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A COMPARISON OF TRANSMISSION-BLOCKING ACTIVITY WITH REACTIVITY IN A *PLASMODIUM FALCIPARUM* 48/45-KD MOLECULE-SPECIFIC COMPETITION ENZYME-LINKED IMMUNOSORBENT ASSAY

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Abstract. Monoclonal antibodies (MAbs) 32F1 and 32F3 react with two independent epitopes of a protein doublet with molecular weights of 48 and 45 kilodaltons (kD) expressed on the surface of *Plasmodium falciparum* (Pfs48/45) macrogametes and zygotes; only 32F3 blocks transmission. These MAbs were used to develop a Pfs48/45-specific competition enzyme-linked immunosorbent assay (ELISA) using 32F1 to capture antigen and labeled 32F3 for quantification and analysis of the contribution of antibodies in human serum to transmission-blocking activity. A comparison analysis was used to determine agreement of competition ELISA titers and transmission-blocking activity as observed in the bioassay in three groups of serum samples: 37 from European travelers with previous exposure to malaria, 56 from gametocyte carriers, and 66 from schoolchildren from a malaria-endemic area in Cameroon. The index of agreement between outcomes of the ELISA and transmission-blocking assay in gametocyte carriers and in travelers was specifically defined as fair-to-moderate; in schoolchildren the agreement was not significant. The combined analysis of all sera showed a significant and fair-to-moderate agreement between the results of the competition ELISA and the transmission-blocking assay, with a relative specificity of 94% (of 105 cases negative in the transmission-blocking assay, 99 were also negative in the competition ELISA) and a relative sensitivity of 44% (of 54 cases positive in the transmission-blocking assay, 24 were also positive in the competition ELISA). This study shows that a positive C48/45-ELISA is indicative for transmission-blocking activity in the mosquito assay, while a negative result does not exclude transmission-blocking activity.

Gametocytes of *Plasmodium falciparum* synthesize 230- and 48/45-kilodalton (kD) molecules that remain exposed on the surface of macrogametes and zygotes.^{1–3} Several monoclonal antibodies (MAbs) directed against these molecules block transmission of the parasite to the mosquito vector. One of the targets of transmission-blocking MAbs is the 48/45-kD glycoprotein doublet (Pfs48/45). Two independent, nonrepetitive epitopes of this protein react respectively with MAbs designated 32F1 and 32F3. The presence of 32F3 in a mosquito blood meal blocks transmission of the NF54 isolate of *P. falciparum* while 32F1 does not.⁴ The conventional assay measuring transmission reduction in mosquitoes using a feeder system, as described by Vermeulen and others,¹ is costly, labor intensive, and thus limits the number of sera that can be tested. Development of a serologic test predicting transmission-blocking activity would greatly simplify epidemiologic studies. A positive correlation between antibody reactivity to Pfs230 and transmission-blocking activity has been found using immunoprecipitation to quantify the antibody response. Such a relation was not found for antibody reactivity to Pfs48/45.⁵

This paper describes the development of a two-site competition enzyme-linked immunosorbent assay (ELISA) based on the 32F3 epitope. This competition ELISA was used to analyze several sets of field sera. In addition, the transmission-blocking activity of these field sera was determined using the feeder assay¹ and the results obtained were compared with those of the competition ELISA using a statistical analysis for comparison of methods.^{6,7}

MATERIALS AND METHODS

Parasites and Pfs48/45 extract. Mature gametocytes of *P. falciparum* (isolate NF54) were produced in an automat-

ed, large-scale, culture system as described by Ponnudurai and others.⁸ Gametogenesis was induced by incubating the mature gametocytes in fetal calf serum (FCS) at room temperature for 30 min. Macrogametes and zygotes were purified as described by Vermeulen and others.¹ Pelleted parasites were stored at -70°C until use. For use in sandwich competition ELISA experiments, Pfs48/45 was extracted in 25 mM Tris-HCl, pH 8.0, containing 0.5% Nonidet P40 (NP40), 1 mM phenylmethylsulfonyl fluoride, and 1 $\mu\text{g}/\text{ml}$ each of DNase and RNase. Insoluble debris was pelleted by centrifugation ($16,000 \times g$ for 5 min at room temperature) and the soluble protein extract was stored at -70°C . The presence and quantity of Pfs48/45 in the extract was determined in a sandwich ELISA using labeled 32F3 and 32F1.

