



Preclinical efficacy testing of three antivenoms against *Naja ashei* venom-induced lethality

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ABSTRACT

This study aimed to determine the efficacy of Inoserp, Vins bioproducts, and South African Institute of Medical Research (SAIMR) polyvalent antivenoms in neutralizing *Naja ashei* venom-induced lethality in mice. The neutralization efficacy of the antivenoms were expressed as effective dose, median effective ratio, potency, normalized potency, volume, and the number of vials of antivenom required to neutralize 100 mg of *Naja ashei* venom (NAV).

Naja ashei is a relatively newly described species of giant spitting cobra and is native to several East African countries including Kenya, Uganda, Tanzania, Somalia, and Ethiopia (Wüster and Broadley, 2007). It is a World Health Organization (WHO) Category 1 venomous snake in Uganda, Somalia, Kenya, and Ethiopia, and a category 2 venomous snake in Tanzania (WHO, 2016). It was previously considered a color variant of *Naja nigricollis*, the black necked spitting cobra (Wüster and Broadley, 2007) but mitochondrial DNA evidence and venom composition studies have refuted this claim (Onyango, 2018; Wüster and Broadley, 2007). *Naja ashei* envenomation may display typical characteristics of spitting cobra envenoming e.g. skin and subcutaneous connective tissue necrosis, blistering, edema (Davidson, 1970; Greenham, 1978; Tilbury, 1982; Warrell et al., 1976), and ophthalmic lesions when the venom is spat in the eyes (Handford, 2018). *Naja ashei* venom (NAV) comprises of three-finger toxins (3FTx), phospholipases A₂ (PLA₂), cysteine rich venom proteins, 5'-nucleotidases, metalloproteinases, cobra venom factor, and venom nerve growth factor (Hus et al., 2018). NAV induces mitochondria-mediated apoptosis in human colorectal

cancer cells (Antolikova et al., 2019). A PLA₂ and 3FTx rich protein fraction of NAV showed good antimicrobial activity against *Staphylococcus epidermidis* (Bocian et al., 2020a). Moreover, a study on the exogenous application of 3FTx from NAV on monocytes (U-937) and pro myelocytes (HL-60) and their effect on cell membranes reported that the mechanism of protein-lipid interactions depends on the presence of lipid polar parts and the degree of membrane saturation (Dyba et al., 2021). Bocian and colleagues compared the protein concentration of venoms from *Naja ashei* and *Agkistrodon contortrix* using the Pierce™BCA Protein Assay Kit, the 2-D Quant Kit, the Qubit® Protein Assay Kit, the Bradford Assay, and the Nanodrop method (Bocian et al., 2020b). Okumu and colleagues reported the lethality and cytotoxicity of NAV (Okumu et al., 2021), and Chowdhury et al. reported the efficacy of varespladib, pronomostat, and marimastat against African spitting cobras including *Naja ashei* (Chowdhury et al., 2021). Furthermore, Okumu and colleagues reported the enzymatic activity and brine shrimp lethality of NAV and its neutralization by two antivenoms (Okumu et al., 2020). Manson and colleagues have recently reported on the development of a prototype

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inhibition enzyme-linked immunosorbent assay for detecting three-finger toxins in African spitting cobra venoms including *Naja ashei* (Manson et al., 2022). Inoserp, Vins bioproducts, and South African Institute of Medical Research (SAIMR) polyvalent antivenoms are available in Kenya and are routinely used to manage snake envenomation including envenomation by cobras. However, not much is known on the efficacy of the available antivenoms in *Naja ashei* envenomation as no preclinical or clinical case reports have been documented. Pre-clinical assays such as the WHO recommended neutralization of lethality assay in mice may provide data which may be useful in guiding decision making by physicians and antivenom purchasers in the country. The aim of the present study was to determine the efficacy of Inoserp, Vins bioproducts, and South African Institute of Medical Research (SAIMR) polyvalent antivenoms in neutralizing NAV-induced lethality in mice. The ethical approval to conduct this work was provided by the Biosafety, Animal Use, and Ethics Committee of the University of Nairobi. REF BAUEC/2019/220. All the animal experiments were conducted in compliance with the Animal Research Reporting for *In vivo* Experiments (ARRIVE) guidelines. Fresh NAV was collected from adult male and female snakes caught in the wild and maintained at the East African Venom Supplies (Malindi, Kenya). A summary of the details on the snakes whose venom was used in this study is available in Table S1. Freshly collected NAV was pooled, freeze-dried, and stored at -20°C . Lyophilized venom was dry triturated then dissolved in phosphate-buffered saline. The protein content of undiluted NAV was 17.32 ± 0.79 mg/mL. Ninety five male CD-1 mice weighing 18–20 g sourced from the animal holding unit of the Department of Public Health, Pharmacology, and Toxicology, University of Nairobi were used for lethality and neutralization of lethality experiments. Five mice were used for LD₅₀ dose range finding tests, 25 mice were used for the main LD₅₀ test, and 65 mice were used for the neutralization of lethality assays. These animals were housed in pathogen free polypropylene cages in groups of five mice, and were provided food and water *ad libitum*. The details of the antivenoms used in this study are summarized in Table 1. The antivenoms were gift samples from the East African Venom Supplies, Laborex Kenya, and one was purchased from a local pharmacy in Kenya. All the antivenoms were diluted in phosphate-buffered saline (PBS) before measuring protein concentration (1/25 dilution). The protein concentration of the three antivenoms was determined using

