The Korean Journal of Parasitology
Vol. 34, No. 4, 239-246, December 1996

Glutamate dehydrogenase antigen detection in *Plasmodium falciparum* infections

Neira de DOMINGUEZ+ and Alexis RODRIGUEZ-ACOSTA*

Universidad Central de Venezuela Tropical Medicine Institute Immunochemistry Department Apartado 47423-Caracas 1041 Venezuela

Abstract: The usefulness of malaria diagnosis by *Plasmodium falciparum*-GDH (NADP+), obtained by affinity chromatography, is demonstrated in ELISA assays, testing IgG antibodies against GDH (NADP+) from patients with acute malaria, who have had two or more episodes of malaria, or from sera of hyperimmune patients. GDH (NADP+) thermal stability was demonstrated in a high heat resistance assay. The immunofluorescence assay demonstrated that anti-culture (*P. falciparum*) supernatant serum and anti-GDH (NADP+) of *Proteus* spp, recognized epitopes in Venezuelan isolates, and Colombian and Brasilian malarial strains. The antigen is soluble, with high specificity, is a potent immunogen and is thermoresistant.

Key words: antigenic enzymes, glutamate dehydrogenase, malaria diagnosis, *Plasmodium berghei, Plasmodium cathemerium, Plasmodium falciparum, Plasmodium vivax,* soluble antigens.

INTRODUCTION

The detection of malaria parasite antigens in the diagnosis of human malaria is an important aspect in the epidemiology of malaria. The development of simple and rapid diagnostic tests that differentiate among active infections, past infections, relapses and recrudescences, as well as measure the immunologic state of protection is a high priority.

The identification of specific malarial parasite enzymes could contribute to a simple technique for rapid malaria diagnosis. While the parasite presents a similar metabolism as the host, the characteristics of many enzymes produced by the malaria parasites are different, kinetical, electrophoretically, specificity of co-factors, substrates, degree of affinity and inmunogenicity (Sherman *et al.*, 1971; Roth *et al.*, 1986; Eckman, 1984). These differences observed in parasite enzymes could contribute to a sensitive, specific and economic diagnostic method based on their enzymatic activity or immunoreactivity. Glutamate dehydrogenase (GDH) (NADP+) was selected for study because many malarial parasites exhibit a NADP-specific GDH activity (Sherman *et al* ., 1971; Walter *et al* ., 1974), malarial parasites produce NADPH in combination with NADP-specific isocitrate dehydrogenase and glucose 6 phosphate dehydrogenase (Van der Jagt *et al* ., 1989). Because this activity is not found in host red blood cells, it is considered as an useful marker enzyme for malaria parasites, including *Plasmodium falciparum* (Heidrich *et al.*, 1982 ; Van der Jagt *et al* ., 1982).

The identification and characterization of the

• Received mar. 28 1996, accepted after revision Aug. 17 1996.

*Corresponding author
+FACULTAD DE FARMACIA-UCV

malaria antigens is essential for development of specific methodologies. Therefore, the central purpose of this study constitutes the identification of enzymatic parasite antigens, frequently associated with the infection of *Plasmodium falciparum*, that could be detected by immunologic methods, using hyperimmune malaria sera from patients or from previously immunized experimental animals.

MATERIALS AND METHODS

Immune sera

Human immune sera were obtained from Yanumana amerindians living in a malaria hyperendemic area, from patients that have suffered 1 or 2 episodes of malaria, and from patients with *Plasmodium vivax* malaria. Immune animal sera were obtained from mice with *Plasmodium berghei* malaria, from Pekinese ducks with *Plasmodium cathemerium* malaria, and from a rabbit polyclonal anti-GDH (NADP+) of *Proteus* spp.

Human sera also were obtained from *Plasmodium falciparum*-infected patients attending the malaria hospital at the Ciudad Bolivar Malariology Division (MSAS). A total of 50 malaria patients was recruited for the study. Corresponding control sera from uninfected individuals (non malaria endemic area) were also obtained.

