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Original Research Paper

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OMNIGEN KIT ACCURACY FOR *PLASMODIUM FALCIPARUM* SALIVA DETECTION IN SENEGAL

Souléye lélo*	Service de parasitologie-mycologie, Faculté de Médecine Université Cheikh Anta Diop Dakar. *Corresponding Author
Magatte Ndiaye	Service de parasitologie-mycologie, Faculté de Médecine Université Cheikh Anta Diop Dakar.
Khadim Sylla	Service de parasitologie-mycologie, Faculté de Médecine Université Cheikh Anta Diop Dakar.
Doudou Sow	Service de parasitologie-mycologie, Faculté de Médecine Université Cheikh Anta Diop Dakar.
Cheikh Binetou Fall	Service de parasitologie-mycologie, Faculté de Médecine Université Cheikh Anta Diop Dakar.
Aida Gaye	Service de parasitologie-mycologie, Faculté de Médecine Université Cheikh Anta Diop Dakar.
Issac Manga	Service de parasitologie-mycologie, Faculté de Médecine Université Cheikh Anta Diop Dakar.
Roger Tine	Service de parasitologie-mycologie, Faculté de Médecine Université Cheikh Anta Diop Dakar.
Babacar Faye	Service de parasitologie-mycologie, Faculté de Médecine Université Cheikh Anta Diop Dakar.

ABSTRACT Malaria is a serious public health burden in Senegal. Accurate diagnosis is essential to avoided unnecessary presumptive treatment. Malaria diagnosis currently relies on identifying malaria parasite using microscopy and detecting soluble parasite antigens by Rapid Diagnostic Tests (RDT). These techniques do not detect low-level, sub-patent malaria infections and are inherently hazardous and invasive. To overcome these obstacles, alternatives diagnostics method were explored. In contrast to blood, saliva presents a reduced biohazard and can be painlessly collected in relatively large quantities by individuals with moderate training. The objective of this study was to use saliva collected in OmnIGEN Kits as an alternative sample for malaria detection. **Methods:** A total of 77 febril patients tested malaria positive by mRDT were enrolled in this study. From each patient, blood sample was collected for dried blood spot and blood smear; and saliva sample on OMNIGEN kit. Parasite density was determined from smear and *Plasmodium falciparum* DNA was extracted from both dried blood spot and saliva samples collected from the same patients. Extracted DNA was amplified by qPCR machine. **Results:** Malaria prevalence using qPCR was 98,7% and 60% respectively with blood and saliva. Compared to blood, saliva showed a sensitivity; specificity; positive predictive value of 60%; 100 %; and 100 %. The concordance between parasites detection from saliva and blood was (p = 0.45). In addition, no difference was found between these two methods and the microscopy counting. **Conclusions:** Saliva could be a non-invasive alternative method for *P. falciparum* detection and epidemiological surveillance in country with limited ressources.

KEYWORDS : Saliva, *Plasmodium falciparum*, PCR, OMNigen Kits, Senegal.

BACKGROUND:

Plasmodium falciparum malaria remains one of the most important infectious diseases in sub-Saharan Africa (SSA). Even with the significant reduction in malaria morbidity and mortality since the beginning of the third millennium, 214 million people (88% living in SSA) acquired malaria and 438,000 people (90% from SSA) died from malaria in 2015. **[1]**

In Sub sahara African countries incuding Senegal malaria diagnosis currently relies on identifying malaria parasite using microscopy and detecting soluble parasite antigens using Rapid Diagnostic Tests (RDT). If performed correctly, these diagnostic tools provide accurate diagnosis for case management **[2]**. However these techniques may have a limit of detection for low parasitémie during malaria infection and are inherently hazardous and invasive. Non-invasive, saliva-based collection and stabilization of *Plasmodium* DNA can allowed country-wide detection of sub-patent parasite reservoirs and tracking the distributions of malaria hotspots and transmission clusters. The self-collection device is robust and easy to use to collect saliva from symptomatic and asymptomatic patients alike in remote endemic settings. Additionally, *Plasmodium* DNA can be stabilized and stored at temperature up to 50°C for up to 1 year, thereby enabling a proactive, surveillance program that does not rely on trained personnel and cool-chain transport. Therefore, in this polite study, we have evaluated the performance of OmnIGEN Kits for *Plasmodium* detection in health facilities in Senegal**. [3]**

This low incidence makes the rapid detection challenging due to the very low parasite biomass. Moreover, RDTs are known to be invasive and difficult for a malaria surveillance. **[4]**. Therefore, there is an urgent need to test and implement other sensitive parasite detection in Senegal. Current malaria diagnostic tools are based on antigen-based Rapid Diagnostic Tests (RDT's)and microscopy. These methods are labor-intensive, require supervision by healthcare workers and examination of thin/thick blood smears by skilled microscopists. Moreover, RDTs and microscopic examinations do not allow the detection of low-level, sub-patent malaria infections. While RDT's have been instrumental in improving speed and precision of malaria diagnosis, blood collection for mRDT and microcoscopy can be affected by several barriers such as pain associated with finger pricking, fear of

contracting blood-borne diseases. Management of wast related to blood collection can also affect the environment. More recently deletion of hrp2 protein has been reported to associated with mRDT falso negative (ref). Despite concerns related to blood samples collection, dried blood spots used for molecular detection of Plasmodium DNA can be affected by humidity and external factors.

