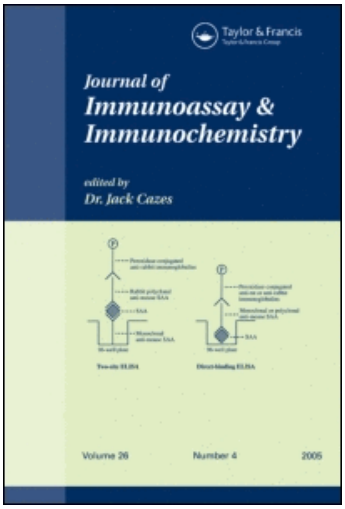

Journal of Immunoassay and Immunochemistry

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

Assessment of Immunometric Parameters in Malaria: Role of Enzyme Immunoassay

Sukla Biswas[a]
[a] National Institute of Malaria Research (ICMR), Delhi, India

To cite this Article Biswas, Sukla(2006) 'Assessment of Immunometric Parameters in Malaria: Role of Enzyme Immunoassay', Journal of Immunoassay and Immunochemistry, 27: 4, 341 — 350
To link to this Article: DOI: 10.1080/15321810600862181
URL: http://dx.doi.org/10.1080/15321810600862181

Journal of Immunoassay & Immunochemistry, 27: 341–350, 2006

ISSN 1532-1819 print/1532-4230 online
DOI: 10.1080/15321810600862181

Assessment of Immunometric Parameters in Malaria: Role of Enzyme Immunoassay

Sukla Biswas
National Institute of Malaria Research (ICMR), Delhi, India

Abstract: Human blood samples collected from local malaria clinics, hospitals, and by cross-sectional surveys in malaria endemic areas were tested by enzyme immunoassay for circulating malarial antigen, antimalarial antibody, and antigen-specific circulating immune complexes. The assays were done in serum and finger-prick blood absorbed on filter paper. The results obtained from the present study suggest their roles as effective immunometric indicators.

Keywords: Malaria, Immunodiagnosis, Antigen, IgG, Enzyme immunoassay

INTRODUCTION

In endemic areas with moderate malaria transmission, age-specific malaria incidences are quite prevalent.[1] Infectivity in non-immune individuals is more frequent and declines after repeated exposures to malaria infection. High immunity usually masks the infection, creating asymptomatic state.

Immunometry in any type of infectious diseases is generally based on a number of tests. The aims and procedures of these tests need to be formulated prior to application. They are used in laboratory based research on the mechanisms of malaria immunity, induction of protective immunity, and other immune phenomena; they are also essential for the study of immune phenomena in individuals and populations which are naturally exposed to malaria. The possible role of immunodiagnosis could be achieved by combining several tests for antibody and antigen. The immunodiagnostic tests could evaluate the development of the immune response in populations where various drug

Address correspondence to Dr. Sukla Biswas, National Institute of Malaria Research (ICMR), 22 Sham Nath Marg, Delhi 110 054, India. E-mail: suklabiswas@yahoo.com or biswass@icmr.org.in

schemes may act as the main and only method of malaria control.[2] The roles of immunometric parameters in malaria epidemiology have been investigated, for the first time, in the Garki Project.[3] The scope of that study was limited because of the paucity of dependable indicators which could represent the wide spectrum of host status, i.e., from a state of susceptibility through grades of partial or imperfect immunity, to a state of protective immunity resisting the manifestation of illness with re-infection or super-infection.[4]

The advent of enzyme linked immunosorbent assay (ELISA) has become established as useful and powerful test systems for detection of malaria antigen, antibody and circulating immune complexes (CIC) with standard defined reagents. The present study describes the use of ELISA in determining circulating antigen, antibodies, and CICs in blood samples collected through a cross-sectional survey in a population belonging to malaria endemic areas. The parameters used for the present investigation may suggest their respective roles as immunometric and serodiagnostic indicators.