Rabbit polyclonal antibody was obtained by immunizing New Zealand rabbits (approximately 2 kg) with GDH (NADP+) from *Proteus* spp. (Sigma, Mo, USA). Two-hundred fifty μg of protein in Freund's complete adjuvant was injected subcutaneously at 5 places on the back of the rabbit, followed by two booster injections, ten days from each other. Rabbits were bled a week after the last injection (Weir, 1978).

Parasite cultures and collection of culture supernatants

FCB2 (Colombia) and Brasilian S/D (FioCruz/Brazil) strains and isolates from *Plasmodium falciparum* infected patients (Bolivar State, Venezuela) were obtained. Parasites were cultured with human erythrocytes at 2% hematocrit in RPMI-1640 media and 10% human serum (Trager and Jensen, 1976). The media were changed every 24 hours, treated with a protease-inhibitor (0.15 mM TPCK, 200 mM PMSF, 0.2 mM EDTA in PBS) cocktail (1 μl/ml of supernatant) and frozen at –70°C until used.

(GDH [NADP+]) immunoaffinity separation

Blood plasma from patients having suffered one or more *Plasmodium falciparum* infections attacks were loaded on Sepharose-4B-rabbit-IgG-anti-GDH (NADP+) columns. The columns were washed with PBS until OD = 0. The fraction linked to Sepharose-4B-rabbit IgG anti-GDH (NADP+) was eluted with 0.1 M glycine HCl, pH 2.8 and concentrated (Fig. 1).

Thermal stability of the antigen (GDH [NADP+]) separated by affinity chromatography (Wilson *et al.*, 1969).

The fraction linked to the Sepharose-4B-rabbit-IgG-anti-GDH [NADP+] of *Proteus* spp, was incubated at temperatures of 60, 80 and 100°C and by periods of 2, 5, 10 and 20 minutes.

Sample percent Residual Antigenicity (%RA) was evaluated by ELISA assay. The % RA was calculated using the relationship:

$$\% \text{ RA} = \frac{A1}{A2} \times 100$$

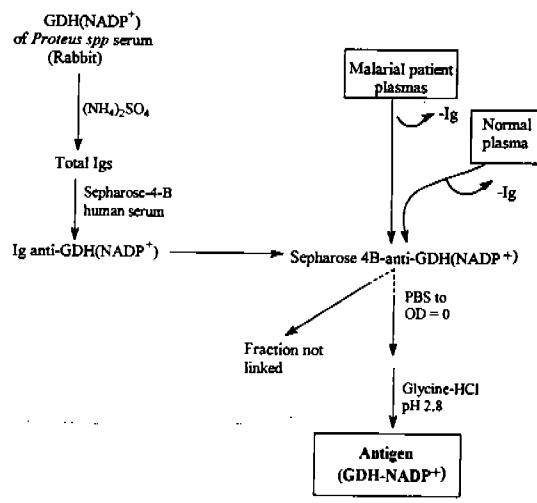


Fig. 1. GDH (NADP+) separation procedure from plasma of malaria patients by affinity chromatography.

% RA: percent of residual antigenicity.
A1: difference of OD_{450} between positive and negative sera, after treatment.
A2: difference of OD_{450} between positive and negative sera, before treatment.

Indirect fluorescence antibody test (IFAT)

Blood films prepared from infected red blood cells washed 6 × with PBS (pH 7.2), were fixed with cold 9:1 (v/v) acetone:methanol for 1 min, rinsed with PBS (pH 7.2) and then were incubated with: a) rabbit IgG anti-*Plasmodium falciparum*-culture-supernatant, B) rabbit anti-GDH (NADP+) *Proteus* spp., and c) IgG anti-human malaria (hyperimmune) (positive control). These were serially diluted (1:10 to 1:60) in PBS 1% Tween 80 with 10 µl of each dilution, aliquoted on each of the blood film preparations and incubated at 37°C for 1 hour in a humid chamber. After incubation, the slides were thoroughly washed in 0.01 M PBS (pH 7.2), incubated in 1:1200 dilution of fluorescein-conjugated anti-human-IgG or anti-rabbit-IgG for 1 hr at 37°C in a humid chamber, and then examined.

