



Evaluation of Loop Mediated Isothermal Amplification (LAMP) for Diagnosis of *Plasmodium falciparum* in Wad Medani City, Gezira State, Sudan

**M. Y. Mohamed¹, A. D. Abakar¹, B. A. Talha¹, Salah Eldin G. Elzaki²,
Y. A. Mohammed³, Usama Abdalla Elsharief⁴, K. A. Mohamed Ahmed⁵
and B. Y. M. Nour^{6*}**

¹Department of Medical Parasitology, Faculty of Medical Laboratory Sciences, University of Gezira, Wad Medani, Sudan.

²Department of Epidemiology, Tropical Medicine Research Institute, National Centre for Research, Khartoum, Sudan.

³Department of Clinical Chemistry, National Cancer Institute, University of Gezira, Wad Medani, Sudan.

⁴Faculty of Medical Laboratory Sciences, University of Gadarif, Sudan.

⁵Department of Hematology and Immunology, Faculty of Medical Laboratory Sciences, University of Gezira, Wad Medani, Sudan.

⁶Department of Parasitology, Blue Nile National Institute for Communicable Disease, University of Gezira, Wad Medani, Sudan.

Authors' contributions

This work was carried out in collaboration among all authors. Authors MYM, ADA and BYMN designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors BAT, SEGE, YAM and KAMA managed the analyses of the study. Author UAE managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/IJTDH/2019/v38i430191

Editor(s):

(1) Dr. Thomas I. Nathaniel, Department of Biomedical Sciences, University of South Carolina, School of Medicine-Greenville, Greenville, SC 29605, USA.

Reviewers:

(1) Mra Aye, Malaysia.

(2) Pranab Goswami, IIT Guwahati, India.

Complete Peer review History: <http://www.sdiarticle4.com/review-history/51785>

Original Research Article

Received 01 August 2019
Accepted 05 October 2019
Published 14 October 2019

ABSTRACT

Plasmodium falciparum considered as the most serious form of species causes malaria compared with other species. Diagnosis of *falciparum* malaria in Sudan remain a major problem, the laboratory diagnosis depends solely on microscopy and RDTs. Loop mediated isothermal

*Corresponding author: Email: bakrinour@gmail.com;

amplification (LAMP) assay is a molecular technique done in isothermal temperature using simple, inexpensive instruments for detection of *falciparum* malaria. The aim of the study is to evaluate the diagnostic performance of loop mediated isothermal amplification (LAMP) assay for detection of *P. falciparum* and compare with microscopic detection. A cross sectional hospital based study conducted on 220 blood samples collected from participants suspected to have falciparum malaria attending Wad Medani Teaching Hospitals and 26 healthy participants during the period November 2018 to January 2019. Thick blood films were done and used for *P. falciparum* detection. The extracted DNA by TE buffer was amplified by LAMP assay targeting 18S rRNA gene. Data were analyzed using Medical calculator (MedCalc) programs (V. 16). The results showed that the sensitivity, specificity, positive predictive value, negative predictive values were 99.1%, 84.6%, 53.2%, 99.8% respectively. Validation of LAMP diagnostic performance revealed that area under the curve is 0.919, while Weighted Kappa is 0.866. The study concluded that the LAMP assay had the identical diagnostic performance compared with microscopy in diagnosis of *Plasmodium falciparum* malaria. This gives a relative effortless application of LAMP assay in Sudan after availing the required logistics.

Keywords: 18S rRNA; LAMP; *Plasmodium falciparum* diagnosis; Sudan.

1. INTRODUCTION

Malaria is a major health problem prevailing in many parts of the world particularly in tropical areas [1-3]. According to WHO, the disease accounts for approximately 219 million cases and 435,000 deaths. In Africa, *Plasmodium falciparum* is the most prevalent species and responsible for 99.7% of malaria cases [4]. In Sudan, overall malaria prevalence is 5.9% in which *P. falciparum* constitute 87.6% of cases and 5% mixed infection with *P. vivax* [5]. Microscopic examination of stained blood film remain the gold standard method for malaria diagnosis, identification of parasite is achieved by thick blood film, whereas as screening and the species differentiation is performed by thin blood film [6]. According to WHO guidelines, the diagnosis of malaria parasites by microscopic examination remain reliable method only if done by trained microscopist [4]. Therefore, accuracy of the microscopy depends on quality of blood film and skill of microscopist's which detect about 10 – 100 parasite/μl [7]. The detection limit increased in expert microscopist's to 5 parasites/μl [8]. Loop mediated isothermal amplification (LAMP) assay is molecular diagnostic test assay, was first reported in 2000 [9]. In malaria diagnosis, the technique detects the conserved 18 small-subunit ribosome RNA (18S rRNA) gene of *P. falciparum* [10]. The reaction endpoint appears as turbidity in reaction tube result from precipitation of magnesium pyrophosphate and reporting results by naked eye [11]. As compared with nested PCR, LAMP assay is easy, simple, cheap, rapid and doesn't required purified DNA for DNA amplification [10]. These advantages lead to considering that LAMP

