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Comparative analysis of genomic DNA amplification yield for *Plasmodium falciparum* extracted from urine, saliva and blood

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Malaria treatment and/or management remains an essential element as well as strategy controlling this pathology. Since 2005, Côte d'Ivoire has adopted Artemisinin-based Combination Therapies (ACTs) as first-line drugs to treat uncomplicated malaria. Malaria diagnostic mechanism involves blood sampling by using finger prick collection or venipuncture which requires very strict aseptic conditions. However, some groups of people refuse to cooperate with these diagnostic methods because of (i) cultural believe forbidding seeing of blood and (ii) trauma related to the needle sting. Here, two non-invasive sampling and one invasive methods to diagnose malaria using molecular method were tried. Blood, urine and saliva samples were collected in three different localities from patients above 2 years of age having simple *Plasmodium falciparum* malaria confirmed by microscopy. Then, *P. falciparum* genomic DNA was extracted and amplified through *Pfcrt*, *Pfdhfr-ts*, and *PfK13 propeller* genes specific primers. Amplification products were processed by electrophoresis and analyzed according to blood, saliva and urine samples. A multivariate statistical analysis based on R software was carried out with the purpose to assess the aptitude and/or performance of each analyzed biological samples in malaria molecular diagnosis procedure. The results revealed the presence of *P. falciparum* DNA in urine (27.57%) of the amplification products, saliva (40.21%) and blood (91.55%). Assuming blood sample as benchmark, the statistical analysis exhibited saliva as a suitable biological sample fitting for malaria molecular diagnosis (p-value ≤ 0.05), and suspected urine as a source of variability analyzing the aforementioned described malaria patient population excluding the latter as a reliable sample in malaria molecular diagnosis. The same results exhibited *Pfdhfr* and *PfK13 propeller* genes amplified from saliva as satisfactory molecular markers for chemo-resistance. The findings suggested *P. falciparum* genomic DNA amplification from saliva sample as closer in comparison to blood and proposed the former (saliva) as an alternative to blood in malaria molecular diagnosis process.

Key words: Molecular diagnosis, malaria, *pfcrt*, *pfdhfr-ts*, *pfK13* propeller, *Plasmodium falciparum,* saliva, urine.

INTRODUCTION

Despite international efforts to stop malaria, unfortunately it is still one of the most common deadly diseases in the world. According to the latest World Health Organization (WHO) statistics, 214 million cases of malaria were

recorded in 2015, of which 348,000 did not survive it. Of these deaths, 80% were from Africa, 71% of whom were children under the age of five (WHO, 2015). Management of malaria is an essential key to the strategy for fighting malaria. This management involves early diagnosis and rapid treatment of the disease with effective antimalarial. Despite remarkable progress being made in the development of malaria diagnostic tools, to date, all these tools require blood sampling at finger prick or venipuncture (Snounou et al., 1993; Murray et al., 2008; Putaporntip et al., 2009). Although blood collection does not generally have serious consequences if carried out under strict aseptic conditions, some groups of the population, such as some pregnant women, infants and some young ones, fail to cooperate when it comes to repeated blood collection (Putaporntip et al., 2009; Sutherland et al., 2009). Indeed, blood sampling requires skill personnel and the biological risk, associated with the inevitable use of needles or sharp objects, may result in poor compliance when repeated sampling is required. Blood collections in some communities is often very difficult to achieve due to cultural or religion believe, therefore sets limits for repeated examinations, particularly in young children who are the main target for most malaria epidemiological surveys. In order to get rid of the obstacles to the problem of blood collection, it is necessary to explore alternative methods that can be substituted for blood samples collection and at the same time useful for all diagnostic, therapeutic or epidemiological investigation of malaria. Urine and saliva of people infected with *Plasmodium falciparum* and *Plasmodium vivax* have been shown to contain plasmodial DNA amplifiable by polymerase chain reaction (PCR) (Mharakurwa et al., 2006; Buppan et al., 2010). Despite the small amount of plasmodial DNA in these biological fluids compared to blood, these two products appear to have a real potential for diagnosing malaria as well as studying antimalarial resistance genes (Nwakanma et al., 2009). The objective of this study was to compare genomic DNA amplification products of *P. falciparum* isolated from the urine, saliva and blood of patients exhibiting simple malaria pathology from different localities, with the purpose to characterize resistance genes to antimalarial drugs.

MATERIALS AND METHODS

Study site

The present prospective study has been realized from February to August 2015 in three different health structures; Anonkoua Kouté (Health Center), Port-Bouët (General Hospital) and Ayamé (General Hospital). Processed sites are located in southern region of Côte d'Ivoire characterized by a tropical climate with annual rainfall exceeding 1700 mm and the temperature oscillating between 27 and 33°C. Malaria is seasonal, predominant during the rainy season from June to September with peak prevalence and incidence in October-November. *P. falciparum* is the dominant species with more than 90% of the total parasite. The main vectors of malaria in this study area are members of the complexes *Anopheles gambiae sl* and *Anopheles funestus sl* (Adja et al., 2011). The Anonkoua-Kouté health center and the Ayamé General Hospital were selected because of the high annual incidence of malaria cases. In addition, these health centers have been used for several years as the main sites for conducting multi-center clinical trials of efficacy by the malaria unit of the Pasteur Institute of Côte d'Ivoire. Port-Bouët site (General Hospital of Port-Bouët) has been included in the present survey because of its swampy environment used for vegetable garden.

Study population and sample collection

Suspected infected malaria patients from our analyzed sites (Anonkoua Kouté health center and both general hospitals of Port-Bouët and Ayamé localities) were recognized as eligible. After obtaining consent, blood, urine and saliva samples were collected in patients over 2 years of age having an axillary or rectal temperature above 37.5°C with malaria *P. falciparum* evidence confirmed by a microscopy test in a thick drop and blood smear. A total of 459 samples from 153 patients were included in this study (Table 1).

Blood, saliva and urine collection

Blood

Two to five milliliters of venous blood was collected in an EDTA tube for each analyzed malaria patients. Next, 50 µl of whole blood was dropped on Whatman 3 MM filter paper using a micropipette with filter cones. The paper containing the blood spots was dried (60 to 120 min) at room temperature and protected from dust. The unused blood after making the confetti and content in the EDTA tube was centrifuged at 2,000 rpm for 10 min. Whole blood constituent, that is, blood plasma, buffy coat and globular pellet, were separated and stored in cryotubes at -20°C for possible subsequent use.

