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Generation of chicken-based IgY polyclonal antibodies against *Dendroaspis polylepis* and preclinical evaluation of envenomation-neutralizing efficacy vis-à-vis selected commercial antivenoms

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ABSTRACT

The Black mamba, *D. polylepis*, is one of the many venomous snakes found in Kenya, and known to account for some snakebite incidents. The Kenyan Ministry of Health data reveals annual 15,000 snakebites occurrences. Also, 1 in 15 people in Kenya gets bitten by a snake, and tragically, 1 in 147 of these individuals die of snakebite yearly. Traditionally, antivenoms for treatment are produced from horse or sheep but have complicated and expensive production issues. Alternative production approaches, such as using IgY antibodies derived from chicken egg yolks, may overcome disadvantages with traditional antivenom manufacturing techniques. In this current study, *D. polylepis* specific IgY polyclonal antibodies were purified from the egg yolks of chickens immunized with *D. polylepis* venom. These antibodies were subsequently assessed for their *in-vivo* neutralizing capacity vis-à-vis commercial antivenoms, PANAF-Premium and VINS. The IgY antibodies were purified by ammonium sulfate precipitation and affinity-chromatography, with quality and specificity determined by SDS-PAGE and ELISA. The LD_{50} of *D. polylepis* was found to be 0.54 mg/kg in chicks, and 0.34 mg/kg in mice, respectively. Pool of extracted IgY yielded 2.8 mg/mL concentration. Purified IgY under non-reducing and reducing conditions on SDS-PAGE exhibited a single-protein band of about 183 kDa and two bands (67 kDa and 25 kDa), respectively. The minimum-edematogenic dose was 0.05 μg. Anti-*D. polylepis* IgY antibodies and two antivenoms demonstrated the capacity to neutralize the toxic activities of *D. polylepis* venom. This study confirms a successful IgY generation against Black mamba venom for the first time, and observed toxic effects of the venom as well as neutralizing capacity of antivenoms.

1. Introduction

On a global basis, snakebite envenoming has a significant negative impact on mortality and disability rates (Gutiérrez et al., 2017). Snakebites were listed by the World Health Organization (WHO) as a category A disease in their list of neglected tropical diseases in 2017 due to their importance to public health (Chippaux, 2017), and the World Health Assembly in 2018 adopted a resolution on the matter (Gutiérrez and Mackessy, 2021). A global strategy to prevent and control envenomings was recently introduced by the WHO, with the goal of halving (reducing by 50%) the incidence of envenoming-related amputations and deaths by the year 2030 (WHO, 2019). One of the four pillars of this strategy is "ensuring safe, effective treatment."

In Kenya, snakebites constitute a neglected emergency, with 15,000 snakebites occurring yearly (MoH-Kenya, 2019), and an estimated 400, 000–600,000 in Africa suffer snakebite envenoming annually (WHO,

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2017). Thus, 1 in 15 people gets bitten by snake, and 1 in 147 people die of snakebite each year in Kenya (Otieno and Gathema, 2021). Among the most common snakes behind this incidence is the *Dendroaspis polylepis* (Black mamba) which is classified as Category 1 snake species (i.e. it is of medical importance and constitute the greatest threat to public health) (WHO, 2017). *D. polylepis* venom primarily causes systemic neurotoxicity, and its neurotoxic venom being extremely potent, making it one of the most feared snakes in the world (Nguyen et al., 2022), (Ochola et al., 2019). It has been indicated as a medically important snake in Kenya, since it causes snakebites in many areas of the region (Ochola et al., 2019), (Ochola et al., 2018). The potential of black mamba venom in the production of antivenom is one of the extensively studied area, but still attracting ongoing investigations for more future prospects.

Antivenoms, which are IgG preparations or IgG fragments made from the plasma of horses or other animals immunized with venoms of one or more snake species (i.e. monospecific or polyspecific antivenoms), are the cornerstone of treatment for snakebite envenomations (WHO, 2017). However, lack of antivenoms access is a significant public health issue in Africa, and when available, it is expensive (Potet et al., 2019), (Larson et al., 2022). Sub-Saharan Africa has only one antivenom producer, situated in South Africa (Gutiérrez et al., 2017). A large portion of the region's antivenom requirements are met by a number of producers in Europe, Asia, and Latin America. Some antivenoms according to earlier report are made from species that are not native to East Africa or Sub-Saharan Africa, and hence their effectiveness upon usage in Africa for treatment turns to be compromised (Harrison et al., 2017).

It has been established that chicken eggs that have been immunized with appropriate antigens are a reliable source of IgY antibodies. The polyclonal antibody Immunoglobulin (Ig-Y) has been taunted as a potential antivenom production system, and has comparable functions as mammalian IgGs (Lee et al., 2021). Egg IgY antibodies have been used in the past to treat bacterial, viral, and other diseases (Abbas et al., 2019), with use in humans through an oral route towards *Pseudomonas aeruginosa* (Nilsson et al., 2007). Numerous studies on IgY based antivenoms have demonstrated its potential as a possible alternative to traditional IgG based antivenoms (Abbas et al., 2019), (Liu et al., 2017).

