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Development of loop-mediated isothermal amplification with *Plasmodium falciparum* unique genes for molecular diagnosis of human malaria

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ABSTRACT

In order to achieve better outcomes for treatment and in the prophylaxis of malaria, it is imperative to develop a sensitive, specific, and accurate assay for early diagnosis of *Plasmodium falciparum* infection, which is the major cause of malaria. In this study, we aimed to develop a loop-mediated isothermal amplification (LAMP) assay with *P. falciparum* unique genes for sensitive, specific, and accurate detection of *P. falciparum* infection. The unique genes of *P. falciparum* were randomly selected from PlasmoDB. The LAMP primers of the unique genes were designed using PrimerExplorer V4. LAMP assays with primers from unique genes of *P. falciparum* and conserved 18S rRNA gene were developed and their sensitivity was assessed. The specificity of the most sensitive LAMP assay was further examined using genomic DNA from *Plasmodium vivax*, *Plasmodium yoelii* and *Toxoplasma gondii*. Finally, the unique gene-based LAMP assay was validated using clinical samples of *P. falciparum* infection cases. A total of 31 sets of top-scored LAMP primers from nine unique genes were selected from the pools of designed primers. The LAMP assay with PF3D7_1253300-5 was the most sensitive with the detection limit 5 parasites/μl, and it displayed negative LAMP assay with the genomic DNA samples of *P. vivax*, *P. yoelii*, and *T. gondii*. The LAMP assay with PF3D7_0112300 (18S rRNA) was less sensitive with the detection limit 50 parasites/μl, and it displayed negative LAMP assay with the genomic DNA samples of *P. yoelii* and *T. gondii*, but displayed positive LAMP detection with *P. vivax*. The positive detection rate of the LAMP assay with PF3D7_1253300-5 was 90% (27/30), higher than that (80%, 24/30) of the positive rate of PF3D7_0112300 (18S rRNA) in examining clinical samples of *P. falciparum* infection cases. The LAMP assay with the primer set PF3D7_1253300-5 was more sensitive, specific, and accurate than those with PF3D7_0112300 (18S rRNA) in examining *P. falciparum* infection, and therefore it is a promising tool for diagnosis of *P. falciparum* infection.

KEYWORDS
Malaria; *Plasmodium falciparum*; unique genes; loop-mediated isothermal amplification

Introduction

Malaria is a fatal and infectious disease, prevalent in tropical and subtropical regions, especially in Africa, Asia, and America. The prevalence and death rate of malaria are much higher in sub-Saharan Africa than in other global regions [1]. It can become a major threat to human health and imported malaria has dramatically increased in China, mainly due to the return of Chinese workers from Southeast Asia (SE Asia) and Africa [2]. In 2015, aproximately 212 million cases of malaria were detected worldwide, with an estimated 429,000 deaths [1].

In the early stage after malaria parasite infection, there occurs symptoms which are similar to the common cold, dengue, yellow fever, and typhoid fever, easily leading to confusion and misdiagnosis. If there is no proper treatment in time, even mild and moderate malaria parasitic infections are fatal to children under five and to pregnant women, particularly those infected by *Plasmodium falciparum*. It has been found that the morbidity and mortality rates of malaria caused by *falciparum* are higher than those of vivax malaria [3,4]. In Africa, *P. falciparum* is the most dangerous malaria parasite and responsible for more than 75% of malaria cases [1]. It is imperative to develop a rapid, specific, sensitive, and inexpensive detection method to identify *Plasmodium* species especially *P. falciparum* infection for malaria control.

Loop-mediated isothermal amplification (LAMP) was developed as a novel nucleic acid amplification method in 2000 [5]. A 10^9-fold amplification of a copy of nucleic acid in less than an hour can be achieved using LAMP

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technology. LAMP reaction is set up under isothermal conditions (60–65 °C) with high specificity. In addition, LAMP assay is simple, convenient, cost-effective, and reliable. Therefore, LAMP technology is being considered for diagnosing infectious diseases including tuberculosis [6], HIV [7], and malaria [8–11], as well as other pathogen infections and diseases such as Q fever and *Salmonella Enterica* Serotype Enteritidis [12,13]. LAMP also holds promise for use in molecular detection of pathogens in malaria [5,9,14,15]. Numerous LAMP assays have been developed for malaria diagnosis based on PfHRP2 [16], 18S rRNA [8,11,15], Pfs16 and Pfs25 [17], and mitochondrial DNA [18] of malaria parasites. These selected genes for conventional polymerase chain reaction (PCR) or LAMP are conserved, resulting in a high false-positive rate of amplification of target sequences. To solve this problem, genes unique to malaria parasites can be selected. The genome database of the *P. falciparum* 3D7 strain provided a vital tool for large-scale unique gene screening [19–21].