Transmission-blocking assay. A bioassay with membrane feeders to feed mosquitoes was used to determine transmission-blocking activity.^{1,9,10} Briefly, while keeping the temperature at 37°C , 14-day-old cultures containing fertile gametocytes were mixed with prewarmed uninfected red blood cells and samples of human sera or MAbs, introduced in prewarmed feeders, and fed to *Anopheles gambiae* mosquitoes. All samples were tested in two experiments in the presence of complement. The minimal obtainable dilution of the sera was three-fold and the IgG preparations of blocking sera were retested using a 10-fold dilution with the malaria-negative control serum. Fully engorged mosquitoes were separated and held at 26°C . Seven days later, 20 mosquitoes per feeder were dissected and the number of oocysts on the stomach wall was counted and William's mean (adjusted geometric mean) oocysts number was calculated.¹¹ An experiment was deemed to have succeeded when at least 90% of the mosquitoes examined carried oocysts in each of three controls. The adjusted geometric mean was used to determine the reduction activity (R) according to the equation: R

$= (T_c - T_i)/T_c \times 100\%$, where T_i is the geometric mean for the test feeder and T_c is the mean of the three controls. If the transmission-reduction value of a given serum was less than 85%, a considerable variation could be observed among independent repeated tests. Values greater than 85% were consistent in subsequent experiments and only those were considered as positive in the transmission-blocking assay in this paper. Reduction of transmission in the feeder assay was confirmed using the immunoglobulin fraction of these sera.

Antibodies. Serum samples were obtained from the following groups: 1) 56 gametocyte carriers (6–36 years of age) recruited at a dispensary in Yaounde, Cameroon,¹² 2) 37 European travelers (6–70 years of age) to malarious regions with antibody titers to *P. falciparum* asexual parasites determined by the indirect immunofluorescence assay, and 3) 66 school-children (8–12 years of age) from Edea, Cameroon.¹³ A panel of individual and pooled sera from Dutch blood bank donors without previous malaria experience was used as negative controls. As a positive control in every test, the serum from one individual who had worked in a malaria area in Tanzania for more than 30 years was included. Complement was acquired from freshly obtained AB blood from Dutch blood bank donors, without previous malaria experience, by allowing the blood to clot at room temperature for 1 hr. Aliquots of the resulting serum were stored at -70°C until used.

Sandwich competition ELISA (C48/45-ELISA). Biotin labeling of MAb 32F3 was performed according to the procedure described by Verhave and others,¹⁴ and horseradish peroxidase (HRPO) labeling following the periodate method using an input HRPO:IgG molar ratio of four was carried out according to the method described by Wilson and Nakane.¹⁵ The labeled MAbs were dialyzed against phosphate-buffered saline (PBS), supplemented with thimerosal (0.01%) and FCS (1%) and stored at 4°C .

