

Evaluation of a Homemade Saliva Kit for the Stabilization of Plasmodium DNA at Room Temperature

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Abstract: Background: Malaria is a tropical disease that continues to have devastating impact on the health and livelihood of people around the world. The good number of new cases and most malaria-related deaths occur in the sub-Saharan African countries especially among children under the age of 5. Most malaria diagnostic methods are invasive and the use of non-invasive alternatives could be of great help in the control of the disease. The use of saliva-based diagnosis has been documented in recent studies. However, long-term storage of saliva also requires a cold chain, which is challenging in poor countries. Current tools to conserve saliva at room temperature are not affordable (~\$16/kit) for malaria endemic countries. Methods: In this cross-sectional study including 83 febrile participants in the Obala district hospital, Cameroon, we evaluated the effectiveness of a cheaper homemade kit for stabilizing *Plasmodium* DNA in saliva at room temperature relative to the OMNIgene®ORAL kit, for the molecular diagnosis of malaria. Results: Of the 83 participants aged between 2 to 77 years included in the study, 24% were males and 76% females. The frequency of malaria in this study was 78.31% (65/83) using microscopy. Saliva PCR-*f*₁ and PCR-S₀ detected 59 (71.08%) and 56 (67.47%) positive malaria samples respectively. Using microscopy as gold standard, the sensitivities of PCR-S₀ and PCR-*f*₁ were 100% while the specificities were 80%, and 85%, respectively. These parameters remained unchanged after 12 months of storage of saliva samples at room temperature. Conclusion: PCR-*f*₁ had a “very good” agreement (kappa 0.81) with microscopy compared to PCR-S₀ (kappa 0.64). We obtained similar results after 12 months of storage of saliva samples at room temperature. Homemade kit could be effective in transportation, preservation and diagnosis of malaria parasite in saliva.

Keywords: Non-invasive, Saliva, DNA, *Plasmodium*, Malaria, Homemade Kit

1. Introduction

Malaria remains a public health concern in many parts of the world [1]. Obligate intra-erythrocytic protozoa of the genus *Plasmodium* cause the disease. Five species are responsible for human malaria, namely *Plasmodium* (*P.*

falciparum, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi*) [2, 3]. According to the latest report, WHO estimated 241 million cases and 627 000 deaths globally in 2020 [4]. Most of cases of deaths still occurred in the World Health Organization (WHO) African Region (90%) [5].

Cameroon is one of 35 countries with a high malaria

burden and almost every Cameroonian reports at least one episode of malaria annually [6]. Cameroon has 3% of all malaria cases in the world; this makes it the third most affected country in central Africa (12.7%) [7]. Suspected cases of malaria constituted 30 % of medical consultations and 21% of visits to health facilities resulted in a laboratory-confirmed diagnosis of malaria (40% among children under five years) [8]. However, like in most endemic countries, early diagnosis of malaria is still quite challenging, requiring the detection parasites in blood samples by microscopy and Rapid Diagnostic Test (RDT). This invasive approach is constraining and challenging, in identifying the asymptomatic carriers with low parasitemia (500 parasites/ μ l), in addition to other blood related customary restrictions in some communities [9]. The risk introduced by blood withdrawal can also cause limitations for repeated measurement and communities can turn to be less cooperative to donate blood during malaria surveillance [10]. RDTs on the other hand also have a detection limit of > 200 parasites/ μ l [11], and the persistence of the *P. falciparum* histidine-rich protein II (*PfHRP-2*) after parasite clearance complicates RDT interpretation by many care givers. Further, recent reports of deletion of *Pfhrp2* and *Pfhrp3* genes leading to false negative results of RDTs also raises concern [12].

It is now been appreciated that non-invasive approaches using other sample such as saliva, coupled with appropriate molecular techniques, could be effective to evaluate the physiological and pathological conditions in humans with malaria [9]. Collection of saliva does not require specialized training nor any special equipment [13]. Like whole blood however, transportation and storage of saliva also requires cold chain to maintain good quality of parasite DNA. Whereas, efforts have been made to produce kits that can stabilize parasite DNA in saliva at room temperature, the average cost of a kit like the OMNIgene[®] ORAL (OM-501) kit (DNA Genotek, Ottawa, Ontario, Canada) is about US\$16. This is not affordable in resource poor countries where their use is intended. To curb this challenge, we sought in this study to evaluate the ability of a cheaper (about US\$2 per sample) homemade kit to stabilize parasite DNA in Saliva.