Lowry's method with Bovine Serum Albumin as protein standard (Practical Biochemistry: Principles and Techniques Wilson and Walker, 2000; Waterborg, 2002). The protein concentration of the antivenom samples was expressed as the mean \pm standard deviation of triplicate determination. One venom LD₅₀ dose was defined as the amount of venom causing death in 50% of injected mice (WHO, 2018). The median murine lethal dose (LD₅₀) of NAV was determined using WHO recommended-protocols (WHO, 2018). Briefly, groups of five mice ($n = 25$) weighing 18–20 g received an intraperitoneal (i.p) injection of varying doses of NAV (1.64 mg/kg–4.00 mg/kg) in 100 μL of phosphate-buffered saline and the number of surviving mice in each group after 24 h was documented (WHO, 2018). The venom LD₅₀ (the amount of NAV that kills 50% of the injected mice and the 95% confidence intervals) was calculated by probit analysis (Finney, 1952). The median effective dose (ED₅₀) of the antivenoms was defined as the volume of reconstituted antivenom corresponding to 50% survival of mice i.e. μL of antivenom per venom challenge dose (WHO, 2016). It was determined by the WHO-recommended median effective dose (ED₅₀) assay. 65 male CD-1 mice were randomly assigned into 13 different groups of five mice per group. One group served as venom control group while other groups comprised of various doses of the antivenoms mixed with a fixed dose (5 venom LD₅₀'s) of NAV and adjusted to a final volume (200 μL) with phosphate-buffered saline (PBS) (WHO, 2016). See Table S2 for a detailed description of individual dosing groups. The venom + antivenom mixtures were incubated at 37°C for $\frac{1}{2}$ an hour and i.p. injected into groups of 5 mice (18–20 g, $n = 5$ per group, 200 μL final volume). The number of dead/surviving mice in each group was recorded after 24 h and the ED₅₀ (with 95% confidence intervals) was determined by probit regression analysis (Finney, 1952). The median effective ratio of the antivenoms (ER₅₀) was defined as the ratio of venom (mg) to the volume dose of antivenom (mL) at which 50% of mice survived (Araujo et al., 2008; Tan et al., 2016). It was determined using the formula described by Morais et al. i.e. $ER_{50} = nLD_{50}/ED_{50}$; where ER₅₀ is the median effective ratio of the antivenoms, nLD_{50} is the number of LD₅₀'s of NAV, and ED₅₀ is the median effective dose of the antivenoms (Morais et al., 2010). The neutralization potency of the antivenoms (P) was defined as the amount of venom (mg) which was completely neutralized by a unit of antivenom (mL) (Araujo et al., 2008; Tan et al., 2016). It was determined using the formula described by

Table 1

Information on the brand name, details of the manufacturer, product characteristics, and efficacy statement of the antivenoms used in the present study.

| Brand name (proprietary name) | Details of the manufacturer | Product characteristics | Efficacy statement ^a | Protein content (mg/mL) ^b | Protein amount (mg) per vial |
|--|---|---|---|--------------------------------------|------------------------------|
| Inoserp™ | Veteria Labs (Mexico City) | Expiry date: June 2022 Batch No: 91T06001, lyophilized, equine (Fab'2), protein content: NMT 1000 mg of proteins | Each vial contains NMT 1000 mg of total proteins that neutralize at least 50DL50 of <i>E. ocellatus</i> , <i>B. arietans</i> , <i>N. nigricollis</i> , and <i>D. polylepis</i> venoms | 45.93 ± 1.66 | ~450 |
| Snake Venom Antiserum (African) | Vins bioproducts (India) | Expiry date: December 2021 Batch number: 07AS18001 Lyophilized, equine immunoglobulin fragments | Each mL upon reconstitution neutralizes not less than 20 LD50 of <i>N. nigricollis</i> venom and 25 LD50 of <i>N. haje</i> , <i>D. polylepis</i> , <i>D. viridis</i> , <i>D. jamesoni</i> , <i>B. gabonica</i> , <i>B. arietans</i> , <i>E. leucogaster</i> , and <i>E. ocellatus</i> | 23.11 ± 0.54 | ~230 |
| SAIMR polyvalent snake antiserum/antivenom | South African Vaccine Producer (South Africa) | Expiry date: October 2019 Lot: BG 03046 Refined equine serum globulins | Effective against rhinkals, mambas, all cobras, and vipers. Inactive against Berg adder (<i>Bitis atropus</i>), the horned adder (<i>Bitis caudalis</i>), the many horned adder (<i>Bitis cornuta</i>), the night adders (<i>Causus</i> spp), the burrowing asps (<i>Atractaspis</i> spp), back fanged snakes (Boomslang, vine snake). Suggested dose by IV injection 50 mL: puff adder, spitting cobras, and Rhinkals 200 mL: Gaboon adder | 67.58 ± 2.81 | ~670 |

