Short Report: Detection of *Plasmodium falciparum* Histidine-rich Protein II in Saliva of Malaria Patients

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Abstract

Detection of *Plasmodium falciparum* parasites in patients with malaria necessitates drawing blood, which increases the risk of accidental infections and is poorly accepted in communities with blood taboos. Thus, non-invasive, cost-effective malaria tests that minimize the need for blood collection are needed. *Plasmodium falciparum* histidine-rich protein II (*Pf*HRP II) levels in plasma and saliva were compared in malaria–positive and –negative patients in Ghana. Plasma and saliva obtained from 30 thick-film positive and 10 negative children were evaluated for *Pf*HRP II by ELISA. Among the 30 children with positive blood smear, 16 (53%) were *Pf*HRP II positive in plasma and 13 (43%) had *Pf*HRP II positive saliva. The sensitivity of *Pf*HRP II detection was 53% for plasma and 43% for saliva. The specificity was 100% with no false positive for both plasma and saliva when compared with blood smear. Thus, rapid detection of *Pf*HRP II antigen in saliva may be a useful non-invasive and cost-effective malaria diagnostic technique.

Malaria transmission and mortality rates remain unchanged in endemic countries lacking adequate health care and malaria control despite the use of preventive measures and treatments against malaria.[1] A major obstacle to effective malaria control is the lack of affordable and accurate malaria diagnostics and treatment, which has led to misuse and abuse of anti-malarial drugs and the development of drug resistance in parasites.

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Microscopic examination of blood smears, the conventional method for *P. falciparum* detection, is currently being augmented with antigen- and PCR-based rapid diagnostic tests (RDTs) for blood. However, inaccurate microscopic evaluation of blood smears have resulted in misdiagnoses and misclassification of malaria severity.[2,3] Blood taboos and increased risk of accidental infections due to needle pricks continue to impact malaria diagnosis negatively. In non-specialized laboratories,[4] microscopic evaluation of blood smears is slow and may lead to late diagnoses and treatment, which contributes to high mortality rates.[5]

Rapid diagnostic tests (RDTs) or "dipstick" are currently being used to detect antigens of *Plasmodium* species in blood or plasma to supplement microscopic evaluation of blood smears to manage tropical febrile disease.[6] The benefits of this approach include the rapid turnaround time and the ease of use, which allows inexperienced laboratory or clinical staff to make on-the-spot diagnoses in the absence of visible parasites. [6] However, issues associated with cultural objections to the collection of blood in communities with blood taboos[7,8] and increased risk of needle injuries and disease transmission must be addressed.[9]

Saliva has been used in surveillance of vaccine-preventable diseases, such as measles, mumps, and rubella,[10,11] and for individual diagnosis of HIV infection[12] by detecting antibodies against the target pathogen. Although *P. falciparum* HRP II antigen has been detected in erythrocytes, serum, plasma, cerebrospinal fluid, and urine,[13,14] detection of parasite antigens in saliva of *P. falciparum*-infected humans has not been reported. The goal of this pilot study was to test the possibility of detecting malaria parasite antigen in saliva in malaria patients. The hypothesis is that *P. falciparum* histidine-rich protein II (*Pf*HRP II) is detectable in saliva in patients with symptomatic malaria.

The study was conducted at the Korle-Bu Teaching Hospital's Child Health Department, Accra, Ghana, after ethical approval by Morehouse School of Medicine and University of Ghana Medical School. Randomly collected samples (plasma and saliva) from children (22 months to 16 years) reporting to the Child Health Department's diagnostic laboratory were retrospectively analyzed for this study. Malaria positive cases were confirmed by thick film slides. Parasitemia was evaluated on the number of parasites per field (+, 1–10 parasites/100 fields, ++, > 10 parasites/100 fields, +++, 1–10 parasites/field, and ++++ > 10 parasites/field) and at least 100 fields/slide were examined to rule out any negative thick film slide. Thirty thick film positive children and 10 negative children were enrolled. Red blood cells (infected and uninfected) and plasma were separated using Vacutainer Cell Preparation Tubes (CPT) with Sodium Citrate (Becton Dickinson, USA). Saliva was collected in sterile containers and aliquoted into microcentrifuge tubes and stored at −20°C. Saliva samples were centrifuged for 3 min at 14,000 rpm and the supernatants were analyzed by ELISA. Both saliva and plasma samples from the same patient were analyzed on the same plate, date, and conditions for *Pf*HRP II antigen levels using a Malaria Antigen ELISA kit (CELISA, Cellabs, Australia). This kit measures HRP II production during growth and multiplication[15] of *P. falciparum* at a specificity of 96% and sensitivity of 98% in whole blood or plasma and can detect *P. falciparum* parasites at a limit of detection of 0.001%[16]; thus incubation periods with reagents were the same for plasma and saliva for the same patient. The plasma samples were tested at a 1:2 dilution and all samples were run in duplicates by ELISA. The incubation period for primary and secondary antibodies with the samples was 1 hr each in a humid chamber and 15 min for enzyme development (substrate) in the dark at room temperature. The minimum limit of detection (cut-off level) of the kit was determined according to manufacturer's instructions.

