Determination of LD_{50} of *Naja ashei* Venom and its Effects on the Heart and Serum Electrolytes in BALB/c Mice through Intraperitoneal, Subcutaneous and Intramuscular Routes

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Abstract

Introduction: *Naja ashei* envenomation causes severe local and systemic toxicity, yet experimental evidence on route-dependent lethality, electrolyte disturbances, and cardiotoxicity remains limited. This study determined the median lethal dose (LD_{50}) of *Naja ashei* venom in BALB/c mice after intraperitoneal, intramuscular, and subcutaneous administration and evaluated electrolyte imbalance and cardiac injury.

Materials and methods: Fresh *Naja ashei* venom was collected, lyophilized, and characterized. One hundred BALB/c mice received venom doses through intraperitoneal, intramuscular, or subcutaneous routes. LD_{50} values were calculated using the Reed-Muench method and confirmed by probit and dose-response analyses. Cardiac injury was assessed using creatine kinase-MB and troponin assays, while histopathological examination of cardiac tissue confirmed venom-induced myocardial damage.

Results: Venom profiling showed predominance of three-finger toxins (~69%) and phospholipase A_2 (~27%), consistent with cytotoxic and systemic activity. Toxicity was route dependent, with intraperitoneal administration producing the lowest LD_{50} (0.70 mg/kg), followed by intramuscular (2.36 mg/kg) and subcutaneous (2.69 mg/kg) routes. Envenomation caused severe hyperkalemia (mean 7.8 mmol/L) with hyponatremia. Mean troponin-I reached 11.7 ± 5.6 ng mL¯1, while creatine kinase-MB averaged 917 ± 122.2 U L¯1, indicating myocardial injury. Histology revealed myocardial necrosis, interstitial edema, and vascular congestion.

Conclusion: *Naja ashei* venom exhibits high lethality and produces electrolyte imbalance and myocardial injury in a route-dependent manner. These findings support antivenom administration, electrolyte correction, intensive cardiac monitoring, and inclusion of *Naja ashei* venom in antivenom development to improve clinical outcomes.

Keywords: *Naja ashei; Snake-venom; LD_{50} -toxicity; Cardiotoxicity; mice*

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INTRODUCTION

Snakebite envenoming remains a major but persistently neglected public health challenge, particularly in low- and middle-income countries where environmental exposure, agricultural livelihoods, and limited access to healthcare increase the risk of human-snake interactions. Globally, snakebite envenoming is responsible for an estimated 2.7 million cases each year, resulting in approximately 81,000-138,000 deaths and a substantial number of survivors living with permanent disability and

socioeconomic consequences.[1] In recognition of this significant burden, the World Health Organization classified snakebite envenoming as a Category A neglected tropical disease in 2017 and subsequently launched a global strategy aimed at reducing snakebite-related mortality and morbidity by 50% by the year 2030.[2]

Sub-Saharan Africa bears a substantial proportion of the global snakebite burden, with an estimated 435,000 to 580,000 snakebite envenoming cases and approximately 20,000-32,000 deaths occurring annually across the region.[3,4] In Kenya, snakebite envenoming remains a significant public health concern, particularly in rural and arid regions where access to antivenom and specialized healthcare services is limited. Current estimates suggest that Kenya records between 15,000 and 20,000 snakebite cases annually, resulting in considerable mortality, disability, and socioeconomic burden.[5,6] These figures likely underestimate the true burden due to underreporting, poor surveillance systems, and reliance on traditional remedies in many affected communities

One of the medically important snake species implicated in cobra envenomation in Kenya is *Naja ashei*, commonly known as the large brown spitting cobra. This species is widely distributed in open, arid, and semi-arid regions of East Africa and is responsible for a substantial proportion of spitting cobra bites.[6] African spitting cobras are classically associated with severe local cytotoxic effects, including tissue necrosis, blistering, and permanent disfigurement, largely mediated by cytotoxins and phospholipase A_2 enzymes.[7] Although neurotoxicity is less prominent compared to non-spitting cobras, emerging evidence indicates that spitting cobra venoms may also induce clinically significant systemic effects including cardiotoxicity, electrolyte imbalance, and inflammatory injury that have not been adequately characterized.[12,13]