The basic procedure was that of the two-site ELISA. All incubations were carried out at room temperature. To capture Pfs48/45, wells of microtiter plates (Hycult, Uden, The Netherlands) were coated with 50 μl of a 10 $\mu\text{g}/\text{ml}$ concentration of 32F1 in PBS for 45 min. Plates were washed three times with PBS. Wells were saturated with 150 μl of 2% bovine serum albumin in PBS for 30 min. After three washes with PBS, the wells were incubated for 2 hr with 50 μl of the NP40 parasite extract (see above) containing the equivalent of 200,000 gametes/zygotes diluted in TNP (25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% NP40). Following three washes with PBS, wells were incubated with 30 μl of test serum sample and 30 μl of labeled 32F3 for 2 hr. For the biotin label, an additional incubation step with streptavidin-biotinylated peroxidase complex (1:3,000 diluted in PBS containing 0.05% Tween-20; Amersham Life Sciences, Hertogenbosch, The Netherlands) preceded the incubation with substrate solution. For both labels, the wells were washed four times with PBS and incubated for 20 min with 60 μl of TMB (0.25 mM 3,3',5,5'-tetramethyl benzidine, 0.7 mM H_2O_2 in 0.1 M sodium acetate, pH 5.5) substrate solution. The peroxidase substrate reaction was stopped by adding 60 μl of 2 M H_2SO_4 and the optical density (OD) was determined in an ELISA reader at 450 nm (Titertek Multiskan MCC/340; ICN, Zoetermeer, The Netherlands).

For optimal immunoreactions, labeled MAbs and antigen

TABLE 1

Guideline for the interpretation of the index of agreement kappa (K)

Value of K	Strength of agreement
≤ 0.20	Poor
0.21–0.40	Fair
0.41–0.60	Moderate
0.61–0.80	Good
0.81–1.00	Very good

were tested in a checkerboard titration. From this antigen-antibody titration, concentrations of labeled MAb and antigen were chosen resulting in $> 60\%$ of the maximal OD of labeled MAb and an OD reading > 1.0 .

Competition was defined as a reduction in the OD reading obtained with the MAb alone in the presence of a negative control serum. The true endpoint was defined as the dilution (titer) at which the positive sample reaches the OD value of the negative control. Sera with true endpoint $\geq 1/20$ were considered positive.

Comparison of the transmission-blocking assay and competition ELISA. The results of the competition ELISA were compared with those obtained by the transmission-blocking assay and analyzed by a statistical test on agreement using a calculated index of observed agreement.^{6,7} The index kappa (K) was calculated from the observed and expected (by chance) frequencies on the diagonal of the 2×2 table of transmission-blocking activity outcomes in the bioassay ($\geq 85\%$, $< 85\%$) and results of the competition ELISA (positive, negative). The index is given by $K = (P_o - P_c)/(1 - P_c)$, where

1) P_o is the observed proportional agreement, i.e., $P_o = \sum_{i=1}^n f_{ii}/n$, where f_{ii} is the number of agreement for category i ($i = 1, 2$) and n is the number of examined sera, and

2) P_c is the expected proportional agreement by chance, i.e., $P_c = \sum_{i=1}^2 r_i c_i / n^2$, where r_i and c_i are the row and column totals for the i^{th} category ($i = 1, 2$).

The approximate error of K is $SE(K) = \sqrt{\frac{P_o(1 - P_o)}{n(1 - P_c)^2}}$ and the approximate 95% confidence interval is given by $K \pm 1.96 SE(K)$.

The index of agreement K has a maximum of 1.00 when agreement is perfect; values of zero or less indicate no agreement better than that due to chance. While no absolute interpretation is possible, the guideline proposed by Altman⁶ was used to determine the strength of agreement (Table 1).

RESULTS

Testing of the labels and competition with serum antibodies. The optimal concentration of labeled MAb and the optimal amount of antigen coated per well as determined in the checkerboard titrations were 2 $\mu\text{g}/\text{ml}$ for the biotin label and 200,000 macrogamete/zygote equivalents/well, and 1 $\mu\text{g}/\text{ml}$ for the HRPO label and 100,000 macrogamete/zygote equivalents/well, respectively. The maximal OD of the

