JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 2007, p. 2521–2528 Vol. 45, No. 8
0095-1137/07/$08.00+0 doi:10.1128/JCM.02117-06

Detection of Four *Plasmodium* Species by Genus- and Species-Specific Loop-Mediated Isothermal Amplification for Clinical Diagnosis▽

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Received 16 October 2006/Returned for modification 6 December 2006/Accepted 31 May 2007

Loop-mediated isothermal amplification (LAMP), a novel nucleic acid amplification method, was developed for the clinical detection of four species of human malaria parasites: *Plasmodium falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*. We evaluated the sensitivity and specificity of LAMP in comparison with the results of microscopic examination and nested PCR. LAMP showed a detection limit (analytical sensitivity) of 10 copies of the target 18S rRNA genes for *P. malariae* and *P. ovale* and 100 copies for the genus *Plasmodium*, *P. falciparum*, and *P. vivax*. LAMP detected malaria parasites in 67 of 68 microscopically positive blood samples (sensitivity, 98.5%) and 3 of 53 microscopically negative samples (specificity, 94.3%), in good agreement with the results of nested PCR. The LAMP reactions yielded results within about 26 min, on average, for detection of the genus *Plasmodium*, 32 min for *P. falciparum*, 31 min for *P. vivax*, 35 min for *P. malariae*, and 36 min for *P. ovale*. Accordingly, in comparison to the results obtained by microscopy, LAMP had a similar sensitivity and a greater specificity and LAMP yielded results similar to those of nested PCR in a shorter turnaround time. Because it can be performed with a simple technology, i.e., with heat-treated blood as the template, reaction in a water bath, and inspection of the results by the naked eye because of the use of a fluorescent dye, LAMP may provide a simple and reliable test for routine screening for malaria parasites in both clinical laboratories and malaria clinics in areas where malaria is endemic.

The rapid and accurate diagnosis of malaria presents a challenge in most countries where it is endemic. Of the four *Plasmodium* species, *Plasmodium falciparum*, infection with which can be fatal, must be identified promptly and differentiated from the other *Plasmodium* species that produce human disease (12). In addition, most regions where malaria is endemic feature infections involving two or more of these species; these mixed infections often go unrecognized or underestimated (30). Failure to detect mixed infections could result in inadequate or incorrect treatment and may result in severe disease (11). There is therefore an urgent need to develop diagnostic methods that are simple, sensitive, and species specific.

Currently, the conventional method for the diagnosis of malaria is microscopic examination of thin and/or thick blood smears. Although light microscopy has a relatively high sensitivity and specificity and also provides parasite density, the stage, and species differentiation, this method is labor-intensive, requires well-trained experts, and may result in therapeutic delays. To improve the speed and precision of malaria diagnosis in areas where standard laboratory diagnosis is not available, researchers have developed malaria rapid diagnostic tests based on antigen-capture immunochromatographic technologies (12, 16). When they are in good condition, some products can achieve sensitivity for the detection of *P. falciparum* infection similar to that of expert microscopists (3, 10). However, the sensitivity can vary between products (14), and a species-specific product is available only for *P. falciparum*. Very long observation times and considerable expertise are required for a correct diagnosis by microscopy under several circumstances: when the level of parasitemia is low, during mixed infection, after drug treatment, and during the chronic phase of the infection. Therefore, this situation can lead to false-negative results or unreliable species determinations (1).

Subsequently, molecular methods based on DNA amplification, such as nested PCR and real-time quantitative PCR, were developed for the diagnosis of malaria. Compared to microscopy, these methods have demonstrated higher sensitivity, detecting one to five parasites/μl of blood, and greater specificity for mixed infection (7, 19, 22, 24–27). However, the long turnaround time, high cost, and availability only in well-equipped laboratories render this technology inadequate for routine diagnosis in hospital laboratories and field clinics in areas where malaria is endemic (4).

Recently, a new, rapid, simple, and sensitive technique called loop-mediated isothermal amplification (LAMP) was developed (17). LAMP is a nucleic acid amplification method that relies on autocycling strand-displacement DNA synthesis performed with Bst DNA polymerase. The amplification products are stem-loop DNA structures with several inverted repeats of the target and structures with multiple loops. The

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▽ Published ahead of print on 13 June 2007.