^a Information was obtained from the product inserts.

^b Protein concentration was estimated by Lowry's method.

Morais et al. i.e. $P = ER_{50} \cdot LD_{50} / ED_{50}$; where ER_{50} is the median effective ratio of the antivenoms, LD_{50} is the median lethal dose of NAV, and ED_{50} is the median effective dose of NAV (Morais et al., 2010). The normalized potency of the antivenoms (n-P) was defined as the amount (mg) of venom neutralized per gram of antivenom protein (mg/g) (Tan et al., 2019a). It was calculated using the formula described by Tan et al. (2020) i.e. $n-P = P / pc$; where p is the potency of the antivenoms and pc is the protein concentration of the antivenoms. The volume and the number of vials of antivenom required to neutralize 100 mg of NAV in mice was determined using the method described by Tan et al. (2020). Normalized potency values; n-P (mg/g) and the protein content of the antivenoms were used in the calculations (Tan et al., 2020). Data on the number of surviving mice in the median lethal dose and median effective dose assays were entered into Microsoft Excel and analyzed by probit regression analysis using GraphPad Prism (version 9.0.0). The median effective ratio, and potency were determined by the method described by Morais et al. (2010). Normalized potency, the volume, and number of vials of antivenom required to neutralize 100 mg of venom were determined by methods described by Tan et al. (2020).

The i.p. LD_{50} of NAV was 3.02 (2.45–3.72) $\mu\text{g/g}$ or 57.38 (46.55–70.68) $\mu\text{g/mouse}$. This value was higher than the i.p. LD_{50} of other spitting cobras including *Naja siamensis* (1.13 $\mu\text{g/g}$), *Naja pallida* (2.00 $\mu\text{g/g}$), *Naja kaouthia*, and *Naja mossambica* (0.083 $\mu\text{g/g}$) (Zhang et al., 2016; Fischer and Kabara, 2016; Schweitz, 1984; Xu et al., 2017). Weakness, incoordination, paralysis, altered respiration, and mortality were observed in the experimental mice. These symptoms were observed from 20 min to 24 h after envenomation. Proteomic analysis of NAV reveals that three-finger toxins (3FTx's) make up much of NAV (Hus et al., 2018). Therefore, it could be argued that the lethality of NAV may be due to post-synaptic neuromuscular blockade mediated through α -neurotoxins (Silva et al., 2018; Tan et al., 2019b). Moreover, Tan and colleagues established a positive correlation between the lethality of cobra venoms and the amount of α -neurotoxins in venom (Tan et al., 2019b). The World Health Organization (WHO) is targeting to lower snakebite associated mortality and morbidity by 2030 (Williams et al., 2019). A key pillar in achieving this target is the supply of safe and effective treatments (Ainsworth et al., 2020). However, there is a paucity of robust, independently-generated preclinical or clinical evidence on the efficacy of many of the antivenoms available in Africa (Ainsworth et al., 2020). The decision on which antivenom is suitable for a particular region is usually based on the results of preclinical efficacy data stated by antivenom manufacturers' (Ainsworth et al., 2020). The preclinical antivenom efficacy of many of the snakes of medical importance in Sub-Saharan Africa are available in the scientific literature. However, *Naja ashei* is an exception despite being a highly venomous snake whose bites are associated with high levels of morbidity, disability, or mortality in Uganda, Somalia, Kenya, and Ethiopia (Ainsworth et al., 2020; WHO, 2016). The present study aimed to fill this gap by providing baseline information that Vins bioproducts polyvalent antivenom has poor efficacy against *N. ashei* envenomation, as shown by the low normalized potency (n-P). Table 2. Moreover, the difference in the normalized potency of Inoserp polyvalent antivenom (53.12 mg/g) and SAIMR polyvalent antivenom (53.27 mg/g) was subtle. Table 2. It could be argued that the lethal components of NAV could be more antigenic and better immunorecognized by Inoserp and SAIMR polyvalent antivenoms. Prospective observational clinical studies are needed to corroborate these findings. Antivenom dosing is a key aspect of snakebite treatment. The South African manufactured antivenom is the only antivenom whose package insert contained clear details on the dosage (number of vials) to be used in the case of snakebite, i.e. five vials in the case of puff adder, rhinkals, or spitting cobra venoms, and 20 vials in the case of a bite from the Gaboon adder. If the example provided by Tan and colleagues is applied (Tan et al., 2020), i.e. where the neutralization of 100 mg of NAV is considered, the antivenom dose of Inoserp, Vins bioproducts, and SAIMR polyvalent antivenoms would be 4, 17, and 3 vials for the respective antivenoms.

