SPECIAL REPORT*

SEROEPIDEMIOLOGICAL STUDIES OF MALARIA IN DIFFERENT ENDEMIC AREAS OF INDONESIA

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Abstract. A total of 618 sera from inhabitants living in various endemic areas in Indonesia were examined for IgG against *Plasmodium falciparum* utilizing young trophozoites and mature schizonts as antigens by the method of ELISA and IFAT. In general, antibodies against trophozites (RESA) based on ELISA and antibodies against schizonts based on IFAT showed a correlation of malarial antibodies with the level of endemicity of the area examined. Anti-RESA antibody, detected either by ELISA or IFAT was more pronounced in the aparasitemic group compared to the parasitemic group. On the contrary, anti-schizont antibody measured by IFAT was more pronounced in the parasitemic group. Malarial antibody levels against the schizont-merozoite fraction of *P. falciparum* as assayed by ELISA appeared to develop more slowly compared to levels based on IFAT.

INTRODUCTION

The significance of seroepidemiological studies in describing the malarial situation in endemic areas has been reported by several authors (McWilson *et al,* 1975, 1976; Tharavanij *et al,* 1986). Determination of antibody levels in a population can be used to estimate the level of endemicity, for evaluation of malaria control programs and for delineation of malarious areas requiring antimalarial activity. However, the host's immune response is complex; thus this study examined the response by utilizing different stages of *P. falciparum* as antigens for the ELISA and IFAT to determine malaria antibodies from populations of various endemic areas.

METERIALS AND METHODS

Surveys were carried out in 1990 in Jelamprang village, Wonosobo, Central Java, and in Sante Piso/Sante Lemarang, Jengkalang villages, in the northern part of Flores, East Nusa Tenggara (Fig 1). Jelamprang is a hypoendemic area (spleen rate 6.3%) where DDT spraying and active as well as passive case detection were in operation. The other two villages are outside Java and had less antimalarial activity. Jengkalang, a hyperendemic area (spleen rate 54.8%) was a model of CHIPS until 1987. Many antimalarial activities such as DDT spraying, radical treatment of fever cases, health education on malaria, blood examination of children between 2-9 years and filling lagoons by community participation were carried out in this area. Sante Piso/Sante Lemarang was a mesoendemic area (spleen rate 27.1%) during the survey.

Spleen examination was done according to the Hackett method. A compound microscope was used to detect malaria parasites in thick and thin blood smears stained with Giemsa.

For serological studies, venous blood was collected and after centrifugal separation the sera were stored at -20°C until used. In Wonosobo, Central Java, 282 sera (80.3%) were collected from the population. Likewise, 155 sera (79.1%) and 181 (74.5%) sera were collected in Sante Piso/Lemarang and Jengkalang, respectively.

The antigen was made from *P. falciparum* Flores strain cultured *in vitro* according to the method of Trager and Jensen (1976). Synchronization was achieved with 5% sorbitol to obtain a single stage of parasite, either mature schizonts or young trophozoites (Lambros and Vandenberg, 1979). When the density of parasitemia reached 5%-10%, either

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ring forms or schizont stages were isolated. For RESA-ELISA, RESA-IFA and IFAT the parasitized erythrocytes diluted to 1% before they were coated and fixed on microscopic slides or ELISA plates. Glutaraldehyde 1% was used as a fixative for RESA-ELISA and RESA-IFAT, whereas cold acetone was used for IRAT. For ELISA purposes the erythrocytes were lysed with 0.1% saponin and then the parasites were disrupted with an ultrasonic desintegrator. After centrifugation at 9000 g for 30 minutes at 4°C, the supernatant was used as antigen after protein determination.

