

Characterization of *Plasmodium falciparum* glutamate dehydrogenase-soluble antigen

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Abstract

The major aim of this study was to characterize a soluble *Plasmodium falciparum* antigen from the plasma of malaria-infected humans and *Plasmodium falciparum* culture supernatants, using immunoabsorbent techniques and Western blotting. An Mr 60-kDa protein was isolated from the plasma of patients with *Plasmodium falciparum* malaria by affinity chromatography using rabbit anti-*Proteus* spp GDH(NADP⁺) serum as ligand. This protein, present in plasma of patients with acute *Plasmodium falciparum* infection, in *Plasmodium falciparum* culture supernatants, and in immune complexes, was tested with *Plasmodium falciparum* malaria hyperimmune serum from patients living in hyperendemic areas and rabbit anti-*Proteus* spp GDH(NADP⁺) serum prepared in the laboratory. In this report, we describe the results of a study showing that parasite GDH(NADP⁺) can be used to detect the presence of *Plasmodium falciparum*. It appears that this technique permits the chromatographic detection of a *Plasmodium falciparum* excretion antigen that may be used in the production of monoclonal antibodies to improve immunodiagnostic assays for the detection of antigenemia, and opens the possibility of its use as a non-microscopic screening method.

Key words

- Glutamate-dehydrogenase
- *Plasmodium falciparum*
- Exoantigens
- Soluble antigens
- Malaria
- Antigenic enzymes

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Introduction

Malaria is still one of the most important and devastating tropical diseases, and *Plasmodium falciparum* kills about 2-3 million people per year (1). A concerted effort is necessary to improve the diagnostic methods to control the disease.

Among the compounds inherent to the parasite there are certain enzymes that could be effective as markers in malaria diagnosis (2). The difficulty encountered in isolating them from the plasma of patients or in cultures of *Plasmodium falciparum* is the fact that only minimal quantities are obtained

and it is difficult to separate the parasitic material from the host products. In general, it may be stated that the parasite has a metabolism similar to the host, but the characteristics of many enzymes compromised in these pathways have been considered to be different. Among these enzymes, this study will concentrate on glutamate dehydrogenase whose presence was suspected on the basis of appearance of an Mr 60-kDa band whose enzymatic activity was confirmed by means of an enzymatic assay (Rodríguez-Acosta A, Gamboa de DN, Aguilar I and Girón ME, unpublished data). In that study we detected the enzymatic activity of *Plas-*

modium falciparum GDH(NADP⁺) in the plasma of acute malaria patients, captured by a rabbit polyclonal anti-*Proteus* spp GDH(NADP⁺), which took advantage of the immunologic relationships existing between the enzymes of the two species (3).

This GDH(NADP⁺) activity of *Plasmodium falciparum* differs from mammalian GDH in several features: kinetics, substrate, specificity of co-factors, degree of affinity and immunogenicity (4-7). This report suggests that *Plasmodium falciparum* glutamate dehydrogenase-soluble antigen may be used as a target antigen in systems designed to detect malarial disease.

Material and Methods

Animals

New Zealand rabbits weighing 2 kg were used to obtain sera against whole parasite, culture supernatant and GDH(NADP⁺).

Sample collection

Blood samples were collected from patients attending the Malaria Consultation Center at the Ciudad Bolívar Malariology Division. Ciudad Bolívar is a city located in the northern part of Bolívar State, Venezuela. Many of the patients are migrant gold miners who routinely spend 20 to 30 days prospecting in the tropical forest where most of their infections originate. A total of 30 patients with acute malaria were recruited for the study as a source of antigens. All the patients gave oral informed consent to participate in the study. Each patient was interviewed and address, place of origin and information about whether or not the patient had been treated with anti-malarial drugs were recorded in a questionnaire.

Blood samples from the patients (approximately 20 ml) were collected into test tubes that contained an anticoagulant and 100 µl of a mixture of proteinase inhibitors (0.15

mM TPCK (Sigma Chemical Co., St. Louis, MO), 200 mM PMFS (Sigma), 0.2 mM EDTA in PBS). The plasma was separated to be used as a source of antigens and stored at -70°C until use.