Saliva

Ten to fifteen minutes after washing the mouth with tap water, 5 ml of saliva was collected per patient in a sterile flask. Then, 50 µl of saliva was dropped on Whatman 3 mm filter paper for each patient. Yielded confetti's were dried for approximately 60 to 120 min at room temperature and protected from dust. After preparing confetti, 1000 µl of absolute methanol was added to 500 µl of saliva (contained in the sterile bottle) in a cryotube and then stored at -20°C (Mharakurwa et al., 2006; Buppan et al., 2010) for subsequent use.

Urine

After blood and saliva collection, 5 to 10 ml of each patient's urine

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Table 1. Samples used for the molecular analysis of resistance markers

Site	Collection period	Age group (t years)	Sample	Confetti collected
Anonkoua-Kouté	February - March 2015	2 to 53	Blood	52
			Saliva	52
			Urine	52
Port-Bouët	April - May - July 2015	2 to 62	Blood	51
			Saliva	51
			Urine	51
Ayamé	June – July - August 2015	2 to 55	Blood	50
			Saliva	50
			Urine	50
Total				459

were collected in a sterile vial. Next, 50 µl of urine were dropped on Whatman 3 mm filter paper by using a micropipette and filter cones. Then, obtained confetti were dried for 60 to 120 min at room temperature and protected from dust. After completion of the confetti, 1000 µl of absolute methanol was added to 500 µl of urine (contained in the sterile flask) in a cryotube and then stored at -20°C for possible subsequent use (Mharakurwa et al., 2006; Buppan et al., 2010).

P. falciparum genomic DNA extraction

(i) Blood plasmodial genomic DNA was extracted by adding methanol to previous prepared blood confetti (Miguel et al., 2013). Indeed, fine cuts of blood confetti were immersed in 1 ml of wash buffer (950 µl of 1X PBS plus 50 µL of 10% saponin) and then incubated at 4°C overnight. The wash buffer was removed and then washed before adding 150 µl of methanol. After 20 min incubation, the methanol was gently removed and the samples were dried at room temperature for 2 h before adding 300 µl of sterile water. The samples were then heated at 99°C in a thermo-mixer for 30 min to elute the DNA. After removing the confetti debris, the DNA extracts were aliquoted in a 1.5 ml Eppendorf tube and stored at -20°C.

(ii) Extraction of plasmodial DNA from urine and saliva confetti was performed by using the Chelex®100 method (Kain and Lanar, 1991; Plowe and Wellems, 1995). 180 µlof 5% (w/v) Chelex-100 solution (Bio-Rad, Catalog No. 1422832) was placed in a 1.5 ml centrifuge tube and brought to the thermal block at 100°C for 5 min. The fine cuts of each confetti were added to the boiling Chelex 100 solution at the thermal block. After centrifugation at 12,000 g for 90 s, the supernatant was collected and then centrifuged again under the same conditions as mentioned earlier. The resulting supernatant was used for PCR.

Amplification of *Plasmodium falciparum* genes resistance

The resistance genes were amplified by a nested PCR using a pair of primers specific for each gene and a commercial kit of DNA polymerase named 5X FIREPol® Blend Master Mix with mM $MgCl_2$. The composition of this kit constituted a pre-mix ready to use solution for the reaction mixture. For primary PCR, the pairs of primers used for *pfK13 propeller*, *pfdhfr* and *pfcrt* genes were K13_PCR_F (5'CGGAGTGACCAAATCTGGGA)/K13_PCR_R (5'GGGAATCTGGTGGTAACAGC), dhfr_M1 (5'TTTATGATGGAACAAGTCTGC)/dhfr_M7 (5'CTAGTATATACATCGCTAACA) and 72_97EF (5'GACCTTAACAGATGGCTCAC)/72_97ER (5'TTTTATATTGGTAGGTGGAATAG), respectively. The primary PCR of these genes was carried out in a reaction volume of 25 µl containing: 0.625 µl of each primer, 3 µl of *P. falciparum* DNA, 0.5 µl of Taq polymerase and 15.75 µl of milliQ water. The mixture was then placed in a PTC-100TM thermocycler (Eppendorf Mastercycler, PTC-100 Peltier Thermal Cycler), programmed as follows: Initial denaturation at 95°C for 15 min, followed by 30 cycles of denaturation at 95°C For 30 s, hybridization at 58°C for 2 min and extension at 72°C for 2 min. Finally, a terminal extension at 72°C for 10 min. The second PCR was carried out on the products of amplification of the primary PCR in a reaction volume of 50 µl containing: 1.25 µl of each primer, 5 µl of amplification product of the first PCR, of 0.5 µl of Taq polymerase and of 37.5 µl of milliQ water. The primer pairs used for the secondary PCR were K13_N1_F (5'GCCAAGCTGCCATTCATTTG)/K13_N1_R (5'GCCTTGTTGAAAGAAGCAGA) for the *pfK13 propeller* gene, dhfr_M9 (5 'CTGGAAAAAATACATCACATTCATATG)/dhfr_M3 (5'TGATGGAACAAGTCTGCGACGTT) for the *pfdhfr* gene, and SecIF (5'GGTAAATGTGCTCATGTGTTTAAACTTATT)/SecIR (5'TTACTTTTGAATTTCCCTTTTTATTTCCA) for the *pfcrt* gene. The secondary PCR was carried out with the following program: Initial denaturation at 95°C for 15 min followed by 30 cycles of denaturation at 95°C for 30 s, hybridization at 60°C for 1 min and extended at 72°C for 1 min. Finally, a terminal extension at 72°C for 10 min.

Detection and analysis of PCR products

The products used for amplification were migrated to a 1.5% agarose gel containing Ethidium bromide (BET). After the migration, the gel was recovered and then observed under a UV lamp using UV translator (Gel DocTMEZ Imager). Presence or absence of bands made it possible to judge the effectiveness of PCR.

