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9. ABSTRACT

The indirect hemagglutination test is being evaluated as a sero-epidemiologic technique for malaria. Employing serology as an epidemiologic method will be especially useful 1) to measure the level of malaria endemicity; 2) to determine whether malaria transmission has been interrupted or reduced; 3) to delineate malarious areas; 4) to detect seasonal changes of malaria transmission; 5) for an independent termination of the intensity and distribution of malaria since it is not necessary to depend on the recorded malariometric surveillance information; 6) for identification of population groups with especially high rates of malaria infection; 7) to assess the coverage of the standard surveillance methods which are used to measure the occurrence of malaria; 8) to determine the role of migrants in the introduction of malaria from malaria endemic areas to receptive areas with little or no malaria; 9) for evaluating sera of malaria infected blood donors; and 10) for surveillance in areas of low endemicity.

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EVALUATION OF THE INDIRECT HEMAGGLUTINATION TEST AS AN EPIDEMIOLOGIC TECHNIQUE FOR MALARIA*

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Most countries have eradicated malaria; others have achieved marked progress through malaria eradication campaigns. However, antimalarial activities in some countries last longer than initially anticipated, and simplified and improved methods for the surveillance of malaria are needed.

The field techniques for epidemiologic evaluation of malaria programs, on which operational decisions are based, consist of blood and spleen surveys, detection of fever cases, and identification of malaria parasites in stained blood films. Epidemiologic surveillance generally depends on obtaining blood slides from individuals with fever. But not all patients with malaria have fever; patients with symptoms frequently do not interpret their symptoms as severe enough to warrant attention, and this attention may not be available at the right moment.

The microscopic examination of blood slides is still the only parasitologic laboratory method used in malaria programs to determine the occurrence of malaria and to measure the effect of anti-malaria activities. This method can only indicate the presence or absence of patent parasitemia at the time of examination; it does not indicate the individual's malaria experience. Furthermore, the absence of patent parasitemia can be misleading, since patency is influenced by immune status and the use of antimalarial drugs, and often occurs only intermittently during malaria infections.

Microscopists in malaria programs examine millions of blood slides each year. In areas with low incidences of infection, few positive blood films are found, and a more sensitive epidemiologic tool is needed. Even in regions with high incidences, where determinations of spleen sizes could indicate the levels of endemicity, a more specific method is required since splenomegaly is

a nonspecific physical finding and may be due to causes other than malaria. To provide an improved epidemiologic tool, we have concentrated on serologic methods to detect the presence of antibodies against malaria.

A practical serologic method for use in malaria programs must meet the following criteria: a) the test has to be simple to perform; b) the interpretation of results must be free of subjectivity; c) the test must be rapid; d) the cost must be minimal; e) the test results have to be sufficiently sensitive and specific, and f) the test must be capable of producing reliable results in various laboratories. The indirect hemagglutination (IHA) test now appears to meet these criteria better than other available serologic methods such as the indirect fluorescent antibody (IFA) test, the soluble antigen fluorescent antibody (SAFA) test, and the complement fixation (CF) test.

The IHA test is well suited for epidemiologic purposes. Large numbers of specimens can be titrated readily at small expense, and blood can be collected by finger prick on filter papers. The specificity is very high. The antigen prepared from *Plasmodium knowlesi* is genus specific and will react equally well with all of the *Plasmodium* species causing human malaria. Storing filter papers and sera at -20°C does not affect the titer. The reproducibility of the IHA antigen is good. Antibodies are detectable 8 to 27 days after the onset of parasitemia, and they can persist for 13 years after a single infection.

Pilot scale studies indicate that the IHA test may have a wide range of applications in epidemiology: 1) to measure the level of malaria endemicity;¹ 2) to verify the absence or presence of transmission from a relatively small number of specimens;² 3) to delineate malarious areas;³ 4) to detect seasonal changes of malaria transmission;⁴ 5) to investigate suspected reintroductions of malaria into consolidation or maintenance phase areas;⁵ and 6) to detect small foci of malaria transmission.³ The test is especially

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useful in areas where the regular malaria surveillance network is limited, and it can be used effectively as a screening mechanism to detect potential carriers of malaria. It can also be used to measure the effect of antimalarial activities on the transmission of malaria and to assess the efficacy of the malaria surveillance system.

