SEROEPIDEMIOLOGIC ASSESSMENT OF MALARIA USING AN ELISA FOR RING-INFECTED ERYTHROCYTE SURFACE ANTIGEN OF PLASMODIUM FALCIPARUM

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ABSTRAK

Zat anti terhadap antigen permukaan dari sel eritrosit yang terinfeksi dengan parasit bentuk cincin (Ring-infected Erythrocyte Surface Antigen/RESA) **Plasmodium falciparum** *telah diukur dari sera yang dikumpulkan di Jawa Tengah, Jawa Timur, dan Irian Jaya.*

Untuk mendeteksi IgG terhadap RESA tersebut digunakan ELISA (Enzyme Linked Immuno Sorbent Assay). Dari pengukuran tersebut menunjukkan adanya korelasi antara RESA ELISA dengan pengukuran epidemiologis transmisi malaria secara konvensional.

Pada tulisan ini diuraikan tentang prosedur standar untuk melakukan RESA ELISA dan membahas hal-hal yang menguntungkan maupun keterbatasan-keterbatasan dalam ekstrapolasi data untuk menggambarkan perkiraan transmisi malaria.

INTRODUCTION

The best means of evaluating malaria transmission in a given area is to conduct long-term surveillance employing both active and passive case detection. In Indonesia, the system of mandatory reporting of malaria provides reliable measures of transmission. This is especially true on Java where the health infrastructure has been closely monitoring malaria since the 1960's when aggressive control programs were instituted.

However, other areas in Indonesia may not have reliable surveillance data as a consequence of either insufficient resources or geographic barriers. When an estimate of malaria transmission is needed quickly from such an area, instituting long-term surveillance may not be practical or immediately helpful. Under these circumstances, a serologic

survey of antibody to malarial antigens, together with point malariometric measurements, may be the best approach to estimating relative levels of malaria transmission.

A seroepidemiologic survey provides a relative measure of the degree of exposure to malaria in a given population. In contrast to conventional point malariometric measurements, the serologic survey provides a view of exposure to malaria which extends over at least several months. When comparing these measurements between different populations or geographic regions, caution must be exercised in insuring comparability of the immuno-assays employed. Standardization of the methodology is required for such comparisons.

In this report, an assay which correlates well with malariometric measures of transmission is described and we recommend it as a standardized method for seroepidemiologic surveys of exposure to malaria. The assay described is an enzyme-linked immunosorbent assay (ELISA) for measuring relative levels of IgG antibody against ring-infected erythrocyte surface antigen (RESA) of *P. falciparum*. RESA refers to a group of parasite antigens which appear on the surface of red blood cells infected by young asexual parasites.[1] Although studies have shown that a particular peptide of 155,000 daltons (Pf-155) is the predominant antigen of RESA,[2,3] the method we describe does not distinguish Pf-155 from any of the other minor

malaria antigens occurring on the surface of ring-infected red blood cells. When considering antigen preparations with which to conduct serologic surveys, this lack of narrow specificity in RESA seems desirable. A highly specific antigen for epidemiologic purposes would allow for greater probability of genetic restriction against the antigen, and thereby confound the results of the survey.[4]

IgG is the most abundant anti-malaria gamma globulin isotype in endemic populations, and its persistance months after parasitemia has subsided provides a view of exposure over many months. Immunoglobulin M against RESA may also be a useful instrument for seroepidemiologic surveys because its transient presence suggests more recent exposure to malaria. Levels of IgG in view of levels of IgM might provide some insight as to recent versus chronic exposure. However, IgM to RESA was not evaluated in this study for lack of sufficient documentation of very recent transmission at the study sites. Nonetheless, evaluation of IgG alone provided an estimate of exposure to malaria which correlated well with conventional malariometric assessments.

MATERIALS AND METHODS

Field Methods

Between November 1987 and October 1989 serum samples were collected from people living in Central and East Java, and in Irian Jaya. Table 1 lists the specific locations. A total of between 100 and 210 people of ages > 1 year (up to 80 years) donated blood in each

village. Ten milliliters of blood was drawn by venipuncture from volunteers and allowed to clot. Serum was aspirated from the samples and stored in a portable liquid nitrogen container after contrifugation at approximately 500 Xg for 10 minutes. The samples were transported to Jakarta for analysis. A thick and thin blood film, along with a standard Hackett score of spleen size, was obtained from each volunteer. All donors were informed of the intent of the study and their right to refuse participation.

