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Evaluation of Multiplex/Nested Polymerase Chain Reaction and Loop-Mediated Isothermal Amplification for Malaria Diagnosis in Southeastern Iran

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Abstract. Malaria is one of the most serious health problems in many countries, including Iran. Accurate diagnosis is important regardless of the elimination status of a country. A cross-sectional study was performed on 105 people who were suspected to be positive for malaria infection in Sistan and Baluchistan, Iran. Blood smears (thin and thick films) were stained with 10% Giemsa. DNA was extracted from the prepared thin and thick films for molecular methods. Multiplex/nested polymerase chain reaction (mn-PCR), loop-mediated isothermal amplification (LAMP), and light microscopy (LM) were compared with nested PCR (nPCR) as a gold standard. Of 105 subjects, 52 (49.5%), 58 (55.2%), 58 (55.2%), and 63 (60%) were positive for malaria by LM, nPCR, mn-PCR, and LAMP, respectively. The sensitivity, specificity, and kappa were 92.1%, 100%, and 0.9 for LAMP and 100%, 100%, and 1 for mn-PCR, respectively. Eight cases of coinfection (*Plasmodium vivax* and *Plasmodium falciparum*) that were not detected by LM method were diagnosed by mn-PCR and LAMP. In the present study, the high sensitivity and specificity of LAMP and mn-PCR indicate that these two tests are good alternatives to nPCR for malaria diagnosis

INTRODUCTION

Malaria is one of the most serious health problems in many countries, including Iran.[1] According to the latest WHO report, malaria deaths have reduced steadily over the period 2000–2019, from 736,000 in 2000 to 409,000 in 2019.[2] A successful program for malaria elimination is mainly dependent on accurate and effective diagnosis.[3] Despite recent advances, accurate and rapid malaria diagnosis is still difficult. The light microscopy (LM) method is used as the gold standard and typically applied for diagnosis in the laboratory setting by experienced technicians.[4,5] Molecular methods such as nested polymerase chain reaction (nPCR) have a relatively high sensitivity and specificity compared with LM but require advanced laboratory equipment, are costly, and require considerable time.[3,6–8] In addition, malaria is often endemic in countries with poor economic conditions, so financing molecular methods is difficult.[8–10] The multiplex/nested PCR (mn-PCR) method is one of the most important techniques used for the correct and timely malaria diagnosis and is similar to the nPCR test but takes less time to implement.[5] The loop-mediated isothermal amplification (LAMP) method is similar to the nPCR test but is more accurate and sensitive.[4,11–13] Furthermore, the LAMP method does not require special laboratory equipment, and the results can be presented to the physician in less than 1 hour by observing them under ultraviolet light.[14] The present study was undertaken to compare the performance of LAMP and mn-PCR for detection of low parasitemia and coinfections of *Plasmodium* species in endemic areas of Iran. In addition, mn-PCR, LAMP, and LM methods were evaluated to find a good substitute for nPCR.

MATERIALS AND METHODS

Study area and sample collection. The samples were prepared from our previous study.[5] This study was a double-blind clinical diagnostic assay of malaria aiming to compare the results of the microscopic method obtained by two microscopists with LAMP, nPCR, and mn-PCR. Some positive and negative blood smears were randomly selected and reexamined by an independent blinded microscopist.

Light microscopy. Thin and thick films of each blood smear were stained with 10% Giemsa and examined for a minimum of 100 high-magnification fields before being recorded as negative for malaria parasites. Blood smears were examined by two experienced microscopists. Expert microscopists were certified by the National Institutes of Health of Iran. The expert microscopists were blind to initial microscopy and PCR results during the study.

DNA extraction from slides. DNA was extracted from the prepared thin and thick films. Genomic DNA was extracted by using Dynabio Blood/Tissue DNA Extraction kit (Bioneer, Korea) according to the manufacturer's protocol and kept at −20°C until used.[5]

nPCR assay. The malaria species in the samples were determined via nPCR assay. These primers (Pishgam Company) were designed[15] based on the 18S ribosomal RNA genes (Table 1) that was used for the identification of different species of *Plasmodium*.[5] PCR products of second round of the PCR were loaded onto a 1.5% agarose gel and the results were compared with standard band markers of *P. vivax*, 120 bp and *P. falciparum*, 205 bp.

mn-PCR. mn-PCR PCR was performed according to previous studies that described by Mirahmadi et al.[5] by using rplu5 and rplu6 primers (Table 1). Briefly, in the first-round ready to use Bioneer, Accupower PCR premix, 96 tubes,

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TABLE 1
Schematic representation of the *Plasmodium* ssrRNA genes and mn-PCR and nPCR protocol

Species	PCR product	Primer	Primer sequence 5'-3'	Reaction
Plasmodium genus–specific	1200 bp	rPLU5	CCTGTTGTTGCCTTAAACTTC	Nested 1
		rPLU6	TTAAAATTGTTGCAGTTAAAAC	
Plasmodium species–specific				
Plasmodium falciparum	205 bp	rFAL1	TTAAACTGGTTTGGGAAAACCAAATATATT	Nested 2
		rFAL2	ACACAATGAACTCAATCATGACTACCCGTC	
Plasmodium vivax	120 bp	rVIV1	CGCTTCTAGCTTAATCCACATAACTGATAC	Nested 2
		rVIV2	ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA	

mn-PCR = multiplex/nested polymerase chain reaction; nPCR = nested polymerase chain reaction; PCR = polymerase chain reaction.

