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Research article

Analysis of the genus *Bothrops* snake venom: An inter and intraspecific comparative study

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ABSTRACT

The genus *Bothrops* are considered Category 1 of medical importance by the World Health Organization, responsible for approximately 85 % of snakebites occurring throughout Brazil. Main factors determining snake venom variations can be genetics, diet, gender, geographic distribution, age, or even seasonality. In this study, we compared the composition of protein profile, biochemical activities, and immunorecognition of toxins present in the venom of eight adults of *Bothrops* species (*B. alternatus, B. atrox, B. jararaca, B. jararacussu, B. leucurus, B. moojeni, B. neuwiedi* and *B. pauloensis*). The following methods were used to analyze the venoms: protein dosage; electrophoresis in polyacrylamide gel containing SDS; High Performance Liquid Chromatography – Reverse Phase; enzymatic activities, western blotting and Enzyme Linked Immuno Sorbent Assay. The results show inter and intraspecific differences in the electrophoretic profile. LAAO and PLA_2 activities, in general, were higher in males than females and proteolytic activity was higher in females than males. The bothropic antivenom produced by Instituto Butantan recognized most of the protein bands in all *Bothrops* species analyzed, with only the regions between 37 and 25 kDa presenting lower intensity. A notable variability in the chromatograms was observed. *Bothrops* venom demonstrated inter-intraspecific disparities in protein composition and biochemical activity.

1. Introduction

Snake envenomation represents a serious public health hazard in tropical and subtropical regions of developing countries (Gutiérrez et al., 2017; Chippaux, 2017) and was reintroduced as a neglected tropical disease by the World Health Organization (WHO) in 2017. Species of the genus *Bothrops* are considered Category 1 of medical importance by WHO and are responsible for approximately 85 % of snakebites occurring annually in Brazil (Sinan, 2020; World Health Organization, 2017; Ministério Da Saúde, 2020) [1,2].

Bothrops venom is a complex mixture of molecules, mainly containing the following families of enzymatic proteins:

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metalloproteases (SVMP), serine proteases (SVSP), phospholipases A_2 (PLA_2) and L-amino acid oxidases (LAAO). Although less abundant, there are also nucleotidases (RNase, DNase and phosphodiesterase) and acetylcholinesterase (AChE). Whereas, proteins with non-enzymatic action are: disintegrins, snake venom factor (CVF), vesprin, C-type lectin (CTL), bradykinin-potentiating peptides (BPP), cysteine-rich secretory proteins (CRISP), endothelial growth factor and nerve growth factor (NGF) (Gutierrez and Rucavado, 2000; Lizano et al., 1997; Kang et al., 2011; Calvete et al., 2007; Fry; Wüster, 2004 [3,4]; Markland, 1998; Serrano et al., 2005).

Toxins present in *Bothrops* venom are directly linked to symptoms caused by the bite. Proteolytic activity determines coagulant-related act in several stages of the coagulation cascade, mainly promoting consumption of coagulation factors. Finally, hemorrhagic activity mainly affects the vascular endothelium ([5]; Secretaria Da Saúde Do Estado De São Paulo, 1993; Gutiérrez et al., 2006; [6,1, 7–14]).

Snake venom composition presents considerable heterogeneity and may vary according to genetics, diet, gender, geographic distribution, age, or even seasonality. This variation occurs due to factors generated by molecular evolution, with diversification in quantitative and qualitative aspects, at interspecific or intraspecific levels [15–24] [25].

Currently, immunotherapy with antivenins is the only available and effective therapeutic method for envenomation resulting from snake bites. Instituto Butantan uses 5 species to produce the polyvalent F(ab')2 bothropic antivenom (antibothropic antivenom—BAV) through hyperimmunization method in horses, namely: *Bothrops jararaca* (50 %), *Bothrops alternatus* (12.5 %), *Bothrops jararacussu* (12.5 %), *Bothrops moojeni* (12.5 %) and *Bothrops neuwiedi* (12.5 %).

Venom toxins are rich sources of peptides, proteins, and other non-protein compounds, with diverse effects requiring clearer elucidation regarding their mechanisms of action. This "gap" encourages novel research to discover new medicines, adjuvants, or diagnostic methods based on molecules found in snake venom (WHO, 2015; 2021) [26].

Taking this into account, this study aimed to investigate the variability of different adult species of *Bothrops* venom, comparing inter- and intraspecific variability (males and females). Since variations may influence the treatment of snakebites, the present study is important for the improvement of antivenom production; and the understanding of symptoms due to toxins' synergy. This study can also contribute greatly to bioprospection, exploiting the venom of various *Bothrops* species and selecting the best ones to isolate and modify certain proteins/enzymes according to their composition.

2. Materials and methods

2.1. Animals and venoms

Venoms were obtained through manual extraction, according to the standardization of the Laboratory of Herpetology at Instituto Butantan and mixed into pools according to sex (GREGO et al., 2021). Adult animals available in the indoor facility used in our study were: *B. alternatus* (*Balt* - 2 males and 7 females), *B. atrox* (*Bax* - 6 males and 5 females), *B. jararaca* (*Bj* - 7 males and 7 females), *B. jararacussu* (*Bju* - 6 males and 6 females), *B. leucurus* (*Bl* - 5 males and 5 females), *B. moojeni* (*Bm* - 6 males and 7 females), *B. neuwiedi* (*Bn* - 4 males and 4 females), and *B. pauloensis* (*Bp* - 6 males and 7 females).

After extraction, the venoms underwent centrifugation at $1700 \times g$ for 15 min, lyophilization, and were stored at −20 C°.

2.2. Protein dosage

The concentration of protein in venom pools was measured based on the Bradford colorimetric assay (1976) using the Bio-Rad Protein Assay reagent and bovine serum albumin (BSA) as a control for the standard curve. These data were the basis for other experiments in order to ensure, with greater precision, the quantity of proteins used in the procedures. Freeze-dried venoms were resuspended in 0.85 % saline solution to a concentration of 1 mg/mL. The experiments were carried out in 96-well with microplates in triplicates of 10 μL of sample (venoms in the same batch) and the absorbances read at SpectraMax at a wavelength of 595 nm. Results were expressed as μg of protein per mg of dry venom.

