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RESEARCH ARTICLE

Enumerating the Yield and Purity of *Pf*DNA from Archived, Newly Used mRDTs and Comparison with DBS from a Malaria-Endemic Focus

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Objective: Archived malaria rapid diagnostic test strips (mRDTs) serves as an important source of plasmodium Deoxyribose Nucleic Acid (DNA) in epidemiological studies. The presence of *Plasmodium falciparum* DNA (*Pf*DNA) in mRDTs (yr. 2016-2017) and newly used ones (yr. 2018) were enumerated with a view to establish the parasite's optimum genomic DNA volume. **Methods**: A retrospective study to determine the yield and purity of used mRDTs was carried out on randomly selected mRDTs (2016 – 2018). Both positive and negative mRDTs samples were analyzed with nested Polymerase chain reaction (nPCR). Dried blood spots (DBS) were obtained from study enrolments and analyzed molecularly. nPCR and Agarose gel electrophoresis were used to determine *P. falciparum* DNA. **Results**: Agarose gel electrophoresis results showed that only 26 out of the 50 samples eligible for screening were PCR positive for *P. falciparum*. The following was observed; yrs.: 2016 - 17(34%) with 2.06 X 103 yield, 1.7235 purity; 2017 - 16(32%) with 1.03 X 103 yield, 1.7619 purity and 2018 - 17(34%) with 1.42 X 103 yield, 1.6194 purity. Molecular analysis (P.f. 18Ss rRNA) was determined to ascertain positive result that appeared negative using mRDTs or microscopy. The DNA yield of the DBS for 2018 was 1.66 X 103 and a purity (Optical Density 260/280) of 1.69. The purity was higher than that of the mRDTs with a DNA yield of 1.42 X 103 and 1.62 purity. **Conclusion**: *Pf*DNA extraction is an important process for malaria PCR screening and the reliability is dependent on pureness and concentration.

Keywords: *Plasmodium falciparum*, mRDTs, DNA, purity, yield

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Introduction

The discovery of suitable malaria genetic markers for the establishment of a promising elimination technique is paramount at this time when it appears all efforts at combating the scourge is fast becoming elusive [1]. Archived mRDTs are important source of *Plasmodium falciparum* DNA (*Pf*DNA) in epidemiological studies, but a number of limiting factors affect the yield and purity [2-5]. The analysis for genetic diversity of malaria among human and *Plasmodium falciparum* populations is akin to monitoring control and elimination [4, 6]. The species *P. falciparum* is said to be the most virulent among the other four types of malaria parasites known to cause physio-pathological syndromes in humans with the transmission rate and the migration of infected and carriers differing from one endemic area to the other [3, 7]. It poses great challenges to malaria elimination, as effective tools are required to detect genomic changes in the parasite populations and subsequently becomes necessary for monitoring the spread of genetic variants [8]. In the past few years, several methods have been developed for eliciting parasite DNA, these methods have allowed the introduction of exogenous DNA into the human malaria parasite [9]. The discovery of parasite transfection as an important technical tool is providing more insights for understanding the function of plasmodium proteins and their roles in biology and disease. This has allowed the analysis of promoter function and helped to establish the role of particular molecules and/or mutations in the biology of plasmodium species. The ability of *P. falciparum* to transfect plasmids and replicate episomally during the merogonic stages is paramount to the survival of the parasite in the presence of lethal antimalarial drugs [9, 10]. Recently, the epidemiology of malaria revealed the discovery of several multiple loci variable tandem repeat analysis (MLVA) aided with microsatellite markers and observed that *P. falciparum* genetics correlate with malaria transmission intensity [1, 6, 11, 12]. In malaria endemic zones that have an uneven atmospheric temperature distribution across the year, collection of occult blood for analysis of DNA probes can prove inconsistent [11], but the advent of malaria rapid diagnostic test (mRDTs) kits for determining clinical malaria infections may provide desired measures to establish past malaria infections and determine variability in DNA sequence of the plasmo-

