Rev. Inst. Med. Trop. Sao Paulo
2016;58:27
http://dx.doi.org/10.1590/S1678-9946201658027

ORIGINAL ARTICLE

MALARIA DIAGNOSIS BY LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) IN THAILAND

Ronja OCKER(1), Yongyut PROMPUNJAI(2), Salakchit CHUTIPONGVIVATE(2) & Panagiotis KARANIS(3)

SUMMARY

The loop-mediated isothermal amplification method (LAMP) is a recently developed molecular technique that amplifies nucleic acid under isothermal conditions. For malaria diagnosis, 150 blood samples from consecutive febrile malaria patients, and healthy subjects were screened in Thailand. Each sample was diagnosed by LAMP, microscopy and nested polymerase chain reaction (nPCR), using nPCR as the gold standard. Malaria LAMP was performed using *Plasmodium* genus and *Plasmodium falciparum* specific assays in parallel. For the genus *Plasmodium*, microscopy showed a sensitivity and specificity of 100%, while LAMP presented 99% of sensitivity and 93% of specificity. For *P. falciparum*, microscopy had a sensitivity of 95%, and LAMP of 90%, regarding the specificity; and microscopy presented 93% and LAMP 97% of specificity. The results of the genus-specific LAMP technique were highly consistent with those of nPCR and the sensitivity of *P. falciparum* detection was only marginally lower.

KEYWORDS: Malaria; *Plasmodium*; Molecular Diagnosis; LAMP; Thailand.

INTRODUCTION

Malaria is a major cause of morbidity and mortality, leading to approximately 198 million cases and 584,000 deaths in 2013[1]. The disease is endemic in a broad band around the equator, placing approximately half of the world's population at risk of infection[1].

Several strategies are currently being used to strengthen malaria control and to optimally lead to malaria elimination. Such reasoning is based on an accurate and prompt diagnosis, measuring the impact of any intervention, as well as being a prerequisite for an effective treatment with anti-malarial drugs[2], especially for the potentially fatal cases of *Plasmodium falciparum* infections[3]. Due to the WHO recommendation of using microscopy and malaria rapid diagnosis tests (RDT) in all suspected malaria cases, they are widely applied for instant parasitological confirmation, especially in regional clinics in endemic areas[4]. However, to obtain acceptable accuracy of the microscopic results, lengthy training sessions and experience are essential.

The molecular diagnostic tools applying DNA amplification have the advantage of distinguishing between similar appearing species and real-time amplification techniques are even able to quantitate the amount of parasites, providing a rapid processing of the samples[5,6]. Nested PCR is considered the most sensitive and specific tool for malaria diagnosis[7]. Several studies have shown that the molecular methods detect up to eight times more *Plasmodium* spp. infections than microscopy, and up to one third of these are mixed infections[8,9]. Therefore, the interpretation of malaria epidemiology has been affected by molecular tools, for instance by revealing large reservoirs of asymptomatic infections[10], by detecting a shift in age distribution of *Plasmodium* spp. infections[11], by facilitating the automation and standardisation along with the ability to differentiate species and detect drug resistance[12,13]. However, disadvantages of nested PCR are the high costs of sophisticated equipment such as the thermal cycler, the time-consuming procedure which delays the release of results to the physician and the need for well-trained laboratory staff. Due to limited economic resources, this diagnosis is not applicable in many endemic areas[14].

The loop-mediated isothermal amplification (LAMP) is a recently developed molecular technique for nucleic acid amplification and it was designed to overcome two disadvantages of PCR by being simpler and faster, while still providing a high level of accuracy. Briefly, a set of four specifically designed primers to recognise six distinct regions of the target DNA as well as a *Bst* polymerase are used for auto-cycling strand-displacement DNA synthesis. The amplification is conducted under isothermal conditions. Therefore, the cost of the technique can be reduced by minimally using a water bath or a heat block[15]. The method can also be less expensive if the assay is conducted with heat-treated blood samples instead of purified DNA[16]. LAMP accumulates approximately 10^9 copies of target DNA within a time frame of less than an hour[17,18].

