Rapid Detection of Lactate Dehydrogenase and Genotyping of *Plasmodium falciparum* in Saliva of Children with Acute Uncomplicated Malaria

Grace O. Gbotosho,* Christian T. Happi, Onikepe Folarin, Ochuko Keyamo, Akintunde Sowunmi, and Ayoade M. J. Oduola
Department of Pharmacology and Therapeutics, College of Medicine, University of Ibadan, Ibadan, Nigeria; Malaria Research Laboratories, Institute of Advanced Medical Research and Training, College of Medicine, University of Ibadan, Ibadan, Nigeria; Stewardship for Research, Tropical Disease Research (TDR), World Health Organization, Geneva, Switzerland

Abstract. The diagnosis of malaria in biological fluids other than blood using non-invasive, rapid diagnostic techniques provides a valuable approach in case management and epidemiological studies of malaria. Rapid detection of *Plasmodium falciparum* lactate dehydrogenase (pLDH) in saliva samples from 130 of 144 children with microscopically confirmed *P. falciparum* infection was evaluated using Optimal-IT dipsticks. Genotyping of parasites was also performed in saliva and blood samples from a cohort of patients by polymerase chain reaction (PCR). The sensitivity of the dipstick in whole-blood, whole-saliva, or supernatant of spun saliva samples was 97.2%, 77.9%, and 48.4%, respectively. The sensitivity of the dipstick in whole-saliva samples was significantly higher than in supernatant of spun saliva samples ($P < 0.0005$). Mutant T76 allele was detectable in 60% and 57% of blood and saliva samples, respectively. This finding shows rapid detection of pLDH in patient saliva.

INTRODUCTION

Malaria remains a major public-health problem in sub-Saharan Africa.¹ Effective management of malaria is focused on use of long-lasting insecticidal nets, indoor residual spraying of insecticides, intermittent preventive therapy in pregnancy, and treatment of acute infections with artemisinin-based combination therapy (ACT).¹ However, effective chemotherapy is dependent on prompt and accurate diagnosis of malaria to avoid unnecessary use of antimalarial agents and selection of resistant parasites.

Light microscopy remains the gold standard for malaria diagnosis, because it can provide information on both the species and parasite density of infection.² Unfortunately, the procedure is not often used at the periphery of the health-care system because of the need for skilled personnel, power supply, good quality microscopes, and reagents. Microscopy is also time-consuming and burdensome in young children during epidemiological surveys where repeated measurements are required. Diagnostic strategies, thus, need to be simple, practical, and applicable, especially in malaria-endemic, resource-poor regions of Africa.

The availability of rapid diagnostic tests (RDTs) based on detection of parasite antigen offers an alternative method to improve malaria diagnosis.^{3,4} The major advantages of RDTs include the ease of use and diagnosis at point of care with minimal infrastructure. However, despite the benefits of the RDTs, the increased risks of needle injuries and disease transmission as well as cultural blood taboos pose limitations. Thus, the development of a non-invasive rapid method of malaria diagnosis using body fluids other than blood would provide a more readily applicable method useful in the periphery of the health-care delivery system.

Saliva is readily available and easily collected on request from patients. It has been used as a diagnostic test fluid for the evaluation of humoral immunity to infectious agents such as hepatitis A, HIV, measles, mumps, and rubella viruses.^{5–7} Recent reports describing polymerase chain reaction (PCR) detection of *Plasmodium falciparum* DNA in human saliva^{8,9}

represents a major, though technically demanding, advancement in diagnosis of malaria. In addition, *P. falciparum* histidine-rich protein II (pfHRP-II) antigen was detected in saliva of malaria patients using a Malaria Antigen enzyme-linked immunosorbent assay (ELISA) kit.¹⁰ Although the assay was time consuming (~2 hours and 15 minutes) with a low sensitivity (43%) and involved the use of expensive instrumentation,¹⁰ the report further showed the potential of a non-invasive approach for malaria diagnosis.

