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Malaria diagnosis: false negative *parasight^{tm}-F* tests in falciparum malaria patients in Nigeria.

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Summary

This study was designed to assess the relative reliability of microscopy, the dipstick technique based on the detection of *Plasmodium falciparum*-specific histidine rich protein II (*HRPII*) (*Parasight^{TM}-F)* and *PCR* assays in diagnosing falciparum malaria infections in Nigerian children. The prevalence of *P. falciparum* infections in enrolled patients was 100% by microscopy. Parasite density ranged from 329 to 81,194 parasites/μL of blood, with a geometric mean parasite density of 5168 parasites/μL of blood. The sensitivity of the *HRPII* based dipstick, *PCR* and microscopy were 80%, 92% and 100% respectively. A false negative rate of 20% was observed with *Parasight^{TM}-F* as compared with microscopy. The parasitemia in patients with false negative *Parasight^{TM}-F* tests ranged from 319 to 54,680 parasites/μL of blood. Detailed *PCR* analysis of Isolates obtained from five out of the eight patients who exhibited a negative *Parasight^{TM}–F* test, showed that the average numbers of *P. falciparum* clones in these five isolates were: 1.7±1.02 with *MSP1*, 3.2±1.3 with *MSP2* and 1.4±1.72 with *GLURP*. Comparison of microscopy and *HRPII* results showed a significant (p=0.009) difference as opposed to microscopy and *PCR* (p=0.239).

This study showed that caution should be exercised when excluding *P. falciparum* infections on the basis of *HRPII* based dipstick results alone. Microscopy or *PCR* diagnosis where possible, should be carried out in order to confirm negative *P. falciparum* HRPII-based dipstick tests.

Keywords: *HRPII-based dipstick test, Plasmodium falciparum, PCR, Microscopy, Malaria, Ibadan, Nigeria*

Résumé

Cette étude était faite pour évaluer la liabilité de la microscopie, de la technique du dipstick basée sur la détection de la protéine spécifique d'histidine riche II (HRPII) du *plasmodium falciparum* (Parasight F) et l'utilisation des analyses PCR pour diagnsotiquer les infections du falciparum malaria aux enfants Nigerians. Le taux d'infection du *P.falciparum* aux enfants recruités était de 100% pour la microscopie. La densité des parasites variait entre 329-81.194 parasites /ul de sang, avec une moyenne géometrique de 5168 parasites par microlitre de sang. La sensitivité du HRPII, PCR et microscopie était de 80%, 92% et 100% respectivement. Un taux de resultat fausse-négative de 20% était enregistré avec le parasight F comparé a la microscopie. La parasitémie des patients fausse-négative au parasight F variait entre 319-54680 parasites /ul de sang. L'analyse détaillé du PCR des isolats obtenu de 5 des 8 patients negative au test du parasight F , montrait une moyenne de clones du *P. falciparum* des isolats de 1.7 ± 1.02 pour MSP1, 3.2 ± 1.3 pour MSP2 et 1.4 ± 1.72 pour le GLURP. La comparaison des résultats de la microscopie et du HRPII montrait une difference significative (P=0.009) opposée a la microscopie et PCR (P=0.239). Cette étude montre que des précautions doivent etre prise quant excluant les infections au *P.falciparum* a base des résultants du HRPII. Autres tests de confirmations ,soit la PCR et/ou la microscopie doivent etre faite aux tests négative du HRPII

Introduction

Microscopy remains the gold standard for diagnosis of malaria. However, this technique can be tedious, time consuming and subjective at times. The recent development and use of rapid non-microscopic tests based on the detection of *Plasmodium falciparum* histidine rich protein II (*HRPII*) antigens (*ParaSight^{TM}-F*; Becton Dickinson Tropical Disease Diagnostics, Sparks, MD, USA) have been useful in the diagnosis and monitoring of malaria infections in many endemic countries [1]. The test is based on an immunochromatographic method to detect the *Plasmodium* specific antigen *HRPII* in a finger prick blood sample. This test in previous evaluation has given consistent specificity and sensitivity at 90% relative to thick blood film examination [1], the gold standard for malaria diagnosis. The detection threshold for the test has been shown to be 40 to 60 parasites per iL of blood [2]. The *ParaSight^{TM}-F* test has also been proposed as a useful tool for monitoring treatment failure in multidrug resistant *P. falciparum* malaria infections, when performed 14 days after treatment [3].

