

EVALUATION OF DIFFERENT METHODS FOR *PLASMODIA* DETECTION, IN WELL DEFINED POPULATION GROUPS IN AN ENDEMIC AREA OF BRAZIL (1).

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SUMMARY

In Brazil, more than 500,000 new cases of malaria were notified in 1992. *Plasmodium falciparum* and *P. vivax* are the responsible species for 99.3% of the cases. For adequate treatment, precece diagnosis is necessary. In this work, we present the results of the traditional *Plasmodia* detection method, thick blood film (TBF), and the results of alternative methods: Immunofluorescence assay (IFA) with polyclonal antibody and Quantitative Buffy Coat method (QBC)[®] in a well defined population groups. The analysis were done in relation to the presence or absence of malaria clinical symptoms. Also different classes of immunoglobulins anti-*P. falciparum* were quantified for the global analysis of the results, mainly in the discrepant results. We concluded that alternative methods are more sensitive than TBF and that the association of epidemiological, clinical and laboratory findings is necessary to define the presence of malaria.

KEYWORDS: Malaria; *Plasmodia* detection; diagnosis; IFA; QBC.

INTRODUCTION

Malaria is one of the most serious public health problems in the world. In Brazil, more than 500,000 new cases per year are reported. Two species of *Plasmodia* are responsible for 95% of the cases: *P. vivax* (59.1%) and *P. falciparum* (40.2%). Cases of *P. malariae* are occasionally diagnosed in the northern areas of the country. Severe *P. falciparum* malaria occurs mainly in non-immune subjects and a rapid clinical intervention is necessary⁴. Rapid diagnosis is also very important in non-endemic areas that receive patients from endemic areas: travellers that develop clinical malaria and transfusion malaria^{6,7,8}. Traditionally, malaria diagnosis is done by the TBF that has low sensitivity and needs skilled microscopists to detect and differentiate the *Plasmodia*

species. However, new methods are appearing²⁵. Immunologic methods for circulating antigen detection have shown limitations in sensitivity and specificity when used in field conditions^{9,12,14,19,20}. Immunologic methods for antibody detection have been useful in the non-endemic areas but are of limited value in endemic areas¹⁰. The attempts to select antigenic bands with activity in the different stages of the disease are still in study in several laboratories². Some bands show interest and may be further studied. The molecular biology methods, such as DNA or RNA probes and PCR, also show limited value in the field^{3,13,15,16,25}. Two tests for *Plasmodia* detection have been studied with success, as alternative methods to TBF, the IFA and QBC[®]

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method^{14, 17, 21, 23}. In this work we present the results of the TBF and of alternative methods, IFA and QBC and analysed them in relation to the presence or absence of malaria clinical symptoms in a well defined clinical and epidemiological population. Also different anti-*P. falciparum* immunoglobulin classes were quantified to establish the probability of malaria in patients with discrepant results.

MATERIAL AND METHODS

Study area

Endemic areas were selected, located in Amazon Region, Mato Grosso State - northern Brazil, that received immigration of people from non-endemic region. In these areas, Alta Floresta, Peixoto de Azevedo and Terra Nova, goldmine and colonization projects are being developed and the transmission of the disease is related to several factors as inadequate mosquito control, inappropriate housing and poverty.

Samples

Two hundred and three subjects were selected for clinical and epidemiological studies and distributed in five groups. Twenty five subjects were the control group. From each subject, venous blood was collected in two tubes, one with anticoagulant and other without. The groups were:

Group 1 - (medium age: 29, 28 females and 61 males) 89 subjects with malaria clinical symptoms, high malaria exposure and positive TBF: *P. falciparum* (36), *P. vivax* (41) and mixed *vivax* and *falciparum* malaria (12);

Group 2 - (medium age: 32, 18 females and 46 males) 64 subjects with malaria clinical symptoms, high and medium malaria exposure and negative TBF;

Group 3 - (medium age: 30, 07 females and 12 males) 19 subjects upon anti-malarial therapeutic with negative TBF;

Group 4 - (medium age: 26, 01 female and 01 male) 2 subjects without malaria clinical symptoms, with high malaria exposure and *P. falciparum* positive TBF.

Group 5 - (medium age: 35, 09 females and 20 males) 29 subjects without malaria clinical symptoms, with high and medium malaria exposure and negative TBF;

Group 6 - samples of 25 blood donors from São Paulo who were never in endemic area and were used as control.