Parasite culture

Plasmodium falciparum parasites (Venezuelan isolates) were cultured with human erythrocytes at 2% hematocrit in RPMI-1640 media and 10% human serum (8). The media were changed every 24 h, treated with a protease inhibitor cocktail and stored at -70°C until use.

Fractionated precipitation of the *Plasmodium falciparum* culture supernatant

Approximately 300 ml of culture supernatant was concentrated (30 times) inside a dialysis bag by dehydration with Aquacide II. The concentrate was subjected to fractionated precipitation with ammonium sulfate using 33, 40 and 50% saturation. The precipitates obtained in each step were collected in PBS and analyzed by Western blotting.

Precipitation of immune complexes

In another group of experiments we analyzed the immune complexes formed *in situ* by a combination of a) plasma from patients with acute *Plasmodium falciparum* infections and Yanumana Amerindian sera (hyperimmune) at a proportion of 5:1 and b) plasma from patients with acute *Plasmodium falciparum* infections with anti-GDH(NADP⁺) antibodies of *Proteus* spp produced in rabbits at a proportion of 20:1.

The precipitation of immunocomplexes was effected in accordance with the protocol utilized by the Laboratório de Malária (Malaria Laboratory), Instituto Oswaldo Cruz, Rio de Janeiro, Brazil (C. Ribeiro, personal communication). The mixtures were treated with polyethylene glycol 8,000 (PEG 8,000)

(Sigma) at 2.5% final concentration and left on ice for 2.5 h. The samples were centrifuged at 2,500 g for 20 min at 4°C and the precipitate was washed with a solution of 2.5% PEG 8,000 in PBS, pH 7.2, and subsequently dialyzed against 1 mM PBS, pH 7.2, for 2 h at 4°C, and absorbance at 280 nm was determined. The material obtained was analyzed by the Western blot method. Normal serum was used as a control and subjected to the same procedure.

Antibody preparation

Antibodies were obtained from plasma of adult Yanumana Indians of both sexes living in hyperendemic areas of malaria (Amazonas State, Venezuela), diagnosed as being infected with *Plasmodium falciparum* and having experienced two or more malaria episodes. Anti-malaria IgG was isolated from the plasma of hyperimmune adult patients by salting out and ion exchange chromatography (DEAE cellulose; Sigma) as described (9).

GDH(NADP⁺) immunoaffinity separation

Blood plasma (400 mg protein) as a source of antigens from patients with acute *Plasmodium falciparum* infections was loaded on columns of Sepharose-4B-rabbit-IgG anti-GDH(NADP⁺) of *Proteus* spp. The columns were washed with PBS until absorbance at 280 nm was equal to 0. The protein absorbed to the column was eluted with 0.1 M glycine-HCl buffer, pH 2.6, concentrated (Figure 1), and analyzed by Western blot. Plasma from normal patients was treated in the same manner as the plasma from malaria patients.

Anti-whole-parasite serum

The infected erythrocytes (20 ml) derived from cultures with a parasitemia of at least 3% were suspended in PBS, pH 7.2, and centrifuged at 300 g for 10 min at 4°C. After being washed with PBS three times, the eryth-

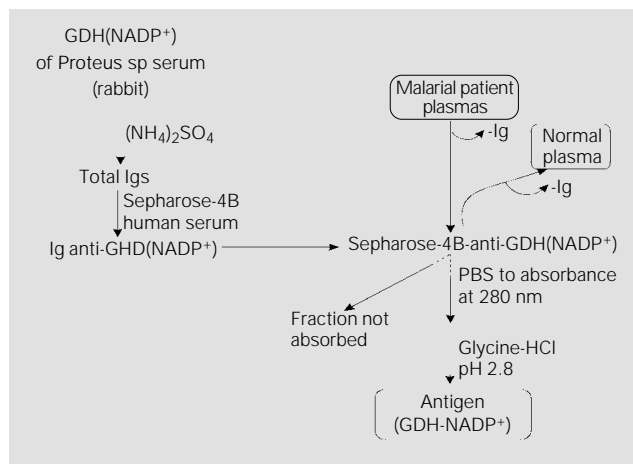


Figure 1 - Procedure for the separation of GDH(NADP⁺) from malarial patient plasmas by affinity chromatography.

rocytes were lysed with saponin (Sigma) at a final concentration of 0.1% for 15 min at room temperature, washed and resuspended again in 0.5 ml of PBS which contained 0.1% of the mixture of protease inhibitors (crude preparation of parasite extracts).