Antibodies to schizont stage of *P. falciparum*

The Indirect Fluorecent Antibody Test was done according to the method described by Collins *et al* (1964), utilizing 12 spot multiwell slides (printed microscope slides, Cel-lincs Associates, Inc cat 10-63). The conjugate used was goat anti-human IgG fluorescein isothiocyanate (Sigma F0132). The serum titer, was the last serum dilution that still showed a clear specific fluoresence. In this study, a titer was considered positive starting at a dilution of 1:200. The other assay, ELISA, was done according to Voller *et al* (1975) in a 96 multiwell Linbro microtitration plate (can no 76-381-04, Flow Laboratories). The starting point for positive titer was 1:40 with an OD value of more than 0.290 at 490 nm. The conjugate used was goat anti-human IgG (gamma chain specific) peroxidase (Sigma A-6029).

Antibodies to ring erythrocyte surface antigen (RESA) of *P. falciparum*

The method described by Perlmann *et al* (1984) was used both for RESA-ELISA and RESA-IFA. The threshold of positivity of RESA-IFA was 1:5, that is when a bright rim of fluorescence restricted to the membrane of the erythrocytes containing ring stages was still visible.

For RESA-ELISA, a serum was considered positive if the difference of OD between ring infected erythrocytes and normal erythrocytes was more than 0.211 at a dilution of 1:5,000. The conjugates used for these last assays were the same as for ELISA and IFA.

Statistical analysis was done by using Student's *t* test and chi square with $p < 0.05$ as a limit of significant difference.

RESULTS

Parasitological findings

As a whole a significant difference in parasite rate was shown only between populations of hypo- and hyperendemic areas (5.3% vs 12.7%) (Table 1). Between hypo- and mesoendemic areas it was shown only in the age group under 15 (6.1% vs 17.6%) (Table 1), but not in the group over 15 (4.8% vs 2.3%) (Table 1). No significant difference in parasite rate was found between meso- and hyperendemic populations in any particular age group < 9% vs 12.7% between 0-70 years, 17.6% vs 23.6% under 15 years, 2.3% vs 5.5% over 15 years) (Table 1).

Serological findings

Seropositive rate in relation to level of endemicity: Seropositive rates based on RESA-ELISA and IFAT showed significant differences between hypo-, meso- and hyperendemic areas (RESA-ELISA 12% vs 40.6% vs 76.2%; IFAT 46.1% vs 77.4% vs 90%) (Table 2). The results of RESA-IFA showed only significant differences between hypo- and mesoendemic areas (35.8% vs 62.6%) (Table 2), as well as hypo- and hyperendemic areas (35.8% vs 72.4%) (Table 2), but not between meso- and hyperendemic areas (62.6% vs 72.4%) (Table 2). The ELISA results showed significant differences between hypo- and mesoendemic areas (6.4% vs 31.6%) (Table 2), as well as hypo- and hyperendemic areas (6.4% vs 16.6%) (Table 2). Although the difference in seropositive rate between meso- and hyperendemic areas was significant, the seropositive rate in the mosoendemic area was higher compared to that in the hyperendemic area (31.6% vs 16.6%) (Table 2).

Geometric mean reciprocal titer (GMRT) and optical density (OD) difference in relation to level of endemicity: The GMRT and OD difference increased significantly according to the level of endemicity based on RESA-ELISA (0.155 vs 0.205 vs 0.311), IFAT (128 vs 731.57 vs 1344.33) and RESA-IFA (2.46 vs 16.33 vs 35.36) (Table 3). Results of ELISA showed a lower significant level of GMRT in the hypo- compared to mesoendemic areas (21.16 vs 31.3) (Table 3), as did GMRT of hypo- compared to hyperendemic areas (21.16 vs 23.04) (Table 3). Although the difference of GMRT between meso- and hyperendemic areas based on EL-

Table 1

Parasite rate of the population in different endemic areas classified by age.