EXPERIMENTAL

Study Subjects

The 117 patients of different age groups, reported with fever in local malaria clinics and hospitals during the transmission season, were checked for the malarial parasite by microscopy. Finger-prick blood samples were collected in Eppendorf tubes and also absorbed on Whatman No. 3 filter paper, as described elsewhere.[5] A group of 240 individuals was also enrolled for the study by a cross-sectional survey; they were the residents of the villages Piyawli and Nanoo, in the Ghaziabad district of Uttar Pradesh in northern India, where malaria is endemic. Blood samples were collected during the transmission of 2002 and 2003, from these individuals, on filter paper, by pricking their fingers. Patients diagnosed with malaria were treated with anti-malarials as per recommendation. The study was initiated after approval of the institutional ethics committee. Each adult subject, and parents or guardians of each child subject gave their informed consent. The sera and filter paper absorbed smears (after drying at ambient temperature) were stored at -20°C until use. Both the sera and filter paper absorbed blood samples were used at 1 : 16 dilution. Two small discs of filter paper smears, containing 10 μl blood, were dipped in 160 μL of isotonic phosphate buffered saline, pH 7.2, giving a dilution of 1 : 16, were kept for elution overnight at 4°C.

Parasite Antigen Preparation

Crude antigen (PfL) was prepared from cultured parasites from established lines of the Indian *Plasmodium falciparum* isolates by the published

method.[5] Briefly, the parasite lines were routinely maintained in vitro using human O+ RBCs and AB+ serum by a candle-jar technique.[6] Crude antigen was prepared from cultures enriched with late trophozoite and schizont stages. The parasites were freed from their host erythrocytes by saponin lysis, then sonicated for 90 seconds at 14 μA in a sonicator (MSE Soniprep, Beckenham, UK); finally the lysed preparation was centrifuged for supernatant solution containing the crude soluble parasite antigens. The antigen was purified by removing host contamination after adsorption with rabbit anti-human erythrocytes.[1]

Raising of Rabbit Anti-PfL Antiserum

Rabbits were immunized by injecting crude PfL antigen adsorbed on alum in their footpads, following the published protocol.[7] The primary immunization of 200-μg proteins was given, followed by two booster injections of 100 μg each on the 15th and 30th days. Rabbits were bled from a central ear artery on the 45th, 52nd, and 60th days after the primary immunization, for determination of the titres of the antisera. Immunoglobulin G (IgG) was fractionated from the high-titre antisera by ammonium sulphate precipitation.

Immune Sera

Finger prick blood samples were collected from a group of 45–50 clinically immune adults, who have recovered recently from *P. vivax* or *P. falciparum* infections after antimalarial treatment and were afebrile and aparasitaemic at the time of blood collection. Sera were tested by ELISA for parasite specific antibodies. Sera with high titres $>1:1{,}000$ by ELISA were pooled and IgG was fractionated by ammonium sulphate precipitation.

Detection of Antimalarial Antibody

An indirect ELISA was performed, following the published method.[5] The 96-well round bottom polystyrene ELISA plates were coated with PfL at a protein concentration of 20 μg/mL (2 μg/well) by incubating for 1 hr at 37°C, then overnight at 4°C. Sera and filter paper (FP) eluates both were tested serially at two-fold dilutions from 1/16 onwards. After addition of samples onto the first layer, they were allowed to react for 1 hr at 37°C. Anti-human IgG conjugated with horseradish peroxidase (HRP) trapped the bound antibody. After addition of enzyme-specific substrate and subsequent termination of the reaction with 8 N sulfuric acid, the end-point titre was determined. In each plate, reference positive and negative samples were tested as controls.

