

Dark-Field Microscopy for Detection of Malaria in Unstained Blood Films

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Pigment present in different forms of *Plasmodium* species infecting humans exhibits light scattering when blood films are viewed by dark-field microscopy. This facilitates the detection of parasite-infected cells owing to their brightly illuminated appearance. The technique was described long ago for the detection of certain highly pigmented forms of malarial parasites such as schizonts and gametocytes but has not found an application in routine diagnosis. Here, modifications are described which allow the detection of all forms of human malarial parasites, including ring forms, in unstained blood films. The technique offers the distinct advantages of rapid diagnosis, increased sensitivity, and adaptability to field work.

Routine laboratory diagnosis of malaria still relies totally on the microscopic examination of blood films stained with the conventional stains such as Giemsa, Wright stain, Leishman stain, etc. These techniques require examination of the slide under oil (magnification, $\times 1,000$), which is time consuming. Conventional thin and thick films both have certain advantages and disadvantages. Thin films only allow the scanning of a relatively small volume of blood and are therefore less sensitive than thick films. They have the advantage, however, of clearly revealing the detailed morphology of the malarial parasites, which is needed for accurate identification of species. Thick films require some experience to prepare and tend to be burdensome for laboratories in areas where malaria is not encountered regularly, especially where automated equipment is used for staining thin films. Moreover, owing to the distortion of certain forms, such as gametocytes and schizonts, accurate identification of species on the basis of the thick films alone may be difficult.

Several new procedures have been recently employed to increase the sensitivity of laboratory diagnosis of malaria. These include, for example, fluorescent-antibody staining of blood films (2, 7), tissue staining with fluorescent antibody or immunohistological techniques (4), and detection of malarial antigen by a modified radioimmunoassay (1) or enzyme immunoassay (5). The use of these techniques, however, seems to be suitable only for specialized laboratories owing to their technical requirements. The culture of the malaria parasite (8) also does not

seem to be feasible at present as a simple diagnostic procedure. The detection of a serological response to malaria (1, 3) may be useful in retrospective diagnosis or survey, but not in the diagnosis of a current infection.

We have attempted to facilitate the microscopic detection of different forms of malarial parasites by using the technique of dark-field microscopy. The technique was described several years ago for the detection of highly pigmented forms of malarial parasites such as schizonts and gametocytes (6, 9). However, none of the described methods has found general recognition in the routine diagnosis of malaria. This may be due to the fact that the technique requires certain modifications to allow the easy recognition of certain forms of malarial parasites, especially ring forms, which contain smaller amounts of aggregated pigmented particles, particularly at early stages of development. These modifications, which are described here, also reduce artifacts such as dust and glass particles, which can be bothersome and confusing. I here describe the use of this technique with different preparations of unstained blood films in which it is possible to detect rapidly all the recognized forms of malarial parasites, including ring forms. The advantages that the technique offers in simplifying and expediting the diagnosis of malaria are discussed.

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MATERIALS AND METHODS

Blood was obtained either directly from a finger prick or from routine hematology tubes of anticoagu-

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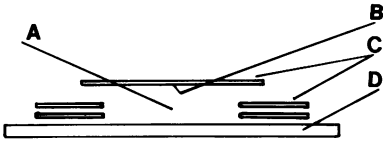


FIG. 1. Use of inverted raised cover slip for viewing thin film present on the lower side of the raised cover slip. The same technique was also used for viewing wet-mount specimens. (A) Mounting medium; (B) specimen; (C) cover slips; (D) slide. If the cover slips supporting the raised specimen are not thick enough, a stack of two or three cover slips can be used.

lated blood. Thin blood films were spread on long cover slips (22 by 50 mm). After drying, each cover slip was placed upside down on a slide on which two blank cover slips (22 by 22 mm) were placed to raise the cover slip containing the specimen in a bridge-like manner (Fig. 1). The specimen was mounted in buffered glycerol (90 ml of glycerol, 10 ml of H₂O, 0.16 g of Na₂CO₃, 0.0715 g of NaHCO₃) by adding the glycerol at one side so that it seeped under the cover slip. This prevented the formation of any bubbles.