2.3. Sodium Dodecyl Sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Analysis of electrophoretic profiles of venom samples was performed according to Laemmli [27]. Twenty μg of protein were applied in 15 % polyacrylamide gels under reducing conditions. The Dual Color Precision Plus molecular mass protein standard (Bio-Rad) was used as reference. The gels were stained using Coomassie Blue G-250 according to manufacturer's specifications and scanned with GE Healthcare ImageScanner III, software LabScan 6.0.

2.4. Phospholipase A_2 activity

The methodology described by Holzer and Mackessy (1996) to measure PLA_2 activity was used, with some modifications. This activity is based on the cleavage of substrate 4-nitro-3-(octanoloxy) benzoic acid (NOBA) in the presence of phospholipases. In a colorimetric microplate, 20 μL of deionized water, 200 μL of 10 mM Tris buffer, 10 mM $CaCl_2$, 100 mM NaCl pH 8.0, 20 μL of venom (1 mg/mL) and 20 μL of NOBA at a final concentration of 4.16 mM in acetonitrile, were pipetted. The reaction was incubated at 37 °C and measurement performed at 425 nm every 10 min. The activity was expressed in units (U - nMol of chromophore released) per minute of incubation per microgram of protein (U/min/mg), an increase of 0.1 unit in the reading is equivalent to 25.8 nMol of chromophore.

2.5. L-amino acid oxidase activity

The LAAO activity of venoms was determined using the methodology described by Kishimoto and Takahashi (2001). The same dilution of protein dosage in 0.85 % saline solution to a concentration of 1 mg/mL were applied in 96-well with microplates in triplicates. 10 μL of sample were pipetted and then incubated with 90 μL of reactive mixture (50 mM Tris pH 8.0, 250 mM L-methionine, 2 mM o-phenylenediamine (OPD) and 0.8 U/mg horseradish peroxidase) for 1 h at 37 °C. Then, the reaction was stopped with 50 μL of 2 M H_2SO_4 and absorbance reading was performed at 492 nm. LAAO activity was expressed by the amount of nMol of H_2O_2 released per microgram of protein per minute of incubation (nMol/mg/min).

2.6. Proteolytic activity

Proteolytic activity on casein was performed according to protocol described by Antunes et al. (2010) with modifications. Ten μL of venom solution (1 mg/mL) and 85 μL of a 4.25 mg/mL azocasein solution (Sigma) solubilized in 100 mM Tris-HCl buffer solution, 10 mM $CaCl_2$ pH 8.8 were added in a microtube. The mixture was incubated for 90 min at 37 °C and the reaction stopped by adding 200 μL of 5 % trichloroacetic acid (TCA). Afterwards, samples were centrifuged at 1700×*g* for 5 min at 4 °C. In a 96-wells microplate, 100 μL of supernatant was added to 100 μL of 0.5 M NaOH. The absorbance was measured in a plate reader using a wavelength of 450 nm. One unit (U) of enzyme activity is defined as the amount of venom causing a 0.005 unit increase in absorbance at 450 nm. Specific activity will be expressed as units per milligram of protein (U/min/mg).

2.7. Western blotting

Individual venoms (20 μg) separated by 15 % SDS-PAGE were electrotransferred at 15 V for 90 min onto polyvinylidene fluoride (PVDF) membranes. Membranes were blocked with Tris-buffered saline containing 5 % fat-free milk (TBS-milk) overnight at 4 °C. The membrane was incubated with 1:1000 antibothropic serum for 2 h at room temperature. After washing the blots with Tris-HCl buffer (10 mM Tris, 150 mM NaCl, pH 7.5) containing 0.1 % Tween 20, membranes were exposed to 1:10,000 peroxidase-labeled anti-horse IgG (Sigma) for 2 h at room temperature. After washing away unbound secondary antibodies, immunoreactive bands were visualized using KPL TrueBlue.

2.8. Enzyme linked immuno sorbent assay (ELISA)

The ability of the antibothropic serum to recognize *Bothrops* venom was evaluated by ELISA. The 96-wells plate was sensitized with 10 μg/mL of antigen triplicate (venom) solution in carbonate buffer (34 mM Na_2CO_3, 15 mM $NaHCO_3$) at pH 9.6 (100 μL/well) and incubated for 18 h at 4 °C in a humid chamber. After fixing the antigen, the plate was washed three times for 5 min using a washing buffer solution (0.77 M NaCl, 1.25 % Tween 20). Blocking was performed by incubating 200 μL/well of blocking solution (3 % fat-free milk) for 2 h at 37 °C. The plate was washed as previously described.

After the second incubation, a serial dilution of antibothropic serum was applied in incubation buffer (1 % fat-free milk and 0.05 % Tween 20–100 μL/well) for 1 h at 37 °C. The immunoenzymatic conjugate (peroxidase-conjugated horse anti-IgG) was applied (100 μL/well at concentration 1:10.000) diluted in the incubation buffer and incubated for another hour at 37 °C. The washing process was repeated again. Then, the substrate (OPD 1 mg/mL, H_2O_2 1 %) diluted in the incubation buffer was applied and incubated at room temperature for 5 min. After this time, the reaction was stopped with a 30 % H_2SO_4 (50 μL/well). The plate was read using a spectrophotometer at A492 nm. The assay was performed in triplicate. The effectiveness of antibothropic antivenom (Instituto Butantan, batch 1305077) in neutralizing the lethal activity of both venoms was tested in mice.

