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INDIRECT IMMUNOFLUORESCENCE USING DIFFERENTIATING ANTIGENS IN DETECTION OF IMPORTED MALARIA

by

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Summary — Results of routine IFA-tests for malaria, performed during a two years period were analysed by systematic comparison with the available data from corresponding blood film examinations. Among the sera tested, 1009 (11.5 %) reacted at significant levels with one or more of the human malarial antigens. Corresponding parasitological results were available in 608 cases. In most of these cases (84.05 %), blood slide examination was negative. However, with increasing serological titers, the percentage of corresponding negative blood slides gradually decreased from 97.14 % for sera reactive at 1/80 to 57.85 % for sera reactive at or above 1/640.

Antibody patterns to human malarial antigens were studied in 118 slide proven cases. Almost 75 % of these parasitological positive cases had sera reactive at or above a dilution of 1/160 with at least one of the human malarial antigens. Both serological results and slide examinations indicated that *P. falciparum* was the most frequent malaria species involved in our series. Moreover, 93 % agreement was observed between the antigen species producing the highest titer in the immunofluorescence test and the parasite species, identified by examination of the corresponding blood film. In 15.3 % of the slide proven cases, serum samples failed to react with any of the antigens, but when the analysis was repeated on a second specimen, taken one or two weeks after demonstration of the parasites, seroconversion was observed.

KEYWORDS : Malaria, Imported; Indirect Fluorescent Antibody Test; Serology.

Introduction

Since its introduction in serodiagnosis of malaria by Kuvin *et al.* (5), the indirect fluorescent antibody test has become the most widely used technique for detection of plasmodial antibodies. However, efforts for standardization of the IFA-test for malaria have not been successfull so far, one of the most important impediments being the lack of standardized antigens. Results from different laboratories are still subject to important variations and in consequence, interpreting serological titers is only possible with accurate information on the sensitivity and the specificity of the applied procedure.

In serodiagnosis of imported malaria the objective is to detect those persons who are or have been infected recently with *Plasmodium* and, if possible, to determine the species involved.

In an attempt to obtain more precise results, the use of more than one human malarial antigen was introduced in our routine procedure for serodiagnosis of malaria in the course of 1982. We present an evaluation of the results obtained during a 2 years period. The purpose was to verify the sensitivity and the specificity of our method in relation to the antigens used, and consequently to facilitate the interpretation of serological titers.

Materials and Methods

Microscope equipment : reading of the slides was performed on a Leitz Ortholux immunofluorescence microscope, equipped with a Ploemopak epi-illuminator (incident light) and 50 × water immersion objective. The light source was a HBO-50 mercury bulb.

Conjugate : wide spectrum goat anti-human conjugate was obtained from Pasteur. The working dilution was 1:100 in PBS containing Evans blue 1:10.000 as counterstain.

Antigens : *P. falciparum* antigen was prepared from *in vitro* cultures of an isolate of unknown geographical origin (isolated from a case infected at Schiphol airport and kindly supplied by Prof. Meuwissen). All other human malarial antigens were prepared from blood of infected patients on different occasions. In total, 5 batches of *P. vivax* antigen, 3 batches of *P. ovale* antigen and 1 batch of *P. malariae* antigen were prepared.

Sera : the serum specimens were sent to our laboratory by different Belgian hospitals, private physicians or blood transfusion centers. The patients were either immigrants from malaria endemic areas or Europeans temporarily exposed to malaria. In total, 8,772 serum samples were tested. During the first sixteen month period, starting on December 1st 1982, all serum samples were screened using both the human malarial antigens *P. falciparum* and *P. vivax,* and a rodent malarial antigen *P. berghei.* From April 1984 on, three human malarial antigens were used, including *P. ovale. P. berghei,* which in general produced much lower titers, was abandoned. The use of *P. malariae* antigen was restricted to the screening of serum samples received from blood transfusion centers.

The serum dilutions studied ranged from 1/20 to 1/640.

