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Full-Length Paper (Revised)

Title: Evaluation of a Loop-Mediated Isothermal Amplification (LAMP) Method as a Diagnostic Tool of Zoonotic Simian malaria parasite *Plasmodium knowlesi* Infection

Running title: A LAMP METHOD FOR PLASMODIUM KNOWLESI INFECTION

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ABSTRACT

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 *Pk*LAMP can be identified in the infectious course of *P. knowlesi* much earlier 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 of molecular diagnosis of *P. knowlesi* infection in endemic areas.

Introduction

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 *P. 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 *P. falciparum* and *P. 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 of *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). 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 FIP and BIP by inserting a restriction enzyme (*Eco R*I) cleavage site between the F1 complementary and F2 and between the B1 complementary and B2, respectively, as shown in Figure 1B.

_Pk_LAMP procedures

The *Pk*LAMP reaction was performed as described previously (10, 12, 13). Briefly, the reaction was performed in 25 µl of a mixture containing 1 µl of the extracted DNA template, 40 pmol each of the FIP and BIP primers, 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 (47-72 °C was also tested) for 60 min and the reaction was

then terminated by heating the mixture at 80 °C for 5 min for termination. For the initial validation study, *Pk*LAMP was confirmed with real-time monitoring of 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 *Eco R*I at 37 °C for 1 h. The non-treated and *Eco R*I digested LAMP product were subjected to electrophoresis on a 2% agarose gel and then visualized under ultraviolet (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, USA). 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 DNA (gDNA) 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 Dr. Takefumi Tsuboi of Ehime University of Japan. The 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 by a QIAamp DNA Blood Mini Kit (QIAGEN, Tokyo, Japan), according to the manufacturer's instruction. The *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 tests, 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 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 reaction was performed at 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 practically evaluated for fluorescence detection of *P. knowlesi* target DNA using blood samples obtained from experimental *P. knowlesi*-infected monkeys. Two monkeys, J58 (male) and J64 (male), which were three-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 $1x10^8$ fresh *P. knowlesi* H strain (ATCC No. 30158) parasitized red blood cells (PRBCs) obtained from another infected Japanese macaque. Monkey J64 was inoculated intravenously with frozen *P. knowlesi* Hackeri strain (ATCC No. 30153) infected blood obtained from ATCC. After infection, Giemsa-stained

thin blood films were prepared daily from peripheral blood obtained by earprick, 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 ribosomal RNA (SSUrRNA) genes, was performed according to a standard protocol as described previously (15). Nest 1 reaction was carried out in a 50µl reaction mixture containing 2x PCR master mix (Ampli Taq Gold PCR Master Mix, Applied Biosystems, New Jersey USA), 250nM of 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 and 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 20µl reaction mixture containing 2x PCR master mix (Applied Biosystems), 250nM of each primer (Pmk8 and Pmkr9) (16) and 2 µl of the nest 1 PCR products used as DNA templates. A 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 and 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 2% agarose gel and illuminated with UV light.

Results

Specificity of *Pk*LAMP primers

The specificity of *Pk*LAMP primers was investigated by using various *Plasmodium* gDNAs as template for *Pk*LAMP. As shown in Figure 2A, a typical ladder pattern was detected in *P. knowlesi* (lane 1) but not from the DNAs of other *Plasmodium* species (Fig. 2A). Moreover, fluorescent detection was also specifically obtained in the reaction tube including gDNA of *P. knowlesi* as shown in Figure 2B. The sizes of *Pk*LAMP fragments digested by *Eco R*I were identical with 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 47-72 °C separately. Positive ladder patterns were observed at 48-71 °C, and strongly at 56-70 °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 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*, AY639984).

Sensitivity of *Pk*LAMP reaction

To examine the sensitivity of *Pk*LAMP, three detection methods of *Pk*LAMP 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 Figure 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 for 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 Loopamp fluorescent detection reagent, as shown in Figure 3C. In contrast, the limit of detection for PCR using 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 methods, was 100-fold higher than that of single-PCR assay.

Evaluation of *Pk*LAMP and nPCR using DNA extracts and whole blood samples as a template

The course of *Macaca* monkeys experimental infected with *P. knowlesi* was monitored by *Pk*LAMP and nPCR reaction for the detecting parasite DNA (Table 1). Both monkeys infected with *P. knowlesi* developed a fulminating acute infection, and finally became lethargic and severely withdrawn just before autopsy. In monkey J58 inoculated with fresh PRBCs of *P. knowlesi* H strain, 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* Hackeri strain, the parasites were first detected by microscopy on day 6; parasite densities then increased sharply to around 58% within 9 days after infection. *P. knowlesi* DNA could be detected by *Pk*LAMP throughout the course of infection, while the earliest detection of parasite DNA by the nPCR assay was the 3rd day after infection (Table 1).

