Interpretation of IHA titres for the study of malaria epidemiology *

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The results of IHA test surveys of persons with malaria parasitaemia in Ethiopia and the Philippines suggest that the antibody response may be influenced by the frequency and intensity of the antigenic stimulations and also be age-dependent. Antibody frequency distribution curves from four different areas suggest that the shape of such curves can provide some information about the endemicity of malaria. Results of similar and of parasitological surveys in Bangladesh, Ethiopia, Haiti, and the Philippines were compared and related to available malaria surveillance information. The results indicate that a serologic population profile may provide an indication of the history and status of malaria. Technical aspects of the IHA test are reviewed ; it may be desirable to use homologous antigens instead of a simian Plasmodium *antigen. To obtain the most useful additional epidemiological information about a malaria situation, serologic data need to be age-related, and longitudinal surveys are usually more informative than a cross-sectional survey.*

Variables influencing the applicability of a serologic method for epidemiological purposes include the sensitivity and specificity of the test, the reproducibility of the test results, the development and persistence of the measured antibodies, and also the laboratory procedures necessary to analyse the specimens.

Little is understood about the complex immunological relationship between the human host and the *Plasmodium* antigens, but considerable progress has already been made in developing tests to detect malaria antibodies. The indirect fluorescent antibody (IFA) method is successfully used by several research laboratories but more adequate standardization is needed.

Progress has also been made in the development of an indirect haemagglutination (IHA) procedure. For epidemiological purposes, the IHA test has two major advantages: a relatively large number of sera can be tested, and the technique can be automated.

The IHA method, as developed by Rogers et al. (14), uses a *P. knowlesi* antigen that reacts with antibodies produced by *P. falciparum*, *P. vivax*, or *P. malariae*. The test results appear to be highly specific (3). Some limited field studies have been carried out with this method to explore its applicability (6, 9, 7, 3).

This paper examines the antibody response in persons with parasitaemia in two different areas and the antibody patterns in populations with different levels of malaria endemicity and relates the results of serologic and parasitologic surveys conducted in 5 different countries to available malariometric information. The studies on which the data are based were carried out between 1968 and 1972.

MATERIALS AND METHODS

The IHA test developed by Rogers et al. (14) was used in the study; *P. knowlesi* was the antigen and human group O erythrocytes tanned with 1 : 20 000 tannic acid solution were the carriers for the titration of eluates of filter paper blood specimens. Antigen was prepared by exsanguinating Rhesus monkeys when the parasitaemia was at a peak and schizonts were the predominant stage. The recovered red cells were washed free of serum proteins and then lysed in distilled water containing 0.01 % Triton

X-100. The parasites were recovered by low-speed centrifugation and washed until they were free of haemoglobin. The washed parasites were frozen at −70°C until ready for further processing. For soluble antigen preparations, parasites were thawed in tepid water and suspended in 5 volumes of antigen diluent, pH 6.4 (equal volumes of pH 6.4 phosphate-buffered saline (PBS) and 4% sodium chloride solution). This suspension was passed through a cooled Ribi Cell Fractionator at 1.17×10^8 Pa pressure. The recovered material was centrifuged at $12\,000 \times g$ for 15 min, and the supernatant stock antigen was decanted and stored at −70°C. The optimum dilution of antigen was determined by sensitizing erythrocytes with dilutions of antigens made up in antigen diluent. Sensitized cells were suspended in PBS, pH 7.2, containing 1% normal rabbit serum and tested against a small battery of sera. Cells sensitized with the optimum antigen dilution were further tested against a larger battery of positive and negative sera to limit the variability of test results.

The capillary blood from a finger-prick (about 0.1 ml) was absorbed onto a preprinted circle of filter paper (Ropaco grade No. 1023-.038). The papers were dried and packaged with " glassine " interleaves for shipment to the laboratory at ambient temperature. In the laboratory, a disk 10 mm in diameter was punched from the blood-soaked circle and eluted for at least 30 minutes in 0.2 ml of PBS. About 0.12–0.14 ml of eluate was expressed from the filter paper disk. The filter paper blood eluate corresponded approximately to a 1 : 11 dilution of serum. In the test procedure, the filter paper eluates were diluted in 2-fold steps with loops in microtitration plates, and erythrocytes sensitized with antigen were added. After at least 1 h, the absence or presence of haemagglutination was determined.

