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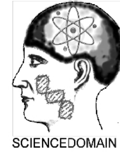

Humoral Response against Native *Plasmodium berghei* Hemozoin and Synthetic Hemozoin in BALB/c Mice

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Authors' contributions

Author LMS was responsible for the design of the study, preparation of antigens (PbHz and SHz), performing immunization experiments and immunological assays, data analysis and writing of the manuscript. Authors AA and ER contributed to preparation of the PbHz and SHz antigens, immunization experiments, immunological assays, and data analysis. Author NGD contributed to preparation of synthetic hemozoin and writing of the manuscript. Author JC contributed to preparation of synthetic hemozoin and author AG contributed to Immunoblotting experiments and data analysis.

Original Research Article

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ABSTRACT

Aims: Malaria is a disease caused by protozoan parasites of the genus *Plasmodium*. One of the malaria mechanisms of adaptation to the host is the digestion of hemoglobin by the trophozoite stage. This mechanism provides the amino acids needed by the parasite and is carried out during the erythrocytics schozogony phase, which results in the formation of in soluble pigment crystals named hemozoin (Hz). Hz is responsible for many of the immune pathological complications of malaria, given that this pigment accumulates in various organs in severe cases of the disease.
Here, we evaluated the humoral response in BALB/c mice against native *Plasmodium berghei* Hz (PbHz) and synthetic Hz (SHz).
Place and Duration of Study: Laboratory of Immunology of Infection Diseases. Department of Cell Biology, Simón Bolívar University, Caracas, Venezuela. This study

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was performed between January 2012 and June 2012.
Methodology: We determined the humoral response of SHz and PbHz by an enzyme linked immunosorbent assay (ELISA), using hyper-immune sera from mice experimentally infected with *P. berghei* or *Plasmodium yoelii*. In addition, SHz was evaluated as antigen by Western Blot and dot-ELISA.
Results: When SHz was employed as antigen, we showed by indirect ELISA that the sera from mice immunized with SHz generated higher titers than sera obtained from mice infected with either *Plasmodium* species. Moreover, the sera from human infections also recognized SHz as antigen, but showed a better recognition by dot- ELISA or Western Blot than by indirect ELISA.
Conclusion: In summary, our results indicated that SHz can be used as a rapid and successful diagnostic antigen for natural malaria infections by indirect ELISA, dot-ELISA and Western Blot techniques.

Keywords: Hemozoin; humoral response; Plasmodium berghei; ELISA and dot- ELISA.

1. INTRODUCTION

Malaria is a disease caused by protozoan parasites of the genus *Plasmodium*, being the species *P. falciparum, P. vivax, P. ovale*, *P. malariae* and *P. knowlesi* responsible for infecting humans, even though *P. falciparum* causes the most severe manifestations of this disease. *P. knowlesi* is the most recent infection reported on humans [1]. World Health Organization reported in 2008 that half of the world's population is at risk of being infected by malaria [2]. Malaria parasites have multiple mechanisms of adaptation to the host; one of the most important is the digestion of hemoglobin. Hemoglobin ingestion and digestion vary with the intraerythrocytic stage. In very early ring stages, the parasite abuts the erythrocyte membrane and may obtain nutrients directly through passageway or "metabolic window" in an apposed membrane [3,4]. There is evidence that host cell cytosol is taken up by micropinocytosis, although hemoglobin digestion and hemozoin production are minimal at this stage [5]. In the more metabolically active trophozoite stage, hemoglobin catabolism is a complex and efficient process. Host cell cytosol is taken up through the cytostome, a pear shaped structure, that is formed by invagination of the parasitoforous vacuolar membrane and parasite plasma membrane [3,6,7].

The food vacuole is an acidic (pH 5.0-5.4) degradative organelle [8,9]. The food vacuole appears devoted to the catabolism of hemoglobin and is probably not involved in general protein degradation [6].