Enzyme linked immunosorbant assay (ELISA)

We followed the Engvall and Perlmann (1971) methodology, modified for microassay (Voller *et al.*, 1974) , using a direct and an indirect (immunoadsorption of the antigen) assay. Different concentrations of *Plasmodium falciparum* antigen fractions obtained by affinity chromatography were used. The direct tests were performed in 96 flat bottom well microtiter plates, incubated overnight at 4°C with the antigen. The blood plasmas were obtained from patients with acute *Plasmodium falciparum* malaria. The conjugated anti-human-IgG-peroxidase was used as a secondary antibody and the absorbance measured at 450 nm. The reaction was stopped at 30 minutes by adding 4N H_2SO_4. All samples were run with both positive and negatives controls.

Indirect tests were performed in 96 flat bottom well microtiter plates. The plates were incubated for 2 hours at 37°C with the rabbit polyclonal anti-GDH (NADP+) of *Proteus* spp., washed three times with a solution of 0.05% PBS-Tween, then incubated with the *Plasmodium falciparum* antigens diluted in carbonate buffer (pH 9.6), overnight at 4°C. The rest of the process was conducted as in the direct test.

Protein determination

The protein determination method followed that of Lowry *et al.* (1951).

Statistical methods

Statistical comparisons were made utilizing unpaired students t-test ($\alpha = 0.05$).

RESULTS

The antigenicity of the GDH (NADP+) by affinity chromatographic isolation and its probable usefulness in malaria diagnosis was demonstrated by ELISA, utilizing IgG antibodies against GDH (NADP+) in serum from acute malaria patients and malaria patients diagnosed with 2 or more episodes of malaria, and carried out with conjugated goat-anti-human-IgG-peroxidase. The mean OD of malaria patients was significantly higher than for healthy patients ($P \leq 0.01$) (Fig.2). However differences between the mean OD values between acute sera and sera from patients with 2 or more episodes (0.3714 ± 0.081 and 0.4039 ± 0.092) respectively were not

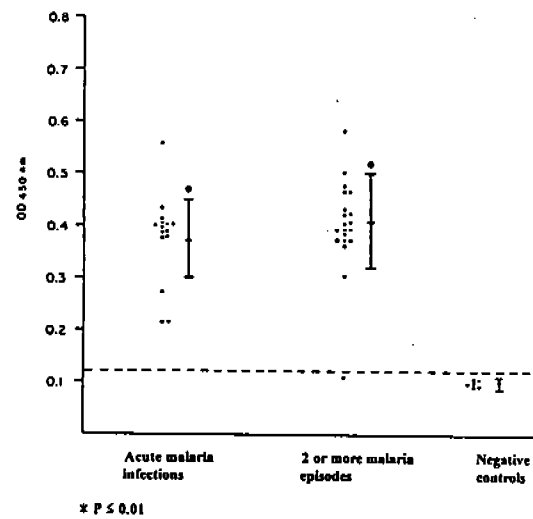


Fig. 2. Determination of IgG anti-GDH (NADP+) in plasma of malaria patients by direct ELISA.

Table 1. Percentages of residual antigenicity of heat treated GDH (NADP+)

Time (min.)	Residual antigenicity (%)[a] in temperature		
	60°C	80°C	100°C
0	100 ± 3.35	100 ± 3.35	100 ± 3.35
2	80 ± 6.05	91 ± 4.19	73 ± 3.22
5	84 ± 8.50	87 ± 5.40	77 ± 5.60
10	86 ± 9.00	81 ± 7.80	
20	84 ± 7.30	80 ± 4.50	

[a]Percentages were calculated as OD values obtained by ELISA (fraction not treated represents 100% of antigenicity). The values represent the X ± SD of 6 determinations.

Table 2. Mean values (X ± S.D) and sensitivity (%) of GDH (NADP+) obtained in two ELISA methods.

