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Diagnosis of *Plasmodium falciparum* infection in man: detection of parasite antigens by ELISA*

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An ELISA method has been developed for the diagnosis of Plasmodium falciparum *infection in man. Parasites from* in vitro *cultures of* P. falciparum *were used as source of antigen for the solid phase and the source of specific antibody was immune Gambian sera; binding of antibody in antigen-coated wells was registered by means of alkaline phosphatase-conjugated anti-human IgG. Parasites were detected on the basis of inhibition of antibody-binding. The test was applied to the detection of parasites in human red blood cells (RBC) from* in vitro *cultures of* P. falciparum *and in RBC from infected Gambians; RBC from 100 Geneva blood donors served as normal, uninfected controls. In titration experiments, the degree of antibody-binding inhibition correlated with the number of parasites in the test RBC. Parasites were detected at a level of 8 parasites/10^6 RBC. Samples of RBC were tested from 126 Gambians with microscopically proven infection; significant antibody-binding inhibition was found in 86% of these cases, where parasitaemia ranged from 10 to 125 000/μl of blood. The presence of high-titre antibody in the test preparations was found to reduce the sensitivity of parasite detection in infected RBC from* in vitro *cultures mixed with equal volumes of different antibody-containing sera. The sensitivity was restored in most cases by recovering the RBC by centrifugation before testing. In a preliminary experiment, there was no significant difference in antibody-binding inhibition using fresh infected RBC and RBC dried on filter-paper and recovered by elution, although there was greater variation in the latter samples.*

A serological method for the diagnosis of current malaria infection would be a valuable tool in epidemiological studies and in malaria control programmes, and such a test will be required for assessment of the efficacy of malaria vaccines that may come to clinical trial in the future (*1*). Important recent progress in research related to the development of experimental malaria vaccines has stimulated efforts to devise a suitable laboratory method that could replace blood-film examination and permit rapid screening of substantial numbers of blood samples for the presence or absence of malaria parasites (*2–6*). Such a test should be sensitive, relatively simple to perform, and inexpensive.

We have previously described a radioimmunoassay for the diagnosis of *Plasmodium falciparum* infection (*4*). The method, based on inhibition of binding of specific antibody in a solid-phase system, detected parasites in lysed red blood cells (RBC) at a level of 8 parasites/10^6 RBC. A similar RIA was also developed by Avraham et al. (*5*). However, a test involving the use of radioactive isotopes may not be readily applic-

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able under field conditions. Recently, ELISA methods have been described for the diagnosis of *P. falciparum* infection by Bidwell & Voller (*6*), who applied the methods to the detection of parasites in blood from experimentally infected *Saimiri* monkeys, using serum from immunized *Aotus* monkeys as source of anti-malaria antibody. ELISA techniques offer the attractive advantages of simplicity, use of inexpensive reagents and apparatus, and objectivity; in addition, tests can be partially automated and many samples tested simultaneously. We have therefore adapted the methodology previously reported (*4*) to an ELISA inhibition test system, using human immune serum and infected RBC. The test was applied to the detection of parasites in RBC from *in vitro* cultures of *P. falciparum* and in RBC from infected Gambians.

MATERIALS AND METHODS

Blood samples

Samples of blood infected with *P. falciparum* were obtained from 126 Gambians in the up-country villages of Keneba and Manduar. The samples formed part of a series collected during a malaria survey carried out in January and February 1979. The age structure and other characteristics of this population have been described by McGregor & Williams (*7*). Samples were taken by finger-prick into 1-ml Durham tubes.

Blood samples to act as normal, uninfected controls were obtained from 100 healthy blood donors in Geneva, Switzerland. These samples were taken into heparinized tubes.

Preparation of red blood cells

Red blood cells (RBC) from the Gambian samples were recovered by elution of the clot obtained after removal of the serum from the Durham tubes. The clots were extruded into physiological saline solution and the RBC were allowed to separate for 1 h. The RBC were recovered and the cells washed twice more in saline. Packed RBC were stored in microtubes at −70 °C.

