

Alternative Malaria Diagnostic Tools: Evaluation of *Plasmodium falciparum* Detection along Thailand's Border by Loop-Mediated Isothermal Amplification (LAMP) and Immunochromatographic Test (ICT)

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Abstract

Introduction: Thailand has a national goal to eliminate malaria from 80 percent of the country by 2020. However, malaria-endemic areas still exist along the forested Thailand's borders. An accurate and prompt diagnosis is the key to effective disease management. This aim of this study was to evaluate the application of LAMP as an alternative tool for falciparum malaria diagnosis of clinical suspicion of plasmodium infection in the border area, compared to those of ICT and microscopy using nested PCR as the reference detection method.

Materials and Methods: One hundred and four of blood samples were obtained from patients who were diagnosed malaria positive by clinical diagnosis or subjected to suffering from malaria as observed from clinical history. The primer set used for LAMP was designed on the basis of nucleotide sequence of 18S rRNA plasmodium gene and the ICT test was performed with the parasite lactate dehydrogease (pLDH) antigen-based lateral flow test.

Results: The LAMP assay has the highest agreement with the reference method (99.04%, $\kappa=0.98$) with a sensitivity (95%CI) of 98.59% (95.85-100.00) comparable to ICT and microscopy. Moreover, LAMP showed specificity 100% compared with 100% of ICT and 96.97% (91.12-100.00%) microscopy. Negative predictive value of LAMP and ICT were 97.06% and 82.50% respectively.

Conclusion: LAMP is useful and more reliable for specific diagnosis in border regions where malaria is endemic but individuals are asymptomatic and hence that LAMP could be the preferred method in resource-limited laboratories to progress towards malaria elimination in Thailand.

Keywords: Loop-mediated isothermal amplification; Immunochromatographic test; Nested polymerase chain reaction

Introduction

Malaria is an endemic disease which is caused by *Plasmodium* parasites. There are five parasite species that cause malaria in humans; *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale* and *Plasmodium knowlesi* [1]. *P. falciparum* and *P. vivax* are the most common but *P. falciparum* is the most deadly. An accurate and prompt diagnosis is needed to deploy the impact of effective treatment especially for potential fatal cases of *P. falciparum* [2,3]. Thailand has experienced a 70 percent decrease in reported malaria cases between 2000 and 2011 [4]. Central Thailand has been malaria-free for more than three decades [5]. Therefore, Thailand has a national goal to eliminate malaria from 80 percent of the country by 2020 [6]. However, malaria-endemic areas still exist along the forested Thailand's borders with Myanmar to the west and Cambodia to the east [5]. Several major outbreaks occurred along Thailand's borders [7]. Moreover, the border area has been the epicentre of multidrug resistant *P. falciparum* [8]. The patient's signs and symptoms of malaria are nonspecific and variable [2,9]. Accordingly, differentiation of clinical diagnosis is sometimes difficult and confirmatory diagnosis using laboratory technologies is needed. An accurate parasite-based diagnosis of malaria is essential for proper treatment of individual patients [10].

Conventional laboratory diagnosis has relied on microscopy. It is an accurate tool but requires well-trained staff. Interpretation of smear requires expertise. Recently, the Immunochromatographic test (ICT) have become available, which are based on the recognition of Plasmodium antigen in the blood circulation of patients. The test is rapid and simple. Following WHO recommendation, ICT were used as rapid diagnosis test for malaria infection [11]. More recently, the

interpretation of malaria diagnosis and epidemiology has changed by using molecular tools, for instance by revealing grand reservoirs of asymptomatic infection and, by detecting distribution of *Plasmodium spp.* infection. According to DNA amplification, nested polymerase chain reaction (nested PCR) method targeting 18S rRNA gene, all species could be identified [12,13]. However, a major drawback of the molecular detection tool is its cost, requiring PCR machine and well-trained staff. The loop-mediated isothermal amplification (LAMP) is a most recently developed molecular technique that overcome disadvantage of PCR by being simpler and faster. The cost of the technique can be reduced by using a water bath or a heat block instead of PCR [14-16].

