Journal of Scientific Research & Reports

27(11): 25-43, 2021; Article no.JSRR.75395
ISSN: 2320-0227

Preclinical Immuno-recognition and Neutralization of Lethality Assessment of a New Polyvalent Antivenom, VINS Snake Venom Antiserum – African IHS®, against Envenomation of Ten African Viperid and Elapid Snakes

Djameh, Georgina I. [a], Nyarko, Samuel [a], Tetteh-Tsifoanya, Mark [a], Marfo, Frances M. [a], Adjei, Samuel [b], Blay, Emmanuel A. [a,c], Anang, Abraham K. [a] and Ayi, Irene [a*]

[a]Parasitology Department, Noguchi Memorial Institute for Medical Research, College of Health Sciences, University of Ghana, Legon, Accra, Ghana.
[b]Animal Experimentation Department, Noguchi Memorial Institute for Medical Research, College of Health Sciences, University of Ghana, Legon, Accra, Ghana.
[c]Department of Pathology and Microbiology, University of Nebraska Medical Center, 985900 Nebraska Medical Center, Omaha, NE 68198-5900, USA.

Authors' contributions

This work was carried out in collaboration among all authors. Authors AI, DGI, AS, BEA and AAK conceptualized and designed the study. Authors DIG, TTM, NS, MFM and AS performed the experiments. Authors AI and DGI prepared the original draft manuscript. Authors AI, DGI and TTM analyzed the data. Authors AI, DGI, TTM, NS, MFM, AS, BEA and AAK viewed and edited manuscript. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JSRR/2021/v27i1130456
Editor(s):
(1) Dr. Ahmed Mohammed Abu-Dief Mohammed, Sohag University, Egypt.
Reviewers:
(1) T. R. Shantala Priyadarshini, India.
(2) Anil Kumar, India.
Complete Peer review History: https://www.sdiarticle4.com/review-history/75395

Original Research Article

Received 01 August 2021
Accepted 09 October 2021
Published 10 November 2021

ABSTRACT

Snakebite envenomation is a major health concern in developing countries causing significant mortality and morbidity. With over 1.2 million cases annually caused by medically important snake

**Corresponding author: E-mail: IAyi@noguchi.ug.edu.gh;*

species belonging to the two families *Viperidae* (*Echis* spp. and *Bitis* spp.) and *Elapidae* (*Naja* spp. and *Dendroaspis* spp.). Several antivenoms are being produced and distributed to western sub-Saharan Africa for treatment of envenomation with the absence of preclinical efficacy studies. The present study evaluated the preclinical efficacy of venoms from *Echis leucogaster, Echis ocellatus, Bitis arietans, Bitis gabonica, Naja haje, Naja melanoleuca, Naja nigricollis, Dendroaspis jamesoni, Dendroaspis polylepis* and *Dendroaspis viridis* against a polyvalent Snake Venom Antiserum - African IHS (lyophilised), manufactured by VINS Bioproducts Limited (Telangana, India). Our *in vitro* results showed that, the SVA- AIHS contains antibodies that are capable of recognizing and binding majority of protein components representative of all eight major protein families of venoms of the snake species tested by double immunodiffusion assay and confirmed by western blot. The venom antiserum exhibited high neutralization efficacy against all the viperid and elapid snake species venoms in *in vivo* studies and confirmed the manufacturer's recommended neutralization capacity. This is clear evidence that the VINS polyvalent SVA-AIHS batch tested has strong neutralizing capacity and will be useful in treating envenoming by most African viperid and some elapid snake species.

Keywords: Snake venoms; venom antiserum; neutralization; elapids; viperids; protein profile.

1. INTRODUCTION

Snakebite envenomation is a neglected tropical disease with developing countries recording the highest number of cases [1]. An estimated 1.2 million snakebite envenomation, 100,000 deaths and over 400,000 cases of morbidity annually are caused by 280 medically important snake species [1–3]. Sub-Saharan Africa records a high burden of more than 1 million bites and 25,000 deaths per year of snake envenoming [4]. Exposure to diverse snake habitats and vulnerability associated with risk occupations account for the high mortality and morbidity rates of snakebites in many parts of sub-Saharan Africa [2,5]. Additionally, poor infrastructure and limited access to appropriate medical treatment and health facilities are major contributing factors to high rate of snakebite mortality and morbidity [2].

Of the venomous snakes that inhabit most parts of Africa, two families, *Viperidae* **(***Echis* spp. and *Bitis* spp.) and *Elapidae* **(***Naja* spp. and *Dendroaspis* spp.) have been reported to be of major medical importance [6]and three others (*Hydrophiidae* – Coral Reef Snakes; *Clubridae* – Colubrid; and *Atractaspididae* - mole vipers, stiletto snakes, or burrowing asps) of minor importance. The saw-scaled vipers, the African cobras and mambas have proved to be the most important cause of morbidity and mortality in Ghana [7]. *Viperidae* snakes induce hemorrhagic effects and coagulatory disturbances with severe necrosis at the bite site as a result of the snake venom-rich metalloproteinases (SVMP) [8,9]. *Echis ocellatus* is responsible for most accidents [2], while *Bitis arietans* has the widest territorial distribution [10].

The lethality of *Elapidae* venoms is characterized by a-neurotoxins, phospholipases A2, and cardiotoxins components that cause neurotoxic effects on preys with little or no damage to the bite site [11]. The most medically important and lethal species of the *Elapidae* are the spitting cobras **(***Naja* spp.) [12,13]. There are 18 widely distributed *Naja* species in Africa and Asia predominantly *N. haje* in deserts**,** *N. nigricollis* **in savannas and** *N. melanoleuca* in forests [14,15]. The mamba (*Dendroaspis*) is an arboreal snake in Africa and the genus consists of four species: the green mambas (*D. angusticeps, D. jamesoni, D. viridis*) and the black mamba (*D. polylepis*). The venom contains phospholipases, dendrotoxins, fasciculins, and a-neurotoxins commonly enhancing nervous transmission [14–16].

Snake venoms are a complex of mixture of proteins, lipids, nucleosides, and carbohydrates that are needed to capture prey, defend and for digestion [17]. Snake venom proteins are grouped into enzymes and toxins. The enzymes mostly have high molecular weights and work by acting on blood coagulation, cytolysis caused by complement activation and activation of metabolism. Conversely, snake venom toxins have varying molecular weights (generally less than 30 kDa) and acts by binding to receptors on membranes of different anatomical site causing various venom-induced pathologies [15]. The variations in venom components have been investigated to understand their toxicological

properties and potentials to aid in antivenom production.

Available medical treatment options for treating snakebite envenomation include antiserum usually polyvalent antiserum that neutralize the enzymes and toxins in the venom. For any snake venom antiserum to be considered suitable for Africa, for that matter sub-Saharan Africa, it must contain components to neutralize venoms of all the major snake species in Africa, especially, Ghana. The challenges with the current antiserum-based therapies are; they are mostly expensive, unspecific and usually unavailable [18,19]. In this study, we present the neutralization ability of Snake Venom Antiserum – African IHS (lyophilized) (SVA-AIHS) (prepared by the VINS Bioproducts Limited, Telangana, India) against venoms of ten (10) snake species found in Ghana: *Bitis gabonica, Bitis arietans, Echis leucogaster, Echis ocellatus, Dendroaspis jamesonii, Dendroaspis polylepis, Dendroaspis viridis, Naja nigricollis, Naja haje* and *Naja melanoleuca*. We evaluated the anti-snake venom activity for: 1) product safety (sterility) evaluation, 2) *In vitro* experimentation for demonstration of immunological- based antibody/toxin precipitation, and 3) *In vivo* demonstration of biological activity of the snake venom anti-serum by neutralization of snake venom toxins in experimental laboratory mice.

2. MATERIALS AND METHODS

2.1 Snake Venoms and Venom Antiserum Samples

Lyophylized venoms of the ten (10) different snake species, namely, *Echis leucogaster, Echis ocellatus, Bitis arietans, Bitis gabonica, Naja haje, Naja melanoleuca, Naja nigricollis, Dendroaspis jamesoni, Dendroaspis polylepis* and *Dendroaspis viridis* were received in clearly labelled respective vials. In addition, there were 10 packs of 5 vials/pack of sealed vials of lyophilized snake venom antiserum parenteral preparations labelled, “Snake Venom Antiserum - African IHS (lyophilised); polyvalent enzyme refined, Equine venom antiserum immunoglobulin fragments; Mfg. Lic. No. 01/MN/AP03/Sera/G; Batch No. 07AS19001; Manufacturing date: 08/2019; Expiry Date: 07/2023” with accompanying packs of sealed 10 ml sterile water as diluents (Antisera Diluent: Batch No. 7505859; Manufacturing date: 07/2019; Expiry date: 06/ 2024”) were delivered for testing. All venoms and Antiserum were received from the manufacturer, VINS BioProducts Limited (Telangana, India) as delivered to the Parasitology Department of the Noguchi Memorial Institute for Medical Research, and stored at 4℃ until used. The SVA-AIHS parenteral preparations were tested in experiments as described in subsequent sections.

