A Novel Approach on the Sensitivity of Preserved Plasmodium falciparum Positive RDT kits for the Molecular Detection of Pfmdr 1 and Pfcrt Genes in Pregnant Women

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Abstract

Storing blood samples for molecular screening have become increasingly difficult due to unstable electricity and high cost of generators fuels. Samples often deteriorate giving false negative upon molecular analysis. This research was aimed at determining the sensitivity of preserved *Plasmodium falciparum* positive RDT kits for the molecular detection of Pfmdr 1 and Pfcrt genes among pregnant women in From February 2020 to May 2020, Sokoto, Nigeria. 200 pregnant patients who visited the hospital for ANC had their blood samples taken, and a questionnaire was given out. To detect *Plasmodium falciparum*, RDT kits tailored to its monoclonal antibody were utilized. *P. falciparum* positive Rapid Diagnostic Test Kits were air tightened with nylon and silica-preserved for three months in a closed cabinet. The preserved positive samples were utilized to extract DNA, and the Pfmdr1 and Pfcrt genes' existence was then checked using a PCR procedure. 4 samples tested positive for both genes, suggesting a 21.05 % prevalence, according to the results of the inquiry into the Pfcrt and Pfmdr1 genes. This study has revealed the sensitivity of preserved RDT kits (with silica) for DNA extraction. Researchers and molecular laboratories should be preserving positive RDT kits meant for DNA extraction and other molecular analysis as it is much easier to preserve and there is no cost involved.

Key words: RDT Kits, DNA extraction, Pfmdr1 gene, Pfcrt gene, pregnant women, *Plasmodium falciparum*.

Introduction

Rapid diagnostic tests (RDTs) are widely employed as a supplementary method to microscopy for the detection of malaria (Wongsrichanalai *et al.,* 2007), and in some cases, they can even be utilized as a point-of-care diagnostic tool (Wiese *et al.,* 2006). The detection of Plasmodium infections is frequently reliant only on RDTs in environments where high-quality microscopy is not accessible (Chilton *et al.,* 2006; Osman *et al.,* 2010). The World Health Organization (WHO) supports the widespread use of RDTs for malaria diagnosis in places where malaria is common and advises using them as part of parasite-based diagnosis (WHO, 2009a; 2009b).

In more rural areas where microscopy services are less accessible, rapid diagnostic

test kits (RDTs) have emerged as the most popular diagnostic technique for detecting patients with *Plasmodium falciparum* malaria (Wongsrichanalai *et al.,* 2007). A possible source of *P. falciparum* DNA is RDTs that test positive for the parasite. Plasmodium DNA may be recovered from RDT using a variety of methods (Wiese *et al.,* 2006; Chilton *et al.,* 2006; Osman *et al.,* 2010), and the quantity and quality of the extracted parasite DNA varies depending on the method used (Wiese *et al.,* 2006). RDT-derived Plasmodium DNA has been evaluated for assessment of 18S rRNA (Wongsrichanalai *et al.,* 2007; Osman *et al.,* 2010; WHO, 2009a), the *P. falciparum* histidine-rich protein-2 and 3 genes (WHO, 2009b), *P. falciparum* polymorphic microsatellite markers (WHO, 2009c), drug resistance markers Pfcrt and Pfmdr1 (Bell *et al.,* 2006) and tRNA methionine-based quantification of Plasmodium parasites (Cnops *et al.,* 2010).
According to a previous work (Wiese *et al.,* 2006), the RDT sample that was kept at room temperature for 36 months (n = 1) was successfully amplified for the 18S rRNA gene. In more rural areas where microscopy services are less accessible, rapid diagnostic test kits (RDTs) have emerged as the most popular diagnostic technique for detecting patients with *Plasmodium falciparum* malaria (Mouatcho *et al.,* 2013). A possible source of *P. falciparum* DNA is RDTs that test positive for the parasite. Plasmodium DNA may be recovered from RDT using a variety of procedures (Veron *et al.,* 2006; Cnops *et al.,* 2011; Ishengoma *et al.,* 2011), and the quantity and quality of the extracted parasite DNA varies depending on the method used (Veron *et al.,* 2006). The most effective extraction method for extracting parasite DNA from RDT samples among the three examined was phenol/chloroform extraction (Veron *et al.,* 2006). RDT derived *Plasmodium* DNA has been evaluated for assessment of 18S rRNA (Veron *et al.,* 2006; Ishengoma *et al.,* 2011; Papa *et al.,* 2016), the *P. falciparum histidine-rich protein-2* and *3* genes (Beshir *et al.,* 2017), *P. falciparum* polymorphic microsatellite markers (Nabet *et al.,* 2016), drug resistance markers Pfcrt And Pfmdr1 (Morris *et al.,* 2013) and tRNA methionine-based quantification of *Plasmodium* parasites (Robinson *et al.,* 2019).
According to a previous work (Veron *et al.,* 2006), the RDT sample that was kept at room temperature for 36 months (n = 1) was successfully amplified for the 18S rRNA gene. For usage in molecular genetics research, however, the RDT blood spots' poor quality and amount of extracted DNA continue to be a significant problem (Suttipat *et al.,* 2020).

