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Comparison of Warmstart colorimetric LAMP using heat-treated samples with two *Plasmodium*-detection methods in Ekiti state, southwestern Nigeria

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ABSTRACT

Background: Malaria remains a deadly disease responsible for numerous deaths worldwide, especially in tropical developing countries. Widely used methods for diagnosis include microscopy, which is laborious, lengthy, and error-prone, and rapid diagnostic tests (RDT), which have recently faced limitations on accuracy. Molecular methods like the polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP) have emerged as accurate, sensitive, and rapid ways of detecting the disease. While PCR requires expensive equipment, LAMP is less expensive to perform. However, contemporary malaria LAMP still requires DNA extraction kits from patient samples. DNA extraction by heating can be used to lower the cost, time, and level of training required from end-users to perform LAMP in low-resource settings.

Methods: Blood samples were screened for *Plasmodium* parasites using microscopy, followed by a commonly used RDT kit. Heat treatment was used for quick DNA extraction from the blood samples, and LAMP primer sets were then used together with Warmstart colorimetric LAMP reagents for isothermal DNA amplification. Agarose gel electrophoresis was used to confirm that the colorimetric observations in LAMP reactions were due to successful DNA amplification. The data obtained were analysed in GraphPad Prism and subjected to chi-square tests with 95% confidence level.

Results: Warmstart colorimetric LAMP using heat-treated blood samples achieved successful DNA amplification and detected Plasmodium parasites in 43.3% of samples, compared to 40% and 30% Plasmodium parasite detection yielded by antigen RDT kit and microscopy respectively. However, the difference between the percentages obtained using the different methods was not statistically significant. LAMP approach further enabled the identification/discrimination of different *Plasmodium* species.

Conclusion: The Warmstart LAMP using heat-treated samples can be used as a rapid, cheaper, and easier-to-perform molecular method for the detection and species-level differentiation of malaria parasites.

Keywords: Malaria, *Plasmodium*, loop-mediated isothermal amplification, heat-treated blood

1.0 INTRODUCTION

Malaria remains one of the world's most prominent killer diseases and is responsible for more than half a million estimated deaths annually worldwide [1]. Diagnosis is one of the critical steps in the fight against this disease. While microscopy is common and remains the gold standard for malaria diagnosis, it is laborious, lengthy, and error-prone and depends on the skills of trained personnel [2]. Antigen-based rapid diagnostic test (RDT) kits are widely employed, but these have limitations on their accuracy and sensitivity [3-7]. The Polymerase Chain Reaction (PCR) is an accurate and sensitive molecular method that requires expensive equipment and reagents, making it unsuitable for use in resource-limited settings.

Loop-mediated isothermal amplification (LAMP) is a molecular method that has also been found to be accurate and sensitive for diagnosing various diseases [8-11]. LAMP is more rapid than PCR and requires less expenses. A colorimetric LAMP method based on pH-sensitive dyes that allow for easy visualization of test results with the naked eye has been developed and recently applied to detect malaria [12,13]. However, contemporary LAMP reactions still require DNA extraction using kits and reagents that adds to the time and cost of the process. Boiling ruptures cells and can be used for quick DNA extraction to lower further the cost, time and level of training required from end-users to perform LAMP. Boiling or heat treatment has been previously used in a non-colorimetric LAMP for *Plasmodium falciparum* [14]. In this study, we employed Warmstart colorimetric LAMP and heat-treated patient blood samples to detect different *Plasmodium* parasites. We compared the performance to those of microscopy and a commonly used RDT kit. This quick and cheaper LAMP approach should facilitate the wider applicability of the LAMP molecular method for malaria diagnosis in areas with limited resources.

