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IDENTIFICATION OF CRYPTIC COINFECTION WITH *PLASMODIUM FALCIPARUM* IN PATIENTS PRESENTING WITH VIVAX MALARIA

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Abstract. In Thailand, ~ 8% of patients treated for vivax malaria are found subsequently to have coinfection with *Plasmodium falciparum.* A *P. falciparum* histidine rich protein 2 (PfHRP-2) dipstick test was evaluated as a predictor of mixed infections with subpatent *P. falciparum* in a prospective study of 238 patients admitted to the hospital with acute vivax malaria. Of these, 23 (10%) had subsequent development of falciparum malaria without reexposure. Patients with cryptic *P. falciparum* infection had a significantly lower mean (standard deviation) hematocrit than those with *P. vivax* alone: 29.6 (7.6%) versus 37.2 (6.4%) ($P < 0.0001$). Using microscopic appearance of *P. falciparum* after the start of treatment as the reference standard, the PfHRP-2 test was 74% sensitive and 99% specific in predicting mixed infections with subpatent *P. falciparum* parasitemia at presentation. The PfHRP-2 dipstick test may be a useful adjunct to microscopy in areas where mixed infections are common.

INTRODUCTION

The 4 species of *Plasmodium* that cause malaria in human are *P. falciparum, P. vivax, P. malariae,* and *P. ovale.* Of these, *P. falciparum* causes the most severe disease and nearly all deaths. In Thailand, *P. falciparum* and *P. vivax* cause approximately equal numbers of malaria cases.[1] *Plasmodium vivax* relapse after curative treatment for *P. falciparum,* or subsequent appearance of *P. falciparum* after *P. vivax* clearance, occurs in ~ 30% of cases.[2–6] Mixed infections with *P. falciparum* and *P. vivax* are clinically unpredictable. In Thailand, as in many parts of the world, *P. falciparum* is highly chloroquine resistant[7] and therefore is not suppressed by conventional treatment of vivax malaria. It has not been possible previously to identify mixed infections with submicroscopic *P. falciparum* in patients diagnosed with acute vivax malaria.

Plasmodium falciparum histidine rich protein 2 (PfHRP-2) is a species-specific antigen derived from asexual and immature sexual stages of *P. falciparum.*[8,9] After chemotherapy-induced parasite clearance, PfHRP-2 antigenemia can still be detectable for up to 3–4 weeks.[10–12] This protein may therefore be found in the circulation of patients with hidden *P. falciparum.* Recently, commercially marketed semiquantitative, rapid dipstick tests based on the detection of PfHRP-2 in whole blood have become available widely for the diagnosis of falciparum malaria. These tests have shown high sensitivity and specificity when compared with microscopy of thick blood films and polymerase chain reaction (PCR) detection methods.[13–19] To evaluate the usefulness of one PfHRP-2–based dipstick test in predicting cryptic *P. falciparum* infection in patients initially diagnosed with *P. vivax* malaria, a prospective study was conducted in adult Thai patients admitted to the hospital with *P. vivax* malaria.

MATERIALS AND METHODS

Patients. Adult Thai patients diagnosed with *P. vivax* malaria by microscopy of thin or thick blood films were studied. These patients were admitted to and remained in the Bangkok Hospital for Tropical Diseases, Thailand, for at least 28 days. These were previously untreated patients entered into prospective studies of antimalarial drug efficacy between 1995 and 1999. The results of some of these studies have been reported.[20] None of the patients had underlying diseases or mixed infections recognized initially (defined as the presence of both asexual and sexual stages of other species) at admission microscopy. All patients gave fully informed consent to the study procedures. The patients were treated with one of the following regimens: chloroquine (3 days) plus primaquine (14 days); chloroquine alone (3 days); primaquine alone (7 days); artesunate (5 days); artemether (5 days); pyrimethamine-sulfadoxine (single dose); halofantrine (1 day); azithromycin (3 days); and tetracycline (7 days). This study was a component of studies approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand.