Recent advances in molecular biology and especially Plasmodium biology has now enabled diagnosis of *P. falciparum* by polymerase chain reaction (*PCR*). However, despite the obvious utility of *PCR* technology in diagnosing malaria infections, it has not been extensively used in malaria endemic areas.

This study was designed to assess the reliance of microscopy, the HRPII based-dipstick assay and *PCR* in diagnosing and monitoring drug response to treatment amongst Nigerian children with *P. falciparum* malaria infections. Results of *PCR* and *HRPII* based dipstick assays were compared with standard microscopy for diagnosis of malaria in this area of intense malaria transmission. In addition, this study used

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polymorphic loci in *P. falciparum* isolates obtained from patients to examine genetic diversity and complexity of parasite populations in Ibadan, Nigeria.

Materials and method

Patient selection criteria and methods:

The study was conducted at the Malaria Clinic of the Malaria Research Laboratories, College of Medicine, University of Ibadan, Nigeria. Malaria is hyperendemic in Ibadan, with transmission occurring round the year. The study protocol was reviewed and approved by the Joint University of Ibadan/ University College Hospital Ethics Committee. Informed consent was obtained from parents or legal guardians of children enrolled into the study.

Forty children aged 1 to 12 years presenting with acute symptoms of *P. falciparum* malaria were enrolled in the study. The criteria for inclusion in the study were: history of fever in the last 24-48 hours preceding presentation; pure *P. falciparum* parasitemia with asexual forms confirmed by microscopy; no history of antimalarial drug ingestion in the 2 weeks preceding presentation; negative urine test for 4 aminoquinoline (Dill-Glazko); absence of concomitant illness and consent from parent or guardians. A child was withdrawn from the study if a concomitant illness developed during the follow-up period, if the parents or guardian desired it or if there was failure to comply with the study protocol. Children with sickle cell anemia were excluded from the study.

A careful history was obtained from an accompanying parent or guardian and a physical examination was performed before enrollment of each patient. Body weight, height and axillary temperature were recorded and Giemsa stained thick and thin blood films were prepared from finger prick blood for quantification of parasitemia and species identification. In addition, dipstick antigen capture assay (*Parasight™-F*) was performed on a drop of blood taken from the same sample as that used to make the thick blood film for identification of malaria parasites [4]. Two drops of blood were also blotted onto 3MM Whatmann filter paper for extraction and analysis of parasite *DNA*.

Each child was treated with chloroquine base (25mg/ kg body weight) over 3 days. The first dose of chloroquine was administered in the hospital with close monitoring for a period of at least 1 hour. Subsequent doses were given to parents or the guardian to administer to the child.

Two different microscopists examined the Giemsa stained blood films under an oil immersion objective (x 100). The parasitaemia in the thick films was estimated by counting the number of asexual forms of *P. falciparum* versus 200 leucocytes. The parasite density was calculated by assuming a leucocyte count of 8000/il of blood. Each microscopist scanned a total of 100 oil immersion fields before a slide was declared negative. The microscopists were unaware of the results of the *Parasight™-F* test or *PCR* results.

Extraction of DNA from samples collected on filter paper.

Parasite genomic *DNA* was extracted from blood samples collected on filter paper using the methanol fixation and heat extraction method as described by Plowe *et al.* [5]

PCR determination of parasite population in patient isolates of P. falciparum.

PCR genotyping of parasite populations using polymorphic loci of *msp1*, *msp2* and glurp genes of P. *falciparum* was performed on samples with negative dipstick results. Briefly, block 2 of *MSP1* (merozoite surface proteins-1), and the Block 3 of *MSP2* (merozoite surface protein-2) and region II of *GLURP* were amplified by two rounds of *PCR* using primers and amplification conditions described previously by Snounou *et al* [6] Ten microliters of the *PCR* products were resolved by electrophoresis on a 2% agarose gel, sized against a 100-base pair molecular weight marker (New England Biolabs, Beverly, MA) and visualized with a tabletop transilluminator UV lamp (ë=302nm).