2.9. High performance liquid chromatography - reversed phase (HPLC)

An aliquot of 75 μg of lyophilized pooled venoms dissolved in 0.85 % saline solution to a concentration of 1 mg/mL, centrifuged at 13,000×*g* for 15 min and were applied to a Teknokroma Europa 300 C-18 25 × 0.4 mm reversed-phase column, previously equilibrated with 0.1 % trifluoroacetic acid solution (TFA; Sigma, 302031; solution A) using the ÄKTA Purifier UPC10 chromatograph (GE Healthcare). Samples were eluted according to Gay et al [28], with some modifications, through a gradient of 0.1 % trifluoroacetic acid and 95 % acetonitrile solution (solution B) under the following conditions: 5 % (solution B) for 5 min, 5–25 % (solution B) for 10 min, 25–45 % (solution B) for 60 min, 45–70 % (solution B) for 10 min, 70–100 % for 10 min and more 10 min of 100 % solution B, at a flow rate of 1 mL/min. Absorbance was monitored with A215 and A280. The proteins presented in different regions in the chromatograms were based on previous studies by the group using the same method, and can be found in the work of Hatakeyama and collaborators (2021).

2.10. Statistical analyses

Turkey Test (T test) was carried out in each experiment, separating males and females, and ANOVA was used to compare different species, in order to visualize groups presenting statistically significant differences.

3. Results

3.1. Sodium Dodecyl Sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The bothropic venoms studied here present variations in composition, number and intensity of protein bands, with the majority components being between 64 and 14 kDa.

Male and female snake venoms SDS-PAGE profiles, under reducing conditions, are shown in Fig. 1. The variations mainly involved the intensity of protein bands that appear in samples at inter and intraspecific levels. When comparing pools of males and females of same species, we observed the absence or presence of certain protein bands, although, as expected, this variation was less marked than between different species. There is a strong presence of the main venom proteins distributed below ~100 kDa. The most prominent reduced protein bands migrated in a region below ~75 and between ~25 and ~15 kDa.

3.2. Enzymatic activities

LAAO enzymatic activity is evidently higher in males than in females in most species. Male pools of *Bj* and *Bn* showed the highest activities ($p < 0{,}0001$), while *Balt* and *Bl* had the lowest activities. Among females, *Bax* and *Bm* presented the highest activities, while *Balt* and *Bj* had the lowest activities in the group. Comparatively, between males and females, *Bax*, *Bl* and *Bp* had no statistical difference in LAAO activities; on the other hand, *Bj*, *Bju* and *Bn* showed statistical difference between males and females ($p < 0{,}0001$), as well as *Balt* ($p < 0{,}003$) and *Bm* ($p < 0{,}0387$) (Fig. 2A).

Similar to the profile we observed in LAAO activity, PLA_2 activity was higher in males than in females, except for the *Balt* and *Bju*. Among males, samples with the highest PLA_2 activity was *Bm*, followed by *Bl*, and those with the lowest activity in the male group were *Balt* and *Bj*. In females, the highest activity was observed in *Bm*, while those with the lowest activity were *Bj* and *Bax*. Comparatively,

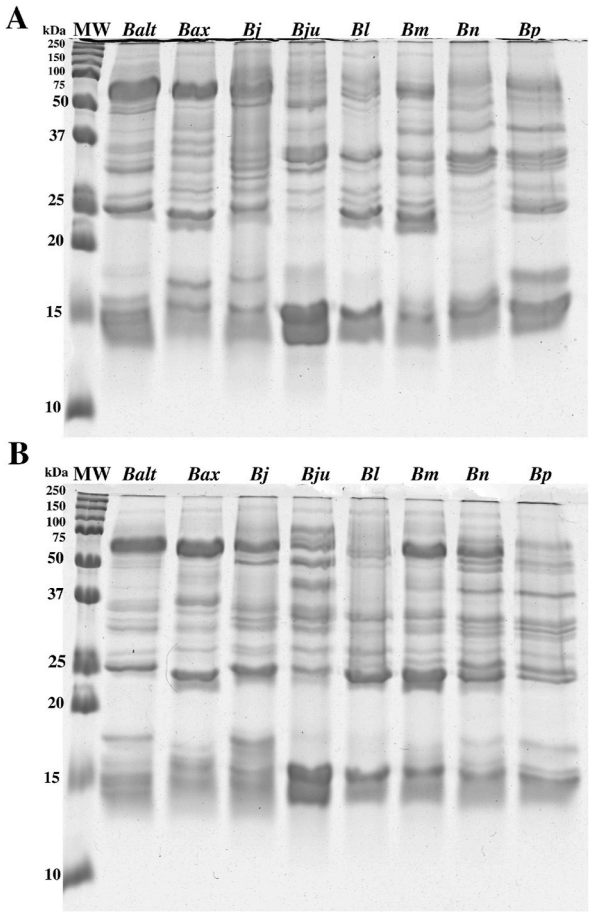

Fig. 1. One-dimensional electrophoresis profile (1-DE, 15 %) of adult males (A) and females (B) of the species *B. alternatus (Balt), B. atrox (Bax), B. jararaca (Bj), B. jararacussu (Bju), B. leucurus (Bl), B. moojeni (Bm), B. neuwiedi (Bn), B. pauloensis (Bp)*. The samples (20 μg) were applied to the gel under reductive circumstances with (β-mercaptoethanol) and the proteins were stained with Coomassie Blue G-250 according to the manufacturer's recommendation. The molecular weight (MW) is shown on the left (Dual Color Precision Plus, BioRad).