Procedures. Venous blood samples (2–5 mL from each patient) were collected prospectively in heparinized tubes at admission and on the day that *P. falciparum* appeared, or that *P. vivax* reappeared, as detected by microscopy. Parasite counts were performed every 12 hr until clearance and daily thereafter. The counts were expressed as numbers of asexual parasites per microliter of blood and were calculated from the numbers of parasitized cells per 1,000 erythrocytes in a thin film, or per 200 leukocytes in a thick film stained with Giemsa or Field stains. Whole-blood samples were stored at −40°C until use.

Testing with PfHRP-2. The ParaSight-F test (Becton Dickinson, San Jose, CA) was used to measure PfHRP-2 in whole blood.[21] The test was performed by a technician who was blinded to the microscopic and clinical results of the patients. The PfHRP-2 dipstick results were scored for intensity by visual comparison with 9 gradations of pink color intensity on a handheld card to give a value between 0 (negative) and 8 (strongly positive). The semiquantitative test has been validated previously for inter- and intraobserver measurement of agreement.[17,21] Blood smears from patients whose initial blood samples were positive for PfHRP-2 were reexamined by an expert microscopist to see if *P. falciparum* had been overlooked. The subsequent appearance of *P. falciparum* parasitemia, detected by microscopic examination at any time after the start of treatment, was taken as the

reference standard to determine the accuracy of predicting mixed infections with *P. falciparum* by use of the PfHRP-2 ParaSight-F test at admission.

Testing using PCR. All patients with admission samples with positive PfHRP-2 tests, and those diagnosed subsequently as having falciparum malaria, were tested for *P. falciparum* by nested PCR.[22] Briefly, parasite DNA was extracted from the whole-blood samples by means of Qiagen columns (Qiagen, Chatsworth, CA). *Plasmodium falciparum* DNA was then amplified by nested rounds of PCR. The PCR products were electrophoresed on 3% agarose gels, visualized by staining with ethidium bromide and ultraviolet fluorescence, and sized against a 100-bp molecular weight ladder standard (Life Technologies, Gibco BRL, Gaithersburg, MD).

Statistical analysis. Data were analyzed by SPSS version 8.0 for Windows computer software (SPSS, Chicago, IL). The sensitivity, specificity, positive predictive value, and negative predictive value of the ParaSight-F test in predicting mixed infections with cryptic *P. falciparum* at admission were calculated according to standard methods.[23] Comparisons between 2 groups were made by the Mann-Whitney *U*, Student's *t*, and chi-square tests, as appropriate. Correlation was assessed by Pearson's method for normally distributed data and Spearman's method for non–normally distributed data.

RESULTS

Of 310 patients admitted with vivax malaria, 238 completed 28 days of in-hospital follow-up and were eligible for the study. Of these, 4 (1.7%) had recognized mixed infections with *P. falciparum* at admission samples detected by microscopy after reexamination. These patients were excluded from analysis. Admission demographic, clinical, and biochemical data of patients are summarized in Table 1.

The parasite clearance time for *P. vivax* was significantly longer ($P = 0.0001$) and the admission hematocrit significant lower ($P < 0.0001$) in the patients who developed subsequent *P. falciparum* parasitemia compared with those who did not. In addition, the admission hematocrit in the patients with subsequent *P. falciparum* appearance was also significantly lower compared with those who had *P. vivax* reappearance ($P = 0.002$). The parasite clearance times were significantly longer in the patients with *P. vivax* reappearance compared with those who did not ($P = 0.0002$). There was no difference in admission vivax parasitemia and other clinical and biochemical parameters between the patients with subsequent *P. falciparum* appearance compared with those who were cured and those with subsequent vivax reappearance. Admission parasitemia, hematocrit, and other biochemical values were similar between the patients with *P. vivax* reappearance and those who did not develop vivax parasitemia reappearance.

***Plasmodium falciparum* appearance and *P. vivax* reappearance.** Of all patients studied, 36 (15%) had reappearance of vivax parasitemia and *P. falciparum* parasitemia appeared in 23 (10%) after the start of treatment for vivax malaria. The overall mean (standard deviation) interval from admission to *P. falciparum* appearance was 10.4 (6.1) days (range, 2–21 days). The time to onset of subsequent *P. falciparum* and the treatment regimens used in these patients are shown in Table 2. The overall mean (standard deviation) time to vivax reappearance was 23.5 (6.0) days (range, 8–41 days).