P. falciparum antigen extract

P. falciparum, Adalberto strain, was cultured *in vitro* in human erythrocytes according the method of TRAGER & JENSEN²⁶. The infected erythrocytes with *P. falciparum* mature forms were centrifuged at 1,500 x g for 10 min at 4°C and the sediment was washed 3 times with phosphate buffer saline 0.01 M, pH 7.2 (PBS), lysed with 0.04% saponin in PBS for 20 min at room temperature, centrifuged at 3,000 x g for 15 min, and washed 3 times with PBS. The antigen extraction was done with 1% Zwittergent in PBS containing protease inhibitors (1mM PMSF, 25 µg/ml leupeptin, 25 µg/ml antipain). The solution was homogenized and centrifuged at 6,000 x g for 30 minutes at 4°C. The supernatant was dialyzed with PBS overnight at 4°C and the protein content was evaluated by the LOWRY method¹⁸.

Antisera

Anti-P. falciparum polyclonal antiserum: *P. falciparum* antigens were emulsified in Freund's complete adjuvant and injected subcutaneously on the dorsal surface of rabbits. After 7 days, new inoculations were made with Freund's incomplete adjuvant, subcutaneously. After 7 days, three inoculations with the antigen diluted in PBS were made intravenously in the peripheral ear vein at 3 days intervals. *Anti-P. falciparum* antibodies were evaluated by Enzyme Linked Immunosorbent Assay (ELISA). The antiserum obtained was purified by caprylic acid precipitation, ammonium sulfate-fractionation, and affinity chromatography CNBR-4B Sepharose column containing non infected red blood cells ghosts, as ligand.

Sheep anti-human IgG, IgM and IgA, specific for heavy chain were acquired commercially (Biolab Diagnostica S. A.).

Conjugates

Sheep anti-rabbit IgG fluorescent conjugate and sheep anti-rabbit IgG conjugated to peroxidase were acquired commercially (Biolab Diagnostica S. A.). Sheep anti-human IgG, IgM, IgA sera were conjugated to peroxidase, as described by WILSON & NAKANE²⁷.

Thick blood film (TBF)

The thick films (N=228) were prepared using 10-25 µl of blood spread over a 10 x 20 mm area of glass slide in duplicate and read independently by two experienced microscopists at x 1000 magnification. The blood film was scanned until 100 microscopic fields had been viewed. The number of parasites and white blood cells

present in 100 microscope fields was determined and the average number of parasites per white blood cells calculated. This figure was multiplied by 6000 (the assumed white blood cells count) to give the parasite density per mm³.

IFA for antigen detection

Two hundred and twenty five blood samples were tested as described by PROU²². The antiserum used was the rabbit anti-*P.falciparum* polyclonal serum (10 µg/ml) diluted in PBS containing 1% Tween 80.

QBC-Method

One hundred and eighty six blood samples were tested by the QBC method as described by SPIELMAN²⁴.

ELISA for antibody detection

Two hundred and twenty eight sera samples were tested as described by FERREIRA et al.¹¹.

RESULTS

The results of the *Plasmodia* detection methods related to the presence or absence of malaria symptoms can be seen in the table 1, with exception of the group 3 (patients upon treatment). All subjects that showed malaria clinical symptoms and epidemiology were considered to have malaria. One can observe low sensitivity (58.2%) but high positive predictive value (97.8%) in the TBF results. The QBC and IFA showed 73.7% and 75.3% of sensitivity and 92.4% and 94.9% of positive predictive value, respectively. The Kruskal Wallis, a non parametric method showed significant differences in the three studied methods (p<0.001). The observed difference was due the results of TBF, but the QBC and

IFA did not show significant difference by multiple range analysis (p<0.001).

The inter-methods results can be seen in table 2 and table 3. In the group 1, one sample was negative in the QBC and 3 samples were negative in the IFA, all with very low *P.vivax* parasitemia. In the group 2, 21 samples were positive in the QBC and 29 in the IFA. This represents an increase of 25.9% and 21.4%, respectively, in the *Plasmodia* detection when compared with the TBF. In the group 3, 10 and 11 were positive in the QBC and IFA, respectively, after TBF became negative. In the group 4, 1 sample tested by the QBC was positive and of the 2 samples tested by the IFA, 1 was positive and other negative. In the group 5, 8 samples were positive in the QBC and 5 samples were positive in the IFA. No subject in the control group (6) was positive in the studied methods.

