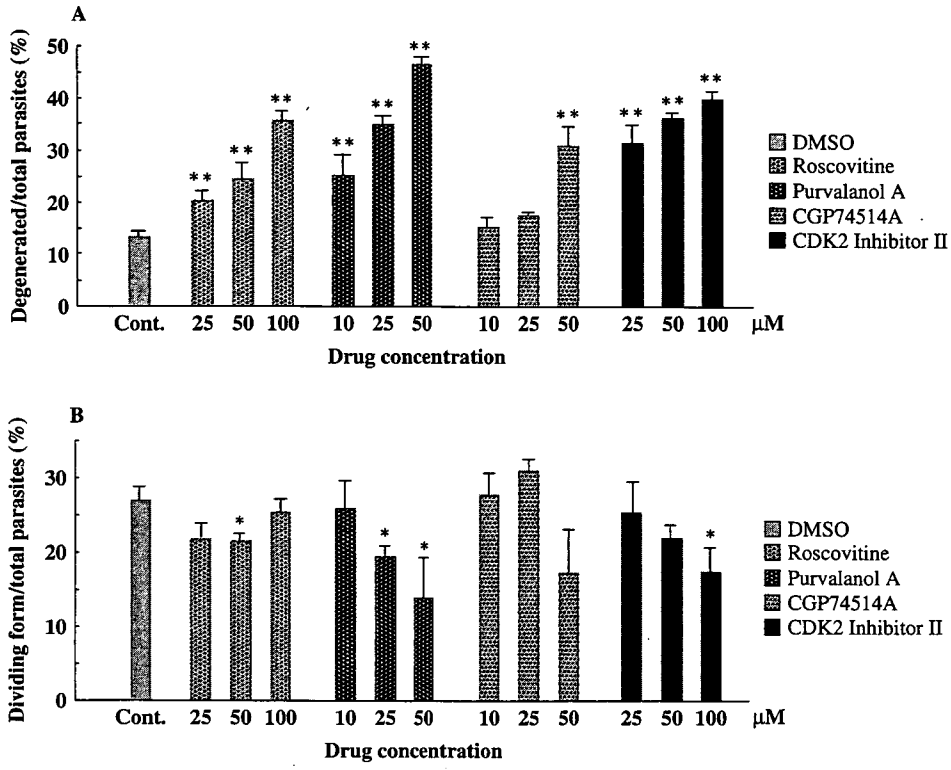

Fig. 4. An increase of degenerated parasites (A), and a decrease of the dividing form parasites (B) by treatment with each of the compounds. Asterisks indicate significant differences (Student's *t*-test; * $P<0{\cdot}05$, ** $P<0{\cdot}01$) between the drug-treated and control cultures. Each value represents the mean ± S.D. in triplicate.

in vitro growth of *B. bovis* in this study. Since this drug shows only moderate inhibitory effect in the *in vitro* kinase inhibition assay (Vesely *et al.* 1994), the concentrations tested in this study were probably too low to have any activity in an *in vitro* culture system.

The IC_{50} values of the inhibitors obtained in this study were considerably higher than the values shown in previous studies. One possible reason for this discrepancy is the low efficiency of transporting the drugs into parasites. The differences in target molecules and/or mode of action of these inhibitors in *B. bovis* from those in higher eukaryotic cells also might explain the higher IC_{50} values. Although the differences between higher eukaryotic CDKs and those of *B. bovis* are not understood, the activity of the inhibitors might be affected by the diversity of CDKs. Further, it has been reported that purvalanol B, an analogue of purvalanol A, interacts with more casein kinase than CDKs as an intracellular target in related protozoa such as *Plasmodium*, *Toxoplasma*, and also *Trypanosoma* and *Leishmania* (Knockaert *et al.* 2000). The compounds used in this study possibly act against completely distinct targets from those reported in higher eukaryotic cells.

Treatment with roscovitine, purvalanol A, and CDK2 Inhibitor II blocked the early stages of intraerythrocytic development of *B. bovis*. These compounds potentially target the molecule(s) involved in intraerythrocytic development of *B. bovis*. Since CDK homologues of *B. bovis* possibly participate in the intraerythrocytic development, they are suspected to be the candidates of intracellular target of these compounds.

Interestingly, CGP74514A was shown to inhibit the RBC invasion of *B. bovis*. Because higher eukaryotic cells need not invade other cells for their replication, the inhibition of the RBC invasion by *B. bovis* is considered to be a very characteristic effect of CGP74514A. Although the reason why CGP74514A showed a different effect than other inhibitors is unclear, CGP74514A could have different targets in *B. bovis*. Inhibition of RBC invasion by treatment with another kind of kinase inhibitor, Staurosporine, has been reported in *B. bovis* as well as *Plasmodium* spp. (Bork *et al.* 2006; Ward *et al.* 1994), indicating that protein phosphorylation necessarily participates in the RBC invasion step of the parasites. CGP74514A may target the kinase family that participates in phosphorylation of the proteins involved in RBC invasion.

