ACTA
PROTOZOOLOGICA

Acta Protozool. (2023) 62: 15–23
www.ejournals.eu/Acta-Protozoologica
doi:10.4467/16890027AP.23.002.17903

Diagnostic performance of loop-mediated isothermal amplification (LAMP) and Ultra-sensitive PCR in diagnosis of malaria in western Saudi Arabia

Rasha H. SOLIMAM[1], Alexandra MARTIN-RAMIREZ[2], Jose M. RUBIO[2,3], Eman A. KHALIFA[1], Bushra E. HUSSEIN[4], Maha M. WAHAB[5], Marta LANZA[2], Yousry A. HAWASH[1]

[1] Department of Microbiology, College of Medicine, Taif University, Saudi Arabia

[2] Malaria & Emerging Parasitic Diseases Laboratory, National Microbiology Center, Instituto de Salud Carlos III, Madrid, Spain

[3] Centro de Investigación Biomédica en Red de Enfermedades Infecciosas, CIBERINFEC, Madrid, Spain

[4] Department of Tropical Medicine and Infectious Diseases, Faculty of Medicine, University of Tanta, Egypt

[5] Microbiology and Parasitology Department, KFMC Taif, KSA

Abstract. Malaria diagnosis continues to be one of the most important steps in the cycle of control specially in endemic countries with low parasitic load infections. Loop-mediated isothermal amplification (LAMP) and ultrasensitive PCR (Us-PCR) are two promising candidates for malaria diagnosis. A cross sectional study performed at King Faisal Hospital, Taif KSA involved patients suffering from signs and symptoms suggesting of malaria, 35 blood samples diagnosed by Nested Multiplex PCR as a reference method (13 *P. falciparum*, 17 *P. vivax*, 3 mixed *P. falciparum* and *P. vivax*) plus two negative controls were selected to be included in this study to analyse the performance of two LAMP methods (LAMP OptiGene® and LAMP WarmStart®) and two ultrasensitive PCRs (Us-PCR TARE-2 and Us-PCR Var-ATS). LAMP OptiGene® and LAMP WarmStart® performances were identical and better than the performance of Us PCR TARE 2 and Us-PCR var-ATS for *P. falciparum*, achieving 93.75% sensitivity, 100% specificity and 97.17% accuracy versus 87.5% sensitivity, 100% specificity and 94.29% accuracy for the Us PCR TARE 2 and 81.25% sensitivity, 94.74% specificity and 88.57% accuracy for the Us PCR var-ATS respectively. In *P. vivax* diagnosis LAMP OptiGene® performed excellently with 100% sensitivity, specificity, and accuracy while LAMP WarmStart® and Us-PCR Cox1 achieved 100% sensitivity, specificity 93.33% and 97.14% accuracy. The study results highlighted the benefits of using LAMP techniques for field diagnosis of malaria in different settings where the need for a more sensitive and reliable molecular tool is mandatory but at the same time removing the high cost, long turnaround time and the need of highly specialized trained technicians to perform more sophisticated molecular techniques.

Keywords: malaria, LAMP, ultrasensitive PCR, diagnosis

Address for correspondence: Rasha H. Soliman, Department of Microbiology, College of Medicine, Taif University, P.O. Box: 11099 Taif 21944, Saudi Arabia; Mobile: +966-560813901; E-mail: y.soliman@tu.edu.sa, dr_rashasoliman@yahoo.com

INTRODUCTION

Malaria is considered the commonest cause of travellers' fever. Although the World Health Organization (WHO) declared that malaria incidence has declined globally, the rate of change has slowed markedly in the last five years (Antinori et al. 2021) causing around 229 million clinical cases and 409,000 deaths globally in 2019 (Dharmaratne et al. 2022). Malaria is responsible for more than 627,000 deaths worldwide in 2020 (WHO, 2021); mostly African children (Abdelmenan et al. 2022). Pregnant women are another target of malaria infection presenting with more severe symptoms (Kourtis et al. 2014). Presently, the risk of malaria transmission exists in about 87 countries and 125 million travellers are yearly at stake (Dharmaratne et al. 2022).

As a result of the non-specificity of its clinical symptoms and signs, malaria diagnosis requires confirmation by parasite-specific laboratory tests spanning from microscopic examination of blood smears, rapid diagnostic tests (RDTs), and Polymerase Chain Reaction (PCR) or other nucleic-acid based assays (Antinori et al. 2021). Microscopy depends on technicians' experience and training, and it consumes more time. RDTs are only qualitative, exhibits low sensitivity when the parasite load is low and false negative results emerge with Histidine Rich Protein 2 (HRP2)-deletion *P. falciparum* malaria diagnosis (Orish et al. 2018, Vernelen et al. 2018). Despite the high-performance indices of PCR, its wide-scale use is restricted by its high cost and prolonged turnaround time (Nijhuis et al. 2018) and it is not considered a practical method in field operations (Selvarajah et al. 2020).