Results

In the first part of this investigation, serological data were chosen as the starting point : all serum samples reacting at significant levels with

TABLE 1

Number of significantly positive reactions in relation to the antigen species

Antigen species producing the highest titer	1/80	1/160	1/320	⩾ 1/640	Total number
P. falciparum	267	57	67	77	468
P. vivax	22	8	3	7	40
P. falciparum-P. vivax	31	10	3	1	45
P. falciparum-P. vivax-P. berghei	—	4	1	3	8
16 months period (December 1st 1982-March 31st 1984)					561
P. falciparum	154	83	61	96	394
P. vivax	4	3	7	5	19
P. ovale	2	8	2	1	13
P. vivax-P. ovale	—	—	—	3	3
P. falciparum-P. vivax	6	—	—	1	8
P. falciparum-P. ovale	2	1	1	3	7
P. falciparum-P. vivax-P. ovale	1	—	—	3	4
8 months period (April 1st 1984-November 30th 1984)					448

one or more of the human malarial antigens were listed, as illustrated on Table 1. Among the sera that were tested, 1,009 (11.5 %) were reactive at or above the dilution of 1/80. In relation to the antigen species, by far most of these samples showed highest reactivity with the *P. falciparum* antigen (85.43 %), while only in 7.43 % of the cases distinct higher reactivity was observed with the *P. vivax* or *P. ovale* antigens. In addition, 7.14 % of the specimens were equally reactive to both the *P. falciparum* and the *P. vivax* or *P. ovale* antigens.

Corresponding results of parasitological examinations, performed at the same period, were available for 608 of these patients. In 84 % of these blood slides no plasmodium parasites were detected. However, with increasing serological titers, the percentage of corresponding negative blood slides gradually decreased from 97.14 % for sera reactive at 1/80 to 57.85 % for sera reactive at or above 1/640 (Table 2).

TABLE 2

Number of significantly positive serological results and corresponding negative blood slide examinations

Highest titer	Number of serum samples	Number of negative blood slides	% of negative blood slides
1/80	280	272	97.14 %
1/160	116	98	84.48 %
1/320	91	71	78.02 %
⩾ 1/640	121	70	57.85 %
Total	608	511	84.05 %

In the second part of the investigation, parasitological data were the starting point: all known cases of slide proven malaria were listed and their corresponding antibody pattern to the different human malarial antigens was studied (Tables 3 and 4).

TABLE 3

Cases with positive parasitological diagnosis and corresponding antibody pattern (December 1st 1982 — March 31st 1984)

Antigen species producing the highest titer	Blood film examination (species involved)							
	P. falciparum		*P. vivax*		*P. ovale*		*P. falciparum* *P. malariae*	
	titer	number	titer	number	titer	number	titer	number
P. falciparum	1/20	1	—	—	—	—	—	—
	1/80	7	—	—	—	—	—	—
	1/160	4	—	—	—	—	—	—
	1/320	12	—	—	1/320	1	1/320	1
	⩾ 1/640	20	—	—	—	—	1/640	1
P. vivax	—	—	1/160	4	1/640	1	—	—
P. falciparum-P. vivax	1/80	1	—	—	1/20	1	—	—
(similar titers)	1/160	1	1/160	1	1/320	1	—	—
Number of sero-negative cases	—	15	—	1	—	—	—	—
Total number	—	61	—	6	—	4	—	2

TABLE 4

Cases with positive parasitological diagnosis and corresponding antibody pattern (April 1st 1984 — November 30th 1984)