Thus, there is an urgent need to develop a more sensitive and less expensive diagnostic test capable of rapidly detecting parasite antigen in saliva at point of care. Availability of such diagnostic tools together with the use of artemisinin-based combination therapy would be valuable in protection of special high-risk groups of children and pregnant women and delaying of development of resistance. In this study, we show detection of *P. falciparum* lactate dehydrogenase (pLDH) in saliva of malaria patients using the OptiMAL-IT dipstick (RDT) (Diamed AG, Cressier s/Morat, Switzerland). In addition, PCR detection of *pfert* K76T polymorphism in parasite DNA from saliva samples is reported.

MATERIALS AND METHODS

Study site. This study was conducted between November 2008 and July 2009 at the Malaria Clinic of the Malaria Research Laboratories, College of Medicine, University of Ibadan, Nigeria as part of an ongoing antimalarial drug efficacy study. Ethical clearance was obtained from the Ethics Committee of the Ministry of Health, Oyo State, Ibadan.

Study design and sample collection. *Patients.* Patients aged 8 months to 13 years were eligible to participate in the saliva study if they had microscopically confirmed pure *P. falciparum* infection $\geq 2,000$ parasites/ μ L blood, a temperature $\geq 37.5^{\circ}\text{C}$ or recent history of fever, and absence of other concomitant illness. Written informed consent was obtained from the parent or guardian before enrollment into the study. Patient enrollment, sample collection, and analysis of samples by microscopy, RDT, or PCR are shown in Figure 1. Matched samples of blood and saliva were collected from patients at presentation before treatment. Ten children with thin- and thick-film negative slides were also enrolled to serve as negative controls, and matched blood and saliva samples were obtained from each of these children.

*Address correspondence to Grace O. Gbotosho, Department of Pharmacology and Therapeutics, College of Medicine, University of Ibadan, Ibadan, Nigeria. E-mail: solagbotosho@yahoo.co.uk

FIGURE 1. Flow chart showing patient enrolment, sample collections, and tests performed on samples.

Malaria diagnosis using thin- and thick-smear films. Thick and thin blood films were prepared from each patient at enrollment and allowed to dry. The blood films were stained with 10% Giemsa stain and analyzed under the microscope at 1,000× magnification for the presence of malaria parasites. Slides were read independently by two expert microscopists. The study was blinded, because results from microscopy were not shared with the individuals performing the RDT until all samples were processed. Parasitemia levels were calculated with results from thick films. Parasites were counted in at least 100 consecutive fields to rule out any negative thick-film slide. Parasite densities were calculated with reference to 6,000 white blood cells/μL and were expressed as parasites per microliter of blood.

Blood and saliva samples for rapid diagnostic tests. Ten microliters of capillary blood samples from a finger prick were obtained from each patient for qualitative diagnosis of malaria using RDT and analyzed immediately. Non-stimulated oral fluid was collected from each patient who was able to expectorate into sterile tubes. In a cohort of patients, whole saliva was analyzed with RDT immediately, whereas in another cohort of patients, saliva samples were stored at 4°C for 24 hours before analysis. The stored samples were thereafter centrifuged at 14,000 revolutions per minute (rpm) for 3 minutes, and 10 μL of the supernatant was used for RDT.

Rapid diagnostic test. Diagnosis of *P. falciparum* in blood was performed using the OptiMAL-IT dipsticks according to the manufacturer's protocol. For diagnosis of *P. falciparum* in saliva, one drop of the buffer provided by the manufacturer was placed in the sample well of the device, whereas three drops of the buffer were placed in the wash well and allowed to stand for 1 minute. Ten microliters of whole saliva or supernatant of spun saliva were transferred into the sample well, mixed

properly, and allowed to stand for 1 minute. The dipstick was placed into the sample well and allowed to stand for 10 minutes. The dipstick was thereafter transferred into the wash well and allowed to stand for another 10 minutes. An RDT result was considered positive when both the internal control band and the test band were stained red. A test result was considered negative if only the internal control was stained. A result was considered invalid if the internal control was not stained, irrespective of the presence of a test band.

