

**INCORPORATING THE 3RS (REFINEMENT,
REPLACEMENT AND REDUCTION OF
ANIMALS IN RESEARCH) INTO THE
PRECLINICAL ASSESSMENT OF SNAKE
VENOM TOXICITY AND ANTIVENOM
EFFICACY**

**Thesis submitted in accordance with the requirements of the
University of Liverpool for the degree of Doctor of Philosophy**

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March 2017



*Noah said to the creatures 'Go forth and multiply'
'We can't' said the snakes 'We're adders.'*

ABSTRACT

Antivenom is the only effective treatment for snakebite and comprise immunoglobulins obtained from venom-immunised horses or sheep. Globally, more than 45 manufacturers make over 120 snake antivenoms; it is a regulatory requirement that the venom-neutralising efficacy of all antivenoms are assessed preclinically. The World Health Organisation (WHO) recommended preclinical tests of efficacy are the median lethal venom dose (LD₅₀) and median effective antivenom dose (ED₅₀) assays performed in mice. They result in substantial pain and suffering to the mice with death/survival as their metric. With NC3R-funding, we sought to apply the 'Refine, Reduce and Replace' principles of animal experimentation to these murine assays.

Pain is a near-universal symptom of snake envenoming, and one of our objectives was to identify an effective analgesic that could be utilised without invalidating the assay results. The Mouse Grimace Scale and Activity scores were used to measure pain. We examined the effects of two opioid analgesics, buprenorphine and morphine, in a range of venom LD₅₀ and ED₅₀ assays. Both were effective at reducing pain scores, but death rates were higher in those which had received buprenorphine, hence morphine is preferable.

We demonstrate that each venom exhibits a distinct set of lesions, the severity of which appears time and dose dependent, and that the observed murine pathological lesions show significant similarities to those reported in envenomed human victims. Applying the 3R principles, we have used pathological observations, in combination with ante mortem observations, to establish more humane end-points, consequently reducing the duration of LD₅₀ and ED₅₀ assays from 24 to 6 hours. In addition, we have implemented a 'dose-staging' element into experimental design in which one dose is given and the next dose(s) selected based on the results of the previous dose, reducing total mice required.

To reduce the numbers of assays, and therefore mice, we have shown an excellent correlation between *in vitro* binding assays, cytotoxicity neutralisation assays and *in vivo* ED₅₀ using antivenoms derived from the same pool of donor animals. Comparison of the results of *in vitro* binding assays between 35 different venom/antivenom combinations showed a poor correlation overall, but the correlation improved when each of five venoms were considered separately.

The possibility of replacing the *in vivo* LD₅₀ and ED₅₀ tests, using a cell-based neutralisation assay was investigated using two cell lines from diverse tissues of origin, namely VERO epithelial-type cells and neural SH SY5Y. All venoms studied produced a cytopathic effect in both cell lines, with the VERO cells being more sensitive to viper venoms and SH SY5Y cells to the effects of elapid venoms, when both cell lines were grown in co-culture. However, variability of results made optimisation of a neutralisation assay inadequate for use as an alternative to *in vivo* tests.

Acknowledgements

I would like to thank my supervisor, Rob Harrison for his help, encouragement and endless patience. I am also indebted to Nick Casewell and Professor John Landon for their guidance and invaluable advice. Special thanks go to my friends and colleagues, past and present, at LSTM – Richard and his family, Rachel, Camilla, Maimonah, Paul Rowley (snake technician extraordinaire), Gareth, Stuart; and at MicroPharm – Brenda, Ibrahim, Caryl, the three Chris's, Dewi, Matt, Sarah, Rossen, Hayley, Elise, Megan, Ruth, Ian, Cheyenne. My husband, David has patiently proof read my thesis and supported me through the difficult times; my family and friends have patiently listened to me pontificating about snakes, venom, snakebites and their treatment. Special thanks to my prosthetist and the team at the Artificial Limb Centre for keeping me mobile. Thanks to Professor Brian Faragher for help with the statistics, to Matt Leach, Newcastle University for explaining how to assess pain in mice and to Lynn McLaughlin and the staff of the BSU, Liverpool University for assistance with the *in vivo* work. Without the help of all these people, and many more, this thesis would never have come to fruition.

I would like to acknowledge the financial support in the form of a PhD studentship from NC3Rs - the National Centre for Refinement, Replacement and Reduction of animals in Research; MicroPharm Ltd and the MRC.

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Abbreviations

(20)WBCT	20 minute whole blood clotting test
°C	Degrees centigrade
3FTx	Three finger toxins
3Rs	Refinement, Replacement and Reduction of animals in research
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ACh	Acetyl choline
AKD	Acute Kidney disease
APS	Ammonium persulphate
ARVRU	Alistair Reid Venom Research Unit
AsV	<i>Aspidelaps scutatus</i> venom (Shield-nosed cobra)
AU	Action units
AV	Antivenom
BaV	<i>Bitis arietans</i> venom (Puff adder)
BK	Banded Krait antivenom
BM	Basement membrane
BPP	Bradykinin potentiating peptide
BS	Block solution
CcV	<i>Cerastes, cerastes</i> venom (Desert horned viper)
CI (cytotoxicity)	Cell Index
CI (statistics)	Confidence intervals
CI₉₅ (statistics)	95% confidence intervals
CNBr	Cyanogen bromide
CNS	Central nervous system
cNTP	Cyclic-nucleotide tri-phosphate
CO₂	Carbon dioxide
COX	Cyclo-oxygenase
CPE	Cytopathic effect
CRISP	Cysteine-rich serine protease
CrV	<i>Calloselasma rhodostoma</i> venom (Malaysian Pit-Viper)
CSL	Commonwealth Serum Laboratories (polyvalent Australian snake antivenom)
CTL	C-Type Lectin
CTZ	Chemoreceptor trigger zone
CV (statistics)	Coefficient of variation
DAB	Diaminobenzamine
DaV	<i>Dendroaspis angusticeps</i> venom
DMEM	Dulbecco's modified Eagles medium
DpV	<i>Dendroaspis polylepis</i> venom
DtV	<i>Dispholidus typus</i> venom
E	Extinction coefficient
EC₅₀	50% CPE neutralisation
(Cytotoxicity)	
EC₅₀ (ELISA)	50% maximum binding
ECG	Electrocardiogram
ECM	Extracellular matrix

EcoIV	<i>Echis coloratus</i> venom
EcsV	<i>Echis coloratus sochurekei</i> venom
ED₅₀	Median effective dose
ELISA	Enzyme linked immunosorbent assay
ELISA EPT	ELISA endpoint titre
EOG	EchiTabG antivenom
EOV	<i>Echis ocellatus</i> venom
EpIV	<i>Echis pyramidum leakeyi</i> venom
ETP	EchiTabPlus-ICP antivenom
F(ab')₂	Prime 2 antibody
Fab	Prime 1 antibody
Fc	Crystallisable fragment of IgG
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
FIX	Factor (coagulation) nine
FPLC	Fast Phase liquid chromatography
FX	Factor (coagulation) ten
GABA	Gamma amino benzoic acid
GIT	Gastrointestinal tract
GMP	Good manufacturing practice
h	Hour
H&E	Haemotoxylin and eosin
H₂O	Water
Hburia	Haemoglobinuria
HCl	Hydrochloride
HEP	Humane endpoint
HPIS	Horse pre-immune (normal) serum
i.m.	intramuscular
i.p	intraperitoneal
i.v.	intravenous
IACUC	Institutional Animal Care and Use Committee
ICP	Instituto Clodomiro Picado
ICT	Immunocytotoxicity
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IR	Infra-red
KC	King Cobra antivenom
kDa	kilodalton
KM (statistics)	Kaplan Meier
KUN	Kunitz-type protease inhibitors
LAAO	L-Amino acid oxidase
LC₅₀	50% cell death
(Cytotoxicity)	
LC₉₀	90% cell death
(cytotoxicity)	
LD₅₀	Median lethal dose
LDL	Lowest detectable limit
LI	Large intestines

LSTM	Liverpool School of Tropical MEdicine
M	Molarity
M₅₀	Molarity of ammonium thiocyanate resulting in 50% disruption of binding by ELISA
MCD	Minimum coagulant dose
MDD	Minimum defibrinogenating dose
MDD-FPT	MDD- fibrin polymerisation time
MGS	Mouse Grimace scale
MHD	Minimum haemorrhagic dose
MND	Minimum necrotic dose
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	Molecular weight
NA	Nucleic acid
NAD	No abnormality detected
NC3Rs	National Centre for the 3Rs
ND	Not done
NE	Not effective
NH₄SCN	Sodium thiocyanate
NhajV	<i>Naja haje</i> venom
NM	Neuromuscular
NMJ	Neuromuscular junction
NnigV	<i>Naja nigricollis</i> venom
NnivV	<i>Naja nivea</i> venom
NnubV	<i>Naja nubiae</i> venom
NP	Natriuretic peptide
NpalV	<i>Naja pallida</i> venom
NR	Neutral red assay
(cytotoxicity)	
NSAIDs	Non-steroidal anti-inflammatory drugs
NT	Nucleotide
O₂	Oxygen (Saturation)
OD	Optical density
P (statistics)	Probability
PAS	Periodic Acid Schiff
PBS	Phosphate buffered saline (Appendix II)
PBW	Phosphate washing buffer (Appendix II)
PCT	Pre-clinical testing (of antivenoms)
PD	Pulse distension (MouseOx)
PIS	Pre-immune (normal) serum
PLA₂	Phospholipase A ₂
PM	Post mortem
PPB	Perl's Prussian blue
PR	Pulse rate
PSS	Pathology Survival score

PT	Prothrombin
QC	Quality control
R²	R-squared – a rank correlation coefficient
RBC	Red blood cell
RCF	Relative centrifugal force
RR	Respiratory Rate
RTCA	Real-Time Cell assay
SAIMR	South African Institute of Medical Research (Now South African Vaccine Producers)
SAIMRb	Boomslang antivenom
SAIMRm	Echis monovalent antivenom
SAIMRp	South African polyvalent snake antivenom
SCS	Citrate buffered saline
SD (statistics)	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEC	Size-exclusion chromatography
SEH	Sub-epicardial haemorrhage
SI	Small intestines
SII	Serum Institute India
SPIS	Sheep Pre-immune (normal)serum
SSAC	Small scale affinity chromatography
SVMP	Snake venom metalloproteinase
(SV)SP	Snake venom serine protease
TA	<i>Trimeresurus albolabris</i> antivenom
TBST	Tris base, salt, tween buffer (Appendix II)
TEMED	<i>N,N,N',N'</i> -Tetramethylethylenediamine
UK	United Kingdom
UV	Ultra violet
V/AV	Venom/antivenom
VbV	<i>Vipera berus</i> venom
(V)CPE	Venom cytopathic effect
VPT	ViperaTab antivenom
VPV	ViperaVet antivenom
VSAb	Venom specific antibody
WB	Western blot
WFI	Water for irrigation (sterile)
WHO	World Health organisation
α-VipH	Anti-Vipera IgG –High VSAb
α-VipL	Anti-Vipera IgG –Low VSAb
α-VipM	Anti-Vipera IgG –Medium VSAb

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Chapter 1. Introduction

The research presented in this thesis investigates ways in which the 3Rs (Refinement, Replacement and Reduction of animals in research) can be implemented into the *in vivo* lethality tests presently recommended to demonstrate preclinical efficacy of snake antivenoms. This chapter covers a brief background and rationale for this work; venomous snakes, venom toxins, their associated lesions and medical importance; antivenoms, their manufacture, efficacy testing, adverse effects, deficiencies in therapy and research to improve them; a definition of the 3Rs and the snake venom species selected for this work.

Venomous snakes occur throughout the world, except in the Antarctic, Greenland, New Zealand and a few other isolated countries, such as Ireland. Few snakes occur at high altitude, especially in areas outside the tropics. Snake-bite envenoming occurs wherever man meets venomous snake, either in the wild or in captivity.

1.1 Background and Rationale

1.1.1 Morbidity and mortality

Snakebite is classified as a neglected tropical disease by the World Health Organisation (<http://www.who.int/mediacentre/factsheets/fs337/en/>)¹. The highest incidence is in the impoverished rural tropics, where income barely covers subsistence, and renders medical treatments such as antivenom, unaffordable. Such areas include South Asia, Southeast Asia and Sub-Saharan Africa (Harrison et al, 2009).

Globally, it is estimated that there are between 1.2 and 5.5 million human snakebite envenomings, with between 25,000 and 125,000 deaths per annum, with an estimated 400,000 amputations and yet more suffering other permanent *sequelae* (Chippaux, 1998; Gutierrez et al, 2016; Harrison et al, 2009; Kasturiratne et al, 2008; Williams et al, 2010). The incidence of human

¹ Website checked 17.04.17

snakebite envenoming is illustrated in Figure 1.1-1 and that of snakebite mortality in Figure 1.1-2.

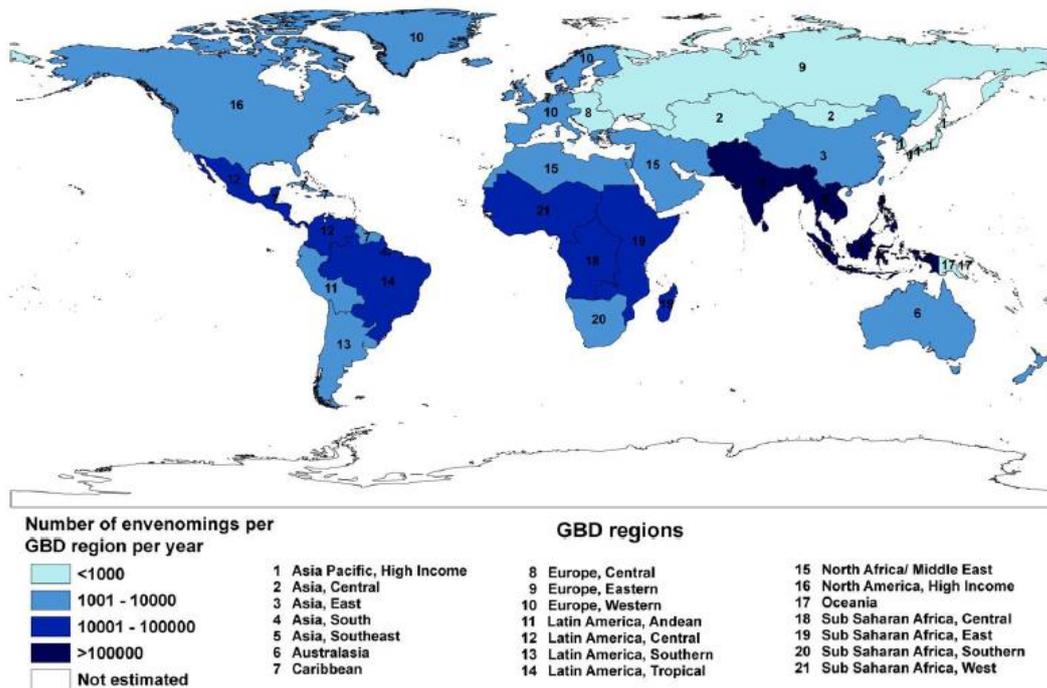


Figure 1.1-1: Regional estimates of envenomings due to snakebite (low estimate) GBD = Global Burden of Disease (Kasturiratne et al 2008)

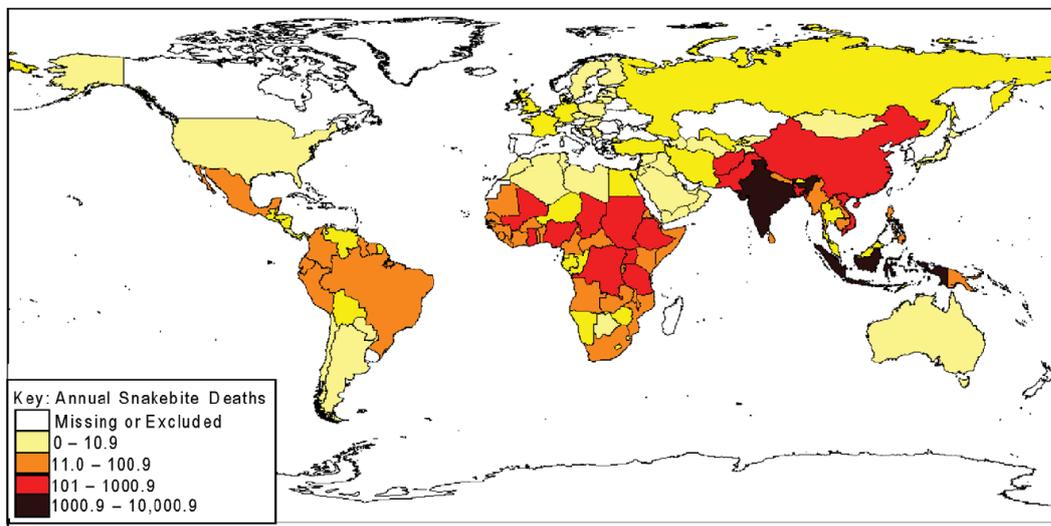


Figure 1.1-2: Annual snakebite mortality: Annual estimates of snakebite-induced deaths for 138 countries were obtained from data published by Kasturiratne et al, 2008, and depicted on a world map using Epi-info; the darker a country's colour, the greater the estimated snakebite mortality – see key for details (reproduced from Harrison et al, 2009).