Antigen species producing the highest titer	Blood film examination (species involved)							
	P. falciparum		*P. vivax*		*P. ovale*		*P. malariae*	
	titer	number	titer	number	titer	number	titer	number
P. falciparum	1/160	3	—	—	—	—	—	—
	1/320	4	—	—	—	—	1/320	1
	⩾ 1/640	22	—	—	—	—	—	—
P. vivax	—	—	1/160	2	—	—	—	—
	—	—	⩾ 1/640	1	—	—	—	—
P. ovale	—	—	1/160	2	1/160	1	—	—
P. vivax-P. ovale (similar titers)	—	—	⩾ 1/640	1	⩾ 1/640	1	—	—
P. falciparum-P. ovale or *P. falciparum-P. vivax* (similar titers)	⩾ 1/640	4	—	—	—	—	—	—
Similar titer with the three antigens	—	—	1/20	1	—	—	—	—
Number of sero-negative cases	—	2	—	—	—	—	—	—
Total number	—	35	—	7	—	2	—	1

In total, 81.36 % of the 118 cases of slide proven malaria were due to *P. falciparum*. All corresponding serum samples were either most reactive to the *P. falciparum* antigen or equally reactive to both the *P. falciparum* and one of the other human malarial antigens.

Blood slide examination revealed 13 cases of vivax malaria (11.02 %) and 6 cases of ovale (5.08 %). When the corresponding serum samples were analysed with only the *P. falciparum* and *P. vivax* antigens (Table 3), all but one of them produced the highest titer with the *P. vivax* antigen or a similar titer with both antigens. When three human malarial antigens were used, including *P. ovale* (Table 4), 4 serum samples were most reactive to the homologous antigen, while 3 others were equally reactive to both the *P. vivax* and the *P. ovale* antigen. However, for 2 serum samples that showed the highest titer with the *P. ovale* antigen, the corresponding blood film examination revealed the presence of *P. vivax*.

In all the cases of *P. malariae* infection (0.85 %) or mixed infections of *P. malariae* and *P. falciparum* (1.69 %), highest titers were observed with the *P. falciparum* antigen.

Finally, 18 cases of slide proven malaria turned out to be seronegative.

Discussion

In our procedure, a serum dilution of 1/20 is considered as the lowest significant titer indicating recent contact with malarial antigen. The proportion of patients with positive blood slide examination that had sera reactive at or above this lowest significant level was 84.7 %. On the other hand, 1/80 is considered as the treshold of specificity for suspicion of clinical cases of malaria. This is in agreement with the observation that almost 75 % of the cases with slide-proven malaria had sera reactive at a dilution of 1/160 or higher with one of the human malarial antigens, while for sera reactive at 1/80, the corresponding slide examination was negative in even more than 97 % of the cases.

Serology is known to be more sensitive than the direct demonstration of parasites, being independent of short term parasitemic fluctuations. Serological titers above 1/80 indicate a recent malaria attack. However, serological tests are not able to differentiate between a current and a past malaria experience. Therefore, residual antibody from cured infections can explain part of the significantly positive reactions that corresponded to negative blood slide examinations, especially in the group of immigrants from endemic areas.

On the other hand, it's highly probable that at least some of the individuals showing high antibody levels were parasitized, but had subpatent parasitaemia at the time slide examination was performed.

In total, 18 sera corresponding to slide proven malaria failed to react with any of the antigens. In order to see whether these samples possibly had been collected at an early stage of primo-infection, health records were reviewed for 12 of these patients and, when possible, serological tests were repeated on a second serum sample, collected at the time of discharge from the hospital. 10 out of 12 of these patients were Europeans who had only temporarily been exposed to malaria. On admission at the hospital, none of them reported previous malaria experience. The two others were a Korean seaman, just returning from West-Africa, and an Indian adopted child. No information on previous malarial contact was available for either of them. The mean time elapsed between onset of symptoms and sampling of the serum specimen was 3 days, with a minimum of 1 and a maximum of 7 days. Moreover, when a second serum sample was analysed within one to two weeks after demonstration of the parasites, significant positive results were obtained (Table 5).