Loop-mediated isothermal amplification (LAMP) is a molecular technique -first introduced in 2000-that relies on the identification of the presence of plasmodium spp. DNA through nucleic acid amplification (Selvarajah et al. 2020). Depending on the primer sequence, different *Plasmodium* species can be identified (Antinori et al. 2021). LAMP comprises of molecular amplification technique occurring in one step that is not needing for cyclical temperature variations allowing the identification of different *Plasmodium* species in a short time with high performance indices that is close to that of PCR (Charpentier et al. 2020). LAMP assay causes the formation of magnesium pyrophosphates that are recognized to cause to an elevation in turbidity when more DNA is amplified. The visualization of amplification products of LAMP can be obtained by densitometry or fluorescence, but it has the drawback of being only qualitative results hence parasitic load cannot be assessed (Lucchi et al. 2016). The WarmStart® colorimetric LAMP reagent is constructed to permit the visual identification of LAMP products through direct observation. The addition of phenol red dye in the master mix induces colour alterations from pink to yellow in case of positive samples where protons are generated as a result of pH drop in the solution, causing a colour change (Lai et al. 2020).

Sub-microscopic but PCR-detectable infections are responsible for almost 70% in low-endemic areas and this percentage decrease to reach 20% of in high-transmission areas (Okell et al. 2012) and so ultra-sensitive molecular methods can maximise test sensitivity (Hofmann et al. 2018) by increasing PCR diagnostic sensitivity through targeting multi-copy genomic sequences which provides more dependable diagnosis for low-density infections (Hofmann et al. 2015).

The telomere associated repetitive element 2 (TARE-2) repeat exists at 24 out of the 28 subtelomeres in the 3D7 culture strain. This reckons to almost 250–280 copies per genome specific to *P. falciparum* strains thus chosen as a target for ultra-sensitive PCR detection of *P. falciparum* (Gardner et al. 2002). Another target is the var gene family as the genome of the 3D7 culture strain harbours 59 var genes, and an estimated 50–150 copies are present encoding *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP1), the intracellular var gene acidic terminal sequence (varATS) covers several well-conserved stretches, hence can opt as a good candidate for ultrasensitive *P. falciparum* PCR (Hofmann et al. 2015).

Mitochondrial DNA (mtDNA) offers relatively conserved regions for primer design as it is present in multiple copies per cell, contained in a single mitochondrion offering higher sensitivity than the single- or low-copy 18S rRNA genes, avoiding the disadvantages of RNA-based amplification. The gains in sensitivity from targeting the mitochondrial genome might be greater for *P. vivax* than for *P. falciparum*. The median gene copy number for *Pv*-mtCOX1 was about ten times higher than for *Pv*18S rRNA as it is present in at least 20 copies per cell (Gruenberg et al. 2020). Malaria infections of very low parasitaemia were detected by *Pv*-mtCOX1 (Gruenberg et al. 2018).

The aim of this study is to investigate the diagnostic performance of two different commercial LAMP and ultrasensitive PCR tools in the diagnosis of Malaria samples collected from western region hospital of Kingdom of Saudi Arabia.

MATERIALS AND METHODS

1. Patient samples. Blood samples were collected from patients visiting King Faisal hospital, Taif, Kingdom of Saudi Arabia (KSA). The study period was for one year starting on the first of March 2021. Patients who were selected to participate in the study were suffering from symptoms that suggest malaria infection (episodes of fever, chills, sweating followed by episodes of normality) and/or symptoms and signs related to blood haemolysis. Patients' consents were obtained before collecting blood samples. Blood spots were collected for DNA extraction on Whatman's filter paper, each paper was separately saved in a sealed zip-lock plastic bag to avoid subsequent contamination.

2. Amplification methods. To perform the DNA extraction a blood circle of 0.5 cm of diameter from the Whatman paper was cut and it was mixed with 180 µl of ATL buffer and 20 µl of proteinase K. This mix was incubated at 56 °C overnight and the next day automatic DNA extraction was performed with the QIAamp DNA Blood Mini Kit (QIAgen, Germany) following the recommendations of the manufacturer in a QIAcube® (QIAgen, Germany). After extraction, the following malaria detection procedures were carried out:

Nested Multiplex PCR (NM-PCR) (Soliman et al. 2018): this is the reference method used in the Malaria and Emerging Parasites laboratory of the CNM (ISCIII), capable of simultaneously detecting *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*. The method involves two multiplex PCR amplifications. The first reaction amplifies *Plasmodium* spp. and includes an internal amplification control, and the second reaction uses amplified DNA of the first reaction to enable the identification of the infecting species of *P. vivax*, *P. falciparum*, *P. ovale*, *P. malariae*, and *P. knowlesi* by the corresponding size of the amplified fragments. Reaction conditions for the first PCR reaction were denaturation at 94 °C for 7 min, followed by 40 cycles at 94 °C for 20 seconds, 58 °C for 20 seconds, and 72 °C for 30 seconds. The final cycle was followed by an extension time at 72 °C for 10 minutes. Conditions for the second PCR reaction were an initial denaturation at 94 °C for 5 minutes, followed by 35 cycles at 94 °C for 15 seconds, 53 °C for 15 seconds, and 72 °C for 20 seconds, finishing with an extension phase at 72 °C for 10 minutes. Electrophoresis of the amplified products was performed by automated DNA electrophoresis (QIAxcel).