The primary intervention for snakebite envenoming is the administration of antivenom, which neutralizes circulating venom toxins and reduces the risk of mortality and severe complications when given promptly.[2] Preclinical evaluation of venom toxicity and antivenom efficacy remains central to improving clinical outcomes and guiding antivenom development. Such evaluations typically rely on standardized animal models, with mice being the most commonly used experimental species due to their genetic uniformity, reproducibility, and well-characterized physiological responses.[8]

Determination of the median lethal dose (LD_{50}) is a cornerstone of venom toxicology, providing quantitative estimates of venom potency and enabling comparisons across different routes of exposure.[10] Prior investigations into *N. ashei* venom toxicity have primarily focused on the intraperitoneal route, leaving important gaps regarding route-dependent variations in lethality that are relevant to real-world envenomation scenarios, where venom is typically delivered via subcutaneous or intramuscular deposition.[11] In the present study, LD_{50} values were determined for *N. ashei* venom using intraperitoneal, subcutaneous, and intramuscular routes in mice, thereby providing a more comprehensive toxicological profile of the venom.[11]

In addition to lethality, snake venoms are known to cause systemic biochemical and physiological disturbances, particularly involving electrolyte homeostasis and cardiovascular function. Alterations in serum electrolytes may contribute to neuromuscular dysfunction and cardiac instability, while cardiotoxic venom components

can lead to myocardial injury, arrhythmias, and circulatory compromise.[7] In this study, serum electrolyte levels and cardiac biomarkers were measured following envenomation, and histopathological examination of cardiac tissue was performed to evaluate venom-induced myocardial damage.

Furthermore, concerns remain regarding the effectiveness of currently available polyvalent antivenoms against *Naja ashei* venom. In Kenya, the currently available antivenoms include AFRIVEN® and PANAF-Premium®, which are primarily developed using immunizing venom mixtures from selected African snake species and may provide limited specific coverage against *Naja ashei* venom.[11] Previous studies have demonstrated partial neutralization of certain venom components using heterologous antivenoms; however, data regarding their effectiveness in counteracting lethality, cardiotoxicity, and systemic toxicity in mammalian models remain limited.[11]

This study therefore presents a comprehensive toxicological evaluation of *Naja ashei* venom based on experimentally generated data. By characterizing route-specific LD_{50} values and documenting associated electrolyte disturbances and cardiac effects, the study provides evidence that is directly relevant to clinical management, antivenom selection, and future antivenom development. The findings contribute to closing critical knowledge gaps and offer a scientific basis for improving outcomes of *N. ashei* envenomation in Kenya and across sub-Saharan Africa.

MATERIALS AND METHODS

Study Site

This study was conducted at the Small Animal Facility for Research and Innovation (SAFARI) at Jomo Kenyatta University of Agriculture and Technology (JKUAT), Juja Town, Kenya. The facility is equipped with standardized animal housing units, biosafety laboratories, and analytical infrastructure suitable for toxicological and biomedical research involving laboratory animals.

Study Design

A controlled laboratory-based experimental study was conducted in two sequential phases. Phase I involved determination of Chemical and physical properties of lethal dose (LD_{50}) of *Naja ashei* venom in mice using three routes of administration: intraperitoneal (I.P.), intramuscular (I.M.), and subcutaneous (S.C.). The Reed and Muench interpolation method (1938) was employed for LD_{50} estimation. Phase II assessed sub-lethal systemic effects of the venom, including electrolyte disturbances, cardiac injury biomarkers, and myocardial histopathological changes, following administration of $0.5\times LD_{50}$ doses via each route. The study design incorporated random allocation of animals, blinded outcome assessment, and predefined humane endpoints in accordance with ethical guidelines.

Animal Model

Ninety male and female BALB/c mice (45 male and 45 female) aged 2-3 months and weighing between 18 and 40 g were used. Animals were acclimatized for five days prior to experimentation under controlled environmental conditions (22 ± 2 °C, 55 ± 10% humidity, 12-hour light/dark cycle). Mice were housed in polycarbonate cages with wood-chip bedding, provided with nesting materials for environmental enrichment, and had *ad libitum* access to commercial pelleted feed and water. Bedding was changed twice weekly. Only healthy mice within the target weight range were included in the study, while animals showing signs of

illness, pregnancy, or prior experimental exposure were excluded. All procedures were approved by the JKUAT Institutional Animal Care and Use Committee and complied with international guidelines for laboratory animal welfare.