1.1.2 Treatment - Antivenom

The only specific treatment for snakebite is antivenom, which consists of purified antibodies to venom components and is species specific in its efficacy (Brown and Landon, 2010). Globally there are 45 antivenom manufacturers making a total of 120 different antivenoms as shown in Figure 1.1-3 (<http://apps.who.int/bloodproducts/snakeantivenoms/database/>).

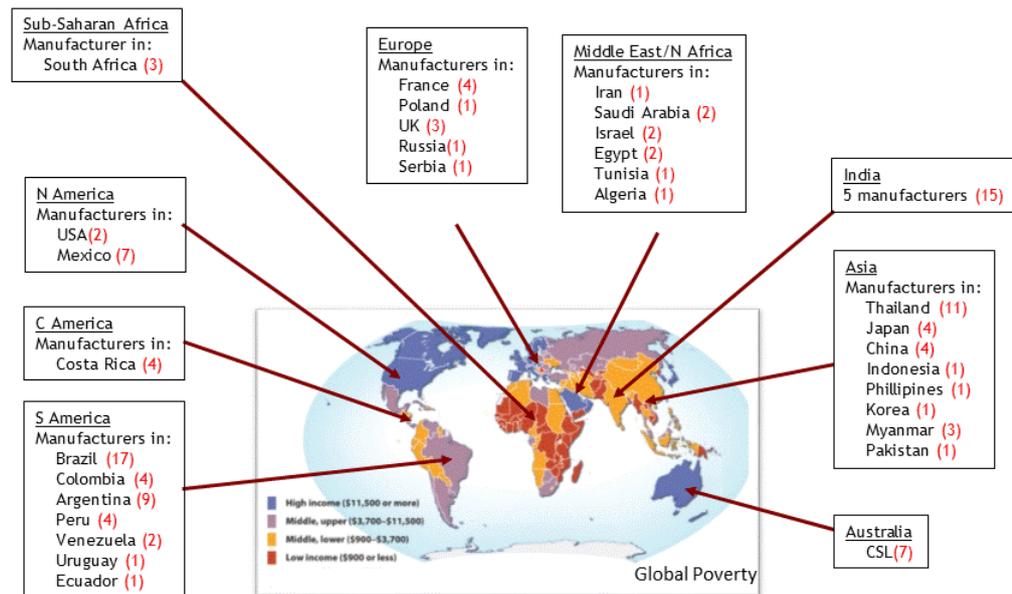


Figure 1.1-3: Antivenom manufacturers globally: Number of antivenoms in red. <http://apps.who.int/bloodproducts/snakeantivenoms/database/> (Harrison, personal communication)

1.1.3 Ensuring antivenom efficacy

It is imperative that antivenom neutralising efficacy is demonstrated for the snakes found in the area in which they are to be used prior to release for treatment of clinical cases.

Theakston and Reid, 1983, defined and described the standard assay procedures for the characterisation of snake venoms and their neutralisation with antivenom. The 'gold standard' assay is the median lethal dose of venom – the dose which kills 50% of a group of mice (LD₅₀) and the median effective

² 06.01.2016

dose of antivenom – the dose which saves 50% of mice receiving a standard lethal dose of venom (ED₅₀). These assays are described in detail in Chapter 2.

1.1.4 The murine cost

At present the World Health Organisation recommend preclinical testing (ED₅₀) of every batch of new or existing antivenom. The assays are of 24 hours duration and require a minimum of twenty mice, divided into one group of five mice which all die, one group of five mice which all survive and two intermediate survival groups. An LD₅₀ may also be required to define the 'lethal dose of venom' (usually 2x to 5x the LD₅₀). Assuming an average of 2 batches of antivenom are made annually, this equates to well in excess of 30,000 mice per annum for regulatory testing of these antivenoms.

1.1.5 Why the need to test antivenom *in vivo*?

The need to preclinically test antivenoms *in vivo* is best illustrated by an example described by Visser et al, 2008. A new antivenom was purchased by the government in Ghana which was 1/10th of the price of FAV Afrique – an antivenom known to be effective in treating cases of *Echis ocellatus* envenoming. It was noted that the fatality from snakebites increased from 1.8% to 12.1% and even larger doses of the new antivenom administered did not adequately neutralise the venom-induced coagulopathy. Had this antivenom been preclinically tested, it would have been shown to be ineffective, without the unnecessary loss of human life and associated morbidity.

1.1.6 Definition of the 3Rs

The 3Rs are: Replacement, Reduction and Refinement of animals in research.

- **Replacement** is the use of methods which avoid or replace the use of animals
- **Reduction** is the use of methods which minimise the number of animals used in an experiment
- **Refinement** is the use of methods which minimise suffering and improve animal welfare

1.1.7 The need for 3Rs

Pain is an almost universal symptom of human snakebite victims and therefore mice undergoing preclinical testing of antivenoms and associated venom characterisation, not unexpectedly, show signs of extreme pain and distress. At present, assay duration is 24 to 48 hours, depending on route of administration, and the metric is death; in the 21st century inflicting this degree of pain and suffering is unacceptable to many. Ethically, we, as scientists, have a duty to implement the 3Rs. The objective of this project is to implement the 3Rs into *in vivo* preclinical testing of antivenoms.

1.2 Literature Review

1.2.1 Classification of snakes

Snakes are vertebrate, ectothermic animals, which possess scales. They belong to the Order *Squamata*, the same Order as lizards. They have their own suborder, *Serpentes*. This Order of animals has a skull which is adapted to enable them to move their upper jaw in relation to the braincase. However, unlike lizards, in the case of snakes the upper and lower jaws are not rigidly attached, enabling them to dislocate their temporomandibular joints and swallow large prey whole (Gilpin, 2008).

Snakes are divided into three superfamilies: *Henophidia* (boas, pythons and primitive snakes), *Typhloplioidea* (blind and thread snakes) and *Xenophidia* (all other snakes, including venomous snakes). There are 19 families of snakes, the largest of which is *Colubridae*. The vast majority of Colubrids are harmless, but the few that are venomous (e.g. the Boomslang, *Dispholidus typus*) have short fangs at the back of their mouth (opisthoglyphs), which are grooved or solid (Figure 1.2-2A). The evolutionary relationship of advanced snakes is shown in Figure 1.2-1.

Most of the medically-important venomous snakes belong to the Families *Elapidae* including *Hydrophiidae* (sea snakes) or *Viperidae*, including *Crotalidae* (pit-vipers). These families all have fangs at the front of their mouths, the elapids' and hydrophiids' fangs being fixed to the maxillary bone

(*proteroglyphs*) (Figure 1.2-2B) whereas those of vipers' are back-folding (*solenoglyphs*) (Figure 1.2-2C) (Vonk et al, 2008).

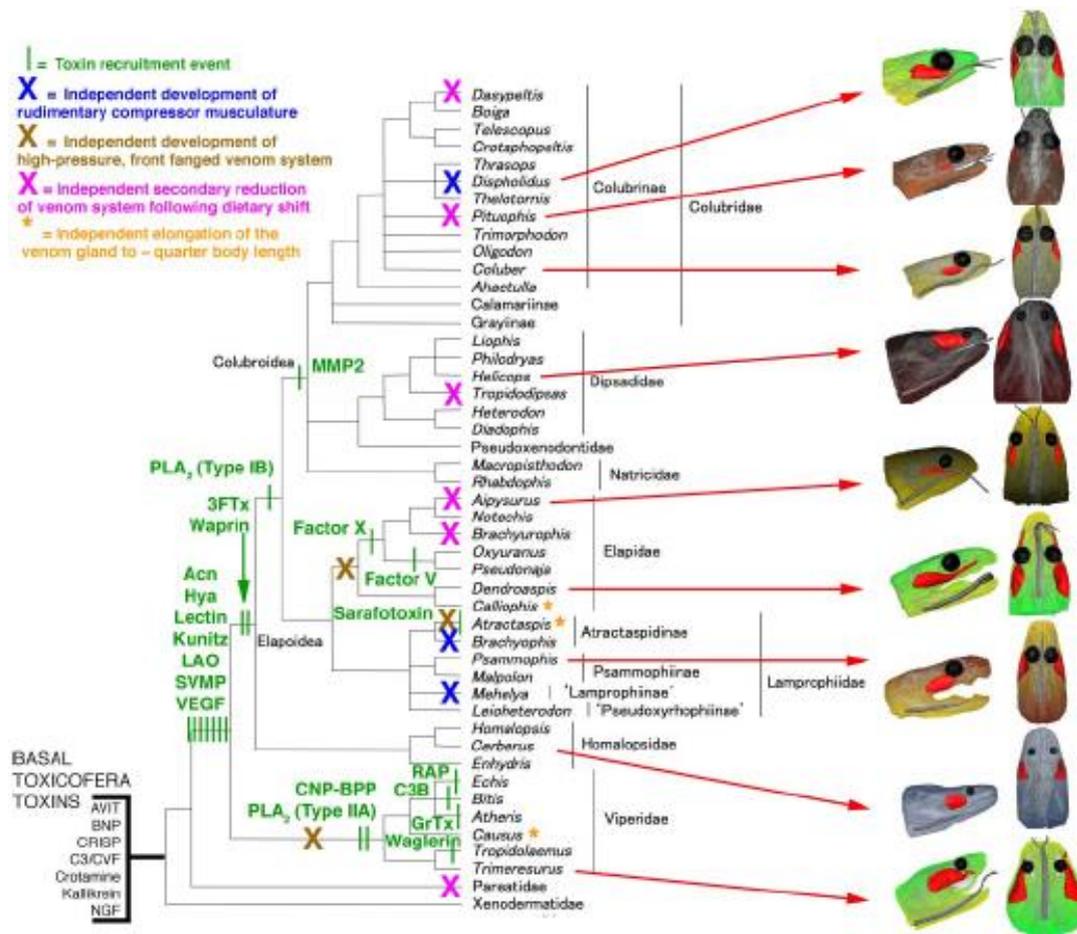


Figure 1.2-1: Cladogram of evolutionary relationships of advanced snakes showing relative timing of toxin recruitment events and derivations of the venom system. MRI images are shown for representatives. Ach, Acetylcholinesterase; LAO, L-amino oxidase; C3B, FAMC3B cytokine; CNP-BPP, c-type naturetic peptide-bradykinin potentiating peptide; GrTx, glycine rich toxin; Hya, hyaluronidase; RAP, renin-like aspartic protease; VEGF, vascular endothelial growth factor (Fry et al, 2008).

Genera of elapids include *Naja* (cobras), *Hemachatus* (rhinkals), *Bungarus* (kraits), *Dendroaspis* (mambas), *Micrurus* (coral snakes) and the Australian snakes (*Acanthophis*, *Pseudoechis*, *Oxyuranus*, and *Pseudonaja*). The venom of these snakes is characterised by neurotoxicity, although the venoms of Australian elapids of the families *Oxyuranus* (Taipans) and *Pseudonaja* (Brown snakes) are also coagulopathic.

Viperidae consist of two subfamilies, *Viperinae* and *Croalinae*; genera of *Viperinae* include *Vipera* (European/Eurasian vipers), *Macrovipera* (Palearctic vipers), *Bitis* (adders), *Cerastes* (horned vipers), *Causus* (night adders), *Atheris* (bush vipers), *Echis* (saw scaled vipers), *Eristicophis* and *Pseudocerastes*; genera of *Croalinae* (pit-vipers) include *Crotalus* (rattlesnakes), *Bothrops* (American lanceheads), *Agkistrodon*, *Trimeresurus* (Asian lanceheads), *Cryptelytrops* and *Sistrurus*.



Figure 1.2-2: Skulls showing fang position of A: Colubrid - rear, B: Elapid –front, fixed; C: Viper – front, hinged (Vonk et al, 2008).

1.2.2 Venom proteins

Venom is produced by two venom glands, which are modified salivary glands that are surrounded by muscle and lined by mitochondria-rich secretory epithelial cells. They lie in the post-orbital region of the maxilla. Venom is a cocktail of enzymes, both toxic and non-toxic proteins, oligopeptides, amines, nucleosides, lipids, carbohydrates and inorganic salts, including metal ions. Toxins generally have a mass less than 150kDa and target specific receptors, which are located mainly on cell membranes (Mackessy, 2010). Enzymes are generally of molecular weight 50-150kDa, although serine proteases, phospholipase A₂s, PI and PII snake venom metalloproteinases are less than 40kDa (Mackessy, 2010). The catalytic properties of which have two clinically-relevant consequences; firstly, the products of substrate degradation, which are not immunogenic, may be toxic in themselves; secondly, the reactions they catalyse are reliant on the enzymatic reaction cycle and are thus time-dependent (Mackessy, 2010). *Viperine* and *Croalaine* venoms are enzyme-rich whereas elapid venoms are richer in toxins (Mackessy, 2010). However, both enzymes and toxins are frequently referred to as 'toxins' and, indeed, some enzymes may exert a toxic effect other than that induced by their enzymatic action e.g. PLA₂s.

Table 1.2-1: Major viper venom enzymes, proteins peptides and toxic components showing their biological action and induced pathological lesions (Mackessy, 2010).

Viper Venom Protein/Component	Biological Action	Pathological lesions
SVMPs	Hydrolysis structural proteins, disrupt vascular BM ^{3,4}	Haemorrhage, tissue destruction, myonecrosis
	Prothrombin activator ^{4,5}	Consumptive coagulopathy
	Activate Fs IX and X	
	Fibrino(gen)olysis	Clot breakdown
Serine Protease	Platelet aggregation	Thrombocytopenia, haemorrhage
Thrombin-like SPs	Catalysis of fibrinogen ⁴	Disruption of haemostasis
Kallikrein-like SPs	Release of bradykinin	Hypotension
PLA₂	Lymphatic obstruction	Oedema
	Platelet aggregation	Haemorrhage
	Myotoxic ⁶	Muscle damage
	Block pre-synaptic ACh release (β -neurotoxins)	Neurotoxicity/ paralysis
C-type Lectin	Bind to Factors IX and X Binds to platelet receptors	Anticoagulant Haemorrhagic Thrombocytopenia ⁴
Disintegrins	Inhibit platelet aggregation	Haemorrhage
LAAO	Generates H ₂ O ₂ Inhibits F IX	Apoptosis and cell damage, Anticoagulant
5' Nucleotidase (NT)	Catabolism of NT	Hypotension ⁷
Phosphodiesterase	Hydrolysis NA and NT	Hypotension
Hyaluronidase	Hydrolysis of hyaluronan 'Tissue spreading factor'	Local tissue damage
BPP/NP	ACE inhibitors	Hypotension
Purines/ Pyrimidines		Hypotension, paralysis, apoptosis, necrosis ⁷

Acronyms in Table 1.2-1 and Table 1.2-2

SVMP	Snake venom metalloproteinase	NP	Natriuretic peptide
SP	Serine Protease	3FTx	Three finger toxin
PLA₂	Phospholipase A ₂	NM	Neuro-muscular
LAAO	L- amino acid oxidase	PT	Prothrombin
ACh	Acetyl choline	KUN	Kunitz-type protease inhibitors
NT	Nucleotide	AKD	Acute kidney disease
NA	Nucleic acid	CRISPs	Cysteine-rich serine protease
F	Factor (clotting)	cNTP	Cyclic nucleotide tri-phosphate
BM	Basement membrane		
ECM	Extracellular matrix		
BPP	Bradykinin potentiating peptide		

³ Gutierrez et al, 2016

⁴ Gutierrez et al, 2009a

⁵ Kini et al, 2005

⁶ Gutierrez et al 2009b

⁷ Aird, 2002

Table 1.2-2: Major elapid venom enzymes, proteins peptides and toxic components showing their biological action and induced pathological lesions (Mackessy, 2010).

Elapid Venom Protein/Component	Biological Action	Pathological lesions
3FTx – α-neurotoxins	Post-synaptic inhibition	Flaccid neuromuscular (NM) paralysis
3FTx – Fasciculins	Acetylcholinesterase inhibitors	Excitatory NM paralysis
3FTx - Cytotoxins		Local tissue destruction
3FTx – aminergic toxin	Cardiotoxin	Inhibits cardiac function Hypotension
3FTx	e.g. Thrombostatin	Antiplatelet function
	Acid-sensing ion channel inhibitor	Analgesic
PLA₂s	Pre-synaptic β -neurotoxins	Flaccid paralysis
	Cytotoxins	Local tissue damage
	Myotoxicity	Myolysis \rightarrow AKD
SP Group C PT activators	Like F Xa-Va complex ⁵	Consumption coagulopathy
SP Group D PT activators	Like F Xa ³	
SVSP KUN –Dendrotoxins	Pre-synaptic ACh release	Excitatory NM paralysis
SVMPs	Hydrolysis of structural proteins	Local tissue damage
CRISPs	?block cNTP-gated channels Mamba intestinal toxin	?Hypothermia Abdominal pain, cramps
Hyaluronidase	Hydrolysis of hyaluronan	'Tissue spreading factor'
5' Nucleotidase (NT)	Catabolism of NT	Hypotension ⁷
Purines/Pyrimidines		Hypotension, paralysis, apoptosis, necrosis ⁷

The principal toxins found in viper venoms are listed in Table 1.2-1 and those found in elapid venoms in Table 1.2-2. The relationship between venom toxin composition and venom-induced pathology is discussed more fully in Section 1.5.