(1) University of Cologne, Medical School, Germany. E-mail: ronjaocker@hotmail.com
(2) Regional Medical Sciences Center 3, Department of Medical Science, Ministry of Public Health Medical Science, Chonburi, Thailand. E-mails: yongyut.p@dmsc.mail.go.th; salakchitc@hotmail.com
(3) One Thousand Talent Plan of the Chinese Government, Center for Biomedicine and Infectious Diseases, Qinghai Academy of Animal and Veterinary Sciences and Qinghai Medical School, Qinghai University 1#Wei'er Road, Qinghai Biological Scientific Garden, Xining City, Qinghai Province 810016, China. E-mail: panagiotis.karanis@uk-koeln.de
Correspondence to: Panagiotis Karanis**.** E-Mail: panagiotis.karanis@uk-koeln.de; Tel./Fax: +86-971-5318-395.

Due to the relative stability of the LAMP reagents at 25 °C and 37 °C, it holds the potential for field application in tropical countries[19]. In an area of low endemicity, a portable LAMP-based assay called RealAmp (Real-time Fluorescence Loop-Mediated Isothermal Amplification) has been validated for the detection of submicroscopic infections[20].

Therefore, it is considered a promising candidate to be used as a diagnostic tool in field studies and in regional clinics, especially in endemic areas where costs must be reduced.

The aim of the present study was to apply the LAMP assay for malaria diagnosis of *P. falciparum* LAMP results were compared to the standard detection methods, microscopy and nested PCR in order to evaluate the sensitivity and specificity.

MATERIALS AND METHODS

Patients and biological material collection. The collection of samples and their analysis was performed from May to July 2011 at the Regional Medical Sciences Center 3, Department of Medical Science, Ministry of Public Health Medical Science, Chonburi, Thailand. DNA was provided by the DNA bank of the Science Center. The samples were previously obtained from various clinics of the mainly central Thailand provinces in May and June 2011, they were received during this period, randomly selected and included in our study. One hundred and thirty two of the samples were obtained from patients following hospital admission, who were either diagnosed with malaria due to a positive microscopy (118 samples) or by signs/ symptoms suggestive of malaria, as observed from the clinical history and geographical origin, but they later proved to be negative by microscopy (14 samples). EDTA-blood samples were taken before patients were treated with anti-malarials. In addition, 18 malaria free blood samples were obtained from the blood bank of Chonburi, drawn from clinically healthy subjects. Only these samples were not analysed by microscopy in the current study.

This study was performed using a protocol approved for medical research on human subjects, Department of Medical Sciences, Ministry of Public Health, Thailand. Informed consent was obtained from all the human adult participants or from the parents or legal guardians of minors.

Microscopy. For the analysis of the samples by microscopy, the hospital's staff collected EDTA-blood samples from the patient's forearm vein upon admission. A drop of this blood sample was placed upon a glass slide and both thin and thick films were prepared[21]. Following this, they were incubated with the Wright stain for 3 min and finally rinsed with water. The hospital's experienced laboratory staff examined the slides under a light microscope using the 100x oil immersion objective, screening more than 100 fields per slide at a pace of approximately 6 min/slide. The microscopy results were compared to the molecular methods (nPCR and LAMP).

DNA preparation. Template DNA for nested PCR and LAMP assays was extracted from 200 µL of EDTA whole blood using the QIAamp DNA Blood Mini Kit (QIAGEN GmbH, Hilden, Germany), according to the manufacturer protocol. The resulting 200 µL aliquots of template genomic DNA was stored at -20 °C.

