COMPARISON BETWEEN MICROSCOPIC EXAMINATION, ELISA AND QUANTITATIVE BUFFY COAT ANALYSIS IN THE DIAGNOSIS OF FALCIPARUM MALARIA IN AN ENDEMIC POPULATION

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Abstract. Monoclonal antibody-based ELISA and QBC (quantitative buffy coat analysis) were tested in two endemic areas with low and high incidence of malaria in Kanchanaburi Province, West Thailand with annual parasite incidence in 1992 of 119 and 5 per 1,000 population, respectively. The numbers of individuals positive by thick blood film examination (TBF) for *P. falciparum* with or without *P. vivax*, and *P. vivax* only were 82 and 69, respectively. The detection limit of ELISA was 10 parasites/10^6 red blood cells (RBC) (0.001% parasitemia). Of 1,095 individuals involved in the study at the beginning of the study, ELISA showed sensitivity, specificity, positive predictive value and negative predictive value of 78.1%, 94.9%, 72% and 98.1%, respectively. Nine of 18 (50%) TBF-positive but ELISA-positive individuals had parasitemia of less than 10 parasites/10^6 RBC. High and low incidence areas did not affect the validity of our result. Regression analysis showed good correlation between log parasitemia and ELISA percent OD increase ($Y = 0 + 64.9*\log X$, $r = 0.65$), and agreement between TBF and ELISA results was 95.9%. In a fortnightly follow-up, in 82 TBF-positive individuals, both ELISA and TBF positive rates correlatively declined with agreement of 96.3%. With samples taken on the first day of the study, the TBF and QBC results were also correlated with agreement of 95.8% for *P. falciparum*, 95.6% for *P. vivax*. During 8 week follow-up involving altogether 191 samples, agreement between TBF and QBC results were 87.4% for *P. falciparum*. QBC detected more cases with *P. falciparum* infections but detected smaller number of cases with *P. vivax* infections.

INTRODUCTION

Malaria is still one of the most important parasitic diseases in the tropics with an estimated incidence of 300-500 million clinical cases each year, 90% of whom are from countries in tropical Africa (WHO, 1993). Among four species of human plasmodia, *P. falciparum* is responsible for most severe, often fatal forms of the disease. The estimated malaria mortality varies from 1.5-3 million deaths worldwide per year, mostly in Africa (WHO, 1993). Resistance of *P. falciparum* to drugs has probably become the most important threat to effective control of the disease. Asymptomatic cases apear to play a major role in the persistence of malaria in endemic foci and are difficult to eradicate. In the malaria control program of Thailand, Giemsa-stained thick blood film examination (TBF) is still the best technique now available, despite its inherent limitations. TBF examination, even by an expert microscopist, is time-consuming and labor-intensive, especially when the parasites are infrequent in the blood or absent at the time of testing. In cases with mixed infections of *P. vivax* and *P. falciparum*, it is difficult to make a correct diagnosis if *P. vivax* is present only in ring stage or the percentage of *P. falciparum*-infected red blood cells is very small. As a consequence, there is a need to develop alternative methods to diagnose malaria cases, especially those with low grade parasitemia, in order to supplement and perhaps in certain situations to replace TBF. Two approaches included in this study were a monoclonal antibody-based two-site sandwich ELISA and a quantitative buffy coat analysis (QBC). QBC has been extensively tested in the field with a claim to be as good as or even better than TBF (Spielman

et al, 1988; Pornsilapatip *et al,* 1990; Wongsrichanalai *et al,* 1991). *P. falciparum*-specific monoclonal antibodies (MAbs) have been used in several recently developed immunological assays for the detection of *P. falciparum* antigens by immunoradiometric assay (IRMA) (Khusmith *et al,* 1987, 1988), and ELISA (Taylor and Voller, 1993; Namsiripongpun *et al,* 1993; Lim *et al,* 1992). In this study, we developed another version of ELISA by using a recently produced MAb to detect malarial parasite antigens in people living in a malaria endemic area in Western Thailand.

