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Immunization with Purine Salvation Pathway Recombinant Enzymes Induces the Production Of Anti- *Schistosoma Mansoni* Immunoglobulines

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Abstract – **Schistosomiasis, a disease caused by the trematodes of the *Schistosoma* genus, affects about 240 million people worldwide. The Praziquantel (PZQ) is the drug used to treat this disease. However, reports of resistant strains reinforce the need to develop a new schistosomicidal drug. The study of new drugs and vaccines that can contribute to the control of this pathology becomes urgent. A new approach can be held by the study of the following *Schistosoma mansoni* enzymes: purine nucleoside phosphorilase 1 (PNP), hypoxanthine guanine phosphoribosyltransferase (HGPRT) and adenylate kinase 1 (ADK). The parasite, incapable of synthetizing purine nucleotides through the *de novo* pathway, has multiple mechanisms to incorporate purine bases through the purine salvage pathway. Our goal was to assess, through immunoenzimatic assay (ELISA-indirect), the production of total IgG, IgE and IgG2a in the plasma after immunization with the PNP, HGPRT and ADK enzymes, using the *S. mansoni* cercariae – infected murine model. Our results showed that the immunization in Balb/c mice with the enzymes mentioned above induced production of the immunoglobulines at the 48th and 85th days, post-infection. Thereby, new assays must be made for a better assessment on how these enzymes modulate an immune response.**

Keywords: *Schistosoma mansoni*, **immunization, recombinant enzymes**, **immunoglobulines**

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I. INTRODUCTION

According to the World Health Organization (WHO), on tropical and subtropical areas, schistosomiasis is the second parasitic disease of greater prevalence, in terms of morbidity and mortality, surpassed only by malaria. Currently, approximately 779 million individuals live at risk areas, in 77 countries where schistosomiasis caused by different species is endemic and 55 countries endemic only to the schistosomiasis mansoni, including Brazil [1]. Schistosomiasis is caused by a platyhelminthes of the *Schistosoma* genus, which affects the poorest communities of underdeveloped and emergent countries [2]. In the last 30 years, no drugs have been developed for schistosomiasis [3,4] being the administration of Praziquantel (PZQ) the current treatment for schistosomiasis mansoni recommended by WHO. However, PZQ is practically inactive against immature schistosomula, besides presenting mutagenic and teratogenic collateral effects and favoring the resistance growth of the lineages [5]. The *S. mansoni* is a complex organism. The study of its genetic and biochemical aspects, besides providing information about its biology, is a great source of interest to the investigations of new vaccines and drugs [6]. During its development, the *S. mansoni* is exposed to different micro-environments, what makes it go through morphological modifications and physiological transformations, as the change in the expression of some genes. The parasite is covered by a syncytium layer, called tegument. The tegument has some proteins that act as a barrier, providing protection to the plasmatic membrane of the parasite against the attack of the immunity of the host [7]. The characterization of the specific proteins of the tegument is especially interesting because these proteins are the first to be presented to the immune system of the host, what makes them a possible target to the development of vaccines [6]. The parasites induce a low natural immunity and they are able to evade or resist to the specific immune responses of the hosts. The dissemination of the disease are even more accentuated by the lack of infrastructures of water resources, absence of sanitary education, and deficiency in people's education concerning the domestic and hygienic habits [8]. Having this context in mind,