The aim of this study was to evaluate the application of LAMP as an alternative tool for falciparum malaria diagnosis of clinical suspicion of plasmodium infection in the Thailand border area, compared to those of ICT using nested PCR as the reference detection method.

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Subjects and Methods

Clinical sample

The blood samples were collected from patients who came to malaria clinic during May to July 2014. One hundred and four of samples were obtained from patients who were diagnosed malaria positive by clinical diagnosis or subjected to suffering from malaria as observed from clinical history. EDTA blood was taken before patients were treated with anti-malarials. The template DNA was extracted from 200 µl of EDTA blood using QIAamp DNA Blood Mini kits (QIAGEN GmbH, Hilden, Germany), according to the manufacturer's protocol. The hospitals in this study were closed to Thailand's border area. Three hospitals were in the province of Chiangmai, Mahongsorn and Kanchanaburi near Myanmar-Thailand border and two hospitals in the province of Trat and Sa Kaeo near the Cambodia-Thailand border. This study was performed using a protocol approved for medical research on human subjects, Department of Medical Sciences, Ministry of Public Health, Thailand.

Microscopy

Microscopy of thick and thin Giemsa-stained smears was considered the traditional standard method to detect malaria infection [17]. The analysis of the sample by microscopy was prepared by trained microscopists upon admission of the patient. Each smear was examined by two microscopists for the presence of parasites per 500 white blood cells counted and for the specie identification. This dependence on microscopist's expertise can be illustrated by the results of external quality control of the Malaria center, Ministry of Public Health, Thailand.

Immunochromatographic Test (ICT)

The ICT test was performed with the parasite lactate dehydrogease (pLDH) antigen-based lateral flow test (DMSc Malaria Pf/PAN Rapid test, Thailand) used according to the manufacturer's instructions. The test detects *P. falciparum* specific and pan-specific antigens. Two trained readers examined the ICT, independently. A test was considered negative only if the internal control was visible. The result of an ICT was considered not valid if the internal control was not visible.

Nested Polymerase Chain Reaction (Nested PCR)

All samples were tested for genus *Plasmodium*, *P. falciparum* by nested PCR. The species-specific nucleotide sequences of the 18S rRNA genes were applied as described previously with slight modifications [18]. For the outer PCR, 1 µl of template DNA was added to a 21 µl PCR mixture that consisted of 0.88 µM each universal primer (P1 forward and P2 reverse primer), 0.44 µM deoxynucleoside triphosphate (10 mM each), 1.5 µM MgCl₂ (25 mM), 2.2 µM 10x ImmoBuffer (100 mM NaCl, 20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 2 mM DTT, 50% Glycerol, stabilizers), 0.25 U IMMUNLASE DNA Polymerase (5 U/µl) and 14.85 µl of distilled water. dNTP, MgCl₂, ImmoBuffer and IMMUNLASE DNA polymerase were obtained from Bioline USA Inc. (Taunton, Ma, USA). 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 outer PCR product was diluted 40-fold in sterile water. One microlitre of this solution was used in the second amplification. The inner 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 and reverse species-specific primer. The amplified products were visualized in 2.5% agarose gels stained with ethidium bromide. The expected band sizes were approximately 160 bp for the outer primer PCR product and approximately 110 bp for the inner primer PCR product.

Loop-Mediated Isothermal Amplification (LAMP)

All blood samples were tested by a primer set for *Plasmodium* DNA and a species-specific *P. falciparum*. The LAMP method was modified by previous study [16,19]. Modifications had to be applied as follows: For the LAMP reaction for genus *Plasmodium*, 1 µl of template DNA was added to a 19 µl LAMP mixture, containing 6.3 µl of DW, 1.3 µl of primer mix, 10.4 µl of 2x Reaction LAMP buffer and 1 µl of Bst-DNA-polymerase (BioLabs Inc., Ipswich, MA, USA). For *P. falciparum* slight modifications had to be applied. 1 µl of Betain was added, the 2x Reaction LAMP buffer was adjusted and DW was reduced to 4.74 µl. The reactions were incubated at 65°C for 60 min; the amplification was performed by water bath. All tubes were analysed by gel electrophoresis to assess the presence of the LAMP DNA product and stained with ethidium bromide (Promega, Madison, WI, USA).