In the IHA test, titres of 1 : 16 or greater were considered positive (3). The serologic indices used included the percentage of specimens with an IHA titre of 1 : 16 or higher (seropositivity rate), the geometric mean titre of the specimens with a titre of 1 : 16 or higher (mean positive titre), and/or the geometric mean titre of all specimens (mean titre). To calculate overall geometric mean titre values, we arbitrarily gave titres of less than 1 : 16 a value of 1 : 2. The sensitivity of the test was defined as the percentage of positive reactors among individuals with patent parasitaemia. The filter paper blood specimens were obtained during studies of populations in Bangladesh,

Ethiopia, Haiti, and the Philippines. Details regarding the characteristics of each population examined are given under " Results " below. Whenever blood was obtained for serologic testing, paired blood slides were made for microscopic examination to detect the presence of *Plasmodium* parasites.

For evaluation of the serologic data, the test results have been compared with the results of the microscopic blood slide examinations and with malaria surveillance information.

RESULTS

Serologic indices and age

To investigate the possibility of an age-related influence on the development of antibodies to malaria, seropositivity rates and mean positive titres were measured in two groups of patients, all of whom had patent parasitaemia as determined by blood slide examination. The IHA titrations were performed from filter-paper blood specimens. One group of 226 patients in Gambela, Ethiopia, came from a population in which the intensity of transmission was high: the parasite rate in 0–4-year-old children was 62.1%. The other group of 197 patients came from populations in the Philippines in which the transmission level was much lower: the parasite rate was 7.4% in 0–4-year-old children.

A total of 75% of the patients in Ethiopia and 65% of the patients in the Philippines were infected with *P. falciparum*. Fig. 1 shows that antibodies to malaria were detected in 70–90% of the malaria patients studied in Ethiopia and the Philippines, regardless of age. In Ethiopia, the mean positive titres were high in all age groups. In contrast, the
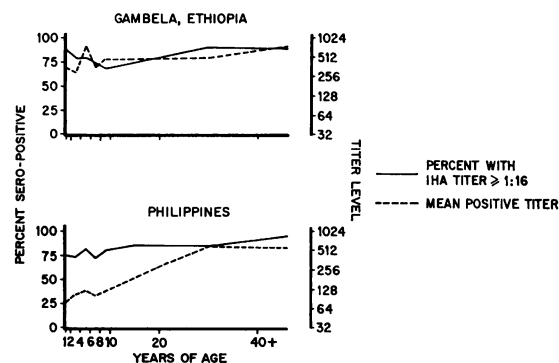

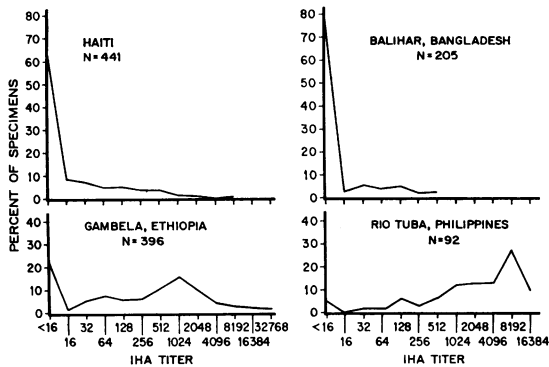Fig. 1. Serologic values in persons with patent parasitaemia, by age.

Fig. 2. Relative frequency distribution of IHA titres.

mean positive titres from the Philippine patients were relatively low in the young age groups; titre levels rose gradually with increasing age but the maximum titre was not reached until adulthood. In this study, an age-related increase of antibody titres could readily be detected in patients with malaria who live in an area with a low level of malaria transmission, but not in patients living in an area with a high level of malaria transmission.

Antibody patterns

Graphic illustration of antibody titre frequency distributions may demonstrate the malaria experience of the examined population.

Four distinctly different frequency distributions of malaria antibody titres were obtained from titrations of filter-paper blood specimens collected in Bangladesh, Ethiopia, Haiti, and the Philippines (Fig. 2). Paired blood slides were examined for the presence of *Plasmodium* parasites.

Only 38.1% of the 441 specimens from Haiti had an IHA titre of 1 : 16 or greater, with a mean positive titre of 1 : 86. The highest titre was 1 : 8 192. The parasite rate was 2.0%, and only *P. falciparum* was diagnosed.