1.2.3 Venom pathogenesis

Venom pathogenesis is generally divided into that which is induced at the bite site (local) and that which is induced by absorbed venom toxins (systemic).

1.2.3.1 Venom induced tissue destruction

Local injury at the bite site (Figure 1.2-3A) results mainly from the direct action of venom components. SVMPs hydrolyse structural proteins such as collagen in the basement membrane of microvessels, and the extracellular matrix (Herrera et al, 2015). This disruption of intercellular junctions results in local haemorrhage as well as allowing further ingress of venom, resulting in signs of systemic envenoming. Disruption of blood supply results in ischaemia,

inflammation and poor regeneration of damaged tissues. Myotoxic PLA₂ lyse skeletal muscle fibres and cause contraction of lymphatic vessels, effectively blocking lymphatic drainage and contributing to the accumulation of oedematous fluids (Gutierrez et al, 2009b). The rapidity of onset of these effects and the release of inflammatory mediators, which influence the outcome of tissue derangement, precludes the use of antivenoms (Gutierrez et al, 1998; Gutierrez et al, 2003; Coppola et al, 1992; Chaves et al, 2005; Trebien et al, 1989; Battellino et al, 2003; Mora et al, 2008). Another study has shown that *Echis carinatus* venom induces neutrophil extracellular trap (NET) formation which, in turn, results in the occlusion of blood vessels thus trapping venom toxins at the bite site and contributing to additional localised tissue destruction (Katkar et al, 2016).

1.2.3.2 Systemic venom pathogenesis

1.2.3.2.1 Haemorrhage and coagulopathy

Viper envenomation commonly results in haemorrhage and coagulopathy (Figure 1.2-3B), often with a multi-pronged attack on the coagulation cascade, platelet function, fibrin(ogen)olysis and blood vessel integrity. For almost every factor involved in coagulation or fibrinolysis there is a venom protein that can inactivate or activate it (Lu et al, 2005). Snake venoms also act on platelets, blood vessel basement membranes and capillaries, contributing to the risk of haemorrhage.

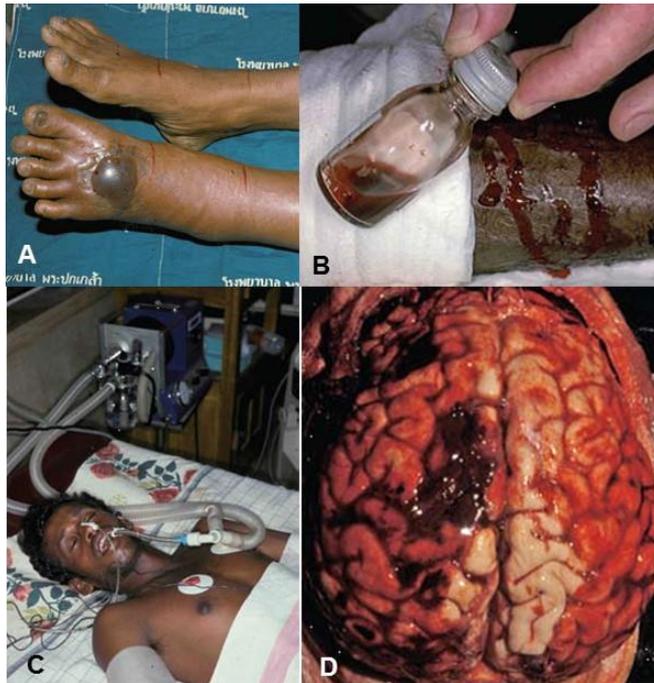


Figure 1.2-3: A. Localised signs of envenoming, showing blistering, necrosis and oedema at the bite site; B. Incoagulable blood after a Saw-scaled viper bite - whole blood fails to clot in a glass tube after 20 minutes – whole blood clotting time (WBCT); C. Flaccid paralysis requiring mechanical ventilation following a Common Krait bite; D. Fatal cerebral haemorrhage following Russell's viper bite. (A-C, Warrell; D, Dr U Hla Mon).

Whether the venom induces a primary coagulopathy, consumptive coagulopathy, thrombocytopenia or blood vessel injury, the result is haemorrhage. In addition to haemorrhage, the resultant hypovolaemia and shock, or thrombotic ischaemia, may result in secondary organ damage (White, 2005; Kini, 2006; Merle et al, 2005; Mosquera et al, 2003; Numeric et al, 2002; Puig et al, 1995; Markland, 1998, Markland and Swenson, 2013).

Haemorrhages may be critical by virtue of their site, such as cerebral (Figure 1.2-3D), intracranial (Mosquera et al, 2003), pericardial (Senthilkumaran et al, 2012) or intrapulmonary (Warrell et al, 1977). Microangiopathy and intravascular thrombi (Casamento et al, 2011) result in ischaemic change in end organs, such as kidney (Puig et al, 1995), myocardium (Blondheim et al, 1996; Saadeh, 2001), lung (Bart et al, 2016) and brain (Numeric et al, 2002, Canas et al, 2016, Merle et al, 2005).

1.2.3.2.2 Cardiotoxicity

Cardiovascular changes are well documented in both man and animals and include myocardial infarction, arrhythmias, ST-depression, T-wave inversion or flattening, atrial tachycardia or fibrillation, ventricular extrasystole, atrio-

ventricular block (Karlson-Stiber et al, 2006; Anlen, 2008; Hoffman et al, 1993; Lervic et al, 2010; Pelander et al, 2010; personal communication, Mark Patteson et al, 2015).

An unusual case of snake-bite by *Vipera aspis* involved a healthy 45-year-old male bitten on the thigh and envenomed intravascularly. He died within minutes before any medical management could be instituted. Autopsy revealed extensive myocardial damage with minimal local manifestations (de Haro et al, 2009).

1.2.3.2.3 Neurotoxicity

Elapid venoms contain pre- and/or post-synaptic neurotoxins which result in descending paralysis. Ptosis, followed by difficulty swallowing, respiratory paralysis and finally, total paralysis may occur within two hours of envenoming (Figure 1.2-3C) and, unless given within 15 minutes, antivenom is ineffective (Harris et al, 2000; Ranawaka et al, 2013, Sharma et al, 2016). Post-synaptic toxins may produce muscle hyperexcitability (fasciculations) or tetany prior to flaccid paralysis (Hodgson and Davidson, 1996). Some Viperid venoms also contain neurotoxins which cause symptoms such as drowsiness, diplopia, strabismus, vertigo and unsteady gait (Reading, 1996; Ferquel, et al, 2007). Secondary neural lesions may result from coagulopathies causing intracranial haemorrhage or thrombotic infarcts (Figure 1.2-3D) (Merle et al, 2005; Mosquera et al, 2003; Numeric et al, 2002).

1.2.3.2.4 Nephrotoxicity

Venom toxins are rarely responsible for primary acute renal failure, but may develop as a complication of profound hypotension, renal casts from myoglobin or haemoglobin and microthrombi from venom-induced disseminated intravascular coagulation (Azevedo-Marques et al, 1985; Paul and Dasgupta, 2012; Martins et al, 1998).

1.2.3.2.5 Myotoxicity

Myonecrosis occurs as the result of the action of myotoxic PLA₂s, but is enhanced by the SVMP-mediated damage to the microvasculature and

haemorrhage, which, in turn, results in poor regeneration in both muscle fibres and nerves (Hernandez et al, 2011).

1.2.4 Medical Importance

The medical importance of snakebite lies not only with mortality, but also the resulting permanent physical and psychological consequences.

Local envenoming can lead to severe tissue necrosis requiring plastic surgery, including extensive skin grafting or amputation (Abubakar et al, 2010; Habib et al, 2015; West et al, 2014). Compartment syndrome, where swelling results in an increase in pressure of oedematous fluid in a distal limb such that vascular supply is compromised, is an uncommon, but significant, complication of viper bites (Evers et al, 2010; Bucarechi et al, 2010). The venom of spitting cobras, projected into its victim's eye, produces corneal erosion and ulceration, which may leave its victim blind due to the residual scarring (Warrell and Ormerod, 1976). Long-term consequences include arthrodesis, chronic ulceration, osteomyelitis and malignant transformation (Warrell, 2010). Other significant complications which may occur following snakebite are: foetal loss, wound infections and tetanus (Habib, 2013). The resulting disfigurement from the local effects of venom can have catastrophic, life-changing sequelae for victims and their families. Victims may become social outcasts and young women may no longer be acceptable as a bride.

The consequences of snakebites can have a profound effect on the local economy. Loss of work days result in a significant loss of income, especially as snakebite accidents tend to occur during the busiest periods of the year (Habib et al, 2013). Having to bear the cost of medical treatment may mean that the victim is left to die or permanently disabled, consigning the family to destitution and the community to a shortage of manpower (Habib, 2013). During planting and harvesting, especially during the rainy season, snakebite victims can occupy over 70% of beds in hospitals which serve these agricultural areas (Theakston and Warrell, 2000).

Envenomation with haemorrhagic venom may require blood or blood product transfusion in addition to antivenom. Venom-induced haemorrhage or microthrombi occurring in a 'vital' site, such as the brain or myocardium may result in fatality or that of prolonged recovery and/or permanent disability (Blondheim et al, 1996; Mosquera et al, 2003).

Acute renal failure is a common complication of snakebite secondary to hypotension, particularly in the Indian subcontinent associated with *Daboia russelli* (Russell's viper) envenomation (Paul et al, 2012) and requires dialysis for management of some patients, which may be unsustainable in areas where snakebite is common.

Neurotoxic venom toxins may cause irreversible paralysis. These toxins are generally of low molecular weight and therefore undergo rapid distribution. Unless antivenom is administered intravenously within hours of the bite, respiratory paralysis is likely and, unless mechanical ventilation is available, death inevitable (Prasarnpun et al, 2005; Pandey et al, 2016).

1.2.5 Treatment: Antivenom

The only specific treatment for snake-bite envenoming is antivenom (Brown and Landon, 2010). However, antivenom cannot neutralise the pathological lesions that have occurred due to irreversible venom-induced changes, or the *sequelae* of its primary effects such as acute renal failure. Administration of antivenom should not replace appropriate supportive therapy (WHO, 2010b).

The first antivenom was produced by Albert Calmette in 1895 against the Indian cobra, *Naja naja* and, since then, the basic principles of antivenom production have changed little. First generation antivenoms, which were produced for over a hundred years, comprised hyperimmune serum from animals immunised with venom (Chippaux et al, 1983). Technologies for antivenom production have improved greatly, and their therapeutic effectiveness has been widely demonstrated, particularly for treatment of systemic envenomings (Lalloo and Theakston, 2003; Theakston et al, 2003;

Gutierrez et al, 2009c). The main active component of antivenom is IgG antibodies or their antibody –containing fragments (see Section 1.2.5.1.2.1); besides having a high affinity for toxicologically relevant venom components, the ideal antivenom would possess a volume of distribution as close as possible to that of the toxins being neutralised (Gutierrez et al, 2003).

Adverse reactions to antivenoms can be divided into immediate Type I-type hypersensitivity and delayed ‘serum sickness’ which may occur 7-10 days after antivenom administration. Early adverse reactions may be caused by complement activation due to the presence of Fc fragments in whole IgG preparations, or the presence of protein aggregates (Otero et al, 1999; Herrera et al, 2005). Delayed reactions are related to the immunogenicity of the antivenom proteins, which elicit an anti-donor IgG antibody response in the recipient (Laloo and Theakston, 2003; Herrera et al, 2005).

Preclinical testing required to demonstrate the efficacy and safety of antivenoms prior to their use in clinically envenomed patients is imperative to avoid failure of therapy and unnecessary fatalities resulting from the use of ineffective antivenom.

1.2.5.1 Antivenom production

1.2.5.1.1 Immunisation

1.2.5.1.1.1 Venom source

The quality of venom used for immunisation of donor animals is important in producing antibodies which are effective in neutralising its pathological effects. Venom composition varies not only between species, but can also vary between individuals of that species and within individuals over time (Chippaux et al, 1991). In order to obtain a representative sample of venom, it is therefore prudent that venom for antivenom production is extracted from multiple specimens differing in terms of:

- geographical origin, relevant to the target areas for antivenom use
- age and sex

Health and welfare of the snakes is vital for the production of high quality venom. Venom is extracted from captive snakes around every 4 weeks, the pooled venoms lyophilised and stored at 2-8°C (Chippaux and Goyffon, 1983). Adjuvants are mixed with the immunogen to hold venom in the donor's tissues to allow slow release, avoiding acute toxicity, and enhancing antibody production. The most commonly used adjuvant is Freund's complete adjuvant (FCA) for primary immunisation and Freund's incomplete adjuvant (FIA) for boosters. FIA contains emulsified mineral oil but FCA contains inactivated *Mycobacterium tuberculosis* in addition to the components of FIA. Other adjuvants include aluminium salts, bentonite and liposomes (WHO, 2010a).

A major problem with the use of adjuvants is the severe localised reactions which may be induced, particularly in horses, and using Freund's adjuvants. This may be overcome either using a low dose, low volume, multi-site procedure (Chowiwathanakun, 2001) or switching to an aluminium hydroxide adjuvant after the primary immunisations (Gutierrez et al, 2005).

1.2.5.1.1.2 Antiserum Donor Animals

The most important criteria for the production of antiserum is the use of healthy individuals as donors. The immunisation schedule depends on the immunogen and adjuvant, donor species, dose of immunogen and immune response of the individual.

i) Horses

Horses have traditionally been the donor of choice due to the large volume of serum that can be obtained from an individual at any one time. Sedimentation of the red blood cells (RBC) is rapid and does not require centrifugation. They are docile and therefore easy to handle and are resistant to trypanosomes in tropical areas. They are not susceptible to transmissible spongiform encephalopathies, the transmission of which is of major concern.

ii) Sheep

A number of factors mean that antiserum production in the UK is predominantly from sheep (Landon et al, 2003). Firstly, they are widely available, relatively inexpensive to purchase and maintain, and produce more

immunoglobulin/acre than horses. Secondly, they have a much less pronounced inflammatory response to venom immunisation than horses and thus do not develop severe reactions at the immunisation sites and, in the UK, where animal welfare is an emotive issue, especially concerning companion animals, sheep are a more acceptable choice than horses. Ovine IgG is less glycosylated than equine IgG, claimed to be less immunogenic, and thus less likely to produce adverse reactions (Sjostrom et al, 1994; Angulo et al, 1997). Clinically, ovine derived EchiTabG caused fewer early reactions than equine derived EchiTab-Plus ICP (Abubakar et al, 2010b). Finally, the use of antiserum pooled from a large number of individuals results in less variation of specific antibody levels than that from a single donor [unpublished results, MicroPharm Ltd]. However, the use of sheep as a donor animal may be limited due to perceived concerns regarding the possible transmission of spongiform encephalopathies from donor to patient (Lalloo and Theakston, 2003).

iii) Other species under investigation

Other sources of serum, such as camelids (Cook et al, 2010a; Cook et al, 2010b; Cook et al, 2010c) chickens (Moussa et al, 2012) and ducks (Criste et al, 2008), are being more widely investigated.

Research has been undertaken to investigate the suitability of using camels as suitable antiserum donors, showing that they are better adapted to tropical climates than conventional donor species, are amenable to handling and bleeding and possess heavy chain only IgG, which is more heat stable than that of other species (Cook et al, 2010c; Cook et al, 2010a, Cook et al, 2010b). Antivenom made from camel antiserum would appear to be less immunogenic than those from horses or sheep (Herrera et al, 2005).

IgY from chicken egg yolk is a cheap source of antivenom polyclonal antibodies and has the added advantage that it does not fix complement and is therefore less likely to induce a hypersensitivity reaction in the recipient (Lalloo et al, 2003; Moussa et al, 2012).

1.2.5.1.1.3 Immunisation procedure

Optimal IgG titres are obtained by the low volume, low dose, multiple site immunisation close to drainage lymph nodes (Figure 1.2-4A) and when injected subcutaneously (Pratanathonet al, 1997; Chotwiwatthanakun et al, 2001; WHO, 2010a).