Extraction of parasite genomic DNA from filter-paper blood samples. Parasite genomic DNA was extracted from filter-paper blood samples using the chelex extraction method as previously described.¹¹ Five microliters of the extraction product were used for PCR detection of *pfprt* K76T polymorphism.

Extraction of parasite genomic DNA from saliva samples. Whole saliva collected was centrifuged for 3 minutes at 14,000 rpm. The supernatant was aspirated while the pellet was resuspended in 1× phosphate buffer saline (PBS)/1% saponin solution by gentle tapping and vortexing. The resuspended pellet was incubated at room temperature for 20 minutes for cell lysis. The sample material was centrifuged for 2 minutes at 14,000 rpm, and the supernatant was discarded. The pellet was washed two times with 1× PBS, resuspended in 20% chelex, and heated for 15 minutes with brief vortexing. The solution was centrifuged for 3 minutes at 14,000 rpm. The supernatant containing DNA was carefully transferred into a pre-labeled microcentrifuge tube, excluding chelex, and 5 μL were used for PCR analysis.

Determination of *P. falciparum* population structures in saliva and blood samples. *P. falciparum* population structure was determined in matched saliva and filter-paper blood samples from 33 consecutive children with microscopically

confirmed *P. falciparum* infection and four healthy volunteers. Parasites' population structure was determined using parasite polymorphic loci as described previously.¹²

Nested PCR and restriction fragment-length polymorphism analysis of *P. falciparum* point mutations in *pfcr* genes in saliva and filter-paper blood samples obtained from patients. The K76T polymorphism on *pfcr* gene (GenBank accession no. AF030694) was detected by nested PCR followed by restriction fragment length polymorphism (RFLP) as previously described.¹³ Parasite DNA from 3D7 and Dd2 laboratories strains of *P. falciparum* served as positive and negative controls, respectively, in all PCR and enzyme-digest procedures. All digest products were resolved on 2% agarose gel and visualized under ultraviolet (UV) transillumination after staining with ethidium bromide. Contamination control measures included the use of dedicated equipment in separate laboratory areas for each assay step.

Statistical analysis. Data entry was performed using Microsoft Excel. Analysis was performed using both Excel STAT and SPSS. Diagnostic performance was evaluated using standard measures of diagnostic accuracy, including sensitivity and specificity. κ coefficient was used to calculate the agreement between the diagnostic tests.

RESULTS

Patients profile. One hundred forty-four patients with microscopically confirmed *P. falciparum* malaria were recruited into the study, and 10 individuals with microscopically negative blood films served as controls; 294 specimens were obtained from all the participants. Geometric mean parasite density in the patients was 59,179 asexual parasites/ μ L blood (range = 2,463–551,614); mean age of the patients was 7.2 ± 3.00 years (range = 0.7–13 years), and mean axillary temperature at presentation was $38.32 \pm 1.21^\circ\text{C}$ (range = 35.8 – 41.0°C). Thirty-four children were ages less than 5 years, and 110 children were older than 5 years. All patients provided blood samples, whereas saliva samples were obtainable only from 130 patients. Fourteen patients did not provide saliva samples (three of these patients were very young and aged ≤ 2 years, whereas the remaining 11 patients aged 4–11 years declined providing saliva samples). All 10 controls provided blood and saliva

samples. Saliva samples obtained from 68 of 130 patients were used as whole saliva for RDT analysis; saliva samples obtained from 62 of 130 patients were spun, and the supernatant was used for RDT analysis.