In Balihar, Bangladesh, only 23.4% of the 205 specimens had a positive serologic reaction. The mean positive titre was 1:78, and no titre was higher than 1 : 512. All blood slides were negative.

The 396 specimens in Ethiopia were collected after the peak of the transmission season and positive IHA titres were found in 77.3% of the specimens, with a mean positive titre of 1 : 530. The highest titre measured was 1 : 32 768. The slide positivity rate was 58.6%.

An outbreak of falciparum malaria had occurred in a lumber camp in Rio Tuba, Philippines. All but 5 of the 92 persons (94.6%) who had had symptoms suggestive of malaria infection had an IHA titre of 1 : 16 or greater. The mean positive titre was 1 : 2 180. Since everyone had been treated with chloroquine several days before the specimens were collected, only 17.4% still had a detectable parasitaemia at the time of the survey.

These data illustrate that some information on the endemicity of malaria can be obtained from the type of the frequency distribution curve.

Malaria surveys

In an effort to determine whether serologic data from population surveys can be used to estimate the occurrence of malaria transmission, serologic and parasitologic surveys were carried out in Gambela, Ethiopia; in a cluster of villages on Palawan, the Philippines; in 2 localities in Bangladesh; and in a group of 6 villages in Haiti.

In one of these localities (Balihar, Bangladesh), the malaria eradication campaign had interrupted the transmission of malaria, and in the other areas, varying levels of malaria transmission were found.
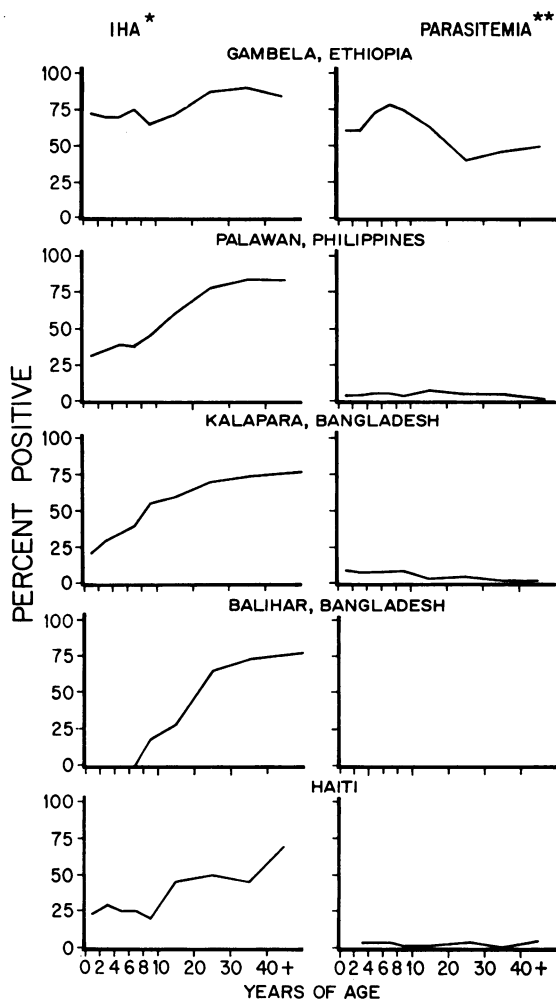
From each individual included in the surveys, a blood slide was obtained for microscopic examination to detect the presence of *Plasmodium* parasites and a filter paper blood specimen was obtained for serologic testing. The parasitologic and serologic survey results were compared, and they were related to the available malaria surveillance information.

The age-related parasite and seropositivity rates are shown in Fig. 3, and Table 1 indicates the mean titre and the mean positive titre values for the age groups 0–4, 5–14, 15–29, and 30 years and older.

A. A survey was conducted in January 1970 in Gambela, Ethiopia, which has a high level of malaria endemicity. The survey included 396 permanent residents of Gambela, i.e., 30% of the population.

Plasmodium parasites were found in 62% of the children under 5 years of age. The parasite rate reached its peak in the 6–7-year-old children and then decreased with increasing age, reflecting the effect of the acquired immunity. *P. falciparum* accounted for 70% of the malaria infections.

The seropositivity rates were 70–75% in young children and increased to a maximum of 90% in adults. The mean positive titre level was high in all age groups: 1 : 333 in the 0–4-year-old group and 1 : 765 in the 30 years and older group.

