original article

evaluation of a new rapid molecular diagnostic system for Plasmodium falciparum combined with DNA filter paper, loop-mediated isothermal amplification, and melting curve analysis

Mariko Yamamura*, Koichi Makimura1, and Yasuo Ota

Department of Internal Medicine, Teikyo University School of Medicine, Tokyo 173-0003, and

Teikyo University Institute of Medical Mycology, Teikyo University, Tokyo 192-0395, Japan

(Received August 25, 2008. Accepted November 12, 2008)

SUMMARY: Falciparum malaria is a fatal infection without immediate diagnosability or treatment. There are shortages of clinicians and examiners skilled in the treatment of malaria in non-endemic countries, including Japan. This study was performed to evaluate a novel rapid molecular diagnostic system consisting of loop-mediated isothermal amplification (LAMP) combined with DNA filter paper (FTA card) and melting curve analysis. Combining LAMP with melting curve analysis enabled diagnosis of Plasmodium falciparum more accurately with relative ease. FTA cards could be used to clarify problems regarding storage, infectivity, and transportation. The LAMP assay was carried out at a constant temperature of 63℃ for 90 min. The diagnostic system (malaria-LAMP) accurately diagnosed malaria (47 samples from Thailand and 50 from Zimbabwe) with 97.8% sensitivity and 85.7% specificity as compared with microscopic methods, indicating the usefulness of this combined system.

INTRODUCTION

Malaria is one of the most important tropical infectious diseases. From 350 to 500 million clinical episodes of malaria occur each year, and the disease is responsible for more than 1 million deaths annually (1). Especially, Plasmodium falciparum causes various complications and may be fatal. The range of areas inhabited by malaria-carrying mosquitoes is currently expanding due to global climate change (2). Therefore, there is a risk of a revival of malaria, not only as a consequence of the increasing number of imported cases with the increase in overseas travelers, but also as a result of the growth of habitats suitable for malaria-carrying mosquitoes. Thus, the rapid diagnosis of this disease is extremely important. Malaria has been diagnosed by microscopic examination using Giemsa stain. However, diagnosis by microscopic examination requires skill, and may be difficult in cases where preventative oral medication was taken before onset, as well as in cases where the level of malaria parasite infection is low (3). In countries where malaria is not endemic, there are shortages of physicians and microscopy-skilled laboratory staff specializing in malaria, and thus the diagnosis of malaria is difficult. In recent years, simple kits have been developed to detect malaria parasite-specific proteins and enzymes as auxiliary diagnostic procedures. However, these kits have a number of problems, such as false positives in patients with rheumatoid factor or in the elderly (4). In addition, these kits are not commercially available in Japan, and are not in common use. Methods for genetic diagnosis of malaria using PCR are also being developed, and there have been reports of cases in which various types of malaria parasites were identified and multiple infections were diagnosed by detecting malaria-specific DNA sequences. However, PCR-based methods require complex procedures, expensive inspections, and several hours before a diagnosis can be made. Therefore, it is necessary to develop a simple, stable, and rapid method for diagnosing malaria. Loop-mediated isothermal amplification (LAMP) (5) meets these requirements and has been put to practical use as a sensitive detection method for many infections (severe acute respiratory syndrome [SARS] coronavirus [6], West Nile virus [7], avian influenza virus [8], norovirus [9], and Legionella bacteria [10]). The LAMP method for P. falciparum malaria has already been reported by Poon et al. (11) and Han et al. (12) The LAMP method is simple to perform and is less influenced by inhibitors than polymerase chain reaction (PCR) is. To identify LAMP products, it is necessary to develop methods such as restriction enzyme digestion and the confirmation of specific DNA ladder formation by electrophoresis (12). However, to avoid contamination from dispersion of the amplification products during electrophoresis, there has been a demand for simpler and safer technology to identify amplification products. To safely distinguish specific amplification products from non-specific primer-dimers often seen in LAMP, melting temperature curve analysis was used. P. falciparum malaria-specific LAMP primers were designed and FTA cards (Whatman, Kent, UK) were used to allow storage, transportation, and extraction of template DNA safely, stably, and easily from blood samples at room temperature. In this study, a new rapid molecular diagnostic system for P. falciparum was developed by combining DNA filter paper (FTA cards), LAMP, and melting curve analysis.