Detection of Circulating Total (TC-Ag.) and Free (Fc-Ag.) Malarial Antigens and Circulating Immune Complexes (CIC)

A double antibody sandwich ELISA was performed to determine circulating free and bound antigens. A solid phase was coated with rabbit anti-PfL IgG at a protein concentration of 10 μg/mL (1 μg/well). Sera and FP eluates were added serially, at two-fold dilutions, from 1/16 onwards, in wells after necessary washings and blocking with 1% bovine serum albumin (BSA). The assays were done in two sets. In one set, after incubation of 1 hr at 37°C, goat anti-human IgG-HRP conjugate was added at the next step for detection of antigen-specific CIC. In the second set, after incubation, the wells were probed with human IgG (1 μg/well) isolated from malaria immune individuals. After further incubation for 1 hr at 37°C, the secondary antibody, goat anti-human IgG conjugated to HRP, was added. The reaction was terminated with 8 N sulfuric acid and the end-point titres were determined. This way, it was possible to determine free circulating malarial antigen by subtracting the ELISA titre values obtained in the CIC set (without immune IgG) from the total antigen (with immune IgG) values. In each set of assays, reference positive and negative samples were tested as controls.

Statistical Analysis

Linear regression analysis (Pearson correlation; 2-tailed Probability test) was done to determine whether the levels of antibody, total and free circulating antigens, and circulating immune complexes correlated with each other in a given time point. The results were considered significant at a P-value of <0.05.

RESULTS

A total of 117 patients were screened for malarial parasite by microscopy; 54 were found to be positive and the remaining 63 were negative at the time of blood collection, but they suffered from malaria in the recent past. The ELISA tests for detection of antimalarial antibody, circulating antigen, and circulating immune complexes were done in 117 sera and FP eluates as a pilot study. The results of sera and FP eluates were comparable (Table 1). Of 54 malaria positive samples, 51 and 52 were detected positive for FC-Ag in sera and FP eluates, respectively. This shows that the test has high diagnostic efficiency. Only 25–28% of the samples of the negative group were detected FC-Ag positive, due to very recent infection in these individuals. Antibody was detected in more than 90% malaria patients, whereas, in blood smears from the negative group, about 86% showed sero-positivity

Table 1. Free circulating malarial antigen (FC-Ag.), antigen-specific antibody (Ab.) and circulating immune complexes (CIC) in serum and filter paper eluates (FP-E) of 117 subjects

Samples (n = 117)			
In Sera	FC-Ag.	Ab.	CIC
	No. + (%)	No. + (%)	No. + (%)
54 + for malaria parasite	51 (94.4)	49 (90.7)	39 (72.2)
63 −	16 (25.4)	54 (85.7)	33 (52.4)
In FP-E	FC-Ag.	Ab.	CIC
	No. + (%)	No. + (%)	No. + (%)
54 + for malaria parasite	52 (96.3)	51 (94.4)	36 (66.7)
63 −	18 (28.6)	54 (85.7)	29 (46.03)

for antimalarial antibody. The bound antigens in the form of CICs were detected more in malaria positive cases than in smear negatives.

The test parameters were applied to 240 FP eluates collected from different age groups (43, 60, 56, and 81 aged $1- < 5$, $5- < 10$, $10- < 15$ and >15 years, respectively). The average antibody titre $-\log_2$ varied from 6–10. Both TC-Ag and CIC were found at lower levels in younger age groups. The FC-Ag levels were almost same in all (Fig. 1). The overall results obtained from 240 individuals are presented as a correlation plot in Figs. 2–7. Figure 2 shows the ELISA antibody titre versus