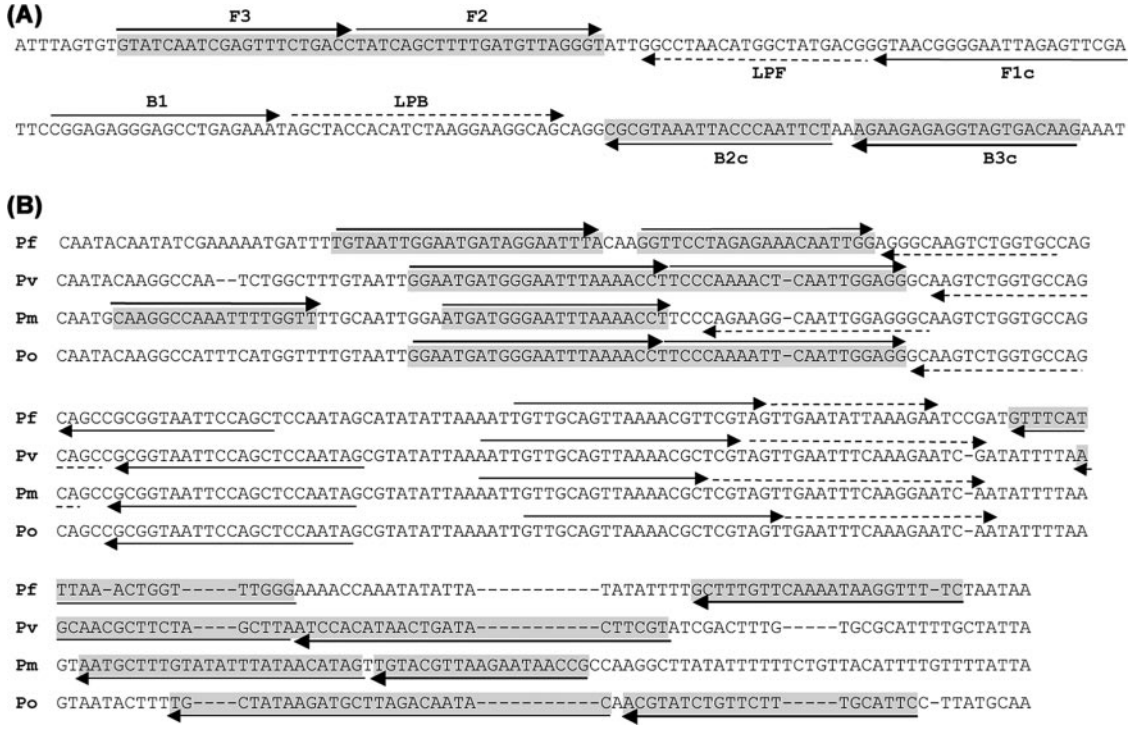


FIG. 1. Location and sequence of LAMP targets and priming sites for *Plasmodium* genus (A) and four *Plasmodium* species (B). (A) The locations of the priming sites by the *Plasmodium* genus-specific primer set in the reference sequence (GenBank accession no. M19173.1) are indicated by arrows. (B) Partial sequence alignment of the 18S rRNA genes of four human malaria parasites, *P. falciparum* (Pf; GenBank accession no. M19173.1), *P. vivax* (Pv; GenBank accession no. U03079), *P. malariae* (Pm; GenBank accession no. M54897), and *P. ovale* (Po; GenBank accession no. L48986), along with the species-specific primer annealing sites.

principal merit of this method is that no denaturation of the DNA template is required (15), and thus, the LAMP reaction can be conducted under isothermal conditions (ranging from 60 to 65°C). LAMP requires only one enzyme and four primers that recognize six distinct target regions. The method produces a large amount of amplified product, resulting in easier detection, such as detection by visual judgment of the turbidity or fluorescence of the reaction mixture (13).

Several investigators have reported on LAMP methods for the rapid identification of *Plasmodium*, *Trypanosoma*, and *Babesia* and have commended the usefulness of the LAMP assay (5, 8, 21, 29). Poon et al. estimated that the cost of running a LAMP assay is about 1/10 of that of the normal PCR method for *P. falciparum* detection (21). The biggest reduction in cost and time came from simple sample preparation, with no requirement for previous DNA extraction (6). Briefly, simple heating of the infected blood at 99°C for 10 min was enough to prepare the DNA template for LAMP without any inhibition of the reaction (21). However, to date, the use of LAMP for the detection of malaria parasites for clinical diagnosis has been validated only with acute falciparum malaria patients (21). Although *P. falciparum* is the most important cause of severe disease, its geographic distribution overlaps those of *P. vivax*, *P. malariae*, and *P. ovale*; and therefore, a method that allows the rapid detection and identification of all four species infecting humans would be preferable.

In the present study, we describe a LAMP for the clinical detection and identification of *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*. The technique was evaluated with blood samples obtained from field clinics. The results of LAMP were compared to those of conventional microscopy and nested PCR.

MATERIALS AND METHODS

Patient samples. Sixty-eight samples that were positive for malaria parasites by microscopy were collected from the malaria clinics of Mae Sod and Mae Kasa in northwestern Thailand. In addition, 53 samples that were negative by microscopy were collected from Kong Mong Tha, a village in Kanchanaburi Province in western Thailand. The blood samples were tested by nested PCR and LAMP. Each of the molecular tests was carried out and interpreted by independent researchers (nested PCR at the Armed Forces Research Institute of Medical Sciences, Thailand, and LAMP at the Cell-Free Science and Technology Research Center, Ehime University, Japan) blinded to the origins of the specimens and the laboratory results.

Conventional microscopy. Thick blood smears were examined under ×1,000 magnification by microscopists with extensive experience in the identification of malaria parasites. The parasite density per 500 leukocytes was counted and was then calculated as the number of parasites per microliter by assuming a leukocyte count of 7,000/μl. The initial thick film was considered negative if no parasites were seen after 500 leukocytes were counted.

