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EVALUATION OF A RAPID DIPSTICK TEST, MALAR-CHECK™, FOR THE DIAGNOSIS OF *Plasmodium falciparum* MALARIA IN BRAZIL

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SUMMARY

The present study was carried out to evaluate the Malar-Check™ *Pf* test, an immunochromatographic assay that detects *Plasmodium falciparum* Histidine Rich Protein II, does not require equipment, and is easy and rapid to perform. In dilution assays performed to test sensitivity against known parasite density, Malar-Check™ were compared with thick blood smear (TBS), the gold standard for diagnosis. Palo Alto isolate or *P. falciparum* blood from patients with different parasitemias was used. The average cut-off points for each technique in three independent experiments were 12 and 71 parasites/mm^3 (TBS and Malar-Check™, respectively). In the field assays, samples were collected from patients with fever who visited endemic regions. Compared to TBS, Malar-Check™ yielded true-positive results in 38 patients, false-positive results in 3, true-negative results in 23, and false-negative result in 1. Malar-Check™ performed with samples from *falciparum*-infected patients after treatment showed persistence of antigen up to 30 days. Malar-Check™ should aid the diagnosis of *P. falciparum* in remote areas and improve routine diagnosis even when microscopy is available. Previous *P. falciparum* infection, which can determine a false-positive test in cured individuals, should be considered. The prompt results obtained with the Malar-Check™ for early diagnosis could avoid disease evolution to severe cases.

KEYWORDS: Malaria diagnosis; *Plasmodium falciparum*; Rapid dipstick test; Immunocapture assay; Malar-Check; Histidine Rich Protein II.

INTRODUCTION

In the last 2 years, 600 thousand of malaria cases/year were notified in Brazil, where most of the *Plasmodium falciparum* infections in the Americas occur[21]. *Falciparum* malaria is increasing 15% a year, causing an annual increment of 10% in the number of registered deaths (www.funasa.gov.br).

A prompt and accurate diagnosis is essential for a reduction of the morbidity and mortality of the disease. Therefore, there is a constant search for new diagnostic alternatives to the Giemsa-stained thick blood smear (TBS), which continues to be used today[17]. Considered as the gold standard, TBS is widely employed because of its efficiency and low cost. However, this technique presents low sensitivity, detecting 10 parasites/µl and requires equipment and trained personnel[4].

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 immunochromatographic tests that spare the use of equipment and highly qualified personnel are currently available. The *Para*Sight™ F test (Becton Dickinson Europe), Malar-Check™ *Pf* test (Cumberland Diagnostics Ltd.) and others[7] use a double sandwich for the detection of the *P. falciparum* Histidine Rich Protein (*Pf*HRPII) in whole blood samples. *Pf*HRPII is a water-soluble protein produced by asexual blood stages and young gametocytes[10] and observed in all *P. falciparum* isolates[16]. The principle of the *Para*Sight™ F test has been described elsewhere[18]. The Malar-Check™ *Pf* test uses a monoclonal anti-*Pf*HRPII antibody conjugated with colloidal gold that complexes with *Pf*HRPII in the lysed sample. This complex moves on the nitrocellulose membrane to the region where it is captured by a monoclonal anti-*Pf*HRPII antibody immobilized on the membrane, leading to the formation of a pink coloured band, which confirms a positive test result. The unreacted conjugate and unbound complex, if any, move further on the membrane and are subsequently captured by anti-mouse antibodies fixed on the membrane in the control region, originating a pink band that serves to validate the test performance (Fig. 1). The OptiMAL® Rapid Malaria test (DiaMed SA) is an immunochromatographic test that detects the presence of *Plasmodium* lactate dehydrogenase (pLDH), an enzyme produced both in the sexual and asexual forms of the parasite. The presence of pLDH is revealed using monoclonal antibodies directed against isoforms of the enzyme and permits to distinguish between *Plasmodium falciparum* and other *Plasmodium* species (*P. vivax*, *P. malariae* or *P. ovale*)[14].