Methodology

Hospital Consent

The management of the Maryam Abacha Women and Children Hospital (MAWCH), Sokoto, Nigeria, gave its agreement prior to the commencement of the research. Patients who gave their consent after being fully informed of the study's purpose and goals donated their blood. Introduction letter was obtained from the Head of Biological Sciences department which was addressed to the management of MAWCH seeking them to assist and give me access to their facilities.

Sample Size Determination

Sample Size Determination Samples from the pregnant women participating in this study, who came to healthcare facility for antenatal treatment using a stratified sampling method, have been collected. The formula below, which was used by Abubakar et al.,2022, was used to determine the sample size. $n = Z^2 p (1-P)/ e^2$ Where; n = Desired sample size, Z = Confidence interval (95%) = 1.96, p = Previous Prevalence, p= 7% e = Degree of precision (0.05) (Ekpereonne et al., 2018).
Therefore, substituting Z for 1.96, P for 0.07 and e for 0.05, then the minimum sample size will be: $n= (1.96)^2 \times 0.07 (1 -0.07) / (0.05)^2$

n= 3.8416 x 0.07 (0.93)/ 0.0025 = 100

The minimum sample size is 100 but it has been adjusted to 200.

Blood Sample Collection

Pregnant women who visited the research area for Anti-Natal Care had two milliliters of blood drawn through the vein with the aid of a qualified nurse using a needle and a 5 ml syringe. The blood samples were placed into an ethylene diamine tetraacetic acid (EDTA) container after collection to prevent the blood from coagulating. The blood samples were transferred in a cold chain to the parasitology lab at the faculty of science at Usmanu Danfodiyo University Sokoto (UDUSok).

***Plasmodium falciparum* Rapid Diagnostic Test Kit (RDT Kit)**

All the 200 blood samples were screened using *Plasmodium falciparum* mono clonal antibody specific RDT kit to confirm the presence of the parasite (*Plasmodium falciparum)*. The RDT (SD BIOLINE Malaria Ag P.f) Kit was used in accordance with the manufacture's instruction.

P. falciparum Positive samples were transported to the central research laboratory of the Faculty of Veterinary Medicine, Usmanu Danfodiyo University Sokoto (UDUSok) for DNA extraction and molecular screening.

Preservation of *Plasmodium falciparum* positive RDT Kits

Five (5 per nylon) *Plasmodium falciparum* positive RDT Kits were placed in air tight nylon and preservative silica (2 packs of silica per nylon). The nylon containing both the RDT kits and silica were place in air tight cabinet and kept for three months. The kits were later transported to central research laboratory, Faculty of Veterinary Medicine for molecular screening.

Molecular Screening

Plasmodium falciparum positive samples were subjected to DNA extraction. The extracted DNAs were further subjected to PCR for the purpose of identifying the drug-resistant genes in *P. falciparum* (Olasehinde *et al.,* 2014). PCR amplicons were loaded in agarose gel electrophoresis (Sharma *et al.,* 2015).