In conclusion, our data revealed that CDK inhibitors had growth inhibitory effects on the erythrocytic stage of *B. bovis* by targeting the molecules that participated in the process of successful RBC invasion and intraerythrocytic development. Further studies for elucidation of the target molecules and mode of action of CDK inhibitors in *B. bovis*, as well as verification of the chemotherapeutic effect of the CDK inhibitors in an *in vivo* rodent model of *Babesia* infection are being pursued.

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SHORT COMMUNICATION

Comparative evaluation of the sensitivity of LAMP, PCR and in vitro culture methods for the diagnosis of equine piroplasmosis

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Abstract The sensitivity of LAMP, PCR and in vitro culture methods for the detection of *Theileria equi* and *Babesia caballi* was evaluated using tenfold serially diluted culture parasites. On day 1 post-culture, both *T. equi* and *B. caballi* parasites could only be observed at 1% parasite dilution from the in vitro culture method, whereas LAMP could detect up to 1×10^{-3}% of both *T. equi* and *B. caballi* parasite dilutions, whilst PCR could detect 1×10^{-3}% *T. equi* and 1×10^{-1}% *B. caballi* parasite dilutions. On day 7 post-culture, the detection limit for *T. equi* and *B. caballi* in the in vitro culture increased up to 1×10^{-6}%, whereas LAMP detection limit increased to 1×10^{-10}% for both parasites, whilst the PCR detection limit increased to 1×10^{-10}% and 1×10^{-6}% for *T. equi* and *B. caballi*, respectively. Furthermore, LAMP and PCR amplified the *T. equi* DNA extracted from the organs of an experimentally infected horse. This study further validates LAMP as an alternative molecular diagnostic tool, which can be used in the diagnosis of early infections of equine piroplasmosis and together with PCR can also be used as supplementary methods during post-mortems.

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Introduction

Equine babesiosis is a severe disease of horses caused by intra-cellular haemoprotozoan parasites, *Babesia equi* (recently re-classified as *Theileria equi*, Mehlhorn and Schein 1998) and *Babesia caballi*. The disease is endemic in many tropical and sub-tropical regions and is transmitted by ticks, including the genera of *Boophilus*, *Hyalomma*, *Dermacentor* and *Rhipicephalus* (Roby and Anthony 1963; Stiller et al. 1980; Battsetseg et al. 2002). Moreover, several earlier studies have documented horses carrying mixed infections of *T. equi* and *B. caballi* (Xu et al. 2003; Alhassan et al. 2005). Along with the appropriate clinical signs and pathological findings of babesiosis, the complement fixation (CF) test was accepted as the standard method for the international transactions of horses (Friedhoff 1982). However, the CF test has low sensitivity and yields false positive and false negative results during the early and latent stages of the disease (Tenter and Friedhoff 1986).

Equine piroplasmosis can be diagnosed by a number of different methods including Giemsa-stained blood smears, in vitro culture method, ELISA and PCR (Nicolaiewsky et al. 2001; Xuan et al. 2001; Rampersad et al. 2003). Recently, we developed a loop-mediated isothermal amplification (LAMP) for the diagnosis of equine piroplasma infections that is highly sensitive and specific (Alhassan et al. 2006). This novel gene amplification method has the advantages of simplicity and rapidity whereby it can be conducted at a constant temperature (63°C) for 1 h using a heat block or a water bath (Notomi et al. 2000). In our efforts to validate LAMP as a reliable diagnostic tool for equine piroplasmosis, the current study conducts a comparative evaluation of the sensitivity between LAMP, PCR

and the in vitro culture method for the diagnosis of *T. equi* and *B. caballi* infections.

Materials and methods

The in vitro cultures of the United States Department of Agriculture (USDA) strains of *T. equi* and *B. caballi* (Avarzed et al. 1997; Ikadai et al. 2001) were adjusted to 1% (180 parasites per ml) and then serially diluted tenfold. Each dilution was cultured in 24-well plates (Nunc, Roskilde, Denmark) with 10% equine RBC, 900 µl of a GIT medium (Wako Pure Chemical Industries, Osaka, Japan) for *B. caballi* and 900 µl of an M199 medium (Sigma-Aldrich, Tokyo, Japan) supplemented with 40% horse serum and 1% hypoxanthin (ICN Biomedicals, Aurora, OH) for *T. equi* in a micro-aerophilous stationary phase culture system as described previously (Avarzed et al. 1997). Parasitemia was examined daily for a period of 7 days by microscopic examination of Giemsa-stained blood smears. In addition, 50 µl of blood samples was collected from the cultures on days 1, 3, 5 and 7 for DNA extraction.