Venom Source, Handling, and Protein Standardization

Crude venom was obtained from six adult *Naja ashei* specimens maintained at Bio-Ken Snake Farm, Watamu, Kenya, and milked by accredited herpetologists. The snakes were housed in secure species-specific enclosures under controlled environmental conditions with regulated temperature, humidity, feeding schedules, hydration, and routine veterinary monitoring in accordance with institutional animal welfare and biosafety guidelines.[12] Bio-Ken Snake Farm is a recognized snake conservation and venom research facility licensed by the Kenya Wildlife Service (KWS) for handling venomous reptiles and venom extraction activities.

Venom extraction was performed using standardized manual milking procedures as previously described for venomous snakes.[12] Briefly, individual snakes were gently restrained using snake hooks and secure handling techniques, after which venom was collected by allowing the snake to bite onto a sterile parafilm-covered collection vessel. The extracted venom was immediately transferred into sterile containers maintained on ice to preserve protein integrity. Venom samples from all snakes were pooled to minimize inter-individual venom variability, flash-frozen in liquid nitrogen, lyophilized, transported on dry ice, and stored at -20 °C until use.

Prior to experimentation, venom was reconstituted in sterile phosphate-buffered saline (PBS, pH 7.4), vortexed, centrifuged to remove particulates, and serially diluted. Protein concentration was quantified using a bicinchoninic acid (BCA) assay with bovine serum albumin as the standard according to the manufacturer's instructions (Thermo Fisher Scientific, USA). All dosing calculations were normalized to the measured protein concentration. Venom-contaminated materials were chemically inactivated and disposed of through incineration according to institutional biosafety guidelines[1].

Precautions While working with venomous Snakes and Venom Samples

All snake specimens were taxonomically verified and handled exclusively by experienced snake handlers and licensed reptile veterinarians. Laboratory personnel received training on venom handling and biosafety procedures. Personal protective equipment, including laboratory coats, eye protection, and double gloves, was always used, and venom preparation was conducted within a biosafety cabinet. Standard operating procedures for accidental exposure were strictly followed.

LD_{50} Determination

Ninety BALB/c mice were randomly assigned to three groups corresponding to intraperitoneal (I.P.), intramuscular (I.M.), and subcutaneous (S.C.) administration routes (n = 30 per group). The sample size was determined based on the World Health Organization guidelines for preclinical venom toxicity testing and previously published LD_{50} studies utilizing the Reed–Muench method, which recommend a minimum of five dose levels with multiple animals per dose group to ensure reliable mortality estimation and statistical validity.[9,13] Each group was subdivided into five dose subgroups (n = 6 per dose). Venom doses were selected based on preliminary range-finding

studies and administered in fixed volumes appropriate for each route. Animals were observed for 24 hours post-injection, and mortality was recorded at predefined intervals. LD_{50} values were calculated using the Reed–Muench method as previously described by Reed and Muench and recommended for venom lethality studies.[9,13], while probit analysis was employed to confirm estimates and generate 95% confidence intervals.

Sub-lethal Dosing and Clinical Observation

Separate cohorts comprising a total of eighteen BALB/c mice were used for sub-lethal toxicity assessment, with six mice assigned to each administration route (intraperitoneal, intramuscular, and subcutaneous). Animals received $0.5\times LD_{50}$ doses to assess sub-lethal systemic effects, as previously described in experimental venom toxicology studies evaluating biochemical and cardiotoxic alterations in murine models. [10,11] Animals were monitored for clinical signs including body weight changes, temperature, posture, locomotion, piloerection, respiratory distress, and responsiveness at regular intervals up to 72 hours post-envenomation. Humane endpoints triggered immediate euthanasia using isoflurane overdose followed by cervical dislocation in accordance with international laboratory animal welfare guidelines.[12]

Blood and Serum Collection

Blood samples were collected from the lateral tail vein under brief anesthesia at 6-, 24-, and 48-hour post-envenomation. Samples were processed to obtain serum, which was either analyzed immediately or stored at -80 °C pending analysis.

Serum Electrolyte and Cardiac Biomarker Assays

Serum sodium and potassium concentrations were measured using an automated clinical chemistry analyzer (Humastar 200, HUMAN Diagnostics, Wiesbaden, Germany) following the manufacturer's protocols and standard laboratory quality control procedures. Cardiac injury biomarkers, including troponin I and creatine kinase-MB (CK-MB), were quantified using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Elabscience Biotechnology Inc., Houston, TX, USA) according to standardized immunoassay procedures previously applied in venom cardiotoxicity studies.[10,11] All analyses were performed in accordance with manufacturer instructions, and internal quality control samples were included in each analytical run to ensure assay accuracy and reliability.