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dium parasite [4]. Additionally, ethical issues surrounding patients consent are easily resolved as ordinarily these used strips/cassettes are discarded once interpreted. The World Health Organization (WHO) [13] advocated the use of mRDTs to detect the presence of circulating malaria parasite specific antigens such as the histidine rich protein 2 (*Pf*HRP2) and lactate dehydrogenase (*Pf*LDH). Earlier studies indicated that used mRDTs potentially harbor *Pf* DNA [5, 14-17). With this development, it is imperative that the detection of antimalarial drug-resistant genes can be achieved through the single nucleotide polymorphism [17]. The abundant contamination of clinical malaria samples for plasmodium species DNA sequencing with human DNA is a major constraint militating against effective and quality *P. falciparum* genome studies [18]. The removal of leukocytes and other blood constituents from infected blood samples [19-21], the use of frozen blood samples [22, 23] and the isolation, genotyping and sequencing of *P. falciparum* from archive clinical blood samples using flow cytometry [18] are germane contributions to its genome sequencing and evolution. In the light of the aforementioned obstacles, our study aims to determine and compare the yield and purity of *Pf* DNA in archived and newly used mRDTs and DBS collected from an area with high malaria transmission.

Methods

Study Design

A retrospective study was carried out on randomly selected used mRDTs in the last three years (between February 2016 and March 2018) at the Molecular Parasitology laboratory of the Department of Zoology, University of Ilorin, Nigeria. Both positive and negative mRDTs samples were analyzed with nPCR. The study was designed to compare and evaluate *Pf*DNA yield and purity of archived and newly used mRDTs using current molecular techniques. The study also evaluated and compared the yield and purity of *Pf*DNA simultaneously derived from 50 samples of dried blood spot (DBS) and newly used mRDTs (2018), respectively. These samples were freshly obtained from study enrolments in private and public hospitals in Kwara State, Nigeria. Peripheral blood finger prick samples (5 – 10ul) were absorbed into mRDTs. Two to three drops of blood were placed on Whatman FTA card (Whatman Inc., Brentford, UK) and stored in air tight desiccant containers and consequently preserved at -80ºC.

Study Area

The study was carried out in Ilorin, an urban location that is the capital of Kwara state, which lies in the North-Central part of Nigeria, located on the longitude 4.33°E - 4.45°E and latitude 8.30°N. It covers an area of about 38 square miles, with an estimated population of 3,192,893million people (2016 projection) [24]. Ilorin is associated with intense rainfall from April to October and daily temperature between 23°C and 37°C with humidity of 70% [25].

Ethical Clearance

Approval for this study was obtained from the University of Ilorin Ethical Consideration Committee. The Directors of Children Specialist Hospital, Centre Igboro and the University Health Services, University of Ilorin, Ilorin were briefed with the details of the aim and objectives of the study. Thereafter, with the assistance of the hospital's laboratory staff, written consent and cooperation of the patients were sought.

DNA Extraction – Qiagen Minikit

Parasite DNA extraction was performed on DBS samples using a QIAamp DNA Mini Kit and the DNeasy Blood and Tissue Kit (Qiagen, Venlo, Netherlands) with strict compliance to the manufacturer's instructions. Extracted genomic DNA was recovered in a final elution volume of 50 µL.

DNA Quantification by Nanodrop 1000

DNA quantification was performed by measuring the UV-induced emission of fluorescence and nucleic acid light absorption at 260nm in two-stranded DNA, having a concentration of 50ng/µl. This was correlated with an Optical Density (OD) of 1 at 260 nm, thereby making it easy to calculate DNA from OD measurements. The light absorbed in the 260nm region was used to determine the concentration of DNA in solution by applying the Beer-Lambert law (Beer-Lambert equation is only linear for absorbance between 0.1 and 1.0 while using the Nanodrop).

DNA concentration was estimated by measuring the absorbance at 260nm; adjusting the A260 measurement for turbidity (measured by absorbance at 320nm), multiplied by the dilution factor, and using the relationship that an A260 of 1.0 = 50µg/ml pure dsDNA.