0.2 mL and 20 μL (Bioneer Co., Daejeon, Korea) were used for the PCR reaction. DNA was amplified using thermal cycler (Eppendorf AG 22331, Hamburg, Germany) under the following conditions: 95°C for 5 minutes and then 25 cycles at 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 1 minute, followed by a final extension at 72°C for 5 minute. Internal primers rFAL.1, rFAL.2, rVIV.1, and rVIV.2 in the second round were applied under the following conditions: 95°C for 5 minutes and then 30 cycles at 94°C for 30 seconds, 58°C for 1 minute, and 72°C for 30 seconds, followed by a final extension at 72°C for 5 minutes.[5] PCR products from a second round were loaded onto a 1.5% agarose gel.

LAMP conditions. The LAMP primer sets, as previously described were used to amplify the gene coding for the 18S rRNA of *Plasmodium genus*.[3] Each LAMP reaction mixture contained 2 mL of both FIP and BIP primers, 0.25 mL F3 and B3 primers, 1 mL LPF and LPB, 1 mL of Bacillus stearothermophilus DNA polymerase, 2 mL MgSO4, 4 mL betaine, 3.5 mL dNTP mix and 1 mL of DNA sample in a total volume of 25 μL. The LAMP reaction mixture was incubated in a water bath at 60°C for 90 min. A LAMP reaction was considered positive for *Plasmodium* spp. if an obvious increase in the turbidity was observed by the naked eye compared with the negative control. The results were evaluated blindly by two researchers.

Statistical analysis. The diagnostic performances of mn-PCR and microscopic method and LAMP were evaluated using nPCR as the gold standard. Analyses were conducted using SPSS 16 software (SPSS Inc., Armonk, NY). Kappa values were determined to observe the consistency of the results among the diagnostic tools. To compare the sensitivity of two methods, the same extracted DNA source was used for LAMP and mn-PCR. Also, the positive results obtained in the LAMP method that were negative in the mn-PCR were tested again by LAMP to reject the false-positive subject.

TABLE 2
Malaria positivity rate of LM, mn-PCR, nPCR, and LAMP in the diagnosis of malaria in Sistan-Baluchestan, Iran

	Diagnostic tool			
Results	LM	Mn-PCR	nPCR	LAMP
Plasmodium falciparum	12	7	7	8
Plasmodium vivax	38	42	42	46
Mix infection (*vivax* and *falciparum*)	2	9	9	9
Total positives	52	58	58	63
Total negatives	53	47	47	42

LAMP = loop-mediated isothermal amplification; LM = light microscopy; mn-PCR = multiplex/nested polymerase chain reaction; nPCR = nested polymerase chain reaction; PCR = polymerase chain reaction.

RESULTS

Demographic characteristics of the study participants. One hundred five individuals were suspected to have malaria. The ratio of men to women was 2.18:1, with the mean age of 45.9 ± 15.2 years. In addition, 96.2% of the smears were prepared from Iranians and 3.8% were from non-Iranians.

The results of LM, nPCR, mn- PCR, and LAMP methods for malaria. Of 105 cases, 52 (49.5%), 58 (55.2%), 58 (55.2%), and 63 (60%) samples were positive for malaria by the LM, nPCR, mn-PCR, and LAMP methods, respectively. LAMP detected highest positive cases of *P. vivax*, and LM detected the most cases of *P. falciparum* (Table 2; Supplemental Materials). The diagnostic differences in these tests are presented in Table 3.

The sensitivity and specificity by using nPCR as the reference standard. When nPCR was selected as the gold standard, the highest sensitivity was found by the mn-PCR method and the highest specificity was seen in the LAMP and mn-PCR methods. The LM and LAMP methods had similar sensitivities (92%), whereas the LM specificity was less than the LAMP specificity (Table 4). The results obtained through the mn-PCR, the nPCR, and the LAMP methods were not similar. Accordingly, five negative cases were detected by the mn-PCR and nPCR methods, but they were found to be positive by the LAMP method. The performance characteristics of different methods are shown in Table 5. The sensitivity for diagnosing *P. falciparum* and *P. vivax* was similar in the LAMP and mn-PCR methods and was higher than the LM method. However, the specificity level in the LAMP method was less than two other methods in term of the diagnosis of *P. falciparum*. The specificity for the diagnosis of *P. vivax* was higher in the LAMP method than the LM method (Table 5).

