Evaluation of lethality and cytotoxic effects induced by *Naja ashei* (large brown spitting cobra) venom and the envenomation-neutralizing efficacy of selected commercial antivenoms in Kenya

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**A R T I C L E  I N F O**

Handling Editor: Ray Norton

**A B S T R A C T**

Neutralization of lethality in mice model at the preclinical level has been established by the World Health Organization as the gold standard for the evaluation of antivenom efficacy. The assessment of the neutralization profiles of antivenoms helps to discern the efficacy or otherwise of these antivenoms at neutralizing the toxic effects induced by medically significant snake venoms. However, for many antivenoms, information on their preclinical efficacy remains limited. Therefore, to strengthen global efforts at reducing the impact of snakebite envenoming, the provision of information on the preclinical efficacy of antivenoms, especially in parts of the world where antivenom availability and accessibility is problematic, including sub-Saharan Africa is crucial. This study presents the lethal and toxic activities of *N. ashei* venom and the neutralizing capacity of two commonly used commercial antivenoms in Kenya; VINS™ and Inoserp™. Median lethal dose (LD\(_{50}\)), minimum necrotizing dose (MND) and minimum edema-forming dose (MED) of *N. ashei* venom as well as the neutralization of these effects were evaluated in mice. The LD\(_{50}\) of *N. ashei* venom was found to be 4.67 (3.34–6.54) mg/kg while MND and MED were 11.00 \(\mu\)g and 0.80 \(\mu\)g respectively. Both VINS™ and Inoserp™ antivenoms demonstrated capacity to neutralize the lethal and toxic effects induced by *Naja ashei* venom albeit at varying efficacies. Our results thus confirm the toxic effects of *N. ashei* venom as previously observed with other *Naja* sp. venoms and also underscore the relevance of para-specific neutralizing capacity of antivenoms in the design of antivenoms.

**Credit author statement**

Ernest Z. Manson: Conceptualization; Methodology; Software; Data curation; Validation; Formal analysis; Investigation; Writing-original draft preparation; Project administration; Fund acquisition. Mutinda C. Kyama: Conceptualization; Validation; Investigation; Writing-review and editing; Supervision. Joseph K. Gikunju: Conceptualization; Validation; Investigation; Writing-review and editing; Supervision. Josephine Kimani: Validation; Investigation; Writing-review and editing. James H. Kimotho: Conceptualization; Validation; Investigation; Writing-review and editing; Supervision; Project administration. All authors have read and agreed to the published version of the manuscript.

1. Introduction

Snakebite envenoming is a neglected disease that is associated with severe outcomes, notably suffering, disability and death. Nearly 2.7 million snakebite envenoming cases are recorded every year. Of these, the consequent deaths are estimated at 81,000–138,000 (World Health Organization, 2019). Given its relevance to public health, snakebite envenoming was designated a category ‘A’ disease in its neglected tropical disease portfolio in 2017 by the World Health Organization (WHO). Thus, in its global strategy for the prevention and control of...
snakebite envenoming, the WHO aims to halve the morbidities, disabilities and deaths resulting from snakebite envenoming by 2030 (Gutiérrez et al., 2021). It is estimated that about 1,000,000 snakebites occur in sub-Saharan Africa alone each year, causing nearly 32,000 deaths (Petras et al., 2011). Estimates of snakebite burden in Kenya indicate that 12,762–18,052 cases of envenoming occur annually, resulting in 502–1444 amputations (Halliu et al., 2019) and close to 1000 deaths (Reid and Gilmour, 2017).

Naja ashei, commonly referred to as the large brown spitting cobra, belongs to the African spitting cobra species and can be found in the open dry areas of sub-Saharan Africa (Hus et al., 2018). The African spitting cobra species are known to cause severe localized tissue damage (cytotoxicity), with very minimal, if any neurological effects. Conversely, envenoming from non-spitting cobras tend to be associated with more severe neurotoxic symptoms and minor tissue damage (Cassasola et al., 2009) (Warrell et al., 1995).

