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

Birgit Pöschl, Jarurin Waneesorn, Oriel Thekisoe, Salakhit Chutipongvivate, and Karanis Panagiotis*

Medical and Molecular Parasitology Laboratory, University of Cologne, Medical School, Center of Anatomy, Institute II, Cologne, Germany; Clinical Pathology Section, Regional Medical Science Center Chiang Mai, Chiang Mai, Thailand; National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido, Japan

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. 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. For definitive species determination, molecular techniques such as polymerase chain reaction (PCR) and microsatellite analysis are needed. 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. 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. Species-specific LAMP assay for P. falciparum and LAMP assays for all four species that infect humans have already been developed. 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.

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.

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

*Address correspondence to Karanis Panagiotis, Medical and Molecular Parasitology Laboratory, University of Cologne, Medical School, Center of Anatomy, Institute II, 50937 Cologne, Germany. E-mail: panagiotis.karanis@uk-koeln.de
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 5 min and then washed with water. The slides were examined by the hospital’s laboratory experienced staff using light microscope with a 100x 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 product was first visualized by gel-electrophoresis. For nPCR, the species-specific nucleotide primer PCR product was subsequently for Nucleic Acid 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 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 were analyzed with a statistical calculation program called S-plus 6 Software for Windows (Insightful Corp., Seattle, WA). The McNemar-χ² 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.
any of the three methods was 25 (24%). The McNemar’s \( \chi^2 \) 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. 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. 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*. For microscopy, Wright stain at the central laboratories and differential staining 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.

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

<table>
<thead>
<tr>
<th>Number of samples</th>
<th>Microscopy</th>
<th>nPCR</th>
<th>LAMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concordant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>42 samples</td>
<td><em>P. falciparum</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14 samples</td>
<td><em>P. falciparum</em></td>
<td><em>P. vivax</em></td>
<td><em>P. vivax</em></td>
</tr>
<tr>
<td>32 samples</td>
<td>Negative</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Discordant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 samples</td>
<td>Negative</td>
<td><em>P. falciparum</em></td>
<td>Negative</td>
</tr>
<tr>
<td>4 samples</td>
<td><em>P. falciparum</em></td>
<td><em>P. vivax</em></td>
<td><em>P. vivax</em></td>
</tr>
<tr>
<td>2 samples</td>
<td><em>P. vivax</em></td>
<td><em>P. falciparum</em></td>
<td><em>P. falciparum</em></td>
</tr>
<tr>
<td>2 samples</td>
<td>Negative</td>
<td><em>P. falciparum</em></td>
<td><em>P. falciparum</em></td>
</tr>
<tr>
<td>2 samples</td>
<td><em>P. falciparum</em></td>
<td><em>P. vivax</em></td>
<td><em>P. vivax</em></td>
</tr>
<tr>
<td>1 sample</td>
<td><em>P. falciparum</em></td>
<td><em>P. falciparum</em> +</td>
<td><em>P. falciparum</em> +</td>
</tr>
<tr>
<td>1 sample</td>
<td><em>P. vivax</em></td>
<td><em>P. falciparum</em> +</td>
<td><em>P. vivax</em> +</td>
</tr>
<tr>
<td>1 sample</td>
<td><em>P. falciparum</em></td>
<td><em>P. falciparum</em> +</td>
<td><em>P. vivax</em> +</td>
</tr>
</tbody>
</table>

*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

<table>
<thead>
<tr>
<th>Species</th>
<th>Method</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. falciparum</em></td>
<td>LAMP</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Microscopy</td>
<td>93%</td>
<td>92%</td>
<td></td>
</tr>
<tr>
<td>PCR</td>
<td>100%</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td><em>P. vivax</em></td>
<td>LAMP</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Microscopy</td>
<td>98%</td>
<td>68%</td>
<td></td>
</tr>
<tr>
<td>PCR</td>
<td>99%</td>
<td>100%</td>
<td></td>
</tr>
</tbody>
</table>

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. 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 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.

Received October 19, 2009. Accepted for publication February 27, 2010. Financial support: This work was supported by funds from the DAAD (Deutscher Akademischer Austausch Dienst), Bonn, Germany, and institutional funds from the Center of Anatomy, Medical School, University of Cologne, Germany, the Regional Medical Sciences Center, Chiang Mai, Thailand, and the National Research Center for Protozoan Diseases at the Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan.

Authors’ addresses: Birgit Pöschl and Karanis Panagiotis, Medical and Molecular Parasitology Laboratory, University of Cologne, Medical School, Center of Anatomy, Institute II, Cologne, Germany. Jarvis Wanesonors and Salakitch Chuttipongvivate, Pathology Section, Regional Medical Sciences Center Chiang Mai, Chiang Mai, Thailand. Oriel Thekisoe, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido, Japan.

REFERENCES