LAMP method using the OptiGene® Isothermal Master Mix from OptiGene® (OptiGene, United Kingdom): OptiGene's Isothermal Master Mix is a commercial assays that allows the detection of amplified DNA just adding the specific primers and template, using real-time fluorescence. Individual reactions with specific primers for *P. falciparum* or *P. vivax* were performed following manufacturer instructions including 15 µL of OptiGene Isothermal buffer, a final primer concentration of 1.6 µM for FIP and BIP, 0.2µ M for F3 and B3 and 0.4 µM for LFP and LBP and 5 µL of extracted DNA, in a final volume of 25 µL. The detection was carried out in a real-time thermal cycler and the result was considered positive if there was a fluorescent signal during the 30 minutes at 65 °C in which the amplification takes place.

LAMP method using the WarmStart® LAMP master mix kit (New England Biolabs, United Kingdom): The WarmStart® Colorimetric LAMP Master Mix is an optimized formulation of Bst 2.0 WarmStart® DNA Polymerase in a special low-buffer reaction solution containing a visible pH indicator for rapid and easy detection of Loop-Mediated Isothermal Amplification (LAMP) reactions. Individual reactions with specific primers for *P. falciparum* or *P. vivax* were performed. The LAMP assay was performed in a 25-µL reaction mixture that consisted of WarmStart® colorimetric LAMP 2 × master mix, Primers FIP/BIP = 1,6 µM, Primers F3/B3 = 0,2 µM, Primers LPF/LPB = 0,4 µM, EvaGreen 20x = 1 ×, DNA = 5 µl. Amplification was performed at 65 °C.

In addition to performing the colorimetric interpretation of the results, a real-time fluorescence reading of results was performed. EvaGreen (1×) was added to the reaction mixture to carry out the detection in a real-time thermal cycler. The results were considered positive if there was a color change from pink to yellow and if there was a fluorescent signal above the threshold during the 50 minutes of isothermal amplification at 65 °C, followed by inactivation at 80 °C for 5 minutes. Target genes of both LAMP reactions were small subunit ribosomal DNA with primer sequences described by the authors (Han et al. 2007).

Ultra-sensitive PCR for detection of *P. falciparum* with target in the high-copy telomere-associated repetitive element 2 (Us-PCR TARE-2) (Hofmann et al. 2015): Conditions for this PCR were previously described by N. Hofmann et al. with the addition of using EvaGreen (1 ×) for the detection in a real-time thermal cycle. Conditions of this PCR consisted of two initial holds of 50 °C for 2 minutes, followed by 95 °C during 10 minutes; then 45 cycles at 95 °C for 15 seconds and 57 °C for 60 seconds; and a final melting from 57 °C to 95 °C, increasing 0.3 °C. The results were considered positive when amplification occurred with a Ct lower than 34 cycles and with a denaturation temperature (Tm) around 79 °C ±1 °C.

Ultra-sensitive PCR for detection of *P. falciparum* with target in the *var* gene acidic terminal sequence (Us-PCR varATS) (Hofmann et al. 2015): It was performed using EvaGreen (1 ×) and carried out in a real-time thermal cycler. Conditions of this PCR were two initial holds of 50 °C for 2 minutes, followed by 95 °C for 10 minutes; 45 amplification cycles at 95 °C for 15 seconds and 55 °C for 60 seconds; and a final melting from 57 °C to 95 °C, increasing 0.3 °C. The analysis of the Ct value was not decisive since there was unspecific amplification with Ct over 25 cycles. Thus, the melting temperature (Tm) also was used as a characterization method (Tm = 76 °C ±1°C).

Ultra-sensitive PCR detection of *P. vivax* with a target in the mitochondrial *cox1* gene (Us-PCR cox-1) (Gruenberg et al. 2018): It was performed using author's description but with the addition of EvaGreen (1 ×) and carried out in a real-time thermal cycler. Conditions of this PCR were two initial holds of 50 °C for 2 minutes, followed by 95 °C for 10 minutes; 45 amplification cycles at 95 °C for 15 seconds and 60 °C for 60 seconds; and a final melting from 57 °C to 95 °C, increasing 0.3 °C. The results were considered positive when amplification occurred with a Ct lower than 30–32 cycles and with a denaturation temperature around 78–79 °C.

Operational characteristics. The time of performing was calculated from the initial moment the sample begins to be processed for the nucleic acid extraction until obtaining the results. The costs per sample were considered, without including the costs of controls included in each run or the duplication of samples. The costs related to the staff were estimated in time of performing the techniques. These costs are limited to performing the technique in Spain; in

other countries the costs of kits may be completely different, including between institutions, although the relative differences will be similar.

1. Data analysis. The laboratory tests results were collected, recorded, and analysed using SPSS V. 21 software (SPSS, Chicago, Illinois, USA). Diagnostic performance was expressed in terms of sensitivity, specificity, accuracy, positive and negative predictive values (PPV and NPV, respectively) and was calculated with the 95% confidence intervals (CI) against the NM- PCR test's results.