the study of the enzymes that participate in the *S. mansoni* essential pathways can be quite promising to develop specific tools to control this disease. The nucleotides of purines are required in all the organisms to DNA and RNA synthesis. These nucleotides can be obtained by the *de novo* synthesis or by the salvage pathway of the purines. The *de novo* pathway uses simple precursors to the synthesis of various nucleotides. On the other hand, the salvage pathway of purines is the reutilization of pathways by which the organism can satisfy its requirement of pre-formed purines [9]. One of the advantages of the salvage pathway is the fact that the *de novo* pathway requires a big quantity of energy to the purine bases [10]. Different from its human host, the *S. mansoni* depends entirely on the salvage pathway of purines to feed its demand of purine bases [11]. Another factor that contributes to this research is the fact that the literature does not have sufficient data to respond to the questions about the mechanisms of action of these enzymes. Concerning the enzymes that act in an intracellular manner, it was not found scientific works that can prove their exposition or secretion by the *S. masoni*. Thus, essential enzymes to the parasite can be promising study subjects. The enzymes purine nucleoside phosphorilase 1 (PNP) (EC 2.4.2.1), hypoxanthine guanine phosphoribosyltransferase (HGPRT) (E.C 2.4.2.8) and adenylate kinase 1 (ADK) (E.C 2.7.4.3) are cytoplasmic enzymes and its exposure in the adult worm's tegument were not yet confirmed. However, there are studies which reports secretion of one of the enzymes constituent of the *S. mansoni* purine salvation pathway, the nucleoside-diphosphate kinase (NDPK), by prokaryote pathogens, as demonstrated by Zaborina [12], in which was observed secretion of this enzyme by *Mycobacterium bovis* e *Pseudomonas aeruginosa* bacteria, as well as Punj [13]. Recently a group of collaborators with this study (IFSC-USP) showed that the structures of the PNP, HGPRT and ADK enzymes do not present any risk regarding the immunization in human beings: the highest percentages of homology were between 30-50% of identity with the same in human beings. Nevertheless, in order to happen an immunological response, it is necessary to have some accessible factors (antigens exposed) and these external antigenic factors are the first to be recognized; with the time being, with the destruction of some of the antigens, more internal factors can be expressed or excrete physiologically. The innate immune system is able to recognize antigenic parts present in the pathogen surfaces known as PAMPs (Pathogen-Associated Molecular Pattern*)* and then the first patterns of immunological response against the pathogen is initiated. The pathogenesis of the schistosomiasis mansoni is dependent on the human interaction with the helminth. Concerning the *S. mansoni*, the strain, the evolutive phase, the intensity and the number of infections are important. In relation to the host, the genomics constitution, organ predominantly damaged, feed pattern, ethnicity, reactivation of the disease, specific treatment, other infections. The acute phase is divided into two evolutive periods: the pré-patent (before the oviposition and the post-patent after the oviposition. Although the immunological mechanisms involved in the response of the acute infection are not yet completely elucidated, some investigations in murine models showed that there is in the beginning a predominance of an immune response type Th1, substituted by Th2 in the post-patent phase[14]. During the pre-patent phase, plasmatic levels considered of tumor necrosis (TNF) and of Interleukins 2 and 6 produced by the mononuclear cells are detected [15]. It is believed that the immune response type Th1 is responsible for the damages in the tissues and the clinic manifestations in the acute phase. Another interesting aspect is the cellular cytotoxicity dependent on the antibodies (ADCC), with effector action on schistosomula. Recently the Oswaldo Cruz Institute (IOC), located in Rio de Janeiro, Brazil developed and patented the first vaccine to the schistosomiasis in the world. The vaccine, approved by the National Agency of Sanitary Supervision (ANVISA/Brazil), proved to be safe and able to immunize human beings against the disease. It also proved to be efficient against fascioliasis and it can be used in the future to avoid the contamination by other types of worms. As other vaccines, the national dose was produced from an antigen Sm14, being part of the fatty acid-binding proteins [16]. Besides the schistosomiasis, many other studies with antigen purified from other parasites are being tested in other experimental models as candidates to the immunizations. Some of them present promising results, as the one that uses the model with primates where the immunization with the protein MSP1 of *Plasmodium cynomolgi* resulted in high levels of antibodies and the decrease of the blood parasite charge after the challenge [17]. The study by PENG [18] that showed in serums of rabbits infected with cercarie *Schistosoma japonicum* a positive immune response with the enzyme adenilato kinase, enzyme that also participates in the salvage pathway of purines. In 2008, the RNAi technique reinforced the importance of the enzyme (HGPRT) to the survival of the parasite. Small RNAs of interference (RNAis) were produced against HGPRT, injected after 70 days of infection with cercariae in mice. The number of worms was counted six days after the injection. The total number of parasites was reduced in approximately 27% after the treatement [19]. The development of a vaccine, together with the treatment, would be a promising way of fighting and controlling schistosomiasis [20]. Two kinds of pathways lead to nucleotide synthesis in the living beings: *de novo* and *salvation* pathways. The *de novo* pathways for purine and pyrimidine biosynthesis are similar in practically every living organism and have as metabolic precursors: amino acids, ribose 5-phosphate, CO_2 e NH_3 [21]. The salvation pathways are the reutilization of nucleosides (without the phosphate group) that were released from the break of nucleic acids. These two kinds of pathways are important for the cellular metabolism [21, 22]. Thus, one of the advantages of the salvation pathway is that it requires less amount of energy for purine bases synthesis, compared to the *de novo* pathway. In *S. mansoni*, [11] have demonstrated, in their work about purine metabolism, a fast adenine incorporation in nucleotides and were not able to establish 14C-glycine and 14C-glucose incorporation in the purine ring. This fact shows great dependence of an external supply of pre-formed bases for nucleotide synthesis, as shown by the loss of the purine synthesis of the *de novo* pathway. The PNP is an enzyme that reversibly catalyzes the phosphorylase purine nucleoside in order to generate the purine base and ribose-1- phosphate and it has been descripted as participant in the *S. mansoni* salvation purine pathway with the sole purpose