DNA extraction. The DNA template for the nested PCR and LAMP was prepared as described previously (20). Twenty-five to 50 μl of human blood was blotted as a single spot and dried on filter paper. A single blood spot from each filter paper was excised and then incubated 4 h at room temperature and/or overnight at 4°C in 1 ml of 0.5% saponin in phosphate-buffered saline (PBS). The filter paper was washed for 30 min in PBS at 4°C and transferred into new tubes containing 200 μl of 5% Chelex-100 (Bio-Rad, Hercules, CA), and the tubes were vortexed for 30 s. The mixture was incubated at 56°C for 15 min, vortexed for 30 s, heated at 100°C for 15 min to elute the DNA, vortexed, and centrifuged

TABLE 1. Primer sets used for amplification of 18S rRNA genes in LAMP

Species	Primer	Sequence (5′→3′)
Plasmodium genus	F3	GTATCAATCGAGTTTCTGACC
	B3c	CTTGTCACTACCTCTCTTCT
	FIP (F1c-F2)	TCGAACTCTAATTCCCCGTTACCTATCAGCTTTTGATGTTAGGGT
	BIP (B1-B2c)	CGGAGAGGGAGCCTGAGAAATAGAATTGGGTAATTTACGCG
	LPF	CGTCATAGCCATGTTAGGCC
	LPB	AGCTACCACATCTAAGGAAGGCAG
P. falciparum	F3	TGTAATTGGAATGATAGGAATTTA
	B3c	GAAAACCTTATTTTGAACAAAGC
	FIP (F1c-F2)	AGCTGGAATTACCGCGGCTG GGTTCCTAGAGAAACAATTGG
	BIP (B1-B2c)	TGTTGCAGTTAAAACGTTCGTAGCCCAAACCAGTTTAAATGAAAC
	LPF	GCACCAGACTTGCCCT
	LPB	TTGAATATTAAAGAA
P. vivax	F3	GGAATGATGGGAATTTAAAACCT
	B3c	ACGAAGTATCAGTTATGTGGAT
	FIP (F1c-F2)	CTATTGGAGCTGGAATTACCGCTCCCAAAACTCAATTGGAGG
	BIP (B1-B2c)	AATTGTTGCAGTTAAAACGCTCGTAAGCTAGAAGCGTTGCT
	LPF	GCTGCTGGCACCAGACTT
	LPB	AGTTGAATTTCAAAGAATCG
P. malariae	F3	CAAGGCCAAATTTTGGTT
	B3c	CGGTTATTCTTAACGTACA
	FIP (F1c-F2)	TATTGGAGCTGGAATTACCGCGATGATGGGAATTTAAAACCT
	BIP (B1-B2c)	AATTGTTGCAGTTAAAACGCCTATGTTATAAATATACAAAGCATT
	LPF	GCCCTCCAATTGCCTTCTG
	LPB	TCGTAGTTGAATTTCAAGGAATCA
P. ovale	F3	GGAATGATGGGAATTTAAAACC
	B3c	GAATGCAAAGAACAGATACGT
	FIP (F1c-F2)	TATTGGAGCTGGAATTACCGCGTTCCCAAAATTCAATTGGAGG
	BIP (B1-B2c)	GTTGCAGTTAAAACGCTCGTAGTGTATTGTCTAAGCATCTTATAGCA
	LPF	TGCTGGCACCAGACTTGC
	LPB	TGAATTTCAAAGAATCAA

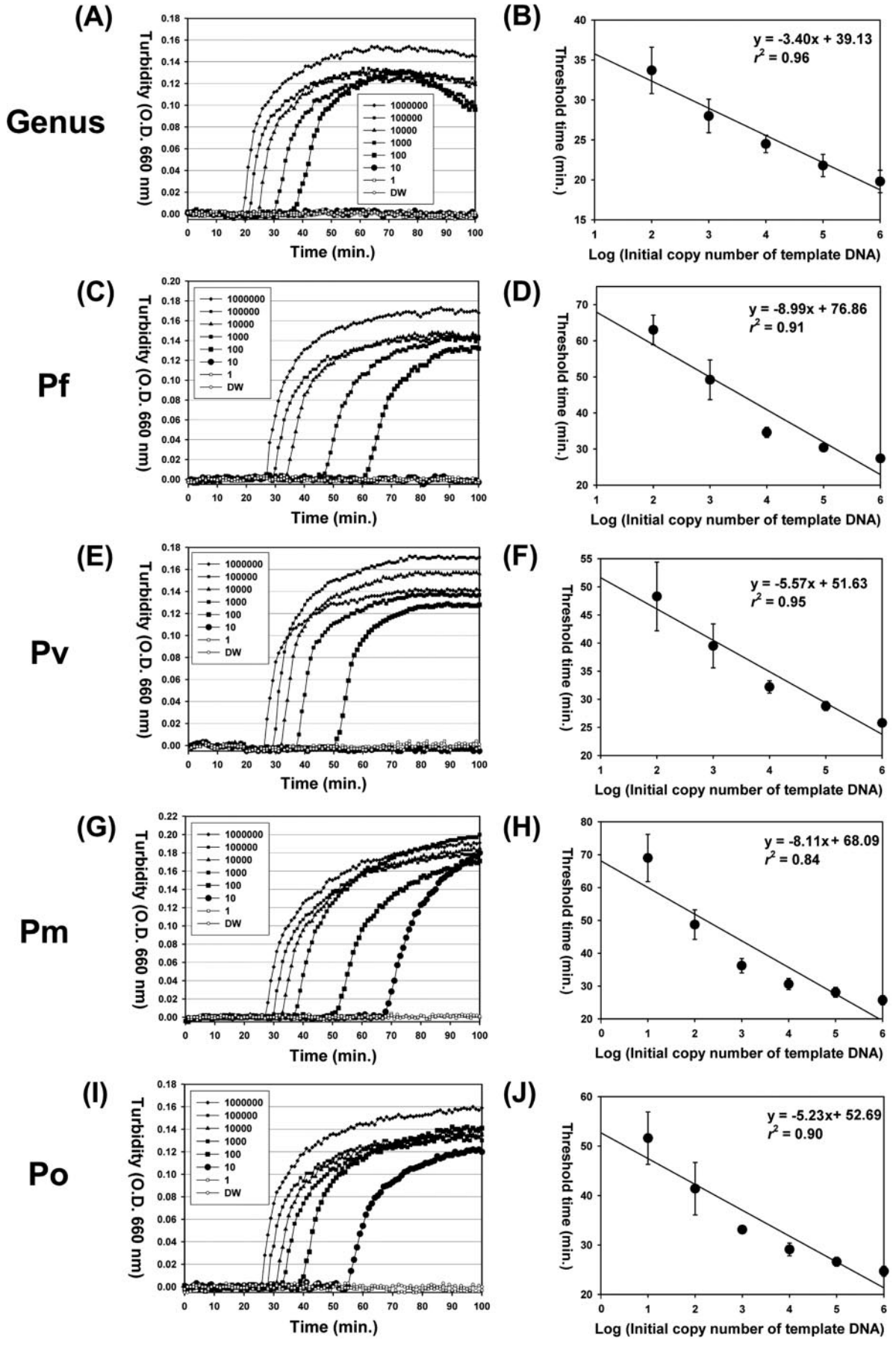
(10,000 × *g* for 5 min). The supernatant was either used immediately in the reaction or stored in aliquots at −20°C.

