

Diagnosis of malaria by detection of *Plasmodium falciparum* HRP-2 antigen with a rapid dipstick antigen-capture assay

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Summary

Two field studies in Kenya and an experimental challenge study in the USA were done to assess the accuracy of a dipstick antigen-capture assay based on qualitative detection of *Plasmodium falciparum* histidine-rich protein 2 (PfHRP-2) in peripheral blood for diagnosis of *P falciparum* infection.

In these studies, the assay was 96.5–100% sensitive for detection of greater than 60 *P falciparum* asexual parasites/ μ L blood, 70–81% sensitive for 11–60 parasites/ μ L blood, and 11–67% sensitive for 10 parasites or less/ μ L blood. Specificity was 95% (95% CI 85–105%; n=20) among naive American volunteers, 98% (96–101%; n=112) among volunteers exposed to the bite of *P falciparum*-infected mosquitoes, and 88% (84–92%; n=285) among Kenyans living in an area with holoendemic malaria. Our results also indicated that PfHRP-2 antigen was not detectable in blood 6 days after initiation of curative chemotherapy, and suggest that such circulating antigens rarely lead to false-positive tests.

The dipstick assay's sensitivity, specificity, simplicity, and speed may make it an important tool in the battle against malaria.

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Introduction

Rapid, simple, and accurate new methods are required for diagnosis of malaria.¹ Antibody capture of circulating *Plasmodium* sp antigens is a technique proposed for rapid diagnosis of malaria,^{2–5} and *Plasmodium falciparum* histidine-rich protein 2 (PfHRP-2), a water-soluble antigen released by blood stages of *P falciparum* from many geographical regions,^{6,7} is a target for an antigen-capture assay. We describe an experimental challenge and field evaluation of a dipstick PfHRP-2 capture assay (ParaSight F, Becton Dickinson Tropical Disease Diagnostics) for diagnosis of *P falciparum*.

Subjects and methods

Study sites and participants

The dipstick antigen-capture assay was evaluated in three groups of subjects. Study protocols received ethical committee approval. The first group (adult field study group) consisted of 40 males aged 15–35 years who had volunteered for a malaria immunology study in Saradidi, western Kenya, during the autumn rainy season in 1992, when malaria prevalence was expected to be greater than 70%.^{8,9} Blood was obtained by fingerprick for the dipstick antigen-capture assay and thick and thin films before subjects received treatment, and daily for the next 6 days. All subjects, regardless of their malaria status, received standard doses of quinine and doxycycline for radical cure.

The second study group (experimental challenge study group) consisted of 20 adult male and female volunteers who participated in a vaccine trial in Baltimore, Maryland, in spring 1993. These individuals were challenged by the bite of five *Anopheles stephensi* mosquitoes which had been infected by membrane feeding on cultured gametocytes of *P falciparum* (NF54 strain or the 3D7 clone of NF54). Blood was drawn by fingerprick from every subject before challenge, and then daily starting on day 6 postchallenge and continuing until day 21 postchallenge or until the individual was assessed positive by blood film.

The third group (child field study group) were 173 girls and boys, aged 9–14 years, who were enrolled in a malaria prophylactic drug study. This study took place during the spring rainy season of 1993 in the same region of Kenya as the initial adult study and involved children from four primary schools.

After children had been screened and excluded from the prophylactic drug study for appropriate medical reasons, about 40 students remained at each of the four schools. Immediately before the start of a 7-day doxycycline and quinine radical cure for malaria, blood was drawn by fingerprick from every child for the dipstick antigen-capture assay and thick and thin blood films were prepared. The same procedures were repeated on day 6 at the conclusion of the radical cure.

Specimen collection and handling

In all three studies, laboratory staff were blinded to the origin of the specimens they tested. In the first two studies, most specimens were evaluated by the dipstick antigen-capture assay within an hour of sample collection, although testing was delayed by a few hours for some of the samples. In the third study, teams of fieldworkers collected the specimens from all students at a particular school within 1 h. Specimens were placed on ice and

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transported to the local health clinic where they were examined by the antigen-capture assay within 4 h of collection.