2.2 Determination of Antiserum Sample Sterility

Two sealed vials containing the SVA-AIHS, purported to be antiserum to snake venom was randomly selected and 3 ml of antisera was dispensed into wells of micro-titre plates and incubated at 37°C in 5% CO_2 in air for up to 7days. In a parallel experiment, sterile enriched Luria Bertani’s medium was prepared and plated to check for contamination. Subsequently, aliquots of antisera were seeded and incubated at 37°C in 5% CO_2 in air for up to 7days. All incubated set ups were examined by microscopy for the growth of contaminating bacteria, if any. For quality control, Luria Bertani’s medium without SVA-AIHS was incubated under similar conditions.

2.3 *In vitro* Experimentations for Demonstration of Immunological-based Antibody/Toxin Precipitation

2.3.1 Determination of protein concentration

Protein quantification in each of the snakes’ venoms and SVA-AIHS vials was estimated using the Bradford Reagent (Sigma, Life Science, USA) and Bovine Serum Albumin (BSA; Sigma-Aldrich™, US) as the standard [20]. A standard curve was generated to estimate the protein concentrations for respective dilutions of venoms and antiserum.

2.3.2 Double-Immunodiffusion assay

Double-immunodiffusion assay was carried out as described by Ouchterlony [21]. Briefly, a 1% (w/v) agarose (AGTC Bioproducts, UK) solution was prepared by melting agarose gel-granules in Phosphate Buffered Saline (PBS; Sigma®, Life Science USA). The molten agarose was poured onto microscope glass slides and allowed to solidify. Wells were then cut into the solid gel and each was filled with approximately 10 µL of appropriate reagent. Snake venom was placed in a central well and test samples (suspected anti-

snake venom and bovine serum albumin) were titrated in the surrounding wells. A precipitin line was formed in-between a sample well and the homologous antiserum in a central well. The precipitin reaction was allowed to develop in up to 48 hours at room temperature in a humid chamber and observed by viewing the gels against light. For preservation, agarose gels with precipitin lines were thoroughly washed with PBS, pH 7.4, followed by distilled water to remove unprecipitated proteins, dried, and stained using Coomassie Brilliant Blue G-250 dye (Pierce, Thermo Fisher Scientific™, USA).

2.3.3 SDS-PAGE analysis of snake venom

The proteins of *Dendroaspis jamesoni*, *Dendroaspis polylepis, Dendroaspis viridis, Naja haje, Naja melanoleuca, Naja nigricolis, Echis leucogaster, Echis ocellatus, Bitis gabonica* and *Bitis arietans* snake venom were analyzed on NuPAGE™ 4 -12% Bis-Tris Gel (Invitrogen™ by Thermo Fisher Scientific, US). The venom samples (5 µg) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis using the NuPAGE™ Tris-Acetate SDS Buffer system kits containing NuPAGE™ Tris-Acetate SDS Running Buffer, NuPAGE™ Sample Reducing Agent, NuPAGE™ Antioxidant and NuPAGE™ LDS Sample Buffer (Invitrogen™ by Thermo Fisher Scientific, US). The samples were mixed with NuPAGE™ LDS Sample Buffer and NuPAGE™ Sample Reducing Agent, boiled for 10 min, and electrophoresed in NuPAGE™ Tris-Acetate SDS Running Buffer at a constant current of 30 mA for 1hour. After electrophoresis, the gel was stained with SimplyBlue™ Safe Stain (Invitrogen™ by Thermo Fisher Scientific, US). Molecular weight (SeeBlue™ Plus2 Pre-stained Protein Standard, Invitrogen™ by Thermo Fisher Scientific, US) standards were co-electrophoresed [22].

2.3.4 Western blot analysis

Western blot analysis was carried out according to the method previously described by [23]. with slight modification. Briefly, following SDS-PAGE, gels were electroblotted onto nitrocellulose membranes (Invitrogen™ by Thermo Fisher Scientific, US). These membranes were blocked with PBS buffer (PBS; Sigma®, Life Science USA) containing 3% BSA at 37°C for 1hour, washed with PBS and treated with and without anti-venom (Snake Venom Antiserum African IHS, Lyophilised) diluted to 1:20,000 in PBS plus 0.05% BSA for 1 hour at room temperature on a horizontal shaker. Each membrane was treated with only one venom antiserum. After being washed three times with PBS plus 0.05% TWEEN®20 (Sigma-Aldrich™ US), the membranes were incubated with Anti-horse IgG (whole molecule) Peroxidase antibody produced in Rabbit (Sigma-Aldrich™ US) diluted to 1:7,500 in PBS plus 0.1% BSA. Then, the membranes were incubated for 1 hour at room temperature on a horizontal shaker (Shaker-XR, Taitec, Japan). The membranes were washed three times with PBS plus 0.05% Tween-20 and placed in Immobilon™ Western Chemiluminescent HRP Substrate (Millipore, USA). Protein bands were visualised and captured in a Chemiluminescence CCD imaging System (ATTO, Japan). The reaction was terminated by washing with distilled water.

2.4 *In vivo* Lethal and Neutralization Experiments for Demonstration of Biological Activity of the Snake Venoms and the SVA-AIHS

2.4.1 Animals

Six to eight-week old mice (ICR strain, 20 – 30 g) of either sex, were housed and maintained at the Department of Animal Experimentation, Noguchi Memorial Institute for Medical Research (NMIMR) where all animal experiments were carried out. The animals were kept in normal rodent cages with sterilized soft wood shavings as bedding, fed rodent feed pellets (AGRIFEEDS, Kumasi), given free access to water, and kept under laboratory conditions (Temperature 25± 2°C, relative humidity 60-70%, and a 12-hour light-dark cycle). Ethical approval was sought from the University of Ghana Institutional Animal Care and Use Committee (UG-IACUC), referenced as UG-IACUC 012-/19-20. All procedures and techniques involving the use of the animals were guided by the UG-IACUC policy document which is premised on the principles and standards outlined in the Principles for use of Animals [24] ,The Guide for the Care and Use of Laboratory Animals [25] and the provisions of the Animal Welfare Acts (P.L. 89-544 and its amendments) [26].

2.4.2 Determination of lethal potency

Five mice per dose level, were each injected by intraperitoneal (i.p.) route with different amounts of venom in 0.15 M NaCl. The number of surviving animals 48 h after the injection were recorded and the median lethal dose (LD_{50}: the

amount of venom that induced the death of 50% of the challenged mice) was calculated by non-linear regression using the GraphPad Prism 8 software (GraphPad, Inc., San Diego, CA).

2.4.3 *In vivo* neutralization assay

This assay was performed as recommended by the World Health Organization [27]. ICR mice (20 – 30 g) were injected i.p. with 3x LD50 of each venom preincubated for 30 min at 37°C with different doses of each venom antiserum (5 mice per dose) in a final volume of 0.5 ml. After 48 h observation, the number of dead mice were recorded and the data analyzed by non-linear regression using the GraphPad Prism 8 software (GraphPad, Inc., San Diego, CA). The neutralizing capacity was expressed in microliters as the median effective dose (ED_{50}: the SVA-AIHS dose which protects 50% of the mice injected).

2.5 Data Analysis

LD_{50} of the venoms and the ED_{50} of venom antiserums are expressed as means ± Standard error mean (SEM) with 95% confidence intervals (CI). LD_{50} (Median lethal dose) and ED_{50} (Median effective dose) were calculated using GraphPad Prism version 8 software, (GraphPad, Inc., San Diego, CA).

3. RESULTS

3.1 Sterility of SVA-AIHS, Protein Content and Lethal Dose 50% (LD_{50}) of Snake Venom

The VINS SVA-AIHS was free from any contaminating bacteria over the period of observation, hence, venom antiserum was sterile. The protein concentration in the vials was found to be 7.7 mg/ml. The concentration of protein in the venoms of the snakes were found to be 1.5 mg/ml (*Echis leucogaster),* 0.6 mg/ml (*Echis ocellatus),* 1.3 mg/ml (*Bitis arietans),* 0.9 mg/ml (*Bitis gabonica),* 1.1 mg/ml (*Naja haje*), 0.4 mg/ml (*Naja melanoleuca*), 2.0 mg/ml (*Naja nigricollis*), 0.6 mg/ml (*Dendroaspis jamesoni*), 0.8 mg/ml (*Dendroaspis polylepis*) and 0.4 mg/ml (*Dendroaspis viridis*). Toxicity was assessed by the mean venom dose ± the standard error of the mean (SEM) for 50% mortality (LD_{50}) of experimental mice inoculated with *Naja melanoleuca*, *Naja haje* and *Naja nigricollis* (cobra) venoms in mg/kg body weight were 0.73 ± 0.01, 0.18 ± 0.04 and 1.83 ± 0.20, respectively, as determined from a dosage-mortality response curve (non-linear regression) with a 24-hour recovery period. The LD_{50} for venom of the mambas in mg/kg body weight were 0.48 ± 0.06 *(Dendroaspis viridis)*, 0.71± 0.1 (*Dendroaspis polylepis)* and 0.99 ± 0.09 (*Dendroaspis jamesoni*). The LD_{50} for the vipers were, 0.92 ± 0.19 (*Bitis arietans*), 1.46 ± 0.205 (*Bitis gabonica*), 2.84 ± 0.302 *(Echis leucogaster)* and 2.17 ± 0.199 (*Echis ocellatus*) (Table 1).