assay is simple and reliable assay for diagnosis of *falciparum* malaria in endemic area as routine diagnostic tool [12]. Need for simple, accurate and reliable molecular technique for diagnosis of *falciparum* malaria in endemic area is importance which helps in improving diagnosis *falciparum* malaria due to limitations of microscopy. The current study was designed to evaluate the performance of Loop mediated isothermal amplification (LAMP) assay for 18S rRNA in diagnosis of *P. falciparum* malaria during high transmission season in Gezira state, Sudan, a known endemic area for *Plasmodium falciparim*.

2. MATERIALS AND METHODS

2.1 Study Design, Area and Population

A hospital based cross-sectional study conducted in 220 patients with *falciparum* malaria from all ages and both gender attending Wad Medani Teaching Hospitals diagnostic by presence of *P. falciparum* and 26 healthy participants, during the period November 2018 to January 2019 in high transmission season. Wad Medani Teaching Hospitals located in Wad Medani city, a capital of Gezira State in East-Central Sudan (Fig. 1).

2.2 Data and Sample Collection

The data were collected using questionnaire. 3 ml of venous blood sample was collected under aseptic condition from each participant into EDTA container. Dry blood spot (DBS) was prepared into Whatt Man filter paper (No. 3) immediately.

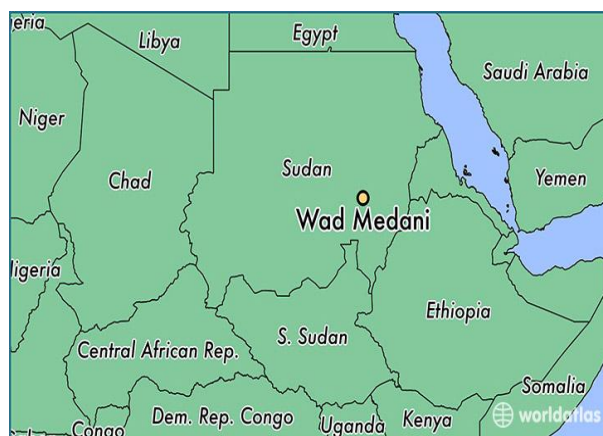


Fig. 1. Location of Wad Medani city, Gezira State, Sudan (World Atlas, 2015)

2.3 Microscopy

The blood films were prepared immediately and stained using Giemsa’s stain and examined under oil immersion lens. The results reported after searching 200 fields and *P. falciparum* stages were identified [13].

2.4 DNA Extraction

The DNA extraction from DBS was done using TE buffer procedure as described by Bereczky with small modifications. Total circle of DBS were punched by a sterile surgical plate and placed into a sterile, clean 1.5 eppendorf. About 100 µl of TE buffer (10 mM Tris, pH 8.0, 0.1 mM EDTA in distilled water) was transferred into tube and incubated at 50°C for 30 minutes. The punches were pushed several times with different pipette tips toward the bottom of the tube and incubated at 97°C for 15 min. The tube was centrifuged at high speed for 30 seconds and the supernatant was used for LAMP assay [14].

2.5 Loop Mediated Isothermal Amplification (LAMP) Assay for 18S rRNA

The reaction was done as previously described by Poon et al., [10]. As shown in Table 2 the

reaction contains primers (FIP and BIP 40 pmol, Loop-F and Loop-B 20 pmol, F3 and B3 5 pmol) Table 1 (Macrogen Europe BV, North Holland). The protocol done according to New England Biolabs; reaction mixture contains (40 mmol/L Tris-HCl, 20 mmol/L KCl, 16 mmol/L MgSO₄, 20 mmol/L NH₄SO₄, 0.2% Tween 20, 1.6 mol/L betaine, deoxynucleotide triphosphates 2.8 mmol/L each), Bst DNA polymerase, template DNA and distilled water to a total volume of 25 µL (New England Biolabs inc., USA). The reaction mixture was incubated in a hot water bath at 65°C for 60 minutes [10,12]. The LAMP product appears as turbidity occurs in reaction tube and the interpretation done by naked eye as described by Mori in 2001; there is positive correlation between the turbidity and the amount of amplified DNA Figs. 2 and 3, Mori et al. [11].