of supplying purine bases used in the ADN and RNA synthesis [11,23] . It is important to notice that, in *Schistosoma* adult worms [23] and schistosomula (immature form) [24], unlike its mammal hosts, the worms are not able to synthesize purine nucleosides and depend solely on the salvation pathway to its purine necessities. Two isoforms were identified in the parasite's genome, named SmPNP1 e SmPNP2. The SmPNP1was the first salvation pathway enzyme which was cloned, expressed, purified, crystallized and had its structure resolved [25,26]. The SmPNP1 has 287 amminoacids, being a trimer in solution and in crystal form. A second coding gene to another isoform for the SmPNP was identified on the *S. mansoni* Genome Project: The SmPNP2, which has 61,8% of identity, when compared to SmPNP1 and coded to a protein with 299 amminoacids, being greater than SmPNP1. The HGPRT enzyme catalyzes the reversible phosphorylation from hypoxanthine and guanine to inosine monophosphate or guanosine monophosphate, respectively, and pyrophosphate using as phosphate and ribose donor the 5-phosphoribosyl-diphosphate (PRPP). Studies on *S. mansoni* schistosomula indicates that organisms basically depends on the purine bases retrieval in order to ensure its purine nucleotide needs [27]. Due to limited interconversion between adenine and guanine nucleotides, the *S. mansoni* HGPRTase provides the main sources of guanine nucleotides for the parasite. The HGPRT enzyme has been found usually as a dimer in solution on *Tritrichomonas foetus* (HGXPRT) [28] *Trypanosoma cruzi*, *Trypanosoma brucei*, *Leishmania donovani* [29] and *Leishmania tarentolae* [30] organisms. It is found in the tetramer form in solution on *Escherichia coli* (HPRT) [31], *Toxoplasma gondii* (HGXPRT) [32], *Plasmodium falciparum* (HGXPRT) [33] and on human enzyme [34]. Three homologous genes for the HGPRT (Smp_148820, Smp_168500, Smp_103560) were found at the parasite's genome, which are related with the parasite's different life stages. This enzyme has 48% of identity, when compared to the human homolog and demonstrated several properties that distinguishes it from mammal's enzymes [27].

Adenylate Kinase (ADK) is an enzyme that is involved in cellular homeostasis, energetic metabolism and nucleotide synthesis. The enzyme catalyzes the ATP (Adenosine triphosphate) + AMP Adenosine monophosphate *ƒ´* 2 ADP (Adenosine diphosphate) reaction. It is known that several ADK are found in mammals, as the skeletal muscle is particularly rich in ADK1, main provider of isoforme citosolica.[35]. There have been reports that its deficiency in humans may cause light Hemolytic anemia [36]. The ADK1 (Smp_071390) codes to a protein with 197 amminoacids and has 54% of identity, when compared to the human ADK1