The administration of antivenoms in a timely manner is considered the mainstay of treatment for snakebite envenoming (Gutiérrez et al., 2021). When administered promptly, antivenoms could potentially reverse the symptoms of systemic envenoming, and hence minimize the incidence of permanent damage to the tissue and prevent mortality (Petras et al., 2011). An assessment of the neutralizing capacity of snake venoms-induced lethality by antivenoms is considered the gold standard in preclinical evaluation of antivenom efficacy and forms part of routine quality control checks by manufacturers and laboratories. However, the complex nature of many snake venoms in terms of composition and toxicological profile, require that the neutralization of venom-induced lethality is complemented with an assessment of the neutralization of other relevant toxic effects including dermonecrosis depending on the venom (Gutiérrez et al., 2021), (Gutiérrez et al., 2013), (WHO, 2017). Mice are the most used experimental animal model for the preclinical assessment albeit other animal models may be used (Theakston et al., 2003).

The determination of median lethal doses (LD50) and effective doses (ED50) is important in basic toxicological work in studying the mechanism of action of toxins and in a more applied field in designing, characterizing and producing antivenoms. Probit and Spearman-Karber methods are currently recommended for determining the LD50 of venoms as well as ED50 of antivenoms (World Health Organization, 1981).

Both VINS™ and Inoserp™ antivenoms are polyvalent antivenoms containing antibodies raised against venoms of snake species belonging to the families Viperidae and Elapidae. Notable among the immunizing venoms of these antivenoms are N. melanoloeuca, N. nigricollis and N. haje. However, Inoserp™ antivenom consist of venoms of a wider variety of Naja snakes including N. pallida, N. rubrae and N. senegalensis, which are not covered in the specific VINS™ antivenom under review. Nevertheless, according to Okumu and colleagues (Okumu et al., 2020), the venom of N. ashei does not form part of the immunizing mixtures of commercially available antivenoms as of yet. Thus, whereas the neutralizing efficacy of Mexican and Indian-made antivenoms against N. ashei venom phospholipase A2 activity and venom-induced lethality in the brine shrimp model has been demonstrated (Okumu et al., 2020), the efficacy of these antivenoms to neutralize N. ashei-induced lethal and cytotoxic effects in mice model is not known. The subject of paraspacific or cross neutralization of several cobra venoms by antivenoms or the lack of it was earlier reported over half a century ago. Subsequently, the paraspacific neutralizing efficacy of commercial antivenoms of venoms of African and Asian cobra species has been addressed in several studies (Hassan and El Hawary, 1976; Khow et al., 1997; Seddik et al., 2002). Here, we have assessed the efficacy of Indian-made VINS™ and Mexican-made Inoserp™ polyvalent antivenoms to abrogate lethality and toxic effects induced by N. ashei venom.

2. Materials and methods

2.1. Snake venom and antivenom

N. ashei venom was procured in its lyophilized form from snakes maintained at the Bio-Ken snake farm in Watamu, Kenya, following which it was reconstituted in distilled water and stored at −20 °C until use. VINS™ (African HIS, B. NO: 07A518005; Expiry date: 04/2022) and Inoserp™ (Inosan, B. NO: 01T101002; Expiry date: 01/2023) polyvalent antivenoms were procured commercially and reconstituted as per the manufacturers’ instructions. Protein concentration of the venom and antivenoms was determined using the BCA kit as described elsewhere (Manson et al., 2022) (VINS™: 98.913 mg/mL; Inoserp™: 28.213 mg/mL).

2.2. Animals

Six-week-old (18–20 g) male and female Swiss mice were used for the study. The animals (n = 315) were maintained at the Kenya Medical Research Centre (KEMRI) and provided with food and water ad libitum.

2.3. Ethical and institutional approvals

The study was approved by the KEMRI Scientific and Ethical Review Unit (SERU) with protocol number KEMRI/SERU/CBRD/229/4340.

2.4. Toxic activities of N. ashei venom

2.4.1. Lethality

Both the LD50 dose-range finding test and median lethal dose were carried out as recommended by the WHO (WHO, 2017). In the former, one mouse per group was used for the five venom doses established (2.0, 3.0, 4.5, 6.8 and 10.0 mg/kg). Venom dose for each mouse was determined according the method described by Ochola and colleagues (Ochola et al., 2019). Briefly, the established doses were constituted in PBS and aliquots of 200 μL were injected intraperitoneally in each mouse. Deaths and survivals within a 24-h period were recorded. In the main LD50 assay, five mice per group were used for the established doses. The resulting deaths and survivals were recorded after 24 h and the LD50 determined by probit analysis (World Health Organization, 1981) using Stats-Direct Statistical Software (version 3.2.109).