This antigen was utilized to immunize the rabbits and obtain anti-whole-parasite serum as described (9). This serum was used in the immunoblotting analysis of the antigens to be studied.

Preparation of IgG anti-culture supernatant

Approximately 300 ml of culture supernatant was concentrated (30 times) inside a dialysis bag by dehydration with Aquacide II (Calbiochem, San Diego, CA). The concentrate was precipitated with 33% ammonium sulfate to eliminate the human serum immunoglobulins present in the culture medium. The protein concentration of the immunoglobulin-free supernatant was determined and the supernatant was then used to prepare anti-culture supernatant serum by inoculating rabbits as described (9). The serum of immunized rabbits was used to prepare IgG by precipitation with 33% ammonium sulfate and DEAE cellulose (Sigma) ion exchange chromatography. This IgG (100 mg

protein/ml) was absorbed by incubation with a mixture of red blood cells (MRBC) type 0⁺, A⁺ and B⁺ (1:1:1), maintaining the relationship of 1 ml MRBC to 3 ml IgG, for 2 h at 4°C, and centrifuged at 200 g for 10 min, and the supernatant was separated out. This supernatant was passed through a column of Sepharose-4B-human serum and the recovered eluate was concentrated by Aquacide II dehydration. This preparation of purified Ig was used to perform the immunoblotting analysis of the antigens to be studied.

Preparation of rabbit peroxidase-conjugated anti-*Proteus* spp GDH(NADP⁺) IgG

The serum of rabbits immunized against *Proteus* spp GDH(NADP⁺) (Sigma) was used to prepare IgG by saline precipitation with 33% ammonium sulfate and ion exchange

DEAE-cellulose chromatography (Sigma). The IgG fraction enriched with anti-GDH(NADP⁺) antibodies was used to prepare Sepharose-4B anti-GDH(NADP⁺) IgG, and the peroxidase conjugate (9). This conjugate was used in the immunoblotting analysis of the different antigen preparations studied.

Polyacrylamide gel electrophoresis

Electrophoresis was carried out by the method of Laemmli (10) using a Dual Mini Slam Kit AE-6450 (Atto Corporation, Tokyo, Japan) chamber. The development gels were prepared at 7.5% or in gradients of 5-15% and in all cases the concentration gel was prepared at 4.5%.

Western blotting

The original immunoblotting technique (11) was used. The electrophoresed proteins were blotted onto nitrocellulose using a semi-dry electrotransfer chamber with graphite electrodes (Atto Corporation). The material transferred was analyzed by immunostaining using specific antibodies.

Results

Complete data was obtained for all patient sera tested. Of these 100% were parasitologically positive for *Plasmodium falciparum* and negative for other *Plasmodium* species. The parasite carriers with acute *Plasmodium falciparum* infections showed a parasite density of 10,000/μl.

To characterize the antigens present in plasma, captured by a column of Sepharose-4B-anti-*Proteus* spp GDH(NADP⁺), we separated the antigens in polyacrylamide gels and electrotransferred them for the Western blot analysis. The result (Figure 2a) shows that the parasite excretes a protein which is recognized by anti-*Proteus* spp GDH(NADP⁺) serum/anti-rabbit IgG peroxidase, that corresponded to a protein with Mr of

