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Immunofluorescence Method Suitable for Large-scale Application to Malaria*

by A. VOLLER¹ & P. O'NEILL²

At the present time, and in the foreseeable future, epidemiological studies of malaria will be based primarily on the results of examinations of blood smears, supported by spleen palpation. This information 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 carried out 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 malaria endemicity in 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.

The test most often used is based on the 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 method that allows multiple tests to be carried out simultaneously and so increases, by a large factor, the processing capability of any laboratory.

Sera for testing

Donors. For sero-epidemiological studies of malaria, it is important that representative serum samples should be taken from all age groups of the population since the antibody response is, at least to some extent, age-dependent. Valid comparisons of the results of different surveys can be made only 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 venepuncture. Alternatively, sera or plasma can be collected from blood obtained from a finger-prick and this is usually more acceptable to populations in developing areas.

Techniques developed by Dr T. Meuwissen of the Catholic University, Nijmegen, Netherlands, and the East African Institute for Malaria and Vector Borne Diseases, at Amani, United Republic of Tanzania, have been adopted and the apparatus required consists of disposable lancets, heparinized capillary tubes, a portable blowlamp, a vacuum flask, a microhaematocrit centrifuge (Fig. 1), modelling clay, and labels (Fig. 1).

Heparinized capillary tubes are filled with blood obtained by puncturing the skin of a finger. The

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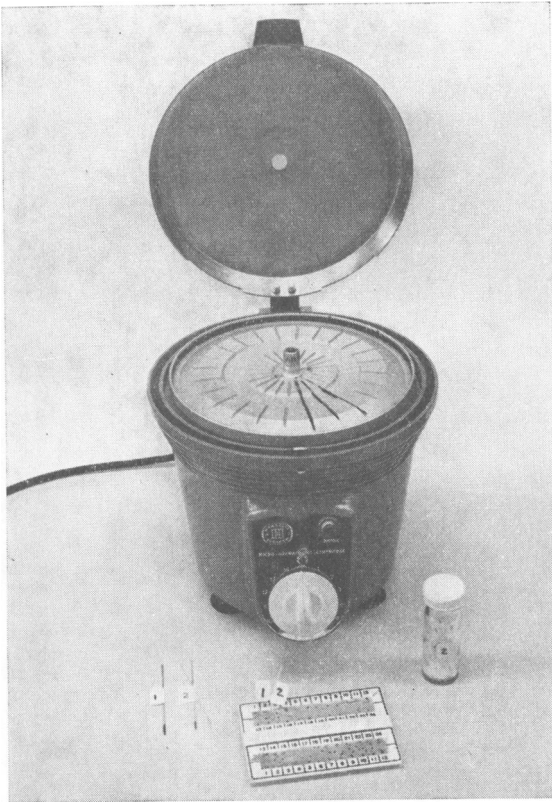


Fig. 1. Microhaematocrit centrifuge, labelled capillary tubes containing blood samples, and labelled storage container.

capillary tubes are labelled with adhesive paper labels or, in the tropics, preferably plastic labels, 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 transported to the laboratory. On arrival, the capillary tubes are centrifuged in a microhaematocrit centrifuge and the packed cell volume can then be noted if these data are required. The tubes are cut at the interface between the plasma and the packed cells and the sections of the tube containing the plasma are sealed with modelling clay at both ends. Batches of capillary tubes are then usually placed in small containers (e.g., "universal" glass or plastic containers). The containers are labelled and stored at -70°C . This storage system permits any particular sample to be obtained from the deep-freeze unit quickly.

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

Preparation of antigen. In most previous studies, various monkey plasmodia have been used as the antigen. This is no longer necessary since human strains of *Plasmodium malariae*, *P. falciparum*, and *P. vivax* adapted to *Aotus trivirgatus* (owl monkey) are now available. It is not easy to establish a new strain of human malaria in *Aotus* monkeys and it is, therefore, advisable to obtain one of the standard adapted strains when the technique is being established at a new centre. The use of homologous antigens avoids the theoretical, and possibly the practical, objections inherent in using heterologous parasites as antigens.

Preservation of Aotus-adapted strains of human plasmodia. Heparinized or citrated blood from heavily infected animals is mixed with an equal quantity of 30% glycerol made up in phosphate-buffered saline. The mixture is distributed in 0.5-ml amounts into ampoules, which are then heat-sealed. The ampoules are slowly cooled to -70°C . This slow cooling can be most simply achieved by placing the sealed ampoules in a large beaker of methanol at room temperature. If this beaker is then transferred to a deep-freeze (-70°C) unit, 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, which is time-consuming and wasteful of monkeys.

