Med. J. Malaysia Vol. XXXI No. 3, March, 1977

Indirect fluorescent antibody tests for *Plasmodium falciparum* infections

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INTRODUCTION

SEROLOGICAL TESTS have been used as an aid for diagnosis of many parasitic diseases in recent years. These tests have also been increasingly used for the detection of parasitic infections and in sero-epidemiological studies especially in infections with malaria. Among the various test methods used for malaria, the Indirect Fluorescent Antibody (IFA) test has been the most investigated test and one of the most reliable and reproducible tests (Wilson *et al.*, 1971; 1975; Collins & Skinner, 1972). Recently, Wilson *et al*, (1975) have described the IFA test as the preferred test throughout the world for obtaining serological evidence of malarial infection.

In the present work, IFA tests were performed on serum samples collected from 3 groups of donors. A single stained thick and thin-film made concurrently with the collection of blood, was examined. Agreement between microscopic diagnosis and IFA titres in these samples was studied. The value of IFA tests in the diagnosis of *P. falciparum* infection and in the sero-epidemiological studies were discussed. The degree of cross-reactivity to heterologous antigens was also estimated using *P. vivax* and *P. brasilianum* (for *P. malariae*) antigens.

MATERIALS AND METHODS

Serum samples

A total of 289 sera were tested. Sera were collected from 3 groups of donors. Group I consisted of 98 patients (Malays, Chinese and Indians) who presented themselves to various clinics in West Malaysia with slide-positive *P. falciparum* infection for treatment. Blood was taken before the treatment was administered. Group II included Orang Asli adults who lived all their lives in endemic areas but who at the time of blood collection showed no patent parasitaemia. Group III comprised of 95 medical students who have never had any history of malaria.

Five to ten ml of blood was withdrawn for the tests. Serum was separated and stored frozen at −20°C. A parasite survey was done by examining a single, stained thick and thin film made concurrently with the collection of blood for sera. Every serum sample was titrated in parallel tests with washed-cell thick-smear antigens of *P. falciparum, P. vivax* (whenever available) and *P. brasilianum* (for *P. malariae*).

Antigens

P. falciparum antigen slides were prepared in Malaysia from *in vitro* cultures using the techniques already described by Thomas and Ponnampalam (1975). The *in vitro* culture techniques used were those of Rieckmann *et al* (1968). *P. vivax* and *P. brasilianum* washed-cell thick-smear antigen slides were supplied by the kind courtesy of Dr. A. J. Sulzer, Center for Disease Control, Atlanta, USA. All antigen slides were stored frozen at −70°C.

P. brasilianum antigen has been shown (Collins *et al*, 1966) to give a high FA response in patients infected with *P. malariae*. Butler *et al* (1973) considered this simian species antigenically identical to *P. malariae*. In this study, *P. brasilianum* antigen is considered antigenically similar to *P. malariae*.

Conjugate

Anti-human-gamma-globulin (rabbit) conjugated with fluorescein-isothiocyanate was prepared at the Center for Disease Control. Measured amounts of 0.02 ml each of the conjugate were stored frozen at −70°C in individual vials until required.

Performance of the tests

The test methods were those described by Sulzer *et al* (1969). The antigen slides were removed from storage, placed in staining racks until they had reached room temperature, and washed for 10 minutes in distilled water on a slowly moving slide rotator. The slides, were then removed from the bath and allowed to dry. After drying these slides were labelled. The serum samples to be tested were diluted with phosphate-buffered saline (PBS) PH 7.6 in 4-fold steps, starting at a dilution of 1:4. A measured quantity of 0.05 ml of each dilution was dispensed on a labelled antigen mount. The antigen slides with various dilutions of antisera were placed in a moist chamber, covered and incubated at 37°C for 30 minutes.

After incubation, the excess serum was removed from each slide with a gentle stream of the phosphate buffered saline. The slides were placed in a staining rack in saline on a slowly moving slide rotator for 15 minutes. They were then dried. Required amount of the conjugate was removed from the freezer and diluted and 0.05 ml of a 1:200 dilution of the anti-human-gamma-globulin conjugate containing Evans blue counter-stain was distributed over every antigen mount. The slides were covered and incubated again at 37°C for 30 minutes in a moist chamber. The excess conjugate was removed by a gentle stream of PBS.

