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Comparative Diagnosis of Malaria Infections by Microscopy, Nested PCR, and LAMP in Northern Thailand

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Abstract. Three methods, microscopy, nested polymerase chain reaction (nPCR), and loop-mediated isothermal amplification (LAMP) have been applied for malaria diagnosis in 105 human blood samples collected in Northern Thailand. Only *Plasmodium falciparum* and *Plasmodium vivax* infections were detected. A total number of 57 positives (54%) could be detected for *P. falciparum* and 25 (24%) for *P. vivax* when all samples that were positive in any of the three methods are counted together. The nPCR was used as a reference standard for comparison with the other methods, microscopy and LAMP. The sensitivity of LAMP for *P. falciparum* was 100%. All nPCR-negative samples for *P. falciparum* were also negative by both microscopy and LAMP (specificity, 100%). For diagnosis of *P. vivax*, microscopy detected 15 of 23 nPCR-positive samples (sensitivity, 65%). LAMP detected 22 of 23 nPCR-positives (sensitivity, 96%). Among the 82 nPCR-negative samples microscopy detected two samples (specificity, 98%). All 82 nPCR-negative were also negative by the LAMP method (specificity, 100%). Both *Plasmodium* genus- and species-specific LAMP primer sets yielded the same results in all samples. There were no significant differences in the prevalence detected by each method. We assume that LAMP was as reliable as nPCR and more reliable than microscopy in the detection of *Plasmodium* DNA tested in the examined Thai field blood samples. This study further validates LAMP as an alternative molecular diagnostic tool, which can be used in the diagnosis of early infections of malaria cases and together with nPCR can also be used as supplementary methods for clinical and epidemiological use.

INTRODUCTION

According to the World Health Organization (WHO) malaria parasites infected 247 million people worldwide in 2006 and caused nearly 881,000 deaths in that year.[1] As most deaths caused by Malaria are because of wrong, late, or unavailable diagnosis, there is a need to find a new alternative diagnostic tool for field diagnosis for malarial infection.[2] For definitive species determination, molecular techniques such as polymerase chain reaction (PCR) and microsatellite analysis are needed.[3] Most countries where malaria is endemic are poor and cannot afford the equipment needed for the PCR method. The process of the PCR is also time-consuming providing the results with a delay to the physician. The loop-mediated isothermal amplification (LAMP) is a molecular method, which in comparison to the PCR is cheaper, simpler, and faster, taking out three disadvantages of the PCR. The LAMP is a nucleic acid amplification method that relies on autocycling strand-displacement DNA synthesis performed with *Bst* DNA polymerase. The principal merit of this method is that no denaturation of the DNA template is required, and thus, the LAMP reaction can be conducted under isothermal conditions.[4] The LAMP method applications have been developed rapidly showing a nearly exponential increase of the publications, because its initial description including applications in the field of parasitological diagnosis, e.g., *Trypanosoma* spp., *Cryptosporidium* spp., *Theileria*, canine and equine piroplasmosis, *Toxoplasma gondii*, *Giardia duodenalis* assemblages A and B, *Microsporidia*, *Taenia* species, and for the detection of *Plasmodia*.[5–25] Species-specific LAMP assay for *P. falciparum* and LAMP assays for all four species that infect humans have already been developed.[21,23] Although this new technique has not yet been established for routine diagnosis in the field, the first studies on *Plasmodium* LAMP highly favor this method because LAMP is almost as specific and sensitive as the nested polymerase chain reaction (nPCR) method for *Plasmodium*-DNA detection in blood. Precipitation of magnesium pyrophosphate as a by-product of the LAMP reaction causes turbidity, which can either be detected visually or by a real-time turbidimeter. The real-time turbidimeter measures the turbidity released during a reaction and provides information not only if the tested sample is positive or negative but also the threshold time when the reaction becomes positive for each sample. Because there is a correlation between the threshold time and the initial concentration of DNA, the LAMP method in combination with a real-time turbidimeter can be used as a quantitative method.[21]

Recently, LAMP was applied for the identification of *Plasmodium*-carrying mosquitoes using rodent malaria parasite (*Plasmodium berghei*) and for filarial parasites detection in the mosquito vectors, suggesting LAMP as more reliable and useful for routine diagnosis of vector mosquitoes in regions where vector-borne diseases such as malaria are endemic.[25,26]

This work further validates the application of LAMP assay as a useful tool for malaria diagnosis in human blood samples from Northern Thailand and compares the findings with those obtained by microscopic and nPCR diagnostic methods.