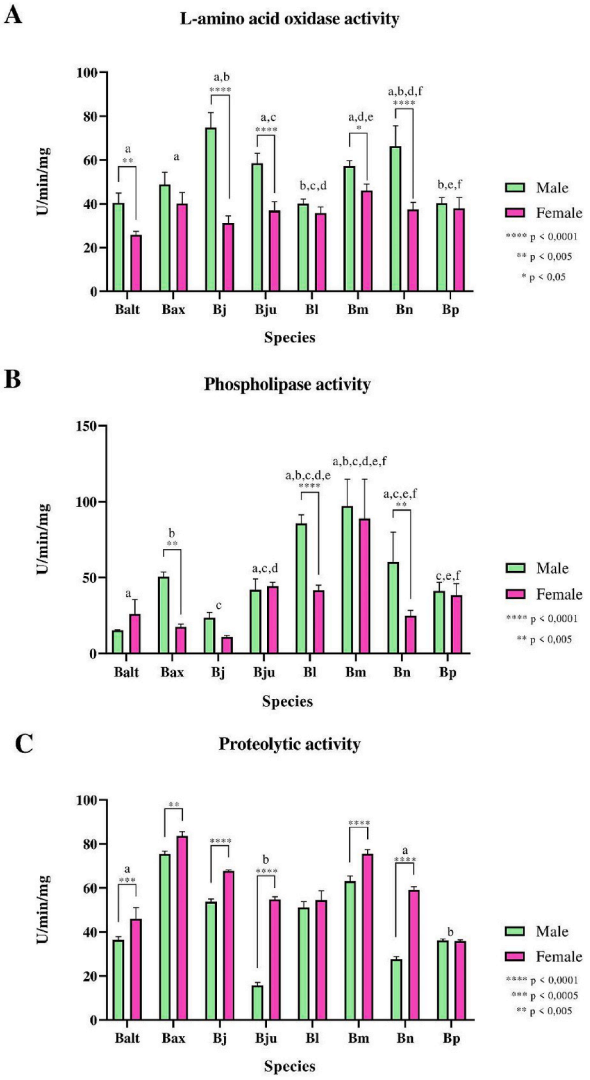

Fig. 2. Enzymatic activities of the enzyme L-amino acid oxidase (A), phospholipase A_2 (B) and proteolytic activity under azocasein (C) present in the venom of the species *B. alternatus (Balt), B. atrox (Bax), B. jararaca (Bj), B. jararacussu (Bju), B. leucurus (Bl), B. neuwiedi (Bn)* and *B. pauloensis (Bp)*. Males are shown in the green columns and females are represented by the pink columns. The lowercase letters above the columns group together the species that had statistical differences between them ($p < 0.0001$), while the asterisks indicate statistical differences between males and females of the same species (*$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$; ****$p < 0.0001$).

between males and females, only *Bl* ($p < 0{,}0001$), *Bax* and *Bn* ($p < 0{,}003$) had statistical differences (Fig. 2B).

In contrast, in proteolytic activity, females showed higher values than males, with only *Bp* samples having similar activities between sexes. *Bax* pools had the highest activity for both males and females, but the lowest activity for males was observed in *Bj* and for females, in *Bp*. Comparatively, between males and females, *Bj, Bju, Bm* and *Bn* showed statistical differences ($p < 0{,}0001$), as well as, *Balt* ($p < 0{,}0005$) and *Bax* ($p < 0{,}003$) (Fig. 2C).

3.3. Western blotting

In western blotting method, the bothropic antivenom produced at Instituto Butantan recognized most protein bands, forming a profile similar to polyacrylamide gels, both in males and females. Below 37 kDa was the only region where weak immunorecognition of bands could be seen in all species, regardless of sex. (Fig. 3).

3.4. Enzyme Linkes Immuno Sorbey Assay (ELISA)

ELISA (Fig. 4) showed that most venoms evaluated here had a similar immunorecognition pattern. Despite this, *Balt*, *Bax*, *Bj* and

