

Immunoreactivity of proteins within 30-40 kDa range during the acute and the recovery phases in rats experimentally infected with *Strongyloides venezuelensis*

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ABSTRACT

In experimental infection with *Strongyloides venezuelensis*, the acute and recovery phases can be distinguished, unlike human infections caused by *Strongyloides stercoralis*. The objective of this study was to evaluate the production of anti-*Strongyloides* IgG antibodies and the recognition of immunogenic protein bands during the acute and the recovery phases in rats experimentally infected with *S. venezuelensis*. Rats were infected subcutaneously with 400 or 4,000 *S. venezuelensis* infective larvae. The acute phase was characterized by elimination of a large number of eggs in the faeces on days 6-14 post infection; the recovery phase was characterized by the resolution of the infection between days 30 and 35 post infection. Differences in IgG levels were observed in the acute and the recovery phases. Different antigenic fractions were recognized in both phases of infection. It is concluded that proteins within the 30-40 kDa range are immunoreactive markers for both the acute and the recovery phases in rats experimentally infected with *S. venezuelensis*, particularly using membrane antigen.

KEYWORDS: *Strongyloides venezuelensis*. Acute and recovery phases. 30-40 kDa, IgG.

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Strongyloides venezuelensis has been used in rodent models of *Strongyloides* infection, particularly in immunological studies¹ related to the production of heterologous antigens in the serological diagnosis of human strongyloidiasis². In experimental infections, it is possible to define the acute and the recovery phases³, unlike human infections caused by *Strongyloides stercoralis*. In this context, antibody production and recognition of immunogenic bands during the acute and the recovery phases have not been well explored. Thus, the objective of this study was to evaluate the production of anti-*Strongyloides* IgG antibodies and the recognition of immunogenic bands produced during the acute and the recovery phases in rats experimentally infected with *S. venezuelensis*.

Male Wistar rats (*Rattus norvegicus*), with four weeks old, were obtained from the Bioterio de Producao de Ratos, Instituto de Ciencias Biomedicas, Universidade de Sao Paulo, Brazil and were kept in the Bioterio do Instituto de Medicina Tropical de Sao Paulo (IMT-SP). Rats received sterilized food and water *ad libitum* and were handled in compliance with the animal ethics guidelines adopted by the Comite de Etica em Experimentacao Animal, IMT (CEUA IMT 317A).

S. venezuelensis infective larvae (iL3) were obtained by charcoal culture of infected rats faeces (CEUA protocol IMT 0356A). The experimental infections were

established in 35 rats divided into three groups: infected subcutaneously with 400 *S. venezuelensis* iL3 (n = 15, 400iL3), infected with 4,000 *S. venezuelensis* iL3 (n = 15, 4000iL3) and uninfected rats (n = 5, negative control, NC).

The number of eggs per gram of faeces (EPG) was obtained daily until day 35 post infection (pi), according to the Gordon and Whitlock method⁴. EPG was performed in 5 samples of 1 gram of faeces randomly collected on each day post infection in each infected group (400iL3 and 4000iL3). The results were determined after five counts (mean \pm standard error). Blood samples (five animals) were collected by cardiac puncture on days 2, 7 and 35 pi after anaesthesia with ketamine/xylazine, and the animals were subsequently euthanized. Blood samples were centrifuged and the serum samples obtained were used in ELISA and Western blotting.

Two antigenic fractions were prepared using approximately 200,000 *S. venezuelensis* iL3. Briefly, iL3 were resuspended in 1 mL of Tris-HCl (25 mM [pH 7.5]) containing protease inhibitors (Sigma-Aldrich, St. Louis, MO, USA) and sonicated on ice (5 cycles of 20 s). The suspensions were centrifuged at $12,400 \times g$ for 30 min at 4 °C, and the supernatant was collected (soluble fraction, SA_g). Pellets were resuspended in 5 M urea, 2 M thiourea and 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) in an ice bath for 30 min, and the supernatant was collected after centrifugation at $12,400 \times g$ for 30 min at 4 °C (membrane fraction, MA_g).

ELISA was performed as described previously⁵, with some modifications. Microplates were coated overnight at 4 °C with 10 $\mu\text{g/mL}$ (to a final volume 50 $\mu\text{L/well}$) of each *S. venezuelensis* antigenic fraction in 0.06 M carbonate-bicarbonate buffer (pH 9.6). Plates were incubated with serum samples (1:20) for 45 min at 37 °C and then with the secondary antibody consisting of peroxidase-labelled goat anti-rat IgG (Sigma-Aldrich) at a dilution of 1:2,000 for 45 min at 37 °C. The assay was developed by adding TMB chromogen solution (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) for 15 min and was stopped by addition of 2 NH_2SO_4 . The optical density (OD) was determined at 450 nm in a plate reader (Thermo Fisher Scientific). Statistical analyses were performed using the GraphPad Prism software version 8.0 (GraphPad Software, San Diego, CA, USA). Statistical significance was determined by ANOVA, followed by Tukey's multiple comparison test ($p < 0.05$).

