

A 33-35 kDa circulating antigen from *Plasmodium falciparum*

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Abstract. Isolation and characterisation of *Plasmodium falciparum* (Welch, 1897) soluble antigens from infected patient plasma, Western blotting, thermal stability and ELISA assays using hyperimmune IgG-antimalaria antibodies was the main objective of this work. A circulating antigen of approximately Mr 33-35 kDa with good specificity and antigenicity, in the plasma of malarial patients was shown. Heating at 100°C did not destroy its antigenicity. When fractions highly enriched in the 33-35 kDa proteins were used in ELISAs, a seroreactivity in plasma obtained from primary-infected individuals was found. Controls from normal patients were always negative. The antigenic characteristics suggest that it may be included within the group of new described *Plasmodium* soluble antigens.

With the development of techniques for *in vitro* cultivation of *Plasmodium falciparum* (Welch, 1897) and the isolation of parasite soluble antigens from malaria patient's plasma, the opportunity presents itself to describe new *Plasmodium* antigens (Chougnat and Deloron 1992). The term "soluble antigen" includes a broad variety of antigens, a majority of which are probably released into the bloodstream of patients *in vivo* or in tissue culture supernatant *in vitro* upon schizont rupture and subsequent merozoite invasion.

Plasmodium falciparum excretion-secretion soluble antigens have been known since 1968 (McGregor et al. 1968). Several authors have studied their importance in protection against disease but they may be implicated in the development of immunopathological complications (Greenwood and Brueton 1974, Facer 1980, Urquhart 1992, Baltahazar-Guedes et al. 1995, Wahlgren et al. 1995). Recent advances in the identification and characterisation of antigens associated with different stages of the parasite suggest new opportunities for vaccination as a strategy for disease control. The potential of these substances as candidates for the development of diagnosis tools must also be indicated. Here we describe a novel antigen recognised by malarial serum. This *P. falciparum* soluble antigen was isolated from infected patient plasma and characterised by Western blotting, thermal stability and ELISA assays using hyperimmune IgG-antimalaria antibodies that could be associated with an active infection.

MATERIALS AND METHODS

Animals. New Zealand strain rabbits were used. All animals were well treated and care taken to avoid distress when blood samples were obtained. The investigation complied with the norms taken from the Guide for the Care and Use of Laboratory Animals, published by the US National Institute of Health (Anonymous 1985).

Sample collection. Blood samples from patients attending the Malaria Hospital at the Malariology Division of Bolívar City were collected. This is a city located in the northern part of Bolívar State, Venezuela. Many of the patients are migrant gold miners. They routinely spend between 20-30 days prospecting in the tropical forest where most of their infections (most assuredly) originate.

The region is a tropical area where the temperature and humidity allows transmission during most of the year. The main vector is *Anopheles darlingi* Root, essentially a non-domestic species that usually does not enter the houses, although when it does, it bites quickly. Malaria generally occurs with moderate incidence, rarely reaching the hyper-endemic levels common in Africa.

A total of thirty patients with acute malaria (primary infection) were recruited for the antigen study. Sera from thirty patients who had suffered two or more malarial episodes were used as source of hyperimmune antibodies. For the investigation of the parasite antigens, a pool of plasma was used (30 ml) belonging to the primary-infected patients included in this study. Parasitaemias of patients were always more than 2%. Before being allowed to participate in the study, all the patients were required to give their oral informed consent.

Twenty ml blood samples were collected from each patient using 3.8% sodium citrate at a ratio of 1:10 ml and 100 µl of a mixture of protease inhibitors (L-1-tosylamide 2-phenylethyl chloromethyl ketone [Sigma, Mo, USA] 0.15 mM, phenylmethylsulphonyl fluoride [Sigma] 200 mM, ethylenediamine-tetraacetic acid 0.2 mM in PBS). Plasma (30 ml) from thirty primary-infected patients were pooled and used to investigate parasitic antigens. The Ig-free plasma (immunoglobulins were previously ammonium sulphate precipitated) was dialysed against PBS pH 7.4 and concentrated to a volume of 5 ml inside a dialysis bag using Aquacide II (Calbiochem, USA). The entire procedure was performed at 4°C.