To date distinct proteases have been purified from food vacuoles and show to act in a semiordered fashion *in vitro* to degrade the hemoglobin tetramer. Two aspartic proteases, termed plasmepsins, appear to initiate the degradative process. Acystein protease, falcipain, plays a vital downstream role. A metalloprotease, falcilysin, acts even further downstream [10,11,12,13]. *In vitro*, plasmepsins I and II are capable of cleaving native hemoglobin, whereas under nonreducing conditions, falcipain will only cleave globin that has been denatured. Falcilysin cleaves at polar residues in contrast to the upstream enzymes, which prefer hydrophobic sites [13]. The complementarity of these specificities ensures efficiency of proteolysis.

Besides amino acids, the hemoglobin degradation process release free heme, a toxic by-product. Free heme can cause enzyme inhibition, peroxidation of membranes, production of free oxygen radicals, and the impairment of leukocyte function [14,15]. *Plasmodium falciparum* has little or no hemeoxigenase (the enzyme used by vertebrates to catabolize heme), although it has been reported in other species [15]. All *Plamodium* species have an unique capability to detoxify heme in the food vacuole by formation of the crystalline structure called hemozoin or malarial pigment [16,17].

There is evidence that hemozoin is responsible for many of the complications in the immunopathology of malaria and in severe cases hemozoin accumulates into leukocytes and organs such as the spleen, liver and brain [18].

Also, hemozoin can be produced synthetically (SHz) by a chemical process in which hemin chloride is incubated under acidic conditions [19]. Some studies have reported that both synthetic and native hemozoin crystals have similar X-ray diffraction patterns [17].

The host immune system is stimulated by hemozoin on macrophages and dendritic cells to produce pro-inflammatory cytokines and chemokines such as TNFα, IL-6 and IL-12. This process is mediated by a Toll-like-receptor-9 (TLR 9), which activates an innate immune response leading to the production of these cytokines [20,21].

Very little is known regarding the stimulation of the humoral response generated by hemozoin. In the present study, we evaluated the humoral response against synthetic hemozoin (SHz) using BALB/c mice. We standardized an Enzyme linked immunosorbent assay (ELISA) using either native hemozoin extracted from *P. berghei*-infected erythrocytes or SHz as antigens. Additionally, we probed by Western blotting and dot-ELISA that SHz serves as a diagnostic antigen for natural infections.

2. MATERIALS AND METHODS

2.1 Hemozoin Preparation and Quantification

Synthetic hemozoin was prepared as described by Frita and colleagues [22]. SHz was resuspended in phosphate buffer saline (PBS) before use.

Native *P. berghei* ANKA strain Hemozoin (PbHz) was purified from schizonts. Ten female BALB/c mice were infected with a cryopreserved sample of *P. berghei* ANKA strain. This sample comes from the Laboratory of Immunology of Infectious Diseases at the Simón Bolívar University which was originally donated by Dr. Oscar Noya from Central University of Venezuela (Caracas, Venezuela). When the parasitemia reached about 40%, mice were sacrificed and parasites were isolated by centrifugation at 800g by 10 minutes through a 60-80% percoll gradient. Parasites (schizonts) were washed with PBS and centrifuged at 500g for 5 minutes. Then, the parasite pellet was resuspended with 1% saponin in ultrapure water (20 mL) for 10 min to lyse the parasites. The resulting lysate was centrifuged at 8.000g for 20 min and the PbHz crystals were obtained in the pellet. The PbHz pellet was washed four times with PBS and finally re suspended in 1 mL PBS. The concentration of PbHz was determined as heme content after solubilization in 20 mM NaOH for 1 hour at room temperature [22,23]. The heme concentration was determined at 400nm by the Quanti Chrom Heme Assay Kit from BioAssay Systems.

2.2 Synthetic Hemozoin Antigen Preparation to Inoculate BALB/c Mice

Since the malaria pigment is highly insoluble in water and organic solvents are not recommended for mice inoculations, the SHz was prepared by sonication [24]. In order to improve the solubilization of the antigen, the SHz (1mg/ml) was dissolved in 0.85% NaCl and 0.2% SDS, and then sonicated 6 times at 8 watts for 1 minute each [25].