Evaluation of RDT in blood and saliva samples. RDT analysis was positive in blood samples from 140 of 144 (97.2%) patients with microscopically confirmed *P. falciparum* malaria (Table 1). Parasitemia in the four RDT negative samples were 17,405, 45,607, 45,711, and 72,115 parasites/ μ L blood. Parasite lactate dehydrogenase was detected with RDT in whole saliva of 53/68 (77.9%) patients (Table 1). RDTs in blood samples from all the patients with saliva-positive RDT were also positive. Parasitemia in the 15 patients with saliva RDT-negative samples ranged from 2,571 to 334,298 parasites/ μ L blood. All blood samples from these 15 patients were positive by RDT. Parasite lactate dehydrogenase was detectable in the supernatant of spun saliva of 30/62 (48.3%) patients (Table 1). The matched blood samples from these patients were also positive by RDT. Parasitemia in the 32 RDT-negative supernatant samples ranged from 3,576 to 237,000 parasites/ μ L blood. Of 32 patients with RDT-negative supernatant samples, 28 patients had RDT-positive blood samples, whereas both saliva and blood samples from the remaining four patients were RDT-negative. The parasite densities in the four RDT-negative saliva and blood samples were 17,405, 45,607, 45,711, and 72,115 parasites/ μ L blood.

Comparative evaluation of the efficiency of RDT in blood and saliva samples. The sensitivities of the RDT for detection of parasite lactate dehydrogenase in whole-blood, matched whole-saliva, or supernatant of spun saliva samples were 97.2%, 77.9%, and 48.4%, respectively (Table 1). Specificity of the RDT was 100% in whole blood or saliva using the negative-control samples. The sensitivity of the RDT in whole blood was significantly higher than in whole saliva or supernatant of spun saliva (97.2% versus 77.9%, $P < 0.000005$; 97.2% versus 48.4%, $P < 0.000001$, respectively). The processing of the saliva sample had a significant effect on the efficiency of RDT, which is evidenced in the significantly higher sensitivity of RDT in whole saliva compared with supernatant of spun saliva (77.9% versus 48.4%, $P < 0.0005$). The agreement between microscopy and the RDT in blood at a threshold parasitemia of $> 2,000$ parasites/ μ L blood was very good ($\kappa = 0.820$), whereas it was

TABLE 1

Comparison of diagnostic accuracy of rapid diagnostic tests in blood, whole saliva, supernatant of spun saliva, PCR assays, and standard microscopy

RDT	Positive	Negative controls	Sensitivity (95% CI)	Specificity (95% CI)	κ
OptiMAL-IT blood					
Positive	140 (97.2%)	0 (0%)			
Negative	4 (2.8%)	10 (100%)			
Total	144 (100%)	10 (100%)	97.2% (94.5–99.9%)	100% (100%)	0.820
OptiMAL-IT whole saliva					
Positive	53 (80%)	0 (0%)			
Negative	15 (22%)	10 (100%)			
Total	68 (100%)	10 (100%)	77.9% (68.1–87.8%)	100% (100%)	0.475
OptiMAL-IT supernatant of spun saliva					
Positive	30 (48.4%)	0 (0%)			
Negative	32 (51.6%)	10 (100%)			
Total	62 (100%)	10 (100%)	48.4% (35.9–60.8%)	100% (100%)	0.207
PCR saliva					
Positive	30 (91%)	2 (50%)			
Negative	3 (9%)	2 (50%)			
Total	33 (100%)	4 (100%)	91% (81.1–100%)	50% (1–99%)	0.369

The gold standard is microscopy. Number positive = 144 (100%). SPSS version and Epi info were used for statistical analysis.

moderate in whole saliva ($\kappa = 0.475$) and slight in supernatant of spun saliva ($\kappa = 0.207$), respectively.

PCR detection of *P. falciparum* in human saliva. Thirty-seven participants were enrolled into this aspect of the study. There were 33 patients with microscopically confirmed *P. falciparum* infection and 4 individuals with negative Giemsa-stained blood films who served as controls. RDTs were positive in supernatant of spun saliva samples of 11/33 (33%) patients. All the four controls had saliva- (supernatant) and blood-negative RDTs.