Gross Necropsy and Cardiac Histopathology

Following the final observation period, animals were perfused with cold phosphate-buffered saline (PBS), and the hearts were carefully excised, examined grossly, weighed, and fixed in 10% neutral-buffered formalin. Histopathological processing and hematoxylin and eosin (H&E) staining were performed according to standard procedures previously described in experimental venom cardiotoxicity studies.[10] Briefly, fixed cardiac tissues were dehydrated through graded alcohol concentrations, embedded in paraffin wax, sectioned at 5 μm thickness using a rotary microtome, and stained with hematoxylin and eosin for microscopic evaluation. Tissue sections were examined by a blinded veterinary pathologist

Protein Electrophoresis

Venom protein composition was analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions as previously described in venom proteomic studies.[14,15] Briefly, lyophilized *Naja ashei* venom was reconstituted in phosphate-

buffered saline (PBS, pH 7.4), and protein concentration was determined using a bicinchoninic acid (BCA) assay prior to electrophoresis. A total of 20 μg of venom protein mixed with Laemmli sample buffer containing 5% β-mercaptoethanol was loaded into each gel well. Samples were heated at 95 °C for 5 minutes to ensure complete protein denaturation before loading.

Electrophoresis was performed using 12% polyacrylamide resolving gels with a 4% stacking gel at a constant voltage of 120 V for approximately 90 minutes alongside a pre-stained molecular weight protein marker (Thermo Fisher Scientific, USA). Following separation, gels were stained with Coomassie Brilliant Blue R-250 for 1 hour and subsequently destained using a methanol-acetic acid solution until clear protein band visualization was achieved. Gel images were captured using a Gel Doc imaging system (Bio-Rad Laboratories, Hercules, CA, USA), and densitometric analysis was performed using ImageJ software (National Institutes of Health, USA) to estimate the relative abundance of major toxin protein families based on molecular weight distribution patterns.[14,15]

Data Handling and Statistical Analysis

Data were analyzed using GraphPad Prism version 9.0 (GraphPad Software Inc., San Diego, CA, USA) and Statistical Package for the Social Sciences (SPSS) version 26.0 (IBM Corp., Armonk, NY, USA). Continuous variables were expressed as mean ± standard deviation (SD). Data was presented using tables, graphs, and descriptive summaries to illustrate variations in venom toxicity, electrolyte disturbances, and cardiac biomarker levels across the different administration routes. Group comparisons were performed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons. Statistical significance was set at $p < 0.05$.

Biosafety and Waste Disposal

All experimental waste was handled according to Jomo Kenyatta University of Agriculture and Technology biosafety policies and National Environment Management Authority (NEMA) regulations. Personal protective equipment was used throughout, and hazardous waste was disposed of through approved institutional channels.

Ethical approval

Ethical approval of this study was granted by Jomo Kenyatta University of Agriculture and Technology (JKUAT) Institutional Scientific and Ethics Review committee (Approval number: *JKU/ISERC/0216/1712*) and data collection permit sought from National Commission for Science, Technology and Innovation - NACOSTI- (License No: *NACOSTI/P/25/4174280*). The study data privacy and confidentiality were adhered to.

RESULTS

Table 1: Physical attributes of crude Naja ashei venom measured in this study and contextualized against published data for African Naja species

Parameter	This study	Comparative data in *Naja* spp.
Appearance and colour	Clear, pale-yellow, viscous fluid	Similar description reported for other African spitting cobras
pH (25 °C)	6.3 ± 0.1 (fresh); 6.8 ± 0.1 (after lyophilization)	Mean 5.77 (range: 5.49–6.02) across 10 *Naja* venoms (Avella et al., 2021).
Total protein (mg mL^{-1})	63.4 ± 2.7 (n = 4; Bradford assay)	51–159 mg mL^{-1} (mean ≈133 mg mL^{-1}) in African *Naja* spp. (Avella et al., 2021).
Dynamic viscosity (mPa·s, 100 s^{-1}, 25 °C)	46 ± 5	44–151 mPa·s for *Naja pallida* venom, representative of African spitting cobras (Avella et al., 2021).
Density (specific gravity)	1.03 ± 0.01 g mL^{-1}	1.084 g mL^{-1} reported for *Naja pallida* venom (Avella et al., 2021).