Age (years)	Hypo	Meso	Hyper
0-70	5.3%/A (15/282)	9%/B (14/155)	12.7%/C (23/181)
< 15	6.1%/A1 (7/115)	17.6%/B1 (12/68)	23.6%/C1 (17/72)
> 15	4.8%/A2 (8/167)	2.3%/B2 (2/87)	5.5%/C2 (6/109)

A and B, B and C : no significant differences (chi-square, $p > 0.05$).
A and C : significant difference (chi-square, $p < 0.05$)
A1 and B1, A1 and C1 : significant difference (chi-square, $p < 0.05$)
B1 and C1 : no significant difference (chi-square, $p > 0.05$)
A2 and B2, A2 and C2, B2 and C2 : no signifícant differences (chi-square, $p > 0.05$)

Table 2

Seropositive rates of the population in different endemic areas based on ELISA, RESA-ELISA, IFAT and RESA-IFA.

Assay	Hypo	Meso	Hyper
ELISA	6.4%/A1 (18/282)	31.6%/A2 (49/155)	16.6%/A3 (30/181)
RESA-ELISA	12%/B1 (34/282)	40.6%/B2 (63/155)	76.2%/B (138/181)
IFAT	46.1%/C1 (130/282)	77.4%/C2 (120/155)	90%/C3 (163/181)
RESA-IFA	35.8%/D1 (101/282)	62.6%/D2 (97/155)	72.4%/D3 (131/181)

A1 and A2, A1 and A3, A2 and A3: significantly different (chi-square $p < 0.05$)
B1 and B2, B1 and B3, B2 and B3 : significantly different (chi-square, $p < 0.05$)
C1 and C2, C1 and C3, C2 and C3 : significantly different (chi-square, $p < 0.05$)
D1 and D2, D1 and D3 : significantly different (chi-square, $p < 0.05$)
D2 and D3 : no significant difference (chi-square $p > 0.05$)

Table 3

Geometric mean reciprocal titer (GMRT) and optical density difference of populations in different endemic areas.

Assays	Hypo	Meso	Hyper
ELISA	21.16 ± 1.29 (A1)	31.31 ± 2.58 (A2)	23.04 ± 1.53 (A3)
RESA-ELISA	0.155 ± 0.043 (B1)	0.205 ± 0.018 (B2)	0.311 ± 0.053 (B3)
IFAT	128 ± 1.28 (C1)	731.57 ± 1.74 (C2)	1344.33 ± 1.44 (C3)
RESA-IFAT	2.46 ± 1.19 (D1)	16.33 ± 1.92 (D2)	35.36 ± 1.62 (D3)

A1, A2 and A3 : significantly different (t test, $p < 0.05$).
B1, B2 and B3 : significantly different (t test, $p < 0.05$).
C1, C2 and C3 : significantly different (t test, $p < 0.05$).
D1, D2 and D3 : significantly different (t test, $p < 0.05$).

ISA was significant, a higher figure was obtained in the population of the mesoendemic area (31.31 vs 23.04) (Table 3).

Seropositive rate and GMRT or OD difference of different endemic areas in relation to parasitemia: In the hypoendemic population, the results of ELISA, RESA-ELISA AND IFAT showed higher levels of antimalarial antibodies in the parasitemic groups compared to the aparasitemic groups (the ELISA seropositive rate was 4.3% vs 1.1%, GMRT : 30.31 vs 20.2; The RESA-ELISA seropositive rate was 46.6% vs 10.1%, OD : 0.192 vs 0.154; The IFAT seropositive rate was 93.3% vs 43.4%, GMRT : 800 vs 116.86) (Table 4). Nevertheless, in the meso- and hyperendemic areas, the humoral immune response measured by RESA-ELISA and RESA-IFA was higher in the aparasitemic groups compared to the parasitemic groups (for RESA-ELISA in the mesoendemic area seropositive rate was 43.9% vs 7.1%, whereas OD was 0.190 vs 0.158) (Table 4). In the hyperendemic area, although the difference in seropositive rates between these two groups was insignificant (76% vs 78.3%) (Table 4) the OD differed significantly (0.313 vs 0.276) (Table 4). For RESA-IFA in the mesoendemic area, although the seropositive rate was not significantly different (61% vs 78.6%) (Table 4), the GMRT of those two groups was significantly different (17.51 vs 9.52) (Table 4). In the hyperendemic area both the seropositive rate and the GMRT of these two groups based on RESA-IFA were significantly different (seropositive rate 75.9% vs 47.8%, GMRT 42.03 vs 13.46) (Table 4).