Nested PCR. For nested PCR, the species-specific nucleotide sequences of the 18S rRNA genes of *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale* were amplified as described previously (7), with slight modifications. In the first PCR, 2 μl of template DNA (corresponding to approximately 0.25 to 0.5 μl of blood) was added to a 20-μl PCR mixture that consisted of 0.4 μM each universal primer (P1 forward primer [5′-ACGATCAGATACCGTCGTAATCTT-3′] and P2 reverse primer [5′-GAACCCAAAGACTTTGATTTCTCAT-3′]), 200 μM each deoxynucleoside triphosphate, 25 mM $MgCl_2$, 1× PCR Gold Buffer II (50 mM KCl, 15 mM Tris-HCl, pH 8.0), and 0.25 U AmpliTaq Gold DNA polymerase. The DNA amplification was carried out under the following conditions: 94°C for 10 min and then 35 cycles at 92°C for 30 s, 60°C for 1.5 min, and 72°C for 1 min, followed by a final extension at 72°C for 5 min. The first PCR product was diluted 20-fold in sterile water. One microliter of this solution was used in the second amplification. The second PCR was performed at 94°C for 10 min and then 20 cycles at 92°C for 30 s, 60°C for 1.5 min, 72°C for 1 min, followed by a final extension at 72°C for 5 min with the P1 forward primer in combination with each species-specific reverse primer (*P. falciparum*, 5′-CAATCTAAAAGTCAC CTCGAAAGATG-3′; *P. vivax*, 5′-CAATCTAAGAATAAACTCCGAAGAGA AA-3′; *P. malariae*, 5′-GGAAGCTATCTAAAAGAAACACTCATAT-3′; *P. ovale*, 5′-ACTGAAGGAAGCAATCTAAGAAATTT-3′). The amplified products were visualized in 2% agarose gels stained with ethidium bromide. The expected band sizes were approximately 160 bp for the first PCR product and approximately 110 bp for the second one. To prevent cross-contamination, different sets of pipettes and distinct work areas were used for DNA template preparation, PCR mixture preparation, and DNA amplification. Moreover, 1 uninfected blood sample was included for every 10 samples processed.

LAMP conditions. The LAMP primer sets for *P. falciparum* were described previously (21). The remaining *Plasmodium* genus- and species-specific LAMP primer sets were designed on the basis of the genus- and the species-specific nucleotide sequences of the 18S rRNA genes of *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale* by use of the LAMP primer design software PrimerExplorer V3 (http://primerexplorer.jp/e/). The location and nucleotide sequence of each primer are shown in Fig. 1 and Table 1, respectively. The LAMP reaction was performed with a Loopamp DNA amplification kit (Eiken Chemical Co., Ltd., Tokyo, Japan). The reaction mixtures (25 μl) contained 1.6 to 2.4 μM each primers FIP and BIP, 0.2 μM each primers F3 and B3c, 0.8 μM each primers LPF and LPB, 2× reaction mixture (12.5 μl), Bst DNA polymerase (1 μl), and 1 to 2 μl of DNA sample (corresponding to approximately 0.125 to 0.5 μl of blood). The LAMP reaction was performed at 60°C for 100 min, and then the enzyme was inactivated at 80°C for 2 min.