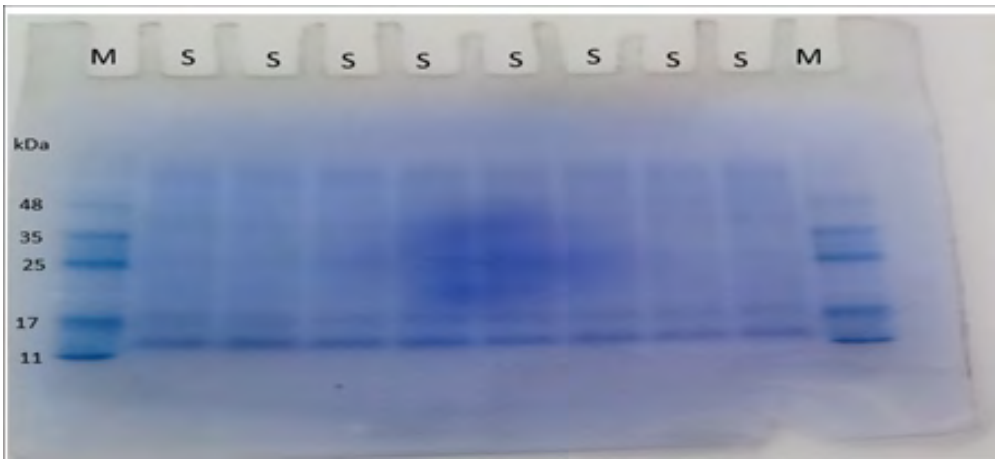

Figure 1: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) pattern of Naja ashei venom proteins

Table 2 : demonstrates that *Naja ashei* venom is predominantly composed of three-finger toxins and phospholipase A_2 enzymes

Protein family	Typical MW range (kDa)	Relative abundance (% of total protein)	Principal biological activity
Three-finger toxins (3FTx)	6–9 and 12–24	69.0	Cytolytic activity and postsynaptic neurotoxicity
Phospholipase A_2 (PLA_2)	13–15	27.0	Phospholipid hydrolysis; myotoxic and cytotoxic synergy
PIII snake venom metalloproteinases (SVMP)	60–90	2.1	Extracellular matrix degradation and local haemorrhage
Venom nerve growth factor (VNGF)	≈13	1.0	Neurotrophic signalling and metalloproteinase inhibition
Cysteine-rich secretory proteins (CRISP)	20–25	0.7	Ion-channel modulation
Cobra venom factor (CVF)	150–200	0.12	Complement C3 depletion
5'-Nucleotidase	55–60	0.014	Adenosine generation and hypotension

Table 3: Mortality profile and LD_{50} estimation of Naja ashei venom following intraperitoneal route administration in BALB/c mice.

Dose (mg kg^{-1})	Dead	Alive	Mortality (%)	Cumulative % Dead (↓)	Cumulative % Survival (↑)
0.50	0	6	0.0	50.0	100.0
0.70	1	5	16.7	62.5	91.7
0.90	3	3	50.0	77.8	77.8
1.10	5	1	83.3	91.7	62.5
1.30	6	0	100.0	100.0	50.0

Table 4: Mortality profile and LD_{50} estimation of Naja ashei venom following intramuscular administration in BALB/c mice.

Dose (mg kg^{-1})	Dead	Alive	Mortality (%)	Cumulative % Dead (↓)	Cumulative % Survival (↑)
0.50	0	6	0.0	53.3	100.0
1.00	1	5	16.7	66.7	91.7
1.50	4	2	66.7	83.3	72.2
2.00	5	1	83.3	91.7	58.3
2.50	6	0	100.0	100.0	46.7

Table 5: Mortality profile and LD_{50} estimation of Naja ashei venom following subcutaneous administration in BALB/c mice.

Dose (mg kg^{-1})	Dead	Alive	Mortality (%)	Cumulative % Dead (↓)	Cumulative % Survival (↑)
1.00	0	6	0.0	56.7	100.0
1.50	2	4	33.3	70.8	83.3
2.00	4	2	66.7	83.3	66.7
2.50	5	1	83.3	91.7	54.2
3.00	6	0	100.0	100.0	43.3

Table 6: Comparative LD_{50} values of Naja ashei venom across different administration routes in BALB/c mice.