Preparation of RDT Kit for DNA extraction

Using a sterile blade, scissors, and forceps, RDT cassettes were opened, and the nitrocellulose strip was extracted from the plastic casing (Nguyen *et al.,* 2019). The test strip's DNA-containing positive section was divided up after the nitrocellulose strip was removed (Nguyen *et al.,* 2019). The strips were placed in microtubes and 200µl of nuclease free water (molecular grade water) was added to dissolve the content of the strip (Nguyen *et al.,* 2019). This was achieved via multiple aspiration and inspiration. The empty strip was later removed and sample was subjected to DNA extraction (Nguyen *et al.,* 2019).

Deoxyribonucleic acid (DNA) Extraction

According to manufacturer's instructions, DNA extraction was performed using a Qiagen DNA easy extraction kit (Qiagen, Krefeld, Germany) (Olasehinde *et al.,* 2014).

Polymerase Chain Reaction (PCR)

From the blood of pregnant women who tested positive for malaria, Polymerase Chain Reaction (PCR) was performed to identify the resistance genes of *Plasmodium falciparum* (Sharma *et al.,* 2015).

PCR amplification of Pfcrt gene

In a 0.2 ml nuclease-free microfuge tube, a 25 µl reaction mix with the following ingredients was used for the PCR: 12.5 µl of Top Taq master mix (qiagen, USA), 2 µl of each 10 µm Pfcrt F and Pfcrt R primers, 2 µl of coral load (both from Qiagen, USA), 1.5 µl of nuclease-free water, and 5 µl of DNA template (Sharma et al., 2015). The tubes were then transferred to the thermocycler of Applied Biosystems ABS 9700, (Sharma et al., 2015).

The cycling conditions used by Vathsala et al. 2004 and Sharma et al. (2015) was adopted, they are; denaturation at 94 °C for 3 min,

followed by 45 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, extension at 72 °C for 1 min, 72 °C for 5 min and post hold at 8°C.

PCR amplification of Pfmdr1 gene

The PCR was carried out in a 0.2 ml nuclease free microfuge tube containing a 25µl reaction mix with the following components; 12.5µl of Top Taq master mix (qiagen,USA), 2µl of each 10µm Pfmdr 1 F and Pfmdr 1 R, 2.5µl of coral load (qiagen,USA), 2µl of Nuclease free water (qiagen,USA) and 4µl of DNA template. The tubes were transferred to Applied Biosystem (ABS) 9700 thermocycler (Sharma *et al.,* 2015). The cycling conditions used by Vathsala et al. 2004 and Sharma et al. (2015) was adopted, they are; denaturation at 94 °C for 3 minutes, followed by 35 cycles of final denaturation at 94 °C for 30 s, annealing at 56 °C for 1 minutes, extension at 72 °C for 2 minutes, 72 °C for 5 min and post hold at 8°C.

Table 1: List of primers used in the study

Primer	Oligonucleotide Sequences (5'- 3')	Expected Band size	
Pfcrt- F	GGCTCACGTTTAGGTGGA	264bp	
Pfcrt- R	TGAATTTCCCTTTTTATTTCCAAA		
Pfmdr 1- F	ATGGGTAAAGAGCAGAAAGA	603bp	
Pfmdr 1 – R	AACGCAAGTAATACATAAAGTCA		

Source: Vathsala *et al.* (2004)

Agarose Gel Electrophoresis

The amplified products were subjected to 1.5% agarose gel electrophoresis pre-stained with ethidium bromide with a 100 bp ladder used as a standard.

The electrophoresis was conducted at 80 volts for 1 hour in a BioRad agarose gel electrophoresis unit. The gel was visualized using a U-V trans-eliminator in a BioRad XRS gel documentation device.

Statistical analysiss

Occurrence of the resistant genes was computed using frequency distribution. Version 4.1.0 of InVivoStat was used for the analysis. The level of significance for all analysis was set at P0.05, with the confidence level being maintained at 95%.