The obstacles associated with using RDT's and dried blood blots impact the implementation of these methods for nationwide malaria surveillance and monitoring programs. Saliva collection is a non-invasive alternative from RDT's for malaria detection and as a surveillance tool. Both *P. falciparum* and *P. vivax* DNA have been detected in saliva samples of malaria patients.[**5- 6 – 7 - 8**] It has been demonstrated that 1 mL of whole saliva samples harbours detectable levels of *Plasmodium* spp. DNA for downstream sequencing of *pfdhfr* and 18S rRNA. [**9 -10**]. Therefore, saliva can be sampled for high sensitivity and specificity molecular-based malaria diagnosis. Additionally, participant compliance is increased with pain-free and easy saliva collection ; therefore providing greater access to *Plasmodium* DNA for improved monitoring of malaria transmission, identification of sub-patent or mixed *Plasmodium* species infections, and patient screening in artemisin in resistance-emerging regions/elimination settings.

The main of this study is to test the accuracy of *Plasmodium falciparum* detection in saliva using the OMNIgen Kits.

MÉTHODES

Study area

This field base study was done in Guédiawaye health district, located in suburb of Dakar. Guédiawaye health district is an semi-urbane and malaria endemic zone located in the Dakar littoral area. This area is characterized by the presence of water paddle, water retention basin favorable to the development of vector mosquitoes of malaria parasites. The population is estimated 349 991 inhabitants. (2015) **[11]**.

Study design and population

The study was carried out in a peri-urban health facility named Guédiawaye located at 10 Km from Dakar. This study was done in 2014 during the malaria transmission season from October-November in Dakar. A total of 77 patients tested malaria positive by RDT were enrolled in this study. Blood samples were collected from patient for Malaria Rapide Test detection, smear preparation for microscopy diagnostic; dried blood spots (DBS) for molecular identification. From the same patient saliva sample was collected into OMNigen Kits for molecular detection.

Data collection methods

Structured questionnaire

A code was given to every child after parents' informed consent. Each eligible child was examined by a physician prior to a biological assessment which included blood and saliva samples. The mother was interviewed directly concerning the child's symptoms as well as well as socio demographic characteristics, history of the fever uptake using a standard questionnaire. Data obtained from physical examination and parents' interview were assigned on a case report form (CRF).

Patients were screened by mRDT, positives patients were given and ACT treatment folloving NMCP guidelines. Blood smears were done and counted in the laboratory by microscopy. Saliva were collected and stored at 20° C for further analyses. All patients who have malaria were subsequently treated with ACTs.

Sample collection and parasites identification

Capillary blood samples were collected from patient for malaria rapide diagnostic test diagnosis, blood smear and dried blood spots collection. From the same patient saliva samples were collected into OMNIgen Kits. Patients with positive mRDTs were treated with ACT following national Malaria Control Programme guidelines. Collected dried blood spots, saliva samples kits and thick and thin film were shipped to Parasitology Laboratory for malaria diagnosis. For microscopy examination, thick and thin smears were stained with Giemsa and read by a laboratory technician. Malaria was defined as any asexual parasite detected on a thick or thin blood smear. Parasitemia was calculated and expressed by number of trophozoites/μL using the following formula: number of parasites counted $\times$8000/200; assuming the average number of white blood cell is 8000. Absence of malaria parasite in 200 high power ocular fields of the thick film was considered as negative.

From dried blood spots and saliva samples parasite DNA was extracted by Chelex-100 **[12- 13]** and Qiagen Kit according the manufacture, respectively.

Briefly for DNA extraction by Chelex-100 method, 1X PBS with 0.5% saponin was added to small pieces of blood-impregnated filter paper, shake for 10mn (150 rpm) and incubated at room temperature overnight. The Supernatant was removed and wash twice with PBS buffer. 150µL milli-Q H2O and 75µl 20% of chelex mix (5g chelex in 25 ml milli-Q H2O) were added in the 96 deep plate wells and gently seal. Plates were boiled for 8mn (2×4) and cool for 10mn at room temperature. Spin down for 5mn, freeze deep well plate with DNA and carefully transpose 50µl of the supernatant to new 96 PCR plates by leaving carefully the chelex in the original deep well plate.

