

MALARIA SEROEPIDEMIOLOGY: COMPARISON BETWEEN INDIRECT FLUORESCENT ANTIBODY TEST AND ENZYME IMMUNOASSAY USING BLOODSPOT ELUATES

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Blood sampling on filter paper is a current practice in malaria seroepidemiological studies by indirect fluorescent antibody test (IFAT). There is, however, scant comparative information about the use of bloodspot eluates for detection of malarial IgG antibodies simultaneously by IFAT and enzyme immunoassay (ELISA). Here we report data obtained by both serological methods done on 219 bloodspot eluate samples collected in a rural community in Brazilian Amazon Basin (Alto Paraíso, Ariquemes municipality) where malaria is endemic. Plasmodium falciparum and P. vivax thick smear antigens were used in the IFAT; a detergent-soluble P. falciparum antigen was prepared for ELISA. Substantial agreement of results (Kappa coefficient $k = 0.686$) was observed when P. falciparum antigen was used in both tests, and IFAT titers were found to be strongly correlated to ELISA antibody units (Spearman correlation coefficient $r_s = 0.818$, $p < 0.0001$). Only moderate agreement ($k = 0.467$) between IFAT with P. vivax antigen and ELISA with P. falciparum antigen was observed. Spearman correlation coefficient value between quantitative results (IFAT titers and ELISA antibody units) in this case was numerically lower ($r_s = 0.540$, $p < 0.0001$). Our results suggest that, with P. falciparum antigen, both IFAT and ELISA performed on bloodspot eluates are equivalent for seroepidemiological purposes.

Key words: ELISA – IFAT – malaria – serology – bloodspot eluates – Brazilian Amazon Region

Blood samples collected on filter paper are currently used in seroepidemiological surveys (Thaver & Draper, 1974; Guimarães et al., 1986; Segui et al., 1990). Antibody detection by indirect fluorescent antibody test (IFAT), the reference method for malaria serodiagnosis (OMS, 1989), was shown to produce consistent results on both serum samples and bloodspot eluates (Wanderley et al., 1982). However, IFAT has been recently replaced by the enzyme immunoassay (ELISA), a serological method more adequate to large field surveys (OMS, 1989). An ELISA method for detection of antibodies to *Plasmodium falciparum* sporozoite recombinant antigen on bloodspot eluates has been assayed in laboratory setting (Esposito et al., 1990). Nevertheless, there is still scant information about the

use of this blood collecting technique in field malaria surveys using ELISA protocols with crude antigen extracts (Pérez & Bolívar, 1989; González-Cerón & Rodríguez, 1991).

Serological tests on 35,000 bloodspot samples will be performed in our laboratory next year, during a multidisciplinary field survey concerning malaria risk factors in a Brazilian endemic area (Ariquemes municipality, Rondônia State, Amazon Basin), sponsored by Pan American Health Organization (PAHO) and Fundação Nacional de Saúde (FNS). A pilot study was designed for standardization of the techniques and comparison of results produced by IFAT and ELISA.

MATERIALS AND METHODS

Blood samples (219) were collected by finger-prick during April 1991 in Alto Paraíso, a rural community near Ariquemes where a previous serological survey had been performed in December 1990. After collection on filter

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paper (Whatman no. 3), the blood samples were dried at room temperature and subsequently stored at 4°C for 2-3 weeks, until extraction in 0.01 M phosphate-buffered saline (PBS), pH 7.2. Bloodspot volume was calculated as described previously (Ferreira & Carvalho, 1982). Eluate samples diluted 1:16 with PBS were stored at -20°C for 30-90 days until used. Giemsa-stained thick smears were simultaneously obtained and examined by field microscopists and at two reference laboratories of FNS. Of them, 8 were *P. falciparum*-positive and 8 were *P. vivax*-positive; mixed infections were not detected. In this preliminary study, data about previous malaria attacks were not collected.

