



## Article/Artigo

# Antigens of worms and eggs showed a differentiated detection of specific IgG according to the time of *Schistosoma mansoni* infection in mice

Antígenos de vermes e ovos demonstraram detecção diferenciada de IgG baseado no tempo de infecção pelo *Schistosoma mansoni* em camundongos

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### ABSTRACT

**Introduction:** The correlation between the immunological assay and the antibody titer can offer a tool for the experimental analysis of different phases of the disease. **Methods:** Two simple immunological assays for *Schistosoma mansoni* in mice sera samples based on specific IgG detection for worms soluble antigens and eggs soluble antigens were standardized and evaluated in our laboratory. Fifty mice were used in negative and positive groups and the results obtained by enzyme-linked immunosorbent assays (ELISA) assays were compared with the number of worms counted and the IgG titers at different times of infection. **Results:** Data showed that ELISA using adult worm antigens (ELISA-SWAP) presented a satisfactory correlation between the absorbance value of IgG titers and the individual number of worms counted after perfusion technique ( $R^2=0.62$ ). In addition, ELISA-SWAP differentially detected positive samples with 30 and 60 days post infection ( $p=0.011$  and  $0.003$ , respectively), whereas ELISA using egg antigens (ELISA-SEA) detected samples after 140 days ( $p=0.03$ ). **Conclusions:** These data show that the use of different antigens in immunological methods can be used as potential tools for the analysis of the chronological evolution of *S. mansoni* infection in murine schistosomiasis. Correlations with human schistosomiasis are discussed.

**Keywords:** Schistosomiasis mansoni. IgG. Worm/egg antigens. Acute/chronic phases.

### RESUMO

**Introdução:** A correlação entre o ensaio imunológico e o título de anticorpos serve como ferramenta para a determinação das diferentes fases da doença. **Métodos:** Dois ensaios imunológicos simples para detecção de IgG específico para antígenos de vermes adultos e ovos do *Schistosoma mansoni* com amostras de soro murino foram padronizados e avaliados em nosso laboratório. Cinquenta camundongos negativos e positivos foram avaliados e os resultados obtidos por *enzyme-linked immunosorbent assays* (ELISA) foram comparados com o número de vermes adultos contados em tempos diferentes de infecção. **Resultados:** Os dados mostraram que a ELISA com antígenos de vermes adultos (ELISA-SWAP) apresentou uma correlação satisfatória entre a absorbância obtida para os títulos de IgG e o número individual de vermes contados por perfusão do sistema porta hepático ( $R^2=0,62$ ). Adicionalmente, a ELISA-SWAP foi capaz de detectar diferencialmente amostras positivas com 30 e 60 dias de infecção ( $p=0,011$  e  $0,003$ , respectivamente), enquanto a ELISA com antígenos de ovos (ELISA-SEA) detectou amostras positivas com 140 dias de infecção ( $p=0,03$ ). **Conclusões:** Estes dados mostram que o uso de antígenos diferentes em métodos imunológicos pode ser usado como ferramentas potenciais para a análise da evolução cronológica da infecção por *S. mansoni* na esquistossomose murina. Correlações com a esquistossomose humana devem ser discutidas.

**Palavras-chaves:** Esquistossomose mansônica. IgG. Antígenos de vermes/ovos. Fases aguda/crônica.

### INTRODUCTION

Schistosomiasis is the most important of the human helminthiasis in terms of morbidity and mortality associated with subtle but persistent morbidities<sup>1-4</sup>. During schistosomiasis progression, schistosomes mature to adults in the hepatic circulation and then in pairs migrate to inhabit in the mesenteric veins, where they mate and lay a large number of eggs in the vessels of the intestinal wall<sup>5</sup> and other tissues, mainly in the liver. The major pathological consequences of chronic schistosomiasis are associated with soluble egg antigens secreted from schistosome egg that produces a vigorous inflammatory response resulting in granuloma formation<sup>6</sup>.