2.4.2. Necrotic activity

Necrotic activity was evaluated in line with the method described by Segura et al. (2010). Briefly, various doses of venom constituted in 100 μL of PBS were injected intradermally in the dorsally shaved skin of five mice per group (18–20 g) (n = 40). Negative control mice received equivalent volume of PBS. All mice were observed for 3 days following which they were sacrificed. For each animal, the skin was removed and the diameter of the resulting necrotic lesion measured using a digital caliper. To determine the minimum necrotizing dose, the mean lesion diameter was plotted against the venom dose and the dose corresponding to a 5 mm diameter estimated using linear regression analysis.

2.4.3. Edematogenic activity

The edematogenic activity of N. ashei venom was evaluated using the method described by Resiere et al. (2018). Five mice per group (18–20 g) (n = 40) were injected subcutaneously in the left footpad with various doses of venom dissolved in 20 μL of PBS. Equivalent volume of PBS was injected into the right footpads. Equal volume of PBS was administered in the left footpad of control mice. One hour post challenge, mice were humanely sacrificed and the thickness or increase in footpad volume immediately measured using a low-pressure spring caliper. The left footpads of the control mice were also measured. The minimum edema-forming dose was determined as the minimum dose of venom that induced a 30% increment in footpad volume or thickness after 1 h of
venom inoculation relative to PBS-only footpad of the control mice.

2.4.4. Neutralization of lethal and toxic effects

The efficacy of the two antivenoms to neutralize the lethal and toxic effects of *N. ashei* venom was conducted in accordance WHO recommendations (WHO, 2017) and in line with previously described experimental approaches (Segura et al., 2010), (Resiere et al., 2018). These procedures involved incubating a fixed amount of venom (challenge dose) with a variety of antivenom dilutions at 37 °C for 30 min. Subsequently, aliquots of the resulting venom-antivenom mixtures were administered and the respective effects were evaluated as previously described. Equivalent volumes of venom and PBS were administered for control mice. The challenge doses for lethality, necrotic and edematogenic activities used were 3 LD$_{50}$, 1 MND and 6 MED respectively. For each effect, the different doses were established based on the ED$_{50}$ dose range finding tests of the two antivenoms. The neutralizing efficacy of the two antivenoms was thus expressed as median effective dose (ED$_{50}$) and represented as the ratio of antivenom (µL) to venom (mg) at which a particular venom effect is reduced by 50% relative to the venom-PBS control.

2.5. Data analysis

GraphPad version 8.4.3 software and Microsoft Excel 2016 were used to analyze data. Differences in group means were determined using ANOVA preceded by Tukey test or unpaired t-test. For statistical significance, a p-value < 0.05 cut-off was used.

3. Results

3.1. Lethal and toxic activities of *N. ashei* venom

*N. ashei* venom was observed to produce lethal effects in mice. Our study found an LD$_{50}$ of 4.67 (3.34-6.54) mg/kg in mice for *N. ashei* venom. Necrotizing effect of *N. ashei* venom was observed at the various doses tested. Following 72 h of exposure to the different doses, 10.60 µg was estimated to be the dose capable of inducing a 5 mm necrotic lesion, hence the minimum necrotizing dose. All mice immunized with *N. ashei* venom also showed signs of increased footpad volume or thickness. At a dose of 0.80 mg, the venom induced edema formation within 1 h, which is considered as 100% activity and thus the minimum edema-forming dose (Table 1). The results confirm previous reports of toxicity among other *Naja* sp. venoms and also the cytotoxic effects mainly induced by other African spitting cobras.