Figure 1.2-4: A – Immunisation in proximity to drainage lymph nodes; B – Bleeding the donor; C – Rotation of harvested blood to accelerate clotting

1.2.5.1.1.4 Harvesting blood

Blood may be collected once peak antibody production has been reached (Figure 1.2-4B), around 16 weeks after primary immunisation (Al-Abdulla et al, 2013), then every 3-8 weeks thereafter (WHO, 2010a). It is essential that the donor animals are healthy when blood is harvested. Venom specific antibody levels are monitored using ELISA or small scale affinity chromatography (Figure 1.2-5).

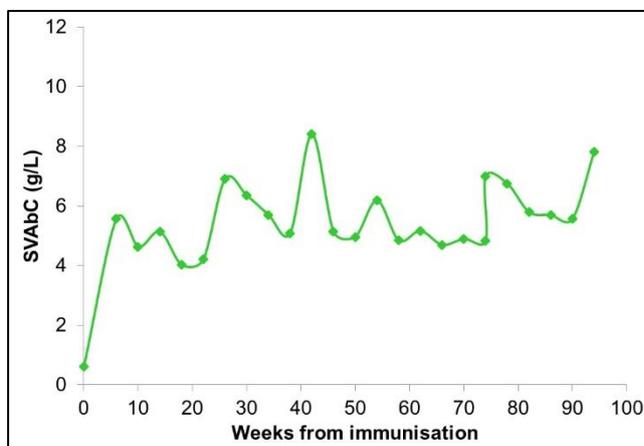


Figure 1.2-5: Specific venom antibody response in a flock of 25 sheep immunised with *Vipera* spp of venom, measured by small scale affinity chromatography (SSAC). (MicroPharm: personal data).

Around 10mls/Kg body weight of blood may be harvested, under aseptic conditions, via the external jugular vein and the red cells removed (Figure 1.2-4B). The RBCs may be re-infused into the donor animal to prevent anaemia, and resulting plasma or serum pooled before storage at -20°C until required.

1.2.5.1.2 Antibody purification

1.2.5.1.2.1 Purification of IgG

Precipitation

Antivenom manufacture from pooled serum or plasma involves purification of IgG from other blood proteins in serum; the purification also concentrates the specific antibodies. The utilisation of highly purified IgG fragments leads to better tolerance and a higher efficacy (Chippaux, 1998).

Immunoglobulins may be purified in a number of ways. They may be precipitated using ammonium sulphate (Lalloo et al, 2003) or sodium sulphate (Smith et al, 1992), then resuspended in appropriate buffer. Caprylic acid precipitates unwanted components of serum, mainly albumin, leaving the IgG in solution (Gutierrez et al, 2005; Rojas et al, 1994). Purification of F(ab')₂ by caprylic acid was compared to that purified by ammonium sulphate precipitation and was found to be free of albumin and other plasma proteins whereas the latter was not (Dos Santos et al, 1989). Caprylic acid purified IgG remains in solution which retains its binding activity better, and is less likely to form aggregates than the resuspended IgG after ammonium or sodium sulphate purification.

Cleavage of IgG

IgG molecules may be cleaved by pepsin (Jones and Landon, 2003) or papain digestion (Smith et al, 1992; Rawat et al, 1994), as shown in Figure 1.2-6 with the advantage that smaller molecules have a larger volume of distribution and, theoretically, lower immunoreactivity, providing superior protection (Al-Abdulla et al, 2003; Laing et al, 1995). Theoretically, cleavage and removal (by anion

exchange) of the Fc fragment translates into a product with fewer adverse reactions.

Fab fragments (MW 50,000) have the largest volume of distribution (equal to that of extra-cellular fluid [ECF]) and the shortest elimination half-life. IgG (MW 150,000Da) has the lowest volume of distribution (equal to the blood volume compartment) and the longest elimination half-life (Gutierrez et al, 2003). F(ab')₂ fragments (MW 100,000Da) are somewhere in between (Ho et al, 1990; Pepin-Covatta et al, 1995; Lomonte et al, 2009). However, these would appear to be inconsistent findings as others have showed that F(ab')₂ was most rapidly distributed, IgG most slowly and Fab between. It was concluded that F(ab')₂ was the most suitable fragment for the treatment of snake and scorpion envenomings (Ismail and Abd-Elsalam, 1998; Riviere et al, 1997). Using smaller fragments would not appear to translate into better neutralisation of local effects of envenomation (Leon et al, 2000; Leon et al, 2001; Burnouf et al, 2004).

Additionally, the pharmacokinetics of IgG and its fragments is altered by the tissue destructive effects of venom toxins. On the one hand, damaged blood supply can impede distribution of antibodies, but on the other hand, damaged blood vessels can allow larger molecular weight compounds to move more easily into the extracellular fluid compartment. Ultimately the choice of IgG or fragment needs to be based on the most lethal venom toxins and their potential distribution (Gutierrez et al, 2003a; Gutierrez et al, 2007).

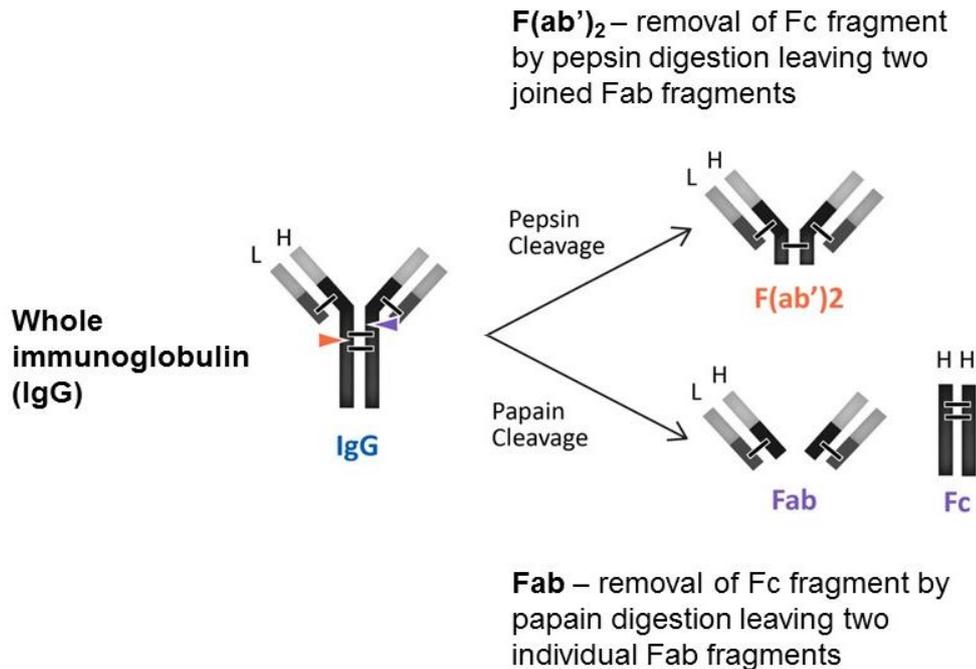


Figure 1.2-6: Cleavage of immunoglobulins to remove the Fc fragment by pepsin digestion yielding F(ab')₂ fragments or by papain yielding Fab fragments. (<http://www.rockland-inc.com/antibody-fragmentation-services.aspx>).

Further purification

Further purification of IgG or its fragments may be achieved by using one or more of the following steps:

- Diafiltration to remove salts by passage through a dialysis membrane, which will remove all molecules smaller than the molecular weight cut-off of the membrane.
- Adsorption onto aluminium hydroxide to remove lipids
- Ion exchange, which involves passage of the product through a chromatography column, exchanging small anions or cations for those that need to be removed.
- Anion exchange to remove the Fc fragment
- Cation exchange to remove papain from Fab formulations.
- Affinity purification, using venom covalently coupled to a cyanogen bromide-activated Sepharose 4B column, may be used to purify specific antibodies (Smith et al, 1992). Here the IgG is passed down the column – only venom-specific antibodies bind to the venom on the column, the

rest are washed off. The electrostatically bound antibodies are eluted by reducing the pH sufficient to disrupt the venom-antivenom binding without damaging them. The resulting antivenom contains only venom-specific antibodies (around 10% of total IgG). The disadvantage is that the cost of manufacture is vastly increased (by around 10 fold).

1.2.5.1.2.2 Viral filtration/removal

To date there have been no recorded cases of viral transmission to patients treated with antivenoms. However, the potential for viral transmission from donor to patient exists and thus steps to remove possible infectious agents are required for all new antivenoms produced in Europe and the USA (Ph. Eur., 2014; WHO, 2010; Theakston et al, 2003; Burnouf et al, 2004).

Caprylic acid is efficient for the removal of enveloped viruses and moderately efficient at removing non-enveloped viruses (Mpandi et al, 2007). This method may be used as a viral inactivation phase prior to viral filtration (WHO, 2010a). The process involves passing the product through a 15-30nm filter. The process also adds a significant cost to the final product because the expensive filters cannot be used more than once, there are losses during filtration, and the product requires re-concentration after filtration, therefore increasing manufacturing time and requirements for validation of the procedures.

1.2.5.1.2.3 Final formulation

The final product is formulated as liquid in an appropriate buffer at a suitable pH. Additives such as Tween (a detergent) or sugars, such as trehalose, may be added to improve stability and prevent aggregate formation.

A liquid formulation of ovine IgG in sodium citrate buffered saline, pH 6.0 was shown to be stable at 4°C or at room temperature for more than 3.5 years, and can be exposed to tropical temperatures for at least a month without losing immunoglobulin binding activity (Al-Abdulla et al, 2003; Al-Abdulla et al, 2013). A freeze-dried product is more stable in tropical climates, however, freeze drying increases cost of production. Reconstitution before administration may

also be slow, sometimes taking more than 20 minutes, which is not ideal in a clinical situation. However, recent technical developments have reduced i) cost and ii) efficiency of resuspension (Cherry et al, 2014).

1.2.5.1.3 Assessment

Both the WHO guidelines (WHO, 2010a) and the European Pharmacopoeia (Ph.Eur, 2014) advocate the use of as wide a range as possible of both *in vivo* and *in vitro* tests to assess quality and efficacy of antivenoms.

1.2.5.1.3.1 Physicochemical properties

The physicochemical properties of the final product are defined in the European Pharmacopoeia (Ph.Eur, 2014) and in the WHO guidelines for antivenom production (WHO 2010a).

- If in liquid form, the product should be free of visible particles (WHO, 2010a).
- Total protein concentration may be measured using a colorimetric method (Bradford, 1976) or by absorption at 280nm (WHO, 2010a).
- Purity may be assessed using SDS-PAGE under reducing or non-reducing conditions, the electrophoretic pattern being compared to a reference preparation (WHO, 2010a).
- Purity may also be assessed using fast-phase liquid chromatography (FPLC) or size exclusion chromatography to demonstrate the proportion of total IgG or its fragments and the presence of impurities such as albumin or small molecular fragments from the digestion process.
- The product must be free of bacteria, viruses and mycoplasma. Validation of virus removal steps is sufficient to show a product free from viruses, but microbiological examination is required to show that there are no bacteria or mycoplasma present.
- Freedom from bacterial lipopolysaccharides (endotoxins) (WHO, 2010a).
- Preservatives such as Phenol or Cresol may be added to the final product to maintain sterility.

1.2.5.1.3.2 Binding

Binding of antivenom to venom is not synonymous with neutralisation of venom toxicity. Toxic venom proteins may be large enough to have more than one potential binding site. If antibody binding does not alter the toxic protein's active site in such a way that it can no longer bind to its biological target, either by binding to its active site or by stoichiometrically altering its active site, then neutralisation of its induced lesions will not occur. Polyclonal antivenom IgGs bind to all venom proteins, not all of which are toxic or cause significant pathological lesions i.e. binding is not necessarily significant.

Enzyme-immunosorbent assays (ELISA)

ELISA is a means of measuring binding of antibodies to antigen (see Chapter 2). It is a very sensitive means of screening antivenoms or antisera for their ability to bind to venom (Sjostrom et al, 1996; Peres et al, 2006; Theakston et al, 1979). It is not a good predictor of antivenom efficacy for the reasons given above. However, it is reported, that by using an ELISA protocol which measures venom-antivenom complexes, antivenom efficacy can be predicted (O'Leary et al, 2014).

One disadvantage of this inherently sensitive test system, especially in tropical areas is that non-specific reactivity, cross-reactivity and the quality of reagents can effect both sensitivity and specificity of venom antigen and antibody detection assays. To exploit its potential as the most versatile immunoassay technique in snake bite research, the test conditions of the ELISA will have to be much more stringently controlled than they are at present (Ho et al, 1986).

Small scale affinity chromatography (SSAC)

The use of SSAC is a method of quantifying venom specific antibodies (VSAb) in antivenoms has been developed, based on affinity purification of antibodies for antivenom manufacture (Smith et al, 1992). Briefly, venom is covalently coupled to a gel (solid phase), antibodies are added to and incubated with the gel. Unbound protein is washed off, then the bound antibodies eluted by lowering the pH. The protein concentration of the eluate is measured at 280nm

and, from this, the VSAb concentration of the original solution calculated (see Chapter 2). The procedure has the advantage over ELISA in that it provides a g/L measurement of venom-specific antibodies and involves fewer steps. Once columns are coupled, they can be re-used in excess of 90 times (Harrison, 2004).

Preparation of the columns are described (Smith et al, 1992) and the assay has been used in assessment of antivenoms and antisera from both ovine and equine origin. The results correlated well with ELISA titres (Al-Asmari et al, 1996; Jones et al, 1999; Rawat et al, 1994 Casewell et al, 2010; Sjostrom et al, 1994).

Disadvantages include a degree of non-specific binding, which may not be constant in the presence of high affinity antibodies, and incomplete elution of very high affinity antibodies. The latter can partially be overcome by reducing the elution pH further, to a level at which the antibodies may be damaged and rendered ineffective. This is not acceptable for manufacture, but produces more consistent results for measurement purposes (MicroPharm – unpublished results). Preparation of the gel column and performing multiple assays can be more time consuming than ELISA.

Western Blot

Immunoblotting permits determination of venom proteins bound by antivenom antibodies. This technique may also demonstrate cross-reactivity between snake venoms and antibodies directed against a heterologous species, and is a useful adjunct to assess the immune reactivity of antivenom IgGs to venom proteins. However, like the above *in vitro* assays, it cannot be used to predict antivenom efficacy at neutralisation of venom toxicity.

Western blotting is a useful technique for demonstrating deficiencies in antivenom binding to separated venom proteins, by identifying missing protein bands in the blot compared to the stained gel, but does not quantify the amount

of binding i.e. it is qualitative, not quantitative. On the other hand, ELISA (and SSAC) measure the total binding of antivenom to all venom proteins.

These binding assays are useful in identifying antivenoms with poor/no venom binding potential and thus eliminating the need to test the efficacy of these antivenoms *in vivo*.

1.2.5.1.3.3 Potency and toxicity

The WHO state that *in vivo* assays are the best test of antivenom efficacy. Standard assays have been developed by the WHO to characterise snake venoms and to assess the neutralising potential of antivenoms. These assays measure lethal, defibrinogenating, procoagulant, haemorrhagic and necrotising properties of snake venoms (Theakston et al, 1983; WHO, 2010a).

Median lethal dose (LD₅₀)

LD₅₀ is the dose of venom, injected into the tail vein, which kills 50% of mice over 24 hours (WHO, 2010a) as shown in Figure 1.2-7. The results are analysed using a Probit analysis (Finney, 1971; Ownby, 1984), Spearman-Kärner or other non-parametric test (WHO, 2010a).

Median effective dose (ED₅₀)

ED₅₀ is the dose of antivenom required to prevent the death of mice, which have been injected with a pre-incubated mixture of venom (2x to 5x LD₅₀) with antivenom, over a period of 24 hours (WHO, 2010a) as shown in Figure 1.2-7. ED₅₀ may be used to predict clinical effectiveness of an antivenom, not only against the species of snake venom against which it is directed, but also against other closely related species (WHO, 2010a; Casewell et al, 2010). The ED₅₀ was used to design a dose-finding human clinical trial of EchiTabG (Abubakar et al, 2010).

Using an ED₅₀ a number of antivenoms have been shown to have an important degree of paraspecific protection within closely related snakes, which has implications for distribution of these antivenoms (Archundia et al, 2011;

Bogarín et al, 2000; Casewell et al, 2010; Segura et al, 2010; Gutierrez et al, 2013a).