Statistical analysis

Data collected from standard questionnaire test were first validated before considered for analysis by Graph Pad Prism 5 software. Then, z-score test based on R software (Core team, 2013) was performed to compare the rates of the amplification products in each analyzed biological sample as well as amplification proportion

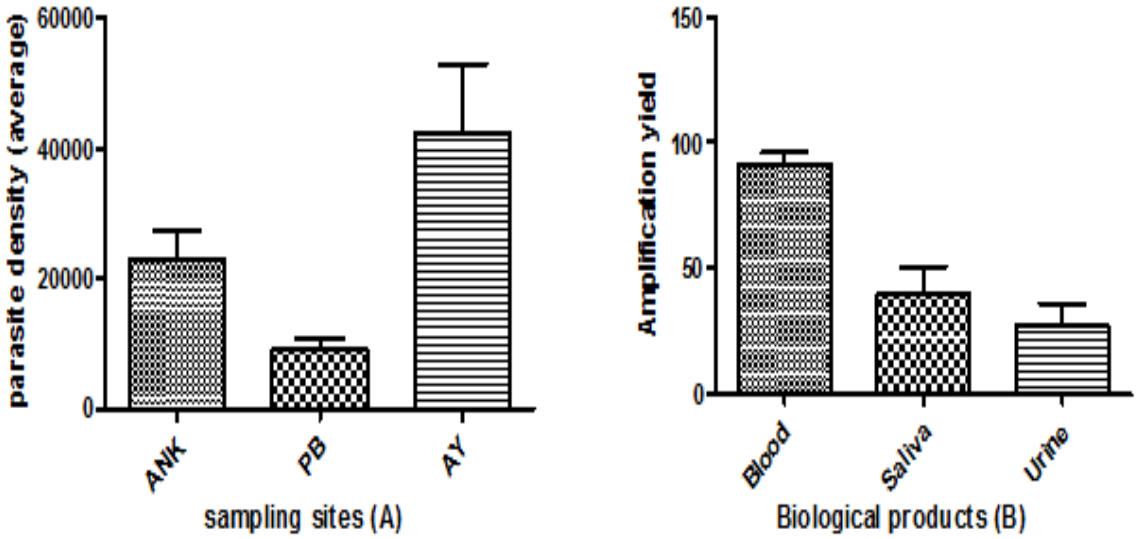

Figure 1. Parasite densities based on the sampling site (A). Amplification yield based on the biological product (B). "ANK", "PB" and "AY*"* acronym indicate urine, saliva and blood samples respectively Anonkoua-Kouté; Port-Bouët and Ayamé.

of each processed *pfK13 propeller*, *pfdhfr* and *pfcrt P. falciparum* genes in blood, saliva and urine biological samples. A difference and/or statistical association and/or correlation was considered significant for p-value<0.05. To carry out these analyses, various functions and/or scripts developed and/or based on R (version 3.2.2) programming environment (Core team, 2013) as well as on our previous developed computational statistical pipeline (Dago et al., 2016) were used. Next, biplot and variance estimation survey and Receiver Operating Characteristic (ROC) analysis (Swets, 1988) have been executed to evaluate the propensity and/or performance (sensibility, specificity and accuracy and positive predictive value) of each considered biological samples (blood, saliva and urine) in malaria molecular diagnosis procedure.

Ethical considerations

The study was conducted in accordance with the Declaration of Helsinki and approval was received from the Ethics and Research National Committee (CNER) of the Health and AIDS Control Ministry of Côte d'Ivoire.

After information and appropriate explanations, adult participants, parents or legal guardians of all children willing to participate in the study gave their written consent before sampling.

RESULTS

Patient profile and rate of amplification product according to biological liquid

In total, 94 patients infected by *P. falciparum* were selected for this study, 58 (61.7%) women and 36 (38.3%) men. Age average of processed patient's was around 17 years (age range 2 to 55 years). Malaria *P. falciparum* parasite density proportion (average) in the present analyzed malaria patient population was estimated to 24682 parasites/µl. However, parasitic densities vary from 1200 to 200000 parasites/µl with an average parasite densities at Anonkoua Kouté, Port-Bouët and Ayamé equal to 22900, 9193 and 42327 parasites/µl, respectively (Figure 1A). Significant and/or substantial difference was observed between (i) Port-Bouët and Ayamé sites in term of malaria *P. falciparum* parasite density (p-value < 0.05) (Figure 1A). Next, saliva, urine and blood extract samples were processed for PCR survey in separate batches by using primers specific to *pfcrt, pfdhfr* and *pfK13 propeller* genes. PCR amplified genes products were loaded into adjacent pathways for each analyzed patient in agarose gel electrophoresis highlighting amplification products. Then, genes amplification results revealed 40.22% (n = 286) and 27.56% (n=284) amplification ratio in saliva and urine samples, respectively against 91.56% (n=258) (amplification products) in blood (Figure 1B). The same analysis suggested a significant difference comparing yielded genes amplification between (i) saliva and blood as well as between (ii) urine and blood biological samples (p-value ≤ 0.05).

Relationship between gene fragments amplification products and blood, saliva and urine samples

The present survey evidenced a substantial presence of amplified DNA fragments in blood sample with the following proportions: (i) *Pfcrt* in 80.66% (n = 94), (ii) *Pfdhfr* in 95.33% and (iii) *PfK13 propeller* in 98.66%. The same analysis processing saliva samples recorded the attendance of both genes *PfK13 propeller* in 49% (n = 94)