3.2. Neutralization of lethal and toxic activities

As can be observed in Table 2, both antivenoms demonstrated neutralization of lethal and toxic effects in mice. In the neutralization of necrotic activity, a characteristic dose-dependent reduction in necrotic activity was observed with *Inoserp*™ antivenom at a median dose of 54.26 (36.13–68.32) µL of VINS antivenom/mg venom; and 95% CI (114.8–217.9), the same effect was achieved by *Inoserp*™ antivenom at a median dose of 54.26 (36.13–68.32) µL of VINS antivenom/mg venom; and 95% CI (114.8–217.9). Our results demonstrate that both antivenoms are capable of neutralizing *N. ashei* venom-induced lethality at different dose levels despite the lack of inclusion of the venom in the immunizing mixtures of these antivenoms. However, *Inoserp*™ antivenom required slightly more the dose of VINS antivenom to achieve the same effect. This finding is surprising in the light of the fact that *Inoserp*™ antivenom has as part of its immunizing mixtures a wider variety of *Naja* venoms including *N. pallida*, *N. nubiae* and *N. senegalensis*, which are not covered in the VINS™ antivenom. When considered in relation to the venom proteomes of African spitting cobras, an analysis by Petras and colleagues showed that the venom proteomes of five *Naja* sp. including *N. nigricollis*, *N. pallida*, *N. katiensis*, *N. mossambica* and *N. nubiae* consist largely of three-finger toxins (3FTxs) and phospholipase A2 (PLA$_2$), with the former believed to account for 67–80% (Petras et al., 2011). *N. ashei*, on the other hand, has 3FTx proteins accounting for 60–80% of its total toxin composition (Hus et al., 2018), (Hus et al., 2020). Three-finger toxins and phospholipase A$_2$, although highly toxic are known to possess limited immunogenicity (Lauridsen et al., 2017), (Bermúdez-Medina & Andrade, 2018). Thus, given the largely similar toxin composition of the *Naja* sp., and the known poor immunogenicity of 3FTxs and PLA$_2$ proteins, the inclusion of more *Naja* species in an immunizing mixture may not necessarily guarantee higher antibody titers.

Also, both antivenoms showed capacity to neutralize edema-forming activity of *N. ashei* venom even though full neutralization of same was hardly attained (Table 2). Whereas 62 ± 14 µL of VINS™ antivenom was able to neutralize edema, a similar neutralization effect was achieved by *Inoserp* antivenom at 90 ± 8 µL. In comparing the % neutralization of edema by the two antivenoms at the volumes tested, a two-tailed unpaired t-test analysis showed that the % neutralization or reduction in edema was not significantly different (p-value = 0.1003).

Table 2

| Antivenom | Toxic activity ΛµL antivenom/ 3 LD$_{50}$ | Toxic activity ΛµL antivenom/ mg venom | Necrotic activity ΛµL antivenom/ 1 MND | Edematogenic activity ΛµL antivenom/6 MED |
|-----------|----------------------------------------|---------------------------------------|----------------------------------------|------------------------------------------|
| VINS™     | 40.06 (29.04–59.61)                     | 151.0 (114.8–217.9)                   | 78 ± 15                                | 62 ± 14                                  |
| Inoserp™  | 54.26 (36.13–68.32)                     | 199.2 (151.6–244.2)                   | 177 ± 21                               | 90 ± 8                                   |

Neutralization is expressed as median effective dose (ED$_{50}$) and refers to the antivenom-venom ratio (µL antivenom/challenge dose of venom) at which a specific venom-induced activity is reduced by 50%. Results are presented as 95% CI (in parenthesis) for lethality and mean ± SD (n = 3) for necrotic and edema-forming activities.
4. Discussion

Previous studies on murine LD$_{50}$ have shown that venoms of known neurotoxic non-spitting cobra species (*N. haje*, *N. melanoleuca*, *N. nivea*) are generally more potent while cytotoxic or spitting species (including *N. nigricollis*, *N. mossambica*, *N. pallida* etc.) are known to possess less toxic venoms (Petras et al., 2011), (Casasola et al., 2009). In a recent study, the LD$_{50}$ of *N. ashei* venom was reported to be 3.02 (2.45–3.72) mg/kg in mice (Otieno et al., 2021). For the same venom, our study found an LD$_{50}$ of 4.67 (3.34–6.54) mg/kg in mice. Although these results vary slightly, differences in toxic activities of venoms from the same species are not uncommon. For instance, Theakston & Reid found that *N. nigricollis* venom from Nigeria induced dermonecrosis at a minimum dose of 14.5 μg (Theakston and Reid, 1983). However, Petras and colleagues in another study reported that the minimum necrotizing dose of the same venom sourced from the same country was 100 μg (Petras et al., 2011). These variations can be attributed among other things to the concentration of the stock venom used for the assays. Chippaux and colleagues observed that clinical variability in envenomation occurs as a result of intraspecific variability of venoms. They noted that venom variability occurs at several levels including interspecies, intergenus, intra-species, attributable among other things to seasonal variation, habitat, diet and sexual dimorphism (Chippaux et al., 1991). The observed variations may also be explained by the inherent diversity in the constitution of snake venoms, due in part to factors including snake family, geographical location, snake size, conventional prey preference, age, genus and others (Sanhajariya et al., 2018). Again, Calvette observed that between and within species, venom variability is a common phenomenon at all taxonomic levels, further buttressing the observed variations as reported earlier (Calvette, 2019).

