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Proteomic and biochemical analyses of short-tailed pit viper (*Gloydius brevicaudus*) venom: Age-related variation and composition–activity correlation☆

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ABSTRACT

We conducted an in-depth analysis of the proteomic and biochemical profiles of the venom of neonate and adult short-tailed pit vipers (*Gloydius brevicaudus*). Identified proteins were assigned to a few main toxin families. Disintegrin, phospholipase A_2 (PLA_2), serine proteinase, cysteine-rich secretory protein, C-type lectin-like protein, L-amino acid oxidase and snake venom metalloproteinase (SVMP) were detected in both venoms, while 5′-nucleotidase was detected only in the adult venom. SVMP was the predominant protein family in both venoms (neonate: 65.7%; adult: 64.4%), followed by PLA_2 (neonate: 13.4%; adult: 25.0%). Antivenomic analysis revealed that commercial *G. brevicaudus* antivenom almost neutralized the chromatographic peaks with medium and high molecular masses in both venoms, but did not completely recognize peaks with low molecular mass. Toxicological and enzymatic activities show remarkable age-related variation in *G. brevicaudus* venom, probably resulting from variation in venom composition. Our data demonstrate age-related variation across venomics, antivenomics and biochemical profiles of *G. brevicaudus* venom, and have implications for the management of *G. brevicaudus* bites, including improving antivenom preparation by combining both venoms.

Biological significance

This study investigates the composition and biochemical activity of neonate and adult *Gloydius brevicaudus* venoms. We found remarkable age-related variation in venom biological activity, likely the result of variation in venom composition. Antivenomics analysis was used to explore difference in neonate and adult *G. brevicaudus* venoms. Our findings have implications for the diagnosis and clinical management of *G. brevicaudus* bites, and the design of venom mixtures that will increase the efficacy of commercial antivenom.

This article is part of a Special Issue entitled: Proteomics of non-model organisms.

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1. Introduction

Venomous snakebites have long been a serious public health problem and it is estimated that 421,000–1,841,000 envenomings and 20,000–94,000 snakebite deaths occur worldwide each year [1]. The highest burden of envenomation is in Africa, Asia and Latin America [1,2]. In East Asia, mainly in China, annual clinical cases of envenoming and death caused by venomous snakes are estimated to vary from 4582 to 218,673 and from 462 to 4829, respectively [1]. This alarming situation has promoted Shanghai Serum Biological Technology (the only antivenom manufacturer in China) to expand its workshop and increase the production capacity of antivenom from 60,000 to 200,000 vials per year.

Small pit vipers, species within the genus *Gloydius* in particular, are responsible for the highest frequency of snakebite envenomations in China where there are seven *Gloydius* species [3]. The short-tailed pit viper *Gloydius brevicaudus* causes more problems for local people than the other six congeneric species because: (1) the abundance of *G. brevicaudus* is high across its distribution; and (2) *G. brevicaudus* is distributed in regions with high human occupancy and is well adapted to anthropically disturbed environments (Fig. 1) [3–6]. The burden of snakebites caused by *G. brevicaudus* is heavy in China. In East China, for example, the incidence rate of envenomation (per 100,000 people) is up to 18 in Jiangxi Province, and 14 in Zhejiang Province [7,8]. The principal clinical symptoms of envenoming by *G. brevicaudus* are local damage characterized by ecchymoma, blistering, edema and occasional tissue necrosis, and serious systemic damage characterized by dizziness, blurred and double vision, followed by chills, fever, myalgia, tachypnea, tachycardia, and in some severe cases, even disseminated intravascular coagulation, cardiovascular shock and actual renal failure [3,7].

Variability in venom composition has been receiving attention from toxinologists and herpetologists because of the association between the complexity of clinical symptoms and the diversity of venom composition. The 'snake venomics' project initiated the in-depth analysis of viperid venom proteomes [9] and, over the past decade, snake venomics analyses by proteomic approaches or venom gland transcriptomic sequencing have been carried out in more than 120 taxa [10–18]. Based on venomics, potential toxin–biochemical/pharmacological correlations and immunological reactivity of snake venoms have been well explained in some species [12,19–21]. Further work could usefully clarify the variability of composition and biochemical characteristics of snake venoms at various levels (e.g., different ages, populations and sexes) to explain complicated symptoms and aid the clinical diagnosis of envenomation and antivenom application.

The venom proteins characterized in adult *G. brevicaudus* mainly belong to six families, but the concentration of each family in total venom proteins has not been explored [22]. In our recent studies, potential age-related variation in venom composition of *G. brevicaudus* has been detected by 1-DE, 2-DE and iTRAQ analyses [14,23]. However, a relative quantitative comparison can only be done when venom proteins are shared in neonate and adult venoms, and cannot be done when each protein family contains more than one venom protein [14]. Thus, information on the whole proteome of neonate *G. brevicaudus* venom as well as age-related variation in the whole proteome or particular toxin families in *G. brevicaudus* venom remains ambiguous. Here, we used a strategy combining proteomic analysis and toxicological and enzymatic characterization of neonate and adult *G. brevicaudus* venoms to assess age-related variation in whole snake venomics and antivenomics. Findings of this study will likely have implications for evaluating relationships between biochemical activities and venom

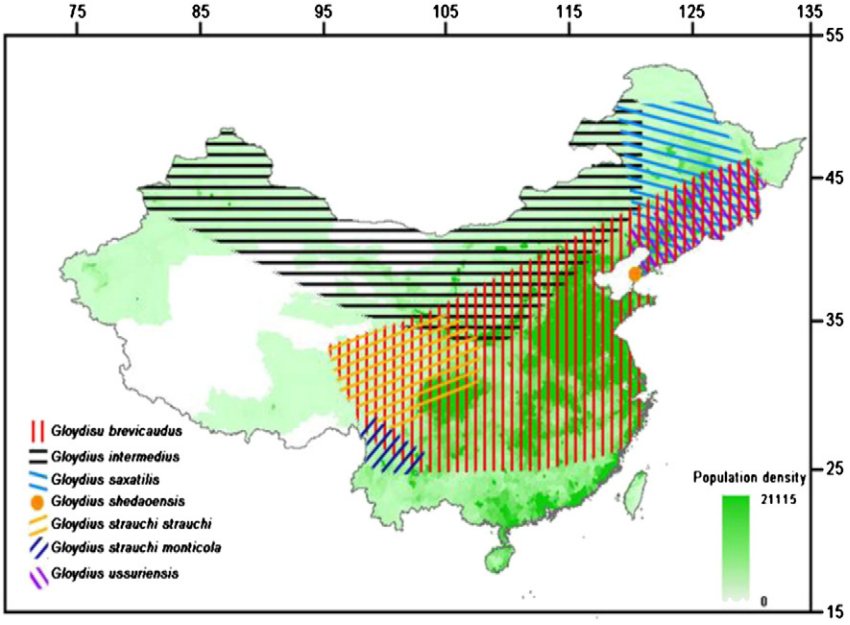

Fig. 1 – Distribution of seven *Gloydius* snakes in mainland China. The scale bar in the figure shows population density as the number of people/km^2.

composition and for optimizing the preparation of antivenom using a mixture of neonate and adult *G. brevicaudus* venom.

2. Materials and methods

2.1. Venoms and antivenoms

A total of 134 adult *G. brevicaudus* (84 females and 50 males) collected in the years of 2008–2011 from Xiaoshan, Zhejiang, China, and 548 neonates (276 females and 272 males) produced by 54 females in the laboratory were used in this study. Pooled venom samples of adults and 3-week old neonates were milked according to Mirtschin et al. [24]. Fresh venoms were centrifuged to remove impurities for 15 min at 10,000 *g* 4 °C, then lyophilized and stored at −80 °C until use. Protein concentrations of venoms were determined according to Bradford [25]. The commercial monospecific anti-*G. brevicaudus* antivenom was purchased from Shanghai Serum Biological Technology Co., Ltd. This antivenom was prepared in horses by conventional hyperimmunization procedures with adult *G. brevicaudus* venom, and consists of purified $F(ab')_2$ fragments generated by digestion with pepsin of ammonium sulfate-precipitated IgGs [26,27]. $F(ab')_2$ fragment concentration was determined spectrophotometrically using an extinction coefficient (ε) of 1.4 for a 1 mg/ml protein concentration at 280 nm using 1 cm light pathlength cuvette.