MATERIALS AND METHODS

Test samples: Blood was taken by cluster random sampling in November 1992 from 1,095 people living in two malaria endemic areas at Tha Ka Noon and Huaya Ka Yeng villages, Thong Pha Phum District, Kanchanaburi, Western Thailand. The slide positive rates in 1991 and 1992 for *P. falciparum* were 5.42% (104/1,916), and 6.14% (211/1,596), respectively for Tha Ka Noon, and 2.37% (38/1,606), and 1.66% (33/1,998), respectively for Huay Ka Yeng. Because of variabilities of the malaria infection rates even in the same villages, the blood collected was regrouped for areas with high and low incidence based on the annual parasite incidence of higher and lower than 8/1,000 involving 611 and 484 individuals, respectively. The blood was subjected to TBF, ELISA and QBC examinations and those positive for *P. falciparum* were subsequently followed fortnightly for a periods of eight weeks.

Thick blood film examination: Each thick blood smear was made and read independently at ×700 magnification by at least two experienced microscopists. Parasitemia was quantitatively determined as described by Rickman *et al* (1089). Briefly, 10 µl of blood was spread evenly over a 10 × 20 mm etched area of glass slide, the volume of blood per field was calculated. With the diameter of an oil immersion field of 186 µm, an area of an oil immersion field is 0.027 mm^2, giving a total area for 200 oil immersion fields of 5.434 mm^2, which is equivalent to 0.072 µl of blood and thus make possible determination of the number of parasites per µl of blood.

Two-site sandwich ELISA

Collection of blood specimens: The blood was processed according to the method of Khusmith *et al* (1988). Briefly, blood-filled capillary tubes were centrifuged in a hematocrit centrifuge, cut at the plasma-cell interface, the pack cell portions were sealed with parafilm, and kept at 4°C for no more than 3 days during transportation to the laboratory in Bangkok to be stored at -70°C until used. The packed cells were flushed with nine volumes of borate buffered saline (0.035M disodium tetraborate, 0.1 M boric acid, 0.075 NaCl, pH 8.6) containing 0.5% Nonidet P-40 (BBS-NP40). The samples were tested at a concentration of 10^6 lysed red blood cells (RBC)/µl. This was accomplished by first counting the parasite by TBF in 200 microscopic fields and then converted to parasites/10^6 RBC according to the formula [Y = 0.907657 + (0.098213*X) when Y = No. of RBC (×10^6)/µl and X = hematocrit value (%)] as previously described (Khusmith *et al,* 1988).

Preparation of standard antigen: The SO strain of *P. falciparum* was used (Tharavanij *et al,* 1982). The parasites were grown in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) with 10% heat inactivated AB serum according to the technique of Trager and Jensen (1976). Parasitized RBC with 6.75% parasitemia of which 80% were ring forms were washed three times with phosphate buffered saline pH 7.2 (PBS) by centrifugation at 500 *g* for 10 minutes at 4°C. One volume of packed infected RBC was mixed with nine volumes of BBS-NP40 and kept in small aliquots at -70°C until used. The parasite extract was thawed and subjected to a serial five-fold dilutions in a diluent made by mixing just before use one volume of lysed human RBC from a healthy control with 9 volumes of BBS-NP40 as to give an initial concentration of 10^6 RBC/µl so that the initial parasite concentration was 67,500/10^6 RBC.

Preparation of human polyclonal anti-*P. falciparum* antibody (PAb): A serum chosen for the study was initially screened from 10 patients who were convalescent from falciparum malaria, and had histories of multiple malaria attacks in the past five years. A chosen serum had an indirect fluorescent antibody (IFA) titer of 1:5,120 as determined by a technique previously described (Tharavanij *et al,* 1982). The serum was inactivated at 56°C for 30 minutes and anti-RBC antibodies were absorbed

three times by mixing 1 ml of the serum with 4 ml of packed group AB erythrocytes, incubated at 37°C for 30 minutes and at 4°C overnight, followed by centrifugation at 500 *g* for 10 minutes at 4°C.

