GENERATION AND FUNCTIONAL CHARACTERIZATION OF CHICKEN-BASED IgY POLYCLONAL ANTIBODIES AGAINST BLACK MAMBA (*Dendroaspis polylepis*) SNAKE VENOM

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DECLARATION

This thesis is my original work and has not been submitted for a degree award in any other University.

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DEDICATION

This work is dedicated to my parents (Mr. Mark Wilson Kpordze and Mrs. Gladys Ntim), Prof. Courage K. S. Saba, Mr. Antwi Mensah and Mrs. Felicia Oppong. May God richly bless you.

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LIST OF ABBREVIATIONS AND ACRONYMS

3FTXs	Three Finger Toxins
ANOVA	Analysis of Variance
CADD	Computer-Aided Drug Design
DAB	Diaminobenzidine tetrahydrochloride
ED	Effective Dose
ELISA	Enzyme-Linked Immunosorbent Assay
FCA	Freund's Complete Adjuvant
FIA	Freund's Incomplete Adjuvant
GBD	Global Burden of Diseases
HAMA	Human Anti-mouse Antibodies
IgYs	Immunoglobulin (Ig)Ys
KEMRI	Kenya Medical Research Institute
KWS	Kenya Wildlife Service
LD	Lethal Dose
NIOSH	National Institute for Occupational Safety and Health
OD	Optical Density
OPD	Ortho-phenylenediamine
PBS	Phosphate Buffer Saline
PEG	Polyethylene Glycol
PLA	Phospholipase A
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SVMPs	Snake Venom Metalloproteinases
TMB	Tetramethylbenzidine
WHO	World Health Organisation
YLLs	Years of Life Lost

ABSTRACT

The life-threatening medical issues associated with snakebites have been a public health concern for decades at the global level. The Black mamba, *D. polylepis*, is one of the many venomous snakes found in Kenya. According to the Kenyan Ministry of Health data, 15,000 snakebites occur annually. For snakebites, antivenom immunotherapy is still the preferred course of action and different antivenoms are needed to treat the venom of different snake species. Traditionally, antivenoms for treatment are produced from horse or sheep but have complicated and expensive production issues. Alternative production approaches, such as using immunoglobulin Y (IgY) antibodies derived from chicken egg yolks, may overcome disadvantages with traditional antivenom manufacturing techniques. In many tropical and subtropical nations such as Kenya, diagnosis of snakebite in the health facilities is imperative. For the purpose of determining which species caused the bite, it is critical to detect and measure snake venom in the blood of envenomed patients. The study presents the generation of IgY antibodies, and development of an enzyme-linked immunosorbent assay (ELISA) against *D. polylepis*. 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. Additionally, the study sought to develop a sensitive ELISA prototype for detection of *D. polylepis* venom in Kenya using generated chicken-based IgY polyclonal antibodies. The IgY antibodies were purified by ammonium sulfate precipitation and affinity-chromatography, with quality and specificity determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and ELISA. Furthermore, serum samples containing specific chicken-based IgY antibodies previously raised against *D. polylepis* venom toxins were used in the assay development. ELISA parameters were optimized, and developed assay assessed for applicability. The LD_{50} of *D. polylepis* was 0.54 mg/kg in chicks (via intramuscular route), and 0.34 mg/kg in mice (via intraperitoneal route), 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. The median effective dose (ED_{50}) for lethality neutralization were 41.36, 35.41 and 46.60 µL/3LD_{50}, and for edema were 80±11.55, 60±18.26, 90±8.16 µL/6MED, respectively for IgY antibodies, VINS and PANAF antivenoms. There was no statistical differences among their neutralizing efficacies (P value = 0.320-0.859) The limit of detection (LoD) of the ELISA for neurotoxic venoms was 0.01 µg/mL. The developed assay identified venoms in blood of BALB/c mice and, distinguished *D. polylepis* venom from that of other snake species, as well as neurotoxic and cytotoxic venoms. This developed assay would be useful in helping physicians to diagnose and manage snakebite cases. The development of an effective IgY antibodies with higher titer represents a significant step towards antivenom production against *D. polylepis*. Diversity of the IgY antibodies development to capture other medically important snake venoms, molecularly characterize IgY, and development of venom detection kits with quick turnaround times based on liquid or lateral flow tests approach are recommended.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Envenomation resulting from snakes' defense mechanisms through bites and venom sprayed into eyes exposes victims to injection of mixtures of different toxins, and leads to potential life-threatening disease (Turner, 2019; World Health Organization-WHO, 2019). This has been a major public health issue across the tropics (thus, most bites occurring in Africa, Latin America and Asia), and from available data at the global level, an estimated 4.5-5.4 million people are bitten by snakes yearly with 2.7 million developing clinical illness, and 81,000-138,000 people dying from several resulting complications (Halilu et al., 2019; WHO, 2019; Gutiérrez, 2021). Permanent disabilities such as blindness, severe scarring, contractures, limited movement, and amputations may also arise from non-fatal envenoming (Gutiérrez et al., 2017; Williams et al., 2019a). Reports have indicated that 2 million people in Asia and an estimated of 400,000-600,000 in Africa suffer snakebite envenoming annually with medical aid as key requirement. In Africa and Asia, study puts burden of snakebites annually at 1.03 million and 391,979 disability-adjusted life years, respectively. In these regions, the burden of the disease rest most on children, women, those with limited access to healthcare, people living in poorly built houses and agricultural workforces in rural parts of such countries (WHO, 2017; Halilu et al., 2019; Williams et al., 2019a; Gutiérrez, 2021).

Snake envenoming is commonly attributed to four families of venomous snakes; Elapidae, Viperidae, Atractaspididae, and Colubridae. The black mambas (*Dendroaspis polylepis*) which belong to the family Elapidae are known to account for the great majority of envenomings, and fatalities. Their venoms often cause systemic neurotoxicity, cytotoxicity, hemotoxicity, and occasionally myotoxicity (Laustsen et al., 2017). However, *D. polylepis* venom primarily causes systemic neurotoxicity. Their neurotoxic venom is extremely potent, making them one of the most feared snakes in the world. In

Kenya, they have been indicated as medically important since they cause snakebites in many areas of the region (Ochola et al., 2018).

The preferred method of treating a snakebite or envenomation is the administration of antivenom. 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). Antivenoms are produced from generated antibodies, and function by enhancing neutralization reaction to a snakebite. They are created by inoculating snake venom into donor animals like horses, sheep, goats, and even camels. Antivenoms of high quality can actually be the difference between life and death. The production of antivenom is a remarkably diverse sector (WHO, 2019). Nevertheless, there has been an increasing interest in creating polyclonal antibodies and/or combinations of monoclonal antibodies which offer better antibody-based therapeutics. Given the complexity of snake venoms, which each comprise a variety of distinct poisons from various protein families, a single monoclonal antibody is unlikely to be able to successfully neutralize a snake venom (Laustsen et al., 2016, 2017).

The polyclonal antibody Immunoglobulin (IgY) has been taunted as the antivenom production system of choice. Chickens, reptiles, amphibians, and other birds are all known to produce immunoglobulin (Ig)Ys. IgYs have comparable functions as mammalian IgGs. IgYs are found in chicken sera and are transferred to the embryo through egg yolk. Egg IgY antibodies have been used in the past to treat bacterial, viral, and other diseases (Abbas et al., 2019). It has been established that eggs of chicken that have been immunized with appropriate antigens are a reliable source of IgY antibodies. Numerous studies on the snake antivenom IgY so far have shown that it could be a potential replacement for the traditional antivenom made from large animals (Liu et al., 2017).

1.2 Statement of the problem

Snakebite is among the leading killers of the 24 diseases and conditions listed as neglected tropical disease by the WHO. Asia, sections of Oceania, Latin America, and Africa and the middle East bear the brunt of snakebite envenomation; recording 1.2-2.0 million (57,000-100,000 deaths), 3,000-5,900 (200-520 deaths), 137,000-150,000 (3,400-5,000 deaths) and 435,000-580,000 (20,000-32,000 deaths) yearly envenomings, respectively (Gutiérrez et al., 2017). Venomous snake bites can cause acute medical emergencies such as severe paralysis, difficult to breath, bleeding issues or fatal hemorrhage, irreparable vital organs damage like the kidney, limb amputation and other permanent deformity (Williams et al., 2019a). The impact of these and associated problems have been particularly severe in Sub-Saharan Africa, where indigenous antivenom production has never been sufficient to meet the needs of the region. Since the last decades, there has been a shortage of effective antivenoms in Africa due to a combining factors such declining demand, incentives for commercial manufacturing, and limited production volumes due to high costs of effective poly-specific antivenoms (Harrison 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. Report has it that some of these antivenoms are made from species that are not native to Africa, and hence its effectiveness upon usage in Africa for treatment turns to be compromised. Sub-Saharan Africa has only one antivenom producer situated in South Africa (Gutiérrez et al., 2017).

In Kenya, snakebites constitute a neglected emergency, and 15,000 snakebites occur yearly according to data from the Ministry of Health (MoH-Kenya, 2019). Among the most common snakes behind this incidence is the 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). Venom from the Black mamba (*D. polylepis*) remains under-explored with respect to antivenom production. According to a study on snakebites epidemiology in selected areas of Kenya by Ochola et al. (2018), the aforementioned snake is widely distributed in areas of the country such as Kakamega Forest, Mau Forest, Lake Naivasha, Masai Mara, Aberdares, Lake Baringo, Mount Kenya, Mount Elgon,

North Eastern province, Coastal regions, Kakuma, Lamu, Ukambani (Makueni), Malindi and Mombasa.

Current snakebite treatments in Kenya and Africa rely on the conventional antivenoms which are produced from immunized horses. However, reports on reactions to these antivenoms among snakebite victims have been observed by the Kenya Ministry of Health (MoH-Kenya, 2019). A previous study Frauches and associates indicated that horses who have been immunized produce unfavorable antibodies that are directed towards non-toxic venom components, with 95% of the immunoglobulins (IgGs) that make up modern antivenoms reportedly being non-therapeutic. Despite $F(ab')_2$ rich antivenoms' potential for effectiveness, they nonetheless have drawbacks, including the propensity to trigger the host complement system (Frauches et al., 2013). In Kenya, preclinical testing of two locally accessible antivenoms revealed very little poly-specific effectiveness (Harrison et al., 2017).

The aim of this study was to generate chicken-based IgY antibodies against *Dendroaspis polylepis* and compare the preclinical assessment of its neutralizing efficacy against two selected commercial antivenoms, and develop an ELISA prototype for *D. polylepis* venom detection.

1.3 Justification

Given that the snake venom proteins utilized in antivenoms used in Kenyan are derived from a foreign snake population, raises the question of whether they are appropriate for use in local clinical settings. The presence of different types of venom in snake populations creates unique challenges for the production of a common antivenom and has reduced the appeal of this class of therapeutic agent on the market. Particularly, it has been well established that variations in venom components across and within species might have an impact on the ability of antivenoms to neutralize the venom (Casewell et al., 2020). Clinical repercussions may include diminished effectiveness against some significant venom activities or even total therapeutic failure in serious envenomation. A study by Harrison et al. (2017) examined the effectiveness of six antivenoms preclinically

and found none to be effective. Hence, the study concluded that some of these developed antivenoms sold for use in Africa have been made using snake venom from snakes that are not native, and are therefore not effective (Harrison et al., 2017).

Majority of parts of the world where snake envenoming occurs rely mostly on broad spectrum antivenoms known to contain immunoglobulins that trigger the host complement system and causes varied adverse effect to the host. Hence, developing less costly, effective and safe antivenoms through IgYs antibodies for medically important snakes remain one key priority, especially in Africa using indigenous snakes (Abbas et al., 2019).

Chicken egg has served as a natural component of the human diet, and hence IgYs from such source are well tolerated. Additionally, because the purified IgY lacks egg albumin, a major catalyst for egg allergy reactions, it can be administered to individuals with egg allergies. Mice and pigs have both been used in earlier studies to investigate IgY's immunogenicity, thus anti-IgY antibodies, mostly of the IgG subclass, were produced in reaction to the IgY. Further inflammation is unlikely because IgY does not interact with human complement system or Fc receptors (Kovacs-Nolan & Mine, 2012; Abbas et al., 2019).

Since then, research on snake venom with a medical purpose has primarily focused on venom protein profiling, and the use of venom in antivenom production (Tan, 2022). The development of IgY antibodies targeting venoms from native snakes would boost the effectiveness of antivenoms and thereby lessen the danger of non-responses associated with the administration of antivenoms generated from horses and sheep. Using chicken will result to cheaper and increased production of antivenom. Thus, advances in IgY based antivenom hold much promise as far as future treatment strategies of snakebite envenoming are concerned.

For an easy and reliable method for determining the species responsible for a snakebite is antibody-based venom protein detection, and undoubtedly, ELISA is one of the best immunoassays in clinical applications (Pawade et al., 2016). It is known in routine diagnosis that ELISA equipment and reagents are inexpensive, and has reportedly been

used to detect several venoms globally (Lakkappa et al., 2015). Developing an IgY based ELISA for diagnosis and treatment options will offer advantages like cost-effectiveness, increased efficacy, sensitivity and specificity for *D. polylepis* envenoming.

Moreover, there is very little information available concerning the potency of available antivenoms to neutralize the toxicity of *D. polylepis* venom. As a result, when information on antivenoms efficacy at the preclinical level is provided, it will not only influence the design or redesign of antivenoms but also strengthens international efforts to lessen the snakebite envenoming (Williams et al., 2011).

1.4 Null hypothesis

Developing chicken-based IgY polyclonal antibodies against *D. polylepis* venom will not yield a sensitive, specific ELISA venom proteins detection and effective antivenom.

1.5 General objective

To generate and characterize chicken-based IgY polyclonal antibodies against black mamba (*Dendroaspis polylepis*) snake venom.

1.6 Specific objectives

i. To generate and functionally characterize chicken-based IgY polyclonal antibodies against *D. polylepis* venom.
ii. To determine the capacity of chicken-based IgY polyclonal antibodies to neutralize *D. polylepis* venom.
iii. To develop an ELISA prototype of IgY polyclonal antibodies for detecting *D. polylepis* venom.

1.7 Scope, assumptions and limitations of the study

It was in the study's interest to consider investigating venoms of *D. polylepis* from other parts of Kenya (apart from Baringo), as well as Africa but however faced with limited funding. Also, due to funds and time constraints, the study could not capture the production of hybridoma and monoclonal antibodies against a specific fraction of *D. polylepis* venom proteins.

Similar to this, the study did not examine additional antivenoms that could be used to treat *Dendroaspis*-related envenomation; instead, it solely assessed the neutralizing capacity of VINS and PANAF-Premium polyvalent commercial antivenoms. The ELISA prototype was developed from IgY polyclonal antibodies raised against *D. polylepis* and other venoms. However, both *Dendroaspis* (*D. angusticeps* and *D. jamesoni*) and non-*Dendroaspis* (*N. ashei* and *B. arietans*) venoms were examined in order to evaluate the assay's specificity.

Finally, the study was able to process the *D. polylepis* venom for proteins to be characterized. However, molecular characterization of *D. polylepis* venom protein was not done in this study due to lack of access to facilities.

CHAPTER TWO

LITERATURE REVIEW

2.1 Global burden of snakebites

Snakes have been feared, loved, or worshipped from the beginning of time. The most frequent medical emergency posing a life-threatening risk is a snakebite, and rural areas are where these incidents are most likely to occur. Unfortunately, snakes continue to do harm to millions of villages on a daily basis (Kamal et al., 2015). Envenomation from snakebite still poses a threat to global health. However, the incidence is overestimated as a result of inadequate epidemiological data (Eslamian et al., 2016). According to the World Health Organization, 2.7 million of the 5.4 million snake bites that happen each year are poisonous, with a 5% fatality rate (137,880 deaths annually) (WHO, 2019). In 2019, there were 4,318 cases of snakebite/envenomation documented in the United States, with between 10% and 44% of venomous snakebite patients enduring permanent impairment, according to the American Association of Poison Control Centers (Vos et al., 2020).

Venom is injected into the victim's body when a venomous snake bites them, resulting in both localized symptoms like minor pain and edema as well as more general ones like tachycardia, dyspnea, mental changes and ptosis. Myocardial infarction, disseminated intravascular coagulation, acute renal failure, and even death can occur in extreme situations (Kang et al., 2016). In warmer areas with an abundance of snakes and where agriculture is the primary industry, snakebites are more common.

From the Global Burden of Disease 2019 study, the burden by region and socio-demographic index, revealed 54,600 deaths (95% UI 31,800-68,300) and 2.54 million years of life lost-YLLs (1.48 million-3.21 million), South Asia bore the most burden, accounting for 86% (76-92) of global deaths and 86% (78-91) of global YLLs. The 3.4 fatalities (2.0-4.2) per 100,000 and 144 YLLs (83-182) per 100,000 age-standardized death and YLL rates, respectively, were reported. The next greatest rates of death from snakebites envenoming were in Western, Central, and Eastern sub-Saharan Africa, with

1.4 (1.0-2.1), 1.3 (0.8-1.8), and 1.2 (0.8-1.6) per 100,000 people, respectively. Central Europe, high-income North America, high-income Asia Pacific, and Western Europe had the lowest age-standardized rates in 2019 (Suraweera et al., 2020; Vos et al., 2020; Roberts et al., 2022). This is depicted in the figure 2.1 below.

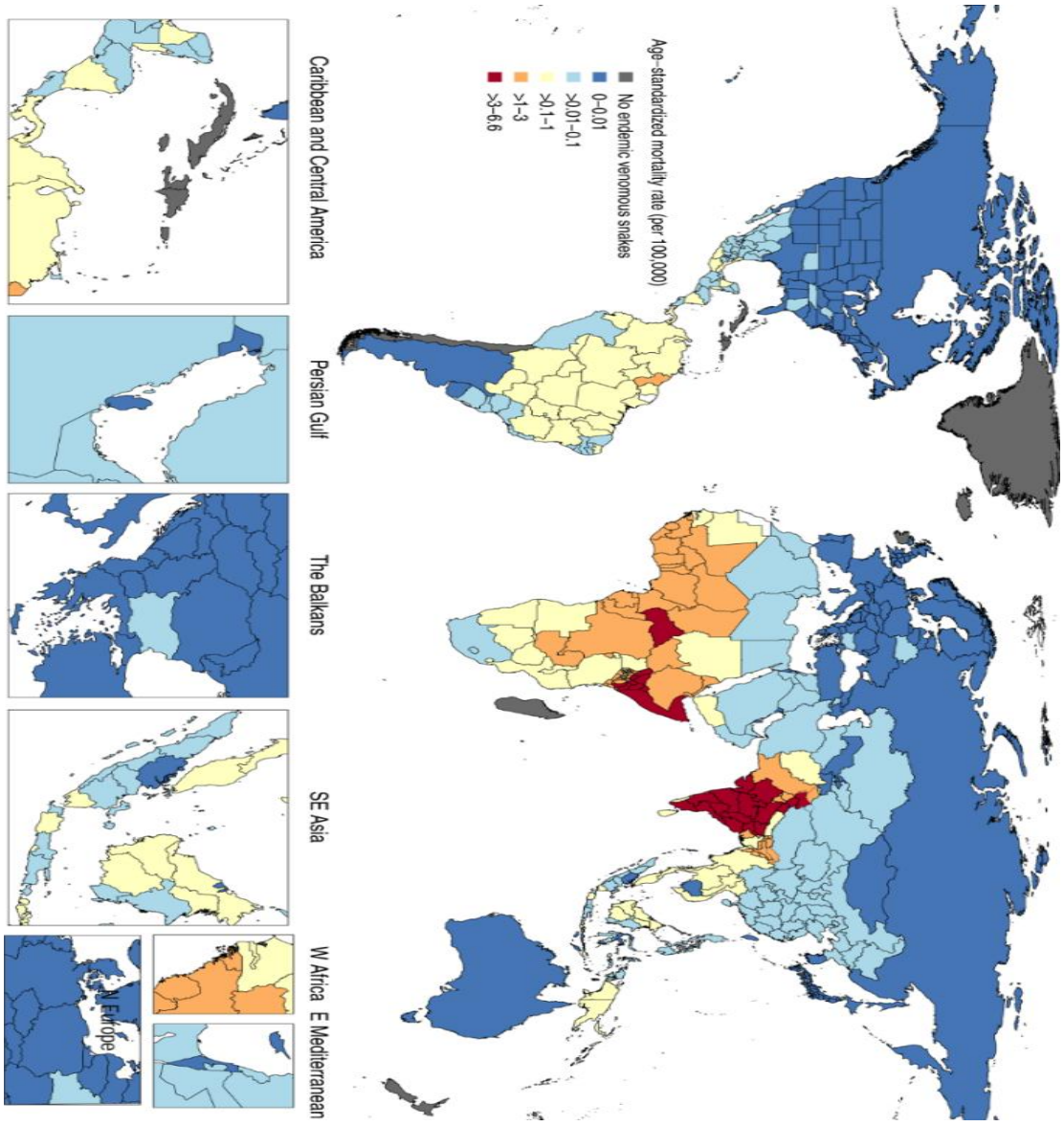


Figure 2. 1: A map showing global rates of mortality resulting from snakebites adapted from the Global Burden of Disease 2019 study by Roberts et al. (2022).

Despite the low death rate after envenomation, subsequent sequelae are frequent and can range from 10% to 44% (National Institute for Occupational Safety and Health-NIOSH, 2021). Local tissue discomfort, cellulitis, edema, infection, muscle contracture, coagulopathy, skin necrosis, compartment syndrome, and other physical abnormalities are only a few of these. The vast spectrum of problems is mostly linked to the scarcity of antivenom, infrastructure of the hospital system, and the socioeconomic position of patients or victims (Tochie et al., 2017; WHO, 2019; Johnson, 2020).

2.1.1 Distributions of venomous snakes worldwide

The majority of severe envenomations are caused by elapids and viperids, two of the three principal families of poisonous snakes, along with colubrids. The boomslang (*Dispholidus typus*) and the twig snake (called vine snake: *Thelotornis mossambicanus, Thelotornis capensis* and *Thelotornis kirtlandii*), both of which belong to venomous and found throughout east and southern Africa, are the only two members of the family Colubridae that are venomous snakes. Despite the fact that many examples of these animals exist in zoos and as exotic pets due to their rarity, beauty, and colour, no colubrid species that are dangerous to humans are found naturally in North America (Asif et al., 2015).

The elapids are a diversified group of snakes that include the coral snakes, sea krait, black mamba (*Dendroaspis polylepis*) and green mamba (*Dendroaspis angusticeps*), which are indigenous to sub-Saharan Africa (genus *Laticaudi*). Three extremely poisonous elapid species, namely the tiger snake (*Notechis scutatus)*, Inland taipan (*Oxyuranus microlepidotus*), and brown snake (*Pseudonaja textilis*), are found in Oceania and Australia. The three species of coral snake (*Micrurus fulvius fulvius*, eastern coral snake; *Micrurus fulvius tenere*, Texas coral snake; and *Micrurus fulvius barbourin*, South Florida coral snake) found in Mexico and the Southern United States are the only members of the family Elapidae that naturally occur in North America. Coral snake bites are uncommon and typically happen when the snakes are handled or mistaken for harmless species like king snakes or milk snakes, whose banding patterns may have developed to resemble the more poisonous coral snakes' more striking coloring. They may be incorrect and result in

misidentification due to variances in banding and color patterns among species and geographical regions (Hessel & McAninch, 2024).

Except for Antarctica, all continents in the world are home to the venomous snake family known as Viperidae. Members of the Viperidae family in North America include the copperhead, cottonmouth, and pit vipers (so named for heat-sensing pits located directly below the eyes), which contain several species of rattlesnakes. These three species accounts for the great majority of venomous snakebite cases in North America, with the copperhead representing about 50% (8,9) of these cases (Ruha et al., 2017). A large variety of venomous snakes are members of the Viperidae family, including the puff adder (*Bitis arietans*), which is indigenous to Eastern and Southern of Africa, the saw scale vipers (carpet vipers, genus *Echis*), which are indigenous to Southern Asia, Arabia, and Sub-Saharan Africa, and *Vipera berus*, the typical adder in Europe.

Three families, including the Atractaspidae (Burrowing Asps, 69 species), the Elapidae (Elapids, 360 species), and the Viperidae, contain nearly all medically significant poisonous snake species (Vipers, 340 species). Viperinae (True vipers, 101 species) and Crotalinae (Pit Vipers, 239 species) are the two subfamilies that make up this last family (data taken from www.reptile-database.org). The three groups of front-fanged snakes are thought to have developed from non-front-fanged poisonous snakes based on the homology of the venom glands in caenophidian (advanced) snakes (Jackson et al., 2017).

2.1.2 Black mamba (*D. polylepis*)

The black mamba (*D. polylepis*) is arguably Africa's most dreaded snake. From the four-membered genus *Dendroaspis, D. polylepis* is an olive-green snake with a black mouth, hence the name "black mamba" (family Elapidae). It lives in open bushland in Eastern and Southern Africa, with rare reports in Western Africa. It typically preys on small mammals (Ochola et al., 2018). According to report, *D. polylepis* specimens can grow to a height of 4.3 meters, can move quickly and repeatedly strike their victims while lifting a sizable portion of their bodies off the ground (Ochola et al., 2019), as shown in Figure 2.2. Adult individuals can spit out 4 to 8 mL of venom from their 3-6 mm-long fangs.

The World Health Organization (WHO) has categorized *D. polylepis* as Category 1 in 17 sub-Saharan African nations. This classification applies to species that are of the "highest medical importance," or those that are "very poisonous snakes, which are widespread/common and frequently cause snakebites, leading to significant levels of disability, mortality or morbidity" (WHO, 2010).

D. polylepis venom is well recognized to be extremely neurotoxic. Paresthesia, a peculiar taste in the mouth, nausea, vomiting, abdominal pain, diarrhea, perspiration, salivation, "gooseflesh," and conjunctival congestion are examples of early symptoms. Additionally, early signs of neurotoxicity include ptosis, diplopia, dysphagia, flaccid paralysis, slurred speech, dyspnea due to paralysis of the respiratory muscles, and involuntary skeletal muscular contractions or fasciculations. Neurotoxicity may start as soon as 15 minutes after exposure (Laustsen et al., 2015).

None of these envenomings are accompanied by hemolysis or local symptoms such edema, bleeding, or necrosis. If the sufferer is not treated after receiving a *D. polylepis* bite, there is reportedly a very high death rate (Závada et al., 2011). On the other hand, it has been demonstrated that using mechanical ventilation or administering adequate antivenoms in a timely manner can stop these envenomings from being fatal (Hilligan, 1987). The most toxic components of the venom have been identified as dendrotoxins, -neurotoxins, and other members of the 3-finger family of toxins (Laustsen et al., 2015).

Figure 2. 2: An image of the Black mamba (*D. polylepis*) on plant and ground with raised head adapted from Ochola et al. (2019)

2.1.2 The burden of snakebites in Kenya

The envenomation issue has worsened as a result of climate change and deforestation, which force snakes out of their normal habitats into the general population. Of the known global cases, more than 70,000 cases are from East Africa. In Kenya, snakebites resulted in 89, 67, and 22 fatalities in the years 1971, 1972, and 1973, respectively (Mbindyo & Okello, 1999). Nineteen percent of the 151 snakebite cases reported in 1994 involved poisonous snakes (Snow et al., 1994). Snakes killed 614 Kenyans between 2007 and 2016, injuring 7,772 others. During this time, there were between 300 and 500 snakebite cases in the Baringo county alone (Kipchumba, 2019; Langat, 2019). When the rainy season begins, snakes often emerge from their hiding places to hunt and reproduce. As a result, between 15 and 25 Kenyans die from snake envenomation per day, and more than 100 others have limbs amputated, leaving them permanently disabled (Koech, 2020). The majority of bites are attributed to unprotected or barefooted victims accidentally treading on the snakes (Nasio, 2016) and to snake ecology (Musah et al., 2019), whereas other

bites are caused by malicious or intoxicated victims (Owuor & Kisangau, 2006; WildlifeDirect, 2019).

Of the more than 400 species of snakes known in Africa, around 200 species are located in East Africa. The most venomous serpents found in Kenya are the puff adder (*Bitis arietans*), Gabon viper (*Bitis gabonica*), black mamba (*Dendroaspis polylepis*), green mamba (*D. angusticeps*) or Jameson's mamba (*Dendroaspis jamesoni*), forest cobra (*Naja melanoleuca*), and black-necked spitting cobra (*Naja nigricollis*) (Harrison et al., 2017; Okumu et al., 2019). Due to their nocturnal habits and superb concealment, puff adders are the primary venomous serpents in Kenya. Their venoms are highly cytotoxic, resulting in excruciating pain, swelling, and burning, and frequently in serious tissue damage (WildlifeDirect, 2019). Another popular species that typically spits its venom in its victims' eyes is the black-necked spitting cobra (WildlifeDirect, 2019).

In Kenya's awareness and handling of snakebite issues, the Kenya Wildlife Service records that 81% of compensation claims involve poisonous snakebites during amendment of Wildlife Conservation and Management Act 2013 (Koech, 2020). Over the years, the number of these claims has risen to billions of Kenyan Shillings, prompting the Tourism and Wildlife ministry to proclaim the situation a catastrophe that poses a threat to a bankrupt nation. It has proposed that the burden caused by snakebites be removed from the zoological list of claims eligible for compensation relating to human-wildlife conflict (WildlifeDirect, 2019; Koech, 2020). Baringo, Tharaka-Nithi, Taita Taveta, Kilifi, Kitui, Wajir, Garissa, Machakos, Marsabit, Isiolo, and Makueni are among the hotspots in Kenya with major instances of snake envenomation (Kihiko, 2013; Ochola et al., 2018; Okumu et al., 2019).