2.2. Proteomic analysis of venoms

About 2 mg of each crude venom was dissolved in 5% acetonitrile containing 0.1% trifluoroacetic acid (TFA, solution A), centrifuged for 15 min at 10,000 *g* 4 °C. Then, the supernatant was collected and loaded on a Kromasil 300 C18 column (250 × 4.6 mm, 5 μm particle) for separation using a Waters E600 chromatography system. The flow-rate was set to 1 ml/min, and venom proteins were separated with a mobile phase system of 0.1% TFA in water (solution A) and acetonitrile (solution B) at the following steps: isocratically (10% B) for 10 min, followed by 10–15% B for 10 min, 15–45% B for 80 min, and 45–60% B for 50 min. Protein detection was performed at 215 nm. Fractions were collected manually, and dried in a Centrifugal Concentrator (CentriVap®, Labconco, USA). The fractions were separated by SDS-PAGE (on 12% or 18% separation gels) under non-reduced and reduced conditions. Protein bands of interest were excised from the Coomassie brilliant blue-stained polyacrylamide gels, and subjected to reduction with dithiothreitol and alkylation with iodoacetamide, followed by automated in-gel digestion. The tryptic peptides were subjected to MALDI-TOF-MS/MS (Autoflex speed™, Bruker Dalton, Germany) or CID-MS/MS (LTQ-Orbitrap, Thermo Electron, Germany) according to the manufacturer's instructions. The MS/MS spectra were interpreted by FlexAnalysis or Xcalibur, and the assignment of protein families was performed against a private protein database deposited in NCBI (strict to the taxa Serpentes; 34,344 entries; downloaded on 12/1/2013) using Mascot search engine (version 2.3.02) or BioTools. The MS/MS mass tolerance was set at 0.6 Da. Carbamidomethyl (C) was set as fixed modification, and Acetyl (N-term) and Oxidation (M) were set as variable modifications. The relative abundance (expressed as % of the total proteins) of each protein family was estimated as described by Calvete et al. [9].

2.3. Antivenomic analysis

Immunoaffinity columns of *G. brevicaudus* antivenoms were prepared according to Pla et al. [28]. The column was packed with 1 ml NHS-activated sepharose 4 fast flow medium (GE Healthcare) according to the manufacturer's instructions, and then washed with 10–15 matrix volumes of 1 mM ice cold HCl, followed by two matrix volumes of coupling buffer (0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3). Thereafter, the matrix was incubated for 4 h at room temperature with ~50 mg $F(ab')_2$ antivenom fragments, the concentration of coupled fragments was estimated measuring the uncoupled antivenom by quantitative band densitometry of SDS-PAGE. Non-reacting groups were then blocked with 0.5 ml 0.1 M Tris–HCl, pH8.0 for 2 h at room temperature using an orbital shaker. The column was then alternately washed with 0.1 M Tris–HCl, pH 8.5 and 0.1 M acetate buffer, 0.5 M NaCl, pH 4.0 with three matrix volumes of at least 6 cycles, then equilibrated with binding buffer (20 mM PBS) for three matrix volumes. About 500 μg venoms dissolved in 0.5 ml PBS were loaded on the affinity columns, and incubated overnight at 4 °C on the orbital shaker. The venom:antivenom ratio used was corresponding to about 40 antigen sites per "25 kDa of toxin molecule". After eluting and collecting the non-retained components with PBS, the retained components were eluted with three matrix volumes of 0.1 M glycine–HCl, pH 2.0, and neutralized with 1 M Tris–HCl, pH 9.0 immediately. The affinity column was then thoroughly equilibrated with PBS for recycling. Both non-retained and retained components were concentrated by a Centrifugal Concentrator (CentriVap®, Labconco, USA), and analyzed by RP-HPLC according to the procedure described above.

2.4. Toxicological and enzymatic activities of venoms

2.4.1. Lethality

Male ICR mice (26 – 28 g) from the Laboratory Animal Center of Hangzhou Normal University were used to determine the median lethal dose (LD_{50}) of venom. Groups of four ICR mice were injected intraperitoneally with various amounts of crude venom dissolved in 100 μl saline, controls only received saline at the same volume. Deaths were recorded over 24 h, and LD_{50} was estimated using the Spearman–Karber method.

2.4.2. Myotoxicity

Groups of four male ICR mice (26 – 28 g) were injected in the right thigh muscle, with 10 μg crude venom dissolved in 25 μl saline. Controls only received 25 μl saline. After 3 h, mice were bled, and the plasma creatine kinase (CK) activity was determined using a biochemical kit (Batch 20131109; Nanjing Jiancheng Bioengineering Institute, China). The CK activity was expressed in U/l.

2.4.3. Hemorrhagic activity

Hemorrhagic activity was determined according to Gutiérrez et al. [29] with some modification. Groups of three male ICR mice (26 – 28 g) were injected intradermically in the dorsal skin, with various amounts of crude venom dissolved in 100 μl saline. Controls only received saline at the same volume. Three hours later, mice were sacrificed and the hemorrhagic

areas were measured. The minimum hemorrhagic dose (MHD) was expressed as the venom dose that induced a hemorrhagic area of 10 mm diameter.

2.4.4. Proteolytic activity

We used casein as the substrate to evaluate the proteolytic activity. Venom (40 μg) was incubated with 0.5 ml substrate solution (0.2 M Tris–HCl, pH 8.5, containing 2% casein) at 37 °C for 2 h. The reaction was ended by adding of 0.6 ml 0.44 M TCA at 37 °C for 30 min. Then, the mixture was centrifuged at 12,000 *g* for 15 min. Aliquots (0.8 ml) of supernatant were mixed with 2.0 ml 0.4 M Na_2CO_3 and 0.4 ml folin reagent (original reagent/water = 1/2), and absorbance was read at 660 nm. We used L-Tyrosine as the standard, and the unit of enzymatic activity was defined as nmol of L-Tyrosine released/min/mg crude venom. For determination of the role of metalloproteinases in hydrolyzing the casein, the venoms were incubated with 40 mM EDTA for 30 min before the addition of substrate solution.

2.4.5. Esterolytic activity

Esterolytic activity was measured following modifications to Tu et al. [30]. The venom (16 μg) was incubated with 180 μl substrate solution (0.1 M Tris–HCl, pH8.0, containing 1 mM BAPNA) at 37 °C for 30 min. Then the reaction was stopped by adding of 18 μl 30% acetic acid, and the absorbance was recorded at 405 nm. We used *p*-nitroaniline as standard; the activity was expressed as nmol of *p*-nitroaniline released/min/mg crude venom. The hydrolytic reaction on TAME was carried out in a buffer system (0.1 M Tris–HCl containing 1.5 mM TAME, pH 8.5) at 25 °C. The absorbance was recorded for 4 min at 253 nm. The activity was expressed as nmol of the substrate degraded min/mg crude venom.

2.4.6. 5′-Nucleotidase activity

5′-Nucleotidase activity was determined according to Dhananjaya et al. [31]. The venom sample (1.6 μg) was added into 90 μl substrate solution (50 mM Tris–HCl, pH 7.4, containing 10 mM $MgCl_2$, 50 mM NaCl, 10 mM KCl and 10 mM 5′ AMP), and incubated at 37 °C for 30 min. Then, 0.42% ammonium molybdate in 1 M sulfuric acid and 10% ascorbic acid were added to stop the reaction. The absorbance was recorded at 660 nm. We used KH_2PO_4 as standard, and defined enzymatic activity as nmol of inorganic phosphate released min/mg crude venom.

2.4.7. L-Amino acid oxidase activity

We used L-Leucine as substrate to evaluate the L-amino acid oxidase activity according to Toyama et al. [32]. The venom (2 μg) was added into 90 μl substrate solution (50 mM Tris–HCl, pH 8.0, containing 0.25 mM L-Leucine, 2 mM o-phenylenediamine and 0.81 U/ml horseradish peroxidase) at 37 °C for 1 h. Then, the reaction was ended by 50 μl 2 M H_2SO_4, the absorbance was recorded at 490 nm. H_2O_2 was used as standard; enzymatic activity was defined as nmol of H_2O_2 degraded/min/mg crude venom.

2.4.8. Phospholipase A_2 activity

Phospholipase A_2 activity was assayed according to Antunes et al. [33] with a slight modification. Aliquots of venoms with various amounts were gently mixed with the substrate system (0.1 M NaCl, 10 mM $CaCl_2$, 7 mM Triton X-100, 0.35% soybean lecithin and 98.8 mM phenol red, pH 7.6). The absorbance was continuously recorded at 558 nm for 2.5 min at room temperature. Enzymatic activity was defined as a change in absorbance of 0.3 unit/min/mg crude venom.

2.4.9. Fibrinogenolytic activity

Human plasma fibrinogen was used as substrate to assay the fibrinogenolytic activity according to Menezes et al. [34] with some modification. The venom (0.4 μg) was mixed and incubated with 20 μl 8 μg/μl human fibrinogen at 37 °C for either 4 min or 180 min. Fibrinogen was also incubated with the venoms pre-treated with 10 mM EDTA for 180 min. The reaction was stopped by the addition of 21 μl denaturing buffer system (1 M urea, 4% SDS and 0.4% β-mercaptoethanol). Then, the mixture was assayed by SDS-PAGE on 7.5% polyacrylamide gel, and the gel was stained in 0.1% Coomassie brilliant blue R250.