Thick films were prepared by making a medium-thick film of blood on a slide. After drying, the film was lysed by dipping in distilled water, allowed to dry, and mounted in buffered glycerol. For more stable preparations, films were fixed for 5 min in methanol before mounting in buffered glycerol. A few minutes were allowed for glycerol to penetrate methanol-fixed thick films so as to subdue background illumination.

Concentrated preparations were made by mixing 0.5 ml of blood with 1.5 ml of distilled water and shaking gently until complete lysis was obtained. The specimen was then centrifuged at 3,000 rpm for 10 min. The supernatant was decanted, and the sediment was suspended, spread on a slide, dried, and mounted in buffered glycerol. For stable preparations the film was

fixed for 5 min in methanol before mounting.

Wet mounts were prepared by mixing a drop of blood with 5 drops of glycerol containing 2.5% sodium dodecyl sulfate and 1% Nonidet P-40 in distilled water. If complete lysis did not occur, the concentration of sodium dodecyl sulfate was increased slightly. Two cover slips were placed on the sides of the specimen, and a third one was placed on top (Fig. 1). This allowed focusing on the middle part of the specimen, away from artifacts present on glass surfaces. If a more concentrated preparation was desired, a drop of blood could be mixed with 10 to 15 μ l of a 10% solution of sodium dodecyl sulfate in water before examination as described above. All of the concentrated preparations described above were suitable for the detection of gametocytes and schizonts but were not particularly reliable for the detection of ring forms, especially the weakly pigmented ring forms sometimes encountered with *Plasmodium falciparum*. For microscopy, a standard transmission light microscope (Reichert, Diavar model) was used for all investigations. Dark-field attachments included a condenser (no. 67297, A-1, 18/1.42) and $\times 40$ and $\times 100$ objectives fitted with iris diaphragms. Viewing was done at magnifications of $\times 250$ and $\times 400$, but photography was done at $\times 1,000$ (under oil) unless specified otherwise. Cover slips prepared in the field could be conveniently transported by taping, face down, to pages of a clean notebook.

RESULTS

Figure 2 shows the appearance of *P. falciparum* gametocytes and ring forms in an unstained thin film prepared on a long cover slip, inverted, and mounted in glycerol while being raised from the slide by the support of two smaller cover slips (Fig. 1). This inverted raised cover slip arrangement avoids most of the artifacts present on the surface of the slide and the mounting medium since it renders these artifacts

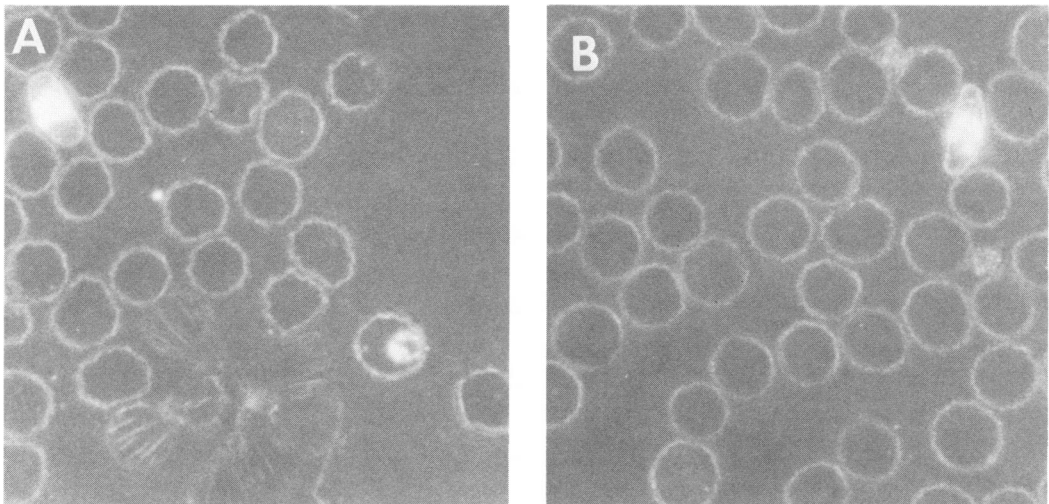


FIG. 2. *P. falciparum* in dark-field microscopy of unstained thin blood films. (A) Ring forms and microgametocytes; (B) macrogametocytes.