The applicability of this system was examined using 97 specimens from Thailand and Zimbabwe where P. falciparum malaria is endemic.

MATERIALS AND METHODS

P. falciparum strain and DNA extraction: (i) P. falciparum strain: The malaria parasite used for DNA extraction was a strain of P. falciparum (FCN-1/Nigeria [13])
maintained at the Department of Microbiology, School of Medicine, Teikyo University, Tokyo, Japan.

(ii) DNA extraction: The DNA of *P. falciparum* was extracted as follows. The strain was cultured for 3 days in flat-bottomed 96-well microtiter plates. The total volume of medium (RPMI1640 with 10% human serum) in each well was 200 µl, each of which contained 10% erythrocytes and 1% infected blood cells. The supernatant fluid in each well was discarded, and the remaining erythrocytes (including *P. falciparum*) were harvested and frozen at −80°C. The contents were thawed to induce hemolysis, after which distilled water and an equal amount of 1.8% NaCl were added. The mixture was centrifuged at 2,500 rpm for 10 min and the supernatant was discarded. These steps were repeated several times until the supernatant changed from pink to colorless. After heating the remaining pellet at 100°C for 15 min, the DNA was extracted by phenol/chloroform extraction and ethanol precipitation, and used as a template for amplification.

(iii) Preparation of plasmid for sensitivity test: The target sequence for LAMP assay was amplified by PCR using a primer pair (MAL-1, 5′-ACAGATTAAGCCATGCAAGTGA-3′; and MAL-2, 5′-AAACTTCCTTGTGTTAGATACAGAATCAC-3′) designed according to the 18S rDNA gene of *P. falciparum* (DDBJ/EMBL/GenBank accession no. AL031746). The PCR products were cloned and transformed using an Original TA Cloning Kit® (Invitrogen, Carlsbad, Calif., USA) in accordance with the manufacturer’s instructions. The cloned plasmids were extracted from the transformed *Escherichia coli* using an Aurum Plasmid Mini Kit® (Bio-Rad Laboratories, Hercules, Calif., USA) in accordance with the manufacturer’s instructions. These plasmids, which were diluted from 10,000 to 5 copies/tube by doubling dilution, were used as control plasmids (c-plasmid) for the sensitivity test to determine the detection limit.

Clinical samples and DNA elution: (i) Clinical samples: Samples of suspected malaria from Thailand (*n* = 47) and Zimbabwe (*n* = 50) were prepared by local staff in each area, who took blood samples from patients and directly deposited a few drops of the sample onto FTA cards for molecular diagnosis. As negative controls, normal whole blood samples on FTA cards from 59 individuals with no history of malaria infection (20 healthy individuals and 39 febrile patients, including those with leukopenia) were used. The study protocol was approved by the corresponding Ethical Committees of Teikyo University School of Medicine. All patients gave their informed consent to participate in the study.

(ii) Preparation of blood smears and microscopic examination: For thin blood smears, a single drop of whole blood was placed on a glass slide, immediately spread using a coverglass, and put aside to dry. After methanol fixation, the smears were stained with Giemsa solution.

For thick blood smears, one or two drops of whole blood were placed on a glass slide. The blood was spread to about 1 cm in diameter and put aside to dry. After drying, these smears were stained with Giemsa solution.

Thin and thick blood smears were prepared from 47 patients with suspected malaria infection in Thailand; a microscopist in Thailand provided the results of microscopic examination. Only thick blood smears were prepared from 50 patients with suspected malaria infection in Zimbabwe, and the results of the microscopic examination were unknown. All 97 blood smears were examined for the presence of malaria parasites at the Department of Microbiology, School of Medicine, Teikyo University. To calculate the infection rate in a thin blood smear, the number of infected erythrocytes was counted in increments of 10,000 erythrocytes. To calculate the infection rate in a thick blood smear, the number of infected erythrocytes was counted in increments of several leukocytes, depending on the smear. The results of microscopic examination were compared with those of malaria-LAMP assay using FTA cards.

