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Analysis of antibodies avidity for *Tityus serrulatus* scorpion venom in antivenom production and its potential for application as a potency test

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ABSTRACT

Antivenoms are the only specific medication for neutralizing toxins present in venom of animals such scorpions and snakes through antigen-antibody binding. Several analyses are carried out throughout its production in order to ensure the quality and effectiveness of the antivenom that will be administered to the patient. One of these is the potency assay, which is performed to assess the ability of antivenoms to neutralize the toxic effects of the venom injected in mice. The substitution of in vivo for in vitro assays such as ELISA has been presented by other authors, bringing several advantages such as the reduction in the use of animals, in costs and in the duration of the assays. However, the avidity index of antivenom antibodies determined by ELISA has not yet been applied for this purpose. Therefore, the objective of this study was to evaluate the avidity of sera from hyperimmunized horses with crude *Tityus serrulatus* venom, a scorpion species associated with the most serious accidents in Brazil, and its potential for application as a potency test replacing the in vivo assay. The avidity ELISA proved to be interesting for monitoring the binding strength of antibodies produced by horses in hyperimmune plasma production programs. It was possible to verify oscillations in antibody avidity that occurred along the immunization cycles, differences between novice and veteran horses, maturation of antibody avidity, and correlation between avidity index and antibody titre. Similar results were obtained for crude venom and purified Ts1 toxin. In addition, the avidity ELISA apparently demonstrated potential for application as a potency test in the initial stage of antivenom production. However, more studies are necessary.

1. Introduction

Scorpion stings constitute an important public health problem in tropical and subtropical countries, including Brazil, where more than two million cases were recorded between 2007 and 2019 (Brasil, 2021). *Tityus serrulatus* is the most medically important scorpion in Brazil, its venom is quite complex, and its main component is the Ts1 toxin, representing 15% of the soluble crude venom (Arantes et al., 1989). This toxin is classified as a beta neurotoxin (Barhanin et al., 1982) with 61 amino acid residues, and is associated with liver degeneration, cardiac lesions, arterial hypertension and hemorrhagic edema in lungs (Corrêa et al., 1997).

The treatment of scorpion stings varies according to the patient's clinical status and may include both the symptomatic approach to relieve symptoms in mild cases, and the administration of antivenom for moderate and severe cases (Brasil, 2001). Scorpion antivenom consists of immunoglobulins obtained from plasma of hyperimmunized animals with *T. serrulatus* venom (Brasil, 2019). These immunobiologicals represent the only specific medication capable of neutralizing the action of venoms through the antigen-antibody interaction (Chippaux and Goyffon, 1998). Therefore, evaluation of their quality and efficacy are essential for the adequate treatment of the patient (Sells, 2003).

The evaluation of quality and effectiveness of antivenom is carried out at various stages of its production process and comprises analyzes such as sterility, pyrogen, pH, proteins and neutralizing potency, by in vivo or in vitro methods (WHO, 2017). The potency of an antivenom is influenced by the specific IgG titre and the affinity of the antibodies against the different antigenic epitopes of a venom (Harrison et al., 2017; Bermúdez-Mendez et al., 2018).

WHO (2017) recommends, whenever possible, in antivenom

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production, the evaluation of antivenom ability to bind to the venom through immunological tests such as ELISA. The use of this methodology was already proposed by Theakston and Reid (1979) and a good correlation was found between ELISA and the in vivo assay for antivenom potency. Pratanaphon et al. (1997) observed a high correlation between the ELISA and in vivo assay for *Naja* venom and Rial et al. (2006) found correlation between ELISA titration and potency assay for *Bothrops* venom. In vitro assays can substitute animals, as recommended by the 3 Rs, require less venom, are faster, simpler to perform, cheaper and demonstrate less variability when compared to in vivo assays (Cajado-Carvalho et al., 2017). It should also be considered that satisfactory results in in vitro immunological tests are an essential prerequisite for the effectiveness of an antivenom in a final in vivo neutralization test (Chaisakul et al., 2019). Therefore, in vitro tests can be properly employed in intermediate stages of antivenom production process when the use of in vivo tests, for ethical and practical reasons, should be avoided (Rial et al., 2006).

Among the in vitro immunological assays for analyzing the potency of antivenom, the avidity ELISA stands out for not only estimating the amount of antibodies present in the sample, but also the strength with which these antibodies are able to bind to the antigens. According to Hedman et al. (1989), avidity is considered ideal to assess the strength of binding between polyclonal antibodies and immunologically complex antigens.

Considering the aspects exposed above, the objective of this study was to develop an in vitro assay, ELISA, which would be able to measure the avidity of antibodies presents in serum of hyperimmunized horses with *T. serrulatus* venom guiding the antivenom production schemes, and evaluate its potential for replacing the initial in vivo potency test.

2. Methods

2.1. Animals

Tityus serrulatus scorpions, horses (400 kg) and Swiss Webster mice (18–22 g), all maintained by the Ezequiel Dias Foundation (Funed), an important center of antivenom production in Brazil, were used in this study. All procedures involving these animals were approved by the Funed Ethics Committee on Animal Use (CEUA), protocol 011/2019.

2.2. Scorpion venom

Tityus serrulatus venom, species recommended for commercial scorpion antivenom production in Brazil by the Brazilian Health Regulatory Agency (ANVISA) (Brasil. Agência Nacional de Vigilância Sanitária, 2019), was provided by Poisonous Animals Service - Funed. Venom was collected through electrical stimulation of the scorpion's telson and subsequently centrifuged, lyophilized, and stored at −20 °C.