Statistical analysis

The data was analyzed to calculate test performances and acceptable evaluation indices using nested PCR results as the reference standard. The indices measured were the number of true positives (TP), number of true negatives (TN), number of false positives (FP), and number of false negatives (FN). Sensitivity was defined as TP/(TP + FN), specificity as TN/(TN + FP), positive predictive value (PPV) as TP/(TP + FP), and negative predictive value (NPV) as TN/(FN + TN). The 95% confidence interval for sensitivity and specificity was calculated using MINITAB 14 software. For analysis of concordant results, Cohen's kappa statistics was used as a reference for two methods. The equation for κ is [P(a)-P(e)]/[1-P(e)]. If the methods are in complete agreement, κ = 1.

Results

One hundred and four samples were tested by microscopy, ICT, LAMP and nested PCR. Nested PCR which was used as the reference method, was positive for *P. falciparum* in 71 (68.27%) patients whereas 70 (67.31%), 64 (61.54%) and 70 (67.31%) were positive by microscopy, ICT and LAMP respectively. The remaining was detected as negative. All of 33 nPCR negative samples were also negative by LAMP and ICT assay (specificity 100%) while microscopy had one false positive (specificity 96.97%).

This result indicated that microscopy gave both false negative and false positive (sensitivity 98.59%. specificity 96.97%). In this study, we evaluate LAMP and ICT for assessing a positive result. Neither of them produced any false positive results, translating into a specificity and positive predictive value of 100% (Table 1). The sensitivity (95% CI)

	Sen	95% CI for sen	Spec	95% CI for spec	PPV	95% CI for PPV	NPV	95% CI for NPV	P(a)	P(e)	Kappa (κ)	95% CI for Kappa
Mic	98.59	(95.85 - 100.00)	96.97	(91.12 - 100.00)	98.59	(95.85 - 100.00)	96.97	(91.12 - 100.00)	0.9808	0.5668	0.9556	(89.47 - 100.00)
ICT	90.14	(83.21 - 97.08)	100.00	(100.00 - 100.00)	100.00	(100.00 - 100.00)	82.50	(70.72 - 94.28)	0.9327	0.5422	0.8530	(74.78 - 95.82)
LAMP	98.59	(95.85 - 100.00)	100.00	(100.00 - 100.00)	100.00	(100.00 - 100.00)	97.06	(91.38 - 100.00)	0.9904	0.5632	0.9780	(93.51 - 100.00)

Mic: Microscopy; ICT: Immunochromatography Test; LAMP: Loop-mediated Isothermal PCR; Sen: Sensitivity; spec: Specificity; PPV: Positive Predictive Value; NPV: Negative Predictive Value; P(a): Percent Agreement; P(e): Probability random agreement.

Table 1: Agreement, Sensitivity, Specificity, PPV and NPV of microscopy, ICT and LAMP for faciparum malaria versus gold standard nested PCR.

		Per dif	95% CI for per dif	P-value
Sen	Mic vs ICT	8.45	(0.99 to 15.91)	0.029
	LAMP vs ICT	8.45	(0.99 to 15.91)	0.029
Spec	Mic vs ICT	-3.03	(-8.88 to 2.82)	0.314
	LAMP vs ICT	0.00		
PPV	Mic vs ICT	-1.41	(-4.15 to 1.33)	0.341
	LAMP vs ICT	0.00		
NPV	Mic vs ICT	14.47	(1.32 to 27.62)	0.049
	LAMP vs ICT	14.56	(1.49 to 27.63)	0.044

Nested PCR was used as reference test. The P-value concerns the difference with the ICT.

Mic: Microscopy; ICT: Immunochromatography Test; LAMP: Loop-mediated Isothermal PCR; Sen: Sensitivity; spec: Specificity; PPV: Positive Predictive Value; NPV: Negative Predictive Value; Per dif: Percent difference.

Table 2: Comparison of LAMP and microscopy with ICT for faciparum malaria in Thailand's border area.

of the LAMP was comparable with ICT: 98.59% (95.8-100%) versus 90.14% (83.2-97.0%: P=0.02, Table 1). The negative predictive value (95% CI) of LAMP was 97.06% (91.38-100.00%). The highest agreement with nested PCR was achieved with the LAMP (99.04%, $\kappa = 0.98$). The LAMP showed the highest negative predictive value and 100% positive predictive value comparing with ICT and microscopy (Table 1).