2.6 Data Analysis

Data was analyzed using MedCalc program (V. 16). Demographic data and test validation was obtained. The validation of LMAP assay was done using receiving operating characteristics (ROC) curve and the sensitivity, specificity, predictive values and area under the ROC curve was generated. Weighted Kappa between LAMP assay and microscopy was calculated to show the agreement.

Table 1. *P. falciparum* species specific 18S rRNA primer for LAMP technique

Primer	Sequence (5´-3´)
FIP (F1c-F2)	AGCTGGAATTACCGCGGCTGGGTTCCCTAGAGAAACAATTGG
BIP (B1-B2c)	TGTTGCAGTTAAAACGTTTCGTAGCCCAAACCAGTTTAAATGAAAC
F3	TGTAATTGGAATGATAGGAATTTA
B3c	GAAAACCTTATTTTGAACAAAGC
LPF	GCACCAGACTTGCCCT
LPB	TTGAATATTAAGAA



Fig. 2. Showed LAMP assay product; 1: Negative control, 2-8: Positive samples

3. RESULTS

The overall patients infected with falciparum malaria were 220 patients and healthy participants were 26 participants. Among patients, males and females was 60.4% and 39.6% respectively. The mean age was 21.4 ± 17.5 years which distributed as 17.3% in children under 5 years, 29.1% in 6 – 15 years, 30% in 16 – 30 years, 12.7% in 31 – 45 years and 10.9% in age > 45 years Table 2. The mean of parasite count was 59096.75 parasite/ μ l \pm (SE=924.097) (range between 79 – 549,333

parasite/ μ l). The common symptoms were fever (99%), fatigue (88.6%) and headache (87.7%) Table 2.

As shown in Table 3, the samples that were diagnosed by thick blood film gave results accounts for 100% *Plasmodium falciparum* positivity among patients (n=220) and one sample of healthy participants (1/26) (3.8%) while all samples were positive by LAMP technique among patients (100%) and two samples were positive among healthy participants (7.6%).

Table 2. Socio-demographic characteristics of study participants

Demographic characteristics		Cases (N=220) No (%)	Healthy (N=26) No (%)
Sex	Male	133 (60.4%)	19
	Female	87 (39.6%)	7
Age (years) Mean \pm SD		21.4 \pm 17.5 Years	-
Age groups (years)	< 5 Years	38 (17.3%)	0
	6 – 15 Years	64 (29.1%)	0
	16 – 30 Years	66 (30.0%)	14
	31 – 45 Years	28 (12.7%)	7
	> 45 Years	24 (10.9%)	5
Mean of parasite count (parasite/ μ l)		59096.75	

Table 3. *P. falciparum* positivity by microscopy versus 18S rRNA gene LAMP assay

Result	Microscopy		18S rRNA gene by nPCR	
	Patients	Healthy	Patients	Healthy
Positive	220	1	210	2
Negative	-	25	-	24

Table 4. AUC, sensitivity, specificity, positive and negative predictive value of LAMP assay for 18S rRNA gene

Diagnostic performance	18S rRNA gene by LAMP assay
Sensitivity	99.1%
Specificity	84.6%
Positive predictive value	53.2%
Negative predictive value	99.8%
Area under the curve (AUC)	0.919
Weighted kappa ¹	0.866

¹ Linear weights (95% CI: 0.770 - 0.972)

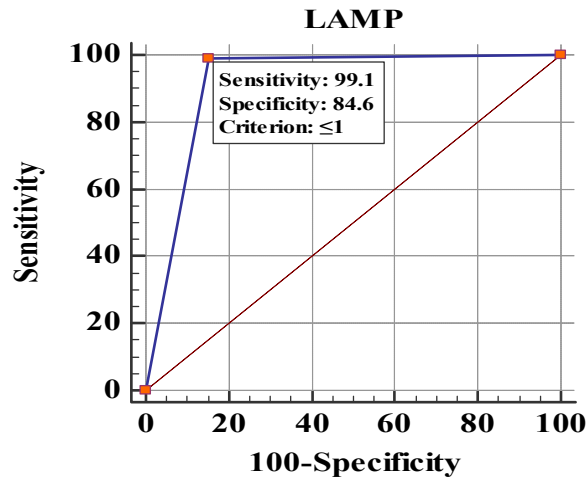


Fig. 3. ROC curve of LAMP assay compared with microscopy

The validity of LAMP assay for 18S rRNA gene were compared with microscopy as reference method showed that the area under the curve (AUC) was 0.919 with 99.1% sensitivity, 84.6% specificity, 53.2% positive predictive value, 99.8% negative predictive value and the agreement between LAMP assay and microscopy by Weighted Kappa was 0.866 (95% CI:0.770 – 0.972) Table 4; Fig. 3.