The PfHRP-2 dipstick sensitivity and specificity in identifying cryptic *P. falciparum* infections. Of 238 patients tested, 19 had positive PfHRP-2 tests at admission specimens but did not have visible *P. falciparum* parasitemia. Of these 19, 17 developed subsequent appearance of *P. falciparum* (true-positive PfHRP-2 tests), and in 2, *P. falciparum* did not appear by Day 28 (both patients had received tetracycline treatment). Of 215 patients with negative PfHRP-2 dipstick tests at admission, 6 had subsequent *P. falciparum* appearance detected by microscopy (falsely negative), and 209 patients had no detectable subsequent *P. falciparum* infections (truly negative). Therefore, the sensitivity and specificity of the PfHRP-2 dipstick test for predicting mixed infections with cryptic *P. falciparum* in the patients diagnosed initially as having *P. vivax* malaria at admission were 74 and 99%, respectively. The positive and negative predictive values of the PfHRP-2 dipstick test in predicting mixed infections with cryptic *P. falciparum* at admission were 89.5 and 97.2%, respectively.

Correlates of PfHRP-2 intensity scores. The PfHRP-2 color intensity scores at admission were correlated inversely with the interval (day) to *P. falciparum* appearance ($r = -0.50$, $P = 0.037$) (Figure 1) but were not associated with *P. falciparum* parasite density at subsequent appearance.

Polymerase chain reaction for *P. falciparum* results. Of 25 patients (17 truly positive, 6 falsely negative, and 2 falsely positive for PfHRP-2 tests) tested by PCR for *P. falciparum*, 13 were positive. All 6 patients with false-negative PfHRP-2 tests had negative PCR results. Of the 2 patients with false-positive PfHRP-2 tests, 1 was positive by PCR, indicating that this was probably a true positive; and the other patient was PCR negative. Of 17 truly positive findings for PfHRP-2, 12 had positive PCR results indicating that 5 patients had false-negative PCR results. The PfHRP-2 dipstick test detected more mixed infections with hidden *P. falciparum* at admission than did PCR (17 of 23 [74%] versus 12 of 23 [52%]), but this difference was not statistically significant ($P = 0.20$). If the PCR results are included in the calculation for the sensitivity and specificity of PfHRP-2 test (that is, one patient without subsequent *P. falciparum* appearance, but both PfHRP-2 and PCR tests were positive), the sensitivity and specificity of the PfHRP-2 test for predicting mixed infections with cryptic *P. falciparum* are 75 and 99.5%, respectively. Overall, the PfHRP-2 dipstick test in combination with PCR was 67% sensitive in predicting mixed infections with hidden *P. falciparum* at presentation.

DISCUSSION

In this study, a PfHRP-2 dipstick test was found to be more sensitive than light microscopy in detecting cryptic *P. falciparum* infection in patients with vivax malaria. In addition, a low hematocrit level was found more commonly in patients with a mixed infection than with vivax malaria alone. In malaria-endemic areas of Thailand, simultaneous infections with both *P. falciparum* and *P. vivax* are not detected commonly by microscopy.[24,25] However, the late ap-

TABLE 1

Admission demographics and clinical and biochemical data of patients with vivax malaria with and without subsequent development of falciparum malaria*