Route	*Ld_{50} (mg kg^{-1})*	*Relative to Intraperitoneal (I.P.)*
I.P.	*0.70*	*1×*
I.M.	*2.36*	*3.4× higher*
S.C.	*2.69*	*3.8× higher*

Table 7: Serum electrolytes and cardiac biomarkers (n = 20 BALB/c mice, 0.5×LD_{50})

Analyte (mouse reference range)	Mean ± SD	Range	Abnormal results (%)
Na^+ (135–155 mmol L^{-1})	144 ± 9.4	133–167	20% >155
K^+ (3.5–6.0 mmol L^{-1})	7.77 ± 2.2	3.9–10.0	65% >6
Troponin-I (<0.05 ng mL^{-1})	11.7 ± 5.6	2.0–23.0	100% elevated
CK-MB (100–400 U L^{-1})	917 ± 122.2	568–>1000	100% elevated

Table 8: Integrated toxicological, biochemical, and histopathological findings following sub-lethal Naja ashei envenomation in BALB/c mice

Variable	Intraperitoneal LD_{50} Benchmark	Sub-lethal Exposure
Cytotoxin/PLA_2 load	0.70 mg kg^{-1} kills 50% of mice (rapid uptake)	0.25–0.35 mg kg^{-1} used in this study
Serum K^+	–	7.8 ± 2.3 mmol L^{-1}
Troponin-I	–	11.7 ± 6.1 ng mL^{-1}
Heart (cardiac tissue)	–	Myocardial necrosis and interstitial oedema

DISCUSSION

This study elucidates the toxicological profile of *Naja ashei* venom by integrating biochemical characterization, proteomic profiling, route-dependent lethality, and systemic cardiotoxic effects in a mouse model. The evidence positions *N. ashei* within the wider context of elapid toxins and provides comparative insights with other

cobra species.[14,15,16,17] The physical properties observed for *N. ashei* venom clear, pale-yellow fluid with mildly acidic pH and moderate viscosity agree with proteomic reports for African spitting cobras and other *Naja* species in which low-molecular-weight toxins predominate.[14] The measured venom pH in this study is similar to previously reported profiles, and slight increases after lyophilization are consistent with loss of labile acidic components rather than substantive chemical changes. Protein concentration (~63 mg/mL) is modest relative to some *Naja* venoms but falls within reported ranges for African spitting cobras, where proteomic studies show dominance of three-finger toxins and phospholipase A_2 enzymes [14,15]. Similar rheological measures, including viscosity and density, have been observed in *Naja pallida* venom, suggesting shared physical properties among African spitting cobras that may facilitate rapid tissue invasion and toxin dissemination.[15]

Proteomic profiling revealed that low-molecular-weight three-finger toxins account for the vast majority of venom proteins, consistent with *N. ashei* and related species.[14,15] This pattern aligns with other African spitting cobra venoms in which three-finger toxins typically constitute 65–75% of the proteome, with phospholipase A_2 as the second most abundant toxin family[16]. The predominance of three-finger toxins explains the potent cytotoxicity and membrane-disrupting effects observed experimentally and clinically, as these toxins form pores in cellular membranes leading to lysis and necrosis.[14] Phospholipase A_2 enzymes, though less abundant, act synergistically by destabilizing phospholipid bilayers and facilitating deeper toxin penetration.[15] The comparatively low abundance of snake venom metalloproteinases aligns with clinical observations that *N. ashei* envenomation typically results in marked cytotoxicity with limited hemorrhagic effects compared with viperid envenomation.[16,23]

A key finding is the significant variation in LD_{50} values across administration routes: intraperitoneal (0.70 mg/kg), intramuscular (2.36 mg/kg), and subcutaneous (2.69 mg/kg). This sequence (I.P. < I.M. < S.C.) reflects differences in venom absorption kinetics, where more vascularized compartments facilitate faster systemic access and greater toxicity at lower doses. Similar route-dependent differences have been reported in other snake toxicity studies, including Chinese elapids, where intraperitoneal and intravenous routes consistently yielded lower LD_{50} values than subcutaneous routes[18]. These findings reinforce that LD_{50} is not an intrinsic fixed property of venom but is influenced by administration context and tissue vascularity.[18,19]

Clinically, bites delivering venom into muscle or highly vascular tissues may lead to faster systemic envenoming and more severe early effects compared with superficial dermal bites, corroborating reports that route and depth of envenomation contribute significantly to clinical severity and should be considered in venom pharmacokinetic modelling.[18,19]