TABLE 5

Malaria antibody titre of 3 control serum samples of patients with patent parasitemia that were seronegative at the first examination

No.	Causative parasite species	Time between first and second serum sample	Serological results obtained with the second serum sample		
			P. berghei	*P. falciparum*	*P. vivax*
1	*P. falciparum*	8 days	1/160	$\geqslant$ 1/640	1/160
2	*P. falciparum*	7 days	1/80	1/320	1/80
3	*P. falciparum*	8 days	1/80	1/80	1/80

Using more than one human malarial antigen, a lot of additional interesting information was obtained, which in many cases permitted to determine the plasmodium species involved.

Using *P. vivax* and *P. falciparum* simultaneously, in 49 out of 52 sera showing different reactivity to these two antigens, test results permitted

TABLE 6

Frequency distribution of fluorescent antibody titers obtained with two series of reference monospecific antisera

Anti-serum	Antigen	Reciprocal titer 0	20	80	160	320	640	GMRT
P. vivax (N = 9)	*P. vivax* 1 (India)	0	0	1	6	1	1	186.64
	P. vivax 2 (Africa)	1	2	4	2	0	0	54.43
	P. vivax 3 (India)	0	0	1	0	7	1	296.28
	P. vivax 4 (India)	0	0	1	3	4	1	235.16
	P. vivax 5 (Solomon Islands)	0	0	3	4	0	2	172.81
	P. falciparum	1	3	5	0	0	0	40.00
	P. malariae	2	5	2	0	0	0	23.33
	P. ovale 1 (Guinea)	1	2	4	2	0	0	54.43
	P. ovale 2 (Cameroon)	0	3	3	2	1	0	68.58
	P. ovale 3 (Cameroon ? Zaire ?)	0	3	3	3	0	0	63.50
P. ovale (N = 7)	*P. vivax* 1	0	3	2	2	0	0	53.83
	P. vivax 2	0	1	1	4	1	0	118.87
	P. vivax 3	0	1	3	2	1	0	97.52
	P. vivax 4	0	3	4	0	0	0	44.16
	P. vivax 5	0	3	4	0	0	0	44.16
	P. falciparum	0	3	1	2	0	1	72.45
	P. malariae	2	2	1	1	1	0	40.00
	P. ovale 1	0	1	1	2	3	0	144.92
	P. ovale 2	0	7	0	0	0	0	20
	P. ovale 3	0	1	1	2	3	0	144.92

correct differentiation between *P. falciparum* infections and the relapsing malarias *P. vivax* and *P. ovale* (Table 3). These observations also show cross reactivity between *P. vivax* and *P. ovale* to be more pronounced than between *P. falciparum* and *P. ovale.* A similar low degree of cross reactivity between *P. falciparum* and *P. ovale* has been reported by Knobloch (4). However, one serum sample was most reactive to the *P. falciparum* antigen, even though the species found by slide examination was *P. ovale.* When this specimen was re-analysed using three human malarial antigens, similar titers were obtained for both the *P. vivax* and the *P. ovale* antigen, while once more, distinct higher reactivity was observed to the *P. falciparum* antigen. However, examination of the health records showed that this patient was an African from Guinea, who had reported previous malaria experience. No data were available on the species previously involved, but it is highly probable that this patient had contact with *P. falciparum* on former occasions. Similar cases have been described by Fisher *et al.* (2) and Hornstein *et al.* (3).

Further differentiation between the causative Plasmodium species was achieved using both *P. vivax* and *P. ovale* antigen (Table 4). However, two test results indicated infection with *P. ovale,* while actually *P. vivax* was found by slide examination. Both results were obtained at about the same period, using the same batches of differentiating antigens. When these sera were re-analysed using different batches of *P. vivax* and *P. ovale* antigen, correctly the highest reactivity was observed to the *P. vivax* antigen.

These observations incited us to set up an experiment in order to compare the serological reactivity of all available batches of antigen using a series of reference antisera to *P. vivax* and *P. ovale.*

Antigens and sera were coded in order to avoid subjective interpretation of borderline fluorescence, and all serum samples were tested simultaneously against all ten antigens.

