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LABORATORY EVALUATION OF THE ICT MALARIA *P.F./P.V.* IMMUNOCHROMATOGRAPHIC TEST FOR DETECTING THE PANMALARIAL ANTIGEN USING A RODENT MALARIA MODEL

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Abstract. We evaluated the ICT Malaria *P.f./P.v.* immunochromatographic test for the detection of the panmalarial antigen (PMA) using a rodent malaria model. Mice were infected with *Plasmodium berghei* by mosquito bite, and blood was examined by microscopy and the ICT test. Treatment with artemether was started when the parasite density exceeded 70,000/μL. The ICT PMA band appeared when the parasite density was more than 2,000/μL, but it continued to be positive after the parasitemia became negative in response to the drug treatment. When all the test results were divided into increasing phase (IP) and declining phase (DP), the sensitivity in the DP was significantly higher than that in the IP, suggesting that the reactivity of the ICT PMA is significantly influenced by persistent and accumulated PMA after drug treatment and longer duration of infection in the DP. Recognizing that the patient population in a clinical situation would be a mixture of individuals in the IP and DP, it should be emphasized that the individual history of recent fever, duration of illness, and drug treatment must be considered carefully for the interpretation of the ICT results.

INTRODUCTION

A prompt and accurate diagnosis is essential for a reduction of the morbidity and mortality of malaria. Considered as the gold standard, microscopic examination of Giemsa-stained blood films is widely used because of its efficiency and low cost. However, the microscopic technique is time-consuming and requires equipment and trained personnel. In many places where malaria is endemic, the lack of laboratory infrastructure and appropriate human resources hinders the use of diagnostic techniques based on microscopy. To overcome this problem, some rapid diagnostic tests (RDTs) that do not require the use of special equipment or highly qualified personnel have been developed, and it has been established that RDTs have the potential for enhancing speed and accuracy in the diagnosis of both *Plasmodium falciparum* and *P. vivax* malaria, especially in nonspecialized laboratories.[1]

The ICT Malaria *P.f./P.v.* immunochromatographic test (ICT test) is based on the detection of histidine-rich protein 2 (HRP-2) from *P. falciparum*, and a genus-specific panmalarial antigen (PMA) that is the *Plasmodium* aldolase[1] present in all four of the *Plasmodium* species that can cause human malaria.[2–5] In addition, Valecha and others[6] have recently reported that the ICT test can detect PMA of rodent malaria species, including *P. berghei*, in mouse blood.

Whereas a number of studies have described the sensitivity for the detection of *P. falciparum* HRP-2 and the reactivity associated with its prolonged persistence after antimalarial treatment,[2,7–11] available data regarding the sensitivity and the persistence of PMA reactivity have been limited.[12–14] Because the reported sensitivities of the ICT PMA test for human malaria parasites are quite variable, particular concern about the usefulness of the ICT test for diagnosing *P. vivax* malaria has been expressed.[15]

The purpose of the present study was to evaluate the performance of the ICT test for detecting PMA in the blood of *P. berghei*-infected mice. Moreover, we examined the persistent reactivity of the ICT PMA test after antimalarial treatment using a rodent model.

MATERIALS AND METHODS

Parasites. The *P. berghei* ANKA strain, clone 2.34 was maintained by cyclic passage through BALB/c mice and *Anopheles stephensi*.

Mosquitoes. The *Anopleles stephensi* SDA500 strain was a gift from Professor R. E. Sinden (Imperial College, London, United Kingdom). Mosquitoes were maintained in our insectary with a 5% fructose and 0.05% *p*-aminobenzoic acid (Nacalai Tesque, Inc., Kyoto, Japan) solution absorbed in filter paper at 26°C, a relative humidity of 50–70%, and a 14-hour light/10-hour dark cycle.

Mice. Female BALB/c and ICR mice were obtained from Japan SLC, Inc. (Shizuoka, Japan). The care and treatment of mice was approved by the Ethics Committee for Animal Experiments at Jichi Medical School.

Infection and drug treatment of mice. On day 0, mice were fed to starved female *An. stephensi* infected with *P. berghei*. Five to ten mosquitoes were allowed to feed on each mouse for three minutes without interruption.[16] Two microliters of tail blood was taken every day, then 1.5 μL and 0.5 μL of the blood was used for the ICT test and a Giemsa-stained thin blood smear, respectively. In three independent experiments, slightly different regimens of drug treatment were given to the infected mice: Experiment 1: From day 7, when the parasite density reached 430,000–700,000/μL, infected mice (BALB/c) were given artemether (Kunming Pharmaceutical Factory, Yunnan, China) intramuscularly (1 mg/mL in olive oil) at a dose of 4 mg/kg once a day for five consecutive days; Experiment 2: From day 8, when the parasite density reached 210,000–280,000/μL, infected mice (ICR) were given artemether, 4 mg/kg once a day for five consecutive days; Experiment 3: From day 7, when the parasite density reached 70,000–190,000/μL, infected mice (ICR) were given artemether, 4 mg/kg once a day for four consecutive days.