The approved remedy for managing snake envenomation is antivenom serum (Janardhan et al., 2014). They may only be helpful if given within 30 minutes to 6 hours of the snakebite and are, regrettably, linked to a number of negative effects. As a result, there are a number of obstacles to addressing the problem of snakebites in Kenya, including lack of antivenom and inadequate antivenom preservation facilities in health centers, as

well as poor road networks, fragmented records, and a lack of public health education (Ochola et al., 2018; Langat, 2019; Okumu et al., 2019). Due to reports on lack of antivenom globally, there has been numerous attempt to create antivenoms from other sources to supplement those generated from venom-immunized horse (Zolfagharian & Dounighi, 2015). However, studies from East Africa suggest that several commercially available antivenom sera were ineffective against some critically relevant snake venoms (Harrison et al., 2017; Ochola et al., 2019). As a result, up to 85% of snakebite victims in Kenya seek treatment from traditional healers or employ crude methods to get rid of the venom (Saya, 2019; Koech, 2020). Within five years, according to the Kenya Snakebite Research and Intervention Center, East Africa will have its first antivenom drug at a price roughly 30% lower than that of currently imported antisera (Shand, 2019), and this needs a consented effort by researchers. The distribution of venomous snakes in Kenya are detailed in Table 2.1 and images of snakes are shown in Figure 2.3.

Table 2. 1: The distributions of medically important snakes in Kenya adapted from Ochola et al. (2018) and MoH-Kenya (2019)

Type of snake	Areas of distribution
Eastern Jameson's mamba (*Dendroaspis jamesoni*)	Masai Mara, Kakamega forest, Lake regions, Mau forest.
Black mamba (*Dendroaspis Polylepsis*)	Masai Mara, Kakamega forest, Mau forest, Lake Naivasha, Aberdares, Lake Baringo, Mount Kenya, Mount Elgon, Kakuma, Coastal regions, Lamu, Malindi, Mombasa, North Eastern province, Ukambani (Makueni).
Eastern Green mamba (*Dendroaspis angusticeps*)	Aberdares, Mount Kenya, Coastal regions, Lake Naivasha.
Puff adder (*Bitis arietans*)	Same distribution as for black mamba
Gaboon viper (*Bitis gabonica*)	Masai Mara, Kakamega forest, Mau forest, Lake regions.
Saw-scaled viper (*Echis carinatus*)	Kakuma, Lake Baringo, Mount Elgon, Tsavo National Park, Makueni, North Eastern Province.
Black-necked spitting cobra (*Naja nigricollis*)	Distribution as for black mamba
Rhino viper (*Bitis nasicornis*)	Masai Mara, Kakamega forest, Mau forest, Lake regions.

Red spitting cobra (*Naja pallida*)	Kakuma, Lake Baringo, Ukambani, North Eastern Province, Mt. Elgon.
Egyptian cobra (*Naja haje*)	Same as for black mamba except it is absent in Coastal regions
Large brown spitting cobra (*Naja ashei*)	Lake regions, Kakamega forest, Masai Mara, Mt. Elgon, Mau forest, Lake Baringo, Kakuma, Aberdares, Coastal parts of Kenya, Mt. Kenya.
Forest cobra (*Naja melanoleuca*)	Masai Mara, Kakamega forest, Mau forest, Lake Naivasha, Lake Baringo, Aberdares, Mt. Kenya, Kakuma, Nairobi, Mt. Elgon, Makueni, Tsavo, Coastal region, Lake regions,
Twig snake (*Thelotornis Kirtlandii*)	Coastal regions
Boomslang (*Dispholidus typus*)	Distribution as for forest or black and white Cobra
Night adders, Causus species	As for black mamba except North Eastern Province
Mole Viper, Atractaspis species	Distribution as for black mamba

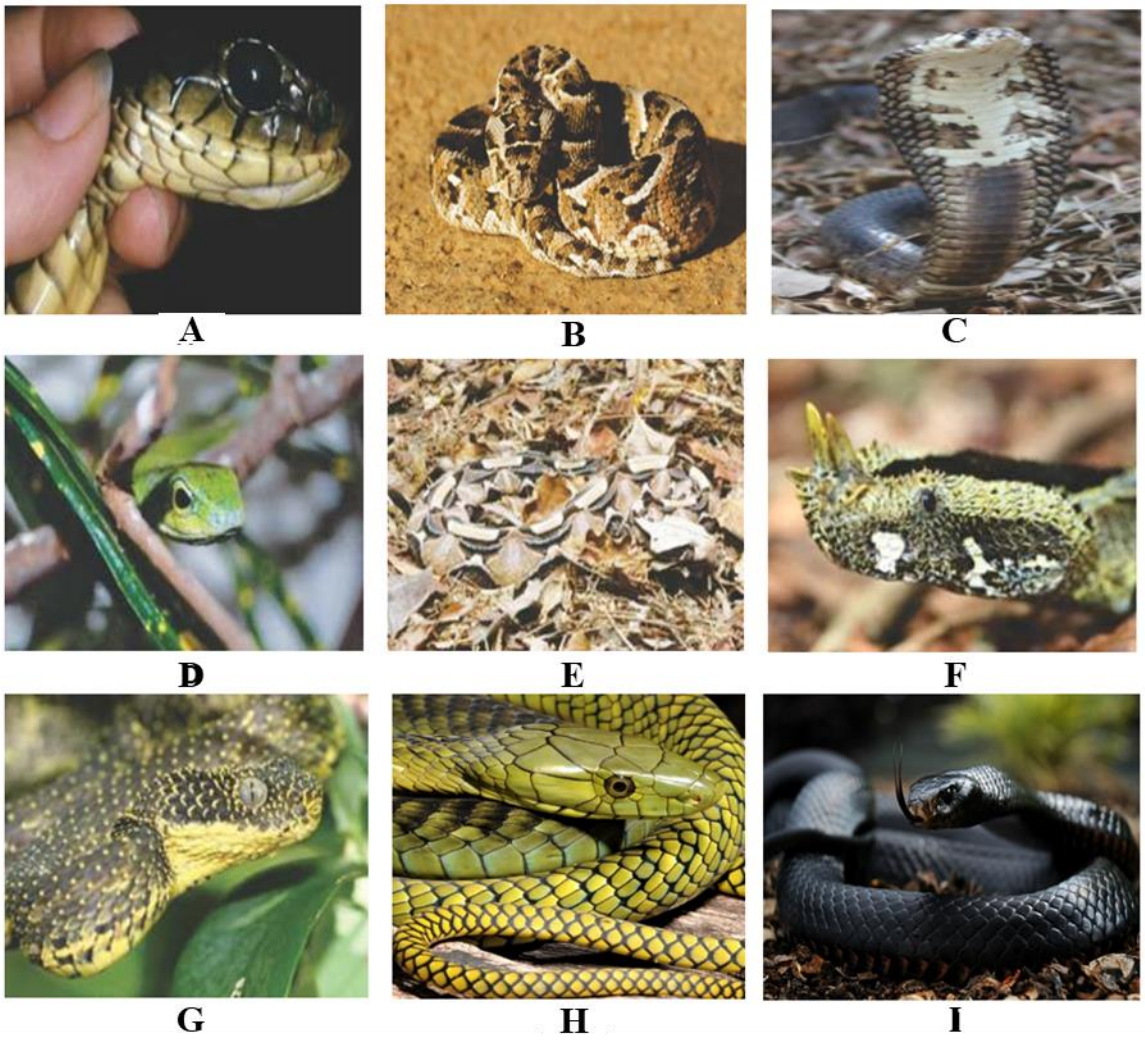


Figure 2. 3: Some venomous snakes implicated as medically important by the Ministry of Health in Kenya and WHO

A- *Gold's tree cobra (Pseudohaje goldii) is only found in Western Kenya and is common in the Kakamega Forest*; ***B-*** *The puff adder (Bitis arietans) is found across Kenya and is responsible for most of the venomous snake bite incidents recorded in the country*; **C-** *The Egyptian cobra (Naja haje) ready to strike;* **D-** *The boomslang (Dyspholidus typus) is often confused with the green mamba or the green bush snake;* **E-** *The Gaboon viper's (Bitis gabonica) skin coloring and patterns blend perfectly with foliage on the ground;* **F-** *The Rhinocerous viper is only found in Western Kenya, mostly in the Kakamega Forest;* **G-** *The Mt. Kenya bush viper (Atheris desaixi);* **H-***The Jameson`s mamba (Dendroaspis jamesoni); and* **I-***The Black mamba (Dendroaspis polylepis). Images adapted from the "Guidelines for Prevention Diagnosis and Management of Snakebite Envenoming in Kenya (2019)".*

2.2 The venom of snake

Snakebites are a common occupational and ecological risk in rural settings. The venom of a snake is often described as a complex mixture; containing combination of benign proteins, including carbohydrates, metals, enzymatic and non-enzymatic compounds (Rana et al., 2015; WHO, 2016). Venom, the deadly weapon of snakes, is made up of roughly 20 toxic enzymes. Along with tiny peptides, carbohydrates, amino acids, lipids, biological amines, nucleosides, proteases, and metal ions, the venom contains phosphodiesterase, cholinesterase, hyaluronidase, ATPases, and other toxins (Kamal et al., 2015). Typically, neurotoxins and cardiotoxins are present in cobra venom. The acetylcholinesterase and vasculotoxins from vipers disrupt coagulation pathways. Neurotoxin in the venom of the Krait causes presynaptic blockade (Rana et al., 2015). Alternately, a neurotoxic effect, in which presynaptic or postsynaptic blockage hinders signal transmission, is found in venom consisting of alpha protein or phospholipase A2 (PLA2). This effect result in respiratory depression, paralysis, dysphagia, diaphoresis, peripheral nerve palsy, reduced reflexes, and visual disturbances (diplopia and ptosis) (Strickland et al., 2018).

2.2.1 Major proteins in venoms

The following protein families, namely; PLA2s and three-finger toxins (3FTxs) for elapids and snake venom metalloproteinases (SVMPs), PLA2s and snake venom serine proteases (SVSPs) for vipers, predominated in both elapid and viper venoms. The venom proteome of elapids and vipers, respectively, contained an average of 83% and 67% of these protein families. PLA2, SVMP, and SVSP are major contributors in venom of vipers, although there is less variation in the proportions of the various protein families than there is in elapids (Tasoulis & Isbister, 2017).

2.2.1.1 Families of dominant proteins

For three-finger toxins (3FTXs) proteins, with the exception of *Crotaline Atropoides nummifer* (venom containing about 0.1%), these toxins are only found in elapids. They account for 95% of the *Micrurus tschudii* venom, and are found in venoms of 98% of

elapids species. Other studies show trace amounts of 3FTxs in *Sistrurus catenatus* (Doley et al., 2008), *Lachesis muta* (Sanz et al., 2008), *Daboia russelii* (Shelke et al., 2002), and *Protobothrops* (Aird et al., 2013), among other viper species.

The phospholipase A2s is the most common protein family found in vipers and elapids is phospholipase A2s. With pancreatic type I in elapids and synovial type II in vipers, the type of PLA2 varied between families. Highest levels reported were *Agkistrodon contortrix* (crotaline) at 51%, *Pseudechis papuanus* (elapid) at 90%, and *Vipera nikolskii* (viperine) at 65% (Fry, 2015). The preganglionic neurotoxins in snake venom are possibly the most dreaded because they cause apneic death, but they also cause edema, tissue damage, and most importantly, pain (Gutiérrez et al., 2017; Jami et al., 2017; Ferraz et al., 2019). Biogenic amines, prostaglandins, bradykinin, and other substances that cause pain are produced as a result of PLA_2 activity on phospholipids at the bite site and elsewhere following release into the circulation (Costa et al., 2017; Ferraz et al., 2019).

Serine proteases are the least significant protein group in terms of quantity. The highest percentage is found in 31% (*Vipera berus*) and 93% in nearly all vipers (*Ovophis okinavensis*). They are only found in 29% of elapids, with *Notechis scutatus* having the highest concentration at 6%. However, given that there have been few proteome studies of Australian elapids. It is generally known that SVSPs play a significant role in Australian snakes as procoagulants. They may potentially contribute 15% in some other elapids, including *Pseudonaja textilis* (Skejić & Hodgson, 2013). Although this group of enzymes is likely best known for causing coagulopathy after a snake bite (Kang et al., 2011), it has been shown that they also significantly increase pain in multiple ways. Serine proteases trigger the activation of protease-activated receptor 2 (PAR2), which in turn causes pain in various contexts (Lam & Schmidt, 2010; Cattaruzza et al., 2014). Human cancer cells have been shown to secrete serine proteases in murine models that cause pain when injected into the hind paw (Lam & Schmidt, 2010).

Metalloproteases are the main protein family found in the venom of all included viper species. For viperines (*Echis ocellatus*), the maximum amount present is 72%, and for

crotalines, it is 85%. They are less significant in elapids. Despite being found in 88% of species, they make up a substantially smaller percentage of the venom; the highest quantities ever found were 19% and 12% for *Calliophis bivirgata* and *Ophiophagus hannah*, respectively. There is evidence implicating metalloproteases with pain from snake bites. In mice, a metalloprotease, batroxase purified from *Bothrops atrox*, triggered the release of IL-6 and the production of PGE2, LTB4, and CysLTs (De Toni et al., 2015; Menaldo et al., 2017). Additionally, metalloproteases found in the venom of *Bothrops jararaca* and BaP1 found in venom of *Bothrops* asper increased hyperalgesia in a mouse model through TNF- and PGE2-dependent pathways, respectively (Zychar et al., 2010). Also, hyperalgesic effects was associated with BpirMP, a metalloprotease isolated from the venom of *Bothrops pirajai*, in a rat model (Bernardes et al., 2015). The cleavage of interleukin-1 by these enzymes activates microglial cells or astrocytes depending on the metalloprotease implicated, which has been linked to neuropathic pain (Kawasaki et al., 2008). Metalloproteases can also activate PAR2 as with serine proteases (Li & Tai, 2014). This group of enzymes can also have a strong procoagulant effects, associated with ischemia discomfort and arterial thrombosis (Kang et al., 2011).

2.2.1.2 Families of secondary proteins

Studies by Tasoulis and Isbister (2017) on database relating to snake venom proteomes revealed the following secondary proteins;

L-amino acid oxidases are most prevalent in crotalines, where in *Rhinocerophis cotiara*, they contribute 20% in venom proteins. About 91% of crotalines have these toxins in their venom. They reach levels of up to 6% in elapids and viperines (Tasoulis and Isbister, 2017).

In black mambas, this family of kunitz peptides contribute 61% of the venom (Oliveira et al., 2022). In viperines, these peptides are found in three genera namely; *Bitis*, *Macrovipera*, and *Daboia*. This implies their lack in crotalines is the result of a reversal or secondary loss. They are found in 13% of elapid species (Tasoulis and Isbister, 2017).

Most snake venoms of different families contain secretory proteins that are high in cysteine, and are prevalent in venoms of vipers (88% of species) and elapids (56% of species). In content wise, rates of 10% for elapids and 16% for vipers are the highest quantities ever found in snake venom samples (Tasoulis and Isbister, 2017).

In the light of disintegrins proteins, elapids do not have these proteins in their venom. About 88% of viperines species and about 68% of crotalines species also have these proteins. For viperines and crotalines venom content, these proteins contribute 18% and 17%, respectively. Also, vipers are significantly more dependent on nephrouretic peptides or protein class than elapids are for bite impact. About 20% elapid species have these proteins, and it is found in 3% of *Dendroaspis polylepis* venom. They are found in 60 and 35% species of crotalines and viperines. It contribute to 11% of *Vipera berus* and 37% of *Bothriechis nigroviridis* venom contents, respectively (Tasoulis and Isbister, 2017).

In elapids, snaclecs/C-type lectins proteins make up just 2% of their venom. However, they are found in all viperine venoms, and *Daboia russelii's* venom contains the highest amount (22% of venom content). They are found in about 88% of crotaline species, and contribute to 31% of *Bothrops insularis'* venom content (Tasoulis and Isbister, 2017).

2.2.1.3 Other significant inter-family venom proteins

The presence of 3FTxs as predominant proteins in venom of elapids distinguishes them from vipers which have very small content. They have been found in several viper venoms, and Aird et al. (2013) showed that these studies used transcriptomics rather than proteomics. In *Agkitrodon bilineatus, Bothropoides pauloensis*, and *Crotalus viridus*-all crotalines, less than 0.5% of vespryns/ohanins contributed to their venom. While DISs are present in almost all viper venoms (ranging from 2-6% average of venom content), they are not present in elapids. CTLs, another protein family, are a small component in elapid venoms. They are only present in roughly a third of the species and in rates no greater than 2%. They are a common component whose contribution ranges between 6-8% in viper venoms. NPs show similar pattern as well (Tasoulis & Isbister, 2017).

2.2.2 Venom characteristics in Elapid family

Two protein families namely PLA2s and 3FTxs contribute more than 75% of the entire venom composition of 90% of the elapids. The difference in venom characteristics demonstrated by New World coral snakes (*Micrurus*) is highlighted by Lomonte et al. (2016). They have shown that varied amounts of PLA2s and 3FTx are found in venom of different species. Afro-Asian cobra venoms, have 3FTxs protein as major contributors. The smaller, less significant *Australo-Papuan* elapids (Elapidae: Hydrophiinae) comprise largely 3FTxs in their venom. According to Lomonte et al. (2016), coral snakes' of the old world may have had mainly 3FTxs in the venom, with PLA2 appearing in the later day proteomics. This pattern has also been observed in the Australia-New Guinea region: sea snakes (Lukoschek & Keogh, 2006; Sanders et al., 2013), Australian terrestrial elapids, and New Guinea terrestrial elapids (Strickland et al., 2016). This finding is based on proteomics studies of a small number of species. Similar Australian elapids, Kraits (*Bungarus*) are dominated by PLA2s. These proteins account for about half the content of the venoms and fewer 3FTxs. In contrast to other elapid groupings, they have higher concentrations of the secondary protein families like kunitzs, LAAOs, and CRiSPs (Tasoulis & Isbister, 2017).

Most cobra (*Naja*) species have more 3FTxs in their venom than PLA2s. CRiSPs, are highly abundant in two non-spitting species, *N. haje* (10%) and *N. melanoleuca* (7.6%). These cobras also lack many of the auxiliary protein families. *Dendroaspis* (Mambas) venom aside from Calliophis lack PLA_2s but contain specialized kunitz. They are dendrotoxins and Kv1 channel blockers. They also have 3FTxs that had been altered to act as both acetylcholinesterase inhibitors (fasciculins) and l-type calcium channel blockers (calciseptines) (Tasoulis & Isbister, 2017).

2.2.3 Implications of venom proteins in health

With the caveat that some significant groupings of snakes have not been thoroughly studied, PLA2s are found in venom of 95% of the two medically significant snake families, elapids and vipers. This raises the possibility of designing an assay that identify the source of snake venom in bodily fluids. This has been earlier demonstrated for vipers and elapids in measurements of phospholipase activity from patient samples (Maduwage et al., 2014). The findings that just a small number of protein families contribute to snake venom from large snake families or subfamilies, encourages effort to develop all-encompassing antivenoms. Such antivenoms will cross-neutralize venoms from different species of snakes (Isbister et al., 2014).

Also, considering a classic example, 3FTxs, which are dominant venom proteins found in the venom of most elapid snakes. The toxin isoforms that have been produced by gene duplication and accelerated evolution share a structure of multiple β-hairpin loops extending from a hydrophobic core stabilized by disulphide bonds, but they also differ significantly in the exposed loops that protrude and interact with target-site receptors (Sunagar et al., 2013). There are those 3FTxs that interact with haemostatic elements like Factor X and platelets, or those that directly cause cytotoxicity and cause local tissue damage (Sunagar et al., 2013). There are comparable levels of functional variability in other venom toxin families. Several SVMP isoforms cause coagulopathy by directly activating or cleaving blood clotting factors, while others work in concert to cause haemorrhage by destroying basement membrane components (Slagboom et al., 2017). Many other toxin families also play a role in systemic diseases by working in concert with pertinent physiological targets. For example, certain PLA2 toxins block presynaptic potassium channels, whereas SVSPs activate Factor V or break down fibrinogen (Sunagar et al., 2013; Slagboom et al., 2017)

2.3 Pathophysiology of snakebite or envenomation

Bites most frequently affect the lower extremity as a result of unintentionally stepping close to the snake (Gutiérrez et al., 2011; Norris et al., 2021). This is especially true in low- and middle-income nations where victims frequently traverse rural trails at night. As cold-blooded snakes go for a warm environment at night, bites happen in areas where it is common to sleep on the ground or in low beds. Also, because more snakes are being kept as pets in the western world, there have been an increasing number of reports of exotic venomous snake bites. Thus, when attempting to handle the snake, victims frequently get bitten on the upper extremity and are frequent when drunk (Norris et al., 2021).

The majority of venomous bites come from species like Viperidae and Elapidae species which have fangs that are anteriorly positioned. Although posterior fanged snake envenoming is uncommon, it can be severe, especially with bites from species like the boomslang snake (*Dispholidus typus*). Even within subspecies, snake venoms are a complicated mixture of peptides, enzymes, and other poisons (Warrell, 2010; Norris et al., 2021). This makes it possible for the venom to cause a number of systemic reactions in potential victim. The toxins that result in tissue necrosis and have negative effects on the circulatory, nervous, and coagulation systems are the most clinically relevant ones (Warrell, 2010). Snake venoms contain a variety of substances that have an impact on the entire body. Pre- and post-synaptic blockers that produce significant local necrosis are among them, for example phospholipase A2 (Warrell, 2010). Since there is still a lot of species-specific variation and the toxicology of snake venom is complex, developing an effective antivenom is challenging (Gutiérrez et al., 2011).

Tissue necrosis, resulting in significant soft tissue loss, is a frequent clinical outcome of snake bite. Viperidae and Elapidae family, which includes species of rattlesnake and puff adder, poison their victims and cause tissue necrosis by secreting cytotoxic chemicals. Cell death, severe local inflammation, and ischaemia resulting from cell lysis, enhanced vascular permeability, and microvascular thrombosis (Warrell, 2010). Various factors can

cause the systemic inflammatory response syndrome, leading to severe local and systemic sepsis. Often, debridement is necessary (Warrell, 2010; Norris et al., 2021). A typical after effect of cytotoxic envenoming that causes abrupt renal failure is snake bite-induced nephropathy. Nephropathy is caused by rhabdomyolysis, cardio-vascular impairment, alterations in the microcirculation, and coagulopathy. Vasculitis, acute tubular necrosis, and glomerulonephritis are three pathological alterations that can affect the kidney and cause a variety of clinical symptoms (Zuliani, 2023).

According to current theories, snake venom only affects the peripheral neural system, with very little or almost no ability to penetrate the central nervous system (Osipov & Utkin, 2015). As it affects both pre- and post-synaptic receptors, toxicology is intricate. The clinical symptoms are very diverse, with respiratory depression and neurogenic shock being the most terrifying. The first signs of neurotoxicity caused by venom from black mamba (*Dendroaspis polylepis*), are a ptosis, metallic taste, and gradually developing bulbar paralysis (Dreyer & Dreyer, 2013). These individuals need to be treated quickly since they have a high chance of dying.

Significant cardio-vascular damage in envenomated patients can result in a range of clinical symptoms with multifactorial etiology. It is believed that increased vascular dilation and permeability involved; this may be due to bradykinin release (Warrell, 2010). Cardiogenic shock is a complication of severe bites due to cardiac-specific myotoxic substances and conduction abnormalities caused by venom. Ischemia owing to coronary artery thrombosis secondary to coagulopathy can make things even more difficult (Gaubert et al., 2020).

Coagulation disorder brought on by a snake bite is a complicated clinical issue. It accounts for a significant fraction of snakebite fatalities and is potentially fatal due to its complicated pathophysiology, however, it is often treated with antivenom (Jeon et al., 2019). Some chemicals that are hypothesized to prevent hemostasis include disintegrins, phospholipases, and lectins. Some snake species' venom contains procoagulant substances such as factor V, X, and XIII as well as pro-thrombin activators, which causes

a pro-thrombotic condition (Abd El-Aziz et al., 2019; Oliveira et al., 2022). Depending on the venom sub-type, platelet aggregation can be either hindered or promoted. Patients' laboratory results frequently show significant derangement without corresponding clinical manifestation. The more frequent causes of disordered clotting must be treated differently than snakebite coagulopathy since conventional therapies may be harmful and ineffectual (Zeng et al., 2022).

2.4 Syndromic management for major snakebite problems

The care of snakebite is challenging due to the global shortage of antivenom, particularly in the remote tropical areas. In some situations where antivenom is unavailable, effective management of the specific clinical state brought on by envenoming can be achieved (Zeng et al., 2022).

2.4.1 Progressive paralysis

Envenoming that is neurotoxic might result in quick decline and death. This is frequently brought on by Elapidae species inhabiting south/east Africa. Minor local tissue injury or severe necrosis with accompanying coagulopathy may be present in certain cases (Gutiérrez et al., 2011). As the initial risk to life from neurotoxicity is significantly greater than that from tissue necrosis, the administration of an arterial tourniquet while awaiting hospital transport is recommended in cases of neurotoxic envenoming. In almost all situations, these patients or individuals require antivenom. If clinical symptoms and indications strongly imply neurotoxicity, antivenom medication should not be postponed due to unknown snake involvement. This involves breathing difficulties, peri-oral paraesthesia, a metallic taste, trouble swallowing, and excessive salivation. Antivenom administration is necessary if individuals are able to support their own respiratory function but experience a generalized weakness with a sudden start (Bickler et al., 2023). Antivenom has a great deal of efficacy in undoing synaptic neurotoxicity, unlike cytotoxic envenoming. Except in cases where the venom also has severe cytotoxic or coagulopathic effects, recovery is achieved if patients are ventilated and anti-venom is administered (Gutiérrez et al., 2011).

2.4.2 Coagulopathy

Coagulopathy and neurotoxic or cytotoxic inflicting are commonly associated with venom, may be the main poisonous impact of some bites. Depending on the venom and the haematological interference it causes, the coagulopathic effects can vary substantially. It is important to keep in mind that although venom's coagulopathic actions might result in incredibly aberrant test findings, however, clinical morbidity or fatality often result. As much as 50% of deaths from snakebites worldwide are caused by snake bite coagulopathy, which often has a hemorrhagic propensity but sporadically can have pro-thrombotic effects (Dreyer & Dreyer, 2013).

Unlike other venom effects, coagulopathy brought on by snakebite does not respond well to conventional therapeutic approaches. Antivenom administration is the sole successful therapy. Antivenom indications include systemic bleeding, intracranial hemorrhage with clinical proof, prolonged bleeding from small skin lesions, and markedly abnormal coagulation test results (Zuliani, 2023). Depending on the clinical responses, patients may need to get antivenom multiple times. A repeat dosage is necessary if blood coagulation profiles are still abnormal six hours following antivenom delivery (Warrell, 2010). It is important for the doctor to remember that cyto- or neurotoxic envenoming frequently coexists with coagulopathy. All clinical bite-related sequelae in these patients should be treated as necessary, including resuscitation.

2.4.3 Localized necrosis and progressing painful edema

Some bites from elapidae (e.g., cobras, mambas, kraits) and viperidae (e.g., puff-adder, diamondback rattlesnake) species have been linked to extremely cytotoxic effects (Gutiérrez et al., 2011). In many regions of the world, notably Africa, this is the most typical snake bite presentation. The venom's cytotoxic effects develop quickly and could be severe in late-presenting patients. Once tissue damage has taken place, administering anti-venom can be relatively ineffectual due to local tissue necrosis and the chemical makeup of cytotoxic venom. These bites can be effectively managed clinically when approached in a systematic fashion (Hamza et al., 2021). It is important to keep in mind

that gradual paralysis caused by venom from African Cobras poses a larger immediate threat to life. Patients presented with progressive tissue necrosis should be given enough analgesia while the afflicted limb is raised. Rehydrating with fluids is a crucial component of management. As a result of the venom's cytotoxic effects, patients may experience fluid loss, and myoglobinuria-related processes put them at risk for acute renal injury (Gutiérrez et al., 2011). It is important to keep a watchful eye on tissue necrosis in the affected limb. Debridement is advised for 5-7 days after the bite if it is necessary. This enables enough demarcation margins to form and helps prevent needless operating room visits for patients who are unstable. Only in cases where sepsis symptoms are present is antibiotic therapy recommended (Smith et al., 2013).

Complications of cytotoxic, acute renal failure, compartment syndrome, rhabdomyolysis, and myoglobinuria are all side effects of cytotoxic envenoming. Compartment syndrome is rare and, if clinically necessary, should be treated via fasciotomy (Garner et al., 2014). Rarely, the inguinal ligament may capture the femoral vessel, leading to an ischemic lower limb. With elevation and analgesia, carpal tunnel pain caused by bites to the upper limb typically improves (Zuliani, 2023).

2.5 Immunology of snake venom

Upon the injection of snake venom, the body goes through two parallel processes; including the development of induced toxic effects by the venom toxins, and activation of innate and adaptive immune responses meant to neutralize and eliminate venom proteins. An envenomation occurs when the animal's ability to absorb and react to the aggression is outweighed by the snake venom's capability to cause both local and systemic harm (Leon et al., 2011). The physicochemical properties of snake toxins enable the host's immune system to recognize them as foreign molecules and trigger an immunological response in addition to giving them the ability to confer toxic effects. The way the toxins are delivered into immune system results in different antibody responses. Therefore, generating effective antivenoms in animals depends on the generation of an antibody

response that is efficient in recognizing and neutralizing the toxic proteins of venoms (Leon et al., 2011).

Snake venoms contain proteins and peptides, making them a cocktail of many bioactive molecules, and these biomolecules disrupt the core physiological processes or systems of envenomed individuals. Snake envenomation affects several physiological systems, including the immune system. Activation of the immune system causes pain, edema, complement system activation, recruitment of neutrophil, and release of cytokines (examples include IL-6, IL-1β, TNF-α and IL-10), as well as release of inflammatory mediators such as LTB4 and PGE2 (Pucca et al., 2022).