We also compared the amplification efficiency of *Pk*LAMP and nPCR using frozen whole blood as template. As shown in Table 1, *Pk*LAMP could amplify the target from whole blood with similar efficiency to DNA extracts throughout the course of infection. These results clearly indicate that *Pk*LAMP could detect even the target DNA from non-purified whole blood. In contrast, nPCR assay using whole blood from J58 and J64 could amplify parasite DNA only on day 3 and day 9, respectively, when parasite densities were markedly increased in the blood (Table 1).

Discussion

The diagnosis of malaria at regional clinics in endemic areas has mainly been performed by microscopic examination of blood smears because of its ease and rapid application. However, the morphology of the asexual stages of the zoonotic simian *Plasmodium* parasites substantially resembles to that of human parasites, particularly on thick blood-films, and laboratory technicians are trained to recognize only the four species of human parasites (16). In fact, numerous human cases of *P. knowlesi* infection have been misdiagnosed by microscopy as *P. malariae* due to their morphological similarities (4, 5, 16). The application of DNA amplification to the diagnosis of malaria can solve these problems. Amplification of parasite DNA using a specific PCR has been applied to various *Plasmodium* species including

four human malarial parasites and *P. knowlesi* (3, 7, 14). However, despite the excellent specificity and sensitivity of PCR and real-time PCR, these methods require complicated procedures and sophisticated instrumentation such as a thermal cycler, and they are often impracticable under conditions requiring field diagnosis. In this regard, the LAMP method has the advantages of simplicity, specificity, and sensitivity of reaction compared to other molecular diagnostic methods. It is thus the LAMP method is a promising candidate for wide use in regional clinics and under field conditions.

In the present study, we presented the successful development of a LAMP method for detecting *P. knowlesi* infection, using a primer set that targets the *β-tubulin* gene of parasites. The specificity of the primers was evaluated using nine species of simian malaria parasites and four species of human malaria parasites. The results showed that the primer set for *Pk*LAMP amplified only the autologous DNA samples of *P. knowlesi* in typical ladder bands. In contrast, no ladder bands were obtained from any other control. These findings indicate that this primer set is specific for *P. knowlesi* and can be used to examine for knowlesi malaria as well as distinguish between it and other types of malaria. The sensitivity of the test was evaluated, and results showed that *Pk*LAMP was 100-fold more sensitive than single-PCR assay using F3 and B3 primers. Moreover, the present study showed that an isothermal reaction time of 1hr was enough to amplify 10^9 of the target DNA in reaction tubes containing from 10^8 to 10^2 copies/µl

of the DNA template, and that results could be easily judged by visual inspection of the turbidity or fluorescence of the reaction mixture (10, 13). These results suggest that the *Pk*LAMP assay is reliable and useful for the diagnosis of knowlesi malaria.

To evaluate the feasibility of using *in vivo* materials, comparisons of *Pk*LAMP and the conventional nested PCR method and microscopic examination were made with blood samples from two infected monkeys. These studies validated *Pk*LAMP as an alternative molecular diagnostic tool, which can be used in the diagnosis of early and advanced infections of *P. knowlesi*. Early species identification in the diagnosis for malaria is very important in preventing disease progression. In particular, early identification of *P. knowlesi* infection is essential, since the unique 24 hr asexual replication cycle among human and simian malaria parasites can rapidly result in high levels of parasitemia with a fatal outcome in humans (4, 5). Although nPCR and sequencing have been applied to species identification for malaria diagnosis, a more rapid diagnostic test such as *Pk*LAMP would be a convenient and powerful tool for enabling the delivery of prompt and adequate medical treatment.

The present study also assessed the detection performance of *Pk*LAMP with different DNA template preparations including frozen whole blood or genomic DNA extracts. The detection efficiency of *Pk*LAMP using whole blood was identical to that of *Pk*LAMP when gDNA extracts were used as the template. However, the detection performance of nPCR using

the whole blood templates was quite poor. It appears that this is due to blood components such as myoglobin, hem-blood protein complexes, and immunoglobulin G that inactivate *Taq* DNA polymerase used in standard PCR (2). In contrast, such inhibitors do not affect the *Bst* polymerase used in LAMP (6). According to previous reports, the specificity and sensitivity of detection appear to be unaffected by LAMP processing conditions or sample type, including whole blood, filter paper or card-processed blood, serum, sputum, and crudely processed tissue samples (8). Furthermore, Poon et al. have reported that *P. falciparum* DNA was detected by LAMP using a promising simple DNA template preparation method from heat-treated blood (14). Further improvement of template production methods for *Pk*LAMP will be required to optimize and simplify template preparation.

In conclusion, *Pk*LAMP can be considered as an efficient candidate for the molecular diagnosis of *P. knowlesi* infection in endemic areas. Thekisoe et al. reported that LAMP reagents are stable at ambient temperature for up to 2 weeks (17). In addition, a recent study of the LAMP method showed that it is able to detect both *Plasmodium* oocysts and sporozoites from an "all-in-one" template using whole mosquito bodies (1). These observations further emphasize the potential usefulness of the LAMP method as a diagnostic and new epidemiological surveillance tool for malaria. Our studies will also provide a powerful method for the diagnosis and monitoring of *P. knowlesi* infection in the field.