The results, summarized in table 6, clearly demonstrate the deviating reactivity of both the *P. vivax* antigen batch N° 2 and the *P. ovale* antigen batch N° 2, which were responsable for the aberrant routine serological results. Geometrical means of reciprocal titers (GMRT) indicate that in the homologous system, batch N° 2 of the *P. vivax* antigens (the only one originating from Africa) was much less reactive than the four other batches of *P. vivax* antigen, while high cross-reactivity to the anti-*P. ovale* sera was observed. Batch N° 2 of the *P. ovale* antigens showed very low sensitivity towards homologous antisera, but cross reactions with the antisera to *P. vivax* were somewhat more intense than with the two other batches of *P. ovale* antigen.

Earlier reports (6, 9) indicated that individual batches of antigen made from one single species of malaria parasites showed considerable variation in sensitivity and that the presence of large trophozoites or schizonts is of the utmost importance if maximum sensitivity is to be achieved. One author (7) called attention to the possibility of important strain related differences, occuring between batches of the same antigen species, even when the stage of development of the malarial parasites used as antigen is comparable for all batches. Regarding the limited number of antigens and antisera that were tested, our results are at present too preliminary to draw any major conclusion as to the possible causes of the observed intra-species differences. However, it is clear that all IFA antigen preparations should be fully characterized and that, when possible, each new batch of antigen should be tested with a choice of reference sera.

The fact that homologous antigens must be used in order to detect the greatest percentage of cases has repeatedly been stressed (1, 8). In addition, it has been reported that the percentage of sera equally reactive to different antigens is significantly lower in the group of temporarily exposed Europeans as compared to patients originating from endemic areas (3, 4).

Considering the serum samples that clearly showed the highest reactivity to only one of the human malarial antigens, we found 93 % agreement between the antigen species producing the highest titer in the immunofluorescence test and the parasite species, identified by blood film examination. Out of the 19 cases with slide proven infections of *P. vivax* or *P. ovale*, 16 (84 %) had sera reactive at or above 1/160 using the homologous antigen. If only *P. falciparum* had been used as antigen, 8 of these sera (50 %) would have been classified as clinically non-significant, showing a titer of only 1/20, while 4 others (25 %) would have been positive only at the treshold dilution of 1/80. In addition, without the use of the *P. vivax* or *P. ovale* antigens, no serologic evidence for the presence of these species would have been obtained.

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L'immunofluorescence indirecte sur des antigènes spécifiques pour le diagnostic de cas de paludisme importé.

Résumé — Les résultats de tests d'immunofluorescence de routine pratiqués pendant une période de deux années ont été confrontés avec les résultats de l'examen des frottis sanguins provenant des mêmes patients. Parmi les sérums testés, 1.009 (11,5 %) ont réagi à des titres significatifs avec un ou plusieurs plasmodiums humains. Les examens parasitologiques correspondants étaient disponibles pour 608 sérums. Dans la plupart des cas, l'examen parasitologique s'est révélé négatif. Cependant, pour des titres de plus en plus élevés le pourcentage de frottis négatifs décroît : de 97,14 % pour le groupe de sérums positifs à 1/80 à 58,85 % pour les sérums positifs à 1/640.

Les anticorps vis-à-vis de plusieurs antigènes humains ont été déterminés chez 118 sujets ayant présenté un examen parasitologique positif. Près de 75 % de ces cas présentaient un titre d'anticorps égal ou supérieur à 1/160 pour au moins un des antigènes proposés.

Aussi bien l'examen sérologique que la recherche du parasite ont montré que l'espèce la plus fréquente dans notre série est *P. falciparum*. De plus, l'espèce plasmodiale ayant donné le titre d'anticorps le plus élevé et l'espèce retrouvée dans les frottis de sang, correspondaient dans 93 % des cas.

Dans 15,3 % des diagnostics parasitologiques positifs, le sérum n'a réagi avec aucun antigène d'origine humaine, mais lorsque l'analyse sérologique était répétée sur un second échantillon prélevé une à deux semaines après la démonstration des parasites, la séroconversion a été observée dans tous les cas.

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