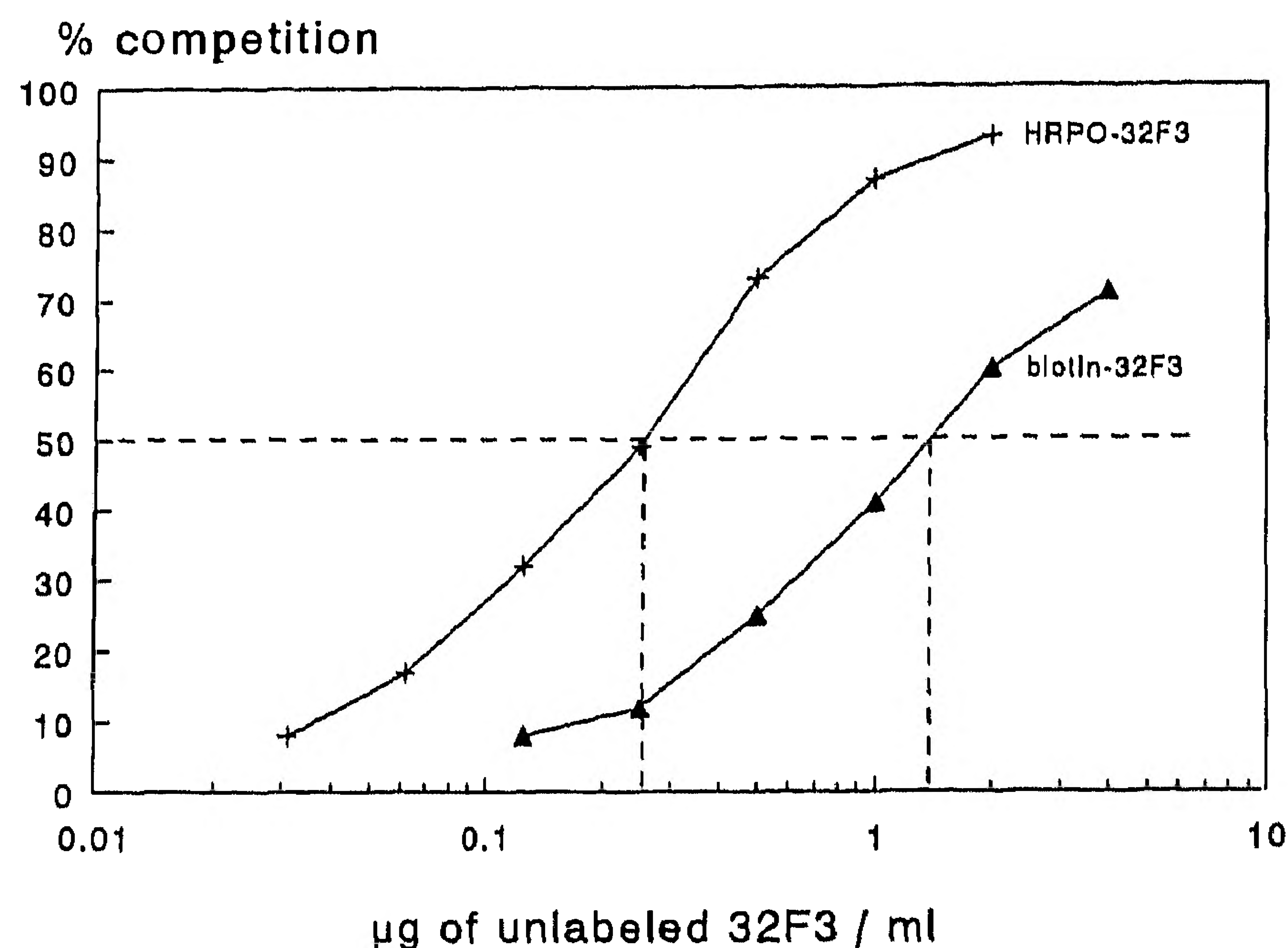


FIGURE 1. Two-site competition enzyme-linked immunosorbent assay. Monoclonal antibody (MAb) 32F1 was used to capture Pfs48/45 from an NP-40 extract. + = horseradish peroxidase (HRPO)-32F3 (1 $\mu\text{g}/\text{ml}$ with 100,000 macrogamete/zygote equivalents/well) and \blacktriangle = biotin-32F3 (2 $\mu\text{g}/\text{ml}$ with 200,000 macrogamete/zygote equivalents/well) were used as labels in competition with serial dilutions of unlabeled 32F3. The horizontal dotted line represents the 50% competition value and the vertical dotted lines represent the concentration of unlabeled MAb needed to arrive at a 50% competition with the labeled MAb.