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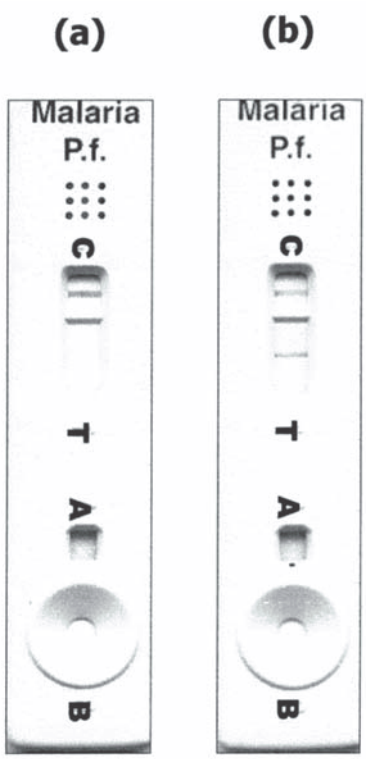

Fig. 1 - Malar-Check™ *Pf* test showing a negative sample (a) and a positive *P. falciparum* sample (b). **C**, Control region; **T**, Test region; **A**, Blood region; and **B**, Reagent region.

The objective of the present study was to determine the performance of the Malar-Check™ *Pf* test in detecting the *P. falciparum* antigen in the blood of Brazilian patients with malaria. Moreover, we assessed the sensitivity of the Malar-Check™ *Pf* test compared with Giemsa-stained TBS as the gold standard.

MATERIAL AND METHODS

Dilution assays

Comparison of the Malar-Check™ Pf test and TBS. The *P. falciparum* Palo Alto isolate was used after culturing by the candle jar method[20]. Parasitized red blood cells were mixed with culture medium to prepare a 50% hematocrit sample with 81,940 parasites/mm^3. By adding a volume of parasitized blood to an equal volume of uninfected donor blood, 14 serial dilutions up to 5 parasites/mm^3 were prepared. TBS and Malar-Check™ *Pf* test were applied to each dilution.

Comparison of the Malar-Check™ Pf test, ParaSight™ F test and TBS. Blood was collected from a patient with 13,400 parasites/mm^3 and diluted with uninfected donor blood to prepare dilutions containing 1,340, 134, 77, 33 and 16 parasites/mm^3. The Malar-Check™ *Pf* test, *Para*Sight™ F test and TBS were performed with each dilution.

Comparison of the Malar-Check™ Pf test, ParaSight™ F test, OptiMAL® Rapid Malaria test and TBS. Blood was collected from a patient with 40,000 parasites/mm^3 and diluted with uninfected donor blood to prepare dilutions with 4,000, 400, 200, 100, 50, 25 and 12 parasites/mm^3. The Malar-Check™ *Pf* test, *Para*Sight™ F test, OptiMAL® Rapid Malaria test and TBS were applied to each dilution.

Field assays

Subjects and sample collection. Blood samples were collected by digital puncture from 65 patients presenting fever (40 collected in São Paulo, São Paulo State, from individuals who were in malaria endemic regions and 25 collected in Santarém, Pará State, Amazon Region). Samples were collected from 7 patients 1, 7, 14 and 30 days after the beginning of treatment to monitor parasitemia clearance. All the blood samples were collected after informed consent from the patients.

Microscopic examination. TBS were prepared for each patient during the blood collection process. After blood films were stained with Giemsa, the species and density of the parasites were determined by two experts in malaria diagnosis. Parasitemia was calculated by the number of parasites per 100 leucocytes based on the assumption of 6,000 leucocytes/mm^3 of blood. A total of 100 oil immersion fields were scanned before a slide was considered negative.

Rapid immunocapture assays. The blood samples were also submitted to the *Para*Sight™ F test, Malar-Check™ *Pf* test and OptiMAL® Rapid Malaria test. The assays were performed in parallel according to manufacturer instructions and independently examined.

Analysis of field data. The sensitivity and specificity of the Malar-Check™ *Pf* test were calculated using microscopy as the gold standard.