Preliminary findings indicate that the IHA test will be a valuable adjunct to the present surveillance methods. With serologic methods, relatively small population samples can be used, and, if necessary, these samples can be obtained independently from the routine surveillance system of the malaria program or the general health services.

The technique of the test is simple. Serum or filter paper eluates are diluted in microplates, and a drop of antigen sensitized red blood cell is added. After 1 hour the presence or absence of agglutination is noted. The antigen is an extract of *P. knowlesi* that has been obtained from infected rhesus monkeys (*Macaca mulatta*).⁵ This *Plasmodium* species was selected because rhesus monkeys can be readily infected and can provide a large volume of parasitized red blood cells for antigen purposes. The parasites are freed from the red cells and are passed through a Ribi Cell Fractionator at 17,000 pounds of pressure. The supernatant fluid is the soluble antigen. Each antigen is then titrated against a standard battery of six positive human malaria sera, one positive monkey serum, and one negative human control. The antigen is then tested at its optimal dilution with a larger battery of 35 positive sera and one negative serum to insure its sensitivity, specificity, and reproducibility. If the antigen qualifies, it is used to sensitize human "O" red cells. The human cells are treated with tannic acid and then sensitized with the soluble antigen. Each lot of sensitized cells is evaluated once again with the small battery of positive and negative sera. The degree of agglutination is read by observing the settling pattern of the antigen sensitized red cells in the wells of microplates. A clear mat of agglutinated cells is considered a positive reaction.

A stable red cell malaria antigen will enhance the usefulness of this technique. Human "O" cells can be stabilized and sensitized by fixation with pyruvic aldehyde, then treated with tannic acid, and fixed with glutaraldehyde.⁷ Such fixed cells have been sensitized with malaria antigen and have remained active for weeks. Once we success-

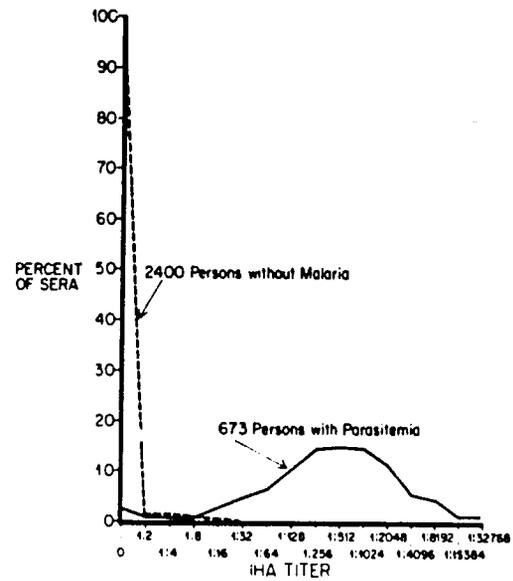


FIGURE 1. Relative frequency distribution of IHA titers in sera from individuals with and without malaria.

fully complete our evaluation studies and such a fixed stable malaria hemagglutination antigen is made available, the test can be used more widely. Fixed cells require no refrigeration and can be produced by a central laboratory for use in the field.

What are the data to substantiate the claims made for use of this technique as an epidemiologic tool? To determine the IHA titer levels which indicate the presence of antibodies to malaria, we made a relative frequency distribution of the IHA titer values found in sera of individuals who had never been exposed to malaria and of those in sera of persons who had a parasitemia at the time of examination (Fig. 1).⁵ On this basis, sera with IHA titers of 1:8 or greater were considered positive, but because the first dilution of filter paper eluates is approximately a 1:16 dilution of serum, this titer level had to be adopted for routine use as the lower limit of positive reactions.