2.3 Preparation of Hyper Immune Ascitic Fluid Sera in Mice

Ten weeks-old female BALB/c mice, bred under specific-pathogen-free conditions, were used for the preparation of hyperimmune sera. Per mouse, SHz was inoculated at a concentration of 100 µg/µl in the presence of 100 µl of either Freud's Complete Adjuvant (FCA) for the first inoculation, and Freund's Incomplete Adjuvant (FIA) for the subsequent four boosters, and 8 days was the time between each boosts. This mixture was administered by intraperitoneal injection in all cases. Another group of ten healthy mice were used as negative controls. Water and food were allowed *ad libitum*. After the last inoculation, animals were sacrificed under deep anesthesia with ether and exsanguinated by cardiac puncture to collect the serum. A similar procedure was implemented for the preparation of hyperinmune sera against PbHz.

In order to obtain ascitic fluid containing antibodies against SHz, a group of six animals, 10 weeks-old female BALB/c mice were inoculated intraperitoneally four times with100 µg/µl of the antigen in the presence of 100 µl FCA, and 8 days was the time between each boosts. The ascitic fluid (AF) was collected and purified as indicated by Harlow and Lane [26].

2.4 Indirect Enzyme-linked Immunosorbent assay (ELISA)

Indirect ELISA was employed to detect the antigenicity of SHz and PbHz using the hyperimmune sera obtained from the inoculated mice. As positive controls, sera from experimentally infected mice with *P. berghei* and *P. yoelii* were also used. Ninety-six well plates (Nunc) were coated with 100 µl of SHz or PbHz at various concentrations (5,10, 20 or 30 µg/ml) per well. Antigens were dissolved in coating buffer (0.1 M sodium carbonate/bicarbonate pH 9.6), and plates were incubated overnight at 4°C and then, washed three times with PBS containing 0.005% v/v Tween 20 (PBS/T). Plates were then blocked by the addition of PBS containing 0.01, 0.015 or 0.02% v/v bovine serum albumin (BSA) for 60 minutes at room temperature, followed by further washing with PBS three times. One hundred µl of a dilution of 1:200 of serum were added to each well and incubated at 37ºC for 1 hour. Plates were then washed three times with PBS and 100 µl/well of horseradish peroxidase (HRP)-conjugated goat anti-mouse poly-valent immunoglobulin (IgG; H+L)(Sigma) were added at a dilution of 1:2000 or 1:3000. After incubation for 1 hour at 37ºC, plates were washed again with PBS. The antigen-antibody reaction was detected by the addition of 100 µl of azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS, Sigma) as substrate, in 0.05 M phosphate-citrate buffer (pH 5.0) containing 0.05% w/v H_2O_2. The reaction was stopped by the addition of 50 µl 2M H_2SO_4 per well. The plates were read at 405 nm in a micro plate reader (Bio-RAD i-Mark) using the Titer-soft Software (Flow) to measure the absorbance. Ten uninfected or normal mice sera (NS) were used as negative controls.

The final settings of the indirect ELISA tests were determined using different concentrations of antigens and conjugates, and the conditions that showed the most significant differences

between NS and hyper-immune sera were selected. The test cut-off point was determined using the mean of the absorbance values from ten uninfected sera plus three standard deviations [27].

2.5 Sodium Dodecyl Sulphate-polyacrylamide gel Electrophoresis (SDS-PAGE)

SHz samples (10 µg) were analyzed by SDS-PAGE on 15% w/v polyacrylamide gels as described by Laemmli [28]. Electrophoresis was carried out at 120 V for 1 hour using a Mighty Small II vertical slab gel unit (Bio Rad Instruments). The pre-stained protein ladder from Invitrogen (170-10 kDa) was used as markers.