In this study, we developed a LAMP assay with the unique genes of *P. falciparum* to diagnose *falciparum* infection. Our findings support the conclusion that LAMP assay with unique genes was a rapid, specific, sensitive, and cost-effective detection method to identify *P. falciparum* infection, and therefore it is useful for malaria control.

Materials and methods

Study design

In order to survey malaria caused by *P. falciparum* on returning Chinese workers from Southeast Asia (SE Asia) and Africa, a LAMP assay based on unique genes was developed. Its sensitivity, specificity, and accuracy in detecting clinically-confirmed *P. falciparum* infection were assessed.

Collection and preparation of human blood samples

This study was approved by the ethics committees of Wuhan Center for Disease Control and Prevention (CDC). Peripheral blood samples were collected from a patient with *falciparum* malaria and a patient with *P. vivax* infection in Wuhan CDC. These two patients were Chinese workers who had returned from Africa. They were diagnosed with One Step Malaria HRP2/pLDH (P.f/Pan) (Wondfo, Guangzhou, China) and microscopic examination of Giemsa-stained thick and thin peripheral blood smear. For quality control, archived malaria positive slides were re-examined and parasitaemia was recorded. The *Plasmodium spp.* was confirmed by *Plasmodium* malaria real time PCR diagnostic kit (Shanghai Liferiver Bio-Tech Corp, Shanghai, China). All these samples were delivered to Hubei University of Medicine at low temperature (–20 °C).

Collection and preparation of mice blood samples

All animal experiments were approved by the Animal Care and Use Committee of the Third Military Medical University. Mice were obtained from the Experimental Animal Center of the Third Military Medical University. Female C57BL/6 mice (5–6 weeks) were infected by *P. yoelii 17XL* 10^6 infected erythrocytes in 200 µl of sterile PBS via intraperitoneal injection. When the parasitaemia reached 5–15%, blood samples were collected using heparin-pretreated 5-ml tube and stored at –20 °C until use, following the published report [22]. Seroperitoneum sample of *T. gondii* was kindly provided by Dr Shuang Shen of Jiangsu Institute of Parasitic Diseases. All these samples were delivered to Hubei University of Medicine at low temperature (–20 °C).

DNA extraction

DNA from blood samples of *P. falciparum, P. vivax*, *P. yoelii,* and *T. gondii* were extracted using TIANamp Blood DNA Kit (Tiangen Biotech Co., Ltd., Beijing, China) following the manufacturer's protocol. All extracted genomic DNA (5 µl) were electrophoretically analysed in a horizontal plate with 2% agarose gels, containing 0.1 µg/ml ethidium bromide, and examined for quality under UV light. The remaining extracted genomic DNA samples were either used immediately or stored at –20 °C.

Selection of unique genes and design of LAMP primers

Unique genes were randomly selected from *P. falciparum* genome database in PlasmoDB [19,23]. Species specificity of target unique genes was verified using OrthoMCL database [24]. 18S rRNA (Gene ID. PF3D7_0112300) was used as a standard reference for unique genes. Nucleotide sequences of target genes were extracted from PlasmoDB (https://plasmodb.org/plasmo/) [23,25]. LAMP primers for target unique genes were designed using the online software PrimerExplorer V4 (https://primerexplorer.jp/elamp4.0.0/index.html) with default parameters. For each target unique gene, 2–5 top-scored sets of LAMP primers were selected and synthesized by Genewiz Company (Soochow, China). The design of LAMP primers was illustrated in Figure 1. The inner primers (FIP/BIP) and outer primers (F3/B3) were dissolved in ultrapure water with concentrations 200 and 100 pmol/µl respectively. A 20 µl working mixture of LAMP primers was made using 4 µl FIP and BIP, 1 µl F3 and B3, and 10 µl of ultrapure water, in which the concentrations of inner primers and outer primers were 40 and 5 µM, respectively.

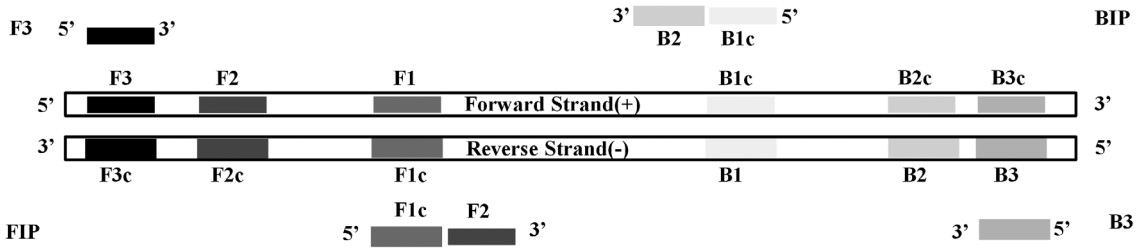

Figure 1. Design of LAMP primers for *Plasmodium falciparum* unique genes.
Note: F3 and B3, outer primer; FIP (F1c, F2) and BIP (B1c, B2), inner primer.