Whilst the mentioned previous studies have demonstrated IgY antivenoms can be developed with efficacy against a range of species, none have thus far been attempted for *D. polylepis* venom. In this study, we generated chicken-based IgY polyclonal antibodies against *D. polylepis*, and evaluated the comparable efficacy with two commercial antivenoms (Indian-made PANAF-Premium™ and VINS™ polyvalent antivenoms) to neutralize the lethal and toxic effects induced by Black mamba venom.

2. Materials and methods

2.1. Ethical approvals

The study was approved by the Jomo Kenyatta University of Agriculture and Technology (JKUAT) Institutional Scientific and Ethics Review Committee (ISERC) (approval number JKU/ISERC/02316/0976) and the Kenya Medical Research Institute (KEMRI) Animal Care and Use Committee (ACUC) (reference number KEMRI ACUC/01.09.2023).

2.2. Reagents

Tween-20, bovine serum albumin (BSA), Freund's incomplete adjuvant (FIA) and Freund's complete adjuvant (FCA) (Sigma, St Louis, Missouri, USA), Tween-20, NHS activated sepharose 4FF, rabbit anti-chicken IgG/HRP conjugated antibody, Amicon ultra-15 centrifugal filter devices and protein marker (Invitrogen, USA), 3,3',5,5'-tetramethylbenzidine (TMB), glycine, non-fat dry milk and precast gel (12% Sodium dodecyl-sulfate polyacrylamide gel electrophoresis, SDS-PAGE) (Solarbio, China), were obtained from the manufacturers (i.e. Invitrogen, sigma and solarbio). Other reagents or chemicals used were purchased from local companies and of analytical grade.

2.3. Animals

One-month-old Isa-Brown chicks (500–700 g each) and seventeen-week-old Isa-Brown hens (weighing 1.7–1.8 kg each), in good health and laying conditions (laying 5–7 eggs per week) were purchased from a local poultry farm, and used for LD_{50} determination and production of IgY against *D. ploylepis* venom, respectively. Chicks (n = 30) were grouped into 5 per cage, whereas, hens (4) were kept in individual cages with standard water and food at the Small Animal Facility for Research and Innovation (SAFARI) on JKUAT main campus. Eight-week-old male and female Balb/c mice (28–30 g each), obtained from Kenya Institute of Primate Research (IPR), were used for venom lethality and its neutralizing determination. Mice were maintained at the Kenya Medical Research Institute with food and water provided *ad libitum* provided.

2.4. Venom and antivenoms

Venom from *D. polylepis* (Black mamba) was obtained through donation from the Kenya Medical Research Institute (KEMRI), lyophilized and stored at −20 °C until use. PANAF-Premium™ (Pan Africa, B. NO: PANAF-027; Expiry date: 08/2027) and VINS™ (African IHS, B. NO: 07AS21006; Expiry date: 07/2025) polyvalent antivenoms were procured commercially and as per the manufacturers' instructions, reconstitution was done, respectively.

2.5. LD_{50} determination in chicks

The median lethal dose and LD_{50} dose range-finding test were conducted in accordance with WHO guidelines (WHO, 2017). Briefly, five venom doses were established (0.25, 0.30 0.40, 0.50 and 0.75 mg/kg), and one chick per group was used for the doses. Each chick's venom dose was calculated using the method outlined by Ochola and associates (Ochola et al., 2019). Thus, the predetermined dosages were prepared in PBS and 0.2 mL aliquots were administered intramuscularly at multiple sites of the breast region of each chick, and within 24-h period, survivals and deaths were recorded. For the full-scale LD_{50} assay, at the established doses, five chicks per group were employed. Control group received only PBS. A 24-h period was used to record the deaths and survivals, and the LD_{50} calculated by probit analysis (WHO, 1981) using IBM SPSS (statistics version 20.). The LD_{50} of *D. polylepis* venom in chicks was estimated by using the Miller method to generate the Log-Dose and probit values for the standard curve. Thus, the estimated LD_{50} of Black mamba venom in chicks was 0.54 (0.49–0.58) mg/kg.

2.6. Chicken immunization schedule

In the first immunization, 0.5 mL of saline (containing sub-lethal dose of 0.341 mg of snake venom, based on the LD_{50} of *D. polylepis* venom in chicks = 0.54 mg/kg) emulsified with an equal volume of Freund's complete adjuvant (FCA) was administered intramuscularly to each of the four hens at various sites in their breast region. Following the initial immunization, the booster doses were administered on the 14th, 35th, 56th, and 77th day using 0.5 mL saline (containing 0.54, 1.08, 2.16, and 2.16 mg of snake venom, respectively) emulsified with an equal volume of Freund's incomplete adjuvant (FIA). Hens in the control group received an intramuscular immunization with 0.5 mL saline only. Daily egg collection was done a week before and sustained after the initial immunization for 22 weeks, labeled individually, and stored at 4 °C until use. Weekly serum samples were collected after first immunization, and stored at −20 °C (Liu et al., 2017).