Nested PCR assay based on 18S rRNA gene for *Plasmodium* species. All 150 samples were tested for the genus *Plasmodium*, *P. falciparum* and *P. vivax* by nested PCR. The species-specific nucleotide sequences of the 18S rRNA genes were applied as described previously by Kimura *et al.*[22], with slight modifications. For the outer PCR, 1 µL of template DNA was added to a 21 µL PCR mixture that consisted of 0.88 µM of each universal primer (P1 forward and P2 reverse primer) (10 mM each), 0.44 µM deoxynucleotide 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. The dNTP, $MgCl_2$, 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 primer in combination with each species-specific reverse primer. The amplified products were analysed in 2.5% agarose gels stained with ethidium bromide. The expected band sizes were approximately 160 bp for the first PCR product and approximately 110 bp for the second. The DNA ladder was from Promega (Madison, WI, USA). The WHO considers nPCR to be the most sensitive and specific diagnostic tool in malaria diagnosis[7,23], for this reason it was used as a reference standard for comparison in this study.

LAMP assay based on 18S rRNA for the detection of the *Plasmodium* genus and *P. falciparum* species. All 150 collected blood samples were tested by a primer set for *Plasmodium* DNA and a species-specific *P. falciparum*. One sample positive for *P. falciparum* nested PCR served as a positive control. A sample of human DNA from a healthy person, obtained from the blood bank of Chonburi served as a negative control. The LAMP assays as performed in this study, were firstly reported by Poon *et al.*[16], and Han *et al.*[18] (Table 1). Few modifications had to be applied as follows: in the LAMP reaction for genus *Plasmodium*, 1 µL of template DNA was added to a 19 µL of LAMP mixture, containing 6.3 µL of distilled water, 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* amplification slight modifications had to be applied: 1 µL of Betain was added, the 2x reaction LAMP buffer was adjusted and distilled water was reduced to 4.74 µL. The reactions were incubated at 65 °C for 60 min; the amplification was performed in a thermal cycler. All tubes were analysed by gel electrophoresis to assess the presence of the LAMP DNA product. Therefore 5 µL of each labelled LAMP product were mixed with 1 µL of loading buffer (RBC BioScience Corp., Taiwan) and added to each lane of 2.0% agarose gel. The gel electrophoresis was run for 50 min at 100 V in 1x Tris-Acetate-EDTA (TAE) buffer and afterwards stained with ethidium bromide (Promega, Madison, WI, USA). The molecular tests were performed by a well-trained staff closely supervised by a medical technologist with long lasting experience. This laboratory personnel has been blinded from the results of the nested PCR while performing the LAMP assays to avoid bias.

Statistical methods. GraphPad Software 2005-2009 (GraphPad Software, Inc., La Jolla, California, USA), an online statistical calculation program, was used to calculate test performances and acceptability

Table 1
Primer sets used for amplification of 18S rRNA genes by LAMP

Species	Primer	Sequence (5'-- 3')
genus *Plasmodium*	F3	GTATCAATCGAGTTTCTGACC
	B3c	CTTGTCACTACCTCTCTTCT
	FIP (F1c-F2)	TCGAACTCTAATTCCCCGTTACCTATCAGCTTTTGATGTTAGGGT
	BIP (B1-B2c)	CGGAGAGGGAGCCTGAGAAATAGAATTGGGTAATTTACGCG
	LPF	CGTCATAGCCATGTTAGGCC
	LPB	AGCTACCACATCTAAGGAAGGCAG
P. falciparum	F3	TGTAATTGGAATGATAGGAATTTA
	B3c	GAAAACCTTATTTTGAACAAAGC
	FIP (F1c-F2)	AGCTGGAATTACCGCGGCTGGGTTCCTAGAGAAACAATTGG
	BIP (B1-B2c)	TGTTGCAGTTAAAACGTTCGTAGCCCAAACCAGTTTAAATGAAAC
	LPF	GCACCAGACTTGCCCT
	LPB	TTGAATATTAAAGAA

evaluation indices using nested PCR results as the standard[24]. The 95% confidence interval for sensitivity and specificity was calculated by the modified Wald method[25].

RESULTS

Overall, the prevalence of *Plasmodium* species in the investigated samples. Out of 150 collected samples, 132 samples were examined by all three tests. The remaining 18 control samples were examined only by nPCR and LAMP. A *Plasmodium* infection was not found in any of these samples. Table 2 shows the results of the prevalence of genus *Plasmodium,* and of *P. falciparum.* In the 132 samples, nPCR detected 117 (88.6%), microscopy 118 (89.4%), and LAMP 116 (87.9%) positive samples for the genus *Plasmodium.* Microscopy showed one false-positive and LAMP showed one false-negative.