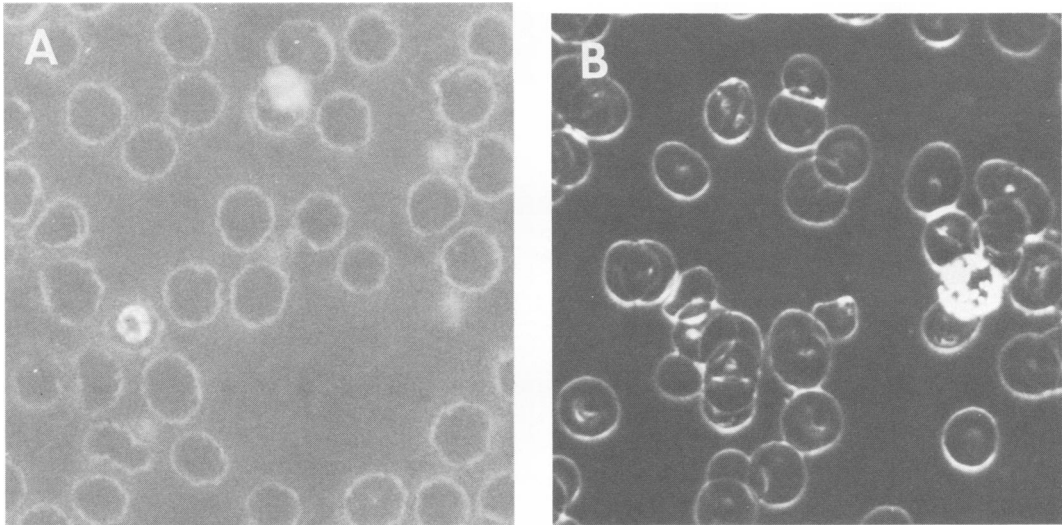


FIG. 3. *P. malariae* in dark-field microscopy of unstained thin blood films. (A) Ring forms; (B) trophozoites. Magnification, $\times 1,000$.

out of focus. Note that the bright pigmentation in the crescent-shaped gametocytes is concentrated in the middle area. In some specimens, *P. falciparum* ring forms may exhibit less pigmentation but can still be clearly seen by this technique. Double rings and appliqué forms are also clearly observed.

Figure 3A shows the ring forms of *Plasmodium malariae* in unstained thin-film preparations. These ring forms are larger than the ring forms of *P. falciparum*. All *P. malariae* ring forms observed so far exhibited bright pigmentation. Figure 3B shows trophozoites of *P. malariae*, which are highly pigmented.

Figure 4 shows schizonts of *Plasmodium vivax*. This form is also highly pigmented, and the pigment is present in discrete rodlets scattered over the whole of the infected erythrocyte. The enlargement of the infected erythrocyte is also evident.

More concentrated preparations such as thick films, wet-mount preparations, and preparations concentrated by centrifugation after lysis of the cells with water were also used to increase the sensitivity of this method in detecting parasites. Highly pigmented forms could easily be recognized in these preparations (Fig. 5). Identification of ring forms, particularly when present in low numbers or when they contained weak pigmentation, was more difficult. The thin-film preparation described above is more suitable for that purpose. In addition, artifacts may occasionally be confused with ring forms, but not with the more characteristic schizont and gametocyte forms, in such concentrated preparations.