2. Ethical consideration. Ethical approval was obtained from Taif University Research Ethics Committee (HAP-02-T-067) The procedures used in this study adhere to the tenets of the Declaration of Helsinki. All participants and patients were informed by the study and signed consents were obtained according to the ethical standards. Prior to examination, relevant information was given to patients, including information about the sampling technique and the benefits of the study.

RESULTS

Nested Multiplex PCR (NM-PCR) is the reference method used in the Malaria and Emerging Parasites laboratory of the CNM (ISCIII). This method identified the 35 samples included in this study as 13 *P. falciparum*, 17 *P. vivax*, 3 mixed *P. falciparum* and *P. vivax* and 2 negative controls.

To assess the performance of the diagnostic techniques: each sample was tested using the different diagnostic techniques and the results are described in detail with cycle threshold (Ct) and melting temperature (Tm) in the case of PCR assays (Us-PCR TARE-2, Us-PCR varATS, Us-PCR cox1) and Ct value in the case of LAMP assays (LAMP OptiGene® and LAMP WarmStart®) in Table 1. The 35 blood samples were diagnosed by NM PCR as the reference method (13 *P. falciparum*, 17 *P. vivax*, 3 mixed *P. falciparum* and *P. vivax*) plus two negative controls. As regards *P. falciparum* samples in comparison to NM-PCR as reference method: LAMP OptiGene® and LAMP WarmStart® performance was identical and better than the performance of Us PCR TARE 2 and Us-PCR var-ATS for *P. falciparum*, achieving 93.75% sensitivity, 100% specificity and 97.17% accuracy versus 87.5% sensitivity, 100% specificity and 94.29% accuracy for the Us PCR TARE 2 and 81.25% sensitivity, 94.74% specificity and 88.57% accuracy for the Us PCR var-ATS respectively (Table 2).

As for the assessment of the performance of LAMP OptiGene®, LAMP WarmStart® and Us-PCR Cox1 in the detection of *P. vivax* in comparison to NM-PCR as reference method: LAMP OptiGene® performed excellently with 100% sensitivity, specificity, and accuracy. LAMP WarmStart® and Us-PCR Cox1 came second with the same results of 100% sensitivity but with specificity 93.3% and 97.1% accuracy (Table 3).

Regarding the operational characteristics, the turnaround time to complete a diagnosis, from the moment the sample is received until the moment the results are provided to clinicians, was estimated to be 2 hours and 35 minutes for the LAMP assays: 1 hour for the management of the samples and DNA purification, 30 minutes for the master mix preparation and LAMP setup, 1 hour to perform the LAMP, and 5 minutes for the analysis of results, and approximately 1 hour of hands-on time.

Meanwhile, for the NM-PCR the diagnosis turnaround time was established in around 6 hours and 15 minutes: 1 hour for the management of the samples and DNA purification, 15 minutes for the first PCR setup, with the tubes ready to use, 2 hours to perform the first PCR, 15 minutes for the second PCR setup, 2 hours to perform the second PCR, 30 minutes to run the automated electrophoresis, and 15 minutes for the analysis of results. The hands-on time for the reference method was calculated to be 2 hours. As for the Us-PCR, the turnaround time was estimated to be 4 hours: 1 hour for the management of the samples and DNA purification, 30 minutes for the master mix preparation and PCR setup, 2 hours to perform the real-time PCR, and 30 minutes for the analysis of results. The hands-on time was much less for the Us-PCR than that of NM-PCR, including only the time of preparation of the samples, the PCR setup and the time of analysis, which was estimated to be 1 hour (Table 4).

Concerning the costs per sample of each method, they varied from 15 to 30 euros for the US-PCR, depending on many circumstances. The cost of each LAMP assay was estimated at 1 euro. The final price can vary depending on the number of LAMP assays performed. The cost for the reference method was calculated at 5 euros (Table 4).

DISCUSSION

The necessity for exceedingly sensitive, field compliant diagnostic techniques that can promptly be used in underprivileged settings is evident, LAMP is a sensitive molecular technique that seems appropriate for malaria diagnosis as a near point-of-care test in the

Table 1. Results of individual samples by different diagnostic techniques in the study.