The venom proteome of *N. ashei* is consistent with the well-known pathophysiological profiles of the other African spitting cobras, that is, the high level of cytotoxins and their role in the widespread tissue damage that typify cobra envenomings. In this study, *N. ashei* venom was able to induce necrosis in the mouse model (Table 1). Our findings concur with those of previous studies (Petras et al., 2011), (Otieno et al., 2021), (Kandiwa et al., 2018), (S à nechez et al., 2018), where several African spitting cobras were found to induce dermonecrosis. Cytotoxins are thought to provoke cell damage and necrosis in vitro and in vivo respectively, a function which is central to localized necrotic effect (Owby et al., 1993), (Fletcher and Lizzo, 1987). Whereas previous studies have reported dermonecrotic activity induced by different *Naja* sp. venoms (Petras et al., 2011) at various doses, our finding compares favorably with that reported by Theakston and Reid in which 14.5 μg of *N. nigricollis* venom was found to be the minimum necrotizing dose (Theakston and Reid, 1983). In most cases of *Naja* envenomation, localized tissue damage provoked by cytotoxic venom components is considered the single most important contributing factor to morbidity and the consequent lasting sequelae. Despite the considerable amount of research, the molecular mechanisms underlying *Naja*-induced cytotoxicity remain inconclusive (Casasola et al., 2009).

Again, consistent with previous studies involving the Indian cobra (*Naja naja*) (Meenatchisundaram et al., 2008), (Meenatchisundaram et al., 2009), *N. ashei* venom was able to induce edema albeit at a lower dose relative to that reported as MED for the Indian cobra. This finding also underscores first of all, the position canvased by Casasola and colleagues to the effect that similar biochemical, antigenic and toxino- logical characteristics are shared by snake venoms that belong to the same genera (Casasola et al., 2009) and secondly, the ability of the venoms to cause localized tissue damage (notably edema among others) as previously reported (Warrell et al., 1995), (Warrell et al., 1976; Tilbury, 1982; WHO Regional Office for Africa, 2010).

Neutralization of lethality at the preclinical level has been established by the WHO as the gold standard for the evaluation of antivenom efficacy. Depending on the venom, additional tests may be required either when new antivenoms are being developed or when existing antivenoms are being distributed to new jurisdictions (World Health Organization, 2018). While the neutralization of viperid venoms-induced defibrinogenating, myotoxic and hemorrhagic activities are recommended, the neutralization of venom-induced dermonecrotic activity by antivenoms indicated for spitting cobra (*Naja* sp.) venoms are highly recommended (Gutiérrez et al., 2013). In this regard, both antivenoms demonstrated capacity to neutralize *N. ashei* venom-induced lethality, necrotic and edematogenic activities although with varying levels of efficacy.

Also, Okumu and colleagues observed in an earlier study that Inoserp™ antivenom was able to better cross-neutralize *N. ashei* venom proteins (notably snake venom phospholipase A$_2$) most likely due to the similarities in the toxicity profiles of *N. ashei* with those of *N. pallida*, *N. nubia* and *N. katiensis* (Okumu et al., 2020), thus suggesting that *N. ashei* may be dissimilar to *N. nigricollis* and *N. haje*. In addition to the fact that experimental set ups for the two studies were completely incomparable (with the former in brine shrimp lethality animal model), our findings suggest a contrary position, particularly given that the Indian-made VINS™ antivenom consistently abrogated the venom ef fects better than Inoserp™. The abrogation of the lethal effect of *N. ashei* venom may thus appear to further confirm the fact that cytotoxins, which are abundant in the venoms of the spitting cobra species are...
responsible for the bulk of envenomation. The observed para-specific neutralization of *N. ashei* venom by the two antivenoms is rationalized by Petras et al. (2011) who observed that the toxin arsenal was highly preserved among the African spitting cobra species despite their huge geographic spread. This, in their opinion was indication that, the potential of an antivenom raised against the venom of a single African spitting cobra species to para-specifically neutralize the venom of other spitting cobras was probable.