For DNA extraction from saliva, 5ml of saliva collected was centrifuge and 200ul of supernatant was collected in sterile tube and incubated 10mn at 55°C with 20ul of proteinase K and 22ul of pure link genomic lysis buffer. The mixture was transferred in QIAamp Mini spin column and centrifuge at high speed (10000 rpm) for 1 minute. Thereafter 200 µl of washing buffer with ethanol were added and the mixture centrifigure in the QIAamp Mini spin column and discard the collection tube. Then 50 µl of Pure link Genomic Elution Buffer were added and centrifugation, purified DNA was used for molecular dectection of malaria parasite by qPCR.

DNA amplification

A quantitave polymerase chain reaction (PCR) was used to amplify 18S (Small subunit ARN ribosomal PfM19173 using the following primers: forward 18SPf GTAATTGGAAT GATAGGAATTTACAAGGT et reverse TCAACTACGAACG TTTTAACTGCAAC. A 133 base pairs corresponding at the PfM19173 expected size 133bp were amplified using the PCR program described in table 1.

The 25ul qPCR mixture consisted of 12,5 ul Universal Probe PCR Master Mixte ; 1.0 µl of each primer (forward and reverse) ; 0.25 µl of 18S MGB probe ; 5,25 µl of sterileilli-Q water and 5 ul of extracted DNA. Plasmide Standards containing the 133bp PCR product cloned into a plasmid from University of Oxford were used as postive contrôle. The qPCR programme used was : 1) AMPerase step : 50°C for 2 minutes ; 2)Hotstart activation : 95°C for 10 minutes ; 3) Cycling : 95°C for 15 seconds (denature) 60°C for 1 minute (annealing and extension) Repeat 'Cycling' 45 times. For the amplification, the ROX reference dye was selected, and the target was FAM-NFQ-MGB.

Data management and data analysis

Data were entered in Excel software and analyzed with Stata software (Stata Corp, Texas). For categorical data, frequency of each outcome with a 95% confidence interval was

determined. For continuous data, mean and standard deviation were calculated to describe normally distributed variables, median and range for other data. Characteristics of all patients included in the study were tabulated. Proportions were compared using chi2 square test or Fisher exact test where appropriated (univariate analysis); significance level of the different tests was set on 0.05, two sided. Sensitivity, specificity, and predictive values of OMNIGEN kits were determined using the following formulas:

Ethical consideration

The study protocol was approved by the Conseil National d'Ethique pour la Recherche en Santé (CNERS) in Senegal. Prior the study, a community sensitization was undertaken in the study area. Informed consent was obtained from adults' participants and parents or children's guardians prior to participants.

RESULTS

Among the 77 patients included in our study, the range age of patients varied from 3 to 74 years with an average of 22.2 ±11.9 years. The sex ratio was 1.75% in favor of men with 63.64% of men against 36.36% of women.

Table I: Distribution of patient socio-demographic parameters

Variable	Distribution
Age (moyenne, écart type)	22,2 ± 11,9 ans
Catégorie d'âge	
Moins de 5 ans	1 (1,3%)
5 – 10 ans	7 (9,1%)
Plus de 10 ans	69 (89,6%)
Sexe	
Masculin	49 (63,64%)
Féminin	28 (36,36%)

Our results shown that, all patient included in our study were positives by mRDT; among them 98.7% were positive for *Plasmodium falciparum* and 1.3% positive for Borrelia by microscopy. From patients positive with *Plasmodium falciparum*, parasitic density varied between 2400 and 15480 trophozoites/µL of blood with a median of 6560 trophozoites/µL. Distribution by parasite load showed that 41.6% of patients had parasitemia below than 5000 trophozoites/µL of blood, 44.2% of subjects had parasitaemia between 5000 and 25000 trophozoites/µL and 14.3% of patients had a parasitaemia greater than 25,000 trophozoites/µL.