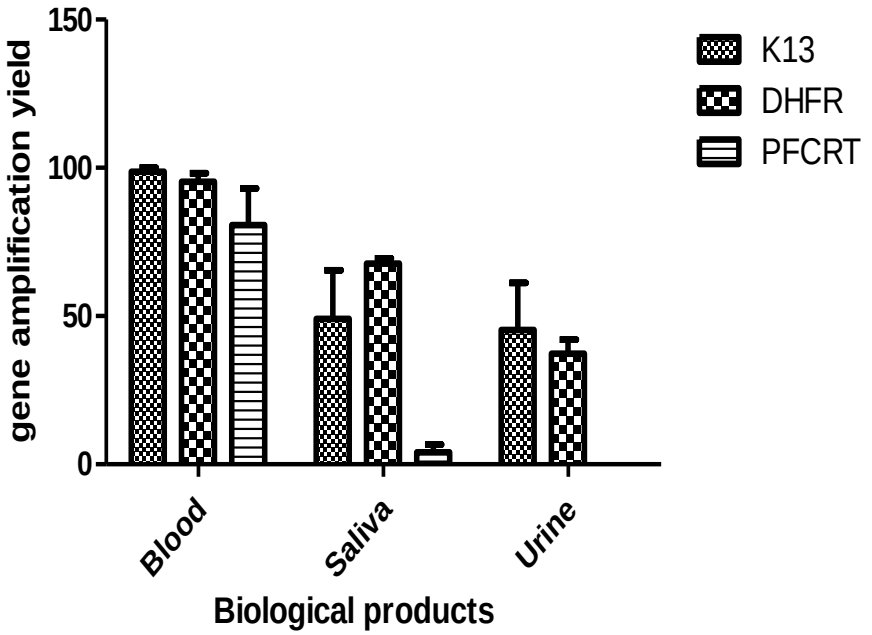

Figure 2. Ratio of *pfK13 propeller*, *pfdhfr* and *pfcrt* genes fragments amplification in blood, saliva and urine biological samples.

and *Pfdhfr* in 67%. It is noteworthy to underline that *Pfcrt* gene was not detectable in urine as opposed to blood sample (Figure 2). Proportion analysis regarding *PfK13 propeller* gene amplification suggested a weak difference (no significant difference) in term of gene expression levels in blood, saliva and urine samples. However, *Pfdhfr* gene fragments amplification yield exhibited a significant difference comparing blood, saliva and urine samples (p-value<0.05). In addition, the results showed a high expression level of *Pfcrt* gene in blood as opposed to both saliva and urine samples (p-value < 0.05). In other word, *Pfcrt* gene is weakly expressed and/or not detectable in saliva and urine samples (Figure 2).

Blood, saliva and urine samples as biological samples in malaria molecular process

A heatmap graphical analysis indicating presence and/or absence of each analyzed *pfK13 propeller*, *pfctr* and *pfdhfr P. falciparum* genes markers in blood, saliva and urine samples discriminating malaria patients is implemented.

Results of this survey advised two tendencies suggesting blood as the best biological system performing malaria molecular diagnosis, since *pfK13 propeller*, *pfcrt* and *pfdhfr-ts P. falciparum* genes are well detected in the latter in a high proportion of processed patient (Figure 1). However, the same analysis showed saliva as an adequate alternative biological sample highlighting a constancy presence of both *pfK13 propeller* and *pfdhfr P. falciparum* genes marker in analyzed malaria patients as opposed to urine sample (Figure 3). Guided by these observations, we performed a variance analysis between blood, saliva and urine biological samples by assessing presence and/or absence of *pfcrt*, *pfdhfr* and *pfK13 propeller P. falciparum* genes marker in malaria molecular diagnosis practice. This survey suggested that blood and saliva could both be used to significantly (p-value=0.032) discriminate malaria patients based on molecular detection of either (i) the set of genes *pfcrt*, *pfdhfr*, and *pfK13 propeller* for blood samples; or (ii) the set of genes *pfdhfr* and *pfK13 propeller* for saliva samples.

Taking together, the present analysis proposed blood and saliva as suitable biological samples to perform malaria molecular diagnosis as opposed to urine (p-value < 0.05).

Variability in malaria patients performing malaria molecular diagnosis in blood, saliva and urine

A comparative enquiry between analyzed patients was performed by introducing parasitic density (PD) parameter through a z-score analysis. Z-score heatmap graphic clustered malaria patients in two distinct groups (Figure 4). Indeed, detected groups, exhibited a relative evident variability among processed patients from Anonkoua Kouté (variance ratio=2.10; p-value=0.04) and Ayamé (variance ratio=1.68; p-value=0.16) districts as opposed to those from Port-Bouët site (variance