Infection of monkeys. Three or four weeks before a batch of antigen slides is to be prepared, a splenectomized *Aotus* is injected with the contents of 1 or 2 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 examining Giemsa-stained thick and thin blood smears. The smears are made at daily intervals early in the infection and, when the parasitaemia reaches about 0.1% (10 parasites/10 000 red blood cells), smears are made both morning and afternoon, until the infection is of a sufficient density to be used as an antigen. It is important to determine the phasing of the de-

velopmental cycle of the parasite because an antigen must contain about 100 parasites/10 000 red blood cells in the schizont or mature trophozoite stage. The *P. malariae* and *P. falciparum* strains that we use predictably undergo schizogony in the afternoon, which 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 1 000 units of heparin are injected into the femoral vein. The needle is left in the vein and, after 2 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 1 500 *g* for 10 minutes, the supernatant fluid is discarded, and the red blood cells are resuspended in phosphate-buffered saline. The centrifuging and washing procedure is repeated 3 times. The red blood cells are then resuspended in a volume of phosphate-buffered saline calculated to yield 1–10 erythrocytes infected with mature trophozoites or schizonts per high power (oil-immersion) microscope field in a thin blood film.

Preparation of antigen slides (Fig. 2). The apparatus necessary consists of a wheeled Perspex reservoir "haemobile",¹ an iron applicator with 12 protruding pegs,¹ and microscope slides sprayed with hydrophobic material in which clear areas have been left.

The bulk of the diluted red blood cell suspension is kept at 4°C, and 20-ml quantities 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 the antigen to be dispensed more quickly.

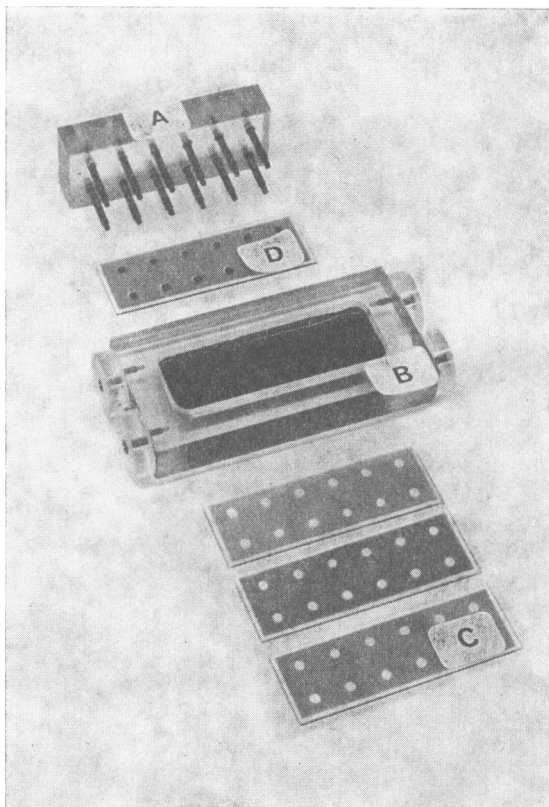


Fig. 2. Apparatus used in the preparation of antigen slides: A, iron applicator; B, "haemobile"; C, microscope slides sprayed with hydrophobic material; D, completed slide with antigen in wells.

The coated slides can be obtained ready prepared² or they can be made by dispensing drops of glycerol on to slides with the applicator and then spraying the slides with Fluoroglide.² After the coating has dried, the slides are washed in water to remove the glycerol and then redried.³

Drops of red blood cell suspension, each approximately 0.2 µlitre, are placed on the slides and allowed to dry; the slides are then wrapped individually in absorbent paper and are packed in groups of 10. These packets are stored in cardboard boxes at –50 to –70°C.

¹ See Annex.

² See also Turner, A. (1971) *Preparation of 8-place slides for multiple testing in fluorescent antibody procedures*, Geneva (unpublished document WHO/Mal/71.743).

¹ This apparatus was constructed in the workshop of the Nuffield Institute of Comparative Medicine.