Concentration (µg/ml) = (A260 reading – A320 reading) × dilution factor × 50µg/ml

Total yield was obtained by multiplying the DNA concentration by the final total purified sample volume.

DNA yield (µg) = DNA concentration × total sample volume (ml)

Polymerase Chain Reaction (PCR) Amplification

PCR is a technique used in molecular biology for amplifying a single copy or a few copies of a segment of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. PCR amplification was carried out after the methods described in Oyola et al. [26] and Papa Mze et al. [2].

Agarose Gel Electrophoresis

The separation of the *P. falciparum* DNA fragments (100 bp to 25 kb) was carried out after the methods of Pie et al., (2012) [27]. After separation, the resulting DNA frag-

ments appeared as clearly defined bands on standardized ladder and separated to a degree that allows for the useful determination of the sizes of sample bands (Figure 1).

Data Analysis

Statistical analysis was performed using Statistical Package for Social Science (SPSS) for windows version 22.0 full version. The mean±SD was also analyzed by comparing means of yield and purity of the samples. Molecular analyses for the determination of yield and purity of *Pf*DNA were carried out in the first part on fifty randomly selected archived mRDTs (2016 – 2018) and same evaluation was simultaneously done on fifty fresh samples of Dried Blood Spots (DBS) and newly used mRDTs (2018). For all tests, the significance level was $\alpha = 0.05$ measured at 95% confidence level.

Results

A total of fifty (50) valid samples were examined for *P. falciparum* DNA obtained from clinically used mRDTs (2016 – 2018). Out of these samples; 24 (48.0%) were truly positive for *P. falciparum* with the following distribution with respect to 2016 – 2018 viz 10 (58.8%); 5 (31.3%) and 9 (52.9%). The distribution for *Pf* DNA positive samples for nPCR was 26 (52.0%) (Table I).

The yield and purity of the sampled *Pf*DNA determined with spectrophotometry (Nanodrop ND-1000) for three consecutive years are as depicted in Table II. The following yield and purity were consecutively recorded in the years' 2016 – 2018, viz; 17(34%) samples with 2.06 X 103 ± 824.55 yield; purity 1.7235 ± 0.2067 (2016), 16(32%) samples with 1.03 X 103 ± 1065.16 yield; purity 1.7619 ± 0.1158 (2017) and 17(34%) samples with 1.42 X 103 ± 1096.64 yield; purity 1.6194 ± 0.1163 (2018). It was observed that archived mRDTs from 2016 had a higher yield when compared to other recently stored mRDTs (i.e. 2017and 2018), but the purity results however showed no obvious differences between 2016 and 2017. A marked downward difference in purity was observed with year 2018 samples as compared to year 2016 and 2017 respectively (Table II).

In another vein, a total of fifty (50) valid samples were separately simultaneously obtained by DBS and mRDTs in 2018 to compare *Pf*DNA yield and purity (Table III). It was discovered that the yield of *Pf*DNA (4.51 X 103 ± 2986.34) obtained was higher than that obtained through the DBS (1.96 X 103 ± 666.15) for the same sample of blood. In fact, the purity obtained through the same means was higher with mRDTs (5.1048 ± 0.4388) than DBS (1.6312 ± 0.09) (Table III).

Agarose gel electrophoregram of *P. falciparum* 18Ss rRNA (205 bp) resolved on 1.2% agarose gel from 50 selected positive mRDTs samples (Fig. 1). Lanes 1= 100bp DNA ladder, lanes 2, 3, 4, 5, and 10, 11, 12, 13, 14, 15 are PCR positive for *P. falciparum*, lane 16 is no template control. Although, the RDT tested negative for samples 9, 12, 13, 17 for year 2016 likewise for year 2017 with samples number 19, 20, 21, 22, 23, 31 and 38, 44 all tested