DISCUSSION

The illuminated appearance of malarial parasites under dark-field microscopy is probably a result of Tindall scattering of the incident light. This property is associated with pigmented par-

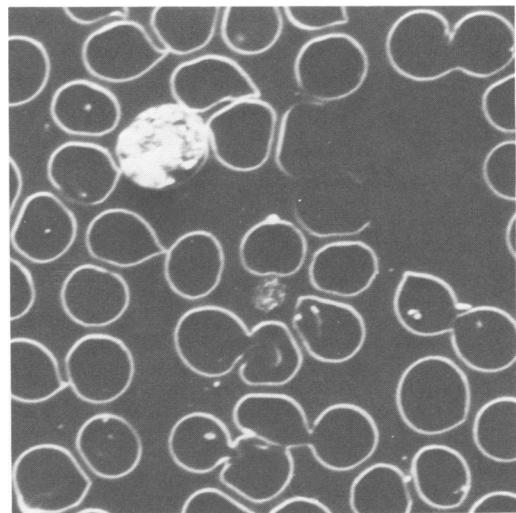


FIG. 4. *P. vivax* schizonts in dark-field microscopy. Magnification, $\times 1,000$. Unstained thin films were prepared as described in the text. If desired, thin films stained routinely with Giemsa or Leishman stain can also be examined by dark-field microscopy for the characteristic schizonts of *P. vivax*. Both stained and unstained preparations give a similar appearance, but staining gives them a sky-blue color.

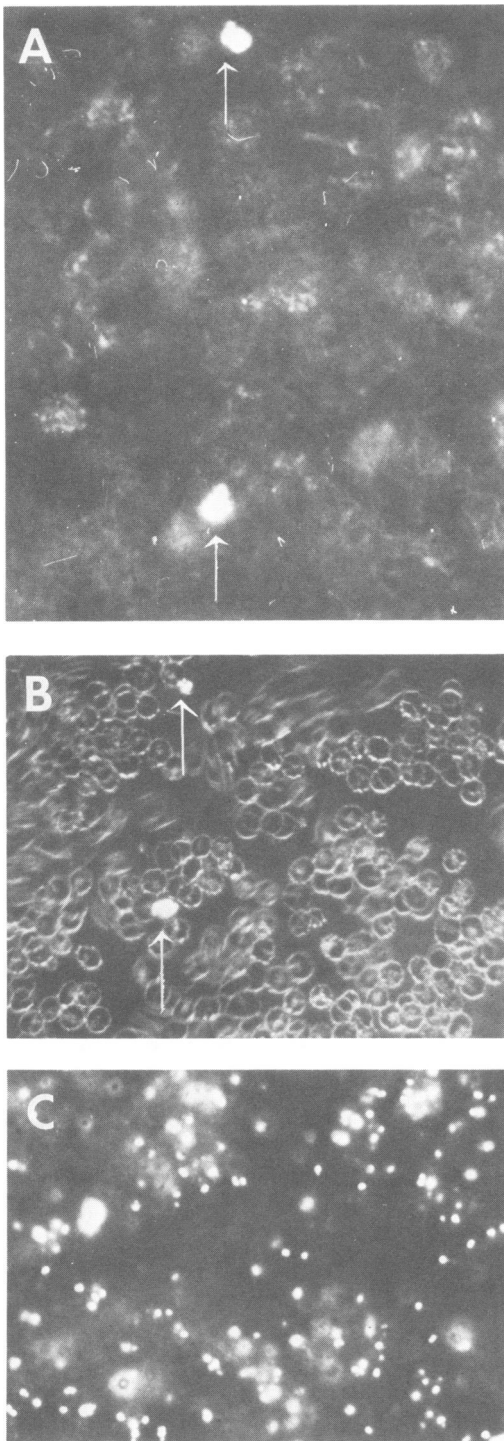


FIG. 5. Various forms of malarial parasites in concentrated preparations of lysed blood. (A) *P. falciparum* gametocytes (thick film); magnification, $\times 1,000$. (B) *P. malariae* trophozoites (wet mount); magnification, $\times 400$. (C) *P. falciparum* ring forms (highly pigmented); magnification, $\times 1,000$.

ticles in the parasites which are seen as brown rodlets with conventional staining. The pigment is known to be due to the presence of hemozoin, a metabolic degradation product of hemoglobin (4).