Monoclonal antibody (MAb): A 3H5 MAb of IgG2b subclass recognizing proteins of 81, 52 and 38 kDa of *P. falciparum* was used. The MAb was raised according to the technique of Galfre *et al* (1977). Briefly, a BALB/c mouse was immunized with three intraperitoneal injections of 500 µl of the SO strain of *P. falciparum* containing 10^8 parasites, mostly in ring stages enriched according to the method of Kutner *et al* (1985) with Freund's complete adjuvant initially and incomplete adjuvant subsequently at an interval of two weeks with an intravenous booster three days prior to fusion. The spleen cells were fused with a X63Ag8.653 cell line, kindly provided by Dr Ananda Nisalak, Armed Forces Research Institute of Medical Science, Bangkok, and cultured in a HAT medium (RPMI-1640 containing 20% fetal bovine serum, 15 mM HEPES, 10^{-2} mM hypoxanthine, 4×10^{4} aminopterin, 1.6×10^{2} mM thymidine, 100 units/ml penicillin, 100 µg/ml of streptomycin, 0.36% glucose, 1.14 mM oxalacetic acid, 0.45 mM sodium pyruvate and 0.2 units/ml of bovine insulin) in a CO_2 incubator. The secreted antibodies were screened by IFA at 7-10 days after fusion. Cell populations positive for *P. falciparum* antibodies were expanded and cloned in soft agar as described by Harlow and Lane, 1988. A large quantity of MAb was prepared as ascites fluid in pristane-prime BALB/c mice as described by Harlow and Lane, 1988.

Preparation of IgG: Human anti-*P. falciparum* sera were first subjected to centrifugation at 500 *g* for 10 minutes at room temperature. The IgG fraction from the supernatant was prepared by protein A agarose (Boehringer Mannheim, Germany) chromatography as described by Harlow and Lane (1988). The MAb IgG from ascites fluid was likewise prepared. The protein content of the IgG fractions was determined by Lowry's method (1951). The IgG-enriched fraction was aliquoted and kept at -20°C until used.

Two-site sandwich ELISA: The assay was done in duplicate. A 96-well flat bottom micro-ELISA plate (Immulon II, Dynatech Laboratories, USA) was coated with 150 µl of 3 µg/ml MAb followed by incubation at 37°C for 3 hours and at 4°C for another 8 hours, washed 5 times with PBS containing 0.05% Tween 20 and 1% non-fat dried milk (PBS-T-1% M) and the non-reactive sites saturated with 200 µl of PBS-T-5% M for 30 minutes at room temperature. The plates were thoroughly washed with PBS-T-1% M to which 100 µl of RBC lysate to be tested or standard antigen in various dilutions was added to each well, followed by incubation at room temperature for 3 hours. After washing, 100 µl of 12.5 µg/ml human anti-malarial PAb in PBS-T-1% M was added and the plates were incubated for another hour at room temperature. After washing, 100 µl of appropriate dilution of alkaline phosphatase-labeled anti-human IgG (H + L) (Kirkegaard and Perry Laboratory, Gaithersburg, USA) was added, and the plates were incubated at 4°C for 8 hours. After washing for five times with PBS-T-1% M, 100 µl of p-nitrophenyl phosphate (PNPP) (Sigma 104) solution was added and the plate kept in the dark at room temperature for 1 hour. The time needed to complete the test was 26.5 hours. Thereafter the reaction was stopped with 50 µl of 3N NaOH, and the OD measured at 405 nm by an ELISA reader (Titertek Multiskan, MCC/340, Flow Laboratories). Lysates of normal RBC and *P. falciparum* infected RBC were used as negative and positive controls, respectively. The result was expressed as percent OD increase calculated according to the following formula:

$$\text{Percent OD increase} = \frac{\text{OD of test sample-OD of negative reference}}{\text{OD of negative reference}} \times 100$$

Quantitative buffy coat analysis (QBC)

A commercially available QBC assay kit (Becton Dickinson Company, Franklin Lakes, NJ) was used and performed according to the manufacturer's instruction. Blood was drawn from each person into the anticoagulant end of the tube, which was slowly tilted and rotated between fingers to dissolve the pre-coated acridine orange, firmly affixed with a cap and the plastic float inserted. Specimens were transported within three hours of collection to the laboratory at a malaria clinic of malaria sector 9 Thong Pha Phum, situated about 20 km from the study sites. The average ambient temperature was 30°C. On arrival, they were centrifuged promptly at 14,000 *g* for 5 minutes and kept upright at room temperature (≈ 30°C). Each specimen was examined independently by the well-trained technicians within 8 hours after centrifugation with a Nikon model SE plan 50 type 102 microscope fitted with a 50x oil immersion lens and a 10x ocular lens.