Although the complement system is known as an important innate immune defense mechanism, overactivation has been associated to the pathology of snake envenomations. Through the use of an ex vivo model of human whole blood, Leonel et al. (2022) demonstrated how the venom of the *B. jararaca* snake stimulates the complement system, which plays a major role in the inflammatory process. Furthermore, the function of innate immune cells during snake envenomations has also been studied. The involvement of mast cells in the inflammatory process caused by *B. jararaca* venom was examined by Kondo et al. (2022) using mice chosen for maximal (AIRmax) and minimum (AIRmin) inflammatory response. The results showed that, mast cells play a role in generation of reactive oxygen species (ROS), pain and neutrophil migration in AIRmax mice which are vulnerable to the venom.

This process in the case of viperid snake venoms is marked by the production of discomfort, an inflammatory cell infiltration, and a noticeable edema. Eicosanoids, cytokines, nitric oxide, matrix metalloproteinases, complement, kinins, and other mediators are among the many that orchestrate this inflammatory response (Maia-Marques et al., 2022).

The venom of some elapids such as *Dendroaspis* sp. and coral snakes, is distinguished by eliciting a limited inflammatory innate immune response, as opposed to the venom of veperids which exhibits a significant inflammatory activity (Baudou et al., 2023).

In the light to adaptive immune response to envenomation, B cells and T cells make up the majority of the adaptive arm of the immune system. The production of antibodies (immunoglobulins; Ig) for humoral defence is the main effector activity of B cells. A variety of subsets of T cells, such as cytotoxic (CD8+) T cells and helper (CD4+) T cells (TH), generate T cell effector functions. While CD4+ T cells preserve homeostasis and influence proinflammatory and regulatory immune responses, cytotoxic CD8+ T cells defend against intracellular pathogens and inhibit the formation of tumours and infectious diseases (Koretzky, 2010).

Venom protein-specific antibodies are produced in response to deliberate venom inoculation or snakebites. Venom activity is successfully neutralized by antibody-mediated neutralization. However, protection against rapid venom activity necessitates an instant reaction by a primary B cell which is slow in response, taking days to weeks to fully activate (Harwood & Batista, 2010). Due to this, antivenom, which is produced in large mammals and refined for medical use, can offer passive immunity to victims of potentially fatal envenomation as an alternative to host antibodies (Gilliam et al., 2013; Restano-Cassulini et al., 2017).

For a primary (thymus-dependent) humoral response towards envenomation, APCs, including DCs, MNCs, and MΦs, capture and process venom proteins at the site of injury after a bite, and facilitate maturation. Via membrane-bound peptide-MHC II protein complexes, matured APCs move to secondary lymphoid tissue to expose naïve TH cells to the venom antigen (Harwood & Batista, 2010). TH activation (signal 1) is triggered in lymph nodes when a T cell receptor (TCR) binds to the relevant peptide-MHC complex. After which, APCs give vital secondary signals that are necessary for full T cell activation. APCs, particularly DCs ligands (such as CD80 and CD86) for T cell co-stimulatory molecules, including CD28 (signal 2), are extensively expressed (Magee et al., 2012). T cell proliferation and differentiation are then induced by circulating and APC-derived cytokines (as well as autocrine IL-2) (signal 3). CD4+ T cells develop a TH2 phenotype in response to external immunological challenges, like envenomation, and perform effector activities, such as activating B cells (Harwood & Batista, 2010).

The immunotherapy of snakebite envenomation is limited by the specificity of antivenom antibodies against a limited set of venoms. Many ramifications for the development and application of antivenoms result from this problem. The choice of venoms for the immunization mixtures must first be thoroughly examined, considering factors like the immunological cross-reactivity of different venoms, clinical manifestation of the various envenomation syndromes, and medical relevance of different species (Leon et al., 2011).

2.5.1 Effect of venoms on the immune system and nature of immunological reactions induced venoms

Snakebite is known to result in cell-specific venom-mediated immune dysregulation such as venom-induced allergic reaction and systemic inflammation. Globally, venom is frequently linked to allergic reactions (Mullins et al., 2016). Immune-mediated respiratory and/or cardiovascular failure can result in fatality, even though the majority of bite reactions are localized and self-limiting (Stone et al., 2013). IgE-mechanisms are primarily responsible for mediating systemic reactions in these cases. Systemic reactions occur in cases of 0.3% to 8.9% of venom-sensitized victims (Pesek & Lockey, 2013).

A process of sensitization precedes the onset of classic IgE-mediated allergy illness. Alarmins, cytokines (IL-4, IL-5, and IL-13), and other proinflammatory mediators necessary for the formation of antibodies are released when keratinocytes and resident immune cells identify damage brought on by harmful substances, such as compounds produced from venom (Curotto de Lafaille et al., 2010). The generation of plasma cell IgE antibodies is triggered by DCs capturing and processing antigen for presentation to naïve T cells in draining lymph nodes (Curotto de Lafaille et al., 2010). After a bite, elevated IgE is a typical physiological reaction, and is not always indicative of illness (Galli et al., 2016, 2017). However, following several bites, systemic IgE levels in certain victims remain high for a prolonged period of time and can cause systemic reactions, including anaphylactic shock (Stone et al., 2013).

During secondary antigen challenges, immediate (Type-1) allergic reaction symptoms appear. The main cause of Type-1 allergy responses' clinical outcomes is histamine.

Histamine, which acts on H1 and H2 receptors, produces an increase in vascular permeability, smooth muscle contraction, airway constriction, and epiglottis edema in allergic diseases (Stitt & Katial, 2016; Thangam et al., 2018).

A potentially fatal immunological disorder brought on by snakebite is systemic inflammation, which includes cytokine release syndrome. Clinical manifestations of systemic inflammations associated with snakebite include neurotoxic and cardiotoxic manifestations, bleeding, acute kidney injury, rhabdomyolysis, nausea, vomiting, diarrhea, diaphoresis, anxiety, confusion, fever, chest pain, difficulty breathing, paresthesias, low blood pressure, and potential death (Zuliani, 2023).

2.6 Preventive measures against snakebite

In this area, management remains a key challenge, even for the most seasoned clinicians, treating venomous snake bites remains difficult. Patients frequently appear later than necessary after the clinical symptoms of envenoming are well established due to the lack of emergency transportation and the rural location of the majority of bites (Potet et al., 2021). According to some studies conducted in Africa, presenting late does not necessarily result in a worse outcome (Michael et al., 2011). However, this inference may be challenged since untreated hemotoxic envenoming can cause fatal coagulopathy, whilst late presentation from neurotoxic bites can cause respiratory collapse and death due to hypoxia.

Through education, snakebites can be prevented amongst people at risk. This include sleeping under bed net and for children in a cot rather than the ground (Ochoa et al., 2021). Also, keep firewood, termite mounds, and garbage away from human habitations since they can attract snakes. In the household areas, efforts should be made to stop rodent growth and reproduction. Snakes like to hide in mud and straw walls and thatched roofs; thus, these structures should be examined frequently. Wearing boots and long pants while engaging in outdoor activities, strolling at night, and using a torch or flash light could all dramatically lower the likelihood of getting bitten (Pe et al., 1998).

2.6.1 Challenges associated with access to antivenoms

The lack of antivenom and cutting-edge medical technology makes it even harder for professionals to treat snakebite victims. The majority of bites take place in low- and middle-income rural tropics and subtropics where resources are scarce and access to healthcare is problematic (Ooms et al., 2021).

Also, the accurate identification of the snake that was involved in an individual's bite is a significant barrier to effective snake bite therapy. Even within nations, the species of snakes that bite differ significantly in geographical regions. This makes creating a regional or national treatment plan challenging. In 40% of cases, the patient is unable to recognize the snake, and it is typical to mistake it for another species (Barnes et al., 2021; Sapkota et al., 2020). Additionally, traditional healers frequently try to treat the bite using unconventional procedures. This exacerbates the situation further due to the cultural beliefs of many rural areas (Warrell, 2010; Michael et al., 2011). Inappropriate first aid procedures frequently worsen the consequences of envenoming in rural areas due to a lack of education among healthcare professionals and the general public (Michael et al., 2011).

Because different antivenoms are required in different regions of the world, producers often develop products for those regions. The World Health Organization reports that there are 46 facilities generating antivenoms derived from animals, 31 of which are government-owned (many of them in Latin America) (Gutiérrez et al., 2011, 2017). There is a therapeutic gap because just one is based in sub-Saharan Africa.

2.6.2 Treatment of snakebites

The two main types of treatment for a toxic snakebite are supportive care and antivenom therapy (Paudel et al., 2015). The amount of time that passes between snakebite envenomation and antivenom therapy has a significant impact on treatment success. The death rate may be 2.6% if antivenom treatment is started within 12 hours, and around 13.5% if started after 24 hours (Natarajan & Natarajan, 2016). Lack of public knowledge, education, and widespread misconceptions about snake bites prevent patients from getting the appropriate care at facilities that treat snake venom (Muller et al., 2012).

Also, antivenom usage is not the only option for treating bites from deadly snakes. Artificial ventilation and careful airway management are necessary to prevent asphyxiation in patients with respiratory paralysis in the event of neurotoxic envenoming (Bickler et al., 2023). In addition, anticholinesterase medications have demonstrated good success in the treatment of cobra bite envenoming and can partially overcome postsynaptic neurotoxic blocking. A few instances of effective anticholinesterase usage in krait bite envenoming have also been documented in India (Anil et al., 2010). At the bite site, bacterial infections can arise and may need antibiotic therapy (Chaudhary et al., 2020). In the event of cobra bites, necrosis of the bitten limb may necessitate surgery and skin grafts (Fujioka, 2015).

2.7 Antivenom: Development of snake antivenom, types and administration

In accordance with WHO guidelines from 2010, those who experienced envenomation symptoms with neurotoxic evidence, such as ptosis, external ophthalmoplegia, respiratory paralysis, and other signs of hemotoxic poisoning, were treated using anti-snake venom (WHO, 2010; Paudel et al., 2015). Antivenom is a pure fraction of immunoglobulin or immunoglobulin fragments segregated from the plasma of animals who have received venom immunization. To get the appropriate antivenom, a hospital should be aware of the most frequent envenomations (Warrell, 2010). Antivenom must be refrigerated and stored in liquid or lipophilic form. Storage temperatures for antivenom range from 2 to 8 °C (Goswami et al., 2014; Kamal et al., 2015). The first antivenom was developed in 1895 by Calmette. He tested his first antivenom against the Indian Cobra in an effort to neutralize the venom poisons (Kamal et al., 2015). Antivenom comes in two different varieties: monovalent and polyvalent (Poudyal et al., 2017).

In the production of antivenom types using venom, a single species of venomous snake or a few closely related species whose venoms exhibit clinically useful cross-neutralization are the only ones that can use monovalent antivenoms. There may not be a clear clinical symptom to guide the use of a monospecific antivenom in the majority of countries, which are home to multiple snake species that are medically significant. In these circumstances,

developing polyvalent antivenoms should be strongly advised (Meissner, 2010). Many nations favor polyvalent antivenoms due to issues with specific diagnosis. Animals can be immunized with a combination of venoms from various snake species to produce polyvalent antivenoms. The antivenom antibodies from numerous snake species will then be present in the final antivenom (WHO, 2016). Horses can be immunized with a combination of snake venoms to produce polyvalent antivenom (Ratanabanangkoon, 2023).

In instances when local symptoms worsen, antivenom is recommended. Included in this is swelling at the digits or toes, that is rapidly growing, or swelling that covers more than half an extremity (Sheikh et al., 2018). Also, at any indication of a systemic disease, including any test abnormalities, antivenom is also administered (Kleinschmidt et al., 2018; Edgerton & Koepplinger, 2019). Reports has it that early intervention (within 4 hours after envenomation) and greater dosages of antivenom have been reported to improve patient outcomes and reduce the likelihood of surgical intervention (Fry, 2018; Hernandez et al., 2019). Antivenom can, however, be administered within 24 hours of envenomation and still have a positive impact (Barish & Arnold, 2022). Antivenom should be administered until clinical and laboratory abnormalities are resolved because there is no maximum dose that can be used (Sheikh et al., 2018; WHO, 2019; Barish & Arnold, 2022). According to a study, an envenomation scale is used to help make this choice (Correa et al., 2014). Thus, antivenom should be infused initially at a rate of 2 mL/min or as a diluted bolus with 5 to 10 mL/kg of isotonic saline given at a constant rate over a period of one to two hours (Warrell, 2010; Barish & Arnold, 2022), and the addition of an indication that adverse effects are less likely to develop with a slower rate of antivenom infusion (Kleinschmidt et al., 2018).

2.8 Antivenom reactions and management

More than 10% of patients experience antivenom reactions, making it a regular occurrence. The severity of adverse reactions might range from minor (discomfort, rash, diaphoresis, and diarrhea) to major or severe issues like hypotension, anaphylactic shock, angioedema, and bronchospasm (Kleinschmidt et al., 2018). However, from early Type I hypersensitivity reactions to late serum sickness-type reactions, are the common reactions range. Patients who have already been exposed to animal serum are particularly at risk because of the usage of animal serum, which causes hypersensitivity. Pre-administration sensitivity testing should not be used since it is ineffective, takes too long, and is performed on critically ill patients (Warrell, 2010).

Antihistamines, corticosteroids, or a combination of the two should be given next if an adverse reaction is discovered after antivenom injection (Silva et al., 2016). In most cases, early reactions are managed with epinephrine; adults are injected with 0.5 to 1ml of 0.1% at the earliest sign of a reaction. If the reaction cannot be controlled, the dose may then be given again. Epinephrine may be administered intravenously (IV) slowly to patients who have severe hypotension, bronchospasm, or laryngeal edema. Antipyretics are used to treat pyrogenic symptoms. Glucocorticoids should be used to treat reactions until all symptoms disappear (Feng et al., 2022).

Furthermore, when a patient has an immediate reaction, intra-muscular adrenaline is the preferred treatment. Anti-histamines and corticosteroids are recommended as with other anaphylactic causes. Anti-venom recipients must be observed for at least two hours after treatment (Warrell, 2010).

2.9 A need for increased production of safe and effective antivenoms

The sole medically-validated remedy for snakebite envenoming has been animal-derived antivenoms since 19th century, and there are laboratories that manufacture antivenom across the continent. The antivenom product is IgG molecules, bivalent F(ab')2 and monovalent Fab (Dias da Silva et al., 2022). An intricate patchwork of public and commercial laboratories of various sizes and capabilities make up the global community of antivenom producers (WHO, 2019). Some of them are modest manufacturing enterprises that are primarily found in public buildings and cater to the demands of particular nations. The production and distribution of antivenoms across numerous nations or regions is handled by other, larger laboratories. These diverse manufacturers have a wide range of technological capabilities. There are laboratories that lack well-trained employees, have subpar facilities, and need to enhance their systems and products. Laboratories exist which have suitable, modern facilities with procedures that conform to good manufacturing standards (GMPs). Some nations or areas produce enough antivenom to meet their national demands. Examples are United States, Europe, Brazil, Mexico, Central America, Japan, Thailand, and India. Others particularly in sub-Saharan Africa, including Kenya are not self-sufficient. Some producers who had provided antivenoms to Africa have stopped doing so, largely due to financial difficulties and pressure from shareholders as these businesses transition to the private sector (WHO, 2019). Implementing a plan to increase the production of secure and efficient antivenoms based on the following crucial elements is vital for these middle-income or underdeveloped nations. Kenya is faced with snakebite issues like other African countries. Shortages and high cost of imported antivenom remains a challenge. Therefore, working towards manufacturing of its own antivenoms from native species is critical in order to address the challenge of accessing effective antivenom.

2.9.1 Established standards for the production, inspection and quality control of antivenoms

In order to develop recommendations for the production and quality control of antivenoms, WHO launched a global consultation process in 2007. As a result, in October 2008, the Expert Committee on Biological Standardization of WHO published "WHO Guidelines for the Production, Control and Regulation of Snake Antivenom Immunoglobulins." The fact that these Guidelines provide in-depth information on the suggested steps for antivenom manufacture and control—including snake collection and maintenance, venom preparation and storage, animal immunization, hyperimmune plasma fractionation, reduction of hyperimmune plasma—represents a significant step forward to towards increasing the quantity and quality of antivenoms. It is very obvious from the guidelines that antivenom design should be based on the prioritization of medically important snake species within the area of intended use (WHO, 2007, 2017).

2.9.2 Designing suitable venom combinations for antivenom synthesis: The interface between toxicological research and antivenom manufacturing

In contrast to diphtheria antitoxin or tetanus antitoxin, which use a single unique immunogen, antivenoms use a variety of antigens depending on the type of snake. This makes them different from other immunotherapeutics. It is becoming more widely acknowledged that intraspecies regional diversity in venom composition and immunogenicity may be significant enough to alter the clinical effectiveness of antivenoms (Warrell, 2010). A great deal of care must be taken to ensure that the venom mixes utilized in animal immunization for the synthesis of antivenom have the proper composition. The development of antivenom products should consider a number of factors, including the accurate identification of the snake species that account for the majority of deaths and snakebite morbidity in the region or country as well as an analysis of geographic, seasonal, and ontogenetic intraspecies venom variability in order to create reflective venom streams. The above problems necessitate strong cooperation between antivenom-producing and toxicology research laboratories. Regrettably, there is

frequently little or no collaboration between industry and academic experts (Rossoni et al., 2024).

2.10 Making antivenoms available where they are most required, and at affordable prices

From the WHO (2017), increased antivenom production and technological advancements in low-income countries laboratories will make it easier to create safe and effective antivenoms, but they will not necessarily guarantee that these vital medications will get to the affected patients in time to be helpful. The insufficient availability of antivenoms reaching the peripheral health posts near to the places where the majority of snakebites occur is a challenge in this area (i.e. the remote and or rural communities in Papua-New Guinea, Africa, Latin America and Asia) (WHO, 2017).

In the majority of low- and middle-income nations, antivenom is expensive and unavailable. Past and current findings have it that due to the high costs associated with the limited volume and sometimes expensive regulatory contexts in which many antivenoms are produced, their prices are exorbitant. As a result, the cost of product may be too costly for the public health systems of low-income nations, even when an adequate quantity of a particular antivenom is available. Antivenoms ex-manufacturer can be purchased for anywhere between $10 to $1,500 USD (McGain et al., 2004; Gutiérrez et al., 2011; Russell et al., 2021), with dealers and distributors frequently pushing these prices higher. An example is a polyvalent antivenom made in India, was sold at US$10 in India, but inflated to US$149 in Cambodia (Gutiérrez et al., 2010), and the same antivenom had its local price also soared to US$940 per vial in Papua-New Guinea (Warrell, 2008). When antivenoms are supplied to low-income nations at unacceptably high prices, one of the results is that it is impossible to purchase enough dosages to ensure the clinical cure of every patient. An adequate supply of anti-venoms cannot be guaranteed by the free market alone, especially in low-income nations. If the cost of antivenoms is to be better suited to customers' budgets, a coordinated plan of action is required. One of the key approaches is the improvement of public laboratories and strengthening of local researchers with the

ability to manufacture antivenoms at reasonable costs, as is the case in many low-income countries of the world (Rossoni et al., 2024).

2.11 Models for developing antivenom: Animal Sources and production associated challenges

2.11.1 Horse, goat and sheep-derived antivenoms

Polyclonal antibodies created in horses following particular immunization, followed by ammonium sulfate fractionation of the pepsin-treated blood plasma, are the current antivenoms utilized as therapies for envenoming by toxic animals. There has been a review of the benefits and drawbacks of using horses as production animals for antivenom (Ratanabanangkoon et al., 2020; Ratanabanangkoon, 2023). Large-scale antisera production in large animals is unquestionably advantageous, though. A different animal model is required for the creation of a secure and affordable antivenom, primarily due to the high costs associated with antivenom manufacturing in horses in developing nations. Antivenoms generated from goats and sheep are similarly likely to cause early adverse reactions and serum sickness (Sjostrom et al., 1994). For those who have previously been sensitive to horse antivenom, they might be helpful. However, caprylic acid fractionation of IgG demonstrated a further method to reduce the side effects (Cheng et al., 2021). This is done through isolating the IgG fraction of antivenom by precipitating other plasma proteins.

2.11.2 Camel-derived antivenom

Currently produced antivenoms for Africa are made from horses or sheep, which are not suitable for the arid climate that dominates much of sub-Saharan Africa. Logistics-wise, camels are a viable alternative to horses and sheep for producing antivenom in these areas because they are equally easy to handle, immunize, and bleed, and their blood yield is comparable to that of horses. Camels are also well adapted to arid environments, making them likely less expensive to maintain. Camelid IgG's distinct physicochemical characteristics present exciting opportunities to raise the efficacy of antivenom therapy (Cook et al., 2010).

According to Herrera et al. (2005), camelid IgG is less immunogenic and less likely to activate the complement cascade than ovine or equine IgG. This means that people receiving camelid IgG antivenom will experience fewer side effects like serum sickness and anaphalactoid, frequently linked to the use of current antivenoms. It is also possible to prepare antivenom that is still effective after being stored at room temperature using camelid IgG, which has an unusually high thermostability (Ratanabanangkoon et al., 2016). This would significantly increase the supply chain for treating snakebites in remote parts of Africa.

2.11.3 Chicken-derived antivenoms

Polyclonal antibodies are found in large quantities in the yolk of chicken eggs, and offer an alternative to mammalian antibodies and the effects they cause (Abbas et al., 2019). From an ethical perspective, using chickens to produce antibodies is appealing, particularly in light of the three Rs principle: refining, replacing and reducing the use of laboratory animals whenever possible (WHO, 2017). It is a significant improvement to be able to extract antibodies without restraint or bleeding of the animals. Despite structural variations, the chicken immunoglobulin (IgY) functions similarly to mammalian IgG. Avian immunoglobulins were originally categorized as IgG-like immunoglobulins, but Leslie and Clem presented experimental evidence demonstrating structural differences and recommended the designation IgY in 1969 (Zhang et al., 2017). IgY is now known to

be an antibody found in lungfish, birds, reptiles, and amphibians. According to a study, IgY does not cross-react with mammalian antibodies or rheumatoid factors, does not attach to cell surface Fc receptors, does not activate mammalian complement, and does not bind to protein-A or protein G (Tabll et al., 2024).

The ability of chicken eggs that have been hyperimmunized to tetanus toxins to reduce the lethality of the toxin in mouse models was first discovered by Klemperer in 1893, and IgY has been used to control tetanus (Selim et al., 2015). IgY is a more advantageous alternative to mammalian IgG due to its advantages high protein yield (Thirumalai et al., 2019), cost, and noninvasive nature of antibody production, convenience for producing a large number of antibodies (Cai et al., 2012), noninterference of IgY with rheumatoid factors, and human anti-mouse antibodies (HAMA) (Pereira et al., 2019). When comparing the costs, practices, and supplies required to maintain horses and hens in Costa Rica, it was found that the prime cost of production could be cut by about 40% (Navarro et al., 2016). Additionally, horses for antivenoms should be between the ages of 3 and 10 (WHO, 2017), while hens begin laying eggs at around 20 weeks (Yuan et al., 2015). This fact, together with the global industrialization of chicken farming, may lower animal upkeep costs and make it easier to provide and replace hens to produce immunoglobulins. Additionally, when egg sample collection replaces animal bleeding, the discomfort and distress of animals are considerably minimized. The current lines of laying hens can produce between 17 and 35 g of IgY annually, of which 1-10% is antigen-specific (Pauly et al., 2011).

2.12 IgY antibodies

Birds, reptiles, amphibians, and lungfish all have IgY. It has been called “avian IgG” and is thought to be the evolutionary ancestor of IgG and IgE. It also functions similarly to IgG in mammals (Pereira et al., 2019). But there are significant structural and functional differences between these two Igs, starting with their molecular weights-IgY is 180 kDa while IgG is roughly 150 kDa (IgG3 is slightly larger) (*Figure 2.4*). IgY possesses four Immunoglobulin (Ig) domains in the constant area of its heavy chain, but IgG only has three aside from the hinge region, demonstrating that IgY shares the same domain design as mammalian IgM and IgE. Both are made up of two light chains and two heavy chains. IgY is highly specific and more resistant to proteolytic degradation because it lacks the hinge region, which restricts its flexibility (Ferreira Júnior et al., 2018; Abbas et al., 2019; Pereira et al., 2019). Because IgY does not bind to Fc receptors, numerous studies suggest that it promotes the phagocytosis of particular pathogens by mammalian cells by a mechanism unrelated to Fc receptors (Zhou & Ma, 2018). IgY, on the other hand, keeps its structural stability between pH 3.5 and 11.0 and between 30 and 70 °C temperatures. It can be stored for years at 4 °C and up to one month at room temperature, which may be advantageous for its usage as a biotechnological reagent in therapy and diagnosis (Karlsson et al., 2019; Wang et al., 2021).

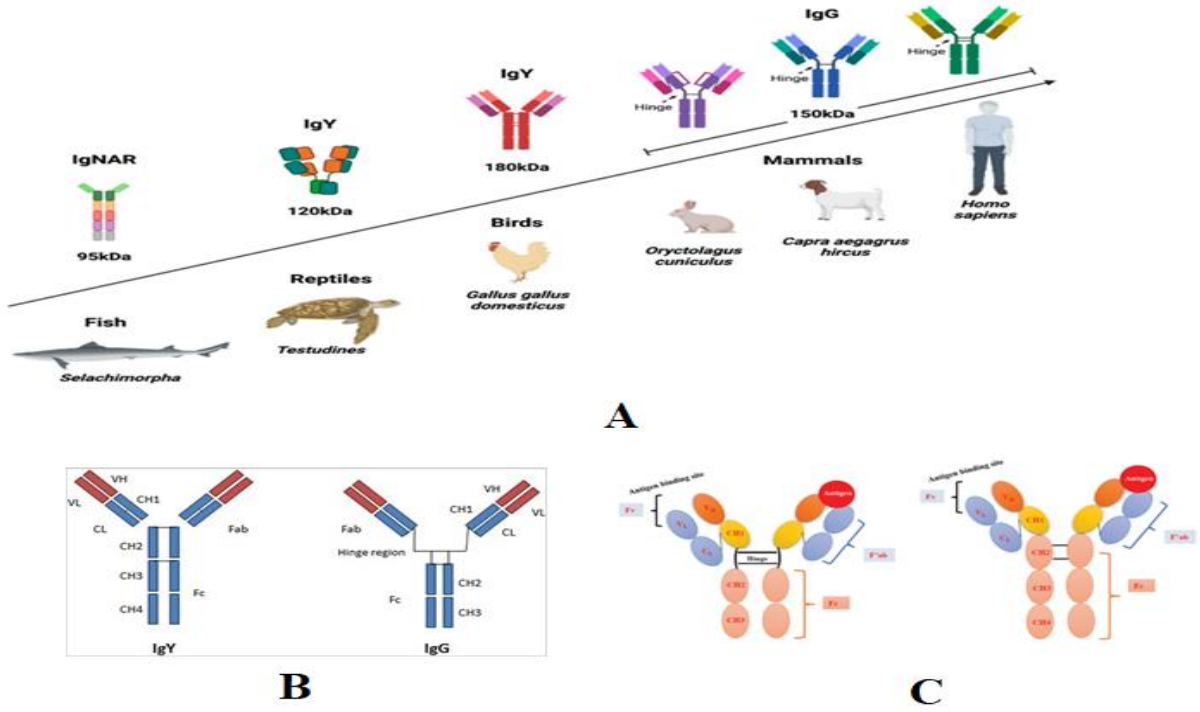


Figure 2. 4: Structural comparison of IgY and the IgG of various species.
***A**-Immunoglobins of different species (León-Núñez et al., 2022); **B**-Structure of IgY and IgG (Pereira et al., 2019); and **C**-IgG and IgY antibodies (Choraria et al., 2022).*

2.12.1 IgY antibody production and purification

Modulators that stimulate B-cells (often called adjuvants) aid in the generation of antibodies, which improves the strength of the immune response despite the fact that synthesis of antibodies is nonspecific and independent of antigen (Harwood & Batista, 2010). Early immunization resulted in the synthesis of IgM being predominant, however with further clonal selection during later immunization, high affinity IgG is found because isotype switching occurs in later stages of B-cell maturation. In hens, class switching is demonstrated where peak IgY concentrations can be seen after 10-25 days after immunization, whereas higher IgM concentrations are seen in the early days following immunization (Lee et al., 2017). The intramuscular route in the chicken's breast muscles is the most popular method of immunization because it results in higher amounts of particular IgY production than the subcutaneous method (Kovacs-Nolan & Mine, 2012). To reduce stress on the bird, intramuscular immunization in the legs should be avoided.

Even 150 days after the initial immunization, booster doses administered at intervals of two weeks have led to a rise in antibody titer (Meenatchisundaram & Michael, 2010).

It is worth noting that poultry, mostly hens but also turkeys, quails, ducks, geese, and ostriches, can produce IgY antibodies (Ge et al., 2021). When made from egg yolk, IgY manufacturing is inexpensive and results in high yields (Pérez de la Lastra et al., 2020). According to some estimates, the average amount of IgY in an egg is about 60 mg, and 50 immunized hens might create up to 1 kg of antibodies in a year (Pauly et al., 2011). The size of the egg determines how much of the refined form is present; ostrich eggs are the largest, weighing about 1.5 kg, and quail eggs are the smallest, weighing around 10 g. The housing situation, egg generation period, and annual egg yield must all be considered (Vieira-Pires et al., 2021). Chickens are still chosen because of these needs, and more recently, the widespread usage of chickens has inspired the development of a transgenic strain for IgY antibodies that has both the human variable region and the avian constant region. This method enables the production of monoclonal antibodies for safe use in therapy by cloning the human variable regions with highly specific IgY antibodies into human constant-region genes (Ching et al., 2018). A chicken produces 28-42 g of IgY per year from laying about 280 eggs per year, with each egg yolk containing 100-150 mg of IgY antibodies (Karlsson et al., 2019).

