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Development of a reverse transcription-loop-mediated isothermal amplification (RT-LAMP) for clinical detection of *Plasmodium falciparum* gametocytes

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ABSTRACT

Plasmodium falciparum gametocytes are usually present in peripheral blood at a very low level, thus requiring a sensitive assay detection method. In this study, reverse transcription-loop-mediated isothermal amplification (RT-LAMP) was developed for clinical detection of *P. falciparum* gametocytes. Transcripts of Pfs16 of sexually committed ring and Pfs25 of mature gametocytes were detected by RT-LAMP in 82 clinical blood samples using nested RT-PCR as a gold standard. RT-LAMP demonstrated a detection limit of 1 parasitized red blood cell (RBC)/500 μl of blood for both Pfs16 and Pfs25 transcripts. For Pfs16 transcript, RT-LAMP detected all 30 samples positive by nested RT-PCR (100% sensitivity) and 1 in 52 samples negative by nested RT-PCR (98.1% specificity). For Pfs25 transcript, RT-LAMP detected all 15 samples positive by nested RT-PCR (100% sensitivity) and none of 67 samples negative by nested RT-PCR (100% specificity). Negative predictive value (NPV) and positive predictive value (PPV) of RT-LAMP for detection of Pfs16 transcript were 100% and 96.8%, respectively, and 100% for both when employing Pfs25 transcript. Detection rate of Pfs16 and Pfs25 transcripts by RT-LAMP in microscopically gametocyte-negative samples was 91.7% and 29.2%, respectively. Compared with nested RT-PCR, RT-LAMP had a higher sensitivity but similar specificity, with the advantage of a shorter assay time. As RT-LAMP requires very basic instruments and the results can be obtained by visual inspection, this technique provides a simple and reliable tool for epidemiological studies of malaria transmission and in gametocyte-targeted control programmes.

1. Introduction

Asexual stages of the malaria parasite, *Plasmodium falciparum*, are responsible for the clinical symptoms, whereas the sexual stages (gametocytes) are transmitted from human hosts to mosquito vectors. The existence and infectivity of mature gametocytes in the blood circulation determine the accomplishment of transmission. Gametocytes arise from only a small fraction of asexual parasites and only a proportion of patients with a primary infection develop patent gametocytaemia [1]. Gametocytes are usually present in the blood circulation at a very low level. Several studies have shown that mosquitoes can be infected with gametocytes at densities lower than those detectable by conventional microscopy (<5 gametocytes/μl of blood) [2–9]. In the field, infected individuals can have *P. falciparum* gametocytaemia of less than 100 gametocytes/μl of blood and 1–10 gametocytes/μl of blood are enough to establish mosquito infection [10]. As *P. falciparum* gametocyte carriers account for a substantial proportion of the human infection reservoir due to an underestimation of such carriers by microscopy, a more sensitive and accurate method for gametocyte detection is needed in order to block transmission by appropriate treatment and in the epidemiological studies

In recent years, new technological methods have been developed as alternatives to microscopy, including nested reverse transcription-polymerase chain reaction (nested RT-PCR). Nested RT-PCR using *P. falciparum* gametocyte-specific genes, including Pfs25, is considered to be the most sensitive technique, capable of detecting 1 gametocyte/μl of blood, as well as being highly specific [11]. Although this technique is time-consuming and requires skilled technician, it can be used as a "gold standard" to evaluate other diagnostic tests.

Recently, a loop-mediated isothermal amplification (LAMP) technique that provides a rapid, simple, sensitive and inexpensive method for DNA amplification has been developed [12]. LAMP depends on autocycling

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strand-displacement DNA synthesis conducted by *Bst* DNA polymerase. The reaction is carried out without denaturation of DNA templates [13] and thus can be performed at an isothermal temperature. The amplified products are a series of stem-loop DNA of various lengths. The amplification result can be determined by visual inspection of a turbid solution due to precipitation of white magnesium pyrophosphate, a byproduct of DNA synthesis [14]. LAMP has been shown to have a similar sensitivity and a greater specificity than conventional microscopy, a gold standard, in clinical detection of four species of human malaria parasites: *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*, with sensitivity similar to that of nested PCR but with a shorter turnaround time [15].

In this study, we report the development of RT-LAMP for clinical detection of *P. falciparum* gametocytes using mRNA of *P. falciparum* gametocyte-specific genes, Pfs16 and Pfs25 as targets. We chose to compare the results of RT-LAMP to those of nested RT-PCR, a gold standard. Pfs16 is a 16.6 kDa protein located in the parasitophorous vacuole membrane. Pfs16 mRNA is firstly expressed in a ring stage and continues to be expressed throughout gametocytogenesis, in both male and female gametocytes [16,17]. Pfs16 mRNA expression indicates circulating sexual stage parasites composed of sexually committed rings and fully mature gametocytes (stage V) as other stages sequester in the vasculature. Pfs16 expression is considered as the earliest marker in *P. falciparum* sexual differentiation process [16–19]. Pfs25 is a 25 kDa major surface protein of zygotes and ookinetes. Pfs25 mRNA is expressed only in stage V gametocytes, making it suitable as a marker of mature gametocytes [11].