Tabel 1. Malariometric features of the study villages.

District	Regency	Province	Point Measurements (1989)		SPR*	Rank
			Prevalence	Spleen		
				(no exposure)		
Jakarta	(negative controls)					0
Wates	Blitar	Jawa Timur	0%	0%	0	1
Dukuseti	Pati	Jawa Tengah	0%	<2%	0.34	2
Sawahan	Madiun	Jawa Timur	2%	0%	0	3
Brunorejo	Purworejo	Jawa Barat	2%	—	3.7	4
Arso PIR	Jayapura	Irian Jaya	35%	75%	40	5

* Slide positive rate in 1986/87.

Laboratory Methods.

***Preparation of Antigen* —** A culture adapted strain of *P. falciparum* (NAMRU # 2300 from Mapurajaya, Irian Jaya) was maintained in petri dish/candle jar cultures according to standard procedures.[5] The sorbitol lysis procedure as described by Lambros and Vanderburg[6] was employed to synchronize parasite cultures. Twenty four hours after the second lysis, cultures contained only ring-stage parasites infecting about 20% of the red blood cells. Cultures were then washed 3 times using Tris buffer/Hank's solution and adjusted to a 0.35% red cell suspension (v/v). A suspension containing uninfected red blood cells from the donor who contributed the blood used to culture the parasites was also prepared. Infected and uninfected red cells were adhered to 96 well microtiter plates and fixed with glutaraldehyde as described by Deloron et al[7] The air-dried plates were then stored et − 25°C.

***Preparation of serum* —** Serum was thawed at room temperature and then heat-inactivated by incubation at 56°C for 30 minutes. An 80 μL aliquot of serum was preabsorbed with 40 μL washed, packed red blood cells from the donor who also provided blood for culture of parasites. The mixture was incubated at 37°C of one hour. This preabsorption step greatly diminished the amount of background reactivity with uninfected red blood cell antigens. The recovered serum was diluted

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1 : 10,000 using phosphate buffered saline, pH 7.2, containing 0.5% bovine serum albumin (fraction V), 0.05% Tween-20 and 5% skim milk prepared from commercially available powder. This mixture was referred to as the working solution (WS). Serum was diluted within 1 hour of use in the assay.

Assay procedure — Figure 1 illustrates the rationale of the RESA ELISA. One hundred microliters of each serum sample was added to each of 3 microtiter wells containing infected red blood cells.
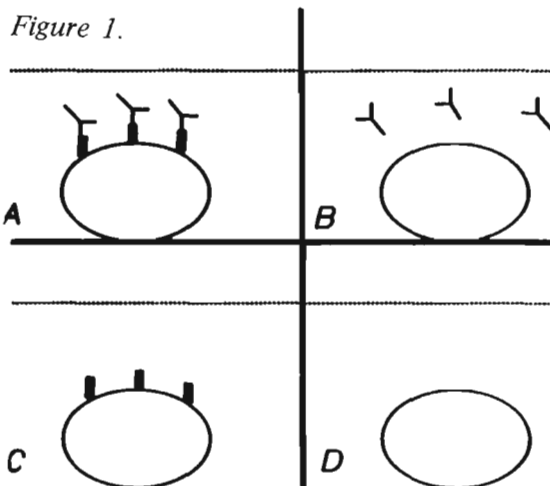
Figure 1.

Figure 1. Rationale of the RESA ELISA. The four ovals represent fixed whole blood cells adhered to the surface of a microtiter well containing serum (dotted line). The dark rectangles on the surface of the red blood cells represent RESA, and the Y-shaped figures represent antibody to RESA. Blocks A & B represent ELISA wells containing immune serum incubated with infected red blood cells (A) and uninfected (B). Blocks C & D represent ELISA wells containing nonimmune

serum incubated with infected red blood cells (C) and uninfected (D). All of these test conditions are represented by the replicate values illustrated in figure 2.