The DNA from in vitro cultures was extracted as previously described (Alhassan et al. 2005). Briefly, 50 µl of RBC infected with each *T. equi* and *B. caballi* parasites was washed three times with cold phosphate-buffered saline by centrifuging at 1,000×*g* for 5 min at 4°C and re-suspended in a DNA extraction buffer (0.1 mM Tris–HCl [pH 8.0], 0.1% sodium dodecyl sulfate, 100 mM NaCl and 10 mM EDTA). The mixture was digested with 100 µg/ml proteinase K (Invitrogen, Carlsbad, CA, USA) for 2 h at 55°C. The parasitic DNA was extracted with phenol-chloroform and precipitated with ethanol. The purified DNA pellets were dissolved in 20 µl of double-distilled water (DDW) for subsequent LAMP and PCR reactions. Un-infected horse blood DNA was obtained from the National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan and used as negative control. DNA from *T. equi* experimentally infected horse organs obtained from the Japan Racing Association (JRA), Tochigi, Japan was extracted using a QIAamp DNA blood mini kit (Qiagen Science, MD, USA) according to the manufacturer's instructions.

For PCR amplification, two pairs of oligonucleotide primer sequences were selected to specifically amplify *T. equi* and *B. caballi* targeting the EMA-2 (accession no. AB01372) and Bc48 (accession no. AB01770) genes, respectively (Table 1). PCR was performed in 50 µl of a reaction mixture containing 2 µl of template DNA, 5 µl each of a 10X PCR buffer and a 2 mM dNTP mixture, 0.25 µl of Taq Gold DNA polymerase (Applied Biosystem, Tokyo, Japan), 2 µl each of 10 pmol *B. caballi*-specific and *T. equi*-specific primers and 33.75 µl DDW. The amplification conditions consisted of initial denaturation at 96°C for 5 min, followed by 96°C for 1 min, primer annealing at 58°C for 1 min, extension at 72°C for 1 min for 35 cycles and a final extension at 72°C for 7 min. The *T. equi*-specific and *B. caballi*-specific LAMP primers targeting the EMA1 and Bc48 genes, respectively were used in LAMP reactions as published by Alhassan et al. (2006). Both PCR and LAMP products were electrophoresed in a 1.5% agarose gel and stained with ethidium bromide for visualization.

Table 1 PCR primers used in this study

Primer	Sequence	Size (bp)
EMA-2F	TCACTGTCTATGCTCATGGTG	260
EMA-2R	CGCTTAGTAGAACAAAGCAACG	
Bc48F	CTGAGGAAATTTCGGCTGTG	300
Bc48R	AGAGTGCAACCGAGCAATGC	

Results and discussion

In the in vitro culture method, less than 1% parasite dilution for both *T. equi* and *B. caballi* could only be observed from day 2 post-culture, whilst on day 7 the detection limit increased to 1×10^{-6}% (Table 2). The in vitro culture method detected *T. equi* parasites with higher sensitivity than *B. caballi* parasites. This is probably due to the fact that *T. equi* parasites propagates faster than *B. caballi* (Holman et al. 1993), which also explains the high prevalence and pathogenicity of *T. equi* than that of *B. caballi* in endemic areas (Alhassan et al. 2005, 2006; De Waal 2000; Schein 1988). Although the culture method is a slow procedure, it is one of the most specific methods for the direct detection of *T. equi* and *B. caballi*, especially in sub-clinical and chronic cases (Holman et al. 1997; Zweygarth et al. 2002). LAMP detected both *T. equi* and *B. caballi* DNA up to 1×10^{-3}% parasite dilutions on day 1 post-culture, whilst PCR detected 1×10^{-3}% for *T. equi* and 1×10^{-1}% for *B. caballi* both on day 1 post-culture (Table 2). On day 7, the detection limits for LAMP increased to 1×10^{-10}% for both *T. equi* and *B. caballi*, whilst PCR detection limit on day 7 was 1×10^{-10}% for *T. equi* (data not shown) and 1×10^{-6}% for *B. caballi* (Table 2). PCR is a highly specific and sensitive method, which is widely used for the diagnosis of equine piroplasmosis (Bashiruddin et al. 1999; Nicolaiewsky et al. 2001); however, its use in resource-poor countries is limited by its high costs. The current study has demonstrated the ability of the LAMP method to detect early stage infection of both *T. equi* and *B. caballi* from in vitro cultures with higher

Table 2 Comparative analysis of detection sensitivity for *T. equi* and *B. caballi* by in vitro culture method, PCR and LAMP