Microscopic examination. Thin blood smears were prepared and stained with Giemsa. To declare a sample negative, smears were read for 300 microscopic fields (50× oil-immersion objective and a 10× ocular lens) without finding a para-

site. If samples were found to be positive, the number of malaria parasites was counted per 5,000–50,000 red blood cells (RBCs) and the percent parasitemia was calculated. Density calculations were based on the approximation of 8×10^6 RBCs/μL in mouse blood at all stages of infection because in our experimental conditions, reduction of the RBC count (< 10%) observed for a short period during drug treatment does not have a serious impact on calculation of parasite density (Arai M, unpublished data).

ICT Malaria *P.f./P.v.* test. The ICT Malaria *P.f./P.v.* test kits (Lot 006530, expiration date November 2002; Binax, Inc., Portland, ME) were used according to the manufacturer's instructions, with a modification of the sample volume. Briefly, 1.5 μL of whole blood, which is 10% of the recommended volume, was taken from a mouse and transferred to the sample pad. Buffer reagent was added to induce cell lysis and allow PMA to bind to colloidal gold-labeled antibodies. Additional buffer caused the blood and immune complex to migrate up the test strip and cross monoclonal antibody lines. Finally, more buffer was added to clear blood from the membrane and facilitate reading.

In this study, when the parasite density was above the detection threshold, *P. berghei*-infected mouse blood resulted in a visible PMA line on the ICT test strip. The test result was scored as - if no line was seen, ± if a faint test line was visible, + if the test line was clear but its intensity was less than that of the control line, ++ if the test line intensity was equal to that of the control line, and +++ if it was greater than the intensity of the control line.

Evaluation of the ICT test for PMA was made by comparison with microscopy used as the gold standard. The variables measured were the numbers of true positives (TP), true negatives (TN), false positives (FP), and false negatives (FN). Sensitivity was calculated as TP/(TP + FN), and specificity was calculated as TN/(TN + FP).[2]

Classification of increasing phase and declining phase. All data were classified into two phases according to the point during the course of infection the blood was examined. The increasing phase (IP) is defined as the period during which the number of parasites increases without influence of the drug. The declining phase (DP) is defined as the period during which the number of parasites is declining because of the drug treatment. When parasites were cleared in the blood smear, the following 48 hours were included in the DP. In treatment-failed cases, the period after 48 hours from parasite clearance was considered as the IP. When decreasing parasitemia was followed by an increase, the turning point was included in the DP, and the subsequent period was taken as the IP.

Statistical analysis. For each ICT PMA test score, the median parasite densities between the IP and DP were compared using the Mann-Whitney U test. All data were again classified by parasite densities and the sensitivities of the ICT PMA test between the IP and DP were compared using the chi-square test.

RESULTS

The reactivity of the ICT test for the detection of PMA was precisely monitored during the course of infection in *P. berghei*-infected mice. Differences in the degree of parasitemia at the first administration of artemether and in the total dose of artemether resulted in different outcomes of the treatment: total failure (Experiment 1, Figure 1A), complete success (Experiment 2, Figure 1B), or partial failure (Experiment 3, Figure 1C). During and after artemether treatment, no prolonged gametocytemia was observed in any experiment.

The ICT PMA test did not detect *P. berghei* with less than 2,000 parasites/μL before drug treatment. When the parasite density reached more than 2,000/μL, the ICT PMA band appeared, and it correlated well with the parasite density (Figure 1). After artemether treatment was started, the intensity of the ICT PMA band was reduced parallel to the parasitemia, but it remained positive after the parasitemia became negative on the blood smear.

All the test results in the mouse experiments were classified into the IP and DP, and parasite density was plotted versus ICT PMA line intensity in each phase (Figure 2). For each test score, a significant difference in the median parasite density was observed between the IP and DP. There were 17 false-negative results (positive by microscopy, but negative by ICT; parasite density range = 40–1,800/μL), all in the IP, and 8 false-positive results (negative by microscopy, but positive by ICT), all in the DP. Overall sensitivity and specificity was 81% and 76%, respectively.