Understanding the dynamic responses of the hosts with schistosomiasis related to the time of infection is important to provide insights into the mechanisms underlying disease progression and thus could be potentially useful for the differential diagnosis in schistosomiasis evolution. Patent schistosome infections are highly immunogenic and there is not difficulty to demonstrate the presence of anti-schistosome antibodies<sup>7</sup>. Together, different levels of immunoglobulin G (IgG) isotypes against *Schistosoma mansoni* antigens have been demonstrated before and after chemotherapy in experimental schistosomiasis<sup>8-10</sup>, but there have been no studies that evaluated the IgG levels against different antigens at different times after infection.

In this work, we standardized and evaluated enzyme-linked immunosorbent assays (ELISA) using soluble worm or egg antigens for detection of IgG levels in different times after mice infection, including acute and chronic phases. The use of different antigens was previously tested in ELISA for human sera showing that the use of soluble antigens is justified by the high degree of sensitivity, specificity and correlation between the quantitative seroreactivity and the faecal eggs count, irrespective of their purity<sup>11</sup>. We also investigated the correlation between the IgG titers and the parasite burden.

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## METHODS

### Mice sera

Thirty swiss female mice (4-6 weeks), purchased at the Animal Facility of René Rachou Research Center, Oswaldo Cruz Foundation (FIOCRUZ), were exposed to an average of 40 cercariae of *S. mansoni* (LE strain) per animal, by subcutaneous route<sup>12</sup>. Serum samples were collected by retro orbital sinus puncture at 30, 60 and 140 days after infection, when mice were sacrificed by cervical dislocation and submitted to perfusion of the hepatic portal system using saline solution (0.85% NaCl) plus 50U/L heparin<sup>13</sup>. Adult worms were counted in a stereoscopic microscope (Zeiss Stemi DV4, New Jersey, USA). Twenty swiss female mice were used as negative control of infection. The use of animals was approved by the Ethical Commission in the Use of Animals, FIOCRUZ, Brazil (CEUA No. L-0023/08).

### Preparation of *Schistosoma mansoni* adult worm soluble antigen

Swiss female mice (4-6 weeks) were subcutaneously infected by syringe injection, with 100 cercariae<sup>12</sup>. Aiming to recover the adult worms, after 45 days the animals were sacrificed by cervical dislocation, and underwent perfusion of the hepatic portal system using saline solution (0.85% NaCl) plus 50U/L heparin<sup>13</sup>. Adult worms were washed three times with 0.15M phosphate buffer saline pH7.2, submitted to mechanical grinding (Virtiz Precisa, Dietikon, Switzerland), and centrifuged at 9,500g for 1h at 4°C (Eppendorf AG, Hamburg, Germany). The supernatant obtained was dialyzed in cellulose membrane (Sigma-Aldrich D9777, St Louis, USA) against saline solution (0.9% NaCl) for 48h at 4°C. The antigen was centrifuged at 1,250g for 15min at 4°C and the supernatant was aliquoted and stored in freezer at -20°C. An aliquot was submitted to protein assessment following Bradford method<sup>14</sup> and the obtained concentration was used as parameter in the standardization of the immunoassay for detection of murine antibody, called here as enzyme-linked immunosorbent assay-soluble adult worm antigens (ELISA-SWAP).

### Preparation of *Schistosoma mansoni* eggs soluble antigen

After performing the perfusion of hepatic portal system of infected mice, the livers of these animals were removed to provide eggs recovery. The antigen used in this study was prepared by the method described by Colley et al.<sup>15</sup>. Eggs of *S. mansoni* were homogenized and ground in Virtiz (Virtiz Precisa, Dietikon, Switzerland) with 0.85% saline solution for 40 minutes. The homogenate was centrifuged at 9,500g for an hour at 4°C. After 48h of dialysis in cellulose membrane (Sigma-Aldrich D9777, St Louis, USA) against 0.9% saline solution, the supernatant was submitted to protein assessment<sup>14</sup>. The final concentration was used as parameter in the standardization of the immunoassay for detection of human and murine antibody, named here as enzyme-linked immunosorbent assay-soluble egg antigens (ELISA-SEA).