Cross-reactivity with rheumatoid factor

A total of 18 positive sera for rheumatoid factor (titers > 160) from patients without malaria history were submitted to the Malar-Check™ *Pf* test. The samples were kindly provided by Laboratório de Investigação em Reumatologia, Faculdade de Medicina, Universidade de São Paulo.

RESULTS

Dilution assays. The dilution experiments were designed to determine the cut-off point for each technique, defined as the highest dilution at which the test was recorded as positive. In a first experiment using the *P. falciparum* Palo Alto isolate, we compared the Malar-Check™ *Pf* test and TBS, which detected 80 and 10 parasites/mm^3, respectively. Also, we compared the Malar-Check™ *Pf* test, *Para*Sight™ F test and TBS using blood from a patient with 13,400 parasites/mm^3. TBS, Malar-Check™ *Pf* test and *Para*Sight™ F test were positive up to 16, 33 and 77 parasites/mm^3, respectively. Additionally, we compared the Malar-Check™ *Pf* test, *Para*Sight™ F test, OptiMAL® Rapid Malaria test and TBS using blood from a patient with 40,000 parasites/mm^3. TBS, Malar-Check™ *Pf* test, *Para*Sight™ F test and OptiMAL® Rapid Malaria test were positive up to 2, 100, 50 and 400 parasites/mm^3, respectively.

Field study. A total of 65 blood samples were tested for *P. falciparum* by the Malar-Check™ *Pf* test and the results were compared to those obtained from TBS. The test was true-positive in 38 patients, false-positive in 3, true-negative in 23 and false-negative in only 1. Sensitivity

was 97.4% (38/39) and specificity 88.5% (23/26). The positive predictive value (PPV) was 92.7% (38/41) and the negative predictive value (NPV) 95.8% (23/24) (Table 1). Of the samples studied, 9 presented *P. vivax* and 1 presented *P. malariae.* Only the Malar-Check™ *Pf* test with *P. malariae* was positive. In seven *P. falciparum* patients, blood was collected 1, 7, 14 and 30 days after the beginning of treatment to monitor parasitemia clearance, and the results showed persistence of the antigen up to 30 days.

Cross-reactivity with rheumatoid factor. All sera with rheumatoid factor were negative to the Malar-Check™ *Pf* test.

Table 1
Comparison of Malar-Check™ *Pf* test results with Thick Blood Smear (TBS) results in field study for the diagnosis of *Plasmodium falciparum* infection in 65 blood samples collected in São Paulo / Santarém, Brazil, from individuals presenting fever, who were in malaria endemic regions

		Malar-Check™ *Pf* test		
		Positive	Negative	Total
TBS*	Positive	19 / 19	01 / 00	20 / 19
	Negative	03 / 00	17 / 06	20 / 06
	Total	22 / 19	18 / 06	40 / 25
Sensitivity = 38/39 = 97.4%		PPV = 38/41 = 92.7%		
Specificity = 23/26 = 88.5%		NPV = 23/24 = 95.8%		

* Diagnosis by two microscopists; PPV = Positive Predictive Value, NPV = Negative Predictive Value

DISCUSSION

New methods for malaria diagnosis that complement or even substitute the TBS would be of great usefulness for the control of the disease. Rapid immunocapture assays would be excellent for this purpose, since intensive training and equipment are unnecessary. However, sensitivity is the main requirement for diagnostic application. Therefore, we tested the Malar-Check™ *Pf* test, a new dipstick assay, using a laboratory isolate with different parasitemias and field samples of *P. falciparum* from Brazil.

The Malar-Check™ *Pf* test was able to detect more than 33 parasites/mm^3, while the TBS was positive up to 10 parasites/mm^3, as described in the literature[4]. When compared to another dipstick test that detects *Pf*HRPII (*Para*Sight™ F test), the Malar-Check™ *Pf* test had a high correlation, although it was more sensitive in one of two experiments. As expected, the Malar-Check™ *Pf* test was four times more sensitive than the OptiMAL® Rapid Malaria test, possibly because the latter detects only viable parasites, while the Malar-Check™ *Pf* test detects *Pf*HRPII, that can be present up to 28 days after treatment and parasitemia clearance[1].