Positive (serum from an adult living in an hyperendemic malarious area) or negative (serum from an adult living in Jakarta) control sera were added to each of 3 microtiter wells containing infected red blood cells, and also to each of 3 wells containing uninfected red blood cells. It should be reemphasized that the red blood cells used in all steps of this assay came from one donor. One set of controls was done for each microtiter plate used. The plates were incubated for 1 hour at 37°C and then rinsed three times with a washing solution prepared as WS, but without the skim milk component. Affinity purified peroxidase conjugated goat anti-human IgG antibody (Toga Inc., Burlingame, Clifornia, USA) was diluted 1:6000 (IgG) in WS and 100 µL added each well. The plates were incubated at 37 C for 1 hour and rinsed three times using the washing solution. One hundred microliters of substrate for the peroxidase reaction (0,55 mM 0-phenylenediamine dihydro- chloride with 0.0003% hydrogen peroxide) was added to each well and incubated at room temperature for 30 minutes. Fifty microliters 4M sulfuric acid was added to each well to terminated the peroxidase reaction. The plates were then read for optical density at 492 nm on an automatic ELISA plate reader.

Calculation of seropositivity score — A seropositivity score was formulated

to provide a relative measure of seropositivity which reflected differences in both percent seropositive and relative intensity of positivity. For example, if two populations both show a 75% positivity rate but the mean RESA ELISA OD in one is 0.680, and 0.150 in another, the seropositivity score would reflect the difference (3.65 vs. 2.51). The following equation was used to derive the seropositivity score :

$$\ln \text{ mean } RESA \text{ OD } \% \text{ seropositive } + 1 = \text{seropositivity score}$$

The natural log function set the seropositivity scores of populations with no evidence of malaria to zero. In this way, populations with slight evidence of transmission may be easily distinguished from populations lacking evidence (e.g., a score of 0 vs. 0.010). Also, the populations with only slight evidence have values well below those from areas having obvious evidence of malaria (e.g., a score of 0.010 vs. 2.75). Adding one to the product of mean RESA OD and percent seropositivity prevented the possibility of a negative seropositivity score (when the product is < 1), or an incalculable score (the natural log of 0 is meaningless).

RESULTS

Malariometric Features.

Table 1 lists the demographic and malariometric features of the areas evaluated for RESA antibody. In general, the study sites represented a relatively wide range of likely malaria transmission levels. Some sites had little or no evidence, others showed evidence of

moderate transmission, and one site had hyper to holoendemic malaria. Thus, it was possible to evaluate the RESA ELISA seropositivity score in light of a range of levels of transmission known from standard malariometric measurements.

The districts of Wates and Dukuseti presented no evidence of malaria transmission. No blood films were positive and splenomegaly was absent except for slightly enlarged spleens in two elderly men living in Dukuseti. In the regency of Blitar as a whole, the slide positive rate (SPR) had been 0% in recent years. Health officials there believed that there had been no active transmission in the past 17 years (personal communication, dr. Widharto, Blitar). In the regency of Pati, recent malaria surveillance suggested active transmission in the area (SPR 0.34%) but there was no evidence of this in Dukuseti from the point measurement.

Two sites showed evidence of low level active malaria. In Sawahan near Madiun, 2 positive blood films were detected among 100 people surveyed. Four other positive blood films were detected, but these were in people who had recently traveled from Irian Jaya. There had been no cases detected in routine surveillance of the district during 1980 to 1987 (data provided by dr. Lani Widiayanti, Sawahan). Low level active transmission in the district of Brunorejo has been documented over the past few years[8], and the point measurement of prevalence in October 1989 revealed 18 parasitemic people among over 1,000 surveyed.

Arso PIR in northeastern Irian Jaya presented characteristics of hyper to holoendemic malaria between 1987 and 1990. Spleen and parasite rates remained high throughout the year. Spleen rates remained above 80% in children and above 60% in adults, and the point prevalence ranged between 33% and 62%.