The slides were dried and a drop of buffered glycerol of pH 9 was placed on each mount and covered with a coverslip. After mounting in the buffered glycerol, labels on all test slides were covered. The slides were then randomised and coded to eliminate all possible prejudice during reading. A positive control serum, a negative control serum and a PBS control were included in each day's test.

The slides were read on a Leitz Ortholux fluorescent microscope using BG 12 and UG 1 exciter filters and a Leitz 470 ocular filter. In this series of tests, only those sera which showed reactions at a titer of 1:64 or at higher dilutions were considered positive.

RESULTS

Table I summarises the results of tests with three species of *Plasmodium* antigens on the serum samples from 3 groups of donors.

Table 1

IFA Titres for Sera From 3 Groups of Donors With *P. Falciparum, P. Vivax* **and** *P. Brasilianum* **Antigens**

Group	Antigen	No. of sera tested	No. and percent of sera with end point titres of					Total No Positive	Percent Positive
			Negative	1:64	1:256	1:1024	1:4096		
I. Donors with slide positive *P. falciparum* infections	*P. falciparum*	98	2 (2%)	35 (35.7%)	38 (38.7%)	17 (17.3%)	6 (6.1%)	96	98%
	P. vivax	98	58 (59.2%)	27 (27.6%)	12 (12.2%)	1 (1%)	–	40	40.8%
	P. brasilianum	98	65 (66.3%)	32 (32.7%)	1 (1%)	–	–	33	33.7%
II. Slide negative donors from endemic area	*P. falciparum*	96	10 (10.4%)	29 (30.2%)	36 (37.5%)	20 (20.8%)	1 (1%)	86	89.6%
	P. brasilianum	96	38 (39.6%)	45 (46.9%)	11 (11.4%)	2 (2.1%)	–	58	60.4%
III. Known negative donors – (Control)	*P. falciparum*	95	95 (100%)	–	–	–	–	–	–
	P. vivax	95	95 (100%)	–	–	–	–	–	–
	P. brasilianum	95	95 (100%)	–	–	–	–	–	–

Out of a total of 98 serum samples from Group I, two samples (2.0%) showed negative reaction. These sera did not react with *P. vivax* or with *P. brasilianum* antigens. The remaining 96 serum samples (98.0%) reacted with the homologous antigens at various dilutions from 1:64 to 1:4096. The number and percentage of sera that reacted with *P. falciparum* antigen is given in the table. The detection rate of known *P. falciparum* infections with the homologous antigen was 98%.

None of the stained blood films from this group showed mixed infection with *P. vivax* or *P. malariae*. However, there was a high rate of cross-reaction with *P. vivax* and *P. brasilianum* antigens. Forty serum samples (40.8%) showed positive reaction with *P. vivax* and 33 samples (33.7%) showed positive reaction with *P. brasilianum* antigens. Of these, 13 sera (13.3%) reacted with all antigens and 27 serum samples (27.6%) with *P. falciparum* and *P. vivax*. Similarly, 20 sera (20.4%) reacted with *P. falciparum* and *P. brasilianum*. Only 36 sera showed a specific positive reaction with homologous *P. falciparum* antigen alone. Among those sera which cross-reacted with the heterologous antigens, none showed higher titres with those antigens, although 8 samples reacted at the same titre levels with the homologous antigen and with *P. vivax* antigen. The remaining 88 sera reacted at higher titres with the homologous antigen. Thus using at least a four-fold greater titres as the minimum difference between the titres obtained with the homologous and the heterologous antigens, it was possible to differentiate correctly about 89.8% of the infections to species.

When sera from Group II were tested, 86 samples (89.6%) showed reactivity with *P. falciparum* antigen and 58 (60.4%) samples with *P. brasilianum* antigen (Table 1). *P. vivax* antigen was not available. Unlike those sera from Group I, two samples (2%) from this group reacted with only *P. brasilianum* antigen; and one sample gave higher titres with this antigen. Two other samples gave the same titres with both *P. brasilianum* and *P. falciparum* antigens.

None of the 95 control sera collected from medical students who were never exposed to malaria, reacted with any of the antigens tested (Table 1).