2.7. IgY isolation and purification

A modified approach by Liu et al. (2017) was used to extract IgY from preimmunized and hyperimmunized egg yolks. Briefly, egg shells were cleaned with 70% ethanol, cracked, and the yolk separated from egg white. After which, the yolk was 10-fold diluted with deionized water, and a magnetic stirrer was used to vigorously swirl the mixture for 30 min. Following a 2-fold dilution with 0.04 M acetate buffer (containing 0.06 M NaCl, pH 5.0), the resultant homogenate was once again homogenized for 30 min during which caprylic acid was added to attain 1% final concentration. The yolk suspension was stored overnight at room temperature. Water-soluble fraction (WSF), the clear supernatant containing the IgY, was collected and centrifuged at 10,000 rpm for 15 min at 4 °C, and then precipitated using a 45% saturated ammonium sulfate. Centrifugation at 10,000 rpm for 20 min at 4 °C was used to recover the salt pellet. The precipitated proteins were then dissolved in phosphate buffered saline (PBS, pH 7.4) and dialyzed against the same solution. An affinity chromatography was then applied to the partially purified antibody (crude extract).

Affinity purification followed the procedure of Liu et al. (2017) with minor modifications. Briefly, venom affinity column was prepared using activated sepharose 4FF coupled with whole *D. polylepis* venom (20 mg/mL) dissolved in coupling buffer, blocked unreacted groups with 0.5 M ethanolamine buffer, sepharose was washed with Tris–HCl buffer and then acetate buffer, alternating for 3-times. After loading the crude IgY extract into the affinity column, the unabsorbed proteins were removed by washing the column with PBS buffer, while bound antibodies were eluted using glycine-HCl buffer. Fractions were pooled and dialysed against PBS, and further concentrated and desalted through ultra-centrifugal filter devices, and finally stored at −20 °C. SDS-PAGE and ELISA were used to assess the preparations' purity and titer, respectively.

2.7.1. Determination of IgY protein concentration, and characterization by SDS-PAGE

The Bradford protein assay kit was used to measure the protein concentration, while following the manufacturer's instructions (ThermoFisherScientific, 2023). Analysis for the purity and molecular weight of the IgY was done using precast gels (12% SDS-PAGE) in line with the manufacturer's recommendations and previously described method (Liu et al., 2017).

2.7.2. Verification of IgY and specificity by ELISA

The activities and specificity of anti-*D. polylepis* IgY antibodies in the serum and yolk was assessed using indirect ELISA approach (Liu et al., 2017), (Duan et al., 2016), with some minor modifications. Briefly, a 5 μg of native *D. polylepis* venom in 100 μL of coating buffer (pH 9.6, 0.05 M carbonate-bicarbonate) was used to coat the microplates and left 4 °C for 16 h. Wells of plate were washed 3-times with rinse buffer (pH 7.4, PBS-0.05% Tween 20). A 200 μL of blocking buffer (rinse buffer plus 5% nonfat dry milk) was used to block the unbound sites, incubated for 1.5 h at 37 °C, and wells washed 3-times. A 200 μL of diluted serum or yolk (1:1000) in dilution buffer (PBS plus 1% nonfat dry milk) was added to the well and incubated at 37 °C for 1 h before 3-times washing. A dilution of 1:5000 for the rabbit anti-IgY peroxidase in blocking buffer was done, and 100 μL added to well, and incubated at 37 °C for 1 h. Wells were washed again, 100 μL of TMB solution added, incubated for 20 min at room temperature, and reaction terminated using 50 μL of 2 M sulphuric acid. Using ELISA plate reader, absorbance was recorded at 450 nm, and results determined by a ratio of positive sample and negative sample giving at least 2.1 (i.e. P/N > 2.1; OD of the positive sample divided by OD of the negative sample), as well as the lowest dilution at which the OD value is significantly higher than the negative control (Mean OD of naïve IgY + 2 times standard deviation) (Senji Laxme et al., 2019). Wells free of venom were used as blanks, and yolk samples from collected eggs or serum before immunization were used as negative control.

2.8. Toxic activities of D. polylepis venom in mice

2.8.1. Lethality (LD_{50})

This assay followed the procedure by Ochola and associates (Ochola et al., 2019), and the WHO guidelines (WHO, 2017) for LD_{50} determination in mice. The doses for range finding test were established at 0.2, 0.3, 0.4, 0.5, and 1.0 mg/kg with one mouse per dose. 200 μL aliquots of established doses was injected intraperitoneally (i.p) into each mouse, and both deaths and survivors recorded in 24 h. For the full scale median lethal dose (LD_{50}), 0.20, 0.3, 0.35, 0.40, and 0.45 mg/kg were established, 5 groups of mice per dose employed, 200 μL of aliquot injected via i. p route, and observed for deaths and survivors in within 24 h. Control groups only received PBS. Observational feature for neurotoxic activity of *D. polylepis* venom such as neuromuscular paralysis leading to respiratory arrest, results from actions of neurotoxins at the neuromuscular junctions, and falling eye lids were observed (Gutiérrez et al., 2017), (Warrell, 2010).