Parasites genotyping. Parasite population diversity by nested PCR was successful in 18 and 16 genomic DNA extracted from blood and saliva samples, respectively. Polyclonality of infections was detected in both blood and saliva samples. Eleven matched blood and saliva samples from the same patients were analyzed for glutamate-rich protein (GLURP). Seventy-three percent (8/11) of the matched samples gave the same parasite clonal size and number, whereas 27% (3/11) showed different parasite population. The rest of the samples not matched were polyclonal and showed amplification either in parasite genomic DNA obtained from blood (7) or saliva (5) alone.

Comparison of microscopy and nested PCR-RFLP in saliva samples. Ninety percent of saliva samples obtained from patients with *P. falciparum* malaria were positive by nested PCR, whereas two saliva samples from the four negative controls were positive by nested PCR. The sensitivity and specificity of PCR-RFLP in saliva was 91% [95% confidence interval (CI) = 81.1–100%] and 50% (95% CI = 1–99%), respectively, with an agreement of 0.369 (Table 1).

PCR-RFLP genotyping of *pfprt* K76T on blood and saliva. Eighty-one percent of parasite genomic DNA extracted from blood samples were successfully amplified by nested PCR and RFLP. Similarly, 89% (33/37) of parasite genomic DNA extracted from saliva were successfully amplified, whereas 11% (4/37; two from malaria patients and two from healthy controls) of the genomic DNA from the saliva were not successfully amplified. In the blood samples, the wild-type K76 allele of *pfprt* gene was observed in 20% of the samples, whereas 60% and 20% harbored mutant T76 and mixed K76 + T76 allele of *pfprt* gene, respectively. In the saliva samples, wild-type K76 allele of *pfprt* gene was present in 18% of the saliva samples, whereas 57% and 25% harbored mutant T76 allele and mixed K76 + T76 allele of *pfprt* gene, respectively.

DISCUSSION

The ideal procedure for obtaining samples for the diagnosis of malaria should be simple, non-invasive, painless, and practicable by all categories of health workers. In addition, presence of parasites or its product should be readily demonstrable in samples obtained by the procedure. Obtaining saliva samples seems to have fulfilled many of these criteria. Rapid detection of pLDH antigen in whole saliva of children with *P. falciparum* infection using OptiMAL-IT dipsticks was shown for the first time in this study with a test sensitivity of 77.9%. This finding raises hope of the potential for the use of non-invasive, non-microscopic yet rapid techniques in the diagnosis of malaria. The present observation supports the limited reports describing potential to diagnose malaria using saliva samples.^{8–10}

The high sensitivity (detection threshold > 2,000 parasites/ μ L of blood) and specificity of the Optimal-IT dipsticks in patient blood at presentation as observed in this study are

consistent with previous reports.^{14–16} Results from the present study show that spinning saliva samples before analysis significantly decreased the sensitivity for antigen detection by the RDT (sensitivity = 48.3%). This finding was consistent with a previous report describing detection of pfHRP-II in supernatant of spun saliva by plate assay at a sensitivity of 43%.¹⁰ One possible explanation for this finding is the fact that centrifugation of saliva samples may have resulted in the sedimentation of parasite proteins and thus, concentrations of parasite antigen detectable in supernatant may have been low. Reports have shown that the apparent accuracy of any RDT in detecting malaria parasites is dependent on various factors, which include concentration of target antigen in host blood, dynamics of antigen-antibody flow along the nitrocellulose strip, and availability of target epitopes to bind antibodies in the test.¹⁷ Storage also seems to be a factor that may have influenced antigen detection in spun saliva samples. Bell and Peeling¹⁷ reported 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.

The approach for analysis of whole-saliva samples from patients as described in this study is a less complex and more sensitive alternative that can be easily applied in malaria-endemic countries of Africa. This approach contrasts with the technique described by Wilson and others,¹⁰ which is cumbersome, time-consuming, and more expensive because it requires centrifugation and two incubation steps as well as the use of a spectrophotometric plate reader; this hinders its routine use in resource-depleted countries where malaria is endemic. In the present study, the enhanced detection of parasite antigen in fresh whole-saliva samples shows potential superiority of whole saliva over spun saliva for rapid detection of malaria infection.