The study of Peng [18] has shown that immunization with the *Schistosoma japonicum* ADK enzyme was capable of stimulating the antibodies production against *S. japonicum*, revealing a positive immune response post-immunization. Thus, reinforcing this hypothesis, our study aimed to assess whether purine salvation pathway enzymes (PNP, HGPRT and ADK), when used as immunizing, associated to the aluminum hydroxide adjuvant, stimulate a specific immune response which favors the *S. mansoni* antigens recognition. Hence, our study, in a novel approach, intends to assess whether *S. mansoni* PNP, HGPRT and ADK enzymes can, in future, be candidates for a vaccine against this disease, since that, to date, no study has been made using such enzymes, isolated from *S. mansoni*, in mice immunization during experimental schistosomiasis mansoni. Thereby, we state the hypothesis that these enzymes, by mechanisms yet unknown, seem to modulate the infection by *Schistosoma* in different species of rodents, used currently as experimental models of this disease. These facts support the hypothesis that the proposed enzymes, although related to the DNA of the parasite, can be expressed in the *S. mansoni* membrane and excrete by some pathway, what can cause a specific activation during the schistosomiasis.

II. MATERIAL AND METHODS

A. Obtention of PNP1, HGPRT and ADK1 recombinant enzymes

The recombinant enzymes were synthesized by the insertion of plasmids in bacterial cultures. To do so, the protein expression methodology was used. The enzymes were purified through the method of affinity chromatography. The used methodology for the obtention of the PNP1 enzyme was according to Pereira [25] and, for the obtention of the HGPRT and ADK1 enzymes, according to Romanello [37] conducted at the Cristallography Laboratory of the São Carlos Institute of Physics – IFSC, located at University of São Paulo, Brazil – USP, São Carlos – SP – Brazil, under the coordination of Dr. Humberto D' Muniz Pereira and the supervision of Prof. Dr. Richard C. Garratt, both belonging to the CBME-IFSC-USP.

B. Animals

Female mice of Balb/c lineage and specific pathogen free (SPF), weighing between 15 and 18 grams, were brought from the CEMIB of the University of Campinas (UNICAMP), Brazil. The mice were kept in proper cages in the experimentation vivarium of the Department of Morphology and Pathology, Federal University of São Carlos, Brazil with free access to potable water and standard commercial diet. The experimental delineation of this work was based upon the recommendations of the Animal Experimentation Ethical Principles, adopted by the Brazilian Society of Laboratory Animals Science (SBCAL) and was submitted and approved by the Ethics Commission in Animal Experimentation (CEEA) of the Federal University of São Carlos, No. 12/2010.

C. Obtention of the *Schistosoma mansoni* infectant larvae (cercariae)

The larvae (cercariae), strain LE (BH-MG), were gently given by Prof. Dr. Vanderlei Rodrigues of the Department of Biochemistry and Immunology, University of São Paulo-(FMRP/USP). The larvae were removed from *S. mansoni*-infected *Biomphalaria glabrata* snails, which were kept in the moluscario of the aforementioned institution.

D. Mice infection with *Schistosoma mansoni* infectant larvae (cercariae)

The mice were infected using subcutaneous route (s.c.) with *S. mansoni larvae.* They were inoculated about 50 cercariae /0,3 mL of 0,9% NaCl/animal physiological solution, with the aid of a 1 ml syringe. This volume and concentration (physiological solution) are unable to affect the cercariae's viability. The method of infection was standardized by the laboratory technician Olinda Mara Brigoto of the Department of Biochemistry and Immunology, University of São Paulo (FMRP/USP).

E. Mice immunization

After the purification of the recombinant enzymes, 100ug (for each enzyme) were added in 200 µL of sterile PBS, containing 100 µg of aluminum hydroxide (Dinâmica lote 35044) and applied into the mice through intraperitoneal route (i.p) thrice in 15-day intervals. After 15 days since last immunization, the mice were infected with 50 *S. mansoni* cercariae/animal. This stage of the research consisted of the creation of the following experimental groups: control group, no immunization of any kind received (n=6/experiment), only infected group and the immunized (PNP1 or HGPRT or ADK1) and infected group (n=10/experiment/each enzyme). Two independent experiments were carried. The mice were euthanized on the 48th and 85th days post-infection with overdose of Thionembutal anesthetic (North Chicago, Illinois, USA). The blood was obtained by cardiac puncture with the aid of a syringe with anticoagulant (EDTA). The serum was obtained after centrifugation of the total extracted blood, in a speed of 1500 rpm, for a 15-minute period.