2. Materials and Methods

2.1. Study Area and Population

This study was conducted in the Obala District Hospital (ODH), in the Lékié Division, Centre Region of Cameroon. Obala is located within the rainforest belt of Central Africa and has the Guinea type equatorial climate. Malaria transmission peaks during and immediately after the two rainy seasons. Only *Anopheles gambiae* and *Anopheles funestus* contribute to malaria transmission in Obala and urban Yaoundé located about 33km away.

2.2. Sample Collection

A total of 83 participants attending Obala District Hospital and suspected of having uncomplicated malaria were

enrolled following the administration of a written informed consent or assent to guardians of minors. Participants having incomplete or missing clinical data or could not provide saliva were not included. Thick blood films and RDT were performed for each individual, and saliva samples (2-4ml) collected in sterile tubes. An experienced microscopist examined all slides, and confirmed malaria cases were treated. Half the quantity (1-2ml) of each saliva sample was introduced into either equal volume of the homemade DNA stabilization buffer (1-2ml) or the OMNIgene[®] ORAL (OM-501) kit (DNA Genotek, Ottawa, Ontario, Canada) standard kit (S_0) buffer, following manufacturer's instruction. Each mixture was further divided into two aliquots. DNA was extracted immediately from one aliquot of each group and 12 months later from the second aliquot which was stored at RT (20 - 25°C) until DNA extractions (Figure 1).

2.3. DNA Extraction

DNA was extracted from each saliva samples using Chelex 100 Resin (BioRad). In the first step, 500 μ l of whole saliva was transferred into a 1.5 ml microcentrifuge tube and centrifuged for 3 minutes at 14,000 rpm. The supernatant was removed up to the 450 μ L mark and discarded. The pellet was gently re-suspended in 500 μ L of 1 \times PBS/(1%) saponin, and incubated at RT for 20 minutes. Samples were centrifuged for 2 minutes at 14,000 rpm and the supernatant was removed up to the 450 μ L mark and discarded. Fifty microliters of the suspensions were mixed thoroughly with 20% Chelex in PCR-grade water. The mixture was boiled for 13 minutes and finally centrifuged for 3 minutes at 14,000 rpm. The resulting supernatant containing DNA was carefully transferred (75 μ L) into a pre-labeled 1.5ml microcentrifuge tube, excluding Chelex, and 5 μ L was used for PCR analysis.

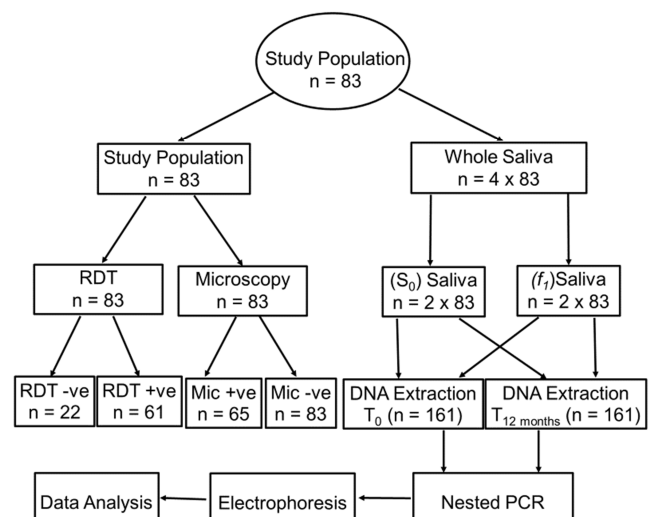


Figure 1. Flowchart of experimental strategy.

2.4. Nested PCR Amplification

Plasmodium DNA and speciation was conducted for each saliva sample by nested PCR as previously described [14]. The outer PCR using *Plasmodium* Genus specific primers in

[15] produced amplicons of 1100 bp, while amplicons from nested PCR with species specific primers in [16] were of sizes 205 bp, 786 bp and 144 bp for *Plasmodium falciparum* (Pf), *Plasmodium ovale* (Po), and *Plasmodium malariae* (Pm) respectively. PCR amplifications were performed in a Thermo Electron® PX2 (HBPX2) thermal cycler. PCR amplicons obtained were resolved by electrophoresis on 2% agarose gels stained with 0.05 µg/mL ethidium bromide and visualized by UV transilluminator (Gel DOC, Bio-Rad).

2.5. Performance of Homemade Compared to the Standard Kit

In order to assess the performance of homemade kit (F_1) compared to the OMNIgene®ORAL (OM-501) kit (DNA Genotek, Ottawa, Ontario, Canada) standard kit (S_0), we compared PCR results using saliva samples collected in the both buffers. To assess the diagnostic accuracy of saliva, we compared saliva PCR to microscopy and RDT.