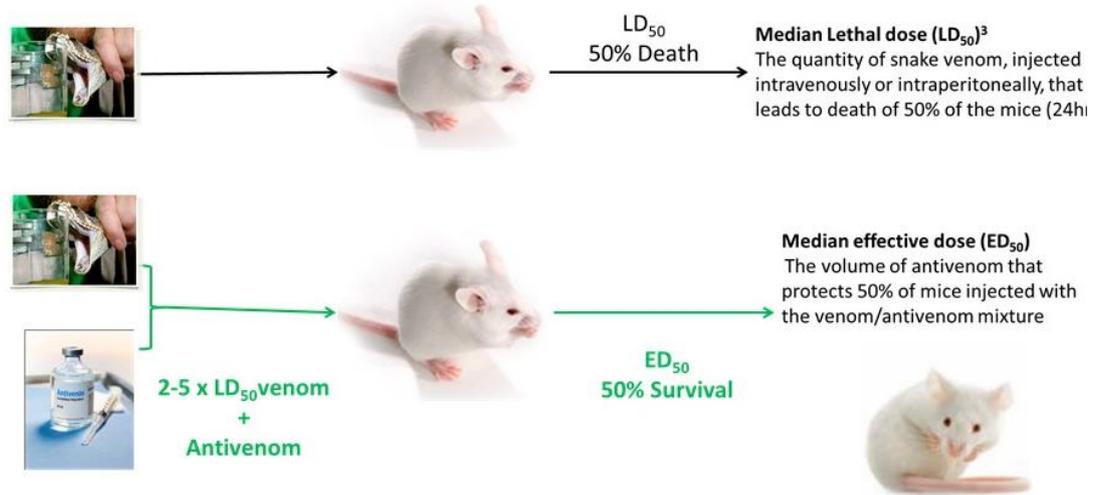


Figure 1.2-7: A diagrammatic representation of median lethal dose and median effective dose (WHO 2010a)

Minimum coagulant dose (MCD)

MCD is defined as the least amount of venom (in mg dry weight per litre test solution) that clots either a solution of bovine fibrinogen (2g/L) in 60 seconds at 37°C (MCD-F) and/or standard citrated solution of human plasma (fibrinogen content, 2.8g/L) in 60 seconds at 37°C (MCD-P). MCD is calculated by plotting clotting time against venom concentration and reading off the level at the 60 second clotting time.

Minimum haemorrhagic dose (MHD)

MHD is defined as the least amount of venom (μg dry weight) which, when injected intradermally into mice or rats, results in a haemorrhagic lesion of 10mm diameter 24 hours later. Results are calculated by plotting mean lesion diameter against venom dose.

An alternative to MHD for venom is performed by injecting venom intramuscularly and measuring the amount of haemoglobin in muscle extract. Results are comparable with conventional MHD (Ownby et al, 1984).

Minimum necrotising dose (MND)

MND is defined as the least amount of venom (μg dry weight) which, when injected intradermally into mice and/or rats, results in a necrotic lesion of 5mm diameter 3 days later. Results are calculated as for MHD

Minimum defibrinogenating dose (MDD)

MDD is defined as the minimum dose of venom which produces non-clotting blood within 60 minutes of intravenous injection into a mouse.

A second method is the fibrin polymerisation time (MDD-FPT). A standard curve prepared from normal mouse blood is prepared and the fibrinogen content of each sample calculated from the standard curve. The MDD-FPT is defined as the dose of venom resulting in a decrease in fibrinogen level to 10% of that for the standard fibrinogen solution.

Results of a combination of these assays, proteolytic and venom-induced oedema showed little or no relationship between the properties of the different venoms and that the determination of one cannot predict the value of the others. Therefore, characterization of all activities of venom must be performed in order to evaluate its toxicity and neutralisation (Sanchez et al, 1992).

Toxicity assay

This assay is used to identify any adverse effects of the antivenom. A group of five mice are injected with antivenom intra-peritoneally and observed for signs of toxicity for a period of 7 days. If any adverse effects are detected, a post mortem examination is performed.

1.2.5.1.3.4 Clinical trials

In vivo and *in vitro* tests of efficacy do not mimic subcutaneous deposition of venom and subsequent intravenous antivenom that occurs in the human victim. Ultimately, clinical testing must be used as the most important determinant of antivenom efficacy. Human clinical trials normally take place in three stages:

Phase I.: Healthy volunteers: In the case of snake antivenoms, treating healthy volunteers is not without risk of adverse effects and sensitisation to the donor species proteins, so is generally not performed. However, *in vivo* toxicity testing is performed in mice or guinea pigs and, in the case of Veterinary antivenoms, safety studies in the target species are performed.

Phase II: Clinical efficacy and dose finding studies. Because of the potentially catastrophic effect of untreated envenoming, withholding effective treatment is not ethical in order to perform the 'gold standard' blinded placebo controlled trial, but a randomised controlled double blind trial comparing two antivenoms can be employed (Abubakar et al, 2010; Abubakar et al, 2010b; Karlson-Stiber et al, 1997; Otero et al, 1999; Otero-Patino et al, 1998). Improvement in clinical score may be used as a means of assessing antivenom efficacy in envenomed patients (Peterson et al, 2011; Smalligan et al, 2004; Woods and Young, 2011).

Phase III: More extensive clinical trials and collection of data relating to efficacy and occurrence of adverse reactions (Ramos-Cerrillo et al, 2008; Petras et al, 2010).

1.2.5.1.3.5 Antivenom stability

Antivenom stability can be defined as maintenance of antibody activity and physicochemical properties, without the formation of aggregates or potentially harmful degradation products. Stability studies are required to show that the antivenom remains stable over the period prior to expiry date, and how stable it is if inadvertently removed from the cold chain and exposed to tropical temperatures (Segura et al, 2009; Al-Abdulla et al, 2013).

1.2.6 Treatment challenges and antivenom alternatives

In areas of the world in which snake-bite mortality and morbidity are highest, there is a deficiency in the supply of effective and affordable antivenoms. This has been addressed to some extent by the development of new antivenoms for these areas (Theakston, 2000; Casewell et al, 2010; Laing et al, 1995; Laing et al, 2003a, Laing et al, 1993b; Abubakar I. et al, 2010, Abubakar S. et al, 2010b; Al-Abdulla et al, 2003; Ramos-Carillo et al, 2008; Chippaux et al,

2007a; Cook et al, 2010a; Cook et al, 2010b; Cook et al, 2010c; Gutierrez et al, 2005; Williams et al, 2011; Sanchez et al 2017). Antivenom production is often driven by cost rather than quality, which led to a crisis in antivenom supply to Africa at the end of the twentieth century (Brown and Landon, 2010; Laing et al, 2003a, Theakston and Warrell, 2000).

Unscrupulous marketing and labelling of antivenom with ambiguous snake names has led to supply of inappropriate antivenoms to treat patients in a region in which the snakes are antigenically distinct from those used in its manufacture (Warrell, 2008). Use of these ineffective products leads to a crisis of confidence in crucial antivenom therapy and an increase in reliance on traditional remedies (Williams et al, 2011). This was illustrated by a study which showed that geographically appropriate antivenom is effective at resolving the coagulopathy induced by *E. ocellatus* envenoming but not against Asian *E. carinatus* envenoming (and *vice versa*) (Habib et al, 2013).

Supportive therapy is important in the management of snake-bite cases and includes wound care, tetanus prophylaxis, infection control and, if appropriate, surgical management of local envenoming. Pain relief should be administered, paracetamol or opiates being the drugs of choice. Non-steroidal anti-inflammatory drugs are contra-indicated due to potential enhancement of primary or secondary venom effects such as coagulopathy or compromised renal function. Patients with respiratory, renal or circulatory failure need resuscitation, as well as antivenom; in cases of neurotoxic envenoming, intubation and mechanical ventilation may be required for several days (WHO 2010b).

Once antivenom has been administered, blood or blood products can be administered, but the benefits need to be weighed against the risk of possible contamination with infections such as Hepatitis B virus or HIV. However, in cases of hyperfibrinolysis, the administration of fresh frozen plasma, without antivenom, may deepen the crisis by adding more substrate, with production

of more degradation products and increased risk of secondary renal failure (White, 2005; WHO, 2010b; Kalyan-Kumar, 2010).

Traditional and herbal remedies are generally ineffective and pose a threat by preventing or delaying the victim's transportation to hospital (Chippaux et al, 2007b). However, some plants, such as *Hibiscus aethopicus L*, may contain endogenous inhibitors of venom-induced haemorrhage (Hasson et al, 2010; Abd-Elsalam, 2011).

Natural inhibitors occur in the plasma and/or muscle of both non-venomous and venomous snakes, as well as some special mammals, such as possums and hedgehogs, which prevent the deleterious action of venom components such as metalloproteinases (Perales et al, 2005; Nobuhisa et al, 1998; Huang et al, 1998).

The use of venomics and antivenomics to identify deficiencies in antivenom efficacy prior to clinical use is a powerful research tool. Traditional antivenoms contain polyclonal antibodies, which are not all venom-specific. Even affinity purified antivenoms contain antibodies to non-toxic components of venom. The use of toxin-specific transcriptomic immunogens and monoclonal antibodies (Kulkeaw et al, 2009) is being investigated, the objective being to produce antivenom which is effective against all the medically important snake venoms in a particular region and which contains only antibodies directed against the significantly pathogenic toxins found in these venoms (Harrison et al, 2011).

Research is being undertaken to investigate specific treatment of the localised effects of venom, which are refractory to antivenom therapy (Gutierrez et al, 2007; Lomonte et al, 1994a; Lomonte et al, 1994b; Melo et al, 1993) and which delay uptake of venom by the lymphatics until treatment can be instigated (Saul et al, 2011).

1.3 The 3Rs (Replacement, Refinement and Reduction of Animals in Research)

The concept of the 3Rs was introduced in 1959 by Russell and Burch to find alternative methods to animal-based testing (Purchase et al, 1998).

“The [principles of the 3Rs](#) were developed over 50 years ago as a framework for humane animal research. They have subsequently become embedded in national and international legislation regulating the use of animals in scientific procedures. Opinion polls consistently show that in the UK support for animal research is conditional on the implementation of the 3Rs.”

Reduction can be defined as the use of methods which reduce the number of animals used per experiment or the number of *in vivo* experiments performed. In this thesis I investigated methods to identify and reject poor quality antivenoms prior to *in vivo* preclinical testing, thus reducing the number of experimental animals required (see Chapters 3 and 4).

Refinement can be defined as methods which minimise suffering and improve animal welfare. Here the use of analgesics and definition of more humane endpoints are investigated. A system of facial grimaces, the Mouse Grimace Scale (MGS) combined with activity, behavioural, physiological and neurological observations were used to assess pain and discomfort, and to define humane endpoints (see Chapters 5 and 6).

During this work, techniques were developed to define pain and humane endpoints more objectively. These are described in detail in Chapter 2A and include scores of activity, neurological function and pathological lesions in relation to survival time (pathology-survival score [PSS])

Replacement is defined as the use of methods which avoid or replace the use of animals. In this project the use of cell based assays is investigated as an *in vitro* alternative to *in vivo* venom assays (see Chapter 8).

During the course of this research, post mortem examinations were performed on mice undergoing venom lethality assays. It became apparent that each venom exhibited a unique set of pathological lesions, the severity of which were dose and time dependent. Gross observations were corroborated with histopathological examination. The relevance of these findings are discussed in Chapter 7.

“Today the 3Rs are increasingly seen as a framework for conducting high quality science in the academic and industrial sectors with more focus on developing alternative approaches which avoid the use of animals. There are a number of reasons for this including the need for better models and tools that more closely reflect human biology and predict the efficacy and safety of new medicines.” (NC3Rs – www.nc3rs.org.uk⁸)

The WHO recommend the preclinical testing of every batch of new or existing antivenom. There are around 45 antivenom manufacturers worldwide, producing in the region of 120 different antivenoms. It is estimated that as the result of murine assays to characterise snake venom and establish the efficacy of these therapeutic antivenoms, well in excess of 30,000 mice undergo testing and therefore suffer pain, distress and death. This also equates to a substantial financial burden, which serves to increase the cost of efficacious antivenoms, which are already beyond the means of many of the victims of snakebite.

1.4 Snakes selected for study

To examine the viability of incorporating the 3Rs into antivenom preclinical efficacy testing, African snakes of distinct genera (two vipers and two elapids), that exhibit variation in venom protein composition and that cause distinct pathological lesions were selected. This strategy was selected because the outputs from this spectrum of snake venoms are intended to be applied to preclinical testing of snake venoms globally. The only venomous snake native

⁸ 06/01/2017

to the UK, namely *Vipera berus* was also selected because antivenoms directed against this snake venom are routinely tested *in vivo*, for regulatory purposes, by the ARVRU and thus this constituted a reduction in the animals required to collect necessary data for this work.

1.4.1 *Echis ocellatus*

Echis ocellatus, the West African saw-scaled viper was chosen because of its haemotoxic venom, which results in a coagulopathy, primarily due to consumption of clotting factors. It is also the snake which is responsible for a higher mortality than any other snake in the world (Theakston, 2000). The venom proteome is illustrated in Section 1.5.1 and its relationship to observed venom-induced lesions discussed.

Local envenoming produces blisters that may become necrotic, with extensive localised swelling and bruising (Figure 1.4-1C). *Sequelae* frequently include the need for skin grafting or amputation (Figure 1.4-1D1-3), resulting in permanent disfigurement and loss of earning capacity.

Incoagulable blood results from a consumptive coagulopathy due to venom prothrombin activators. This is manifested clinically by persistent local bleeding, even from old wounds, and spontaneous systemic haemorrhage, especially from the gingival sulci (Figure 1.4-1E).

In addition to the procoagulant and other toxins affecting clotting and platelet function, the tendency to haemorrhage is further aggravated by lysis of collagen in the basement membrane of blood vessels. This multi-pronged attack has profound clinical consequences, which commonly include epistaxis, blood stained saliva, melaena and haematomas, subconjunctival, gut, retroperitoneal, extrapleural, genito-urinary bleeding and intracerebral haemorrhage – a rare cause of fatalities. Intra-uterine and post-partum bleeding is life-threatening to both pregnant women and their unborn foetus. Renal failure is uncommon, but may occur secondarily after profound

hypotension and haemorrhagic shock (WHO, 2010b; Warrell et al, 1977; Markland, 1998).



Figure 1.4-1: A - *Echis ocellatus* is a small venomous viper with characteristic 'eye-spots' along its length (A - <http://snakedatabase.org/species/Echis/ocellatus>). It is usually 30-50cm long, rarely more than 60cm and has specialised scales which, when rubbed together, produce a sawing sound – a warning behaviour prior to striking. It lays around 6-20 eggs, usually at the end of the dry season, the hatchlings are 10-12cm. It inhabits the agricultural areas of West Africa – Mauritania, Senegal, Guinea, Mali, Ivory Coast, Burkina Faso, Ghana, Togo, Benin, southern Niger, Nigeria, northern Cameroon and south-western Chad (B: the distribution of *E. ocellatus* is shown in blue, and the related species *E. pyramidum* in red, *E. coloratus* in green and *E. carinatus* in purple (Casewell et al, 2010)). C: local envenoming (WHO, 2010b); D1-3: Necrosis resulting in above-knee amputation (Abubakar et al, 2010); E: Bleeding from mouth and gums (Warrell – personal communication).

1.4.2 *Bitis arietans*

Bitis arietans – the puff adder – was selected because it causes haemorrhage with no coagulopathy. It is a medically important snake with a much wider distribution than, but overlapping with that of *E.ocellatus* and is thought to be

responsible for the majority of serious envenomations throughout Africa (WHO, 2010b). The venom proteome and its relationship to observed lesions is discussed in Section 1.5.2.



Figure 1.4-2: *Bitis arietans* is a large, thick snake with the typical triangular viper head (A <http://www.biolib.cz/en/image/id30446/>) found throughout much of Africa (B - https://en.wikipedia.org/wiki/Bitis_arietans). Its average length is 90 – 110 cm and its orange and brown colouration with black chevron-like markings in combination with heavily keeled scales give it a rather dull appearance. It is an ambush predator, preferring rodents or birds and, because it has few natural predators of its own, can often be seen in the open, particularly at night. Despite being a generally sluggish reptile it is one of the fastest striking snakes in the world. It delivers in excess of 100-300 mg of cytotoxic venom per bite, sufficient to cause death in a human victim. As part of its warning display it inflates and deflates its body, causing a hissing sound and hence its common name (Puff Adder). It is ovoviviparous, giving birth to 20-50 young after a gestation period of 7-9 months (Sprawls). Figures C (Warrell in WHO, 2010b), D C (Warrell in WHO, 2010b) and E (<http://ktmc.info/puff-adder-bite>) illustrate the effects of its venom cytotoxins.

Local swelling of the bite site is often extensive and may involve the entire bitten limb, extending to the trunk. Characteristic blistering (Figure 1.4-2C) and necrosis (Figure 1.4-2D) occur at the bite site with extravasation of plasma,

which may result in hypovolaemic shock. Compartmental syndrome may develop, which, in turn, may lead to ischaemic necrosis.

In systemic envenomation, there may be myocardial effects and arrhythmias, commonly sinus bradycardia, which may contribute to the profound adenosine-induced hypotension. Hypotension may be further exacerbated by the presence of 5'-nucleotidase, which hydrolyses nucleotides generating more purines, such as adenosine (Aird, 2002).