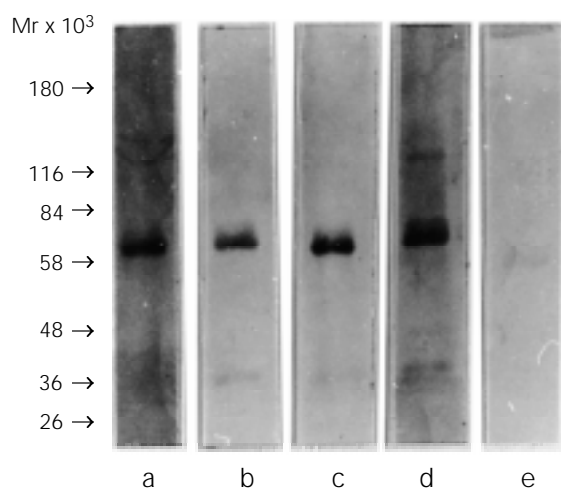


Figure 2 - Western blot analysis of the antigenic fraction present in malarial plasmas of patients infected with *Plasmodium falciparum* separated through columns of Sepharose-4B linked to anti-*Proteus* spp GDH(NADP⁺) IgG. The electrophoretic separation was carried out in 7.5% SDS-polyacrylamide gels under reducing conditions. Immunologic detection was obtained by incubation with:

- Anti-*Proteus* spp GDH(NADP⁺) serum/anti-IgG rabbit-peroxidase.
- Anti-culture supernatant of *Plasmodium falciparum*/anti-IgG rabbit-peroxidase.
- Anti-whole-parasite (*Plasmodium falciparum*)/anti-IgG rabbit-peroxidase.
- Hyperimmune (Yanumana) serum/anti-IgG-human-peroxidase.
- Normal patient serum/anti-IgG-human-peroxidase.

approximately 60 kDa.

The polyclonal anti-culture supernatants and anti-whole-parasite, as well as the hyperimmune serum of the Yanumana Indians, were also able to recognize the 60-kDa band (Figure 2b,c,d).

In the Western blot analysis of crude preparations of parasites, the complex composition of the sample becomes evident by the use of an antiserum produced against the proteins of the whole parasite. A band with the same electrophoretic migration (approximately 60 kDa) is also recognized by the hyperimmune serum of Yanumana Indians and by the anti-*Proteus* spp GDH(NADP⁺)-peroxidase conjugate (Figure 3).

The Western blot analysis of the 40% ammonium sulfate-precipitated fraction of the culture supernatant of *Plasmodium falciparum* is shown in Figure 4. When developed with the polyclonal anti-*Proteus* spp GDH(NADP⁺)-peroxidase, a band with a molecular mass of approximately 60 kDa appears, similar to that obtained from the plasma of acute *Plasmodium falciparum* infections.

The Western blot analysis of immune-peroxidase-conjugated anti-*Proteus* spp GDH(NADP⁺) IgG indicated the presence of a 60-kDa protein (Figure 5).

Discussion

The identification and description of new malarial antigens should contribute to improving both the individual and mass diagnosis of this endemic disease, as well as the incorporation of antigens relevant to the preparation of new vaccines. To date, studies focused in this direction have not been consolidated. In this regard, the main purpose of the present study was to identify the products of excretion that could be associated with infection by *Plasmodium falciparum*. The discovery of a 60-kDa protein in the plasma of acute *Plasmodium falciparum* infections, in the culture supernatant of *Plas-*