2.4.10. Fibrinolytic activity

Bovine fibrinogen was used as substrate to assay the fibrinolytic activity according to Aguilar et al. [35] with some modification. Aliquots (1 ml) of sterile saline (containing 0.4% bovine fibrinogen, 0.5 U thrombin and 25 mM $CaCl_2$) were evenly poured into a 12-well plate, and agglutinated at room temperature for 30 min. The venom (16 μg) was slightly added on the fibrin surface. The reaction system was incubated at 37 °C for 3 h, and the clearance zone was measured. The activity was defined as mm^2 of the clearance zone formed/μg crude venom.

2.5. Statistical analyses

The LD_{50} was calculated by Trimmed Spearman–Karber 1.5, and expressed as 95% confidence limits. Statistical analyses of other toxicological and enzymatic activities were performed using Statistica 8.0 (StatSoft Inc., USA). We used Student's *t*-test to analyze the corresponding data. Descriptive statistics are presented as mean ± standard error (SE), and the significance level is set at $\alpha = 0.05$.

3. Results and discussion

3.1. Characterization of the venom proteome of neonate and adult G. brevicaudus

Age-related variation in venomics has been demonstrated in a number of snake species, and has provided useful information for snakebite management and understanding the adaptive evolution of venom proteins [36–43]. In an earlier study of *G. brevicaudus*, we examined a neonate-to-adult transition of snake venomics mainly by iTRAQ [14], a technique used to label each tryptic peptide with isobaric tag, and increase the sequencing coverage of peptides, including those from low abundant, highly acidic/basic and hydrophobic proteins [44]. iTRAQ is a LC-based proteomic technique, presenting higher sensitivity and reproducibility as compared with gel-based techniques (e.g. 2-DE). Moreover, iTRAQ can work together with mass spectrometer directly, thus making automatic operation more likely to realize. Ideally, iTRAQ can execute absolute quantitative analysis of a

special protein using unique peptides labeling with isobaric tags as internal standard [45]. However, it is complex, difficult and expensive for absolute quantitation of mixed samples, like snake venoms. Therefore, iTRAQ was mostly used for relative quantitative comparison of the proteins that shared in the similar samples under different experimental conditions, like neonate and adult venoms of *G. brevicaudus* [14] and *Bothrops jararaca* [42]. Our previous study only revealed relative quantitative differences in several important venom components between neonates and adults, but not age-related variation in whole snake venomics or each protein family. A combined proteomic strategy including RP-HPLC separation followed by SDS-PAGE analysis and protein band identification by MALDI-TOF-MS/MS and CID analyses was proposed by Calvete and his colleagues [9,46], and was widely used in whole proteome analysis of snake venom. Although the strategy cannot avoid shortcomings of gel-based proteomic techniques, it can estimate the relative amount of each component and protein family in the total venom sample and has advantages in absolute quantitative comparison of similar venom components or protein families.

In this study, the proteins of crude venoms pooled from neonates and adults were fractionated by RP-HPLC, followed by an analysis of each fraction using SDS-PAGE, MALDI-TOF/TOF and ESI mass spectrometry. The RP-HPLC procedure resolved 37 fractions from the neonate venom and 27 fractions from the adult venom (Figs. 2 and 3), and two fractions from each venom could not be characterized by mass spectrometric analysis (Tables 1 and 2). Eight protein families [disintegrin, phospholipase A_2 (PLA_2), serine proteinase, cysteine-rich secretory protein (CRISP), C-type lectin-like protein (CTL), L-amino acid oxidase,

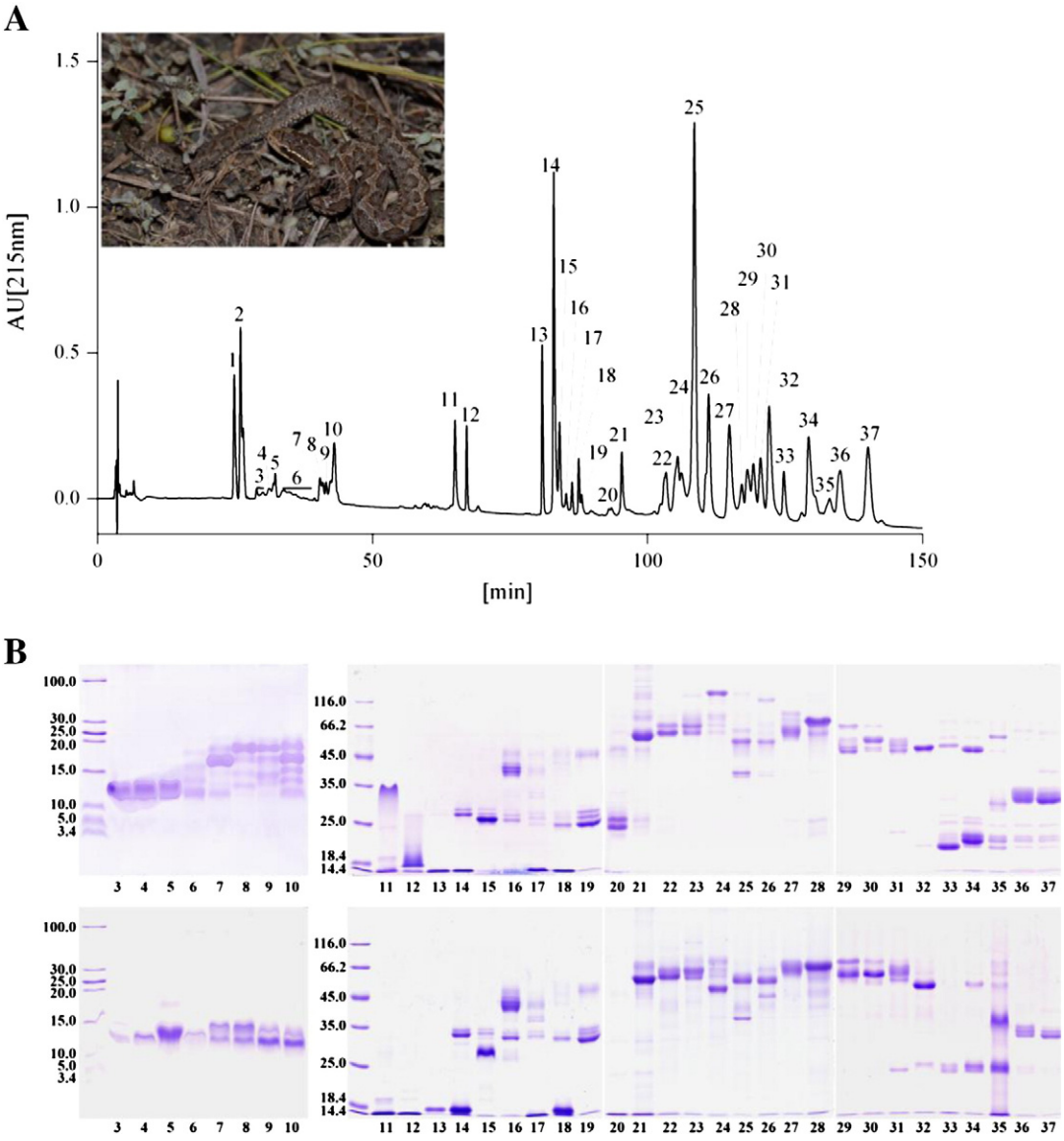

Fig. 2 – Characterization of venom proteins of neonate *Gloydius brevicaudus* from China. A: Separation of venom proteins by reverse-phase HPLC. Two milligrams of pooled venom proteins was fractionated on a Kromasil 300 C18 column, as described in the Materials and methods. B: Fractions were collected and analyzed by 12% or 18% SDS-PAGE under non-reduced (upper panels) and reduced (lower panels) conditions, and characterized by in-gel tryptic digestion followed by MALDI-TOF/TOF or nESI-MS/MS identification. Results are shown in Table 1.

5′-nucleotidase, and snake venom metalloproteinase (SVMP)] were identified in the adult venom; all protein families except 5′-nucleotidase were also identified in the neonate venom (Fig. 4). Our results support the conclusion that snake venom is mainly comprised of the proteins belonging to a few toxin families [47,48]. Using adult *G. brevicaudus* venom, Li and his colleagues identified six protein families: disintegrin, PLA_2, serine proteinase, CTL, L-amino acid oxidase and SVMP [22]. In addition to these six families, we have identified here another two protein families, CRISP and 5′-nucleotidase. Disintegrin and 5′-nucleotidase are two protein families not detected in a previous study using the same venom samples [14]. Four toxin families (hyaluronidase, nerve growth factor, phospholipase and peptidase) and some non-toxin components found in the previous study were not detected here, and these toxins also were not detected in the protein spots separated from adult *G. brevicaudus* venom by 2-DE [22]. These toxins were not observed in gels stained by Coomassie brilliant blue because they were expressed with low abundance. However, they could be identified in our previous study using the more sensitive iTRAQ technique [14]. Given different outcomes between studies, it is clear that a strategy that combines various proteomic approaches will arrive at a more comprehensive recognition of snake venomics.