Of the 30 children testing positive for blood smear, 16 (53%) had detectable *Pf*HRP II antigens in their plasma (Table 1). Thirteen (43%) patients of the 30 positive blood smears were *Pf*HRP II positive for saliva samples (Table 1). All patients that were *Pf*HRP II positive for saliva were also positive for plasma. Three patients (P006, P008, and P011) were *Pf*HRP II positive in

plasma but negative for saliva samples. Surprisingly, P006 had a mean OD reading (0.144) that is slightly below the cut-off level of 0.161 compared with the other 2 (P008 and P011) *Pf*HRP II negative saliva. This observation suggests that P006 may have *Pf*HRP II in the saliva that is undetectable in the kit used for this study. The 10 negative blood smears were also negative for *Pf*HRP II antigen in both plasma and saliva. In our study the minimum limit of detection (cut-off level) was an OD reading of 0.161, which was determined according to the manufacturer's instructions. In addition, all 13 saliva specimens had lower titers (OD, 0.166– 0.427) of *Pf*HRP II with a mean of 0.209 ± 0.07. The sensitivity of *Pf*HRP II detection test for plasma was 53% and 43% for saliva whereas specificity was 100% for both specimens when compared with blood smears.

Rapid and accurate malaria diagnosis enables effective malaria control by eliminating malaria-associated morbidity and mortality in resource-poor countries. In Africa, fevers are treated presumptively as malaria in the absence of laboratory-confirmed diagnosis,[17] which results in the extensive overuse of anti-malaria drugs.[18] Light microscopy, the conventional method for malaria diagnosis, remains unavailable to a huge segment of patients.[18,19] Thus, integrating detection of circulating antigens of malaria parasites in saliva with RDT technology will considerably improve parasite detection and diagnosis of malaria.[1]

Malaria detection and epidemiological surveys in developing countries often require collection of blood samples from severely anemic children and communities with blood taboos. In central Africa, blood is considered an essential constituent of the "vital force" and an object of greed, "devoured" by sorcerers.[7] Therefore, collection of blood specimens—regardless of the volume for definitive or confirmatory diagnosis—is poorly accepted.[7] Thus, a non-invasive approach will greatly enhance cooperation of patients.

PCR methods have been used to detect malaria parasites in the blood.[8,20] Although PCR-based methods are more sensitive and specific than existing techniques, the process is lengthy and requires specialized, costly equipment and reagents, as well as laboratory conditions that are not possible in the field.[21] Sensitivity of detection in saliva was not enhanced in this study due to limitations of the commercially available kit used, which is designed to detect higher levels of *Pf*HRP II in whole blood or plasma than is found in saliva. Therefore, development of a kit or test that is sensitive enough to detect lower levels of the antigen present in saliva could be a more appropriate approach to malaria diagnostics and in epidemiological surveys, thus, substituting blood samples with saliva specimens.

The detection of *Pf*HRP II in saliva offers a practical alternative to *Pf*HRP II detection in blood for malaria diagnosis and offers some distinct advantages over blood. Collection of saliva is non-invasive, simple, safe, stress free, painless, and can be done by individuals with limited training, including patients. It will not require blood cell lysis that diminishes HRP II antigen availability and detection. No special equipment is needed for collection and it allows for multiple or serial collections outside of the hospital.

Detecting parasite antigens in saliva to determine presence or absence of parasites could be valuable for communities with blood taboos and reduce compliance problems associated with collection of blood.[22,23] Furthermore, it will provide a cost-effective approach for the screening of large populations in epidemiological surveys while being affordable, rapid, non-invasive, and safe for patients and technicians in resource-poor environments.