The specificity (i.e., the proportion of negative reactors in persons who had never had malaria) was measured by testing sera from 2,400 individuals, including military recruits in the United States; Eskimos on St. Lawrence Island, Alaska; tuberculosis and syphilis patients in the United States; and Indians in North Carolina who were

TABLE 1
Specificity of the IHA test. Number of persons with negative serum titers ($\leq 1:16$)

Source of sera	Number tested	Percent negative
U. S. recruits	2,237	99.7
Alaska	61	100.0
Tuberculosis and syphilis patients	54	98.1
North Carolina Indians*	48	97.9
Total	2,400	99.6

* Forty-four were infected with one or more intestinal parasites.

heavily infected with intestinal parasites (Table 1). Only 9 of these 2,400 individuals (0.4%) had a serologic reaction to the *P. knowlesi* antigen of 1:16 or higher.⁵ For a hemagglutination test, the malaria antigen has an exceedingly high specificity which makes it exceptionally valuable for epidemiologic studies.

The sensitivity (i.e., the proportion of positive reactions in individuals with recent malaria infection) was determined by testing 1,202 specimens from patients who were determined by microscopic blood-slide examination to have malaria (Table 2). This group included patients with induced infections in the United States and individuals who had been infected in malaria-endemic areas, such as United States military personnel who had acquired the infection in Vietnam, and people living in Brazil, Ethiopia, Haiti, New Guinea, Pakistan, Honduras, and the Philippines. The sensitivity of the test was 91% for vivax infections, 86% for falciparum infections, and 94% for malariae infections.⁵

Reproducibility studies of the IHA test showed that within-day variations of one dilution or less were obtained for 88% of 235 serum determinations. Day-to-day variations of one dilution or less were obtained for 76% of the 560 duplicate serum determinations (Table 3).

Because using filter papers to collect and handle blood specimens is of great practical advantage in field studies, we have developed a filter paper blood collection method for use in malaria serology. A number of types of absorbent filter paper were evaluated for strength, inertness, absorbency, and availability, and one grade of paper, ROPACO #1023-038, was selected.

The filter paper is cut into 1" \times 3" rectangles

TABLE 2
Sensitivity of the IHA test. Number of persons with positive serum titers ($\geq 1:16$)

<i>Plasmodium</i> species	Number tested	Percent positive	Source of sera
<i>Induced infections</i>			
<i>vivax</i>	19	95	U. S.
<i>falciparum</i>	13	85	U. S.
<i>malariae</i>	2	100	U. S.
<i>Naturally acquired infections</i>			
<i>vivax</i>	58	83	Vietnam†
	130	99	Honduras
	100	95	New Guinea
	19	100	Brazil
	62	82	Philippines*
	8	88	Ethiopia*
	26	92	Brazil*
	58	78	Pakistan*
Total	461	91	
<i>falciparum</i>	17	94	Vietnam†
	9	89	Haiti
	27	100	Brazil
	162	91	New Guinea
	3	67	Pakistan*
	195	79	Ethiopia*
	36	97	Brazil*
	119	84	Philippines*
Total	598	86	
<i>malariae</i>	117	96	New Guinea
	22	86	Ethiopia*
Total	139	94	
TOTAL	1,202	89	

* Sera collected on filter paper.

† U. S. military.

and imprinted with circles 12 mm in diameter. Capillary blood is collected from a finger prick by placing the paper, with the circle uppermost, over the bleeding point and allowing the circle to fill with blood. Approximately 0.1 ml is required to saturate the circle. Identifying data is written on one end of the paper. The papers are dried at ambient temperature, packaged together with glassine interleaves, and kept cool and dry for shipment. For laboratory storage, freezing at -20°C is recommended.

Preliminary studies were conducted with paired serum and filter paper blood specimens from infected monkeys. These studies indicated that the eluate from the filter paper represented approximately a 1:16 dilution of serum. Filter paper specimens kept dry at temperatures up to 40°C for 15 days and specimens kept at 37°C and

TABLE 3
Agreement of duplicate titrations in the IHA test for 1968 to 1971

Reagents		Within day			Day-to-day		
Antigen	Cells	Duplicate determinations	Percent differing by		Duplicate determinations	Percent differing by	
			≤1 dil.	≤2 dil.		≤1 dil.	≤2 dil.
Same*	Same	185	89	96	96	70	92
Same	Different	—	—	—	96	88	96
Different	Same	50	86	92	24	87.5	100
Different	Different	—	—	—	344	75	90
Total		235	88	95	560	76	91

* Same, antigen from a single monkey or red blood cells from a single donor.