Variable	Without subsequent falciparum malaria (n = 211)	With subsequent falciparum malaria (n = 23)	P value
Sex, M/F	191/20	18/5	–
Age (yr), mean ± SD	25.0 ± 9.6	22.9 ± 8.1	0.32
Weight (kg), mean ± SD	53.3 ± 8.6	47.2 ± 8.6	0.001
PCT for *Plasmodium vivax* (hr), mean ± SD	82.7 ± 42.0	125.8 ± 86.4	0.0001
PCT for *Plasmodium falciparum* (hr), mean ± SD	–	72.4 ± 31.8	–
FCT for *P. vivax* (hr), mean ± SD	43.2 ± 33.6	54.9 ± 43.0	0.17
FCT for *P. falciparum* (hr), mean ± SD	–	31.7 ± 24.8	–
Onset of *P. falciparum* appearance (day), mean ± SD	–	10.4 ± 6.1	–
Onset of *P. vivax* reappearance (day), mean ± SD	23.5 ± 6.0	–	–
Vivax parasitemia (parasites/μL), geometric mean (95% CI)	6,077 (4,968–7,432)	5,441 (2,516–11,765)	0.74
Subsequent *P. falciparum* parasitemia (parasites/μL), geometric mean (95% CI)	–	133 (76–235)	–
Hematocrit (%), mean ± SD	37.2 ± 6.4	29.6 ± 7.6	<0.0001
Serum total bilirubin (mg %), mean ± SD	1.7 ± 2.9	1.2 ± 0.8	0.42
Serum alkaline phosphatase (U/L), median (range)	48.3 (15.8–536.0)	42.0 (20.8–164.0)	0.19
Serum aspartate amino transferase (U/L), median (range)	27.0 (9.0–735.0)	25.0 (14.0–112.0)	0.55
Serum alanine amino transferase (U/L), median (range)	22.0 (5.0–873.0)	19.0 (10.0–82.0)	0.22
Serum creatinine (mg %), median (range)	1.0 (0.6–25.2)	1.0 (0.7–1.3)	0.32
Blood urea nitrogen (mg %), mean ± SD	14.5 ± 5.9	13.1 ± 5.0	0.30

* FCT = fever clearance time; PCT = parasite clearance time; SD = standard deviation.

TABLE 2

Onset of *Plasmodium falciparum* appearance time and treatment regimens in 23 patients who later developed falciparum malaria

Patient no.	Day of *P. falciparum* appearance	Treatment*
1	2	CQ
2	2	CQ
3	2	CQ
4	21	CQ
5	17	CQ + PQ
6	17	CQ + PQ
7	21	CQ + PQ
8	14	CQ + PQ
9	4	PQ
10	2	PQ
11	4	PQ
12	9	PQ
13	9	PQ
14	9	PQ
15	8	PQ
16	8	AZI
17	12	AZI
18	12	AZI
19	11	AZI
20	19	AZI
21	16	AZI
22	13	HAL
23	7	PS

* AZI = azithromycin; CQ = chloroquine; HA = halofantrine; PQ = primaquine; PS = pyrimethamine-sulfadoxine.

pearance of one species after curative therapy for another (without reexposure) is common.[2–6] Approximately 10% of the patients in this study experienced subsequent *P. falciparum* parasitemia after *P. vivax* clearance. This finding concurs with our earlier observations.[4–6] The majority (85%) of these mixed infections were still negative for *P. falciparum* on microscopy, even when rechecked by an experienced mi-

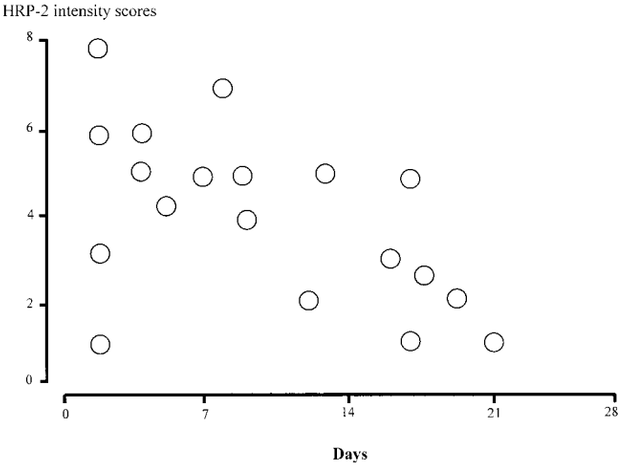

FIGURE 1. Correlation between admission histidine rich protein 2 (HRP-2) intensity scores and interval (days) to *Plasmodium falciparum* appearance.

croscopist. This might in part have resulted from the difficulty in differentiating the young ring forms of *P. falciparum* and *P. vivax.*