### Enzyme-linked immunosorbent assays

**ELISA-SWAP:** microtiter plates MaxiSorp™ Surface (NUNC Brand Products, Roskilde, Denmark) were sensitized with 100µl/well of 1µg/mL of SWAP diluted in buffer 0.05M carbonate-bicarbonate pH 9.6 for 16h at 4°C. The plates were washed three times with 0.15M phosphate buffer saline pH 7.2 with 0.05% of

Tween 20 (PBS-T) (LGC Biotecnologia São Paulo, SP, BR) and the non specific sites were blocked with 10% fetal bovine serum in PBS-T 0.05% at 37°C for an hour. After new washing steps, 100µl of serum samples diluted 1:100 in PBS were added in triplicate into each well and the plates were incubated at room temperature for one hour. Then, the plates were submitted to washing steps and incubated at room temperature for one hour with conjugated anti-IgG mouse-peroxidase (Southern Biotech, Birmingham, USA) diluted 1:5,000 in PBS-T 0.05%. The plates were again washed and 100µl of substrate 3,3',5,5-tetramethylbenzidine solution (TMB (Invitrogen, Carlsbad, USA) were added to each well. The reaction was stopped after 20 minutes of incubation in the dark by addition of 50µl/well of 2N sulfuric acid. The results were obtained as absorbance values at 450nm in microplate reader (Model 3550, Bio-Rad Laboratories, Tokyo, JA). The cut off value was determinate as 0.250 of absorbance, using Roc curve (area=0.997). Positive and negative sera were added to the plates, and wells without antigen and antibodies were kept as controls of the assay. The standard dilution used in each technique was determined by a dilution curve, performed with the same reagents and equipment<sup>16</sup>.

**ELISA-SEA:** Microtiter plates MaxiSorp™ Surface (NUNC Brand Products, Roskilde, Denmark) were sensitized with 100µL/well of 3µg/mL of SEA antigen diluted in buffer 0.05M carbonate-bicarbonate buffer pH 9.6 for 16h at 4°C. The plates were washed three times with 0.15M phosphate buffer saline pH 7.2 with PBS-T 0.05% of Tween 20 and the non specific sites were blocked with 10% fetal bovine serum in PBS-T 0.05% at 37°C for 1h. After new washing steps, 100µL of serum samples diluted 1:100 in PBS were added in triplicate into each well and the plates were incubated at room temperature for one hour. Following, the plates were submitted to washing steps and incubated at room temperature for one hour with conjugated anti-IgG mouse-peroxidase (Southern Biotech, Birmingham, USA) diluted 1:15,000 in PBS-T 0.05%. The plates were washed and the results were obtained as described for ELISA-SWAP. The cut off value was determined as 0.544, using Roc curve (Area=0.828). Positive and negative sera were added to the plates, and wells without antigen and antibodies were used as controls of the assay. The standard dilution used in each technique was determined by a dilution curve, performed with the same reagents and equipment<sup>16</sup>.