Controls	Direct Assay	Indirect Assay
	C[a]: 0.1116	C: 0.1346
Acute malaria infection	X: 0.3714 ± 0.081 S[b]: 100%	X: 0.4192 ± 0.077 S: 100%
2 or more malaria episodes	X: 0.4039 ± 0.092 S: 95%	X: 0.4626 ± 0.086 S: 95%

[a]C, cut off (X + 3 S.D); [b]S, sensitivity

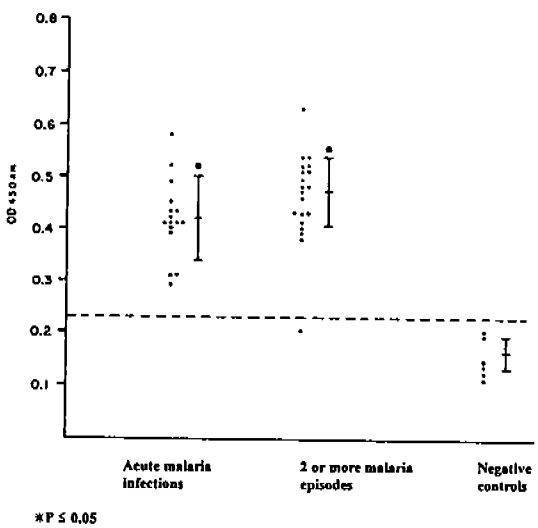


Fig. 3. Determination of anti-GDH (NADP+) IgG antibody in plasma of malaria patients by indirect ELISA

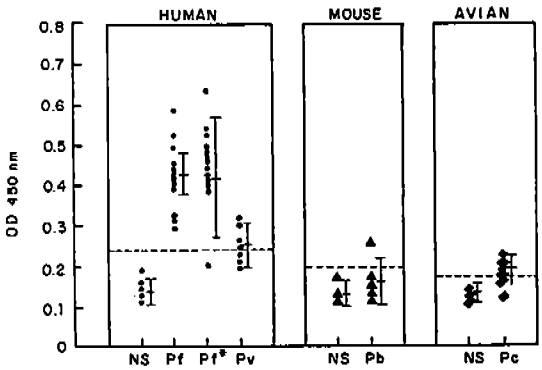
significantly different. The sensitivity for acute sera from patients was 100%, but was only 95% for the group of patients with two or more episodes of malaria.

In the indirect test (immunoadsorption of the antigen) for IgG anti-GDH (NADP+) detection, there was an observed increase in the OD cut off value (OD 0.2245). The experimental group mean values of infected and non-infected patients were significantly different ($P \leq 0.05$), but there were no observed differences between the two infected groups ($P \leq 0.2$) (Fig. 3). The sensitivity of this test was similar to the previous.

In Table 2 is shown a comparison of the results obtained in the two modalities of ELISA. The positivity limits for each modality were very similar, but in the indirect test the mean values were higher.

Criteria for the thermal stability test of GDH (NADP+) followed that of Wilson *et al.*, (1969). Values of % RA are shown in Table 1. The antigenicity in all cases was > 77% of the control activity at 100°C for 5 minutes.

To establish immunologic relationships between isoenzymatic forms of the GDH (NADP+) of different species of *Plasmodium*, we conducted ELISA tests, using as the capture antibody, the polyclonal rabbit anti-GDH (NADP+) of *Proteus* spp. and GDH (NADP+) (prepared by immunoaffinity) (positive control) as antigen. The following sources of IgG anti-GDH (NADP+), were evaluated: a) Patient plasmas with acute *Plasmodium vivax* malaria,



NS: Normal serum.
Pf: Acute serum from patients with *P. falciparum*.
Pf*: Serum from malaria patients diagnosed with 2 or more episodes of *P. falciparum*.
Pv: Acute serum from patients with *P. vivax*.
Pb: Acute serum from mice infected with *P. berghei*.
Pc: Acute serum from Pekinese ducks infected with *P. cathemerium*.

Fig. 4. Indirect ELISA evaluation of the GDH ($NADP^+$) specificity, compared to other *Plasmodium* species.

b) mice plasmas with acute experimental infections of *Plasmodium berghei*, and c) Pekinese duck plasmas with acute experimental infections of *Plasmodium cathemerium*. The mean OD values obtained for samples of each experimental group were compared with the mean OD values obtained from patient plasmas with *Plasmodium falciparum* malaria (Fig. 4).