2.6 Immunoblotting and dot-ELISA

Following SDS-PAGE, SHz (10 µg) was electrophoretically transferred to nitrocellulose paper (NCP, Schleicher & Schuell, 0.45 µm pore size), at 120 mA in a transblotting chamber (Bio-Rad, Instruments), for 1 h at 40ºC as described by Towbin et al. [29]. Since SHz possesses a positive charge, the transfer was carried out in an inverted manner, placing the gel on the side of the positive electrode. For dot-ELISA, SHz (5 µg) was directly spotted onto nitrocellulose membranes and air-dried at room temperature.

Nitrocellulose filters used for Western blot and dot-ELISA were blocked by incubation with a solution of 3% w/v non-fat milk in PBS for 30 min at room temperature, and washed three times with PBS/T containing 0.05% v/v Tween-20. Membranes were then incubated for one hour at room temperature with a solution of the hyper immune serum as primary antibody (dilution 1:200 in PBS). Then, washed three times with PBS and incubated in a solution of affinity purified goat anti-mouse immunoglobulin conjugated to horse radish peroxidase at a 1:2000 dilution (Sigma) for 1 hour. The blots were washed again three times with PBS and antibody binding was detected by incubation in a solution of 3 mg/ml 4-chloro-1-napthol in methanol, mixed with 50 ml of 50 mM Tris-HCl pH 7.5 and 30 µL of 30% H_2O_2. The color reaction was stopped by washing with H_2O.

2.7 Statistical analysis

Each experimental value is presented as the mean of three replicates ± standard deviation. Once normality and homogeneity criteria were satisfied, statistical analyses were carried out by a one-way ANOVA, taking $\alpha = 5\%$ ($p<0.05$) as significant.

3. RESULTS AND DISCUSSION

3.1 Results

Initially, we standardized an indirect ELISA using SHz as antigen and sera from mice inoculated with SHz. The anti-IgG conjugate was diluted 1:3000 as previously determined [27]. Fig. 1 shows the evaluation of sera from two groups of SHz-immunized mice using either 20 or 30 µg/ml of SHz as antigen, under two concentrations of BSA (0.03 and 0.04%) as blocking solution. Following the ELISA standardization, the optimal conditions found were 20 µg/ml of antigen and 0.03% of BSA to block the plates.

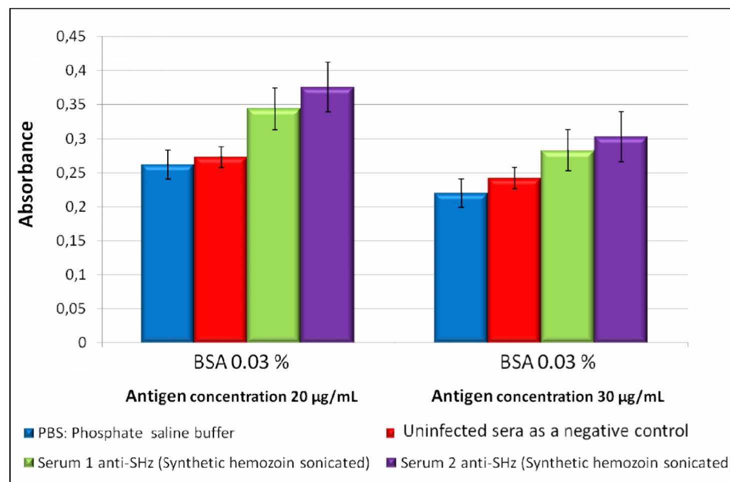

Fig. 1. Indirect ELISA using 20 and 30 µg/ml of sonicated SHz as antigens and 0.03% of BSA to block the spaces not occupied by the antigen. Sera anti-SHz1 and 2 represent the mean values of absorbance from two different groups of immunized mice, each group contained tree mice. Bars represent the standard error for each treatment.

Polyclonal anti-SHz antibodies from different sources were examined by ELISA to establish the immunogenicity of SHz (Fig. 2). All evaluated IgGs recognized the SHz antigen with high titers. In particular, hyperimmune sera (Fig. 2, red and green) which resulted in an absorbance about 4 times higher than normal sera. An absorbance of about 3 and 4 times higher than uninfected was obtained when sera from *P. berghei* and *P. yoelii* infected mice were employed, respectively.