100% humidity for 5 days showed only a slight loss of antibody activity; the eluate was equivalent to a 1:11 dilution of serum.

With serum obtained by venipuncture from 673 persons with slide-proven parasitemia, the sensitivity of the test was 93.9%. With blood collected by the filter-paper absorption method, the sensitivity was 82.6% (Table 4).

To obtain a direct comparison of IHA titers of filter paper blood specimens with those of sera from venous blood, we collected paired specimens by both methods from 335 individuals in Haiti and 70 persons in the State of Mato Grosso, Brazil.⁵ The positive and negative IHA test results of these 405 paired specimens are summarized in Table 5. Both sera and filter paper blood specimens of 203 of the 405 examined individuals (50.1%) were positive, whereas 135 of the paired specimens (33.3%) had a negative titer.

"False negative" titers, i.e., those that were

TABLE 4
Sensitivity of the IHA test ($\geq 1:16$) by method of specimen collection

<i>Plasmodium</i> species	No. sera tested	Percent positive
SERUM		
<i>vivax</i>	326	94.8
<i>falciparum</i>	228	91.7
<i>malariae</i>	119	95.8
Total	673	93.9
FILTER PAPER BLOOD		
<i>vivax</i>	154	82.5
<i>falciparum</i>	353	82.4
<i>malariae</i>	22	86.3
Total	529	82.6

positive by serum titer but negative by filter paper titer, were found in 57 of 260 (21.9%) paired specimens. Also, 10 of 145 (6.8%) persons with negative IHA serum titers had positive titers when their filter paper specimens were examined. The greatest loss of sensitivity occurs with specimens that have serum titers of 1:16 to 1:32. These specimens are often negative by the filter paper method.

The level of malaria endemicity in a population can be measured by plotting the geometric mean reciprocal titer (GMRT) of the IHA test against the observed per cent of the sample seropositive.⁴ With such a graph, one can depict the experience of a population with malaria, and also detect changes in malaria endemicity especially when malaria has been interrupted since the GMRT levels fall more rapidly than the percentage of positives and the plotted points would deviate from the normal pattern.

In a study carried out in Mindanao, Philippine Republic, State of Catobato,³ a cluster of 11 communities was tested (Fig. 2). Communities 27 and 29 stand out as areas where more transmission may have taken place because the GMRT values and the percentages of positives are higher than those in the surrounding communities.

TABLE 5
Positive and negative IHA titers* in 405 paired sera and filter paper blood specimens

Filter paper Sera	Negative	Positive	Total
Negative	135	10	145
Positive	57	203	260
Total	192	213	405

* Negative titer $< 1:16$; positive titer $\geq 1:16$.

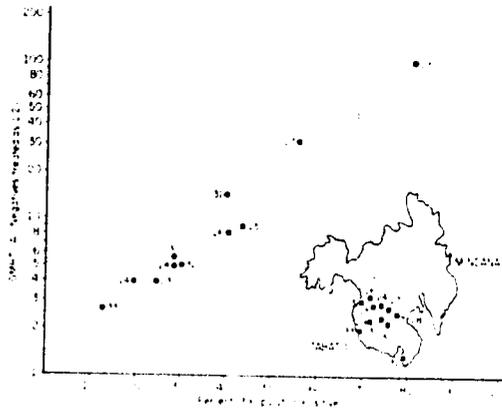


FIGURE 2. Geometric mean reciprocal titer (GMRT) versus the observed per cent of samples positive by the IHA test for malaria, 11 Philippine communities.

The IHA test can be a useful tool in the delineation of malarious areas, which is important in the direction of malaria operations. In many programs the decision to carry out antimalarial activities in areas above a certain elevation may be based on very slight evidence. Serologic studies can support or modify this position. In Nepal, for example, there is no spraying at elevations above 4,000 feet. The question of whether or not malaria was being transmitted above 4,000 feet was evaluated with the IHA test by collecting sera from 163 individuals living in villages above this altitude.⁴ Twenty-two (13.5%) were positive. Of the 22 positives, 19 were males with a history of travel to areas below 4,000 feet. The other three were young adolescent girls who may have gone to the valley although they denied leaving the village. We concluded from this small study that malaria was not being transmitted above 4,000 feet because none of the stationary population had evidence of malaria antibody.