A maximum of 100 microscopic fields were scanned and the parasite densities graded as 1 +, 2 +, 3 + or 4 + for < 1, 1-2, 3-5 and > 5 parasites/field.

Quality control

Each of the three assays was examined independently by well-trained technicians who were unaware of each other's result. TBF and QBC examinations were done on the same day by rotating technicians. TBF was checked in 200 microscopic fields (0.272 µl blood). QBC was examined for a maximum of 200 fields and ELISA was assayed in duplicate for every blood specimen.

Statistical analysis

Chi-square and Fisher's exact tests were used to determine association of two variables *eg* TBF and ELISA, TBF and QBC, and a regression analysis [Y = a + b*log(x)] was used to determine correlation between percentage OD increase and parasitemia. All calculation was facilitated by a TRUE EPISTAT computer software (Epistat Service, Richardson, Taxas, USA). Sensitivity, specificity, positive and negative predictive values were calculated as described by Griner (1981).

RESULTS

Reproducibility of ELISA

Based on an arithmetic mean plus three standard deviations in 74 healthy controls, an OD increase of 12 [0.779 + (3 × 3.7458)] was used as a cutoff. The assay performed against a reference standard showed high level of reproducibility (Fig 1, panel A) with coefficients of variation in 19 runs of 2.11% and 12.9% at parasite concentrations of 62,500 and 12,500/10^6, respectively.

The detection limit, sensitivity, specificity, positive and negative predictive values of the ELISA

The detection limit of the assay was 10 parasites/10^6 RBC (Fig 1, Panel B). Of 1,095 people examined by TBF in November 1992, 82 were positive for *P. falciparum* and 69 for *P. vivax,* respectively. Among

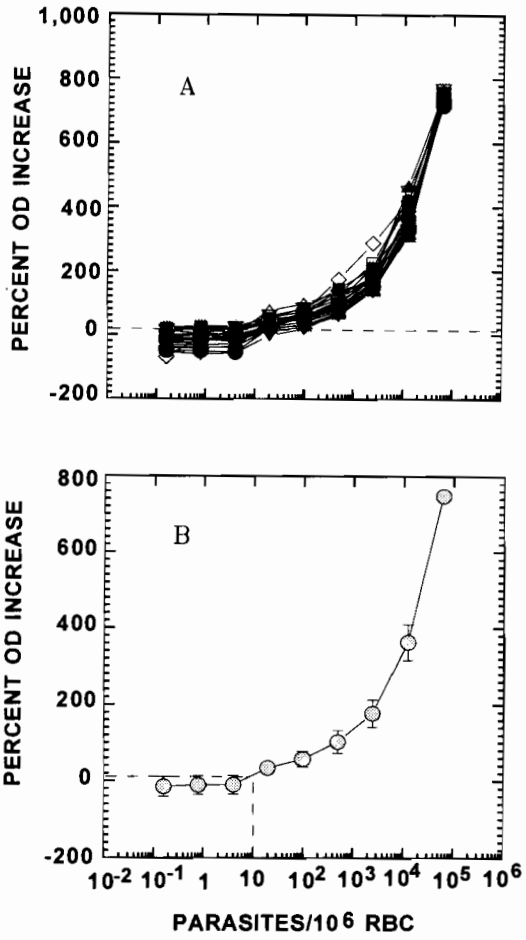

Fig 1–Reproducibility of the assay in 19 experiments (A) and the mean ± SD (B) showing a good correlation between mean percent OD increase and the parasite count with a detection limit of 10 parasites/10^6 RBC.

82 TBF positive individuals for *P. falciparum,* 64 were positive by ELISA thus giving a test sensitivity of 78.1% (Fig 2). Nine of 18 (50.0%) TBF positive but ELISA negative individuals had parasitemias of less than 10 parasites/10^6 RBC. In 74 healthy controls, two were marginally positive with a percent OD increase of 17.6% and 13.0%, giving specificity of 97.3%. Positive and negative predictive values were 97.0% and 80.0%, respectively. Twenty seven of 69 (39.1%) *vivax* cases were also positive.