Fig. 3. Serologic and parasitologic values in 5 areas, by age.

the oldest age group. The mean positive titre was 1 : 91 in the 0–4-year-old group and 1 : 420 in the 30 years and older group.

The surveillance methods of the malaria eradication programme on Palawan included active and passive case detection (ACD and PCD). In 1970 a total of 832 blood slides had been examined in this population of 1 643 (51%) and in 1971 a total of 1 641 slides were examined (100%). The number of detected malaria cases per 1 000 population (annual parasite incidence, API) was 94.9 in 1970, as compared with 153.8 in 1969. *P. falciparum* was diagnosed in 83.3% of the cases found in 1970.

C. A survey was carried out in February 1970 in Kalapara, Bangladesh (population 5 300), which included 995 individuals (19% of the population).

Of the children in the 0–4-year age group, 10% had a patent parasitaemia and the parasite rates were somewhat lower in the older age groups. Only 5% of the infections were with *P. falciparum*.

Antibodies to malaria were found in 30% of the children under 5 years old, and the seropositivity rate increased gradually to 76% in the 30 years and older group. The mean positive titre was 1 : 80 in the 0–4-year age group and 1 : 223 in those 30 years and older.

In December 1968 the malaria eradication programme conducted a parasitologic survey of 1 315 children under 10 years of age; the survey showed a parasite rate of 9.4%. The malaria eradication campaign started in May 1970 with residual indoor insecticide spraying.

D. The serologic and parasitologic survey in Balihar, Bangladesh (population 1 400) was conducted in February 1970. No *Plasmodium* parasites were found in any of the 205 persons (15% of the population) examined during the survey.

Antibodies to malaria could not be detected in children under 8 years of age. The seropositivity rates then rose rapidly with increasing age, and 77% of the persons older than 30 had antibodies to malaria. The mean positive titre was 1 : 90 in this age group.

The malaria surveillance activities had been initiated in Balihar in 1964 with ACD. Between 1964 and 1969 the number of slides examined annually per 100 population ranged between 7.4 and 26.7. The API declined from 2.8 in 1964, to 1.4 in 1965, 0.1 in 1966, 0.1 in 1967, and 0.07 in 1968. No malaria cases were found in 1969. *P. falciparum* accounted for 61.5% of the infections in 1964 and 10% of the infections

B. The survey conducted in September 1970 on the coast of Palawan, Philippines, included a cluster of 7 adjacent villages with a population of 1 643, and almost the entire population was tested.

Of the children under 5 years of age, 6% had a patent parasitaemia, and similar parasite rates were found in the older age groups. *P. falciparum* accounted for 65% of the infections.

The seropositivity rate was 39% in children under 5 years of age and increased gradually to 84% in

Table 1. Parasitologic and serologic survey data, by ages of persons sampled

Area	Age	Parasitology		IHA test results		
		Number examined	Parasite rate (%)	Percent positive [a]	Mean titre	Mean positive titre
Gambela, Ethiopia	0–4	95	62.1	70.5	1 : 74	1 : 333
	5–14	101	78.2	68.3	1 : 121	1 : 405
	15–29	100	46.0	83.0	1 : 229	1 : 657
	30+	100	48.0	87.0	1 : 350	1 : 765
	Total	396	58.6	77.3	1 : 149	1 : 530
Palawan, Philippines	0–4	206	5.7	38.8	1 : 9	1 : 91
	5–14	572	7.2	47.9	1 : 14	1 : 114
	15–29	326	7.4	74.5	1 : 107	1 : 417
	30+	440	2.9	84.3	1 : 182	1 : 420
	Total	1545	5.8	62.7	1 : 42	1 : 257
Kalapara, Bangladesh	0–4	115	9.6	29.6	1 : 6	1 : 80
	5–14	477	6.7	51.9	1 : 12	1 : 65
	15–29	190	5.3	64.7	1 : 28	1 : 121
	30+	213	2.8	75.6	1 : 71	1 : 223
	Total	995	5.9	55.7	1 : 18	1 : 105
Balihar, Bangladesh	0–4	94	0	0	1 : 2	—
	5–14	51	0	13.7	1 : 3	1 : 29
	15–29	26	0	57.7	1 : 19	1 : 97
	30+	34	0	76.5	1 : 37	1 : 90
	Total	205	0	23.4	1 : 5	1 : 78
Haiti	0–4	89	3.4	29.2	1 : 5	1 : 49
	5–14	214	1.9	29.9	1 : 5	1 : 55
	15–29	65	1.5	52.3	1 : 14	1 : 77
	30+	73	1.4	60.3	1 : 36	1 : 244
	Total	441	2.0	38.1	1 : 8	1 : 86

[a] IHA titre ⩾1 : 16.

in 1965; no falciparum parasites were detected in any of the subsequent years.