PCR efficacy from saliva samples

Our results have shown that among 76 samples *P.falciaprum* positives by microscopy, 60.52% (46/76) were positive by qPCR performed from saliva samples; while 100% of samples were positive by qPCR from dried blood spots. By comparing qPCR performed from DBS samples and microscopy (Gold standard), our results show 100% (sensitivity and specificity) of concordance for both technics. However, qPCR results from saliva samples showed sensitivity of 60.52% ((IC :50-70), specificity of 100% (IC: 62.9 -100) compare to microscopy. In addition, our results have shown that the performance of qPCR conducted on saliva samples increases with parasitic density. The amount of parasite DNA quantified in peripheral blood samples from infected patients was greater than that of saliva samples. (Table II)

Table II: Microscopy and PCR Results

Microscopie	
Echantillons positifs	76 (98,7%)
Echantillons négatifs	01 (1,3%)
Densité parasitaire (trophozoites/ µL)	Médiane : 6560
	Q75%* : 15480
	Q25%** : 2400
Classe de densité parasitaire	
Moins de 5000 tropho/ µL	32 (41,6%)
5000 – 25 000 tropho/ µL	34 (44,2%)
Plus de 25000 tropho/ µL	11 (14,3%)
PCR	
Positif PCR salive	46 (60,52%)
Positif PCR sang	76 (98,7%)

Q75% : Quartile 75% ; Q25% : Quartile 25%.

DISCUSSION

Saliva is increasingly being recognized as a potential non-invasive alternative to blood for malaria diagnosis. Sample handling methods that do not compromise the quality of samples and do not require cold chains are ideal for field settings in low and middle-income countries. This study was conducted to assess the diagnostic performance of qPCR for detection of *Plasmodium falciparum* DNA in saliva collected into OMNIgene®ORAL (OM-501) kit and stored at room temperature. Nested PCR-saliva was compared with two well-established blood-based methods for malaria diagnosis, i.e., TFM and qPCR-blood. The prevalence of malaria in the study population detected by qPCR-blood (35%) was higher than prevalence found by TFM (24%), agreeing with well-known increased sensitivity of nPCR-blood for diagnosis of malaria [**14–15, 16–17**].

With the decline of *Plasmodium falciparum* malaria in most African countries including Senegal, there is a real need to develop and optimize rapid diagnostic tools for Plasmodium parasites detection. Malaria diagnostic relay on parasites identification by microscopy, parasites antigens detection by RDTs and more recently parasites DNA detection by molecular tools. These technics have been shown to be accurate for malaria diagnosis if performed correctly [**2**]. However blood samples collected for these technics could be harmful and invasive. We propose an innovative idea of shared value to develop a non-invasive, thermo stable, saliva-based, field adaptable molecular method for nation-wide surveillance of malaria in Senegal.

Our study has shown that Plasmodium falciparum can be detected by PCR from saliva collected into OMNigen Kits. This study suggested that Non-invasive, saliva-based collection and stabilization of *Plasmodium* DNA allows multiple temporal and spatial detections of sub-patent parasite reservoirs and tracking the distributions of parasites-resistance hotspots and transmission clusters. However we noted a sensitivity of 60, 52% (IC95%=50-70). Davis et al found similar results; authors have noted a sensitivity of 73% (IC95%=58-84) [**4**].

In this same study the authors showed different sensitivities according to the extraction kit used 63.27% on the Zymo kits and according to the DNA extracted from the salivary base or the supernatant which was respectively 61.9% and 73.83%.

In terms of specificity, our results 100% specificity are similar to those of other studies. Davis et al in 2009 in The Gambia found 97% specificity in a study of the quantitative detection of Plasmodium falciparum in saliva, blood and urine [**4**].

J Pooe et al found 97% specificity in Zambia in 2010 by evaluating PCR detection of Plasmodium falciparum from salivary samples on Qiagen kits [**18**].

CONCLUSION

The results of this study support the conclusion that nPCR-saliva is as sensitive as TFM for malaria diagnosis, at least in patients with fever. Furthermore, the results demonstrate that nPCR on saliva collected from individuals of different ages and stored in the OMNIgene®•ORAL (OM-501) kit at room temperature are equally, or more accurate than results

previously-reported for nPCR on saliva stored at 4 °C or – 20 °C. With the capacity to store *Plasmodium* DNA at ambient temperature and the non-invasive sample collection procedure, OMNIgene® • ORAL, saliva-based nPCR presents a new diagnostic tool for clinical trials and epidemiological surveillance studies where submicroscopic infections need to be detected.

Saliva can potentially be used as an alternative non-invasive sample for the diagnosis of malaria and the OMNIgene® • ORAL kit is effective at transporting and preserving malaria parasite DNA in saliva at room temperature. The technology described in this study for diagnosis of malaria in resource-limited countries adds on to the armamentarium needed for elimination of malaria.

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Authors Contribution

Souléye Lélo ,Magatte Ndiaye and Babacar Faye were responsible for the concept and the design of the study.SLelo collected samples for the study.Slélo Aida Gaye and Magatte Ndiaye were responsible for PCR analysis.Roger Tine analyse the data.Slélo and Magatte Ndiaye Prepare the manuscript.All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used during the current study are available from the corresponding author on reasonable request

Conflict of interest: The authors have no conflicts of interest concerning the work reported in this paper.

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