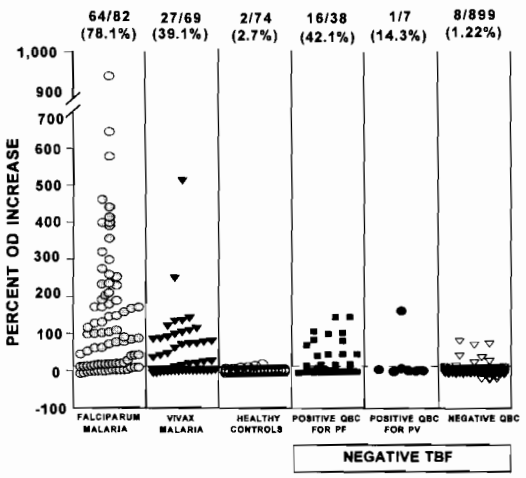

Fig 2–Field testing of MAb-ELISA in individuals in endemic areas and healthy controls.

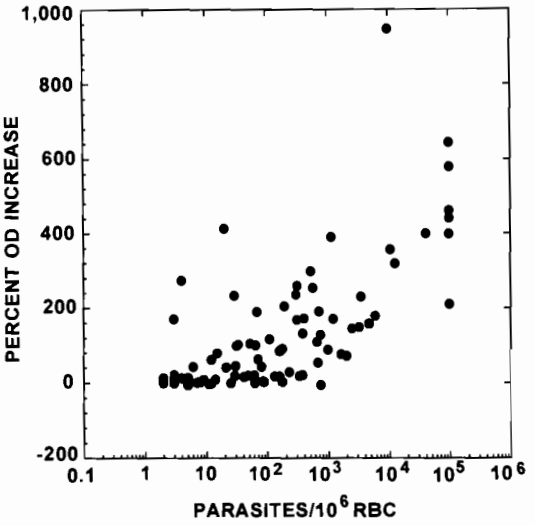

Fig 3–Regresson analysis of the percent OD increase by ELISA and parasite counts in 82 field collected samples with TBF positive for *P. falciparum* on day 0. Log parasite count is correlated with the percent OD increase (Y = 0 + 64.9*logX; r = 0.65).

Application in high and low incidence areas

TBF positive rate for *P. falciparum* of 12.8% (72/562) in the high incidence area was significantly different from 2.2% (10/464) in the low incidence area (chi-square = 37.8, $p < 0.000001$). Likewise, ELISA positive rates in the high incidence area of 13.0% (73/562) was significantly different from 3.5% (16/464) in the low incidence area (chi-square = 28.0, $p < 0.000001$).

Correlation between parasitemia and ELISA

Regression analysis of the percent OD increase by ELISA and the log parasite count from 96 TBF positive samples (82 samples on day 0 and 14 samples during the follow-up) showed a positive Pearson linear corrlation (Y = 0 + 64.9*logX; r = 0.65), and the regression account for 63.3% of variation (Fig 3). Agreement between microscopic examination and ELISA was 95.9% (Table 1A). When all 96 TBF-positive samples were stratified according to parasitemia into 4 groups of 1-9, 10-99, 100-999 and ≥ 1,000, it was clearly shown that the higher the parasitemia the higher the ELISA positive rates (Fig 4).

Follow-up after treatment

Follow-up in 82 TBF positive individuals showed a decline in parasitemia as well as a decline in ELISA positivity on weeks 2, 4, 6 and 8 after treatment (Fig 5) with TBF and ELISA positive rates of 13.3% and 8.9%, respectively on week 2; 6.1% for both assays on week 4; 8.9% and 11.1%, respectively on week 6; and 5.8% and 7.7%, respectively on week 8. TBF and ELISA positivities showed agreement of 96.3% (Table 1B).

Table 1A

Agreement between TBF for *P. falciparum* and MAb-ELISA in samples collected on day 0.