Table 2

Summary of the capacity of three antivenoms to neutralize *Naja ashei* venom-induced lethality in mice.

| Parameter | Antivenom | | |
|--|------------------------|---------------------------|------------------------|
| | Inoserp | Vins bio products | SAIMR |
| ED_{50} (μL) ^a | 94.14 (92.24–96.42) | 389.67 (382.02–395.75) | 63.77 (62.48–66.10) |
| ER_{50} (mg/mL) ^b | 3.05 | 0.74 | 4.50 |
| P (mg/mL) ^c | 2.44 | 0.59 | 3.60 |
| n-P (mg/g) ^d | 53.12 | 25.53 | 53.27 |
| Volume of antivenom required to neutralize 100 mg of NAV ^e (mL) | 40.93 | 169.62 | 27.88 |
| Number of vials required to neutralize 100 mg of NAV ^e | ~4 | ~17 | ~3 |

^a Effective dose: volume (μL) of reconstituted antivenom per challenge dose of venom.

^b Median effective ratio: ratio of venom (mg) to the volume dose of antivenom (mL) at which 50% of mice survived.

^c Potency: amount of venom (mg) neutralized completely by a unit of antivenom (mL).

^d Normalized potency: the milligrams of venom neutralized per gram of antivenom.

^e NAV: *Naja ashei* venom.

Table 2. When the cost of these antivenoms is considered (based on estimates provided by a previous study (Harrison et al., 2017), 4 vials of Inoserp polyvalent antivenom would translate to ~US\$480, 17 vials of Indian manufactured antivenom would translate to ~US\$816, and 3 vials of SAIMR polyvalent antivenom would translate to US\$945. These are damning figures especially considering that most snakebite victims are the rural poor (Harrison et al., 2009; Okumu et al., 2019). Such astronomical sums of money may well be beyond their means. The findings of this study suggest that Vins bioproducts polyvalent antivenom has low potency; therefore, many vials of antivenom are required to neutralize 100 mg of NAV completely. Table 2. Administering up to 17 vials of antivenom during an emergency may not be practical and may expose victims to adverse effects such as urticaria and anaphylactic shock. Ultimately, these findings suggest that Vins bioproducts antivenom has poor efficacy in experimental *Naja ashei* envenomation. The findings also suggest that there may be subtle differences in the efficacy of Inoserp and SAIMR polyvalent antivenoms in *Naja ashei* envenomation.

Credit author statement

MO, FO, and JG were responsible for the concept; MO carried out the statistical analysis; all authors participated in the investigation; MO provided the resources and the software; this work was under the supervision of JM, JG, PM, and VM; and was validated by FO, JM, JG, PM, and VM; MO prepared all the tables in the manuscript; MO and FO wrote the original manuscript draft; all authors were involved in writing review and editing; All authors read and approved the final manuscript.

Ethical statement

The ethical approval to conduct this work was provided by the Biosafety, Animal Use, and Ethics Committee of the University of Nairobi. REF BAUEC/2019/220. All the animal experiments were conducted in accordance with the Animal Research Reporting for *In vivo* Experiments (ARRIVE) guidelines.

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Declaration of competing interest

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

Abbreviations

| | |
|------------------|---|
| NAV | <i>Naja ashei</i> venom |
| LD ₅₀ | Lethal dose (corresponding to 50% death in mice) |
| ED ₅₀ | Median effective dose (volume of reconstituted antivenom corresponding to 50% survival of mice) |
| ER ₅₀ | Median effective ratio (ratio of venom (mg) to the volume dose of antivenom (mL) at which 50% of mice survived) |
| P | Potency (amount of venom (mg) neutralized completely by a unit of antivenom (mL)) |
| n-P | normalized potency (the milligrams of venom neutralized per gram of antivenom) |
| PBS | phosphate-buffered saline |
| 3FTxs | three-finger toxins |
| UV-VIS | ultraviolet and visible |
| CI | Confidence interval |
| SD | standard deviation |

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.toxcx.2022.100124>.

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