N	NM-PCR	LAMP OptiGene *P. falciparum* (Ct)	LAMP Opti-Gene *P. vivax* (Ct)	LAMP Opti-Gene	LAMP WarmStart *P. falciparum* (Ct)	LAMP WarmStar *P. vivax* (Ct)	LAMP WarmStar	Us-PCR {Citation} var-ATS *P. falciparum* (Ct/Tm)	Us-PCR var-ATS *P. falciparum*	Us-PCR cox1 *P. vivax* (Ct/ Tm)	Us-PCR cox1 *P. vivax*	Us-PCR TARE-2 *P. falciparum* (Ct/Tm)	Us-PCR TARE-2 *P. falciparum*
	F	15.3	N	F	26.8	N	F	16.6/76.1	F	N	N	17,5/79,1	F
	F	21.1	N	F	27.2	N	F	24.4/76.2	F	N	N	26,9/78,8	F
	F	15.1	N	F	19.6	N	F	16.9/76	F	N	N	17,1/79,3	F
	VF	27.1	15.3	VF	32.2	17.7	VF	N	N	18.4/78.1	V	N	N
	VF	25.5	17.8	VF	30.9	14.86	VF	N	N	20.7/78.1	V	32/79,1	F
	F	16.1	N	F	19.7	N	F	17.5/76.2	F	N	N	17,9/79,9	F
	V	N	19	V	N	15.1	V	N	N	25.4/78.2	V	N	N
	V	N	15.6	V	N	14.57	V	N	N	19.3/78.2	V	N	N
	V	N	17.3	V	N	16.35	V	N	N	22.4/78.3	V	N	N
	F	22.6	N	F	30.6	N	F	25.9/76	F	N	N	29,1/79,6	F
	V	N	16.5	V	N	12.96	V	76.3	F	16/78.3	V	N	N
	V	N	17.9	V	N	14.28	V	N	N	19.8/78.3	V	N	N
	V	N	17.7	V	N	14.58	V	N	N	21.3/78.3	V	N	N
	V	N	17.2	V	N	15.56	V	N	N	17.5/78.2	V	N	N
	F	15.9	N	F	23.1	N	F	17.2/76.2	F	N	N	17,8/78,8	F
	F	19.9	N	F	26.6	N	F	22.6/76.2	F	N	N	24,2/79	F
	F	23.2	N	F	35.6	N	F	27.7/76.4	F	N	N	30,3/79,1	F
	F	17.2	N	F	21.7	N	F	18.8/76.5	F	N	N	18,4/79,4	F
	V	N	16.2	V	N	16.82	V	N	N	20.3/78.1	V	N	N
	V	N	19.1	V	N	21.29	V	N	N	21.5/78.3	V	N	N
	V	N	14.7	V	N	17.21	V	N	N	18.4/78.3	V	N	N
	F	16.9	N	F	22.1	N	F	19/76.2	F	N	N	20,8/78,8	F
	F	10.7	N	F	21.7	N	F	23.2/76.1	F	N	N	25,5/78,6	F
	V	N	10.7	V	N	15.74	V	N	N	21.9/78	V	N	N
	V	N	8.4	V	N	14.55	V	N	N	20.2/78.1	V	N	N
	F	13.9	N	F	22.8	22.9	FV	22.9/76.1	F	N/78.2	V	25,5/79,2	F
	FV	N	11.1	V	N	19.5	V	N	N	24.2/78.1	V	36,7 /N	N
	V	N	10.4	V	N	21.4	V	N	N	24.2/78.2	V	N	N
	F	16.9	N	F	28.8	N	F	27.6/76.1	F	N	N	30,1/79,5	F
	V	N	6.8	V	N	17.8	V	N	N	19.2/78.2	V	N	N
	V	N	8	V	N	13.5	V	N	N	20.6/78.3	V	N	N
	V	N	9.5	V	N	17.8	V	N	N	21.9/78.2	V	N	N
	V	N	7.1	V	N	18.4	V	N	N	19.3/78.3	V	N	N
	N	N	N	N	N	N	N	N	N	N	N	N	N
	N	N	N	N	N	N	N	N	N	N	N	N	N

N = negative, Ct = cycle threshold, Tm = meting temperature.

Table 2. Performance of LAMP OptiGene®, LAMP WarmStart®, Us-PCR var-ATS and Us-PCR TARE-2 in detection of *P. falciparum* in comparison to NM-PCR as reference method.

	NM-PCR+ve	NM-PCR-ve	Sensitivity, specificity, accuracy, PPV, NPV, Cohen's k
LAMP OptiGene®+ve	15	–	Sensitivity: 93.7% (95% CI 69.7–99.8%) Specificity: 100% (95% CI 82.3–100%) PPV: 100% NPV: 95% (95% CI 74–99.2%) Accuracy 97.14% (95% CI 85–99.9%) Cohen's k: 0.9
LAMP OptiGene®-ve	1	19	
LAMP WarmStart®+ve	15	–	Sensitivity: 93.7% (95% CI 69.7–99.8%) Specificity: 100% (95% CI 82.3–100%) PPV: 100% NPV: 95% (95% CI 74–99.2%) Accuracy 97.14% (95% CI 85–99.9%) Cohen's k: 0.9
LAMP WarmStar®-ve	1	19	
Us-PCR var-ATS+ve	13	1	Sensitivity: 81.25% (95% CI 54.3–95.9%) Specificity: 94.74% (95% CI 73.9–99.8%) PPV: 92.8%95% CI 65.5–98.8%) NPV: 85.71% (95% CI 68.2–94.3%) Accuracy 88.57% (95% CI 73.2–96.8%) Cohen's k: 0.7
Us-PCR var-ATS-ve	3	18	
Us-PCR TARE-2+ve	14	–	Sensitivity: 87.5% (95% CI 61.6–98.4%) Specificity: 100% (95% CI 82.3–100%) PPV:100% NPV: 90.48% (95% CI 72.2–97.2%) Accuracy 94.29% (95% CI 80.8–99.3%) Cohen's k: 0.8
Us-PCR TARE-2-ve	2	19	
Total	16	19	

Table 3. Performance of LAMP OptiGene®, LAMP WarmStart® and Us-PCR Cox1 in detection of *P. vivax* in comparison to NM-PCR as reference method.