2.8.2. Edematogenic assay

The Resiere et al. (2018) approach was employed to assess the edematogenic activity of *D. polylepis* venom. Five mice per group were used, and each group received subcutaneous injections of different doses of venom diluted in 50 μL of PBS in the left footpad. The right footpads were injected with an equivalent volume of PBS. Control mice received an equal volume of PBS in their left footpad. The mice were euthanized an hour after the challenge, and a low-pressure spring caliper was used to measure the thickness or rise in footpad volume. Measurements were also taken of the control mice's left footpads. The least amount of venom that caused a 30% increase in footpad thickness or volume after 1 h of venom inoculation in comparison to the control mice's footpad that was only given PBS was determined as the minimum edema-forming dose (MED).

2.9. Neutralization studies for the lethal and toxic effects induced by D. polylepis venom in mice

The neutralizing assay for the *D. polylepis* venom's lethal and induced toxic effects to determine the efficacy of IgY antibodies and two commercial antivenoms (VINS™ and PANAF-Premium™) was carried out using the WHO guidelines along with other literatures (WHO, 2017), (Resiere et al., 2018), (Segura et al., 2010). In these tests, a set dose of venom challenge was incubated for 30 min at 37 °C with various antivenom dilutions. The ensuing venom-antivenom combinations were then administered in aliquots, and the corresponding effects were assessed as previously mentioned. A 3 LD_{50} and 6 MED challenge dosages were employed for lethality and edematogenic assays, respectively. For positive and negative control mice groups, equivalent volumes of venom and PBS were injected, respectively. The median effective dose (ED_{50}) range-finding tests of the IgY antibodies and the two antivenoms were used to determine the various doses for each effect. Doses were 15, 30, 45, 60 and 75 μL for the neutralization of lethality, and 20, 40, 60, 80 and 100 μL for edema neutralization, respectively. Consequently, the neutralizing effectiveness (ED_{50}) of the IgY antibodies and two antivenoms was calculated following probit analysis using StatsDirect 3 software.

2.10. Data analysis

Data analysis was done using Microsoft Excel 2019, OriginPro 2023b version and StatsDirect 3 software. ANOVA was used to find differences in group means, followed by either an unpaired *t*-test or a Tukey test. The cut-off for statistical significance was set at p-value <0.05.

3. Results

3.1. IgY extraction, purification, specific activity and biochemical identification

The water-soluble extraction method was used to obtain partially purified crude extract of antibody preparation from egg yolks, and then fractionated by thiophilic affinity chromatography. Through the three steps fractions (water dilution-WSF, ammonium sulfate precipitation or salting-out-SOF, and thiophilic affinity chromatography-TACF). In the different fractions (WSF, SOF and TACF), the venom-specific activities of the IgY from 328 eggs was assayed on an ELISA for comparison. The average recovery was 19.1% for venom-specific IgY. Titer of TACF was a two-fold higher than that of SOF, and approximately 16 times (15.93) higher in comparison to WSF. The results indicated a clear enrichment of venom-specific IgY through the three purification steps (Table 1).

Also, the IgY obtained from the egg yolk under reduced conditions presented two bands approximately 67 kDa and 25 kDa, representing the heavy and light chain, respectively. However, under non-reducing conditions, they exhibited one band of about 183 kDa (Fig. 1).

3.2. Monitoring the concentrations of venom-specific chicken-based IgY

The concentrations were determined using the Bradford protein assay kit (Thermo Fisher Scientific InC., USA). Least yield of extraction was found for week 1 and the highest for week 13 with 10.03 and 3,164.926 μg/mL, respectively (Fig. 2).

3.3. Antibody response: immune response against D. polylepis venom in Layer chicken and antibody titers determination

To evaluate the immune response for the immunization process, an ELISA assay was carried out to determine the IgY-antibody titers. Assayed samples included both pre-immune and booster immunization serum samples alongside PBS (saline)-only immunized samples. Following the primary immunization, varied immune (antibody) responses were elicited against *D. polylepis* venom toxins in chicken. Antibody response was detected in serum of chicken by day 7. Despite the low pre-booster response in serum after the initial booster, a sharp increase in both serum and egg yolk for antibody titer was observed. At week 3, antibodies transference which is specific to *D. polylepis* venom was observed from serum to egg yolk after the primary immunization. The highest level of antibody response was reached at week 4 in both serum and egg yolk (titers of OD_{450} = 3.381 and 3.356), and the observed secondary response was maintained afterwards for the remaining weeks by the second booster injection. At week 6, there was a sharp drop in antibody production peaks (titers of OD_{450} = 2.659 and 2.622) (Fig. 3). The PBS-only immunized chicken however showed no immune response.