(iii) DNA extraction from FTA card samples, and transport of FTA card samples: FTA cards with whole blood deposits were dried and stored at room temperature, and were then mailed to Japan. Disks 2.0 mm in diameter were cut from the bloodstained areas with a Harris Micro Punch (2.0 mm; Whatman). Each disk was washed three times with 200 µl of FTA purification reagent and twice with 200 µl of TE-1 buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). The disks were dried and used directly as the DNA template.

Oligonucleotide primers for LAMP reaction: The 18S rRNA gene nucleotide sequences were obtained for eight *Plasmodium* spp., including four species of human malaria parasite (*P. falciparum*, accession no. AL031746, NC004325; *P. ovale*, AB182489, AB182493; *P. vivax*, U03079, AY579418; *P. malariae*, AF145336; *P. knowlesi*, AY327557) from DDBJ/EMBL/GenBank Database, and by using DNA analysis software (GENETYX®-Mac ver.13; GENETYX, Tokyo, Japan). Six sites of specific nucleotide sequences of *P. falciparum* were determined as the primer sets. One pair of primers (F3 and B3) was designed based on the sequences at two sites in the outermost region, while another pair of primers (FIP and BIP) was designed to connect the sequences at two sites in the inner region. Thus, four primers were prepared for LAMP assay to detect *P. falciparum* genes. In addition, one pair of loop primers corresponding to this malaria-LAMP system was also designed. The location and nucleotide sequence of each primer are shown in Table 1.

The results of BLAST (Basic Local Alignment Search Tool) (14) analysis against DDBJ/EMBL/GenBank databases indicated that the sequences of malaria-LAMP primers were specific only for *P. falciparum*. Species-specific LAMP primer sets for *P. falciparum* (11) and *P. vivax* (12) were also used. The specificity of malaria-LAMP primers was tested with each of four species of human malaria DNA. Template DNAs for three malaria parasites, *P. vivax*, *P. malariae*, and *P. ovale*, were provided by Dr. Tsuibo, Ehime University, Japan (12).

Malaria-LAMP condition and melting curve analysis: The LAMP reaction was performed with a Loopamp DNA amplification kit (Eiken Chemical Co., Ltd., Tokyo, Japan). The reaction mixtures (25 µl) contained 40 pmol of primers FIP and BIP, 40 pmol of primers loopF and loopB, and 10 pmol of primers F3 and B3, 2 × reaction mixture (12.5 µl), 1 µl Bst DNA polymerase, 0.5 µl of YO-PRO®-1 iodide (Molecular Probes, Eugene, Oreg., USA), 1 to 3 µl of DNA sample, and distilled water (made up to a final volume of 25 µl) in a 0.2 ml Eppendorf tube. The LAMP reaction was performed at 63°C for 90 min. As negative controls, several tubes containing distilled water supplied with the kit were also prepared per the reaction. The c-plasmid was used as a positive control at 10 copies/reaction. LAMP products were identified by melting curve analysis. Melting temperature curves were obtained using the default settings recommended by the manufacturer in the presence of a fluorescent intercalator (YO-PRO®-1 iodide). Amplification and melting curve analysis were carried out using a Genopatter Analyzer GP1000 (Yamato Scientific Co., Ltd., Tokyo, Japan).
RESULTS

Sensitivity and specificity: The malaria-LAMP system was able to detect c-plasmid at 10 copies/tube, but not at 5 copies/tube (Fig. 1). All of the melting curve peaks (10 - 10^4 copies/tube) obtained in this sensitivity test with c-plasmid were consistent (Fig. 2). The time required to amplify 10^4 copies/tube was 35 min, and that to amplify 10 copies/tube, which was decided as the detection limit, was 60 - 80 min.

The malaria-LAMP system was specific only for *P. falciparum* genomic DNA and c-plasmid, not for the three other human pathogenic malaria parasites. The peaks of the melting temperature curves were identical in genomic DNA and c-plasmid products. However, no melting temperature curves were obtained from other species of malaria parasite, because no products were amplified from their template DNAs (Fig. 3).