Analysis of LAMP products. The LAMP reaction causes turbidity in the reaction tube proportional to the amount of amplified DNA. Therefore, the turbidity was observed with the naked eye. To confirm the sensitivity and the possibility of real-time LAMP quantification of *Plasmodium* parasites, the turbidity was also monitored with a Loopamp real-time turbidimeter (RT-160C; Eiken Chemical Co.). For further confirmation, 5 μl of the LAMP product was electrophoresed at 100 V in a 3% agarose gel, followed by staining with ethidium bromide and the use of a MassRuler DNA ladder marker (Fermentas Inc., Hanover, MD). The amplified LAMP product was validated by restriction analysis. On the basis of the restriction maps of the target sequences of each LAMP product, DdeI was selected for use for restriction analysis of genus *Plasmodium* products; HpyCH4V was selected for *P. falciparum*, *P. vivax*, and *P. malariae*; and AluI was selected for *P. ovale*. Following overnight digestion at 37°C, the digested products were analyzed by agarose gel electrophoresis.

Diagnostic threshold of LAMP results. LAMP was monitored through nephelometric analysis by recording the scattering light intensity reflected from a light source (wavelength, 660 nm) every 6 s with the help of the Loopamp real-time turbidimeter (RT-160C). The threshold value was defined as the value halfway between the mean maximum differential value (which represents a maximum velocity of the turbidity increment) − 3 standard deviations (SDs) for positive samples and the mean maximum differential value + 3 SDs for negative samples. Threshold values were as follows: for the *Plasmodium* genus, 0.0041; for *P.*


(A)
Genus
Turbidity (O.D. 660 nm)
Time (min.)
1000000
100000
10000
1000
100
10
1
DW
(B)
y = -3.40x + 39.13
r2 = 0.96
Threshold time (min.)
Log (Initial copy number of template DNA)
(C)
Pf
Turbidity (O.D. 660 nm)
Time (min.)
(D)
y = -8.99x + 76.86
r2 = 0.91
Threshold time (min.)
Log (Initial copy number of template DNA)
(E)
Pv
Turbidity (O.D. 660 nm)
Time (min.)
(F)
y = -5.57x + 51.63
r2 = 0.95
Threshold time (min.)
Log (Initial copy number of template DNA)
(G)
Pm
Turbidity (O.D. 660 nm)
Time (min.)
(H)
y = -8.11x+ 68.09
r2 = 0.84
Threshold time (min.)
Log (Initial copy number of template DNA)
(I)
Po
Turbidity (O.D. 660 nm)
Time (min.)
(J)
y = -5.23x+ 52.69
r2 = 0.90
Threshold time (min.)
Log (Initial copy number of template DNA)

falciparum, 0.0036; for *P. vivax*, 0.0040; for *P. malariae*, 0.0043; and for *P. ovale*, 0.0045. The threshold time represented the time for the turbidity to increase to the threshold value. Most of the positive samples tested multiple times showed positivity within 1 h. Therefore, a sample with a turbidity greater than or equal to the threshold value within 1 h, as determined with the turbidimeter, was considered positive.

Positive control plasmid DNA and sequencing. For sensitivity assessment, plasmids containing the target region of the 18S rRNA gene were constructed for each species for use in the LAMP reaction. The target DNA sequence was amplified with two LAMP primers (primers F3 and B3c) by PCR with ExTaq DNA polymerase (Takara Bio Inc., Otsu, Japan) and was then cloned into the pCR2.1-TOPO TA cloning vector (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Plasmid DNA purification was performed with a QIAprep Miniprep kit (QIAGEN, Hilden, Germany). The nucleotide sequences were determined with a BigDye Terminator sequencing kit (Applied Biosystems, Foster City, CA) by using an automated DNA sequencer (ABI PRISM 310 genetic analyzer; Applied Biosystems), according to the manufacturer's instructions. The resulting sequences were aligned by using the 18S rRNA sequences for the four species of *Plasmodium* deposited in GenBank to confirm that the target sequences were correct.

Analytical sensitivity and specificity of LAMP. To establish the minimum copy number (lower detection limit) of the target gene sequence detectable by LAMP, positive control plasmid DNAs were used. The standard curve for LAMP was constructed by using 10-fold serial dilutions of plasmid DNA (10^6 copies to 1 copy) to sterile water. For each standard, the copy number was plotted against the threshold time. The resulting plots were analyzed by linear regression, and the statistical significance of the r^2 values was analyzed by analysis of variance. Probabilities of less than 0.05 were considered statistically significant. The specificities of the genus- and the species-specific LAMP assays were evaluated with each control plasmid DNA and *P. falciparum* genomic DNA (gDNA) purified from strain NF54, *P. vivax* gDNA purified from strain Sal-I, *P. malariae* gDNA purified from strain Uganda, *P. ovale* LS type gDNA purified from a Thai isolate, *P. ovale* CDC type gDNA purified from a CDC strain, *P. knowlesi* gDNA purified from strain H, and *P. yoelii* gDNA purified from strain 17XNL.

Clinical sensitivity and specificity. The clinical sensitivity and specificity of the *Plasmodium* LAMP were calculated by using 121 whole-blood samples and microscopy as the reference standard method. Sensitivity was calculated as (number of true positives)/(number of true positives + number of false negatives), and specificity was calculated as (number of true negatives)/(number of true negatives + number of false positives).