IgY antibodies can be isolated in the lab using a variety of techniques, including water dilution, PEG precipitation, anionic polysaccharide treatments, organic solvent treatments, and other treatments with particular chemicals. However, each of these techniques has to overcome the problem of removing yolk lipid. Specifically, the structure, charge state, and microheterogeneity, each of which needs to be determined by at least two analytical methods, must all be characterized for antibodies aimed at treating human diseases, and the purities allowed are high (Staub et al., 2011; Zhang et al., 2021). Two techniques-the polyethylene glycol (PEG) extraction approach and the water dilution method—have been employed often to separate antibodies from the yolks of hyperimmunized eggs. Despite the fact that there are various techniques for isolating IgY

antibodies, including those involving chloroform, caprylic acid, phenol, and carrageenan (Ren et al., 2016), the aforementioned techniques yield antibodies with a high degree of purity. The water dilution method calls for a six-fold dilution of egg yolk in water, a pH adjustment to 5.0, and a 6-hour incubation period at 4 °C. After that, ammonium sulfate (60% w/v) precipitation is done, which is then preceded by ultrafiltration and ion exchange chromatography. PEG 6000 is used in various concentrations in the PEG extraction process. The egg yolks are, in a nutshell, diluted twice with PBS (pH 7.0), added 3.5% (w/v) of PEG6000, and centrifuged at 10,000 g for 20 mins at 4 °C. After centrifuging and filtering the supernatant, 8.5% PEG is added. The following step entails removing the supernatant, adding PEG with 10 ml of PBS to a final concentration of 12%, and centrifuging. Using Ion exchange chromatography or Bio-affinity purification, the resulting pellet is further purified after being dissolved in PBS (Pauly et al., 2011).

According to Akita and Nakai (1993), the majority of hens who receive immunization are White leghorn hens and hybrids of Rhode Island red and white. The effective dose for developing antivenom antibodies is based on the LD_{50} of the specific venom utilized to immunize the chickens.

2.12.2 IgY antibodies' capacity for neutralization of snake venoms

Lethal elements found in snake venom are crucial for capturing and killing prey. Phospholipases (PLA2), L-amino acid oxidases, C-type lectins, metalloproteases, serine proteases, hyaluronidase, cysteine rich secretory proteins (CRiSP), esterase, disintegrins, three finger toxins (3FTx), and myotoxins, that produce pro-coagulant, hemorrhagic, defibrinogenating, myolitic, necrotizing and neurotoxic activity are some of these elements (Gasanov, 2014; Kularatne & Senanayake, 2014; Choudhury et al., 2017; Pla et al., 2019). Several studies have assessed the ability of IgY antivenoms to neutralize a number of immunologically significant snake proteins and enzymes. The first team to produce IgY antivenom against *Crotalus atrox* venom was Thalley and Carroll (1990). They showed that antibody concentration might reach 1 mg/mL of yolk even four months after the original inoculation. Literatures on trend the IgY ability to neutralize snakes

venom are illustrated as follows; Almeida et al. (1998) investigated the possibility of utilizing IgY antivenoms to counteract the PLA2-dependent hemolysis and hemorrhagic activity of *Bothrops* and *Crotalus* snake venoms. The components of the venom from *Crotalus* snakes (Crotoxin and crotamine) are mild immunogens in horses, but when administered to hens, a robust immunological response was elicited. After two weeks from the first dosage, which included the complete Freund's adjuvant, IgY antivenom antibodies were initially detected in the blood, and there was an increase in titration following the administration of booster doses. Additionally, they noted significant antibody titers 168 days following the vaccination. They verified that IgY antivenoms could, in a dose-dependent way, neutralize PLA_2 and hemorrhagic activities when pre-incubated with the venoms of *Bothrops* and *Crotalus* snakes (Almeida et al., 1998).

In Vietnam, IgY antibodies against the venom of *Calloselasma rhodostoma* were given to patients (N=30) who had been bitten by the specific snake in a study done by Kiem in the year 2000 (Kiem, 2000). As far as the clinical studies of IgY antivenom are concerned, this is the sole report that is currently accessible. Sadly, there are currently no leads concentrating on the use of IgY antivenom in clinical trials. Currently, equines are used to develop and manufacture the *C. rhodostoma* antivenom (Díaz et al., 2014). IgY anti-viper venom was created by Maya and colleagues. Their findings demonstrated that antivenom antibodies were produced after 12 days of immunization, and specific antibodies were found in egg yolks for up to 90 days as confirmed by immunodiffusion experiments. A yield of 80–100 mg of antibodies per day from a single egg was also noted (Maya et al., 2002).

In order to minimize the venom's toxicity and prevent any tissue damage at the vaccination site without compromising the venom's immunogenicity, Brunda et al. (2006) employed detoxified *Naja naja* venom for the synthesis of rabbit IgG and IgY antivenoms. Due to this, antivenom was produced at high titers (1:10,000), as determined by immunodiffusion and ELISA experiments (Brunda et al., 2006). Manjula et al. (2006) carried out toxicological tests using rat models and created IgY anti-*E. carinatus* snake

venom. They tested the potential for acute oral, intraperitoneal, intramuscular, and mucous membrane irritation as well as the allergenicity and mutagenicity of IgY antibodies in rats for a total of 28 days. IgY from egg yolks does not exhibit any clinical indicators of toxicity or mortality in experimental animals, according to the first study to use parenteral injection of IgY antibodies. This opens the door for IgY antibodies to be used as a therapeutic alternative (Manjula et al., 2006). A study from Paul et al. demonstrated that hens inoculated with the venom of *Echis carinatus* showed a stronger response with a rise in antibody titer in both serum and yolk after administration of booster doses, with IgY in sera being identified after 8 days post-immunization in lower amounts (once in 4 weeks). Western blotting was used in their investigation to validate the dose-dependent specificity of IgY antivenoms, and *in vivo* tests provided additional confirmation (Paul et al., 2007).

From the "Big Four" snakes (*Daboia russelii, Echis carinatus, Naja naja*, and *Bungarus caerulus*) studies of India, the neutralization of phospholipase, hemorrhagic, edem, and pro-coagulant forming activity were observed (Meenatchisundaram et al., 2008a, 2008b). Thus, following the immunization of chickens with venoms from *Daboia russelii* and *Echis carinatus*, the research team discovered an enhanced titer of 1:10,000 and protein concentrations of 0.3-6.7 mg/ml and 0.5-6.9 mg/mL for *Daboia russelii* and *Echis carinatus* venoms, respectively. Chiou (2008) isolated IgY antivenom antibodies from duck egg yolks that had been injected with cobra (*Naja atra*) and krait (*Bungarus multicinctus*) venom that had been detoxicated. The pepsin-digested Fab component of IgY was used instead of the entire molecule, and its potential to neutralize was investigated. Duck IgY (ΔDFc) and Fab fragments both exhibit equal neutralizing efficacy in his trials, supporting the use of duck IgY from duck egg yolks as a substitute source for the production of antivenoms (Chiou, 2008). By immunizing hens with the venom, Chinese cobra (*Naja naja atra*) IgY antivenoms have been created. After the initial vaccination, 10 days later, antibodies were found. Utilizing ELISA, titer examination revealed a rise after giving chickens a booster, there was a change in titer readings (Liu et al., 2010). One of the most lethal snake venoms in the world is that of the Australian taipan

snake, *Oxyuranus scutellatus*, and from which Navarro et al. (2016) created anti-*O. scutellatus* IgY. Also, after 15 days post-immunization, Duan et al. (2016) produced IgY antivenoms against the venom of *Trimeresurus albolabris*, and these in-house IgY antivenoms demonstrated good specificity when isolated from egg yolks.

Recently, a team assessed the ability of IgY antivenoms to neutralize the venom of *Daboia russelii* and *Echis carinatus* snakes (Choraria et al., 2021). Even 90 days after the initial dose of immunization, they found high titers of 1:32,000 for *D. russelii* venom and 1:64,000 for *E. carinatus* venom. These IgY antivenoms demonstrated good neutralizing capacity against a challenge dosage of $3LD_{50}$ when they were further compared with rabbit IgG antivenoms and commercial equine antivenom (Choraria et al., 2021). A paper-based micro-fluidic diagnostic kit for the detection of *Daboia russelii* snake venom was recently undertaken by Lin et al. (2020). The IgY antivenom used in this kit were produced from goose eggs. The Limit of Detection (LoD) of the venom in clinical snakebite samples was shown to be as low as 10 ng/ml.

2.13 Detection methods for snake venoms

An easy and reliable method for determining the species responsible for a snakebite is antibody-based venom protein detection. From reports, several immunoassays for identifying venom proteins in bodily fluids have been described (Kulawickrama et al., 2010; Stone et al., 2013), including enzyme-linked immunosorbent assays – ELISAs (Nong et al., 2023), radioimmunoassays, fluorescence immunoassays (Gilliam et al., 2013), and agglutination assays (Kulawickrama et al., 2010). Immunology-based biosensors have also been investigated for the purpose of detecting snakebite in addition to immunoassays (Teja et al., 2018; Choowongkomon et al., 2024). Undoubtedly, the two best immunoassays for identifying snakebites are ELISAs and lateral flow assays (Hung et al., 2014; Pawade et al., 2016). ELISAs, the most popular and all-purpose immunoassays in clinical application, are sensitive to their target at picogram per levels of milliliter (Gilliam et al., 2013). For routine diagnosis, ELISA equipment and reagents are inexpensive despite the comparatively high cost of antibodies.

2.14 ELISA-based techniques for snake venom detection

Using the double sandwich approach and a microliter plate with 96 wells, the use of enzyme immunoassays (EIAs) or ELISAs, in detecting individual venoms and the indirect detection of specific venom antibodies (including antivenom), was first described in 1977 (Theakston & Laing, 2014). The ELISA has reportedly been used to detect numerous venoms all over the world, and was first used to detect a range of 1 to 5 ng of venom/mL in 3 hours (Lakkappa et al., 2015). The kinetics of snake venoms in blood, the severity of envenomation, and the effectiveness-or lack thereof-of antivenom serotherapy have all been studied using ELISAs, which are now frequently employed. In comparison to other immunoassays, ELISA is still thought to provide more practical value in terms of detecting venom antibodies, snake venoms, and toxins in bodily fluids.

From Theakston and Laing (2014), the principle behind ELISAs is that soluble antigens are coated to ELISA plate wells, which maintains the individual components' reactivity. In the double sandwich approach, a given venom antibody is coated to the plate well. The unbound material is removed, and test antigen (venom-specific) added. Finally, the reaction is allowed to set for several hours. An antigen-specific antibody conjugated with enzyme such as alkaline phosphatase or horseradish peroxidase, is used to detect the complex formed between the venom and antibody after additional washing. A subsequent washing step is followed by the addition of an enzyme-specific substrate, and the resulting hydrolysis or color change is measured spectrophotometrically or visually and is considered proportionate to the amount of venom or antigen present in the test sample. It has become possible to detect venom concentrations at picogram levels due to major advancements in ELISA sensitivity. An increase in the affinity of antibodies, which is possible by lengthening the immunization period and speeding up the administration of booster injections, can be used in conjunction with avidin-biotin amplification to improve the sensitivity of ELISA even further. Additionally, venom-specific antibodies purified using affinity chromatography are frequently utilized to achieve species specificity of an ELISA, with the latter appearing to be the most effective for venom detection (Lakkappa

et al., 2015). Several examples of studies on ELISA development and its sensitivity and/or specificity enhancement for venoms detections have been enumerated with reports below;

In South of Vietnam, for the purpose of detecting venoms of four common snake species (*Calloselasma rhodostoma*, *Trimeresurus popeorum*, *Ophiophagus hannah*, and *Naja naja*), an avidin-biotin ELISA (AB-ELISA) assay was developed. An ELISA test kit for venom detection was developed using the study's three-step affinity purification technique to generate species-specific antivenom antibodies. The ELISA kit was able to distinguish between the venoms from the four snakes in the various sample types tested based on the results. The study's AB-ELISA was very sensitive, with the majority of analyzed samples having venom detection limits that were less than 1.6 ng/mL. As a result, the choice of high titer serum samples with high affinity and avidity antibodies as well as antibody purification were credited with increasing the sensitivity of the AB-ELISA assay. Furthermore, experimentally envenomed rats were used to successfully demonstrate the ELISA kit's effectiveness in identifying snakebite envenoming. Furthermore, the results showed that the kit successfully detected venom in each of the 140 human samples (blood, urine, wound exudates, and blister fluids) that were tested for venom (Van Dong et al., 2004).

Also, a study conducted by Núñez Rangel et al. (2012) in Columbia which aimed at developing a sensitive enzyme immunoassay (ELISA) for the identification of *Lachesis acrochorda* venom reported efficiency in a concentration range of 3.9 to 1000 ng/mL. However, the results did not show a cross-reaction with *Bothrops, Portidium, Botriechis* and *Crotalus* venoms. In addition, the venom of an Indian cobra (*Naja naja naja*) was detected in several tissues (brain, heart, lungs, liver, spleen, blood, kidneys, and tissue at the site of injection) using developed sandwich enzyme-linked immunosorbent assay (ELISA). The developed kit was able to detect up to 2.5 ng/mL of tissue homogenate for *N. n. naja* venom, and the venom could still be detected 24 hours after injection (Venkatesan et al., 2014).

A sandwich ELISA test and lateral flow assay were developed as a strategy to improve the clinical management of snakebites in Taiwan in a study by Liu et al. (2018). These assays were designed to identify the four principal snake species that account for more than 90% of all clinical envenomation cases for both hemorrhagic and neurotoxic venom proteins. A two-step affinity purification process was used in the study to produce species-specific antibodies from antivenoms. The biotinylated antibodies against the neurotoxic and hemorrhagic species were then utilized to create the two (sandwich and lateral flow) assays. The interaction of the biotinylated antibodies with the streptavidin conjugated to alkaline phosphatase and using 4-methyl umbelliferyl phosphate as substrate led to the development of the sandwich ELISA assay. The fluorescence was subsequently determined using a SpectraMax M5 microplate reader. The study's findings demonstrated that both diagnostic tests could distinguish between venoms of neurotoxic and haemorrhagic effects. The haemorrhagic and neurotoxic venom limits of quantification for the ELISA were established at 0.78 ng/mL and 0.39 ng/mL, respectively, whereas the lateral flow assay identified the venoms at concentrations lower than 50 ng/mL and 5 ng/mL, respectively, within 10-15 minutes.

Similarly, an indirect double antibody sandwich enzyme linked immunosorbent test (ELISA) based on immunoglobulin Y (IgY) was developed for the purpose of detecting the venom of the Indian cobra (*Naja naja naja*) in biological samples used in forensic investigations. From chick egg yolk and rabbit serum, polyclonal antibodies were produced and purified. In the study, the cobra venom was sandwiched between immobilized affinity purified IgY and the rabbit IgG, and found cobra venom detection with the developed ELISA kit at concentrations between 0.1 and 300 ng (Brunda et al., 2006). Moreover, a study by (Choraria et al., 2018) developed sandwich ELISA for the detection of Russell's viper (*Daboia russelii*) venom using antivenoms (antibodies) from chicken and rabbits. The venom of the Russell's viper was detected using these antibodies, and from the results a range of 10-300 ng of samples of the snake venom could be detected on a plate covered with IgY antibodies.

2.15 Antivenoms and antibodies efficacy in venom neutralization

2.15.1 Snakes venom characterization with standard test techniques or procedures

It was advised to create standardized tests to be utilized in assessing the biological activity of medically significant snake venoms and the potential for antivenoms to neutralize these venoms at a World Health Organization (WHO) coordination workshop on venoms and antivenoms held in 1979 (WHO, 2017). According to the recommendations, common tests for evaluating hemorrhagic, lethality, defibrinogenating, procoagulant, and necrotizing characteristics of venoms were developed. These methods were then used to study about 53 venoms from 30 different medically significant snake species worldwide. This study was intended to develop more potent IgY antibodies for antivenom production for usage in both developed and developing nations. The WHO standardized tests were used to evaluate the neutralizing potency of novel and currently available antivenoms on the commercial markets. Additionally, these tests were anticipated to improve knowledge of snakebites and how they are handled globally (Afroz et al., 2023).

2.15.2 Lethality assessment and neutralization (Median lethal dose-LD_{50})

The median lethal dosage (LD_{50}) of snake venom is crucial for precisely determining if a venom is toxic or not, as well as frequently for the selection of pertinent antivenom batches and the determination of the antivenoms' potential to neutralize in an animal's immune system. The WHO defines venom lethality as the median lethal dose (LD_{50}), which is the amount of venom (or substance) that will cause 50% of injected mice to die. The ability of snake antivenoms to counteract the toxic effect (LD_{50}) of a certain snake venom serves as evidence of their potency. Therefore, the median effective dose (ED_{50}), which is the lowest dose of snake antivenom that neutralizes and protects 50% of the population of injected mice, aids in determining the effective titer of snake antivenoms (Parveen et al., 2017).

In a study by Laustsen et al. (2015), the LD_{50} of the Black mamba (*D. polylepis*) was evaluated in France using pooled venom. From the study, groups of mice (weighing 18-20 g) were injected intravenously, and observation for deaths was done after 24 hours,

and LD_{50} was calculated by probits. The LD_{50} was found to be 0.68 mg/kg for the whole venom. In addition to this, two Indian and SAIMR antivenoms were evaluated for neutralization of lethality, and found to be effective at 95% confidence level with ED_{50} as 0.76 mg/mL (limits of range 0.57-1.32 mg/mL for VINS African), 0.97 mg/mL (limits of range 0.68-1.44 mg/mL for VINS Central Africa), and 5.26 mg/mL (limits of range 3.54-9.02 mg/mL for SAIMR). However, it was observed in the study that SAIMR has 3-times higher protein levels compared to the other two Indian antivenoms leading to the highest efficacy record.

In another study by Ainsworth et al. (2018), the WHO method was followed for venom median lethal dose (LD_{50}) determination using intravenous injection of varying doses of venom in 100 µL in CD-1 mice (18-20 g). Venoms from species of *Dendroaspis* from different origin were considered and the doses were 2-14 µg/mouse (*D. polylepis*; Tanzania), 2-40 µg/mouse (*D. j. kaimosae*; Uganda), 10-45 µg/mouse (*D. j. jamesoni*; Cameroon), 12-40 µg/mouse (*D. viridis*; Togo), and 27-60 µg/mouse (*D. angusticeps*; Tanzania). After 24-hour of administration, survival of mice was observed and at 95% confidence limits for each snake species, the LD_{50} was calculated using probits. Based on venom LD_{50} statistics from murine model, the black mamba (*D. polylepis*) venom poses the greatest medical risk (with LD_{50} 6.2 µg/mouse; 0.33 µg/g). Amidst being closely related to *D. jamesoni*, and sharing both *D. jamesoni* and *D. viridis'* overall venom composition. The venom of *D. kaimosae* is typically two times more potent (with mean intravenous LD_{50} 10.1 µg/mouse; 0.53 µg/g) than that of *D. jamesoni* (22.8 µg/mouse; 1.2 µg/g), and *D. viridis* (21.63 µg/mouse; 1.14 µg/g), respectively.

Also, Ochola et al. (2019), calculated the acute intraperitoneal median lethal dosage (LD_{50}) of black mamba venom in mice (weighing 20-25 g) using the Weil's moving average interpolation method. In addition, two polyvalent antivenoms (antivenom I and II) efficacies were examined. The study recorded the acute intraperitoneal LD_{50} of the whole venom in mice to be 0.341 mg/kg, and for antivenom I with $3LD_{50}$ recording 80%

protection, while at the same $3LD_{50}$, antivenom II afforded 60% protection. However, 4 LD_{50} dose of black mamba venom, the antivenom II afforded 40% protection.

In a study by Menzies et al. (2022), the lethal activity of venoms from five Eswatini snake species; *Bitis arietans, Dendroaspis polylepis, Hemachatus haemachatus* and *Naja annulifera* were evaluated in murine mice weighing 18-20 g using the recommended method by (WHO, 2017). A total volume of 100 µL mixtures were prepared and intravenous administered via the tail vein, and mouse monitored over 6 hours. After which, the LD_{50} and 95% confidence intervals were determined using probit analysis. From the LD_{50} calculated, venoms of *D. polylepis* proved to be the highly potent among the other snake species (9.8 µg/mouse; 8.8-11.0), thus of all the 5 venom samples tested. LD_{50}s for the remaining four species assessed were 32.5 (28.3– 37.2) µg/mouse for *B. arietans*, 26.0 (24.3– 27.7) µg/mouse for *H. haemachatus*, 25.9 (22.4–30.0) µg/mouse for *N. annulifera*, and 17.4 (15.8–19.3) µg/mouse for *N. mossambica*. Additionally, Panafrican-ICP, SAIMR Polyvalent-SAVP and PANAF-PS&V antivenoms were evaluated for venom neutralizing efficacy using ED_{50} and eMND assays, along with a refined version of the WHO recommended antivenom ED_{50}, in which the venom/antivenom combinations were given intravenously after being pre-incubated with 3x or 5x the LD_{50} dosage at 37 °C for 30 minutes with various quantities of each antivenom. In neutralization tests, *B. arietans* venom was most effectively neutralized by SAIMR Polyvalent (6.4; 6.1-6.6 µL/mouse), Panafrican (19.4; 18.4-20.6 µL/mouse) and PANAF (47.5; 46.6-48.4 µL/mouse), followed by *D. polylepis* in the order of 22.1 (21.2–23.0) µL/mouse for SAIMR, 56.1 (53.8–58.6) µL/mouse for PANAF, 79.8 (76.3–83.6) µL/mouse Panafrican antivenoms, respectively. The ED_{50} for the other snake species were *H. haemachatus* (33.5, 125.5, 52:7), *N. annulifera* (77.8, 122.3, 108.4) and *N. mossambica* (80.3, 82.6, 68.0) µL/mouse for the SAIMR, Panafrican and PANAF antivenoms, respectively.

2.15.3 Necrotic activity assessment and neutralization

For venoms, such as those of mambas (*Dendroaspis* species) and others like the cobras found in Africa and Asia, that cause cutaneous necrosis, neutralization of dermonecrosis or necrotic activity in the skin is important. The procedure involves injecting liquids containing various venom doses intradermally in the belly region of rodents, primarily mice or rats. After 72 hours, animals are killed, their skins are removed, and an estimate of the extent of the necrotic lesion in the interior section of the skin is made (Gutiérrez et al., 2017). Liu et al. (2020) defined necrotic activity, expressed as minimal necrotizing dose (MND), as the smallest amount of venom (μg dry weight) that 3 days after intradermal injection (i.d.) of venom into rats, causes a 5-mm diameter necrotic lesion. The procedure for calculating the MND is similar to that for calculating the minimum hemorrhagic dose (MHD), with the exception that for the MND, the dorsal skin is removed three days after intradermal injection of venom samples. Some of the MND studies recorded for mambas and other snake species are given below;

In the same previously described study by Menzies et al. (2022), through utilizing doses of venom in PBS to a total of 50 μL, it was possible to establish the minimal dosage (MND) of venom to induce a lesion of 5 mm diameter. Lesion size and general health of the animals were tracked three times daily for 72 hours. Lesions were removed from the skin and assessed with callipers after the animals had been exposed to increasing carbon dioxide concentrations (euthanized). From the study findings, necrotic activity for *D. polylepis* and *N. annulifera* were not detected. However, the venom MND μg/mouse (average lesion size in mm^2) for the remaining species were 32.0 (22.4 mm^2) for *B. arietans*, 26.0 (15.0 mm^2) for *H. haemachatus*, and 44.0 (25.2 mm^2) for *N. mossambica*, respectively. However, the efficacy (μL) of each antivenom to neutralize lesion after injection with 1 x MND dose were *B. arietans* (10 μL; 1.1, 0.0, 3.9 mm^2 lesion), *H. haemachatus* (15 μL; 0.0, 0.0, 0.0 mm^2 lesion), and *N. mossambica* (45 μL; 3.6, 0.8, 0.3 mm^2 lesion), for SAIMR, ICP and PANAF, respectively. The highest studied doses of *D. polylepis* and *N. annulifera* venoms did not result in necrotic lesions, making it impossible to assess the neutralization of dermonecrosis.

2.15.4 Edema-forming activity assessment and neutralization

Edema at the site of venom injection is typically a defining characteristic of envenomings by several cytotoxic elapids as well as vipers. Experimental studies on edematogenic activity can be conducted by injecting different dosages of venom into the skin of rat or mouse feet. The increase in pad volume is then measured at various time intervals either by plethysmometry or by gauging the thickness of the injected pad with a low-pressure spring caliper. Because prior approaches could only determine edema once, these two procedures have mostly supplanted those that relied on calculating the weight of mice's footpads after euthanized. The amount of venom that causes thickness or a 30% increase in footpad volume an hour after injection is therefore regarded as the minimal edematogenic dosage (MED). Antivenom efficacy (ED_{50}), which is the ratio of venom to antivenom or the volume of antivenom that reduces the edema brought on by venom alone by 50%, has been used as the challenge dose for neutralization, at a dose of 6 MED (Gutiérrez et al., 2017).

2.16 Models for determining toxicity

According to Kasper et al. (2015), toxicity is described as "the degree to which a substance (a toxin or poison) can harm humans or animals." To assess the detrimental effects of chemicals on the human body, toxicity models have been developed. Three main categories of toxicity models—in silico, in vivo, and in vitro (Summerfield & Dong, 2013)—as well as one category—divided into three phases—are now used in preclinical research. This model, clinical toxicity, is managed as necessary by the current drug approval procedure (WHO, 2019).

2.16.1 In silico toxicity model

In silico translates to "performed on a computer or via computer simulation". Drug interactions with the body are primarily predicted using this toxicity model. In silico toxicity models have several benefits, but their principal benefits are their lowered costs and fast testing times (Summerfield & Dong, 2013). There are various in silico toxicity models with various methodologies, and as a result, there are various ways to classify

them (Bassan et al., 2021). Some of which includes; the “art of toxicology”, or “eminence-based” specialist knowledge, is formalized by in silico techniques. In the worst situation, the evaluators’ ‘‘expert knowledge” is nothing more than their instincts. Expert systems at least define them systematically and use the programme to apply them to chemicals of interest. Modelling tools are the next category of in silico technology. More and more people are using modelling tools that were developed from computer-aided drug design (CADD) methods to model receptors and examine how well new structures suit them. Crystal structures are used to model the fit of the test compound into the reactive site of the receptor and to determine the likelihood that it will trigger a response. Typical examples come from protein modelling, such as models of the oestrogen receptor or various P-450 enzymes. These models are normally three dimensional, but it’s important to note that there are also four-dimensional models that consider the induced fit of the drug on the receptor (Dutkiewicz & Mikstacka, 2018).

2.16.2 In vivo toxicity model

Tests conducted on live things are known as in vivo tests. The capacity to examine how medications affect physiological and biochemical processes in living things as well as the accessibility of sizable and well-coordinated databases are the main benefits of in-vivo models (Summerfield & Dong, 2013). It is considered that, if adequately evaluated, any compound’s effect seen in lab animals will also apply to people. The “No Observed Adverse Effect Level (NOAEL)” is one of many in-vivo toxicities testing endpoints that must be established before the start of the clinical phase (Klaassen et al., 2018).

This is only model that considers an organism as a whole, including all physiological reactions and biochemical interactions. As a result, it is the only model that offers details on drug distribution in the organism and potential drug interactions with non-target organs. Therefore, it is possible that in-vivo models offer the most accurate models for assessing toxicity of medicines or antigens. The current in-vivo toxicity models do, however, have considerable drawbacks, particularly in light of the substantial biological variations between humans and other organisms (Madorran et al., 2020).

2.16.3 In vitro toxicity model

In vitro tests isolate a particular component of a given organism-such as an organ, tissue, or cell-and investigate it in a controlled setting with little to no interference from the external or internal environment. As they are produced utilizing the cells or tissues of the target organism, in vitro toxicity models have the advantages of being representative and taking up less time and cost (Summerfield & Dong, 2013). A typical example is the cell-based toxicity models. Comparatively to other in vitro models, cell-based toxicity models are more likely to take into consideration the recognized metabolic networks at the cellular level (Astashkina et al., 2012).

In some circumstances, in vitro cell-based toxicity models are more realistic than in vivo toxicity models due to the intricacy of these models, which now span many organs (Wang et al., 2023). The bulk of the current in vitro cell-based toxicity models, however, do not adequately represent the target organism because they have been overly simplified and have lost one or more essential properties of the organism. The majority of in vitro cell-based organ toxicity models do not include a variety of cell lines specific to the target organ. Therefore, a drug may still be hazardous to other cell lines in the target organ even if it does not cause toxicity in a specific cell line (Astashkina et al., 2012).

2.16.4 Clinical toxicity model

It is undeniable that the target organism itself is the model that most closely resembles the target organism, making clinical testing of drug toxicity the final step in the drug approval processes. The primary objective of clinical research, which keeps track of the body's biochemical equilibrium and metabolism is homeostatic imbalance. These macromolecules are employed as benchmarks in the monitoring of homeostasis since several clinical investigations have demonstrated that they are present in varying degrees in various patients, with some having higher or lower concentrations than others (McPherson & Pincus, 2021).

CHAPTER THREE
MATERIALS AND METHODS

3.1 Study area

The layer hens were raised for eggs and blood collection at the Small Animal Facility for Research and Innovation (SAFARI) at Jomo Kenyatta University of Agriculture and Technology. The extraction and purification of IgY antibodies was done in the Molecular and Biotechnology Laboratory of the Pan African Institute (PAUSTI) at JKUAT campus (Juja, Nairobi-Kenya).