LAMP assay and determination of sensitivity and specificity

LAMP assay was developed according to the published reports [15,23] with modifications. Briefly, the LAMP assay was performed with a DNA amplification kit (Deaou, Guangzhou, China). The reaction mixture in a 0.2-ml PCR tube was set up by adding 12.5 µl 2× reaction buffer, 1.0 µl Bst DNA polymerase, 1.0 µl genomic DNA, 1.0 µl working LAMP primer mixture, and 9.5 µl sterile ultrapure water. Finally, isometric mineral oil (20 µl) was added into the PCR tube, which was then centrifuged for 30 s. The LAMP reaction tubes were incubated at 63 °C for 60 min in a T100™ Thermal Cycler (BIO-RAD, Singapore). SYBR Green was used to indicate the results of LAMP assay. The light green mixture in PCR tubes indicated successful amplification, and the brown mixture indicated negative results of LAMP assay. To assess the sensitivity of LAMP assay, the genomic DNA sample was extracted from parasitaemia 50,000 parasites/µl (1% parasitaemia), which was adjusted from 58,241 *P. falciparum* parasites/µl (1.16% parasitaemia) of the returned Chinese worker. The DNA sample was then serially diluted in 10-fold (10^0–10^{-6}) and used as templates. The most sensitive primers were selected for specificity evaluation using the genomic DNA sample of *P. vivax*, *P. yoelii*, and *T. gondii*. For comparison, the 18S rRNA-based LAMP assay was also performed using the same samples. System quality control (positive, negative, and blank) was carried out using the DNA amplification kit.

Validation of the unique gene-based LAMP assay with clinical samples of P. falciparum *infection cases*

This study was approved by the ethics committees of Malabo Regional Hospital on Bioko Island, Equatorial Guinea. The informed consents were obtained from all participants. Blood samples were collected from Chinese workers with uncomplicated *P. falciparum* infection at Malabo Regional Hospital on Bioko Island, Equatorial Guinea from March 2014 to September 2015. The initial diagnosis of *P. falciparum* infection was conducted by microscopic examination of blood smears and a rapid diagnostic assay with the ICT malaria *P.f.* Cassette Test (ICT Diagnostics, South Africa). Approximately 300 µl of each whole blood sample was aliquoted on 3MM Whatman® filter paper (Whatman International Ltd., Maidstone, England), and air dried. These dried filter papers with blood spots (DBS) were then stored individually in Ziplock bags containing silica desiccant beads and kept at −20 °C. All these DBS were delivered to Hubei University of Medicine at low temperature (−20 °C). Among these DBS, thirty pieces were randomly selected. All these 30 patients were diagnosed with *P. falciparum* infection with 135,111 ± 216,492 parasites/µl. DNA was extracted from 30 DBS following the Chelex-100 extraction procedure described in our previous study [26]. The DNA samples were subjected to LAMP assay. For comparison, PF3D7_0112300 (18S rRNA) based LAMP assay was also performed.

Results

Selection of P. falciparum *unique genes and LAMP primers*

To develop unique gene-based LAMP assay, we randomly selected nine unique genes of *P. falciparum* from genome database of *P. falciparum* 3D7 strain, including one pseudogene PF3D7_1253300 and eight protein-coding genes (Table 1). PF3D7_0702300, PF3D7_1001900, PF3D7_1253300, and PF3D7_1301700 were only found in *P. falciparum* but not in other *Plasmodium spp.* according to OrthoMCL database (Table 1). A total of 31 sets of top-scored LAMP primers for nine unique genes were selected from the pools of primers designed by the online software PrimerExplorer V4 (Table 1).

LAMP assays with primers from P. falciparum *unique genes and their sensitivity and specificity*

We first examined whether the primers from *P. falciparum* unique genes can be used to develop LAMP assay using the DNA of a *P. falciparum*-infected blood sample (parasitaemia: 50,000 parasites/µl). The results showed that 25 sets of LAMP primers from eight genes displayed positive LAMP assay, and six sets of LAMP primers from five genes displayed negative LAMP assay. Notably, all five sets of LAMP primers from the gene PF3D7_1253300 displayed positive amplification (Table 2). Four sets of LAMP primers from PF3D7_0202200, PF3D7_1002000,

Table 1. The LAMP primers of Plasmodium unique genes.