3.2 *In vivo* Neutralization of Venom Lethality by VINS SVA-AIHS

The VINS SVA-AIHS was capable of neutralizing three times the median lethal concentrations ($3LD_{50}$) of venom from each of the 10 snake species in the experimental mice at different effective doses (ED_{50} in µL), whilst all control mice injected with the $3LD_{50}$ of each venom only, died within 12 hours. The neutralizing efficacy of the SVA-AIHS in terms of both experimental ED_{50} and the capacity of 1 mL to specifically neutralize each of the snake species venoms in descending order was as follows: *Bitis arietans, Echis ocellatus, Dendroaspis viridis, Dendroaspis jamesoni, Echis leucogaster, Bitis gabonica, Naja nigricollis, Naja melanoleuca, Dendroaspis polylepis* and *Naja haje.* Thus, 1 mL of SVA-AIHS is capable of neutralizing the lowest of three times to the highest of seventeen more of the recommended $25LD_{50}$ venom of *Naja haje* and *Bitis arietans,* respectively, by the manufacturer (Table 1).

3.3 *In vitro R*eactivity of the VINS SVA-AIHS with Specific Snake Species Venoms

The polyvalent SVA-AIHS strongly recognized the venoms from *Echis leucogaster, Echis ocellatus, Bitis arietans, Bitis gabonica, Najahaje, Naja melanoleuca, Dendroaspis polylepis, Dendroaspis viridis, Dendroaspis jamesoni and Naja nigricolis* (Fig. 1). The reaction between the SVA-AIHS and each of the 10 snakes' venoms produced multiple precipitin lines. No precipitin lines occurred between the SVA-AIHS and the control protein, BSA used.

3.4 SDS-polyacrylamide Gel Electrophoresis and Western Blot

The electrophoretic profile showed differences between the venoms. The venoms from

Table 1. Median Effective Dose and neutralizing efficacy of VINS Snake Venom Antiserum – African HIS

Snake Species	LD_{50}[1] in mg/Kg (95% CI[2])	ED_{50}[2] (in µL) / $3LD_{50}$ in mice (95% CI)[3]	Estimated venom LD_{50} neutralized by 1 mL of SVA-AIHS[4]	Recommended venom LD_{50} neutralized by 1 mL of VINS SVA-AIHS	Neutralizing Efficacy of 1 mL of VINS SVA-AIHS[5]
Bitis arietans	0.92 (0.337 - 1.286)	7.25 (1.469 - 19.14)	414.0LD_{50}	25LD_{50}	16.56
Echis ocellatus	2.17 (1.646 - 2.650)	7.83 (3.060 - 17.58)	383.14 LD_{50}	25LD_{50}	15.33
Dendroaspis viridis	0.48 (0.1054- 0.7357)	8.69 (2.636 - 20.59)	345.38 LD_{50}	25LD_{50}	13.82
Dendroaspis jamesoni	0.99 (0.1190- 0.6861)	11.16 (4.723 - 22.96)	268.82 LD_{50}	25LD_{50}	10.75
Echis leucogaster	2.84 (0.617 - 3.678)	13.81 (4.672 - 34.11)	217.23 LD_{50}	25LD_{50}	8.69
Bitis gabonica	1.46 (0.979 - 1.892)	16.73 (4.794 - 49.24)	179.32 LD_{50}	25LD_{50}	7.17
Naja nigricollis	1.83 (0.02540-0.4635)	24.87 (10.76 - 58.41)	120.63 LD_{50}	20LD_{50}	6.03
Naja melanoleuca	0.73 (0.06600-0.3472)	25.93 (10.12 - 106.4)	115.70 LD_{50}	20LD_{50}	5.79
Dendroaspis polylepis	0.71 (0.1067- 0.5591)	45.30 (19.32 - 129.1)	66.23 LD_{50}	25LD_{50}	2.65
Naja haje	0.18 (0.2668 to 1.398)	46.51(18.57 - 124.0)	64.50 LD_{50}	25LD_{50}	2.58

[1]LD_{50}: The median lethal doses of the snake species venoms in mice [2]ED_{50}: The median effective doses that neutalize $3LD_{50}$snake species venom challenge in mice [3]CI: Confidence Interval
[4]SVA- AIHS: VINS Snake Venom Antiserum – AfricanHIS [5]Neutralizing Efficacy: the number of times the estimated venom LD_{50} is neutralized by 1 mL of the VINS SVA-AIHS over the manufacturer's recommended LD_{50}

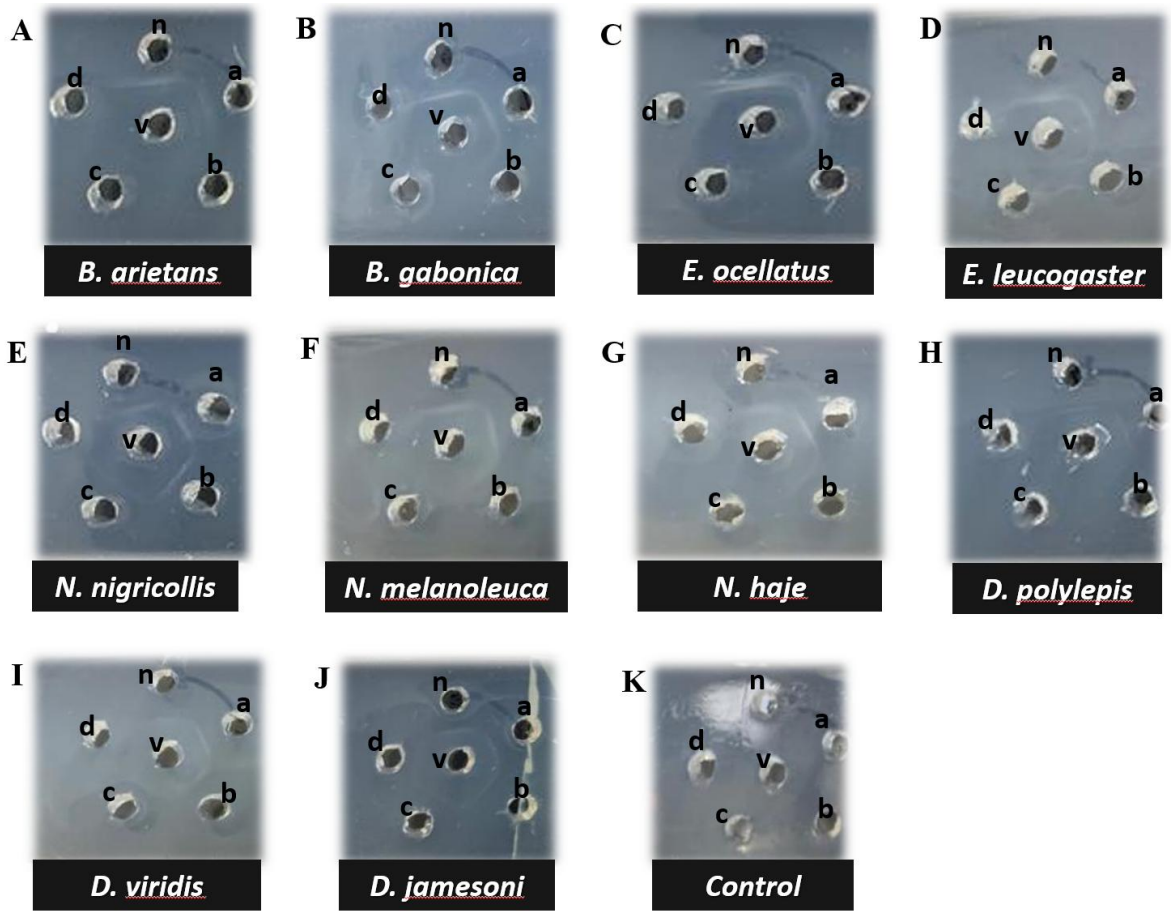

Fig. 1. Pictures of double immunoprecipitation (Ouchterlony technique in 1% agarose gel) of the venoms of ten *Viperidae* and *Elapidae* snake species SVA—AIHS. A. *Bitis arietans,* B.*Bitis gabonica,* C. *Echis ocellatus* D. *Echis leucogaster,* E. *Naja nigricolis,* F. *Naja melanoleuca,* G. *Najahaje,* H. *Dendroaspis polylepis,* I. *Dendroaspis viridis,* J. *Dendroaspis jamesoni* and K. Bovine Serum Albumen (control). The central wells (v) were each filled with 10 µL of 1 mg/ml of venom. The peripheral wells were filled with 10 µL of dilutions of the SVA-AIHS (1/1 (n), 1/2 (a), 1/4 (b), 1/8 (c), 1/16 (d)).