The preclinical evaluation of antivenoms and ELISA-based assay development was conducted at Innovation and Technology Transfer Division (ITTD) of the Kenya Medical Research Institute (KEMRI).

3.2 Study design and project schema

The design of the study followed first by immunizing chickens with *D. polylepis* venom and collection of eggs for the extraction and purification of IgY polyclonal antibodies as well as collecting blood samples for serum extraction. The efficacy of generated IgY antibody was then tested against two selected commercial antivenoms for toxicity neutralization in mice, whereas antibodies generated in serum samples were used in ELISA prototype optimization and development for venom toxins detection. The diagrammatic flow is shown in Figure 3.1.

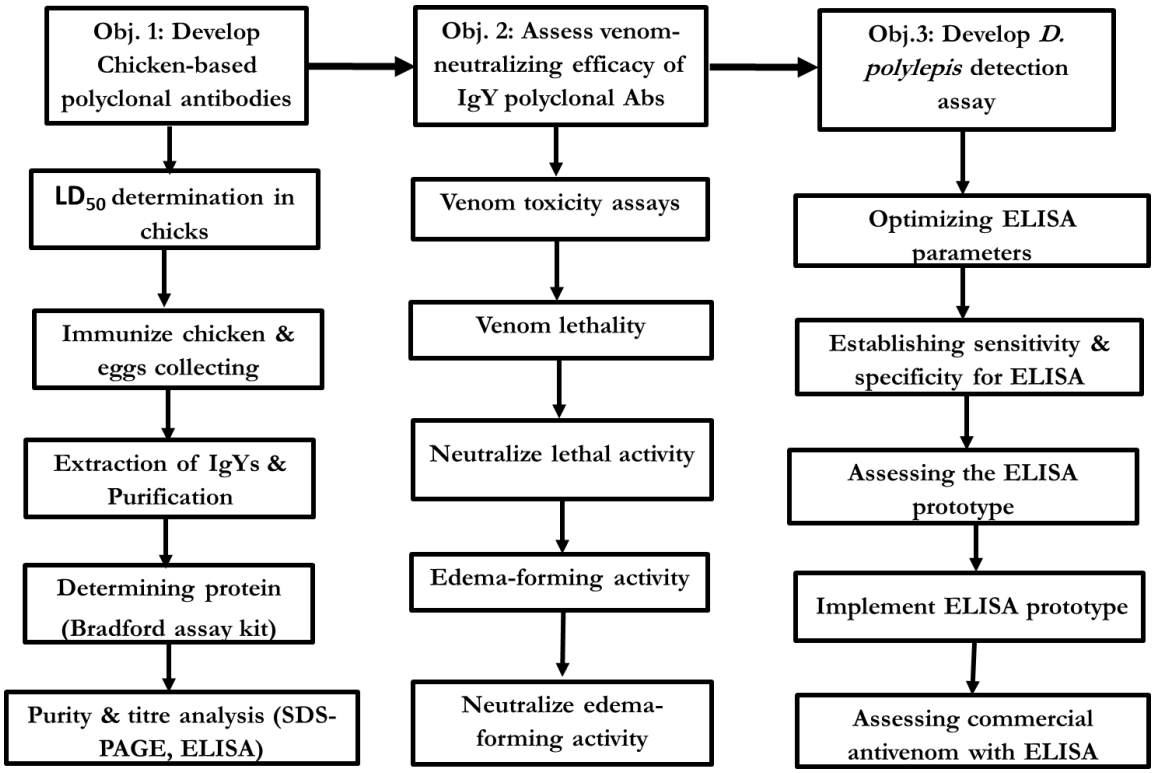


Figure 3. 1: Project method schema.

3.3 Animals, and study population

One-month-old Isa-Brown chicks weighing 500-700 g each (n=30) and seventeen-week-old Isa-Brown hens (weighing 1.7-1.8 kg each, n=5), 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. polylepis* venom, respectively. The one-month old chicks (n=30) were grouped into 5 per cage, whereas, layer hens were kept in individual cages with standard water and food at the Small Animal Facility for Research and Innovation (SAFARI) on JKUAT main campus *(Plate 3.1).* Both female and male BALB/c mice weighing between 28-30 g each and at eight-week-old were obtained from the Kenya Institute of Primate Research (IPR), and used for venom lethality and its neutralizing determination, venom-challenged assay as well as sampling blood for spike samples during ELISA assays. Purchased mice were maintained at the Animal house in the Kenya Medical Research Institute with provision of food and water *ad libitum (Plate 3.4).*

3.3.1 Study inclusion and exclusion criteria

Inclusion criteria: Matured healthy layer hens that had started laying were used for the IgY production, and mice used for laboratory experiment. The health statuses of all animals were determined by veterinarian and animal facility experts.

Exclusion criteria: Pre-mature and unhealthy layer hens showing sings such as labored breathing, deformed eggs, reduced rate of egg production, poor appetite, hard abdomen, behavioral changes, comb and wattle issues among others were excluded.

3.4 Snake venoms and commercial antivenoms

Venoms used in this study were from *D. polylepis*, 2-homologous *Dendroaspis* sp. (*D. angusticeps* and *D. jamesoni*) and 2-heterologous *Dendroaspis* sp. (*B. arietans* and *N. ashei*). The *D. polylepis* venom was used in raising the IgY antibodies in chicken and for the ELISA assays. Other homologous and heterologous snake venoms were used for ELISA specificity and toxins detection assays. Venoms sourced from Baringo County were obtained through donations from the Kenya Medical Research Institute (KEMRI), and archives of Prof. Joseph K. Gikunju at the Department of Medical Laboratory Science, JKUAT, Nairobi-Kenya. Crude venoms were 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.

3.5 Ethical approvals and research license

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). Additionally, a research license or permit (license number NACOSTI/P/23/31716) was obtained from the Kenya National Commission for Science, Technology and Innovation (NACOSTI) for the study (Appendices 1-3).

Objective I: Generation and molecular characterization of chicken-based IgY polyclonal antibodies

3.6 Generation of chicken-based IgY polyclonal antibodies against the *D. polylepis* snake venom

3.6.1 Determination of LD_{50} in chicks

The median lethal dose and LD_{50} dose range-finding test was 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-hour 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. After 24-hour period, the deaths and survivals were recorded, 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 LogDose and probit values for the standard curve.

3.6.2 Immunization schedules for chicken

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; Sigma-Aldrich, St. Louis, Missouri, USA) 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 14^{th}, 35^{th}, 56^{th}, and 77^{th} 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; Sigma-Aldrich, St. Louis, Missouri, USA). Hens in the control group received an intramuscular immunization with 0.5 mL saline only. Daily egg collection was done for 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). Plate 3.1 below depicts housing and immunization of chicken.

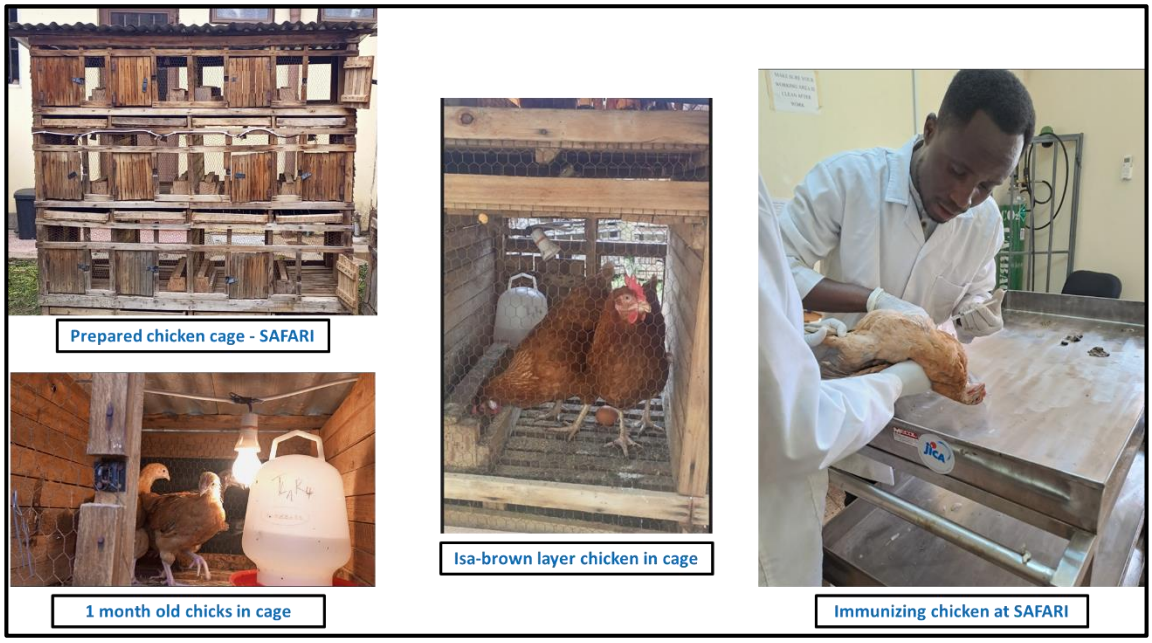


Plate 3. 1: Housing and immunizing chicken for anti-*D. polylepis* polyclonal antibodies production.

3.6.3 Isolation and purification of IgY

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 minutes. 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 minutes 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 minutes at 4 °C, and then precipitated using a 45% saturated ammonium sulphate. Centrifugation at 10,000 rpm for 20 minutes 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 (Sloarbio, China). 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 dialyzed against PBS. It was 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. Plate 3.2 shows the extraction of IgY antibodies from egg yolk, and purification.

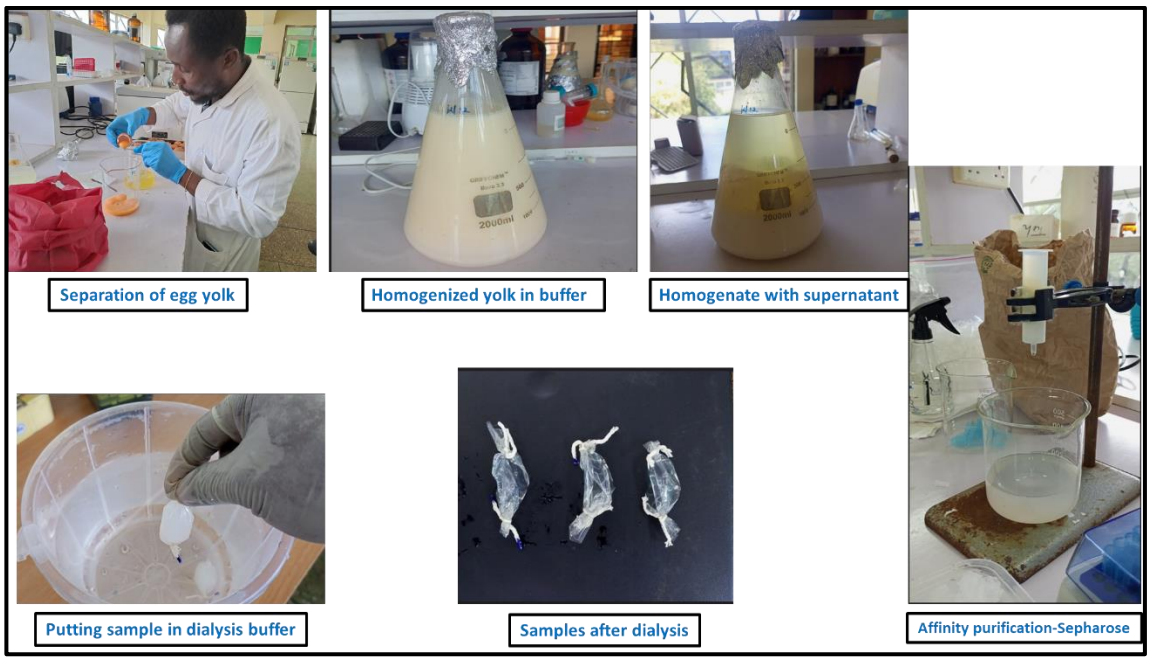


Plate 3. 2: Separating egg yolk, extraction and purification of anti-*D. polylepis* IgY polyclonal antibodies.

Here, the water-soluble extraction method was used to obtain partially purified crude extract of antibody preparation from egg yolks, and then fractionated by venom-column affinity chromatography (Solarbio, Hong Kong, China). Through the three steps fractions (water dilution-WSF, ammonium sulfate precipitation or salting-out-SOF, and venom-column affinity chromatography-VACF). In the different fractions (WSF, SOF and VACF), the venom-specific activities of the IgY from 328 eggs was assayed on an ELISA for comparison.

3.6.3.1 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; Solarbio, China) in line with the manufacturer's recommendations and previously described method (Liu et al., 2017). The electrophoresis system was set up and gel run as in Plate 3.3 below.

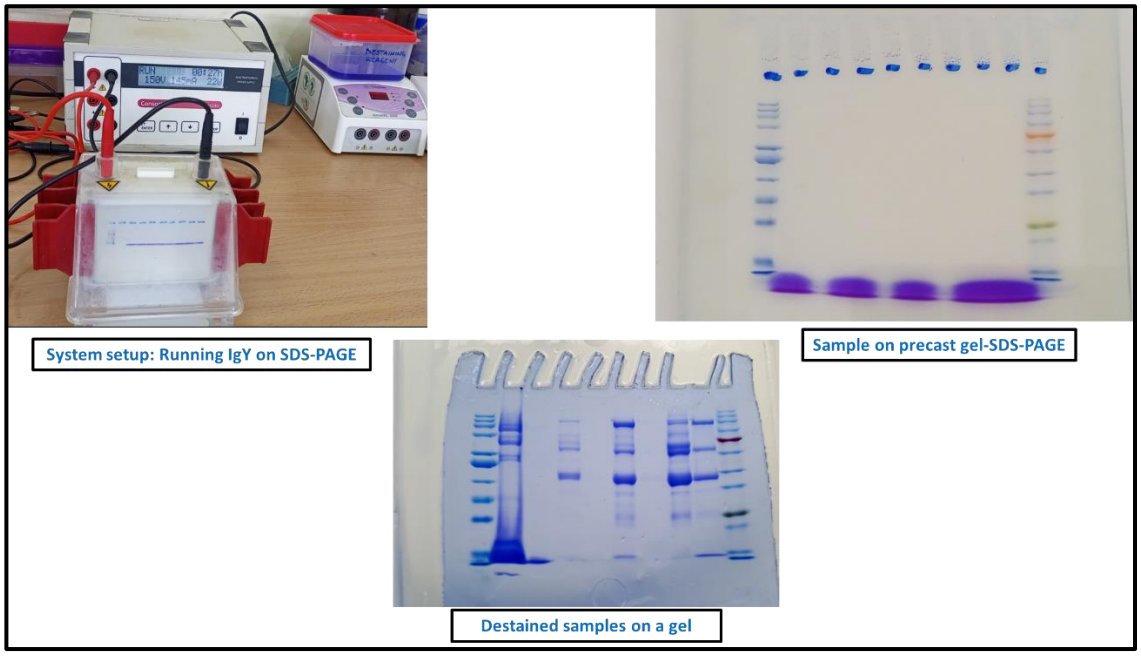


Plate 3. 3: Characterization of purified antibodies on SDS-PAGE using precast gel.

3.6.3.2 Verification of IgY and determination of its specificity by ELISA

The activities and specificity of anti-*D. polylepis* IgY antibodies in the serum and yolk was assessed using indirect ELISA approach (Duan et al., 2016; Liu et al., 2017), 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 hours. 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 hours at 37 °C, and wells washed 3-times. A 200 µL of diluted serum or yolk (1:1,000) in dilution buffer (PBS plus 1% nonfat dry milk) was added to the well and incubated at 37 °C for 1 hour before 3-times washing. A dilution of 1:5,000 for the rabbit anti-IgY peroxidase in blocking buffer was done, and 100 µL added to well, and incubated at 37 °C for 1 hour. Wells were washed again, 100 µL of TMB (3,3',5,5'-tetramethylbenzidine, TMB; Solarbio, China) solution added, incubated for 20 minutes 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.

Objective II: Neutralization efficacies of Chicken-based IgY polyclonal antibodies and commercial antivenoms

3.7 Determining chicken-based IgY antibodies efficacy vis-à-vis commercial antivenoms

3.7.1 Toxic activities of *D. polylepis* venom in mice

3.7.1.1 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 hours. For the full scale median lethal dose (LD_{50}), 0.20, 0.30, 0.35, 0.40, and 0.45 mg/kg were established, a group of 5 mice per dose employed, 200 µL of aliquot injected via i.p route, and observed for deaths and survivors in within 24 hours. 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 (Warrell, 2010; Gutiérrez et al., 2017).

3.7.1.2 Edematogenic assay

The Resiere *et al.* (2018) approach was employed to assess the edematogenic activity of *D. polylepis* venom. A group of 5 mice per dose were used, and each group received subcutaneous injections of a venom dose 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 one hour 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) (Resiere et al., 2018). Images of mice in cages, and observing for edema are shown in Plate 3.4 below.

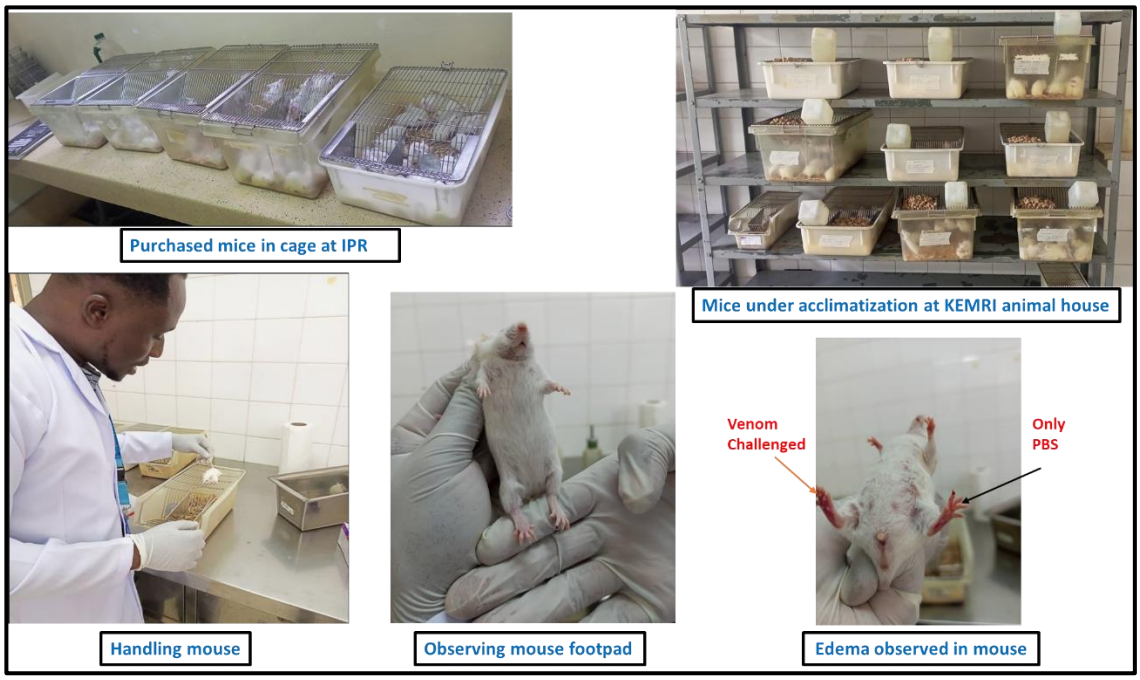


Plate 3. 4: Purchased mice, housing and handling of mice for venom toxicity testing.

3.7.2 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 published procedures (Segura et al., 2010; WHO, 2017; Resiere et al., 2018). In these tests, a set dose of venom challenge was incubated for 30 minutes 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 $3LD_{50}$ and 6MED 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 (StatsDirect, 2013).

3.7.3 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.

Objective III: Development of an ELISA prototype

3.8 Development of an IgY polyclonal antibodies ELISA for *D. polylepis* toxin detection

3.8.1 Optimization ELISA parameters

An indirect ELISA approach, as reported by Islam & Jones (1988) and Manson et al. (2022) with some modifications was used to optimize for the rabbit anti-chicken IgG/HRP conjugated antibody, antigen coating concentration, and substrate sensitivity.

3.8.1.1 Optimizing for secondary antibody

Following the methods for ELISA from reports as indicated earlier in Islam & Jones (1988) and Manson et al. (2022), Lyophilized *D. polylepis* (Black mamba) venom was diluted in coating buffer (0.1 M carbonate-bicarbonate, pH 9.6) to obtain 1 µg/mL concentration. From this preparation, 100 µL was added to each well of a 96-well plate (Maxisorp plate, NUNC, Denmark), covered and incubated overnight at 4 °C. The antigen-coating buffer was removed, washed 3-times with 3 mins in-between each wash using 200 µL of washing buffer (PBS, containing 0.05% Tween-20 $_{v/v}$, pH 7.4), and excess water blotted on a tissue paper. Each well of washed plate was then blocked with 200 µL blocking buffer (2% BSA $_{w/v}$ + washing buffer), incubated at room temperature for 1 hour and washed as previously described. Pooled serum (containing primary antibody) sample

was diluted at 1:1,000 in dilution buffer (PBS, containing 0.5% BSA $_{w/v}$, pH 7.4), and 100 µL of diluted samples added to wells in duplicates before incubating plate at 37 °C for 1 hour, sample removed and washed. A 2-fold serial dilution was performed for the rabbit anti-chicken IgG/HRP secondary antibody (from 1:2,000 to 1:256,000), and 100 µL of diluted antibody added to wells, incubated for 1 hour at 37 °C, removed and washed 3-times. A 200 µL of TMB substrate solution was added to each well, incubated at room temperature in the dark for 15-20 mins, and reaction stopped upon adding 50 µL of 2 M H_2SO_4 solution. Absorbance was measured at 450 nm. However, preliminary secondary antibody dilutions were tried before settling on the range indicated.

3.8.1.2 Antigen coating optimization

Based on the 1-10 µg/mL antigen (*D. polylepis* venom) concentration recommended range (Islam & Jones, 1988; Manson et al., 2022), using a checkerboard approach, different antigen concentrations were tested in parallel. A 1, 2, 4, 6, 8 and 10 µg/mL concentrations of *D. polylepis* venom in coating buffer were used to coat plates, and tested while evaluating two secondary antibody dilution (1:5,000 and 1:1,0000) combinations towards achieving optimal antigen concentration - detection antibody dilution combinations. Serum sample dilutions, blocking and washing of plate, substrate addition and absorbance reading were all done as previously described (section 3.8.1.1).

3.8.1.3 ELISA substrate optimization

The previously described indirect ELISA method was employed to compare tetramethylbenzidine (TMB) and o-phenylenediamine dihydrochloride (OPD) substrates performance. The antigen coating (1 µg/mL *D. polylepis* venom in coating buffer), pooled serum sample (containing primary antibody, dilution 1:1,000), and rabbit anti-chicken IgG/HRP secondary antibody (dilution 1:5,000), blocking and washing were done as previous. A 100 µL of TMB substrate solution was added to each well and incubated in the dark at room temperature for 15-20 mins, whereas, 200 µL of OPD substrate solution (containing 0.1 M Citric acid monohydrate, plus 0.2 M di-Sodium hydrogen orthophosphate 12-hydrate, pH 5.0; 5 mg OPD tablet, 30% hydrogen peroxide) was added

to wells of OPD plate followed by 20-30 mins incubation in the dark at room temperature. After reaction was stopped, absorbance was read at 450 nm and 492 nm, respectively, for TMB and OPD plates.

3.8.1.4 Primary antibody (serum) dilution optimization

All other procedures for the ELISA were same as described previously, except for coating plate with 1 µg/mL of antigen, pooled serum sample dilutions (1:500, 1:1000, 1:2000 and 1:4000), secondary antibody diluted at 1:5,000, and use of the optimized substrate (OPD).

3.8.2 ELISA specificity and sensitivity (cut-off point) determination

To evaluate the ELISA's ability to distinguish between closely related and unrelated venoms, procedures from references were modified and employed (Islam & Jones, 1988; Sharma et al., 2019; Manson et al., 2022). Briefly, two heterologous (*B. arietans* and *N. ashei*) and two homologous (*D. angusticeps* and *D. jamesoni*) venom samples were used to measure the assay's specificity using different sample antigen concentrations (6.6-0.003 µg/mL). A 3-fold serial dilutions of sample antigens were performed in coating buffer for various venom concentrations, and a 100 µL of the aliquot used to coat 96-well plate. 'No antigen control (NAC)' row was included for each snake species venom. Blocking, serum sample diluted at 1:2,000, rabbit anti-chicken IgG diluted at 1:8,000 and OPD substrate solution, done using previously described procedure. Percent inhibition was then calculated across all inhibitor concentrations for venoms, and patterns compared between homologous and heterologous venom samples.

Additionally, sensitivity determination for the ELISA was assayed by coating plate with crude *D. polylepis* venom with initial concentration from 44 µg/mL to a final of 0.02 µg/mL. A 3-fold serial dilution was made, and samples analyzed in duplicates. During which 16-pre-immuned serum samples and sample buffer were analyzed as controls (negative controls, devoid of analyte of interest), and were used in determining the limit of detection (LoD). Consequently, the assay's limit of detection (LoD) was ascertained utilizing the formula below, which is based on the signal-noise approach (Islam & Jones, 1988);

LoD = concentration of antigen in well with % inhibition > mean + 2×Standard Deviation of negative controls

Plate 3.5 below shows the images of specificity and sensitivity assay reactions during inoculation and terminated reactions.

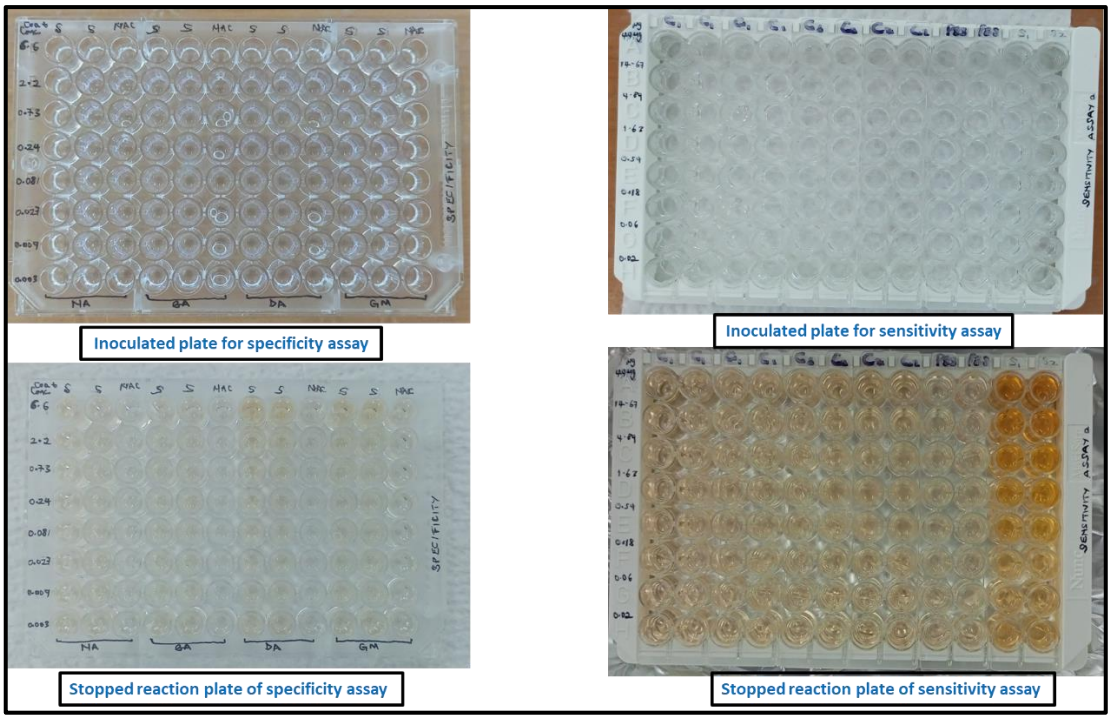


Plate 3. 5: Prepared specificity and sensitivity assay plates.

3.8.3 Inhibition ELISA for detecting toxins in crude *D. polylepis* venom

A modified procedure from Sharma et al. (2019) was employed for this assay. Briefly,

Step 1: a 96-well plate (1) was coated with 100 µL of 1 µg/mL *D. polylepis* venom constituted in coating buffer and incubated overnight at 4 °C. In the following day, solution was removed, plate washed 3-times as previously described and each well blocked using 200 µL of blocking buffer. The blocked plate was incubated at room temperature for 1 hour, washed as previous, and extra water blotted out using tissue paper.

Step 2: a 44 µg/mL final concentration of sample antigen (*D. polylepis* venom) was prepared in blocking buffer, and 150 µL of the aliquot added to wells of row A of a new uncoated plate (2). A volume of 100 µL blocking buffer was added to each well from row B up to H. Next, a 3-fold serial dilution was carried out, thus, 50 µL was transferred from row A to row B, and through until row G, with the extra 50 µL discarded. At dilution of 1:2,000, serum sample (containing the primary antibody) was prepared in dilution buffer, and 100 µL each added to wells including row H of the same plate (2). This was done in in duplicates. The plate (2) was incubated for 1 hour at 37 °C.