Reviewing all of the available malariometric data from all of these sites, the following ranked order (from least to greatest) of likelihood of exposure to malaria was formulated: 1) Wates, 2) Dukuseti, 3) Sawahan, 4) Purworejo, and 5) Arso. The ranking value (1 through 5) was referred to as the ranked malariometric score.

Standardization of the RESA ELISA.

The reproducibility and specificity of the RESA ELISA for antigens presented on the surface of red blood cells infected by young rings of *P. falciparum* is demonstrated in Figure 2. Positive control sera were consistently positive on infected red blood cells, and negative on uninfected red blood cells in parallel tests. Conversely, negative control sera failed to react positively with either infected or uninfected red blood cells. Using many replicates of negative control sera, it was determined that an optical density value of > 0.060 was to be considered positive for anti-RESA IgG. The value was the mean RESA ELISA O.D. from negative control sera plus 3 standard deviations.
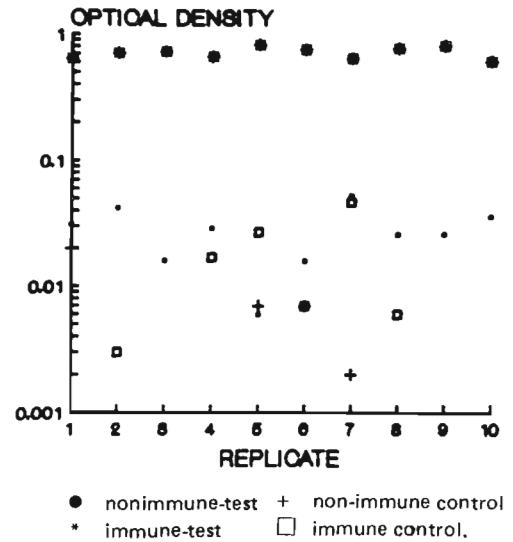
Figure 2.

Figure 2. Results of repeated RESA ELISA evaluation of immune and nonimmune sera incubated with infected (test) and uninfected red blood cells (control). Ten replicates were completed for each group, but for some tests a negative optical density precluded its presentation on the log scale above. Negative optical density values did not exceed − 0.050.

Figure 3 illustrates increasing mean RESA ELISA O.D. as a function of age among the 210 people surveyed in Arso. This was a consideration in selecting volunteers for the seroepidemiologic evaluation. It was clear that a comparison of RESA antibody among different villages would require age-matched groups. Because adults had higher levels of RESA antibody, and because they generally are more willing to donate blood, people between the ages of 20 and 35 were selected for seroepidemiologic analysis of each village.
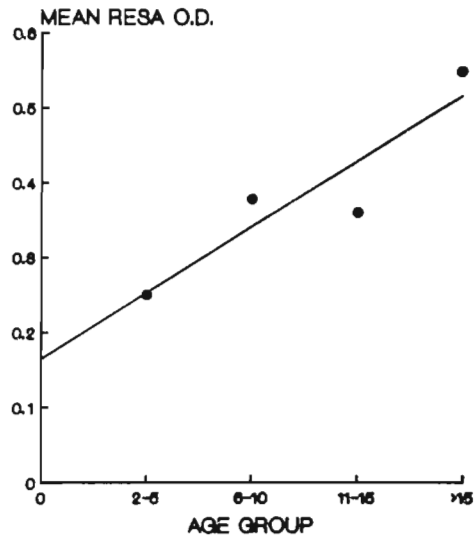
Figure 3.

Figure 3. Increasing mean RESA ELISA optical density among age groups of 210 people living in Arso, Irian Jaya.

Seroepidemiologic Analysis.

Table 2 lists the mean RESA ELISA O.D., the percent positive for RESA, and the seropositivity score. The table

Tabel 2. Summary of mean RESA ELISA O.D., percent RESA seropositive and the seropositivity score for the study sites.