After the inter-method study, we observed in the symptomatic cases (groups 1 and 2) the highest positivity for IFA, followed by the QBC and lowest for the TBF. In these groups the concordance of the methods was: TBF/IFA= 78.6%, TBF/QBC= 83.5%, and QBC/IFA= 82.3%. In the non-symptomatics cases (groups 4 and 5) we observed the highest positivity for the QBC, followed by the IFA and TBF. The concordance was: TBF/IFA= 80.6%, TBF/QBC= 42.8%, and QBC/IFA = 71.4%. The concordance between QBC and IFA in the group 3 was 71.4%.

The results of the TBF, QBC and IFA and the level of antibodies in the non-symptomatic group with discrepant clinical and laboratorial results are shown in table 4. All tested patients were positive in the QBC, two not having been done. We observed high levels of *Plasmodia* IgG antibodies in almost all patients. With these results we concluded that all patients had malaria, with exception of two cases. The samples 31, 32, 34, 35, 36 and 37/M3 were from individuals living in a goldmine where was occurring cases of malaria: two individuals with clinical symptoms and *P.falciparum* positive TBF (samples 26 and 39/M3- group 1) and one without symptoms, but *P.falciparum* positive TBF (sample 31/M3).

DISCUSSION

The Ministerial Conference on Malaria in Amsterdam, Netherlands, on 26-27 October 1992, have adopted a World Declaration on the control of malaria,

TABLE 1
 Binary combination between individuals (groups 1, 2, 4, 5 and 6) with and without malaria clinical symptoms and the TBF, QBC and IFA results.

Methods	Malaria Symptoms		Total
	present	absent	
TBF	+	89	91
	-	64	118
	Total	153	209
QBC	+	98	106
	-	35	66
	Total	133	172
IFA	+	113	119
	-	37	87
	Total	150	206

TABLE 2
 Binary combinations between defined population groups
 (subjects with malaria symptoms and non-symptomatic, patients on treatment and controls) and the IFA and QBC results when
 the TBF was positive and negative.

Methods	TBF	Malarial Clinical Symptoms						Antimalarial Therapy			Controls								
		Present			Absent			3			6								
		1	2	total	4	5	total	+	-	total	+	-	total						
		Pf	Pv	Pf Pv				Pf											
IFA	+	34	38	12	29	113		1	5	6	0	11	11	0	0	0			
	-	0	3	0	34	37		1	24	25	0	8	8	0	25	25			
	total	34	41	12	63	150		2	29	31	0	19	19	0	25	25			
QBC	+	31	34	12	21	98		1	8	9	0	10	10	0	0	0			
	-	0	1	0	34	35		0	5	5	0	4	4	0	25	25			
	total	31	35	12	55	133		1	13	14	0	14	14	0	25	25			

Pf=*P.falciparum*
 Pv= *P.vivax*
 PfPv= mixed malaria

pointing 4 elements which are applicable to any local or national conditions:

- provision of early diagnosis and prompt treatment;
- planning and implementation of selective and sustainable preventive measures, including vector control;
- detection, containment or prevention of epidemics;
- strengthening of local capacities in basic and applied research coupled with regular assessment of the malaria situation at country level ²⁸.

The first element basic has been our principal interest.

The confirmation of malaria disease is normally performed by the TBF. Interfering factors may reduce its application in large scale for epidemiological diagnosis. Organizational, personnel and technology deficiencies contribute to the problems associated with the TBF ²¹.

The development of highly sensitive methods are in study in different laboratories as alternative for TBF. Immunologic methods for antibody detection are not available for endemic areas and do not differentiate between recent and past malaria. In this study we tested all sera patients for anti-*P.falciparum* IgG, IgM and IgA antibodies. The geometric means obtained did not permit the definition of the studied groups of patients (data do not published). Well defined antigens of the parasite, in association with the detection of different classes of immunoglobulins are in study and could contribute for the question, as pointed by AVILA ¹. Immunologic tests for detection of soluble antigens in blood or molecular biology methods, showed low sensitivity in field conditions ^{5,25}. The detection of *Plasmodia* by different methods appears to be an alternative solution for TBF.

An important aspect of this work was the selection criteria for the population groups and of endemic areas

TABLE 3
 Binary combinations between subjects with malaria symptoms and non-symptomatics, patients on treatment and controls,
 and IFA results when QBC was positive and negative.