In line with many studies that show SVMPs present in high amounts in total venom proteins in viperid snakes [15,37,40,43,49–57], the concentrations of SVMPs from neonate and adult *G. brevicaudus* venoms were respectively up to 64.4% and 65.7% of total venom proteins, and was estimated as the major protein family in both venoms (Fig. 4). The analysis of

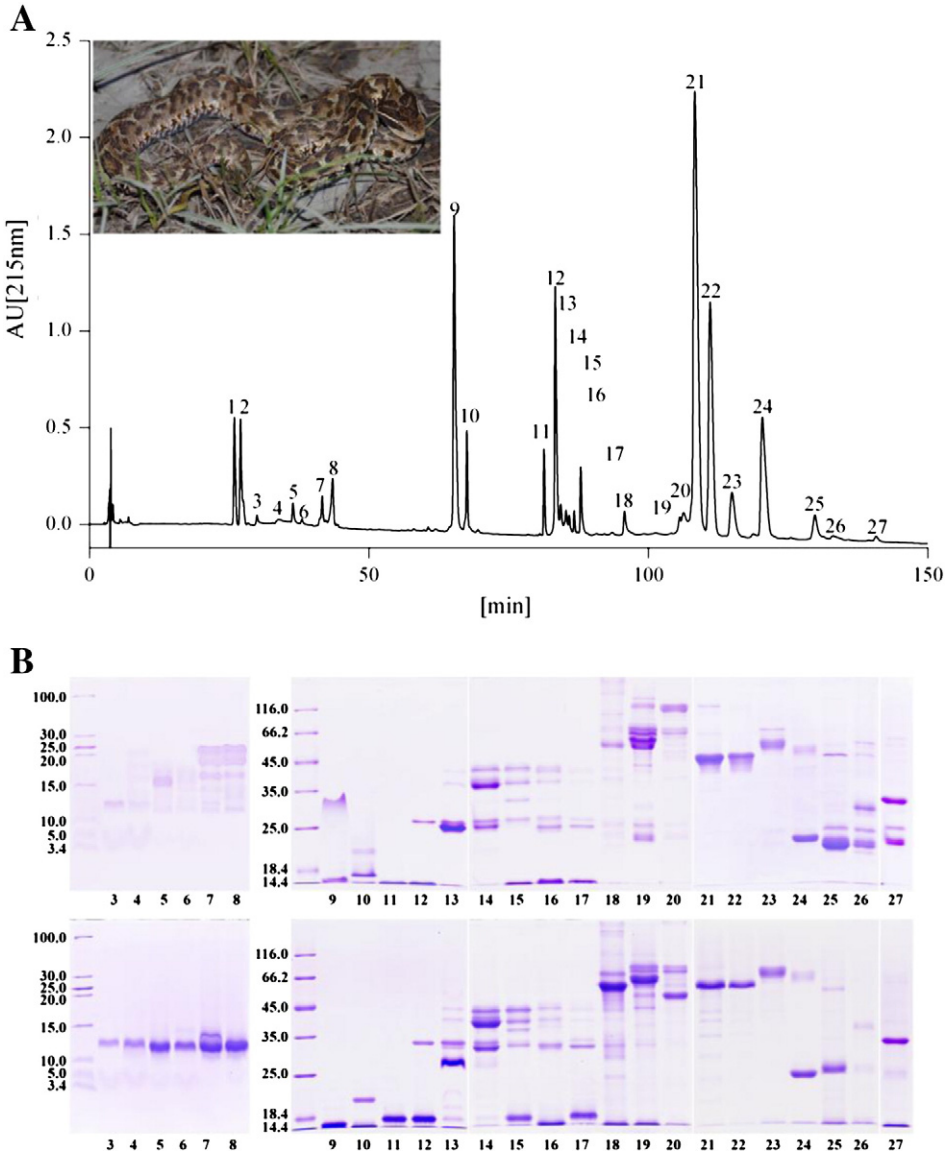

Fig. 3 – Characterization of venom proteins of adult *G. brevicaudus* from China. A: Separation of venom proteins by reverse-phase HPLC. Two milligrams of pooled venom proteins was fractionated on a Kromasil 300 C18 column, as described in the Materials and methods. B: Fractions were collected and analyzed by 12% or 18% SDS-PAGE under non-reduced (upper panels) and reduced (lower panels) conditions, and characterized by in-gel tryptic digestion followed by MALDI-TOF/TOF or nESI-MS/MS identification. Results are shown in Table 2.

Table 1 – Assignment of the reverse-phase chromatographic fractions from neonate venom to protein families by MALDI-TOF/TOF and nESI-MS/MS of selected peptide ions from in-gel digested protein bands separated by SDS-PAGE.

Peak	%	MW (kDa)	Peptide ion		MS/MS-derived sequence	Protein family
			m/z	z		
1,2	n.d.					
3–6	2.8	13▼	906.8	2	BGABCAEGXCCDBCR	Disintegrin; *Gloydius brevicaudus*; Q698K8
			951.9	2	GDDMDDYCNGXSAGCPR	
7a–10a	1.8	14▼	951.9	2	GDDMDDYCNGXSAGCPR	Disintegrin; *G. brevicaudus*; AAP20644
			959.9	2	GDDMDDYCNGXSAGCPR	
			689.0	3	KXRPGABCAEGXCCEBCR	
7b–10b	2.8	13▼	634.9	3	GDDMDDYCNGXSAGCPR	Disintegrin; *Gloydius halys*; Q9DGH6
			951.9	2	GDDMDDYCNGXSAGCPR	
			634.9	3	GDDMDDYCNGXSAGCPR	
			959.9	2	GDDMDDYCNGXSAGCPR	
			684.3	3	BXRPGABCAEGXCCDBCR	
			1025.9	2	BXRPGABCAEGXCCDBCR	
11	2.3	14▼	2028.8	1	EPVVSYAFYGCYCGSGGR	PLA_2; *Agkistrodon halys*; 1JIA_A
			2446.0	1	MTGBEPVVSYAFYGCYCGSGGR	
12	1.4	14▼	852.4	1	VAAECFR	PLA_2; *A. halys*; 1BJJ_A
			876.5	1	NXXBFNK	
			1008.5	1	VAAECFRR	
			1075.5	1	SDXYSYSXK	
			1533.5	1	CCFVHDCCYGR	
			1670.7	1	NXDTYNNGYMFYR	
			2541.1	1	NAXPFYAFYGCYCGWGGBGBPK	
13,14a	8.4	16▼	1322.7	1	SXXBFETXXMK	PLA_2; *G. halys*; O42191
14b	2.7	29■	1215.6	1	BVPNEDEBTR	Serine protease; *G. halys*; 4E7N_A
			1477.7	1	APYPEFEXPATSR	
			1526.7	1	XXGGDECNXNEHR	
		28■	1069.6	1	FXVAXYTSR	Serine protease; *G. halys*; 4E7N_A
			1477.7	1	APYPEFEXPATSR	
			1526.7	1	XXGGDECNXNEHR	
15	2.5	26■	1537.7	1	MEWYPEAAANAER	CRISP; *Gloydius blomhoffi*; Q8JI40
			1755.8	1	WTDXXHXWHDEYK	
			1905.0	1	BPEXBNEXVDXHNSXR	
16	0.7	42■	1512.7	1	VXGGDECNXNEHR	Serine protease; *G. halys*; Q802F0
17	0.8	15■	1785.8	1	BXCECDKDAAXCFR	PLA_2; *G. blomhoffi*; O42192
18a, 19	1.1	25■	1003.6	1	DKDXMXXR	Serine protease; *G. brevicaudus*; Q9YGJ8
			1117.5	1	XXNEDEQTR	
18b	0.5	15■	1785.8	1	BXCECDBDAAXCFR	PLA_2; *G. blomhoffi*; O42192
20	0.5	25■/24■	1918.7	1	DCPSDWSSYEGHCYR	C-type lectin; *G. halys*; CCH35770
21	1.7	56■	1514.7	1	ETDYEEFXEXAR	L-Amino-acid oxidase; *G. halys*; Q6STF1
			1708.8	1	NDBEDWYANXGPMR	
			2092.0	1	DCADXVXNDXSXXHBXPR	
22–24a	1.5	73■	2225.9	1	XHSWVECESGECCEBCR	SVMP-III; *Viridovipera stejnegeri*; Q3HTN2
22–24b	2.2	65■	2011.8	1	XTPGSBCAEGXCCDBCR	SVMP-II; *Protobothrops jerdonii*; P0C6E4
22–24c	2.9	59■	2224.9	1	XHSWVECESGECCBBCR	SVMP-III; *Crotalus adamanteus*; AEJ31991
24d	1.5	129■	1685.6	1	AATDECDMADXCTGR	SVMP-III; *G. halys*; Q8AWI5
			1812.6	1	BGABCAEGXCCDBCR	
		54▼	1113.6	1	BEHBAFXXK	SVMP-III; *G. halys*; Q8AWI5
			1269.5	1	SAECTDRFBR	
			1379.5	1	ACSNGBCVDVNR	
			1685.6	1	AATDECDMADXCTGR	
			1739.9	1	MYDXVNVXNVXYBR	
			1755.9	1	MYDXVNVXNVXYBR	
			1812.7	1	BGABCAEGXCCDBCR	
			2401.0	1	SCVMAGTXSCEASYXFSDCSR	
25,26a	21.7	60▼	1052.4	1	GNYYGYCR	SVMP-III; *G. brevicaudus*; P0C7B0
			1132.6	1	FVEXVXVADK	
			1180.5	1	KGNYYGYCR	
			1628.8	1	MYEXANNXNDXYR	
			1664.9	1	FVEXVXVADKGMVTK	
			2051.0	1	XTVTPNVDDTXSSFAEWR	
			2082.9	1	MFYSNEDEHKGMVXPGTK	