Time required to perform different methods to provide the test results. The results showed the LM method required the minimum time for preparation and obtaining results (60 minutes) compared with other tests. In addition, the time required for DNA extraction (30 minutes) by the LAMP, mn-PCR, and nPCR methods was the same (Table 6). In addition, the LM and LAMP method, which do not require specific laboratory facilities, required the minimum time to obtain the results

DISCUSSION

Malaria is one of the most common infectious diseases in the tropical regions of the world.[16] Therefore, identification

TABLE 3
Comparison of nPCR, mn-PCR, LM, and LAMP in the diagnosis of malaria in in Sistan-Baluchestan, Iran

	LM				mn-PCR				LAMP				
nPCR	*P. falciparum*	*P. vivax*	Mix	Neg.	*P. falciparum*	*P. vivax*	Mix	Neg.	*P. falciparum*	*P. vivax*	Mix	Neg.	Total
P. falciparum	7	0	0	0	7	0	0	0	7	0	0	0	7
P. vivax	0	31	1	10	0	42	0	0	0	42	0	0	42
Mix	4	4	1	0	0	0	9	0	0	0	9	0	9
Neg.	1	3	0	43	0	0	0	47	1	4	0	42	47

LAMP = loop-mediated isothermal amplification; LM = light microscopy; Mix = mixed infection *(vivax* and *falciparum*); mn-PCR = multiplex/nested polymerase chain reaction; Neg. = negative; nPCR = nested polymerase chain reaction; PCR = polymerase chain reaction.

of the best diagnostic method that can accurately diagnose all cases of malaria will be helpful for malaria elimination in endemic areas. In our past studies, we showed that microscopic examination of blood smears does not reliably distinguish *Plasmodium* species in the presence of mix infection. Therefore, it seems that mn-PCR is a good candidate for examing the presence of malaria parasite in clinically suspected but microscopically negative cases. In the second part of study, our goal was to compare the mn-PCR and LAMP tests with the n-PCR as a gold standard. This study was performed for evaluation of two important goals: first, the examination of LAMP to find a good alternative test for malaria diagnosis in the field; second, to evaluate the mn-PCR method as a suitable substitute of nPCR to save time and consumable materials. Our findings indicated the LAMP and LM methods had similar sensitivity (92%), whereas the LAMP specificity was greater than the others. The LAMP method provided better results for the diagnosis of *P. vivax* and *falciparum* than the mn-PCR and nPCR methods. These findings were consistent with the studies conducted in Thailand, which reported high LAMP sensitivity and specificity.[3,11] The results of these studies indicated that the LAMP method is a useful and sufficiently sensitive tool for the malaria diagnosis in cases where parasite density is low and asymptomatic. However, the nPCR method requires high cost, more materials, and trained staff for molecular testing.[11,17] Currently, countries pay much attention to planning for malaria elimination, especially in populations with a lower awareness about the disease.[18] The results of studies in Ethiopia and Thailand showed that the LAMP method was faster and more user-friendly than the nPCR method for malaria diagnosis.[11,14] Also, the nPCR method can enable the diagnosis in the field without the need for thermal cyclers and a high level of technical skills. Moreover, the LAMP can be performed with a simple incubator, such as a thermal block or a water bath (60°C), and SYBR green I and at a much lower cost than the nPCR and mn-PCR methods.[19–21]

The results of the present study are contrary to a study conducted in Bangladesh, which reported that LAMP specificity was lower than the LM[22] but similar to the observations made by Sattabongkot et al.,[21] who revealed high LAMP sensitivity and specificity compared with the LM method. This study showed that the LAMP and nPCR results were different in five cases that were positive by the LAMP method but negative with the nPCR method. Of these, one case was related to *P. falciparum* and four cases were related *P. vivax*. These findings are similar to those reported by Ocker et al.[23] and Tegegne et al.,[14] who reported more positive cases by the LAMP method than the LM method. A comparison of the LAMP and mn-PCR results showed that the specificity of these two methods is much higher than that of the LM method. These results are exactly the same as those of investigations done in previous years.[3–5,20] DNA extraction is one of the important steps in molecular experiments, but it can be performed by simple boiling through the LAMP and nPCR. This method can be cheap, fast, and appropriate in the field.[8,20,21,24,25] In this study, the minimum time required from the beginning of the sampling to obtaining the results of the patient's test was related to the LM method (less than an hour), but this method may yield false-positive results. However, the required time in the LAMP method was 105 minutes, which is acceptable in the field. This finding was similar to the results of a study conducted in Ethiopia that reported the shortest time when the LM method was used.[14] The present study found 10 false-positive cases that were negative in LM but positive in nPCR. Ebrahimzadeh et al.[26] also reported too many false-positive cases produced in the LM method. The malaria diagnosis gold standard is LM, but this method does not have high sensitivity and specificity in cases with low parasite density (no less than 20 parasites/μL) and asymptomatic patients. In addition, several factors (e.g., blood lam quality; meeting the required pH when preparing the Giemsa stain; the use of fresh colors and buffers; the technician's inability