Regarding *P. falciparum,* nPCR detected 77 positive samples (58.3%). Among these there were four samples with mixed infection, also containing *P. vivax*. Microscopy also detected 77 *P. falciparum* positive samples (58.3%), including two false-positive and two false-negative results. The false-negative samples were found to be of mixed infection by nPCR, but were only diagnosed positive for *P. vivax* by microscopy. Similarly, for the other four samples of mixed infection, *P. vivax* was also dominant by microscopy, and diagnosed as *P. falciparum* only. LAMP also detected 77 positive samples (58.3%), including, however, four false positive and four false negative results.

Concerning *P. vivax,* nPCR detected 46 positive samples (34.8%), including two samples that also contained *P. falciparum.* Microscopy detected 42 (31.8%) positive results, including two samples that also contained *P. falciparum* that were not detected and one false positive result. All of the 15 samples of patients with fever of unknown origin were tested negative by all diagnostic tools.

Comparison of sensitivity and specificity, of microscopy, nested PCR and LAMP is shown in Table 3: the 117 nPCR-positive samples for the genus *Plasmodium* were also detected by microscopy (sensitivity of 100%; 95% CI: 96.1-100%), 116 were detected by LAMP (sensitivity of 99%; 95% CI: 94.8-99.9%). All 15 nPCR-negative samples for the genus *Plasmodium* were also negative by the LAMP assay (specificity of 100%; 95% CI: 76.1-100%), by microscopy 14 were negative (specificity of 93%; 95% CI: 68.2-100%).

Table 2
Comparison of nPCR, microscopy, and LAMP for the *Plasmodium* genus detection and species identification*

Parasite(s) detected by each method (nº. of samples) †		
nested PCR	**Microscopy**	**LAMP**
genus *Plasmodium* (117)	genus *Plasmodium* (118)	genus *Plasmodium* (116)
		negative (1)
P. falciparum (77)	*P. falciparum* **(72)**	*P. falciparum* **(69)**
		negative (4)
	P. vivax **(5)**‡	*P. falciparum* (4)
negative (15)¶ negative (18)°	negative(15)	negative(15) negative (18)

*n PCR=nested polymerase chain reaction; LAMP = Loop-mediated isothermal amplification. † Each row shows results obtained from identical blood samples. Microscopy + LAMP results that were not concordant are shown in **bold**. ‡ Four of these samples were not detected due to mixed infections. ¶ Samples of patients with fever of unknown origin. Samples provided by the blood bank

Regarding the 77 nPCR-positive samples for *P. falciparum*, 72 were positive by microscopy (sensitivity of 95%; 95% CI: 87.0-98.4%), and 69 by the LAMP assay (sensitivity of 90%; 95% CI: 80.6-94.9%). Among the 55 nPCR-negative samples for *P. falciparum*, LAMP was also negative in 51 (specificity of 93%; 95% CI: 82.3-97.6%), and microscopy in 53 nPCR-negatives (specificity of 97%; 95% CI: 87.0-99.7%).

Table 3
Sensitivity, specificity of microscopy and LAMP for genus *Plasmodium* and *P. falciparum* detection. Nested PCR results were used as the reference (gold standard) for comparison

	Method	Sensitivity	95% CI Sensitivity	Specificity	95% CI Specificity
Genus *Plasmodium*	Microscopy	100%	96.-1-100%	93%	68.2-100%
	LAMP	99%	94.8-99.9%	100%	76.1-100%
P. falciparum species	Microscopy	95%	87.0-98.4%	97%	87.0-99.7%
	LAMP	90%	80.6-94.9%	93%	82.3-97.6%

In all 6 samples containing mixed infections, microscopy detected only one *Plasmodium* species.