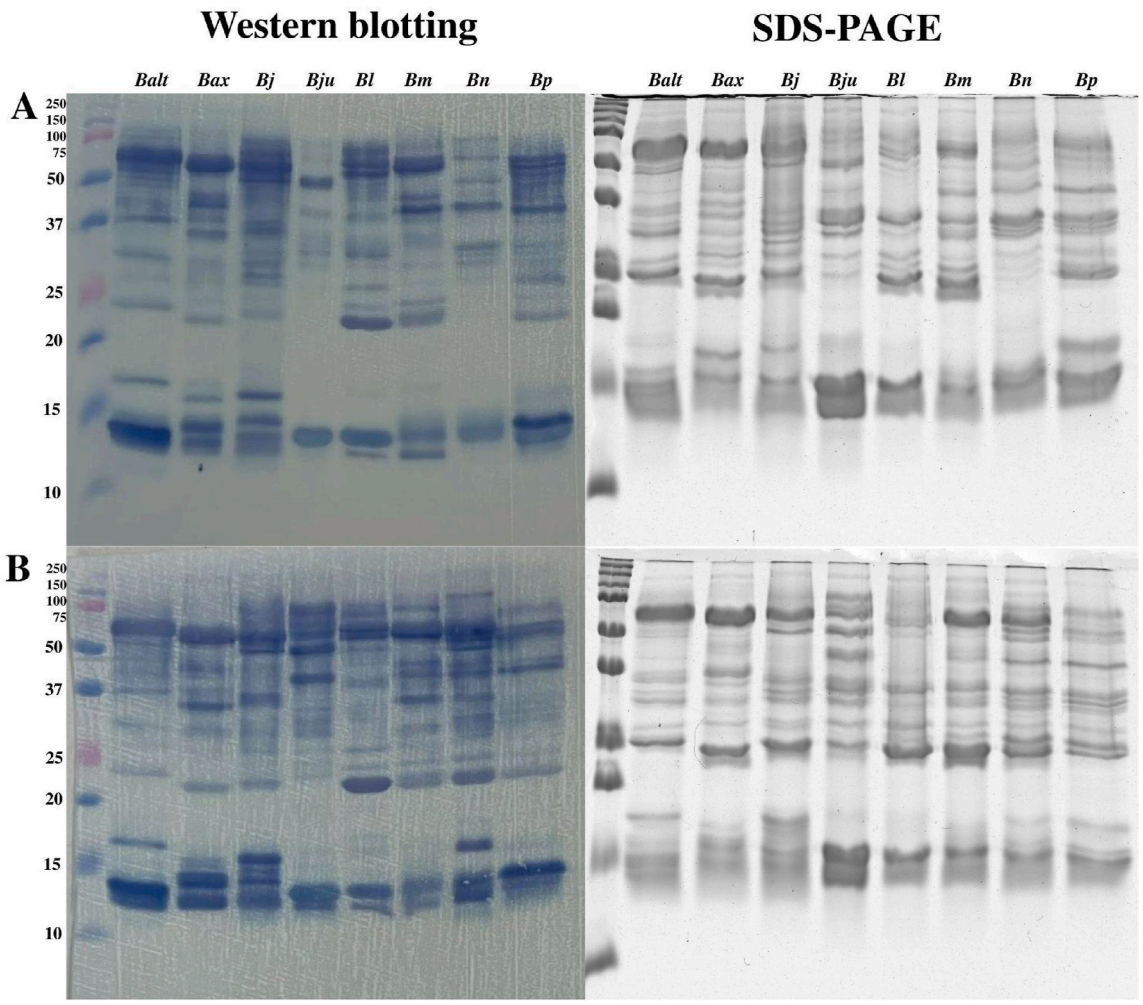

Fig. 3. Immunorecognition between venoms from males (A) and females (B) of the species *B. alternatus (Balt), B. atrox (Bax), B. jararaca (Bj), B. jararacussu (Bju), B. leucurus (Bl), B. moojeni (Bm), B. neuwiedi (Bn), B. pauloensis (Bp)* and commercial antibothropic serum. The interaction of venom proteins with the antivenom produced at the Institute Butantan was evaluated by western blotting. The samples were subjected to 1-DE, the proteins were electrotransferred to a PVDF membrane and incubated with the antibotropic serum.

female *Bju* had lower recognition within the species evaluated. The titer of *Balt* and females of *Bax* and *Bju* was lower than other species. In addition, we call attention to the difference observed between males and females of *Bn*, in which the female venom pool was strikingly more recognized compared to the variation between both sexes in other species.

3.5. High performance liquid chromatography - reversed phase (HPLC)

The chromatogram profiles showed notable variability and some quantitative differences among the venoms studied. Proteins groups highlighted in the chromatograms were based on the study by Hatakeyama et al. [19].

Male venoms had more intense peaks than females in different regions, with the exception of DIS elution region (dotted box shaded in blue) for *Bax* and *Bj*, and also PIII SVMP elution region (dotted box shaded in light purple) where females of the species *Bj, Bl, Bm, Bn* and *Bp* showed more intense peaks than males. The chromatograms of *Bn* and *Bp* showed lower intensity of peaks than other species (Fig. 5).

It was also notable the absence of non-enzymatic PLA_2 in *Balt* and *Bax* venoms while *Bju* showed the most intense peak in this region, indicated with a dotted box shaded in yellow (Fig. 5).

4. Discussion

The knowledge on snake venom intra- and interspecific variation is fundamental for a better understanding of envenomation and its clinical manifestation; to understand biochemical mechanisms behind venom function; to improve the efficiency of antivenom serums;