Comparative analysis of detection sensitivity for *T. equi* and *B. caballi*

Theleria equi

	Day 1							Day 2							Day 3							Day 4		
	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{0}	10^{-1}	10^{-2}
In vitro[a]	+	−	−	−	−	−	−	+	+	+	−	−	−	−	+	+	+	+	+	−	−	+	+	+
PCR	+	+	+	+	−	−	−	nd	nd	nd	nd	nd	nd	nd	+	+	+	+	+	−	−	nd	nd	nd
LAMP	+	+	+	+	−	−	−	nd	nd	nd	nd	nd	nd	nd	+	+	+	+	+	+	−	nd	nd	nd

Babesia caballi

	Day 1							Day 2							Day 3							Day 4		
	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{0}	10^{-1}	10^{-2}
In vitro[a]	+	−	−	−	−	−	−	+	+	−	−	−	−	−	+	+	+	−	−	−	−	+	+	+
PCR	+	+	−	−	−	−	−	nd	nd	nd	nd	nd	nd	nd	+	+	+	+	+	−	−	nd	nd	nd
LAMP	+	+	+	+	−	−	−	nd	nd	nd	nd	nd	nd	nd	+	+	+	+	+	+	−	nd	nd	nd

Comparative analysis of detection sensitivity for *T. equi* and *B. caballi*

Theleria equi

	Day 4				Day 5							Day 6							Day 7						
	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
In vitro[a]	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
PCR	nd	nd	nd	nd	+	+	+	+	+	+	+	nd	nd	nd	nd	nd	nd	nd	+	+	+	+	+	+	+
LAMP	nd	nd	nd	nd	+	+	+	+	+	+	+	nd	nd	nd	nd	nd	nd	nd	+	+	+	+	+	+	+

Babesia caballi

	Day 4				Day 5							Day 6							Day 7						
	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
In vitro[a]	+	−	−	−	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+
PCR	nd	nd	nd	nd	+	+	+	+	+	+	−	nd	nd	nd	nd	nd	nd	nd	+	+	+	+	+	+	+
LAMP	nd	nd	nd	nd	+	+	+	+	+	+	−	nd	nd	nd	nd	nd	nd	nd	+	+	+	+	+	+	+

+: parasites observed in Giemsa-stained smears or detection of parasite DNA, −: no parasites observed in Giemsa-stained smears/no positive reaction, *nd*: not done.

[a] The Giemsa-stained smears were used for the observation of parasites from the in vitro cultures.

sensitivity than PCR and in vitro culture method. The high sensitivity of LAMP is due to four primers used in the reaction, which target six distinct sequences on the target DNA and can detect DNA at as few as six copies (Notomi et al. 2000).

The possible invasion of equine piroplasma parasites into the parenchyma of organs was not reported yet; however, *T. equi* DNA was amplified by both LAMP and PCR from the liver, spleen, groin lymph node, lung, heart, bone marrow and brain of the experimentally infected horse. This may be due to the engorgement of parasites in the small blood capillaries during the acute phase of the disease. These results suggest that the LAMP and PCR methods can also be useful during post-mortem examinations where *T. equi and B. caballi* are suspected as the causal agents of death. In addition to the in vitro culture and PCR techniques, which are already established diagnostic methods for equine piroplasmic infections; this study further validates LAMP as an alternative molecular diagnostic method because it is cost effective, simple, rapid and highly sensitive.

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ORIGINAL ARTICLE

Comparison of polymerase chain reaction methods for the detection of *Theileria equi* infection using whole blood compared with pre-extracted DNA samples as PCR templates

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Abstract Rapid, efficient, and reproducible procedures for isolating DNA before PCR gene amplification are essential for the diagnosis of piroplasms. In this study, we evaluated the ease and reliability of detecting *Theileria equi* by PCR using pre-extracted DNA samples (by QIAamp DNA Mini Kit and phenol-chloroform methods) compared with blood spotted on FTA cards as PCR templates. Although minimal variations in limit of detection were observed among the methods compared, overall, the use of pre-extracted DNA samples and blood spotted on FTA cards had comparable detection limits. These results indicate that *T. equi* infection can be efficiently detected directly from FTA cards by PCR without the need for pre-extraction of DNA from blood samples.

Keywords Diagnosis · Epidemiology · Piroplasmosis · *Theileria equi* · Whatman FTA cards

Abbreviations

PBS	phosphate-buffered saline
PCR	polymerase chain reaction
RBC	red blood cell
EMA-1	equi merozoite antigen 1
EMA-2	equi merozoite antigen 2

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Introduction

Equine piroplasmosis, caused by *Theileria equi*, is an economically important tick-borne protozoan disease of horses (Schein 1988; Melhorn and Schein 1998). The parasite is distributed worldwide, and its prevalence is directly related to the distribution of ticks capable of transmission (Friedhoff and Soule 1996). *T. equi* parasites are usually shown during the acute phase of infection by Giemsa-stained blood smears (Bose et al. 1995). However, during low parasitaemia, it is extremely difficult to detect parasites in blood smears (Holbrook 1969).