RBC from the Geneva blood samples were recovered after removal of plasma and buffy coat from the heparinized blood samples. The RBC were washed three times in phosphate buffered saline (PBS), pH 7.2, and the packed RBC were stored at −70 °C.

RBC were also taken from *in vitro* cultures of *P. falciparum* maintained according to the candle-jar method of Trager & Jensen (*8*). The RBC were collected when parasitaemia reached at least 10%; they were washed three times with PBS, and stored in aliquots at −70 °C.

For use in the immunoassay, the RBC were thawed and samples were mixed with an equal volume of saline solution containing 1% Nonidet 40 (NP40). This mixture was then diluted 1 in 5 with PBS containing 0.05% Tween 20 (PBS-Tween) to give a final dilution of lysed RBC of 1 in 10. In titration experiments using RBC from *in vitro* cultures of *P. falciparum*, series of five-fold dilutions were prepared using as diluent a solution containing normal lysed human RBC from a pooled sample from blood donors. The diluent was prepared by lysing the washed RBC with an equal volume of NP40-NaCl solution, then diluting 1 in 5 with PBS-Tween.

Preparation of antigen-coated plates

RBC recovered from *in vitro* cultures of *P. falciparum* were mixed with an equal volume of NP40-NaCl for 1 min. The mixture was centrifuged at 200 *g* for 4 min to remove any intact blood cells. The supernatant fluid was recovered and centrifuged at 6000 *g* for 10 min. This supernatant was then discarded and the parasite fraction resuspended and washed three times in PBS; it was then suspended in 1 ml of 0.1 mol/litre sodium carbonate-bicarbonate buffer, pH 9.6, and sonicated for 1 min. The suspension was then centrifuged at 6000 *g* for 10 min and the supernatant recovered. The protein content was estimated by spectrophotometry and the solution diluted to 50 μg of protein/ml of carbonate-bicarbonate buffer. 100-μl samples of this solution were placed in the wells of flat-bottom, disposable, 96-well, polystyrene microtitration plates.[a] The plates were covered and incubated at 37 °C for 3 h, then stored at 4 °C for up to 2 weeks. Immediately before use, the plates were emptied and washed three times with PBS-Tween.

Preparation of sera

Serum samples were selected from adult Gambians having a specific IgG anti-malaria antibody titre of at least 1:2000 as measured by indirect immunofluorescence. A pool of 8 such sera was used as source of anti-malaria antibody. Normal serum was obtained from blood donors in Geneva.

Goat anti-human IgG purified by affinity chromatography[b] was conjugated with alkaline phosphatase by the glutaraldehyde method (*9*). The working dilution (1:400) was determined by titration in plates coated with purified human IgG (100 ng/ml in coating buffer).

[a] Flow Laboratories Inc., Zug, Switzerland.

[b] Tago Inc., Immunodiagnostic Reagents, Burlingam, California, USA.

Test procedure

To 100-μl volumes of the test RBC preparations, at each dilution to be tested, were added 100 μl of diluted immune serum or of diluted normal serum.[c] Controls included RBC from *in vitro* cultures of *P. falciparum* at 10% parasitaemia incubated with immune and normal sera, pooled normal RBC from Geneva blood donors incubated with immune and normal sera, and immune and normal sera incubated with PBS instead of RBC. The tubes were incubated for 30 min at 37 °C, then centrifuged at 6000 *g* for 5 min. From the supernatant, 80 μl were placed in each of two antigen-coated wells and incubated at room temperature for 3 h. Two wells were incubated with PBS only. The wells were then washed three times with PBS-Tween. To each well was added 100 μl of diluted conjugated anti-human IgG. The plates were covered and incubated at 4 °C overnight. The wells were then washed three times with PBS-Tween and 100 μl of substrate (4-nitrophenyl phosphate in diethanolamine buffer) were added to each well. The reaction was stopped after 20 min by addition of 50 μl of 1 mol/litre NaOH solution and the absorbance read at 405 nm in a multichannel automatic photometer.[d]