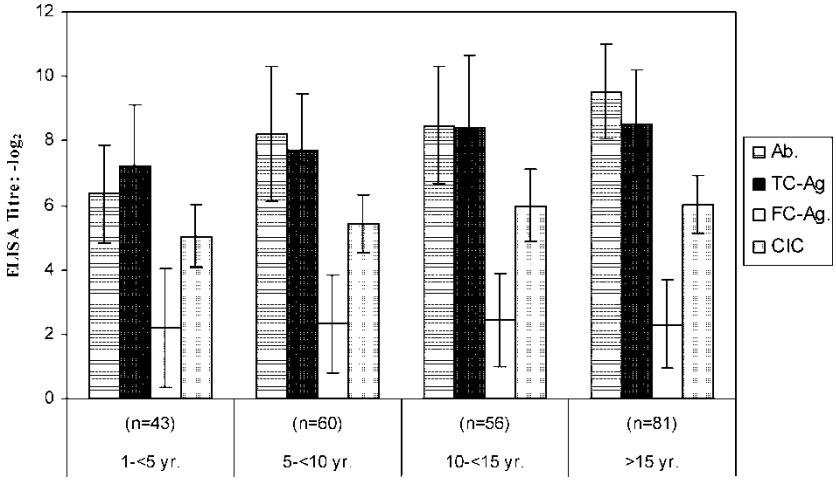

Figure 1. Antibody, total circulating antigen, free circulating antigen, and circulating immune complex profile in the study subjects (n = 240). The bar diagram is plotted with mean ELISA −log2 titre of each group.

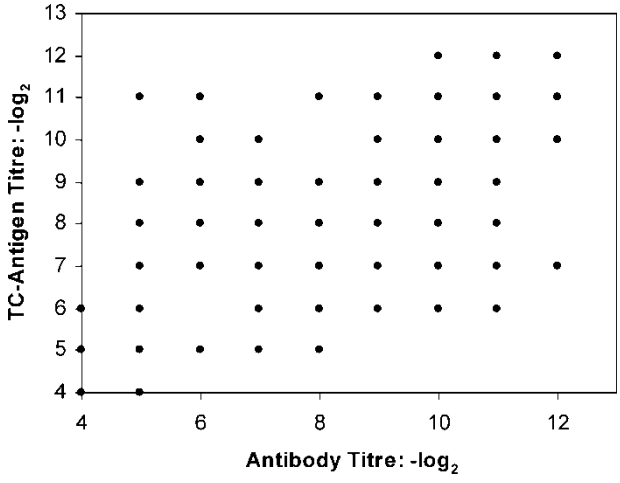

Figure 2. Atibody vs TC-Ag.

total antigen in circulation. The scattered plot shows some degree of correlation between these two parameters ($r = 0.67$; $P < 0.0001$), as if degree and status of infection may be controlled by the humoral immune responses. Antibody titres versus CIC are shown in Fig. 3. It shows a tendency towards a positive correlation ($r = 0.48$; $P < 0.0001$). In Fig. 4, the antibody titre versus FC-Ag shows significant correlation between these two parameters ($r = 0.46$; $P < 0.0001$). Figure 5 depicts the TC-Ag versus

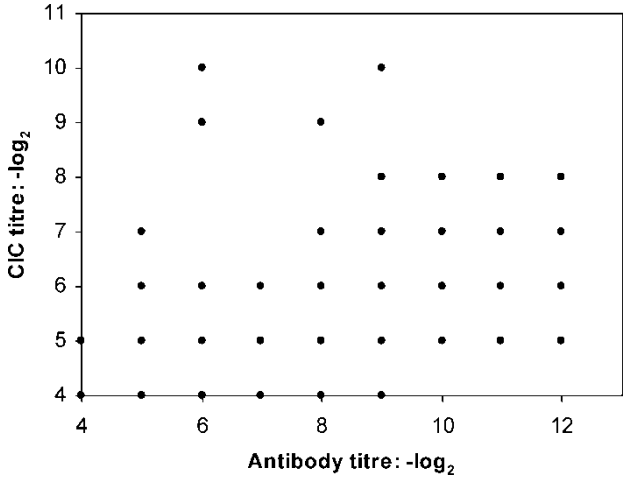

Figure 3. Antibody vs CIC.

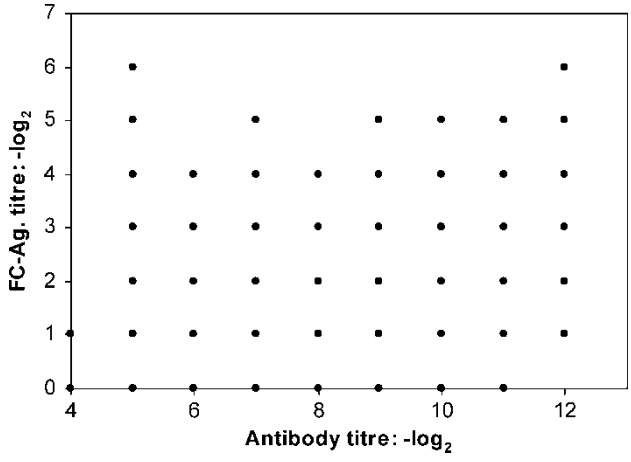

Figure 4. Antibody vs FC-Antigen.