(continued on next page)

Table 1 (*continued*)

Peak	%	MW (kDa)	Peptide ion		MS/MS-derived sequence	Protein family
			m/z	z		
26b	1.3	49▼	985.5	1	EHBAFXXK	SVMP-III; *G. halys*; Q8AWI5
			1058.5	1	XACEPBNVK	
			1113.6	1	BEHBAFXXK	
			1379.6	1	ACSNGBCVDVNR	
27	5.4	72▼	1623.8	1	XYBXANXXNEXYR	SVMP-III; *Echis coloratus*; ADI47644
			2225.7	1	XHSWVECESGECCBBCR	
28	1.8	67■	1006.2	3	YVEFXXVVKPVECTXNTTMXQXHXR	SVMP-III; *Echis ocellatus*; ADW54344
29,30	5.4	61▼	1327.7	1	YXEXVXVADHR	SVMP-II; *P. jerdonii*; P83912
31	3.1	64▼	1327.7	1	YXEXVXVADHR	SVMP-II; *P. jerdonii*; P83912
32	6.3	87■	1194.6	1	VTXDSFGNWR	SVMP-III; *C. adamanteus*; AEJ31991
33a	0.5	91■	1194.6	1	VTXDSFGNWR	SVMP-III; *C. adamanteus*; AEJ31991
33b	1.4	22■	1096.6	1	YDSNXDTXR	SVMP-II; *G. halys*; AAD02655
34a	1.3	83■	1024.4	1	GBGHFYCR	SVMP-III; *Trimeresurus flavoviridis*; BAK64383
			1174.6	1	XFPCAPBDVK	
			1606.9	1	XFEXXNVXTXXYR	
34b,35a	4.3	26■	1918.7	1	DCPSDWSSYEGHCYR	C-type lectin; *G. halys*; CCH35770
			1972.8	1	EMTWEEAEBFCTBBR	
		24■	1235.6	1	TEXYVWVGXR	C-type lectin; *G. halys*; Q7SZV0
			1244.5	1	WFNXYCGER	
		23■	1235.6	1	TEXYVWVGXR	C-type lectin; *G. halys*; CCH35771
			1244.5	1	WFNXYCGER	
			1372.6	1	BWFNXYCGER	
			1701.6	1	TWEDAESFCTEBAK	
			1890.6	1	DCPSDWSSYEGHCYK	
			2061.8	1	WFNXYCGERNPFVCEA	
34c,35b	1.5	22■	1096.5	1	YDSNXDTXR	SVMP-II; *G. halys*; AAD02655
			1721.8	1	YXEXVVVADHGMFTK	
			1906.9	1	TWVHEXVNSXNEFYR	
36,37	8.0	33■	1188.7	1	XFEXVNXVNK	SVMP-III; *Atractaspis microlepidota andersoni*; AAF01040

n.d.: not determined.
▼/■: reduced/non-reduced conditions.
X: Leu/Ile; B: Lys/Gln. Methionine oxidation is underlined.

chromatographic and electrophoretic profiles reveals a more complex composition of SVMPs in neonate than in adult venoms, confirmed by the MS analysis (Tables 1 and 2). This was consistent with our previous study [14] where the SVMPs identified in both neonate and adult *G. brevicaudus* venoms could be assigned to classes P-II and P-III. Generally, the ontogenetic shift in the expression pattern of SVMPs with high (SVMP-III) to low molecular masses (SVMP-I) is a remarkable feature in some species [37,41,42], while it has not been revealed in *Sistrurus miliarius barbouri* where the SVMPs with high molecular mass are at higher abundance than those of low molecular mass during ontogenetic development [40]. Similar to the expression pattern in *S. m. barbouri* venom, either neonate or adult *G. brevicaudus* venom in our study presented higher abundance in SVMP-III than SVMP-II (Fig. 4). The total concentration of SVMP-III or SVMP-II showed almost no age-related difference in *G. brevicaudus* venom.

PLA_2 was estimated as the second major component in adult *G. brevicaudus* venom metalloproteinase (Fig. 4). Our previous work revealed that both neonate and adult *G. brevicaudus* venoms contain acidic, neutral and basic PLA_2s [14]. This was confirmed in the present study. Both venoms presented similar abundance of acidic and neutral PLA_2s, while the abundance of basic PLA_2 was 5-fold higher in adult venom than neonate venom (Table 1: peak 11; and Table 2: peak 9 and peak 10). The concentration of PLA_2 and snake venom metalloproteinase together accounted for 89.4% of total proteins in the adult venom, and only 79.1% in the neonate venom due to the relative low concentration of PLA_2. Six protein families (disintegrin, serine proteinase, CRISP, CTL, L-amino acid oxidase and 5′-nucleotidase) presented low concentrations (<5%), and together accounted for 10.6% of total venom protein in the adult venom. Except for disintegrin with a concentration higher than 5%, the other four protein families (serine proteinase, CRISP, CTL and L-amino acid oxidase) with concentrations lower than 5% together accounted for 13.5% of total venom protein in the neonate venom. Age-related variation in the proteomic profile of *G. brevicaudus* venom is evident, but it is less of a change than has been observed in some South American *Bothrops* species [37,39].

3.2. Antivenomics assessment of commercial antivenom against both venoms

Clinically, antivenom is the most available medicine in the treatment of snakebite envenomings and it is critical that victims are treated with appropriate antivenoms. Therefore, it is important to explore the immunological basis of antivenom and

Table 2 – Assignment of the reverse-phase chromatographic fractions from adult venom to protein families by MALDI-TOF/TOF and nESI-MS/MS of selected peptide ions from in-gel digested protein bands separated by SDS-PAGE.

Peak	%	MW (kDa)	Peptide Ion		MS/MS-derived sequence	Protein family
			m/z	z		
1,2	n.d					
3	0.2	13▼	942.9	2	GDDXDDYCNGXSAGCPR	Disintegrin; *Gloydius brevicaudus*; AAC08997
			1056.4	2	ARGDDXDDYCNGXSAGCPR	
			704.6	3	ARGDDXDDYCNGXSAGCPR	
4	0.4	13▼	942.9	2	GDDXDDYCNGXSAGCPR	Disintegrin; *G. brevicaudus*; AAC08997
			1056.4	2	ARGDDXDDYCNGXSAGCPR	
			704.6	3	ARGDDXDDYCNGXSAGCPR	
5,6	0.8	12▼	942.9	2	GDDXDDYCNGXSAGCPR	Disintegrin; *G. brevicaudus*; AAC08997
			1056.4	2	ARGDDXDDYCNGXSAGCPR	
			704.6	3	ARGDDXDDYCNGXSAGCPR	
7,8	3.2	12▼	942.9	2	GDDXDDYCNGXSAGCPR	Disintegrin; *G. brevicaudus*; AAC08997
			1056.4	2	ARGDDXDDYCNGXSAGCPR	
			704.6	3	ARGDDXDDYCNGXSAGCPR	
9	12.3	16▼	1221.7	1	AAAXCFRDNXK	PLA_2; *G. blomhoffi*; P04417
			1263.6	1	WDDYTYSWK	
			1390.7	1	YMAYPDXXCSSK	
10	2.5	17■	876.4	1	NXXBFNK	PLA_2; *Gloydius halys*; P14421
			1075.5	1	SDXYSYSXK	
		21▼	1221.7	1	AAAXCFRDNXK	PLA_2; *G. halys*; O42187
			1263.6	1	WDDYTYSWK	
			1419.7	1	YMTYPNXXCSSK	
11	1.9	18▼	1322.8	1	SXXBFETXXMK	PLA_2; *G. halys*; O42191
12a	2.1	27■	1061.5	1	TXCAGXXEGGK	Serine protease; *G. halys*; AFM29142
			1069.5	1	FXVAXYTSR	
			1087.4	1	VPNEDEBTR	
			1215.5	1	KVPNEDEBTR	
			1469.6	1	XXGGDECNXNEHR	
			1477.6	1	APYPEFEXPATSR	
			1937.8	1	HXAPFSXPSSPPSVGSVCR	
12b	5.8	18▼	1322.8	1	SXXBFETXXMK	PLA_2; *G. halys*; O42191
13	1.1	26■	1755.7	1	WTDXXHXWHDEYK	CRISP; *G. blomhoffi*; Q8JI40
14	1.1	44■/38■	1455.7	1	VXGGDECNXNEHR	Serine protease; *G. brevicaudus*; P85109
15a	0.2	33■	1455.7	1	VXGGDECNXNEHR	Serine protease; *G. brevicaudus*; P85109
15b	0.3	18▼	1322.8	1	SXXBFETXXMK	PLA_2; *G. halys*; O42191
16a	0.3	26■	1103.5	1	VXNEDEBTR	Serine protease; *Gloydius saxatilis*; Q7SZE1
			1231.6	1	BVXNEDEBTR	
16b	0.3	15■	958.6	1	YWFFPAK	PLA_2; *Gloydius shedaoensis*; AAR11860
			1097.6	1	DNXDTYDNK	
			1308.9	1	SXVBFETXXMK	
			2037.1	1	DNXDTYDNKYWFFPAK	
17	1.9	19▼	1322.8	1	SXXBFETXXMK	PLA_2; *G. halys*; O42191
18	0.9	77▼	1165.7	1	XKFEPPXPPK	L-Amino oxidases; *G. blomhoffi*; Q90W54
			1281.6	1	SAGBXYEESXGK	
			1402.7	1	KFWEDEGXHGGK	
			2246.1	1	XYFAGEHTAEAHGWXDSTXK	
		60▼	872.5	1	VVEEXBR	L-Amino oxidases; *G. halys*; Q6STF1
			1266.5	1	RFDEXVGGMDK	
			1281.6	1	SAGBXYEESXGK	
			1402.6	1	KFWEDEGXHGGK	
			2246.0	1	XYFAGEHTAEAHGWXDSTXK	
			2463.0	1	FGXBXNEFSBENDNAWYFXK	
			2591.1	1	BFGXBXNEFSBENDNAWYFXK	
			3054.2	1	YAMGGXTTFTPYBFBHFSESXTASVDR	
			3251.5	1	HVVVVGAGMSGXSAAYVXSGAGHBVTVXEASER	
			3362.3	1	EGNXSPGAVDMXGDXMNEDSGYYVSFPESXR	
			3683.5	1	WSXDKYAMGGXTTFTPYBFBHFSESXTASVDR	
19a	0.1	91▼	1110.5	1	BAFEHSVHR	5′-Nucleotidase; *G. brevicaudus*; B6EWW8
			1577.8	1	HANFPXXSANXRPK	
			1589.7	1	XXAXGHSGFFEDBR	
			2689.2	1	ETPVXSNPGPYXEFRDEVEEXBK	