3.4. Lethal and toxic activities of D. polylepis venom

Lethal activities of *D. polylepis* was observed in the venom-challenged mice. The LD_{50} for *D. polylepis* venom in mice was found to be 0.34 (0.29–0.38) mg/kg. Following 1-h injection with *D. polylepis* venom, all the mice under investigation showed signs of edema (increase footpad thickness or volume). The induced edema formation from the venom was at a dose of 0.052 μg which is thus the minimum edema-forming dose, and considered 100% activity (Table 2). The venom showed no necrotic and hemorrhagic activities in mice. However, paralysis and ptosis (falling-eye lids) were observed.

Table 1
IgY fractions titer and the recovery ratio of proteins.

Fractions	Titer of IgY by ELISA ($x10^4$)	Total proteins in yolk-328 eggs (g)	Recovery ratio of proteins (%)
WSF	8.3	79.10	100
SOF	66.1	32.38	40.94
TACF	132.2	15.09	19.10

Key; WSF: water-soluble fraction, SOF: Salting-out fraction, TACF: Thiophilic affinity chromatography.

3.5. Lethal and toxic activities neutralization assays

As shown in Table 3 below, the neutralizing ability in inducing *D. polylepis* lethality was demonstrated by both the extracted chicken-based IgY antibodies and antivenoms. VINS™ antivenom was able to neutralize the lethality at a median dose of 35.41 μL as most effective, followed by 41.36 μL for Chicken-Based IgY antobodies, and 46.6 μL for the PANAF-Premium™ *(Table 3)*. Relatively, the neutralizing capacities observed had a means close-range effect across, and similar observations made for the edema-forming neutralization assays. There were no statistical differences among their neutralizing efficacies (P = 0.320-0.859).

4. Discussion

The Black mamba (*D. polylepis*) is endemic to Sub-Saharan Africa, and it is Africa's deadliest snake, with untreated bite fatality rate of 100 percent (Quarch et al., 2017). The venom is extremely toxic, causing postsynaptic blockade of neuromuscular junctions. Despite having low concentrations of necrotic-causing proteins (Laustsen et al., 2015), the venom typically does not result in necrosis and tissue destruction (Strover, 1967), (Hilligan, 1987). It is possible for neurotoxic symptoms to appear as soon as 10 min after a Black mamba bite (Tan et al., 1991), and treatment choice is usually through the use of antivenoms. However, the production of conventional antivenom derived from the blood of immunized large animals like sheep or horses can slow down venom's toxicity and pathophysiologic effects as demonstrated in some neutralization studies (Amro et al., 2018), (Rojas et al., 2005), as well as speeding up recovery. This current research to the best of our knowledge, is the first to describe the generation of anti-*D. polylepis* chicken-based IgY polyclonal antibodies and assess the neutralizing efficacy vis-à-vis commercial antivenoms for the lethal and toxic effects induced by *D. polylepis* venom.

In accordance with other reports, the study followed the three principal steps to obtain highly purified IgY (Liu et al., 2017), (Duan et al., 2016), (Araújo et al., 2010). The total yield of 15.09 g of pure IgY, with an average recovery of 19.10% for venom-specific IgY was notably higher in our study compared to other reports; and may be due to differences in venom potency, as well as number of eggs used (Liu et al., 2017), (Duan et al., 2016). In addition, it was discovered that IgY activity in TCF was 2 and 16-times higher than that of SOF and WSF, respectively (Table 1). The antibody production response induced by the black mamba venom is similar to reports from other snake venoms (Liu et al., 2017), (Duan et al., 2016), (Araújo et al., 2010), (Amro et al., 2018), (Rojas et al., 2005), (Paul et al., 2007).

Venom from mamba species are generally potent, and neurological effects of the black mamba venom appear within 2 h of the onset of bite or disease (Aalten et al., 2021). These neurotoxic effects have been observed in several researches on mice (Ochola et al., 2019), (Ainsworth et al., 2018), (Ratanabanangkoon et al., 2020). Our revealed LD_{50} of 0.54 mg/kg in chicks which was a first-time report, and 0.34 mg/kg in mice (Table 2). The LD_{50} in mice was not consistent with some previous reports which had 0.28 (0.16–0.51) μg/g (Ratanabanangkoon et al., 2020), 6.2 μg/mouse; 0.33 μg/g (Ainsworth et al., 2018) and 0.32 (0.16–0.54) μg/g (Laustsen et al., 2015) for the same D. polylepis venom. On the other hand, our result was comparable to Ochola et al. (2019) who reported 0.341 mg/kg in mice and also fell within the range of 0.055–0.940 mg/kg that other studies have noted (Harrison et al., 2017), (Laustsen et al., 2015). It is not unusual for venoms from the same