	TBF +	TBF -	Total
ELISA +	64	25	89
ELISA -	18	919	937
Total	82	944	1,026

Agreement = 95.9%

Correlation between TBF and QBC positivities

QBC and TBF positivities showed 96.1% agreement in 1,095 samples on day 0 (Table 2A), of which

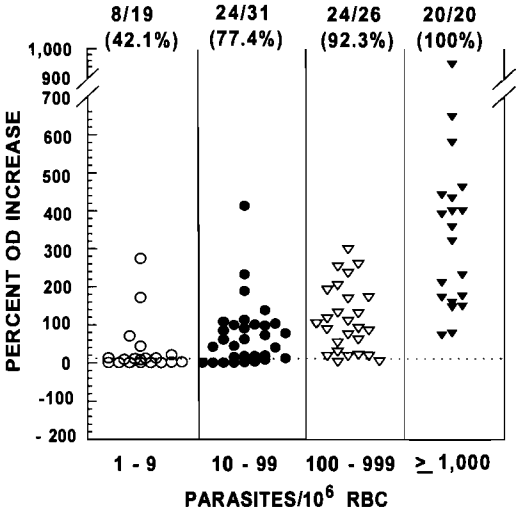

Fig 4–Correlation between parasite count and the ELISA OD increase in 96 TBF positive samples collected during the 8 week period. It is clearly seen that the higher the parasitemia the higher the ELISA OD increase.

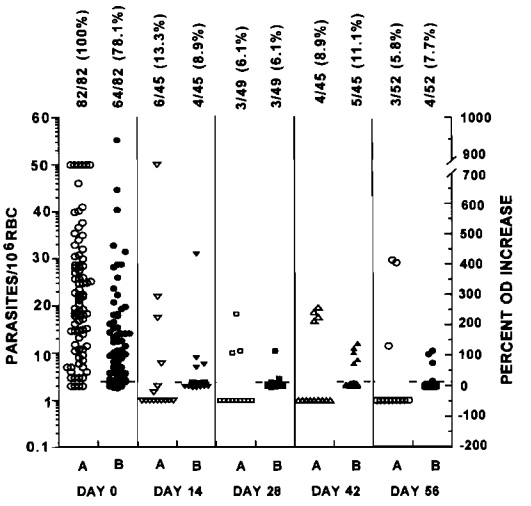

Fig 5–Thick blood film (TBF) positivity (column A) and ELISA positivity (column B) in field collected blood samples from 82 individuals positive on day 0 for *P. falciparum*. There was a decline in both TBF and ELISA positive rates. Cases positive by TBF were often positive by ELISA.

115 were positive by QBC and only 82 by TBF. Similar finding was obtained with 191 follow-up samples in which 87.4% agreement between QBC and TBF positivities was found with a higher

Table 1B

Agreement between TBF for *P. falciparum* and MAb-ELISA in samples collected on weeks 2-8.

	TBF +	TBF -	Total
ELISA +	13	4	17
ELISA -	3	171	174
Total	16	175	191

Agreement = 96.3%

number of QBC positive cases (34) than that of TBF positive cases (16), (Table 2B). In 82 TBF positive individuals with parasitemia of 1-9, 10-99, 100-999 and > 1,000/10^6 RBC, QBC positive rates were 88.2% (15/17), 100% (23/23), 100% (23/23) and 100% (19/19), respectively. Parasitemia in two QBC negative individuals was 3 and 4 per 10^6 RBC, respectively.

Table 2A

Agreement between TBF for *P. falciparum* and QBC in samples collected on weeks day 0.

	TBF +	TBF -	Total
QBC +	77	38	115
QBC -	5	906	911
Total	82	944	1,026

Agreement = 95.9%

Table 2B

Agreement between TBF for *P. falciparum* and QBC in samples collected on weeks day 2-8.

	TBF +	TBF -	Total
QBC +	13	21	34
QBC -	3	154	157
Total	16	175	191

Agreement = 87.4%

In contrast to *P. falciparum*, the number of QBC positive samples on day 0 for *P. vivax* (35) was less than that of TBF (69), despite high degree of agreement (95.6%). In follow-up of 191 individuals, two were positive by QBC and none by TBF.