Anti-whole-parasite serum. The infected erythrocytes (20 ml) derived from cultures with at least 3% parasitaemia were suspended in PBS, pH 7.2, and were centrifuged at 300 g for 10 minutes at 4°C. After being washed with PBS three times, the erythrocytes were lysed with saponin (Sigma, Mo, USA) in a final concentration of 0.1% for 15 minutes at room temperature, washed and resuspended again in 0.5 ml of PBS containing 0.1% of the mixture of protease inhibitors (crude preparation of parasite extracts). This antigen was used to immunise the rabbits and obtain anti-whole-parasite serum, as described (Hudson and Hay 1980).

Immune sera. Human anti-*P. falciparum* immune sera from ten Yanumana Amerindians living in a malaria-hyper-endemic area (Amazonas State, Venezuela), and sera (30 ml) from thirty patients who had suffered two or more malarial episodes were pooled and used to isolate parasite antigens. These mixtures were treated with the appropriate volume of saturated ammonium sulphate solution to precipitate immunoglobulins. Corresponding control sera from uninfected individuals at non-malaria-endemic area were obtained. Control eluate attained by affinity chromatography in the SDS gel and the ELISA analysis were tested.

Malarial antigen immunoaffinity separation. Immunoglobulin-free plasma (400 mg protein) from patients with acute *P. falciparum* infection as a source of antigens was loaded on Sepharose-4B-hyperimmune human-IgG-anti-*P. falciparum* columns. The columns with PBS were washed until OD₂₈₀ = 0. The fraction bound to the column was eluted with 0.1 M glycine-HCl buffer, pH 2.6 and concentrated (Fig. 1).

Parasite culture. Isolates from *P. falciparum* infected patients (Bolívar State, Venezuela) were procured. Parasites were cultured in non-synchronised cultures of all stages with human erythrocytes (0 group) at 2% haematocrit in RPMI 1640 medium and 10% human serum (Trager and Jensen 1976). The medium was changed every 24 hours, then treated with a cocktail of protease-inhibitors as previously mentioned, centrifuged to remove red blood cells (RBC) and debris and stored at -70°C until used.

Preparation of IgG anti-culture supernatant. A volume of approximately 300 ml of culture supernatant was concentrated 30 times by dehydration inside a dialysis bag with Aquacide II. The concentrate was 33% ammonium sulphate precipitated, to eliminate the human serum immunoglobulins present in the culture medium. The Ig-free supernatant was dialysed against 0.05 M Tris-HCl buffer, pH 7.6 for 16 hours at 4°C, concentrated and used to prepare anti-culture supernatant serum by inoculating rabbits as described

(Hudson and Hay 1980). The sera from immunised rabbits were used to prepare IgG by 33% ammonium sulphate precipitation and DEAE cellulose (Sigma, Mo, USA) ionic exchange chromatography (Hudson and Hay 1980). This IgG (100 mg protein/ml) was absorbed on a mixture of red blood cells (RBCM) type 0⁺, A⁺ and B⁺ (1:1:1) (1 ml RBCM : 3 ml IgG) for 2 hours at 4°C, and centrifuged at 200 g for 10 minutes. This supernatant was exhaustively absorbed on a column of Sepharose-4B-human serum and the eluate concentrated using Aquacide II. Purified immunoglobulins were used to perform the ELISA analysis of the antigens.

Electrophoresis. Electrophoresis was carried out using a Dual Mini Slam Kit AE-6450 (Atto Corporation, Tokio, Japan) chamber (Laemmli 1970).

Gels at 7.5% with stacking gel at 4.5% were prepared. The samples (antigenic fraction) to be analysed were dissolved in a proportion of 1:1 in the solubiliser solution: 0.5 M Tris-HCl, pH 6.8, with 10% (w/v) SDS, 10% (v/v) β-mercaptoethanol, 10% (v/v) glycerol and 0.05% (w/v) bromophenol blue, and heated at 100°C for 10 minutes. Gels were stained with Coomassie blue.