D. polylepis showed few stained bands less than 25 kDa and between 50 kDa to 90 kDa whereas the venom from *D. viridis* showed cluster of bands between 50 kDa to 100 kDa and a prominent band at around 120 kDa. Venoms from *Naja melanoleuca* showed cluster of bands below 35 kDa and prominent bands clustered around 50 kDa to 55 kDa whereas venoms from *N. haje* and *N. jamesoni* showed prominent stained bands around 50 to 100kDa with differences in intensity between both venoms. *Echis leucogaster* venoms showed prominent stained bands around 25k Da and 60 kDa with minor bands between 30 kDato 50 kDa whilst *E. ocellatus* showed two prominent bands approximately 37 kDa and 45 kDa with minor bands between 50 kDa to 70 kDa. Venoms from *Bitis arientans* on the other hand showed strong stained bands around 25 kDa, 35 kDa and between 60 to 70 kDa. However, all venoms showed bands below 15 kDa (Fig. 2A) and Elapid venoms showed bands ≤6 kDa (Fig. 2A). The protein bands correspond to antibodies in SVA-AIHS, as confirmed by Western Blot analysis using rabbit anti-horse IgG as the secondary antibody (Fig. 2B). The Western Blot without SVA-AIHS incubation was included as control to confirm or otherwise the neutralization capability of the SVA-AIHS (Fig. 2C).

A list of some major and minor enzymatic and non-enzymatic venom protein families that correspond to the range of band sizes revealed in the SDS-PAGE and WB analysis are presented in Table 2, as compared with existing literature (Tables 3 and 4) adapted from [25][28].

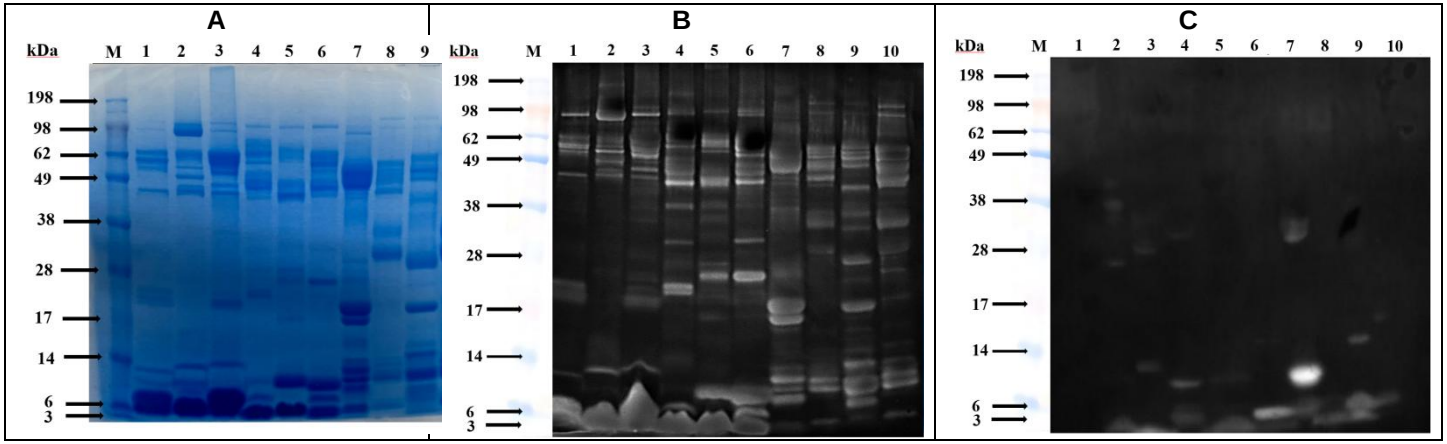

Fig. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) protein profiles of 10 *Elapidae* and *Viperidae* crude snake species venom samples and Snake Venom Antiserum–African IHS (SVA-AIHS) recognition of snake species venom proteins by Western Blotting (WB). A. Stained SDS-PAGE gel showing the protein profiles of snake species venoms. B. WB profile of SVA-AIHS recognition of the different snake species venom proteins. C. WB without SVA-AIHS. In A, B&C, 5 µg of crude venom was applied under reducing conditions. SVA-AIHS was diluted to 1:20000. Lane labels for A, B&C: Lane M: molecular mass marker (kDa); Lane 1: *Dendroaspis jamesoni;* Lane 2: *Dendroaspis polylepis*;Lane 3: *Dendroaspis viridis*; Lane 4: *Naja haje*; Lane 5: *Naja melanoleuca*; Lane 6: *Naja nigricollis*; Lane 7: *Echis leucogaster*; Lane 8: *Echis ocellatus*; Lane 9: *Bitis arietans*: Lane 10: *Bitis gabonica* venoms

Table 2. Summary of damaging effects of the ten Elapid and Viperid snake species venoms and some venom proteins neutralized by the SVA-AIHS in current study

Snake Family	Snake species	Common name	Damaging effect	Previously identified Venom Proteins	Proteins resolved in SDS-PAGE	Proteins recognised by SVA-AIHS in WB
Elapidae	*Naja haje*	Egyptian Cobra	Neurotoxic	PLA_2, SVMP, LAAO, 3FTxs, KUN, CRiSP [29]	PLA2, SVMP, LAAO, 5'-NT, AChE, HYA, 3FTxs, KUN, CRiSP	PLA2, SVMP, LAAO, 5'-NT, AChE, HYA, 3FTxs, KUN, CRiSP,
	N. melanoleuca	Forest Cobra	Neurotoxic	PLA_2, SVMP, 3FTxs, KUN, CRiSP [30]	PLA2, SVMP, LAAO, 5'-NT, AChE, HYA, 3FTxs, KUN, CRiSP	PLA2, SVMP, LAAO, 5'- NT, AChE, HYA, 3FTxs, KUN, CRiSP
	N. nigricollis	Spitting Cobra	Cytotoxic	PLA_2, SVMP, 3FTxs, CRiSP [31]	PLA2, SVMP, LAAO, 5'-NT, HYA, 3FTxs, KUN, CRiSP	PLA2, SVMP, LAAO, 5'-NT, HYA, 3FTxs, KUN, CRiSP
	Dendroaspis polylepis	Black Mamba	Neurotoxic	SVMP, 3FTxs, KUN, NP [32]	PLA2, LAAO, 5'-NT, HYA, 3FTxs, KUN, CTL,	PLA2, 3FTxs, LAAO, 5'-NT, HYA, KUN, CTL
	D. jamesoni	Jameson Mamba	Neurotoxic	3FTxs, SVMP, PLA_2, KUN [33]	PLA2, LAAO, 5'-NT, HYA, 3FTxs, KUN	PLA2, LAAO, 5'-NT, HYA, 3FTxs, KUN
	D. viridis	Green Mamba	Neurotoxic	3FTx, KUN, SVMP, NP, CRiSP, PLA2, ProKin, HYA [34]	PLA2, LAAO, 5'-NT, HYA, 3FTxs, KUN, CTL	PLA2, LAAO, 5'-NT, HYA, 3FTxs, KUN, CTL
Viperidae	*Bitis arientans*	Puff Adder	Hemorrhagic/ coagulopathy	PLA_2, SVSP, SVMP, CTL, DIS, KUN, CYS [35]	PLA2, SVMP, SVSP, LAAO, 5'-NT, KUN, CRiSP, CTL, DIS, NP	PLA2, SVMP, SVSP, LAAO, 5'=NT, KUN, CRiSP, CTL, DIS, NP
	B. gabonica	Gaboon Viper	Hemorrhagic/ coagulopathy	PLA_2, SVSP, SVMP, LAAO, CRiSP, CTL, DIS, NP, KUN, VEGF, CYS[35]	PLA2, SVMP, SVSP, LAAO, 5,'-NT, HYA, KUN, CTL, DIS, NP	PLA2, SVMP, SVSP, LAAO, 5'-NT, HYA, KUN, CTL, DIS, NP
	Echis ocellatus	Saw-scaled Viper	Hemorrhagic/ coagulopathy	PLA_2, SVSP, SVMP, LAAO, CRiSP, CTL, VEGF [36]	PLA2, SVMP, SVSP, LAAO, 5'-NT, HYA, CRiSP, DIS	PLA2, SVMP, SVSP, LAAO, 5'-NT, HYA, CRiSP, DIS
	E. leucogater	White-bellied Carpet Viper	Hemorrhagic/ coagulopathy	PLA_2, SVSP, SVMP, LAAO [37]	PLA2, SVMP, SVSP, LAAO, 5'-NT, HYA, CRiSP, CTL, DIS	PLA2, SVMP, SVSP, LAAO, 5'-NT, HYA, CRiSP, CTL, DIS

Abbreviations: PLA2, phospholipase A2; 3FT, three-finger toxin; SVSP, snake venom serine protease; SVMP, snake venom metalloprotease; LAAO, L-amino acid oxidase; 5'-NT, 5'-Nucleotidases; HYA, Hyaluronidase; AChE, Acetylcholinesterase; CRiSP, Cysteine-Rich Secretory Protein; CTL, C-type lectins; DIS, disintegrin; NP, natriuretic peptides; KUN, Kunitz peptides; VEGF, vascular endothelial growth factor; CYS, cystatin; ProKin, Prokineticin

Table 3. Characteristics of common enzymatic venom components of Elapidae and Viperidae snake families