Antigen-capture assay

50 μ L blood was placed in a labelled polypropylene tube and three drops (about 100 μ L) of a red blood cell lysing reagent were added. After gently agitating the tubes, specimens were ready to be tested by the antigen-capture assay.

Assay test sticks were made of cellulose fibre and contained an immobilised IgG1 monoclonal antibody directed against the synthetic peptide (AHH[AHHAAD]₂)¹⁰ from PfHRP-2. Test sticks were packaged with dessicant in foil pouches (unrefrigerated). One drop of a lysed blood specimen was dispensed into one well of a ten-specimen test-stick-holder platform. The end of a test stick was then placed in the drop of lysed blood. The drop of blood was absorbed by the test stick along its entire length, a process that required 2–10 min depending on the specimen. PfHRP-2 antigen-detector reagent (containing polyclonal antibodies against PfHRP-2 that were conjugated to liposomes containing pink dye) was then added to every specimen well. After the antigen-detector reagent had been absorbed by the dipstick, a wash reagent was added and absorbed. Results were then read immediately and recorded. A positive test result (presence of PfHRP-2) was evident when a thin, solid pink band appeared on the dipstick. Results were recorded as either "positive" or "negative", unless the result was difficult to interpret—such as when there was a suggestion of a pink line in the appropriate position—in which case an "intermediate" result was recorded. A control dot near the top of every dipstick showed whether the test was conducted properly and the reagents were functional. In the third study group, the two technicians performing the assay exchanged each group of ten dipsticks immediately after reading so a second independent interpretation of each specimen test result could be recorded.

Microscopy and parasite-density determination

Thick blood films were stained with 5% Giemsa and examined at a magnification of 1000. In the adult field study, one slide reader counted 200 white blood cells (WBCs) before classifying a slide as negative. Later, slides initially called negative were reread by counting up to 4000 WBCs if the corresponding dipstick result had been positive. In the child field study, all blood films were first examined by two independent slide readers who counted 200 WBCs before classifying a slide as negative. When either of the two slide readers reported a slide as negative at 200 WBCs, the slide was reread by counting 2000 WBC before it was finally classified as negative, regardless of the dipstick result. In the experimental challenge study, a microscopist counted 500 WBCs before classifying a slide as negative. No slides were reread in the experimental challenge study.

Parasite density (parasites/ μ L blood) was calculated for each positive slide assuming 4300 WBCs/ μ L blood for the Kenyan adult field study and 6000 WBCs/ μ L blood for the child field study. The former WBC estimate was derived from 26 study volunteers (mean [SD] 4300 [780] WBCs/ μ L) and the latter estimate was derived from the study population's WBC counts (5981 [1453] WBCs/ μ L) obtained from blood taken during initial work-up. In the experimental challenge study, individual's WBC counts done on the same day as the slide was made were used.

Data analysis

Sample sizes were not calculated for the adult field and experimental challenge studies. These studies provided a preliminary estimate of the dipstick's sensitivity and specificity when measured against the standard of thick blood films. Before starting the child field study, sample sizes were calculated according to the method for a descriptive study of a dichotomous variable.¹¹ These calculations showed that about 200 *P. falciparum*-infected specimens and 140 uninfected specimens were required to estimate within $\pm 5\%$ a sensitivity of 85% and specificity of 90% with a 95% level of confidence.

On day 0 of the child field study, many volunteers were expected to be infected with *P. falciparum*. To provide negative control specimens for the slide readers and dipstick assay technicians to examine, 4 individuals known to be *P. falciparum* negative each provided 10 specimens. 10 of these specimens were allocated to each of the four schools on day 0 and randomly combined with the study group specimens. These specimens were processed and labelled in a manner indistinguishable from specimens from study volunteers to ensure blinding of the dipstick assay technicians and blood-film readers.