Table I. Retrospective distribution of *P. falciparum* DNA in used mRDTs stored at room temperature

mRDTs	Years of Enrolment			Total (%)
	2016	2017	2018	
Total No. examined/year	17 (34.0%)	16 (32.0%)	17 (34.0%)	50 (100%)
No. positive	10 (58.8%)	5 (31.3%)	9 (52.9%)	24 (48.0%)
No. negative	7 (41.2%)	11 (68.8%)	8 (47.1%)	26 (52.0%)
Positive PCR for *P. falciparum*, n (%)	10 (58.8%)	9 (56.3%)	7 (41.2%)	26 (52.0%)

mRDTs – malaria rapid diagnostic tests; nPCR – nested Polymerase Chain Reaction

Table II. Comparison of the *Pf*DNA yield and purity obtained by Nanodrop 1000 with respect to the years under study (2016 – 2018)

Year under study	Sampling type	No. Examined	No. Positive	Mean ± St. Dev	Minimum	Maximum
2016	mRDTs					
	Yield(ng/µL)	17	10	2.06 X 103 ± 824.55	1152.70	3833.30
	Purity			1.7235 ± 0.2067	1.15	1.91
2017	mRDTs					
	Yield(ng/µL)	16	5	1.03 X 103 ± 1065.16	102.90	3638.70
	Purity			1.7619 ± 0.1158	1.58	1.97
2018	mRDTs					
	Yield(ng/µL)	17	9	1.42 X 103 ± 1096.64	487.90	5466.00
	Purity			1.6194 ± 0.1163	1.51	1.96

mRDTs – malaria rapid diagnostic tests

Table III. Comparison of *Pf*DNA yield and purity obtained by Nanodrop 1000 with respect to mRDTs and Dried Blood Spot (DBS) from the study enrolments in 2018

Sampling type	No. Examined	No. Positive (%)	Mean ± St. Dev	Minimum	Maximum
mRDTs	50	26 (52.0)			
Yield(ng/µL)			4.51 X 103 ± 2986.34	1743.5	12938
Purity			5.1048 ± 0.4388	4.24	5.84
DBS	50	22 (44.0)			
Yield(ng/µL)			1.96 X 103 ± 666.15	1021.20	3424.90
Purity			1.6312 ± 0.09	1.52	1.76

mRDTs – malaria rapid diagnostic tests, DBS - dried blood spots.

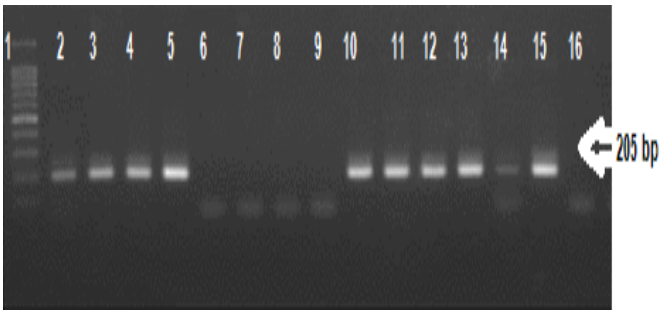

Fig. 1. Agarose gel electrophoregram of P. falciparum 18Ss rRNA resolved on 1.2% agarose gel

negative with mRDT but tested positive during agarose gel electrophoresis which is an indication of the low sensitivity of RDT in diagnosis. A total number of 50 Dried Blood Spot (DBS) for year 2018 were analyzed in agarose gel electrophoregram having 20 positives 2, 3, 4, 15, 17, 21, 22, 23, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 50.