The use of nucleic-acid-amplification techniques for the detection of infectious agents in clinical specimens continues to expand, and these techniques promise to play an increasing role in diagnostic laboratories (Fahle and Fischer 2000). While a great number of reports have been published regarding PCR applications for equine piroplasmosis, less information is available addressing the issue of DNA recovery prior to amplification. This issue is of critical importance, since an optimal DNA extraction procedure would offer a high degree of efficiency for use in routine diagnostic procedures. However,

many of the PCR techniques developed for equine piroplasmosis in recent years are based on complex protocols requiring the use of different target genes and sample preparations, making standardization difficult. Furthermore, these methods have never been compared. Additionally, it is important to consider the challenges associated with establishing laboratories with PCR capabilities, especially in developing countries, where economic resources and sophisticated technology are limited (Hanscheid and Grobusch 2002).

The conventional method for DNA extraction from animal tissues is phenol-chloroform extraction followed by ethanol precipitation (Sambrook et al. 1989). Although the cost of the reagent may be lower than that of commercially available DNA extraction kits, it takes about 3 h to obtain DNA with the phenol-chloroform extraction method. The recent development of simplified sample collection matrices, such as the FTA classic cards, which permit direct PCR amplification, may overcome these problems (Boom et al. 1990; Picozzi et al. 2002). Incorporation of these improved sample collection techniques, together with properly designed PCR-based screening protocols, is required for the direct analysis of field samples.

In the present study, we evaluated the ease and reliability of detecting *T. equi* by PCR with various primer pairs using DNA samples extracted by the standard phenol-chloroform method and QIAamp DNA blood mini kit in comparison to direct PCR amplification using whole blood-spotted FTA discs as the templates.

Materials and methods

In vitro culture parasites

Theileria equi (USDA strain) was grown in horse red blood cells (RBCs) in a continuous microaerophilous stationary-phase (MASP) culture as reported previously (Bork et al. 2003).

Template preparation

The infected RBCs were washed twice in 1× phosphate-buffered saline (PBS), and a thin smear was stained with Wright stain to determine the percentage of parasitaemia. Fifty-μl cell suspensions were used for DNA extraction using the phenol-chloroform method (Alhassan et al. 2005) and the QIAamp DNA blood mini kit (Qiagen Science, Germantown, MD, USA) method. In addition, 50 μl of the cell suspensions were spotted on FTA classic cards (Whatman, UK), dried at room temperature (RT) and subsequently used for PCR template preparation.

To determine the detection limit of the PCR methods, *T. equi*-infected erythrocytes with 1% parasitaemia were subjected to 10-fold serial dilutions using uninfected horse RBCs, and DNA was extracted from each diluted sample and processed for PCR amplification. The approximate number of parasites corresponding to each dilution was determined as described previously (Birkenheuer et al. 2003; Alhassan et al. 2005). Furthermore, the diluted RBC suspensions were spotted onto the FTA cards for DNA amplification. For the FTA cards, 3 mm discs were punched out from the FTA cards using a hole puncher and placed in 1.5 ml microcentrifuge tubes. To avoid contamination, the puncher was cleaned with 70% alcohol after every punch. Each FTA disc was washed 3 times with 200 μl of an FTA purification buffer (Whatman) for 5 min per wash and then twice with 200 μl of a TE buffer (10 mmol/L Tris-HCl (pH 8.0), 0.1 mmol/L EDTA (pH 8.0)) for 5 min and dried at RT for 10 minutes. The washed FTA discs were used directly as the DNA template in PCR amplification.

Evaluation of field samples on FTA cards

To evaluate the utility of FTA cards as a suitable device for blood sampling and recovery of parasite DNA, 20 blood samples spotted on FTA cards were obtained from domestic horses in Amasaman district, Ghana. In addition, 6 blood samples were collected from healthy horses with no history of equine piroplasmosis in Japan, spotted on FTA cards, and used as controls.

Primer design and PCR amplification

The nucleotide sequences of the PCR primers used in this study are shown in Table 1. To ensure the detection of all of *T. equi* strains, PCR primers were designed by the alignments of all registered sequences derived from *T. equi* 18S rRNA, EMA-1, EMA-2, and Be82 genes retrieved from the GenBank using a Mac Vector (Oxford Molecular, Ltd., Oxford, UK). The

Table 1 Primers used for the amplification of *T. equi* DNA

Primer	Sequence (5′–3′)	Size (bp)
EMA-1F	GCATCCATTGCCATTTCGAG	750 bp
EMA-1R	TGCGCCATAGACGGAGAAGC	
EMA-2F	GCTGGCAAGGTTGTCTATACC	670 bp
EMA-2R	CATCTGGCTTGTAGAGGTACC	
Be18S-F	CTCACCAGGTCCAGACAGAG	590 bp
Be18S-R	TTCTGCAGGTTCACCTACGG	
Be82-F	ATGCACCTGTAGACACAAGG	570 bp
Be82-R	ACGTGCCAGAGTTGGCTCAT	

specificity of the designed primers was tested against DNA extracted from *Babesia caballi*-infected and uninfected equine RBC.