	NM-PCR+ve	NM-PCR-ve	Sensitivity, specificity, accuracy, PPV, NPV, Cohen's k
LAMP OptiGene®+ve	20	–	Sensitivity: 100% (95% CI 83.1–100%) Specificity: 100% (95% CI 78.2–100%) PPV: 100% NPV: 100% Accuracy 100% (95% CI 90–100%) Cohen's k: 1
LAMP OptiGene®-ve	–	15	
LAMP WarmStart®+ve	20	1	Sensitivity: 100% (95% CI 83.1–100%) Specificity: 93.3% (95% CI 68.1–99.83%) PPV: 95.2% (95% CI 75.1–99.2%) NPV: 100% Accuracy 97.1% (95% CI 85–99.9%) Cohen's k: 0.9
LAMP WarmStart®-ve	–	14	
Us-PCR Cox 1+ve	20	1	Sensitivity: 100% (95% CI 83.1–100%) Specificity: 93.3% (95% CI 68.1–99.8%) PPV: 95.2% (95% CI 75.1–99.2%) NPV: 100% Accuracy 97.1% (95% CI 85–99.9%) Cohen's k: 0.9
Us-PCR Cox 1-ve	–	14	
Total	16	19	

Table 4. Time and cost estimation for the different techniques.

Technique	Turnaround time	Hands-on work	Cost per determination
NM_PCR	6h, 15min	2h	5 euros
Us-PCR	4h	1h	15–30 euros
LAMP	2h, 35min	1h	1 euro

aforementioned settings (Morris and Aydin-Schmidt 2021). Current meta-analyses assessing the diagnostic performance of LAMP for malaria have described high sensitivity and specificity of LAMP when compared to other diagnostic techniques including microscopy, PCR, and RDTs in both endemic and non-endemic settings reaching more than 95% whichever the comparator demonstrates that malaria LAMP is a test with excellent diagnostic performance (Picot et al. 2020, Selvarajah et al. 2020). The authors concluded that LAMP is a robust tool for malaria diagnosis in both symptomatic and asymptomatic individuals (Picot et al. 2020), and that LAMP is one of the best encouraging new diagnostic techniques for administration in malaria endemic venues (Selvarajah et al. 2020). LAMP performance was very promising either in endemic settings, asymptomatic and low-density infections, low resource settings, screening in pregnancy and in returning travelers in non-endemic settings (Morris and Aydin-Schmidt 2021).

For the diagnosis of *P. vivax*, LAMP technique proved 100% sensitivity compared to NM- PCR as reference method with LAMP OptiGene® achieving 100% specificity, PPV, NPV and accuracy. On the other hand, LAMP WarmStart® achieved 100% sensitivity, although specificity was lower (Table 2 and 3). This was due to a sample that was identified as positive for this LAMP assay, but negative for the reference technique. As for *P. falciparum* diagnosis, the performance indices for the two LAMP tests were lesser in sensitivity than that for *P. vivax* (Table 2 and 3). This lower sensitivity obtained with *P. falciparum* LAMP assays is due to one sample identified as a mixed infection of *P. falciparum* and *P. vivax*. with NM-PCR but the *P. falciparum* Us-PCR TARE-2 and the Us-PCR var-ATS failed to identify it as well.

These findings come in accordance with those of other researchers conducting a systematic reviews and meta-analysis for diagnostic test accuracy where they concluded that P. *falciparum* LAMP exhibited high sensitivity of 96% in 14 studies, (95% CI 0.9 to 0.9) and specificity of 99% (95% CI 0.96 to 1.00). Six studies showed that *P. vivax* LAMP achieved a sensitivity of 96% (95% CI 0.9 to 1.0) and 99% specificity (95% CI 0.5 to 1.0) (Selvarajah et al. 2020). Others concluded that LAMP as a tool for malaria diagnosis had a 97.3% sensitivity and 99.6% specificity (Charpentier et al. 2020). In another study, the WarmStart® LAMP technique achieved an overall 98% sensitivity and 100% specificity. For *P. falciparum* diagnosis; it achieved 100% sensitivity (95% CI 69.2–100%) and 92.7% specificity (95% CI 86.2–96.8%) but the sensitivity decreased for *P. vivax* diagnosis: 90.5% (95% CI 69.6–98.8%) with 100% specificity (95% CI 96.3–100%) (Lai et al. 2020).

Greater diagnostic sensitivity of PCR can be gained using Us-qPCR assays that target multi-copy sequences in the parasite genome rather than a single-copy gene even while using minute amount of genetic material like finger prick blood samples The authors concluded that the relative benefits in the detection of *P. falciparum* and *P. vivax* infections using Us-PCR were significant (Gruenberg et al. 2020).