The choice of antimalarial drug depends on the species of malaria parasite identified as the cause of infection. Delayed or missed diagnosis of falciparum malaria increases the risk of complicated or severe disease, which may be fatal, especially in nonimmune hosts. This study shows that a simple, generally available, rapid dipstick technique based on detection of PfHRP-2 will detect approximately three-quarters of the mixed infections with cryptic *P. falciparum* in patients diagnosed initially with *P. vivax* malaria with high specificity. Indeed, in this study, the specificity almost certainly exceeded 99%, as 1 of the 2 apparent false-positive findings was PCR positive and the patient had received tetracycline treatment, which is active against *P. falciparum.* Whether it is necessary to apply this method to screen for mixed infections with *P. falciparum* in every patient diagnosed as having vivax malaria at presentation depends on the prior probability (i.e., the geographic distribution of malaria species and the entomological inoculation rates), and the cost of the test compared with the risks and cost of managing the subsequent falciparum malaria.

In endemic areas where both *P. falciparum* and *P. vivax* are prevalent, it may be useful to screen all patients diagnosed microscopically as vivax malaria at presentation for *P. falciparum* infection. Dipsticks are now available to do this. In malaria-endemic areas of Thailand, although the inoculation rate is low (approximately one infectious bite per person-year)[26] double infections with *P. falciparum* and *P. vivax* detected after parasite clearance for one species are common[2–6]; approximately 1 in 10 patients with vivax malaria developed subsequent *P. falciparum* malaria,[4–6] and ~ 1 in 3 with falciparum malaria developed subsequent vivax malaria.[2,3] In Thailand, the standard recommended regimen for vivax malaria treatment is chloroquine for 3 days, followed by primaquine for 2 weeks to eradicate exoerythrocytic forms.[4,27] For mixed infections, the recommended regimen is the same as in treatment for falciparum malaria: 3 days artesunate plus mefloquine or quinine plus tetracycline for 7 days. The cost of diagnosing and treating vivax malaria is ~ US$0.50 and US$2–3 for subsequent falciparum malaria. Therefore, the cost of antimalarials for the first episode of vivax and subsequent falciparum malaria is US$3.50, without taking into account supplemental therapy or hospitalization costs. The use of a dipstick method to screen for hidden *P. falciparum* approximately doubles the cost of vivax diagnosis and treatment (the cheapest available PfHRP-2 test is currently US$0.50 per test).

Mixed infections with *P. vivax* and *P. falciparum* have been associated with anemia.[28,29] Our findings here confirm this: the admission hematocrit levels were significantly lower in patients who developed subsequent *P. falciparum* compared with those who did not.

The blood stages of asexual and immature sexual *P. falciparum* secrete PfHRP-2 into the host's circulation.[8,9] The finding that PfHRP-2 dipstick tests were positive initially in admission blood samples of patients with vivax malaria who developed subsequent *P. falciparum* parasitemia indicates that asexual parasites of *P. falciparum* were present in the circulation in sufficient numbers to yield a positive test when the patients were admitted. This was confirmed by positive PCR results in the majority of these samples. Although clinical observations and artificial infection experiments in humans suggest that *P. falciparum* suppresses *P. vivax* malaria,[2,30–33] the converse may also occur.[4,34–36] Drug treatment of vivax malaria also contributes to suppression of *P. falciparum* parasitemia.

There are 3 possible explanations for the 6 patients who had negative examination microscopy and PfHRP-2 dipstick and PCR tests but subsequently developed *P. falciparum* parasitemia. Newly acquired infections with *P. falciparum* after admission may occur but can be ruled out in this study because all of the patients were monitored in the hospital, where there is no malaria transmission, and none of them received blood transfusion during this hospitalization. Second, the preerythrocytic hepatic stage of *P. falciparum* maturation may have been present only. Third, asexual blood stages of *P. falciparum* may have been present, but the number of parasites was below the level detectable by all 3 methods. The latter 2 possibilities cannot be differentiated.

In Thailand, where patients admitted with acute vivax malaria are commonly coinfected with *P. falciparum,* and this is often cryptic, a PfHRP-2 dipstick test may be useful for patient management. Because subsequent appearance of *P. falciparum* parasitemia can herald the onset of complicated or severe malaria, it may be clinically important to screen for subpatent *P. falciparum* in vivax malaria patients in geographic regions where mixed infections are common.

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