Sublethal envenomation resulted in prominent hyperkaliemia in the majority of mice, whereas serum sodium remained largely unaffected. This selective potassium elevation suggests direct membrane disruption and ion efflux rather than dehydration or renal dysregulation. Cobra cytotoxins form non-selective pores in cell membranes, leading to efflux of intracellular cations, including potassium, a mechanism likely underlying the observed hyperkaliemia.[14] Comparable electrolyte imbalances have been described in other toxin-mediated injuries where pore-forming agents induce potassium shifts that

increase arrhythmogenic risk.[12] Such findings are clinically important, as hyperkaliemia exceeding 6.0 mmol/L is associated with cardiac conduction disturbances and potential sudden cardiovascular collapse.[12,13]

This study provides clear evidence of venom-induced cardiotoxicity. Elevated cardiac biomarkers troponin I and creatine kinase-MB were accompanied by histopathological lesions, including myofiber necrosis and interstitial oedema. These findings align with broader venom research showing that elapid venom components, particularly cardiotoxins and phospholipase A_2, directly impair cardiomyocyte integrity and function.[12,23] Although cardiotoxicity has been under-recognized in snakebite pathophysiology, clinical reports document electrocardiographic abnormalities following envenomation, including conduction disturbances and arrhythmias, indicating significant cardiovascular involvement in severe cases.[20,22,24] The combination of direct membrane injury and electrolyte imbalance creates a pro-arrhythmic environment that likely contributes to the profound myocardial injury observed in this model.

While this study did not include coagulation assays, existing evidence indicates that PLA_2 toxins in cobra venoms can exert anticoagulant effects by interfering with factor Xa and prothrombinase activity, contributing to systemic pathophysiology when venoms reach the circulation. The combined effects of cytotoxicity, cardiotoxicity, and anticoagulation provide a coherent mechanistic framework explaining the patterns of tissue damage and systemic failure observed in severe envenomation's.

CONCLUSION

This study demonstrates that *Naja ashei* venom possesses a highly potent and complex toxicological profile capable of causing severe local and systemic effects. Proteomic analysis confirmed that the venom is predominantly composed of three-finger toxins and phospholipase A_2 enzymes, accounting for its marked cytotoxicity and systemic toxicity. Lethality was shown to be strongly dependent on the route of venom administration, with intraperitoneal exposure producing the lowest LD50, followed by intramuscular and subcutaneous routes, indicating that deeper tissue or internal exposure results in significantly higher toxicity. Envenomation also induced pronounced electrolyte disturbances, particularly hyperkalemia and mild hyponatremia, consistent with extensive muscle damage and cellular disruption. In addition, clear cardiotoxic effects were observed, evidenced by myocardial necrosis, edema, vascular congestion, and elevated cardiac biomarkers, confirming direct venom-induced cardiac injury. Collectively, these findings highlight the potential for rapid cardiovascular compromise following *N. ashei* envenomation and emphasize the need for prompt, aggressive clinical management, even in cases initially presenting with predominantly local tissue involvement.

Limitations of the study

This study was conducted in a murine model, and while BALB/c mice provide a standardized and reproducible system for venom toxicology, extrapolation of the findings to human envenomation should be made with caution due to interspecies physiological differences. Venom effects were assessed over a limited observation period, which may not fully capture delayed or chronic cardiotoxic and systemic outcomes. Electrocardiographic monitoring and coagulation parameters were not evaluated,

limiting comprehensive cardiovascular and hemostatic assessment. In addition, the study did not assess antivenom neutralization efficacy against *Naja ashei* venom, which would have strengthened translational relevance to clinical management.