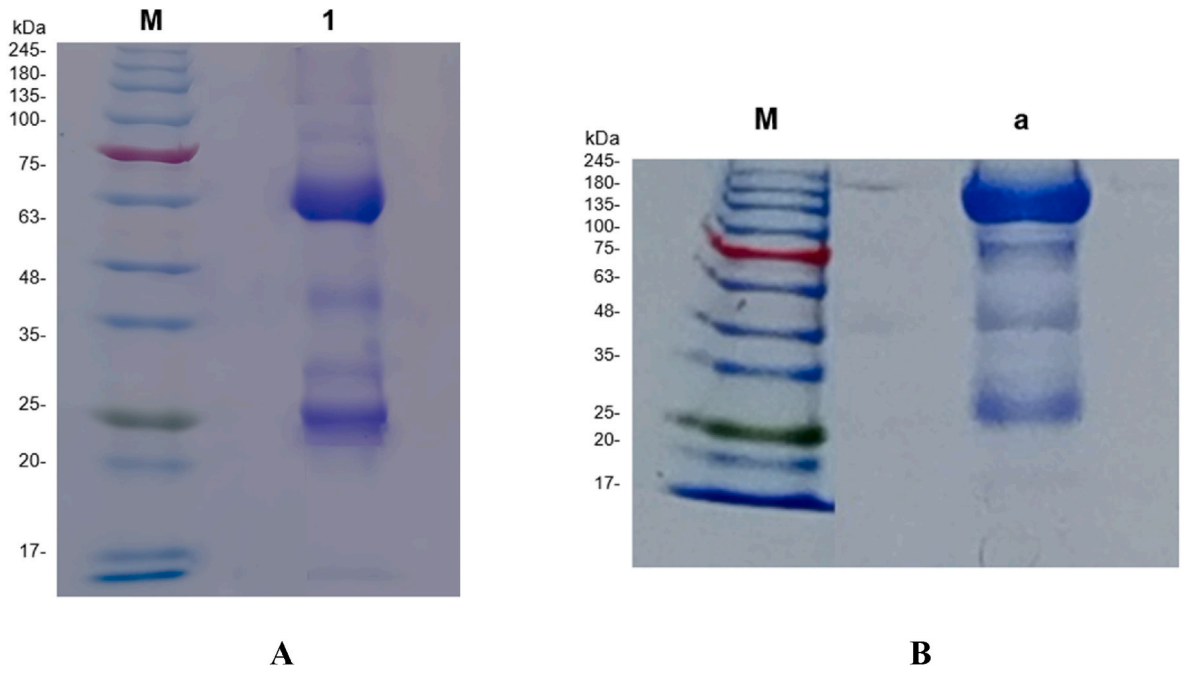

Fig. 1. Lane **1**: IgY under reducing conditions (1A) and lane **a**, IgY under non-reducing conditions (1B) Lane M: molecular weight marker.

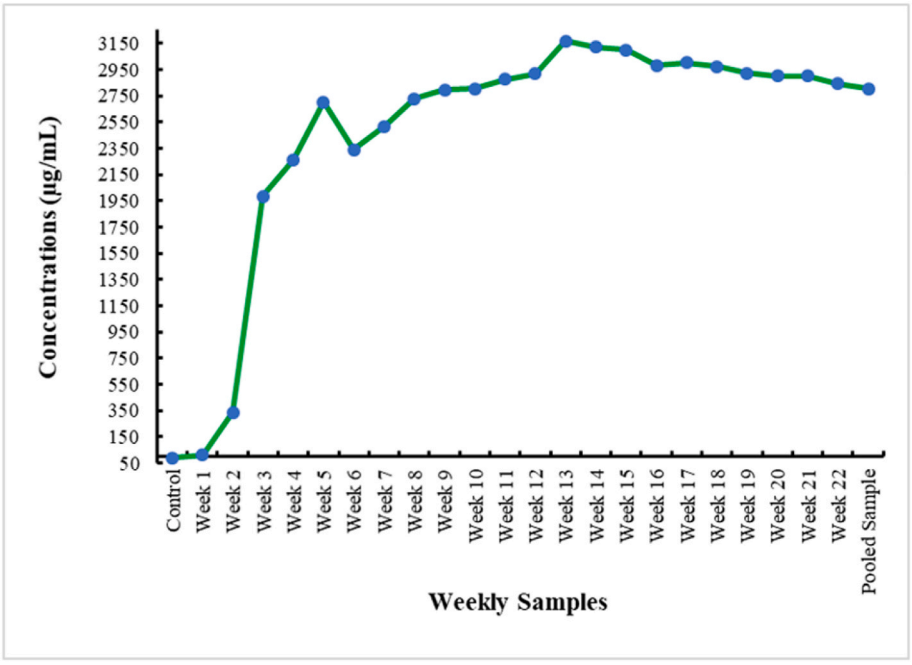

Fig. 2. Concentrations of the affinity purified antibodies yield per week.

species to have different toxicity actions, and a previous research by Calvete noted that venom variability is a common phenomenon both between and within species at all taxonomic levels (Calvete, 2019). The variations in the mouse breed as well mice slightly bigger/heavier than the standard 18–20 g recommended by the WHO used in this work could be the cause of the disparities in LD_{50} result and those published elsewhere. Also, the observed discrepancies could be attributed to venom-composition variations, sex-specific proteome differences, collection and pooling variations of black mamba venom (Menezes et al., 2006), (Mackessy et al., 2003), (Chippaux et al., 1991), (Daltry et al., 1996).