	Target region			LAMP primer		
Gene ID.	Name	Start to end	Length (bp)	Name	Oligo (nt)	Sequence (5′-3′)
PF3D7_0202200	PF3D7_0202200-1	921-1121	201	F3	18	GAACAAGTTTCAAAAGCA
				B3	18	GTCATCTTGTTCCCTAAG
				FIP	48	CATACGTTTTTGCATATCCATATTACCTTTTATATTACCT-TTCGTTCC
				BIP	39	TTCATGCTATGTTTAAATGGGC-CTTCATTTCTGCGTTGT
	PF3D7_0202200-2	604-798	195	F3	17	TTGGCGCTATAAGAGAA
				B3	20	GTTTATCTTTTCCTTCATCA
				FIP	40	AGTTATACTTGCAGGTAATGGAAATAACGATTCCTTTGGT
				BIP	46	AAGGAGATTCTGAAGCAGAAGTATCATCATTTGAA-GAACTTACAGC
	PF3D7_0202200-3	927-1121	195	F3	19	GTTTCAAAAGCATTACCTT
				B3	18	GTCATCTTGTTCCCTAAG
				FIP	38	AACATTACATACGTTTTTGCATTACCTTTCGTTCCTCC
				BIP	37	TTCATGCTATGTTTAAATGGCTTCATTTCTGCGTTGT
	PF3D7_0202200-4	597- 798	202	F3	17	AAAAGAATTGGCGCTAT
				B3	20	GTTTATCTTTTCCTTCATCA
				FIP	45	TGCAGGTAATGGTAAATGATTTGAGAAATGA-TAAATAACGATTCC
				BIP	41	ATAACTAAAGGAGATTCTGAAGATTTGAAGAACTTA-CAGCT
	PF3D7_0202200-5	660-883	224	F3	18	CATTTACCATTACCTGCA
				B3	20	TGTAAATTCGATTTACCTGA
				FIP	45	TGATGTATTACGTGAATCATAGTATAACTAAAGGAGAT-TCTGAAG
				BIP	42	TGAAGGAAAAGATAAACGTCCACATATTTTTGA-TAATCCCAT
PF3D7_0702300	PF3D7_0702300-1	338-543	206	F3	20	ATCAGAATATCAATCGAACT
				B3	19	TTACTTTTGTAGTGCTTGT
				FIP	34	TGTTCCGGATTTTTTACTATTCTTGGTGGGGGAT
				BIP	44	TATACCAGAAAGTAGTAGTACAGTTGTACTGTTAT-TTGCTGCTA
	PF3D7_0702300-2	305–544	240	F3	20	ATCAACATACGTTATAAAGC
				B3	19	GTTACTTTTGTAGTGCTTG
				FIP	43	GTTCCGGATTTTTTACTATTTACATATCAATCGAACT-TTCTTG
				BIP	44	TATACCAGAAAGTAGTAGTACAGTTGTACTGTTAT-TTGCTGCTA
	PF3D7_0702300-3	347–543	197	F3	18	TCAATCGAACTTTCTTGG
				B3	19	TTACTTTTGTAGTGCTTGT
				FIP	41	ACTTTGTATTTACATTTGTTCCGGAGGGATATAGTG-CAGCT
				BIP	41	TATACCAGAAAGTAGTAGTACAGTTGTACTGTTATTTGCTG
PF3D7_1001900	PF3D7_1001900-1	691–873	183	F3	16	CGGAAGAAGGTGAAGA
				B3	18	TCAAAAGTATTGCTGTCA
				FIP	44	CCAATTTCTCCTTTATTTGGATATACCTAAACCTTATA-CAGTTG
				BIP	45	TTCGTAGAAGTGTTATTACTTTGTAATAAGAAAAGTAG-GTGACGT
	PF3D7_1001900-2	681–895	215	F3	17	GTTGAAAATGCGGAAGA
				B3	20	GCTGTCTTAAAATTGTCATT
				FIP	47	CCAATTTCTCCTTTATTTGGATTAGGTGAAGAAAT-ACCTAAACCTTA
				BIP	43	TTCGTAGAAGTGTTATTACTGTCATTGTAATAAGAAAA-GTAGG
PF3D7_1002000	PF3D7_1002000-1	707–930	224	F3	18	AATCAAATGTGACAAACG
				B3	19	GGTCTTTCTCTTTTTCCTT
				FIP	48	GTCTGCTTTTATTTCTTCAATTGAAAAGTGTGAAATTT-GAAGAACCAC
				BIP	43	TAAACGTGGATGAAAAAAAGGTGTTTTTTCTTCTTCACT-GGAA
	PF3D7_1002000-2	720–930	211	F3	17	AAACGTGAAAAGTGTGA
				B3	19	GGTCTTTCTCTTTTTCCTT
				FIP	39	TGTCTGCTTTTATTTCTTCAAATTTGAAGAACCACAAGA
				BIP	43	TAAACGTGGATGAAAAAAAGGTGTTTTTTCTTCTTCACT-GGAA
	PF3D7_1002000-3	666–892	227	F3	19	TACCTATTCTAGACGAGAA
				B3	19	TTTTTTCTTCTTCACTGGA
				FIP	41	CTCATCTTGTGGTTCTTCTAGTAAATCAAATGTGA-CAAACG
				BIP	39	AAGCAGACAATAATGATTCAAATCCTTTTTTTCATCCAC
	PF3D7_1002000-4	744–969	226	F3	18	AGAACCACAAGATGAGAA
				B3	20	ATAAGATGTGTTACTGTCTT
				FIP	43	TCATCCACGTTTATATTAGTCAATTGAAGAAATAAAAG-CAGAC
				BIP	42	AGTGTAATTTCCAGTGAAGAAGATTCTTATGAGACCT-TTTGG