Although this study clearly shows the potential to use saliva as a non-invasive body fluid for rapid diagnosis of malaria, there are still many challenges in establishing saliva as a reference fluid for diagnosis. For instance, in this study, RDT failed to detect parasite antigen in some whole-saliva samples, despite high parasitemia (2,571–334,298 parasite/ μ L blood) and positive RDT in matching whole-blood samples from the same patients. The reasons for this disparity are unclear. However, it is possible that differences in concentration of parasite antigen in whole blood and saliva may be responsible for the lack of detection of parasite antigen in saliva, despite high parasite densities and positive RDT in matching blood samples. In a recently reported study using quantitative PCR analysis, it was shown that the amount of parasite DNA quantified in peripheral blood samples from infected patients was ~600-fold greater than in saliva samples, although a statistically significant correlation between parasite density and amount of parasite DNA in saliva was observed.⁹ A full understanding of biological processes leading to the release of parasite antigen in saliva would provide more insight into these disparities. Furthermore, matched blood and saliva RDTs of four patients with microscopically confirmed *P. falciparum* malaria were negative despite high parasite densities. The exact reason for this is not immediately known, although the possibility of gene deletion may explain, in part, the reason for the false-negative RDT. Gene deletion has been postulated in isolates that do not express pfHRP-II and yield false-negative results with Parasight-F dipsticks.^{18,19} Further investigation for these and other non-expressed antigens need to be considered.

The additional challenge with the use of saliva as a diagnostic fluid is the fact that the sensitivity of the RDT in whole saliva reported in our study is still below optimum. The World Health Organization recommends a minimum standard of 95% sensitivity at parasite densities of 100/ μ L.¹⁷ Obviously, one of the reasons for the low sensitivity of detection using Optimal-IT dipsticks in saliva may be the fact that this RDT was commercially designed to detect high levels of lactate dehydrogenase in whole blood and not in saliva. Therefore, proteomic analysis and quantification of pLDH or other potential *Plasmodium* antigens circulating in saliva is needed to develop an RDT specifically for saliva samples.

The results from this study also confirm detection of *P. falciparum* DNA by PCR in saliva of patients with *P. falciparum* malaria.^{8,9} *P. falciparum* population structure in most saliva samples was identical to that found in corresponding finger-prick blood samples of the same individual. The polyclonal nature of most infections in both saliva and blood samples is consistent with previous reports^{12,13} from the same study site. Nested PCR with RFLP techniques are frequently used to detect polymorphisms in parasite genes that serve as molecular markers of antimalarial drug resistance.^{20–22} This approach was used in saliva samples to detect K76T polymorphism on the *pfert* gene that is associated with chloroquine resistance. Similar K76T *pfert* genotypes were observed in the parasite genomic DNA extracted from filter-paper blood samples and saliva samples. The high prevalence of the mutant *pfert* K76T allele in both saliva (57%) and blood (60%) specimens is consistent with previous reports^{20–22} from genomic DNA extracted from blood sample.

Overall, this study has shown the rapid detection of malaria parasite antigen in saliva. Improvement of the sensitivity of this non-invasive method would provide a potentially valuable adjunct to microscopy, especially at the time of emergency for rapid diagnosis of malaria. However, expert microscopy will still be required for species identification and confirmation of a negative blood and saliva RDT result. Furthermore, there is a need to evaluate the value of using RDTs in detecting parasite antigen in saliva during longitudinal studies to monitor and evaluate the efficacy of antimalarial drugs with lengthy follow-up periods and low levels of parasitemia. Such studies would provide an opportunity to study the time course of appearance and dissipation of parasite antigen in saliva.

Received March 18, 2010. Accepted for publication April 26, 2010.