Electrophoresis and Western blotting were performed as previously described⁶. Briefly, approximately 140 μg (2 $\mu\text{g/mm}$ of gel) of the antigenic fractions (SA_g and MA_g) underwent electrophoresis in 12% polyacrylamide gel (SDS-PAGE) for 2 h (20 mA). A molecular mass standard (10-260 kDa; Bio-Rad Laboratories, Hercules, CA, USA)

was used to quantitate the relative protein bands. After electrophoresis, the proteins on the gel were transferred to a polyvinylidene difluoride (PVDF) membrane (0.2 μm) (Bio-Rad Laboratories). In the Western blotting, after blocking (50 mM Tris-HCl [pH 7.5]; 3% Tween 20, and 3% milk), the membranes were incubated with sera diluted 1:50 in T buffer (50 mM Tris-HCl [pH 7.5], 100 mM NaCl, 0.1% Tween 20 and 5% milk). The secondary antibody (anti-rat IgG conjugated with peroxidase; Sigma-Aldrich) was then diluted 1:2,000 in T buffer and added to the membrane. Binding was detected using ECL Prime Western Blotting detection reagents (GE Healthcare Life Sciences, Little Chalfont, UK). The antigenic components were visualized in a Luminescent Image Analyzer (Fujifilm, Minato, Tokyo, Japan) and analysed by the VisionWorks LS Analysis Software (Analytik Jena, Jena, Germany).

The egg count showed the development of the experimental infection (Figure 1A). The acute and the recovery phases in the experimental infection with *S. venezuelensis* were well characterized by Chiuso-Minicucci *et al.*³ The acute phase was identified by elimination of a large number of eggs in the faeces on days 6-14 pi (Figure 1A). On day 8 pi, the egg excretion peaked with $14,600 \pm 1,702.9$ and $43,870 \pm 801.5$ EPG in the rats infected with 400iL3 and 4000iL3, respectively. The recovery phase was defined by a reduction in egg excretion and resolution of infection between days 30 to 35 pi (Figure 1A). The increase in the EPG on day 8 pi and the reduction after day 13 pi have been reported in the literature in rats experimentally infected with *S. venezuelensis*^{3,5,7}. Although infection with *S. venezuelensis* is well tolerated by rats,¹ infections with 400iL3 and 4000iL3 can be considered moderate and severe, respectively^{8,9}.

The immune response during the acute and the recovery phases in experimental infection with *S. venezuelensis* was shown by Chiuso-Minicucci *et al.*³ In this study, the specific IgG for *S. venezuelensis* was lower in the acute phase than in the recovery phase. Our results showed differences in IgG levels in the acute and the recovery phases (Figure 1B). There was a considerable increase in the OD values for the SA_g during the experimental infection, suggesting the differentiation of the acute and the recovery phases, independent of the inoculum. Increased production of antibodies in the recovery phase in rats infected with 2000 iL3 has been observed previously⁵. On the other hand, previous studies have shown higher OD values on day 8 pi compared with other days pi in animals infected with *S. venezuelensis*⁷ and *S. ratti*¹⁰.

Comparing the infections with 400iL3 and 4000iL3, a statistical difference ($p < 0.05$) was observed for OD values only on day 7 pi, considering the two antigens (Figure 1B). The results showed a significant increase in OD values