The venom of this species contains toxins which inhibit platelet aggregation and prolong bleeding time. In addition, other toxins which induce platelet aggregation, making platelets unavailable, resulting in thrombocytopenia. This manifests clinically as petechiation of serosal surfaces and skin (White, 2005), and spontaneous bleeding and bruising may occur. Whilst no coagulopathy has been observed in Nigerian bite victims, in East and South Africa, both coagulopathy and cerebral thrombosis has been recorded, which suggests that there may be more than one species of puff adder (WHO, 2010b).

1.4.3 *Naja nigricollis*

Naja nigricollis – the black-necked spitting cobra (Figure 1.4-3B) - was chosen as an elapid snake with a powerfully cytotoxic venom, which may be projected into the eye of its victim, resulting in corneal necrosis and even permanent blindness (Figure 1.4-3G).

The venom is cytotoxic, cardiotoxic and necrotising but, unlike other elapids, shows little signs of neurotoxicity in man. Bites cause local necrosis (Figure 1.4-3, E and F) and there is immediate pain at the bite site with enlarged and tender lymph nodes. This is followed by vomiting, extensive local swelling, blistering and necrosis of the bite site, involving only the skin and superficial connective tissues. Spread of venom via the lymphatics may result in 'skip' lesions – strips of affected skin separated by strips of normal skin.

Haematological changes include neutrophilia and evidence of complement activation. Chronic ulceration, osteomyelitis, arthrodesis, hypertrophic scars, keloid formation and, after several years, malignant transformation or Marjolin's ulcer which is a type of squamous cell carcinoma (Figure 1.4-3C) are some of the long-term *sequelae* of bites by this species (WHO, 2010b; Warrell, 1976).

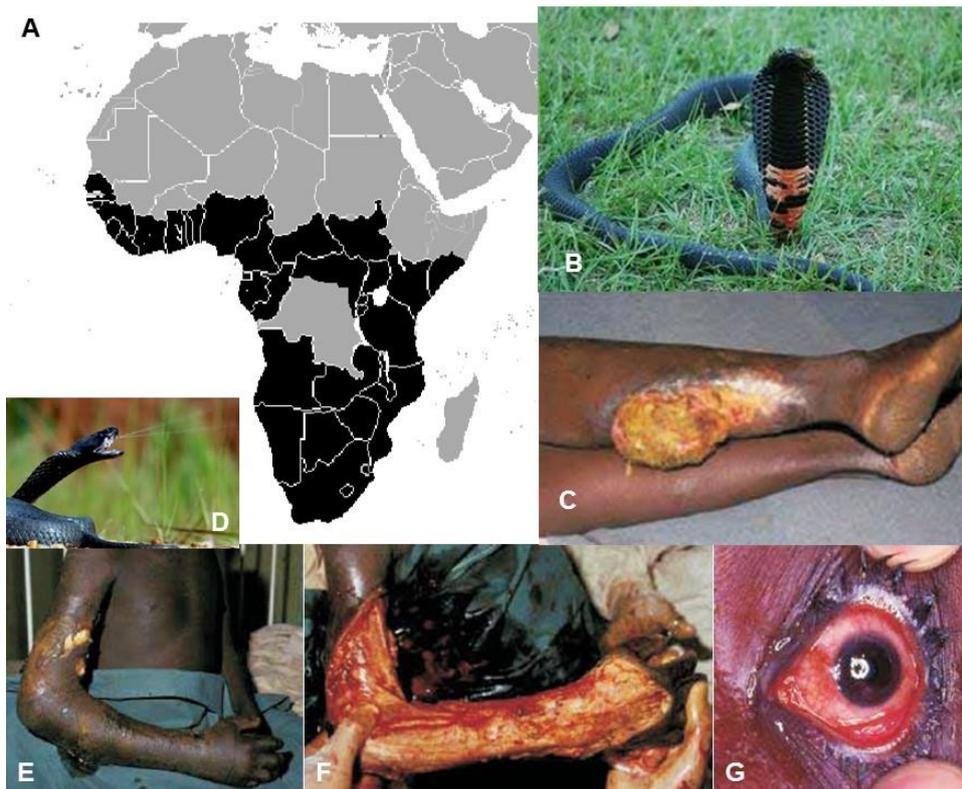


Figure 1.4-3: *Naja nigricollis* (B <http://www.venomstreet.com/Naja%20nigricollis.htm>)

occurs in Sub-Saharan Africa

(A https://commons.wikimedia.org/wiki/File:Naja_nigricollis_range.png). It grows to 1.2 – 2.2m in length and preys primarily on rodents. It is oviparous, laying 10-15 eggs, which take 60-70 days to hatch. Human victims are usually bitten in their huts at night whilst the snake is hunting rodents. When threatened, like other cobras, it rears up and expands its hood using modified ribs. It is from this position that it sprays venom over some considerable distance at its aggressor (D - Wuster) thus causing irritation on contact with the skin or cornea. If venom enters the eye corneal necrosis resulting in temporary or permanent blindness (G Warrell in WHO, 2010b) may occur. Local necrosis at the bite site (E Warrell in WHO, 2010b) with subsequent debridement (F Warrell in WHO, 2010b) is shown. In the long term, malignant transformation may occur (C Warrell in WHO, 2010b).

1.4.4 *Dendroaspis angusticeps*

Dendroaspis angusticeps – the Eastern green mamba (Figure 1.4-4A) - was chosen because it is an elapid with a primarily neurotoxic venom, the effects of which are irreversible, unless antivenom is rapidly administered. The resultant descending paralysis, including the muscles of respiration, is inevitable, and, without access to assisted ventilation for 5-7 days post envenoming, the prognosis is poor.



Figure 1.4-4: *Dendroaspis angusticeps* inhabits the East coast of Africa (D). It is an arboreal snake, feeding on small birds, mammals, amphibians and lizards. It is bright green in colour and grows to a length of up to 2.5m (A <http://picssr.com/tags/gr%C3%BCnemamba/page2>). It has a lifespan of 10-15 years and is oviparous, with a gestation period of around two and a half months. They are elusive snakes therefore human envenoming is relatively rare. Its neurotoxic venom causes paralysis, starting with ptosis (B Iain Thirsk in WHO, 2010b) and descending to the respiratory muscles, death is inevitable unless the patient can be artificially ventilated (C Warrell, 1999).

The venom contains small proteins, called fasciculins, unique to this species and which bind to acetylcholinesterase, prolonging the presence of acetylcholine in the neuromuscular junction, leading to muscle fasciculations (Lauridsen et al, 2016). Dendrotoxins, which are unique to the *Dendroaspis* genus and homologous to Kunitz-like serine proteinase inhibitors, cause

paralysis by binding to voltage-gated potassium channels resulting in a depolarising neuromuscular blockade. (Barrett et al, 1979; Lauridsen et al, 2016). The result is paraesthesia, signs of autonomic nervous system stimulation (salivation and sweating) and muscle fasciculations, which may be confused with shivering. The venom also contains a number of other toxins of the 3FTx family. Notably the venom does not contain α -neurotoxins, unlike its close relative, *D. polylepis*, the black mamba (Lauridsen et al, 2016).

Rapid descending muscular paralysis (Figure 1.4-4B&C) may progress to respiratory failure, which is inevitably fatal without assisted ventilation. Patients often complain of severe local pain, excessive salivation, a strange taste in the mouth, diarrhoea, involuntary muscle contractions and recurrent paralysis, despite antivenom treatment. Unlike other mambas, *D. angusticeps* envenoming can cause local tissue swelling and coagulation disturbances. (WHO, 2010b). Recently, the venom has been shown to contain SVMPPs, CRISP, galactose-binding lectins, hyaluronidase, peptidases and nerve growth factors, which could explain these other symptoms of envenoming (Lauridsen et al, 2016), and a cardiotoxin has also been described (Mbugua et al, 1988).

1.4.5 *Vipera berus*

Vipera berus, the European adder, is the only venomous snake found in the UK. Envenomations rarely result in fatality, but can produce life-threatening complications. This is particularly true in children and the elderly, and may therefore incur significant cost both for immediate life-saving treatment as well as long term *sequelae*. Only 14 fatalities have been recorded in the UK between 1876 and 1976 (Reid, 1976), but up to 50% of recorded cases have significant local or systemic signs (Harborne, 1993).



Figure 1.4-5: *Vipera berus* is a small, stout-bodied snake with a triangular head and a striking black zig-zag pattern along its back (A <http://www.naturephoto-cz.com/common-viper-photo-1704.html>). It is usually slow moving and tends to lie in wait until its prey comes within striking distance. Its diet consists mainly of small rodents, and, depending on climate, hibernates from October to April, mating on emergence from hibernation. It is ovoviviparous and live young are produced in summer or autumn. It inhabits an area from just within the Arctic Circle extending throughout Europe, apart from the most southerly parts of Spain, Italy and Greece (E https://en.wikipedia.org/wiki/Vipera_berus). Severe localised swelling occurs at the bite site in a human (B <http://www.dailymail.co.uk/news/article-1296520/Adder-bite-leaves-father-blinded-choking-just-minutes-death-walk-family.html>) and dogs (C <https://terriermandotcom.blogspot.co.uk/2012/07/death-before-discomfort-says.html> & D Kate Kenyon, personal communication) (bitten hand/limb compared to normal).

Its venom is rapidly lethal to mice and causes significant local tissue damage – oedema, haemorrhage and moderate myonecrosis (Calderon et al, 1993). The venom components and their relationship to observed pathological lesions is discussed further in Section 1.5.3.

In humans, snake bites by this species result in localised pain and swelling extending up the bitten limb to the trunk (Figure 1.4-5C). If there is systemic envenomation, the most common symptoms are nausea, vomiting, diarrhoea and abdominal pain. Hypotension and shock, angioedema of the face, lips, gums, tongue and throat, central nervous system (CNS) depression, electrocardiogram (ECG) changes – ST depression, T wave inversion or flattening, atrial fibrillation, ventricular extrasystoles and atrio-ventricular block

may also occur. Haematological and biochemical changes include leucocytosis (neutrophilia), thrombocytopenia, anaemia and creatine kinase elevation (Karlson-Stiber, 2006; Reid, 1976; Warrell, 2005). Rapidly progressive symptoms and early leucocytosis may serve as a warning signal for higher probability of severe reactions (Gronlund, 2003).

Rare complications include compartment syndrome (Evers et al, 1982) cardiac arrest, acute myocardial infarction and cerebrovascular accident (Aravansis, 1982), acute gastric dilatation, paralytic ileus and acute pancreatitis (Warrell, 2005). Disseminated intravascular coagulation has been described in humans and animals (Audebert, 1992; personal communication, Cambridge University Veterinary School, 2012). Rarely, neurological symptoms are described (Malina, 2008). Local envenoming may require skin grafting or even amputation of the affected digit (personal communication, Welsh Burns and Plastics Unit, Morriston Hospital, Swansea, 2014).

A similar pattern is seen in envenomed dogs, which occurs considerably more frequently than in humans (Personal communications, MicroPharm ViperaVet clinical trial data). Mortality in the dog is relatively low (4.6% in the UK [Sutton et al, 2012]), with death usually occurring due to fatal arrhythmias (Figure 1.4-6), disseminated intravascular coagulation or euthanasia (Sutton et al, 2012; personal communication, Cambridge University Veterinary School, 2012).



Figure 1.4-6: ECG trace from a dog bitten by *V. berus* showing a life-threatening arrhythmia (Personal communication).

Clinical and biochemical changes in dogs bitten by *V.berus* in Sweden were recorded (Lervic et al, 2010). All dogs had local swelling (Figure 1.4-5C&D) 73% had affected mental status and 6 of 53 dogs developed cardiac

abnormalities. Elevated CK levels indicating transient muscle damage was observed in 50% of the dogs sampled. Several dogs had transient, minor increases in hepatic test results (Lervic et al, 2010). Other surveys have recorded extensive swelling, refractory hypotension, cardiac arrhythmias, lymphomegaly, leucocytosis, thrombocytopenia and coagulopathy (Sutton et al, 2012; Segev et al, 2004). Myocardial damage has been shown to occur in both dogs and horses (Segev et al, 2008; Pelander et al, 2010; Anlen, 2008; Hoffman et al, 1993).

1.5 Toxin/pathology relationship

In this section, published data on venom proteomes and toxin composition is examined in the context of pathological lesions observed in envenomed mice and humans.

1.5.1 *Echis ocellatus* venom

The proteome of *E. ocellatus* venom (Wagstaff et al, 2009) is dominated by snake venom metalloproteinases (SVMP), and of these, over 50% comprises the PIII class. SVMPs are responsible for local tissue and vascular damage by means of extracellular matrix and basement membrane destruction, respectively (Gutiérrez et al, 2009a). Ecarin is a PIII SVMP found in *E. ocellatus* venom, which is prothrombin activating, and therefore plays a key role in the consumptive coagulopathy induced by this venom.

Disintegrins, which may be incorporated as a domain in a PII SVMP, inhibit platelet aggregation and thus contribute to the haemorrhagic nature of the whole venom. Other haemorrhagic components found in this venom are:

- Serine proteases, which affect platelet aggregation and catalyse fibrinolysis
- C-Type Lectins bind to Factors IX and X
- Phospholipase A₂ (PLA₂) which may also inhibit platelet aggregation

PLA₂s may also play a role in local tissue damage, oedema and myonecrosis (Gutiérrez et al, 2009b).

The toxin composition and examples of the venom induced lesions is illustrated in Figure 1.5-1.

Toxin key (Figure 1.5-1 to Figure 1.5-5):

SVMP: Snake venom metalloproteinase	AP: Unknown proteins
LA(A)O: L-amino acid oxidase	NP: Natriuretic peptide
CTL: C-type lectin	AspP: Aspartic protease
Ser-Prot: Serine protease	KPI: Kunitz-type protease inhibitors
CRISP: Cysteine-rich serine protease	3FTx: 3 finger toxins
DC-fragments: Disintegrin/cysteine-rich	KUN: Kunitz inhibitors
PLA ₂ : Phospholipase A ₂	HYA: Hyaluronidase
SVMPi: SVMP inhibitor	GAL: Galactose binding enzymes
5'NT: 5' nucleotidase	PEP: Peptidases
DISI: Disintegrin	KTC: Prokineticins
BPP: Bradykinin potentiating peptide	NGF: Nerve growth factors

References for images:

Figure 1.5-1

Top left – West African Carpet Viper – Huetter, Arco Images

Top middle: Warrell, WHO, 2010b

Bottom three: Warrell, WHO, 2010b

Figure 1.5-2

Top left – Fotonatura.org

Middle left - <http://www.factzoo.com/reptiles/snakes/snake-venom-bite-first-aid.html>

Bottom left – Warrell

Figure 1.5-3

Top left - http://www.biopix.com/common-viper-adder-vipera-berus_photo-42529.aspx

Bottom left, dog and human - See Figure 1.4-5

Figure 1.5-4

Bottom left and middle – Warrell in WHO, 2010b

Figure 1.5-5

See Figure 1.4-4

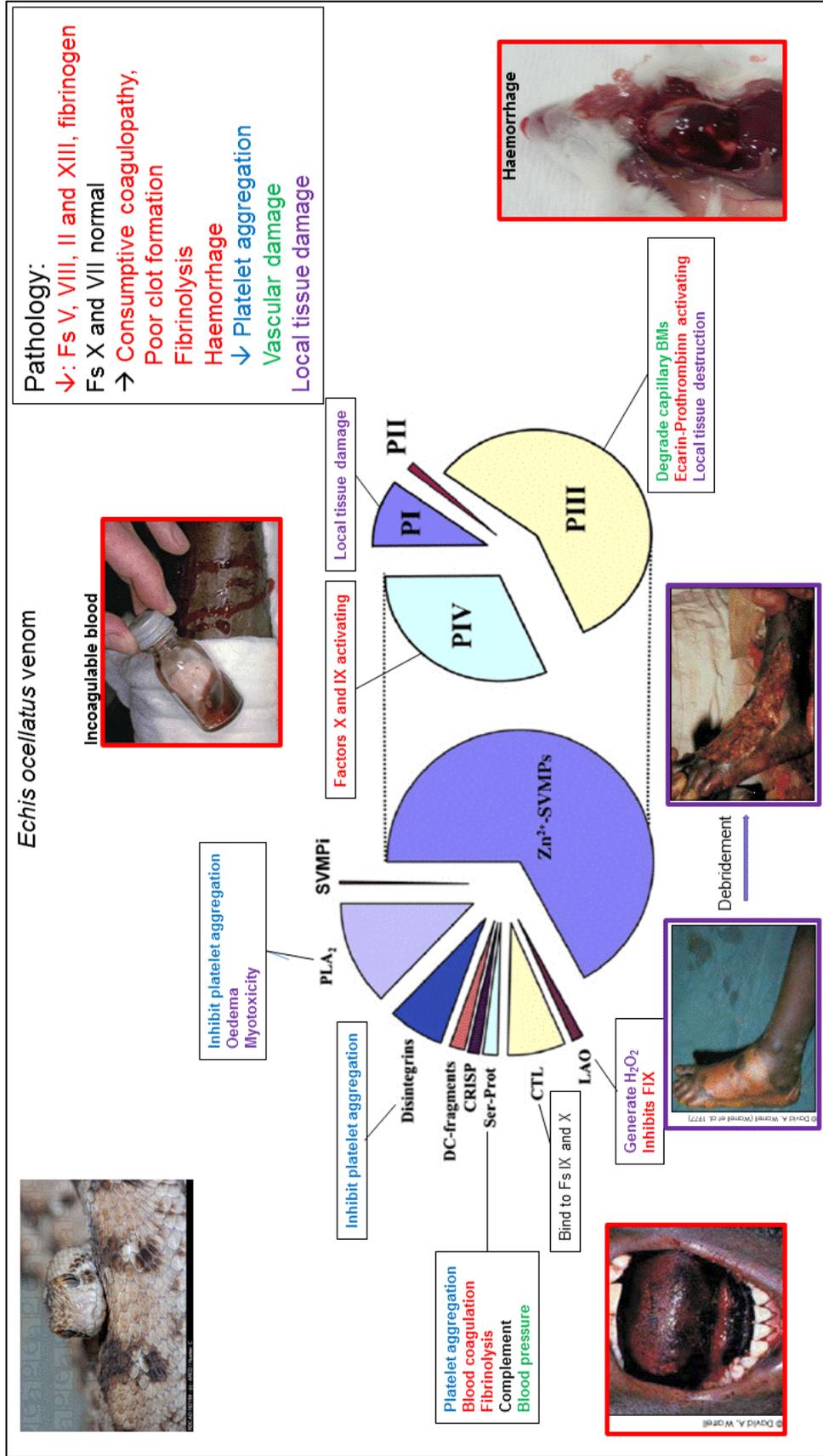


Figure 1.5-1: Interaction between venom proteome and observed pathology of envenomation by *Echis ocellatus*. References: Warrell et al. 1977; Waastaff et al. 2009; Casewell et al. 2014.