On day 6 of the child study, most subjects were expected to be cured of their malaria infection and negative by blood film and the dipstick assay. To provide positive controls, 29 specimens were collected from volunteers of varying ages who visited the outpatient clinic of the Saradidi Rural Health Clinic 1 day before collection of the study group specimens. These control specimens were placed on ice immediately after collection and later stored in a refrigerator for the night. The next morning, identity numbers were generated randomly and assigned to the control specimens. The control specimens were then combined with the study group specimens and blindly evaluated with the dipstick procedure. The control blood slides were combined with the slides collected from the children and sent to the laboratory for examination.

To calculate sensitivity and specificity, the first dipstick reader's interpretation and the results of the two microscopists' blood-film examinations were used. When both slide readers read a slide as positive at 200 WBCs, the average of these two results was used in calculating parasite density. When a slide was reread to 2000 WBCs to verify a negative slide reading by one or both microscopist at 200 WBCs, the result obtained by the final reader was used.

Since all dipsticks were independently examined and interpreted by two individuals, the inter-rater agreement of the visual outcome was assessed with the kappa statistic.¹² Concordance between the two dipstick technicians was also evaluated by stratification according to parasite level.

Results

Adult field study

121 specimens were positive by blood film, of which 74 were also positive by dipstick. In addition, 2 specimens with positive blood films had indeterminate results by dipstick. Table 1 shows several strata of parasite densities, the number of specimens that were positive by dipstick and by blood film within each stratum, and the resulting sensitivity. Of 91 specimens negative by blood film, 82 were negative by dipstick, resulting in a specificity of 90% (95% CI 84–96%). In 8 of the 9 cases positive by dipstick, but negative by blood film, a positive blood film was reported at least once within the previous 3 days. In the ninth case, the individual had a positive blood film the day following the positive dipstick result. These data indicated that persistence of circulating antigen after treatment might have accounted for the discrepancies between dipstick and blood-slide results. We therefore assessed results in 11 individuals who were positive by both dipstick and blood film either on the day treatment was started or the day after and who were followed long enough to have negative blood films subsequently on 2 consecutive days. In 6 of these

Parasites/ μ L	Samples positive by dipstick	Samples positive by blood film	Sensitivity (%)
1–10	3	27	11
11–60	63	86	73
61–100	3	3	100
101–500	5	5	100

Table 1: Sensitivity of dipstick assay by level of parasitaemia in adult field study

Parasites/ μ L	Samples positive by dipstick	Samples positive by blood film	Sensitivity (%)
1-10	2	3	67
11-20	2	3	67
21-40	4	6	67
41	1	1	100

Table 2: Sensitivity of dipstick assay by level of parasitaemia in experimental challenge study

individuals, the dipstick became negative on the same day as the blood film. In 1 individual, the dipstick became negative the day before the blood film. 4 individuals had a positive dipstick after the blood film became negative; 3 remained positive for only 1 day and 1 remained positive for 3 additional days.

Experimental challenge study

Before challenge with infected mosquitoes, 1 of the 20 subjects was positive by dipstick but negative by blood film. The other 19 subjects were negative by both diagnostic methods, resulting in a prechallenge specificity of 95% (85-105%).

13 of the 20 subjects became blood-film positive after challenge. 8 of these 13 individuals had positive dipstick results on the same day that parasites were detected by blood film and 1 had a positive dipstick result 2 days before parasites were detected by blood film. These 9 positive dipstick results and the corresponding parasite levels are shown in table 2. Of the 125 samples tested, 112 specimens were negative by blood film. Of these 112 negative specimens, 110 were negative by dipstick also, resulting in a specificity of 98% (96-101%). 1 subject was positive by dipstick before being assessed positive by blood film, leaving only 1 truly false-positive result.

The subject whose blood was positive by the dipstick assay before challenge with infected mosquitoes continued to have positive specimens by dipstick (8 in all) after challenge even though the individual never developed an infection detectable by blood film. Only the false-positive results obtained from subjects who had negative results before challenge were used in calculating overall specificity.