In the present study, *P. falciparum* Us-PCR TARE-2 achieved Lower performance indices than that of LAMP OptiGene® and LAMP WarmStart® which achieved 93.7% sensitivity, 100% specificity and 97.1% accuracy, but the Us-PCR var- ATS did not perform as good as the previously mentioned tools. On the other hand, in *P. vivax* diagnosis Us-PCR Cox 1 had near excellent results that are identical to those of LAMP OptiGene® and LAMP WarmStart®.

With regards to the operational characteristics, the time to obtaining the results in LAMP assays was much shorter than with the both reference method and US-PCR. This is also reflected in staff performance time, around 1 hour more for the reference method. Moreover, the cost of LAMP assays is lower than the price of the NM-PCR, and much lower than US_PCR. For this reason, it is recommended to use the LAMP assays on wider scales specially as a screening method.

In our opinion: WarmStart® LAMP has the advantage of changing of color (pink to yellow) if there is amplification in the tube and, in addition, it can be visualized in the amplification curves (Ct) with EvaGreen in the real-time thermal cycler equipment. On the other hand, Us-PCR TARE-2, Us-PCR varATS and Us-PCR cox-1 methods had the inconvenience of producing nonspecific amplifications with EvaGreen that can only be resolved using a non-resolute method as the analysis of melting temperature (Tm) or using more specific one, such as specific probes which are very expensive.

In addition, these Us-PCR assays, as other PCR assays, hold drawbacks to their use in endemic settings, because of the necessity of specific and expensive devices and long turnaround times.

CONCLUSIONS

The results of the present study shed the light on the huge potential of using LAMP techniques for the field diagnosis of malaria in different settings where the need for a more sensitive and reliable molecular tool is mandatory but at the same time removing the high cost, long periods needed to reach confident results and the need of highly specialised trained technicians to perform more sophisticated molecular techniques especially when performance indices of LAMP are more or less identical with those of ultrasensitive PCR assays used in the detection of low parasitaemic infection in many endemic areas of the world.

Acknowledgment. The authors would like to acknowledge The Deanship of Scientific Research, Taif University, Taif, Saudi Arabia for funding of the present research.

Statements and Declarations. The authors declare no conflict of interest related to this study.

Authors contribution. All authors contributed to the study conception and design. Clinical examination and patient sampling by Bushra Hussein and Maha Wahab. Material preparation, data collection and analysis were performed by Jose Miguel Rubio, Alexandra Martin-Ramirez and Marta Lanza. The first draft of the manuscript was written by Rasha Soliman, Eman Khalifa and Yosry Hawash and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

REFERENCES

Abdelmenan S., Teka H., Hwang J., Girma S., Chibsa S., Tongren E., Murphy M., Haile M., Dillu D., Kassim J., Behaksra S., Tadesse F. G., Yukich J., Berhane Y., Worku A., Keating J., Zewde A., Gadisa E. (2022) Evaluation of the effect of targeted Mass Drug Administration and Reactive Case Detection on malaria transmission and elimination in Eastern Hararghe zone, Oromia, Ethiopia: A cluster randomized control trial. *Trials* **23**: 267–278

Antinori S., Ridolfo A. L., Grande R., Galimberti L., Casalini G., Giacomelli A., Milazzo L. (2021) Loop-mediated isothermal amplification (LAMP) assay for the diagnosis of imported malaria: A narrative review. *Infez. Med*. **29**: 355–365

Charpentier E., Benichou E., Pagès A., Chauvin P., Fillaux J., Valentin A., Guegan H., Guemas E., Salabert A.-S., Armengol C., Menard S., Cassaing S., Berry A., Iriart X. (2020) Performance evaluation of different strategies based on microscopy techniques, rapid diagnostic test and molecular loop-mediated isothermal amplification assay for the diagnosis of imported malaria. *Clin. Microbiol. Infect.* **26**: 115–121

Dharmaratne A. D. V. T. T., Dini S., O'Flaherty K., Price D. J., Beeson J., McGready R., Nosten F., Fowkes F. J. I., Simpson J. A., Zaloumis S.G. (2022) Quantification of the dynamics of antibody response to malaria to inform sero-surveillance in pregnant women. *Malar. J.* **21**: 75–87

Gardner M. J., Hall N., Fung E., White O., Berriman M., Hyman R. W., Carlton J. M., Pain A., Nelson K. E., Bowman S., Paulsen I. T., James K., Eisen J. A., Rutherford K., Salzberg S. L., Craig A., Kyes S., Chan M.-S., Nene V., Shallom S.J., Suh B., Peterson J., Angiuoli S., Pertea M., Allen J., Selengut J., Haft D., Mather M. W., Vaidya A. B., Martin D. M. A., Fairlamb A. H., Fraunholz M. J., Roos D. S., Ralph S. A., McFadden G. I., Cummings L. M., Subramanian G. M., Mungall C., Venter J. C., Carucci D. J., Hoffman S. L., Newbold C., Davis R. W., Fraser C. M., Barrell B. (2002) Genome sequence of the human malaria parasite *Plasmodium falciparum. Nature* **419**: 14–48