Table 1. (*Continued*).

	Target region			LAMP primer		
Gene ID.	Name	Start to end	Length (bp)	Name	Oligo (nt)	Sequence (5′-3′)
PF3D7_1148900	PF3D7_1148900-1	48–267	220	F3	17	CGTGAAAACATTGATGA
				B3	18	CGTTACATAAAATGCTGA
				FIP	35	CTTTGCCAAAAACTTTCAGAAAATTCCTTTCAAGG
				BIP	39	TTCTGCCGTTGTTTTATTTTTTGACTAAAAGTGCTTTGA
	PF3D7_1148900-2	56–267	212	F3	19	CATTGATGAAGAAAATTCC
				B3	18	CGTTACATAAAATGCTGA
				FIP	28	GAAGGGCTTTGCCTTTCAAGGGTCCAAG
				BIP	39	TTCTGCCGTTGTTTTATTTTTTGACTAAAAGTGCTTTGA
PF3D7_1240100	PF3D7_1240100-1	25–236	212	F3	20	ATTTTATATCTCATTGCTGC
				B3	18	AGTCCTATACCATATGCA
				FIP	48	CCTTTCTTTTTTTTTTTTCCCTC-TTGGCAATTAATTTAAT-AGCTCCCA
				BIP	42	TTCAGAAAAAAACATCATAAGGCCAAATAAAAGAGCGA-TAGC
	PF3D7_1240100-2	41–228	188	F3	17	CTGCCTTATTGGCAATT
				B3	17	ACCATATGCAGTACCAA
				FIP	42	GAGGACATCCTTTCTTTTTTTTTATTTAATAGCTCCCAGT-GT
				BIP	47	TTCAGAAAAAAACATCATAAGGCTAAAAGAGCGATAG-CAGAAACAAC
	PF3D7_1240100-3	41- 228	188	F3	17	CTGCCTTATTGGCAATT
				B3	17	ACCATATGCAGTACCAA
				FIP	34	AGAGGACATCCTTTCTTTCCCAGTGTTTGTAACG
				BIP	47	TTCAGAAAAAAACATCATAAGGCTAAAAGAGCGATAG-CAGAAACAAC
PF3D7_1253300	PF3D7_1253300-1	359–570	212	F3	17	TTGACAAGAGAGGAGTT
				B3	18	CCATGTGATTTTAGGAGG
				FIP	45	ACCATTTTGTGATTCCATATTTGATTTATTAGTTCAAG-TACCACC
				BIP	42	ATGTAGACAAGGAACAAAGGATTTAATTTGGGTTACCCG-TAT
	PF3D7_1253300-2	328–532	205	F3	18	AGATAGCAAATTCGAGAG
				B3	16	GGTTACCCGTATGTCT
				FIP	41	GTGGTACTTGAACTAATAAATCACGTAACTGAACAGTT-GAC
				BIP	35	TATGGAATCACAAAATGGTAGGTGCAATTCCTCTT
	PF3D7_1253300-3	542–738	197	F3	18	ATGGTAAATCACCTCCTA
				B3	21	CTTCACATATAAATCGGTATG
				FIP	38	TGTTTCCAAAGTAGAAACCAAATCACATGGCAAGGATG
				BIP	45	ATAACCTACTTAATAAAAAGGCTCCCATCTATACAT-GAACGAATG
	PF3D7_1253300-4	521–738	218	F3	17	ATACGGGTAACCCAAAT
				B3	21	CTTCACATATAAATCGGTATG
				FIP	43	CAAAGTAGAAACCATCATATTACTCACCTCCTAAAATCA-CATG
				BIP	42	ACCTACTTAATAAAAAGGCTCTATACATGAACGAAT-GAAACT
	PF3D7_1253300-5	507–703	197	F3	20	ACTTACATATAGACATACGG
				B3	20	CATCTATACATGAACGAATG
				FIP	45	AATCATACGAACATCCTTGCACCCAAATCAATATGG-TAAATCACC
				BIP	47	ATATGATGGTTTCTACTTTGGATGGAGCCTTTTTATTAA-GTAGGTTA
PF3D7_1301700	PF3D7_1301700-1	688–913	226	F3	21	TCAATGCACTCAAATCACAAT
				B3	21	ACATATAACATAGCTGGGACA
				FIP	45	TGCAGCTACTGCTGAAATAGTTAATTAATGTTAGAG-CAGCTACCA
				BIP	46	ATATGCTTGCTATTGCAGGAGTTAATAATAATGCCATAC-CAGGTAA
	PF3D7_1301700-2	722–913	192	F3	19	TGTTAGAGCAGCTACCATT
				B3	21	ACATATAACATAGCTGGGACA
				FIP	46	ATGTTGCAGCTACTGCTGAAAATAGCAGGATTCTTAT-CAATCTTTG
				BIP	46	ATATGCTTGCTATTGCAGGAGTTAATAATAATGCCATAC-CAGGTAA
PF3D7_1334700	PF3D7_1334700-1	351–553	203	F3	20	ACAAGAATGTCAATCAATCA
				B3	17	TTTGGCTAGTTCCTCTT
				FIP	46	GTAGAGAAAGGAGTATTAACATTATAAAAGAACTTTT-GAGTGCTGC
				BIP	40	GCATTAGAAAAAGATGAATTCCTGTATGTCCACATAGTGC
	PF3D7_1334700-2	235–441	207	F3	19	AGTTTATTAGACCAACCAA
				B3	19	AGTTGTAGAGAAAGGAGTA
				FIP	40	GGATCATTTGGATTTGTTAAATTTCTACATCAACACAAGG
				BIP	42	CAAGAATGTCAATCAATCAATGTTCATCATTTGTAGCAG-CAC