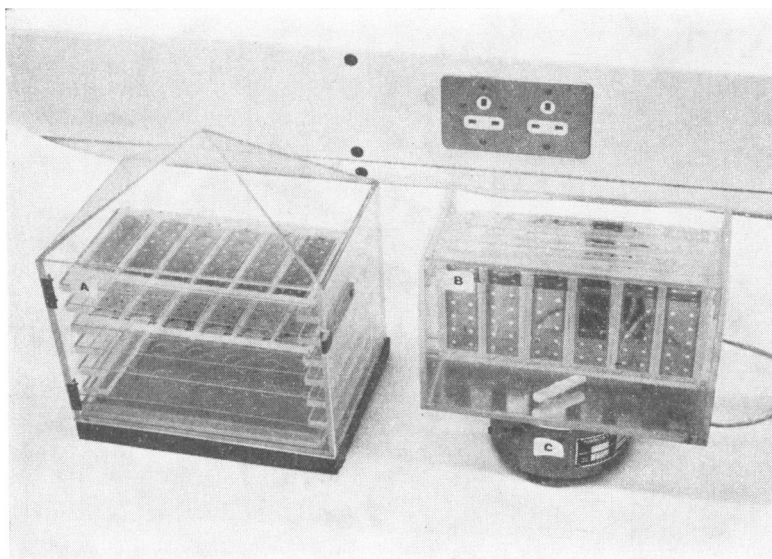


Fig. 3. Apparatus used for making dilutions and dispensing plasma samples. A, micropipette; B, Takatsy loops for dilutions; C, haemagglutination plate; D, E, transfer applicators; F, slide tray.

Performance of the indirect fluorescent antibody test

Apparatus. The following apparatus is required (Fig. 3, 4): (1) Perspex trays holding 6 slides;¹ (2) a micropipette to deliver 10 μ litres; (3) a Perspex haemagglutination plate with a well pattern to match the well pattern on the slides;¹ (4) Takatsy loops for dilutions; (5) transfer applicators (these consisting of 6 or 12 hollow steel tubes set in small Perspex chambers to which suction can be applied)¹; (6) a humid chamber, consisting of a Perspex box containing shelves, on which the trays of slides can be fitted, and a well in the base, which is flooded with warm water to provide the necessary humidity;¹ and (7) a washing trough, consisting of a Perspex tank with an internal ridge on either side from which the trays of slides can be suspended. The solution is agitated with a magnetic stirrer.¹

When the tests are to be carried out, packets of slides are removed from the deep-freeze unit and are allowed to warm to room temperature in a desiccator or plastic bag before being unwrapped. The slides are then fitted face upwards into the Perspex trays where they remain during the subsequent processing.

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

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 that transfer 25 μ litres. For 2-fold dilutions, 25 μ litres of diluent are placed in each well and for 3-fold dilutions 50 μ litres of diluent are used.

The samples are transferred to the slides in groups of 6 or 12 by means of the transfer applicators, alternatively the serum dilutions can be transferred individually by means of micropipettes. (Glass Pasteur pipettes can also be used but this takes much longer.)

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

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

(2) Rinse with phosphate-buffered saline, using a wash-bottle. Wash with phosphate-buffered saline in the trough for 15 minutes.

(3) Pour off the phosphate-buffered saline and shake the slides until they are almost dry.

¹ Constructed in the workshop of the Nuffield Institute of Comparative Medicine.

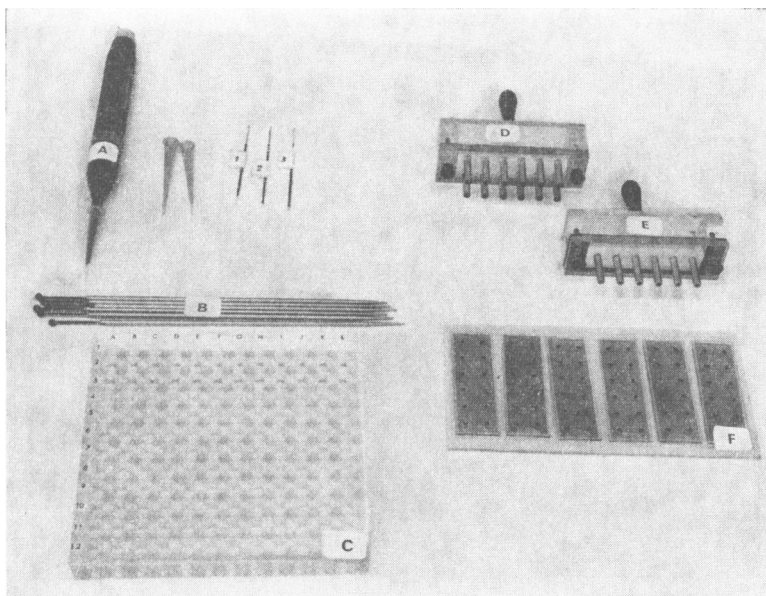


Fig. 4. Apparatus used for immunofluorescence test. A, humid chamber; B, washing trough; C, magnetic stirrer.