CIC. The pattern indicates linearity, as if an increase in total infection load may cause retention of immune complexes in circulation ($r = 0.60$; $P < 0.0001$). Figure 6 shows the ELISA titre of CIC versus FC-Ag ($r = -0.02$; $P = 0.8142$). The pattern shows a negative correlation, though not significant, suggesting that the CICs seem to bear a role in controlling infection load, whereas FC-Ag indicates active infection. The TC-Ag versus FC-Ag titres show positive correlation ($r = 0.77$; $P < 0.0001$) as if there may be the tendency of forming complex in antigen excess with available specific antibodies (Fig. 7).

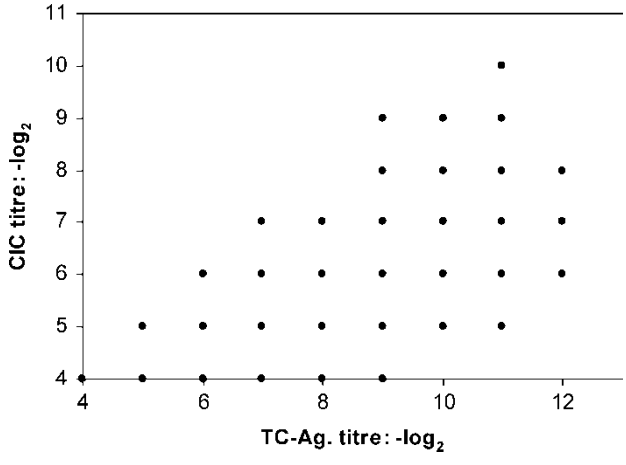

Figure 5. TC-Antigen vs CIC.

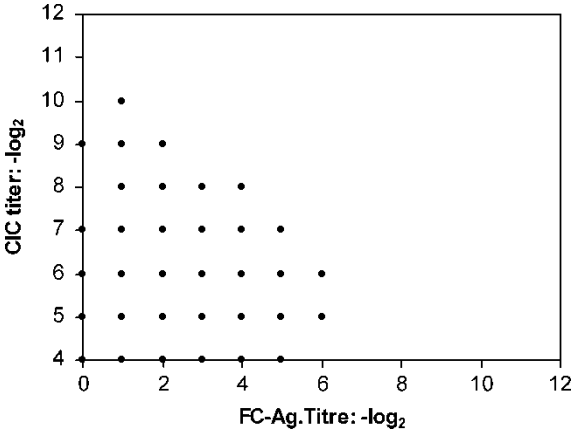

Figure 6. FC-Antigen vs CIC.

DISCUSSION

People living in malaria endemic areas acquire immunity to the disease. However, acquisition of immunity is slow, requires years of exposure, and is not long lasting.[8,9] It is suggested that the precise age-specific pattern of disease depends upon the intensity of transmission in a given community such that, under conditions of intense stable transmission, younger children are more at risk from disease to death.[10] It was observed that, while parasite rates and densities declined gradually after 6 months till one year age, positivity rates to malarial antigens increased rapidly within this

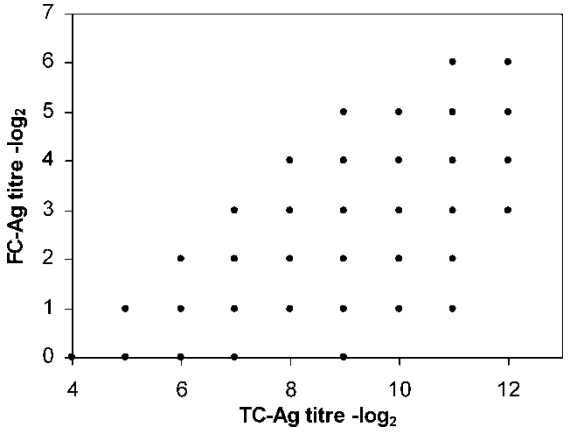

Figure 7. TC-Ag vs FC-Ag.