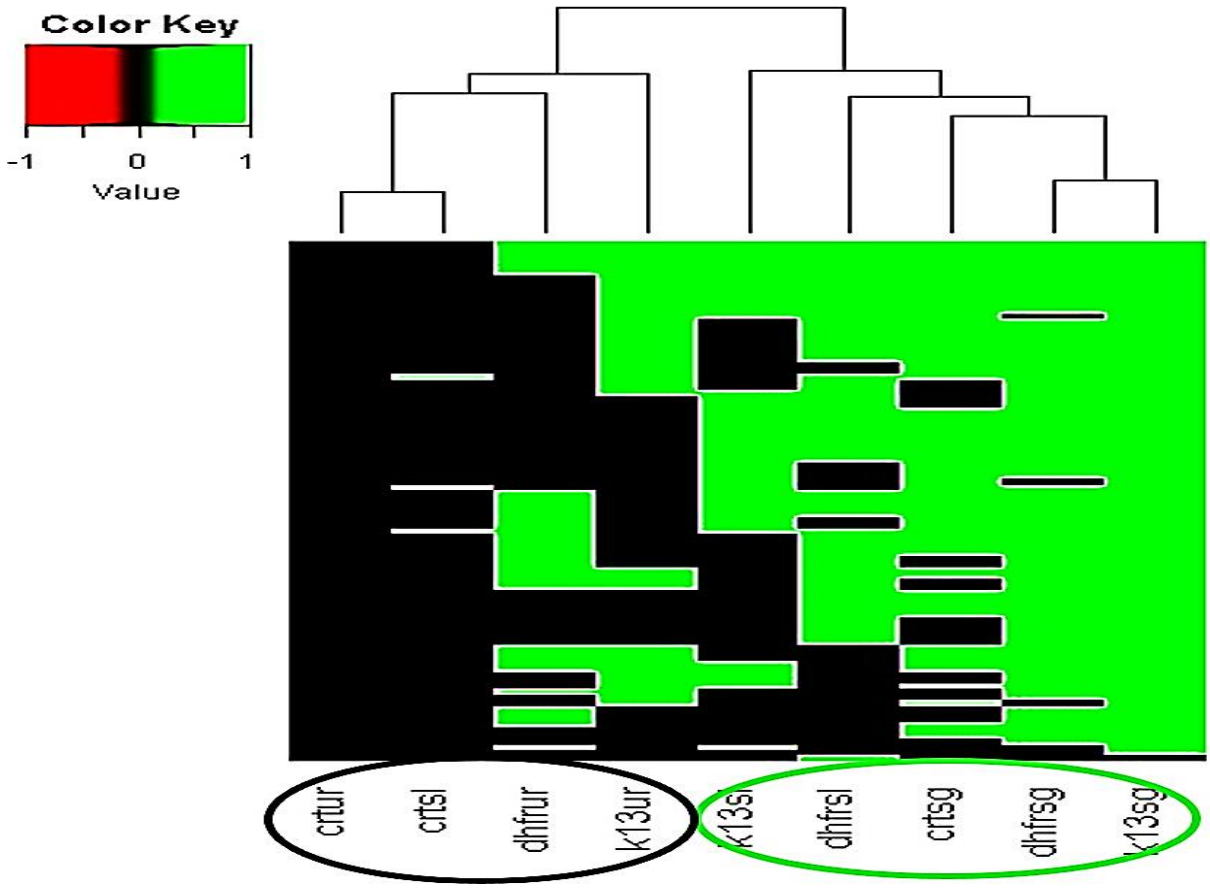

Figure 3. Heatmap graphic monitoring *P. falciparum pfK13*, *pfdhfr* and *pfcrt* genes expression in blood, saliva and urine samples in malaria molecular diagnostic process. "*ur", "sl"* and "*sg"* acronym indicate urine, saliva and blood samples, respectively.

ratio=0.66; p-value=0.26). However, z-score heatmap showed reasonably homogeneous and constancy prevalence of green color merging *pfcrt*, *pfdhfr* and *pfK13 propeller* genes prevalence in blood samples (variance ratio<0.001; p-value<0.05). Interestingly, the same tendency has been observed considering *pfdhfr* and *K13 propeller P. falciparum* gene expression in saliva samples by processing the same malaria patients (Figure 4). In addition, our statistical analysis exhibited high concordance between *pfcrt P. falciparum* genes expression in blood liquid and both *P. falciparum pfdhfr* and *pfK13 propeller* genes expression in saliva sample (variance ratio<0.02; p-value < 0.05). Also, the present survey (Figure 4) suspected (i) *pfdhfr* and *pfK13 propeller P. falciparum* genes detected in urine and (ii) *pfcrt P. falciparum* gene marker detected in saliva sample as potential source of variability between analyzed malaria patient population as opposed to (i) *pfcrt*, *pfdhfr* and *pfK13 propeller* genes and (ii) *pfdhfr* and *pfK13 propeller* genes in (i) blood and (ii) saliva samples, respectively (Table 2) as well as to parasite density (PD) parameter (Figure 4). Moreover, the present results suggested a low sensitivity of PD parameters in malaria diagnosis process when molecular diagnosis methodology based on *pfcrt, pfdhfr and pfK13 P. falciparum* genes screening in blood and saliva samples was assumed as reference (p-value<0.05). Considering as a whole, the present survey proposed both *pfdhfr* and *pfK13 propeller P. falciparum* gene markers screening in saliva as an adequate molecular diagnosis methodology discriminating malaria patients.

Biplot PCA analysis assessing the relationship between *pfdhfr, pfcrt and pfK13 propeller* in blood, saliva and urine samples

The results showed an apparent concordance between (i) *pfdhfr, pfcrt* and *pfK13 propeller P. falciparum* genes in blood sample by PC1 component and/or factor, (ii) *pfdhfr* and *pfK13 propeller* P. *falciparum* genes in urine and saliva and parasitical density (PD) parameter evaluating malaria patient distribution (Figure 5). The same analysis based on PC1 component suspected a discrepancy

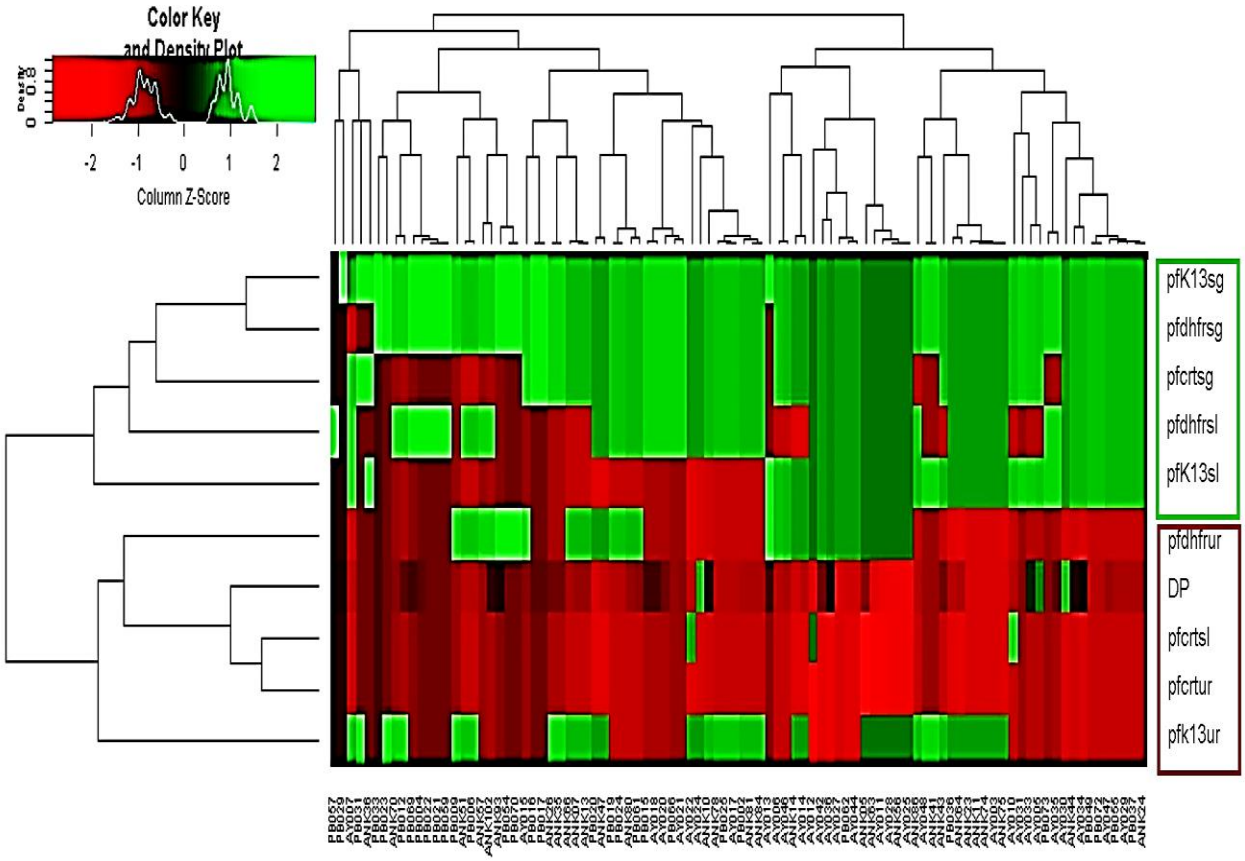

Figure 4. Performance assessment of variability in malaria patient populations performing z-score clustering analysis in malaria molecular diagnosis process by detecting *pfdhfr, pfcrt* and *pfK13 P. falciparum* genes markers in blood, saliva and urine samples. *ur, sl* and *sg* acronyms indicate urine, saliva and blood, respectively.