MATERIAL AND METHODS

Material collection in Thailand. A total of 130 EDTA-blood samples were collected in April and May 2008 in the Upper North Region of Thailand. Sixty-five samples were provided by 10 different hospitals whereby they tested positive for *Plasmodium* infections by microscopy during admission of the patient. Two hospitals in the province of Mae Hong Son provided 13 samples from patients that were suspected to suffer from malaria, as observed from clinical symptoms and geographical history, but were negative by microscopic

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analysis. Two samples were obtained from already treated malaria patients in a follow-up examination at the Malaria Center of Chiang Mai. Twenty-five samples of healthy persons were provided by the blood bank of the Upper North Region of Thailand and were not analyzed by microscopy in the current study. An additional 25 samples of patients with fever of unknown origin were provided by the Chomthong Hospital of Chiang Mai. These patients had been tested once for malaria using Giemsa-stained smears, but as the symptoms were not typical for malaria, no further testing on malaria was undertaken by the hospital.

Microscopy. For microscopic diagnosis a blood drop either from the fingertip or from the EDTA blood taken from the vein was placed on a glass slide. Slide results were designated based on reported results from the contributing clinical laboratories. They were not reviewed by expert microscopists, because we chose clinical microscopy as a comparator diagnostic method. Using the samples that were collected at the hospitals, both thick film and thin film were prepared. At the Malaria Center only a thick film was prepared. The Malaria Center of Chiang Mai, which specializes only in malaria diagnosis, used the Giemsa method for staining because it is the standard staining method for *Plasmodium* detection by microscopy. Thus, the slides obtained from the Malaria Center of Chiang Mai were prepared as follows: The thick blood film slide was incubated with 20% Giemsa solution (1 volume Giemsa: 4 volumes PBS-Buffer [phosphate buffered saline]) for 5 min and then dipped into water to wash off the stain excess. At the hospitals the Wright stain was used, because it allowed examination for various diseases, such as leishmaniosis and tuberculosis at one time. Both thin and thick film were incubated with Wright stain for 3 min and then washed with water. The slides were examined by the hospital's laboratory experienced staff using light microscope with a 100× oil immersion objective. More than 100 fields per slide were examined; approximately 6 min/slide.

nPCR based on the 18S rRNA gene of *Plasmodium* spp. The DNA of all samples was purified using the Qiagen PCR purification kit (Qiagen Inc., Valencia, CA) according to manufacturer's protocol. Altogether 130 samples were tested for *P. falciparum* and *P. vivax* infections in Thailand and subsequently for *Plasmodium ovale* and *Plasmodium malariae* at the Unit for Diseases Control and Genetics of the National Research Center for Protozoan Disease (NRCPD) in Japan. For those samples that were negative by microscopy the outer primer PCR product was first visualized by gel-electrophoresis and only when found positive subjected to the inner primer PCR as well. For nPCR, the species-specific nucleotide sequences of the 18S rRNA gene of *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale* was amplified as described previously with slight modifications.[27] For the outer primer PCR, 1 μL of template DNA was added to a 21-μL PCR mixture that consisted of 0.4 μM each universal primer (P1 forward and P2 reverse primer), 200 μM each deoxynucleoside triphosphate, 25 mM MgCl2, 1× PCR Gold Buffer II (50 mM KCl, 15 mM Tris-HCl, pH 8.0), 0.25 U AmpliTaq Gold DNA polymerase, and 14.85 μL of distilled water. 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 primer PCR product was diluted 40-fold in sterile water. One microliter of this solution was used for the inner PCR 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 primer in combination with each species-specific reverse primer. The amplified products were visualized in 2.5% agarose gel 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.