The sensitivity of the *ParaSight™-F* dipstick assay was compared with the microscopic diagnosis and *PCR*. The Chi-square and Fisher's exact tests (2 tails) were used to evaluate the sensitivity of the three methods. $P < 0.05$ was considered significant.

Results.

The prevalence of *P. falciparum* infections in enrolled patients was 100% by microscopy. Parasite density ranged from 329 to 81,194 parasites / µL of blood, with a geometric mean parasite density of 5168 parasites/ µL of blood. The *HRPII* based dipstick had a sensitivity of 80% as 32 of 40 slide-positive samples were positive with the *ParaSight™–F* test. *ParaSight™-F* test had a significant false negative rate (20%), as determined by both microscopy and the *PCR*. False negatives *ParaSight™–F* test occurred in samples from eight patients with parasitaemia as determined by microscopy. The parasitemia in these eight patients ranged from 329 to 54,680 parasites / µL of blood (Table 1), and were at least one order of magnitude above the detection threshold for this dipstick test.

Table 1: Comparative diagnosis of malaria infections using microscopy, *Parasight™-F* or PCR.

Patient ID	Diagnosis		
	Microscopic diagnosis: Parasite /ul of blood	Parasight™-F	PCR
009/2000	34,565	-	+
016/2000	329	-	+
019/2000	4,680	-	+
036/2000	3,235	-	-
041/2000	54,680	-	+
078/2000	1,320	-	-
089/2000	7,201	-	-
098/2000	6,722	-	+

Comparison of the dipstick assay and microscopy showed that microscopy was more significantly sensitive ($+^2 = 8.89$; $P = 0.0053$). *PCR* amplification of blood samples from these eight patients showed the presence of *P. falciparum DNA* in five of the samples. Parasitemia in the three *PCR* negative

samples ranged from 1,320 to 7,201 parasites/μL of blood (Table 1). Comparison of *PCR* and *ParaSight™-F* results did not show any significant difference ($\div^2 = 2.64$; p=0.104). No significant difference ($\div^2 = 1.39$; p=0.239) was also observed between microscopy and the *P. falciparum*-specific HRPII-based dipstick diagnosis results.

Polymorphisms of MSP1, MSP2 and GLURP reflect population diversity.

Isolates from 37 out of 40 patients positively diagnosed with malaria by microscopy were positive by *PCR*, while three of the isolates were negative by this technique. Genotyping of all isolates positive by *PCR* showed that the allelic families of *MSP1* and *MSP2* were often represented in parasite *DNA* derived from a single patient, indicating a polyclonal infection. Specifically, three allelic families of *MSP1* were assessed. Alleles were classified according to the size of PCR fragments. Amplification products of the *MSP1* allelic family *K1* was positive in 28 of 37 (72%) isolates, and yielded two different fragments (90-290 bp). The *MSP1* allelic family *MAD20* was detected in 18 of 37 (49%) isolates and produced 2 major bands (190-250 bp). The *MSP1* RO33 allele was detected in 32% (12/37) of the isolates, and produced amplification products of 3 different sizes with a predominant 190bp fragment. Thirty-five isolates (95%) were positive for the *MSP2* IC1 and/or FC27 alleles and produced up to 10 different fragment sizes (IC1: 300-950 bp, FC27: 190-800 bp). The region II of *GLURP* was present in 22 of 37 (59%) isolates and produced three different fragments (225 bp-900 bp). These results are summarized in Table 2. Comparison of the prevalence between the allelic families of *MSP1* and *MSP2* showed a significant difference (*P*=0.000) indicating a very diverse *Plasmodium* population per infection. The number of different alleles per infection detected with *MSP-2* was higher than that obtained with *MSP-1* or *GLURP* or the combination of both markers. All patients showed multiple alleles of *MSP-1*, *MSP-2* and *GLURP*.

Table 2: PCR genotyping of *P. falciparum* isolates obtained from 37 Nigerian children diagnosed by parasight-F test, and microscopy.

Loci	No. positive by PCR (%)	No. of distinct alleles and sizes (bp)
MSP1		
K1	28 (72%)	2 (90-290 bp)
MAD20	18 (49%)	2 (190-250 bp)
RO33	12 (32%)	3 (190bp predominant)
MSP2		
IC1/3D7	35 (95%)	6 (300-950 bp)
FC27	35 (95%)	4 (190-800 bp)
GLURP	22 (59%)	3 (225bp-900bp)

Complexity of infections in patients isolates.