The results of the indirect immunofluorescence are shown in the Fig. 5. In the parasite preparations of different strains (FCB2-Colombia, S/D-Brasilian, Venezuelan-*P. falciparum* isolates) the antigen recognition can be observed on the cell membrane and cytoplasma.

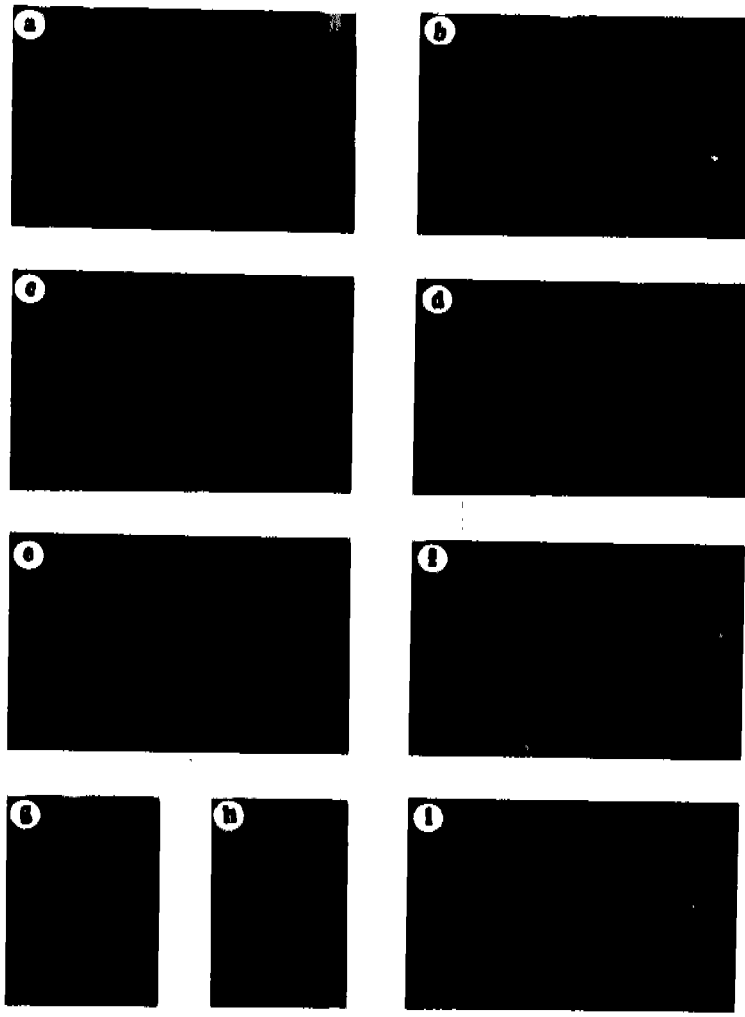
DISCUSSION

One of the initial objectives of this work, was to corroborate the antigenicity of GDH ($NADP^+$) produced by *Plasmodium falciparum*, and its possible utilization in *Plasmodium falciparum* malaria inmunodiagnosis. To determine this, ELISA and sandwich ELISA immunoassays were performed, which demonstrated the detection of IgG antibodies against the antigenic fractions isolated from patient plasmas with acute malaria. The results from direct immunoassay, and from antigen immunoadsorption, indicated the presence of IgG against the GDH ($NADP^+$). The comparison of detected OD levels in each assay did not indicate significant differences between the two groups of malaria patients (acute infections or patients with two or more malaria episodes) (Figs. 2 and 3). The sensitivity of the two ELISA methods, was similar. However, the sensitivity of the test for the group of patients with two or more episodes of malaria was only 95%, when compared to acute infections. Genetic restriction may be a factor for the lack of response (Benacerraf, 1981) and may be responsible for a higher degree of susceptibility to the reinfection. (Butterworth, 1987).