Western blotting. Immunoblotting technique (Towbin et al. 1979) was used. The electrophorised proteins were blotted onto nitrocellulose filter using a semi-dry electrotransfer chamber with graphite electrodes (Atto Corporation, Tokio, Japan). The filters were analysed by immunostaining, using serum containing specific antibodies (human IgG-anti-*P. falciparum*). Once the transfer had been completed, the nitrocellulose sheets were blocked with 0.03% solution of Tween 20 in PBS for one hour. After washing with 0.01% Tween 20 in PBS (4 × 15 minutes), the strips were incubated for one hour on a rocker at room temperature with the antibodies diluted in 5% foetal calf serum (FCS) in PBS, as indicated earlier. After washing (4 × 15 minutes), the strips were incubated with the secondary antibody (anti-human-IgG-peroxidase) appropriately diluted in PBS for two hours at room temperature, then washed and developed with diaminobenzidine (DAB, Sigma, USA) for 1 or 2 minutes until the immunoreactive proteins were stained clearly. To stop the reaction, distilled water was used.

Thermal stability of the antigen 33-35 kDa separated by affinity chromatography. The fraction Sepharose-4B-hyperimmune-IgG-malaria linked was incubated at temperatures of 60, 80 and 100°C for periods of 2, 5, 10 and 20 minutes (Wilson et al. 1969).

Sample percentage residual antigenicity (RA) was evaluated by ELISA assay (Engvall and Perlmann 1971).

The % RA was calculated using the relationship:

$$\% \text{ RA} = \frac{A_1}{A_2} \times 100$$

% RA = residual antigenicity.

A₁ = difference of OD₄₅₀ between positive and negative sera, after treatment.

A₂ = difference of OD₄₅₀ between positive and negative sera, before treatment.

Enzyme-linked immunosorbent assay (ELISA). We followed the methodology of Engvall and Perlmann (1971) and Hudson and Hay (1980), as modified for microassay (Voller et al. 1978), using a direct (Assay 1), and an indirect (Assay 2) (immunoabsorption of the antigen) assay. Different concentrations of *P. falciparum* antigen fractions obtained by affinity chromatography purification were used. The direct tests were carried out with the antigen (6 µg/ml) in 96 flat-bottom-well microtiter plates placed overnight at 4°C. Plasma from patients with acute (> 2% parasitaemia) *P. falciparum* malaria were obtained. The conjugated anti-human-IgG-peroxidase as a secondary antibody was used. 3,3'-diaminobenzidine peroxidase (Sigma, Mo, USA) was used as substrate. The reaction was stopped by adding 4N H₂SO₄ after 30 minutes and the absorbance was measured at 450 nm. All samples were with both positive and negative controls.

Indirect tests in 96 flat-bottom well microtiter plates were incubated for 2 hours at 37°C with the rabbit polyclonal anti-whole *P. falciparum* antibodies. After washing three times with a solution of 0.05% PBS-Tween, plates were incubated overnight at 4°C with *P. falciparum* antigens at 6 µg/ml in carbonate buffer pH 9.6. The rest of the process was performed as for the direct test.

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

Statistical methods. Statistical comparisons were made utilising unpaired Student's t-test ($P = 0.05$).

RESULTS

The antigenic fraction separation by affinity chromatography with Ig-antimalaria ligands from hyper-immune patients is shown in Fig. 1.

Fig. 2 shows the polyacrylamide gel electrophoresis analysis of antigens retained by a Sepharose-4B-IgG-antimalaria column and indicates the two bands of Mr 33-35 kDa seen in a pool of samples of *P. falciparum* primary-infected individuals.

Western blot analysis of the antigens retained by a Sepharose-4B-IgG-antimalaria column is shown in Fig. 3. Peroxidase-conjugated-IgG anti-*P. falciparum* immunological analysis shows the two bands of Mr 33-35 kDa seen in a pool of samples of *P. falciparum* primary-infected individuals analysed.

To corroborate the presence of antibodies against the 33-35 kDa proteins in sera from primary-infected patients, two types of immunoassays (ELISA) were conducted:

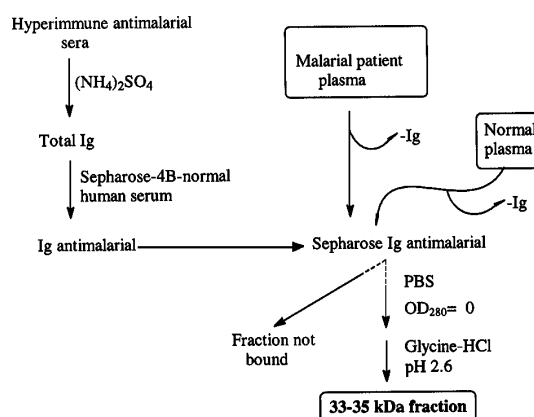


Fig. 1. Procedure for separation of antigenic fractions from malarial patient plasma by affinity chromatography.