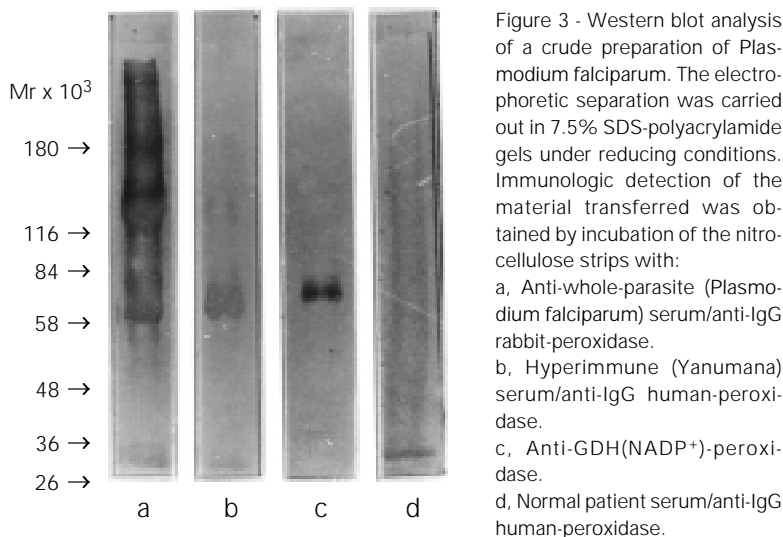


Figure 3 - Western blot analysis of a crude preparation of *Plasmodium falciparum*. The electrophoretic separation was carried out in 7.5% SDS-polyacrylamide gels under reducing conditions. Immunologic detection of the material transferred was obtained by incubation of the nitrocellulose strips with:
a, Anti-whole-parasite (*Plasmodium falciparum*) serum/anti-IgG rabbit-peroxidase.
b, Hyperimmune (Yanumana) serum/anti-IgG human-peroxidase.
c, Anti-GDH(NADP⁺)-peroxidase.
d, Normal patient serum/anti-IgG human-peroxidase.

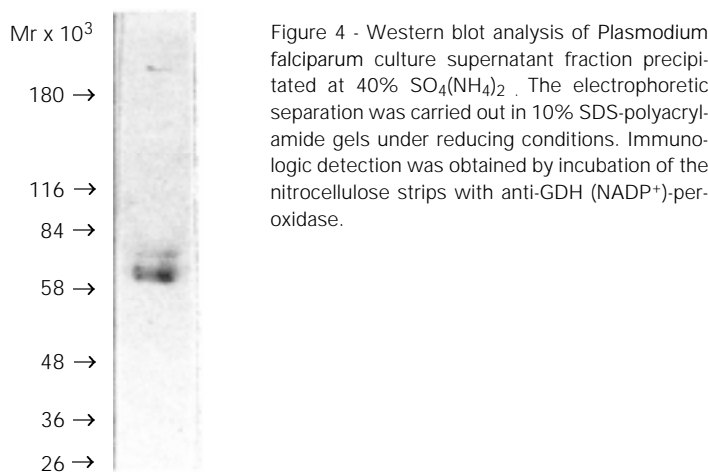


Figure 4 - Western blot analysis of *Plasmodium falciparum* culture supernatant fraction precipitated at 40% SO₄(NH₄)₂. The electrophoretic separation was carried out in 10% SDS-polyacrylamide gels under reducing conditions. Immunologic detection was obtained by incubation of the nitrocellulose strips with anti-GDH (NADP⁺)-peroxidase.

