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Rapid evaluation of malaria parasite density and standardization of thick smear examination for epidemiological investigations

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Abstract

The results of epidemiological surveys and the follow-up of malaria endemicity are often difficult to compare due to the high frequency of low-density parasitaemia in semi-immune populations. Detecting malaria parasites is dependent on the conditions and methods of parasitological examination. A standardized method of examination in epidemiological surveys is proposed: this includes the systematic rapid determination of parasite density.

Introduction

Although the standard thick smear examination readily permits a diagnosis of malaria infection in non-immune subjects because parasite density is usually high, it is often inadequate for diagnosing those living in endemic areas whose parasite density is usually lower. This is especially true of adults, who have acquired a high level of immunity. The significance of a negative result varies according to the conditions of the examination and the immune status of the subject. In epidemiological studies, the parasite rate will depend upon the method used (thin or thick films), the time devoted to examining the film, and the motivation and training of the microscopist, in addition to the prevalence of infection (ROSS & THOMSON, 1910; SCHWETZ, 1938; COVELL *et al.*, 1953; BRUCE-CHWATT, 1963).

Although the importance of the parasite density has been recognized for a long time (CHRISTOPHERS, 1924; WILSON, 1936; EARLE *et al.*, 1939; SCHWETZ, 1941; WILSON *et al.*, 1950; PARROT & CATANEI, 1950; MILLER, 1958), few field studies have used it systematically. This, in our opinion, is because most of the proposed counting methods are time-consuming and unsuitable for mass screening.

Based on a review of the literature and a series of complementary investigations, we have tried to define a method permitting a relatively precise measure of parasite density that is simple and rapid enough to be used during large field surveys.

Standardization of techniques

Amount of blood examined

The probability of detecting a malaria infection is a function of the amount of blood examined. In a thick smear 20 times the amount of blood in a thin smear can be examined in the same time. Hence, almost all investigations use the thick smear technique. However, in most early and in some recent works thin films were used, which explains some paradoxically low prevalence rates found in highly endemic areas. During equal times of examination, the difference in efficiency between these two methods varies with the length of the examining time, the plasmodial species and forms present, and the immune status of the population studied. For the time usually allotted to examine a slide, these differences are always important. In a comparative study performed recently in Nigeria, thick and thin films were examined from 403 individuals of all ages for 10 min. The thick film was 1·4 times more sensitive in detecting *Plasmodium falciparum* trophozoites, eight times more sensitive in detecting *P. falciparum* gametocytes and five times more sensitive in detecting *P. malariae* infections (STOREY *et al.*, 1973).

To ensure that the diagnosis of a given species or form is independent of the presence of other species or forms, it is necessary to examine the slide systematically for a predetermined number of fields or interval of time. We prefer to use a predetermined number of fields, since the amount of blood examined during an interval of time varies widely according to the examiner, the parasite density, the quality of the film, and the diagnostic difficulties encountered.

The examination of 100 oil immersion fields is usually adopted for field studies (WHO, 1961). However, the examination of 200 fields was adopted for the Garki Project (STOREY *et al.*, 1973) and several recent studies.

In a study of 245 schoolchildren aged 6 to 16 years conducted at Linzolo village (Congo), we compared the results obtained by the examination of 100 fields with those found after 200 fields.

The global prevalence was 75·9% for 100 fields and 80% for 200 fields (Table I). Ten individuals who were negative after 100 fields were positive after 200, showing *P. falciparum* infections (trophozoites in seven cases and gametocytes in three). In 25 indi-

Table I—Comparative results of the examination of 100 and 200 fields of the thick film in 245 schoolchildren aged 6 to 16 years from Linzolo (Congo)

Parasite	Slides found positive: In 100 fields	Slides found positive: In 200 fields
All species	186 (75·92%)	196 (80%)
P. falciparum all forms	184 (75·10%)	194 (79·18%)
P. falciparum gametocytes	52 (21·22%)	69 (28·16%)
P. malariae	58 (23·67%)	65 (26·53%)
P. ovale	9 (3·67%)	13 (5·31%)

viduals positive by the examination of 100 fields, an additional species or *P. falciparum* gametocytes were identified after the examination of 200 (*P. malariae* = seven cases; *P. ovale* = four cases; *P. falciparum* gametocytes = 14 cases). In conclusion, examination of 100 fields of the thick film was inaccurate for 35 individuals, or 14·3% of the total.

Although the examination was not continued beyond 200 fields, the rather high frequency of low parasite-density infections shows that the true prevalence is certainly higher in this group of schoolchildren.