The absorbance readings were used to calculate the percentage inhibition of specific antibody binding, as follows:

% specific antibody-binding inhibition = $(c/a) \times 100$ where:

a = maximum specific binding of antibody, i.e., the difference between ELISA values obtained from immune and normal sera incubated with pooled normal RBC before reaction in antigen-coated wells,

b = specific binding of antibody, i.e., the difference between ELISA values obtained from test RBC incubated with immune and normal sera[c] before reaction in antigen-coated wells,

c = specific inhibition of antibody binding, i.e., $a - b$.

RESULTS

The assay was applied to the detection of parasites in lysed RBC taken from *in vitro* cultures of *P. falciparum* and from blood samples from infected Gambians. Inhibition of binding of specific antibody in antigen-coated microtitration wells was measured after incubation with the test RBC.

The optimal concentration of parasite antigen for coating the wells of the microtitration plates was determined by testing the fixation of specific antiserum and of normal serum, as well as the background readings obtained with PBS, in wells coated with parasite antigens at concentrations ranging from 1 to 200 μg/ml. In the wells coated with antigen at each concentration, were incubated 100 μl of pooled antisera diluted 1:5, 100 μl of normal serum 1:5, or 100 μl of PBS, followed after washing by incubation with conjugated anti-IgG and addition of substrate as described above. The results are shown in Fig. 1. The working concentration selected was 50 μg of parasite protein/ml; at this concentration there was maximum fixation of antibody and almost minimum fixation of normal serum, and low background with PBS.

The working dilution of the pooled antisera was established by titration in wells coated using 50 μg of parasite protein/ml; maximum ELISA values were obtained at dilutions up to 1:80. The working dilution selected was 1:40. Normal serum was diluted 1:10.

The ELISA assay for antibody-binding inhibition was carried out on RBC samples from 100 randomly selected Geneva blood donors. The mean antibody-binding inhibition was $7 \pm 6.9\%$. The results did not differ significantly with blood group.

To establish the sensitivity of parasite detection, the test was carried out on serially diluted RBC samples taken from *in vitro* cultures of *P. falciparum* and on pooled normal RBC from 10 blood donors.

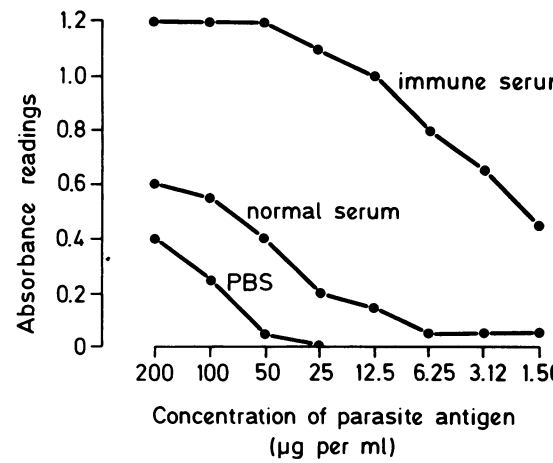

Fig. 1. Absorbance readings obtained in wells coated with different concentrations of *P. falciparum* antigen (μg of protein/ml) on reaction with immune Gambian sera, normal sera, and PBS, followed by conjugated anti-IgG.

[c] In practice, there was no significant difference between ELISA values obtained after incubation of normal serum with test RBC and with RBC from *in vitro* cultures of *P. falciparum*. It therefore proved unnecessary to react the test samples with normal as well as immune serum and the value obtained with RBC from cultures can be used in the calculation of specific inhibition.

[d] Titretek Multiskan, Flow Laboratories Inc., Zug, Switzerland.