The serologic method is especially useful as an epidemiologic tool since only a relatively small number of individuals in a population need to be examined to obtain a serologic profile of the malaria history of the group, because malaria antibodies can persist for many years.

In a study of 49 Nigerian students who had lived in the United States uninterruptedly for periods ranging from 1 week to 7 years, examination of blood slides obtained simultaneously with the filter paper blood specimens did not reveal the presence of *Plasmodium* parasites. On the

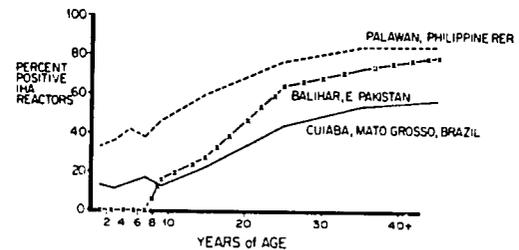


FIGURE 3. Per cent of three populations with malaria antibodies by the indirect hemagglutination $\geq 1:16$ by age.

other hand, examination of their filter paper blood specimens with the IHA test indicated that 92% of the students had a positive IHA titer. The geometric mean titer of the reactors declined from 1:1,112 for students in the U. S. for less than 6 months to a titer of 1:71 for the students who had resided in the U. S. at least 4 years.

To determine the serologic profile in different areas, filter paper blood specimens were obtained from all age groups in Palawan, Philippines; in the Cuiabá sector, State of Mato Grosso, Brazil; and in Balihar, East Pakistan (Fig. 3).⁵ Palawan represents an area of relatively high endemicity and Cuiabá, Mato Grosso, an area of low endemicity. In Balihar, East Pakistan, no antibodies were detected in children under 7 years of age, but the seropositivity rates in adults increased to levels comparable to those observed in Palawan and Brazil. In the East Pakistan locality, active malaria surveillance for 5 years had not detected a single case in this formerly malarious area.

If eradication in the East Pakistan locality had failed, antibodies should have been detected in the young children. We demonstrated this by measuring antibody profiles in two communities in Afghanistan.⁶ Both in Saidabad and Bulla Quchi, malaria was reported to have been eradicated. A survey made 3 years ago showed the absence of antibodies in the 1- to 4-year-old group in Saidabad but the presence of antibodies in this age group in Bulla Quchi. After this study, recurrence of malaria transmission in the Bulla Quchi area in northeastern Afghanistan was confirmed, and antimalaria measures had to be reinstated.

The more advanced the stage of a malaria program, the greater is the need for alertness and vigilance. But, as malaria becomes a less prominent problem, the financial resources needed to carry out surveillance are often increasingly

limited. At this stage, the use of serologic methods will be especially advantageous. Certain epidemiologic tasks, such as surveillance in areas of high vulnerability and receptivity, identification and coverage of populations at special risk to malaria, and screening of potential carriers to prevent introduction of malaria, can then be carried out more effectively.

The seroepidemiologic method can be usefully applied in areas where the endemicity of malaria is very low or where years of attack measures have not completely interrupted transmission. In such communities, it should be used as a surveillance tool. Fewer blood specimens will need to be examined, and the many technicians who each year examined large numbers of slides can be diverted to areas where malaria is still a significant problem.

Another application of the serologic technique is for evaluation of the status of malaria. Evaluation is usually based on analyzing recorded malariometric data rather than on direct measurement of the level of malaria transmission. Serology offers an independent method which can be used in assessing malaria programs by examining a relatively small sample of the population.

The value of the seroepidemiologic method is that it provides a sensitive tool to determine the population's experience with malaria. Properly applied, it will permit interpretation of the level of transmission and identification of the subpopulations that are principally affected and will make possible the assessment of the effects of antimalaria measures. Use of this method requires only a small laboratory staff. One trained serologist and a small staff of technicians can operate a serologic laboratory that can process several thousand blood specimens a day.