Also, the differences in the ED₅₀ of the two antivenoms is in agreement with observations made by Resieri and colleagues who also found that a new batch of a monospecific antivenom showed higher efficacy compared to an earlier batch, demonstrated in differences in the ED₅₀ values. Among other things, they conjectured that the differences could be due to variations in the protein concentrations of the antivenom batches (Resieri et al., 2018). As far as neutralizing venom-induced lethality is concerned, 40.06 (29.04–59.61) (ED₅₀ of 151.0 µl antivenom/mg venom; 95% CI (114.8–217.9) and 54.26 (36.13–68.32) µl (ED₅₀ of 199.2 µl antivenom/mg venom; 95% CI (151.6–244.2) were found to be the effective dose of VINS™ and Inoserp™ antivenoms respectively. The protein concentration of the batches of VINS™ and Inoserp™ antivenoms was found to be vastly different in the current study (98.913 and 28.213 mg/mL respectively). To that extent, the varied levels of neutralization of *N. ashei* venom induced lethality and cytotoxic effects could be due to the vastly different protein concentration of the two antivenom batches. The differences in efficacy between the two antivenoms may also be supported by Gutierrez and colleagues (Gutierrez et al., 2017) who observed that disparities in snakes whose venoms are used for immunization may give rise to IgG specificities in the venom-immunized animal. Consequently, the proportion of IgGs targeted at any one snake may be small, for which reason more vials may be needed to achieve clinical cure. However, the results presented only relate to these specific batches, hence, any interpretation should be guided by caution.

The antivenom field has not benefited significantly from the broad knowledge available on the composition and toxicological profiles of snake venoms. Particularly, for many antivenoms, information on their preclinical efficacy remains limited. The assessment of the neutralization profiles of antivenoms at the preclinical level will help to discern the efficacy or otherwise of these antivenoms at neutralizing the toxic effects induced by medically significant snake venoms. These endeavors, not only help to identify the strengths and limitations of existing antivenoms, but also lead to the design and redesign of appropriate mixtures of venoms for immunization, with the overall aim of improving the neutralization potential of antivenoms (Gutiérrez et al., 2013). It is anticipated that the provision of information on the preclinical efficacy of antivenoms, especially in parts of the world where antivenom availability and accessibility is problematic, including sub-Saharan Africa and parts of Asia, will strengthen global efforts at reducing the impact of snakebite envenoming (Williams et al., 2011).

5. Conclusion

Our findings highlight the lethal and toxic activities of *N. ashei* venom and also add to earlier reports of the neutralizing efficacy of VINS™ and Inoserp™ antivenoms (with different batch numbers) of *N. ashei* phospholipase A₂-induced enzymatic effects in the brine shrimp model (Okumu et al., 2020). This study reports for the first time, the full spectrum of cytotoxic effects induced by *N. ashei* venom and the efficacy of these two commercial antivenoms in neutralizing these effects in a mouse model. It is noteworthy to stress that any attempt to extrapolate the basic neutralization of mouse lethality by these antivenoms to clinical effectiveness, especially cytotoxicity resulting from *Naja* envenomation may be farfetched (Casasola et al., 2009). As far as antivenom efficacy is concerned, the variable para-specific neutralization of lethality and other toxic effects observed with VINS™ and Inoserp™ antivenoms raises the question of whether or not many species are needed to achieve almost complete or full spectrum specific/para-specific neutralization. To the extent that the initial recommended dosage of VINS™ and Inoserp™ antivenoms in human envenoming are 2 vials and ≥ 1 vial (with the possibility of a further 2 vials based on evaluation) respectively, the results reflect the findings of the study but cannot be said to be definitive as far as human envenomation is concerned. Nevertheless, the study results suggest that the para-specific neutralizing capacity of antivenoms remains relevant in the design of antivenoms.

Funding

This research was supported with funding from the Pan African University under the African Union Commission scholarship.

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.

Acknowledgments

The authors acknowledge the immense support of Lucy W. Nungari and Stephen G. Kaniaru of the Animal facility, Kenya Medical Research Institute. We also acknowledge VINS Bio-products Limited for donating a vial of antivenom for the study.

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