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

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Authors' contributions

This work was carried out in collaboration among all authors. Authors AI, DGI, BEA and AAK conceptualized and designed the study. Authors DGI, TTM, NS, MFM and ACER 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, ACER, BEA and AAK viewed and edited manuscript. All authors read and approved the final manuscript.

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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

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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 envenomation [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 Atractaspidae – 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, cytology 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
2. MATERIALS AND METHODS

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.

2.2 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-litre plates and incubated at 37°C in 5% CO₂ in air for up to 7 days. 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% CO₂ in air for up to 7 days: 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 polyolepis, Dendroaspis vindis, 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 1 hour. 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 1 hour, 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, Taipec, 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 (LD₅₀) the
amid 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 (ED50: the SVA-AIHS dose which protects 50% of the mice injected).

2.5 Data Analysis

LD50 of the venoms and the ED50 of venom antiserums are expressed as means ± Standard error mean (SEM) with 95% confidence intervals (CI). LD50 (Median lethal dose) and ED50 (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% (LD50) 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 was 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 (LD50) 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 LD50 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 LD50 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 (3LD50) of venom from each of the 10 snake species in the experimental mice at different effective doses (ED50 in µL), whilst all control mice injected with the 3LD50 of each venom only, died within 12 hours. The neutralizing efficacy of the SVA-AIHS in terms of both experimental ED50 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 25LD50 venom of Naja haje and Bitis arietans, respectively, by the manufacturer (Table 1).

3.3 In vitro Reactivity 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, Naja haje, Naja melanoleuca, Dendroaspis polylepis, Dendroaspis viridis, Dendroaspis jamesoni and Naja nigricollis (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$_{50}$* in mg/Kg (95% CI) | ED$_{50}$* (in µL) / 3LD$_{50}$ in mice (95% CI) | 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*  | 0.46 (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                                          |

*LD$_{50}$: The median lethal doses of the snake species venom in mice
*ED$_{50}$: The median effective doses that neutralize 3LD$_{50}$snake species venom challenge in mice
*Confidence Interval
*SVA-AIHS: VINS Snake Venom Antiserum – African HIS
*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 kDa to 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 arietans* 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 | PLA₂, SVMP, LAAO, 3FTx, KUN, CRiSP [29] | PLA₂, SVMP, LAAO, 5'-NT, AChE, HYA, 3FTx, KUN, CRiSP | PLA₂, SVMP, LAAO, 5'-NT, AChE, HYA, 3FTx, KUN, CRiSP |
|              | N. melanoleuca | Forest Cobra  | Neurotoxic | PLA₂, SVMP, 3FTx, KUN, CRiSP [30] | PLA₂, SVMP, LAAO, 5'-NT, AChE, HYA, 3FTx, KUN, CRiSP | PLA₂, SVMP, LAAO, 5'-NT, AChE, HYA, 3FTx, KUN, CRiSP |
|              | N. nigricollis | Spitting Cobra | Cytotoxic | PLA₂, SVMP, 3FTx, CRiSP [31] | PLA₂, SVMP, LAAO, 5'-NT, HYA, 3FTx, KUN, CRiSP | PLA₂, SVMP, LAAO, 5'-NT, HYA, 3FTx, KUN, CRiSP |
|              | D. polylepis   | Black Mamba  | Neurotoxic | SVMP, 3FTx, KUN, NP [32] | PLA₂, LAAO, 5'-NT, HYA, 3FTx, KUN, CTL, PL₂A, LAAO, 5'-NT, HYA, 3FTx, KUN | PLA₂, LAAO, 5'-NT, HYA, 3FTx, KUN, CTL |
|              | D. jamesoni   | Jameson Mamba | Neurotoxic | 3FTx, SVMP, PLA₂, KUN [33] | PLA₂, LAAO, 5'-NT, HYA, 3FTx, KUN, CRiSP, DIS | PLA₂, LAAO, 5'-NT, HYA, 3FTx, KUN, CRiSP, DIS |
|              | D. viridis     | Green Mamba  | Neurotoxic | 3FTx, KUN, SVMP, NP, CRiSP, PL₂A, ProKIn, HYA [34] | PLA₂, LAAO, 5'-NT, HYA, 3FTx, KUN, CTL | PLA₂, LAAO, 5'-NT, HYA, 3FTx, KUN, CTL |
| Viperidae    | Bitis arietans | Puff Adder   | Hemorrhagic/coagulopathy | PLA₂, 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 | PLA₂, SVSP, SVMP, LAAO, CRiSP, CTL, DIS, NP, KUN, VEGF, CYs [35] | PLA₂, SVMP, SVSP, LAAO, 5'-NT, KUN, CRiSP, CTL, DIS, NP | PLA₂, SVMP, SVSP, LAAO, 5'-NT, KUN, CTL, DIS, NP |
|              | Echis ocellatus | Saw-scaled Viper | Hemorrhagic/coagulopathy | PLA₂, SVSP, SVMP, LAAO, CRiSP, CTL, VEGF [36] | PLA₂, SVMP, SVSP, LAAO, 5'-NT, KUN, CRiSP, CTL, DIS | PLA₂, SVMP, SVSP, LAAO, 5'-NT, KUN, CRiSP, DIS |
|              | E. leucogater | White-bellied Carpet Viper | Hemorrhagic/coagulopathy | PLA₂, SVSP, SVMP, LAAO [37] | PLA₂, SVMP, SVSP, LAAO, 5'-NT, HYA, CRiSP, DIS | PLA₂, SVMP, SVSP, LAAO, 5'-NT, HYA, CRiSP, 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 (type I PLA2); Viperidae (type II PLA2)[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] | Induce local and 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 Elapidae and Viperidae 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]                            |
| Kunitz peptides (KUN)                  | -6[59]; 7[60]                     | Disruption of haemostasis                                            | Elapidae and Viperinae (absentin Crotalinae)[41]                  |
| Cysteine-rich secretory 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]                                 |
| Natriuretic peptides (NP)              | 3.5–4[65]; −4.6 [66]              | Vasodilation, diuresis, and natriuresis leading to hypotension, and promote 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 antivenin 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 polyplepis 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 3LD₅₀ (Table 1). This study found the neutralizing capabilities of 1 mL of the SVA-AIHS to be highly efficacious against multiple LD₅₀s and times above the manufacturer’s recommended 20LD₅₀ or 25LD₅₀ from the most lethal snake species venoms to the least (as found in mice) as follows: Naja haje (Egyptian cobra; 65LD₅₀; 3x); ... 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). Among the viper species venoms, the VINS SVA-AIHS showed strongest neutralizing efficacy against B. arietans 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. melanoleuca, D. polyplepis and last, but not the least, N. haje (the most lethal among the 10 venoms).
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 LD50 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 vperid 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 vperid 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 venom components with high and low molecular masses. Major advances in snake venom proteomics (venomics) over the years has broadened 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 all the venoms suggest the presence of cytotoxins and neurotoxins [72, 75, 87], among these, three-finger toxins (3FTxs) [31, 88], Phospholipase A2 (PLA2) [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 PLA2 is highly abundant and generally conserved [41, 75][38, 72] whilst the presence of PLA2 without 3FTx in observed in Echis and Bitis species, which is the major difference between elapids and vperids venoms. This phenomenon was revealed in the venoms of elapids and vperids tested. Furthermore, higher molecular mass (> 20 - 120 kDa) proteins was revealed in the venoms of elapids and vperids. (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; defibrinogenation and hypotension, respectively [44]. Metalloproteases 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 vperids (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 vperids (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.

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