2.0 METHODOLOGY

2.1 Ethical Consideration

Ethical approval for this study was obtained from the ethical committee of the Federal Teaching Hospital (FTH), Ido-Ekiti, Ekiti State, Nigeria (ERC/2022/06/02/792B). Awosolu *et al.,* [15] had earlier shown a malaria infection rate of greater than 90% from symptomatic individuals irrespective of gender in a study from several different communities in Ekiti State. Consequently, blood samples were obtained from symptomatic individuals within the age of consent at FTH, Ido-Ekiti. The blood samples were collected in Ethylene Diamine Tetra-Acetic Acid (EDTA) tubes and then subjected to microscopy examination before transfer to the Department of Animal and Environmental Biology laboratory, Federal University Oye-Ekiti. After that, the blood samples (n=30) were screened for malaria parasites using the CareStart Malaria Pf (HRP2) Ag RDT kit (Access Bio, NJ, USA) according to the manufacturer's instructions.

The LAMP primers (Table 1) were purchased from Inqaba Biotec (Pretoria, South Africa). The sets of primers for differentiation of malaria due to different *Plasmodium* species malaria targeted the 18sRNA gene of *Plasmodium falciparum* and *Plasmodium malariae* [11], which are the two most dominant types of malaria parasites in Nigeria [16, 17]. A PAN-malaria set of primers also targeted a conserved region in the 18sRNA gene of the five human-infecting *Plasmodium* species [18]. The blood samples were heated in a tube containing 20ul of water and 10ul of blood (ratio 2:1) at 99°C for 10 minutes before being used in a LAMP reaction. LAMP assay reaction was performed on the blood samples using WarmStart Colorimetric LAMP 2X Master Mix reagent (New England Biolabs, MA, USA) according to the manufacturer's instructions. Each reaction tube contained 12.5µl of the WarmStart Colorimetric LAMP 2X Master Mix, 2.5µl of the LAMP primers mix, 1µl of DNA template, and sterile water up to a total volume of 25µl. Specifically, a single LAMP assay for each blood sample has two reaction tubes: the test reaction tube to which 1µl of the heat-treated blood sample was added as a template and the negative reaction tube to which 1µl of water was used as the template. The reaction tubes were put in a thermocycler and incubated at 65°C for 30 minutes. A colour change from pink to yellow indicated that a sample was positive, while a lack of colour change (pink) indicated a negative result. To confirm that the observed colour changes in the reaction tubes were because of DNA amplification, agarose gel electrophoresis and visualization of the LAMP amplification products were performed under a blue light transilluminator.

2.2 Data Analysis

Data obtained from the diagnostic methods were analysed by Chi-Square using the GraphPad Prism version 5.01. Observations were considered to be statistically significant at $p<0.05$.

Table 1. List of Primers Used in the Study

Primer set	Primer sequence (5' – 3')	Remarks
Genus (PAN-LAMP)	F3: GGCGCGTAAATTACCCAA	[18]
	B3: AGCGTTTTAACTGCAACAA	
	FIP: CCATCATTCCAATTACAAAGCCAGAAGAAGAGAGGTAGTGACAAG	
	BIP: AACCTTCCCAAAACTCAATTGGATTTTAATATACGCTATTGGAGCT	
	LB: GCAGCCGCGGTAATTCC	
Plasmodium falciparum	FIP: AGTAGTCCGTCTCCAGAAAATCTTACTTTGGGGGCATTCGTATT	[11]
	BIP: GCGAAAGCATTTGCCTAATCTATTTAAGATTACGACGGTATCTGATC	
	FLP: TCACCTCTGACATCTG	
	BLP: GTTAAGGGAGTGAAGACG	
	F3: GCTTAGTTACGATTAATAGGAGTA	
	B3: AGTCGGCATAGTTTATGGT	
Plasmodium malariae	FIP: GCTTTCGCAGTTGCTTGTCTCGGGGGCATTTGTATTCAGA	[11]
	BIP: ACGAAAGTTAAGGGAGTGAAGACAGTCGGCATAGTTTATGGTT	
	FLP CTAAGAATTTCACCTCTGAC	
	BLP GATCAGATACCGTCGTAATC	
	F3: AGTTACGATTAATAGGAGTAGCT	
	B3: TTACACTATCATCCAACACCT	

