IMMUNOFLUORESCENCE METHODOLOGY SUITABLE FOR LARGE SCALE APPLICATION TO MALARIA

by
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At the present time and for the foreseeable future malarial epidemiological studies will be based primarily upon the results of blood smear examinations supported by spleen palpation. The information yielded enables the current status of malaria in a population at the time of the survey to be evaluated with considerable accuracy. However, unless several blood surveys are done at different times of the year and are combined with spleen rates it is not possible to predict with any degree of certainty the amount and intensity of perennial malarial endemicity of a given area. It is in the hope of obtaining additional knowledge on the malarial experience of populations by simple methods that attempts have been made to detect and measure malarial antibodies.

To date the test most often used has been based on indirect fluorescent antibody technique. The main limitation of this technique has been the time consuming process of slide manipulation. The present paper describes, in detail, a methodology which allows multiple tests to be carried out simultaneously and so increases by a large factor the processing capability of any laboratory.

SERAS FOR TESTING

Donors

For malarial sero-epidemiological studies it is important that representative serum samples are taken from all age-groups of the population, as the antibody response is, at least to some extent, age-dependent. Valid comparisons of the results of different surveys can only be made on similar age-groups. The pattern of the malarial antibody titres in the young age-groups (e.g. under 1 year, 2-4 years, 5-9 years) gives, perhaps the best indication of malaria transmission levels.

Collection of serum or plasma

Serum can be collected in the usual manner from blood samples obtained by venipuncture. Alternatively, sera or plasma can be collected from blood obtained from a finger-prick and this is usually more acceptable to populations of developing areas of the world and renders return visits more rewarding.

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We have adopted techniques developed by Dr Th. Meuwissen of the Catholic University, Nijmegen, Netherlands and the East African Institute for Malaria and Vector Borne Diseases, at Amani, United Republic of Tanzania.

The apparatus required is shown in Plate 1 and consists of the following:

1. Disposable lancets
2. Heparinized capillary tubes
3. Portable blowlamp
4. Thermos flask
5. Micro-haematocrit centrifuge
6. Plasticine and labels.

Heparinized capillary tubes are filled with blood obtained by puncturing the skin of the finger. The capillary tubes are labelled with adhesive paper or preferably plastic labels (in the tropics) and are heat sealed at one end by means of the blowlamp. The tubes are then transferred to a flask, previously cooled in a domestic refrigerator, and can be transported to the laboratory. On arrival the capillary tubes are centrifuged on a micro-haematocrit centrifuge and the packed cell volume can then be noted if required. The tubes are cut at the plasma-packed cell interface and the sections of the tube containing the plasma are sealed with plasticine at both ends. The capillary tubes are then usually placed in small containers (e.g. "universal" glass or plastic containers) in small groups. The containers are labelled and stored at -70°C. This storage system permits any particular sample to be obtained from the deep freeze within a matter of minutes.

Alternatively blood can be taken directly on to filter paper discs which are then dried. The serum is obtained from the discs by elution in phosphate buffered saline (P.B.S.) pH 7.2 in the laboratory where the tests are being carried out. This method is feasible but storage conditions of the discs are extremely critical, which reduces the reliability and accuracy of the method.

Preparation of antigen

In most past studies species of monkey malaria parasites have been used as antigen. This is no longer necessary as human strains of Plasmodium malariae, Plasmodium falciparum and Plasmodium vivax adapted to Aotus trivirgatus (Owl monkeys) are now available. It is not easy to establish a new strain of human malaria in Aotus monkeys and it is, therefore, best to obtain one of the standard adapted strains when establishing the technique at a new laboratory centre.

The use of the homologous antigens avoids the theoretical (and possibly practical) objections inherent in using heterologous parasites as antigens.

Preservation of Aotus adapted strains of human malaria parasites

Heparinized or citrated blood from heavily infected animals is mixed with an equal quantity of 30% glycerol made up in P.B.S. The mixture is then distributed in 0.5 ml amounts into ampoules which are heat sealed. The ampoules are then slowly cooled to -70°C. This slow cooling can be most simply achieved by placing the sealed ampoules in a large beaker of methyl alcohol at room temperature. If this beaker is then transferred to a deep freeze (-70°C) cabinet a satisfactory slow rate of cooling will occur over a period of several hours.

The maintenance of the strains in the deep frozen condition avoids the necessity for frequent passaging, expensive in terms of time and wasteful of monkeys.
Infection of monkeys

Three to four weeks before the date when it is planned to make a batch of antigen slides, a splenectomized Aotus is injected with the contents of one or two ampoules of the preserved parasites which have been quickly warmed to room temperature. Intravenous injection via the femoral vein is the most convenient route.

The course of the infection is monitored by examination of thick and thin blood smears stained with Giemsa. These are taken at daily intervals early in the infection and, when the parasitaemia reaches about 0.1% (10 parasites/10 000 rbc) smears are made both morning and afternoon, until the infection is of a sufficient density to be used as antigen. It is important to determine the phasing of the parasites developmental cycle because an antigen to be suitable must contain about 100 parasites/10 000 rbc in the schizont or mature trophozoite stage. The P. malariae and P. falciparum strains which we use predictably undergo schizogony in the afternoon, a convenient characteristic, as this allows antigen to be made in the morning when maturing parasites are numerous.