Venom component	Approximate molecular mass (kDa)	Representative Biological effect	Snake families
PhospholipaseA2 (PLA2)	12–14 [38] 13 – 15 [39]	Myotoxicity, oedema formation, anticoagulant effects, hypotension, presynaptic neurotoxicity [39,40]	Elapidae (typeIPLA2); Viperidae (type IIPLA2)[41]
Snake-venom metalloproteases (SVMP)	Classified into 3 groups based on domain organization [42];P-I:20–30;P-II:30–60;P-III:60–100 [43–45]	Inducelocaland systemic bleeding and disrupt haemostasis through its pro-/anticoagulation properties. Extravasation of blood, inflammation and tissue necrosis [45,46]	Major protein family in viper venoms, but less abundant in elapid venom[41]
Serine proteases (SVSP) e.g.,thrombin-like enzymes	26–67 [47]; 25 – 75 [48]	Disruption of haemostasis and hypotension[47]	Almost all Viperidae, uncommon in Elapidae except Australian snakes[41]
L-aminoacidoxidases (LAAO)	50–70 when measured by SDS/PAGE method under reducing and non-reducing conditions[49,50]	Effects on platelet aggregation, inducing cell apoptosis, and antimicrobial activities[51]	Both Elapidae and Viperidae. Most common in Crotalinae[41]
5'-Nucleotidases (5'-NT)	53–82 [52]	Platelet aggregation inhibition[53,54]	Both Elapidae and Viperidae[53]
Acetylcholinesterases (AChE)	55–60 [52]	Termination of neurotransmission by acetylcholine[55,56]	Elapidae except Dendroaspis genus [56]
Hyaluronidases (HYA)	33–110 [57]	"Spreading factor" alters the structural, rheological, and chemical properties of the extracellular matrix [57]	Both Elapidae and Viperidae[53]

Table 4. Characteristics of common non-enzymatic venom components of ElapidaeandViperidae snake families

Venom component	Approximate molecular mass (kDa)	Representative Biological effect	Snake families
Three-fingertoxins (3FTx) e.g.,α-neurotoxins	6–9[52,58]	Post synaptic neurotoxicity	Elapidae and very rare in Viperidae[41]
Kunitzpeptides (KUN)	~6[59]; 7[60]	Disruption of haemostasis	Elapidae and Viperinae (absentinCrotalinae)[41]
Cysteine-richsecretory proteins (CRiSP)	20–30[61]	Inhibit smooth muscle contraction[61]	More common and abundant in Viperidae[41]
C-typelectins (CTL)	Composed of two subunits[62]; α (A chain): 14–15; β (B chain):13–14	Anticoagulation, promote or inhibit platelet aggregation[63]	More abundant in Viperidae[41]
Disintegrins (DIS)	5–10[64]	Inhibit platelet aggregation[64]	Viperidae, absent in Elapidae[41]
Natriureticpeptides (NP)	3.5–4[65];~4.6 [66]	Vasodilation, diuresis, and natriuresis leading to hypotension, andpromote sodium and water excretion[67]	Both Elapidae (atrial-type and brain-type) and Viperidae (C-type). More common and abundant in Viperidae than Elapidae[41]
Prokineticin (ProKin)	~ 8 [68]	Potent constriction of intestinal smooth muscle and induction hyperalgesia[69]	Elapidae and Viperidae [69]
Vascular endothelial growth factor (VEGF-F) e.g, Barietin	11 kDa under reducing conditions; 22 kDa under non-reducing conditions, 22.07 kDa when purified and ~28.2 kDa as a homodimer [70]	Potent hypotensive effect and strong enhancement of vascular permeability	Viperidae[70]

4. DISCUSSION

Antivenoms remain the only effective therapy against envenomation [71]; however, the demand for snakebite treatment in Africa is far less than the present antivenom production [19]. Effective management of snakebite is hindered, as almost 80% of the culprit snakes are unidentified [72], which becomes a challenge if only specific, non-cross-neutralizing monovalent antivenoms are accessible [73]. Many antivenom manufacturing companies produce and distribute antivenoms for the use in sub-Saharan Africa [74], whereas information on the preclinical efficacy of some antivenoms against panel of medically significant African snake venoms is limited [7,75]. This current study assessed the preclinical neutralization efficacy of the polyvalent VINS Snake Venom Antiserum – African IHS (SVA-AIHS) against medically relevant snake venoms from *Elapidae* (*Naja* spp. and *Dendroaspis* spp.) and *Viperidae* (*Echis* spp. and *Bitis* spp.) families.

In double-diffusion precipitation experiments, preparations containing several antigens give rise to multiple lines which is indicative of the complexity of the antigen [76]. The mixing of potent antibodies with multivalent soluble antigens results in the classical precipitation reaction which appears visible in agar gel [21,77,78]. Snake venoms contain many soluble proteins in the form of enzymes and polypeptide toxins most of which are multivalent and may therefore be expected to react with potent antisera [78]. In this study, the snake venom antiserum produced precipitation in reaction to the venoms of all the 10 snake species thereby suggesting the presence of anti-snake venom antibodies in the SVA-AIHS parenteral preparations. The absence of reactivity with the irrelevant control BSA protein also suggests that the observed reactions between the SVA-AIHS and the snake venoms may be specific. Furthermore, titration of either the venom antiserum or the snake venoms tested resulted in the expected decrease in precipitation products and subsequent abolition of precipitation thus, agreeing with reported observations that precipitation would occur only in a narrow optimum concentration range for either antigen or antibody [77]. The occurrence of multiple precipitin lines between the snake venom antiserum and each of the ten snake venoms tested, therefore, confirm the heterogeneity of the venoms, as well as, the presence of specific antibodies in the SVA-AIHS (prepared by the VINS Bioproducts Limited, Telangana, India) to the different antigens.

The antivenom from VINS (SVA-AIHS) was raised against six common African elapids (*Naja haje, Naja melanoleuca, Naja nigricollis, Dendroaspis jamesoni, Dendroaspis polylepis and Dendroaspis viridis)* and four African viperids (*Echis leucogaster, Echis ocellatus, Bitis arietans* and *Bitis gabonica)* venoms. Result from the *in vivo* experiment using ICR mice revealed that the batch of SVA-AIHS tested was effective in the neutralization of lethality of venoms of elapids (*Dendroaspis* spp. and *Naja* spp.) and viperids (*Echis* spp. and *Bitis* spp.) at varying neutralization potency with the corresponding $3LD_{50}$. (Table 1). This study found the neutralizing capabilities of 1 mL of the SVA-AIHS to be highly efficacious against multiple LD_{50}s and times above the manufacturer's recommended $20LD_{50}$ or $25LD_{50}$ from the most lethal snake species venoms to the least (as found in mice) as follows: *Naja haje* (Egypian cobra; 65LD50;3x); ... to 65LD50; 3x);

345LD50;14x); ... to 345LD50; 14x);

cobra ... *to* 66LD50; 3x); *Naja melanoleuca* (Forest cobra;

414LD50;17x); ... to 414LD50; 17x);

269LD50;11x); ... to 269LD50; 11x);

179LD50;7x); ... to 179LD50; 7x)

121LD50;6x); ... to 121LD50; 6x);

383LD50;15x); ... to 383LD50; 15x);

217LD50;9x). to 217LD50; 9x).
. Among the viper species venoms, the VINS SVA-AIHS showed strongest neutralizing efficacy against *B. arientans* which happens to be most lethal followed by *E. ocellatus*, *E. leucogaster* and *B. gabonica.* Considering the elapid snake species venoms, the VINS SVA-AIHS showed the strongest neutralizing efficacy against *D. viridis* (third most lethal among the 10 snake venoms) followed by *D. jamesoni*, *N. nigricollis*, *N. melenoleuca*, *D. polylepis* and last, but not the least, *N. haje* (the most lethal among the 10 venoms).

Previous preclinical studies using VINS venom antiserum exhibited variations and inconsistency in neutralization capacity of the venom

antiserum. This may be due to the differences in the source of venom used in the experiment, or with experimental protocol employed, especially, regarding the multiples of LD_{50} used as a 'challenge dose' in the lethality experiments [75,79,80]. This assertion was supported by varying reports on the effectiveness of snake venom neutralization potency of VINS venom antiserum products as follows:Wong et al [75] study showed that VINS African Polyvalent Antivenom (VAPAV) was able to cross-neutralize the lethal effect of *N. senegalensis* (Senegalese cobra) which is a homologue of *N. haje* (Egyptian cobra). In our current study, the effectiveness of the SVA-AIHS against the *Viperidae* and *Elapidae* snake species venoms supports the hypothesis that immunizing horses with a mixture of the *Viperidae* and *Elapidae*venoms generates antibodies capable of recognizing the majority of components of medically relevant homologous and heterologous viperid and elapid venoms of the genera *Bitis*, *Echis, Dendroaspis* and*Naja* from sub-Saharan Africa.