Table 2. Descriptive statistic weighing *DHFR, CRT and K13 Plasmodium falciparum* genes abundance in blood, saliva and urine samples by screening malaria patients.

Biological Samples	Blood *CRT Gene*	Blood *DHFR Gene*	Blood *K13 Gene*	Saliva* *CRT Gene*	Saliva *DHFR Gene*	Saliva *K13 Gene*	Urine* *CRT Gene*	Urine *DHFR Gene*	Urine *K13 Gene*	Parasitical Density (DP)
Median	1.36	1.07	1.01	0.00	1.45	0.00	0.00	0.00	0.00	0.41
¼ Quartile	0.00	1.07	1.01	0.00	0.00	0.00	0.00	0.00	0.00	0.20
¾ Quartile	1.36	1.07	1.01	0.00	1.45	2.04	0.00	2.67	2.18	0.93
Minimum	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.049
Maximum	1.36	1.07	1.01	31.33	1.45	2.04	0.00	2.67	2.18	8.10
Variance	0.37	0.07	0.01	30.66	0.46	1.05	0.00	1.70	1.20	2.51
Standard Deviation	0.61	0.26	0.10	5.54	0.67	1.02	0.00	1.30	1.10	1.59

*****Not suitable for following descriptive as well as inferential statistical analysis because of data's null dispersion (inter-quartile distance is null).

between PD and urine sample as well as between urine and saliva samples assessing processed malaria patient population dispersion and/or distribution (p-value<0.05). In addition, performed biplot PCA survey subtly exhibited *pfdhfr* and *pfcrt P. falciparum* genes in urine as a potential source of the variability observed in the analyzed malaria patient population (Figure 5 and Table 2). Also, correlation analysis based on Pearson's test displayed lowest agreement between *pfdhfr* and *pfK13 propeller P. falciparum* gene markers expressed in both blood and urine samples (p-value=0.3) as opposed to *pfcrt P. falciparum* gene abundance in blood and saliva (p-

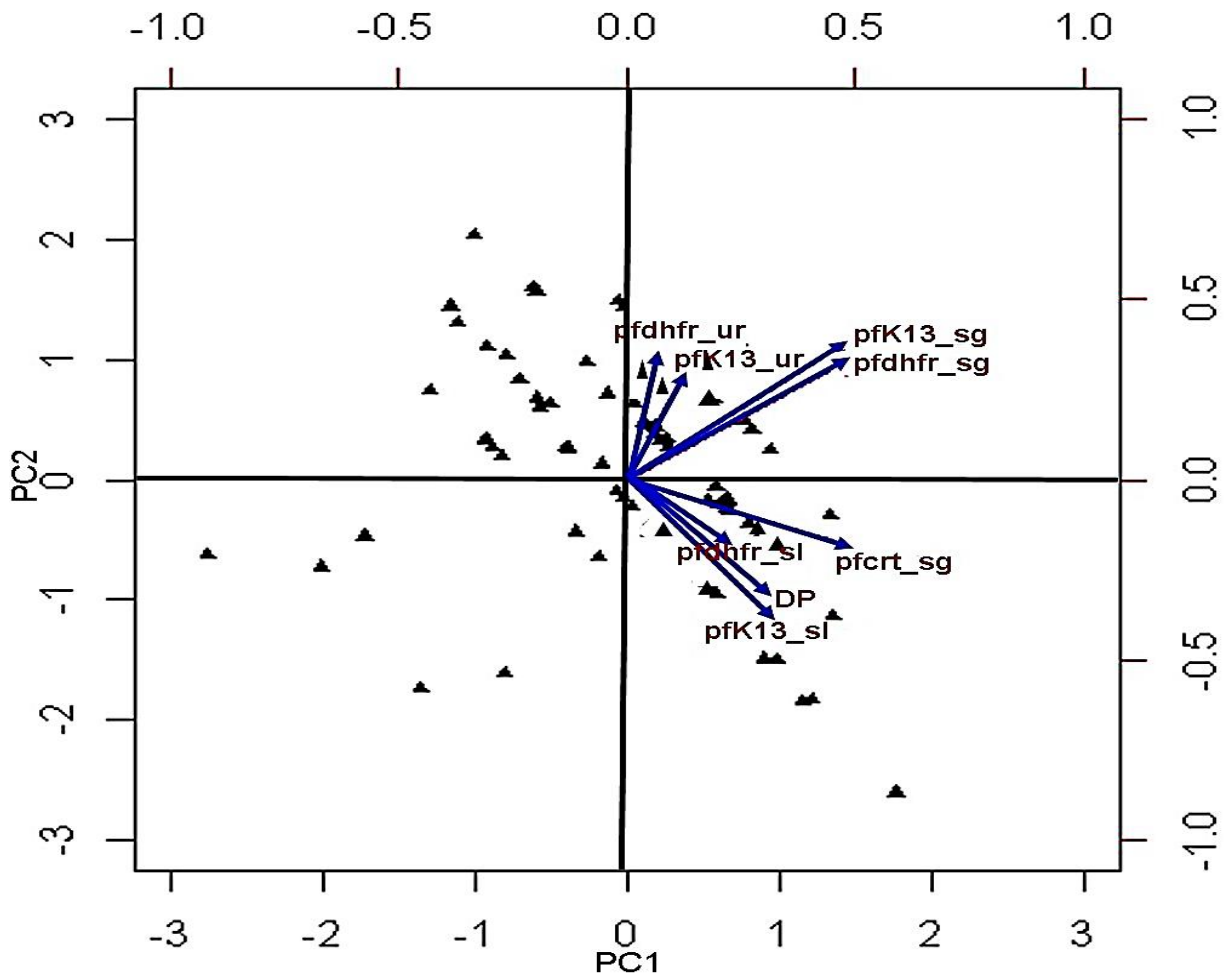

Figure 5. PCA comparative analysis assessing relationship between *pfdhfr*, *pfcrt* and *pfK13 Propeller* genes of *P. falciparum* in malaria molecular diagnosis. *ur, sl* and *sg* acronym indicate urine, saliva and blood liquid, respectively.