DISCUSSION

The risk of malaria transmission is considered to be low in most central parts of Thailand, but remains to be a burden in rural, forest areas at the border of Myanmar and Cambodia. Multidrug-resistant *P. falciparum,* in particular, is constraining malaria control programs. In order to provide an accurate treatment, clinical suspected malaria cases need to be confirmed by adequate laboratory diagnosis. In this study we compared a basic LAMP assay for malaria diagnosis of *P. falciparum* infections, to microscopy and to nested PCR as the gold standard.

Since the initial description of the LAMP method, genus and species-specific LAMP assays for the malaria parasites that infect humans have been developed[18,26]. Several studies currently working on the establishment of routine diagnosis are highly favouring this method[27,28,29]. The LAMP technique aims at combining an accuracy close to PCR with basic reagents, low technical requirements, and accomplishment by minimally-trained health workers. A rapid turnaround time produces results in about one hour[30]. Furthermore, recent techniques such as a variety of non-instrumented nucleic acid amplification (NINA) heater configurations have been developed for assays such as LAMP, even providing independence from electricity and/or instrumentation[31,32].

The genus-specific LAMP assay in our study provided highly consistent results, concurring with those of a previous study using the same primer set[33]. However, the species-specific *Plasmodium* detection performed marginally less accurately, presenting lower sensitivity and specificity than microscopy. This test yielded lower results in comparison with those of a previous study that used the same LAMP assay for malaria diagnosis in Northern Thailand and had detected falciparum malaria parasites in 48 out of 48 nPCR positive samples (100% sensitivity)[34]. In that study, a real-time coupled to a turbidimetric assay was used, possibly improving the accuracy.

The study held by Patel *et al*.[20], evaluated the RealAMP system in low transmission areas of India and Thailand. The study group from India presented a similar study group size (141 patients) compared to our study. The test presented 95% of sensitivity and 100% of specificity compared to nPCR, respectively. For low-density asymptomatic infections in the Thai study group, this system showed an explicit higher sensitivity than microscopy. In the RealAMP protocol, DNA is extracted from dried blood spots, real-time detection is carried out with fluorescence, thereby simplifying and shortening the time of analysis.

A possible explanation for the four cases of false-positive *P. falciparum* results via LAMP should be contamination (carry over) due to the high sensitivity of the LAMP assay. Despite preparing the reagent in a separated location and working according to standards to minimize the risk of contamination, it might have occurred when the post-LAMP microtubes were opened. To reduce the risk of contamination a LAMP combined with DNA filter paper (FTA card) for the diagnosis of *P. falciparum* has been developed, using a melting curve analysis instead of gel electrophoresis to visualize specific amplicons[35]. Furthermore, several methods of visual detection of the post-LAMP product in closed tubes without the need of opening them have been employed[33,36,37,38]. In order to increase sensitivity, the use of mitochondrial targets for LAMP-based detection of any *Plasmodium* genus parasite and of *P. falciparum* in particular, as recommended by Polley et al.[39], could be applied instead of the 18S rRNA *Plasmodium* gene. It has been claimed that mitochondrial primer sets can detect as few as 5 parasites per µL, opposed to around 100 parasites per µL as applied in this study. Incorporated into a LAMP kit the time to release a final result can be reduced, still providing similar accuracy to nPCR[40]. The detection of low parasitaemia favours the LAMP method as a point-of-care test for the treatment and follow-up of patients[41]. But taking in account that the LAMP technique has been proven to be very sensitive in other studies[34], it should also be considered that some of the false positive results by LAMP might have been real positives.

The application of the molecular tests in this study had some limitations, resulting firstly from the fact that the DNA for both PCR and LAMP was extracted with a commercial kit and secondly because both techniques were coupled to electrophoresis. This resulted in a high workload, the need for well-trained staff and the requirement of special reagents as well as equipments. The costs were not lowered. The turnaround time remained high because the genus-specific primers were firstly tested and afterwards each sample was screened with the species-specific primers. Therefore, the disadvantages of the PCR method could not be solved by the LAMP method in the present study, but these limitations can be eliminated in future investigations[42].