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TABLE 1

Results of analysis of specimens collected from children confirmed malaria positive or negative with blood smear

Sample ID	Age in months	Gender	Parasitemia (Microscopic Evaluation)	P. falciparum HRPIIAg ELISA test			
				Plasma OD (SD)	+/−	Saliva OD (SD)	+/−
P001	156	F	++	3.640 (0.214)	+	0.180 (0.026)	+
P002	192	M	+	0.063 (0.003)	−	0.118 (0.043)	−
P003	84	M	++	0.059 (0.004)	−	0.095 (0.003)	−
P004	144	F	+	3.296 (0.047)	+	0.167 (0.001)	+
P005	192	M	++	0.070 (0.003)	−	0.061 (0.001)	−
P006	60	M	++	2.976 (1.174)	+	0.144 (0.016)	−
P007	192	F	++	0.073 (0.001)	−	0.087 (0.037)	−
P008	120	M	++	2.621 (0.023)	+	0.064 (0.006)	−
P009	144	F	++	0.223 (0.011)	+	0.257 (0.011)	+
P010	30	F	+++	3.464 (0.165)	+	0.427 (0.051)	+
P011	192	M	++	1.565 (0.013)	+	0.062 (0.003)	−
P012	144	F	++++	3.625 (0.144)	+	0.185 (0.011)	+
P013	152	F	+	2.809 (0.089)	+	0.179 (0.008)	+
P014	192	F	+++	3.672 (0.249)	+	0.195 (0.023)	+
P015	168	F	++++	3.426 (0.172)	+	0.191 (0.031)	+
P016	108	F	++	0.039 (0.001)	−	0.021 (0.004)	−
P017	36	M	−	0.125 (0.001)	−	0.097 (0.025)	−
P018	72	M	−	0.090 (0.018)	−	0.058 (0.001)	−
P019	96	F	−	0.043 (0.006)	−	0.034 (0.007)	−
P020	84	M	++	3.274 (0.061)	+	0.251 (0.017)	+
C001	22	F	+	0.061 (0.001)	−	0.064 (0.004)	−
C002	72	F	+	0.054 (0.005)	−	0.049 (0.003)	−
C003	44	M	++++	3.438 (0.165)	+	0.176 (0.010)	+
C004	96	F	+	0.060 (0.001)	−	0.063 (0.001)	−
C005	48	M	+	0.061 (0.001)	−	0.061 (0.001)	−
C006	72	F	++	0.087 (0.005)	−	0.076 (0.001)	−
C007	72	F	++	3.099 (0.041)	+	0.169 (0.008)	+

P. falciparum HRPIIAg ELISA test

Sample ID	Age in months	Gender	Parasitemia (Microscopic Evaluation)	Plasma OD (SD)	+/−	Saliva OD (SD)	+/−
C008	96	F	+	0.061 (0.001)	−	0.077 (0.026)	−
C009	108	F	++++	3.201 (0.103)	+	0.168 (0.004)	+
C010	23	F	+	0.061 (0.002)	−	0.073 (0.001)	−
C011	192	M	−	0.058 (0.001)	−	0.063 (0.003)	−
C012	180	F	−	0.065 (0.006)	−	0.050 (0.004)	−
C013	22	F	−	0.064 (0.003)	−	0.088 (0.006)	−
C014	48	M	+	0.062 (0.001)	−	0.063 (0.001)	−
C015	48	M	++++	3.392 (0.157)	+	0.166 (0.015)	+
C016	48	F	+++	0.065 (0.003)	−	0.053 (0.001)	−
C017	82	M	−	0.024 (0.006)	−	0.014 (0.006)	−
C018	24	M	−	0.057 (0.002)	−	0.038 (0.003)	−
C019	132	F	−	0.042 (0.004)	−	0.029 (0.001)	−
C020	72	M	−	0.083 (0.014)	−	0.047 (0.007)	−

Parasitemia were evaluated on the number of parasites per field (+, 1–10 parasites/100 fields, ++, > 10 parasites/100 fields, +++, 1–10 parasites/field, and ++++, > 10 parasites/field). Both saliva and plasma samples from the same patient were analyzed on the same plate, date, and conditions in parallel for *P*HRP II antigen levels using a Malaria Antigen ELISA kit (CELISA, Cellabs, Australia). The plasma samples were tested at a 1:2 dilution and all samples were run in duplicates on the ELISA (+, positive; −, negative). The procedures and the minimum limit of detection (mean OD 0.161) of the kit were determined according to manufacturer's instructions. The mean OD and standard deviation (SD) of each patient were recorded. Accurate upper limit of detection for plate reader is 3.0. Note that Optical Density reading above 2.5 may not be linear.