### Statistical analysis

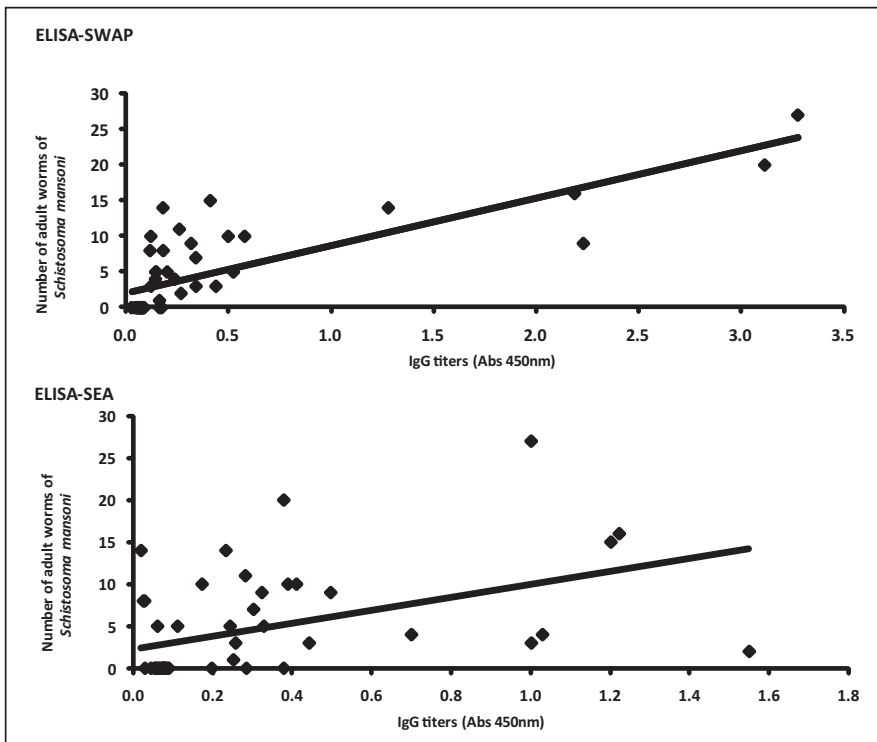
Data deriving from absorbance values were analyzed with Minitab software by Kolmogorov-Smirnov normality test. Normal distributed data were analyzed by Student's *t* test and non-normal distributed data were analyzed by Mann-Whitney test,  $p \leq 0.05$  as significance level. Significance levels for percentages were determined by Chi-square test ( $\chi^2$ ). The cut off values and likelihood ratio were determined with Prism 4.0 software.

### Ethical considerations

All the experiments on animals reported were performed after the Oswaldo Cruz Foundation ethics committee approval (L-0023/08).

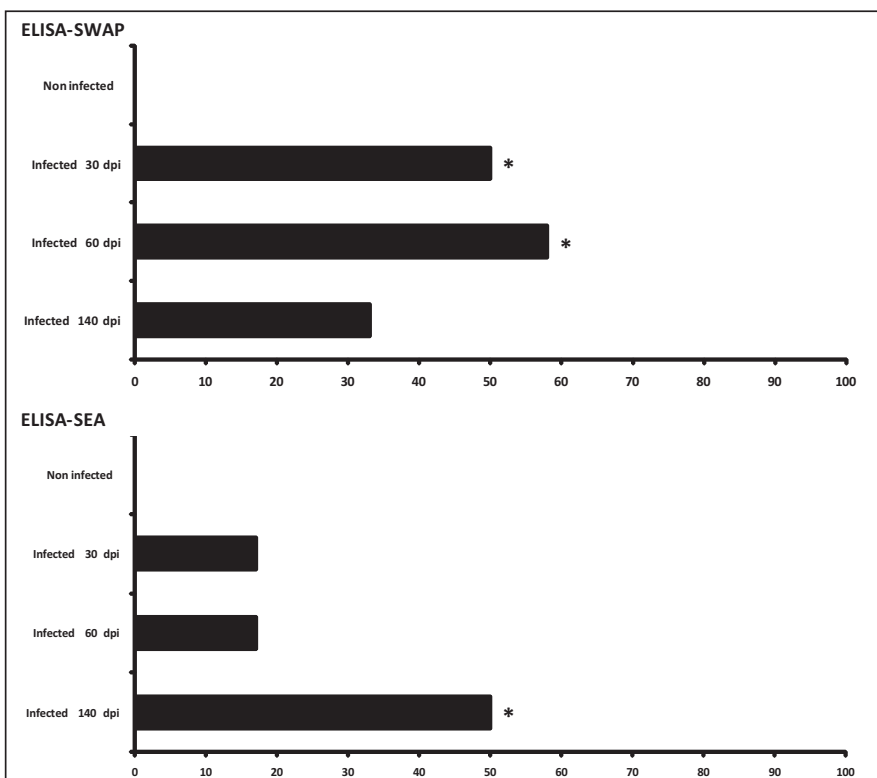
## RESULTS

Serum samples from 30 mice infected with *S. mansoni* were tested by the ELISA methods (ELISA-SWAP and ELISA-SEA). Together, 20 mice were used as negative control. Comparison of the results obtained by the ELISA-SWAP and ELISA-SEA systems was firstly



**FIGURE 1 - Relationship between individual values of the number of worms after perfusion of hepatic portal system and IgG levels in mice serum detected by ELISA assays at 140 days after infection.** ELISA-SWAP with a cut off value of 0.250 showed a linear regression equation of  $y = 6.683x + 1.923$  ( $R^2=0.62$ ). ELISA-SEA with a cut off value of 0.544 showed a linear regression equation of  $y = 7.755x + 2.266$  ( $R^2=0.21$ ).