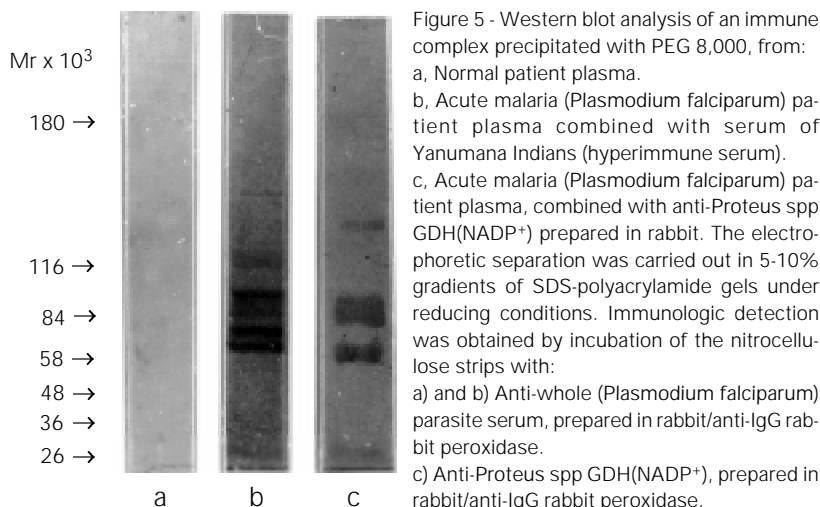


Figure 5 - Western blot analysis of an immune complex precipitated with PEG 8,000, from:
a, Normal patient plasma.
b, Acute malaria (*Plasmodium falciparum*) patient plasma combined with serum of Yanumana Indians (hyperimmune serum).
c, Acute malaria (*Plasmodium falciparum*) patient plasma, combined with anti-*Proteus* spp GDH(NADP⁺) prepared in rabbit. The electrophoretic separation was carried out in 5-10% gradients of SDS-polyacrylamide gels under reducing conditions. Immunologic detection was obtained by incubation of the nitrocellulose strips with:
a) and b) Anti-whole (*Plasmodium falciparum*) parasite serum, prepared in rabbit/anti-IgG rabbit peroxidase.
c) Anti-*Proteus* spp GDH(NADP⁺), prepared in rabbit/anti-IgG rabbit peroxidase.

modium falciparum and in the isolated immune complexes allows us to make an inference regarding its high level of immunogenicity and distribution. The inclusion of anti-whole-parasite polyclonal antibodies in our Western blotting experiments has proved to be a valuable tool in the characterization of this antigen. As commented in the Introduction (Rodríguez-Acosta A, Gamboa de DN, Aguilar I and Girón ME, unpublished data) we have detected the GDH(NADP⁺) enzymatic activity of this 60-kDa protein and evidence is provided that GDH reactivity correlates with parasite burden.

Following a strategy similar to that used by Riha et al. (12) we decided to analyze from an immunological viewpoint the immune complexes generated when the plasma of acute *Plasmodium falciparum* infections reacted with polyclonal anti-GDH(NADP⁺) and also with the hyperimmune serum from Yanumana Indians. The immunological development of the immune complexes precipitated by PEG 8,000 from prior mixtures (Figure 5) using the polyclonal anti-whole-parasite prepared in rabbits allowed us to observe the presence of a series of bands (absent in normal plasma), including one which has a molecular weight of approximately 60 kDa. Considering the heterological nature of the antiserum used in immunodevelpment (anti-whole-parasite prepared in the rabbit), the parasitological nature of this antigen is once again confirmed.

Another important discovery in the present study was the fact that the antigen under study was recognized by the naturally acquired antibodies of the Yanumana Indians (Figures 3 and 4). The serum of these patients, although it may prove to be heterogeneous, had the advantage of being an indicator of those antigens that produce a good immunological response.

Van Der Jagt et al. (13), who purified the GDH(NADP⁺) of *Plasmodium falciparum* using HPLC, report a band of close to 230 kDa. Our results, obtained under reducing

conditions, led to the interpretation that native GDH(NADP⁺) could correspond to a protein consisting of 4 subunits. GDH (NADP⁺) shows no cross-reaction with the enzyme of mammals (14), but rather with *Proteus* spp.

From a practical point of view, and in keeping with the findings of others (15-17), we would like to state that GDH should be considered to be the most important marking enzyme for malarial parasites, including *Plasmodium falciparum*.

The characteristics mentioned earlier with reference to the enzyme allow us to suggest its possible use in the implementation of a methodology for *Plasmodium falciparum* malaria diagnosis. Its double characteristic of being a highly immunogenic product of excretion and an enzyme has the potential to be developed into a useful format for a diagnostic test for malaria. Similarly, others (18) have proposed the measurement of *Plasmodium falciparum* lactate dehydrogenase (pLDH) activity. In our case, GDH(NADP⁺) and proteases currently being studied in our laboratory would allow GDH to be used in both an immunological and an enzyme-type method, a methodology which is already being used with different enzymes (alkaline phosphatase) in other parasites (19). In conclusion, it appears that this technique permits the chromatographic detection of a *Plasmodium falciparum* excretion antigen that may be used for the production of monoclonal antibodies to improve immunodiagnostic assays for the detection of antigenemia, and opens possibilities as a nonmicroscopic screening method. Furthermore, the GDH isoenzymes of different *Plasmodium* species (*falciparum*, *vivax*, *berghei* and *cathemerium*) have shown to be immunologically different (20).