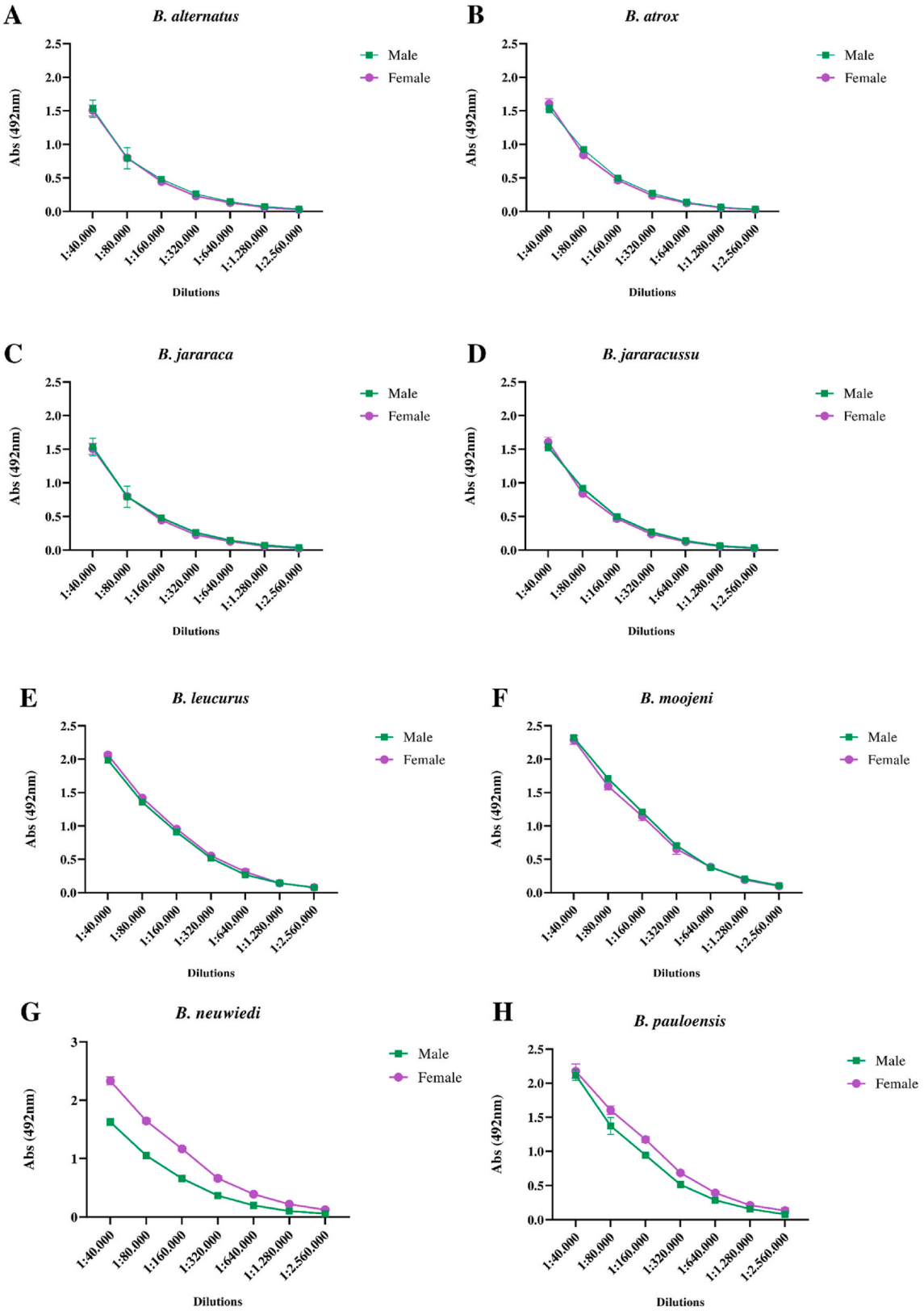

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Fig. 4. – Enzyme Linkes Immuno Sorbey Assay (ELISA) was made to see cross-reactivity of the antibothropic serum and eigth *Bothrops* sp venoms (*B. alternatus, B. atrox, B. jararaca, B. jararacussu, B. leucurus, B. moojeni, B. neuwiedi, B. pauloensis*). Males are shown in the green squares and females are represented by the pink circles. ELISA plates were coated with 1 μg of *Bothrops* venoms and incubated with different dilutions of horse anti-bothropic serum, followed by immunoenzymatic conjugate (peroxidase-conjugated horse anti-IgG) diluted 1:10000.

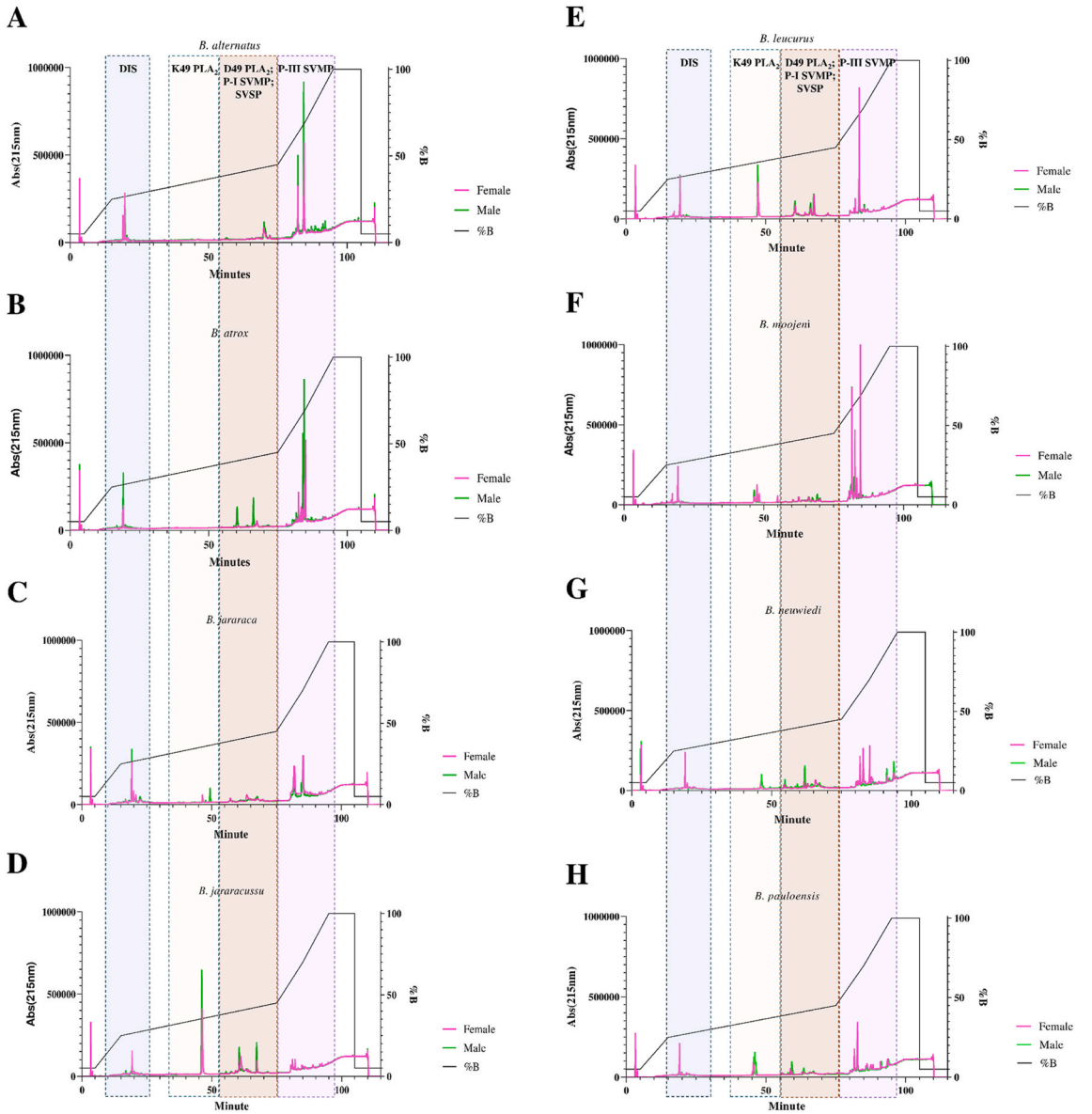

Fig. 5. Venom pool profile of males (green) and females (pink) of *B. alternatus* (A), *B. atrox* (B), *B. jararaca* (C), *B. jararacussu* (D), *B. leucurus* (E), *B. moojeni* (F), *B. neuwiedi* (G) and B. *pauloensis* (H), by RP-HPLC. Samples of 1 mg of lyophilized venom diluted 1:1 in PBS were subjected to RP-HPLC on a C-18 column. Elution was done at 1 mL/min with application of solution B (95 % acetonitrile containing 0.1 % TFA) in the gradient: 5 % for 5 min; 5–25 % for 10 min, 25–45 % for 60 min, 45–70 % for 10 min, 70–100 % for 10 min, and 100 % for 10 min. Absorbance was measured at 215 nm to identify peptide bonds. Males are shown in the green lines and females are represented by the purple lines. DIS: disintegrin; K49 PLA_2: non-enzymatic phospholipase A_2; D49 PLA_2: enzymatic phospholipase A_2; SVSP: snake venom serine protease; SVMP: snake venom metalloprotease [19].

and to support the search for new biotechnological products of interest (Chippaux; Williams & White, 1991; Girón et al., 2018; Pla et al., 2017b; Yu; Yu; Casewell et al., 2020; Li, 2020). Due to the wide geographic distribution of this genus' species, intra- and interspecific variations are expected between, especially considering the sex of the animal ([29]; Chippaux et al., 1991; [30–32]). Therefore, in our study, methodologies performed aimed to comparatively investigate the protein composition profile, biochemical

activities and immunorecognition of toxins present in *Bothrops* adult venom s of the species: *B. alternatus, B. atrox, B. jararaca, B. jararacussu, B. leucurus, B. moojeni, B. neuwiedi* and *B. pauloensis*.