The results in each phase were again classified by parasite density: <1,000/μL, 1,000–10,000/μL, or >10,000/μL. The calculated sensitivity of the ICT PMA test for the detection of *P. berghei* is shown in Table 1. The sensitivity in the DP was significantly higher than that in the IP (100% versus 70%; $P < 0.001$). Such differences in sensitivity between the DP and IP were more apparent at lower parasite densities (100% versus 0% at <1,000/μL; $P < 0.01$ and 100% versus 50% at 1,000–10,000/μL; $P < 0.05$). In both phases, all samples with more than 10,000/μL parasites resulted in positive results. If phase was not considered, the sensitivity was 46% for parasite density <1,000/μL, 72% for 1,000–10,000/μL, and 100% for >10,000/μL.

DISCUSSION

Valecha and others[6] reported that the ICT PMA test can detect rodent malaria parasites, including *P. berghei*, in mouse blood, which encouraged us to evaluate the ICT kit for the detection of PMA using a *P. berghei*-infected mouse model.

In the present study, the sensitivity for PMA was significantly higher in the DP than that in the IP, and all the false-positive results were observed only in the DP. Our findings in mice have been previously reported in a study of *P. falciparum* malaria. Tjitra and others[14] demonstrated that the ICT test sensitivity for both PMA and HRP-2 was lower in recrudescent infections (comparable with the IP in mice) than in the initial clinical presentation with a similar level of parasitemia (comparable to a mixture of the IP and the DP).

One explanation of our findings is the persistence of PMA after clearance of parasitemia. This possibility is based on the findings that HRP-2 used in the ICT test for the detection of *P. falciparum* has been shown to persist and be detectable for up to four weeks after the parasites have been cleared by antimalarial treatment,[2,7–11] resulting in high sensitivity at low parasitemia and false-positive results.[17] However, the avail-