HRPO-32F3 label was higher than that of the biotin-32F3 label using the same concentration of antigen. Competition of labeled MAb with increasing concentrations of unlabeled MAb is depicted in Figure 1. The 50% competition values of the corresponding unlabeled MAb were 0.25 $\mu\text{g}/\text{ml}$ for the 1.0 $\mu\text{g}/\text{ml}$ HRPO label and 1.3 $\mu\text{g}/\text{ml}$ for the 2.0 $\mu\text{g}/\text{ml}$ biotin label. In addition, it appeared that the biotin-32F3 competition ELISA was positive, with seven of 19 sera having positive transmission-blocking activity as observed in the bioassay, whereas the HRPO-32F3 competition was positive for nine of 19 sera. Due to the limited sensitivity of the biotin-32F3 competition ELISA, human sera could not be diluted more than 1:10, preventing a complete titration of the sera. The MAb 32F1 (anti-Pfs48/45) and MAbs against the 25-kD and 230-kD antigens were negative in the competition test up to a concentration of 330 $\mu\text{g}/\text{ml}$.

Figure 2 shows examples of the competition of serial di-

lutions of sera with the HRPO-32F3 label. Data of the negative serum are the mean of 14 individual sera tested on three separate occasions. Calculation of the overall intra-assay variation resulted in an average deviation of the mean OD of 0.08 (10%). The overall interassay variation resulted in an average deviation of the mean OD of 0.11 (14%). The titration values of three sera that were positive in the competition ELISA are depicted using the overall mean of duplicate determinations of two independent tests.

Control experiments with 14 sera from Dutch blood bank donors exhibited a nonspecific reduction up to a maximum of 15% of the OD of the HRPO-32F3 label compared with reactions without competing serum antibodies. This nonspecific reduction was not seen with IgG preparations of these sera. Nevertheless, the competition titer found for sera and their corresponding IgG preparations were the same.