Results

Using microscopy and an RDT kit, 200 pregnant women were examined for the presence of malaria parasites. The results for microscopy revealed 3 samples positive for *Plasmodium falciparum* representing 1.50% prevalence. The results of this study with respect to *P. falciparum* mono clonal antibody specific RDT kit revealed 19 samples positive for *P. falciparum* representing 9.50% prevalence (Table 2). Out of the 19 samples tested for Pfmdr1 and Pfcrt genes, 17 samples were positive for Pfmdr1 (89.50%) and 5 samples (26.30%) were positive for Pfcrt gene. While 4 samples (sample 6, 10, 13, and 14) were positive for both genes representing 21.05% prevalence (Table 2).

Table 2: Malaria, **Pfmdr1 and Pfcrt genes** Prevalence

Testing Tools	**Examined**	**Positive**	**Percentage Positive (%)**
RDT kit	200	19	9.50
Microscopy	200	3	1.50
***P. falciparum* drug Resistant genes**			
Pfmdr1	19	17	89.5
Pfcrt	19	5	26.3

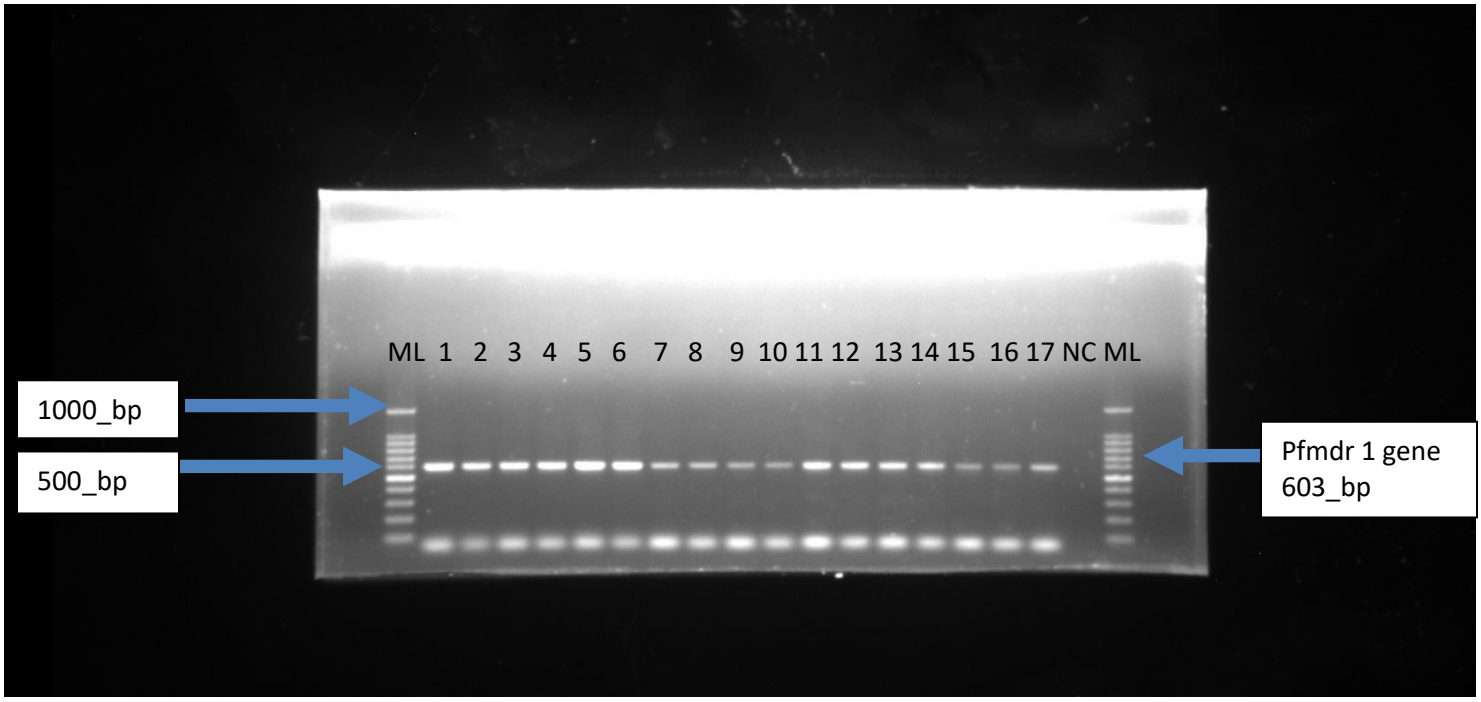