**ELISA-SWAP:** enzyme-linked immunosorbent assay-soluble adult worm antigens; **ELISA-SEA:** enzyme-linked immunosorbent assay-soluble egg antigens.



**FIGURE 2 - Percentage of positive mice sera for the detection of IgG levels by ELISA-SWAP and ELISA-SEA.** Mice were exposed to 40 cercariae of *S. mansoni* and serum samples were collected at 30, 60 and 140 days after infection. Each column represents the percentage of mice sera that were positive for the presence of specific IgG anti-SWAP (\* $p=0.011$ ,  $0.003$  respectively for 30 and 60 days after infection) and anti-SEA (\* $p=0.029$  for 140 dpi) for Chi-square test. The Cut off values were 0.250 and 0.544, respectively.

**ELISA-SWAP:** enzyme-linked immunosorbent assay-soluble adult worm antigens; **ELISA-SEA:** enzyme-linked immunosorbent assay-soluble egg antigens.

performed using cut off values determined by the ROC curve, and compared with worms recovered by perfusion technique. The cut off values of 0.250 and 0.544 were determined for ELISA-SWAP and ELISA-SEA, respectively. The geometric mean of the number of worms per mouse previously infected with 40 cercariae, estimated by the perfusion technique, was 8.

Analysis of results showed that 7 samples of infected mice were negative for both ELISA-SWAP and ELISA-SEA assays. Four of these samples were from infected mice with low parasite burden (3-5 worms recovered) ( $p=0.001$ ) while 1 sample was from an infected mice with absence of worms. Two other samples were from mice with 10 and 14 worms recovered after perfusion technique.

The relationship between individual values of the number of worms after perfusion and specific IgG levels in mice sera after both ELISA assays was evaluated. As showed in **Figure 1**, ELISA-SWAP showed a more confident association with a  $R^2$  of 0.62 in comparison with ELISA-SEA with a  $R^2$  of 0.21.

To analyze the capability of both assays in detecting IgG titers in different times of infection by *S. mansoni*, replicates of the same samples were assessed on three different days post infection (30, 60 and 140 dpi). As shown in **Figure 2**, ELISA-SWAP was capable of detecting specific IgG antibodies in 50% of serum samples from mice with 30 days of infection ( $p=0.01$ ) and in 58% of samples with 60 days ( $p=0.003$ ). On the other hand, only 33% of the positive serum from mice after 140 days of infection was detected.

Whereas, ELISA-SEA was sensible for detecting specific IgG titers in only 17% of serum samples from mice with 30 or 60 days of infection, but showed a much better result for 140 dpi samples with 50% of positive detection ( $p=0.03$ ). All negative samples were also negative for both ELISA assays.

## DISCUSSION

Patent schistosome infection is highly immunogenic and it is easy to demonstrate the presence of anti-*Schistosoma* antibodies response in infected patients and experimental schistosomiasis. Many different assays have been used to display such immunological reactivity, including skin hypersensitivity reactions against



injected antigens, complement fixation, indirect immunofluorescence, indirect haemagglutination, radioimmunoassay, and various flocculation and precipitation tests<sup>17-20</sup>. But thus far, all these methods revealed low sensitivity, demonstrating the remaining inefficiency correlation between results from direct and indirect methods.

In this study, we evaluated the efficiency of two different ELISA assays based on the detection of IgG antibodies (against worm soluble antigens and egg soluble antigens) as a tool to confirm the results obtained by the portal system perfusion technique considering the number of worms from mice infected with 40 cercariae. Worm antigens are most abundant and easily obtained source of antigenic material. Crude extracts of worms work well in ELISA<sup>11,21</sup>, and worm antigens generally give higher sensitivity and specificity than those from larvae<sup>16</sup>. Collectively, antigens from schistosome eggs are highly immunogenic. Their exit from the host after all depends on it<sup>21</sup> and, in consequence, anti-*Schistosoma* antibody titers rise after the onset of infection patency, as defined by the detection of eggs in clinical specimens.