4. DISCUSSION

Plasmodium falciparum is a fatal pathogenic *Plasmodium* species that cause malignant malaria in humans. *Falciparum* malaria represent 99.7% of estimated cases in the WHO African Region [4] and 87.6% of malaria cases in Sudan [5]. Microscopy is the standard method; it's simple, rapid, inexpensive and allow to determine

stages, degree of parasitemia and species differentiation but required an expert microscopist to give a reliable results. LAMP assay may solve these problems and filling the gaps, this molecular assay is sensitive and not require instrument as needed to other molecular assay.

The tests used for diagnosis of *falciparum* malaria in Sudan are microscopy and RDTs and showed varying sensitivity and specificity of when compared with nested PCR [15]. Moreover, RDTs haven't ability to be efficient diagnostic tool when compared to microscopy in Gezira state [16]. The distribution of *falciparum* malaria in Gezira state among suspected cases is generally about 30.3% [17]. The study aim to detect diagnostic performance of LAMP technique in diagnosis of *Plasmodium falciparum* malaria.

In the current study, regarding high sensitivity, specificity, area under the curve and agreement (kappa) of LAMP assay compared with microscopy indicate that LAMP assay for 18S rRNA gene gave reliable results for screening of *P. falciparum* in suspected patients in study area. Similar findings were also reported by other authors elsewhere [10,12,18]. However, Vincent *et al.*, [19]; Pöschl *et al.*, [20]; Sirichaisinthop *et al.*, [21] reported a higher specificity (100%) when compared to our results. Moreover, Weighted Kappa (0.86) is higher when compared with finding of Poblete *et al.*, [22] (Weighted Kappa = 0.84). In their study, Paris and his colleagues [23], reported both lower sensitivity (76.1%) and Weighted Kappa (0.65), this could be related to small sample size (n = 115) and different method of DNA extraction adopted in their study. For more comparison: indicated the finding of this study is higher in its sensitivity and lower in its specificity when compared with that found by Panel Rambabu Surabattulaab *et al.* [24] 95% and 93.3% respectively.

The two false positive results by LAMP assay among healthy participants may be due to asymptomatic falciparum malaria infection a trend, which may be presents in endemic areas.

5. CONCLUSION

The study concluded that the LAMP assay had high sensitivity, specificity and had identical diagnostic performance compared with microscopy in diagnosis of *Plasmodium falciparum* malaria. This gives a s relative effortlessness implementation of LAMP assay in Sudan, Teaching Hospitals or even private laboratories to reduce the false negative and positive results after availing the required logistics.

CONSENT AND ETHICAL APPROVAL

The ethical approval and permission obtained from Ministry of Health, Gezira State and written informal consent from each participant.