In adults, the level of parasitaemia is usually low, resulting in greater differences depending on the number of fields examined. In Nigeria, DOWLING & SHUTE (1965) showed that only 43% of infections in adults were detected by examining 200 fields, 61% by examining 600 fields and 70% by examining 1000 fields. In this study, the mean result of a 100-fields examination of single thick films was a parasite rate of 38%; by making use of serial samples, the parasite rate in the series was doubled, i.e., raised to 77% when 14 consecutive drops of blood from a single finger prick were prepared and examined as 14 thick films. In the Garki Project, the prevalence observed by the examination of 400 compared to 200 fields was increased 10% for *P. falciparum*, 24% for *P. malariae* and 21% for *P. ovale* (MOLINEAUX & GRAMICCIA, 1980).

However, it is necessary to limit the time spent in examination. It is impractical to examine each slide to find every infection in the sample of blood slides investigated. Epidemiologically, it is not useful to extend the time spent in examination beyond that necessary to detect the minimum gametocytaemia that is theoretically sufficient to infect the vector, i.e., about 2·5 gametocytes/µl of blood for *P. falciparum*—male gametocytes of *P. falciparum* are on an average three times less numerous than female gametocytes, and a blood meal of *Anopheles gambiae* and of the main malaria vectors is about 1·6 µl (LACAN, 1958). Since the volume of blood examined in 200 fields of a thick film is usually 0·4 to 0·5 µl (WHO, 1961; DOWLING & SHUTE, 1966), examining 200 fields is probably the best compromise between the need to limit the number of false negatives and reducing the time for examining each slide.

Parasite count

Numerous counting methods have been proposed (THOMSON, 1911; SINTON, 1924; EARLE & PEREZ, 1932; see also BOYD *et al.*, 1949; FIELD *et al.*, 1963; RUSSEL *et al.*, 1963). We have rejected those that require a separate count of leucocytes or the addition of a given amount of bird red cells. These methods are more precise but impractical in mass surveys because they are time-consuming or cannot be used on finger prick samples. We also rejected the method that determines the ratio of infected red cells, because it cannot be used with a thick film.

The remaining methods are of two types; one is based on the parasite/leucocyte ratio in the thick smear, the second on the mean number of parasites per oil immersion field. The latter requires standardization of the thickness of the smears, which is usually difficult to achieve. A variant of this method was used in the Garki Project (MOLINEAUX & GRAMICCIA, 1980), the parasite density being expressed as a percentage of positive fields. However, this has the disadvantage of collecting in a single class all the films with a parasitaemia above about 500 per µl. It is important to give a more precise evaluation of these parasite densities, mainly for diagnostic and clinical application (TRAPE *et al.*, in press).

Evaluation of parasitaemia

The methods based on the parasite/leucocyte ratio in the thick smear avoid the problem of the heterogeneity of the thickness of the smear. However, in order to express the parasitaemia per µl of blood, it is necessary to know both the leucocytaemia and the loss of parasites during the staining of the thick film.

Loss of parasites during the staining process: This parasite loss was described by DOWLING & SHUTE (1966). The authors have compared the parasite/leucocyte ratio on paired thick and thin films. They observed a considerable loss of parasites in thick smears, amounting to 60% for trophozoïtes and 90% for *P. falciparum* gametocytes. Subsequently Dowling in 1968 specified that the loss of parasites varied with the staining technique used, the age of the slide, the use of pre-staining in the field, and environmental conditions such as temperature and humidity, etc., but the results of this study have not been published.

In a study at Linzolo we compared the parasite/leucocyte ratio on paired thick and thin films from 80 schoolchildren. These schoolchildren were selected following a first examination of the thick smear:

- 50 schoolchildren with at least one *P. falciparum* trophozoïte per oil immersion field.
- 30 schoolchildren with at least three *P. falciparum* gametocytes per 200 oil immersion fields.

We counted the trophozoïtes for 200 leucocytes both on thick and thin films and the gametocytes for 1,000 leucocytes in the thin film and 3,000 leucocytes in the thick film.

A relative count of parasites/leucocytes is difficult in the thin film because the leucocytes are heterogeneously distributed. Many of them are forced toward the edges of the slide, especially in thinner films. In order to minimize this disadvantage, all the films were examined transversally, including the corresponding edges, in a fairly thick portion of the slide where the red cells do not overlap enough to conceal parasites.

The results obtained are as follows:

- *P. falciparum* trophozoïtes: in total, 7,489 trophozoïtes were counted in thick films and 7,854 trophozoïtes in thin films, a difference less than 5%
- *P. falciparum* gametocytes: the number of gametocytes was an average of three times higher in the thick film examined for 3,000 leucocytes than in the thin film examined for 1,000 leucocytes (457 gametocytes in thick films and 141 in thin films), namely, an equivalent number of gametocytes as related to the number of leucocytes with the thick film.