In the direct assay (Fig. 4), IgG antibodies were evaluated against the antigens present in the pooled plasma of the group of patients chosen; OD = 0.139 as a positive limit ($\bar{X} \pm \text{S.D.}$) was established. The mean value obtained from the group of patients (primary-infected patients) was 0.351 ± 0.075 , and it was significantly higher than for healthy patients (control sera) ($P \leq 0.01$). The sensitivity for acute sera (primary-infected) from patients was 100% (Table 1).

In the indirect assay (Fig. 5) a value of 0.172 units OD was established as positive limit ($\bar{X} \pm \text{S.D.}$). As in Assay 1, differences with respect to mean values of the experimental group between infected and non-infected patients (control sera) were significantly different ($P \leq 0.01$). The serum sensitivity for the group of primary-infected patients was also 100% (Table 1). Control eluate obtained by affinity chromatography tested in the SDS gel and the ELISA analysis were always negative.

Regarding thermal stability of the antigens, the percentage of residual antigenicity (% RA) values are shown in Table 2. These results indicate that the antigenicity of the preparation is maintained higher than the 80% of the control activity, even in the case of the 33-35 kDa fraction treatment at 100°C for 5 minutes. These results indicate the thermal stability of the antigens under analysis.

Table 1. 33-35 kDa antigen mean values ($\bar{X} \pm \text{S.D.}$) and sensitivity (%) obtained by two ELISA methods.

	Direct assay		Indirect assay	
Controls	C: 0.139		C: 0.172	
Primary-infected patients	$\bar{X} : 0.351 \pm 0.075$ n = 16	S: 100%	$\bar{X} : 0.417 \pm 0.076$ n = 16	S: 100%

C – cut off ($\bar{X} + 3 \text{ S.D.}$); S – sensitivity; n – number of patients

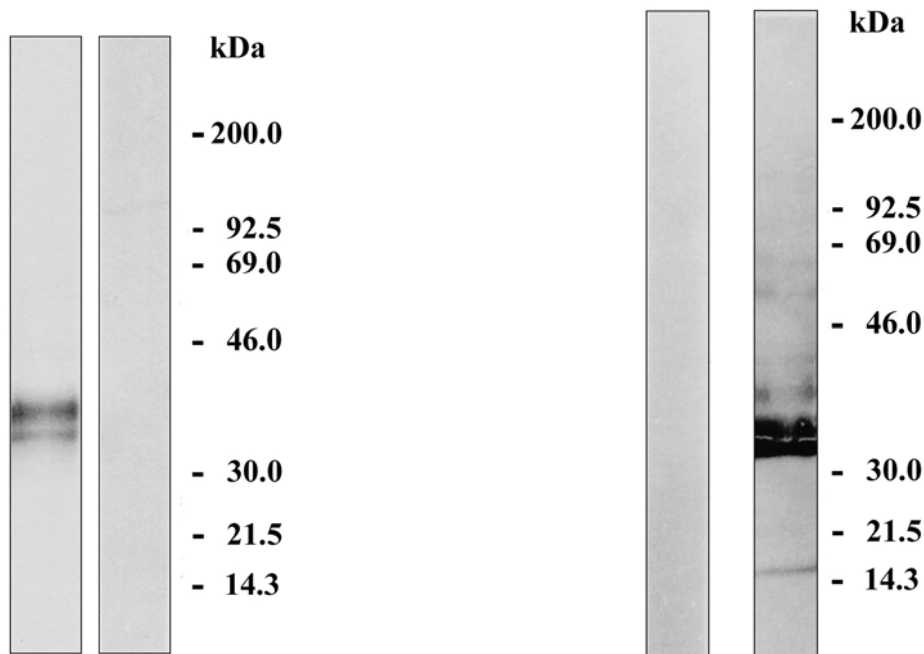


Fig. 2. Polyacrylamide gel electrophoresis of the antigenic fraction (AF) obtained by affinity chromatography from *Plasmodium falciparum* primary-infected patient plasma. The AF electrophoretic separation was performed in polyacrylamide-SDS gels at 7.5% under reduced conditions: **a** – 33-35 kDa antigenic fraction; **b** – normal plasma.