Step 3: After that, the sample antigen and primary antibody-containing contents of plate (2) were transferred to plate (1), which had been coated, and incubated for 1 hour at 37 °C. Again, the plate was washed 3-times and 100 µL of diluted rabbit anti-chicken IgY/HRP in block buffer (1:8,000) was added to each well, incubated for 1 hour at 37 °C, and washing step repeated. After washing, 200 µL of OPD substrate solution was added to each well, incubated at room temperature in the dark for 20-30 mins, and reaction stopped upon adding 50 µL of 2 M H_2SO_4 solution. The plate was read using a plate reader (Multiscan EX reader, Thermo Scientific, Massachusetts, MA, USA) at 492 nm absorbance *(Plate 3.6).* Row H which had no sample antigen but contained block buffer and primary antibody was considered 'No antigen control (NAC)'. Against the NAC absorbance, percentage inhibition for each well that contained a different concentration of the sample antigen was calculated as;

$$Percent\ Inhibition = \frac{(NAC\ OD - Test\ Sample)}{NAC\ OD} * 100$$

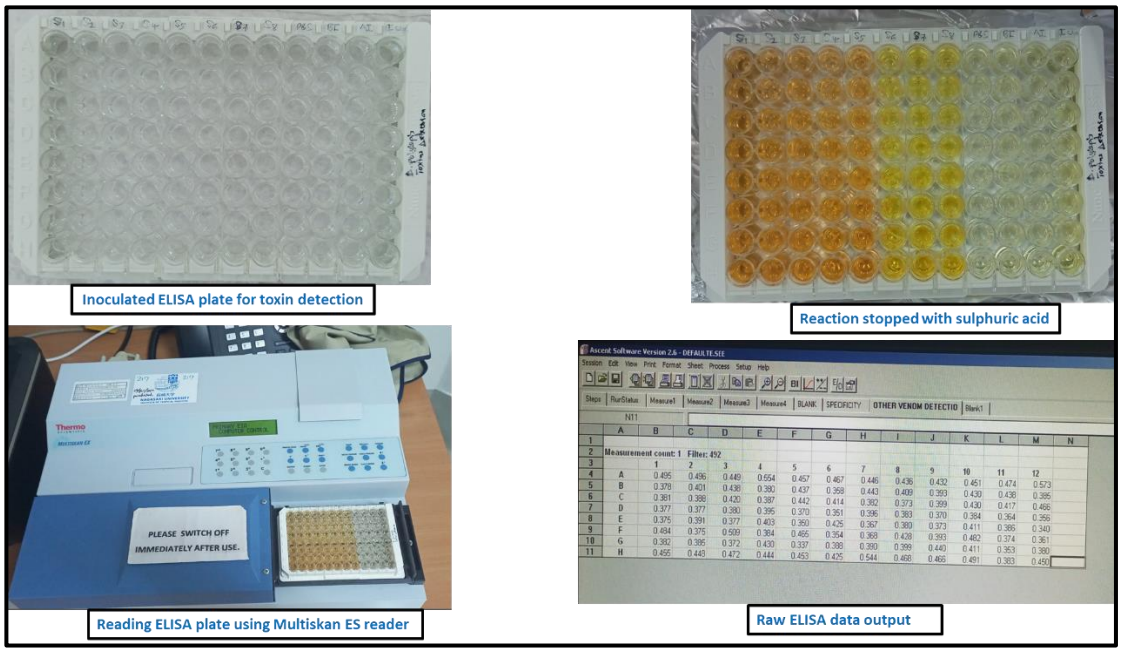


Plate 3. 6: Prepared ELISA plate for toxin detection and reading of absorbance using the Multiskan ES reader.

3.8.4 Evaluating inhibition ELISA's ability for toxin identification in other *Dendroaspis* sp. and non-*Dendroaspis* sp. venom analysis

Crude venoms from two different *Dendroaspis* sp. (*D. angusticeps* and *D. jamesoni*) and two non-*Dendroaspis* sp. (*B. arietans* and *N. ashei*) were used to assess the inhibitory ELISA's capacity to distinguish between venoms containing toxins of *Dendroaspis* sp. and those without. The above procedure (section 3.8.3) was employed for this assay. However, sample antigen concentrations were prepared through serial dilution from 6.6 µg/mL to final of 0.003 µg/mL. Incubation, washing, blocking, addition of serum samples and secondary antibody, and OPD substrate buffer solution were all carried out as described previously. Reaction was stopped adding 50 µL of 2 M H_2SO_4 solution, absorbance read at 492 nm, and determining of percentage inhibition per well was done.

3.8.5 Toxins detection in *D. polylepis* venom-injected mice

In this assay, the inhibition procedure described previously was followed (Section 3.9.3). Mice injected with crude *D. polylepis* venom were used to investigate the suitability of the inhibition ELISA in snakebite envenoming diagnosis. Five mice (BALB/c) were injected with *D. polylepis* venom (concentration of 0.1 mg/mL of venom in PBS). Blood samples were collected before injection, and used as negative control. After the venom-challenge, mice were bled in the tail vein at 0.5, 1, 2, 4, 6, 8, and 24 hours intervals. Samples were collected on ice, stored at 4 °C and immediately used after the last sample collection. Using the optimized parameters, inhibition ELISA method was used as previous and percentage inhibition calculated based on values of the optical density (OD) measured.

3.8.6 Toxins of *D. polylepis* venom detection in spiked samples

The inhibition ELISA procedure described previously was implemented to test the ELISA's ability to detect toxins in blood samples which are spiked with *D. polylepis* venom. Despite similar conditions, steps and components of the assay as previous, sample collection and processing were different. Briefly, initial blood samples were collected from five BALB/c mice and used as negative control or 'No antigen control (NAC)'. To spike the samples, blood samples collection in mice tail vein were sustained at 0.5, 1, 2, 4, 6, 8, and 24 hours intervals. An equivalent volume of reconstituted crude *D. polylepis* venom (concentration of 1 mg/mL) was added to blood samples right after they were collected. Collected samples were stored and assayed as previously indicated. Considering both the individual OD values and the NAC, the percentage inhibition was calculated.

3.8.7 Detection of commercial *D. polylepis* snake antivenom using developed ELISA

Using modified indirect ELISA assay method by Liu et al. (2018) to assess its capacity to identify antibodies produced against toxins of *D. polylepis* and other snake venoms in two commercial antivenoms; PANAF-Premium^{TM} and VINS^{TM} polyvalent antivenoms. Briefly, a 96-well plate was coated using 100 µL of a 1 µg/mL antigen of *D. polylepis* venom in coating buffer, left overnight at 4 °C incubation. Plate wells were emptied after

incubation, washed and blocked with 200 µL blocking buffer following 1-hour incubation at room temperature, as previous. Block plate was emptied and washed 3-times at 3 mins intervals. For each antivenom, a 9-point of 2-fold serial dilutions (from 1:1,000 to 1:256,000) were performed in dilution buffer, and 100 µL of each aliquot was added to respective well in duplicates, and finally incubated for 1 hour at 37 °C. The washing step was repeated after incubation, and 100 µL of diluted (1:8,000) anti-equine IgG/HRP in blocking buffer added to wells. Plate with secondary antibody suspension was incubated at 37 °C for 1 hour, emptied and washed as previous. After which, 200 µL of prepared OPD substrate solution was added to each well, covered with adhesive seal and incubated in the dark at room temperature for 20-30 mins. The reaction was stopped after incubation using 50 µL of 2 M H_2SO_4. Plate was read at absorbance of 492 nm. Rabbit anti-horse IgG/HRP antibody was used in this case because the commercial antivenoms were produced from horse plasma.

3.8.8 Data management

Data obtained from assays were entered into Microsoft excel 2019, and analyzed using OriginPro version 2024 (https://www.originlab.com/myOriginDownload). Substrate sensitivity analysis was done using paired sample t-test. Group mean differences from the detection of other snake venoms, venom detection in spiked blood and blood of venom-challenged mice were ascertained using a one-way ANOVA. Tukey's multiple comparison test was used to compare the means within the comparisons, and statistical significance determined (where p-value < 0.05).

CHAPTER FOUR
RESULTS

4.1 Preliminary assays, generation, extraction and purification of IgY polyclonal antibodies

4.1.1 Lethality (LD_{50}) of *D. polylepis* venom in chicks

The LD_{50} of *D. polylepis* venom in chicks was estimated by using the Miller method (Randhawa, 2009) to generate the LogDose and probit values for the standard curve *(Figure 4.1)*. From the equation below for the regression, the response variable (probit of % death) represented by 'y', the predictor variable (LogDose) as 'x', and 'R^{2}` estimates the percentage variation in the response. From the equation y = 4.4025x + 6.1751, the value of 'x' was determined to be -0.2669. Therefore, the LD_{50} which is antilog(x) was then calculated to be 0.541. Thus, the estimated LD_{50} of Black mamba venom in chicks was 0.541±0.04. LD_{50} results of 0.54 (0.49-0.58) mg/kg was observed.

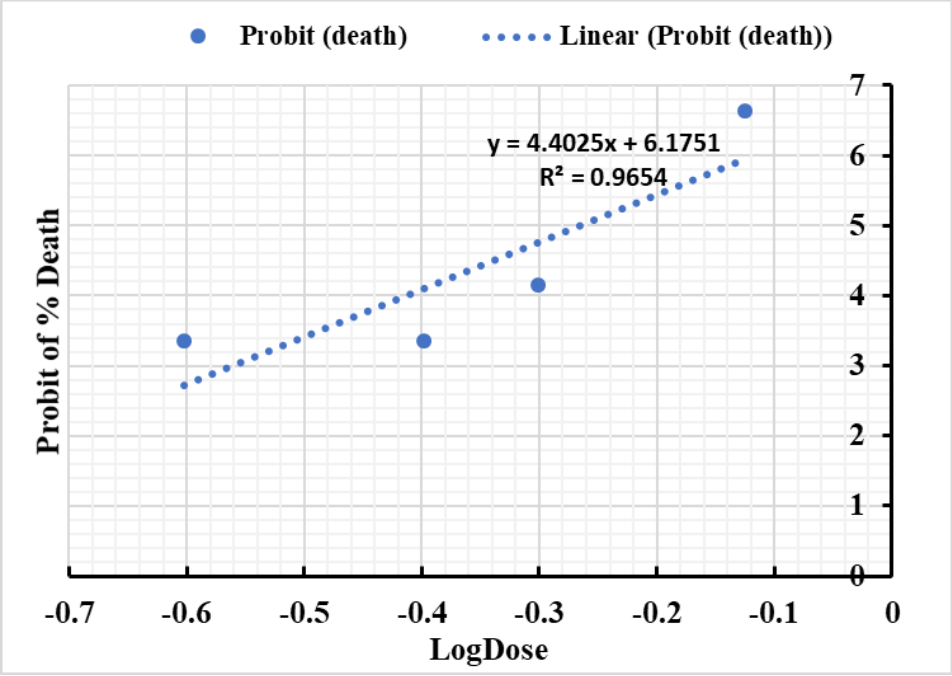


Figure 4. 1: Probit standard curve for estimating LD_{50} of *D. polylepis* venom in chicks.

4.1.2 IgY extraction, purification, specific activity and biochemical identification

The average recovery was 19.1% for venom-specific IgY. Titer of VACF 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 4.1)*.

Table 4. 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
VACF	132.2	15.09	19.10

Key; WSF: water-soluble fraction, SOF: Salting-out fraction, VACF: Venom affinity chromatography

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 (*Figure 4.2A*). However, under non-reducing conditions, they exhibited one band of about 183 kDa (*Figure 4.2B*).

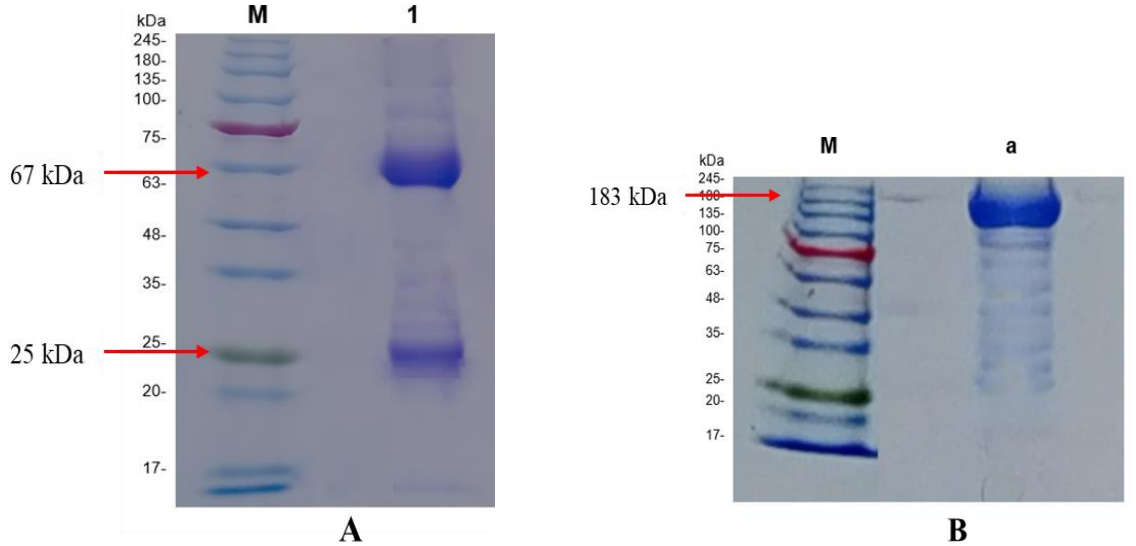


Figure 4. 2: A, Lane 1: IgY under reducing conditions and B, lane a, IgY under non-reducing conditions. Lane M: molecular weight marker.

4.1.3 Concentrations of venom-specific chicken-based IgY yield per week

The protein concentration of the IgY fraction was estimated from a seven-point standard curve generated by using bovine serum albumin-BSA (protein standard) *(Figure 4.3)*. From the equation below for the regression, the response variable (absorbance) represented by 'y', the predictor variable (concentration) as 'x', and 'R^2' estimates the percentage variation in the response. From the equation, y = 0.00059x + 0.84481, the 'x' value was determined to be 1,401.713 and since samples were diluted 2 times, the calculated protein concentration of the pooled sample fraction was found to be 2,803 µg/mL. Thus, IgY antibodies extracted for collected eggs per each week were pooled together.

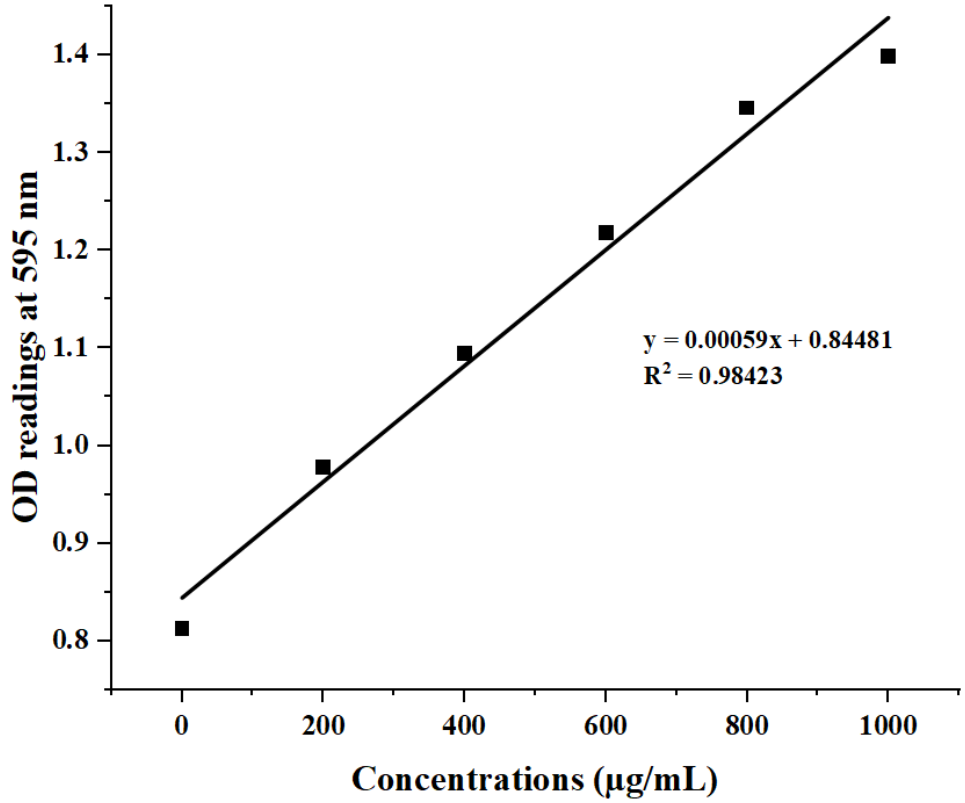


Figure 4. 3: Bovine serum albumin-BSA (protein standard) standard curve for quantifying the fraction of chicken-based IgY protein concentration.

The OD readings of the samples per week of extraction were found as in the Figure 4.4 below. 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 *(Figure 4.4)*.

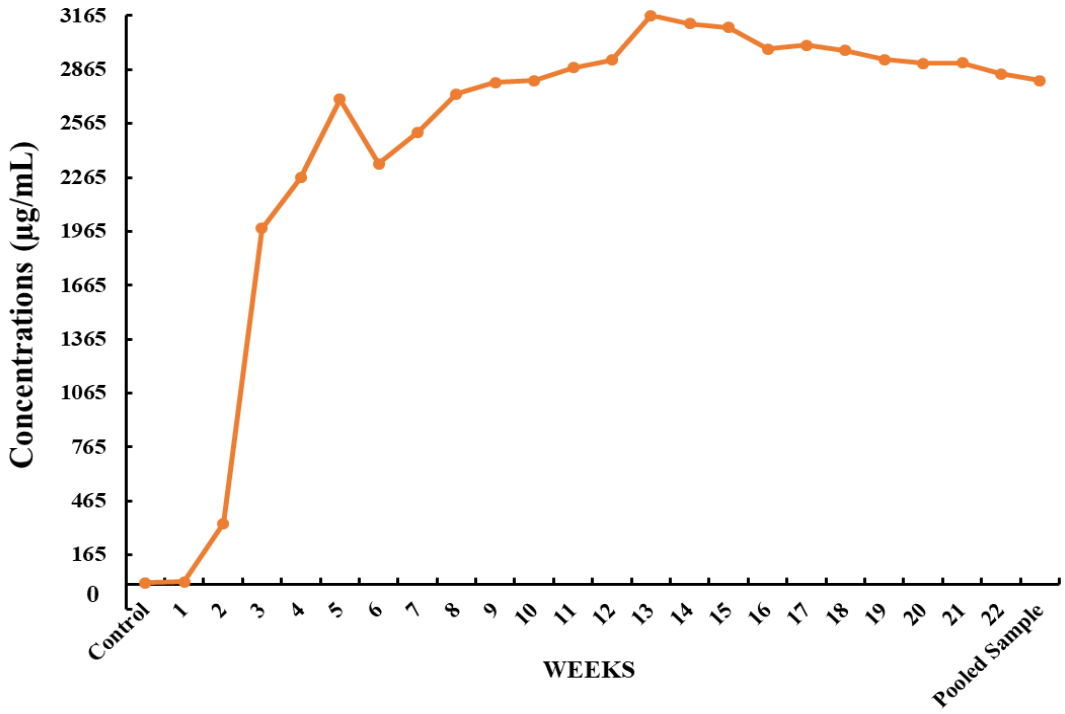


Figure 4. 4: Concentrations of the affinity purified antibodies yield from egg yolk per week.

4.1.4 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, and PBS (saline)-only immunized samples. Following the primary immunization, varied immune (antibody) responses were elicited against *D. polylepis* venom 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, an 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) *(Figure 4.5)*. The PBS-only immunized chicken however showed no immune response.

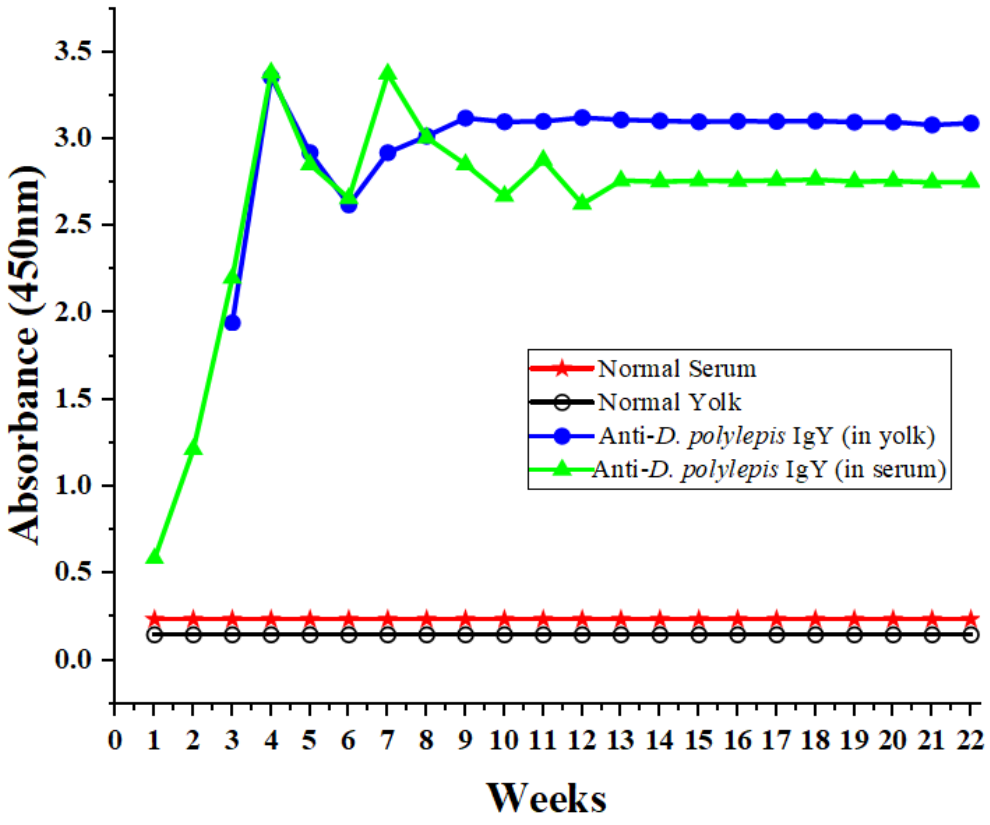


Figure 4. 5: Immune response for primary and secondary antibody in serum and egg yolk of immunized Isa-Brown chicken with *D. polylepis* venom.

4.2 *D. polylepis* venom lethality, and envenomation-neutralizing efficacies of IgY polyclonal antibodies and commercial antivenoms

4.2.1 Lethal and toxic activities of *D. polylepis* venom

Lethal activities of *D. polylepis* was observed in BALB/c mice. The LD_{50} for *D. polylepis* venom in mice was found to be 0.34 (0.29-0.38) mg/kg. Following 1-hour 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 4.2)*. The venom showed no necrotic and hemorrhagic activities in mice. However, paralysis and ptosis (falling-eye lids) were observed.

Table 4. 2: *D. polylepis* 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 one hour of injection, caused a 30% increase in footpad volume. Results are presented as mean ± SD (n = 5) for edema-forming activities.

4.2.2 Lethal and toxic activities neutralization assays

As shown in Table 4.3 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 antibodies, and 46.6 µL for the PANAF-Premium™ *(Table 4.3)*. 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 value = 0.320-0.859).

Table 4. 3: Neutralization of lethal and edematous effects of *D. polylepis* 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.

4.3 ELISA prototype

4.3.1 Optimization assays of ELISA parameters

From the ELISA parameters optimization assays, immune response was detected at OD of 2.067 value for venom-immunized chicken and OD value of 0.505 for negative control sample at the lowest rabbit anti-chicken IgG/HRP dilution of 1:2,000. It was revealed that the detection of response as in the case of target antibody-containing serum sample was dilution-dependent. The negative control sample showed very little variation in detection, and had saturation starting at the 1:32,000 dilution *(Figure 4.6)*.

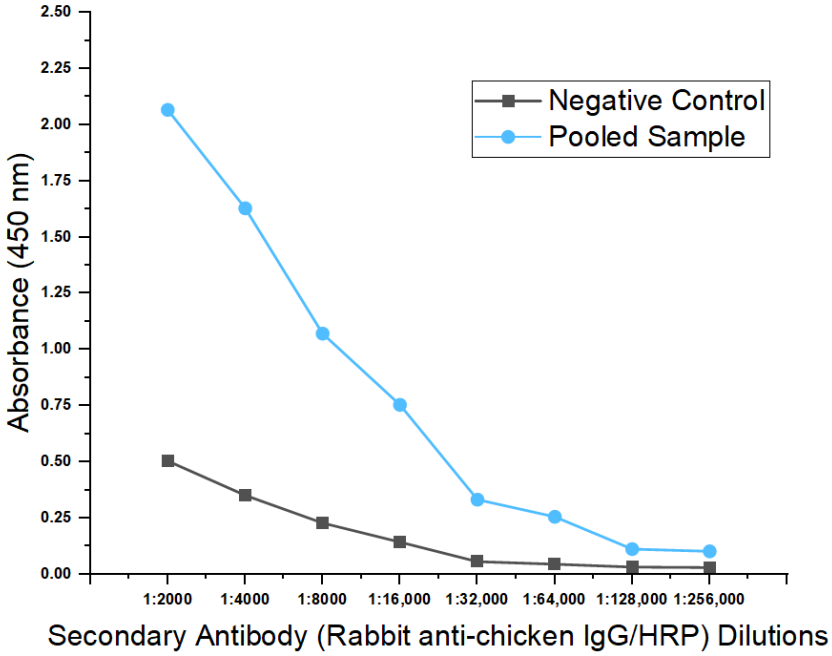


Figure 4. 6: Optimal dilution for secondary antibody determination.

However, the highest signal-noise ratio for the secondary antibody conjugate was obtained at 1:8,000 dilution, and was determined by computing ratios of the absorbance readings of sample-negative control for each dilution *(Figure 4.7)*.

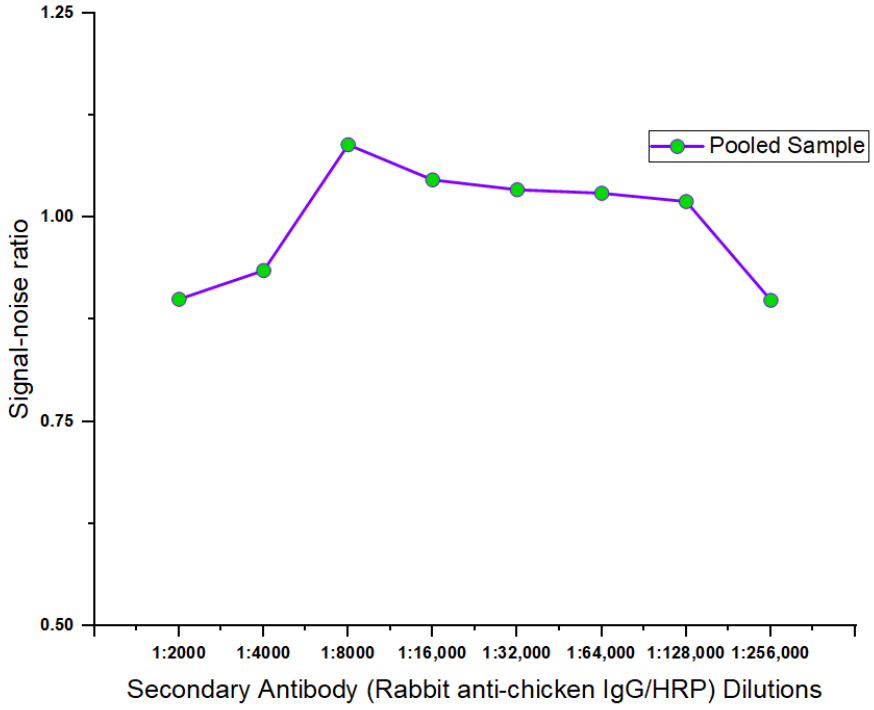


Figure 4. 7: Determination of signal-noise ratio at each level of secondary antibody dilution for the optimum.

The established antigen coating concentrations for this study showed increasing detection with a peak at 6 µg/mL and decreased with increasing concentration. However, at a coating of low antigen concentration of 1 µg/mL, detection of significant levels was achieved and considered the optimal due to its economical representation *(Figure 4.8)*.

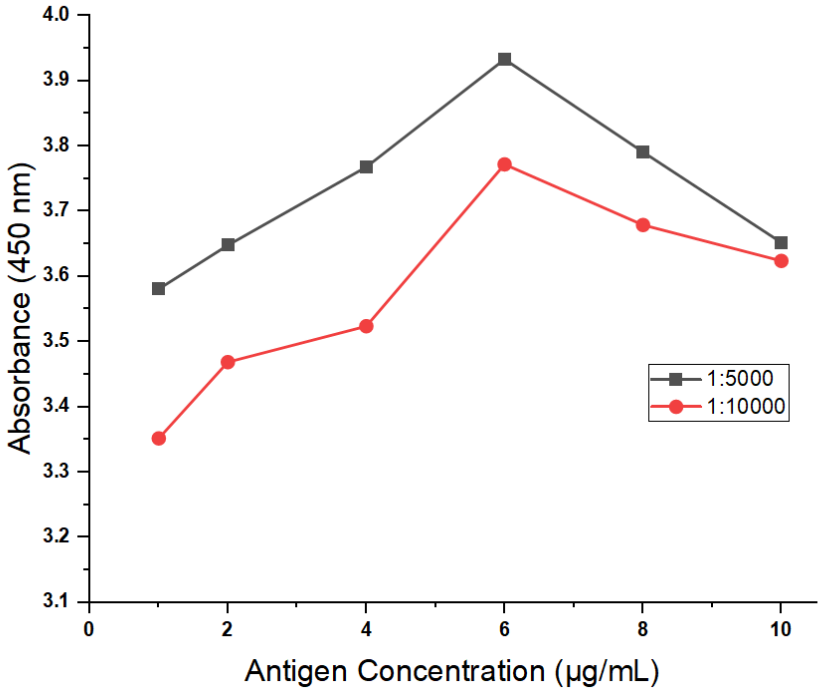


Figure 4. 8: Optimal concentration of *D. polylepis* venom (antigen) for coating.