(continued on next page)

Table 2 (*continued*)

Peak	%	MW (kDa)	Peptide Ion		MS/MS-derived sequence	Protein family
			m/z	z		
19b	0.6	84▼	1698.8	1	YVEFVVVXDHGMYK	SVMP-III; *Bothrops erythromelas*; Q8UVG0
			1826.9	1	BYVEFVVVXDHGMYK	
		65▼	1605.8	1	YXEXVXVADNVMVK	SVMP-III; *Agkistrodon piscivorus leucostoma*; C9E1S0
20	1.6	133■	985.6	1	EHBAFXXK	SVMP-III; *G. halys*; Q8AWI5
			1001.6	1	XACEPBNVK	
			1113.7	1	KEHBAFXXK	
			1212.6	1	SAECTDRFBR	
			1458.9	1	XVMVADYXMYXK	
			1740.1	1	MYDXVNVXNVXYBR	
		54▼	985.5	1	EHBAFXXK	SVMP-III; *G. halys*; Q8AWI5
			1113.6	1	BEHBAFXXK	
			1458.8	1	XVMVADYXMYXK	
			1739.9	1	MYDXVNVXNVXYBR	
			2067.1	1	MNXHVAXVGXEXWSNBDK	
21	32.3	52■	1299.5	1	MFYSNEDEHK	SVMP-III; *G. brevicaudus*; P0C7B0
			1628.8	1	MYEXANNXNDXYR	
			2051.0	1	XTVTPNVDDTXSSFAEWR	
			2083.0	1	MFYSNEDEHKGMVXPGTK	
			2195.1	1	YMYXHVAXVGVEXWSDGDK	
22	15.3	59▼	1132.6	1	FVEXVXVADK	SVMP-III; *G. brevicaudus*; P0C7B0
			1628.7	1	MYEXANNXNDXYR	
			1664.8	1	FVEXVXVADKGMVTK	
			2050.9	1	XTVTPNVDDTXSSFAEWR	
23	3.3	69▼	1673.9	1	YVEXVXVADHGMVTK	SVMP-II; *Protobothrops mucrosquamatus*; E9NW26
24	9.3	25■	1067.5	1	YNGDSDKXR	SVMP-II; *G. brevicaudus*; Q90WC0
			1583.8	1	GSTGVVBDHSTINLR	
			1735.9	1	YXEXVXVADHGMFTK	
25a	0.3	55■	1628.8	1	MYEXANNXNDXYR	SVMP-III; *G. brevicaudus*; P0C7B0
25b	1.3	23■	860.5	1	ETDXXNR	SVMP-II; *G. halys*; AAD02655
			1096.6	1	YDSNXDTXR	
			1722.1	1	YXEXVVVADHGMFTK	
			1907.1	1	TWVHEXVNSXNEFYR	
26	0.3	27■	978.4	1	EGTXCQEAK	SVMP-II; *G. brevicaudus*; O93516
		37▼	1327.7	1	YXEXVXVADHR	SVMP-II; *Crotalus adamanteus*; AEJ31986
			1357.6	1	YEGDKTEXCSR	
27a	0.1	32■	942.9	2	GDDXDDYCNGXSAGCPR	SVMP-II; *G. brevicaudus*; Q90WC0
			704.6	3	ARGDDXDDYCNGXSAGCPR	
27b	0.2	23■	1701.7	1	TWEDAESFCTEBAK	C-type lectin; *G. halys*; Q7SZV0
			1890.7	1	DCPSDWSSYEGHCYK	
		15▼	2726.3	1	GGHXVSXESDGEADFVABXVAENXBK	C-type lectin; *G. halys*; Q7SZV0

n.d.: not determined.
▼/■: reduced/non-reduced conditions.
X: Leu/Ile; B: Lys/Gln. Methionine oxidation is underlined.

assess the efficacy of antivenom before clinical application. The traditional evaluation of commercial *G. brevicaudus* antivenom efficacy is only based on the assessment of the capacity to neutralize the lethality of snake venom [58], and the protocol is recognized as the gold standard for quality control of antivenom at the preclinical level for many snake venoms [59]. However, the complexity of venom composition and toxicological profiles demands expanded protocols for evaluation of antivenom efficacy, such as neutralization efficacy of lethality, hemorrhage, myotoxicity, procoagulant and defibrinogenation on experimental animals [59]. Moreover, the application of antivenomics, which is firstly coined (first-generation antivenomics) by Lomonte et al. [60] and developed to its current state (second-generation antivenomics) by Pla et al. [28], can complement the preclinical evaluation of antivenom efficacy and quality control of antivenom preparation [57,59]. Over the past five years, both antivenomics approaches have been used to evaluate immunological relationship between venom and antivenom [15,21,28,49,55–57,60,61]. A second-generation antivenomics approach has two major advantages, the possibility of analyzing $F(ab')_2$ antivenoms and the reusability of the affinity system [28]. Moreover, this approach can reduce the use of experimental animals [59]. Here, a second-generation antivenomics analysis was selected to assess whether *G. brevicaudus* antivenom produced by immunizing the horse with adult venom effectively neutralizes both neonate and adult venom.

Nearly all fractions with medium and high molecular masses in both neonate and adult *G. brevicaudus* venoms could completely be captured by immunoaffinity column (Fig. 5). The remaining fractions with medium and high molecular masses

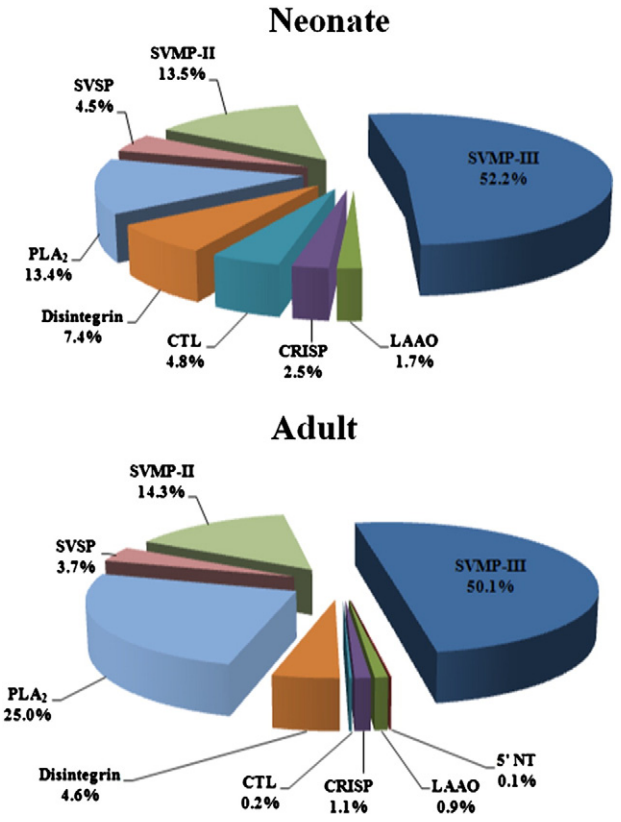

Fig. 4 – Comparison of total protein composition of venoms from neonate and adult *G. brevicaudus*. SVMP-PII and -PIII, snake venom metalloproteinase of classes PII and PIII; PLA_2, phospholipase A_2; SP, serine proteinases; LAAO, L-amino acid oxidase; CTL, C-type lectin-like protein; CRISP, Cysteine-rich secretory protein; 5′ NT, 5′ nucleotidase. The details of each protein are listed in Tables 1 and 2.