This study is the first study to use Pfs16 and Pfs25 for RT-LAMP. The RT-LAMP method developed here allowed detection of *P. falciparum* gametocytes in clinical blood samples with higher sensitivity than that achieved with nested RT-PCR or microscopy. Moreover, RT-LAMP detected gametocytes in over 29% of the blood samples missed by microscopy.

2. Materials and methods

2.1. Blood samples

EDTA-treated blood samples, 32 positive and 20 negative for *P. falciparum* infection by microscopy, were collected from participants at malaria clinics in Mae Sot and Mae Kasa, Tak, northwestern Thailand. Thirty additional blood samples negative for *P. falciparum* by microscopy were collected from residents in Bangkok, Thailand. Thick and thin blood films were made of all samples. Two milliliter of the remaining blood samples was centrifuged at 600 ×*g* for 5 min at room temperature and approximately 0.8 ml of packed red blood cells (RBCs) was suspended in 1 ml Trizol reagent and kept at −80 °C until used for RNA extraction. All participants signed informed consent forms before enrollment. Ethical approval for this study was obtained from Mahidol University ethics committee, Mahidol University, Bangkok, Thailand.

2.2. Conventional microscopy

Thick blood films were examined under 1000× magnification by an expert microscopist to identify malaria parasites. The results were confirmed from thin blood films by another microscopist. Percent of parasitized RBCs was determined by counting 10,000 RBCs and approximate number of parasites/μl of blood was calculated by assuming a RBC count of 5×10^6/μl of blood [20].

2.3. Total RNA isolation

Total *P. falciparum* RNA was isolated from infected blood samples using Trizol reagent (Invitrogen, USA) according to the manufacturer's instruction and treated with RNase-free DNase I (Fermentus Life Sciences, USA), followed by phenol-chloroform extraction. Any remaining parasite genomic DNA was eliminated by digestion with *Hinc*II and *Pci*I, and the samples were kept at −80 °C. Total RNA from gametocyte-producing *P. falciparum* laboratory strain AMB47 was used as a positive control. A negative control was total RNA from blood samples negative for *P. falciparum* by microscopy.

2.4. Detection of expression of gametocyte-specific genes, Pfs16 and Pfs25, by nested RT-PCR

Expression of A (asexual) type and S (sporozoite) type 18S rRNA was used as an internal positive control to confirm the presence of total RNA of the parasite, and for the presence of *P. falciparum* gametocytes, respectively [21]. Primers and amplification conditions were as described previously [11,22,23] with slight modifications. Details are given in the online Data Supplement (Table A1).

For the first PCR, 2 μg of RNA was added into a mixture containing 0.4 μl of random primers (500 μM) and 0.5 μl of RNase inhibitor (40 U/μl). Then, the mixture was incubated at 70 °C for 10 min and rapidly cooled on ice for 5 min to denature RNA secondary structures. For the RT step, 11 μl of the mixture was added to an RT-reaction mixture containing 4 μl of 5× Bart's buffer, 2 μl of 0.1 M DTT, 2 μl of 10 mM dNTPs, and 1 μl of AMV reverse transcriptase (RTase) enzyme (10 U/μl). To test for carry-over of genomic DNA, a negative control, without RTase, was included. RT-reaction was performed at room temperature for 10 min followed by incubating at 42 °C for 1 h, followed by 95 °C for 10 min to inactivate the enzyme. Two microliters of cDNA was used for the first RT-PCR, using GoTaq® Green Master Mix (Promega, USA) in Eppendorf Mastercycler gradient thermal cycler (Eppendorf, Hamburg, Germany). The amplicon was diluted 100-fold in nuclease-free water and 2 μl was used in the second amplification as described previously [11,22,23]. Amplicons were separated by electrophoresis in 1.5% agarose gel, stained with ethidium bromide and visualized under an ultraviolet light.

Table 1
Primer sets used for amplification of Pfs16 and Pfs25 genes in RT-LAMP.

Gene	Primer	Sequence (5′→3′)
Pfs16	F3	CCAGGAAGTTCTTCAGGT
	B3	TCATATTTGTGACAGCAGTAC
	FIP (F1c–F2)	GCAGAATCTTTTCCAGAAAGACCTTGCCTCTCTTCATGCTGTTG
	BIP (B1–B2c)	TGCCTTTAGAAACTCAGCTAGCTATAAGTTTCTGTTAACTGTCGTT
	LPF	GAGATAGTCCACCTAGATTAGG
	LPB	AGAAGAAATCAAGAGCTTATC
Pfs25	F3	TTTTAATTCAGATGAGTGGTCAT
	B3	CTTACATTCATTTGGTATACAAACA
	FIP (F1c–F2)	GTCTTTTCGTCACATTTCAGAACTTATGTAAATGTGAAAATGATTTGGTG
	BIP (B1–B2c)	ACCATGTGGAGATTTTTCCAAATGTCAAGATTACATTTACAAGCGTATG
	LPF	CTTCACATGTTTCTTCATTTACTAA
	LPB	ATTAAAATAGATGGAAATCC