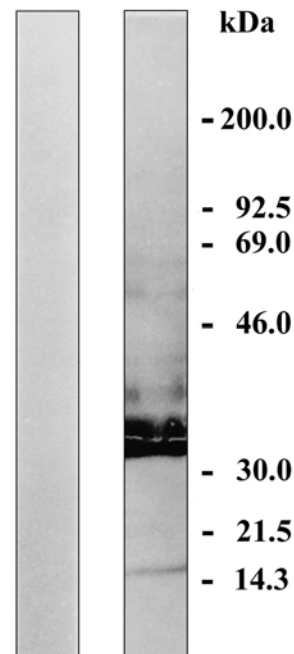


Fig. 3. Western blot analysis of the antigenic fraction (AF) present in *Plasmodium falciparum* primary-infected patient plasma linked to Sepharose-4B-IgG (hyperimmune selected) antimalaria. The AF electrophoretic separation was carried out in polyacrylamide-SDS gels at 7.5% under reduced conditions. The material transferred to nitrocellulose was revealed by incubation with: **a** – normal human serum/anti-human-IgG-peroxidase; **b** – hyperimmune serum/anti-human-IgG-peroxidase.

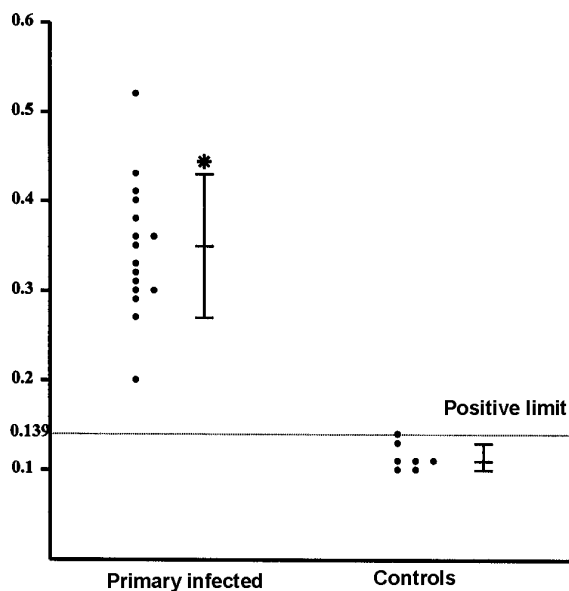


Fig. 4. Determination of IgG anti-antigenic fraction in the plasma of malaria patients by direct ELISA. Left: primary-infected patients, $n = 16$. Right: controls, $n = 7$. * $P \leq 0.01$.

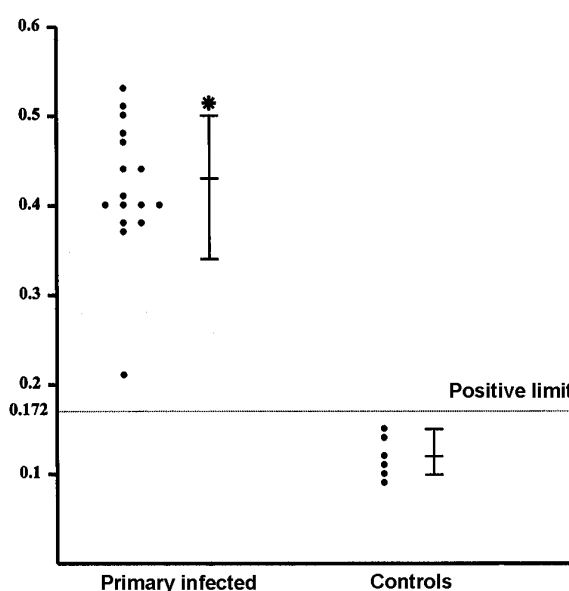


Fig. 5. Determination of IgG anti-antigenic fraction in the plasma of malaria patients by indirect ELISA. Left: primary-infected patients, $n = 16$. Right: controls, $n = 6$. * $P \leq 0.01$.

DISCUSSION

The antigen captured by the rabbit polyclonal anti-*P. falciparum* antibodies in the ELISA indirect tests provided important evidence about the parasitic nature of the antigen. The inclusion of anti-whole-parasite polyclonal antibodies has proved to be a valuable tool in the characterisation of this parasite antigen.