period, suggesting the development of naturally acquired immunity to malaria.[11]

Although microscopic demonstration of malarial parasite in blood film is the method of choice to diagnose acute malaria, detection of circulating malarial antigens, antimalarial antibodies, and immune complexes may help as supplemental tools for immunodiagnosis. When applied in a community-based study, these tests would be able to monitor infection dynamics. Diagnosis of acute infection by FC-Ag detection was achieved in 95% of malaria positive cases; this indicates that this simple test parameter may be further improved before its application in large-scale surveys. The improvement in the technique may be achieved by using stage- and species-specific monoclonal or polyclonal antibodies. Longitudinal and sequential followup at different transmission seasons would help in analysis of malaria endemicity. Malaria antigen specific CICs detection by ELISA in areas may help to locate the endemic foci. The total infection load, level of antibodies, and the degree of superinfection may help in maintaining "herd immunity" in a given community. With proper planning of sampling in various seasons, infection dynamics could be computed. The collation of these parameters, along with parasitological data, could help in categorization of infection dynamics in an actual field situation.

ACKNOWLEDGMENTS

This study received financial support from the Indian Council of Medical Research. Thanks are due to Dr. Aparup Das for valuable suggestions and statistical analysis.

REFERENCES

1. Biswas, S.; Saxena, Q.B.; Roy, A.; Kabilan, L. Naturally occurring Plasmodium-specific Immunoglobulin A antibody in humans from a malaria endemic area. J. Biosci. **1995**, *20*, 453–460.
2. Molineaux, L. Essential parameters in seroepidemiological assessment for epidemiological analysis of serological data. **1981**, WHO/MAL/81.969.
3. Molineaux, L.; Gramiccia, G. The Garki project: Research on the epidemiology and control of malaria in the Sudan Savanna of West Africa. WHO publication: Geneva, 1980.
4. Chaudhuri, S.N. Immunometric parameters as a feedback for control operation in malaria. J. Commun. Dis. **1985**, *17*, 11–19.
5. Biswas, S. Inter test comparison between filter paper absorbed blood eluate and serum for malaria serology by enzyme immunoassay: an operational feasibility. J. Immunoassay. Immunochem. **2004**, *25*, 397–408.
6. Trager, W.; Jensen, J.B. Human malarial parasites in continuous culture. Science **1976**, *193*, 673–675.

7. Biswas, S.; Tomar, D.; Rao, D.N. Investigation of the kinetics of histidine-rich protein 2 and of the antibody responses to this antigen, in a group of malaria patients from India. Ann. Trop. Med. Parasitol. **2005**, *99*, 553–562.
8. Greenwood, B.; Marsh, K.; Snow, R. Why do some African children develop severe malaria? Parasitol. Today **1991**, *7*, 277–181.
9. Troye-Blomberg, M.; Perlmann, P.; Mincheva Nilson, L.; Perlmann, H. Immune regulation of protection and pathogenesis in *Plasmodium falciparum* malaria. Parasitologia **1999**, *41*, 131–138.
10. Brooker, S.; Guyatt, H.; Omumbo, J.; Shretta, R.; Drake, L.; Ouma, J. Situation analysis of malaria in school-aged children in Kenya – what can be done? Parasitol. Today **2000**, *16*, 183–186.
11. Achidi, E.A.; Perlmann, H.; Salimonu, L.S.; Perlmann, P.; Walker, O.; Asuzu, M.C. A longitudinal study of seroreactivities to *Plasmodium falciparum* antigens in Nigerian infants during their first year of life. Acta Trop. **1995**, *59*, 173–183.

Received February 1, 2006
Accepted March 7, 2006
Manuscript 3196