For the pooled serum sample dilution, an OD value of 2.310 as the highest was obtained at 1:2,000 dilution level *(Figure 4.9)*.

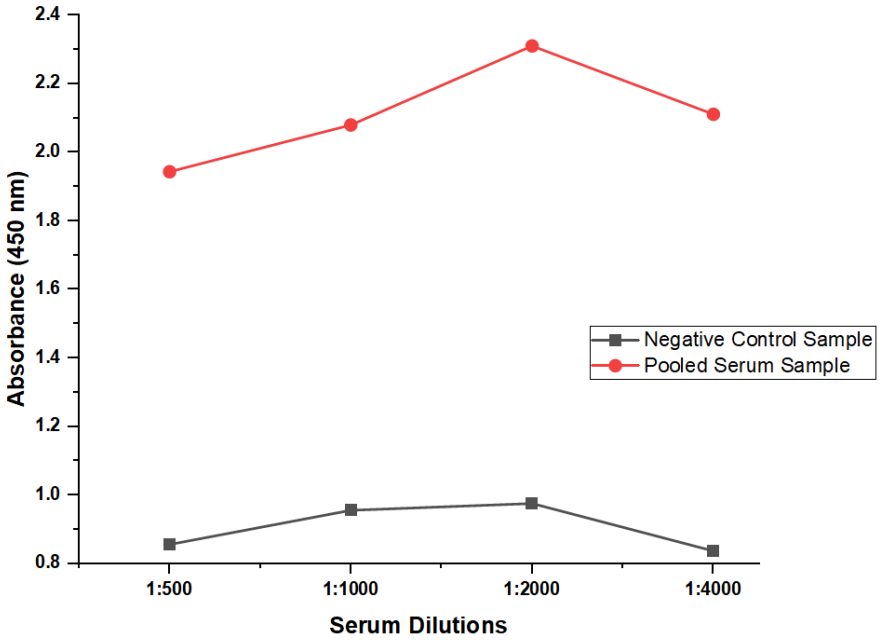
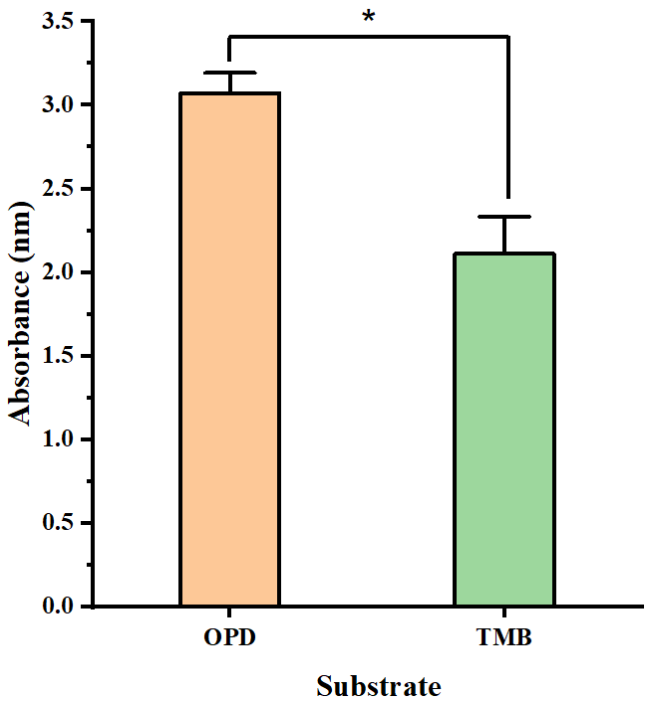


Figure 4. 9: Optimal serum dilution observed at highest peak.

Significantly, OPD solution produced higher OD values than of TMB for the substrate sensitivity analysis (as shown by the two-tailed t-test analysis with *p*-value = 0.0001) *(Figure 4.10)*. These optimal parameters were employed for the remaining assays procedure.



*-indicates statistical difference

Figure 4. 10: Observed response of OPD highest sensitivity in comparison to TMB substrate.

4.3.2 Cut-off point determination for the ELISA: Specificity and sensitivity inhibition

Following the use of homologous and heterologous venoms for the specificity assay, tested inhibitor concentrations showed percent inhibition greater than 40% for all homologous venoms (specifically, 49.67 and 52.72% for *D. jamesoni* and *D. angusticeps*, respectively). However, heterologous venoms revealed a percent inhibition less than 19% across all tested inhibitor concentrations *(Table 4.4)*. The specificity assay criteria for identifying the presence of analyte of interest was set to 30% for both homologous and heterologous venom samples using the deductions of Sharma et al. (2019).

Table 4. 4: Detected percent inhibition by venoms of *D. jamesoni*, *D. angusticeps*, *B. arietans* and *N. ashei*.

Inhibitor Concentration (µg/mL)	*Dendroaspis jamesoni*		*Dendroaspis angusticeps*		*Bitis arietans*		*Naja ashei*	
	OD	% Inhibition	OD	% Inhibition	OD	% Inhibition	OD	% Inhibition
6.600	0.065	89.31	0.092	86.82	0.166	18.62	0.179	18.63
2.200	0.079	87.01	0.105	84.96	0.172	15.68	0.182	17.27
0.730	0.094	84.54	0.086	87.68	0.170	16.67	0.181	17.73
0.240	0.183	69.90	0.196	71.92	0.173	15.19	0.187	15.00
0.081	0.195	67.93	0.208	70.20	0.175	14.21	0.193	12.27
0.027	0.207	65.95	0.210	69.91	0.181	11.27	0.187	15.00
0.009	0.214	64.80	0.238	65.90	0.190	6.86	0.198	10.00
0.003	0.306	49.67	0.330	52.72	0.193	5.39	0.213	3.18
NAC	0.608		0.698		0.204		0.220	

NAC-No antigen control, OD-Optical density

Furthermore, at various different crude *D. polylepis* venom concentrations, the sensitivity of inhibition ELISA was determined, and serum samples from pre-immunized chickens (as negative controls) were analyzed. The Limit of Detection (LoD) was determined to be 0.01 µg/mL, using 0.973 and 0.202 recorded for the mean and standard deviation (of negative control), respectively *(Table 4.5)*. The determined OD was 0.966, and thus fell in between the lowest assay's concentration (0.02 µg/mL) and the NAC (No Antigen Control), given the 0.01 µg/mL as LoD. Hence, the ELISA was able to discriminate both positive and negative samples at a 0.01 µg/mL minimal concentration. Comparably, the assay could verify whether the specific toxins were present in a venom sample or not at the same cut-off. Lower optical density (OD) values indicate higher concentrations of the target analyte, which leads to increased binding and a lower signal (OD), and vice versa, as is the case with inhibition ELISAs.

Table 4. 5: Percent inhibition for sensitivity assay determination.

Percent (%) inhibition for ELISA sensitivity determination				
***Dendroaspis polylepis* venom**				
Concentration (µg/mL)	**Replicate 1**	**Replicate 2**	**Negative controls (n=16)**	
44.00	76.00	80.61	50.06	48.28
14.67	68.85	72.65	47.08	46.95
4.89	67.06	65.51	44.97	44.70
1.63	65.87	59.79	42.58	44.10
0.54	64.88	57.96	34.77	37.02
0.18	50.20	54.49	32.18	34.44
0.06	42.06	50.82	20.46	21.39
0.02	39.08	45.92	12.58	11.26

4.3.3 Assessment of performance, functionality and overall effectiveness of the developed ELISA prototype

4.3.3.1 ELISA for crude *D. polylepis* and other venoms toxin screening

Following the three-fold dilution, the results showed a significant inhibition of the sample antigen in a dose-dependent manner, with the highest inhibition (72.66%) observed at a concentration of 44.00 µg/mL and the lowest inhibition (29.78%) at 0.02 µg/mL *(Figure 4.11).* The corresponding absorbance values were 0.538 and 1.382, respectively. In contrast, the 'No Antigen Control (NAC)' sample showed a much higher absorbance value of 1.968 *(Table 4.6),* indicating a strong binding response in the absence of the antigen. The inhibition of toxins in the crude *D. polylepis* venom was observed across all concentrations, with the highest antigen concentrations showing the greatest reduction in OD values compared to the No Antigen Control (NAC). This suggests that the primary

antibody bound more efficiently to the toxins at higher concentrations, resulting in increased percent inhibition as observed in Figure 4.11 below.

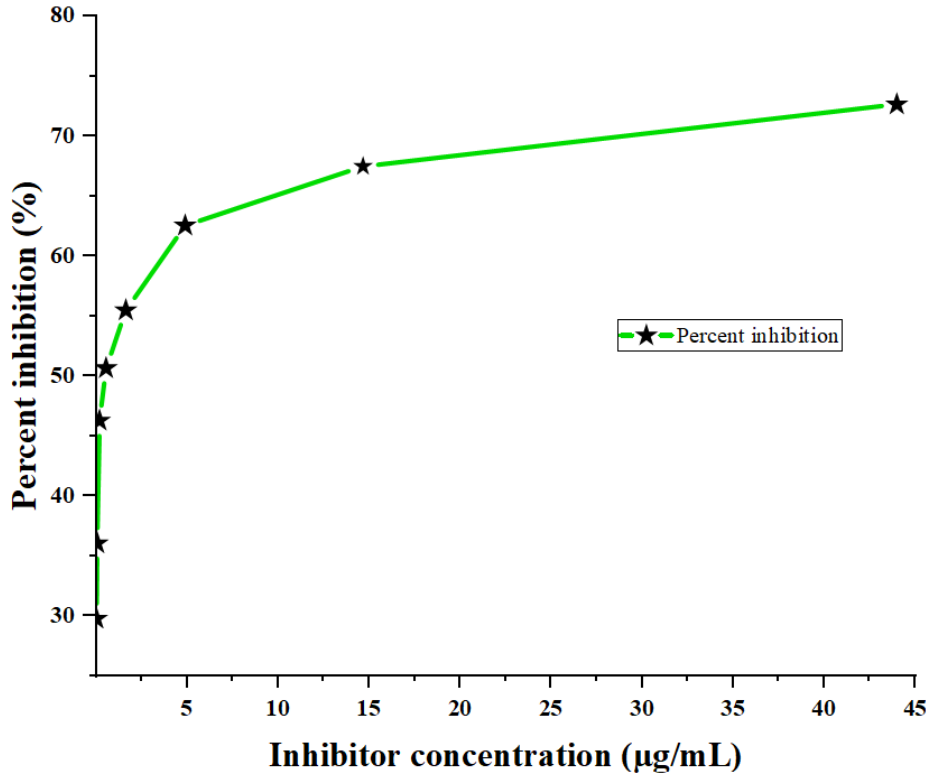


Figure 4. 11: Dose-dependent ELISA inhibition curve showing percentages at sample-containing antigen levels of the inhibitor (crude *D. polylepis* venom).

Table 4. 6: Antigen-mediated inhibition of toxins from crude *D. polylepis* venom.

Inhibitor concentration (µg/mL)	OD (492 nm)	Percent inhibition (%)
44.00	0.538	72.66
14.67	0.640	67.48
4.89	0.737	62.55
1.63	0.876	55.49
0.54	0.971	50.66
0.18	1.057	46.29
0.06	1.258	36.07
0.02	1.382	29.78
NAC	1.968	-

NAC-No antigen control, OD-Optical density

In light with the toxin's detection in venoms of *Dendroaspis* sp. and non-*Dendroaspis* sp., the inhibition ELISA assay revealed toxins presence in all assayed crude venoms and at different tested concentrations, the percent inhibition due to sample antigens in various crude venoms determined. Means of the sample antigen-induced inhibition were subjected to ANOVA and the result was statistically significant (*p value* = 0.0001) as shown in the table of ANOVA summary in Appendix 10. Given the F-value, a higher variation between the sample means in relative to variation within the venom samples was observed. Additionally, the analysis showed R-squared = 0.6814, meaning that sample antigen presence in crude venoms caused 68% of the variation in inhibition.

The tukey's test results for multiple mean comparisons revealed significant difference in inhibition caused by toxins-containing samples between *Dendroaspis* sp. and non-*Dendroaspis* sp. Moreover, no significant difference was observed among all *Dendroaspis* species, as well as induced inhibition between *N. ashei* and *B. arietans (Appendix 11)*.

The study further revealed low ODs for homologous species (*D. jamesoni* and *D. angusticeps*) at higher inhibitor concentration resulting in high induction of percent inhibition, and OD increases with observed inhibitor-concentrations reduction *(Figure 4.12)*. The ODs of 'No antigen control (NAC)' for all venoms tested had the highest values recorded, and was a sign of no inhibition in contrast to wells with various concentrations of inhibitor. Since the venoms of *N. ashei* and *B. arietans* either contain little or no of the target antigen, the ODs observed for these two species indicate that there was either no inhibition or very little inhibition.

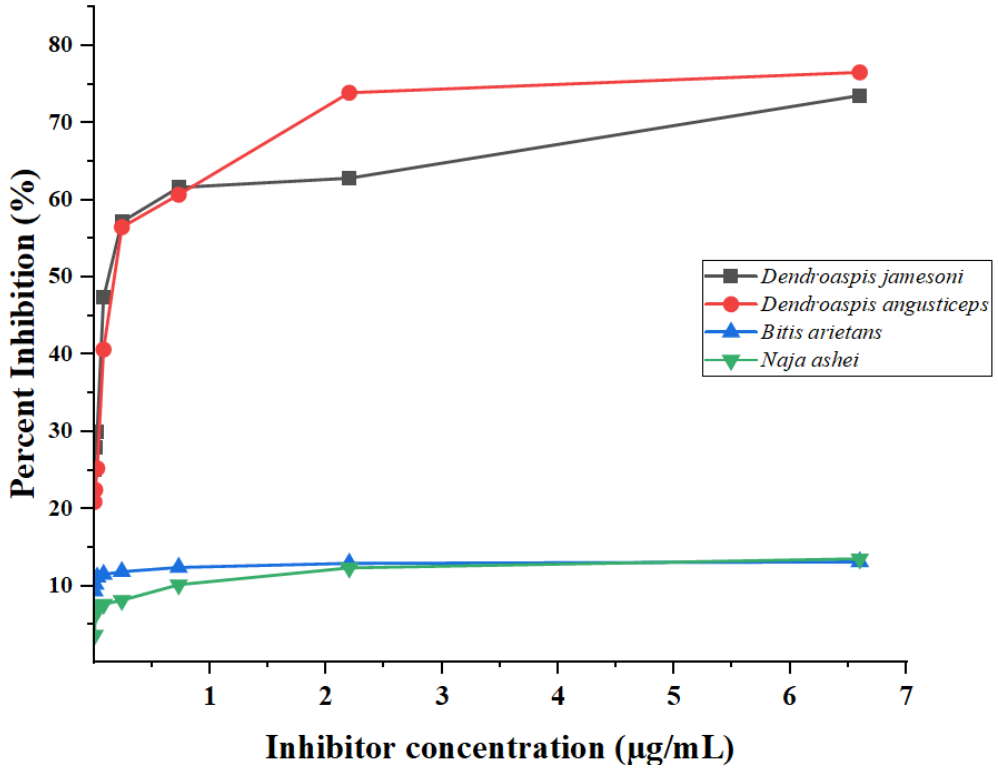


Figure 4. 12: An ELISA inhibition curve showing percentages at sample-containing antigen levels of the inhibitors (crude *D. jamesoni, D. angusticeps, N. ashei* and *B. arietans* venoms at different concentrations).

4.3.3.2 Toxin detection in *D. polylepis* venom-challenged mice

This assay assessed the applicability of the inhibition ELISA potential to detect snakebite envenoming in mice using *D. polylepis* venom. It was clearly revealed by the ELISA ability to detect the toxins with observed inhibitions in wells containing the samples. There was no statistical difference (*p-value* = 0.3392) between the samples and negative control, and same observed for comparison test through the one-way ANOVA. Besides, the assay was able to detect toxins with observed percent inhibition *(Figure 4.13)*.

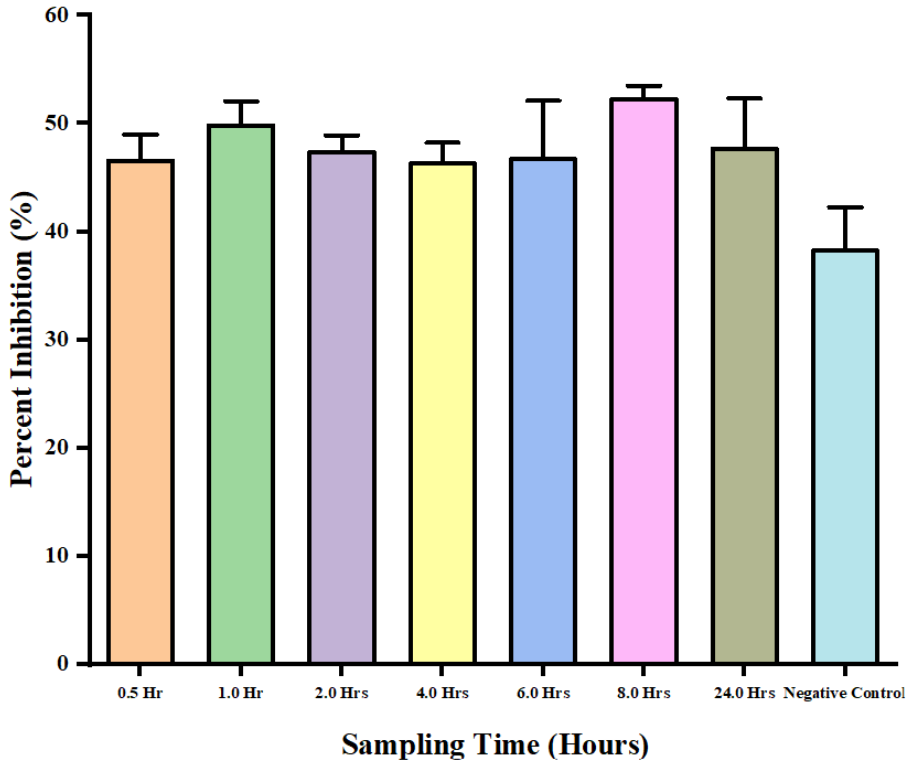


Figure 4. 13: Toxins detection in venom-challenged mice, and percent inhibition comparisons for different time interval sampling.

4.3.3.3 Toxin detection in spiked samples

Blood samples collected and spiked with *D. polylepis* venom at different times were assayed to assess the ELISA's ability to detect venom toxins in blood. Control sample of previously collected serum (pre-immune) samples was assayed along blood samples. From the assay results, inhibition was observed but not for the negative control due to the absence of target antigen or antibodies. Like that of previous assay, no significant difference (*p-value* = 0.0602) was observed from the ANOVA analysis in respect to the sampling times. However, the ELISA was able to detect the toxins in blood samples with some level of percent inhibition *(Figure 4.14)*.

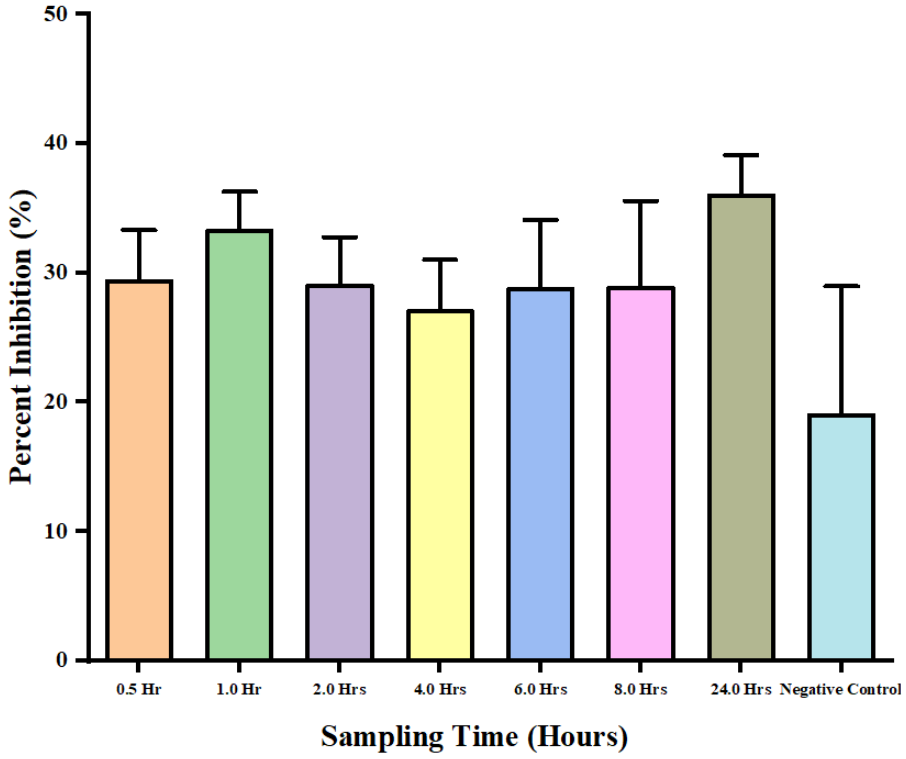


Figure 4. 14: Different times sampling comparison for percent inhibition of spike samples collected.

4.3.3.4 Detection of antibodies in commercial antivenom products

Upon analyzing the two commercially available polyvalent antivenoms (PANAF-Premium™ and VINS™) and generated IgY antibodies, the ELISA assay was able to detect the presence of toxins-specific antibodies in them. Results showed highest detections all dilutions level for the generated chicken-based IgY. Despite the results showing higher detection for VINS™, second to the IgY antibodies, there was no varying detections observed beyond 1:16,000 dilution. It was revealed that antibodies in the antivenoms could be detected by the assay even at the 1:256,000 dilution level *(Figure 4.15).* However, comparison between the detection efficacy amongst IgY antibodies, PANAF-Premium™ and VINS™ showed significant differences at all dilution levels (*p-value* = 0.0001).

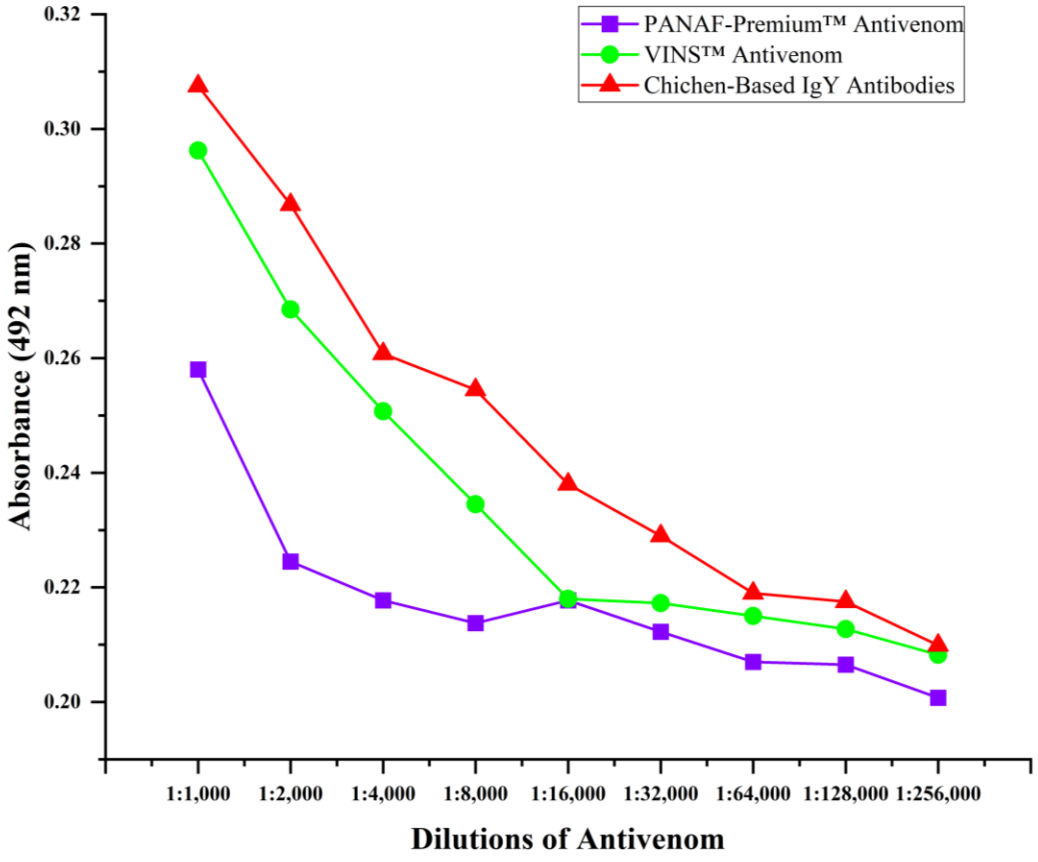


Figure 4. 15: Anti-*D. polylepis* antibodies detection in two commercial antivenoms and generated IgY by the developed ELISA assay.

CHAPTER FIVE
DISCUSSION

5. Summary

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 to half (50%) by 2030 (Williams et al., 2019a). However, snakebite-prone areas worldwide often have to settle for broad-spectrum antivenoms, which unfortunately have a low concentration of the specific antibodies needed to effectively treat individual snakebites (Williams et al., 2019a). Thus, breakthroughs in producing targeted antibodies against specific snake toxins could revolutionize the treatment of snakebite envenoming, offering new hope for improved outcomes. In line with this, current study aimed to characterize and develop chicken-based IgY polyclonal antibodies against black mamba (*Dendroaspis polylepis*) snake venom.

The study isolated IgY antibodies from chicken and further compared their efficacy with commercial antivenoms used to neutralize toxins of *D. polylepis* venom. In addition, the study developed an ELISA prototype that showed promise in detecting venom in both spiked blood samples and in mice challenged with *D. polylepis* venom, paving the way for potential diagnostic applications. This is in line with the urgent call from Williams and associates call for developing novel strategies to aid snakebites diagnosis and treatments or managements (Williams et al., 2019b).

5.1 Generation and functional characterization of chicken-based IgY antibodies against *D. polylepis* toxins

The Black mamba (*D. polylepis*) is endemic to Sub-Saharan Africa, and 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 at neuromuscular junctions. Despite having low concentrations of necrotic-causing proteins (Laustsen et al., 2015), the venom typically does not causes necrosis and tissue destruction (Quarch et al., 2017). It is possible for neurotoxic symptoms to appear as soon as 10 minutes 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 (Rojas et al., 2005; Amro et al., 2018), as well as speeding up recovery. This study describes the generation of anti-*D. polylepis* chicken-based IgY polyclonal antibodies and compares the neutralizing efficacy with commercial antivenoms for the lethal and toxic effects induced by *D. polylepis* venom.

Guided by previous reports, the study purified IgY (Araújo et al., 2010; Duan et al., 2016; Liu et al., 2017). 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 (Duan et al., 2016; Liu et al., 2017). In addition, it was shown that IgY activity in VACF was 2 and 16-times higher than that of SOF and WSF, respectively. The antibody production response induced by the black mamba venom was similar to that in reports for other snake venoms (Rojas et al., 2005; Paul et al., 2007; Araújo et al., 2010; Duan et al., 2016; Liu et al., 2017; Amro et al., 2018).

5.2 Comparison of *D. polylepis* venom neutralizing-efficacy of the isolated chicken-based IgY polyclonal antibodies with commercial antivenoms used in the Kenya

Venom from mamba species are generally potent, and neurological effects of the venom appear within 2 hours after the bite (Aalten et al., 2021). These neurotoxic effects have been observed in several researches on mice (Ainsworth et al., 2018; Ochola et al., 2019; Ratanabanangkoon et al., 2020). The current study showed LD_{50} of 0.54 mg/kg in chicks, and 0.34 mg/kg in mice. The LD_{50} reported in this study was higher than 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. These results were 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 noted (Laustsen et al., 2015; Harrison et al., 2017). It is not unusual for venoms from the same species to have different toxicities. 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 as mice slightly bigger/heavier than the standard 18-20 g recommended by WHO and 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; Ochola et al., 2019).

The clinical signs and symptoms that were observed in both chicks and the mice were consistent with neurotoxic venom. These included paralyses, ptosis (drooping or falling eyelids), and respiration patterns which varied from shallow to rapid (more pronounced with accompanying unusual sound). The results agree with those of Ochola et al. (2019). Blanchet et al. (2014) and Ratanabanangkoon et al. (2020) had observed that venom of black mamba has α-neurotoxins which attach to nicotinic receptors at the motor end plate, and cause failure in respiration.

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 of edema. This study observed edematogenic activity of *D. polylepis* venom in mice may be inked to presence of metalloproteinases earlier reported by Laustsen and colleagues in the venom. Further, major toxins of the elapid venoms include metalloproteinases (SVMPs), snake venom serine proteases (SVPs) and L-amino acid oxidases (LAAOs), all contributing an average of 6% of elapid venom (Kang et al., 2011; Ullah et al., 2018; Ferraz et al., 2019; Hiu & Yap, 2020; Olaoba et al., 2020; Ullah, 2020; Oliveira et al., 2022). Serine protease and LAAOs though present in small quantity, are prevalent in many snake venoms, and in myocytes, can cause edema development, hemolysis, toxicity, and platelet aggregation (Kaur et al., 2012; Hiu & Yap, 2020; Gutiérrez et al., 2021). The presence of edema forming activity in this study could be that our venom had significant amount of LAAO or serine protease (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 from among *Dendroaspis*, attention must be placed on type of antivenoms for treating snakebite victims in sub-Saharan Africa. In order to achieve this, the study compared the neutralizing-efficacy of generated IgY antibodies with to two commercial *Dendroaspis* antivenoms that are currently used in sub-Saharan Africa against *D. polylepis* venom in mice. Both antivenoms and the chicken-based IgY polyclonal antibodies showed ability to neutralize the lethal and edematogenic effects of *D. polylepis* venom but with varying efficacy levels. The effectiveness of VINS^{TM} antivenom in neutralizing lethality of black mamba venom in mice has been documented by other researchers conducting similar studies (Laustsen et al., 2015; Ochola et al., 2019). Antivenoms have been reported to be effective when they immunocapture 20-25% of the components of venom (Calvete et al., 2014). According to the WHO guidelines for the manufacture, control, and regulation of antivenoms (WHO, 2017), immunocapture of ≥25% of venom proteins is a positive result of neutralization. This result confirms the

manufacturers' inscription on antivenom containers that each mL neutralizes at least ≥ 25LD_{50} for *D. polylepis* venom.