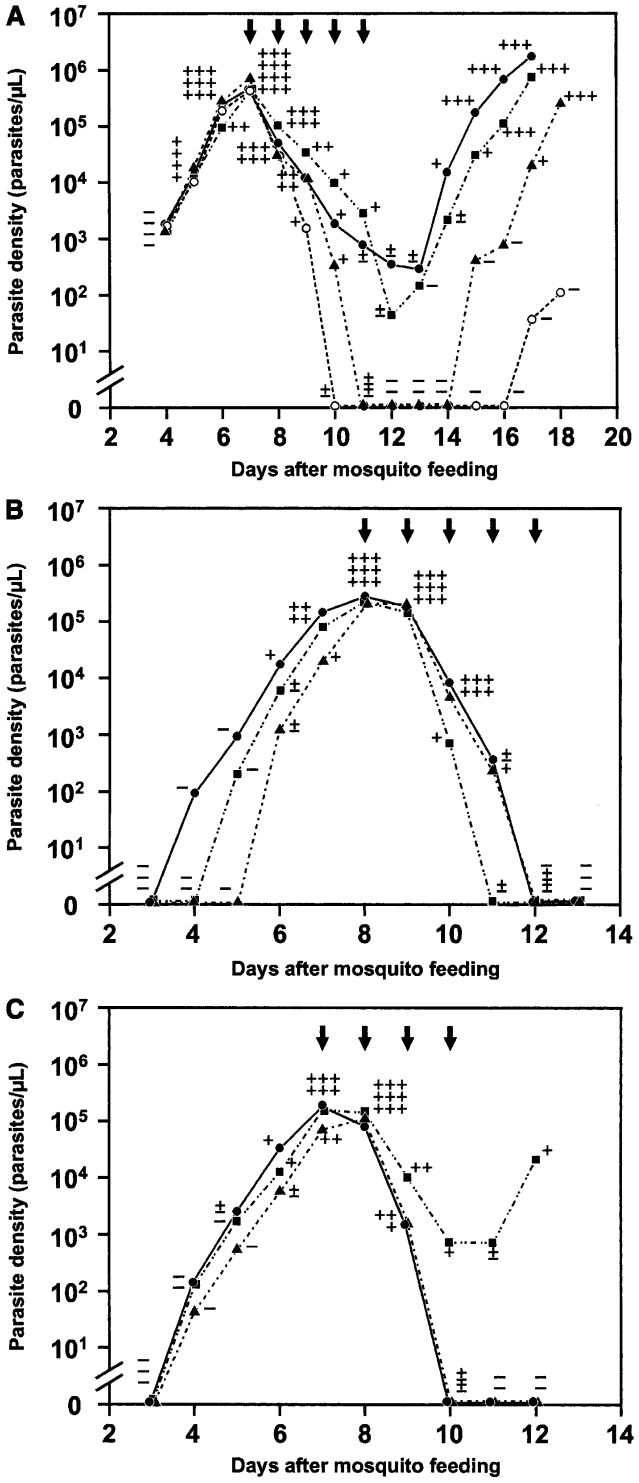

FIGURE 1. Results of microscopy and the ICT Malaria *P.f./P.v.* test with the blood of *Plasmodium berghei*–infected mice. Mice infected with *P. berghei* by infectious mosquito feeding were examined by microscopy and the ICT panmalarial (PMA) antigen test. **A**, Experiment 1: Treatment was started on day 7 when the parasite densities were 430,000–700,000/μL. **B**, Experiment 2: Treatment was started on day 8 when the parasite densities were 210,000–280,000/μL. **C**, Experiment 3: Treatment was started on day 7 when the parasite densities were 70,000–190,000/μL. Artemether was injected intramuscularly at a dose of 4 mg/kg, once a day for four (**C**) or five (**A** and **B**) consecutive days. Each line indicates an individual course of parasite density. The ICT PMA test results are shown adjacent to each point. **Arrows** represent intramuscular injections of artemether.

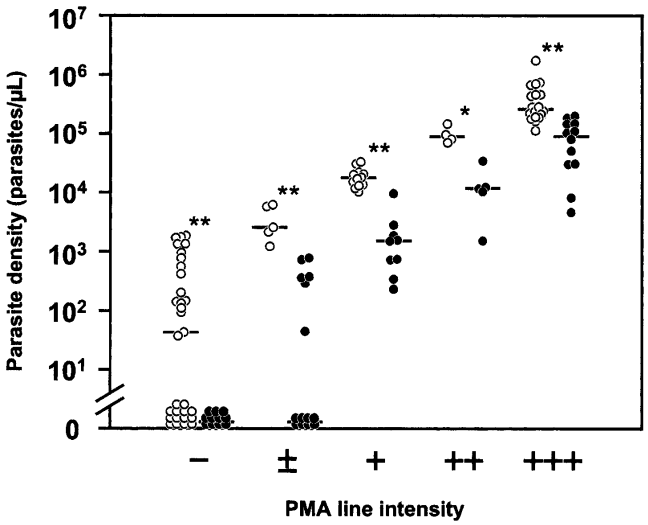

FIGURE 2. Plot of parasite density versus line intensity of the ICT Malaria *P.f./P.v.* test for the panmalarial antigen (PMA). All test results were divided into two phases, increasing phase (○) and declining phase (●), according to the point during the course of infection the blood was examined (see Materials and Methods for the classification criteria). Horizontal lines indicate medians. $*P < 0.05$ and $**P < 0.01$ indicate significant differences from corresponding results in the declining phase by the Mann-Whitney U test.

able information about PMA reactivity after drug treatment and clearance of parasitemia is relatively limited.[12–14] Eisen and Saul[12] demonstrated that PMA reactivity in the ICT test paralleled the decline of parasitemia of *P. falciparum* and *P. vivax*, suggesting only minor prolongation of PMA after clearance of parasitemia in the absence of gametocytes. In contrast, Tjitra and others[13] reported that persistent reactivity of the ICT PMA test for *P. falciparum* was observed up to four weeks after treatment with chloroquine or sulfadoxine-pyrimethamine, which was probably due to post-treatment gametocytemia. These investigators also demonstrated that therapy with artesunate plus sulfadoxine-pyrimethamine was followed by rapid clearance of PMA, which paralleled the rapid clearance of *P. falciparum* gametocytemia. This is consistent with the findings of our present study in which no prolonged *P. berghei* gametocytemia was observed.

Another possible factor affecting the sensitivity of the ICT PMA test is duration of infection. Bechem and others[18] sug-

TABLE 1

Sensitivity of the ICT Malaria *P.f./P.v.* test for detection of the panmalarial antigen in *Plasmodium berghei*–infected mouse blood at different phases

Parasite density (/μL)	Sensitivity (%)†		
	Increasing phase	Declining phase	Overall
<1,000	0 (0/12)	100 (10/10)‡	46 (10/22)
1,000–10,000	50 (5/10)	100 (8/8)§	72 (13/18)
>10,000	100 (34/34)	100 (14/14)	100 (48/48)
Overall	70 (39/56)	100 (32/32)¶	81 (71/88)

* See Materials and Methods for the classification criteria.
† Values are the no. ICT positive/no. microscopy positive.
‡ Significantly different from increasing phase ($P < 0.01$) by chi-square test.
§ Significantly different from increasing phase ($P < 0.05$) by chi-square test.
¶ Significantly different from increasing phase ($P < 0.001$) by chi-square test.