Table 3. Pearson's correlation between the *pfdhfr, pfK13 propeller* and *pfcrt* genes in blood, saliva and urine for the molecular diagnosis of malaria

Correlation	PfK13_sg	PfK13_sl	Pfdhfr_sg	Pfdhfr_sl	Pfcrt_sg	DP	Pfk13_ur	Pfdhfr_ur
Pfk13_sg	1	-	-	-	-	-	-	-
PfK13_sl	0.10	1	-	-	-	-		-
Pfdhfr_sg	0.40*	-0.01	1	-	-	-	-	-
Pfdhfr_sl	-0.07	0.05	0.20*	1	-	-	-	-
Pfcrt_sg	0.20*	0.30*	0.14	0.12	1	-		-
DP	0.05	0.17*	0.11	0.06	0.21*	1	-	-
PfK13_ur	0.10	-0.04	0.07	0.01	0.12	-0.11	1	-
Pfdhfr_ur	0.08	-0.01	0.11	-0.10	0.02	-0.07	-0.0004	1

*Significant at a p-value <0.1.

value=0.09) excluding urine as competitive biological sample in malaria molecular diagnosis process (Table 3). Interestingly, our analysis reinforced *pfdhfr* and *pfK13 propeller P. falciparum* genes expression in saliva as acceptable system in malaria molecular diagnosis process when blood sample was assumed as reference. Moreover, our findings evidence a positive correlation between PD parameter and all analyzed *P. falciparum* gene

Table 4. Performance assessment of urine and saliva samples in malaria molecular diagnosis based *pfdhfr*, *pfcrt* and *pfK13 P. falciparum* genes.

Parameter	Saliva			Urine		
	pfk13	pfdhfr	pfcrt	pfk13	pfdhfr	pfcrt
Sensitivity	0.46	0.64	0.05	0.45	0.38	0
Specificity	0.2	0.5	0.5	0.5	0.5	0.01
Accuracy	0.43	0.54	0.05	0.45	0.38	0.01
Positive predictive value	0.91	0.98	0.8	0.95	0.94	0

markers discriminated in both blood and saliva samples evoking data normalization process as a suitable statistical practice allowing parasitical density (PD) parameter to be considered and/or accepted as potential metric tool controlling malaria molecular diagnosis by both blood and saliva samples (Figure 5). Based on both PC1 and PC2 parameters our findings revealed both *pfdhfr* and *pfK13 propeller P. falciparum* genes expression in saliva sample as a satisfactory alternative system to *pfcrt P. falciparum* gene marker expressed in blood sample discriminating malaria patient (Figures 4 and 5) and excluded urine sample as adequate biological sample for malaria molecular diagnosis (Figure 5).

Assessment of *P. falciparum* genes markers discriminating malaria patients by sensitivity, specificity, accuracy and positive predictive parameters

The aptitude of both saliva and urine samples exhibiting *P. falciparum pfdhfr, pfcrt* and *pfK13 propeller* gene markers discriminating accurately malaria patients by a receiver operational characteristic (ROC) analysis is evaluated. Data revealed a high sensitivity of saliva with respect to urine sample in malaria molecular diagnosis process (Table 4). Indeed, *pfdhfr* in saliva sample (pfdhfr_sl) recorded the highest sensitivity with respect to the other's analyzed gene markers. All analyzed *pfdhfr*, *pfcrt* and *pfK13 propeller P. falciparum* gene markers detected in both saliva and urine samples (except *pfcrt* gene marker in urine sample) claimed to perfectly predict malaria infection, when blood was assumed as reference (positive predictive value > 0.80). Moreover, saliva sample exhibited a relative best accuracy and specificity parameters to evaluate attendance performance of processed *P. falciparum* gene markers analyzing the present malaria patient population (Table 4). In addition, the present ROC survey endorsed and confirmed and/or emphasized *P. falciparum pfK13 propeller* and *pfdhfr* gene markers detection in saliva as an efficient molecular system discriminating malaria patients in alternative to blood sample. Finally, our survey suspected *pfdhfr* presence in saliva as the best parameter predicting positively malaria pathology occurrence in our process patient populations when blood sample was assumed as reference (Table 4).

DISCUSSION

Malaria disease represents a major public health issue in several tropical areas worldwide. Malaria cure remains an essential strategy controlling this disease, since efficient malaria management involves early diagnosis and effective treatment. Generally, currently developed malaria diagnostic tools and/or protocols need blood sampling. This practice obfuscates patients collaboration for malaria diagnosis procedure because of some cultural believe regarding blood taboo (for some people) as well as fear related to sting trauma, especially when blood sampling has to be repeated. In order to contrast these tendencies, we evaluated more than a few palliative methods based on urine and saliva sampling performing malaria molecular diagnosis. Indeed, the present study proposed a comparative analysis by assessing *P. falciparum* genomic DNA amplification yield extracted from urine, saliva and blood samples with the purpose to find an alternative to blood sampling executing malaria diagnosis protocol. Thus, we quantified malaria patient's *P. falciparum* genomic DNA amplification yield, extracted to blood, saliva and urine samples. This survey suggested different aptitudes and/or performances of previous mentioned biological samples in term of presence and/or absence of *P. falciparum* genomic DNA amplification product. Without a doubt, despite the low levels of plasmodial DNA amplification yield in urine (27%) and saliva (40%) compared to blood (91%), the present analysis suggested a good propensity as well as a real potential of both saliva and urine samples in malaria molecular diagnosis process advising their suitableness for antimalarial resistance genes study. However, it is noteworthy to underline that the proportion of amplified *P. falciparum* genomic DNA in blood, saliva and urine samples by handling our malaria patient population is comparable with those obtained by Ghayour-Najafabadi et al. (2014) in Iran (95, 47 and 29% respectively in blood, saliva and urine), where malaria transmission level is similar. Also, our findings resulted to be in agreement with those of Mharakurwa et al. (2006)

in Zambia and Nwakanma in the Gambia (Nwakanma et al., 2009) suggesting that the sensitivity of molecular methods discriminating malaria patients via saliva and urine samples was affected by several factors, such as DNA extraction methods, target gene size, sample fraction and/or type and sample conservation methodology (Nantavisai, 2014). A commercial *Qiagen* kit saliva extraction had a 2.6-times success rate compared to the Chelex, which we have used.