Standard PCR

The PCR amplifications were performed under the following conditions: a 25 μl mixture containing 5 μl of the extracted template DNA, 2.5 pmol of each primer, a 0.2 mmol/L dNTP mixture, 2.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Tokyo, Japan), and 2.5 μl of a buffer (10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, and 1.5 mmol/L $MgCl_2$). The mixture was heated for 10 min at 96°C to activate the AmpliTaq Gold DNA polymerase, followed by 35 cycles of denaturation for 1 min at 96°C, annealing for 1 min at 58°C, and extension for 1 min at 72°C, followed by a final extension for 7 min at 72°C. A 10 μl PCR product from each reaction mixture was run on a 1.5% agarose gel (FMC Bioproducts, Rockland, ME, USA) with a 1% Tris borate-EDTA buffer. Gels were stained with ethidium bromide and visualized under UV light.

Direct PCR amplification

The washed FTA discs were used directly as the DNA template in PCR amplification using an ampdirect protocol as described previously (Okamoto et al. 1999; Nishimura et al. 2000) with some modifications. Briefly, a 50 μl reaction mixture contained 10 μl each of 5× ampdirect buffer-B and Amp Addition-2 (Shimadzu Corporation, Tsukuba, Japan), a 2.5 mmol/L dNTP mixture, 2.5 pmol of each primer, and 0.25 μl of Taq polymerase (TaKaRa Corporation, Shiga, Japan) and PCRs were done with the following conditions: 35 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and extension for 1 min at 72°C, followed by a final extension for 7 min at 72°C. Agarose gel analysis was conducted as described above.

Results and discussion

In recent years, several conventional PCR tests have been developed for the diagnosis of piroplasms in equine blood (Bashiruddin et al. 1999; Nicolaiewsky et al. 2001; Rampersad et al. 2003). These tests have been shown to be highly sensitive and specific for the diagnosis of equine piroplasmosis. However, the conventional PCR tests are technically cumbersome for large-scale applications. The objective of the present study was to compare simple methods for DNA preparation requiring minimal sample manipulation that would allow PCR amplification from blood without the need for organic extraction of DNA.

In an attempt to identify appropriate combinations of blood sampling and PCR protocol for the easy diagnosis of *T. equi* infection, we compared different DNA sample preparation procedures and PCR amplification protocols using four target genes as described in the Materials and Methods section. To further determine the efficiency of the DNA extraction and PCR methods, DNA samples extracted from *T. equi*-infected RBCs and blood spotted on FTA cards were used for PCR amplification. The PCR methods specifically amplified the respective DNA fragments from all of the extracted *T. equi* DNAs but did not

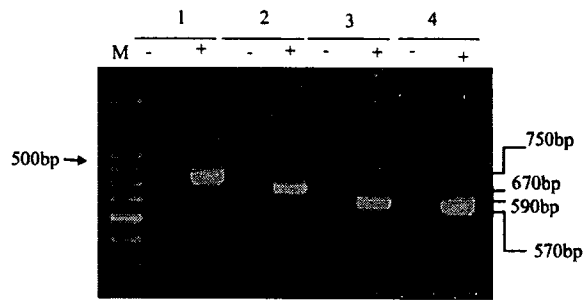

Fig. 1 Standard PCR amplification of *Theileria equi* DNA extracted from infected RBC by the phenol-chloroform method. The specific bands amplified with 18S rRNA, EMA-1, EMA-2 and Be82 primers were detected on a 1.5% agarose gel stained with ethidium bromide and visualized under UV light. Lane M, 100 bp DNA ladder marker; lane 1, 18S rRNA primers; lane 2, EMA-1 primers; lane 3, EMA-2 primer; lane 4, Be82 primers. *B. caballi* DNA (−) and *T. equi* DNA (+)

Table 2 Detection limits of *T. equi* DNA using different target genes and template preparations

% Parasitaemia	PCR assay			
	18S RNA	EMA-1	EMA-2	Be82
1	$+^a +^b +^c$	$+^a +^b +^c$	$+^a +^b +^c$	$+^a +^b +^c$
0.1	+ + +	+ + +	+ + +	+ + +
0.01	+ + +	+ + +	+ + +	+ + +
0.001	+ + +	+ + +	+ + +	+ + +
0.0001	+ + +	+ + +	+ + +	+ + +
0.00001	+ + +	+ + +	+ + +	+ + +
0.000001	− + +	+ + +	+ + +	− + +
0.0000001	− + +	− + +	− + +	− + −
0.00000001	− − −	− − −	− − −	− − −
0.000000001	− − −	− − −	− − −	− − −