Site	Mean RESA O.D.	Percent RESA Seropositive	Seropositivity Score
Jakarta	0.013	0	0
Wates	0.005	4	0.02
Dukuseti	0.006	0	0
Sawahan	0.067	29	1.08
Purworejo	0.222	78	2.91
Arso	0.550	100	4.03

illustrates the utility of the seropositivity score in more easily understanding

the outcome of the test. The score detected a few seropositive people in Wates who would have been unnoticed in a reporting of the mean RESA O.D., Likewise, the score separated by a wide margin the estimated levels of transmission in Purworejo and in Arso, when the difference in intensity of transmission (point prevalence of 2% versus 40%) may not have been appreciated by a reporting of the percent seropositive (78% versus 100%). Figure 4 compares the seropositivity score with the point prevalence measurement, and Figure 5 shows good correlation ($r = 0.898$) between the seropositivty score and the ranked malariometric score.
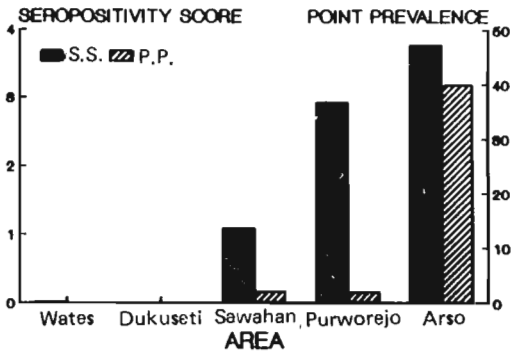
Figure 4.

Figure 4. RESA ELISA seropositivity score and point prevalence at the five study sites.

DISCUSSION

The epidemiologic and serologic measurements compared in this study show that the RESA ELISA is a suitable instrument for seroepidemiologic surveys. The seropositivity score was derived

from both the relative intensity of seropositivity and the proportion of seropositives in people 20 to 35 years old, and it correlated well with conventional measures of malaria endemicity.
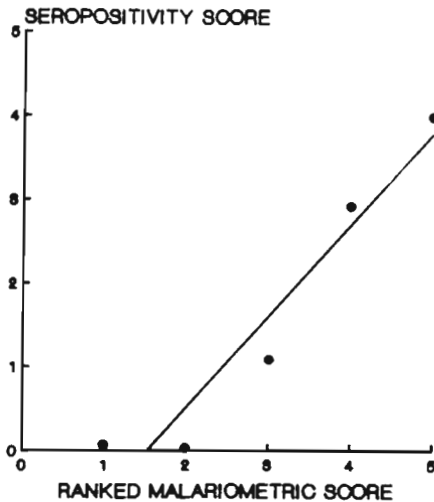
Figure 5.

Figure 5. Correlation (r = 0.898) between the RESA ELISA seropositivity score and the ranked malariometric score for the five study sites.

Appropriate interpretation of the seropositivity score requires caution. Any laboratory employing this assay must develop its own standars of endemicity versus seropositivity. There is much variation between laboratories in any ELISA and to compare a seropositivity score derived from another laboratory to those described in this report could invite erroneous conclusions. Once a laboratory has adopted a standardized assay procedure with internal controls, and obtained its own standards

of endemicity versus seropositivity, then seroepidemiologic estimates of malaria transmission may be obtained.

Interpretation of the estimate should proceed with consideration given to the results of the simultaneous point prevalence in the area under examination. For example, if P. vivax predominates in a given area, the RESA ELISA will probably greatly underestimate relative overall transmission because it detects only antibody to P. falciparum. Direct comparisions of different areas should be restricted to those with similar proportions of malaria species. Seropositivity scores should not be considered absolute measures of transmission. The relative nature of ELISA O.D. values prohibit this. The investigator should think of the seropositivity as a strictly relative measure; e.g., three areas having seropositivity scores of (a) 0.10, (b) 0.80, and (c) 2.5 may be considered as low, higher, and highest, but it can not be concluded that malaria transmission in are (a) is 8-fold less than in area (b).

In summary, the RESA ELISA seropositivity score was compared to conventional malariometric measures of exposure to malaria, and good correlation was observed. The RESA ELISA, with appropriate standardization, can provide a reliable relative measure of malaria endemicity. The test must be interpreted in light of point prevalence measurements obtained at the time of serum collection. Also, the seropositivity score must be understood in the context of scores from areas of known

levels of transmission, i.e., each lab conducting the assay must construct its own standard curve of seropositivity versus endemicity.

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