Additionally, in this study, protein profiles of venoms of the medically significant African venomous snakes were ascertained by SDS-PAGE. Species of *Elapidae* notably *Dendroaspis polylepis, Dendroaspis viridis, Naja haje, Naja melanoleuca, Naja nigricollis;* and *Viperidae* species notably*Echis leucogaster, Echis ocellatus* and *Bitis arietans* venoms elicited diverse protein band patterns.Thus, both elapid and viperid snake species venoms showed slight disparities in diverse protein patterns. Although similar proteins were observed in some venom studies [72,81–84] diversity in snake venom may be linked to ecological variance amongst population and neutral evolution whereby venom system works in tandem with positive selection [85,86]. In addition, western blot analysis of the SVA-AIHS against the tested snake species venoms revealed bands at molecular mass sizes (Fig. 2B) very similar to the venom protein patterns as the SDS-PAGE (Fig. 2A), whilst immunoblotting without SVA-AIHS showed no such bands (Fig. 2C). Although with different intensities, SVA-AIHS was able to recognise the various venoms components with high and low molecular masses. Major advances in snake venom proteomics (venomics) over the years has broaden our understanding to the significance of venom protein families in relation to pathophysiology caused by envenomation and antivenom production. The current study revealed bands of low and high molecular mass proteins in the presence of SVA-AIHS for the families of *Elapidae* (*Naja* spp. and *Dendroaspis* spp) and *Viperidae* (*Echis* and *Bitis*). Low molecular mass proteins (< 17 kDa) observed in the all venoms suggest the presence of cytotoxins and neurotoxins [72,75,87], among these, three-finger toxins (3FTxs) [31,88],Phospholopase A2 (PLA_2) [89], Kunitz-type protease inhibitors (KUNs)[59,87] and Natriuretic peptides (NP) [66]. Recent enzymatic and proteomic studies demonstrated that among mambas and cobras, 3FTxs and PLA_2 is highly abundant and generally conserved [41,75][38,72] whiles the presence of PLA_2 without 3FTx in observed in *Echis* and *Bitis* species, which is the major difference between elapids and viper venoms. This phenomenon was revealed in the venoms of elapids and viperids tested. Furthermore, higher molecular mass (> 20 - 120 kDa) proteins was revealed in the venoms of elapids and viperids. (Figs. 2A and 2B). These are likely to correspond with zinc-dependent snake venom metalloproteinase (SVMP), snake venom serine proteases (SVSP), L-amino acid oxidase (LAAO), cysteine-rich secretory proteins (CRiSP) (Tables 2, 3 and 4). Viperids reveal a predominance of SVMPs and SVSPs with variations within species whereas they are mainly responsible for haemorrhage and coagulopathies; defribrinogenation and hypotension, respectively [44]. Metalloproteseases and Serine proteases are of lesser importance in elapids, although are made up of much smaller proportion of the venom composition [41]. This current study demonstrated the various proteins responsible for pathological effects in venoms of elapids (*Dendroaspis* spp. and *Naja* spp.) and viperids (*Echis* spp. and *Bitis* spp.) and recognition of venom component in the presence of the VINS SVA-AIHS.

5. CONCLUSION

The development of highly purified and lyophilized polyvalent venom antiserums of appropriate neutralizing capacity or efficacy is the ideal choice for many African countries. Our findings have presented clear evidence on the capacity of the VINS SVA-AIHS to immune-react and recognise several protein components of the snake venoms from viperids (*Echis* spp. and *Bitis* spp) and elapids (*Dendroaspis* spp. and *Naja* spp.) from Africa *in vitro* and effectively neutralized same *in vivo*. The results presented clearly indicate that the Snake Venom Antiserum – African IHS (Lyophilised) (SVA-AIHS) prepared by VINS Bioproducts Limited, Telangana, India,

contains antibodies capable of neutralizing *Viperidae* and *Elapidae* venoms *in vivo* and will, therefore, be useful clinically as post-exposure prophylaxis in snake bite victims in many African countries. Our results are promising, and provide the basis for polyvalent venom antiserum that could prove viable for widespread use.

ETHICAL APPROVAL

Ethical approval was sought from the University of Ghana Institutional Animal Care and Use Committee (UG-IACUC), referenced as UG-IACUC 012-/19-20.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. World Health Organisation. Global snakebite burden; 2018. DOI:https://doi.org/10.1038/nrdp.2017.63.1
2. Musah Y, Ameade EPK, Attuquayefio DK, Holbech LH. Epidemiology, ecology and human perceptions of snakebites in a savanna community of northern Ghana. PLoS Neglected Tropical Diseases. 2019;13:e0007221. DOI:https://doi.org/10.1371/journal.pntd.0007221
3. Williams DJ, Faiz MA, Abela-Ridder B, Ainsworth S, Bulfone TC, Nickerson AD, et al. Strategy for a globally coordinated response to a priority neglected tropical disease: Snakebite envenoming. PLoS Neglected Tropical Diseases. 2019;13. DOI:https://doi.org/10.1371/journal.pntd.0007059.
4. Chippaux JP. Estimate of the burden of snakebites in sub-Saharan Africa: A meta-analytic approach. Toxicon. 2011;57:586–99. DOI:https://doi.org/10.1016/j.toxicon.2010.12.022
5. Harrison RA, Hargreaves A, Wagstaff SC, Faragher B, Lalloo DG. Snake envenoming: A disease of poverty. PLoS Neglected Tropical Diseases. 2009;3. DOI:https://doi.org/10.1371/journal.pntd.0000569.
6. Alirol E, Sharma SK, Bawaskar HS, Kuch U, Chappuis F. Snake bite in south asia: A review. PLoS Neglected Tropical Diseases. 2010;4:e603. DOI:https://doi.org/10.1371/journal.pntd.0000603.
7. Gutiérrez JM, Warrell DA, Williams DJ, Jensen S, Brown N, Calvete JJ, et al. The Need for Full Integration of Snakebite Envenoming within a Global Strategy to Combat the Neglected Tropical Diseases: The Way Forward. PLoS Neglected Tropical Diseases. 2013;7:e2162. DOI:https://doi.org/10.1371/journal.pntd.0002162.
8. Ferraz CR, Arrahman A, Xie C, Casewell NR, Lewis RJ, Kool J, et al. Multifunctional toxins in snake venoms and therapeutic implications: From pain to hemorrhage and necrosis. Frontiers in Ecology and Evolution. 2019;7:1–19. DOI:https://doi.org/10.3389/fevo.2019.00218.
9. Slagboom J, Kool J, Harrison RA, Casewell NR. Haemotoxic snake venoms: their functional activity, impact on snakebite victims and pharmaceutical promise. British Journal of Haematology. 2017;177:947–59. DOI:https://doi.org/10.1111/bjh.14591.
10. Habib AG. Public health aspects of snakebite care in West Africa: Perspectives from Nigeria. Journal of Venomous Animals and Toxins Including Tropical Diseases. 2013;19:1. DOI:https://doi.org/10.1186/1678-9199-19-27.
11. Osipov A V., Utkin YN. Snake Venom Toxins Targeted at the Nervous System. Snake Venoms, Springer Netherlands; 2015; 1–21. DOI:https://doi.org/10.1007/978-94-007-6648-8_23-1.
12. Méndez I, Gutiérrez JM, Angulo Y, Calvete JJ, Lomonte B. Comparative study of the cytolytic activity of snake venoms from African spitting cobras (Naja spp., Elapidae) and its neutralization by a polyspecific antivenom. Toxicon. 2011;58:558–64. DOI:https://doi.org/10.1016/j.toxicon.2011.08.018.
13. Bougis PE, Marchot P, Rochat H. Characterization of Elapidae Snake Venom Components Using Optimized Reverse-Phase High-Performance Liquid Chromatographic Conditions and Screening Assays for α-Neurotoxin and Phospholipase A2 Activities. Biochemistry. 1986;25:7235–43. DOI:https://doi.org/10.1021/bi00370a070.

14. Chippaux J-P. Snakebite in Africa. Handbook of Venoms and Toxins of Reptiles, CRC Press. 2009;453–73. DOI:https://doi.org/10.1201/9781420008661.ch22.
15. Kularatne SAM, Senanayake N. Venomous snake bites, scorpions, and spiders. 1st ed. Elsevier B.V. 2014;120. DOI:https://doi.org/10.1016/B978-0-7020-4087-0.00066-8.
16. Marsh N. Snake Venoms and Envenomations. By Jean-Philippe Chippaux; translated by , F W Huchzermeyer. Malabar (Florida): Krieger Publishing. $58.50. xii + 287 p; ill.; index. ISBN: 1-57524-272-9. [Originally published in French as Venins de Serpent et Enveninmati. The Quarterly Review of Biology 2007;82:61–61. DOI:https://doi.org/10.1086/513371.
17. El-Aziz TMA, Soares AG, Stockand JD. Snake venoms in drug discovery: Valuable therapeutic tools for life saving. Toxins 2019;11. DOI:https://doi.org/10.3390/toxins11100564.
18. Vineetha MS, Bhavya J, More SS. Inhibition of pharmacological and toxic effects of echis carinatus venom by tabernaemontana alternifolia root extract. Indian Journal of Natural Products and Resources. 2019;10:48–58.
19. Guidolin FR, Caricati CP, Marcelino JR, da Silva WD. Development of equine IgG antivenoms against major snake groups in mozambique. PLoS Neglected Tropical Diseases. 2016;10:1–17. DOI:https://doi.org/10.1371/journal.pntd.0004325.
20. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry. 1976;72:248–54. DOI:https://doi.org/10.1016/0003-2697(76)90527-3.
21. Ouchterlony OT. Immunochemistry. vol. 15. Pergamon Press; 1978.
22. He F. Laemmli SDS PAGE Fanglian He Carnegie Institution at Stanford. Bio-ProtocolOrg. 2011;1:3–6.
23. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proceedings of the National Academy of Sciences of the United States of America. 1979;76:4350–4. DOI:https://doi.org/10.1073/pnas.76.9.4350.
24. National Institute of Health N. Guide for Grants and Contracts. vol. 14. No.8. Special Ed. Laboratory Animal Welfare; 1985.
25. Institute of Laboratory Animal Resource (US) Committee on care use of laboratory A. Guide for the care and use of laboratory animals. US: US Department of Health and Human services, Public Health Service, National Institute of Health; 1986.
26. Act AW. Public Law 89-544 Act. 89th Congr; 1966. DOI:https://www.nal.usda.gov/awic/animal-welfare-act-public-law-89-544-act-august-24-1966 (accessed October 6, 2021).
27. World Health Organisation. WHO guidelines for the Production, Control and Regulation of Snake Antivenom Immunoglobulins. 2016:17–21.
28. Sanhajariya S, Duffull SB, Isbister GK. Pharmacokinetics of snake venom. Toxins. 2018;10. DOI:https://doi.org/10.3390/toxins10020073
29. Malih I, Ahmad rusmili MR, Tee TY, Saile R, Ghalim N, Othman I. Proteomic analysis of moroccan cobra naja haje legionis venom using tandem mass spectrometry. Journal of Proteomics. 2014;96:240–52. DOI:https://doi.org/10.1016/j.jprot.2013.11.012
30. Lauridsen LP, Laustsen AH, Lomonte B, Gutiérrez JM. Exploring the venom of the forest cobra snake: Toxicovenomics and antivenom profiling of Naja melanoleuca. Journal of Proteomics. 2017;150:98–108. DOI:https://doi.org/10.1016/j.jprot.2016.08.024.
31. Petras D, Sanz L, Segura Á, Herrera M, Villalta M, Solano D, et al. Snake venomics of African spitting cobras: Toxin composition and assessment of congeneric cross-reactivity of the Pan-African EchiTAb-Plus-ICP antivenom by antivenomics and neutralization approaches. Journal of Proteome Research. 2011;10:1266–80. DOI:https://doi.org/10.1021/pr101040f.
32. Laustsen AH, Lomonte B, Lohse B, Fernández J, Gutiérrez JM. Unveiling the nature of black mamba (Dendroaspis polylepis) venom through venomics and antivenom immunoprofiling: Identification of key toxin targets for antivenom