IFAT was performed essentially as described by Sulzer et al. (1969) and Ferreira & Carvalho (1973). *P. falciparum* and *P. vivax* thick smear antigens were prepared with blood samples obtained from patients with high parasitaemia from Rondônia and stored at -20°C for 30-90 days until used. A fluorescein-conjugated rabbit anti-human IgG (Behring, Germany) was used at a 1:160 dilution. All reactions were read under an Olympus FLM fluorescence microscope by the same microscopist. Samples were first assayed at 1:16 dilution; the positive ones were further serially diluted with PBS until 1:4096. Quantitative results were expressed as titers (reciprocals of last sample dilution having fluorescence).

Antigen from a *P. falciparum* isolate from Rondônia State (S-20) (Kimura, 1991), maintained in continuous culture (Trager & Jensen, 1976), was used as a detergent-soluble preparation for the enzyme immunoassay. At 10% parasitaemia, red blood cells (RBC) from culture were washed with incomplete RPMI-1640 medium (Sigma, USA) and infected erythrocytes were concentrated in discontinuous Percoll gradients. Two ml of a 1:6 RBC dilution with RPMI-1640 were applied on the top of each 9-ml gradient column. Each column consisted of three layers of, respectively, 40%, 70% and 80% Percoll (Pharmacia, Sweden) dilutions with incomplete RPMI-1640 medium plus sorbitol 5% (w/v). After centrifugation (16000 g for 30 min at room temperature), schizont-infected RBC were collected and washed with incomplete RPMI-1640 medium. Final concentration of infected RBC was assessed on Giemsa-stained thin smears and only preparations with nearly all RBC infected were used. Infected RBC diluted to 1:5 with sterile distilled water

were added to equal volume of NET-Triton buffer, pH 7.5 (0.4 M NaCl, 10 mM ethylenediamine tetracetate (EDTA), 10 mM Tris and 2% v/v Triton X-100) (Braun-Breton et al., 1986). After incubation for 15 min at 4°C and sonication (15 W for 3 sec), the antigen preparation was centrifuged (16000 g for 10 min at 4°C). The supernatant was aliquot and stored in liquid nitrogen until used.

For enzyme immunoassay, 96-well microplates (C. E. B., France) were coated with a 1:2000 antigen dilution with 0.06 M carbonate-bicarbonate buffer, pH 9.6, 50 µl per well. This optimal antigen dilution was determined by titration of known positive and negative controls. Microplates were incubated for 1 h at 37°C and washed five times with 0.01 M PBS, pH 7.2 with 0.1% v/v Tween 20 (PBST) (one washing cycle). Blocking solution (PBST with 5% w/v low-fat powdered milk) was then added (incubation for 1 h at 37°C). After a washing cycle, eluate samples (50 µl) diluted to 1:64 with PBST were added. Following an one-hour incubation period at 37°C and an additional washing cycle, 50 µl per well of horseradish peroxidase-conjugated goat anti-human IgG (Bio-Sys, France), diluted to 1:4000 with blocking solution, were added. After a further one-hour incubation period at room temperature and a washing cycle, enzymatic reaction was assayed using 0.4 mg ortho-phenylenediamine (Sigma, USA) per ml of 0.05 M citrate-phosphate buffer, pH 5.0, with 0.5 µl of 30% v/v hydrogen peroxide (50 µl per well). Fifteen min later, the reaction was stopped by adding 2N HCl. Absorbance values at 492 nm were measured in a Dynatech MR-5000 microplate reader. The cut-off point was set at three standard deviations above the mean absorbance value of 14 negative controls (bloodspot eluates from healthy people without malaria history, inhabiting a nonendemic region) included in each plate. The cut-off value calculated for each plate varied between 0.2 and 0.3. Quantitative results were expressed as antibody units (ABU), the sample absorbance value divided by the cut-off absorbance value for each microplate (Knobloch & Hermentin, 1987). Samples with ABU > 1.0 were considered positive.