The technique described here relies on a routinely used method of microscopy and requires very few additions of equipment or material. One of the main advantages of the technique is its speed since examination is immediate and does not require any fixation or staining. This makes the technique particularly suitable for field work. The technique also allows speedier scanning of relatively larger volumes of blood under lower magnifications ($\times 250$ or $\times 400$), which should make it more sensitive than routine bright-field methods. Although a detailed comparison between the two techniques is still under way, our experience so far indicates that the dark-field technique is very sensitive in detecting malaria-infected specimens. Although the criteria applied for species identification are the same as those used with conventional staining methods, the ease of detection of certain pigmented forms such as schizonts and gametocytes may facilitate species identification with specimens containing only a few of these forms. Of the different methods described above for viewing blood specimens, only the thin-film preparation clearly reveals the morphology of the parasite and of the infected erythrocyte. This preparation is most suitable for use while learning the technique and for identification of the infecting species, and therefore, it is most suitable for routine use. *P. vivax*, for example, is characterized by its brightly pigmented schizonts filling the enlarged erythrocytes. *P. malariae* is characterized by its brightly pigmented trophozoites, which occupy erythrocytes of normal size. *P. falciparum* is distinguished by its crescent-shaped gametocytes, in which the pigment is often concentrated in the middle, and also by its smaller and finer rings. Double infections and high multiplicities of infection are characteristic of *P. falciparum*. Our experience with *Plasmodium ovale* is still minimal, but parasites in a suspected infection of this species also exhibited bright forms.

Although the technique described here is simple, it no doubt requires familiarity with dark-field microscopy, the proper microscopic setup, and some experience to avoid the confusion caused by artifacts. The examination of a number of known positive and negative specimens is therefore initially required.

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LITERATURE CITED

1. Avraham, H., J. Golenser, D. T. Spira, and D. Sulitzeanu. 1981. *Plasmodium falciparum*: assay of antigens and antibodies by means of a solid phase radioimmunoassay with radioiodinated staphylococcal protein A. *Trans. R. Soc. Trop. Med. Hyg.* 75:421-425.
2. Brooke, M., M. Melvin, and G. R. Healy. 1961. Staining *Plasmodium berghei* with fluorescein tagged antibodies. *Proceedings of the Sixth International Congress on Tropical Medicine and Malaria*.
3. Collins, W. E., G. M. Jeffrey, and J. C. Skinner. 1964. Fluorescent antibody studies in human malaria. I. Development of antibodies to *Plasmodium malariae*. *Am. J. Trop. Med. Hyg.* 13:1-5.
4. Galbraith, R. M., W. P. Faulk, G. M. P. Galbraith, and T. W. Holbrook. 1980. The human materno-foetal relationship in malaria. I. Identification of pigment and parasites in the placenta. *Trans. R. Soc. Trop. Med. Hyg.* 74:52-60.
5. Mackey, L. J., I. A. McGregor, N. Paounova, and P. H. Lambert. 1982. Diagnosis of *Plasmodium falciparum* infection in man: detection of parasite antigens by ELISA. *Bull. W.H.O.* 60:69-75.
6. Packer, H. 1945. The use of dark field illumination in studies of malarial parasites. *J. Natl. Malaria Soc.* 4:331-340.
7. Tobie, J. E., G. R. Coatney, and C. B. Evans. 1961. Fluorescent antibody staining of human malaria parasites. *Exp. Parasitol.* 11:128-132.
8. Trager, W., and J. B. Jensen. 1976. Human malaria parasites in continuous culture. *Science* 193:674-675.
9. Wolter, A. 1932. Über die Schnellidiagnose der Maralia mit Hilfe des Dunkelfeldes. *Dermatol. Z.* 63:69-72.