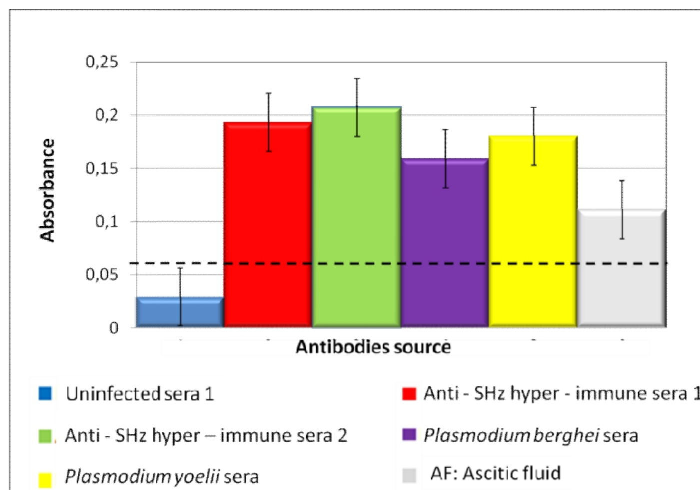

Fig. 2. Evaluation of different sources of polyclonal antibodies against SHz. ELISA was carried out using 20 µg/ml of SHz. Bars represent the mean of the absorbances read at 405 nm, and the discontinue line symbolizes the cut-off value (0.05 D.O). The cut-off was determined using era from 10 normal or uninfected mice.

We also prepared native hemozoin from *P. berghei* (PbHz) as soluble antigen in order to compare both sources of hemozoin by indirect ELISA. Firstly, the optimal concentration of PbHz was determined. Fig. 3 shows the results obtained when PbHz was utilized as antigen against the different sources of polyclonal anti-SHz antibodies. As illustrated in Fig. 3, the highest difference in absorbance value between the hyper immune sera and NS was acquired when 10 µg/ml of PbHz was used.

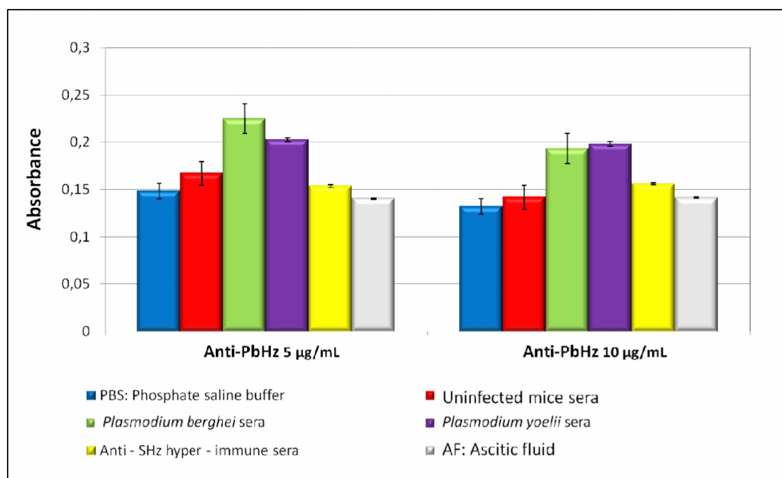

Fig. 3. Indirect ELISA using PbHz (5 or 10 µg/ml) as antigen, and 0.03% of BSA to block the plates. Bars represent the mean values of absorbance (Sera 1 and 2 anti-SHz) from two different groups of immunized mice, each group containing three mice.

Pb Hz and SHz were also evaluated by indirect ELISA in order to discriminate which source of hemozoin was better recognized by the polyclonal antibodies that were generated. Both antigens were assayed with sera and ascitic fluids from mice inoculated with SHz, and with sera from mice infected with either *P. yoelii* or *P. berghei* at a parasitemia of 30 to 50%. As seen in Fig. 4, the purified antibodies from the as citic fluid (AF) showed the lower absorbance values, when compared to the rest of the antibody samples in ELISA with PbHz as antigen. This lower response was probably due to the low yield of antibodies obtained following their purification. The values of absorbances with the different sources of antibodies were about 4 and 7 times higher than uninfected mice sera, when PbHz and SHz were utilized as antigens, respectively (Fig. 4). Although both PbHz and SHz were recognized by all antibody samples, our results clearly showed that SHz was a better antigen than PbHz for diagnostic purposes.