2.6. Data Analysis

We used Microsoft Excel 2010 to enter all data and R-software® version 3.0.2 to calculate sensitivity and specificity to diagnostic measure the diagnostic performance. Kappa value of association was used to evaluate the degree of agreement of each test. Kappa was interpreted as: poor (<0.20) to very good (0.81 to 1) based on Kappa interpretation (Cohen, 1968). *p*-value of less than 0.05 was considered statistically significant at a 95% confidence interval (CI).

3. Results

A total of 83 samples (paired blood and saliva) were collected and analyzed from consenting participants. Participants' age range was 2 -77 (mean = 27) years. All the participants, 20 (24.1%) males and 63 (75.9%) females had a body temperature reading between 35 and 38°C on the day of examination. Using RDT, 61 (73.4%) were positive while 22 (26.5%) were negative and with Microscopy, 65 (78.3%) were positive (parasitemia ranging from 120 to 28480 parasites/µl) while 18 (21.7%) were negative. Of the 83 samples, PCR- f_1 (formulation) recorded 59 (71.1%) positive cases while PCR- S_0 (GENOTEK) recorded 56 (67.5%) positive cases.

Diagnostic test performance of PCR-saliva and microscopy as gold standard.

Of the 56 positive samples detected by PCR- S_0 , 40 samples were infected by *Plasmodium falciparum*, 13 by *Plasmodium ovale* and 15 by *Plasmodium malariae*. Of the 59 positive samples detected with PCR- f_1 we identified 45 samples infected by *Plasmodium falciparum*, 18 by *Plasmodium ovale* and 35 by *Plasmodium malariae* (represented in Figure 2), identified as DNA amplicons of molecular weights of about 205bp, 786bp and 145bp respectively. Similar results (100% of positive samples were still positive while 100% of negative samples remained

negative) were obtained after 12 months of storage at RT.

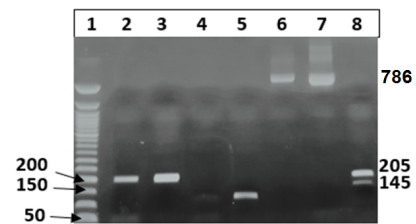


Figure 2. Identification of Pf, Pm and Po in saliva.

Legend: Lane: (1) 50bp Molecular Weight Markers, (2) *P. falciparum* amplicon from PCR- f_1 (205 bp), (3) *P. falciparum* amplicon from PCR- S_0 (205 bp), (4) *P. malariae* amplicon from PCR- f_1 (144 bp), (5) *P. malariae* amplicon from PCR- S_0 (144 bp), (6) *P. ovale* amplicon from PCR- f_1 (786 bp), (7) *P. ovale* amplicon from PCR- S_0 (786 bp), (8) Positive control of *P. falciparum* and *P. malariae*, f_1 : Homemade Formulation 1, S_0 : Standard kit. Numbers on the right and left of the gel represent sizes of bands in base pairs.

4. Discussion

The main finding from this study is that we have a cheaper homemade formulation f_1 which can stabilize parasite DNA in saliva even after 12 months of RT storage. Comparing PCR- f_1 /PCR- S_0 , we showed that the same samples were positive in each group after a 12-month storage at RT. The sensitivity was 100% and Kappa value was 0.814. Moreover, for PCR- f_1 /MIC, sensitivity was 100% and Kappa value was 0.641. The sensitivity and Kappa values found by [17] were 94% and 0.907 respectively. Valid concerns about this study could include the method of extraction of DNA and parasite density. However, since we treated both samples in the same way after collection, these aspects are non-consequential to our finds. Though, Fung and collaborators [18] suggested that saliva samples preserved in ethanol yielded superior positive PCR results when compared to samples kept on ice. However, such preservation cannot last for up to 12 months and besides, the absence of ethanol preservation in this study does not appear to have negatively affected PCR amplification. A very good agreement ($\kappa = 0.814$) was observed for DNA derived from saliva using the Chelex extraction method compared to DNA purified from blood samples. Besides, this method is very cheap ~\$ 0.5 compared to other saliva extraction methods which cost ~\$4.10/ saliva sample [19].

A study in Gambia found that the sensitivity of nested PCR increased from 73% to 82% in samples with a parasite density of >1000 parasites/µl [20]. Although the study by [21] found that the positive rates of nested PCR of saliva samples increased with parasite density for *P. falciparum*, this was not the case with our results. Our results did not appear to be influenced by the parasitemia of 120 to 28480 parasites/µl.