By IFAT the parasitemic group had more antimalarial antibodies in hypo-, meso-, and hyperendemic populations. In the mesoendemic area the difference in seropositive rate between the 2 groups was insignificant (85.7% vs 76.6%) (Table 4), but the GMRT was significantly different (1055.62 vs 721.01) (Table 4). In the hyperendemic area the seropositive rate was not significantly different (95.6% vs 89.2%) (Table 4) between those 2 groups, but the GMRT was significantly different (1804.99 vs 1121.5) (Table 4).

Results of ELISA in the meso- and hyperendemic areas showed insignificant differences either in seropositive rate or in GMRT between aparasitemic compared to parastemic groups (in the mesoendemic area. The seropositive rate was 33.3% vs

Table 4

Serological results of populations of 3 different endemic areas with or without *P. falciparum* parasitemia based on ELISA, RESA-ELISA, IFAT and RESA-IFA.

Assay	Hypo		Meso		Hyper	
	PAR +	PAR -	PAR +	PAR -	PAR +	PAR -
ELISA						
Rate	4.3% (5/15) (A1)	1.1% (3/267) (A2)	14.3% (2/14) (A3)	33.3% (47/141) (A4)	8.7% (2/23) (A5)	17.7% (28/158) (A6)
GMRT	30.31 ± 0.4 (B1)	20.2 ± 0.01 (B2)	24.37 ± 0.25 (B3)	32.05 ± 0.68 (B4)	23.24 ± 3.07 (B5)	23.01 ± 0.1 (B6)
RESA-ELISA						
Rate	46.6% (7/15) (C1)	10.1% (27/267) (C2)	7.1% (1/14) (C3)	43.9% (62/141) (C4)	78.3% (18/23) (C5)	76% (120/158) (C6)
OD	0.192 ± 0.07 (D1)	0.154 ± 0.04 (D2)	0.158 ± 0.04 (D3)	0.190 ± 0.08 (D4)	0.276 ± 0.08 (D5)	0.313 ± 0.05 (D6)
IFAT						
Rate	93.3% (14/15) (E1)	43.4% (116/267) (E2)	85.7% (12/14) (E3)	76.6% (108/141) (E4)	95.6% (22/23) (E5)	89.2% (141/158) (E6)
GMRT	800 ± 1.31 (F1)	116.86 ± 1.19 (F2)	1055.62 ± 1.69 (F3)	721.01 ± 1.75 (F4)	1804 ± 1.28 (F5)	1121.5 ± 1.43 (F6)
RESA-IFA						
Rate	46.6% (7/15) (G1)	36.2% (94/267) (G2)	78.6% (11/14) (G3)	61% (86/141) (G4)	47.8% (11/23) (G5)	75.9% (120/158) (G6)
GMRT	2.9 ± 1.27 (H1)	2.4 ± 1.19 (H2)	9.5 ± 1.52 (H3)	17.5 ± 1.92 (H4)	13.4 ± 1.67 (H5)	42 ± 1.62 (H6)