Comparison of competition ELISA and transmission-

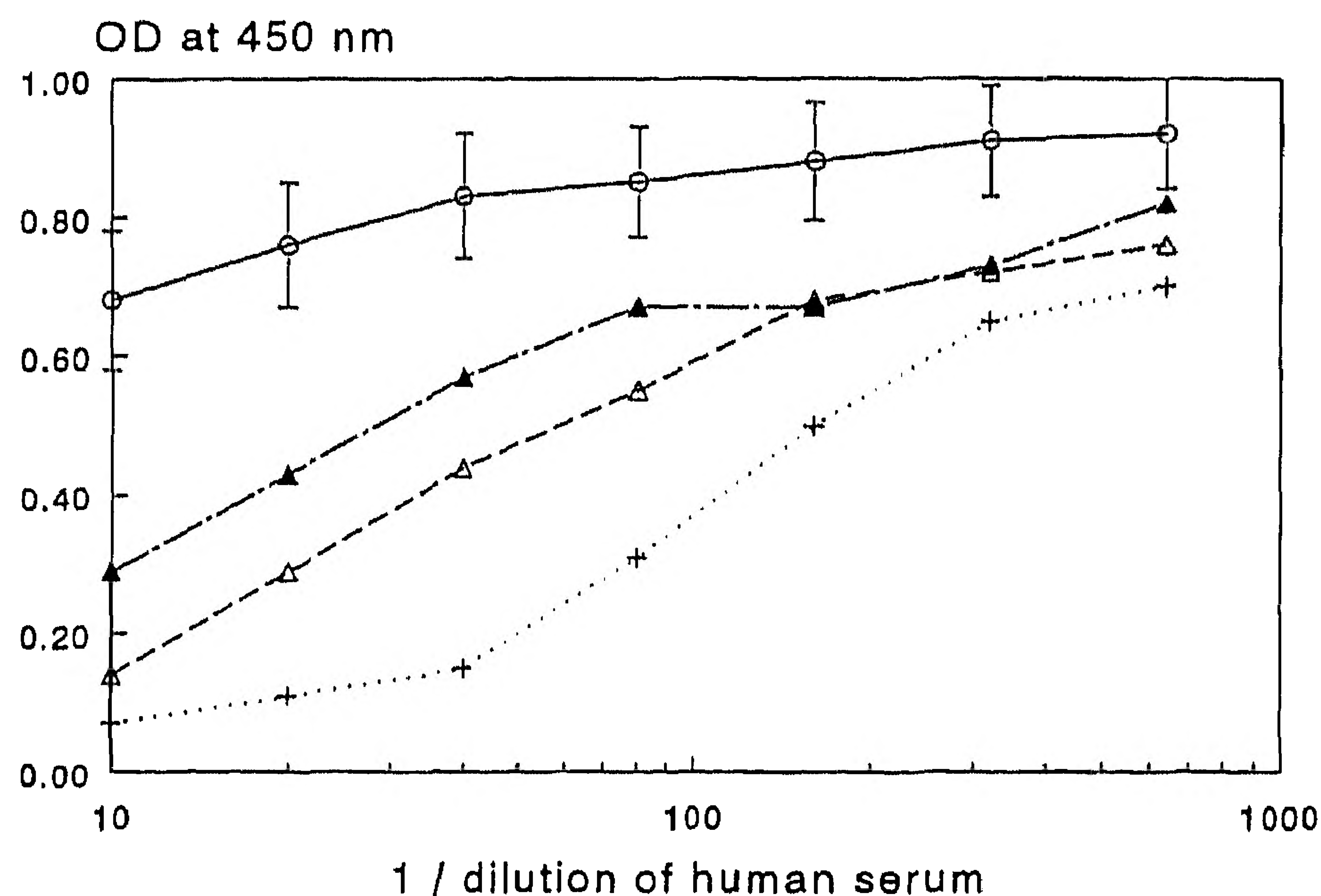


FIGURE 2. Inhibition of binding of horseradish peroxidase (HRPO)-32F3 in the presence of serial dilutions of human serum. Negative serum (\circ); each data point represents the mean \pm SD of 14 determinations performed on three different days. (\blacktriangle , \triangle , and $+$) = three sera positive in the competition enzyme-linked immunosorbent assay. Each data point represents the mean of duplicate determinations performed on two days. OD = optical density. For concentrations of antigen and HRPO-32F3, see Figure 1.

TABLE 2

Activity in the Pfs48/45 competition enzyme-linked immunosorbent assay (C48/45-ELISA) and in the transmission-blocking (TB) assay in sera of gametocyte carriers, travelers, and schoolchildren

TB assay	C48/45-ELISA results					
	56 gametocyte carriers		37 travellers		66 school children	
	No. positive	No. negative	No. positive	No. negative	No. positive	No. negative
≥85%	13	12	9	10	2	8
<85%	4	27	0	18	2	54
Total	17	39	9	28	4	62