In the field study, the Malar-Check™ *Pf* test showed sensitivity up to 240 parasites/mm^3. Malar-Check™ *Pf* test failed to detect *P. falciparum* in one patient with a mixed infection with *P. vivax*; however, the TBS showed *P. falciparum* only in the stage of mature gametocyte. In this slide, all the parasites in the young trophozoite stage presented Schüffner's dots. In this study, three tests showed false-positive results, but we may assume that this probably occurred because of circulating antigen that remained after a recent *falciparum* infection. In the case of *P. malariae*, a previous infection with *Plasmodium* was described by the patient less than one month before, but the species was not identified. In the other two cases the patients were not able to report their last *Plasmodium* species.

As reported in the literature, the OptiMAL® Rapid Malaria test showed sensitivity ranging from 88.5 to 94%[11,19]. For the tests based on the detection of *Pf*HRPII, such as the *Para*Sight™ F test, sensitivity has been reported to range from 84.2 to 96.5%[3,15], in agreement with that found in the present study using the Malar-Check™ *Pf* test (97.4%). Previous studies have revealed that the OptiMAL® Rapid Malaria test presented specificity ranging from 92 to 99.4%[5,11]. However, the *Para*Sight™ F test showed values from 72.1 to 97%[6,18], similar to the results obtained in the present study using the Malar-Check™ *Pf* test (88.5%).

Many studies have described false-positive reactions when *Pf*HRPII-based immunocapture diagnostic assays were applied to rheumatoid factor-positive sera[2,8,9,12,13]. This prompted us to assay the Malar-Check™ *Pf* test in samples collected from patients with high titers of rheumatoid factor. Using the Malar-Check™ *Pf* test none of these samples showed false-positive results, differently from the other findings.

We conclude that Malar-Check™ *Pf* test should aid the diagnosis of *P. falciparum*, since the ready execution of the test for early diagnosis in places where TBS cannot be performed could avoid evolution of the disease to severe cases and death. Moreover, the use of rapid dipstick tests could reduce the spread of drug resistance, eliminating the need for presumptive treatments. Some antimalarials are expensive, and therefore using dipsticks instead of treatment based on clinical diagnosis will be more cost effective for malaria control programs. Also, the test could improve the routine diagnosis (TBS), detecting mixed infections when microscopy would not be able to differentiate among young trophozoites of different species. However, when tests that detect *Pf*HRPII are used, previous *P. falciparum* infection should be considered, since it may cause a false-positive test in cured individuals.

RESUMO

Avaliação de um teste rápido em fita, Malar-Check™, para o diagnóstico de malária por *Plasmodium falciparum* no Brasil

Este trabalho avaliou o Malar-Check™ *Pf* test, ensaio imunocromatográfico que detecta a proteína rica em histidina de *Plasmodium falciparum*, dispensa uso de equipamentos, é rápido e de fácil execução. Ensaios de diluição com o isolado Palo Alto ou sangue de pacientes com *P. falciparum*, foram realizados para testar a sensibilidade em diferentes densidades do parasita. Malar-Check™ foi comparado à gota espessa (GE), padrão ouro para diagnóstico de malária. A média do limiar de sensibilidade para cada técnica em três experimentos independentes foi de 12 e 71 parasitas/mm^3 (GE e Malar-Check™, respectivamente). Em ensaios de campo, amostras foram coletadas de pacientes febris de áreas endêmicas. Comparado à GE, Malar-Check™

foi verdadeiramente positivo em 38 pacientes, falso positivo em 3, verdadeiramente negativo em 23 e falso negativo em um. Malar-Check™ realizado com sangue de pacientes com *P. falciparum* após tratamento mostrou persistência do antígeno durante 30 dias. Malar-Check™ pode ser útil no diagnóstico de *P. falciparum* em áreas remotas e auxiliar a rotina diagnóstica, mesmo quando a microscopia está disponível. Deve ser considerada infecção pregressa por *P. falciparum*, que pode determinar testes positivos em indivíduos curados. A rapidez do Malar-Check™ para o diagnóstico precoce pode evitar evolução para casos graves.

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