TABLE 4
Performance characteristics of mn-PCR, LM, and LAMP by malaria case in comparison to the gold standard (nPCR) in Sistan-Baluchestan, Iran

Test	Sensitivity% (95% CI)	Specificity% (95% CI)	PPV% (95% CI)	NPV% (95% CI)	Agreement between tests, kappa
All positive by nPCR vs. all positive by LM	92.31 (81.46–97.86)	81.13 (68.03–90.56)	82.76 (73.20–89.40)	91.49 (80.6–96.53)	0.734
All positive by nPCR vs. all positive by mn-PCR	100 (92.45–100)	100 (91.59–100)	100 (0.9–100)	100 (0.89–100)	1
All positive by nPCR vs. all positive by LAMP	92.06 (82.44–97.37)	100 (91.59–100)	100 (0.92–100)	89.36 (78.37–95.12)	0.903

CI = confidence interval; LAMP = loop-mediated isothermal amplification; LM = light microscopy; mn-PCR = multiplex/nested polymerase chain reaction; nPCR = nested polymerase chain reaction; NPV = negative predictive value PPV = positive predictive value.

TABLE 5
Performance characteristics of mn-PCR, LM, and LAMP by species identified in comparison to the gold standard (nPCR) in Sistan-Baluchestan, Iran

	Plasmodium falciparum			*Plasmodium vivax*		
nPCR	LM	mn-PCR	LAMP	LM	mn-PCR	LAMP
Sensitivity% (95% CI)	87.5 (47.35–99.68)	100 (59.04–100)	100 (91.59–100)	91.18 (76.32–98.14)	100 (91.59–100)	100 (91.59–100)
Specificity% (95% CI)	100 (91.78–100)	100 (92.45–100)	87.5 (47.35–99.68)	81.13 (68.03– 90.56)	100 (92.45–100)	91.3 (79.21–97.58)
PPV% (95% CI)	100 (100–100)	100 (100–100)	97.67 (87.04–99.62)	75.61 (63.72–84.55)	100 (100–100)	91.3 (80.45–96.4)
NPV% (95% CI)	97.73 (87.30–99.63)	100	100	93.48 (82.84–97.7)	100	100
Agreement between tests, kappa	0.922	1	0.922	0.697	1	0.902

CI = confidence interval; LAMP = loop-mediated isothermal amplification; LM = light microscopy; mn-PCR = multiplex/nested polymerase chain reaction; nPCR = nested polymerase chain reaction.

to detect the parasite; and the microscopist's specialization, motivation, and experience) will affect the accuracy of the diagnosis.[5,27] In the present study the LM was able to detect only 22.2% of coinfection samples. One reason all coinfection cases could not be detected by the LM method was that the parasitic density of a species varies from the other, in which case the microscopist mistakenly reports only one species. Therefore, the length of time taken by the technician to examine the sample is important.[1,5,20,28–30] The mn-PCR results were completely similar to the nPCR results, and their sensitivity, specificity, positive predictive value, and negative predictive value were all equal to 100%. These two methods are performed differently, but the same primer is used in both methods.[5] Other advantages of mn-PCR include the reduced testing time, the need for less consumable materials, and less potential contaminants compared with the nPCR method.

CONCLUSION

This study showed that the mn-PCR methods could be effectively used for a large number of samples in contrast to LM. This procedure can even examine dried and old specimens. We have demonstrated that malaria diagnosis by the LAMP method can be performed at a field clinic. The minimum equipment required is a water bath or a heat block for carrying out the LAMP reaction. The LAMP method, which is simpler and cheaper than the nPCR and possesses high sensitivity and specificity, along with LM can be effective in the field and also for people who present for malaria diagnosis several times. The use of LAMP is a good solution in coinfection cases. In addition, mn-PCR can be a good alternative to nPCR, reducing testing time and cost.

TABLE 6
Total time required for the diagnosis of malaria with diagnostic tools

	Time in minute from sample preparation to reporting		
Diagnostic tool	Sample preparation (min)*	Testing and reporting (min)	Total time (min)
LM	40	20	60
LAMP	30 ± 10	60	105
mn-PCR	30 ± 5	150	185
nPCR	30 ± 5	215	250

LAMP = loop-mediated isothermal amplification; LM = light microscopy; mn-PCR = multiplex/nested polymerase chain reaction; nPCR = nested polymerase chain reaction.
*The time required to extract DNA.

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