2.3. Ts1 purification and characterization

Due to the relevance of Ts1 in scorpion envenomation, it was considered important to evaluate the avidity of antibodies produced by horses immunized with crude *T. serrulatus* venom specifically for this toxin. According to Heneine et al. (1998), differences in avidity and specificity of antibodies and the immunogenicity and antigenicity of toxic and non-toxic venom fractions need to be considered in these analyses.

The purification of Ts1 was performed through a single step of cation exchange chromatography, as described by Arantes et al. (1989). For this purification, a Mono S column, Pharmacia Biotech, was used in the AKTA Pure liquid chromatography system, General Electric.

A 5 mg sample of *T. serrulatus* venom was suspended in 1.0 mL of 0.01 M ammonium bicarbonate buffer, pH 7.8 at 4 °C, vortexed and centrifuged for 10 min at 10,000 g at 4 °C in a Megafuge 40 refrigerated centrifuge, Thermo Scientific. The clear supernatant was filtered through a 0.45 μm porous PTFE syringe filter, and then applied to a Mono S column (5 × 50 mm), previously equilibrated with the same ammonium bicarbonate buffer. Protein elution was performed by a convex concentration gradient of ammonium bicarbonate buffer 0.01 M–1.0 M (buffer B) and the absorbance reading at 280 nm. Used a flow of 1.0 mL/min and collected a volume of each fraction equal to 0.5 mL. Samples of interest were collected, pooled, lyophilized, and stored at −20 °C.

The characterization of the toxin and confirmation of its purity were performed using mass spectrometry, MALDI-TOF/TOF Autoflex III Smartbeam, Bruker.

2.4. Equine serum samples

Serum samples, 20 mL, were collected before each immunization and before the first bleeding from eight horses used in the hyperimmune plasma production at Funed. This group consisted of three veteran horses (more than ten years old and more than five years of plasma production) and five novice horses (less than ten years old and starting to produce plasma at that time).

Plasma production process adopted at the institution consisted of cycles of four immunizations (days 01, 10, 13 and 16) followed by four bleedings (days 24, 26, 28, 30). In the first immunization was used 6 mg of venom in Incomplete Freund's Adjuvant, and in the others three were used 2 mg of venom in saline. After each bleeding, reinfusion of blood cells was carried out. After the production cycle, animals were kept at rest for at least 30 days.

Four immunization cycles (A, B, C and D) were analyzed. For cycle A, exceptionally, only the sample that corresponds to the first bleeding after immunizations was analyzed. This was the beginning of the research. In the other cycles (B, C and D) samples were collected before each immunization (days 01, 10, 13 and 16) and before the first bleeding (day 24), already described above. Sixteen serum samples were collected from each horse at different times in the immunization scheme.

2.5. In vivo neutralizing capacity test

This in house potency test is carried out during the production of antivenom at Funed in order to assess the ability of equine serum samples to neutralize the effects of venoms. Five Swiss Webster mice (18–22 g) were used for each sample and five for the control group, totaling 45 mice per production cycle. Based on the LD 50 of the reference venom and the required minimum potency of 0.25 mg/mL for the *Tityus serrulatus* venom in this assay, the sample dose was calculated, Dose = 5 X LD 50/minimum potency.

An amount corresponding to 5 X LD 50 of *T. serrulatus* venom was mixed with each dose of equine serum sample collected before the first bleeding (day 24) and incubated at 37 °C for 1 h. Next, 0.5 mL of this mixture was injected intraperitoneally in mice. The control group was inoculated only with a solution of 5 LD 50 of venom in sterile saline. Deaths were recorded at 24 and 48 h and results were defined as positive (potency $\geq$0.25 mg/mL) when at most one death occurred, and negative (potency <0.25 mg/mL) when two or more deaths occurred. Power of the test (80%), significance level (0.05). Biological Control Service – Funed carried out this assay.

The use of 5 LD 50 challenge and the intraperitoneal route were the Brazilian Health Regulatory Agency (ANVISA) recommendations and were in accordance with the World Health Organization (WHO) for the antivenom potency analysis.

2.6. Avidity ELISA for crude Tityus serrulatus venom and Ts1 toxin

Avidity ELISA was performed according to Wiuff et al. (2002) with modifications, using two microplates in parallel, with and without addition of urea, as a chaotropic agent. This avidity ELISA was adapted

to our laboratory's indirect ELISA protocol and preliminary tests allowed us to define the best serum dilutions, amount of antigen, urea concentration and other ELISA details according to the intended use for the assay. In these preliminary tests, serum from a horse never immunized was used as a negative control. Regarding the use of the chaotropic agent, the literature cites different agents in different concentrations depending on the intended use for the test. Although in the study of avidity between venom and antivenom NH_4SCN, 0–8 M, is one of the most used chaotropic agents, as can be seen in Ibrahim et al. (2013), Harrison et al. (2017), Chaisakul et al. (2019), Choraria et al. (2021), and Alsolaiss et al. (2023), it was decided to use urea, which also acts as a chaotropic agent, and due to its availability. Prior to the avidity assays the concentration of urea was standardized by testing 4 M, 6 M and 8 M urea in ELISA assays. As shown in the supplementary material the concentration of 8 M urea yielded results of higher dissociation of the antigen-antibody immune complex (Urea standardization - S1).