For that propose, infected mice were submitted to perfusion technique after 140 days after infection and the number of worms were counted. We notice that ELISA-SWAP showed a better correlation between the number of adult worms and the titer of IgG for each individual mouse with  $R^2=0.62$ . All the non infected mice samples analyzed were diagnosed as real negative reaching a 100% of specificity. Two samples showed incoherent results with 10 and 14 worms and low absorbance for IgG specific for SWAP antigens.

The same analysis was performed for ELISA-SEA that showed a very low correlation with the individual number of adult worms ( $R^2=0.21$ ). Seven serum samples from mice with 10-20 worms recovered from perfusion technique presented disjointed correlation with IgG titers for soluble egg antigens.

Pathologic lesions of schistosomiasis are mainly caused by the eggs deposited in various tissues<sup>22-24</sup>. Host immune response to antigens excreted from the embryonated mature eggs results in the formation of granulomas that in chronic infections lead to fibrotic changes. The female adult worms start laying eggs 35 days postinfection, and about 5 weeks postinfection, the eggs may be seen in host liver tissues, and 6 weeks postinfection, egg granulomas appear in infected liver. If *S. mansoni* infections could be detected in the early stage the chemotherapy could abort the pathology<sup>8,25</sup>.

In order to determine the ability of both assays in detecting IgG titers in different times of the infection, we assessed the titers of IgG on three different days post-infection (30, 60 and 140dpi). Data showed that IgG against adult worms antigens were easily detected in most of the positive samples 30 and 60 days post-infection. In this case, 50% of serum samples from mice infected with 40 cercariae after 30 days of infection ( $p=0.01$ ) and 58% of samples with 60 days ( $p=0.003$ ) were properly detected. In contrast, ELISA-SWAP assay was able to detect positive serum samples collected after 140 days of infection in only 33% of the cases. When we checked the IgG levels against eggs antigens by ELISA-SEA, the assay showed low detection capability for serum after 30 and 60 days of infection since only 17% of serum samples were detected as positive samples. On the other hand, the ELISA-SEA showed a much better result for 140 dpi samples with 50% of positive detection ( $p=0.03$ ). Accordingly, all negative samples were also negative for both ELISA assays.

Results from individual laboratories and, from multicentre trials, suggest that egg antigens provide greater diagnostic sensitivity and

specificity than worm antigens for the detection of an infection<sup>11,16,26</sup>. In the present study, we show that diagnostic tests based on egg antigens should be postponed until egg laying is started. To obtain a positive result, the parasite cycle must be completed within the definitive host with the development of males and female adult worms, which reproduce and produce the oviposition<sup>27</sup>. Also, although extracts prepared by homogenizing *Schistosoma* eggs contain a large number of molecules, only a minority of the constituents of SEA might be released by viable eggs *in vivo*, as demonstrated *in vitro*<sup>28</sup> which can explain the low detection capability of ELISA-SEA for the detection of IgG from positive mice sera. On the contrary, others have shown that during the acute phase of the disease there is an increase in anti-worm antibody titers and this fact may be due to the production (at least initially) of antibodies specific for glycanic epitopes which schistosome larvae and worms, and probably also other parasites<sup>29</sup>, have in common.

Briefly, the use of ELISA-SWAP or SEA as a possible tool for the diagnosis of *S. mansoni* infection has been reaffirmed. Importantly, although both methods showed limitations, a higher ratio of anti-*Schistosoma* antibodies could be useful in discriminating between acute and chronic phases of the infection using antigens from different stage forms of the helminth in murine model. It is worth to note that these findings are not seen in the diagnosis of human schistosomiasis and further analysis is recommended. Grenfell et al. (unpublished results) found that immunological assays using SWAP antigens showed high sensitivity even in chronic phase of human infection.

## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## FINANCIAL SUPPORT

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