Due to the fact that microscopy is the standard method of malaria diagnosis, it has been applied for comparison in this study. However, microscopy has the disadvantage of a poor performance in low parasitaemia cases, as well as when the patients have already been treated or have taken anti-malarial drugs. Patients previously diagnosed with malaria and treated with anti-malarials within a week before admission,

were excluded from this study. In this study, the examiner did not diagnose by microscopy the six mixed infections identified by nested PCR, and has also detected two false-positive results for *P. falciparum* and one false-positive result for *P. vivax* (data not shown). These are common problems associated with microscopy. Both, the non-detected mixed infections, as well as the over-reporting of positive findings, lead to an either insufficient treatment or over-prescription of antimalarial drugs, thereby delaying the differential diagnosis of other febrile illnesses[33]. In addition, microscopy yielded results similar to those of nPCR. However, the results obtained from the various hospitals have probably limited the comparison with other methods due to the existence of differences in skill, concentration, and motivation of the microscopists. Therefore, the validity of such work, should be optimized in clinical trials in which microscopy will be strictly standardised. However, this effort is rarely found in most settings of malaria diagnosis.

The LAMP assay is a promising methodology for molecular diagnosis and molecular screening of malaria.The significant utility of the LAMP assay for the *Plasmodium* genus-specific detection developed by Han *et al*.[18], has been confirmed. Hence, it could be used as a diagnostic tool for malaria infection instead of a standard PCR detection. In conclusion, implementation of innovative research is necessary to ensure a more accurate molecular diagnosis of falciparum malaria in a resource limited setting.

CONCLUSIONS

Any method with a potential to improve the malaria diagnosis will be beneficial to human health and it is worthy and valuable to work in the field. The development of simple and effective molecular methods with the characteristics of LAMP for malaria diagnosis and *Plasmodium* infections in humans and mosquito vectors will be useful. Implementation of innovative research is necessary to ensure a more accurate molecular diagnosis of *Plasmodium* malaria in a resource limited setting.