The 96-well microplates were sensitized with 100 μL of *T. serrulatus* crude venom (2.5 μg/mL) or Ts1 toxin (1 μg/mL) in carbonate buffer, pH 9.6, and incubated overnight (2–8 °C). After that, the plates were washed with phosphate buffer, pH 7.4, and 1% (w/v) albumin blocking solution (100 μL) was added and kept at 37 °C for 1 h. Then, plates were washed and 100 μL of serum samples diluted in PBS plus Tween 20, pH 7.4, were added in duplicate, performing 11 two-fold serial dilutions from 1/200 to 1/204,800. For Ts1 toxin was done five two-fold serial dilutions (1/200 to 1/3200) because of the limited amount of purified toxin. Plates were incubated for 1 h at 37 °C and washed again. After this step, corresponding plates were incubated for 8 min with the phosphate buffer, pH 7.4, plus 8 M urea or phosphate buffer alone and washed. This step was repeated three times. 100 μL of anti-IgG equine conjugate from Sigma Life Science with dilution of 1/10,000 in PBS, tween 20 and dried albumin 1%, pH 7.4, solution was added to plates and incubated for 1 h at 37 °C. The substrate with OPD and H_2O_2 (80 μL) was added and incubated for 1 h at 37 °C. Interruption of color reaction was performed with 40 μL of H_2SO_4 30%. The absorbance at 492 nm was read using a Multiskan GO, Thermo Scientific.

The mean of duplicates was calculated after subtracting the blank (wells without serum samples). Then, optical density (OD) obtained in the plate that was treated with urea was divided by OD from the plate without urea for each dilution.

$$\text{Avidity index by dilution} = \text{OD}_{(\text{dilution } x, \text{ with urea})} / \text{OD}_{(\text{dilution } x, \text{ without urea})}$$

Avidity index was obtained after calculating the mean of the results obtained for each dilution. Only dilutions with OD above the blank and OD with urea lower than OD without urea were considered.

2.7. ELISA titration for crude T. serrulatus venom

This test was carried out to assess the oscillation of antibody titres over immunization cycles and the possible correlation between avidity index and antibody titre. The protocol used was the same as for the avidity ELISA, with the exception of steps for using urea. A microplate was prepared for each horse: test serums and negative control in duplicate at dilutions ranging from 1/6400 to 1/819,200, and blank. Titre was defined as the dilution at which the measured OD was greater than the OD mean of the negative control plus two standard deviations. Titre was expressed as the inverse value of serum dilution.

2.8. Statistical analyses

Comparison of avidity index between samples was made by paired Student's t-test. In the evaluation of Ts1 toxin was used linear regression. Pearson correlation between avidity index and antibody titre. The ROC curve was used for the analysis between in vivo and in vitro and definition of a cutoff. P values < 0.05 were considered significant.

All data analyses were performed using the R Language and Environment for Statistical Computing, version 4.3 (https://www.r-project.org/).

3. Results

3.1. Purification and characterization of Ts1 toxin

Chromatographic profile of crude soluble scorpion venom is shown in Fig. 1. Based on results presented by Arantes et al. (1989), the fraction marked with an **X** in the figure was collected for mass spectrometry analysis and a molecule with 6872.80 Da of molecular mass was found. The molecular mass obtained is very close to the molecular mass described for Ts1 (6882 Da) according to Martin-Eauclaire et al. (2018). The result obtained indicated that this fraction had a high degree of purity, being suitable for ELISA tests (see Fig. 2).

3.2. Avidity index – crude T. serrulatus venom

Avidity index was calculated for each sample totaling 128 indices (16 samples from each horse) which are represented in boxplot, Fig. 3. On the first day of sampling, the mean avidity index for the eight horses was equal to 51.2% (95% CI 41.2–61.2%) while on the last day this index was 74.1% (95% CI 71.7–76.5%). This growth was statistically significant ($p = 0.0017$). The veteran horses showed a non-significant increase, while the novice horses showed a significant increase in avidity of 30.2% ($p < 0.05$). The mean avidity of the veteran horses for the period was equal to 76.6% (95% CI 73.2–80.1%) while for the novice it was 65.8% (95% CI 62.2–69.5%), $p < 0.05$. The mean difference between minimum and maximum avidity index for the veteran horses was 42.7% and for the novice was 58.5%. It is important to consider that the first sample of each horse was collected after all immunizations of the first cycle (cycle A).

Regarding the three cycles followed in full (B, C and D), for the eight horses, it was observed in cycle B an initial mean avidity index of 50.4% (95% CI 34.8–66.0%) and final of 62.7% (95% CI 51.2–74.2%), in cycle C initial index of 53.0% (95% CI 44.5–61.4%) and final of 80.8% (95% CI 73.0–88.5%), and in cycle D initial index of 82.4% (95% CI 77.8–87.1%) and final of 74.1% (95% CI 71.8–76.5%), all with $p < 0.05$, Fig. 4.

3.3. Avidity index – Ts1 toxin

Due to the limited quantity of purified Ts1, only pooled serum samples (veteran and novice horses) collected on the first bleeding dates (sample 1, 6, 11 and 16) were analyzed. It was observed that the specific avidity for Ts1 toxin increased over time. The avidity of the veteran horses was significantly ($p < 0.05$) higher than those of the novice only in the first sample, Fig. 5. Linear regression analysis of two sample pools showed an increasing trend of avidity index over immunization cycles ($p < 0.05$), Fig. 6. The indices obtained in each of the five dilutions (1/200 to 1/3200) were considered in these analyses, increasing the number of observations.

3.4. ELISA titration

Growth in mean antibody titre was observed over time and it was similar to that observed for avidity for crude scorpion venom, Fig. 7.

Results obtained in avidity and titration assays suggested the existence of an interdependence between them. A Pearson correlation index between the variables avidity and titre of 0.43 was obtained.