Discussion

The corner stone of malaria control is prompt diagnosis, which remains the basis for effective anti-malarial treatment. A rational therapy avoids non-target effects and saves costs on alternative drugs. Given the extent of multidrug-resistant *P. falciparum* infections and the resulting need for more expensive drugs unaffordable to resource-limited countries, an accurate diagnosis is essential. In this study, we assessed which method, is best suited for diagnosis of clinical suspicion of malaria within a low or unstable endemic area. For this purpose, blood samples with the clinical symptoms of malaria from border hospitals were analyzed with microscope, ICT, LAMP and nested polymerase chain reaction (PCR) as the gold standard.

In Thailand, microscopy is the mainstay of malaria diagnosis. However, the sensitivity and specificity of microscopic technique varied depending on the blood staining, the amount of parasite density, quality of the microscope, well-trained competent microscopists and rigorous maintenance of effective quality control and quality assurance [20]. In addition, the impaired microscopy-based diagnosis in hospital laboratories is common [21]. In this study, microscopy gave both false positive and false negative. These errors seem like inherent limitations of microscopy for malaria diagnosis. Example of the false positive case by microscopy is misidentifying the stain granules and/or platelets superimposed on erythrocytes as the malarial parasite. Even among local laboratories with similar equipment, equal training and quality of essential laboratory supplies, abilities vary significantly [22,23].

Current ICT tests are manufactured with different target antigens. Histidine-Rich-Protein 2 (HRP-2) and pLDH are most common malaria antigens targeted and specific for *P. falciparum*. HRP-2 tests gave *P. falciparum* sensitivity of 90% or more in clinical cases [24,25]. However, the sensitivity of pLDH assays was varied. Sensitivity for *P. falciparum* is excellent (>95%) in some studies and poorer (80%+) in the others [26,27]. In this study, ICT using pLDH assays showed the lowest value of sensitivity (90.14%) which is a failure to correctly identify malaria infected person. Moreover, ICT gave a high rate of false negative comparing with LAMP. Several factors in manufacturing process as well as environmental conditions may affect ICT performance [25]. In addition, exposure of the test kit to high temperature during transport

and storage is commonplace in tropical countries and can degrade the test and bias the result [20]. In this study, the ICT storage and transport conditions were controlled throughout the study, and the storage temperature was that recommended by the manufacture.

The recently described LAMP technique has the potential of combining the accuracy of molecular technique with low cost and simple technical requirements. To perform the test only water bath and gel electrophoresis are needed. Water bath commonly used in routine work and gel electrophoresis are not expensive and can be used in a laboratory of a rural border hospital. In this study, 104 blood samples from malaria endemic border Thai provinces were collected for comparative malaria diagnosis. These samples were diagnosed using microscopy, ICT, LAMP and nested PCR as the gold standard. Both LAMP and ICT showed specificity and positive predictive value of 100% but the LAMP showed higher sensitivity, PPV and NPV than ICT. Furthermore, LAMP gave the highest agreement with nested PCR. Although the use of simple techniques, together with common instruments, has the advantage that they can be deployed in a resource-limited setting, a strictly separated area is needed to perform the test. Avoidance of contamination should have priority when conducting the assay, which can be obtained by changing gloves between every assay and strict separation of reagent. Accuracy of LAMP is sufficient to promote its use as a diagnostic tool because the test currently shows acceptably high rates of sensitivity and specificity. Furthermore, LAMP gave a low rate of false negative results which is particularly important to clinicians using diagnostic tests because failing to treat a true case of faciparum malaria may result in progression to a severe disease and death. This is a preliminary study. Hence, this method needs further study and validation using a larger sample size before it can be adopted for malaria diagnosis in the field. In conclusion, LAMP is a promising malaria diagnostic tool to enable molecular diagnosis of *P. falciparum* in settings with limited technical resources especially laboratories in hospital along border where malaria are endemic and unstable to progress towards malaria elimination in Thailand.

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