REFERENCES

1. World Health Organization. WorldMalariaReport2014. WHO; 2015. [cited 2015 Jul 11]. Available from: http://www.who.int/malaria/publications/world_malaria_report_2014/wmr-2014-no-profiles.pdf?ua=1
2. Na-Bangchang K, Karbwang J. Current status of malaria chemotherapy and the role of pharmacology in antimalarial drug research and development. Fundam Clin Pharmacol. 2009;23:387-409.
3. Clark IA, Alleva LM, Mills AC, Cowden WB. Pathogenesis of malaria and clinically similar conditions. Clin Microbiol Rev. 2004;17:509-39.
4. World Health Organization.. Malaria: diagnostic testing. WHO; 2014. [cited 2014 Mar 5]. Available from: http://www.who.int/malaria/areas/diagnosis/en/
5. Snounou G, Viriyakosol S, Zhu XP, Jarra W, Pinheiro L, do RosarioVE, et al. High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. Mol Biochem Parasitol. 1993;61:315-20.
6. Rougemont M, Van Saanen M, Sahli R, Hinrikson HP, Bille J, Jaton K. Detection of four *Plasmodium* species in blood from humans by 18S rRNA gene subunit-based and species-specific realtime PCR assays. J Clin Microbiol. 2004;42:5636-43.
7. Banoo S, Bell D, Bossuyt P, Herring A, Mabey D, Poole F, et al. Evaluation of diagnostic tests for infectious diseases: general principles. Nat Rev Microbiol. 2006;4(9 Suppl):S21-31.
8. Singh B, Cox-Singh J, Miller AO, Abdullah MS, Snounou G, Rahman HA. Detection of malaria in Malaysia by nested polymerase chain reaction amplification of dried blood spots on filter papers. Trans R Soc Trop Med Hyg. 1996;90:519-21.
9. Snounou G, Pinheiro L, Goncalves A, Fonseca L, Dias F, Brown KN, et al. The importance of sensitive detection of malaria parasites in the human and insect hosts in epidemiological studies, as shown by the analysis of field samples from Guinea Bissau. Trans R Soc Trop Med Hyg. 1993;87:649-53.
10. Alves FP, Durlacher RR, Menezes MJ, Krieger H, Silva LH, Camargo EP. High prevalence of asymptomatic *Plasmodium vivax* and *Plasmodium falciparum* infections in native Amazonian populations. Am J Trop Med Hyg. 2002;66:641-8.
11. Kasehagen LJ, Mueller I, McNamara DT, Bockarie MJ, Kiniboro B, Rare L, et al. Changing patterns of *Plasmodium* blood-stage infections in the Wosera region of Papua New Guinea monitored by light microscopy and high throughput PCR diagnosis. Am J Trop Med Hyg. 2006;75:588-96.
12. Singh B, Bobogare A, Cox-Singh J, Snounou G, Abdullah MS, Rahman HA. A genus- and species-specific nested polymerase chain reaction malaria detection assay for epidemiologic studies. Am J Trop Med Hyg. 1999;60:687-92.
13. Dong J, Olano JP, McBride JW, Walker DH. Emerging pathogens: challenges and successes of molecular diagnostics. J Mol Diagn. 2008;10:185-97.
14. Hanscheid T, Grobusch MP. How useful is PCR in the diagnosis of malaria? Trends Parasitol. 2002;18:395-8.
15. Chutipongvivate S, Prompunjai Y, Neadruengsang W, Wangsiricharoen S. Alternative malaria diagnostic tools: evaluation of *Plasmodium falciparum* detection along Thailand's border by Loop-Mediated Isothermal Amplification (LAMP) and Immunochromatographic Test (ICT). J Trop Dis. 2014;2:147.
16. Poon LL, Wong BW, Ma EH, Chan KH, Chow LM, Abeyewickreme W, et al. Sensitive and inexpensive molecular test for falciparum malaria: detecting *Plasmodium falciparum* DNA directly from heat-treated blood by loop-mediated isothermal amplification. Clin Chem. 2006;52:303-6.
17. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, et al. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. 2000;28:E63.
18. Han ET, Watanabe R, Sattabongkot J, Khuntirat B, Sirichaisinthop J, Iriko H, et al. Detection of four *Plasmodium* species by genus- and species-specific loop-mediated isothermal amplification for clinical diagnosis. J Clin Microbiol. 2007;45:2521-8.
19. Thekisoe OM, Bazie RS, Coronel-Servian AM, Sugimoto C, Kawazu S, Inoue N. Stability of loop-mediated isothermal amplification (LAMP) reagents and its amplification efficiency on crude trypanosome DNA templates. J Vet Med Sci. 2009;71:471-5.
20. Patel JC, Lucchi NW, Srivastava P, Lin JT, Sug-Aram R, Aruncharus S, et al. Field evaluation of a real-time fluorescence loop-mediated isothermal amplification assay, RealAmp, for the diagnosis of malaria in Thailand and India. J Infect Dis. 2014;210:1180-7.
21. Warhurst DC, Williams JE. ACP Broadsheet nº 148. July 1996. Laboratory diagnosis of malaria. J Clin Pathol. 1996;49:533-8.
22. Kimura M, Kaneko O, Liu Q, Zho M, Kawamoto F, Wataya Y, et al. Identification of the four species of human malaria parasites by nested PCR that targets variant sequences in the small subunit rRNA gene. Parasitol Int. 1996;46:91-5.
23. World Health Organisation. Focused screening and treatment. WHO; 2011. [cited 2011 Nov 5]. Available from: http://www.who.int/malaria/diagnosis_treatment/arcp/focused_screening/en/
24. GraphPad.Software. La Jolla: Graph Pad Software, Int. [cited 2011 Dec 1]. Available from: http://graphpad.com/quickcalcs/index.cfm

25. Agresti A, Coull BA. Approximate is better than "Exact" for interval estimation of binomial proportions. Am Stat. 1998;52:119-26.

26. Iseki H, Kawai S, Takahashi N, Hirai M, Tanabe K, Yokoyama N, et al. Evaluation of a loop-mediated isothermal amplification method as a tool for diagnosis of infection by the zoonotic simian malaria parasite *Plasmodium knowlesi*. J Clin Microbiol. 2010;48:2509-14.