Child field study

338 blood specimens were collected from the study participants on two occasions. In addition, there were 40 specimens from 4 negative-control volunteers and 29 control specimens from clinically ill individuals. Of these 407 specimens, 356 had matching dipstick and blood film results. Among study participants, 141 of 165 (85%) had positive blood films on day 0, and 0 of 162 had positive blood films on day 6. 18 of 29 clinically ill controls had positive blood films on day 6. The combination of these results included 159 positive blood films, 137 of which were positive by dipstick. In addition, 1 specimen positive by blood film had an indeterminant result by dipstick. Table 3 shows the distribution of positive dipstick results and the resulting sensitivities stratified by parasite density. Both dipstick readings that were negative at parasite densities greater than 1000 parasites/ μ L were from clinically ill controls.

194 blood films were characterised as negative by thick blood film examination of 2000 WBCs and 168 of these specimens were negative by dipstick, resulting in a specificity estimate of 87% (82-91%). In addition, 2 specimens that were negative by blood film had

Parasites/ μ L	Samples positive by dipstick	Samples positive by blood film	Sensitivity (%) (95% CI)
1-10	9	23	39 (19-59)
11-60	17	21	81 (64-98)
61-100	14	16	88 (71-104)
101-500	57	57	100
501-1000	12	12	100
1001-5000	17	18	94 (84-105)
>5000	11	12	92 (76-107)

Table 3: Sensitivity of dipstick assay by level of parasitaemia in child field study

indeterminant results by dipstick. Concordance between microscopy and the dipstick assay was 86% (83-90%; n = 353).

To evaluate the role of persistent antigen in affecting the frequency of false positives on day 6, results from children studied on both days 0 and 6 were compared. Of the 157 children meeting this criterion, 134 had blood films that were positive on day 0 and negative on day 6. 16 (11.9%) of these 134 individuals were positive by dipstick on day 6, and 1 (4.3%) of the 23 individuals negative by blood film on both day 0 and day 6 was positive by dipstick on day 6. The difference in frequency of false positives on day 6 in these two groups was not significant ($p = 0.47$, Fisher's exact test, 2-tailed).

PfHRP-2 antigen is reported to be present in immature gametocytes, but not in mature circulating gametocytes (P Millet, unpublished observations). To see if presence of gametocytes was associated with false positivity, frequency of gametocytaemia among the 17 subjects with false-positive dipstick results on day 6 was compared with the frequency in 139 subjects with true-negative dipstick results on day 6; 3 (17.6%) and 27 (19.4%) subjects, respectively, had gametocytaemia ($p = 1.0$, Fisher's exact test, 2-tailed). Thus, this analysis did not provide any indication that gametocyte presence was responsible for false positivity.

Because all dipsticks were independently examined and interpreted by two technicians, the inter-rater agreement of visual outcome (coloured band) was assessed with the kappa statistic. Among 400 paired dipstick test results, both technicians characterised 159 (39.75%) samples as positive and 211 (52.75%) as negative. The technicians disagreed on 30 (7.5%) pairs; in 15 of these pairs one technician called the sample positive whereas the other technician called it negative, and in 15 of the pairs one technician read the sample as positive or negative whereas the other technician called it indeterminant. Taking outcomes from all 400 pairs of results gave kappa statistics of 0.85 (0.82-0.89) overall, 0.90 (0.87-0.93) for the positive value, and 0.87 (0.84-0.90) for the negative value, all of which represent, beyond chance, excellent agreement between the two independent interpretations of the dipstick's visual outcome.¹² When parasite density was greater than 100/ μ L (n = 99), the kappa was 1.0, which represents excellent agreement between the two dipstick technicians. There was still good agreement at 100 parasites/ μ L or less (n = 61), where the kappa was 0.68 (0.56-0.80).

In the child field study, the prevalence of *P falciparum* infection among 9-14-year-olds was 86% (81-91%; n = 164) when data from day 0 were considered. With this point prevalence, the dipstick assay had a positive predictive value of 96% (93-99%; n = 129) and a negative predictive value of 51% (35-68%; n = 35). Among the controls on day 6, when prevalence was 64% (47-82%,

n=28), the positive predictive value was 93% (80–106%; n=14) and the negative predictive value 64% (40–89%; n=14). These estimates of predictive values will vary according to the *P falciparum* prevalence in the area where the assay is used.