Application of malaria-LAMP to clinical samples: (i)

Results of microscopic examination: Thailand (Table 2): A local microscopist examined all 47 samples of clinically suspected malaria infection, and found that 29 samples indicated the presence of *P. falciparum* parasites, while 4 samples

---

**Table 1. Malaria-LAMP primers**

| A | F3                       | gaggigaatcattagattctt |
|   | FIP(F1c+F2)              | cacctaggtggataggtaattgtagcatactcttccaatt |
|   | F1c                      | ttgcctaatctttggcactaggtgaagactactgcgaaagctactgcgaaagcat |
|   | F2                       |
| B | B3c                      | ttccgtaattctttactttc |
|   | BIP(B1+B2c)              | gtagcatttcttgagaatgttgccgccagacaaggctactgcgaaagcattga |
|   | Loop F                   | ggtatatcgtgatctctactct |
|   | Loop B                   | gaattgctactctctctct |

A: Sequences of malaria-LAMP primers FIP, BIP, F3, B3, LoopF, and LoopB are used in this study.

B: Locations and sequences of malaria-LAMP target sequence and primer binding sites of *P. falciparum*. The locations of the primer binding site in the reference sequence (18S rRNA, GenBank accession no. AL031746) are underlined. Base numbers in the reference sequence are indicated at both ends.
showed the presence of \textit{P. vivax} parasites in both thick and thin blood smears. Twelve samples (\#9, \#28, \#30 - 32, \#35 - 37, \#40 - 43) showed the presence of \textit{P. falciparum} parasites only in thick blood smears (i.e., they were absent in thin blood smears). The local microscopist was not able to detect parasites in 2 samples (\#2, \#33), but a microscopist at Teikyo University detected \textit{P. falciparum} parasites in both of these samples. The diagnoses of the malaria parasite species were the same between Thailand and Teikyo University, although the infection rate in thin blood smears differed between the microscopists.

Zimbabwe (Table 3): A microscopist at Teikyo University examined 50 samples of clinically suspected malaria infection, and found that 47 samples were positive for the presence of \textit{P. falciparum} parasites in thick blood smears, whereas 3 samples (\#95 - 97) did not have any parasites.

\textbf{Results of malaria-LAMP of clinical samples:} Malaria-LAMP was performed using template DNA extracted from FTA card specimens of thin and thick blood smear samples from which \textit{P. falciparum} was detected (90 cases; 43 from Thailand, 47 from Zimbabwe). \textit{P. vivax} was detected in samples from 4 cases. However, remaining 3 cases were negative on
microscopic examination. Melting temperature curves were used to identify the amplification product. The samples were defined as negative if the peaks of the melting temperature curves did not reach 2/5 the height of the peaks of the positive control using c-plasmid. All 90 cases of falciparum malaria showed gene amplification, but the peaks of the melting temperature curves of 2 cases (#43, #69) differed from those of *P. falciparum*, and the sensitivity of malaria-LAMP was 97.8%. Case #43 showed a very low infection rate of 0.0000654% on the thin blood smear sample.

In addition, 1 (#47) of the 4 cases of vivax malaria showed gene amplification, but the peaks on the melting temperature curves differed from those of *P. falciparum*, and thus could be distinguished from falciparum malaria. *P. vivax* DNA was amplified from this sample with *P. vivax*-specific LAMP reaction (12). Of the 3 cases that were negative on microscopic examination, 1 case (#97) showed gene amplification, while the remaining 2 (#95, 96) did not. *P. falciparum* DNA was also amplified from #97 with another *P. falciparum*-specific LAMP reaction (11). Assuming that the 3 cases that were negative on microscopic examination as diseases other than falciparum malaria, the specificity compared to microscopic examination was 85.7%.

The results of the microscopic examinations, as well as those of malaria-LAMP for specimens from Thailand and Zimbabwe, are shown in Tables 2 and 3, respectively.

The time required to confirm the reaction in clinical specimens using FTA cards was 33–85 min (average, 49 min).

Non-specific amplification and characteristics of melting temperature curves: When this reaction was performed for FTA cards from 59 non-malaria cases, amplification was confirmed in 7 cases and in the negative control in one reaction. However, none of the melting temperature curves matched that for *P. falciparum*, so they were classified as non-specific reactions. These non-specific amplifications had at least one of the following characteristics: (i) no reproducibility of amplification; (ii) no reproducibility of peak positions of the melting temperature curves.