Although several broods of parasites frequently develop, one brood usually predominates. The minority group of parasites at an earlier stage of development can be ignored. Antigen should be made before crisis occurs in P. malariae infections and should be made at least one developmental cycle before death occurs in P. falciparum infections. The known characteristics of the strains allow these events to be forecast with some accuracy.

A thin blood film is made and examined immediately before the antigen is made. If the film is satisfactory the donor monkey is tranquillized with phencyclidine (2 mg/kg intramuscularly) and 1000 units of heparin are given into the femoral vein. The needle is left in the vein and after two minutes, gentle suction is applied and blood is withdrawn into a syringe previously rinsed with heparin. Usually 25-35 ml of blood can be obtained from an adult Aotus before it dies as a result of exsanguination. The blood is centrifuged at 1500 g for 10 minutes, supernatant discarded and the red cells resuspended in P.B.S. The centrifuging and washing procedure is repeated three times. The red cells are then resuspended in a volume of P.B.S. calculated to yield 1-10 erythrocytes infected with mature trophozoites or schizonts per high power (oil immersion) microscope field of a thin blood film.

Preparation of antigen slides

The necessary apparatus is shown on Plate 2 and consists of the following:

1. Wheeled perspex reservoir "haemobile" 1
2. Iron applicator with 12 protruding pegs
3. Microscope slides sprayed with hydrophobic material in which clear areas have been left.

Fig. 1b Plate 2
Fig. 1a
Fig. 1c

The bulk of the diluted red cell suspension is kept at 4°C. Twenty ml aliquots are transferred to the "haemobiles". The antigen is then dispensed by means of the applicator to the clear wells on the coated slides. Wheels are fitted to the "haemobile" because this allows much faster dispensation of the antigen.

The coated slides can be obtained ready prepared from Shandon Scientific Co., Runcorn, Cheshire, England. Alternatively they can be made by dispensing drops of glycerol on to slides with the applicator and then spraying the slides with Fluoglide (Chemplast Inc., United States of America) After the coating has dried the slides are washed in water to remove the glycerol and are then redried. 2

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1 Constructed in the Nuffield Institute of Comparative Medicine workshop.

2 See also Turner, A., "Preparation of 8-place slides for multiple testing in fluorescent antibody procedures", WHO/Mal/71.743.
The drops of red cell suspension, approximately 0.2 μl are placed on the slides and allowed to dry; the slides are then wrapped individually in absorbent paper and are packeted in groups of 10. These packets are stored in cardboard boxes at -50°C to -70°C.

PERFORMANCE OF THE INDIRECT FLUORESCENT ANTIBODY TEST

Apparatus

(1) Perspex trays holding six slides ¹

(2) Micropipette to deliver 10 μl.

(3) Perspex haemagglutination plate with well pattern which matches well pattern on slides ¹

(4) Takatsy loops for dilutions

(5) Transfer applicators. Consisting of six or 12 hollow steel tubes set in small perspex chambers to which suction can be applied ¹

(6) The humid chamber consists of a perspex box containing shelves on to which the trays of slides can be fitted. A well in the base of the box is flooded with warm water to provide the necessary humidity ¹

(7) The washing trough consists of a perspex tank with an internal ridge on either side on to which the trays of slides can be suspended. Agitation of the solution is achieved by a magnetic stirrer ¹

Fig. 2F Plate 2

Fig. 2A

Fig. 2C.

Fig. 2B

Fig. 2D

Fig. 2E

Fig. 3A

Fig. 3B

On the day when the tests are to be carried out, packets of slides are removed from the deep-freeze and are allowed to warm to room temperature in a dessicator or plastic bag before being unwrapped. The slides are then fitted face upwards in the perspex trays where they remain for subsequent processing.

The capillary tubes containing the plasma samples to be tested are warmed to room temperature and measured amounts (10 μl) of plasma are removed from them by breaking one end of the tube and inserting the tip of a micro-pipette.

The initial dilution is made in the top row of wells in the perspex haemagglutination plates and subsequent dilutions are made by means of loops which transfer 25 μl. For two-fold dilutions 25 μl diluent is placed in each well and for threefold dilutions 50 μl diluent in each well.

The samples are transferred to the slides in groups of six or 12 by means of the transfer applicators, alternatively the serum dilutions can be transferred individually by means of micro-pipettes or with glass Pasteur pipettes but this takes much longer.

The subsequent processing of the trays of slides is as follows:

(1) Incubate with serum dilutions in a humid chamber for 40 minutes.

(2) Rinse with P.B.S. using a wash bottle. Wash with P.B.S. in the trough for 15 minutes.

¹ Constructed in the Nuffield Institute of Comparative Medicine workshop.
(3) Pour off P.B.S. and shake slides until almost dry.