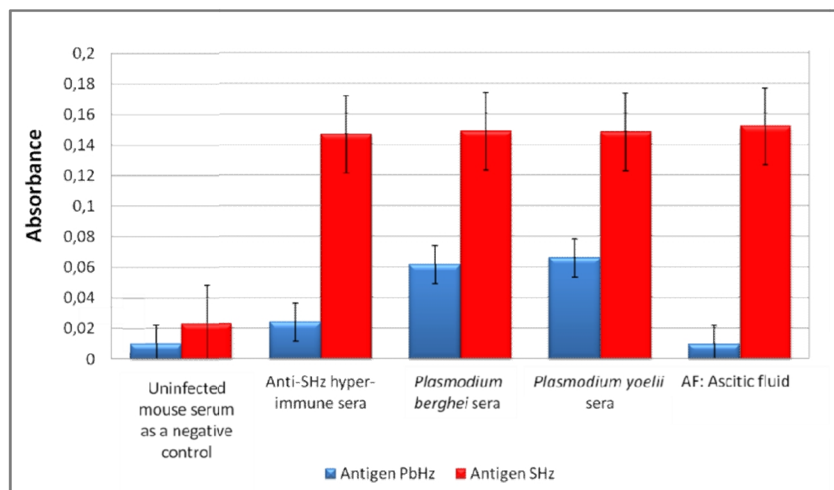

Fig. 4. Detection of polyclonal antibodies against PbHz (blue bar) and SHz (red bar) antigens by indirect ELISA. PbHz and SHz were used at a concentration of 10µg/ml and plates were blocked with 0.03% BSA. Sera were diluted at 1:200 and the conjugate was diluted at 1:3000. Bars represent the mean values of absorbance from different antibodies sources.

ANOVA tests showed the distribution of the results when normal and positive sera were employed were statistically different ($p<0,05$).

Following SDS-PAGE, the synthetic SHz migrated on a 15% polyacrylamide slab gel as a band that corresponded to the β-haematin (Fig. 5). The SHz band possessed the characteristic brown color of the malaria pigment. Immunoblots showed that sera from SHz-inoculated mice recognized the band related to β-haematin (Fig. 6). Additionally, sera of infected mice with *P. berghei* also recognized the SHz band (Fig. 6).

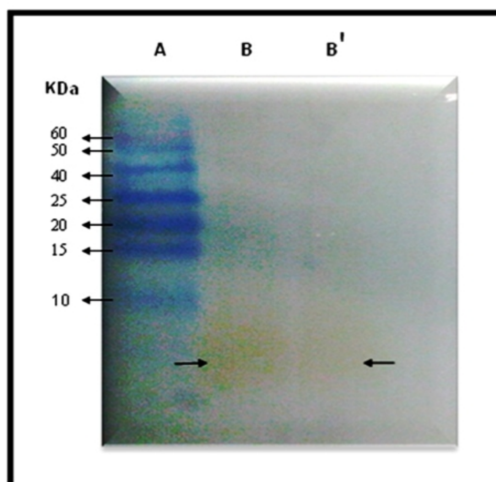

Fig. 5. SDS-PAGE of synthetic hemozoin on a 15% polyacrylamide slab gel. A, molecular weight markers; B and B', duplicate samples of SHz.