ACKNOWLEDGEMENT

The authors thank all patients who participated in this study and acknowledgements extended to the laboratory staff of molecular labs in the Faculty of Medical Laboratory Sciences in University of Gezira and Tropical Institute for Medical Research for their technical assistance.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Adam I, Khamis AH, Elbashir MI. Prevalence and risk factors for *Plasmodium falciparum* malaria in pregnant women of eastern Sudan. *Malaria Journal*. 2005;4(1):18.
2. Girma S, Cheaveau J, Mohon AN, Marasinghe D, Legese R, Balasingam N, et al. Prevalence and epidemiological characteristics of asymptomatic malaria based on ultrasensitive diagnostics: A cross-sectional study. *Clinical Infectious Diseases*; 2018.
3. Khan S, Jamil S, Jamil M, Alam I, Ubaid M. Changing seasonal trends of plasmodium vivax and plasmodium falciparum malaria. *Journal of Medical Sciences*. 2019;27(1):16-19.
4. World Health Organization. World Malaria Report. Geneva Licence: CC BY-NC-SA 3.0 IGO; 2018.
5. Federal Ministry of Health. Sudan Malaria Indicator Survey 2016. CNCD/CD, Federal Ministry of Health, Khartoum, Sudan. 2017;8.
6. Bharti AR, Patra KP, Chuquiyauri R, Kosek M, Gilman RH, et al. Polymerase chain reaction detection of *Plasmodium vivax* and *Plasmodium falciparum* DNA from stored serum samples: Implications for retrospective diagnosis of malaria. *Am J Trop Med & Hyg*. 2007;77:444–446.
7. Wongsrichanalai C, Barcus MJ, Muth S, Sutamihardja A, Wernsdorfer WH. A review of malaria diagnostic tools: microscopy and Rapid Diagnostic Test (RDT). *Am J Trop Med Hyg*. 2007;77(6): 119–127.
8. Payne D. Use and limitations of light microscopy for diagnosing malaria at the primary health care level. *Bull World Health Organ*. 1988;66:621–628.
9. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, et al. Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res*. 2000;28:63.
10. Poon LL, Wong BW, Ma EH, Chan KH, Chow LM, Abeyewickreme W. 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: 303–306.
11. Mori Y, Nagamine K, Tomita N, Notomi T. Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. *Biochemical and Biophysical Research Communications.* 2001;289(1):150-154.
 12. 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;45:2521–2528.
 13. Cheesbrough, Monica. *District laboratory practice in Tropical Countries (Part 1).* Cambridge University Press. UK. 2009;239–258.
 14. Bereczky S, Martensson A, Gil JP, Farnert A. Short report: Rapid DNA extraction from archive blood spots on filter paper for genotyping of *Plasmodium falciparum*. *Am J Trop Med Hyg.* 2005; 72:249–251.
 15. Abdalla ZA, Rahma NA, Hassan EE, Abdallah TM, Hamad HE, Omer SA, Adam I. The diagnostic performance of rapid diagnostic tests and microscopy for malaria diagnosis in eastern Sudan using a nested polymerase chain reaction assay as a reference standard. *Transactions of the Royal Society of Tropical Medicine and Hygiene.* 2019;1-5.
 16. Nour BY, Almobark MA, Talha AA, Elgaili EM, Abuidris D, Habour AB, et al. Performance of rapid diagnostic test for malaria diagnosis at the different specialized hospitals in Wad Medani, Gezira State, Sudan. *International Journal of Tropical Disease.* 2016;12(4): 1-7.
 17. Elgoraish AG, Elzaki SEG, Ahmed RT, Ahmed AI, Fadlalmula HA, Abdalgader Mohamed, et al. Epidemiology and distribution of *Plasmodium vivax* malaria in Sudan. *Transactions of the Royal Society of Tropical Medicine and Hygiene.* 2019; 1–8.
 18. Ocker R, Prompunjai Y, Chutipongvivate S, Karanis P. Malaria diagnosis by Loop-mediated Isothermal Amplification (LAMP) in Thailand. *Revista do Instituto de Medicina Tropical de São Paulo.* 2016;58:27.
 19. Vincent JP, Komaki-Yasuda K, Iwagami M, Kawai S, Kano S. Combination of PURE-DNA extraction and LAMP-DNA amplification methods for accurate malaria diagnosis on dried blood spots. *Malaria Journal.* 2018;17(1):373.
 20. Pöschl B, Waneesorn J, Thekisoe O, Chutipongvivate S, Panagiotis K. Comparative diagnosis of malaria infections by microscopy, nested PCR and LAMP in northern Thailand. *The American Journal of Tropical Medicine and Hygiene.* 2010;83(1):56–60.
 21. Sirichaisinthop J, Buates S, Watanabe R, Han ET, Suktawonjaroenpon W, Krasaesub S, Takeo S, Tsuboi T, Sattabongkot J. Evaluation of Loop-mediated Isothermal Amplification (LAMP) for malaria diagnosis in a field setting. *The American Journal of Tropical Medicine and Hygiene.* 2011;85(4):594–596.
 22. Poblete JB, Tongol-Rivera PN, Yano K, Kano S, Kawazu SI. The validity and applicability of Loop-Mediated Isothermal Amplification (LAMP) in the Diagnosis of Malaria in the Philippines. *Philippine Journal of Health Research and Development.* 2015;19(1):6.
 23. Paris DH, Imwong M, Faiz AM, Hasan M, Yunus EB, Silamut K, Lee SJ, Day NP, Dondorp AM. Loop-mediated Isothermal PCR (LAMP) for the diagnosis of *falciparum* malaria. *The American Journal of Tropical Medicine and Hygiene.* 2007;77(5):972–976.
 24. Panel Rambabu Surabattulaab, Manju Pradeep Vejandlaa, Prudhvi Chand, Mallepaddia, Konrad Faulstichb, Rathnagiri Polavarapua; Simple, rapid, inexpensive platform for the diagnosis of malaria by Loop Mediated Isothermal amplification (LAMP). *Experimental Parasitology.* 2013;134(3):333-340.

© 2019 Mohamed et al.; This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/4.0>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Peer-review history:

The peer review history for this paper can be accessed here:
<http://www.sdiarticle4.com/review-history/51785>