Acknowledgments: We are grateful to all the patients who participated in the study. We deeply appreciate Dr. Michael Obaro for clinical assistance and Mr. Abayomi Sijuade, Mrs. Sola Oyetade, Miss Wunmi Lawal, Miss Titi Okuboyejo, Mr. Rotimi Olatunde, and Mr. Gbenga Akinola for technical support. The American Society of Tropical Medicine and Hygiene (ASTMH) assisted with publication expenses.

Financial support: This study was supported by grants from the European Union and Developing Countries Clinical Trials Partnership (EDCTP), the Fogarty International Center/National Institutes of Health (NIH), and the Multilateral Initiative for Malaria in Africa (MIM)/Tropical Disease Research (TDR). G.O.G. is supported by the MIM/TDR projects ID A20239 and A20244. C.T.H. is supported EDCTP Grant Award no. TA2007/40200016 for Senior Research Fellowship and Fogarty International Research Collaboration Award (FIRCA) NIH no. RO3TW007757-03. A.S. is supported by a Swiss Pharma Nigeria PLC Grant.

Authors' addresses: Grace O. Gbotosho, Christian T. Happi, Onikepe Folarin, Ochuko Keyamo, and Akintunde Sowunmi, Department of

Pharmacology and Therapeutics and Malaria Research Laboratories, Institute of Advanced Medical Research and Training, College of Medicine, University of Ibadan, Ibadan, Nigeria, E-mails: solagboto sho@yahoo.co.uk, christianhappi@hotmail.com, onikepefolarin@yahoo.com, ochukzee@yahoo.com, and akinsowunmi@hotmail.com. Ayoade M. J. Oduola, Strategic and Discovery Research, World Health Organization/Tropical Disease Research, Geneva, Switzerland, E-mail: oduolaa@who.int.

REFERENCES

1. World Health Organization, 2008. *World Malaria Report. Technical Report*. Geneva: World Health Organization.
2. Murray CK, Gasser RA Jr, Magill AJ, Miller RS, 2008. Update on rapid diagnostic testing for malaria. *Clin Microbiol Rev* 27: 97–110.
3. Moody A, 2002. Rapid diagnostic tests for malaria parasites. *Clin Microbiol Rev* 15: 66–78.
4. Piper R, Lebras J, Wentworth L, Hunt-Cooke A, Houze S, Chiodini P, Markler M, 1999. Immunocapture diagnostic assays for malaria using *Plasmodium* lactate dehydrogenase (pLDH). *Am J Trop Med Hyg* 60: 109–118.
5. Oba IT, Spina AM, Saraceni CP, Lemos MF, Senhoras R, Moreira RC, Granato CF, 2000. Detection of hepatitis A antibodies by ELISA using saliva as clinical samples. *Rev Inst Med Trop Sao Paulo* 42: 197–200.
6. Thieme T, Piacentini S, Davidson S, Steingart K, 1994. Determination of measles, mumps, and rubella immunization status using oral fluid samples. *J Am Med Assoc* 272: 219–221.
7. Hunt AJ, Connell J, Christofinis G, Parry JV, Weatherburn P, Hickson FC, Coxon AP, Davies PM, McManus TJ, Sutherland S, 1993. The testing of saliva samples for HIV-1 antibodies: reliability in a non-clinic setting. *Genitourin Med* 69: 29–30.
8. Mharakurwa S, Simoloka C, Thuma PE, Shiff CJ, Sullivan D, 2006. PCR detection of *Plasmodium falciparum* in human urine and saliva samples. *Malar J* 5: 103.
9. Nwakanma DC, Gomez-Escobar N, Walther M, Crozier S, Dubovsky F, Malkin E, Locke E, Conway D, 2009. Quantitative detection of *Plasmodium falciparum* DNA in saliva, blood and urine. *J Infect Dis* 199: 1567–1574.
10. Wilson NO, Adjei AA, Anderson W, Baidoo S, Stiles JK, 2008. Detection of *Plasmodium falciparum* histidine-rich protein II in saliva of malaria patients. *Am J Trop Med Hyg* 78: 733–735.
11. Plowe CV, Djimde AA, Bouare M, Doumbo O, Wellems TE, 1995. Pyrimethamine and proguanil resistance-conferring mutations in *Plasmodium falciparum* dihydrofolate reductase: polymerase chain reaction methods for surveillance in Africa. *Am J Trop Med Hyg* 52: 565–568.
12. Happi CT, Gbotosho GO, Sowunmi A, Falade CO, Akinboye DO, Gerena L, Kyle DE, Milhous W, Wirth DF, Oduola AMJ, 2004. Molecular analysis of recrudescence *Plasmodium falciparum* malaria infections in children treated with chloroquine in Nigeria. *Am J Trop Med Hyg* 70: 20–26.
13. Happi TC, Thomas SM, Gbotosho GO, Falade CO, Akinboye DO, Gerena L, Sowunmi A, Kyle DE, Milhous W, Wirth DF, Oduola AM, 2003. Point mutations in *pfert* and *pfmdr1* genes of *Plasmodium falciparum* and clinical response to chloroquine among patients from Nigeria. *Ann Trop Med Parasitol* 97: 439–451.
14. Palmer CJ, Lindo JF, Klaskala WI, Quesada JA, Kaminsky R, Baum MK, Ager AL, 1998. Evaluation of the OptiMAL test for rapid diagnosis of *Plasmodium vivax* and *Plasmodium falciparum* malaria. *J Clin Microbiol* 36: 203–206.
15. Moody A, Hunt-Cooke A, Gabbett E, Chiodini P, 2000. Performance of the OptiMAL malaria antigen capture dipstick for malaria diagnosis and treatment monitoring at the Hospital for Tropical Diseases, London. *Br J Haematol* 109: 891–894.
16. Hunt-Cooke A, Chiodini PL, Doherty T, Moody AH, Ries J, Pinder M, 1999. Comparison of a lactate dehydrogenase-based immunochromatographic antigen detection assay (OptiMAL) with microscopy for the detection of malaria parasites in human blood samples. *Am J Trop Med Hyg* 60: 173–176.
17. Bell D, Peeling RW, 2006. Evaluation of rapid diagnostic tests: malaria. *Nat Rev Microbiol* 4: S34–S38.