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Figure legends

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

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

Figure 3: Comparison of sensitivities among 3 methods of detection of *Pk*LAMP and conventional single-PCR for the detection of *P. knowlesi β-tubulin* gene. Template DNA was prepared on serial dilutions of plasmid DNA (10^8 to 1 copies per reaction) containing a *β-tubulin* gene for each assay. Panel A, Real-time LAMP assay was monitored by real-time

measurement of turbidity. Panel B, Agarose gel electrophoresis of LAMP products. Panel C, visual detection of LAMP products under UV light using Loopamp fluorescent detection reagent. Panel D, Agarose gel electrophoresis of single-PCR products using F3 and B3 primers. Lane M indicates 200-bp ladder size markers (Panel A) and 100-bp ladder size markers (Panel B); lane 1 to 9, 10^8 to 1 copies of plasmid; lane 10, distilled water (Panel B-D).

Table 1: Comparison *Pk*LAMP and nPCR and microscopic examination for detection of *P. knowlesi* using two infected monkeys

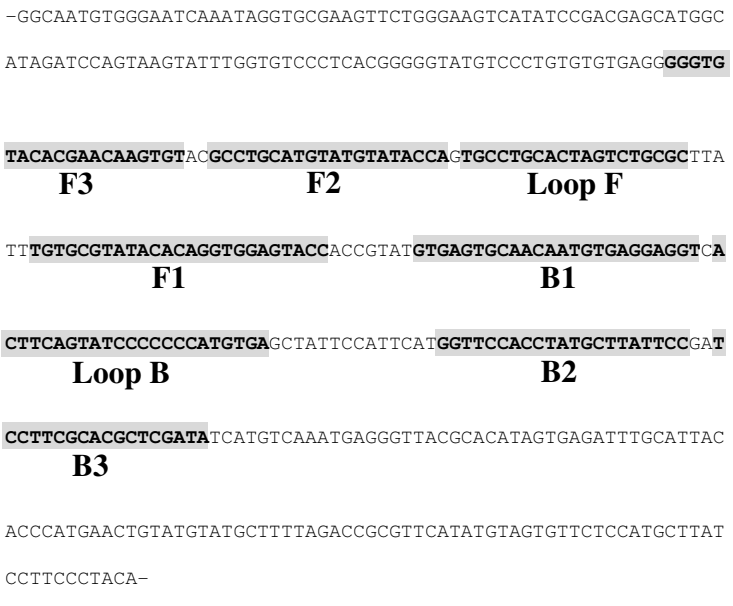
Table 1

day after infection	Monkey J58 (*P.k*, H strain)					Monkey J64 (*P.k*, Hackeri strain)				
	Parasitemia (%)	*Pk*LAMP (DNA ex.)	*Pk*LAMP (w-blood)	nPCR (DNA ex.)	nPCR (w-blood)	Parasitemia (%)	*Pk*LAMP (DNA ex.)	*Pk*LAMP (w-blood)	nPCR (DNA ex.)	nPCR (w-blood)
day 0	-	-	-	-	-	-	-	-	-	-
day 1	<0.01	+	+	+	-	-	+	+	-	-
day 2	0.2	+	+	+	-	-	+	+	-	-
day 3	10.8 (autopsy)	+	+	+	+	-	+	+	+	-
day 4						-	+	+	+	-
day 5						-	+	+	+	-
day 6						0.01	+	+	+	-
day 7						0.1	+	+	+	-
day 8						2.0	+	+	+	-
day 9						58.0 (autopsy)	+	+	+	+

DNA ex.: DNA extract w-blood: whole blood

Figure 1

A



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.

Figure 2

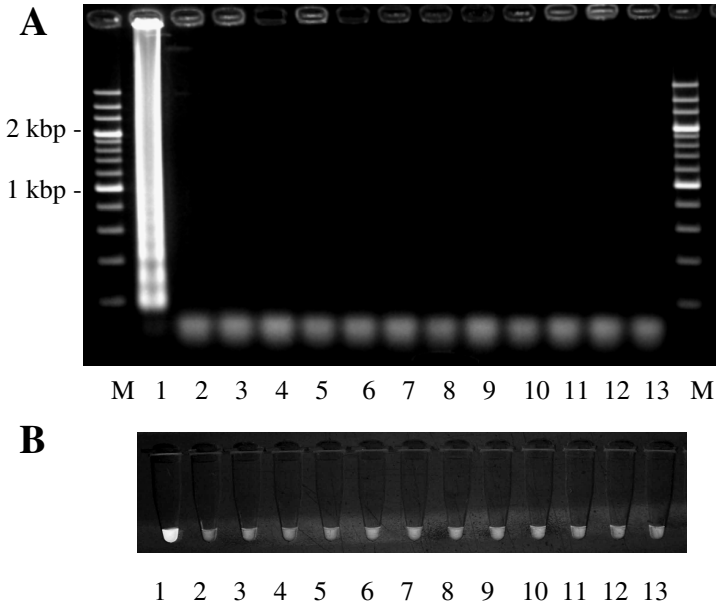


Figure 3