3.0 RESULTS

Microscopy on the 30 blood samples that were used in this study showed that 9 (30%) of them were positive for malaria, while RDT tests yielded 12 (40%) positive samples for malaria (Table 2). Examples of positive and negative RDT results are shown in Figure 1. Warmstart colorimetric LAMP combined with heat-treated blood samples demonstrated a colour change from pink to yellow in test reaction tubes to indicate a positive reaction for the presence of *Plasmodium* as shown by a representative result in Figure 2A, while there was no colour change in test reaction tubes that were negative for *Plasmodium* as represented in Figure 2B.

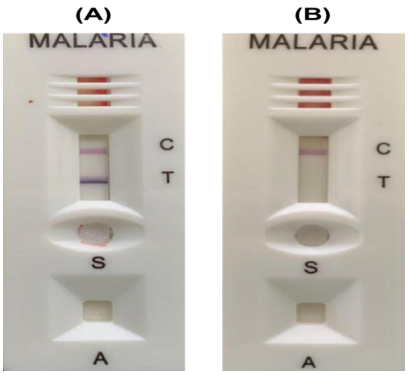

Figure 1: Examples of test results using the Carestart Malaria Pf Ag RDT kit. On the kit cassette, C is the control, while T is for the test. A positive result is shown in (A), while a negative result is shown in (B).

Table 2. Summary of Microscopy, Antigen RDT, and Warmstart Colorimetric LAMP Using Heat-treated Blood Samples for Detection of *Plasmodium* parasites

Sample	Microscopy	Ag RDT	LAMP		
			PAN	PF	PM
1	-	-	+	-	-
2	-	-	-	-	-
3	+	+	+	+	+
4	-	-	+	-	+
5	+	-	-	-	-
6	+	+	+	+	+
7	+	+	+	+	+
8	+	+	+	+	+
9	-	-	-	-	-
10	-	+	+	+	+
11	-	-	-	-	-
12	-	+	-	-	-
13	-	-	-	-	-
14	+	+	+	+	-
15	+	-	-	-	-
16	-	-	-	-	-
17	-	-	-	-	-
18	-	-	-	-	-
19	-	-	-	-	-
20	-	+	+	+	+
21	-	-	-	-	-
22	-	-	-	-	-
23	+	+	+	+	+
24	+	+	+	+	+
25	-	-	-	-	-
26	-	+	+	+	+
27	-	+	+	+	+
28	-	-	-	-	-
29	-	-	-	-	-
30	-	-	-	-	-

- = negative; **+** = positive; **Ag RDT** = Antigen Rapid Diagnostic Test; **PAN** = *Plasmodium* genus; **PF** = *Plasmodium falciparum*; **PM** = *Plasmodium malariae*

Agarose gel electrophoresis confirmed that successful DNA amplification occurred in the LAMP test reaction tubes that showed colour change (malaria positive) and there was lack of DNA amplification in the LAMP test reaction tubes that showed no colour change (malaria negative) (Figure 3).

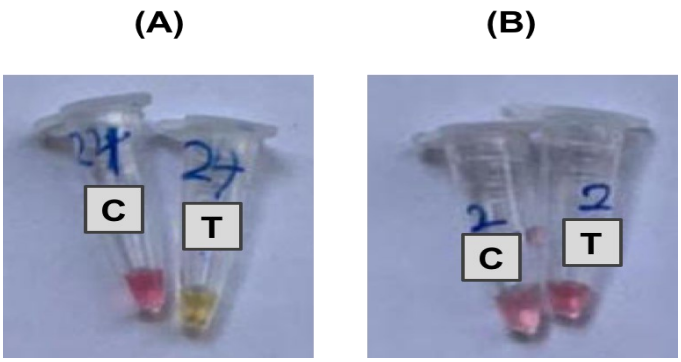

Figure 2: Examples of test results using Warmstart colorimetric LAMP assay. For each sample assay, C is the control tube without a DNA template, and T is used for the test. Control and negative LAMP reactions stay pink in colour, while positive LAMP reactions have a colour change to yellow. A positive colorimetric LAMP test result is shown in (A), while a negative LAMP result is shown in (B).