could not be thoroughly captured, and these non-retained fractions mainly refer to PLA_2, serine proteinase and SVMP (peaks 11, 12, 17 and 25 in Fig. 3) in adult venom, and PLA_2, serine proteinase, CTL and SVMP (peaks 14, 25 and 34 in Fig. 2) in neonate venom. In marked contrast, fractions with low molecular mass were poorly captured in both venoms (Fig. 5). Except for peaks (peak 1 and 2 in Figs. 2 and 3) not identified in these regions with low molecular mass, all other peaks belonged to the disintegrin family and was slightly more abundant in neonate than in adult venom. Disintegrin purified from *G. brevicaudus* venom can inhibit platelet aggregation, angiogenesis and tumor growth [62]. Therefore, neonate *G. brevicaudus* venom may have a stronger ability to prolong bleeding time in victims.

Observations from a number of elapid and viperid snakes demonstrate that the immunogenicity of venom proteins tends to be enhanced as molecular mass and structural complexity increase [15,39,55,56,60,63]. Our results reveal similar traits and confirm that commercial *G. brevicaudus* antivenom can appropriately be used against venom components with medium and high molecular masses in neonate and adult *G. brevicaudus*. However, components with low molecular mass, which could not be captured by the affinity system, might possess potential biochemical function (e.g. inhibit platelet aggregation) and even cause severe damage to patients. In further design of antivenom it is necessary to improve immunization schemes to increase immunological activity against these components, even if their immunogenicity seems intrinsically poor. Moreover, expanded protocols on neutralization of toxic effects should be employed in evaluating the preclinical efficacy of antivenom, and the antivenomics analysis should be employed in assessing the efficacy of antivenom [59].

3.3. Toxicological and enzymatic activity of both venoms

Compared to adult snakes, bites by neonate venomous snakes have not received much attention. However, differences in the biochemical characteristics between neonate and adult venoms may result in remarkable differences in clinical manifestation and severity. For example, the recovery of hemostatic disturbances after antivenom treatment is slower in patients bitten by young *B. jararaca*, because the venom of young snakes possesses a more severe coagulopathy than the venom of adult snakes [64]. Elucidating age-related differences in the biochemical characteristics thoroughly will help reveal the potential damage of snakebites. Here, the venoms of neonate and adult *G. brevicaudus* show different toxicological activities: the former is more toxic and hemorrhagic than the latter by intraperitoneal routine injection (Table 3). Further, our findings showed that both venoms can induce evident respiratory failure in mice, and that neonate venom is more severe than adult venom.

A general trend in venoms from nine subspecies of *Crotalus viridis* is that high toxicity and high metalloproteinase activity are incompatible [65]. A similar feature has been observed as an ontogenetic shift in venoms in *Daboia russelli siamensis* and *Boiga irregularis* [66,67]. However, such a trend cannot be detected in some species. In *Bothrops asper*, for example, venoms from newborn animals are more proteolytic, hemorrhagic, edema-forming and lethal than that from adults [66]. On the contrary, adult *B. jararaca* venom is more proteolytic and lethal than neonate venom [33]. Moreover, a form of venom pedomorphosis has been found in *Crotalus oreganus concolor*: juvenile and adult venoms present high toxicity whereas both of them present low metalloproteinase activity [67]. In our study, crude neonate venom presented higher activity (hydrolysis of casein) than adult venom (Table 3). When pre-incubated with EDTA–Na_2, proteolytic activity was markedly reduced by 84% in neonate venom and 79% in adult venom. It is therefore clear that hydrolysis of casein was mainly induced by SVMPs with proteolytic activity, and SVMPs in neonate *G. brevicaudus* venom seem more active than in adult venom. In agreement with patterns found in *B. asper* venom [66], neonate *G. brevicaudus* venom presented higher hemorrhagic activity and higher lethality than adult venom. Patterns of variation in snake venom described above may represent a trade-off between the metabolic costs of venom production and increment of foraging efficiency [68], or an adaptation to ecological niches or competition for resources [40,53].

Except for SVMPs, the serine proteinase of snake venom is also responsible for proteolytic activity [69]. In this study, there was no doubt that the remaining proteolytic activity in neonate and adult *G. brevicaudus* venoms was induced by serine proteinase, while SVMP proteolytic activity was inhibited by a metal chelator, and respectively accounted for 16% and 21% of total activity (Table 3 and Fig. 4). However, the abundance of

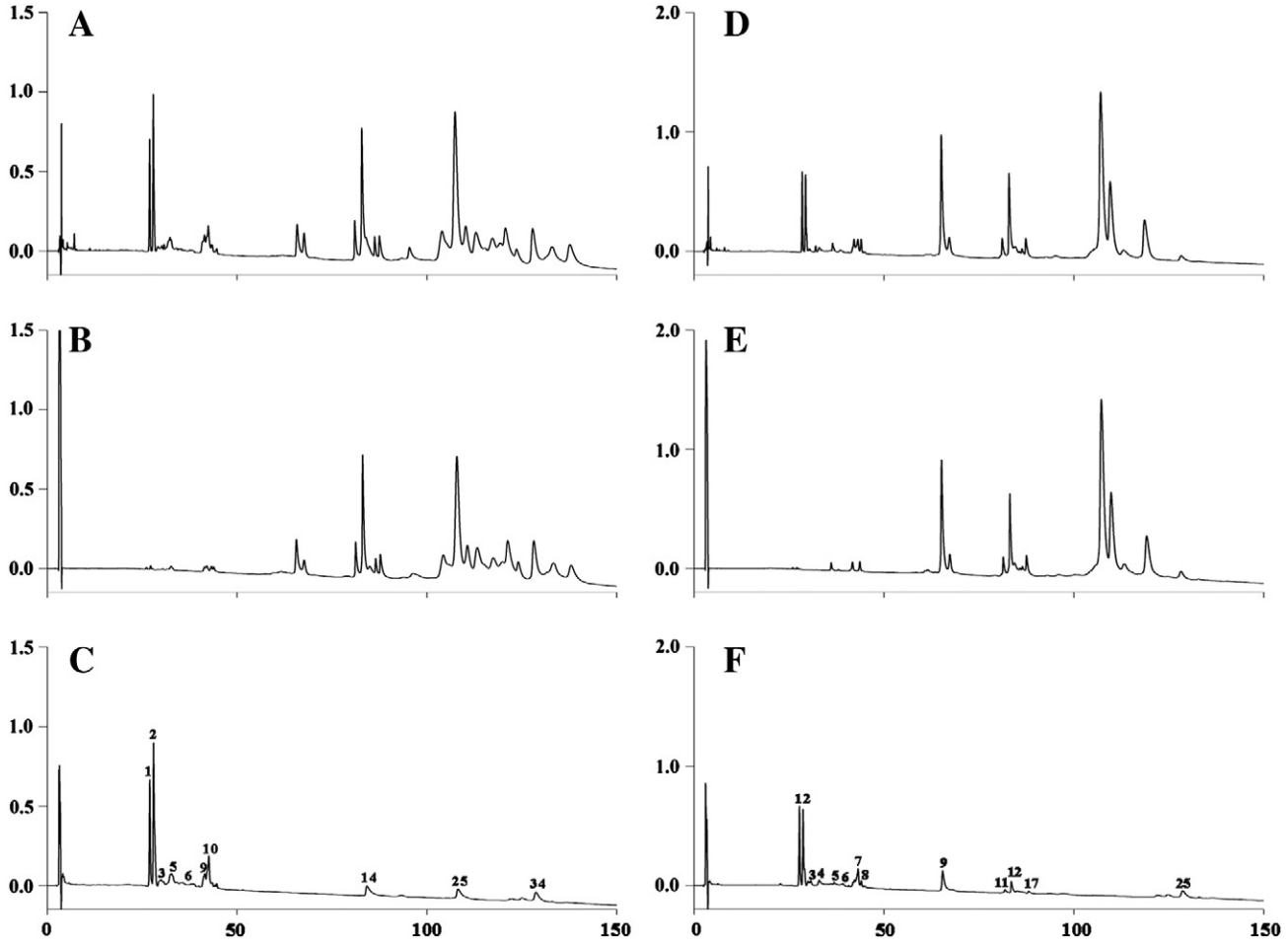

Fig. 5 – Immunocapture efficacy of commercial *G. brevicaudus* antivenom towards neonate and adult *G. brevicaudus* venoms by reverse-phase HPLC. Panels A–C show, respectively, the chromatographic profiles of whole components, the components retained then recovered from the affinity column, and non-retained components of neonate venom. Panels D–F display, respectively, the separations of whole components, the components retained then recovered from the affinity column, and non-retained components of adult venoms.

serine proteinase in both venoms was nearly identical (Fig. 4), and adult *G. brevicaudus* venom still showed greater hydrolysis of BAPNA and TAME (Table 3). This indicates that the arginine esterolytic activity of serine proteinase is more active in neonate venom than in adult venom.