The effective neutralization doses of PANAF-Premium™, IgY antibodies, and VINS™ antivenoms (ED_{50}) shown in this study are consistent with those reported earlier on *D. polylepis* venom neutralization (Laustsen et al., 2015; Engmark et al., 2016; Ainsworth et al., 2018; Ochola et al., 2019; Menzies et al., 2022). A report on differences in ED_{50} regarding different batches by Resiere and others 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. Gutierrez et al. indicated that this could potentially provide credence to the discrepancies in efficacy between antivenoms (Gutiérrez et al., 2017). The results from this study, however, are limited with regard to looking at particular batches, because they were the available and accessible ones by the time.

5.3 Development of an ELISA prototype of IgY polyclonal antibodies for detecting toxins of *D. polylepis* venom

The optimal concentration for antigen coating determined in this study is comparable to that used in previous reports (Laustsen et al., 2017; Manson et al., 2022b). John-Goka & Farthing (1987) and Hosoda et al. (1986) showed TMB substrate to be more sensitive. However in this study, higher absorbance was associated with OPD substrate compared with TMB significantly which agrees with report by Manson et al., (2022b). Whereas the optimal dilution for secondary antibody was consistent with Manson and others, the observed optimal primary antibody dilution found in this study was not, and could be attributed to the differences in animal models used in generating the antibodies.

The developed ELISA assay exhibited better sensitivity with lower limit of detection (LoD) when compared to earlier studies. The increased sensitivity demonstrated is partly attributed to the use of an easy-to-afford steps employed for the assay development. More so, apart from the closely related *Dendroaspis* species, there was an observed minimum (less than 4%) of cross-reactivity between heterologous venoms. Despite this, the assay still enables the offending snake venom to be identified. Also, it is worth noting that since all *Dendroaspis* species induce identical clinical symptoms, there is little clinical or practical benefit to distinguishing them (Aalten et al., 2021). Therefore, when employed in clinical samples, this assay will continue to be helpful in clinical decision making even when antivenom for *Dendroaspis* becomes accessible. ELISA results of the inhibition showed that, in comparison to "No Antigen Control (NAC)", the toxins found in the crude *D. polylepis* venom were inhibited at all tested concentrations as reflected by reduced ODs linked to high antigen-containing samples. In short, the high percent inhibition can be explained by the fact that there was more antigen in these wells which led to increased binding of the primary antibody with the antigen.

Conversely, since the venoms of *N. ashei* and *B. arietans* either contain little or no target antigen, the ODs reported in this study for these two species indicated low inhibition. Studies have established that toxins in venoms of these two species is highly cytotoxic,

and not neurotoxic which distinguishes the *Dendroaspis* species (Blanchet et al., 2014; Laustsen et al., 2015). Additionally, the observation by Casasola and others that snake venoms from same genera of snakes exhibit similar biochemical, antigenic, and toxicological features supports the results on inhibition amongst the three *Dendroaspis* (*D. polylepis, D. jamesoni* and *D. angusticeps*) snakes (Casasola et al., 2009). The overall one-way ANOVA analysis showed R-squared (0.6814), implying that sample antigen present in crude venoms caused 68% of the variation in inhibition. Also, the observed high F-value was an indication of a higher variation between the sample means relative to variation within the venom samples amounting to the significant differences (p-value = 0.0001).

The developed ELISA protocol also detected toxin proteins in blood samples of venom-challenged mice and those spiked with crude *D. polylepis* venom. However, the spiked and venom-challenged blood samples when compared to the control blood samples was not statistically significant, but comparable to results of Manson et al. (2022b). This could be attributed to the large similarity of the observed percent inhibition across the various sample collection times for spiked blood samples. In addition, the no significant difference seen between spiked samples and control blood samples could be that the test run was just unable to detect the difference. Also, highest detections were shown by the assay for chicken-based antibodies at all dilution levels compared to the commercial antivenoms, and this is because it contained purified antibodies raised specifically against *D.* polylepis. The ELISA was able to detect the existence of black mamba venom-specific antibodies in two polyvalent antivenoms which are for treating bites from varied snake species including *D. polylepis*. These observations are quite significant because the assay was not only able differentiate venoms but help implicate offending snakes, and when applied to clinical samples, could help to administer treatments targeted at specific venom toxins (Williams et al., 2019b). Reports on *D. polylepis* neutralization assays have indicated VINS product to be more effective among other polyvalent antivenoms (Ainsworth et al., 2018; Menzies et al., 2022), and is however consistent with our antibodies detection test for antivenoms. Comparison between the antivenoms displayed higher detections for

VINS^{TM} significantly, and could be 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).

Since ELISA's initial description in 1977 (Theakston et al., 1977), ELISA-based snake venom detection has been applied in several laboratory, preclinical and clinical investigations (Maduwage et al., 2013; Manson et al., 2022b). ELISA has proven to be a useful tool when examining snake venom kinetics in blood, the extent of envenomation, and the suitability of antigen-based serotherapy. Out of all the immunoassays, ELISA is thought to have greater practical usefulness and is still the preferred approach for identifying poisons, venoms, and venom-antibodies in bodily fluids (Kulawickrama et al., 2010). The low LoD values found in this study is comparable to that reported for African spitting cobra venom (Manson et al., 2022b). This means that when small amount of venom of *D. polylepis* is injected comparatively, it can be detected in preclinical serum samples due to the high sensitivity of the ELISA method reported in this study. It is possible too with this method to identify the envenoming species.

One major restriction with the developed ELISA is cross-reactivity of the chicken-based IgY antibodies with venom of closely related species. The observed cross-reactivity is not out of the norm since similar *in-vivo* toxic effects, venom characteristics, and clinical envenoming signs have all been documented for homologous venom samples (Silva et al., 2012; Maduwage et al., 2013). As a result, there was a significant high amount of cross-reactivity between the venoms of the two *Dendroaspis* species and the *Dendroaspis polylepis* antibodies in this study. Nonetheless, this cross-reactivity was very minimal for the unrelated species which suggest that the ELISA could readily distinguish venom from *D. polylepis* and other non-related species. Hence the study null hypothesis was rejected. Since *D. polylepis* is responsible for majority of snakebite cases in Kenya, the developed ELISA protocol is potentially useful in diagnosis of snakebite culprits particularly those of *Dendroaspis* species showing similar clinical symptoms.

Despite the mamba being medically important snake especially in the Sub-Saharan Africa, and presenting many fatality issues, no documented report on the development of immunoassay for *Dendroaspis* species (mamba snakes' venom) have been found yet. This study reports the detection of venom from *D. polylepis* resident in Kenya for the first time.

For epidemiological reasons, it is critical to identify the kind of offending snake's venom present in patients' blood. Also, antivenom dosing rely most importantly on the detection of snake venom in serum. A study from Sri Lanka describes a syndromic technique or presentation of images frequently used to identify the snake, and only approximately 25% of victims presenting in hospitals are able to identify the snake that cause the bite (Ariaratnam et al., 2009). The validity of epidemiological investigations is limited since none of these methods is precise enough. Because there is a chance of bites, guidelines for the treatment of envenomation from snakebite do not advise catching the offending snake and transporting it to the hospital. In numerous clinical investigations, the identification of snakebite cases has been hampered by the absence of venom confirmation (Kularatne et al., 2011; Rathnayaka et al., 2019). Thus, enrolling envenomed patients in clinical investigations would be made easier with the use of venom detecting ELISA, thereby increasing the chance of effective therapy and snake identification. However, the lengthy and labor-intensive procedure for detecting snake venom antigens that is disclosed in this work must be carried out in a laboratory by a qualified technician, which presents a limitation. Therefore, in a clinical environment, the test proposed does not permit the prompt detection of venom. To identify the venom at the point of care, it would be feasible to design a quick venom detection kit, like an immunochromatography tests or lateral flow assay. Notwithstanding, this study offers evidence that it is possible to identify venom of the most medically important snake in Kenya with precision. Also, the study focused on preclinical animal sera samples and encourages use of human sera for further studies. In future, it is possible to design a rapid point-of-care venom detection kit in Kenya using the knowledge obtained from this study.

CHAPTER SIX
CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

To conclude;

i. Immunization of chickens with *D. polylepis* snake venom resulted in production of higher titre IgY antibodies against *D. polylepis* venom toxins in egg yolk.
ii. The lethal effects and edematogenic activities of *D. polylepis* venom toxins were effectively neutralized by the generated IgY antibodies.
iii. The developed ELISA was capable of distinguishing venom from the species under study due to its analytical specificity and sensitivity, making it a potentially useful tool for the diagnosis and clinical treatment of envenomation caused by *D. polylepis* snakebite in Kenya.

6.2 Recommendations

Based on the findings from this study, the following are therefore recommended:

i. Diversify IgY antibodies development to capture other medically important snake venoms, and further characterize antibodies molecularly.
ii. Extend the comparison of generated IgY antibodies efficacy to other locally-made and foreign antivenoms.
iii. Further verification of the assay's specificity using more different venoms is recommended.

6.3 Suggestions/recommendations for further studies

i. Development of rapid diagnostic kits with quick turnaround times.
ii. Assess the ELISA assay using both preclinical and human sera for a greater emphasis of its usefulness in human snakebite diagnosis and management.
iii. Identifying proteins and peptides of *D. polylepis* venom is recommended.

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reaction induced by *Bothrops jararaca* crude venom in mice. *Toxicon*, *55*(2–3), 227–234. https://doi.org/10.1016/j.toxicon.2009.07.025

APPENDICES

Appendix 1: Animal care and use committee approval

KENYA MEDICAL RESEARCH INSTITUTE

Center for Virus Research P.O. Box 54628-00200, NAIROBI, Kenya,
Tel: 254 2722541, Fax: 2720030, Email: Director@cvr-kemri.org Website: www.kemri.org

12Th September 2023

KEMRI ACUC/01.09.2023

Stephen Wilson Kpordze
PAUSTI

Kpordzei,

RE: Animal Use Approval for "Molecular Characterization and Development of Chicken-Based Ig-Y Polyclonal Antibodies Against Black Mamba (Dendroaspis Polylepis) Snake Venom" protocol

The KEMRI ACUC committee acknowledges the resubmission of the above-mentioned protocol. It has been confirmed that all concerns raised earlier have been addressed appropriately and that the use of the mentioned mice and avian species is justified in achieving the study objectives.

Approval is granted for a period of one year starting from when the final SERU approval will be obtained. The committee expects the study to provide an annual report on the progress of animal use simultaneously with the annual continuing review report to SERU.

The committee expects you to adhere to all the animal handling procedures as described in the protocol.

The committee wishes you all the best in your work.

Yours sincerely,

Dr. Konongoi Limbaso
Chairperson KEMRI ACUC

In Search of Better Health

Appendix 2: Ethical approval.

JOMO KENYATTA UNIVERSITY OF AGRICULTURE AND TECHNOLOGY
P.O BOX 62000(00200) NAIROBI, Tel:(067) 58700001-4
(Office of the Deputy Vice Chancellor, Research Production and Extension Division)

JKUAT INSTITUTIONAL SCIENTIFIC AND ETHICS REVIEW COMMITTEE

REF: JKU/2/4/896B **Date: 17th August, 2023**

STEPHEN WILSON KPORDZE
PAN AFRICAN UNIVERSITY INSTITUTE FOR BASIC SCIENCES TECHNOLOGY AND INNOVATION, JKUAT

Dear Mr. Kpordze,

RE: MOLECULAR CHARACTERIZATION AND DEVELOPMENT OF CHICKEN-BASED Ig-Y POLYCLONAL ANTIBODIES AGAINST BLACK MAMBA (*Dendroaspis polylepis*) SNAKE VENOM

This is to inform you that JKUAT Institutional Scientific and Ethical Review Committee has reviewed and approved your above research proposal. Your application approval number is **JKU/ISERC/02316/0976.** The approval period is **17th August 2023 to 16th August 2024.**

i. Only approved documents including (informed consents, study instruments, MTA) will be used
ii. All changes including (amendments, deviations, and violations) are submitted for review and approval by JKUAT ISERC.
iii. Death and life threatening problems and serious adverse events or unexpected adverse events whether related or unrelated to the study must be reported to JKUAT ISERC within 72 hours of notification
iv. Any changes, anticipated or otherwise that may increase the risks or affected safety or welfare of study participants and others or affect the integrity of the research must be reported to JKUAT ISERC within 72 hours
v. Clearance for export of biological specimens must be obtained from relevant institutions.
vi. Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. Attach a comprehensive progress report to support the renewal.
vii. Submission of an executive summary report within 90 days upon completion of the study to JKUAT ISERC.

Prior to commencing your study, you will be expected to obtain a research license from National Commission for Science, Technology and Innovation (NACOSTI) https://oris.nacosti.go.ke and also obtain other clearances needed.

Yours sincerely

Dr Patrick Mburugu
CHAIR, JKUAT ISERC

INSTITUTIONAL ETHICS REVIEW COMMITTEE
17 AUG 2023
APPROVED
P.O. Box 62000-00200 NAIROBI
www.jkuat.ac.ke
JKUAT

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Setting Trends in Higher Education, Research, Innovation and Entrepreneurship

Appendix 3: Research license.

REPUBLIC OF KENYA

NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Ref No: **314421**

Date of Issue: **12/December/2023**

RESEARCH LICENSE

This is to Certify that Mr.. Stephen Wilson Kpordze of Jomo Kenyatta University of Agriculture and Technology, has been licensed to conduct research as per the provision of the Science, Technology and Innovation Act, 2013 (Rev.2014) in Kiambu on the topic: Molecular characterization and development of chicken-based Ig-Y polyclonal antibodies against black mamba (Dendroaspis polylepis) snake venom. for the period ending : 12/December/2024.

License No: **NACOSTI/P/23/31716**

314421

Applicant Identification Number

Director General
NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Verification QR Code

NOTE: This is a computer generated License. To verify the authenticity of this document, Scan the QR Code using QR scanner application.

See overleaf for conditions

Appendix 4: Published paper in 'Toxicon: X' Elsevier.

Toxicon:X 23 (2024) 100201

Contents lists available at ScienceDirect

Toxicon: X

journal homepage: www.journals.elsevier.com/toxicon-x

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

Stephen Wilson Kpordze [a,f,*], Victor Atunga Mobegi [b], Gideon Mutie Kikuvi [c], Joseph Kangangi Gikunju [d], Courage Kosi Setsoafia Saba [e,f], Jackan Moshe [d], James Hungo Kimotho [g]

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[e] Department of Microbiology, Faculty of Biosciences, University for Development Studies, Tamale, Ghana
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[g] Kenya Medical Research Institute, Nairobi, Kenya

ARTICLE INFO

Handling Editor: Dr. Ray Norton

Keywords:
Antivenom
Anti-*D. polylepis* IgY antibodies
Snake venom
Neutralizing efficacy

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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Available online 28 June 2024

Appendix 5: Published paper in 'Antibodies' MDPI.

antibodies

MDPI

Article

Development, Optimization and Evaluation of a Sensitive Enzyme-Linked Immunosorbent Assay (ELISA) Prototype for Detection of Chicken-Based IgY Polyclonal Antibodies against Toxins of *D. polylepis* Venom

Stephen Wilson Kpordze [1,2,*], Gideon Mutie Kikuvi [3], James Hungo Kimotho [4] and Victor Atunga Mobegi [5]

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Abstract: Life-threatening medical issues can result from snakebite, and hence this is a public health concern. In many tropical and subtropical nations such as Kenya, where a wide variety of poisonous snakes are prevalent, diagnosis of snakebite in health facilities is imperative. Different antivenoms are needed to treat the venom of different snake species. Nonetheless, it might be difficult for medical professionals to identify the exact snake species that envenomated a patient due to the similarities of several snake envenomations' clinical symptoms. Therefore, the necessity for an assay or technique for identifying venomous species is critical. The current study sought to develop a sensitive ELISA prototype for the detection of *D. polylepis* venom in Kenya using generated chicken-based IgY polyclonal antibodies. Serum samples containing specific chicken-based IgY antibodies previously raised against *D. polylepis* venom toxins were used in the assay development. ELISA parameters were optimized, and the developed assay was assessed for applicability. The limit of detection (LoD) of the ELISA for neurotoxic venoms was determined to be 0.01 µg/mL. Successful discrimination between neurotoxic and cytotoxic venoms was achieved by the ensuing inhibition ELISA assay. The developed assay showed the capability of identifying venoms in blood samples (from spiked and venom-challenged blood samples) of BALB/c mice, providing compelling evidence of the strategy's usefulness. This assay could help physicians diagnose and manage victims of snakebites through the evaluation of clinical samples.

Keywords: chicken-based IgY; ELISA; optimization; *D. polylepis* venom; antivenoms

check for updates

Citation: Kpordze, S.W.; Kikuvi, G.M.; Kimotho, J.H.; Mobegi, V.A. Development, Optimization and Evaluation of a Sensitive Enzyme-Linked Immunosorbent Assay (ELISA) Prototype for Detection of Chicken-Based IgY Polyclonal Antibodies against Toxins of *D. polylepis* Venom. *Antibodies* **2024**, *13*, 50. https://doi.org/10.3390/antib13030050

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1. Introduction

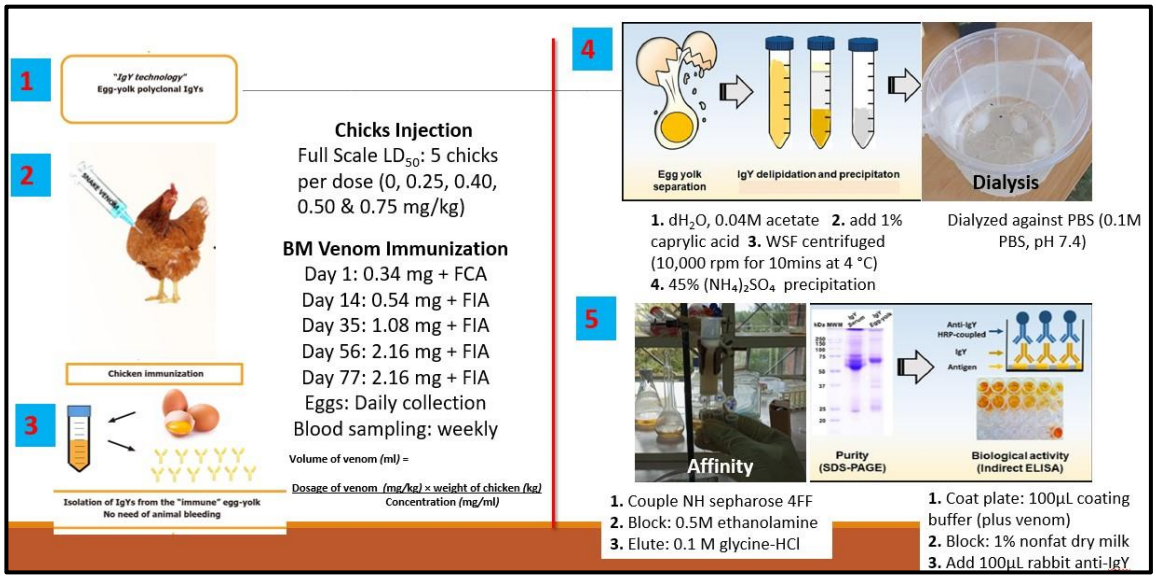
In the tropical agrarian world, snakebite represents a significant medical and public health concern, with an estimated worldwide burden of 1.8 to 2.7 million yearly, with more than 138,000 fatalities and more than 400,000 victims with permanent disabilities annually [1,2]. Yearly, 314,000 cases of snakebite envenomations resulting in 5900–14,600 amputations and 7000–32,000 fatalities in sub-Saharan Africa are recorded [1]. Given its impact on world health, snakebite is recognized by the World Health Organization as a category A neglected tropical disease [3].

Appendix 6: List of snakes cited in the thesis, their scientific and common name (s)

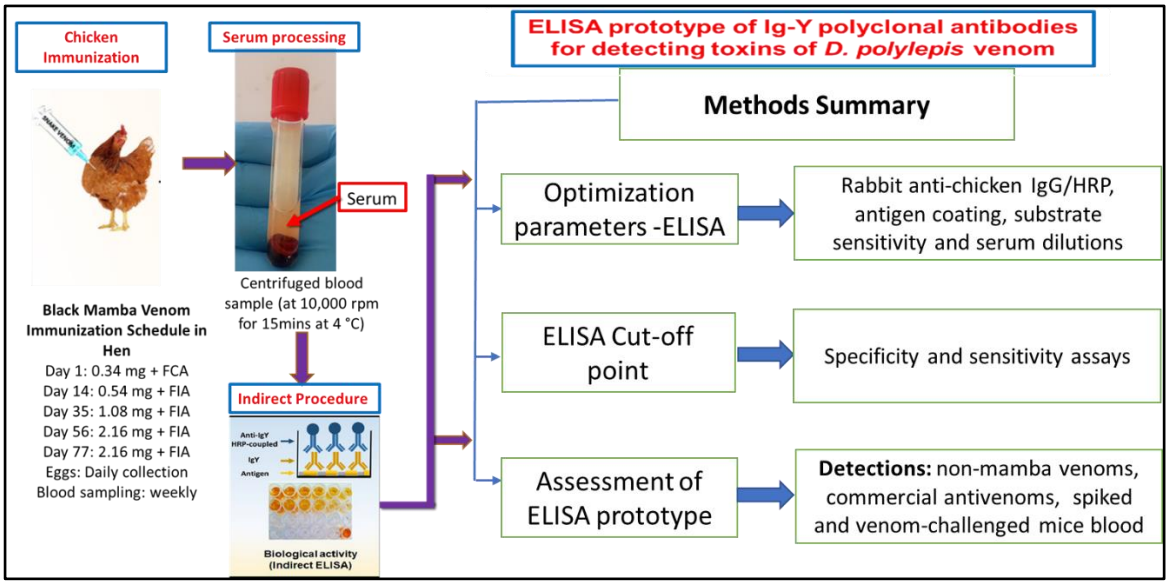
Scientific name	Common name (s)
Agkistrodon contortrix	Copperhead; Eastern copperhead
Agkistrodon piscivorus	Cottonmouth
Agkitrodon bilineatus	Cantil; Mexican cantil; Mexican ground pit viper; Cantil viper; Black moccasin, Mexican moccasin
Atheris desaixi	Mount Kenya bush viper; Ashe's bush viper; Desaix's bush viper
Atropoides nummifer / Metlapilcoatlus nummifer	Mexican jumping pit viper; Jumping viper
Bitis arietans	Puff adder
Bitis gabonica	Gaboon viper or adder
Bitis nasicornis	*Rhinocerous* viper
Bothriechis nigroviridis	Black-speckled palm-pit viper; Speckled palm viper; Black-spotted palm viper; Yellow-spotted palm viper
Bothropoides pauloensis	Boca-de-Sapo; Bocuda; Jararaca-Pintada
Bothrops asper	Terciopelo
Bothrops atrox	Lancehead; Fer-de-lance; Barba amarilla; Mapepire balsain
Bothrops insularis	Golden lancehead
Bothrops jararaca	Jararaca; Yarara
Bothrops pirajai	Piraja's lancehead
Bungarus caerulus	Common krait; Bengal krait
Bungarus multicinctus	Many-banded krait; Taiwanese krait; Chinese krait
Calliophis bivirgata / Calliophis bivirgatus	Blue coral snake; Blue Malayan coral snake
Calloselasma rhodostoma	Malayan pit viper
Crotalus viridus	Prairie rattlesnakes; Western rattlesnake; Great Plains rattlesnake
Daboia russelii	Russell's viper
Dendroaspis angusticeps	Green mamba; Eastern green mamba
Dendroaspis jamesoni	Jameson's mamba
Dendroaspis polylepis	Black mamba
Dispholidus typus	Boomslang
Echis carinatus	Saw-scaled viper; Indian saw-scaled viper; Little Indian viper
Echis ocellatus	West African carpet viper; Ocellated carpet viper
Hemachatus haemachatus	Rinkhals; Ringhals; Ring-necked spitting cobra
Lachesis muta	Southern American bushmaster; Atlantic bushmaster
Micrurus tschudii	Desert coral snake

Naja annulifera	Snouted cobra; Banded Egyptian cobra
Naja ashei	Large brown spitting cobra
Naja atra	Chinese cobra; Taiwan cobra
Naja haje	Egyptian cobra
Naja melanoleuca	Forest cobra; black cobra; black and white-lipped cobra
Naja mossambica	Mozambique spitting cobra
Naja naja	Indian cobra; Spectacled cobra; Asian cobra; Binocellate cobra
Naja nigricollis	Black-necked spitting cobra
Naja pallida	Red spitting cobra
Notechis scutatus	Tiger snake
Ophiophagus hannah	King cobra
Ovophis okinavensis	Hime habu; Ryukyu Island pit viper; Okinawan pitviper
Oxyuranus microlepidotus	Indian taipan; western taipan; small-scaled snake, or fierce snake
Oxyuranus scutellatus	Coastal taipan; Common taipan
Pseudechis papuanus	Papuan black snake
Pseudohaje goldii	Gold's tree cobra
Pseudonaja textilis	Eastern brown snake
Rhinocerophis cotiara / Bothrops cotiara	Boicoatiara; Boicotiara; Boiquatiara; Coatiara; Jararaca-de-barriga-preta; Jararaca-preta; Quatiara
Sistrurus catenatus	Eastern massasauga
Thelotornis capensis	Savanna vine snake; Southern vine snake
Thelotornis kirtlandii	Twig snake; Forest vine snake; Forest twig snake
Thelotornis mossambicanus	Eastern vine snake; Eastern twig snake
Trimeresurus albolabris	White-lipped pit viper; White-lipped tree viper
Trimeresurus popeorum	Pope's pit viper; Pope's green pit viper; Pope's tree viper; Pope's bamboo pit viper
Tropidolaemus wagleri	Wagler's pit viper
Vipera berus	Common European adder or viper
Vipera nikolskii	Nikolsky's adder; Forest-steppe adder

Appendix 7: Graphical representation of the generation and development of anti-*D. polylepis* IgY antibodies



Appendix 8: Graphical abstract representation of the ELISA prototype development



Appendix 9: Reagents Used in the Study

Reagents

Rabbit anti-chicken IgG/HRP conjugated antibody, 3,3',5,5'-tetramethylbenzidine (TMB), and bovine serum albumin (BSA) (Solarbio, China), o-phenylenediamine dihydrochloride (OPD) 5mg-tablet, Tween-20, Freund's incomplete adjuvant (FIA) and Freund's complete adjuvant (FCA) (Sigma-Aldrich, St. Louis, Missouri, USA), NHS activated sepharose 4FF, Amicon ultra-15 centrifugal filter devices and protein marker (Invitrogen, USA), glycine, non-fat dry milk and precast gel (12% Sodium dodecyl-sulfate polyacrylamide gel electrophoresis, SDS-PAGE) (Solarbio, China), OPD-powder (ThermoFishers, USA), citric acid monohydrate ($(HOC(COOH)(CH_2COOH)_2 \cdot H_2O$, mw=210.14), di-Sodium hydrogen orthophosphate 12-hydrate ($Na_2HPO_4 \cdot 12H_2O$, mw=358.14), hydrogen peroxide (H_2O_2), sulfuric acid, were obtained from the manufacturers (i.e. ThermoFishers, Invitrogen, Sigma and Solarbio). The other chemicals or reagents used were analytical grade and were bought from local companies.

Appendix 10: Overall of the one-way ANOVA results.

A table showing the ANOVA summary results[m]

	DF	Sum of squares	Mean square
Model	4	1.86281	0.4657
Error	35	0.8709	0.02488
Total	39	2.7337	-
F value		18.7159	
p value		< 0.0001	
p value summary		****	
Significant difference among means ($p < 0.05$)?		Yes	
R-squared		0.6814	

[m] The overall ANOVA table depicting statistical significance exists or not for the inhibition caused by *Dendroaspis* sp. and non-*Dendroaspis* sp. venoms containing samples. The goal of this experiment was to assess the assay's capacity for specific detection of homologous (*D. jamesoni, D. angusticeps, and D. polylepis*) venom samples, while maintaining a high degree of discrimination against heterologous ones (*N. ashei and B. arietans*). DF-means the degrees of freedom in the source.

Appendix 11: Tukey's multiple comparison test results.

A table detailing the multiple comparison test results[n]

Tukey`s multiple comparison test	**Adjusted *p* value**
D. jamesoni vs. *D. angusticeps*	0.99829
D. jamesoni vs. *B. arietans*	0.03341
D. angusticeps vs. *B. arietans*	0.02264
N. ashei vs. *D. jamesoni*	0.00206
N. ashei vs. *D. angusticeps*	0.00132
B. arietans vs. *N. ashei*	0.67821
B. arietans vs. *D. polylepis*	< 0.0001
D. polylepis vs. *D. jamesoni*	0.34015
D. polylepis vs. *D. angusticeps*	0.09557
D. polylepis vs. *N. ashei*	< 0.0001

At adjusted *p* value < 0.05 is significantly different.

[n] the venom samples of homologous species and heterologous species were compared against one another, and was carried out using the Tukey's multiple comparison test from the ANOVA to examine the sample-induced inhibition differences.