1.5.2 *Bitis arietans* venom

The proteome of *Bitis arietans* venom (Calvete et al, 2007; Fasoli et al, 2010) contains fewer SVMPs (33.4% of venom proteins) than *E. ocellatus* venom, and nearly as many serine proteases (25.2%) and C-type lectins (22.1%) as SVMPs. It also contains significant amounts of 5'nucleotidase, L-amino oxidase and disintegrins. This mix of toxins induces local tissue destruction, including oedema and myotoxicity, haemorrhage, thrombocytopenia and profound hypotension.

The relationship of venom proteins with observed pathological lesions is illustrated in Figure 1.5-2. One venom component missing from the proteome is the purine (i.e. not a protein), adenosine, which is thought to be partially responsible for the profound hypotension associated with envenomation by this species of snake (Aird, 2002). The action of 5'nucleotidase also contributes to the presence of adenosine in the snakebite victim, thus adding to the hypotensive effect (Aird, 2002; 2005).

1.5.3 *Vipera berus* venom

The venomics of *Vipera berus* was compared to that of *V. ammodytes* (Latinovic et al, 2016) and the results used to explain the differences in lesions observed following envenoming by the two species. The proteome and induced pathological lesions are illustrated in Figure 1.5-3.

Clinical signs of envenoming by *V. berus* are mild in comparison to the other two Vipers described above. The proteome is dominated by serine proteases and SVMPs, which, along with disintegrins, PLA₂s, C-type lectins and possibly L-amino acid oxidases (LAAO), form a toxic arsenal which disrupt haemostasis. Frank haemorrhages are rare, but pulmonary haemorrhage and bruising are commonly observed in envenomed patients (Warrell, 2005).

As a practicing veterinary surgeon, my overwhelming memory of snakebite is the almost pathognomic extensive oedema. This observation is supported by

descriptions in the literature of both human and animal snakebite victims (Karlson-Stiber et al, 1994; Anlen, 2008; Lervik et al, 2010; Reid, 1976).

Local tissue damage and oedema is likely to be induced by snake venom metalloproteases, phospholipase A₂s, L-amino acid oxidases, and other proteases. Hypotension is the most notable systemic sign of envenomation in humans, and it is likely that a natriuretic peptide is largely responsible. Neurotoxicity is a rare symptom of *V. berus* envenoming, and tends to be confined to certain isolated populations (Malina et al, 2008). The venom studied by Latinovic was not from a neurotoxic population, but genes encoding for A and B subunits of Vaspin (a neurotoxic PLA₂) were found. This suggests that *V. berus* venom has the potential to contain a neurotoxin, but that this gene is rarely expressed.

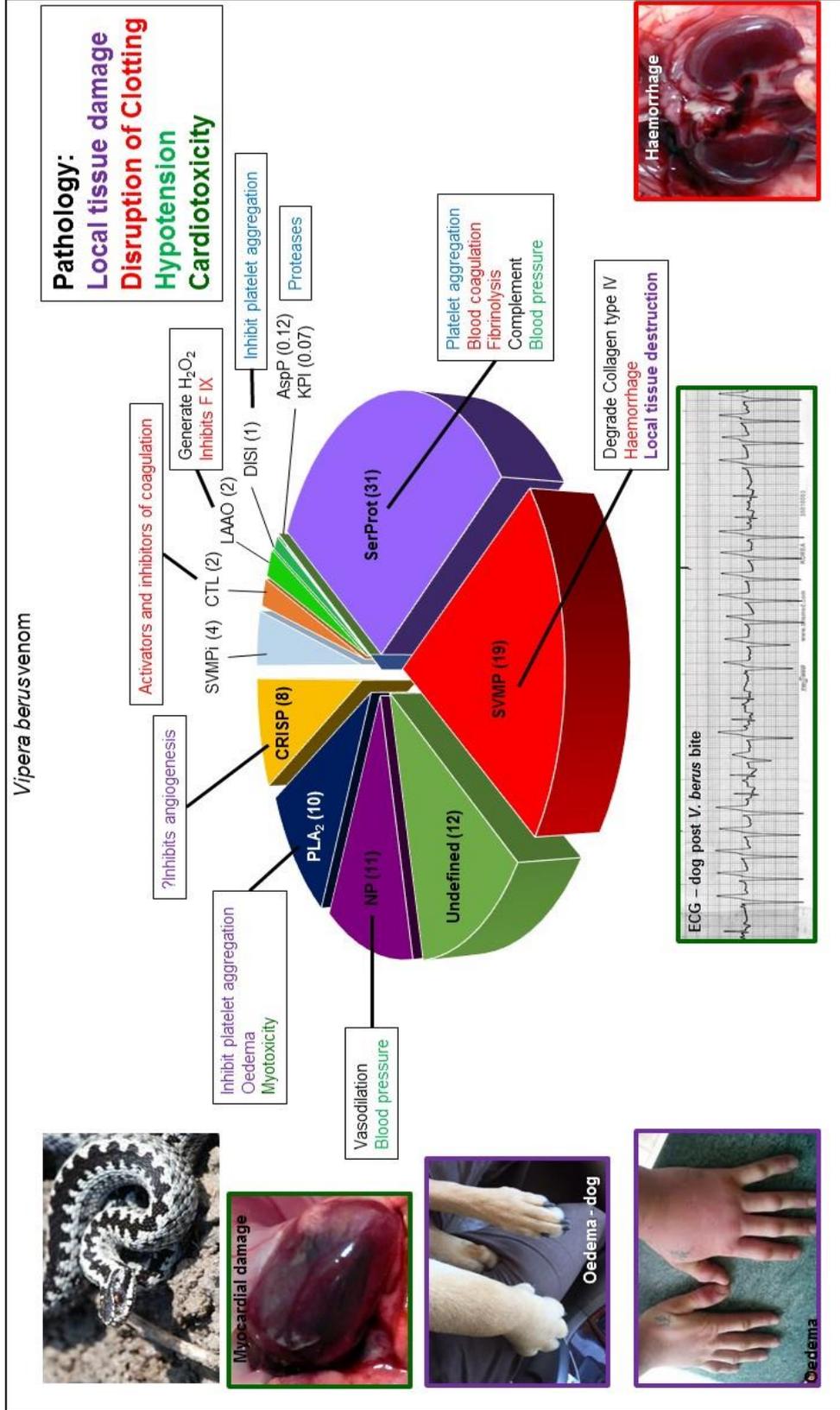


Figure 1.5-3: Interaction between venom proteome and observed pathology of envenomation by *Vipera berus*. References: Latinovik et al, 2016

1.5.4 *Naja nigricollis* venom

Figure 1.5-4 illustrates the relationship between the venom proteome (Petras et al, 2011) and observed pathological lesions.

The venom of *Naja nigricollis* is rich in cytolytic three finger toxins (3-FTxs) (Petras et al, 2011), which is reflected in the severe tissue destruction seen in local envenoming. Damage to the cornea when venom is projected into the victim's eye can be so severe that blindness results. This tissue destructive effect is exacerbated by cytotoxic PLA₂s.

N. nigricollis venom also contains PLA₂s which have myotoxic, cardiotoxic and anticoagulant activities (cited by Petras et al, 2011), as well as 3FTxs which are post-synaptic α -neurotoxins (0.4% of venom proteins). Experimentally the cardiotoxins reproduce the ocular effects resulting from venom 'spitting' (Petras et al, 2011). Although the venom contains SVMPs, the relative paucity of this toxin in this venom means that its contribution to tissue damage and necrosis is likely to be minimal (Petras et al, 2011). Endonucleases are present in small amounts, but however in sufficient concentrations to catalyse the breakdown of nucleotides into purines, such as adenosine, which may explain the hypotension and immobility associated with envenoming (Aird, 2002). The biological function of other venom constituents, such as nawaprin, are yet to be elucidated (Petras et al, 2011).

It may be concluded that the toxins in *N. nigricollis* venom correspond well to the observed dominance of cytotoxic and necrotic effects of local envenoming, with little evidence of neurotoxicity in man (Petras et al, 2011). However, in the mouse model, evidence of neurotoxicity, such as respiratory and progressive paralysis, was observed.

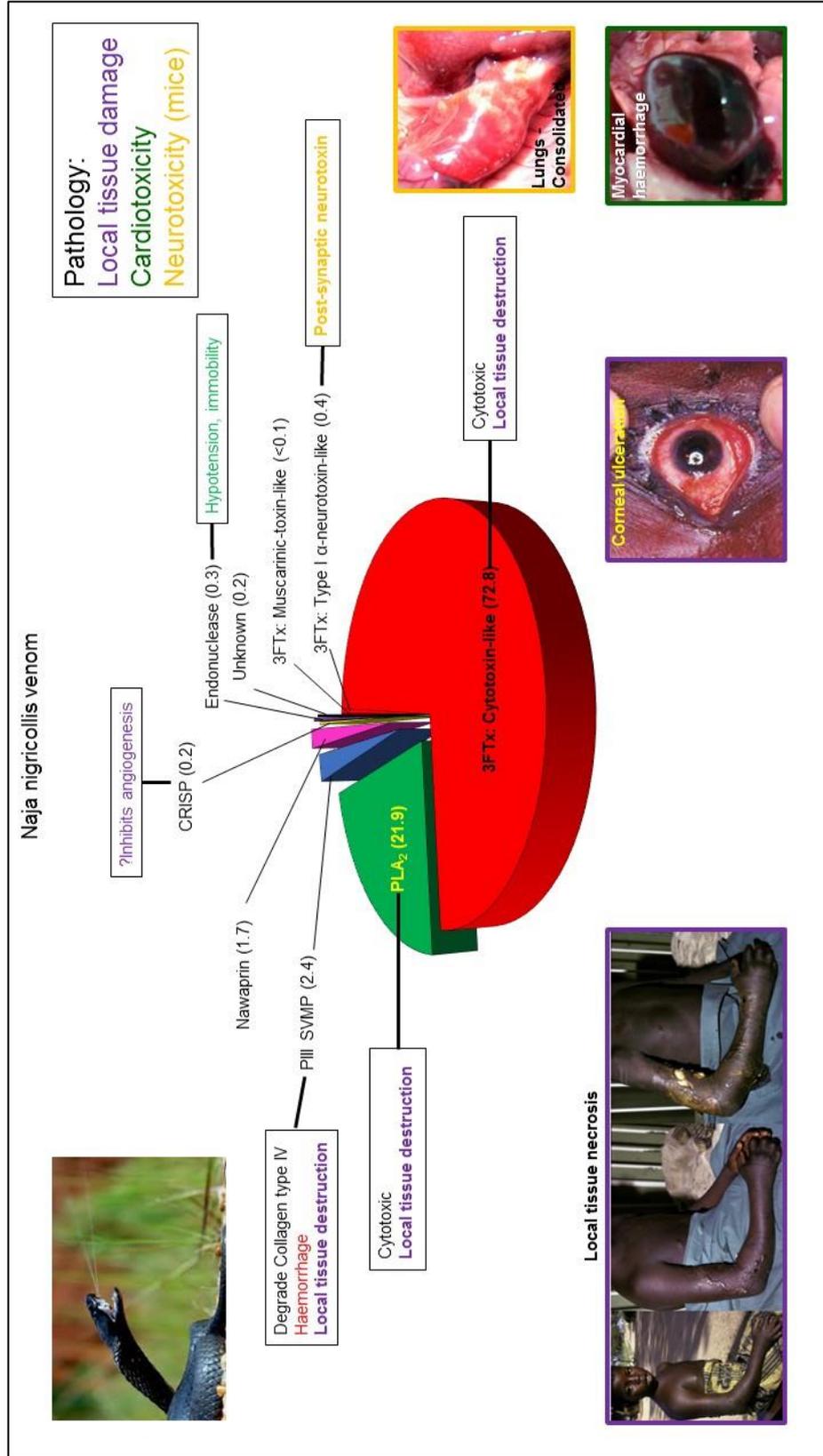


Figure 1.5-4: Interaction between venom proteome and observed pathology of envenomation by *Naja nigricollis*. References: Petras et al, 2011; Warrell, 1976

1.5.5 *Dendroaspis angusticeps* venom

Figure 1.5-5 illustrates the proteome of *D. angusticeps* venom (Lauridsen et al, 2016) and resultant observed venom-induced lesions this species of snake. In addition to the proteins and peptides, the nucleoside adenosine, was also identified (Lauridsen et al, 2016).

D. angusticeps venom is dominated by 3FTxs and Kunitz-type serine protease inhibitors (KUN) (Dendrotoxins). The 3FTxs can be subdivided into a number of families, including Fasciculins (unique to *D. angusticeps*), Orphan group XI, and aminergic toxins. The net result of this cocktail of toxins is excitatory depolarisation of the neuromuscular junction, with dendrotoxins acting presynaptically to enhance acetyl choline release and Fasciculins blocking acetyl cholinesterase (Lauridsen et al, 2016). Clinically, this is seen as prolonged muscle fasciculations, which was observed in mice to continue after death (defined as cardiac arrest).

Aminergic toxins affect muscarinic receptors and thus clinically induce the cardiovascular changes (arrhythmias) and gastrointestinal signs (nausea). Acid-sensing ion channel inhibitors (Mambalgesins) block pain sensation (Diochet et al, 2012).

Other venom components are of questionable significance, although the presence of SVMPs may be involved in the local inflammation observed at the bite site. This assumption is debatable, given that the SVMPs isolated from this venom has little activity, experimentally. It is highly likely that some of the venom components act synergistically, such as dendrotoxins and fasciculins, but may not, in themselves, be toxic (Lauridsen et al, 2016).

The most significant difference between this arboreal mamba venom and the much-feared terrestrial black mamba (*D. polylepis*) is that *D. angusticeps* venom is completely devoid of α -neurotoxins (Lauridsen et al, 2016). This group of toxins act post-synaptically to block neuromuscular transmission which results in flaccid paralysis (Mackessy, 2010).