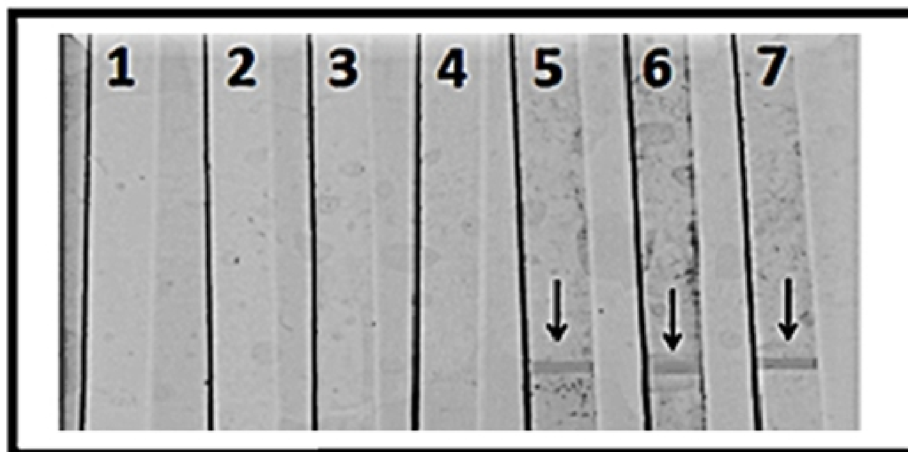

Fig. 6. Recognition of SHz by hyper immunesera from SHz-inoculated mice. Lanes 1 and 2, immunoblots developed with uninfected serum; lanes 3 and 4, immunoblots developed with sera from mice inoculated with PBS as a control; lane 5, immunoblot developed with sera from mice infected with *P. berghei*; lanes 6 and 7, immunoblots developed with sera from mice inoculated with SHz. Nitrocellulose filters were developed with chloronaphthol. The positive reactions are indicated with arrows.

Since our results indicated that the SHz was recognized by the hyperimmune sera, we also evaluated the response of the SHz antigen by dot-ELISA as a rapid diagnostic test (Fig. 7). As shown in the figure, SHz was also detected by dot-ELISA (Fig. 7, strips C,D, and F). These results suggest that dot-ELISA using SHz as antigen could be used to detect malaria infection in humans, especially in areas of high malaria prevalence, where this test can be a good alternative for malaria diagnosis. This test is easy and rapid and can be used to detect antibodies from the host without sophisticated instruments.

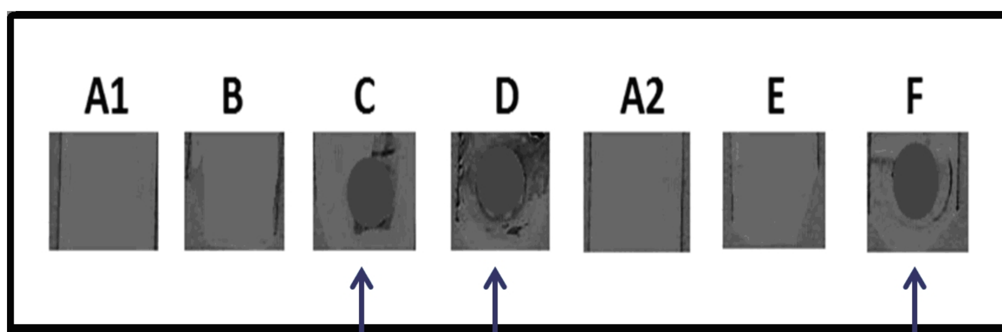

Fig. 7. Detection of SHz by dot-ELISA. SHz was spotted onto nitrocellulose membranes (0.22 µm). A1 and A2,dots developed with sera from mice inoculated with PBS; B, dot developed using healthy mouse serum; C, dot developed using hyper-immune sera from mice immunized with SHz; D, dot developed with sera from mice infected with *P. berghei*; E, dot developed with healthy human serum; F, dot developed with a *P. falciparum*-infected human serum.

3.2 Discussion

The native hemozoin and synthetic hemozoin act similarly when activating the cell cytokine response [23]. Our results showed that synthetic and native hemozoin could generate a humoral antibody response against both molecules because the antibodies could recognize SHz and PbHz in serological tests. In this study, carried out by ELISA, dot-ELISA and Western blot, we showed evidences that revealed the presence of antibodies against SHz and PbHz.