Discussion

New methods for the diagnosis of malaria that complement or supplant the blood smear would be of great use in the diagnosis and treatment of patients with uncomplicated and complicated malaria, in epidemiologic studies, and in field trials of vaccines and new chemotherapeutic agents.¹ Our results indicate that the dipstick antigen-capture assay would be useful in many of these settings. Like a qualitative blood smear, the dipstick assay will not be particularly useful for determining the cause of illness in holoendemic rural areas of sub-Saharan Africa where more than 75% of children always have *P falciparum* parasitaemia. The assay will be most useful for diagnosis of the cause of illness in urban areas of sub-Saharan Africa and in other parts of the world where a smaller percentage of febrile patients have *P falciparum* parasitaemia.

The results from our two field studies show that when *P falciparum* asexual parasitaemia is greater than 60 parasites/ μ L the dipstick test is 96.5–100% sensitive. At lower levels of parasitaemia the sensitivity decreases; however, at 11–60 parasites/ μ L the assay still detects 70–81% of infections, and at 10 parasites/ μ L or less the assay detects 11–67% of infections. Since most individuals with symptomatic *P falciparum* infections have greater than 60 parasites/ μ L blood, the dipstick assay will be of particular use in rapid diagnosis of febrile patients and in epidemiologic field studies. Indeed, in a recent assessment of the cross-sectional prevalence of *P falciparum* in 1007 children aged less than 6 years in western Kenya, most of whom were symptom-free, 951 (94%) were infected and of these 96% had more than 60 parasites/ μ L blood (P McElroy, unpublished observations). Furthermore, because inexperienced microscopists often have difficulty in detecting less than 60 parasites/ μ L, assessment of comparative sensitivity of blood film and dipstick among such technicians may indicate that the dipstick has greater sensitivity than the blood smear.

In the experimental challenge study, we assessed specificity in two ways. First, we tested sera from all 20 volunteers before exposure to sporozoites. Serum from 1 volunteer was positive at this time and remained positive when tested on eight occasions after challenge. We have not determined what factors in this individual's blood accounted for the consistent false positivity. More importantly, since our study of only 20 individuals gave a quite wide 95% confidence interval for specificity (85–105%), larger population-based studies are clearly needed to assess specificity in naive populations. Studies on 50 naive individuals in France indicate that false positivity is not a common finding (M Maret, personal communication). The second way of assessing specificity was to test specimens from the 19 individuals who were not initially positive daily after challenge. The resulting specificity of at least 98% indicates that operator or test error will account for few if any false positives with the dipstick. It is difficult to assess specificity in a country such as Kenya where malaria is holoendemic, because one can never be certain that an individual with a single negative thick blood film is truly parasite free. Nonetheless, in this setting the minimum specificity of the test was 88%

(84–92%) when results from both field studies were combined. Comparable results have been obtained with PfHRP-2-based enzyme-linked immunosorbent assay of fresh and frozen blood⁵ and western blot and dot-blot analyses of plasma.⁷

A potential problem with the dipstick test is that circulating antigen will be detectable after elimination of viable *P falciparum* from the blood stream. Our data (child field study) showed clearly that by day 6 after the start of treatment, there were few such cases. There was no significant difference in the frequency of dipstick false positives on day 6 among the individuals who were positive or negative by blood film on day 0. Furthermore, in the adult field study, in which blood films were read for 6 consecutive days after the start of treatment, only 4 of the 11 individuals so assessed were positive after the blood film was negative (3 for 1 day and 1 for 3 days). These data indicate that PfHRP-2 antigen is not detectable in blood 6 days after starting curative chemotherapy, and suggest that such circulating antigens do not often lead to false-positive tests. In addition, our data provide no evidence that gametocytes are responsible for false-positive results.