Fig. 1: Pfmdr1 gene gel
Key: ML = Molecular ladder, NC = Negative control

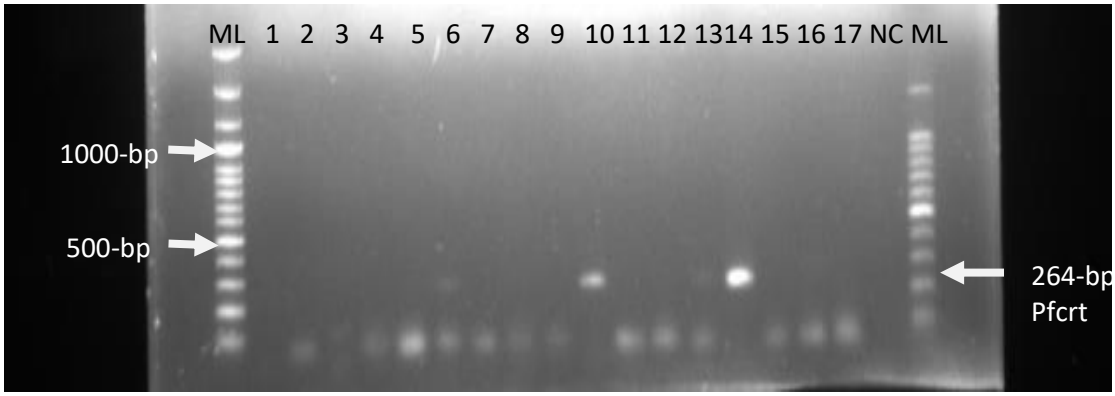

Fig. 2: Pfcrt gene gel
Key:

Positive wells = well 6, 10, 13 and well 14
ML = Molecular ladder, NC = Negative control

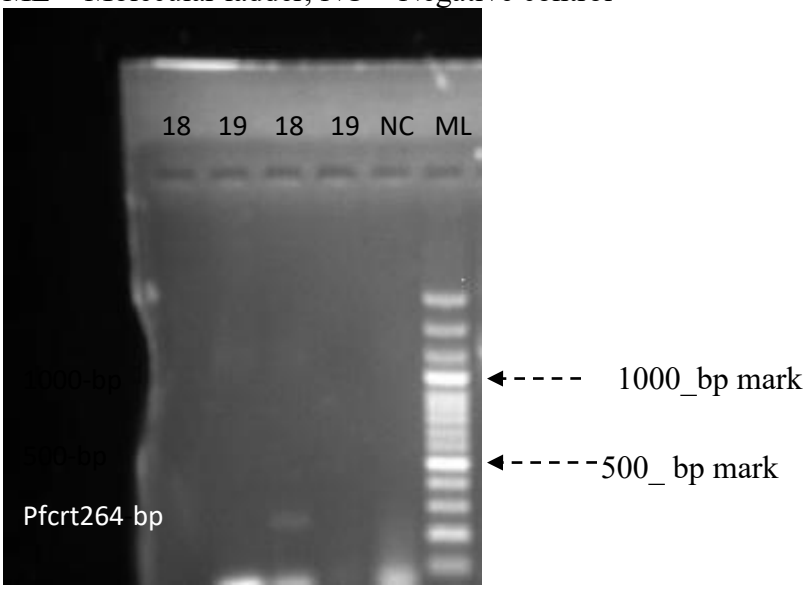

Fig. 3: Pfmdr1 and Pfcrt genes gel
Key:
Positive sample: Pfcrt = 18
Negative samples: Pfmdr1 = 18 and 19, Pfcrt = 19
ML = Molecular ladder, NC = Negative Control

Discussion

This study shows that the Qiagen DNA easy (Qiagen, Krefeld, Germany) extraction kit successfully recovered *Plasmodium falciparum* DNA from the nitrocellulose strip of *P. falciparum* positive RDT Kits. The results of this study concur with those of Cnops *et al.* (2011), who found that accurate Plasmodium species identification by a sensitive real-time PCR assay was possible for the four Plasmodium species with varying parasite densities on RDTs stored for weeks to months after typical laboratory diagnosis in a reference setting.