Storage conditions have been shown to be a factor that may potentially influence antigen detection in spun saliva samples [22, 23]. A study showed that stored blood can lose antigen activity, and early lysis and protein coagulation can

inhibit flow, thus influencing the results of RDT-based malaria diagnosis [16]. Although this study and many others [24, 25] clearly show the potential to use saliva as a non-invasive body fluid for rapid diagnosis of malaria, there are still many challenges in establishing it as a reference. Prominent among these challenges are failure of current RDTs to detect parasite antigen in some whole-saliva samples despite high parasitemia of >1000 parasite/ μ L blood [26]. The reasons for this disparity are unclear. Quantitative PCR showed up to ~600-fold greater DNA quantity in blood compared to saliva samples from infected patients, and a statistically significant correlation between parasite density and amount of parasite DNA in saliva was observed [20]. We will understand these disparities better when we have a clearer mechanism of the biological processes leading to the release of parasite antigen in saliva. Nonetheless, saliva still has its merits as we and others [27] have shown that it is capable of identifying submicroscopic parasitemia in both clinical and nonclinical settings even with archived saliva samples, especially when analyzed by an appropriate molecular method.

Also, it is known that malarial products such as *P. falciparum* histidine-rich protein II (*PfHRP-II*) or *P. falciparum* lactate dehydrogenase (pLDH) released upon schizont rupture into circulation may get into saliva through pericellular ultrafiltration from the surrounding vasculature [18]. The study by [20] detected *PfHRP-II* in whole saliva at 43% sensitivity, while [18] achieved a sensitivity of 100%. The difference in the sensitivities was probably due to the method of storage and stabilization of the samples [21]. Unfortunately, only DNA downstream analysis are possible in saliva samples stored in our buffer.

The study by [28] detected both *P. falciparum* and *P. vivax* in urine and saliva albeit with a low sensitivity compared blood through nested PCR of all three types of samples. Similar results were later reported by [29]. However, reports of the presence of *P. ovale* and *P. malariae* in non-blood samples like saliva and urine are infrequent. We show the presence of *P. ovale* and *P. malariae* in saliva preserved in both our homemade kit and the OMNIgene®ORAL (OM-501) kit (DNA Genotek, Ottawa, Ontario, Canada) commercial kit, although the band for *P. malariae* was almost invisible after 12 months of room temperature storage in our kit.

5. Conclusion

We obtained significantly high sensitivities and specificities for detecting *Plasmodium* DNA in saliva samples stored in our homemade Formulation (f_1) even after 12 months of RT storage. Thus, if standardized, it will greatly reduce inconveniences associated with transportation and storage of samples during large-scale malaria surveillance.

Limitations

Our Kit is only designed for DNA stabilization and thus, stored saliva samples cannot be used for any other downstream analysis apart from DNA based detection. DNA extraction using one of the standard DNA extraction kits may

have an influence on our results. The content of the homemade buffer could not be provided as it is under consideration for a patent. Similarly, the reference buffer is under patent by DNA Genotek, Ottawa, Ontario, Canada, thus, its content is also unavailable in this manuscript.

Abbreviations

f_1 : homemade Formulation 1; S₀: OMNIgene®ORAL (OM-501) kit (DNA Genotek, Ottawa, Ontario, Canada) standard kit; RT: room temperature; *Pf.*: *Plasmodium falciparum*, *Po.*: *Plasmodium ovale*; *Pm.*: *Plasmodium malariae*; PCR: Polymerase chain reaction. CI: confidence interval; WHO: World Health Organization.

Authors' Contribution

PMN conceived the idea and designed the experiments, and together with EBT participated in all phases of the study including writing the proposal, submission to ethics committee, data collection and writing of the manuscript. SDK and J-PC participated in sample collection and laboratory experiments respectively under the supervision of PMN and WFM who approved the manuscript. All authors have read, and confirm that they meet, ICMJE criteria of authorship. All authors read and approved the final manuscript.

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Availability of Data and Materials

The dataset(s) supporting the conclusions of this article can be provided upon reasonable request to the corresponding author.

Ethics Approval and Consent to Participate

Ethical clearance was obtained from the National Ethics Committee on Research for Human Health (CNERSH) of the Cameroon's Ministry of Public Health. Clearance N° 2015/06/602/CE/CNERSH/SP. A Research Authorization was obtained from the Director of the District Hospital of Obala. Participants were enrolled following the administration of a written informed consent while a written informed assent was obtained from guardians of minors.

Competing Interests

The authors declare that they have no competing interests.

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