The estimated average number of genetically distinct parasite population in isolates obtained from all patients in this study was 2.2±1.43 with *MSP1*, 4.2±0.21 with *MSP2* and 2±1.11 with *GLURP*. There was a significant difference in mean multiplicity of infection between *MSP2* and *MSP1* or *GLURP* (p=0.000). Detailed *PCR* analysis of Isolates obtained from five out of the eight patients who exhibited a negative *ParaSight™–F* test, showed that they all yielded positive amplification products at all three *MSP1*, *MSP2* and *GLURP* loci. The average numbers of *P. falciparum* clones detected by *PCR* in these five isolates were: 1.7±1.02 with *MSP1*, 3.2±1.3 with *MSP2* and 1.4±1.72 with *GLURP*. Three out of the isolates obtained from the eight patients with negative dipstick results also showed a negative *PCR* results, although they were positive by microscopy.

Discussion

This study showed that the *ParaSight™-F* dipstick assay could be falsely negative in patients with *P. falciparum* infections. Failure by *ParaSight™-F* to detect *P. falciparum* in one (patient ID. 016/2000, Table 1) of the eight samples could be due to low parasitemia. False negative *ParaSight™-F* results have been reported in patients with parasite density over 1000 parasites /μL of blood [7,8,9]. Whether failure to detect parasites by *ParaSight™-F* could be due to antigenic variation in *HRPII* is unknown. Polymorphisms within the coding or non-coding sequences of the *HRPII* gene could result in a change in the structure of the *HRPII* protein or its expression level, respectively. It has been reported that some isolates of *P. falciparum* do not express *HRPII* antigens, which may account for the false negative results [1]. Studies are currently being carried out to investigate the existence of polymorphisms in *HRPII* sequences in *P. falciparum* isolates from Nigeria.

Three of the eight isolates that were negative using the *P. falciparum* spefic-HRPII dipstick assay were also negative by *PCR*. However, these three isolates were confirmed positive microscopically by two independent microscopists. The reasons for the negative PCR results remain unclear despite the fact these assays were repeated. However, inhibition of *PCR* during the amplification process or contamination of with DNase during the extraction process may explain these negative results.

The population structure of *Plasmodium falciparum* infections in the children analyzed for the polymorphic markers, *MSP1*, *MSP2* and *GLURP* showed extensive diversity in parasite populations in Ibadan. *MSP1*, *MSP2* and *GLURP* showed 7, 10 and 3 allelic families respectively.

A catalogue of genetically distinct parasite populations co-infecting Nigerian children, based on *PCR* amplification of the *GLURP*, *MSP1* and *MSP2* markers, showed that multiplicity of infection was very common. However, *MSP2* was shown to be the most informative of the markers of multiplicity of infections as it showed more clones than other markers. The multiclonality of infections is a common feature in most malaria endemic areas.[5,10-14] and may explain the false negative results observed with the *P. falciparum* HRPII based-dipstick assay. Polymorphisms within the coding or non-coding sequences of the *HRPII* gene of major *P. falciparum* population in a polyclonal infection could result in a change in the structure of the *HRPII* protein or its expression level of this population. Since the major population in such infection overshadows other parasitedsub-populations the result of the HRPII based dipstick assay could appear negative. It has

been reported that some isolates of *P. falciparum* do not express *HRPII* antigens, which may account for the false negative results [1]. Host factors could have also played an important role in the outcome of *Parasight™-F* test. It is also possible that the presence of blocking antibodies can result in failure to detect parasites by *Parasight™-F*. However, this remains to be determined.

Rapid non-microscopic based malaria diagnostic tests are a major advance in malaria diagnosis in endemic countries. However, caution should be exercised when excluding *P. falciparum* infection on the basis of dipstick results alone. This study showed that although a negative *Parasight™-F* with high parasitemia is rare, it cannot be ruled out. Confirmation of negative dipstick tests by microscopy, or *PCR* where possible should be carried out. Taking into consideration the small sample size used for this study, further studies are required to determine the extent of this problem in Nigeria and other malaria endemic countries.

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