It is of concern that 2 individuals with greater than 1000 parasites/ μ L blood were negative by dipstick assay. Variation in PfHRP-2 could not account for the false-negative results. A computer search of GeneBank revealed no isolates of PfHRP-2 that do not include the (AHH[AHHAAD]₂) sequence used to produce the monoclonal antibody attached to the dipstick. Nevertheless, studies of the sensitivity and specificity of the dipstick assay are underway in other areas of Africa and in South America and Asia.

The dipstick assay meets many of the criteria for an ideal diagnostic test: it is sensitive and specific, easy to perform, does not require electricity or equipment, and requires only a small amount of unprocessed whole blood. Furthermore, analysis of a single specimen can be completed within 20 min or of a group of 10 specimens within 30 min. Although additional work is required to establish sensitivity and specificity throughout the world, decrease the time taken to complete the assay, and make the assay available in poor areas of the developing world where it is most needed, our results suggest that the dipstick method for detecting PfHRP-2 antigen in blood may prove to be an important tool in the battle against malaria.

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Pressure sores and pressure-decreasing mattresses: controlled clinical trial

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Summary

Pressure sores are a problem, especially in elderly patients. Our study was designed to determine the effectiveness in pressure-sore prevention of a new interface-pressure decreasing mattress.

In a prospective randomised controlled clinical trial we tested the Comfortex DeCube mattress (Comfortex, Winona, USA) against our standard hospital mattress in 44 patients with femoral-neck fracture and concomitant high pressure-sore risk score. In addition both groups were treated according to the Dutch consensus protocol for the prevention of pressure sores. On admission and 1 and 2 weeks after admission, pressure sores were graded. The two groups were similar in patient characteristics and pressure-sore risk factors. At 1 week, 25% of the patients nursed on the DeCube mattress and 64% of the patients nursed on the standard mattress had clinically relevant pressure sores (grade 2 or more). At 2 weeks the figures were 24% and 68%, respectively. The maximum score over the several body regions of the pressure-sore grading, measured on a 5-point scale, was significantly different in favour of the DeCube mattress at 1 week ($p=0.0043$) and 2 weeks ($p=0.0067$) postoperatively.

We show that the occurrence of pressure sores and their severity can be significantly reduced when patients at risk are nursed on an interface-pressure decreasing mattress.

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Introduction

Pressure sores are an increasing problem in the ageing society of the western world. In the Netherlands, the incidence of pressure sores in bedridden patients in hospital is reported to be as high as 6–20%, and the hospital cost of treatment is estimated at 750 million guilders yearly.¹ A similar situation is reported for the UK.²

Prevention of pressure sores leads to a reduction in suffering, morbidity, bed occupancy, work load, and the cost of health care. Pressure sores result from both intrinsic and extrinsic factors. Among the intrinsic factors, malnutrition,³ anaemia, and hypoproteinaemia are major contributing factors. Among the extrinsic factors, excessive compression of the soft tissues between a bony prominence and the surface of the bed (interface pressure) is well recognised.⁴

The manufacturer of the Comfortex DeCube mattress (Comfortex, Winona, USA) claims that this mattress is effective in preventing pressure sores because the peak interface pressure is reduced.^{5,6} This benefit is achieved by a special surface covering and by the possibility of removing small cubes of the mattress under the bony prominences of the patient. To judge the effectiveness of the DeCube mattress, we assessed in a prospective controlled randomised clinical trial the development of pressure sores on either our standard hospital mattress or the DeCube mattress in patients with a femoral-neck fracture and a concomitant high risk for the development of pressure sores.

Patients and methods

The study was approved by the hospital ethics committee. The pressure-sore grading was as follows:

0 = Normal skin

1 = Persistent erythema of the skin

2 = Blister formation

3 = Superficial (sub)cutaneous necrosis

4 = Deep subcutaneous necrosis

This grading and the pressure-sore risk score (table 1) were assessed according to the Dutch consensus meeting for the prevention of pressure sores in 1985.⁷ For each patient the