27. Surabattula R, Vejandla MP, Mallepaddi PC, Faulstich K, Polavarapu R. Simple, rapid, inexpensive platform for the diagnosis of malaria by loopmediated isothermal amplification (LAMP). Exp Parasitol. 2013;134:333-40.

28. Tao ZY, Zhou HY, Xia H, Xu S, Zhu HW, Culleton LR *et al*. Adaptation of a visualized loop-mediated isothermal amplification technique for field detection of *Plasmodium vivax* infection. Parasit Vectors. 2011;4:115.

29. Abdul-Ghani R, Al-Mekhlafi AM, Karanis P. Loop-mediated isothermal amplification (LAMP) for malarial parasites of humans: would it come to clinical reality as a point-of-care test? Acta Trop. 2012;122:233-40.

30. Major Breakthrough: LAMP test opens new paths to malaria elimination. Geneva: Finddiagnostics.org. [cited 2014 Mar 10]. Available from: http://www.finddiagnostics.org/media/press/130517.html

31. Sema M, Alemu A, Bayih AG, Getie S, Getnet G, Guelig D, et al. Evaluation of non-instrumented nucleic acid amplification by loop-mediated isothermal amplification (NINA-LAMP) for the diagnosis of malaria in Northwest Ethiopia. Malar J. 2015;14:44.

32. LaBarre P, Gerlach J, Wilmoth J, Beddoe A, Singleton J, Weigl B**.** Non-instrumented nucleic acid amplification (NINA): instrument-free molecular malaria diagnostics for low-resource settings. Conf Proc IEEE Eng Med Biol Soc. 2010;2010:1097-9.

33. Lucchi NW, Demas A, Narayanan J, Sumari D, Kabanywanyi A, Kachur SP, et al. Real-time fluorescence loop mediated isothermal amplification for the diagnosis of malaria. PLoS One. 2010;5:e13733.

34. Pöschl B, Waneesorn J, Thekisoe O, Chutipongvivate S, Karanis P. Comparative diagnosis of malaria infections by microscopy, nested PCR, and LAMP in northern Thailand. Am J Trop Med Hyg. 2010;83:56-60.

35. Yamamura M, Makimura K, Ota Y. Evaluation of a new rapid molecular diagnostic system *for Plasmodium falciparum* combined with DNA filter paper loop-mediated isothermal amplification, and melting curve analysis Jpn J Infect Dis. 2009;62:20-5.

36. Tomita N, Mori Y, Kanda H, Notomi T. Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. Nat Protoc. 2008;3:877–82.

37. Mori Y, Hirano T, Notomi T. Sequence specific visual detection of LAMP reactions by addition of cationic polymers. BMC Biotechnol. 2006;6:3.

38. Mohon AN, Elahi R, Khan WA, Haque R, Sullivan DJ Jr, Alam MS. A new visually improved and sensitive loop mediated isothermal amplification (LAMP) for diagnosis of symptomatic falciparum malaria. Acta Trop. 2014;134:52-7.

39. Polley SD, Mori Y, Watson J, Perkins MD, González IJ, Notomi T, et al. Mitochondrial DNA targets increase sensitivity of malaria detection using loop-mediated isothermal amplification. J Clin Microbiol. 2010;48:2866-71.

40. Polley SD, Gonzalez IJ, Mohamed D, Daly R, Bowers K, Watson J, et al. Clinical evaluation of a loop-mediated amplification kit for diagnosis of imported malaria. J Infect Dis. 2013;208:637-44.

41. Lee PW, Ji DD, Liu CT, Rampao HS, do Rosario VE, Lin IF, et al. Application of loop-mediated isothermal amplification for malaria diagnosis during a follow-up study in Sao Tome. Malar J. 2012;11:408.

42. Han ET. Loop-mediated isothermal amplification test for the molecular diagnosis of malaria. Expert Rev Mol Diagn. 2013;13:205–18.

Received: 17 May 2015
Accepted: 17 September 2015