Gruenberg M., Moniz C. A., Hofmann N. E., Koepfli C., Robinson L. J., Nate E., Monteiro W. M., de Melo G. C., Kuehn A., Siqueira A. M., Nguitragool W., Bassat Q., Lacerda M., Sattabongkot J., Mueller I., Felger I. (2020) Utility of ultra-sensitive qPCR to detect *Plasmodium falciparum* and *Plasmodium vivax* infections under different transmission intensities. *Malar. J.* **19**: 319–328

Gruenberg M., Moniz C. A., Hofmann N. E., Wampfler R., Koepfli C., Mueller I., Monteiro W. M., Lacerda M., de Melo G. C., Kuehn A., Siqueira A. M., Felger I. (2018) Plasmodium vivax molecular diagnostics in community surveys: Pitfalls and solutions. *Malar. J.* **17**: 55–64

Han E.-T., Watanabe R., Sattabongkot J., Khuntirat B., Sirichaisinthop J., Iriko H., Jin L., Takeo S., Tsuboi T. (2007) Detection of four *Plasmodium* species by genus- and species-specific loop-mediated isothermal amplification for clinical diagnosis. *J. Clin. Microbiol*. **45**: 2521–2528

Hofmann N., Mwingira F., Shekalaghe S., Robinson L. J., Mueller I., Felger I. (2015) Ultra-sensitive detection of *Plasmodium falciparum* by amplification of multi-copy subtelomeric targets. *PLOS Med*. **12**: e1001788

Hofmann N. E., Gruenberg M., Nate E., Ura A., Rodriguez-Rodriguez D., Salib M., Mueller I., Smith T. A., Laman M., Robinson L. J., Felger I. (2018) Assessment of ultra-sensitive malaria diagnosis versus standard molecular diagnostics for malaria elimination: An in-depth molecular community cross-sectional study. *Lancet Infect. Dis*. **18**: 1108–1116

Kourtis A. P., Read J. S., Jamieson D. J. (2014) Pregnancy and infection. *N. Engl. J. Med.* **370**: 2211–2218

Lai M. Y., Ooi C. H., Jaimin J. J., Lau Y. L. (2020) Evaluation of WarmStart colorimetric loop-mediated isothermal amplification assay for diagnosis of malaria. *Am. J. Trop. Med. Hyg.* **102**: 1370–1372

Lucchi N. W., Ljolje D., Silva-Flannery L., Udhayakumar V. (2016) Use of malachite green-loop mediated isothermal amplification for detection of *Plasmodium* spp. parasites. *PLOS ONE* **11**: e0151437

Morris U., Aydin-Schmidt B. (2021) Performance and application of commercially available loop-mediated isothermal amplification (LAMP) kits in malaria endemic and non-endemic settings. *Diagnostics* **11**: 336–353

Nijhuis R. H. T., van Lieshout L., Verweij J. J., Claas E. C. J., Wessels E. (2018) Multiplex real-time PCR for diagnosing malaria in a non-endemic setting: A prospective comparison to conven-

tional methods. *Eur. J. Clin. Microbiol. Infect. Dis*. **37**: 2323–2329

Okell L. C., Bousema T., Griffin J. T., Ouédraogo A. L., Ghani A. C., Drakeley C. J. (2012) Factors determining the occurrence of submicroscopic malaria infections and their relevance for control. *Nat. Commun*. **3**: 1237–1245

Orish V. N., De-Gaulle V. F., Sanyaolu A. O. (2018) Interpreting rapid diagnostic test (RDT) for *Plasmodium falciparum*. *BMC Res. Notes* **11**: 850–855

Picot S., Cucherat M., Bienvenu A.-L. (2020) Systematic review and meta-analysis of diagnostic accuracy of loop-mediated isothermal amplification (LAMP) methods compared with microscopy, polymerase chain reaction and rapid diagnostic tests for malaria diagnosis. *Int. J. Infect. Dis.* **98**: 408–419

Selvarajah D., Naing C., Htet N. H., Mak J. W. (2020) Loop-mediated isothermal amplification (LAMP) test for diagnosis of uncomplicated malaria in endemic areas: A meta-analysis of diagnostic test accuracy. *Malar. J.* **19**: 211–220

Soliman R. H., Garcia-Aranda P., Elzagawy S. M., Hussein B. E.-S., Mayah W. W., Martin Ramirez A., Ta-Tang T.-H., Rubio J. M. (2018) Imported and autochthonous malaria in West Saudi Arabia: Results from a reference hospital. *Malar. J.* **17**: 286–292

Vernelen K., Barbé B., Gillet P., Van Esbroeck M., China B., Jacobs J. (2018) Photo-based External Quality Assessment of Malaria rapid diagnostic tests in a non-endemic setting. *PLOS ONE* **13**: e0201622

WHO (2021) Malaria report

Received on 20th September, 2022; revised on 14th February, 2023; accepted on 14th March 2023