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

(5) Rinse in phosphate-buffered saline using a wash-bottle; then wash for a further 15 minutes. (Rinse in acetone if Evans blue is used in step 4.)

(6) Remove the slides from the trays, mount in phosphate-buffered saline (or in 10% glycerol in phosphate-buffered saline if there is a delay in reading) under a coverslip or transparent plastic film.

(7) Examine under the 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 γ -globulin can be employed. The suitable dilution of the conjugate (in phosphate-buffered saline) must be determined by trial and error, but it is usually in the range of 1:10–1:40 for the preparations commercially available. 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 rinse in acetone.

Reading the result. The slides are examined by fluorescent microscopy. The excitation is usually obtained by means of 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 UV-blue illumination. Barrier filters with transmission above 420–450 nm 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 ones. The fluorescence of the mature parasites and schizonts is noted and the last serum dilution to yield readily detectable fluorescence is the endpoint. At low dilutions other forms of the parasite (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 are also suitable for use with *Toxoplasma gondii* and *Entamoeba histolytica*. Freeze-dried organisms of

these parasites were kindly donated by Dr G. Kane, Wellcome Reagents. This material was reconstituted in half the recommended volume of phosphate-buffered saline and was dispensed from a master slide or shallow well by means of the antigen applicator, to large numbers of coated slides. The best results were obtained when Evans blue was used as a counterstain.

ACKNOWLEDGEMENTS

The authors are grateful to Mr G. Ray for constructing the apparatus used in this work, and to Dr C. C. Draper and Mrs D. G. Green for testing the equipment.

Annex

SUPPLIES OF MATERIALS AND REAGENTS

The following sources were used by the authors:

(1) *Conjugates* (fluorescein-labelled antiglobulins): Nordic Pharmaceuticals, Langestraat 57-61, P.O. Box 22, Tilburg, The Netherlands; and Wellcome Reagents Ltd., Beckenham, Kent, England.

(2) *Plastic sprays* (Fluoroglide): Chemplast, 100 Dey Road, Wayne, N.J., USA. (UK agent: Marshall Howlett, 293 Main Road, Sidcup, Kent, England.)

(3) *Ready-prepared coated slides*: These will shortly be available from Shandon Southern Instruments, Ltd, Camberley, Surrey, England.

(4) Details of *Aotus*-adapted strains of *P. falciparum* and *P. malariae*, and of slide-processing apparatus, may be obtained from Dr A. Voller, Nuffield Institute of Comparative Medicine, The Zoological Society of London, Regent's Park, London N.W.1., England.

Aedes aegypti and *Aedes simpsoni* Breeding in Coral Rock Holes on the Coast of Tanzania*

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Aedes aegypti (L) and *Aedes simpsoni* (Theobald) are closely related species of the subgenus *Stegomyia*. Both species are widely distributed throughout Africa (Stone et al., 1959; van Someren, 1968). *Ae. aegypti* and *Ae. simpsoni* are both proved vectors of yellow fever in Africa, particularly in Uganda (Mahaffy et al., 1942; Smithburn & Haddow, 1946) and in Nigeria (Beeuwkes & Hayne, 1931). The recent Ethiopian epidemic of 1960-62 illustrated the potential of epidemics transmitted by *Ae. simpsoni* (Metselaar et al., 1970). The importance of these two mosquitos in particular as potential vectors of yellow fever makes it imperative to know the full extent of their breeding habits.

During the course of ecological studies on species of *Aedes* in the area of Dar es Salaam, Tanzania, *Ae. aegypti* and *Ae. simpsoni* were both found to be

breeding in holes in coral along the coast. Wiseman et al. (1939) had reported *Ae. aegypti* breeding in such holes on the Kenya coast near Mombasa. However, to the best of our knowledge, the present study is the first to record *Ae. simpsoni* breeding in holes in coral.

Description of the biotope

Msasani peninsula (6°45'S, 39°17'E) is located 8 km north of Dar es Salaam, Tanzania, and extends about 3 km into the Indian Ocean. The entire peninsula is an old elevated coral reef, with a surface up to about 12 m above sea level, which is mostly covered with a thin layer of sandy soil. The underlying rock is coral limestone with numerous cavities and embedded debris such as mollusc shells. The rock surface is frequently exposed and in such places many of the rock cavities are open and collect rain water.

A continuation of the elevated reef at Msasani occurs as the uninhabited offshore Bongoyo Island. On the island the vegetation is rank and luxurious with an almost closed canopy at 10-20 m, and both tree holes and rock holes are common. The peninsula was formerly cleared for the growing of sisal but the plantations have been neglected for several

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