Methods	QBC	Malarial Clinical Symptoms						Antimalarial Therapy (group 3)			Controls (group 6)		
		Present (groups 1 and 2)			Absent (groups 4 and 5)								
		+	-	total	+	-	total	+	-	total	+	-	total
IFA	+	86	14	100	5	0	5	7	1	8	0	0	0
	-	9	21	30	4	5	9	3	3	6	0	25	25
	Total	95	35	130	9	5	14	10	4	14	0	25	25

TABLE 4

Level of anti-*P. falciparum* IgG, IgM and IgA antibodies obtained by the ELISA test in the non-symptomatic patients (groups 4 and 5) with discrepant results among the TBF, QBC and IFA.

Sample	TBF	QBC	IFA-Ag	ELISA-Antibodies			Probability of malaria
				IgG	IgM	IgA	
31/M3	<i>P. falciparum</i>	+	+	4993	132	153	*
99/M3	<i>P. falciparum</i>	ND	ND	1334	negative	negative	*
25/M3	negative	+	-	417	negative	negative	inconclusive
82/M3	negative	ND	+	157	negative	56	+
32/M3	negative	+	+	1371	negative	negative	+
34/M3	negative	+	-	4549	301	negative	+
35/M3	negative	+	+	4339	990	negative	+
36/M3	negative	+	+	4834	2000	negative	+
37/M3	negative	+	+	5625	321	negative	+
61/M3	negative	+	-	5332	4176	negative	+
63/M3	negative	+	-	181	negative	negative	inconclusive
cut-off for ELISA- antibodies				100	100	25	

ND - not done

* true positive

by clinical and epidemiological methods. All selected subjects completed a history and were examined by specialized clinicians in malaria and bled for laboratory tests. Our study shows the discrepancy of the results between the TBF and the other methods. In the symptomatic cases, table 1, the sensitivity of the TBF was 51.7% with high positive predictive value, 97.8%, which shows the correlation of the diagnosis with the clinical signs.

The sensitivities of the QBC and the IFA was 73.1% and 72.7%, respectively, with positive predictive values of 91.6% and 95.7%. We conclude for the highest sensitivity is attained by IFA, followed by QBC and then TBF. In the non-symptomatic group, the QBC method showed the highest efficiency. The inter-test study in the different groups showed concordance of results between IFA and QBC (Kappa Index = 0.69). We observed in the non-symptomatic subjects and in patients on treatment a low positivity or negativity of the TBF. The QBC method was positive in 10/14 patients on treatment (71.4%) and the IFA was positive in 11/19 (57.8%). This is an important data to use to allow the discharge of the patient with malaria without risking transmission in areas where there is crowding and large quantities of mosquitoes. A significant case was a patient who lived in an endemic area for 15 years and who never had clinical malaria. This subject was negative by TBF and IFA, but was positive in the QBC and had anti-*P. falciparum* IgG antibodies. Table 4 shows the importance of the alternative methods in patients with discrepancies between clinical and the TBF. It is important to observe

that the QBC method detected all cases that were studied, and in agreement with the IFA and the anti-*Plasmodia* antibody level. In conclusion, we observed great advantages for alternative methods for *Plasmodia* detection. The QBC method is more practical and could be useful not only in the field but also in the industrialized centers of non-endemic areas as well as in blood donors screening.

RESUMO

Avaliação de diferentes métodos para detecção de plasmódios em grupos populacionais bem definidos em uma área endêmica do Brasil.

Mais de 500.000 casos novos de malária foram notificados no Brasil, em 1992. *P. falciparum* and *P. vivax* são as espécies responsáveis por 99,3% dos casos. O diagnóstico precoce é indispensável para início do tratamento adequado. Neste trabalho, apresentamos os resultados do método tradicional para detecção de plasmódios, gota espessa, e os resultados de métodos alternativos estudados: imunofluorescência indireta com anticorpo policlonal anti-*P. falciparum* e QBC-método, em grupos populacionais bem definidos. A análise dos resultados foi feita em relação à presença ou ausência de sintomas clínicos de malária. Também, diferentes classes de imunoglobulinas anti-*P. falciparum* foram quantificadas para auxiliar na análise global dos resultados, principalmente nos resultados discrepantes. Nós concluímos que os métodos alternativos são mais sensí-

veis do que a gota espessa e que a associação das informações epidemiológicas, clínicas e laboratoriais é necessária para definir a presença de malária.

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