[a] DNA extracted by the phenol-chloroform extraction method combined with standard PCR amplification buffer.
[b] DNA extraction by QIAamp DNA mini kit combined with standard PCR amplification buffer.
[c] Direct PCR amplification of FTA disc without DNA extraction with Ampdirect buffer.

produce any amplicons from DNA extracts from *B. caballi*-infected RBCs or uninfected equine whole blood (Fig. 1). The most efficient extraction and PCR method was defined as that which could extract DNA from a sample with the lowest parasite concentration and give a positive reaction for *T. equi* per PCR amplification with the sets of primers described in Table 1. All samples generated signals of the appropriate size. Signals were similar whether the DNA was eluted from FTA cards or extracted by other methods, demonstrating that PCR amplification of *T. equi* was possible from samples preserved on FTA cards in a manner equal to conventional DNA isolation techniques.

FTA® cards are a recent invention for easy sample collection and preservation of DNA (Hsiao et al. 1999) and have been used successfully for whole-blood storage for the diagnosis of malaria and animal trypanosomosis by PCR (Zhong et al. 2001; Picozzi et al. 2002). FTA cards are easy to handle and transport without the need for a cold-chain in comparison with other sampling methods. These cards can be stored at room temperature and are easy to handle and transport for further analysis without the need for cold-chain and biohazard precautions (Dobbs et al. 2002). In this study, the comparison shows that direct PCR amplification with FTA discs was as sensitive as the standard PCR amplification. The whole procedure, including drying and washing to get the DNA template ready for PCR amplification, takes about 30 min. In addition to its sensitivity, the FTA cards method is simple and can be used with large numbers of samples at a time. One possible disadvantage of this method is that, for future PCRs, new punched discs will be needed in contrast to other DNA extraction protocols. However, despite this disadvantage, FTA cards have been used successfully for the recovery of DNA or RNA for the diagnosis of various pathogens such bacteria, parasites and viruses (Boom et al. 1990; Dobbs et al. 2002). As shown in Table 2, the extraction efficiency of the method was apparent. At high concentrations (10^0 to 10^{-5}), there were no significant differences, as revealed by PCR amplification. Blood dilutions between 10^0 and 10^{-5} were clearly positive, with a diminishing signal as the target DNA concentration decreased in the respective PCR reaction (data not shown). Consistent detection of the target molecule per reaction was 10^{-5} with 0.00001% parasitaemia by all the extraction and PCR methods (Table 2). All PCR protocols revealed high specificity (Fig. 1). The

Table 3 Evaluation of direct PCR amplification of *T. equi* DNA on FTA cards from Ghana

Sample	Target genes			
	18S RNA	EMA-1	EMA-2	Be82
1	+	+	+	+
2	−	−	−	−
3	+	+	+	−
4	+	+	+	+
5	−	−	−	−
6	+	+	+	+
7	+	+	+	+
8	−	−	−	−
9	−	−	−	−
10	+	+	+	−
11	−	−	−	−
12	+	+	+	+
13	−	−	−	−
14	+	+	+	+
15	+	+	+	−
16	−	−	−	−
17	+	+	+	−
18	+	+	+	+
19	−	−	−	−
20	+	+	+	+

detection limit of the assay reported in this study compares favourably with other PCR methods described for the detection of *T. equi* (Bashiruddin et al. 1999; Nicolaiewsky et al. 2001; Alhassan et al. 2005). The presence in blood of components that inhibit PCR amplification has been a major problem associated with direct amplification of DNA in cell lysates. However, in this study, we observed an increase in the detection sensitivity when FTA cards in combination with the Ampdirect buffers were used for amplification rather than the standard PCR buffer (Table 2). The active reagents in the ampdirect buffers allow the neutralization of inhibitory substances that bind to DNA polymerase and/or template DNA. The binding of these inhibitors by the reagents, thus, liberates the DNA polymerase and template DNA for amplification (Nishimura et al. 2000).

The choice of target genes and the design of oligonucleotide primers are critical elements in determining the specificity and sensitivity of PCR. Currently, many different PCR targets, including EMA-1, EMA-2, 16S rRNA, and 18S rRNA genes, have been used for the identification of *T. equi* (Rampersad et al. 2003; Alhassan et al. 2005). The ribosomal RNA and merozoite surface antigen genes are well conserved within the genome of *T. equi* strains (Kappmeyer et al. 1993). In contrast, very little is known about the *T. equi* Be82. However, it has been reported to be suitable for the detection of antibodies against *T. equi* infection (Hirata et al. 2002). Primers derived from these genes and different PCR protocols were evaluated using field samples.