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References

1. Klayman DL (1989). Weeding out malaria. *Natural History*, 10: 18-91.
2. Saxena N, Pandey VC, Dutta GP & Ghatak S (1986). Characterisation of lactate dehydrogenase of *Plasmodium knowlesi*. *Molecular Biochemistry and Parasitology*, 21: 199-202.
3. Schmidt E (1974). Glutamate dehydrogenase U-V assay. In: Bergmeyer HU (Editor), *Methods of Enzymatic Analysis*. Academic Press, New York.
4. Shermann IW, Peterson I, Tanigoshi L & Ting IP (1971). The glutamate dehydrogenase of *Plasmodium lophurae* (avian malaria). *Experimental Parasitology*, 29: 433-439.
5. Roth Jr EF, 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.
6. Bernard F, Mayer R, Picard I, Deguercy A, Monsigni M & Schrevel B (1987). *Plasmodium berghei* and *Plasmodium chabaudi*: A neutral endopeptidase in parasite extracts and plasma of infected animals. *Experimental Parasitology*, 64: 90-103.
7. Aikawa M, Miller LH, Johnson J & Rabbage J (1988). Erythrocyte entry by malarial parasites: A moving junction between erythrocyte and parasite. *Journal of Cell Biology*, 77: 72-82.
8. Trager W & Jensen JB (1976). Cultivation of malarial parasites. *Nature*, 237: 621-622.
9. Hudson L & Hay FC (1980). *Practical Immunology*. Blackwell Scientific Publications, Oxford, England.
10. Laemmli UK (1970). Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature*, 227: 680-685.
11. Towbin H, Staehelin T & Gordon J (1979). Electrophoretic transfer of proteins for polyacrylamide gels to nitrocellulose sheets. Procedure and some applications. *Proceedings of the National Academy of Sciences, USA*, 76: 4350-4354.
12. Riha I, Haskova V, Kaslik J, Maierova M & Stransky J (1979). The use of polyethylene glycol for immune complex detection in human serum. *Molecular Immunology*, 16: 489-493.
13. Van Der Jagt DL, Hunsaker LA, Kibirige M & Campos NM (1989). NADPH production by the malarial parasite *Plasmodium falciparum*. *Blood*, 74: 471-474.
14. Ling IT, Cooksley S, Bates PA, Hempelmann E & Wilson RJM (1986). Antibodies to the glutamate dehydrogenase of *Plasmodium falciparum*. *Parasitology*, 92: 313-324.
15. Brett M, Chambers GK, Holder AA, Fincham JRS & Wootton JC (1976). Mutational amino acid replacements in *Neurospora crassa* NADP⁺-specific glutamate dehydrogenase. *Journal of Molecular Biology*, 106: 1-22.
16. Hempelmann E & Wilson RJM (1982). Immunoprecipitation of malarial enzymes. *Protozoology*, 29: 637-642.
17. Eldford BC, Hayes JD, Chulay JD & Wilson RJM (1985). Stage-specific transport and metabolism of L-glutamine induced by *Plasmodium falciparum* in human red cells. *Molecular Biochemistry and Parasitology*, 12: 267-282.
18. Makler MT, Ries JM, Williams JA, Bancroft JE, Piper RC, Gibbins BL & Hinrichs DJ (1993). Parasite lactate dehydrogenase as an assay for *Plasmodium falciparum* drug sensitivity. *American Journal of Tropical Medicine and Hygiene*, 48: 739-741.
19. Pujol de Freychet FH (1988). Evaluación de la fosfatasa alcalina y otros antígenos para el diagnóstico inmunológico de la Esquistosomiasis mansoni, mediante el uso de anticuerpos policlonales y monoclonales. Doctoral thesis, Instituto Venezolano de Investigaciones Científicas, Caracas, Venezuela.
20. Domínguez N & Rodríguez-Acosta A (1996). Glutamate dehydrogenase antigen detection in *Plasmodium falciparum* infections. *Korean Journal of Parasitology*, 34: 239-246.