The indirect hemagglutination test in its present stage of evaluation is not ready for routine use in malaria eradication programs. Another year or two will be needed to overcome problems of antigen production. We do not understand why some infected monkeys give us excellent antigen while other animals yield antigen of poor quality. Is it the monkey or the way we process the antigen? It is not a question of whether or not the antigen is sensitive and specific, or whether the test results are reproducible. We have been titrating collections of sera for over 4 years, and when we retitrate an old collection, we obtain very similar results. These are technical

problems, and only further laboratory study will resolve them.

In addition to the laboratory program, we need to evaluate the use of the test in countries with different types of malaria in order to better define its optimal application in different situations. Toward this end, we have entered into longitudinal studies in Brazil, El Salvador, and Nigeria. Malaria is not the same disease the world over, and one cannot assume that the techniques used in determining the transmission of malaria in El Salvador, where the slide positivity rate in the area under study is low, are going to be useful in Nigeria, where the level of endemicity is very high.

The serologic method has to be given full evaluation and pilot implementation. It provides a new and sensitive tool for the surveillance of malaria and represents an additional factor for the guidance of malaria eradication programs.

SUMMARY

The indirect hemagglutination test is being evaluated as a seroepidemiologic technique for malaria. Employing serology as an epidemiologic method will be especially useful 1) to measure the level of malaria endemicity; 2) to determine whether malaria transmission has been interrupted or reduced; 3) to delineate malarious areas; 4) to detect seasonal changes of malaria transmission; 5) for an independent determination of the intensity and distribution of malaria since it is not necessary to depend on the recorded malariometric surveillance information; 6) for identification of population groups with especially high rates of malaria infection; 7) to assess the coverage of the standard surveillance methods which are used to measure the occurrence of malaria; 8) to determine the role of migrants in the introduction of malaria from malaria endemic areas to receptive areas with little or no malaria; 9) for evaluating sera of malaria infected blood donors; and 10) for surveillance in areas of low endemicity.

The IHA test is well suited for epidemiologic purposes. Large numbers of specimens can be titrated readily at small expense, and blood can be collected by finger prick on filter papers. The specificity of the test with sera from 2,400 individuals was 99.6% and the overall sensitivity with 1,202 specimens was 89%. The antigen prepared from *Plasmodium knowlesi* is genus

specific and will react equally well with all the *Plasmodium* species causing human malaria. Antibodies are detectable 8 to 27 days after the onset of parasitemia and they can persist for many years after a single infection. With serologic methods, relatively small population samples can be used to measure epidemiologic parameters.

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REFERENCES

1. Kagan, I. G., Mathews, H. M., Rogers, W. A., Jr., and Fried, J. A., 1969. Seroepidemiological studies by indirect hemagglutination tests for malaria. Military recruit collections from Argentina, Brazil, Colombia, and the United States of America. *Bull. Wld. Hlth. Organ.*, *41*: 825-841.
2. Mathews, H. M., Fisher, G. U., and Kagan, I. G., 1970. Persistence of malaria antibody in Tobago, West Indies, following eradication, as measured by the indirect hemagglutination test. *Am. J. Trop. Med. Hyg.*, *19*: 581-585.
3. Mathews, H. M., Fried, J. A., and Kagan, I. G., 1970. A seroepidemiologic study of malaria in the Republic of the Philippines by the indirect hemagglutination test. *Am. J. Epidemiol.*, *92*: 376-381.
4. Kagan, I. G., Mathews, H. M., and Sulzer, A. J., 1969. The serology of malaria; Recent applications. *Bull. N. Y. Acad. Med.*, *45*: 1027-1042.
5. Lobel, H. O., Mathews, H. M., and Kagan, I. G. The indirect hemagglutination test and its role in malaria surveillance. (In preparation.)
6. Rogers, W. A., Jr., Fried, J. A., and Kagan, I. G., 1968. A modified, indirect microhemagglutination test for malaria. *Am. J. Trop. Med. Hyg.*, *17*: 904-909.
7. Kagan, I. G., 1972. Malaria: seroepidemiology and serologic diagnosis. *Exp. Parasitol.*, *31*: 126-135.
8. Buck, A. A., Anderson, R. I., Kawata, K., Sasaki, T. T., Amin, F. M., and Abrahams, I. W., 1972. *Health and Disease in Rural Afghanistan. An Epidemiologic Study of Four Villages*. Johns Hopkins University, Baltimore, Md., 225 pp.