LAMP assay based on 18S rRNA *Plasmodium* gene. All 130 samples collected in Thailand were tested by using five primer sets reported by Han and others,[21] whereby one set amplifies *Plasmodium* genus DNA and the four species specific for each of four human infecting *Plasmodium* species. To confirm the specificity of LAMP primers under our laboratory conditions, DNA of *Plasmodium* (*P. falciparum*, *P. vivax*, and *P. berghei*) and DNA from other protozoan parasites (*Trypanosoma cruzi*, *Trypanosoma brucei gambiense*, *Cryptosporidium parvum*, *Toxoplasma gondii*) were subjected to LAMP with *Plasmodium* genus primer set. The LAMP assay in this study was conducted as reported previously[21] with minor modifications. Briefly, 2 μL of template DNA was added to a 23-μL LAMP mixture that consisted of 1.3 μL of primer mix (40 pmol of each FIP and BIP, 20 pmol of each LF and LB, 10 pmol of each F3 and B3), 12.5 μL of 2× reaction buffer (40 mM Tris-HCl [pH 8.8], 20 mM KCl, 16 mM $MgSO_4$, 20 mM $[NH_4]_2SO_4$, 0.2% Tween 20, 1.6 M Betaine, 2.8 mM of each dNTP), 1 μL of *Bst* DNA polymerase and 8.2 μL of distilled water. The reactions were incubated at 60°C for 60 min. The amplification was performed by a real-time turbidimeter (LA-200, TERAMECS, Tokyo, Japan) at the NRCPD, Obihiro, Japan.

Statistical methods. The results of all three methods, microscopy, nPCR, and LAMP were analyzed with a statistical calculation program called S-plus 6 Software for Windows (Insightful Corp., Seattle, WA). The McNemar-χ^2 test was used to investigate whether there is a significant difference in accuracy between the three methods. The 25 samples of healthy persons were excluded from the statistical calculations because they had not been tested by microscopy. For analysis of the diagnostic sensitivity and specificity, a composite diagnosis for each sample (two out of three tests giving the same result) was created and used as a reference for all three test modalities.

RESULTS

Prevalence of *Plasmodium* species in the Thai samples. In total 130 field samples have been investigated. All three methods, microscopy, nPCR, and LAMP only detected *P. falciparum* and *P. vivax* in the examined field samples. No infections with *P. malariae* and *P. ovale* were found. The 25 samples of healthy persons were excluded from the calculations of prevalence because they had not been tested by microscopy. The prevalence of *P. falciparum* and *P. vivax* in the remaining 105 samples varies according to the method used. Although microscopy and LAMP both detected 48 positives (46%) for *P. falciparum*, nPCR detected 53 positives (50%). When all samples that were positive for any of the three methods are counted together, a total number of 57 positives (54%) could be detected. The McNemar's test showed no significant differences in the accuracy of all three methods. Microscopy detected 17 *P. vivax* positives (16%), nPCR 23 (22%), and LAMP 22 (21%). The total number of *P. vivax* infections for

any of the three methods was 25 (24%). The McNemar's χ^2 test showed no significance differences in the accuracy for all three methods.

Comparison of sensitivity and specificity of LAMP, nPCR, and microscopy for malaria diagnosis of the investigated samples. A composite diagnosis for each sample, based on two out of three methods giving the same result, was created and used as a reference to measure sensitivity and specificity of all three test modalities. Out of 48 positive samples for *P. falciparum,* 44 were positively detected by microscopy (sensitivity, 92%), whereas nPCR and LAMP assay positively detected all 48 positive samples (sensitivity, 100%). All negative samples for *P. falciparum* were also negative by the LAMP assay (specificity, 100%). Four of the 57 negative samples were positively detected by microscopy (specificity, 93%) and five samples were positively detected by nPCR in the first trial (specificity 91%). The samples were re-tested twice and both times gave negative results by nPCR, which meant there might have been contamination during the first trial. Using the result of the retested trial, the specificity of nPCR is 100%.

For diagnosis of *P. vivax*, microscopy detected 15 of 22 positive samples (sensitivity, 68%). The LAMP and nPCR detected 22 of 22 positive samples (sensitivity, 100%). Among the 83 negative samples for *P. vivax*, microscopy detected 2 samples (specificity, 98%) and nPCR detected 1 sample (specificity 99%). All 82 negative samples for *P. vivax* were also negative by the LAMP method (specificity, 100%). Both *Plasmodium* genus- and species-specific LAMP assays yielded the same results in all samples (Table 1 and 2).