RESULTS

Analytical sensitivity and specificity of *Plasmodium* genus- and species-specific LAMP. The sensitivity of LAMP for the genus *Plasmodium* and four species of malaria parasites, *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*, is shown in Fig. 2A, C, E, G, and I, respectively. An increase in the quantity of the initial template plasmid DNA shortened the threshold time, i.e., the amplification time required to exceed the threshold turbidity value. The detection limits for a positive turbidity signal were 10 copies for *P. malariae* and *P. ovale* and 100 copies for the genus *Plasmodium*, *P. falciparum*, and *P. vivax*. A plot of the threshold time versus the log of the initial template copy number showed a linear regression, with statistically significant regression coefficients: $r^2 = 0.96$ ($P = 0.004$) for the genus *Plasmodium*, $r^2 = 0.91$ ($P = 0.012$) for *P. falciparum*, $r^2 = 0.95$ ($P = 0.005$) for *P. vivax*, $r^2 = 0.84$ ($P = 0.010$) for *P. malariae*, and $r^2 = 0.90$ ($P = 0.004$) for *P. ovale* (Fig. 2B, D, F, H, and J, respectively).

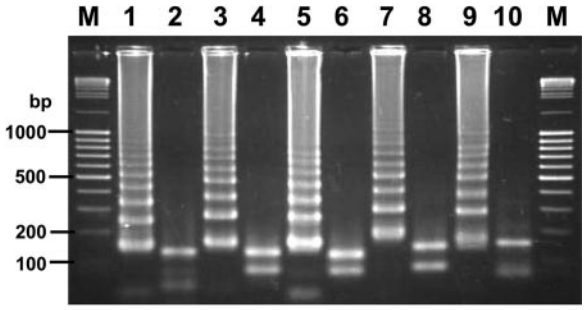


FIG. 3. Restriction analysis of *Plasmodium* genus- and species-specific LAMP products amplified from plasmid DNA containing each target 18S rRNA gene. The digestion products were run on a 3% agarose gel. Lane M, DNA ladder marker; lane 1, genus *Plasmodium* LAMP product; lane 2, DdeI digestion of genus *Plasmodium* product (123-, 44-, and 20-bp bands were expected); lane 3, *P. falciparum* LAMP product; lane 4, HpyCH4V digestion of *P. falciparum* product (130- and 79-bp bands were expected); lane 5, *P. vivax* LAMP product; lane 6, HpyCH4V digestion of *P. vivax* product (121- and 77-bp bands were expected); lane 7, *P. malariae* LAMP product; lane 8, HpyCH4V digestion of *P. malariae* product (142- and 84-bp bands were expected); lane 9, *P. ovale* LAMP product; lane 10, AluI digestion of *P. ovale* product (152- and 69-bp bands were expected).

The specificity of the *Plasmodium* species-specific LAMP was confirmed by using the gDNA of seven species of *Plasmodium*. Each species-specific LAMP amplified only the target species. The specificity of the amplification was further confirmed by restriction enzyme digestion of the LAMP products. As depicted in Fig. 3, the sizes of the resultant digestion products were in good agreement with the predicted sizes. The *Plasmodium* genus-specific LAMP amplified all four human malaria parasites (*P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale* LS and CDC types), the rodent malaria parasite (*P. yoelii* 17XNL), and the simian malaria parasite (*P. knowlesi*); all were detected within a 1-h reaction time (data not shown).

FIG. 2. Sensitivities of *Plasmodium* genus- and species-specific real-time LAMP assays performed with serial dilutions of plasmid DNA (10^6 copies to 1 copy per reaction) containing an 18S rRNA gene. (A) Amplification with a *Plasmodium* genus-specific primer set. One representative result of four replicates is shown. Samples contained a plasmid harboring the *P. falciparum* 18S rRNA gene. (B) Plot of the mean threshold time of the *Plasmodium* genus-specific LAMP. The error bars represent the standard errors of the mean values from four replicates. The plot of the mean threshold time against the log of the input DNA fit a linear function ($r^2 = 0.96$). (C) Amplification with the *P. falciparum* species-specific primer set. One representative result of four replicates tested with the plasmid harboring the *P. falciparum* 18S rRNA gene is shown. (D) Plot of the mean threshold time of the *P. falciparum* species-specific LAMP from four replicates, which fit a linear function ($r^2 = 0.91$). (E) Amplification with a *P. vivax* species-specific primer set. One representative result of four replicates tested with plasmid harboring *P. vivax* 18S rRNA gene is shown. (F) Plot of the mean threshold time of the *P. vivax* species-specific LAMP from four replicates, which fit a linear function ($r^2 = 0.95$). (G) Amplification with a *P. malariae* species-specific primer set. One representative result of four replicates tested with a plasmid harboring the *P. malariae* 18S rRNA gene is shown. (H) Plot of the mean threshold time of the *P. malariae* species-specific LAMP from four replicates, which fit a linear function ($r^2 = 0.84$). (I) Amplification with a *P. ovale* species-specific primer set. One representative result of four replicates tested with a plasmid harboring the *P. ovale* 18S rRNA gene is shown. (J) Plot of the mean threshold time of the *P. ovale* species-specific LAMP from four replicates, which fit a linear function ($r^2 = 0.90$). Genus, genus *Plasmodium* LAMP; Pf, *P. falciparum* LAMP; Pv, *P. vivax* LAMP; Pm, *P. malariae* LAMP; Po, *P. ovale* LAMP; O.D., optical density.