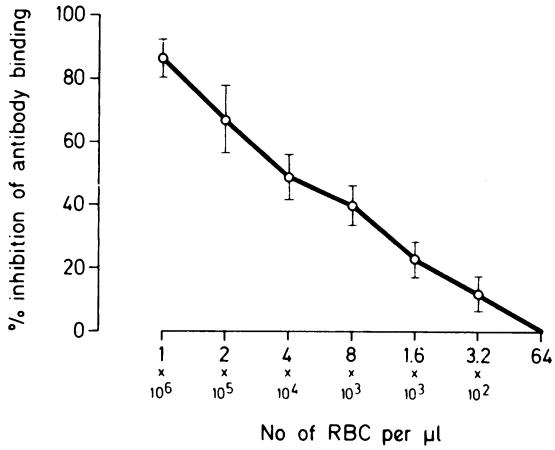

Fig. 2. Specific antibody-binding inhibition as measured by ELISA on serially diluted samples of RBC from *in vitro* cultures of *P. falciparum* at 12% parasitaemia. Ten samples were tested at each concentration of RBC. The concentrations shown refer only to the test RBC and do not include normal RBC used as diluent. The results were corrected in relation to values obtained with pooled normal RBC.

The results, corrected in relation to values obtained with normal RBC, are shown in Fig. 2. Significant antibody-binding inhibition was found in preparations containing 3.2×10^2 test RBC/µl and 38.4 parasites/µl. This is equivalent to a level of detection of approximately 8 parasites/10^6 RBC in whole blood.

Experiments were carried out using the IgG fraction of the Gambian immune serum, and using the immune serum absorbed with pooled normal RBC. In neither case did the results differ significantly from those obtained using untreated immune serum.

Samples of RBC from 126 Gambians infected with *P. falciparum* were tested at a concentration of 10^6 lysed RBC/µl. The levels of parasitaemia determined microscopically ranged from 10 to 125 000 parasites/µl of blood. Significant antibody-binding inhibition was found in 108 (86%) of these cases; levels of parasitaemia and ELISA test results are shown in Table 1.

To determine whether the relationship between the level of parasitaemia and the degree of antibody-binding inhibition using naturally infected RBC followed the standard curve established using RBC from *in vitro* cultures of *P. falciparum*, a titration experiment was carried out. RBC from 5 Gambian blood samples of relatively high parasitaemia were tested at each of a series of five-fold dilutions (in diluent containing 10% normal RBC). The results are shown in Fig. 3. The corresponding numbers of parasites at each dilution were equivalent to parasitaemia

Table 1. Examination of RBC by microscopy and by ELISA for evidence of *P. falciparum* infection

Parasitaemia (per µl)	No. of cases	No. positive by ELISA	Percentage antibody-binding inhibition[a] (mean ± SD)
0	100[b]	3 (3%)	7 ± 7
$< 10^2$	49	41 (84%)	19 ± 14
$10^2 - 10^3$	43	36 (84%)	16 ± 12
$10^3 - 10^4$	24	21 (88%)	26 ± 18
$> 10^4$	10	10 (100%)	35 ± 23

[a] Results corrected in relation to values obtained with pooled normal RBC.

[b] Geneva blood donors.

levels in whole blood ranging from 125 000 parasites/µl at dilution 1, decreasing by one-fifth at each dilution to 8 parasites/µl at dilution 7. The results show a similar relationship between the level of parasitaemia and degree of antibody-binding inhibition using RBC from *in vitro* cultures of *P. falciparum* and RBC from naturally infected patients, with a minor reduction in sensitivity with some of the latter samples.