The GDH ($NADP^+$) is a widely distributed enzyme among species of Plasmodium infecting mice, birds and man (Sherman *et al.*, 1971; Roth *et al.*, 1986; Eckman, 1984). In spite of the fact that this enzyme fulfills the same physiological properties in different species (contribution of $NADP^+$), in our experiments, immunologically, it behaved differently. On the other hand, the GDH of vertebrate tissues differs from *Plasmodium falciparum* GDH in its activation by purine nucleotides, reversible association and dissociation and utilization of both NAD(H) and NADP(H) as coenzymes (Smith, 1975).

Polyclonal antisera against *Proteus* spp. GDH ($NADP^+$) cross-reacted with *Plasmodium falciparum* GDH ($NADP^+$) but did not react with GDH ($NADP^+$) from bovine liver (Hempelmann and Wilson, 1982; Ling *et al.*, 1986).

The results obtained in ELISA-sandwich assay using *Plasmodium falciparum* GDH ($NADP^+$) and serum from mice infected with *Plasmodium berghei*, Pekinese ducks infected with *Plasmodium cathemerium* and human infected with *Plasmodium vivax* and *P. falciparum*, appear to support the hypothesis of enzyme immunologic differences between *Plasmodium* species. The anti-*Plasmodium cathemerium* and anti-*Plasmodium vivax* antibodies, include some IgG cross reaction for *Plasmodium falciparum* GDH ($NADP^+$) while there was no cross reactivity for anti-*Plasmodium berghei*. This possible inmunologic



Venezuelan (Bolivar State) isolates
a) Anti-culture supernatant serum, dilution (1: 60)
b) Anti-GDH (NADP+) of *Proteus* spp., dilution (1: 20)
Colombian FCB2 strain
c) Anti-culture supernatant serum, dilution (1: 40)
d) Anti-GDH (NADP+) of *Proteus* spp., dilution (1: 20)
Brazilian S/D strain
e) Anti-culture supernatant serum, dilution (1: 40)
f) Anti-GDH (NADP+) of *Proteus* spp., dilution (1: 20)
Positive Control
g) Venezuelan isolates/IgG anti-human malaria, dilution (1: 200)
h) Venezuelan isolates concentrated by 37% Percoll ®/IgG-anti-human malaria
Negative Control
i) Venezuelan isolates/normal human serum, dilution (1: 200).

Fig. 5. Indirect immunofluorescence assay of different preparations.

relationship is not surprising, since *Plasmodium falciparum* exhibits a high homology with *Plasmodium* species that infect birds.

The lack of information about the molecular characteristics of the *Plasmodium vivax* GDH do not allow for speculation, except that we can assume the IgG antibodies, recognize a

related epitope. We are not able to determine with 100% certainty that some of the patients with two or more episodes of malaria did not have a past *Plasmodium vivax* infection, or a mixed infection that was not recorded.

If criteria for positive *P. falciparum* infections are based on X ± 3 SD (standard deviation) (Fig. 4) of the negative controls, then the group of sera with *Plasmodium vivax* infections, would be false positives. However, the mean OD values observed in this group is significantly different ($P \leq 0.05$) from the means observed for *Plasmodium falciparum* infected groups, allowing differentiation between the infections of both species.

Based on thermal stability, *Plasmodium falciparum* GDH ($NADP^+$) can be classed within the group of the Type S excretion antigen. In this case, treatment at 100°C for 5 minutes (most extreme condition) conserved 77% of its antigenicity. Heat stability would be of great importance when developing diagnostic kits used in tropical areas.

Indirect immunofluorescence assays (Fig. 5) were positive for isolates of *Plasmodium falciparum* from Bolivar State (Venezuela), as well as the Colombian strain (FCB2) and the Brasilian strain (S/D). This demonstrates that the IgG polyclonals anti-*Plasmodium falciparum* culture supernatants and IgG anti-GDH ($NADP^+$) of Proteus spp. recognized epitopes in these cellular preparations, indicating the parasite origin of these antigens and antigen similarities among strains from different geographical locations. In conclusion, GDH (NADP+) is an excretion antigen, with good specificity, is a potent immunogen and is thermoresistant. It would be very useful diagnostic tool in epidemiological and limited clinical studies for the diagnosis of current and past *Plasmodium falciparum* infections; It would be a very useful clinical tool if we test antigenemia, because the antigen is always present in current infections.