F. Immunoenzimatic assay – Indirect ELISA – Antibodies detection

An ELISA (BD KIT) was performed to detect immunoglobulins. To detect IgG/ IgG2a, 5 µg/mL of the total antigen's protein was added to the 90-well plate's sensitization and 1:64, IgE, 10 mg / ml of total protein antigen of adult worm and sample dilution 1:4). The protein extract of *S. mansoni* antigens was gently prepared by the student Ricardo Oliveira Correia (LAP-DMP-UFSCar), in accordance with Rofatto [38]. Thus, for the detection of immunoglobulin in plasma, 96-well micro plates were used, coated with 5 or 10 µg/mL of *S. mansoni* total antigen's protein (diluted in carbonate buffer 0.1M and applied 100 µL/well). After the plates have been washed with PBS-Tween 20 0,05%, 200 µL of blocking buffer (PBS-BSA 1%) was added and kept for one hour at room temperature. The plates were then washed once again and 50 µL of the samples were added, diluted in carbonate buffer (1:64 for IgG/IgG2a and 1:4 IgE), being kept at room temperature for 2 hours. Once again, the plates were washed and 100 µL of biotin conjugated secondary antibody (4 µL Ac + 10 mL of carbonate buffer) was added. After 1 hour, the plates were washed again and 100 µL of streptavidin enzyme were added (1:200 dillution). After 30 minutes, the plate was washed and 100 µL of the substratum, a 1:1 mixture of H_2O_2 and tetramethylbenzidine (TMB) (BD-OpTEIA), were added. The reaction was then blocked with 50 µL/well of H_2SO_4 1M. The absorbance reading was performed in 450nm wave length in ELISA reader (**MicroQuant**-Sellex,Inc.).

G. Statistical analysis

The results were expressed as means and standard deviations. Data from pairs of groups were analyzed using the Mann-Whitney U test; and data from multiple groups were analyzed using the ANOVA test followed by a multiple comparison test (Bonferroni's Multiple Comparison Test) (Prism 5.0 software).

III. Results

A. Humoral response evaluation at 48th day post-infection

Results of imunoenzimatic assay – ELISA indicate that levels of antibodies were produced at 48th day post-infection. Figure 1 represents the optical density (O.D.) of IgG, IgG2a and IgE antibodies, reactive to adult worms antigens, present in the plasma of the animals of the following groups: no immunization/infected, immunized with PNP enzymes/infected, immunized with HGPRT/infected and immunized with ADK/infected, at the 48th day post-infection. An IgG increase was observed in the immunized with HGPRT enzyme/infected group, when compared to the control group. As for the detection of IgG2a production in the immunized with HGPRT enzyme/infected group and ADK groups presented significant increase when compared to the control group. Relative to detection of IgE production, the immunized with the HGPRT enzyme/infected and ADK enzyme/infected groups showed a significant increase when compared to the control group. The PNP, however, didn't present a significant increase when compared to the control group.

B. Humoral response evaluation at 85th day post-infection

At the 85th day post-infection (figure 2), it was observed that the immunized with PNP enzyme/infected and HGPRT enzyme/infected groups presented significant increase of IgG when compared to the control group. As for the immunized with ADK enzyme/infected group, no significant increase of IgG was observed, when compared to the control group.
For the detection of IgG2a, the immunized with HGPRT enzyme/infected and ADK enzyme/infected groups showed significant increase when compared with the control group. The immunized with PNP enzyme/infected group, however, didn't present a significant increase of IgG2a when compared to the control group. For the detection of IgE production, the immunized with HGPRT enzyme/infected and ADK enzyme/infected groups presented significant increase when compared to the control group. The immunized with PNP enzyme group, however, didn't present significant increase in antibodies production in this period.