3.5. Comparison of in vivo and in vitro assays

In vivo tests for the neutralizing capacity were performed for each sample collected on the first bleeding dates (sample 1, 6, 11 and 16), as part of the antivenom production process at Funed. Of the 32 samples

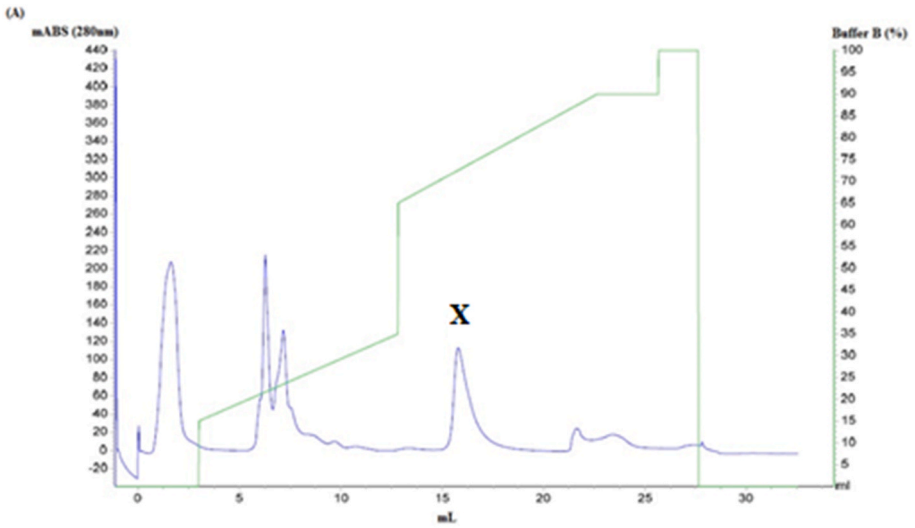

Fig. 1. Chromatographic profile of the soluble crude *T. serrulatus* venom. The peak identified with X refers to the fraction collected for analysis.

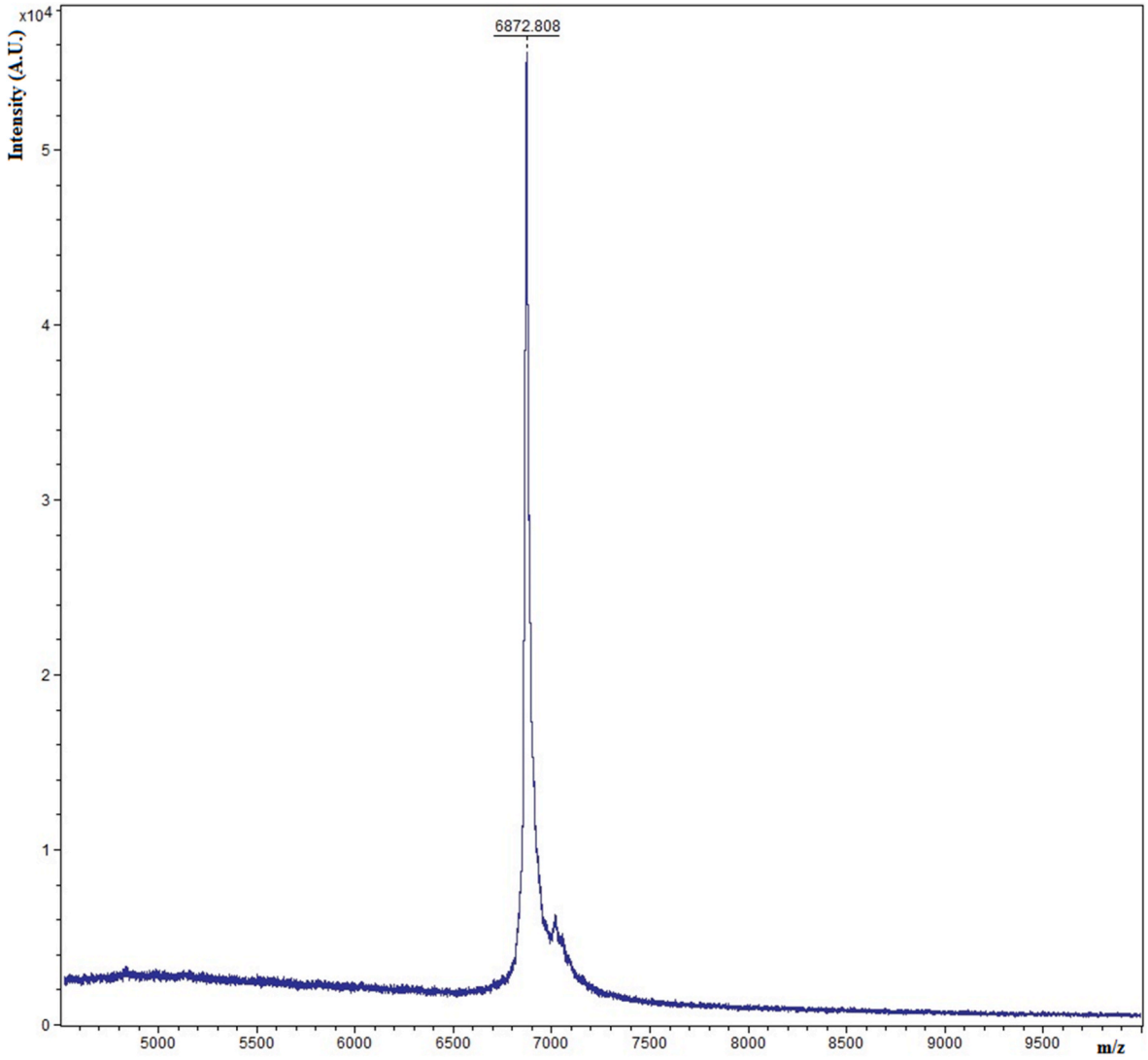

Fig. 2. Mass spectrometry of the highlighted fraction. Identified molecule with 6.872,80 Da.