development. Journal of Proteomics. 2015;119:126–42. DOI:https://doi.org/10.1016/j.jprot.2015.02.002.

33. Williams HF, Layfield HJ, Vallance T, Patel K, Bicknell AB, Trim SA, et al. The urgent need to develop novel strategies for the diagnosis and treatment of snakebites. Toxins. 2019;11:1–29. DOI:https://doi.org/10.3390/toxins11060363.
34. Ainsworth S, Petras D, Engmark M, Süssmuth RD, Whiteley G, Albulescu LO, et al. The medical threat of mamba envenoming in sub-Saharan Africa revealed by genus-wide analysis of venom composition, toxicity and antivenomics profiling of available antivenoms. Journal of Proteomics. 2018;172:173–89. DOI:https://doi.org/10.1016/j.jprot.2017.08.016.
35. Calvete JJ, Escolano J, Sanz L. Snake venomics of Bitis species reveals large intragenus venom toxin composition variation: Application to taxonomy of congeneric taxa. Journal of Proteome Research. 2007;6:2732–45. DOI:https://doi.org/10.1021/pr0701714.
36. Casewell NR, Harrison RA, Wüster W, Wagstaff SC. Comparative venom gland transcriptome surveys of the saw-scaled vipers (Viperidae: Echis) reveal substantial intra-family gene diversity and novel venom transcripts. BMC Genomics. 2009;10:1–12. DOI:https://doi.org/10.1186/1471-2164-10-564.
37. Calvete JJ. Proteomic tools against the neglected pathology of snake bite envenoming. Expert Review of Proteomics. 2011;8:739–58. DOI:https://doi.org/10.1586/epr.11.61.
38. Toyama MH, Soares AM, Wen-Hwa L, Polikarpov I, Giglio JR, Marangoni S. Amino acid sequence of piratoxin-II, a myotoxic Lys49 phospholipase A2 homologue from Bothrops pirajai venom. Biochimie. 2000;82:245–50. DOI:https://doi.org/10.1016/S0300-9084(00)00202-9.
39. Huang MZ, Gopalakrishnakone P, Chung MCM, Kini RM. Complete amino acid sequence of an acidic, cardiotoxic phospholipase A2 from the venom of Ophiophagus hannah (King cobra): A novel cobra venom enzyme with "pancreatic loop." Archives of Biochemistry and Biophysics. 1997;338:150–6. DOI:https://doi.org/10.1006/abbi.1996.9814.
40. Arni RK, Ward RJ. Phospholipase A2 - A structural review. Toxicon. 1996;34:827–41. DOI:https://doi.org/10.1016/0041-0101(96)00036-0.
41. Tasoulis T, Isbister GK. A review and database of snake venom proteomes. Toxins. 2017;9. DOI:https://doi.org/10.3390/toxins9090290.
42. Bjarnason JB, Fox JW. Hemorrhagic metalloproteinases from snake venoms. Pharmacology and Therapeutics. 1994; 62:325–72. DOI:https://doi.org/10.1016/0163-7258(94)90049-3.
43. Tosin O, Karina P, Selistre-de-araujo HS, Helena D, Souza F De. Toxicon : X snake venom metalloproteinases (SVMPs): A structure-function update. Toxicon: X. 2020;7:100052. DOI:https://doi.org/10.1016/j.toxcx.2020.100052
44. Gutiérrez JM, Vargas M, Segura Á, Herrera M, Villalta M, Solano G, et al. *In Vitro* tests for assessing the neutralizing ability of snake antivenoms: Toward the 3Rs principles. Frontiers in Immunology 2021;11:1–13. DOI:https://doi.org/10.3389/fimmu.2020.617429
45. Takeda S, Takeya H, Iwanaga S. Snake venom metalloproteinases: Structure, function and relevance to the mammalian ADAM/ADAMTS family proteins. Biochimica et Biophysica Acta - Proteins and Proteomics. 2012;1824:164–76. DOI:https://doi.org/10.1016/j.bbapap.2011.04.009.
46. Gutiérrez JM, Rucavado A. Snake venom metalloproteinases: Their role in the pathogenesis of local tissue damage. Biochimie. 2000;82:841–50. DOI:https://doi.org/10.1016/S0300-9084(00)01163-9.
47. Serrano SMT, Maroun RC. Snake venom serine proteinases: Sequence homology vs. substrate specificity, a paradox to be solved. Toxicon. 2005;45:1115–32. DOI:https://doi.org/10.1016/j.toxicon.2005.02.020.
48. Roldan-Padron O, Castro-Guillen JL, Garcia-Arredondo JA, Cruz-Perez SM, Diaz-Pena LF, Saldaña C, et al. Snake venom hemotoxic enzymes : Biochemical

Comparison between Crotalus Species from. Molecules. 2019;24:1–16. DOI:https://doi.org/10.3390/molecules24081489.

49. Du XY, Clemetson KJ. Snake venom L-amino acid oxidases. Toxicon. 2002; 40:659–65. DOI:https://doi.org/10.1016/S0041-0101(02)00102-2
50. Tan KK, Bay BH, Gopalakrishnakone P. L-amino acid oxidase from snake venom and its anticancer potential. Toxicon. 2018;144:7–13. DOI:https://doi.org/10.1016/j.toxicon.2018.01.015.
51. Guo C, Liu S, Yao Y, Zhang Q, Sun MZ. Past decade study of snake venom l-amino acid oxidase. Toxicon. 2012;60:302–11. DOI:https://doi.org/10.1016/j.toxicon.2012.05.001.
52. Mackessy S. Section I. Reptile Toxinology, Systematics, and Venom Gland Structure. Structure 2010:1–13.
53. Iwanaga S, Susuki T. Enzymes in Snake Venoms. Venomous Animals and their Venoms, Springer, Berlin, Heidelberg; 1979;62–157. DOI:https://doi.org/10.1016/b978-1-4832-2949-2.50015-1.
54. Aird SD. Ophidian envenomation strategies and the role of purines. Toxicon. 2002;40:335–93. DOI:https://doi.org/10.1016/S0041-0101(01)00232-X.
55. Kang TS, Georgieva D, Genov N, Murakami MT, Sinha M, Kumar RP, et al. Enzymatic toxins from snake venom: Structural characterization and mechanism of catalysis. FEBS Journal. 2011; 278:4544–76. DOI:https://doi.org/10.1111/j.1742-4658.2011.08115.x.
56. Frobert Y, Créminon C, Cousin X, Rémy MH, Chatel JM, Bon S, et al. Acetylcholinesterases from Elapidae snake venoms: Biochemical, immunological and enzymatic characterization. Biochimica et Biophysica Acta - Protein Structure and Molecular Enzymology. 1997;1339:253–67. DOI:https://doi.org/10.1016/S0167-4838(97)00009-5.
57. Fox JW. A brief review of the scientific history of several lesser-known snake venom proteins: L-amino acid oxidases, hyaluronidases and phosphodiesterases. Toxicon. 2013;62:75–82. DOI:https://doi.org/10.1016/j.toxicon.2012.09.009.
58. Kini RM, Doley R. Structure, function and evolution of three-finger toxins: Mini proteins with multiple targets. Toxicon 2010;56:855–67. DOI:https://doi.org/10.1016/j.toxicon.2010.07.010.
59. Mukherjee AK, Mackessy SP, Dutta S. International Journal of Biological Macromolecules Characterization of a Kunitz-type protease inhibitor peptide (Rusvikunin) purified from Daboia russelii russelii venom. International Journal of Biological Macromolecules. 2014;67:154–62. DOI:https://doi.org/10.1016/j.ijbiomac.2014.02.058.
60. Willmott N, Gaffney P, Masci P, Whitaker A. A novel serine protease inhibitor from the Australian brown snake, Pseudonaja textilis textilis: Inhibition kinetics. Fibrinolysis and Proteolysis. 1995;9: 1–8. DOI:https://doi.org/10.1016/S0268-9499(08)80040-9.
61. Yamazaki Y, Morita T. Structure and function of snake venom cysteine-rich secretory proteins. Toxicon. 2004;44:227–31. DOI:https://doi.org/10.1016/j.toxicon.2004.05.023.
62. Morita T. Structures and functions of snake venom CLPs (C-type lectin-like proteins) with anticoagulant-, procoagulant-, and platelet-modulating activities. Toxicon. 2005;45:1099–114. DOI:https://doi.org/10.1016/j.toxicon.2005.02.021.
63. Ogawa T, Chijiwa T, Oda-Ueda N, Ohno M. Molecular diversity and accelerated evolution of C-type lectin-like proteins from snake venom. Toxicon. 2005;45:1–14. DOI:https://doi.org/10.1016/j.toxicon.2004.07.028.
64. Kamiguti AS, Zuzel M, Theakston RDG. Snake venom metalloproteinases and disintegrins: Interactions with cells. Brazilian Journal of Medical and Biological Research. 1998;31:853–62. DOI:https://doi.org/10.1590/S0100-879X1998000700001.
65. St Pierre L, Flight S, Masci PP, Hanchard KJ, Lewis RJ, Alewood PF, et al. Cloning and characterisation of natriuretic peptides from the venom glands of Australian elapids. Biochimie. 2006;88:1923–31.