PAR + : Parasitemia positive; PAR - : Parasitemia negative ELISA; seropositive rate : A1 and A2 were significantly different (chi-square, $p < 0.05$), A3 and A4 and A5 and A6 were not significantly different (chi-square $p > 0.05$). GMRT : B1 and B2 were significantly different (t test, $p < 0.05$), B3 and B4 and B5 and B6 were not significantly different (t test, $p > 0.05$). RESA-ELISA, seropositive rate : C1 and C2, C3 and C4 and C5 and C6 were significantly different (chi-square $p < 0.05$). OD difference : D1 and D2, D3 and D4 and D5 and D6 were significantly different (t test, $p < 0.05$). IFAT, seropositive rate : E1 and E2 were significantly different (chi-square, $p < 0.05$), E3 and E4 and E5 and E6 were not significantly different (t test, $p > 0.05$). GMRT : F1 and F2, F3 and F4 and F5 and F6 were significantly different (t test, $p < 0.05$). RESA-IFA, seropositive rate : G1 and G2 and G3 and G4 were not significantly different (chi-square $p > 0.05$), G5 and G6 was significantly different (chi-square, $p < 0.05$). GMRT : H1 and H2 were not significantly different (t test, $p > 0.05$), H3 and H4 and H5 and H6 were significantly different (t test, $p < 0.05$).

14.3%, GMRT was 32.05 vs 24.37, whereas in the hyperendemic area the seropositive rate was 17.7% vs 8.7% and GMRT was 23.01 vs 23.24) (Table 4).

DISCUSSION

Parasite rate is one of the important measurements for determination of malaria prevalence in an endemic area. Our serological study showed no significant differences in this parameter among hypo-, meso- and hyperendemic populations as a shole, except in the group under 15 years (Table 1). The impact of naturally acquired immunity, developed after years of exposure to the parasites, would tend to reduce the parasite density; consequently parasitemia becomes a parameter which is less reliable in describing the real malaria situation in an endemic area.

Results of serological assays showed the superiority of this cross-sectional seroepidemiological study in differentiating the level of endemicity. In general RESA-ELISA and IFA both qualitatively and quantitatively increased in accordance with the degree of endemicity. Quantitatively, RESA-IFA showed similar results (Table 2, 3).

Dissimilarities of antibody levels measured by different assays with different antigens have been reported by other authors (Tharavanij *et al,* 1986; Marsh *et al,* 1989). It seems that the humoral immune response or antibodies assessed in this study antibodies against antigens of schizontmerozoite fractions detected by ELISA were the last to appear during infection; conversely, IFA utilizing schizont whole antigen, showed antibody beginning early in infection (Table 2).

As a result of malaria control activities, malaria antibodies decrease, but then increase again gradually in relation to the intensity of transmission (Otoo *et al,* 1988). Perhaps the discrepancy of ELISA results with respect to seropositive rate and to GMRT between meso- and hyperendemic populations (Table 2, 3) was due to the different malaria control activities applied to these two areas in the past; 3 years seemed to be insufficient to lead to a schizont merozoite fraction antibody level relevant to the degree of endemicity. Entomological studies during the survey showed that the infection rate of *A. subpictus* as the vector in the hyperendemic area was only 0.29% (Hoedojo, 1992). The significance of IFA as an indicator of the presence of infection was supported by the tendency of parasitemic group to exhibit a higher level of malarial antibodies compared to the aparasitemic group, in hypo-, meso-, or hyperendemic areas. (Table 4).

In the meso- and hyperendemic areas, RESA-ELISA and RESA-IFA demonstrated higher levels of humoral immune response in the aparasitemic group compared to the parasitemic group. This inverse relationship suggested that RESA antibody might play a role in antibody-mediated immune protection against malaria.

In the hypoendemic area, the parasitemic group had more anti-malarial antibodies based on ELISA, RESA-ELISA and IFAT compared to the aparasitic group; with RESA-IFA the subjectivity of this assay might influence the results.

In conclusion, this study showed the value of seroepidemiological study using four types of serological tests, with two kinds of antigens. It appeared that RESA-ELISA and IFAT could be useful in the determination of endemicity of malarious areas. In addition it is proposed that RESA-ELISA may be useful as an indicator of protective immunity at a certain level of endemicity, whereas IFAT is a better indicator of infection.

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