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

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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.

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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
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 effortlessness 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 falciparum.

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 What Man filter paper (No. 3) immediately.
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) | AGCTGGAATTACCGCGGCTGGGTTCCTAGAGAAACAAATTGG |
| BIP (B1-B2c) | TGGTGCAGTTAAAACGTTCGTAGCCCAAACCAGTTTAAATGAAAC |
| F3 | TGTTAATTGGAATGATAGGAATTTATGG |
| B3c | GAAAACCTTTATTTGAACAAAGC |
| LPF | GCACCAGACTTGCCCT |
| LPB | TTGAATTTAAAGAA |
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 $21.4 \pm 17.5$ 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/µl ± (SE=924.097) (range between 79 – 549,333 parasite/µl). 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 ± SD       | 21.4 ± 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/µl) | 59096.75        |                       |
Table 3. \textit{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 \textsuperscript{1} | 0.866                        |

\textsuperscript{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

\textit{Plasmodium falciparum} is a fatal pathogenic \textit{Plasmodium} species that cause malignant malaria in humans. \textit{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 \textit{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 \textit{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 \textit{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.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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