IV. DICUSSION

Schistosomiasis is an infection of worldwide importance, affects a great number of people and promotes socioeconomic impairment. Despite the existence of drugs against adult worms, there is deficiency of drugs which can control the morbid effects of infection or even vaccines which can control the parasite's dissemination. Even though the Health Ministry has made available a vaccine for tests in humans, in 2012, its efficiency is still debatable. It is known that the parasites induce weak natural immunity and are capable of evading or resisting the host`s specific immune responses active. This persistence leads to chronic immune reactions, resulting in tissue injury and pathologic complications. These complications are more related to the host's immune response to the parasite and eggs than to the infection itself [39]. During the infection, the host's immune response against cercarie and schistosomula is initially measured by Th1 type cells. As the infection progresses, the response is modulated by Th2 cells, induced particularly during the formation of granulomas, which were generated by antigens of eggs that were retained in the host's tissues [40].

Considering the humoral immune response, it has been shown that the classes and subclasses of anti-Schistosoma vary with the age, infection intensity and, possibly, with the duration of infection [41]. Some studies have shown that the composition of isotypes of antibodies against *Schistosoma* may strongly influence on the efficiency of effector mechanisms of antibody-dependent cellular cytotoxicity (ADCC) and, consequently, on the expression of protective immunity or pathology [42]. Antibody-dependent, cell-mediated cytotoxicity is considered an important defense mechanism against schistosomula, which involves IgG and IgE antibodies and various cell types, including T cells, eosinophils and neutrophils [43, 44 apud 45].

One of the strategies to produce vaccines against *S. mansoni* considers the use of the recombinant proteins. Already in clinical testing, the membrane protein Sm TSP-1 found in the tegument apical membrane of the parasite, after immunization, induced a reduction of 30-50% in the parasite load against adult worms and a reduction in more than 50% against the eggs [46]. The paramyosin Sm97, a protein from the schistosomula tegument and from the musculature of adult worms, induced an action against adult worms, decreasing by 30-50% in the parasite load and promoting the production of IgG and IgE [47,48]. Thus, these data support our hypothesis that using recombinant proteins from the *S. mansoni*, independently of the parasite origin, may assist us in the search for tools that effectively contribute in the control of schistosomiasis mansoni.

Anaphylactic antibodies have been associated with the expression of immunity. These antibodies of isotypes IgE and some of IgG subclasses are directly involved in the killing of schistosomules, in vitro, in association with populations of effector cells, such as macrophages, eosinophils and platelets. Furthermore, these antibodies induce a high level of protection against new exposures to cercariae, when passively transferred to mice not infected [49]. The studied enzymes present different identities, compared to humans' enzymes, being the PNP enzyme the closest, with an identity of 50%. Recently, this study's collaborator group (IFSC-USP) showed that the enzymes' structures (PNP/HGPRT/ADK) don't pose a risk in human immunization, as the highest percentages of homology were of 30-40% of identity with the same enzymes in humans. The obtained results suggest that the enzymes were immunogenic in order to stimulate an increase on the production of the anti- *S. mansoni* antibodies in the schistosomiasis experimental model. There is a family of *S. mansoni* proteins, with molecular weight of 20 kDa, which was associated with the host's resistance against reinfection, being the resistance correlated with IgE production induced by these antigens. In our tests for detection of IgE production in the immunized animals' plasma, we verified that the immunized with HGPRT enzyme/infected and ADK enzyme/infected groups increased IgE production at the 48th and 85th days post-infection (Figure 1C/2C). There are reports relating the age-acquired resistance of individuals from endemic regions and reinfection during schistosomiasis mansoni with an IgE-mediated response [50]. In our tests for detection of IgE in the plasma of animals from the immunized with HGPRT enzyme/infected and ADK enzyme/infected groups, we verified a significant IgE production at the 48th and 85th days post-infection, when compared to the control group. In this respect, this suggests that these enzymes can be associated to the Th2 response pattern induced in the host by the parasites, and that immunization with these enzymes could contribute to that Th2 response. However, IgE levels against certain antigens is a characteristic in resistant individuals from endemic area, and it is worth mentioning that some studies report the idea that an antigen, to be effective against schistosomiasis mansoni, must also be capable of increasing IgE antibodies [51]. Moreover, it was described that the eggs from *S. mansoni* lead to the release of IL-4, contributing to the IgE secretion [52]. The immunized with HGPRT enzyme/infected and ADK enzyme/infected groups (Figure 2) presented an IgG2a production only at the 85th day post-infection. In face of these facts, it emerged the hypothesis that the proposed enzymes, despite being related to the parasite's ADN can be expressed in the *S. mansoni* membrane and secreted by some pathway. The results presented in this study suggest that these antigens, the *S. mansoni* PNP and HGPRT recombinant enzymes, can play a role in the schistosomiasis prevention. It is necessary, however, further studies in order to comprehend the immune mechanism involved in the antibodies production from the mentioned enzymes in this experimental model.