The clinical signs and symptoms that we observed in both chicks and the mice were consistent with neurotoxic venom. This included paralysis, ptosis (drooping or falling eyelids), and respiration patterns which varied from shallow to rapid (more pronounced in the chicks with accompanying unusual sound). The observation was in agreement with a report by Ochola et al. (2019). From reports, venom of black mamba has α-neurotoxins that can attach to nicotinic receptors at the motor end plate, and cause failure in respiration (Ratanabanangkoon et al., 2020), (Blanchet et al., 2014).

Despite the trace amount of metalloproteinase found by Laustsen and colleagues (Laustsen et al., 2015) in black mamba venom, it was not sufficient in our venom samples to elicit the required effect. However, we recorded edematogenic activity (minimum edematogenic dose =

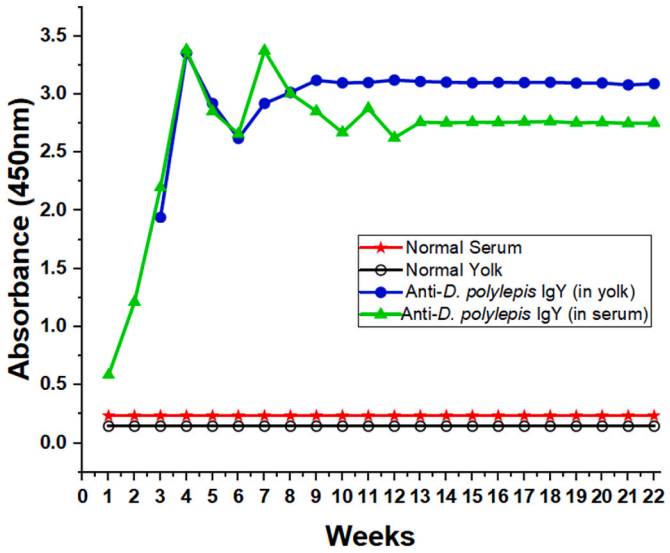

Fig. 3. Immune response for primary and secondary antibody in serum and egg yolk of immunized Isa-Brown chicken with *D. polylepis* venom. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2
D. ploylepis venom's lethal and edematogenic activities.

Venom	Lethality (LD_{50}; mg/kg)[a]	Edema-Forming Activity (MED; μg)[b]
D. polylepis	0.34 (0.29–0.38)	0.052 ± 0.11

[a] Using i. p route, LD_{50} (median lethal dose) was determined in Balb/c mice (28–30 g); 95% CI in parenthesis.

[b] The term "minimum edematogenic dose" (MED) describes the venom dosage that, after 1 h of injection, caused a 30% increase in footpad volume. Results are presented as mean ± SD (n = 5) for edema-forming activities.

Table 3
Lethal and edematous effects of *D. polylepis* neutralization by IgY antibodies, PANAF-Premium™ and VINS™ polyvalent antivenoms[a].

Antivenoms & Antibody	ED_{50} of lethal activity 3 LD_{50}/ antivenom (μL)	ED_{50} of lethal activity venom (mg)/ antivenom (μL)	Edema-forming activity antivenom (μL)/6 MED
Chicken-Based IgY	41.36 (28.57–52.01)	151.85 (119.89–185.90)	80 ± 11.55
VINS™	35.41 (21.14–45.79)	133.48 (83.56–167.40)	60 ± 18.26
PANAF-Premium™	46.60 (30.27–64.03)	175.65 (127.03–234.06)	90 ± 8.16

[a] The antivenom-venom ratio (μL antivenom/challenge dosage of venom) at which a certain venom-induced activity is reduced by 50% is known as neutralization, and it is expressed as median effective dose (ED_{50}). Results are shown as mean ± SD (n = 5) for edema-forming activities and 95% CI (in parenthesis) for lethality.

0.05 ± 0.11 μg) in mice for the black mamba venom (Table 2). This observation is the first report with edema activities found in association to black mamba venom and may be arguable just as the metalloproteinase traces report by Laustsen and colleagues in the venom. Additionally, literatures have it that among the major toxins of the elapid venoms also lies others like metalloproteinases (SVMPs), snake venom serine proteases (SVPs) and L-amino acid oxidases (LAAOs), all representing an average of 6% of elapid venom (Ullah et al., 2018), (Hiu and Yap, 2020), (Kang et al., 2011), (Ferraz et al., 2019), (Ullah, 2020), (Olaoba et al., 2020), (Oliveira et al., 2022). Besides serine protease and LAAOs not present in large quantity, it is prevalent in many snake venoms, and in myocytes, can cause edema development, hemolysis, toxicity, and platelet aggregation (Gutiérrez et al., 2021), (Hiu and Yap, 2020), (Kaur et al., 2012). Observation of edema forming activity in this study could be that our venom had significant amount of LAAO or serine protease which can elicit the edema effect (Resiere et al., 2022).