The malaria eradication campaign had been initiated in Balihar in 1964 with bi-annual cycles of residual indoor insecticide spraying.

E. In April 1970 a parasitologic and serologic survey was conducted in 6 localities (population 4 775) on Haiti that had a history of unstable malaria.

A total of 441 persons were examined, and the average parasite rate was 2.0%, without a marked concentration in any of the age groups. The seropositivity rate was 29% in children under 5 years of age; it increased gradually to 60% in the oldest age group. The mean positive titres were less than 1 : 80 up to the age of 30 years and reached a maximum of 1 : 244 in those aged 30 years and over. The surveillance activities in these localities included ACD and PCD. During the preceding 3 years, the number of detected cases had varied from 25 to 261 per year.

The antimalaria measures had consisted of residual indoor insecticide spraying and mass drug administration.

A comparison of the age-related population profiles in the 5 study areas (Fig. 3) indicates that differences in the malaria experience are detectable from the serologic observations, in contrast to the information provided by the parasitologic examinations.

DISCUSSION

Serologic observations on malaria infections in adults indicate that the antibody response is related to the frequency, level, and duration of parasitaemia. However, no data are available on the influence of age on the development and persistence of antibodies. The serologic studies of people with parasitaemia in Gambela, Ethiopia, and in the Philippines suggest that the possibility of an age-related influence on the antibody development may

have to be considered in the interpretation of sero-logic indices. The malaria antibody levels of the children with parasitaemia in the two areas complement the blood-slide findings of the populations from which the individuals were drawn and suggest that the level of exposure was much higher for the subjects with parasitaemia in Gambela than for those in the Philippines. The relatively low antibody levels of the infected children in the Philippines are in contrast to the high titres of the adults in the same population. Similar observations have led to the suggestion by Voller et al. (16) and J. Lelyveld (unpublished observations, 1971) that the antibody levels in adults may be influenced by the cumulative effect of multiple infections and that adults may also have a greater immunological capacity than children to respond to malaria infections.

The most notable parasitological difference between the two populations described was that the parasite rates of the children were much higher in Gambela than in the Philippines; this difference indicates that the Gambela population was subject to more frequent antigenic challenges. This could explain why the children with malaria in Gambela had higher antibody titres than those in the Philippines. It may be that not only the frequency but also the variety of antigenic stimulations determines an age-related increase in the antibody titres, or that an age-dependent change in the ability to produce antibodies after infection is more apparent in individuals who live in areas with a relatively low level of transmission. For instance, the high infection rates with the accompanying high antibody titre levels in children in an area such as Gambela may obscure an age-dependent immunological capability.

The frequency distribution curves obtained in Haiti and in Balihar, Bangladesh, are characteristic of an area where a parasitic disease is absent or where the prevalence of infection is very low (5). These curves consist of a single low-titred peak resembling an exponential curve. The practically unimodal curves obtained for Gambela, Ethiopia, and for Rio Tuba, Philippines, indicate that these populations experienced a high level of exposure. The peak of the curve for Rio Tuba was at a higher titre level than that for Gambela, which suggests more recent or frequent infections in the Philippine individuals.

The data from the serologic and parasitologic malaria surveys in 5 separate areas suggests that a serologic population profile can provide an indication of the history and status of malaria in a popula-tion. Relating the survey results to the available malaria surveillance data is difficult because the surveillance information from these different areas is not readily comparable. However, the absence of malaria transmission during several years in Balihar, Bangladesh, is evident from both the serologic data and the surveillance information. In Gambela, Ethiopia, the serologic and the parasitologic population profiles indicated a high level of malaria transmission. The serologic profiles from Kalapara, Bangladesh, from Palawan, and from Haiti, fall between these two extremes, and it is possible that, especially in such areas, with a " moderate " amount of malaria, the serological technique will be a useful complement to the parasitological parameters for characterizing the intensity and distribution of malaria transmission.