It is difficult to explain the difference observed between our results and those of DOWLING & SHUTE (1966). In our study, blood was collected by finger prick, the time for defibrination was 30 seconds to one minute, staining took place between three and six days after. The mean temperature and relative

humidity were 25°C and 80% respectively. The main difference from the study made by Dowling and Shute seems to lie in the time interval before staining, three to six days instead of only 24 hours.

Classification of the parasitaemia: The classification of parasite density by geometric progression was introduced by BRUCE-CHWATT (1958). This method has two advantages for epidemiological surveys. First, in most situations the decision designating parasitaemia to its appropriate class can be made quickly, without requiring an exact count of the parasite/leucocyte ratio and after only reading several fields. Second, classification permits characterization of the epidemiological situation by analysing the distribution of parasite density and its evolution with age. This can be used to define a threshold of parasitaemia where the clinical symptoms are likely to be caused by the malaria infection, thereby facilitating the differential diagnosis of febrile episodes in an endemic zone of malaria (TRAPE *et al.*, 1982 and in press).

To maximize the advantages of this method, we have decided to use a geometric progression of a factor of 10 (Table II) instead of the factor of two used by BRUCE-CHWATT (1958). This results in an important reduction of time required to read each slide, since in four slides out of five the class distribution can be made very quickly, without increasing the time spent in examination. The relative loss of precision occurs mainly in cases of low and moderate parasitaemia, for which a precise determination is of little interest, at least for children. Finally, the choice of a factor of 10 limits the importance of the problem of individual variation in the leucocytaemia and the variation by age of the mean leucocyte count.

Mean standard leucocyte count: The separate counting of leucocytes per µl of the blood enables a better quantitative result to be given. However, since this is impracticable for mass surveys, it is necessary to adopt an average value of leucocytaemia as standard. This approximation is acceptable for a series of subjects, since the individual variations tend to cancel themselves out.

The mean number of leucocytes and its variation with age have been investigated in many populations. Usually, it is approximately 7000 per µl in adults (WINTROBE, 1967). In children the average values are higher: about 10,000 per µl between two and four years, 8,500 per µl between five and nine years and 8,000 per µl between 10 and 14 years (CARTWRIGHT, 1963).

In tropical Africa, a tendency toward leucopenia is seen in adults: most studies detect an average number of leucocytes between 5000 and 6500 per µl (ACKER *et al.*, 1967; BLISTEIN, 1950; EZEILO, 1971; HAGWOOD, 1969; LINHARD, 1958; ROUGEMONT *et al.*, 1975; SHAPER & LEWIS, 1971).

A scheme for age grouping for malaria studies is recommended by WHO (1963). The best solution would be the adoption for each age group of the corresponding standard leucocyte count. However, on selecting 8,000 leucocytes per µl as the average value for the community, the distribution among classes of parasitaemia is in fact little influenced since a factor of 10 separates each class as compared with a factor of 2, which includes the range of average values.

Conclusion

The method of searching for malaria parasites in epidemiological surveys is not standardized as is the spleen measurement, which is another important malaria parameter (HACKETT, 1944), or the search for blood microfilariae, which is another parasitic diseases in which diagnosis is based on the thick smear (WHO, 1962). The advantages of such a standardization are obvious, making studies in varying epidemiological situations easier to compare and making it easier to follow the evolution of malaria endemicity in a given area.

Based on a review of the literature and our experience in Congo, we think that a satisfactory method for mass surveys, a compromise between the need for precision and rapidity, could consist of the systemic examination of 200 oil immersion fields of the thick smear (0·5 µl of blood), the evaluation of parasitaemia in relation to leucocytes on the basis of 8000 leucocytes per µl, and the classification of parasite density by geometric progression of a factor of 10.

The correspondence between the parasite/leucocyte ratio in the thick smear and the actual parasitaemia raises the problem of the loss of parasites in the course of staining. However, such a loss can be avoided.

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Table II—Classification of parasitaemia and corresponding parasite count for a mean standard leucocyte count of 8000 per µl

Class	Parasitaemia (per µl)	Parasite count
0		*no parasite observed in 200 oil immersion fields**
1	<50	Less than 1 parasite for 160 leucocytes
2	50-<500	1 to 9 parasites for 160 leucocytes
3	500-<5000	1 to 9 parasites for 16 leucocytes
4	5000-<50000	10 to 99 parasites for 16 leucocytes
5	≥50000	100 parasites and over for 16 leucocytes

*In thick film of normal thickness a single parasite in 200 oil immersion fields corresponds to about 2 parasites per µl.

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