ACKNOWLEDGMENTS

This work received financial support (Grant No.09.34.0173.93) from the Consejo de Desarrollo Cientifico y Humanistico and the Instituto de Investigaciones Farmaceuticas de la Universidad Central de Venezuela. We thanks to LTC Terry A. Klein, Professor Soon-Hyung Lee and Dr. Stephen Tillet for their hepful comments and for reviewing the english of the manuscript.

REFERENCES

Benacerraf B (1981) Role of MHC gene products in immune regulation. *Science* **212:** 1229-1238.

Butterworth AE (1987) Immunity in human schistosomiasis. *Acta Trop* **44:** 31-40.

Eckman JR (1984) Malaria and the Red Cell. Progress in Clinical and Biological Research Vol 165, p1-12 GJ Brewer, Allen R. Liss A (Eds) New York.

Engvall E, Perlmann P (1971) Enzyme-linked immunosorbent assay (ELISA) quantitative assay for immunoglobulin *G. Immunochemistry* **8:** 871-876.

Heidrich HG, Mrema JEK, Van der Jagt DL, Reyes P, Rieckmann KH (1982) Isolation of intracelullar parasites (*Plasmodium falciparum*) from culture using free-flow electrophoresis: separation of the free parasites according to stages. *J Parasitol* **68:** 443-449.

Hempelmann E, Wilson RJM (1982) Immunoprecipitation of malarial enzymes. *Protozoology* **29:** 637-642.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. *J Biol Chem* **193:** 265-272.

Ling IT, Cooksley S, Bates PA, Hempelmann E, Wilson RJM (1986) Antibodies to the glutamate dehydrogenase of *Plasmodium falciparum. Parasitology* **92:** 313-324.

Roth EF Jr, Schulman S, Vanderberg J, Olson J (1986) Pathways for the reduction of oxidised glutathione in the *Plasmodium falciparum*-infected erythrocyte: can parasite enzymes replace host red cell glucose-6-phosphate dehydrogenase? *Blood* **67:** 827-830.

Sherman IW, Peterson Y, Tanigoshi L, Ting IP (1971) The glutamate dehydrogenase of *Plasmodium lophurae* (Avian malaria). *Exp Parasitol* **29:** 433-439.

Smith A (1975) Glutamate dehydrogenases In: The Enzymes, Vol 11 p293-367 PD Boyer (Ed) New York Academic Press.

Taithong S, Beale GH, Fenton B, McBride J, Rosario V, Walker A, Walliker D (1984) Clonal

diversity in a single isolate of the malarial parasite *Plasmodium falciparum*. *Trans Roy Soc Trop Med Hyg* **78:** 242-245.

Trager W, Jensen JB (1978) Cultivation of malarial parasites. *Nature* (London) **237:** 621-622.

Van der Jagt DL, Intress C, Heidrich JE, Mrema JEK, Rieckmann K.H, Heidrich HG (1982) Marker enzymes of *Plasmodium falciparum* and human erythrocytes as indicators of parasite purity. *J Parasitol* **68:** 1068-1074.

Van der Jagt DL, Hunsaker LA, Kibirige M, Campos NM (1989) NADPH production by the malarial parasite *Plasmodium falciparum*. *Blood* **74:** 471-474.

Voller A, Bidwell B, Holdt G, Engvall E (1978) A microplate method of enzyme-linked immunosorbent assay and its applications to malaria. *Bull WHO* **51:** 209-211.

Walter RD, Nordmeyer JP, Konig E (1974) NAD-specific glutamate dehydrogenase from *Plasmodium chabaudi Hope-Seyler's Zeitschrift fur Physiologische Chemie* **335:** 495-501.

Weir DM (1978) Immunochemistry. In: Application of Immunological Methods, Vol 1; Blackwell Scientific Publications.

Wilson RJM, McGregor IA, Hall P, Williams K, Bartholomew R (1969) Antigens associated with *Plasmodium falciparum* infections in man. *Lancet* **2:** 201-205.

[Korean J. Parasitol. 34(4): 239-246, December 1996]