**DISCUSSION**

A number of reports have indicated that PCR’s minimal detection level of PCR is from 1 to 10 copies/reaction (15-18). Therefore, the sensitivity of the system presented here is equivalent to that of PCR. The levels of microscopic examination by skilled laboratory staff are 10-30 parasites/μl in thick blood smear samples under optimal conditions (15), and theoretically the present method is equivalent to or better than microscopic examination. When samples of 125 μl of blood, a feasible sample volume according to the manufacturer’s literature, are applied to FTA cards, the expected erythrocyte number on the disc is 3.6 × 10⁴, and the detectable infection rate of malaria is theoretically as low as 0.000278% when the detection limit in the present system is 10 copies. The sensitivity for the clinical sample compared to the microscopic examination was 97.8%. The LAMP method is superior to PCR-based methods in that the procedures involved are less complex. The LAMP method is also superior to the microscopic method, which is affected by the skill of the individual microscopist, in its reproducibility. The present method could detect parasites in cases where the infection was undetectable on microscopic examination in thin blood smears. The specificity of this system compared to microscopic examination was 85.7%, because only 1 sample (#97) was shown to be negative on microscopic examination but was positive in malaria-LAMP. However, sample #97 was positive in another *P. falciparum*-specific LAMP reaction reported by Poon et al. (11). Therefore, considering #97 to be from a falciparum malaria patient, the specificity of the present method would be 100%.

Turbidimetry to visual observation (11,12), with which the malaria-LAMP products are identified, is both simple and accurate, but identification is expected to be difficult in some clinical samples. In addition, as the present paper indicated, there is a possibility of non-specific amplification even for the LAMP method, and therefore the amplification products should always be checked. Melting curve analysis can automatically differentiate between specific and non-specific amplification using commercially available real-time PCR analyzers. As reported previously (19), the process of agarose gel electrophoresis tends to cause severe contamination by dispersion of LAMP products, so melting-curve analysis was used rather than electrophoresis to confirm specific malaria-LAMP products. Melting temperature curves have been used to identify PCR products in diagnosing malaria or in identifying the species of malaria parasite (15,16). Although the application of melting curve analysis for LAMP products has already been reported to identify pneumocystis (19), this is the first report to identify the LAMP products of malaria using melting curves.

The cells in a sample are destroyed after they are dropped onto the FTA cards (specially processed filter paper) with DNA fixation. The cards can therefore be stored at room temperature and mailed. Indeed, the cards were obtained by mail from two countries in the present study (Thailand and Zimbabwe). In hospitals with no examination apparatus or skill for identifying malaria, mailing the FTA card presents a useful diagnostic opportunity. Zhong et al. (20) reported the usefulness of the FTA card in detecting malaria parasite DNA by PCR. Mas et al. (21) reported that the DNA recovery rate in samples of FTA cards was better in the presence of ethylenediaminetetraacetic acid (EDTA), and tubes containing EDTA were used to collect blood samples. The DNA synthetic enzyme in the LAMP method is unaffected by the presence of hemoglobin or excessive salt concentration, etc., while Taq DNA polymerase used in PCR (22) is affected by these conditions. Poon et al. (11) reported that they were able to use heat-treated whole-blood specimens directly as a DNA template for LAMP assay. However, in a preliminary experiment, we could not obtain stable results through DNA extraction by heat treatment, unlike the case using FTA cards. If pretreatment of FTA cards can be performed in 30 min, it would take approximately 2 h from blood sampling to obtain the results. Due to its rapidity and ease of use, as well as the lack of a requirement for specialized skill, this system will be useful in countries where malaria is not endemic, such as Japan. Using this system in combination with microscopic examination, it will be possible to obtain a more accurate diagnosis of malaria in clinical practice.

**ACKNOWLEDGMENTS**

We wish to express our gratitude to Prof. Kiseko Kamei of the former Department of Microbiology and Immunology, Teikyo University School of Medicine (currently Teikyo Heisei University), Dr. Ryuichi Fujisaki and Associate Prof. Hajime Nishitani of Internal Medicine, Teikyo University School of Medicine, and Mr. Masato Ishihara of Yamato Science Co. Ltd. for their valuable support. In addition, we wish to thank Prof. Takafumi Tsuboi, Cell-Free Science and Technology Research Center and Venture Business Laboratory, Ehime University, for providing malaria DNA.
REFERENCES