Regarding the protein composition of venom pools analyzed by SDS-PAGE and HPLC, it is pertinent to state that interspecific variability was predominant and we found some differences between sexes of the same species, as observed in several studies ([32]; Da Silva Aguiar et al., 2020; Guércio et al., 2006 [19]; López-Lozano et al., 2002; Saldarriaga et al., 2003; Santoro et al., 2015; Zelanis et al., 2009; Zelanis; Travaglia-Cardoso & Furtado, 2008).

In the electrophoretic profile resulting from the 15 % SDS-PAGE with samples in a reduced state, analyzing species individually, the pattern is similar to what was reported in other studies ([33]; Galizio et al., 2018 [18,19,21], Tasima et al., 2021).

Concerning differences found between males and females, some previous studies corroborate our findings, as reported for *B. atrox* by Hatakeyama and collaborators (2020), where few differences between pools of males and females were observed, except for bands by around 37-35 kDa, which were stronger in females. In *B. jararacussu*, Aguiar and collaborators (2020) showed that females had an electrophoretic profile with stronger bands than males, which is similar to our study.

Hatakeyama and collaborators (2021) also showed that *B. moojeni* females have a protein band more intense than males. Tasima and collaborators (2021) found differences between males and females of *B. pauloensis*, the opposite of what was found in the present study, where the results between sexes were very similar.

It is known that the existence of a cofactor present in LAAO, called riboflavin, is what characterizes the yellowish color of the venoms and, due to this, the enzymatic variation may be hypothesized by the color of the samples. Despite this, *in vitro* assay is indispensable (Izidoro et al., 2014). LAAO activity was measured based on the cleavage reaction of the substrate, L-methionine, with consequent production of H_2O_2.

The data obtained suggests greater activity in male group in all species analyzed, mainly in species with sexual dimorphism, such as *Balt*, *Bj*, *Bju* and *Bn*. Previous studies focusing on ontogenetic variation in other *Bothrops* species have already portrayed greater LAAO activity, not only in groups of older snakes, but also greater activity in males, as seen in the present work ([18,19,21]; Marín et al., 2021; Tasima et al., 2021).

Even though in all SDS-PAGE there were intense presence of bands in the 16–13 kDa region, generally associated with PLA_2 (Kini, 2003; [34]), the result of PLA_2 activity showed a diversity in the intensity of their action between species and sex. This fact may be related to the fact that, in Viperidae family, PLA_2s from snake venoms are divided into enzymatic and non-enzymatic, with Asp49 - D49 having catalytic activity, and the Lys49 - K49 (or S49, R49, Q49 e N49) without catalytic activity (Rodrigues et al., 2020; Yamazaki; Hyodo; Morita, 2003). The replacement of amino acid residue D49 by K49 causes interference in the binding of calcium, which is an essential cofactor for the enzyme's activity (Kini, 2003).

Additionally, PLA_2s present great versatility and are related to several biological functionalities, which have pre-synaptic action as β-neurotoxins and are also involved in the inflammatory process characterized by increased microvascular permeability, formation of edema, recruitment of leukocytes in tissues, nociception and release of inflammatory mediators (Dutta et al., 2019; Miyoshi; Tu, 1996; Trento et al., 2019). This protein family is responsible for myotoxicity, platelet aggregation, neurotoxic effect and hemolysis (Pla et al., 2019 [35]; Gutiérrez e Lomonte, 1995; [34]) seen in envenomation.

B. jararacussu samples in SDS-PAGE had a strong presence of proteins in the 15 kDa region, but the result of PLA_2 activity of this species was the third lowest. When analyzing the chromatogram, the region before 50 min, where PLA_2 K49 is eluted, *B. jararacussu* presented the highest peak within all sample groups. As it is widely known, this species is rich in this isoform of PLA_2 (Kini, 2003; Calvete et al., 2011). Thus, the activity *in vitro* does not reflect the composition observed in SDS-PAGE, nor the chromatographic profile.

On the other hand, the chromatogram of *B. moojeni* male, which was the sample pool with the highest PLA_2 activity, was not the one that obtained the highest elution peak in the region between 50 and 75 min, as well as *B. leucurus* male, which, despite having more peaks in the region of PLA_2 - D49, also had a large peak before 50 min. These data lead to the feasibility that the quantity of proteins in samples are not necessarily related to their potential action [10,12].

The measurement of proteolytic activity of venoms was evaluated using casein as a substrate, which is commonly associated with both serine proteases and metalloproteases. In this assay, the enzymatic activity of females was higher than males, similarly to what was evidenced in other studies with *B. jararaca, B. jararacussu, B. moojeni* and *B. pauloensis*, ([19]; Tasima et al., 2021; [21,30]). Focusing on *B. atrox*, a study by Hatakeyama and collaborators (2020) showed proteolytic activity with a similar pattern between males and females. Sanz and collaborators (2020), in a comparative study between two subspecies (*B. b. bilineatus* and *B. b. smaragdinus*), provided evidence that females of *B. b. smaragdinus* also have a higher caseinolytic activity than males, which is corroborated with this study.

Comparing the chromatograms, not all females of all species showed larger or more peaks than males in SVMP or SVSP elution regions. Similarly, in SDS-PAGE we observed that males had more intense bands than females in certain regions where these proteins might be located, leading us to believe that their quantity may not reflect their potency.