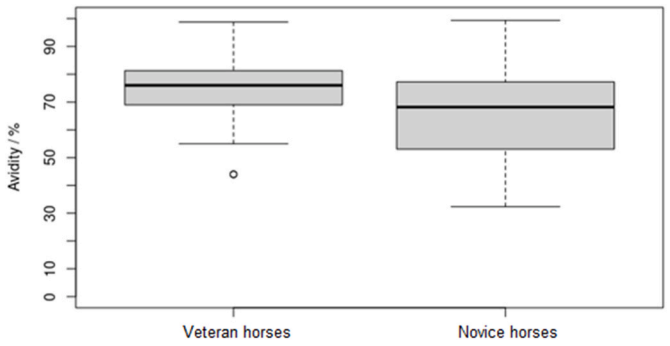

Fig. 3. Avidity index, boxplot. Veteran horses (48 samples) and novice horses (80 samples).

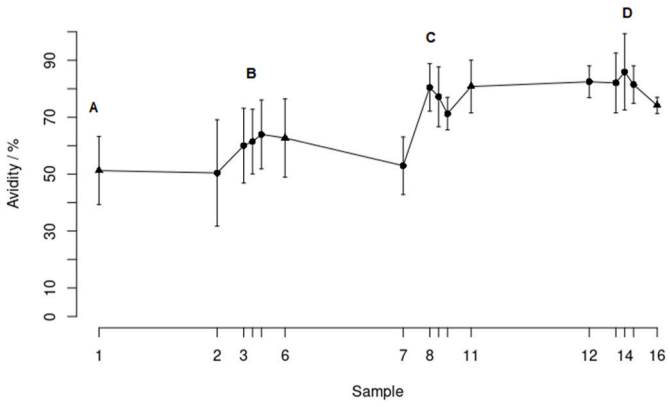

Fig. 4. Mean avidity index and standard deviation of equine group over time (sample 1 to 16) for *T. serrulatus* venom. Production cycles represented A, B, C and D. Immunizations (circles) and first bleedings (triangles).

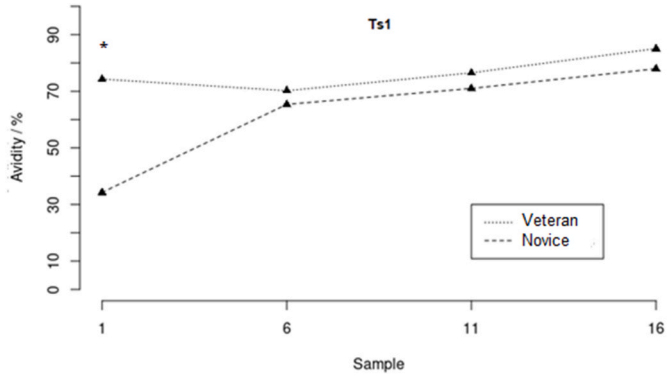

Fig. 5. Evolution of avidity index for Ts1 toxin during immunization cycles. * Significant difference ($p < 0.05$) between veteran and novice horses was observed only for the first sample.

tested, only four showed negative results, two in cycle A and two in cycle B. Three negative samples were from novice horses and one from a veteran horse. In order to facilitate the analysis between in vitro and in vivo assays, the results were organized in Table 1.

Avidity indices were ordered from lowest to highest with differentiation between indices with positive and negative results in the neutralizing capacity test, in order to facilitate interpretation of possible relationships between them, Fig. 8. The sample with the lowest avidity index showed a negative result in the neutralizing capacity test, and the four in vivo negative samples were among the thirteen with the lowest avidity indices. In addition, from an avidity of 70.0%, there was no negative in vivo sample.

In the analysis of the ROC curve, the cutoff obtained by the Youden index (J) was 69.7%. At this cutoff, specificity was equal to 1.00 and sensitivity equal to 0.68. For the intended purpose of this work, it was considered important to obtain a high specificity to reduce false positive results. The area under the curve (AUC) was 0.81 (95% CI 0.62–1.00). Fig. 9.

4. Discussion

Antibodies avidity represents a measure of the strength with which they bind to antigens. It depends in part on affinity, but also involves other factors such as the valence of antigens and antibodies. Its analysis by ELISA is very interesting for application in antivenom production because this in vitro assay not only estimates the amount of antibodies present in the sample but also the strength with which they bind to the venom toxins, and this may be directly related to its neutralizing capacity (Steward and Lew, 1985). In addition, it is highly recommended in the production of antivenoms to reduce the use of animals and replace in vivo tests with in vitro assays (Gutiérrez et al., 2017). Thus, the use of avidity index as an in vitro assay of neutralizing potency to replace the in vivo test, promoting reduction in cost, time and animal use, should be evaluated.

ELISA test proved to be suitable for monitoring avidity index of antibodies in the hyperimmune plasma production. Rial et al. (2006) had already reported that ELISA was suitable for monitoring antibody titres in serum of horses immunized with *Bothrops* venom. This in vitro monitoring of the antibody avidity can be applied for the selection of horses with strong immune response against scorpion venom, replacing the weak responders. A panel of horses composed with the best responders should yield antivenoms with higher effectiveness. In this study, a significant avidity growth of 22.9% between the first and the last sample was observed. This increase in antibody avidity over time, measured under different methodologies, both in natural and experimental infections, has already been demonstrated by: Hedman and Seppala (1988), Hedman and Rousseau (1989), Hedman et al. (1989), Bjorkman et al. (1999) and Bin et al. (2016).