The literature describes differences in synthetic Hemozoin molecules depending on the origin of their chemical synthesis and this could suggest that the antibodies response could be different due to Hz molecules presenting different protein structures [22]. However, our results demonstrated that SHz presented a similar structural conformation with PbHz, because antibodies from hyper-immune sera recognized both hemozoins in serological assays. This suggests a similarity in the chemical structures, at least in the recognition of molecules by antibodies. We also performed an experiment by an analytical chemistry technique as High Pressure Liquid Chromatography (HPLC) to determine the integrity of HzS molecule after sonication (data not shown). Our results showed that the integrity of molecule of HzS after the sonication process was maintained. This result was similar to that reported by Orjih and Fith [30].

Once established the conditions for the ELISA assay the cut-off value for test was determined using the average of the absorbances obtained from ten healthy mice sera ± two standard deviations. The OD value obtained was 0.06, which means that any absorbance value above this cut-off is considered a positive hyper immune serum in ELISA assays. These results demonstrate that immunization of mice was effective to obtain positive absorbance with this serological test.

In order to compare the SHz and PbHz molecules, an ELISA assay was performed under the same standardized conditions, finding that ascetic fluid or sera from immunized mice (antibodies to synthethic Hemozoin) had higher values of absorbances by ELISA with SHz as antigen than antibodies from mice infected with *Plasmodium berghei and P. yoelii* (antibodies to native Hemozoin). These results showed that SHz is a good antigen for ELISA tests.

Other authors studied the native hemozoin by electron microscopy, revealing that the pool obtained from purified Hz contained cellular debris, impliying that the purification was not complete and there might be contamination which interferes in assays using this hemozoin as antigen. In consequent the synthetic hemozoin is obtained chemically and it is free of cell membranes or other cellular debris [31]. We showed the haematin migration by SDS-PAGE gel and the recognition of this molecule by hyper-immune sera using Western blots. Our results agree with other authors reports, who demonstrated that SHz has a potent adjuvant effect with some malaria antigens, when immunized mice and obtained IgG response [18].

A rapid assay was performed to check for the formation of antigen-antibody complexes between the SHz and hyper-immune sera by dot-ELISA. These results demonstrated that the dot-ELISA could be a simple, quick and economical test using SHz as antigen. Moreover, this kind of the test could be used in endemic areas, due to the fact that dot-ELISA can be easily performed and interpreted to diagnose the disease.

This trial demonstrates the recognition of natural *P. berghei* infections using SHz as antigen. This suggests that the SHz could be a useful diagnostic tool in malaria endemic areas. However, the most recent research has led to the detection of Hz in leukocytes and parasite erythrocytes using flow cytometry [22,32,33], but this technique is not available in endemic areas when a rapid diagnosis is necessary.

This work represents one of the few evidences that showed an experimental immunization of the SHz molecule in a murine model and its evaluation in serological assays proving that it could be an useful tool for malaria diagnosis in endemic areas where active transmission is possible.

4. CONCLUSION

Synthetic hemozoin showed to be a good antigen for the evaluation of a humoral response. Immunized BALB/c mice with SHz produced antibodies which can be detectable in indirect ELISA, in a dot-ELISA and Western blot assays. Sera from immunized mice with SHz produced higher titer values by ELISA than PbHz as antigen and sera from mice infected with *Plasmodium* species, such as *P. berghei* and *P. yoelii*, demonstrating the immunogenicity of this molecule when performing an indirect ELISA.

In addition, serum of a patient infected with *Plasmodium falciparum* recognized SHz by dot-ELISA in a clear manner, and the same results were obtained with sera of mice infected with *P. berghei* showing an evident precipitate reaction, suggesting that the dot-ELISA assay can be an useful tool in the diagnosis of malaria.

CONSENT

Not applicable.

ETHICAL APPROVAL

All the animal experimentation was approved by the ethical review board of Simón Bolívar University.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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