DOI:https://doi.org/10.1016/j.biochi.2006.06.014

66. Zhang Y, Wu J, Yu G, Chen Z, Zhou X, Zhu S, et al. Toxicon A novel natriuretic peptide from the cobra venom. Toxicon. 2011;57:134–40. DOI:https://doi.org/10.1016/j.toxicon.2010.10.014.
67. Levin ER, Gardner DG, Samson WK. Natriuretic Peprides. The New England Journal of Medicine. 1998;339:321–8. DOI:https://doi.org/10.1300/J123v27n02_05.
68. Zhao Y, Wu J, Wang X, Jia H, Chen DN, Li J Da. Prokineticins and their G protein-coupled receptors in health and disease. 1st ed. Elsevier Inc.; 2019;161. DOI:https://doi.org/10.1016/bs.pmbts.2018.09.006.
69. Fry BG. From genome to "venome": Molecular origin and evolution of the snake venom proteome inferred from phylogenetic analysis of toxin sequences and related body proteins. Genome Research. 2005;15:403–20. DOI:https://doi.org/10.1101/gr.3228405.
70. Yamazaki Y, Matsunaga Y, Tokunaga Y, Obayashi S, Saito M, Morita T. Snake venom vascular endothelial growth factors (VEGF-Fs) exclusively vary their structures and functions among species. Journal of Biological Chemistry. 2009;284:9885–91. DOI:https://doi.org/10.1074/jbc.M809071200.
71. Chippaux JP, Goyffon M. Venoms, antivenoms and immunotherapy. Toxicon. 1998;36:823–46. DOI:https://doi.org/10.1016/S0041-0101(97)00160-8.
72. Katali O, Shipingana L, Nyarangó P, Pääkkönen M, Haindongo E, Rennie T, et al. Protein identification of venoms of the African spitting cobras, *Naja mossambica* and *Naja nigricincta* nigricincta. Toxins. 2020;12. DOI:https://doi.org/10.3390/toxins12080520.
73. Chotwiwatthanakun C, Pratanaphon R, Akesowan S, Sriprapat S, Ratanabanangkoon K. Production of potent polyvalent antivenom against three elapid venoms using a low dose, low volume, multi-site immunization protocol. Toxicon. 2001;39:1487–94. DOI:https://doi.org/10.1016/S0041-0101(01)00108-8.
74. Potet J, Smith J, McIver L. Reviewing evidence of the clinical effectiveness of commercially available antivenoms in sub-saharan africa identifies the need for a multi-centre, multi-antivenom clinical trial. PLoS Neglected Tropical Diseases. 2019;13:1–17. DOI:https://doi.org/10.1371/journal.pntd.0007551
75. Wong KY, Tan KY, Tan NH, Tan CH. A Neurotoxic Snake Venom without Phospholipase A2: Proteomics and Cross-Neutralization of the Venom from Senegalese Cobra, *Naja senegalensis* (Subgenus: Uraeus). Toxins. 2021;13. DOI:https://doi.org/10.3390/toxins13010060.
76. Williams CA, Chase MW. Precipitation analysis by diffusion in gels. Methods in immunology and immunochemistry, vol. 3, Academic Press New York. 1971;103–374. DOI:https://doi.org/10.1016/b978-0-12-754403-8.50008-3.
77. Oudin J. [9] Immunochemical Analysis by Antigen-Antibody Precipitation in Gels. Methods in Enzymology. Academic Press, Inc., New York. 1980;70:166–98. DOI:https://doi.org/10.1016/S0076-6879(80)70048-4.
78. Bailey GS. Ouchterlony double immunodiffusion. Humana Press Inc., Totowa, NJ; 1996. DOI:https://doi.org/10.1007/978-1-60327-259-9_135.
79. Calvete JJ, Arias AS, Rodríguez Y, Quesada-Bernat S, Sánchez L V., Chippaux JP, et al. Preclinical evaluation of three polyspecific antivenoms against the venom of Echis ocellatus: Neutralization of toxic activities and antivenomics. Toxicon. 2016;119:280–8. DOI:https://doi.org/10.1016/j.toxicon.2016.06.022.
80. Pla D, Sanz L, Quesada-Bernat S, Villalta M, Baal J, Chowdhury MAW, et al. Phylovenomics of Daboia russelii across the Indian subcontinent. Bioactivities and comparative in vivo neutralization and in vitro third-generation antivenomics of antivenoms against venoms from India, Bangladesh and Sri Lanka. Journal of Proteomics. 2019;207:103443. DOI:https://doi.org/10.1016/j.jprot.2019.103443.
81. Casewell NR, Cook DAN, Wagstaff SC, Nasidi A, Durfa N, Wüster W, et al. Pre-clinical assays predict Pan-African Echis

viper efficacy for a species-specific antivenom. PLoS Neglected Tropical Diseases. 2010;4. DOI:https://doi.org/10.1371/journal.pntd.0000851.

82. Paixão-Cavalcante D, Kuniyoshi AK, Portaro FCV, da Silva WD, Tambourgi D V. African adders: Partial characterization of snake venoms from three bitis species of medical importance and their neutralization by experimental equine antivenoms. PLoS Neglected Tropical Diseases. 2015;9:1–18. DOI:https://doi.org/10.1371/journal.pntd.0003419.
83. Andrejccaronáková Z, Petrilla V, Tomečková V, Tóth Š, Pekárová T, Komanický V, et al. New approaches in monitoring venom of genus Dendroaspis. Spectroscopy Letters. 2015;48:462–72. DOI:https://doi.org/10.1080/00387010.2014.906471.
84. Currier R. Investigating venom synthesis: exploring the composition, variation and gene expression dynamics of Bitis arietans venom. PhD Thesis, LSTM 2012.
85. Casewell NR, Jackson TNW, Laustsen AH, Sunaga K. Europe PMC Funders Group Causes and Consequences of Snake Venom Variation. 2020;41:570–81. DOI:https://doi.org/10.1016/j.tips.2020.05.006.Causes.
86. Giorgianni MW, Dowell NL, Griffin S, Kassner VA, Selegue JE. The origin and diversification of a novel protein family in venomous snakes. 2020;117. DOI:https://doi.org/10.1073/pnas.1920011117.
87. Post Y, Puschhof J, Beumer J, Richardson MK, Casewell NR, Clevers H. Article Snake Venom Gland Organoids. Cell. 2020;180:233-247.e21. DOI:https://doi.org/10.1016/j.cell.2019.11.038.
88. Tanaka GD, Furtado MDFD, Portaro FCV, Sant'Anna OA, Tambourgi D v. Diversity of Micrurus snake species related to their venom toxic effects and the prospective of antivenom neutralization. PLoS Neglected Tropical Diseases. 2010;4:1–12. DOI:https://doi.org/10.1371/journal.pntd.0000622.
89. Tanaka GD, Pidde-Queiroz G, Furtado M de FD, van den Berg C, Tambourgi D V. Micrurus snake venoms activate human complement system and generate anaphylatoxins. BMC Immunology. 2012;13. DOI:https://doi.org/10.1186/1471-2172-13-4.

Peer-review history:
The peer review history for this paper can be accessed here:
https://www.sdiarticle4.com/review-history/75395