DISCUSSION

Malaria is scarce in most parts of the Upper Northern Region of Thailand but remains a major public health issue at the border line to Myanmar. In particular, multidrug-resistant (MDR) *P. falciparum* is a big obstacle in malaria control programs. Malaria is a curable disease if treatment is provided promptly and accurately. Therefore, the diagnosis of malaria needs to be highly specific and sensitive. Even though microscopy is currently the standard method of malaria diagnosis it has the disadvantages of poor sensitivity and specificity, especially during low parasitemia. Furthermore, it is often difficult to distinguish between species, especially if the patient has already been treated or taken preventive anti-malarial drugs. The molecular method, nPCR, is recognized as the most sensitive and specific method of all diagnostic tests for malaria that are currently available.[28] The nPCR has the disadvantage of being time-consuming and having many steps in the diagnostic procedure. Furthermore, it is expensive because of complex equipment needed to run the PCR assay. These disadvantages prevent the implementation of nPCR as an alternative diagnostic test in poor countries and remote areas where malaria is mainly distributed. The new molecular method, LAMP, is a solution to the two disadvantages of the nPCR because it does not require complex machines and the running time of the DNA amplification is shorter. Furthermore, the LAMP does not need the laborious step of DNA purification. As shown previously, even simple heat-treated blood can be used for the assay without further purification as it has been reported for falciparum malaria.[22] Furthermore, in larger hospitals where a real-time turbidimeter is available, the LAMP could also be used as a quantitative method. The LAMP assays have already been successfully developed for detection of other protozoan parasites, such as *Cryptosporidium* species, *Trypanosoma* species, *Theileria* species, *Babesia* species, *Toxoplasma gondii*, and *Giardia duodenalis*.[8–11,16,17] Moreover, LAMP successfully identified just a single oocyst in the midgut of a *Plasmodium*-carrying mosquito, a level that can be easily overlooked in conventional microscopic analysis.[25] The robustness of the LAMP reaction was revealed by its ability to detect both *Plasmodium* oocysts and sporozoites from an "all-in-one" template using whole mosquito bodies.[25]

In our study, blood samples of the Northern Region of Thailand were collected for comparative malaria diagnosis using three diagnostic methods: microscopy, LAMP, and nPCR. Microscopy has been applied because it is the standard method for malaria diagnosis. The differential staining procedures used at different facilities, Wright stain at the contributing clinical facilities and Giemsa smear at the Chiang Mai Malaria Center, and likely differences in skill of microscopists are limiting the comparability to other methods in this study.

However, microscopy is very rarely rigidly standardized, except in the most stringent pivotal clinical trial environments, and this is done at great expense and trouble not realistically transferred to most settings in malaria research. It may not be reasonable to hold the validity of the current work to such a standard. The same sentiment applies to the discordant PCR findings. Laboratory errors occur and tend to be detected as

TABLE 1

Comparative details of microscopy, nPCR, and LAMP for *Plasmodium* detection and species identification

Number of samples	Microscopy	nPCR	LAMP
Concordant			
42 samples		*Plasmodium falciparum*	
14 samples		*Plasmodium vivax*	
32 samples		Negative	
Discordant			
4 samples	Negative	**P. falciparum*	Negative
4 samples	*P. falciparum*	*P. vivax*	*P. vivax*
2 samples	*P. vivax*	*P. falciparum*	*P. falciparum*
2 samples	Negative	*P. falciparum*	*P. falciparum*
2 samples	Negative	*P. vivax*	*P. vivax*
1 sample	*P. falciparum*	*P. falciparum* + *P. vivax*	*P. falciparum* + *P. vivax*
1 sample	*P. vivax*	**P. falciparum* + *P. vivax*	*P. vivax*
1 sample	*P. falciparum*	*P. falciparum* + *P. vivax*	*P. falciparum*

*These 5 samples were retested 2 months after extraction; 1 week after the loop-mediated isothermal amplification (LAMP) was carried out. At that time all of the 5 samples were negative for *P. falciparum* by nested polymerase chain reaction (nPCR).