18. Happi CT, Gbotosho GO, Sowunmi A, Falade CO, Akinboye DO, Oladepo O, Oduola AMJ, 2004. Malaria diagnosis: false negative Parasight-F tests in falciparum malaria patients in Nigeria. *Afr J Med Sci* 33: 15–18.
19. Baker J, McCarthy J, Gatton M, Kyle DE, Belizario V, Luchavez J, 2005. Genetic diversity of *Plasmodium falciparum* histidine rich protein-2 (pfHRP2) and its effect on the performance of pfHRP2-based rapid diagnostic tests. *J Infect Dis* 192: 870–877.
20. Djimde A, Doumbo OK, Cortese JF, Kayentao F, Doumbo S, Diourte Y, Dicko A, Su XZ, Nomura T, Fidock DA, Wellems TE, Plowe CV, Coulibaly D, 2001. A molecular marker for chloroquine-resistant falciparum malaria. *N Engl J Med* 344: 257–263.
21. Happi CT, Gbotosho GO, Folarin OA, Akinboye DO, Yusuf BO, Ebong OO, Sowunmi A, Kyle DE, Milhous W, Wirth DF, 2005. Polymorphisms in *Plasmodium falciparum* dhfr and dhps genes and age related *in vivo* sulfadoxine-pyrimethamine resistance in malaria-infected patients from Nigeria. *Acta Trop* 95: 183–193.
22. Folarin OA, Gbotosho GO, Sowunmi A, Olorunsogo OO, Oduola AMJ, Happi TC, 2008. Chloroquine resistant *Plasmodium falciparum* in Nigeria: relationship between pfcr1 and pfmdr1 polymorphisms, *in vitro* resistance and treatment outcome. *Open Trop Med J* 1: 74–82.