In Ghana, haemoparasites, such as *Theileria* and *Trypanosomes* spp., are believed to be widely present (Assoku 1979; Bell-Sakyi et al. 2004; Mahama et al. 2004). However, to date, there is no molecular evidence of equine piroplasmosis in Ghana. This work provides the first molecular report of the presence of *T. equi* in this country. As observed in the present study, 60% of the examined animals were positive for *T. equi* infection by at least one of the primers used. Of these, 4 blood samples were negative using the Be82 primers but positive using 18S rRNA, EMA-1, and EMA-2 primers (Table 3). The reason for this discrepancy is not known. Furthermore, there is not much information available about the Be82 gene, thus antigenic variation can be considered since many of the babesial genes have been suggested to express antigenic variation (Allred 2001).

Therefore, the design of primers from this gene for diagnostic purposes must be undertaken with special care. Owing to the small sample size used in this study, further analysis with more samples would be required to determine the distribution of the disease in Ghana.

In conclusion, our results indicate that *T. equi* can be detected directly from whole blood samples in PCR amplification using FTA cards without DNA extraction. Because blood sampling with FTA cards is simpler to perform, and relatively cost-effective, this method can be potentially beneficial in regions where technology resources are limited for clinical diagnosis and in epidemiological studies of equine piroplasmosis.

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Comparación de métodos de reacción en cadena de la polimerasa para la detección de infección por *Theileria equi* utilinzado sangre entera comparado con muestras de ADN preextraído como plantillas PCR

RESUMEN – Los procedimientos rápidos, eficaces y reproducibles para aislar ADN antes de la amplificación genética de PCR (siglas en inglés de Reacción en Cadena de la Polimerasa) son esenciales para el diagnóstico de los piroplasmas. En este estudio, evaluamos la facilidad y fiabilidad de detectar *Theileria equi* mediante PCR utilizando muestras de ADN previamente extraídas (por métodos con "QIAamp DNA Mini Kit" y Fenol-cloroformo) comparado con sangre observada en tarjetas FTA como plantillas de PCR. Aunque se observaron mínimas variaciones en el límite de detección entre los métodos comparados, en general el uso de muestras de ADN previamente extraídas y de sangre observada en las tarjetas FTA tenían límites de detección comparables. Estos resultados indican que la infección de *T. equi* puede ser eficazmente detectada directamente a partir de las tarjetas FTA por PCR, sin necesidad de extraer previamente ADN de muestras sanguíneas.

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Development of two immunochromatographic tests for the serodiagnosis of bovine babesiosis

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Abstract

In this study, we developed two immunochromatographic tests (ICTs), which are nitrocellulose membrane-based immunoassays for the convenient and rapid serodiagnosis of bovine babesiosis caused by *Babesia bovis* (BoICT) and *Babesia bigemina* (BiICT). The efficacy of two ICTs was evaluated using 13 positive sera from experimentally infected cattle with *B. bovis* or *B. bigemina*. Clear results showed that the BoICT and ELISA detected antibodies in sera collected from 14 to 93 days post-infection, while BiICT and ELISA detected from 13 to 274 days post-infection. In additon, non-infected cattle, *Neospora caninum*, and *Cryptosporidium parvum* were negative in two ICTs. To evaluate the field utility of the ICTs, we tested 186 field bovine sera collected from cattle living in Yanbian (China) and Mato Grosso do Sul (Brazil). The results of ICTs were compared to those of classical serodiagnostic methods, enzyme-linked immunosorbent assay (ELISA) and the indirect immunofluorescence assay (IFAT). The overall concordances of BoICT were determined as 92.5 and 90.3% when the results of ELISA and IFAT were set as the reference standards, respectively. In contrast, those of BiICT showed 96.8 and 92.5% relative to the results of standard ELISA and IFAT, respectively. Conventional and rapid diagnosic devices for bovine babesiosis may provide a valuable tool in clinical and field applications.

Keywords: *Babesia bovis*; *Babesia bigemina*; Immunochromatographic test; ELISA; IFAT

1. Introduction

Bovine babesiosis, a tick-transmitted infectious disease caused by bovine *Babesia* parasites, remains a major threat to livestock animals in tropical and subtropical regions of the world. Two species, *Babesia bovis* and *Babesia bigemina*, have a significant economic effect in cattle (McCosker, 1981). The disease is characterized by fever, hemolytic anemia, jaundice, hemoglobinuria, and in acute cases, death (Ristic, 1981). It is estimated that bovine babesiosis may endanger half a billion cattle worldwide (McCosker, 1981). Cattle that recover from the *B. bovis* and *B. bigemina* infections generally results in parasite persistence and protection against disease following reinfection (Bose et al., 1995).

Routine clinical diagnosis for babesiosis is usually based on the microscopic detection of parasites from the collected blood smears. However, this technique is relatively laborious when large numbers of blood smear samples need to be simultaneously quantified. Furthermore, the detection limit of microscopy may not always be sufficiently sensitive (Almeria et al., 2001). An alterative for the diagnosis of *Babesia* infection is the indirect identification of the parasites through the

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