The increase of venom proteolytic activity as age advances has already been observed in generalist species present in this study and in other species of snakes (Andrade; Abe, 1999; [23,36]). Some authors suggest this differentiation may be due to the shift in animals' diet (Andrade; Abe, 1999; [23,36]). However, a change in this activity relative to sex may perhaps be related to the fact that females generate offspring and might need greater defense mechanisms, since the main symptomatology caused by proteases (SVMP and SVSP) involves tissue degradation, coagulant, hemorrhagic and fibrinogenolytic action ([5]; Secretaria Da Saúde Do Estado De São Paulo, 1993 [7]; Andrade; Abe, 1999 [36]; Gutiérrez et al., 2006; [6]).

The western blotting assay showed that immunological recognition of all venoms presented similar results. There was a lower intensity in the recognition of proteins between 37 kDa (SVSP has low immunogenic potential due to their glycosylation sites) and an

above 15 kDa (PLA_2), which leads us to the hypothesis that, as in other studies, higher molecular weight molecules are more likely recognized by the immune system, while smaller ones have low immunogenicity, as shown in *B. atrox* and *Lachesis muta*, in which bands between 46 and 14.3 kDa have low immune recognition (Colombini et al., 2001; Da Silva Aguiar et al., 2020 [18,19]; Marín et al., 2021; Tasima et al., 2021; Abbas, 2007).

In the study by Queiroz et al. [33] proteins with molecular weight between 50 and 14 kDa had a stronger and more intense recognition by the antivenom produced at Instituto Butantan, than components around 15 kDa, present in *B. taeniata* venom. However, in the present study, all species showed strong recognition for low molecular weight proteins in western blotting. In the same study by Queiroz et al. [33] there were samples of *B. leucurus* and *B. neuwiedi* that presented a possible weaker recognition, while in the results obtained here, both species showed good immune recognition, with only males having a slightly lower intensity.

ELISA shows a good immune interaction between proteins present in all *Bothrops* venoms, and even with the interspecific quantitative difference between *Bothrops* species, bothropic antivenom produced at Institute Butantan is effective. Five of the eight species used in the study are used in the venom pool to produce bothropic antivenom. The polyspecificity of Latin American antivenom sera often results in cross-recognition of proteins present in the venom of different snake's species [37]. Given that, recognition assay must be complemented by an *in vivo* neutralization test to validate the efficacy of the antivenom.

The most recent works affirm that the variation in a specific group of enzymes activities, evaluated by enzymatic assays, might not correspond to the real effect of the venom on its prey, since, despite each protein having its own action, there is a synergistic effect when envenomation occurs (Tasima et al., 2021; Ferraz et al., 2019; Xiong & Huang, 2018). In some species the variation in diet according to age, might be important to occupy different ecological niches and reduce competition (Surm & Moran, 2021). In general, there are studies that do not show major variations in protein composition depending on the gender of snakes (Lira-Da-Silva, 2009; Da Silva 2017), while other studies on peptidomic evaluation describe gender-specific molecular markers in the venom of males and females of *B. jararaca* ([31]; Zelanis et al., 2016).

Such inter- and intraspecific variations found in several studies, including ours, highlight the importance of characterizing biochemical and protein profiles of snakes' venoms,. since, when we understand venom as a valuable and rich source of proteins, it is easier to map, with the aid of this kind of study, which species and sex have the greatest potential for certain activities, and target venom extractions to purify important enzymes with biotechnological interest.

It is noteworthy that, in the present study, all animals were kept in captivity at the Laboratório de Herpetologia at Instituto Butantan, with a diet based solely on mice and rats. Thus, minimizing the influence of diverse prey consumption in the venom.

When we understand the venom as a valuable source of proteins, it is easier to map, with the aid of studies like ours, which species and sex have the greatest potential for certain activities and then target venom extractions to purify important enzymes with biotechnological interest.

5. Conclusion

In general, the venom of *Bothrops* genus demonstrated a sex correlation in some analyzes which could lead to the conclusion that there are interspecific disparities in protein composition and biochemical activity, which was already expected. Possible variations in protein composition and enzymatic activity between males and females of the same species, at different intensities, were also observed. When comparing profiles of electrophoresis gels and the chromatograms generated by HPLC, it is plausible to consider that main fluctuations are found in SVMP and PLA_2.

The antibothropic antivenom showed similar immune recognition both between species and gender, with a deficiency in the recognition of medium molecular mass proteins, raising the hypothesis of the necessity of improving serum therapy for envenomations caused by *Bothrops*.

Considering all the results, there may be fluctuations in the symptoms of envenomation and the way in which the body reacts to antibotropic serum therapy due to intraspecific and interspecific variation in *Bothrops* venom. However, this protein diversity observed in this species highlights the relevance of studies focused on venom variability for ecological, evolutionary, and medical purposes.

Data availability statement

This study hasn't data associated deposited into a publicly available repositor and data will be made available on request.

Ethical statement

All procedures involving the use of animals were performed in accordance with the Guide for the Care and Use of Laboratory Animals (1996) and were approved by the Ethical Committee for the Use of Animals of Instituto Butantan - CEUAIB (protocol n° 7967310720).

CRediT authorship contribution statement

Thais Almeida de Godoy: Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Eduardo Oliveira Venancio de Lima:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology. **Giovanni Perez Machado Silveira:** Resources. **Fabíola Souza Rodrigues:** Resources. **Sávio Stefani Sant'anna:** Writing – review & editing, Resources. **Daniela Miki Hatakeyama:**

Writing – review & editing, Writing – original draft, Visualization. **Kathleen Fernandes Grego:** Writing – review & editing, Resources. **Anita Mitico Tanaka-Azevedo:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Thais Almeida de Godoy reports financial support was provided by Sao Paulo State Department for Health Coordination of Science Technology and Strategic Health Supplies. Anita Mitico Tanaka-Azevedo reports was provided by State of Sao Paulo Research Foundation. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e37262.

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