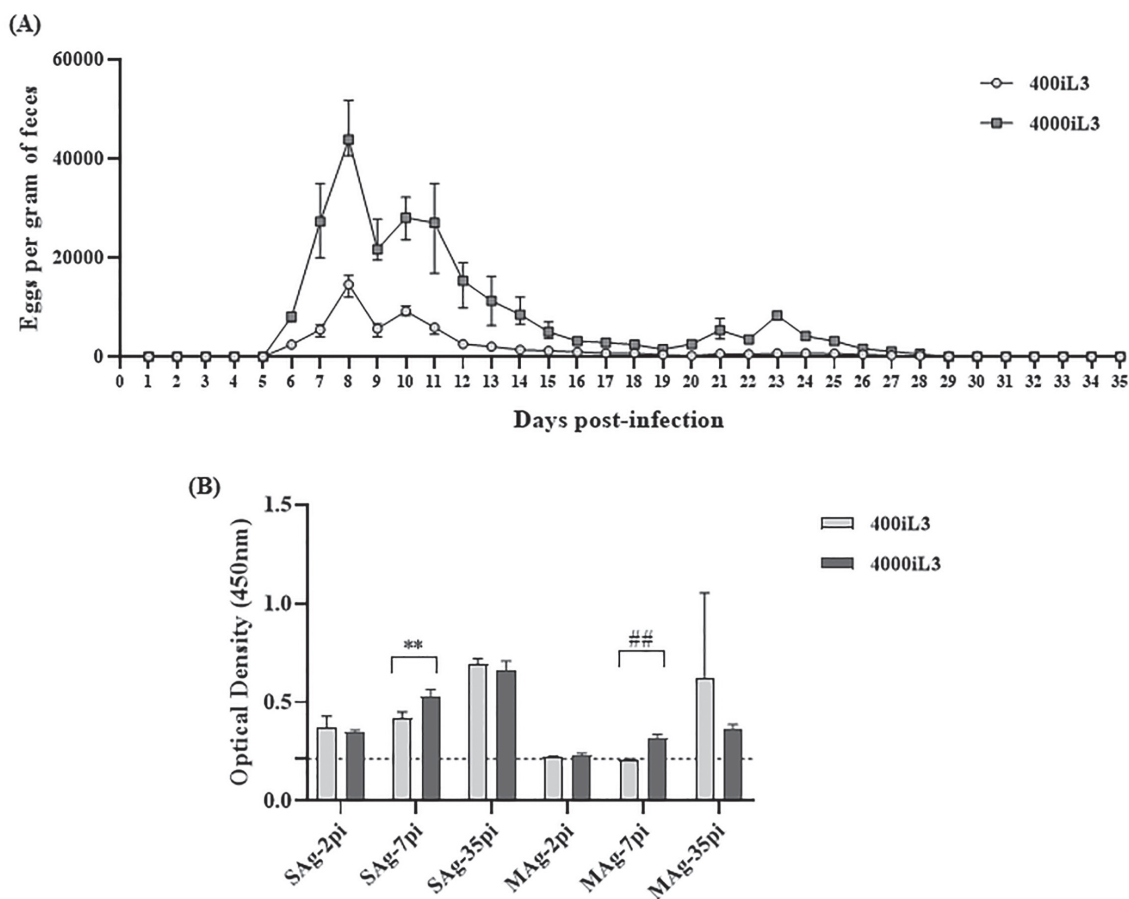


Figure 1 - Eggs per gram of faeces (A) and IgG anti-*S. venezuelensis* in serum samples (B) from rats experimentally infected with 400iL3 and 4000iL3 *S. venezuelensis*. (A) Results are expressed as means \pm SEM of five rats per group. (B) ELISA test using soluble (SAg) and membrane (MAg) antigens from *S. venezuelensis* filariform larvae on days 2, 7 and 35 post infection (pi). Data are expressed as means \pm SEM (n = 5), **p = 0.0055, ## p = 0.0024. Dashed lines indicate the mean optical density of the negative control \pm SEM (n = 5).

in rats infected with 400iL3 on day 2 pi versus day 7 pi independent of antigens; and on day 35 pi versus 2 pi and 7 pi, only the SAg showed a difference. In addition, for rats infected with 4000iL3, there were statistically significant differences on days 2, 7 and 35 pi for the two antigens and on day 2 pi versus 7 pi for each antigen. Analysing the OD values, we can observe that the 4000iL3 group showed lower values in relation to 400iL3 by MAg. These results could be related to variations in the group, because *S. venezuelensis* infection is well tolerated by rats¹.

Few studies have evaluated immunogenic bands during experimental infection¹⁰⁻¹². In the present study, immunogenic bands were recognized (Figure 2), independent of the antigenic fraction and the phase of infection. Bands of ~100-140 kDa were recognized in two groups independently, in the infection phase by SAg and in the recovery phase by MAg. We observed the ~65-70 kDa bands in the recovery phase in the 400iL3 and 4000iL3 groups, mainly using SAg. In addition, there was greater staining intensity of protein bands visualized in MAg. In a recent study of experimental

strongyloidiasis¹¹, immunoreactive bands at 17, 38 and 50 kDa were demonstrated using *S. venezuelensis* iL3 alkaline extract. In addition, 36, 68, 76, 83 and 102 kDa bands for a saline extract were considered antigenic in another experimental investigation with *S. venezuelensis*¹².

The 30-40 kDa band was recognized in the acute and recovery phases of infection in both groups. In agreement with previous reports¹⁰⁻¹², this mass range has been considered an immunogenic band in experimental strongyloidiasis. In addition, 30-40 kDa bands are considered important for the diagnosis of *S. stercoralis* infections^{6,13,14}. Recognition of this protein range by IgG antibodies from the serum of rats infected with 400iL3 may reinforce its potential diagnostic value. In addition, it is difficult to define the phases in *S. stercoralis* infection, which makes recognition of the 30-40 kDa band, mainly by MAg, an important diagnostic tool.