The above experiments were carried out using washed RBC in order to standardize as far as possible the test conditions and to compare reactions with RBC from *in vitro* cultures and from naturally infec-

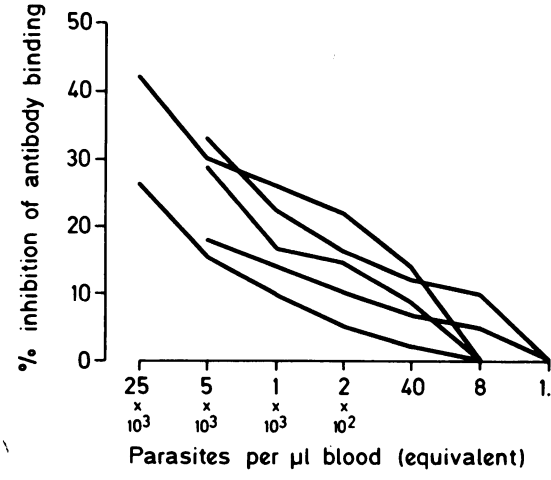

Fig. 3. ELISA results obtained with serially diluted samples of RBC from Gambians infected with *P. falciparum*; the test RBC were diluted with normal RBC.

ted individuals. Since it would be much more convenient to use whole blood for diagnostic purposes, experiments were carried out to determine the effect of the presence of antibody in the test sample. Sera from adult Gambians with low, medium, and high anti-malaria antibody titres (as measured by indirect immunofluorescence) were mixed with equal volumes of washed, packed RBC from *in vitro* cultures of *P. falciparum* at 10% parasitaemia. These samples were then lysed and incubated with antiserum and reacted in antigen-coated wells as usual. Controls included (*a*) infected RBC mixed with normal human serum (NHS) from Geneva blood donors and incubated with antiserum, (*b*) normal RBC mixed with NHS and incubated with antiserum, (*c*) normal RBC mixed with NHS and incubated with NHS. The specific inhibition was calculated and the results are summarized in Table 2. It can be seen that the sensitivity of parasite detection was reduced in the presence of antibody, particularly at high titre.

The results of the above experiment indicate that the level of parasite detection by ELISA in whole blood may be reduced in the presence of a high titre of antibody, particularly at low levels of parasitaemia. A relatively simple alternative to whole blood would be packed, unwashed RBC, in which the amount of antibody in the test preparation would be substantially reduced. An experiment was therefore carried out to assess the efficiency of parasite detection in RBC exposed to antibody and recovered by centrifugation before testing. Samples of packed, washed RBC from *in vitro* cultures of *P. falciparum* at 10% parasitaemia were mixed with equal volumes of 10 different Gambian sera, which had completely prevented antibody-binding inhibition in the preceding experiment. One of these sera had an antibody titre < 1:200, four had titres < 1:1000, and five had titres > 1:3000. Control samples were mixed with NHS from Geneva blood donors. The samples were incubated at 37 °C for 30 min and the RBC were then packed and recovered and tested by ELISA without prior washing. Nine of the ten samples showed significant antibody-binding inhibition, with results ranging from 8.3% to 47.6%. The one negative result was from a sample of RBC exposed to a serum with an antibody titre of 1:800.

Table 2. Parasite detection by ELISA in presence of anti-malaria antibody in test samples

Antibody titre	No. tested	No. showing significant inhibition	No. showing reduced or zero inhibition[a]
< 1 : 200	7	6	3
< 1 : 1000	11	8	7
> 1 : 3000	12	7	9
Total	30	21	19

[a] Compared with control sample of infected RBC mixed with NHS before incubation with antiserum.

The field application of immunodiagnostic tests would be simplified if blood spots dried on filter-paper could be used rather than fresh blood or RBC. A preliminary experiment was therefore carried out to test the efficiency of the ELISA method when applied to RBC samples dried on filter-paper and recovered by elution. Samples of RBC from *in vitro* culture of *P. falciparum* at 10% parasitaemia were washed and mixed with equal volumes of NP40; 20-μl samples of RBC-NP40 mixture were placed on each of 20 1-cm diameter discs of filter-paper (Werthemann, Basel) and allowed to dry at room temperature. After 24 h, elution was carried out by placing the discs in 300-μl volumes of PBS-Tween overnight at 4 °C. Control samples of normal RBC were treated in the same way. The eluates were then tested by ELISA along with samples of fresh, infected and normal RBC at the same dilutions. The results showed no significant difference in specific antibody-binding inhibition between fresh and dried, eluted RBC. At this dilution, the fresh infected RBC gave 23.4% inhibition and the dried eluted samples gave 26.3 ± 5.5% inhibition.