Table 1. (*Continued*).

Gene ID.	Target region			LAMP primer		
	Name	Start to end	Length (bp)	Name	Oligo (nt)	Sequence (5′-3′)
	PF3D7_1334700-3	211–428	218	F3	17	AATTCCCTTGAAGAAGC
				B3	21	GGAGTATTAACATGTTCATCA
				FIP	48	GAAGTATTATTTTCACTACCTTGTGACAAAAGAGTTTATT-AGACCAAC
				BIP	41	TAACAAATCCAAATGATCCAACACCAGCACTCAAAA-GTTCT
	PF3D7_1334700-4	247–441	194	F3	23	CAACCAAATATTTCTACATCAAC
				B3	19	AGTTGTAGAGAAAGGAGTA
				FIP	45	GGATCATTTGGATTTGTTAACAAGGTAGTGAAAATAAT-ACTTCAG
				BIP	42	CAAGAATGTCAATCAATCAATGTTCATCATTTGTAGCAG-CAC
	PF3D7_1334700-5	278–509	232	F3	23	GTGAAAATAATACTTCAGGTGTT
				B3	18	CCACATAGTGCTTCTGTT
				FIP	45	GCAGCACTCAAAAGTTCTTTTATAATAACAAATCCAAAT-GATCCA
				BIP	41	ATGATGAACATGTTAATACTCCCGTGATACTATCACGGAAT
PF3D7_0112300	18S rRNA	506-725	220	F3	24	TGTAATTGGAATGATAGGAATTTA
				B3	23	GAAAACCTTATTTTGAACAAAGC
				FIP	41	AGCTGGAATTACCGCGGCTGGGTTCCTAGAGAAA-CAATTGG
				BIP	45	TGTTGCAGTTAAAACGTTCGTAGCCCAAACCAGT-TTAAATGAAAC

or PF3D7_1334700 gene displayed positive LAMP detection. Three and two sets of LAMP primers between PF3D7_1240100 and PF3D7_1001900 displayed positive LAMP assay, respectively. Only one set of LAMP primer from PF3D7_0702300, PF3D7_1148900, and PF3D7_1301700 displayed positive reactions.

We subsequently assessed sensitivity of the LAMP assay with primers from unique genes with serial diluted DNA template. The results showed that the LAMP assay with PF3D7_1253300-5 was the most sensitive, as shown by the positive LAMP assay at the maximum dilution (10^{-4}) of DNA template (Table 2), indicating that the *P. falciparum* detection limit of the LAMP assay with PF3D7_1253300-5 was 5 parasites/μl. The LAMP assay with PF3D7_1001900-2 was the second most sensitive (positive LAMP reaction at the maximum dilution 10^{-3}) among all LAMP primers from unique genes (Table 2), indicating its *P. falciparum* detection limit was 50 parasites/μl. The sensitivity of PF3D7_0112300 (18S rRNA) was the same as that of PF3D7_1001900-2.