Other authors (Wilson and Ling 1979, Anders et al. 1983) have also found the main characteristics of exoantigens such as solubility, thermostability and molecular weight. In the present study some of these characteristics were verified for the 33-35 kDa exo-antigen of *P. falciparum*. Molecular weight comparisons among exoantigens are very difficult to procure because they can vary from 120 to 250 kDa (WHO 1984). Jepsen (1983) reported lower molecular weight ranging from 97 kDa to 37 kDa. In our work, a molecular weight of 33-35 kDa was found by SDS-PAGE. ELISA analysis searching for antigens in patient plasma showed the presence of 33-35 kDa molecules.

As was described in the Western blot results, the fraction retained by Sepharose-4B-antimalaria indicated the two bands of Mr 33-35 kDa in the plasma of *P. falciparum* primary-infected patients and recognised by hyperimmune anti-*P. falciparum* serum.

The thermostability study showed that this antigen is probably related to S antigens (Wilson and Ling 1979). The results obtained by direct and indirect ELISA indicate the presence of IgG antibodies against the 33-35 kDa proteins. The adequate correlation obtained with SDS-PAGE and ELISA indicates that 33-35 kDa antigens are closely related to total precipitating exo-antigens. Control eluate obtained by affinity chromatography tested in the SDS gel and the ELISA analysis were always negative, confirming the 33-35 kDa protein as a malarial antigen. ELISA is a technique that can be used to follow the dynamics of this antigen release into the patient plasma without the requirement to partially purify each sample. Several authors have demonstrated that antigens are released during the later stages of the parasitic cell cycle (Wilson et al. 1969, Wilson and Bartholomew 1975). We found that 33-35 kDa proteins are released during 24 hours period that corresponds to free merozoites and erythrocyte reinvasion.

Identification and characterisation of new malaria antigens should contribute to the improvement of malaria diagnosis in individuals and population groups, in anti-malaria vaccine evaluation and in the measurement of the impact of malaria control campaigns. Affinity chromatography using Ig-anti-malaria ligands of hyperimmune patients enabled the separation of the antigenic fractions retained by these specific antibodies, from plasma of the malarial patients selected for this study. The immune-adsorbents were pre-run by

Table 2. Percentages of residual antigenicity of heat-treated antigenic fraction, calculated as OD values obtained by ELISA ($\bar{X} \pm$ S.D. of 6 determinations; antigenicity of untreated fraction = 100%).

Time (min)	Residual antigenicity (%)		
	Temperature (°C)		
	60	80	100
0	100 \pm 6.0	100 \pm 6.7	100 \pm 6.6
2	96 \pm 5.8	89 \pm 5.8	89 \pm 5.0
5	93 \pm 5.0	83 \pm 6.4	81 \pm 3.5
10	98 \pm 5.0	90 \pm 4.0	
20	88 \pm 7.0	82 \pm 4.0	

affinity in a column of Sepharose-normal human serum to eliminate those autoantibodies directed against elements of the normal human tissues in the malaria patient plasma (Ritter 1993). Studies of *P. falciparum* cultures indicate that the antigens excreted by the intraerythrocytic schizont stage are immunochemically similar to those detected in host infected serum (Wilson and Bartholomew 1975, Ramos et al. 1989), but there are very few reports concerning their characterisation. Based on thermal stability, *P. falciparum* 33-35 kDa proteins may be classified within the group of the Type S excretion antigens. The only antigen with a relative mass of 33 kDa that has been found was described by Ramos et al. (1989). However, this antigen is different from the 33-35 kDa proteins since its antigenicity was damaged by treatment at 65°C, and it was classified as a Type La soluble antigen (Ramos et al. 1989). Its similarity to known Type S antigen and the presence of two bands (33-35 kDa) suggest that the antigen described here is definitely a different molecule to that of similar molecular weight characterised previously. If this antigen were to be used in a diagnostic assay, it would have the advantage of thermoresistance, so making it attractive to commercial development in a kit for use in tropical countries under extreme conditions of temperature.

In conclusion, the reactivity of 33-35 kDa proteins in ELISA assay suggests that this is a parasite-excreted antigen as it is not found in normal serum or in the serum of malaria patients without active infection. The characteristics of this soluble antigen, with good antigenicity and thermoresistance, indicate it could be useful as antigenic marker of active *P. falciparum* infections.

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