DISCUSSION

Plasmodium falciparum antibodies were detected in about 98% of the samples collected from slide-positive patients. Cross-reactions with *P. vivax* and *P. brasilianum* antigens were also detected in many sera. Similar incomplete cross-reactivity to heterologous antigens in *Plasmodium falciparum* infections has been demonstrated in earlier studies (Diggs & Sadun, 1965; Gleason, *et al* 1971). Sixty-two serum samples cross-reacted with heterologous antigens. Due to this phenomenon of cross-reactivity, only 37.5% of the infections were diagnosed with certainty to *P. falciparum* species. However, if a minimum of a 4-fold difference in end-point titre levels between homologous (higher) and heterologous antigens is considered as the criterion for identification of species, about (89.8%) of active *P. falciparum* infections were correctly identified. Comparable results were reported earlier by Gleason *et al* (1971).

In a small proportion of cases, the titre levels of heterologous antigens were the same as with homologous antigens. Similar results were obtained in earlier studies by Diggs & Sadun (1965) and Gleason *et al* (1971) who have contributed this phenomenon to the residual effects of previous attacks by these species. Since many of these patients under present study came from areas where all three species are present, a previous infection probably with *P. vivax* or even with *P. malariae* species was possible.

Although malarial parasites were absent in thin blood films, 89.6% of the donors in group 2 from an hyperendemic group (Thomas & Dissanaike, 1977) had significant levels of antibodies to *P. falciparum* and 60.4% to *P. malariae*. Most of the sera, showed stronger reaction with *P. falciparum* antigen. This indicated that the predominant species among Orang Asli donors was *P. falciparum* which showed cross-reactivity with *P. brasilianum* antigens. However, a small number of those sera showed the same or higher degree of reactivity with the latter, indicating a small focci of *P. malariae* infection among Orang Asli as reported earlier by Sandosham (1965).

None of the sera from known negative donors showed reactivity or "false positive" reactions with any of the antigens tested. This showed the specificity of *Plasmodium* antigens and the reliability of the IFA tests.

The IFA technique seems to be very useful in detecting malarial antibodies, not only in slide-positive current malaria infections, but also in sera from donors who have had past infections. However, it should be emphasised that it was not possible to differentiate a past infection from a current infection. Neither was it possible, in most cases, to identify the species with certainty. Therefore IFA test is not a very efficient technique in diagnosing

individual cases of slide-positive malaria. For this purpose, microscopical examination is still the method of choice. However, the IFA technique seems to be a very useful aid in estimating the malaria experience of a population and the type of endemicity especially when the parasites are present at very low-levels. The value of IFA in the study of malarial endemicity rates, including species prevalence, has already been shown by Bruce-Chwatt, *et al*, 1972; WHO, 1974 & 1975. These tests were shown (WHO, 1974) to be of immense value to exclude malaria in patients with symptoms such as pyrexia of unknown origin, hepato-splenomegaly, anaemia and other syndrome.

Thus the IFA technique is a very useful tool in detecting malaria antibodies and malaria infection. However, those who use this technique must be aware of the inherent limitations of these tests which have already been discussed above.

SUMMARY

Sera from 289 donors were tested with *P. falciparum, P. vivax* and *P. brasilianum* (for *P. malariae*) antigens. Thick and thin blood-films which were made concurrently with collection of blood for sera were also examined. The donors included 98 patients with slide-positive *falciparum* infection and 96 Orang Asli adults who lived in endemic areas, but who at the time of serum collection, showed no parasitaemia. Sera from 95 medical students who had never been exposed to malaria were also tested as negative control. Results were tabulated and conclusions made. Detection rate was high, but there was high rate of cross-reactions with heterologous antigens and therefore identification of species was not easy. False negative rate was about 2% only among known positive sera. The sera from group two were also reactive with both the antigens. None of the serum from the negative control group however, gave positive reactions. The value of IFA technique for malaria has been discussed.

ACKNOWLEDGEMENT

The authors are indebted to Dr. A.J. Sulzer, Center for Disease Control, Atlanta, Georgia, USA for providing antigen slides and other reagents as well as for the critical review of the manuscript and his valuable suggestions. We are also grateful to Professor A.S. Dissanaike of this Department for his constant encouragement. Special thanks are due to the University of Malaya for providing financial aid under Vote F for carrying out this study. The authors also wish to record their thanks to Dr. J.T. Ponnampalam, Senior Malariologist, Institute for Medical Research, Kuala Lumpur for supplying a number of sera from slide-positive individuals.

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