The IHA method permits the testing of a large number of specimens in a relatively short period of time by a small laboratory staff. A recent paper (10) contained a report on several of the technical aspects that may affect the IHA test results for malaria. A relatively low degree of reproducibility (3, 15) is an inherent limitation of many IHA tests. Some factors influencing reproducibility include the anti-serum diluent and the microtitration loops used; variations in erythrocyte lots and their fragility and susceptibility to lysis and metabolic changes during storage (12, 1, 8); the concentration of the erythrocytes added to the diluted serum; the type, molarity, and pH of the buffer; the nature and concentration of the protein stabilizer; and the length and temperature of the incubation. The use of complex antigenic material for sensitization also influences the test results. Variability of results can be reduced by using several groups of positive and negative control sera, but this is time-consuming and inefficient. Farshy & Kagan (2) reported that red blood cells that are treated with pyruvic aldehyde, tanned, and fixed with glutaraldehyde,[a] and subsequently sensitized with a batch of antigen can be stored for several months without any substantial change in reactivity. The use of such a method in the IHA test for malaria may reduce the variability of the test results.

A single nonhuman primate antigen, *P. knowlesi*, was used in the test. However, the use of homologous antigens would be desirable to increase the reactivity of the test, especially since the filter paper blood collection method results in some loss of sensitivity. A multi-species antigen would then gen-

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[a] 1,5-pentanedial.

erally have to be used, because in population surveys it is seldom possible to know which *Plasmodium* species is responsible for, or has contributed to, the development of malaria antibodies. However, a single homologous antigen could be used to test specimens from areas where one *Plasmodium* species is known to predominate markedly.

Serologic data reflect the accumulated malaria experience, i.e., the period prevalence (11), and the presence of antibodies can only indicate that the examined population is, or has been, infected with malaria. More meaningful estimates of malaria transmission can be obtained from age-related serologic population profiles.

The ability of serologic surveys to detect short-term or temporary changes in the level of malaria transmission needs to be determined. A decline in the transmission level may be especially difficult to detect by serologic means and may necessitate periodic surveys of very young children or surveys to determine the seroconversion rates.

The experience with the IHA test for malaria suggests that it may become an important procedure in the armamentarium of malaria epidemiologists. Further laboratory investigations will be necessary to improve the standardization of test results. Field studies need to be carried out in areas with different malariological conditions to permit better interpretation and understanding of the results in relation to other malariometric measurements and to determine the optimum ways in which the method can be applied to the study of malaria epidemiology.

RÉSUMÉ

INTERPRÉTATION DES TITRES D'HÉMAGGLUTINATION INDIRECTE DANS L'ÉTUDE DE L'ÉPIDÉMIOLOGIE DU PALUDISME

Les auteurs exposent la technique de l'épreuve d'hémagglutination indirecte au moyen d'un antigène *Plasmodium knowlesi* et examinent les résultats obtenus au cours d'une série d'enquêtes épidémiologiques sur le paludisme. L'étude comparative des données sérologiques et parasitologiques conduit à admettre une influence de l'âge sur la production des anticorps dont il faut tenir compte dans l'interprétation des résultats. On établit un parallèle entre les réponses en anticorps et les taux de positivité des examens parasitologiques et autres données paludométriques dans des populations du Bangladesh, d'Éthiopie, de Haïti et des Philippines. Il semble, d'après les courbes de répartition des anticorps en fonction de l'âge, que la sérologie puisse fournir des informations intéressantes sur les antécédents et la situation présente en matière d'infection paludéenne dans une population.

Les critères épidémiologiques utilisés au cours des programmes de lutte antipaludique sont fondés sur une méthode parasitologique dont les possibilités sont limitées : elle n'indique en effet que l'absence ou la présence de parasitémie à un moment déterminé. On a donc proposé d'avoir recours aux épreuves sérologiques pour l'étude de l'épidémiologie du paludisme. Plusieurs techniques de recherche des anticorps peuvent être utilisées à cet effet. Leur avantage principal est leur aptitude à déterminer l'intensité et la répartition de la transmission du paludisme au sein d'une population. La collecte des échantillons de sang sur papier filtre facilite beaucoup les enquêtes sur le terrain.

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