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REFERENCES

1. World Health Organization. Snakebite envenoming: A strategy for prevention and control. Geneva: World Health Organization; 2019.
2. Benjamin JM, Chippaux JP, Sampson HA, Warrell DA. Venom composition and clinical effects of African cobra envenomation. Clin Toxicol (Phila). 2020;58(7):641-650.
3. Chippaux JP. Snakebite envenomation turns again into a neglected tropical disease. J Venom Anim Toxins Incl Trop Dis. 2017;23:38.
4. Dyba J, Offor BC, Piater LA, Muller B. Proteomic characteristics of African spitting cobra venom profiles. Sci Rep. 2024;14:69459.
5. Dyba J, Offor BC, Piater LA, Muller B. Three-finger toxin pore-forming dynamics and cytolytic mechanisms in cobra venom. Sci Rep. 2024;14. In press.
6. Dyba J. Venom absorption and systemic toxicokinetics in elapid envenomation. Sci Rep. 2024;14. In press.
7. Farooq U, Rashid MH, Rashid MI, Akram M, Ahmad N. Snakebite envenoming in sub-Saharan Africa: Burden, challenges, and management strategies. Trop Med Infect Dis. 2022;7(10):278.
8. Gutiérrez JM, Calvete JJ, Habib AG, Harrison RA, Williams DJ, et al. Snakebite envenoming. Nat Rev Dis Primers. 2022;8(1):1-23.
9. Halilu S, Iliyasu G, Hamza M, Chippaux JP, Habib AG. Snakebite burden in sub-Saharan Africa: Estimates from available data. Toxicon. 2019;159:109-115.
10. Harrison RA, Hargreaves A, Wagstaff SC, Faragher B, Lalloo DG. Snake envenoming: A disease of poverty. PLoS Negl Trop Dis. 2009;3(12):e569.
11. Kalita B, Patra A, Mukherjee AK. Cardiotoxic and biochemical alterations induced by cobra venoms: Experimental evidence from murine models. Toxicol Rep. 2022;9:1120-1129.
12. Khourcha S, El Hattimy F, Chgoury F. Cardiovascular complications of snakebite envenomation: Mechanisms and clinical implications. Front Cardiovasc Med. 2023;10:1154321.
13. Longbottom J, Shearer FM, Devine M, Alcoba G, Chappuis F, et al. Vulnerability to snakebite envenoming: A global mapping of hotspots. Lancet. 2018;392(10148):673-684.
14. Manson P, Wüster W, Broadley DG, Malhotra A. Phylogeography and venom variation in African spitting cobras. Toxins (Basel). 2022;14(2):118.
15. Ochola FO, Okumu MO, Muchemi GM, Mbaria JM. Epidemiology of snakebite envenomation in Kenya: Current status and future perspectives. Toxicon X. 2021;

11:100074.
16. Offor BC, Piater LA, Muller B. The proteome of African spitting and non-spitting cobra venoms and cytotoxicity against pancreatic cancer cells. J Appl Toxicol. 2025;45(10):2055-2067.
17. Okumu MO, Nyongesa AW, Wamola BM. Toxicity profiling and antivenom neutralization of Naja ashei venom. Toxicon. 2020;181:84-92.
18. Otieno MO, Nyongesa AW, Wamola BM. Toxicity and proteome analysis of Naja ashei venom: Evidence of major three-finger toxins and phospholipase A2 influence. Toxicon X. 2022;11:100077.
19. Patra A, Mukherjee AK, Chatterjee U. Determination of LD50 and ED50 of snake venoms and antivenoms: Statistical approaches and applications. J Venom Anim Toxins Incl Trop Dis. 2021; 27:e20200155.
20. Potet J, Smith J, McIver L. Reviewing evidence of the clinical effectiveness of antivenoms in snakebite management. Toxicon X. 2021;11:100077.
21. Pouokam I, Akoachere MB. Cardiac sequelae and troponin elevations in snakebite patients. Pan Afr Med J. 2020;36. In press.
22. Pouokam I, Akoachere MB. Electrocardiographic and biochemical effects of snake envenomation: Clinical enlargements. Pan Afr Med J. 2020;36. In press.
23. Saaiman Engelbrecht A, Piater LA, Offor BC. Comparative proteomics of African spitting cobra venoms highlights cytotoxic and enzymatic toxin families. J Appl Toxicol. 2024;44. In press.
24. Theakston RDG, Warrell DA, Griffiths E. Report of a WHO workshop on the standardization and control of antivenoms. Toxicon. 2003;41(5):541-557.
25. Zhao LX, et al. Administration route influences venom lethality: Comparative toxicokinetics in murine models of snakebite. Toxins (Basel). 2025;17(3). In press.

Wafula E, Gikunju J, Kamanja I, Shiundu BM. Determination of LD50 of Naja Ashei Venom and its Effects on the Heart and Serum Electrolytes in Balb C Mice Through I.P, S.C, and I.M Routes. Afr. J. Trop. Med. & Biomed. Res. 2026; 9(1) 101-113
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