Figure 3: Agarose gel electrophoresis of LAMP reaction products. Sample 2 is negative for *Plasmodium* parasites as can be seen from the lack of DNA amplification, while samples 8 and 24 were positive for *Plasmodium* parasites as seen by the high amount of amplified DNA in the test reactions.

The collection and short-term storage (up to 7 days at 2° C) of the blood samples in EDTA vials did not interfere with successful WarmStart colorimetric LAMP assay outcomes. Samples 2 were used as a representative sample that tested negative for the presence of *Plasmodium*, while samples 8 and 24 were used as representative samples for those that tested positive for the presence of *Plasmodium*. Our colorimetric LAMP assay approach showed that 13 out of the 30 blood samples (43.3%) tested were positive for malaria (Table 2). However, the differences between the total positive and negative malaria samples obtained with the three diagnostic methods were not statistically significant (chi-square = 1.2290, df = 2, p = 0.5409).

Corresponding positive malaria diagnosis was obtained in 7 samples for both microscopy and RDT and also for both microscopy and LAMP (PAN *Plasmodium* used for these comparisons), while 11 samples were correspondingly positive for both RDT and LAMP (Table 2). These corresponding and the non-corresponding diagnostic outcomes between the methods were not significantly different (chi-square = 4.079, df = 2, p = 0.1301). There were 2 cases of positive malaria diagnosis from microscopy, which proved negative in LAMP and RDT, whereas five samples shown to be negative for malaria by microscopy tested positive for malaria in LAMP and RDT, respectively. On the other hand, there was one positive malaria diagnosis from RDT, which showed a negative diagnosis in microscopy and LAMP, and two positives from LAMP, which were negative in microscopy and RDT, respectively (Table 2). The outcome obtained for LAMP using additional species-specific LAMP primer sets further indicated the species of the *Plasmodium* parasites (*P. falciparum* or *P. malariae*) that were present in the samples (Table 2).

4.0 DISCUSSION

This study evaluated a combination of Warmstart colorimetric LAMP assay using heat-treated blood samples as a simplified, yet rapid and accurate alternative method for malaria diagnosis. In all the LAMP reactions, blood samples were heat-treated or boiled for 10 minutes instead of conventional DNA extraction, thus reducing the cost of the LAMP procedure. Isothermal DNA amplifications during LAMP normally yield DNA of several sizes and they appeared on agarose gels as a continuous smear-like products. Our LAMP results, in comparison to the microscopy and RDT malaria diagnosis methods utilized in this study, has shown that this boil-and-use method worked well with the Warmstart colorimetric method to give a simplified yet accurate and malaria diagnostic outcome. The outcome also showed that collection and short-term storage of up to a week of the blood samples in EDTA vials does not interfere with WarmStart colorimetric LAMP assay outcomes, though EDTA may inhibit some other enzymatic reactions.

Fresh blood samples were used in this study and were not centrifuged after boiling to mimic the field, outpost or resource-limited settings where centrifuges could be lacking. A small amount of boiled sample was rather taken directly and added straight to the LAMP reaction tube. This may sometimes result in clumps of cells from the boiled blood in some of the final LAMP reaction assays, but these do not prevent visualization of the final colour of reagents in the reaction tubes. For convenience we used a thermocycler for heating our LAMP reactions in this study, but a heating bath/block with a thermometer to keep to desired temperature ranges should also work well and can be used in resource-limited settings.