DISCUSSION

The diagnosis of malaria by an ELISA method could provide a convenient alternative to blood-film examination in any study, whether epidemiological or clinical, where large numbers of samples have to be processed. In previous studies, we developed, using a murine malaria model (*2*), a radioimmunoassay (RIA) which was subsequently modified and applied to the detection of *P. falciparum* infection in man (*4*). We have now adapted the method, which is unchanged in principle, to an ELISA system with no loss of sensitivity of parasite detection.

Applied to RBC from *in vitro* cultures of *P. falciparum*, the test proved to be reproducible and capable of detecting parasites at a level of 8 parasites/10^6 RBC, a degree of sensitivity close to that achieved by microscopy. In this case, where parasites from the same source were used both to coat the microtitration wells and to react with the specific antibody, optimal sensitivity and consistency can be expected. When applied to RBC from 126 naturally infected Gambians, the test gave positive results in 86% of cases, where levels of parasitaemia ranged from 10 to 125 000/μl of blood. The minor reduction in sensitivity of parasite detection and the greater variability in degree of test positivity with the Gambian

samples could be related to antigenic differences between the cultured parasites used to coat the wells and those in the test RBC. It is also possible that the Gambian samples had undergone some alteration during two years' storage at −70 °C.

These results indicate that the ELISA inhibition method can detect parasites with a degree of sensitivity equal to that previously achieved by RIA (*4*). In both studies, the basic reagents were the same and the tests proved equally sensitive when applied to RBC from *in vitro* cultures of *P. falciparum*. The samples from the Gambia used in the two studies formed part of the same series, but owing to shortage of material, the two methods could not be carried out on identical samples. However, in randomly selected samples with microscopically proven infection, the overall detection rates of 82% by RIA and 86% by ELISA were comparable.

Most of our experiments were carried out using washed RBC in order to test cells infected *in vitro* and *in vivo* in comparable conditions. The presence of antibody in the test sample may reduce the sensitivity of parasite detection, as was shown by testing infected RBC mixed with antibody-containing sera of different titres. Under natural conditions, it can be envisaged that this effect would be particularly important in the case of blood samples with high antibody titres and low levels of parasitaemia. The test may not therefore be readily applicable to whole blood. However, the results obtained with infected RBC exposed to antibody, then recovered by centrifugation and tested without washing, indicate that removal of most of the serum may be enough to reduce the antibody content of the preparation to a level that would be unlikely to impair parasite detection. For application of the test on a large scale, unwashed RBC could provide a simple and relatively convenient alternative to whole blood. The efficiency of parasite detection in unwashed RBC from whole blood should be investigated in natural infections.

Preliminary results using RBC dried on filter-paper and recovered by elution suggest that dried samples may be suitable, provided that the elution does not introduce too great a dilution factor. The slightly greater variation in test positivity with the dried, eluted samples may have been due to irregular antigen trapping in the filter-paper. Further work is in progress to test different methods of drying and storing the test samples.