It is interesting to observe that in cycles monitored in full, there was 12.3% increase in avidity between the first and last sampling in cycle B, 27.8% increase in cycle C and a decrease of 8.3% in cycle D. This result suggests the trend of increasing avidity until reaching a plateau associated with a reduction in the amplitude of its variation over time, demonstrating the process of maturation of antibody avidity in response to successive immunizations. Our results are in accordance with data in the specific literature. Ibrahim et al. (2013) observed growth in the titre and avidity of antibodies in rabbits immunized with *Walterinnesia aegyptia* venom until the sixth week of the immunization program. After that, avidity remained at the plateau and the titre fell regardless of new immunizations. Chotwiwatthanakun et al. (2001) evaluating antibody titre during immunizations of equines with elapid venom, observed its growth until reaching a plateau followed by a slow decrease after its end. Pratanaphon et al. (1997) observed, in the immunization of horses with *Naja kaouthia* venom, that after reaching the plateau, antibody titre slowly dropped even with immunizations and, after months of rest, a new immunization resulted in an immediate increase. This plateau represents a physiological limit for the animal.

Veteran horses had a higher mean avidity index with less oscillation than the novice for the studied period. In addition, veteran horses did not show a significant increase in avidity between the first and last sample, which has already occurred with novice. This result can be explained by the fact that veteran horses, due to various immunizations they have already gone through, remain close to the avidity plateau. Avidity maturation observed in an immunization program can be understood as a result of antibody affinity maturation, and the increase in titre and variety of antibodies produced over time.

Avidity decrease in cycle D suggests the possibility of adjustments in

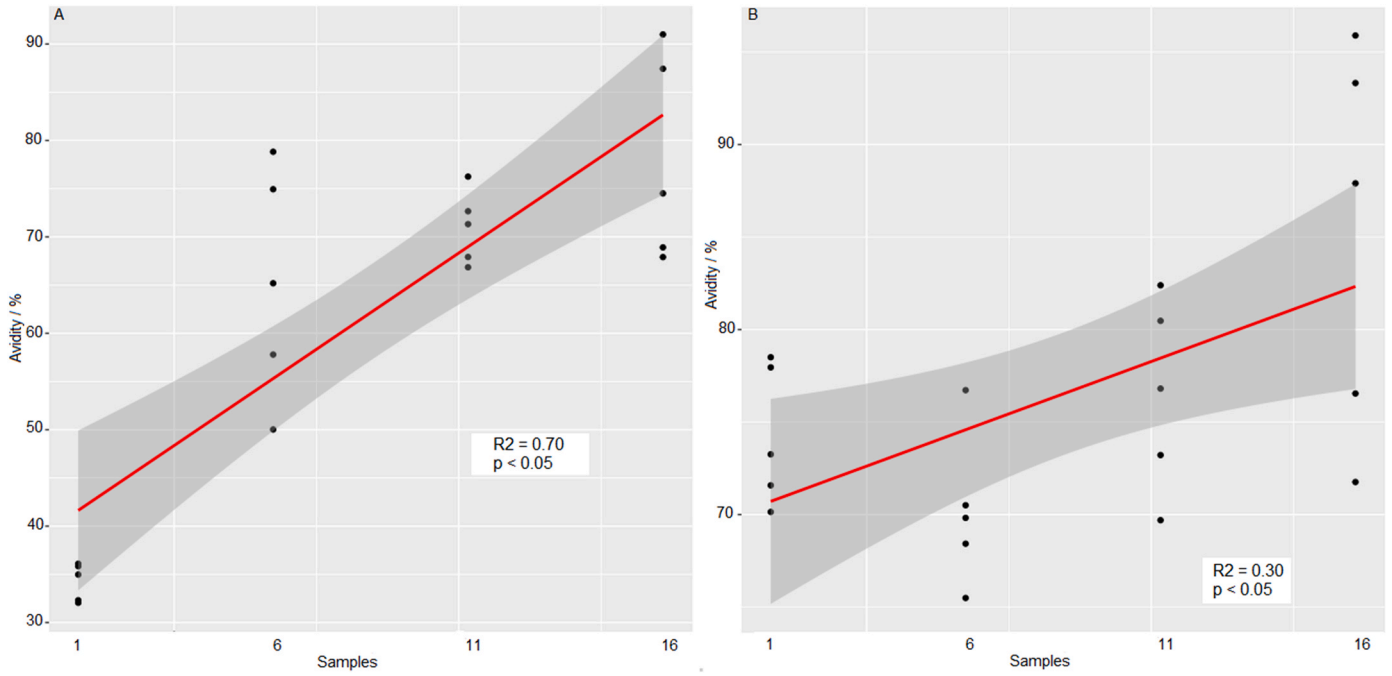

Fig. 6. Avidity growth over immunization cycles. The novice horses (A) showed greater growth than the veteran horses (B). The effect of the number of immunization cycles on antibody avidity was greater in the novice.