Using our approach, more positive malaria samples totalling 43.3% were detected compared to 40% and 30% that were obtained when microscopy and a more commonly used RDT kit were employed. RDT and LAMP agreed to most samples more than microscopy, suggesting that human error could be affecting the outcome of microscopy [2]. The exceptions where RDT and LAMP did not agree were in samples 1, 4 and 12. For sample 1, LAMP revealed that *Plasmodium* parasites were present but neither *P. falciparum* nor *P. malariae*. This case could be due to any of the 3 other human-infecting Plasmodium species and an appropriate primer set would be required for its detection using LAMP. For sample 4, LAMP showed that *P. malariae* was present, but *P. falciparum* was absent. The RDT kit applied in this study was CareStart Malaria Pf (HRP2) Ag RDT kit and it was not surprising nor out of place that this *P. falciparum*-specific antigen RDT kit could not detect the non-*falciparum* cases of *Plasmodium* infections detected by LAMP. The case of sample 12 is rather more interesting as it was positive for *P. falciparum* using the antigen RDT kit, but tested negative when all 3 LAMP primer sets for the *Plasmodium* genus, *P. falciparum* and *P. malariae* respectively were utilized (Table 2). In addition to LAMP, it tested negative using microscopy. This observation is probably a case of a false positive reaction from the antigen RDT kit. Reports have shown that cases of false positives are one of the limitations of the antigen RDT kit as the protein antigen from the *P. falciparum* histidine-rich protein 2 (HRP2) gene could be found in the blood of recovered patients for several weeks after recovery [3,19].

The results are in agreement with those of other studies on the detection of Plasmodium parasites from the blood samples of patients using LAMP. In this study, we focused and detected PAN-Plasmodium, P. falciparum and *P. malariae* using Warmstart colorimetric LAMP. Lai *et al.* 2020) 13]used Warmstart colorimetric LAMP to successfully detect the five known human-infecting *Plasmodium* parasites with 100% specificity and 98% sensitivity [13]. However, our study was more cost-effective than that of Lai *et al.,* [13] by employing heat-treatment instead of DNA extraction kits. The results from our study has also validated the findings of other authors who employed heat-treatment (boil and spin) in colorimetric or non-colorimetric LAMP assays for malaria [14, 20-22]. Besides Warmstart colorimetric LAMP which is based on pH-sensitive dyes, other colorimetric LAMP assays using SYBR Green fluorescence or malachite green have been shown to be accurate and sensitive to detect low parasitemia of *Plasmodium* parasites [20, 23]. Similar to this study, other studies in African countries such as Angola and Ethiopia also compared microscopy, RDT and LAMP (accompanied by PCR validations) for detection of *Plasmodium* parasites and found that LAMP performed better than microscopy and RDT [22, 24].

Taken together, our results highlight the accuracy, rapidness and ease of Plasmodium detection using the Warmstart LAMP with heat-treated samples method. However, this study did have some limitations. The number of blood samples that could be included in the study was limited as the consent of individuals for their blood samples to be used in the study was not something in our control. Also, strategies similar to those of other researchers in several developing countries could be explored in future studies to improve sample or reagent preservation [25-27].

We have shown that WarmStart Colorimetric LAMP can be utilized with heat-treated blood samples as a quick, easy-to-use and accurate molecular method to detect different Plasmodium parasites such as *P. falciparum* and *P. malariae* from human individuals. The boil-and-use approach employed further cuts down the cost for LAMP and should be easy to perform by healthcare users who may not have specialized training in molecular biology. Our approach could also benefit the diagnosis of other blood-borne pathogens.

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Conflicts of Interest

The authors declare that there is no conflict of interests.

Authors' Contributions

CO conceived and designed the study, contributed to data analysis tools, analysist of data and manuscript writing. **AA, TO, TA, OO, TA, SA** performed data collection, contributed to data analysis tools and reagents. **IO** contributed to data collection . All authors approved the final copy of the manuscript.

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