Considering the difference in protein extracts and the intrinsic variability of the reaction, it can be suggested that the OD values and the recognition of immunogenic bands

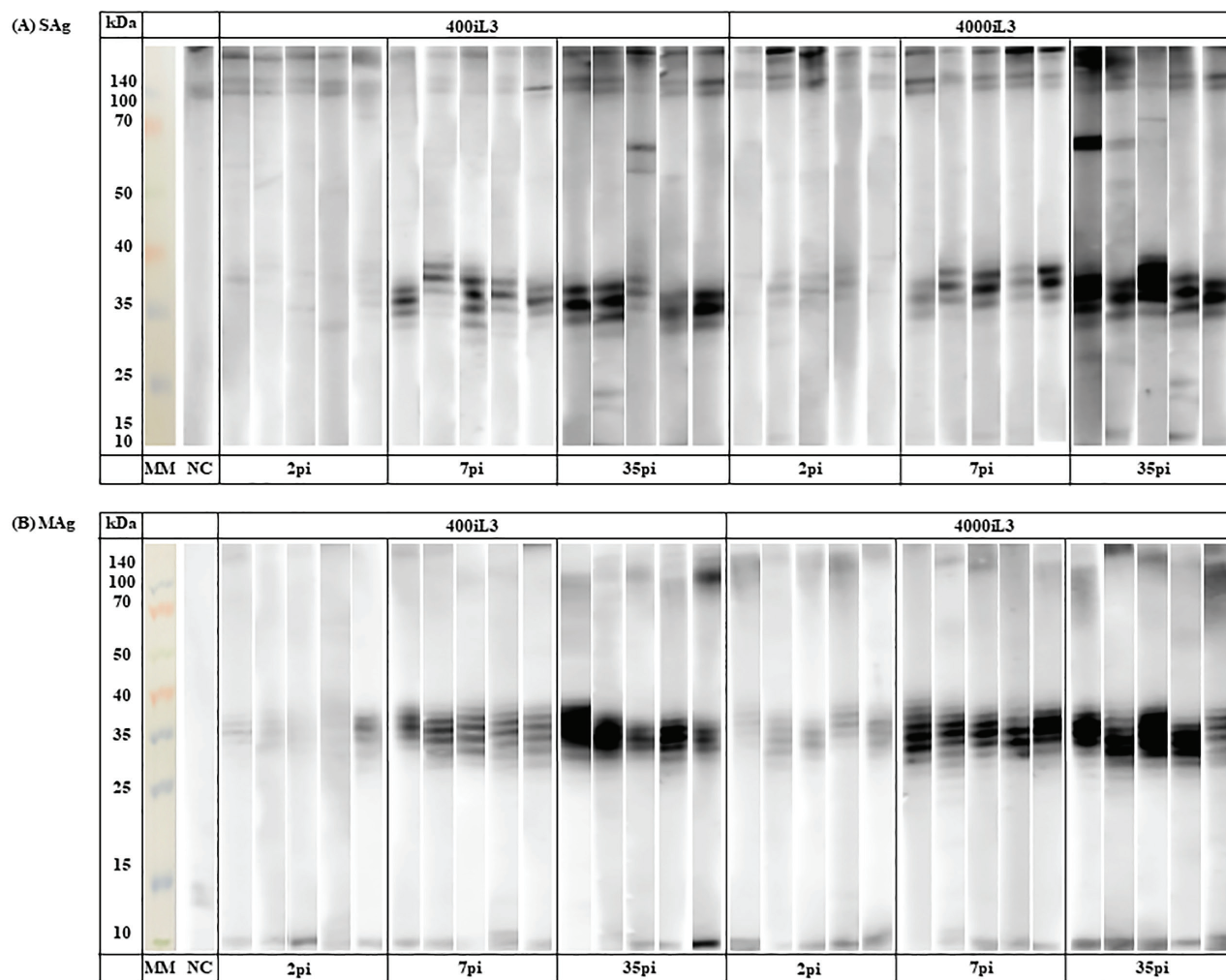


Figure 2 - Recognition of antigenic bands (soluble fraction [Sag, A] and membrane fraction [Mag, B]) in serum samples from rats experimentally infected with *S. venezuelensis* (400iL3 and 4000iL3) on days 2, 7 and 35 post infection (pi) (n=5).

occurred according to the phases of the infection. Based on the literature¹⁵, the increase in OD values was related to the increase in the number of immunogenic bands recognized.

Therefore, experimental *S. venezuelensis* infection is an important tool for diagnostic evaluations for use in human strongyloidiasis. Thus, the present study points to the use of SAg in ELISA and highlights the proteins within the 30-40 kDa range in MAG as a tool for the diagnosis of the acute and the recovery phases in experimental infection by *S. venezuelensis*.

CONFLICT OF INTERESTS

None.

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