Double-antibody-sandwich ELISA and inhibition ELISA methods for the diagnosis of *P. falciparum* have recently been developed by Bidwell & Voller (*6*) with parasitized cells detected by these methods in blood from infected *Saimiri* monkeys at levels of $1/10^3$ RBC and $1/10^4$ RBC, respectively. The differences in sensitivity observed in the latter experiments and in our own may be related to three factors. Firstly, the RBC used in the present study were lysed by detergent before testing, thereby exposing the antigens of the intracellular parasites to the action of specific antibody. Secondly, Bidwell & Voller reacted conjugated immune serum with infected RBC, whereas we employed a second antiserum in the form of conjugated anti-IgG, which could be expected to enhance the reaction and therefore the sensitivity of parasite detection. Thirdly, differences in the source of specific antibody may be important. Sera from adults in an area hyperendemic for malaria, who have undergone repeated natural infection throughout life, may contain antibodies of greater affinity than serum from monkeys immunized by experimental infection and challenge. It has been shown that sera from people who have had numerous malaria infections react with a larger number of *P. falciparum* polypeptides than sera from patients recovering from a first infection (*10*).

Since our experiments were carried out using undefined reagents, the test should be regarded as semi-quantitative. Greater precision can be expected when purified malaria antigens and suitable monoclonal antibodies become available for use in immunodiagnosis. However, lack of monoclonal antibodies need not present a barrier to the field application of currently available methods for malaria diagnosis. The use of sera in the present study from the local population under investigation as source of antibody, illustrates the considerable degree of sensitivity that can be achieved with high-titre sera to the particular parasite strains in the region concerned. Furthermore, the test requires only minimal quantities of reagents; for instance, using immune serum diluted 1:40 as in our study, 10 ml of serum would be sufficient to carry out 40 000 assays, with each sample tested in duplicate. The ELISA methods that have been developed recently could therefore be readily applied in areas endemic for malaria by using immune sera from individuals in the local community as source of anti-malaria antibody.

RÉSUMÉ

DIAGNOSTIC DE L'INFECTION À *PLASMODIUM FALCIPARUM* CHEZ L'HOMME: DÉTECTION IMMUNO-ENZYMATIQUE (ELISA) DES ANTIGÈNES PARASITAIRES

On a mis au point une méthode immuno-enzymatique de type ELISA pour le diagnostic des infections à *Plasmodium falciparum* chez l'homme. L'antigène constituant la phase solide provenait de parasites de l'espèce *falciparum* cultivés *in vitro*, la source d'anticorps spécifique étant constituée par des immunsérums de Gambie. La fixation de l'anticorps dans les cupules enduites d'antigène était mise en évidence au moyen d'un conjugué phosphatase alcaline-immunoglobuline anti-IgG humaine préparée sur chèvre. La détection des parasites s'effectuait en observant l'inhibition de la fixation de l'anticorps. Cette épreuve a été appliquée à la recherche des parasites présents dans des hématies humaines provenant de cultures *in vitro* de *P. falciparum* ainsi que dans des hématies provenant de sujets gambiens infectés. Des hématies prélevées sur 100 donneurs de sang de Genève ont été utilisées comme témoins normaux non infectés. Les expériences de titrage ont permis de corréler le degré d'inhibition de la fixation de l'anticorps avec le nombre de parasites présents dans les hématies d'épreuve. Le seuil de détection se situait à 8 parasites pour 10^6 hématies. On a éprouvé des échantillons d'hématies provenant de 126 Gambiens présentant une infection confirmée par examen au microscope; une inhibition significative de la fixation des anticorps a été constatée chez 86% de ces cas, dont la parasitémie allait de 10 à 125 000 par μl de sang. Il est apparu qu'en présence de titres élevés d'anticorps dans les préparations d'épreuve, la sensibilité de la détection des parasites dans des hématies infectées provenant de cultures *in vitro* mélangées à des volumes égaux de divers immunsérums, était quelque peu réduite. Dans la plupart des cas, on a pu rétablir la sensibilité en récupérant les hématies par centrifugation avant l'épreuve. Lors d'une expérience préliminaire, on n'a pas constaté de différence significative dans l'inhibition de la fixation des anticorps selon que l'on utilisait des hématies infectées fraîches ou des hématies séchées sur papier filtre et récupérées par élution, encore que ces derniers échantillons aient présenté une plus grande variabilité.

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