TABLE 2

Sensitivity and specificity of LAMP and microscopy for *Plasmodium vivax* and *Plasmodium falciparum* detection*

Species	Method	Specificity	Sensitivity
P. falciparum	LAMP	100%	100%
	Microscopy	93%	92%
	PCR	100%	100%
P. vivax	LAMP	100%	100%
	Microscopy	98%	68%
	PCR	99%	100%

*A composite diagnosis for each sample based on two out of three methods giving the same result was used as reference.
LAMP = loop-mediated isothermal amplification; PCR = polymerase chain reaction.

discordant findings in a quality assurance round of assays, which we did. The nPCR was chosen because it is the most sensitive and specific method of all methods available for Malaria diagnosis. Of 130 collected samples, 105 samples were examined by all three tests. The remaining 25 samples were obtained from the blood bank and only examined by nPCR and LAMP. Both tests showed no *Plasmodium* infections in any of the 25 samples. As the blood donors were not suspected to suffer from malaria by physical examination and individual geographical history, their blood was not examined for malaria by microscope at the blood bank. Of the 105 samples that were tested by all three methods, the reference method nPCR detected *P. falciparum* in 53 samples and *P. vivax* in 23 samples. The specificity of microscopy was lower than previously reported.[22] Only one sample was non-concordant for *P. vivax* between LAMP and nPCR. This sample was diagnosed with a mixed infection of *P. vivax* and *P. falciparum* by nPCR and a single *P. falciparum* infection by LAMP. The results that were inconsistent for *P. falciparum* appeared to be consistent after they were retested in the laboratory in Japan, leading to a specificity of 100% for LAMP. Three out of 25 samples from patients with fever of unknown origin were positive by nPCR in Thailand but negative by LAMP. After retesting those samples by nPCR in the laboratory in Japan they were also negative. In both laboratories in Thailand and in Japan, we used the same nPCR conditions. An explanation for discrepancies might be that the DNA had been in a very low concentration and degraded during transportation from Thailand to Japan or that the original positive PCR results could reflect laboratory contamination. Laboratory errors occur and tend to be detected as discordant findings in a quality assurance round of assays, which we did. Therefore the validity of this work is possibly limited. But there also might be other unknown reasons for those discordant PCR findings. The same finding occurred for one sample that was negative by microscopy but positive for *P. falciparum* by the nPCR in Thailand. Furthermore, another sample was a mixed infection of *P. falciparum* and *P. vivax* by nPCR in the laboratory in Thailand, but it had been diagnosed as *P. vivax* infection by microscopy and LAMP. This sample was also diagnosed a single *P. vivax* infection by PCR when retested in Japan. Hence, we can assume there was no *P. malariae* or *P. ovale* in any of the 105 samples. Because 99% of the results of the LAMP assay were consistent with those of nPCR, we can propose that LAMP is as reliable as nPCR for the clinical detection of two species of malaria parasites, as reported here for *P. falciparum* and *P. vivax*.

As a molecular diagnostic method, LAMP has the advantage of allowing distinctions between physically similar appearing species. This has become an important issue as the zoonotic simian species *Plasmodium knowlesi* is now starting to spread throughout Southeast Asia, frequently infecting humans.[29] The molecular methods PCR and LAMP also have the potential to detect MDR *Plasmodium* species. Though, further development is still needed. If the LAMP method can be applied to Thai samples, it could be a useful diagnostic method to identify MDR *P. falciparum* in the future. The microscopic diagnosis is a simple and cheap test; nevertheless, to examine a blood smear slide to acceptable accuracy, lengthy training sessions and a lot of experience are needed. However, for LAMP diagnostics personnel can be trained within a day or two. With the development of diagnostic kits, the examiner would only have to follow the indicated steps without any training. Another advantage of the molecular tests is that they give objective results. In contrast, the microscopic examination depends on individual subjective judgment. An advantage of microscopic diagnosis is that the slide can be examined for different species at the same time, whereas the molecular methods currently can only examine the specimen for one species or *Plasmodium* in general in one assay. Altogether, the listed advantages and disadvantages of the LAMP method rather favor an incorporation of the LAMP method in routine diagnosis and field studies of malaria in the future. LAMP may have real potential as a diagnostic method for clinical use, but this work does not show it.

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