(4) Apply fluorescein labelled antoglobulin (with or without 0.1% Evans Blue) to each well by means of the applicator. Incubate in humid chamber for 30 minutes.

(5) Rinse in P.B.S. using a wash bottle. Then wash for a further 15 minutes.

Rinse in acetone if Evans Blue is used in step 4.

(6) Remove slides from trays. Mount in P.B.S. (or 10% glycerol in P.B.S. if there is delay in reading) under coverslip or transparent plastic film.

(7) Examine on fluorescent microscope.

Antiglobulin conjugates

Fluorescein labelled antisera reactive against the individual human immunoglobulins, especially IgG and IgM, can be used or a crude labelled antiserum against human gamma globulin can be employed. The suitable dilution of the conjugate (in P.B.S.) must be determined by trial and error, but it is usually in the range of 1:10 - 1:40 for the available commercial preparations. To the working dilution of the conjugate Evans Blue can be added to a final concentration of 0.1%. The washing should then include a brief acetone rinse.

Reading the result

The slides are examined by fluorescent microscopy. The excitation is usually obtained using a high pressure mercury vapour lamp (e.g. Osram H.B.O. 200) filtered (e.g. by BG 38 and BG 12 filters) to give U.V. blue illumination. Barrier filters with transmission above 420-450 μm are used to provide a dark background for observation.

Six successive dilutions of a particular serum are tested along one side of each slide in the series of six adjacent antigen wells. Reading commences with the strongest serum dilution and proceeds to the progressively weaker samples. The fluorescence of the mature parasites and schizonts is noted and the last serum dilution which yields readily detectable fluorescence is the end point. At low dilutions other forms of the parasites (e.g. ring forms) also fluoresce. These should be ignored because reading the result on the schizonts is more sensitive.

Using the technique outlined above it is possible for a single person to set up and read the results on 300-400 antigen spots per day.

Application to other protozoal diseases

We have found that the techniques described for malaria in the above sections are suitable for use with *Toxoplasma gondii* and *Entamoeba histolytica*. Freeze dried organisms of these parasites were kindly donated to us by Dr G. Kane of Wellcome Reagents. This material was reconstituted in half the recommended volume of P.B.S. and was dispensed from a master slide or shallow well by means of the antigen applicator, to large numbers of the coated slides. Best results were obtained using Evans Blue as counter-stain.

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APPENDIX

Supplies of materials and reagents

The following sources have been used by the authors:

1. CONJUGATES (Fluorescein labelled antiglobulins)
   
   (a) Nordic Pharmaceuticals
       Langeestraat 57-61
       P.O. Box 22
       Tilburg, The Netherlands

   (b) Wellcome Reagents Ltd.
       Beckenham
       Kent, BR3 3BS
       England

2. PLASTIC SPRAYS (Fluoroglide)

   Chemplast
   100 Dey Road
   Wayne
   New Jersey
   United States of America

   (United Kingdom Agents)
   Marshall-Howlett
   293 Main Road
   Sidcup, Kent
   England

3. READY-PREPARED COATED SLIDES

   These will shortly be available from:

   Shandon Scientific Co. Ltd.
   Brindley 73
   Astmoor Industrial Estate
   Runcorn, Cheshire
   England

4. For details of Aotus adapted strains of P. falciparum and P. malariae and for details of slide processing apparatus, contact:

   Dr. A. Voller
   Nuffield Institute of Comparative Medicine
   The Zoological Society of London
   England
RESUME

Ce document expose en détail une méthode permettant d'effectuer simultanément de multiples épreuves d'immunofluorescence indirecte pour le diagnostic du paludisme et d'accroître ainsi considérablement la capacité de travail du laboratoire.

Il décrit la collecte du sérum ou du plasma sur le terrain et la préparation de l'antigène à partir d'espèces de Plasmodium infectantes pour l'homme et adaptées à Aotus trivirgatus. Il explique la manière d'infecter le singe nyctipithèque et donne des explications détaillées au sujet de l'appareil à utiliser pour la préparation des lames d'antigène. L'exécution de l'épreuve et la lecture des résultats sont également décrites, ainsi que l'application de cette technique à Toxoplasma gondii et Entamoeba histolytica.

Grâce à cette technique, une personne peut préparer et lire de 300 à 400 lames d'antigène par jour.¹

¹ (Voir également "Preparation of 8-place slides for multiple testing in fluorescent antibody procedures", par Turner, A. - WHO/MAL/71.743.)
PLATE 1

Obtaining plasma samples from a finger prick

Fig. 1. Blood being taken into labelled capillary.
Fig. 2. Heat sealing of capillary tubes in the field.
Fig. 3. Centrifuging and final sealing of capillary tubes in the laboratory.
PLATE 2

Apparatus for multiple immunofluorescence tests for malaria

Fig. 1. Apparatus used in preparation of antigen.
Fig. 2. Apparatus used for making dilutions and dispensing plasma samples.
Fig. 3. Apparatus used for actual immunofluorescence test. For details see text.