TABLE 2. Detailed comparison of microscopy, nested PCR, and LAMP for malaria parasite detection and species identification

Parasite(s) detected by each method (no. of samples)[a]		
Microscopy	Nested PCR	LAMP[b]
P. falciparum (12)[c]	*P. falciparum* (12)	*P. falciparum* (12)
P. falciparum + *P. vivax* (5)	*P. falciparum* + *P. vivax* (4), *P. vivax* (1)	*P. falciparum* + *P. vivax* (4), *P. vivax* (1)
P. vivax (34)	*P. vivax* (30), *P. vivax* (1), *P. vivax* (1), *P. ovale* (2)	*P. vivax* (30), *P. vivax* + *P. falciparum* (1), negative (1), *P. ovale* (2)
P. malariae (12)	*P. malariae* (8), *P. malariae* + *P. vivax* (1), *P. ovale* (1), *P. malariae* (1), negative (1)	*P. malariae* (8), *P. malariae* + *P. vivax* (1), *P. ovale* (1), *Plasmodium* spp.[d] (1), *P. malariae* (1)
P. ovale (5)	*P. ovale* (5)	*P. ovale* (5)
Negative (53)	Negative (50), *P. falciparum* (1), *P. vivax* (2)	Negative (50), *P. falciparum* (1), *P. vivax* (2)

[a] Results that were nonconcordant between nested PCR and LAMP are underlined.
[b] LAMP assays were run twice; in all cases the two experiments gave the same results.
[c] Each row provides the results obtained with identical DNA samples.
[d] Positive for the genus *Plasmodium* by LAMP but negative by all four species-specific LAMPs.

Clinical sensitivity and specificity: comparison of microscopy, nested PCR, and LAMP. The results of microscopy, nested PCR, and LAMP are given in Table 2. Among 121 patients in whom malaria was suspected, 68 (56.2%) were positive by microscopy (mean ± SD, 6,007 ± 5,761 parasites/μl; range, 210 to 24,164 parasites/μl). Of these, 12 patients (9.9%) were diagnosed with *P. falciparum* infection (812 to 24,164 parasites/μl), 34 (28.1%) with *P. vivax* infection (210 to 20,678 parasites/μl), 12 (9.9%) with *P. malariae* infection (294 to 5,253 parasites/μl), 5 (4.1%) with *P. ovale* infection (513 to 8,124 parasites/μl), and 5 (4.1%) with mixed *P. falciparum* and *P. vivax* infection (924 to 3,710 parasites/μl). The remaining 53 samples were negative. Microscopy was used as the reference standard for comparison with the other methods.

Nested PCR detected malaria parasites in 67 of 68 microscopically positive samples (sensitivity, 98.5%). LAMP with the genus-specific primer set also detected 67 of 68 samples positive by microscopy (sensitivity, 98.5%). Among the 53 samples that were negative by microscopy, both nested PCR and LAMP detected malaria parasites in 3 samples (specificity, 94.3%). The three samples positive by LAMP but negative by microscopy were reread by an expert microscopist; however, no parasite was detected in the slides. These samples were also retested by both nested PCR and LAMP. All three samples were again positive by LAMP and PCR; one was found to contain *P. falciparum* and the other two were found to contain *P. vivax.* Because of the agreement between PCR and LAMP, we believe that these three samples are true positives. Overall, both LAMP and nested PCR yielded results very similar to those of microscopy; the exceptions were 11 nonconcordant results (9.1%) each for nested PCR and for LAMP. These were mainly in the cases of *P. vivax*, *P. malariae*, *P. ovale*, or mixed infections. In contrast, only four samples (3.3%) had nonconcordant results between nested PCR and LAMP (Table 2).

LAMP yielded results in about 25.7 ± 4.9 min (mean ± SD; range, 19.4 to 52.9 min) for detection of the genus *Plasmodium* in the 67 samples that were positive by microscopy. The detection times were 31.7 ± 4.8 min (range, 25.8 to 44.9 min) for 17 *P. falciparum* samples, 30.6 ± 5.2 min (range, 25.4 to 46.6 min) for 37 *P. vivax* samples, 34.8 ± 4.8 min (range, 30.5 to 46.6 min) for 10 *P. malariae* samples, and 36.1 ± 6.8 min (range, 29.9 to 49.8 min) for 8 *P. ovale* samples. The average copy numbers of the target genes in the clinical samples were calculated on the basis of the linear regression formulas from the analysis of serial dilutions of plasmid DNA (Fig. 2B, D, F, H, and J). The average log copy numbers in clinical samples were 3.95 for the *Plasmodium* genus, 5.02 for *P. falciparum*, 3.78 for *P. vivax*, 4.10 for *P. malariae*, and 3.17 for *P. ovale*.

DISCUSSION

In regions where malaria is endemic, cases of mixed infections with malaria parasites are expected, so there is need for a test for species-specific identification that is more reliable than microscopy. Nested PCR is one such method; we have developed a simpler method for the diagnosis of clinical malaria diagnosis, including species identification, based on LAMP. The LAMP developed in this study has a sensitivity similar to that of microscopy and a specificity that is better than that of microscopy, and it yielded results similar to those of nested PCR for the detection of four species of human malaria parasites.