Then, the low level of *P. falciparum* genomic DNA amplification yield in saliva and urine samples could be related to DNA fragment size to be amplified. Indeed, the possibility of detection of amplification yield increases with shorter fragments (Mharakurwa et al., 2006; Pooe et al., 2011). The low level of amplification yield in urine and saliva can also be explained by the fact that either the plasmodial DNA in these biological products (saliva and urine) was not enough to provide a useful amplification model and/or that new genomic DNA isolation, purification or concentration methods are necessary. However, while *pfcrt*, *pfdhfr* and *pfK13 propeller* antimalarial resistance genes were clearly detectable in blood and saliva, only two of these genes were measureable in the urine. These results suggested blood and saliva as suitable biological samples detecting *pfdhfr* and *pfK13 propeller* genes in malaria patients. Nevertheless, previous studies using specific primers of antimalarial resistance genes have shown that infection detected in saliva or urine samples was consistently identical to that found in the corresponding peripheral blood of the same individual and/or malaria patient advising the former's as potential biological samples for the molecular diagnosis of malaria (Mharakurwa et al., 2006; Nwakanma et al., 2009, Putaporntip et al., 2011). Also, we performed a multivariate statistical analysis *via* a z-score test with the purpose to establish an association and/or link between processed biological samples and antimalarial resistance genes detection in saliva, urine and blood by processing our above described malaria patient populations. This survey highlighted (i) *pfK13 propeller*, *pfdhfr* and *pfcrt* and (ii) *pfK13 propeller* and *pfdhfr* gene groups, detected in blood and saliva samples respectively as a satisfactory system discriminating malaria patient and/or malaria pathology through molecular methodology. In addition, merging our descriptive statistical results reported in Table 2 with those of Pearson's correlation test by weighing *pfdhfr*, *pfcrt* and *pfK13 propeller P. falciparum* genes abundance and/or proportion in blood, saliva and urine samples discerning malaria patients, the present study indexed urine sample as a potential sources of variability in the studied malaria population as opposed to saliva and blood. In the same tendency we performed a biplot dispersion analysis. Considering as a whole, the same survey suggested a comparable performance between blood and saliva in terms of malaria patient population dispersion by measuring the aptitude of *pfdhfr*, *pfcrt* and *pfK13 propeller P. falciparum* genes in the previous mentioned biological samples (blood and saliva). These results and/or tendency were relatively confirmed by processed Pearson correlation test, which at the same time suggested a negative correlation between urine components and those of both blood and saliva samples. Taking together, these results indicate saliva as the best alternative to blood for malaria molecular diagnosis procedure. Our results are consistent with those of Nantavisai (2004) who observed that molecular detection of plasmodial DNA in malarial subjects' urine was less sensitive than in saliva. This is probably due to the small amount of DNA matrix in the urine compared to blood and saliva. Furthermore, a quantitative real-time PCR survey showed that plasmodial DNA amount in blood sample was 600 and 2,500 fold higher than in saliva and urine respectively (Nwakanma et al., 2009). Next, parasite density (PD) parameter was integrated as control element discriminating malaria patients by molecular diagnosis. Thus, our statistical analysis evoked a low propensity of PD parameter assessing malaria molecular diagnosis methodology since exhibiting high variance and/or variability assessing malaria patient. Indeed, PD average of our considered experimental sites (see material and methods chapter) is greater than 500 parasites/µl of blood; threshold below which the effect of parasite density is perceptible in the molecular diagnosis of malaria (Bousema et al., 2014). Also, our findings suggested that the detectability of both *pfdhfr* and *pfKk13 propeller* genes in saliva sample was poorly correlated with the prevalence of PD. However, it is interesting to note that PD parameter showed the same trends in Pearson's correlation survey with both blood and saliva as opposed to urine sample confirming the susceptibility of saliva as a biological sample for processing malaria molecular diagnosis alternatively to blood sample. Our performed receiver operational characteristic (ROC) analysis evaluating *P. falciparum* gene markers in discriminating malaria patients by sensitivity, specificity, accuracy and positive predictive parameters assuming blood sample as reference, evidenced the high performance and/or tendency of saliva sample to substitute blood in malaria molecular diagnosis procedure as opposed to urine. Interestingly it is noteworthy to underline that the same analysis exhibited *pfdhfr-ts* gene as the best molecular marker discerning and predicting accurately malaria patients since displaying the highest sensitivity, specificity, accuracy as well as positive predictive value with respect to urine sample.

Conclusion

Assuming blood sample as biological reference product, our findings revealed high levels of *pfcrt*, *pfdhfr* and *pfK13 propeller* antimalarials resistance-conferring genes amplification yield in saliva with respect to urine sample.

Also, we were able to easily detect *pfcrt*, *pfdhfr* and *pfK13 propeller* genes in both blood and saliva, while the *pfcrt* gene was not detectable in the urine sample. Next, performed multivariate statistical analysis proposed saliva as the best alternative to blood for the molecular diagnosis of malaria as well as for the study of molecular markers of antimalarial resistance. The same investigation revealed that blood and saliva could both be used to significantly discriminate malaria patients based on molecular detection of either (i) the set of genes *pfcrt*, *pfdhfr*, and *pfK13 propeller* for blood samples; or (ii) the set of genes *pfdhfr* and *pfK13 propeller* for saliva samples. Finally, this study suggested saliva as a valid alternative to blood sample to detect malaria patients by molecular approach as well as emphasized the recurrence of *pfdhfr* gene in saliva as a reliable biomarker calling accurately malaria patient.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interest.

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