blocking assay in several groups of sera. Table 2 shows the results of the competition ELISA and the transmission-blocking assay for the sera of gametocyte carriers, travelers, and schoolchildren. Using the HRPO-32F3 label, 17 (30%) of 56 of the gametocyte carriers exhibited significant competition in the ELISA. These results were compared with the corresponding transmission-blocking activity observed in the bioassay. By shifting the transmission-blocking assay cutoff point in the gametocyte carrier group in the range of 10% to 90%, it appeared that the maximum degree of agreement between the transmission-blocking assay and the C48/45-ELISA was at the 85% reduction point. Furthermore, it appeared that the agreement between the transmission-blocking assay and the C48/45-ELISA was not greater than chance if the cutoff point of the transmission-blocking assay was set at a reduction lower than 70% (95% confidence interval of K included the value 0). At the 85% reduction point, the index K assumed the value 0.40, indicating fair-to-moderate agreement between the outcomes of the competition ELISA and the transmission-blocking assay (Table 3). Four of the 17 positive sera from gametocyte carriers in the C48/45-ELISA test had oocyst counts that did not meet the 85% reduction standard. Of the 25 sera that were positive in the transmission-blocking assay, 13 had antibodies competing with the HRPO-32F3 MAb, indicating a relative sensitivity of 52%. Of 31 sera considered negative by the transmission-blocking assay, 27 were also found to be negative by the C48/45-ELISA, showing a relative specificity of 87% for the competition ELISA.

Nine (24%) of 37 travelers exhibited significant activity in the ELISA. Nine (47%) of 19 sera positive in the transmission-blocking assay had antibodies competing with the HRPO-32F3 MAb. All 18 sera considered negative by the transmission-blocking assay were also negative by the C48/45-ELISA. The index of agreement K between the transmission-blocking assay and the C48/45-ELISA for the group of travelers assumed a value of 0.47, indicating moderate agreement (Table 3).

TABLE 3

Comparison of activity by index of agreement K in the Pfs48/45 competition enzyme-linked immunosorbent assay (C48/45-ELISA) and the transmission-blocking assay in sera of gametocyte carriers, travelers, and schoolchildren

Group	n	Index of agreement K		
		K	95% confidence interval	Strength
Gametocyte carriers	56	0.40	0.15–0.65	Fair to moderate
Travelers	37	0.47	0.19–0.75	Fair to moderate
Schoolchildren	66	0.22	–0.23–0.67	Not significant

TABLE 4

Comparison of activity in the transmission-blocking (TB) assay and in the Pfs48/45 competition enzyme-linked immunosorbent assay (C48/45-ELISA) in all field sera combined*

TB assay	C48/45-ELISA results		
	No. positive	No. negative	Total
≥85%	24	30	54
<85%	6	99	105
Total	30	129	159

* Relative specificity = 94% (99 or 105); relative sensitivity = 44% (24 or 54); index of agreement K = 0.44.

Four (6%) of 66 sera of schoolchildren showed significant competition in the ELISA. Only 2 (20%) of 10 sera positive in the transmission-blocking assay were positive in the competition ELISA. The relative specificity was 96% (54 of 56). The index of agreement K between the results of the C48/45-ELISA and the transmission-blocking assay was low with a wide confidence interval (K = 0.22, 95% confidence interval = –0.23, 0.67). Since the confidence interval contains zero, chance can explain the observed level of agreement.

A summary of the data of the three groups combined is given in Table 4. The index of agreement K between the results of the C48/45-ELISA and the transmission-blocking assay was K = 0.44; with a 95% confidence interval of 0.28, 0.60, indicating fair-to-moderate agreement (Table 1). The ELISA outcomes show a relative specificity of 94% (99 of 105) and a relative sensitivity of 44% (24 of 54).

DISCUSSION

An MAb-based competition ELISA was developed to study the natural antibody reactivity to Pfs48/45 in field sera in comparison with transmission-blocking activity as observed in a bioassay. The 32F3-labeled MAb was used in this competition ELISA because of its blocking activity in the transmission-blocking assay. Competition of antibodies in field sera with labeled 32F3 for binding to Pfs48/45 might therefore be relevant for transmission-blocking activity. Transmission blockade has not been found in serum of rabbits immunized with recombinant Pfs48/45 (Roeffen W and others, unpublished data). Therefore, Pfs48/45 extract from parasites was used as antigen in the competition ELISA.