Since 96.7% of the results of LAMP were consistent with those of nested PCR and the sensitivity (98.5%) and specificity (94.3%) of both LAMP and nested PCR compared with the results of microscopy were equally high, we can propose that LAMP is as reliable as nested PCR for the clinical detection of four species of malaria parasites. Among five samples positive for both *P. falciparum* and *P. vivax* by microscopy, one was positive only for *P. vivax* by both nested PCR and LAMP. The level of *P. falciparum* parasitemia (approximately 0.01%) was lower than that of *P. vivax* parasitemia (approximately 0.05%) in this sample. Since the level of parasitemia was within the level detectable by nested PCR or LAMP, there may be another reason why this sample was negative for *P. falciparum* by both nested PCR and LAMP, such as the use of a smaller amount of blood or a lower efficiency of extraction of DNA from this specimen. Even though the LAMP results were in good agreement with those of nested PCR, there were some nonconcordant results. Among 12 samples positive for *P. malariae* by microscopy, 1 was negative by nested PCR and another 1 was negative by the species-specific LAMP. However, these two samples were successfully amplified by the *Plasmodium* genus-specific LAMP. One explanation is that the target sequences of the rRNA gene of *P. malariae* have variant sequences (9); thus, new primer sets for both nested PCR and

LAMP would be required to target regions that are universally conserved among *P. malariae* variants for reliable diagnosis. Among the samples found to contain *P. ovale* by both LAMP and PCR, microscopy had detected *P. ovale* in five of them, *P. vivax* in two samples, and *P. malariae* in the last sample. In contrast, both LAMP and nested PCR could successfully detect *P. ovale* in all eight samples, including both the LS type (six samples) and the CDC type (two samples), as confirmed by the *P. ovale* type-specific PCR method (28) (data not shown). On the basis of the comparable results between the LAMP and the nested PCR methods, as described above, the overall specificities of LAMP and nested PCR may be greater than that of microscopy.

The LAMP method can be used to quantify parasites in infected blood, although it is not a perfect assay for quantification. To quantify the parasites in infected blood by LAMP, we applied the average threshold time for the clinical blood samples to the formula of linear regression for each genus- and species-specific analysis. While there was a statistically significant linear correlation between the threshold time and the log of the initial copy number of template DNA, the linear correlation was rather poor for low copy numbers of template DNA (Fig. 2B, D, F, H, and J). Possible explanations are that (i) the longer incubation time before the detectable amplification caused by the lower template copy number makes the enzyme activity lower and (ii) the lower template DNA concentration, which decreases the annealing efficiency of the primers, may cause a delay in the threshold time. Although further improvement is needed, the statistically significant negative correlations between the threshold time and the parasite count by microscopy were obtained for the genus-specific LAMP-positive samples ($n = 67$), the samples positive for *P. vivax* only ($n = 30$), and the samples positive for *P. falciparum* only ($n = 12$) by using Spearman's rank correlation test (genus-specific LAMP, $P = 0.0002$; *P. vivax*-specific LAMP, $P < 0.0001$; *P. falciparum*-specific LAMP, $P = 0.0034$).

Diagnosis by LAMP does not require expensive reagents for DNA extraction, a turbidimeter, a thermal cycler, or skilled technicians. The template can be prepared by direct heat treatment of blood samples, without time-consuming and expensive DNA extraction with a commercial kit (21). Moreover, LAMP requires only a simple incubator, such as a heat block or a water bath that provides a constant temperature of 60°C, which makes it more economical and practical than nested PCR and real-time PCR. The white turbidity that results from magnesium pyrophosphate accumulation as a by-product of DNA amplification can be detected with the naked eye or a turbidimeter (13). Alternatively, inspection for amplification can be simply be performed with the naked eye by using SYBR green I, which turns green in the presence of amplified DNA. The results obtained by use of SYBR green I were consistent with those deduced by use of the real-time turbidimeter (18). Since the turbidity assay can be carried out in a closed system, the risk of contamination is lower than that when agarose gel electrophoresis is used. This is an additional advantage of LAMP for clinical use (2, 21, 23). Screening by genus-specific LAMP and then confirmation by a species-specific LAMP may provide a simple and reliable test for epidemiological surveillance. However, the routine screening for malaria parasites in both clinical laboratories and malaria clinics in areas where malaria is endemic may require a species-specific LAMP. Background epidemiological information will be helpful for local governments to decide how to apply LAMP for the diagnosis of malaria in each area.

In conclusion, the LAMP methods developed in this study can be useful for clinical diagnosis and active surveillance of malaria parasites in countries where malaria is endemic because it has a sensitivity and a specificity similar to those of nested PCR, requires minimal laboratory facilities, and is simpler and less expensive to perform than nested PCR. The on-site performance of LAMP at malaria clinics in the field will be required for further evaluation of this technique for the rapid diagnosis of malaria.

ACKNOWLEDGMENTS

We are grateful to the technical staff at the Entomology Department, AFRIMS, and the malaria clinic in Mae Sod, Tak Province, for support with blood collection, blood preparation, and microscopic examination and to Akira Oshima, Eiken Chemical Co., Ltd., Japan, for useful discussions.

This work was supported in part by Grants-in-Aid for Scientific Research 16406009 and 18390129 and Scientific Research on Priority Areas grant 16017273 from the Ministry of Education, Culture, Sports, Science and Technology of Japan and a Grant-in-Aid of the Ministry of Health, Labor and Welfare (grant H17-Sinkou-ippan-019), Japan, and was partially supported by U.S. Military Infectious Diseases Research Program.

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