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Application of loop-mediated isothermal amplification assay combined with lateral flow dipstick for detection of *Plasmodium falciparum* and *Plasmodium vivax*

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ABSTRACT

Malaria is largely a preventable and curable disease. However, a delay or an inappropriate treatment can result in serious adverse outcomes for patient. Rapid, simple and cost-effective diagnostic tests that can be easily adapted and rapidly scaled-up at the field or community levels are needed. In this study, accelerated detection methods for the *Plasmodium falciparum* (*Pf*) and *Plasmodium vivax* (*Pv*) dihydrofolate reductase–thymidylate synthase were developed based on the loop-mediated isothermal amplification (LAMP) method. The developed methods included the use of species-specific biotinylated primers to amplify LAMP amplicons, which were then hybridized to specific FITC-labeled DNA probes and visualized on a chromatographic lateral flow dipstick (LFD). The total LAMP–LFD assay time was approximately 1.5 h. The LAMP–LFD assays showed similar detection limit to conventional PCR assay when performed on plasmid DNA carrying the malaria *dhfr-ts* genes. The LAMP–LFD showed 10 folds higher detection limit than PCR when performed on genomic DNA samples from *Pf* and *Pv* parasites. The *dhfr-ts* LAMP–LFD assays also have the advantages of reduced assay time and easy format for interpretation of results.

1. Introduction

Malaria is a serious mosquito-borne infectious disease of human and other animals caused by parasitic protozoan from the genus *Plasmodium*. In 2010, 219 million people were afflicted with malaria resulting in an estimated 660,000 deaths, mostly in children younger than 5 years old [1]. The disease is widespread in tropical and subtropical regions and most of human morbidity is caused by *Plasmodium falciparum* (*Pf*) and *Plasmodium vivax* (*Pv*) parasites. Early diagnosis at the onset of fever and appropriate treatment are key strategies to reduce clinical complications and possible death.

Traditionally, diagnosis of malaria infection is done by microscopic examination of thick and thin blood-films prepared from suspected patient, but the sensitivity and specificity of this method are highly dependent on the skill of an individual microscopist. The average detection limit for examination of a thick blood film by an experienced microscopist has been reported to be around 50 parasites per microliter (P/μL) of blood, while the average sensitivity of field microscopy technicians has shown to be around 0.01% infected RBC or 500 P/μL during routine clinical examination [2].

Rapid diagnostic tests (RDTs) based on antibody detection of specific malaria antigens are now widely used for diagnosis in endemic countries. Most brands of RDTs have been shown to perform well with low false positives and false negatives when used on samples with high parasite density (2000–5000 P/μL) [3]. The disadvantages of these antibody-based tests are that they cannot quantitate parasite burden and may give false positive results due to the long half-life of the target protein antigen, Histidine Rich Protein II (HRP II), found in blood circulation of patients even after parasite clearance [4].

A number of nucleic acid-based detection methods such as polymerase chain reaction (PCR), multiplex PCR, and real-time PCR have subsequently been developed and on average showed a detection limit range of around 1–5 P/μL [5–7]. The multi-step protocols for PCR-based methods are not conducive for most field applications or for routine point-of-care services in most health facilities. Rapid detection of target DNA by loop-mediated isothermal amplification (LAMP) represents a promising malaria detection method with robust performances and is more adaptable for field application.

LAMP technique allows the amplification of a few copies of target nucleic acid under a constant temperature by self-recurring strand-

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displacement DNA synthesis using only a simple water bath or a heating block [8]. The endpoint detection of LAMP amplicons can be visualized by several methods: by agarose gel electrophoresis stained with ethidium bromide, by observing white precipitates that formed as by-product when LAMP amplicons are in the presence of magnesium pyrophosphate, or by measuring fluorescence from the integration of an intercalating dye [8,9]. Visual detection of the turbidity change from precipitated LAMP product is a common end-point detection method, but it is rather subjective and can result in low detection limit. The use of a spectrophotometer may be required for the detection of precipitated LAMP product. SYBR Green I intercalating dye has been used in the endpoint detection for better visualization of LAMP amplicons [10]. Several studies have reported the successful development of LAMP assays to detect protozoan parasites using one of these end-point detection methods [11–13].

More recently, the chromatographic lateral flow dipstick (LFD, Milenia® GenLine HybriDetect) was adopted to help improve the specific and rapid detection of the LAMP products. Briefly, the biotinylated LAMP-amplicons are allowed to be hybridized with FITC-labeled DNA probes that specifically recognize the loop region of the LAMP amplicons. The hybridized products will form complexes with the gold-labeled anti-FITC antibody on the conjugated pad of the LFD to generate a signal following a streptavidin–biotin interaction. Non-target products would not hybridize with the FITC-probes or form complexes with the gold-labeled anti-FITC antibody to react with streptavidin on the test line. The total LAMP–LFD assay time is reduced when compared to gel electrophoresis detection while maintaining comparable sensitivity. In this study, the combination of LAMP–LFD was developed for detection of malaria dihydrofolate reductase–thymidylate synthase (*dhfr-ts*) gene, a target for antifolate class of antimalarial drugs.