DISCUSSION

In the present study, the detection limit by ELISA for *P. falciparum* was 10 parasites/10^6 RBC. Giving an average RBC count in malaria patients of 4×10^6/μl, the detection limit of our assay was 40 parasites/μl of blood. This level of sensitivity is still 4 times less than that of TBF which has been claimed to detect by good microscopists less than 10 parasites/μl (WHO 1988). In the present field study, as few as 2 parasites/10^6 RBC or 8 parasites/μl were observed. Nevertheless, this level of sensitivity by TBF is rarely encountered. In typical field conditions in areas of unstable malaria, the detection limit by TBF is 100 parasites/μl. By comparison with conventional practice of TBF examination in which only 100 microscopic fields were examined in about 5 minutes (unpublished observation from the Malaria Division, Thailand), our assay would be 2.5 times more sensitive than TBF. In comparison with published reports on parasite detection by DNA hybridization or antigen detection by ELISA (Tharavanij 1990; Taylor *et al*, 1993; Namsiripongpun *et al*, 1993), their detection limits were in a similar order as our ELISA. In term of sensitivity, our assay was only 78.1% sensitive. This apparently low level of sensitivity could be due to two factors: 1) the number of parasites was less than the detection limit of 10 parasites/10^6 RBC which was found in 50% (9/18) of TBF-positive but ELISA-negative individuals. 2) our MAb did not react with gametocytes by IFA. One of 18 ELISA-negative individuals was positive only for gametocytes (62/10^6 RBC).

Our assay was not specific for *P. falciparum*, since 39% of 69 *vivax* malaria cases were positive. These observed false positive reactions could be due to two possibilities: 1) our MAb crossreacted with some epitopes in *P. vivax* as detected by ELISA and not by IFA and 2). Some *P. vivax* samples were mixed with *P. falciparum* and could not be distinguished by TBF examination alone because of the difficulty in differentiating between ring stages of these two malarial species by this technique. With PCR, it has been reported that *P. falciparum* could be detected in 4.8% (5/104) of microscopically diagnosed *P. vivax* infections (Brown *et al*, 1992).

In comparison with QBC, ELISA was less sensitive with sensitivity of 78.1% compared with 94% by QBC. This could be due to low parasitemia mentioned above. QBC was, on the other hand, negative in 5 of 82 TBF positive individuals, two of whom had parasitemia of only 3 and 4 parasites per 10^6 RBC, indicating that the QBC limit of detection was approximately 5 parasites/10^6 RBC, which was equivalent to 20 parasites/μl. Three other individuals had mixed infections with *P. falciparum* and *P. vivax*, with parasitemia of 29, 31 and 52 and were scored by QBC as *P. vivax* only. QBC was also positive in 4% (38/944) of TBF-negative samples collected on day 0. At present, it is difficult to assess in relation to TBF whether this number is true or false positive. Concomitant use of PCR could solve this problem. If this number is true positive, it follows that QBC has higher sensitivity of detection than TBF and that the whole concept of TBF-based gold standard could be changed by using QBC as a new standard.

As regards *P. vivax* infections, QBC showed lower sensitivity than TBF, since only 27 of 69 (39.1%) TBF-positive individuals were QBC-positive and only 7 of 944 TBF-negative individuals were QBC-positive. Failure to detect *P. vivax* in TBF-positive cases was most likely due to the fact that mature *P. vivax* trophozoites, schizonts and gametocytes with similar buoyant density as that of human leucocytes are segregated in the buffy coat, and are therefore difficult to be distinguished from leukocytes (Rickman *et al*, 1989).

The time taken to complete the ELISA was 26.5 hours. In practice, the MAb-plate could be prepared in advance, thus the time actually needed after applying blood sample would be 12.5 hours. The entire process could be further reduced by cutting short the time of reaction with alkaline phosphatase anti-human globulin to be less than 8 hours. Nevertheless, such duration is still too long to be of practical value for diagnosing acute malaria. With sensitivity of only 78.1%, our ELISA cannot substitute TBF made by well-trained microscopists in the malaria control program. In area with low but persistent transmission where thousands of slides have to be examined, our assay could be considered as a supplement to TBF on the ground that the assay could be automated and could

process a larger number of blood samples and provide unbiased diagnosis of malaria. It could also serve as a quality control for slide examination.

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