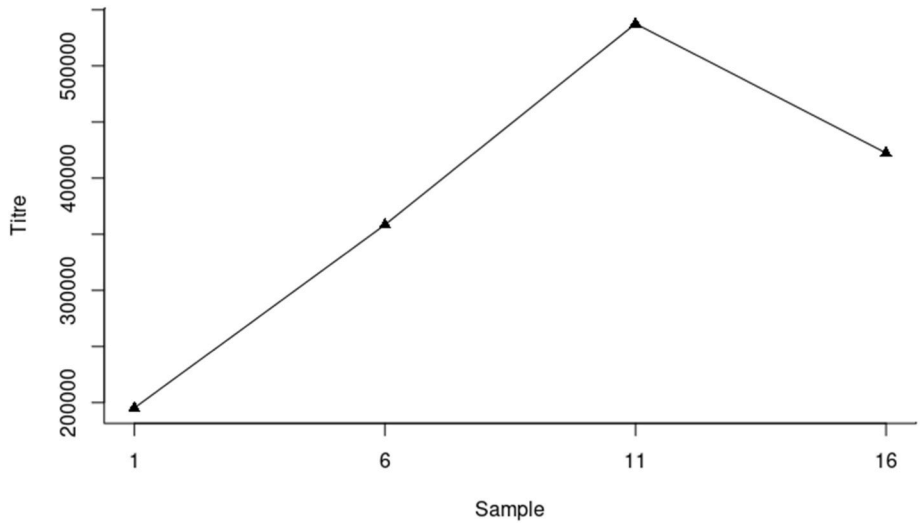

Fig. 7. Representation of the antibody titre growth over the immunization cycles. Mean antibody titre of the eight horses for crude *T. serrulatus* venom. Values expressed as the inverse of dilution.

number of immunizations or in interval between them according to the number of cycles that horses have already been submitted. This could result in reduction of time, quantity of venom, financial resources and, mainly, of immunological stimuli on animals.

Tityus serrulatus venom is very complex, and its main toxin is the Ts1. This toxin is related to most of the toxic effects of the venom (Corrêa et al., 1997; Bertazzi et al., 2003). Due to the importance that this toxin has on scorpion envenomation, associated with low immunogenicity characteristic of low molecular weight toxins (Sells et al., 1994) and the importance of evaluating specific toxic fractions of venoms in in vitro potency tests (Heneine et al., 1998), it was deemed necessary to evaluate the response of antibodies produced by horses specifically against this toxin in avidity test. Results obtained for crude venom and Ts1 toxin were similar. Avidity index of veteran and novice horses increased significantly over immunization cycles. Novice horses showed greater growth than the veteran, and avidity obtained by veteran horses were greater than obtained by novice in the first sample. In the last three samples there were no significant difference between groups. This also demonstrates the occurrence of avidity maturation process over time and successive immunizations. Horses immunized with crude *T. serrulatus* venom were able to produce antibodies that recognized and bound to Ts1, which is highly relevant and must be considered when analyzing the efficiency of antivenoms.

According to Hedman and Seppala (1988), in the study of avidity it is necessary to consider that it is influenced by the amount of antibodies present in the sample. Thus, it was decided to evaluate the antibody titre and its relationship with avidity index. It was observed that titre increased over time and successive immunizations, as observed by De La Rosa et al. (2019) with recombinant elapid antigen. Results of antibody titre were similar to that of avidity index, with growth up to cycle C and

Table 1
Avidity index for crude T. serrulatus venom and Ts1 toxin, antibody titres, and in vivo results of first bleeding samples from each cycle (A – D).

Cycle	Horse	In Vivo	Avidity (%)	Titre	Avidity Ts1 (%)	Group
A	407	Positive	71,0%	102,400	74,3%	Veteran
A	540	Positive	55,1%	102,400		
A	655	Positive	62,0%	819,200		
A	658	Negative	32,3%	204,800	34,3%	Novice
A	660	Positive	52,0%	25,600		
A	666	Positive	43,3%	51,200		
A	672	Positive	43,7%	204,800		
A	674	Negative	50,5%	51,200		
B	407	Positive	79,7%	409,600	70,2%	Veteran
B	540	Negative	69,6%	204,800		
B	655	Positive	79,6%	819,200		
B	658	Negative	64,6%	102,400	65,4%	Novice
B	660	Positive	39,7%	204,800		
B	666	Positive	53,1%	204,800		
B	672	Positive	60,9%	819,200		
B	674	Positive	54,3%	102,400		
C	407	Positive	92,1%	204,800	76,5%	Veteran
C	540	Positive	85,8%	819,200		
C	655	Positive	90,4%	819,200		
C	658	Positive	88,6%	204,800	71,0%	Novice
C	660	Positive	72,5%	204,800		
C	666	Positive	71,9%	409,600		
C	672	Positive	74,0%	819,200		
C	674	Positive	70,8%	819,200		
D	407	Positive	72,8%	409,600	85,1%	Veteran
D	540	Positive	69,7%	409,600		
D	655	Positive	77,5%	819,200		
D	658	Positive	75,6%	204,800	77,9%	Novice
D	660	Positive	71,6%	102,400		
D	666	Positive	77,3%	204,800		
D	672	Positive	75,9%	409,600		
D	674	Positive	72,6%	819,200		

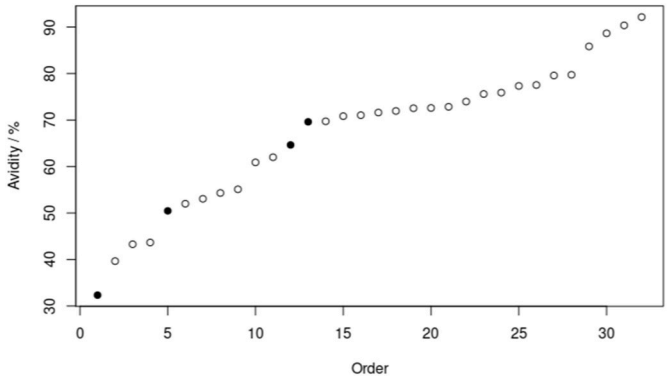

Fig. 8. Avidity index for crude T. serrulatus venom ordered from lowest to highest (32 samples). Black dots represent the four in vivo negative samples. White dots are the positive results in the in vivo test for the remaining 28 samples.

subsequent reduction in cycle D. Similar results were obtained by Pratanaphon et al. (1997), Chotwiwatthanakun et al. (2001), and Ibrahim et al. (2013), as presented above. According to León et al. (2018), there is a limit of antibody production by animal that varies according to its physiological state and that cannot be crossed, regardless of immunization program used. Pearson correlation index between titre and avidity was equal to 0.43.