2. Materials and methods

2.1. Recombinant plasmid construction

To construct pUC18-*Pfdhfr-ts* (pUC18-*Pf*) and pUC19-*Pvdhfr-ts* (pUC19-*Pv*), we obtained the respective *dhfr-ts* genes from pET17 vector by restriction digest, kindly provided by Dr. Penchit Chitnumsub [14]. The *dhfr-ts* genes were prepared by restriction endonuclease digestions of pET17b-*Pfdhfr-ts* and pET17b-*Pvdhfr-ts* to release the target genes and resolve on agarose gel electrophoresis. The *dhfr-ts* genes were then purified by gel extraction according to the manufacturer's protocol (Geneaid Biotech, Ltd., Taipei, Taiwan) and cloned into the 5′-*Hind*III/*Kpn*I-3′ sites and 5′-*Nde*I/*Hind*III-3′ sites (New England Biolabs, Ipswich, MA, USA) to generate recombinant plasmids pUC18-*Pfdhfr-ts* and pUC19-*Pvdhfr-ts*, respectively. Each recombinant plasmid was prepared by a ten-fold serial dilution to obtain samples with final concentrations ranging from 2×10^9 to 2 copies/μL. Diluted samples of various concentrations were subjected to LAMP reaction to establish the optimal condition for the LAMP–LFD assay.

The recombinant pET17*Pmdhfr-ts* plasmid and pET17*Podhfr-ts* plasmid were gifts from Dr. Ubolsree Leartsakulpanich [15]. The specific *dhfr-ts* gene fragment was released from the respective pET17 plasmid, and then recloned into pUC18 at the *Hind*III/*Nde*I restriction sites to generate either pUC18-*Pm* or pUC18-*Po*. The recombinant plasmid pUC18-*Pk* was obtained in similar manner, by re-cloning the *dhfr-ts* fragment from pET17-*Pkdhfr-ts* into pUC18 at the *Hind*III/*Nde*I sites to generate pUC18-*Pk*.

2.2. Parasite culture and parasite blood smears

The *Pf* (strain 3D7) parasites were cultured in human RBC (5% hematocrit) in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 0.3 g/L, L-glutamine, 5 g/L hypoxanthine and 10% human serum under an atmosphere of 1% O_2 and 5% CO_2 [16]. Thin blood films were prepared according to the standard protocol and read independently twice. Percent parasitemia was calculated by counting the number of infected RBCs per 1000 total RBCs under microscopy at 100× magnification.

2.3. Preparation of genomic DNA samples and crude lysate samples from in vitro Pf culture and Pv field samples

Freshly unsynchronized *Pf* parasite culture was collected and subjected to genomic DNA extraction [17]. Briefly, infected red blood cells were lysed following treatment with 0.15% saponin and washed in PBS. Parasite pellet was resuspended in extraction buffer (20 mM Tris–HCl (pH 7.5), 1 mM EDTA, 10% SDS, and 20 μg Proteinase K) and allowed to incubate at 50 °C for 4 h. DNA was extracted with phenol: chloroform, and subjected to ethanol precipitation. Sample was washed in 70% ethanol and resuspended in 1× TE buffer (10 mM Tris–HCl (pH 7.5), 1 mM EDTA). Field sample of *Pv* parasites were obtained from Mae Sa Riang Hospital, Thailand. Fresh *Pv* parasite samples were washed with RPMI-1640 medium and then subjected to genomic DNA extraction in the same manner as the *Pf* parasite samples. The genomic DNA sample was diluted to a concentration of 100 ng/μL. Samples were further diluted and used as templates for conventional PCR and LAMP assays, as needed.

For preparation of crude *Pf* lysate samples, parasite culture of known parasitemia was serially diluted in 50% RBC in culture media to final concentrations ranging between 1% and 0.00001% parasitemia. One microliter of crude lysate samples were directly used as template for the LAMP and PCR amplification reactions without an additional sample preparation step. For preparation of *Pv* crude lysate, fresh *Pv* samples from Mae Sa Riang Hospital were diluted in distilled water at a ratio of 1:2, 1:3, 1:7, and 1:15. One microliter of the diluted sample was subjected to LAMP and PCR amplification reactions.

2.4. Blood sample collection and preparation from Mae Sa Riang Hospital, Thailand

Out-patients diagnosed with uncomplicated *Pv* infection were informed of the study's objectives, procedures, and outcomes, as well as their reserved rights to decline participation. Patients who agreed to participate in the study were asked to sign a consent form. Then, prior to receiving treatment, approximately 5 mL heparin anti-coagulated blood sample was collected by a qualified medical technologist at Mae Sa Riang Hospital. After sample collection, volunteer patients were provided with treatment according to the national guidelines for uncomplicated malaria infection. Samples were transported under room temperature to the laboratory at BIOTEC within the same day. Parasite samples were prepared as described earlier and tested by PCR and LAMP assays. The study protocol was approved by the *Research Ethics Committee on Research Involving Humans* (No. 187/2011) at the Faculty of Medicine, Chiang Mai University, Thailand.

2.5. Primers and conditions of the LAMP reactions

Two LAMP primers sets were designed manually according to the published gene sequences of *Pfdhfr-ts* (GenBank accession number J03772.1) and *Pvdhfr-ts* (GenBank accession number X98123.1). The details for the primer sets are shown in Fig. 1A–B and Table 1. To optimize the reaction conditions, the LAMP reactions were carried out at 60 °C, 63 °C or 65 °C for 15, 30, 45 or 60 min in various combinations. The reactions were performed in a 25 μL of total reaction mixture containing: 2 μM each of FIP and BIP, 0.2 μM each of F3 and B3, 2 μM each of LF and LB primers, 1× thermopol-supplied reaction buffer, 0.4 M betaine (USB Corporation, Cleveland, OH, USA), 6 mM $MgSO_4$ (Sigma-Aldrich, St. Louis, MO, USA), 1.4 mM dNTP mix (Promega, Madison, WI, USA), 8 Units of *Bst* DNA polymerase (New England Biolabs,