Thrombin-like enzymes in *G. brevicaudus* venom can induce the degradation of Aα and Bβ chains of fibrinogen directly, metalloproteinase fibrinolytic components and plasmiogen activators can induce the degradation of fibrin and Aα chains of fibrinogen directly or indirectly, and these enzymes belong to serine proteinases and SVMPs [70–72]. Furthermore, the serine proteinases of snake venom evolve from glandular kallikrein, and present a structure not belonging to the thrombin gene family [69,73]. The thrombin-like enzymes mentioned above actually belong to the kallikrein-type family. Here, neonate and adult venoms induced a rapid degradation of Aα chains and a slower degradation of Bβ chains of human fibrinogen, and neonate venom hydrolyzed Aα chains more quickly than adult venom in the first 4 min (Fig. 7). Both venoms also induced degradation of fibrin generated from bovine fibrinogen, but the clearance zone induced by adult venom was larger than that induced by neonate venom (Table 3). Fibrinogenolytic activity collectively caused by thrombin-like enzymes, plasminogen activators and metalloproteinase fibrinolytic components was stronger in the neonate venom, while fibrinolytic activity caused by plasminogen activators and metalloproteinase fibrinolytic components was stronger in the adult venom. The inhibition of fibrinogenolytic and fibrinolytic activities by both venoms by EDTA–Na_2 indicates that SVMPs play an important role in the hydrolysis of fibrinogen and fibrin.

Three types of PLA_2 from *G. brevicaudus* venom exert a variety of toxic and pharmacological effects, including neurotoxicity, myotoxicity, cytotoxicity and hemolyticity [74]. Mostly, PLA_2 activity can be evaluated by the hydrolysis of lecithin or phosphatidylcholine. Here, adult *G. brevicaudus* venom presented higher activity in hydrolyzing soybean lecithin than neonate venom (Fig. 6), a likely result of the relative higher abundance of PLA_2 in adult venom. The basic PLA_2 of *G. brevicaudus* venom can induce the strongest hemolytic activity [74] and, in this study, adult venom contained a higher abundance of basic PLA_2 than neonate venom. Accordingly, we may suppose that adult *G. brevicaudus* venom be more hemolytic than neonate venom. It has been considered that basic Asp49 PLA_2s and Lys49 PLA_2s homologs are largely responsible for the myotoxicity in viperid

Table 3 – Toxicological and enzymatic activities of pooled venoms from neonate and adult snakes.

Activity Animal/substrates	Neonate venom	Adult venom	Statistical results
Lethality (LD_{50})[a]			
ICR mice (μg/g)	1.26 (0.95–1.67)	1.62 (1.12–2.35)	N > A
Myotoxicity (CK)[b]			
ICR mice (U/l)	4032 ± 203	5224 ± 389	$P < 0.05$, N < A
Hemorrhage (MHD)[b]			
ICR mice (μg)	0.82 ± 0.04	1.28 ± 0.05	$P < 0.01$, N > A
Proteolytic activity[b]			
Casein (nM/min/mg)	53.5 ± 0.5	41.8 ± 0.1	$P < 0.0001$, N > A
Casein[c] (nM/min/mg)	8.7 ± 0.1	8.9 ± 0.4	$P = 0.67$
Esterolytic activity[b]			
BAPNA (nM/min/mg)	57.5 ± 0.5	73.1 ± 3.0	$P < 0.01$, N < A
TAME (nM/min/mg)	21.4 ± 1.3	38.5 ± 0.6	$P < 0.0001$, N < A
5′-Nucleotidase activity[b]			
AMP (nM/min/mg)	60.3 ± 1.9	96.6 ± 3.7	$P < 0.001$, N < A
L-Amino oxidase activity[b]			
L-Leu (nM/min/mg)	85.7 ± 0.3	65.3 ± 0.5	$P < 0.0001$, N > A
Fibrinolytic activity[b]			
Fibrin (mm^2/μg)	1.2 ± 0.1	2.5 ± 0.1	$P < 0.01$, N < A

[a] LD_{50}: dose of venom that induces death in 50% of injected mice. Values in parentheses are 95% confidence limits.
[b] Data are expressed as mean ± SE (n = 3).
[c] Venoms pre-incubated with 40 mM EDTA.

venoms [75]. In this study, mice injected with both neonate and adult venoms showed significant increase in CK levels of plasma because of the basic PLA_2s expressed in both venoms. Since the basic PLA_2s were more abundant in adult *G. brevicaudus* venom, the statistical analysis showed adult venom presents higher myotoxicity than neonate venom (Table 3).

Despite the fact that 5′-nucleotidase was detected only in adult *G. brevicaudus* venom, both neonate and adult venoms displayed activity in hydrolyzing AMP (3 and Fig. 4). It is possible that the abundance of 5′-nucleotidase is lower in neonate venom than in adult venom, and the abundance of 5′-nucleotidase is too low to be detected by the techniques used here. However, neonate venom displayed higher LAAO

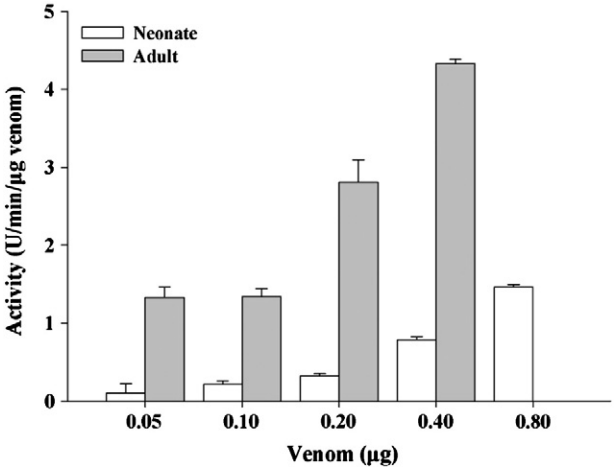

Fig. 6 – Phospholipase A_2 activity of venoms from neonate and adult *G. brevicaudus* determined using soybean lecithin. Data are expressed as mean ± SE (n = 3). The substrate was greatly degraded in the reaction system by 0.4 μg adult venom, thus the phospholipase A_2 activity of adult venom at 0.8 μg decreased significantly in the reaction system, and it has not been considered in the comparison.

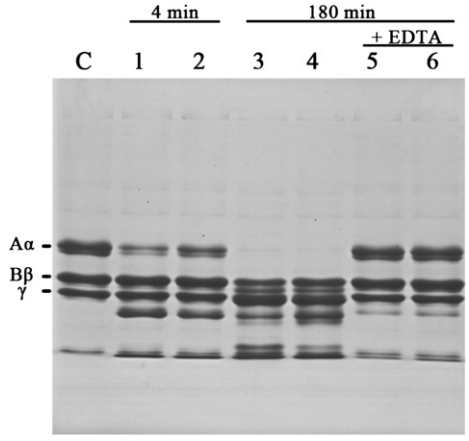

Fig. 7 – Fibrinogenolytic activity of venoms from neonate and adult *G. brevicaudus* evaluated using human fibrinogen. Lane C is the control of fibrinogen incubated without venom; lanes 1, 3 and 5: reaction incubated with neonate venom; lanes 2, 4 and 6: reaction incubated with adult venom. Letters (Aα, Bβ, γ) at left indicate three chains of fibrinogen.

activity than adult venom, in agreement with the estimation of LAAO in neonate (0.9%) and adult (1.7%) venoms.

4. Conclusions

Age-related variation in the proteomic profile of *G. brevicaudus* venom is evident, although it is less of a change than what has been observed in some South American *Bothrops* species. Age-related variation in toxicological and enzymatic activities is correlated with age-related variation in venom composition. Antivenomics analysis shows that proteins with medium and high molecular masses in both neonate and adult venoms can be well recognized by commercial *G. brevicaudus* antivenom, but proteins with low molecular mass cannot be recognized completely, especially in the neonate venom. Our data will aid discrimination of the clinical symptoms caused by neonate and adult *G. brevicaudus*, as well as methods for improving antivenom preparations by combining both venoms. Our work has an implication that, even if the venom yield is low in neonate snakes, envenomation by neonates requires medical attention and clinical evaluation.

Acknowledgments

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