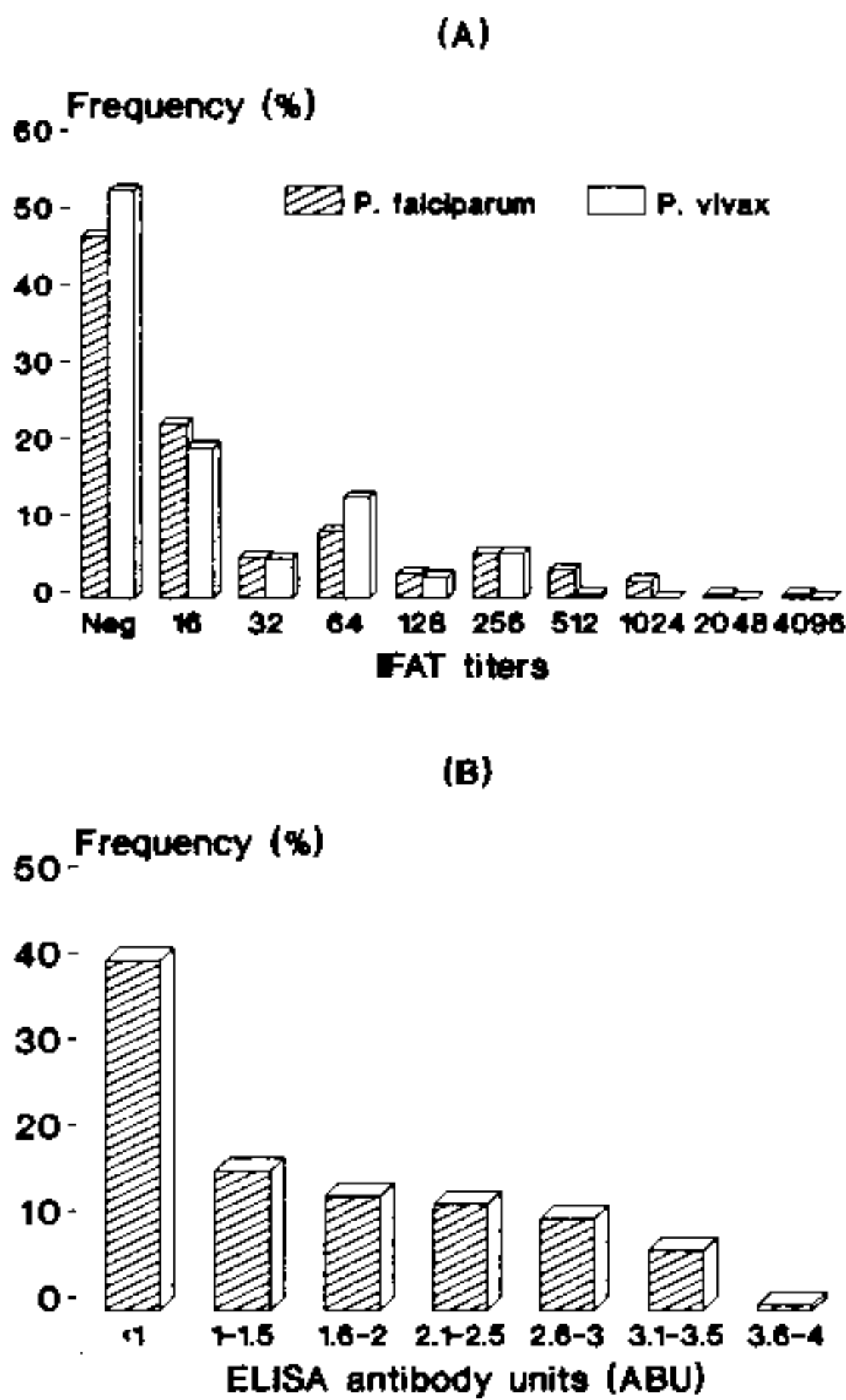
Agreement of quantitative results (positive/negative) by IFAT and ELISA was assessed using the Kappa statistic (Cohen, 1960). Correlation between quantitative results (IFAT titers and ELISA ABU) was estimated using

the nonparametric rank test proposed by Spearman (Siegel, 1977).