V. CONCLUSION

Ours results suggested that the recombinant enzymes immunization was helpful the increase for the specific immune response by antibody, in the future has been possibility as immunogenic proteins target against schistosomiasis protection. Future work may concentration on evaluation of the use of theses enzymes on the schistosomiasis murine model for the cooperation with other studies by eradication schistosomal infection in the world.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interests associated with this paper.

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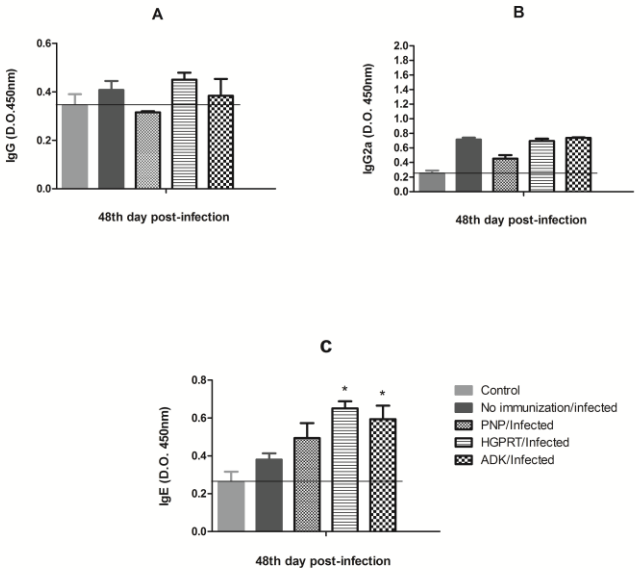

Figure 1. Detection of antibodies in the plasma of immunized animals at 48th day post-infection. Detection of IgG (A); IgG2a (B) and IgE (C) in plasma. Data represent mean ± EPM (n= 10 animals) of 2 independent experiments. The symbol ***** ($p < 0,05$) represents the significant difference between the results obtained from the immunized/infected groups when compared to the control group, using the nonparametric one-way ANOVA Tukey's test.

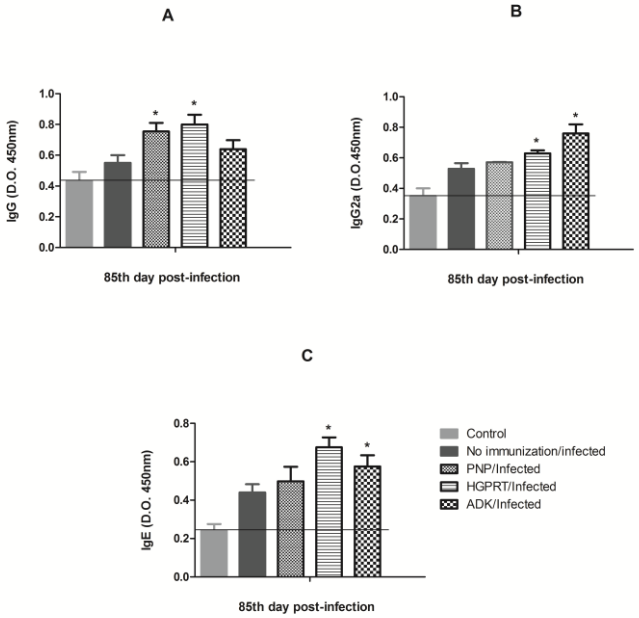

Figure 2. Dosage of antibodies in the plasma of immunized animals at 85th day post-infection. Dosage of IgG (A); IgG2a (B) and IgE (C) in plasma. Data represent mean ± EPM (n= 10 animals) of 2 independent experiments. The symbol ***** ($p < 0,05$) represents the significant difference between the results obtained from the immunized/infected groups when compared to the control group, using the nonparametric one-way ANOVA Tukey's test.