The molecular identification of *P. falciparum* chloroquine resistant transporter gene (Pfcrt) and *P. falciparum* multidrug resistant gene 1 (Pfmdr1) using DNA recovered from archived (for 3 months) *P. falciparum* positive RDT Kits was clearly demonstrated. The results of this work are consistent with those of Nguyen *et al.* (2019), who noted the value of archival RDTs with a large sample size for molecular characterisation of *P. falciparum* population diversity, MOI, and the distribution of CQ-resistant haplotypes. *P. falciparum* positive RDT Kits can therefore be stored overtime (for as long as 3 months) in areas with unstable electricity until required for molecular screening/analysis (DNA extraction and PCR).

PCR protocol was used to detect the occurrence of Pfmdr1 and Pfcrt genes of *Plasmodium falciparum*. Prevalence of Pfmdr1 gene observed in this study (89.50%) was relatively higher than previously reported (45.0%) by Simon-oke *et al.* (2018), in Akure, Ondo State and 18.90% Pfmdr1 gene prevalence reported in Benin metropolis, Edo State by Okungbowa and Mordi (2013). The prevalence of the Pfcrt gene in this study (26.30%) was also greater than that previously reported by Okungbowa and Mordi (2013) (24.0%). High indiscriminate use of drugs (without a prescription) for the

treatment of malaria by pregnant women, the partial withdrawal of chloroquine from the treatment of malaria, variations in the environmental conditions, or the presence of *Plasmodium falciparum*, the disease's causative agent throughout the nation, which may have been caused by people moving from one location to another for recreational activities, could all contribute to the high prevalence of these mutant genes in the study area.

Four samples were found to have both of the two mutant Pfcrt and Pfmdr1 genes. They therefore have tendency of conferring resistance to Chloroquine in addition to Mefloquine (MQ), Halofantrine (HF), and Lumefantrine (LM). While the remaining 13 samples (1-5, 7-9, 11, 12, 15, 16 and 17) with only Pfmdr1 gene have tendency of conferring resistance to Mefloquine (MQ), Halofantrine (HF), and Lumefantrine (LM) as earlier reported (Price *et al.,* 2004, Rohrbach *et al.,* 2006).

Prevalence of malaria infection observed by RDT in this study (9.5%) is lower compared to previous researches conducted among pregnant women attending ante-natal care in Sokoto by Udomah *et al.* (2015) with 52.2% prevalence and 10.6% malaria infection prevalence reported also in Sokoto by Buhari *et al.* (2016). This low prevalence of malaria among pregnant women in the study area may be attributed to period of the study (no rainfall) (Patricia *et al.,* 2014), use of antimalarial drugs (WHO, 2020) and the fact that the pregnant women are going for ante natal care (ANC) (Fana *et al.,* 2015). Earlier Udomah *et al.* (2015) reported malaria prevalence to be low due to the period of the study (dry season).

This study is limited to the use of preserved *P. falciparum positive* RDT kits (for 3 months) for molecular analysis of Pfmdr 1 and Pfcrt genes. Feature studies should test the sensitivity of preserved *P. falciparum* positive RDT kits over a long period of time (more than 3 months).

Recommendations

1. We therefore recommend preservation of *P. falciparum* positive RDT kits if required for feature use since samples can last for as long as 3 months and the procedure is user friendly, free/not expensive as the RDT kits comes with the silica in the pack.
2. Researchers, students, laboratory technologist and research centers/institutes should be educated on the use of silica in preservation of *P. falciparum* positive RDT kits since the protocol prevent deterioration of samples and does not affect the sensitivity during molecular screening.

Conclusion

This research has provided evidence of the effectiveness and sensitivity of *P. falciparum* Positive RDT kits preserved for 3 months in the molecular detection of Pfmdr 1 and Pfcrt genes and also contributed in the reduction of risk factors for global warming caused by generators (carbon emission) used in the laboratories that power up freezers/refrigerators.

Conflict of interest

No conflict interests.

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