The concentration of unlabeled antibody necessary to reduce binding of the labeled MAb in the competition ELISA was lower for HRPO-labeled 32F3 than for biotin-labeled 32F3. This was supported by the observation that in the competition ELISA with HRPO-32F3 as label, nine of 19 blocking field sera were positive in contrast to seven of 19 when biotin-32F3 was used. Therefore, HRPO-labeled 32F3

was used in the competition ELISAs of subsequent experiments.

Transmission-blocking activity was found in a high proportion of sera from gametocyte carriers and travelers to malarious regions. In the group of travelers, all sera positive in the C48/45-ELISA blocked transmission, but 10 of 19 blockers were negative in the C48/45-ELISA. The Pfs48/45 epitope has several, surface-exposed domains recognized by MAbs.^{16,17} Since only one domain potentially involved in blocking activity could be analyzed in this study, reactivity to other domains might explain why 10 of 19 blockers were negative in the C48/45-ELISA. Since sera from people living in endemic areas react to all epitopes tested on the Pfs230,¹⁸ several alternative explanations may be considered. It may be that blocking reactivity is targeted to molecules other than that of Pfs48/45, or that blocking does not only depend on antibody, but may also be caused by nonspecific factors as observed in *P. vivax* and *P. falciparum* infections.¹⁹ In our analyses, it appeared, however, that IgG fractions of sera with transmission-blocking activity also blocked transmission. Although this does not exclude the presence of nonspecific transmission-blocking activity, specific activity can explain the results. It is also possible that the C48/45-ELISA is less sensitive than the transmission-blocking assay.

The results for the gametocyte carriers and schoolchildren were also problematic because four of 17 and two of four sera positive in the C48/45-ELISA, respectively, were in the nonblocking group (< 85%). It should be noted that the category $R < 85\%$ in the transmission-blocking assay does not necessarily mean that there is no transmission reduction. It only shows that repeated experiments with sera from this group exhibited considerable variation of the transmission-reduction value, making it impossible to subdivide the group with R values < 85% in different subclasses.

A statistical test for comparison of proportions or association is not considered appropriate for the comparison of the outcomes of the transmission-blocking assay and the C48/45-ELISA. Besides sensitivity and specificity, measurement of agreement rather than association is required. The kappa (K) measure, which measures the amount of agreement beyond chance, seems to fit our purpose. In two of three groups of sera, agreement was significant, (fair-to-moderate for gametocyte carriers and travelers) and only in the group of schoolchildren could agreement between the outcomes of the two methods be explained by chance. Nevertheless, overall a fair-to-moderate agreement was found. In addition, the C48/45-ELISA exhibited reasonable levels of sensitivity ($\geq 44\%$) and specificity ($\geq 94\%$).

In a Papua New Guinea study, an MAb (MAb IIC5B10) specifically reacting with an epitope of Pfs48/45 was used to compete with a panel of field sera in a Pfs48/45 competition ELISA. The results of this competition ELISA did not correlate with either the capacity of these field sera to precipitate Pfs48/45 from a radiolabeled gametocyte extract or the transmission-blocking activity observed in these same field sera.²⁰

On the other hand, the analyses of the Papua New Guinea sera revealed a correlation between serologic reactivity to Pfs230, but not to Pfs48/45, and transmission-blocking activity.⁵ Our competition ELISA is specific (94%), but only positive in 44% of the sera with transmission-blocking ac-

tivity. This leaves the possibility that the competition ELISA is insensitive or that the transmission-blocking activity in these sera is directed to other sexual stage molecules, e.g., Pfs230 as observed by Graves and others.⁵

In summary, the transmission-blocking bioassay is a costly, comparatively labor-intensive, and time-consuming test. A Pfs48/45 epitope-specific competition ELISA has a significant predictive value to the transmission-blocking activity, which is limited by the fact that only 44% of all transmission-blocking sera are positive in the competition ELISA. This indicates the need to increase the sensitivity of the test and/or to define additional transmission-blocking targets.

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