In vivo analyzes showed an improvement in the neutralizing capacity of antibodies throughout production cycles of hyperimmune plasma. In the first two cycles there were two negative results, while in the last two there were none. These results are in agreement with what was observed

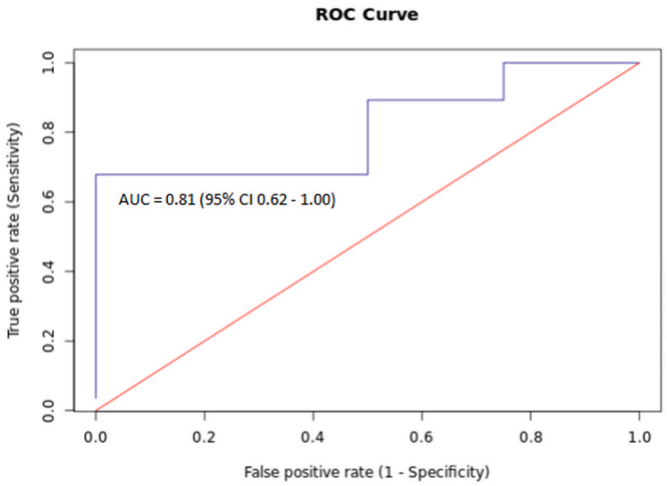

Fig. 9. ROC analysis for avidity index, 32 samples.

for avidity and titre in ELISA, and demonstrate the efficiency of the immunization program to which horses were submitted. Chavez-Olórtegui et al. (1997) and Mendes et al. (2008), working with antigens derived from scorpion venom, obtained antigen-antibody interaction in ELISA and satisfactory protection in potency assays. Casewell et al. (2014) obtained satisfactory results both in the indirect ELISA, and in the in vivo assay, for snake venoms. Avidity index obtained in samples considered from cycles C and D remained above 70%, with only one exception, Table 1. This relatively high avidity tends to be indicative of a serum with high efficiency. Chaisakul et al. (2019), working with *Daboia siamensis* antigen, found correlation between avidity and in vivo potency assays.

According to WHO (2017), ELISA assays for antivenom analysis must be defined internally, according to the characteristics, objectives and needs of each institution. In this study, a cutoff for the avidity index equal to 69.7% was obtained with a specificity of 1.00. This cutoff seems to be interesting for the intended purpose because it reduces the occurrence of false positive results, which would have unsatisfactory potency. However, it is also necessary to consider the occurrence of false negative results due to the low sensitivity (0.68). One option for this would be to perform the avidity ELISA first for all samples, and use the neutralizing capacity test as a confirmatory test only for samples with negative results in the first. In this way, it would be possible to reduce costs and the number of mice used in the assays. In these analyses, the existence of individual variations in the immune response and the uncertainties of in vitro and in vivo assays need to be considered.

A limitation of this study is related to the number of samples. Only 32 samples were available for analysis between in vivo and in vitro and this, probably, increased the confidence interval obtained in the ROC curve. Despite this limitation, the results obtained are promising, which justifies further studies to apply the antibody avidity index in the initial analysis of antivenom potency.

This avidity ELISA proved to be applicable and suitable for monitoring binding strength of antibodies produced by horses in hyperimmune plasma production. This feature probably offers greater security than simply quantifying antibody titre when considering replacing an in vivo with an in vitro test for potency analysis. Avidity results obtained for Ts1 toxin were similar to those obtained for crude *T. serrulatus* venom, demonstrating the presence of specific antibodies for this toxin in plasma of immunized horses. Thus, an avidity test for crude venom without the need for toxin purification apparently proves to be sufficient for the intended purpose. Finally, the avidity ELISA seems to be promising for replacing the in vivo test of neutralizing capacity, despite the limited number of samples used. The substitution of techniques that use animals for others that do not, one of the 3 Rs principles, has been

encouraged by the World Health Organization and has become a worldwide trend, which justifies new studies for development and implementation of avidity tests in antivenom production.

It is also important to mention that horses used in this research maintained good body and health condition throughout the study, with no noteworthy clinical intervention being necessary.

Ethical statement

This article is derived from MSc thesis of Lucas Tadeu Silva under the coordination of Luiz Guilherme Dias Heneine.

Título: Aplicação do teste de potência in vitro específico para a toxina Ts1 do veneno de escorpião em soros de equinos hiperimunizados.

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CRediT authorship contribution statement

Lucas Tadeu Silva: Conceptualization, Formal analysis, Investigation, Methodology, writing, Visualization, Project administration. **Robson Silva Junior:** Methodology, Investigation. **Thiago Xavier Teixeira de Carvalho:** Methodology, Investigation. **Luiz Carlos Moutinho Pataca:** Conceptualization, Formal analysis, Investigation, Methodology, writing. **Luiz Guilherme Dias Heneine:** Conceptualization, Formal analysis, Investigation, Methodology, writing, Visualization, Project administration, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.toxicon.2023.107315.

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