We further examined the specificity of PF3D7_1253300-5 in detecting *P. falciparum* infection using the genomic DNA samples of *P. vivax*, *P. yoelii*, and *T. gondii*. The results showed that the LAMP assay with PF3D7_1253300-5 displayed negative LAMP assay with the genomic DNA samples of *P. vivax*, *P. yoelii*, and *T. gondii*, and the LAMP assay with PF3D7_0112300 (18S rRNA) displayed negative amplification with the genomic DNA samples of *P. yoelii* and *T. gondii*, but displayed positive LAMP assay with *P. vivax*. These results indicated that LAMP assay with the primer PF3D7_1253300-5 unique to *P. falciparum* had higher sensitivity and specificity than those with PF3D7_0112300 (18S rRNA). The genomic location and sequence of the LAMP primer set PF3D7_1253300-5 was illustrated in Figure 2.

Validation of the unique gene-based LAMP assay with clinical samples of P. falciparum *infection cases*

We further validated the unique gene-based LAMP assay with the primer PF3D7_1253300-5 using clinical samples of *P. falciparum* infection cases. The results showed that the positive detection rate of PF3D7_1253300-5 was 90% (27/30), higher than that (80%, 24/30) of the positive rate of PF3D7_0112300 (18S rRNA), suggesting that the unique gene-based LAMP assay with the primer PF3D7_1253300-5 was more accurate than PF3D7_0112300 (18S rRNA)-based LAMP assay in examining *P. falciparum* infection cases.

Discussion

In the current study, we developed LAMP assays using primers of *P. falciparum* unique genes to diagnose *P. falciparum* infection. We found that the LAMP assay with the primer set PF3D7_1253300-5 was more sensitive, specific, and accurate than those with PF3D7_0112300 (18S rRNA) in examining *P. falciparum* infection, and therefore it is a promising tool for diagnosis of *P. falciparum* infection.

It has been reported that LAMP assay is a highly specific method to detect various pathogens [6–9,11–13]. In the current study, the LAMP assay with the primer set PF3D7_1253300-5 displayed negative LAMP assay with all the genomic DNA samples of *P. vivax*, *P. yoelii*, and *T. gondii*, and the LAMP assay with PF3D7_0112300 (18S rRNA) displayed negative amplification with the genomic DNA samples of *P. yoelii* and *T. gondii*, but displayed positive reaction with *P. vivax*. These results indicate that the LAMP assay with the primer set PF3D7_1253300-5

Table 2. LAMP assays with primers from P. falciparum unique genes and their sensitivity.

	Sensitivity (10-fold serial dilutions) specificity						
LAMP name	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
PF3D7_0112300	P	P	P	P	N	N	N
PF3D7_0202200-1	P	N	N	N	N	N	N
PF3D7_0202200-2	P	P	P	N	N	N	N
PF3D7_0202200-3	P	P	P	N	N	N	N
PF3D7_0202200-4	N	N	N	N	N	N	N
PF3D7_0202200-5	P	P	P	N	N	N	N
PF3D7_0702300-1	N	N	N	N	N	N	N
PF3D7_0702300-2	N	N	N	N	N	N	N
PF3D7_0702300-3	P	P	P	N	N	N	N
PF3D7_1001900-1	P	N	N	N	N	N	N
PF3D7_1001900-2	P	P	P	P	N	N	N
PF3D7_1002000-1	P	P	P	N	N	N	N
PF3D7_1002000-2	P	P	P	N	N	N	N
PF3D7_1002000-3	P	P	N	N	N	N	N
PF3D7_1002000-4	P	P	P	N	N	N	N
PF3D7_1148900-1	N	N	N	N	N	N	N
PF3D7_1148900-2	P	P	N	N	N	N	N
PF3D7_1240100-1	P	P	N	N	N	N	N
PF3D7_1240100-2	P	N	N	N	N	N	N
PF3D7_1240100-3	P	N	N	N	N	N	N
PF3D7_1253300-1	P	P	N	N	N	N	N
PF3D7_1253300-2	P	P	N	N	N	N	N
PF3D7_1253300-3	P	P	N	N	N	N	N
PF3D7_1253300-4	P	P	P	N	N	N	N
PF3D7_1253300-5	P	P	P	P	P	N	N
PF3D7_1301700-1	P	P	N	N	N	N	N
PF3D7_1301700-2	N	N	N	N	N	N	N
PF3D7_1334700-1	P	P	N	N	N	N	N
PF3D7_1334700-2	P	P	P	N	N	N	N
PF3D7_1334700-3	P	P	N	N	N	N	N
PF3D7_1334700-4	P	P	P	N	N	N	N
PF3D7_1334700-5	N	N	N	N	N	N	N