The WHO has established that neutralization of lethality at the preclinical stage is the gold standard for assessing antivenom efficacy. Further testing can be necessary depending on the venom, whether existing antivenoms are being supplied to new countries or new ones are being produced (WHO, 2017), (Durán et al., 2021). Examining the possible effects of the heterogeneity in intrageneric venom patterns among *Dendroaspis* on the use of antivenoms for treating snakebite victims in sub-Saharan Africa was made possible by this valuable opportunity. In order to achieve this, the study evaluated the neutralizing-efficacy of generated IgY antibodies in comparison to two commercial *Dendroaspis* antivenoms that are currently used in sub-Saharan Africa against the effect induced by *D. polylepis* venom in mice. Both antivenoms and the chicken-based IgY polyclonal antibodies showed the ability to neutralize the lethal and edematogenic effects of *D. polylepis* venom but with varying efficacy levels. The effectiveness of VINS™ antivenom in neutralizing the lethality of black mamba venom in mice has been documented by other researchers conducting comparable investigations (Ochola et al., 2019), (Laustsen et al., 2015). Antivenoms have been reported to be effective when they can immunocapture 20–25% of the components of venom (Calvete et al., 2014), and having an immunocapture capability of at least ≥25% of venom proteins is often correlated with a positive result in in-vivo neutralization tests, according to the WHO guidelines for the manufacture, control, and regulation of antivenoms (WHO, 2017). This result confirms the manufacturers' inscription on antivenom containers that each mL neutralizes at a minimum of ≥25LD_{50} for *D. polylepis* venom.

Regarding the determined neutralization of venom-induced lethality, the effective doses of PANAF-Premium™, IgY antibodies, and VINS™ antivenoms; the observations of mean differences in the ED_{50} of the antivenoms are consistent with those from other studies on *D. polylepis* venom neutralization (Ochola et al., 2019), (Laustsen et al., 2015), (Ainsworth et al., 2018), (Engmark et al., 2016), (Menzies et al., 2022). It further shares in the light of research by Resieri and associates when they indicated that a new batch of an antivenom had higher efficacy compared to an earlier batch as seen by differences in the ED_{50} values. They speculated that the variances might be caused by differences in the protein concentrations of the batches of antivenom, among other factors (Resiere et al., 2018). In addition, antibody specificities in the venom-immunized animal may result from variations in the venoms of snakes used for the immunization, and Gutierrez et al. indicated that this could potentially provide credence to the discrepancies in efficacy between antivenoms (Gutiérrez et al., 2017). The results from our study, however, are limited to these particular batches, thus caution should be exercised when interpreting them.

According to the WHO, snakebite envenomation is under category A Neglected Tropical Diseases, meaning it is an extremely serious disease. As a result, the World Health Organization (WHO) has initiated a global campaign to cut the illness burden of snakebite envenomation in half (50%) by 2030 (Williams et al., 2019). Therefore, we anticipate a significant expansion in the advantages of IgY technology and its widespread application in medical and scientific fields. Upcoming diagnostics, immunotherapy and research are anticipated to place a greater emphasis on IgY as demonstrated by Kpordze et al. (2024).

5. Conclusions

In conclusion, immunizing chickens with *D. polylepis* snake venom resulted in the successful raising of IgY antibodies against *D. polylepis* venom toxins for the first time in egg yolk with high purity and titer. The lethal effects and edematogenic activities of *D. polylepis* venom toxins

were effectively neutralized by the generated IgY antibodies in relative comparison as the commercial antivenoms (even a very lower concentration than the commercial antivenoms preparations recommended by the manufacturers which had higher concentrations). The IgY has proven effective and may be a useful tool for creating human or animal-specific alternative treatments for snakebite sufferers. Nonetheless, more research to acquire data on safety and efficacy in this regard is necessary.

In addition to supporting previous reports on the neutralizing effectiveness of IgY antibodies, PANAF-Premium™ and VINS™ antivenoms, our results emphasize the lethal and toxic activities of *D. ploylepis* venom. The study reports for the first time of raising chicken-based IgY antibodies and its efficacy against *D. polylepis*.

Declarations

The authors declare that none of the work reported in this study could have been influenced by any known competing financial interests or personal relationships.

Ethical statement

The authors wish to state that the study was approved by the Jomo Kenyatta University of Agriculture and Technology (JKUAT) Institutional Scientific and Ethics Review Committee (ISERC) (approval number JKU/ISERC/02316/0976) and the Kenya Medical Research Institute (KEMRI) Animal Care and Use Committee (ACUC) (reference number KEMRI ACUC/01.09.2023).

A scanned copy of these documents can be made available upon request.

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CRediT authorship contribution statement

Stephen Wilson Kpordze: Writing – original draft, Validation, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Victor Atunga Mobegi:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Gideon Mutie Kikuvi:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Joseph Kangangi Gikunju:** Writing – review & editing, Project administration, Conceptualization. **Courage Kosi Setsoafia Saba:** Writing – review & editing, Project administration, Methodology, Conceptualization. **Jackan Moshe:** Writing – review & editing, Project administration, Conceptualization. **James Hungo Kimotho:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

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.

Data availability

All data used are in the manuscript.

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