RESULTS

Of 219 samples examined, 106 (48.4%) were positive and 79 (36.1%) negative by both ELISA and IFAT with *P. falciparum* antigen. Kappa coefficient indicated substantial agreement of these results ($k = 0.686$). There were 24 samples ELISA-positive and IFAT-negative and 10 samples IFAT-positive and ELISA-negative. All patients ($n = 8$) with *P. falciparum*-positive Giemsa-stained thick smears were serologically positive by both methods.

Figure shows the frequency distribution of IgG antibody titers by IFAT (A) and antibody units (ABU) by ELISA (B) in all samples assayed.



Frequency distribution of IFAT titers (A) and ELISA antibody units (ABU) (B) in 219 bloodspot eluate samples from Alto Paraíso, Ariquemes municipality, Brazilian Amazon Basin. For definition of ABU see text (Materials and Methods).

A strong correlation, as estimated by non-parametric Spearman rank test, was observed between IFAT titers and ELISA ABU values when *P. falciparum* antigen was used in both methods (correlation coefficient $rs = 0.818$, p

< 0.0001). However, only moderate agreement ($k = 0.467$) was observed when qualitative results of ELISA with *P. falciparum* antigen and IFAT with *P. vivax* antigen were compared. In this case, Spearman correlation coefficient value calculated for ELISA ABU values and IFAT titers was numerically lower ($rs = 0.540$, $p < 0.0001$). Seven of 8 patients with *P. vivax*-positive thick smears were also IFAT-positive, but only 5 of them were ELISA-positive when *P. falciparum* antigen was used.

DISCUSSION

Higher positivity frequency of antibodies against *P. falciparum* found by ELISA may be partially due to detection, by this method, of specific antibodies at a concentration undetectable by IFAT using 1:16 sample dilution. Of 24 samples positive by ELISA only, 10 were obtained from patients IFAT-positive (with *P. falciparum* antigen) in the previous serological survey, December 1990. For adequate blood extraction from filter paper, a 16-fold volume of PBS is usually necessary, which results in a minimal samples dilution of 1:16. This initial eluate dilution may reduce antibody detection sensitivity by IFAT, but not by ELISA, which can be performed on higher sample dilutions.

Our results suggests that ELISA using the described detergent-soluble antigen can replace IFAT in *falciparum* malaria serodiagnosis on bloodspot eluate samples. However, homologous antigen must be used in ELISA for detection of *vivax* malaria endemicity (Pérez & de la Rosa, 1990; González-Cerón & Rodríguez, 1991). Interspecific cross-reactions are expected in human malaria serodiagnosis (Diggs & Sadun, 1965), but their frequency may be variable in different antigen preparations. Following a *P. vivax* malaria outbreak among intravenous drug addicts without either previous malaria infection or a stay in endemic areas (Barata et al., 1990), we performed both IFAT and ELISA in sera of 18 thick smear-positive patients. All of them were positive by IFAT using *P. vivax* antigen, but only 6 (33.3%) were ELISA-positive with detergent-soluble *P. falciparum* antigen. There were no samples positive by *P. falciparum* IFAT (Carvalho et al., unpublished data). In regions where *P. falciparum* and *P. vivax* infections are equally prevalent, as in Brazilian Amazon Basin, the observed "cross-reactions" in parasitologically positive patients may reflect previ-

ous infections due to the other *Plasmodium* species (González-Cerón & Rodríguez, 1991). For this reason, true cross-reactivity with heterologous antigen in human malaria serology should be evaluated on samples from first-infected patients.

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