Note: P and N represented positive and negative, respectively. 10^{0} represented the extracted DNA from blood sample of *P. falciparum* infection (parasitaemia: 50,000 parasites/μl). The 10^{-1} represented 10-fold dilution of 10^{0}

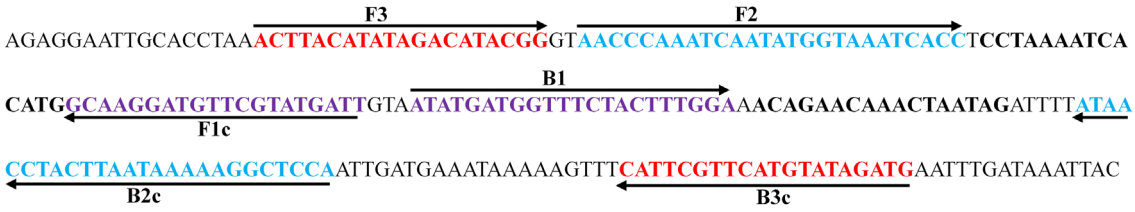

Figure 2. The genomic location and sequence of the primer set PF3D7_1253300-5.

displayed higher specificity than PF3D7_0112300 (18S rRNA) did, and there is false-positive amplification with 18S rRNA in LAMP assay. It seems that selection of target genes in LAMP is critical for high specificity of LAMP detection.

The conserved gene 18S rRNA has been a conventional target gene in diagnosing malaria [27]. There are both benefits and pitfalls using 18S rRNA as a target to diagnose malaria. On the one hand, 18S rRNA is a conserved gene, and it is easy to identify and design primers for LAMP assay in eukaryotes [27]. On the other hand, there are only seven copies of 18S rRNA gene in *P. falciparum* while there are many copies of 18S rRNA gene in other eukaryotes [21]. For this reason, the limit of LAMP-based detection is not as good for *P. falciparum* as that for other eukaryotes. Furthermore, the LAMP detection often generates false-positive results using conserved genes as templates, as indicated in previous studies using PfHRP2 [16], 18S rRNA [8,11,15], Pfs16 and Pfs25 [17], and mitochondrial DNA [18]. In the current study, we found that the detection limit of the LAMP assay with the primer set PF3D7_1253300-5 was as low as 5 parasites/μl, more sensitive than that of the primer set from PF3D7_0112300 (18S rRNA) (50 parasites/μl). The LAMP assay with the primer set PF3D7_1253300-5 displayed higher specificity than PF3D7_0112300 (18S rRNA) did. Further, the unique gene-based LAMP assay with the primer PF3D7_1253300-5 was more accurate than PF3D7_0112300 (18S rRNA)-based LAMP assay in examining *P. falciparum* infection cases. Therefore, it is likely that the LAMP assay with the primer PF3D7_1253300-5 unique to *P. falciparum* is superior to the conserved PF3D7_0112300 (18S rRNA).

It is desirable to diagnose *P. falciparum* infection at an early stage so that better outcomes can be achieved.

In the current study, the detection limit of the LAMP assay with the primer set PF3D7_1253300-5 was 5 parasites/μl, lower than that of the primer set from PF3D7_0112300 (18S rRNA) (50 parasites/μl). However, it is not as low as those in some other studies. The detection limit of a LAMP assay with primers from the apicoplast genome achieved 2 parasites/μl [27]. This is probably due to that the apicoplast is semi-autonomous with its own genome and it has 15 copies of genome in *P. falciparum* [27]. In a recent study, the detection limit of a commercial malaria LAMP assay was reported to be ≤2.0 parasites/μl, reaching the detection limit of a real-time LAMP test [28]. Therefore, the LAMP assay with the primer from unique genes needs to be improved, as also suggested by previous studies [18,29,30]. As far as our LAMP assay system, it can be improved at least in the following aspects. First, genes with a high copy number are selected. Second, loop primers of LAMP (LPF and LPB) are used, because they may improve detection limit by 10-fold [15]. Third, LAMP reaction conditions can be optimized by screening primers, concentrations of Mg^{2+}, reaction buffer, polymerase, and DNA template, and reaction temperature and time. This deserves further investigation.

In conclusion, we have established the LAMP assay with the primer set PF3D7_1253300-5, which is more sensitive, specific, and accurate than that with PF3D7_0112300 (18S rRNA) in examining *P. falciparum* infection. Since the LAMP assay is rapid, simple, sensitive, specific, and cost-effective, and it can be applied without the aid of sophisticated equipment, the LAMP assay with the primer set PF3D7_1253300-5 is a promising tool widely used to diagnose *P. falciparum* infection in the regions where malaria is prevalent.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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