TABLE 2
Reported sensitivities of the ICT Malaria *P.f./P.v.* test for the detection of *Plasmodium vivax*

Study site	Patient population		Parasite density (/μL)	Sensitivity (%)	Reference
	Symptomatic or asymptomatic	Patients with history of recent malaria treatment			
Eastern Indonesia	Symptomatic	Included	<499	29	Tjitra and others, 1999[2]
			≥500	96	
Central India	Symptomatic	Included	≤1,500	60	Singh and others, 2000[19]
			>1,500	100	
Australia	Symptomatic (inpatients)	All	≤1,000	38	Eisen and Saul, 2000[12]
			>1,000	83	
Southern Vietnam					Huong and others, 2002[20]
Dac Lac	Symptomatic	Excluded	≤1,000	0	
			1,000–10,000	38	
			10,000–100,000	100	
Binh Phuoc	Symptomatic	Excluded	≤1,000	0	
			1,000–10,000	14	
			10,000–100,000	89	
Western Thailand	Mainly asymptomatic	Not specified	<100	0	Coleman and others, 2002[17]
			100–499	0	
			≥500	67	
Australia	Symptomatic (returned travellers)	Not specified	<100	0	Playford and others, 2002[21]
			100–1,000	7	
			1,000–10,000	52	
			>10,000	100	

gested that the ability to accurately diagnose low parasitemia with antigen tests may depend on not only the number of parasites, but also the length of infection, i.e., the level of antigen accumulating in plasma. Tjitra and others[14] also suggested that low sensitivity of the ICT test for both PMA and HRP-2 early in recrudescence is due to a shorter duration of recrudescent infection relative to the initial clinical illness. When one recognizes that duration of infection in the present study is necessarily longer in mice DP than in the IP, higher sensitivity observed in the DP may reflect accumulation of PMA during the infectious course.

The reported sensitivity of the ICT PMA test for human malaria parasites varies considerably depending on the study. Eisen and Saul[12] reported that the threshold level for PMA detection was approximately 100 parasites/μL for *P. falciparum* and *P. vivax*. Iqbal and others[15] demonstrated that the sensitivity was only 23% at <500 parasites/μL for *P. falciparum* and *P. vivax*. To understand this variation, we reviewed previous studies reporting sensitivities of the ICT PMA test for *P. vivax* (Table 2). If the patient population consisted mainly of symptomatic individuals, and those with history of recent malaria treatment were not excluded, the observed sensitivities of the ICT PMA test were relatively high.[2,12,19] It is likely that a considerable number of patients in these studies were in the DP and had temporarily prolonged or accumulated PMA in plasma, which may have increased the apparent sensitivity.[17] Conversely, in the studies in which patients with a recent history of malaria were excluded, or the patient population consisted mainly of asymptomatic individuals, the sensitivities for PMA were relatively low.[17,20] It may be presumed that most of the patients in these studies were in the IP, and the observed sensitivity reflected the true sensitivity of the ICT test. It should be emphasized that in clinical situations, the patient population would be a mixture of individuals in either the IP or the DP. Moreover, the lower sensitivity of the ICT PMA test in a study of febrile returned travelers[21] likely reflects the short duration of infection in non-immune travelers relative to residents of endemic areas.[13,14] In endemic areas, a degree of immunity results in higher fever thresholds and allows for greater chronicity before seeking treatment,[2,22] which likely contributes to a higher sensitivity for antigen tests. We therefore hypothesize that the detection sensitivity of the ICT PMA test may depend on both the infection phase (IP or DP) and the duration of infection.

All the ICT PMA tests in the present study were performed with 1.5 μL of mouse blood, which is 10% of the recommended volume. Such a reduced sample volume was used because 1) the available volume of mouse blood is very limited, and 2) in a pilot experiment, we did not see any case in which the ICT PMA band was visible with 5 μL or 15 μL of blood but was invisible with 1.5 μL of blood, although a 1.5-μL sample always gave a weaker PMA band than those of the 5-μL or 15-μL samples (Arai M, unpublished data). When we recognize that we cannot exclude the possibility that using smaller volumes of blood samples may have lowered the sensitivity of the ICT PMA test, it should be noted that the findings in the mouse model cannot be directly extrapolated to human malaria.

In conclusion, ICT kit users should be aware that 1) antimalarial treatment results in temporarily prolonged circulation of PMA, which causes inappropriately high sensitivity and false-positive results, 2) the true sensitivity for PMA may be approximately 2,000 parasite/μL, and 3) individual history of recent fever, duration of illness, and drug treatment must be carefully considered for the interpretation of the test results.

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