Discussion

In many malaria endemic regions, mRDTs have gradually replaced routine microscopy for the clinical detection of suspected symptomatic malaria infections [5, 13]. The general acceptability of mRDT as a simple diagnostic procedure will certainly give credence to a wide array of molecular approach in plasmodium genome studies. A promising novelty in the current efforts for malaria elimination in Africa is the ability to elute malaria parasite DNA from archived, mRDTs and DBS. From our findings, we established that used mRDTs harbour *Pf*DNA, as reported by other authors [4, 5, 14, 15, 17]. The majority of the eluted DNA were positive for *P. falciparum*, which makes the detection of antimalarial drug-resistant genes achievable even in our own settings. The aforementioned is made possible through the identification of the parasite's strains from single nucleotide polymorphism, an important finding earlier reported by Morris et al. [17]. Again, a major hindrance to the progress observed in the present study was the contamination of human DNA with PfDNA during sequencing which definitely affected its quality for further genomic studies [18]. Earlier, the yield and purity of archived blood samples were used for determining the amount of protein contamination left from nucleic acid isolates [4, 28]. The yield and purity of the sampled specimen for *Pf*DNA however was dependent on the storage facility and the longevity of the storage bearing in mind the ambient temperature of the room where the archived mRDTs were kept. From the foregoing, the highest yield and purity of the *Pf*DNA was obtained from the samples obtained in 2016. However samples analyzed from year 2018 showed a decrease in yield and purity, a value lower than the standard purity value (1.8-2.0) which may due to excess of PCR inhibitors [29, 30]. The current findings are dissimilar to that of Sylwia et al. [31] that reported 57-94 ng/µl of yield and -1.76-1.86 purity. Similarly, other studies were able to identify certain limitations in the yield and purity of *Pf*DNA which the authors suggested might be as a result of one or more of the following; viz, sample collection by in-experienced technicians, infection risk, presence of certain interfering proteins and PCR inhibitors in blood [32].

The variation observed between the samples that tested positive by mRDTs and negative for qPCR may be interpreted as false-negative due to the presence of PCR inhibitors or errors in the DNA extraction process as it was the case in other regions [4, 5, 14, 15]. Inadequate parasitic material can also lead to false-negative result due to low volume of blood dropped inside the RDT kit compared to higher volume of blood spotted on the filter paper which is comparable to earlier reports [33]. However, it was observed that increased parasitaemia demonstrated a significant association with increased *Pf*DNA. A similar case was reported by Auburn et al., (2011) where the authors observed that lower human DNA levels resulted in greater relative representation of *Pf*DNA [19]. From the foregoing, it then suggests that there is a strong correlation between parasite density and specific DNA probes. Barka et al. (1986) enunciated a general method for the development of highly species specific DNA probes for the diagnosis of *P. falciparum* and established sequences that recognize the difference between drug-resistant and drug-sensitive strains [34].

Conclusion

The assessment of *Pf*DNA quality and integrity is strongly dependent on both the storage medium and conditions. Obtaining an optimum parasite yield and purity in laboratory/field conditions is certainly a way to go in genomic and malaria vaccine studies. Eliminating malaria in endemic populations can be achieved when there is an understanding of the evolution of the malaria parasite in response to the human immune system and interventions such as drugs and vaccines. It is however difficult to draw general conclusions regarding the suitability of extracted genomic DNA for molecular biological analysis. Indeed, the assessment of DNA quality and integrity may depend on the techniques used for the evaluation. The current findings may provide multifaceted platforms in guiding malaria control programs and also facilitate the monitoring of drug resistance via genotyping of resistance genes. mRDTs are becoming more popular during the course of evaluation in the case management of malaria in hyperendemic regions and our study identified with these kits as potential source of *Pf*DNA in diverse and large population studies. In situations where there are difficulties associated with ethical and clinical approval for blood samples for genomic studies, used mRDTs may just be the ideal, as they are in conformity with the recommendations of the World Health Organization. mRDTs also provide easy transportation from field to standard laboratories with minimal to no health associated challenges. Genetic diversity is easily achievable with mRDTs collected from diverse environmental foci, in order to guide and monitor current control strategies.

Authors' contributions

OS (Conceptualization; Data curation; Writing – original draft)
OAI (Formal analysis; Methodology; Writing – review & editing)
OAO(Conceptualization; Data curation)
OA (Formal analysis; Investigation)
GIC (Methodology; Project administration)
MIA (Methodology; Project administration)
OF (Investigation; Methodology; Supervision)
EA (Formal analysis; Investigation; Methodology)
MRF (Investigation; Methodology; Project administration)

Conflict of interest

None to declare.

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