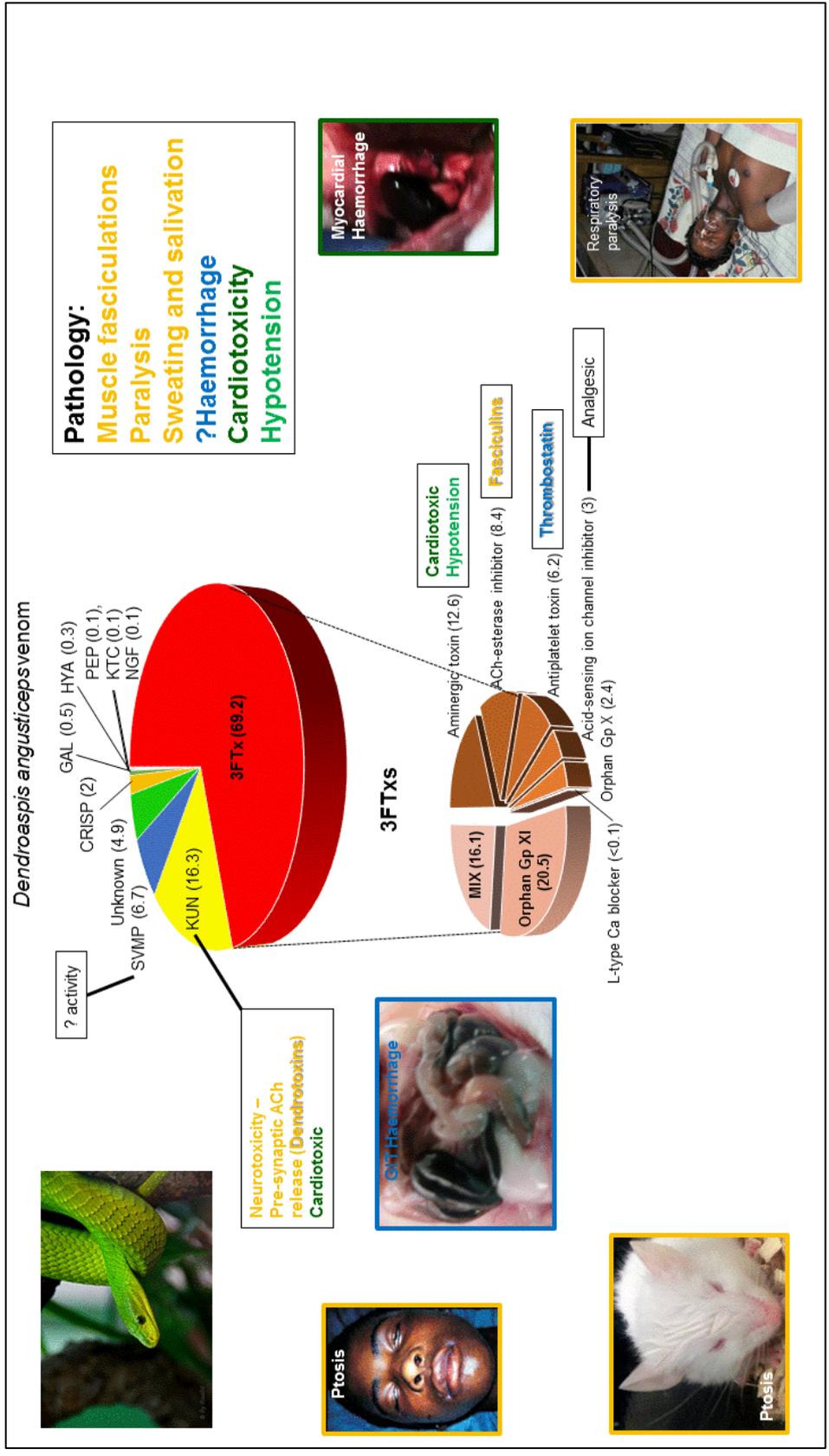


Figure 1.5-5: Interaction between venom proteome and observed pathology of envenomation by *Dendroaspis angusticeps*.
 References: Lauridsen et al 2016; Petras et al, 2016.

1.5.6 Toxicovenomics

Investigation of venoms other than the five which are core to this work and by consulting the literature, it became obvious that patterns of envenoming fell into 'syndromes' and that these syndromes could be correlated with venom toxin composition. Major pathological lesions of envenoming, with the main toxin families thought to induce this pathology, are listed below and references cited as footnotes:

- Haemorrhagic
 - Coagulopathic
 - Procoagulant (SVMP IIIs, SVSPs)^{9,10}
 - Anticoagulant (C-type lectins – Factor IX/X inhibitors)⁹
 - Fibrin(ogen)olysis (SVSPs)⁹
 - Platelets
 - Aggregation (SVSPs)⁹
 - Inhibition of aggregation (Disintegrins, PLA₂s)⁹
 - Vascular damage⁹
 - Basement membrane (SVMP III)¹¹
- Cytotoxic
 - Local tissue damage (SVMPs, PLA₂s)¹¹
 - Blistering (SVMPs)¹²
 - Bruising (SVMPs)¹²
 - Oedema (PLA₂s)¹²
 - Local tissue damage caused by cytotoxic 3FTxs in spitting cobra venom.¹³
 - Myotoxicity (PLA₂s and SVMP III)¹⁴
- Cardiotoxicity

⁹ Lu et al, 2005

¹⁰ Kini, 2005

¹¹ Herrera et al, 2015

¹² Gutierrez et al, 2009b

¹³ Petras et al, 2011

¹⁴ Gutierrez et al, 2009a

- Cardiomyotoxicity (3FTxs, PLA₂s)¹⁵
- Hypotension (adenosine¹⁶, natriuretic peptide¹⁷)
- Neurotoxicity
 - Presynaptic
 - Destruction (PLA₂s – β neurotoxins)¹⁸
 - Excitatory (Dendrotoxins)¹⁹
 - Postsynaptic – (3FTXs – α neurotoxins)²⁰
 - Acetylcholinesterase (Fasciculins)²¹

The simple post mortem examination (PM) which has been developed has the potential to complement the antivenomics profiling of venom-antivenoms. The latter technique may be used to demonstrate which venom components are bound by antivenom, but cannot demonstrate neutralisation of bound toxins. PM examinations may be used to reveal which pathological lesions are neutralised by an antivenom, and, using toxicopathology, venom components which have not been neutralised identified. Alternatively, toxicopathology could be augmented by detection of unneutralised circulating toxins.

1.6 Conclusions

Snake-bite envenoming is one of the world's neglected tropical diseases, causing significant mortality and morbidity as well as having a considerable socio-economic impact, particularly in the poverty-stricken rural tropics (Harrison et al, 2009). The only specific and effective treatment is antivenom, which must be demonstrated to be safe, effective and affordable to those who need it (Brown and Landon, 2010). Currently, the best single model for efficacy testing is the *in vivo* murine lethality tests, recommended by the WHO (Theakston and Reid, 1983, WHO, 2010a). These assays are expensive, both financially and in the use of research animals, as well as causing considerable

¹⁵ Mackessy, 2010

¹⁶ Aird, 2005

¹⁷ Latinovic et al, 2016

¹⁸ Gutiérrez and Lomonte, 2013

¹⁹ Lauridsen et al, 2016

²⁰ Petras et al, 2016

²¹ Rodriguez et al, 1983

distress and suffering to the subjects. Implementation of the 3Rs in these assays is long overdue, so here we seek to redress this problem. We will investigate the use of a matrix of cells from anatomically-distinct origins, reflecting the multiplicity of pathological lesions encountered in snake envenoming, to replace the *in vivo* murine assays. To reduce the numbers of animals required results from the present *in vitro* immunoassays will be compared with *in vivo* results to demonstrate that routine batch testing of manufactured antivenoms of proven clinical efficacy may be performed using an *in vitro* assay. Finally, assays will be refined using the MGS score to assess pain in envenomed mice and thus the efficacy of analgesia. Better humane endpoints will be defined using behavioural observations, surface body temperature and other physiological assessments, leading to a reduction in assay duration.

Chapter 2. Materials and Methods

2.1 Materials

2.1.1 Equipment

See Appendix I

2.1.2 General reagents

See Appendix II

2.1.3 Buffers

See Appendix II

2.1.4 Specific materials:

See Appendix II

2.1.5 Venoms:

Venoms were obtained from wild-caught snakes maintained in the Alistair Reid Venom Research Unit Herpetarium at the Liverpool School of Tropical Medicine. The snakes underwent venom extraction approximately every 6 weeks. The venom was freeze-dried immediately, pooled and stored at 2-8°C. *Vipera berus* (European Adder) venom was not available from the LSTM herpetarium and was obtained from Latoxan (France) from wild-caught Russian snakes.

2.2 In vivo assays pre-clinical assays of venom toxicity and antivenom efficacy

Experiments were conducted in accordance with World Health Organisation guidelines and under licenced approval of the UK Home Office.

The murine lethality assays are the 'gold standard' pre-clinical assays of venom toxicity and antivenom efficacy and are recommended by the WHO to test every new batch of antivenom produced prior to use in envenomed patients, either as part of a clinical trial or therapeutically (WHO, 2010a).

These assays inflict a great deal of suffering on the large number of mice involved and it is the purpose of this project to replace, refine and reduce the numbers of mice used in these experiments. However, it is imperative that the efficacy of antivenom is demonstrated prior to their clinical use, so, until a suitable robust and practical replacement has been found these assays remain obligatory.

2.2.1 Median lethal dose: venom LD₅₀

The experiment was carried out using five groups of five mice receiving different concentrations of venom identified in the range-finding experiment or prior experiments using the same venom. For statistical calculation of a venom's LD₅₀ one group requires 100% survival, one group requires 100% mortality with the remaining groups demonstrating partial survival.

Lyophilised venom extracted from the species of choice was reconstituted to a stock solution (typically 10mg/mL) in phosphate buffered saline (PBS) and stored at -80°C. The different doses of the venom were made up in 1.5mL tubes, with an injectable volume of 100µl per mouse in PBS, sufficient for 8 mice per group of 5 mice (to allow for dead-space), before temporary storage at 4°C. Prior to injection, doses were briefly warmed to room temperature to avoid shock to the mouse, and pre-warmed, male CD-1 mice (18-20g) received an intravenous (iv) injection via the tail vein – 5 mice per dose group.

Regular observations of the mice were recorded every 30 minutes for the first 4 hours and every hour thereafter. Evidence of extreme distress and severe envenoming, or a significant decrease in the animal's response or health resulted in implementation of humane endpoints and euthanasia by rising concentrations of carbon dioxide. Euthanasia was carried out to reduce suffering to the test animals without compromising the validity of the results. After 7 or 24 hours the numbers of surviving mice in each group were recorded. Table 2.2-1 shows the summary table of experiment MV178, an LD₅₀ of *Bitis arietans* venom.

Numbers of mice were kept to a minimum by careful selection of dose groups, then waiting until the likely outcome became obvious, then selecting the next dose group accordingly.

Table 2.2-1: LD₅₀ of *Bitis arietans* venom

Doses per mouse (µl)			Gp/# mice	Preparation of injection (8 doses)			Time: Injection	# mice		
Antivenom	Diluent	Venom µl		Antivenom	Diluent	Venom		F - Dead	Sch - 1	# deaths
-	98	2	B#5	-	784	16	11.25	-	-	0/5
-	96	4	D#5	-	768	32	11.30	-	2	2/5
-	95	5	E#5	-	760	40	11.16	-	4	4/5
-	93	7	O#5	-	930	70	11.40	-	5	5/5

The LD₅₀ (the amount of venom that kills 50% of the injected mice) and 90% confidence limits were calculated using Probit analysis (Finney, 1971) of surviving mice. Figure 2.2-1 shows the output from the Probit analysis obtained using *Stats Direct* programme.

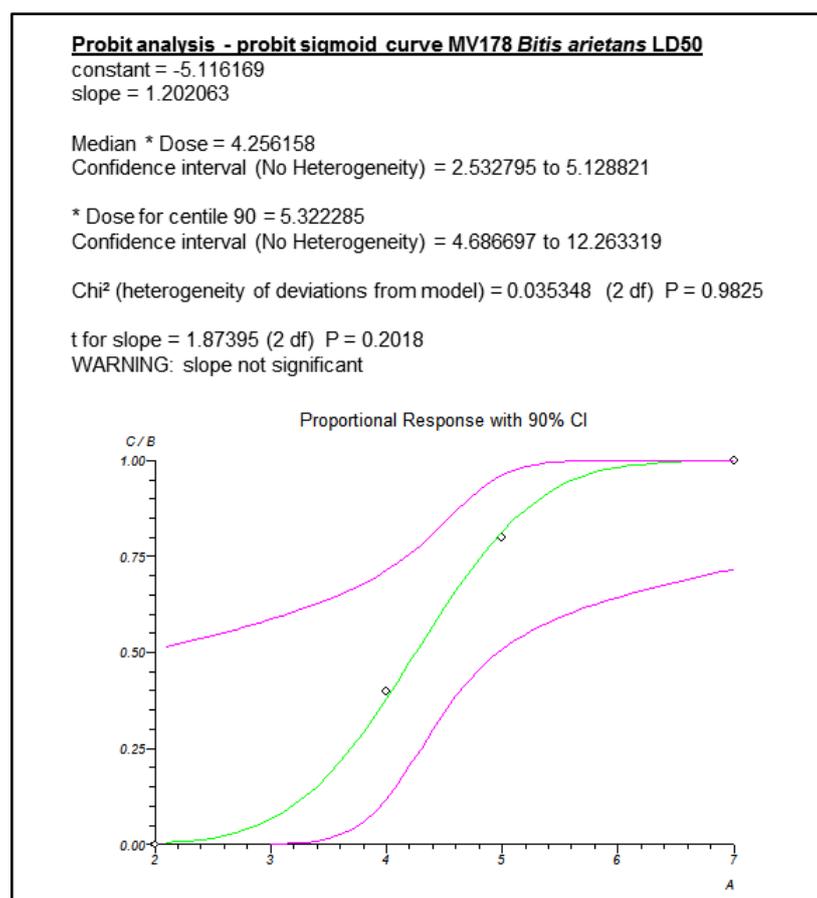


Figure 2.2-1: Probit analysis output.

2.2.2 Median effective dose: ED₅₀

The experiments were carried out with appropriate doses of antivenom that neutralised two to five LD₅₀ of venom (determined in the LD₅₀ experiment). The experiment is carried out with varying doses of antivenom (typically 5 different doses identified by range-finding experiments or previous experiments using the same antivenom), which are pre-incubated with venom for 30 minutes at 37°C, and then intravenously injected into the tail vein of 5 mice per dose. For statistical calculation of an antivenom's ED₅₀ one group requires 100% survival, one group requires 100% mortality with the remaining groups demonstrating partial survival.

Lyophilised venom extracted from the species of choice was reconstituted to a stock solution (typically 10g/L) in phosphate buffered saline (PBS) and the stock solution stored at -80°C. The stock solution of venom was the same as that which was used in the LD₅₀ experiment.

For the experiment, 2-5 times the LD₅₀ dose of the venom was made up in 2mL tubes, and mixed with various doses of antivenom. The different doses were made up to a volume of 200µl per mouse with PBS, sufficient for 8 mice for each 5 mouse group (to allow for dead space), before incubation for 30 minutes at 37°C and then temporary storage at 4°C. Prior to injection, doses were briefly warmed to room temperature, before five, pre-warmed male CD-1 mice (18-20g) received an intravenous (i.v.) injection via the tail vein for each dose. The experiment was then carried out as per LD₅₀.

2.2.3 Analgesia

On day 'Minus One' mice were marked for individual identification and baseline observations performed – weight, behaviour, activity and facial grimace score (MGS).

On day 'One' activity and MGS were recorded for each mouse. The mice were given appropriate analgesia or placebo treatment approximately 30 minutes prior to venom or venom/antivenom injection. Activity, behavioural

observations and facial grimace were recorded every 30 minutes, where possible, following intravenous venom injection (as described for LD₅₀ and ED₅₀ experiments), until termination of the experiment. Post mortem examinations were performed on all mice, and, where appropriate, samples taken for histopathology (see Chapter 2A for details).

2.3 Binding Assays

Binding assays demonstrate the ability of antibodies to bind to antigens but do not predict whether this binding results in neutralisation of antigen activity. However, if there is little or no binding this is an indication that there will be minimal neutralisation of antigen activity. The assays consist of antigen bound to a solid phase, addition of antibody then visualisation or measurement of the amount of bound antibody. I used SDS-PAGE followed by Western blotting, end-point enzyme-linked immunosorbent assay (ELISA), Avidity ELISA and small scale affinity chromatography.

2.3.1 SDS-PAGE

SDS-PAGE gel electrophoresis is a method of separating proteins according to their molecular weight. Highly folded proteins run through the gel more slowly than their MW would dictate due to electrostatic forces. Samples may be reduced by cleaving the disulphide bonds responsible for its folded structure by heating with β -mercaptoethanol. If proteins are being separated prior to immunoblotting changing its folding could affect the configuration of the antibody binding site and therefore it may be preferable to run them in their native state.

Method:

Gels were made up using the recipes shown in tables 2.3.1 and 2.3.2.

Two sets of 2.5mm gel plates and cover plates were cleaned and dried using washing up liquid followed by 60% ethanol. This process is important to prevent the gels from sticking to the glass once the samples have been run. The plates were assembled, a 15% resolving gel (Table 2.3-1) was poured between the plates and allowed to polymerise.

Table 2.3-1: 15% Resolving gel.

Reagent	Volume	
	2 gels	4 gels
H ₂ O (WFI – water for irrigation)	3.75 mL	7.5mL
Tris pH 8.8, 1.5M	2.5mL	5.0mL
40% bis acrylamide	3.75mL	7.5mL
10% SDS (sodium dodecyl sulphate)	100µL	200 µL
10% APS (ammonium persulphate) (made fresh in WFI)	60 µL	120 µL
TEMED (Tetramethylethylenediamine)	7 µL	14 µL

Table 2.3-2: Stacking gel.

Reagent	Volume	
	2 gels	4 gels
H ₂ O (WFI)	2.5mL	5.0mL
Tris pH 6.8, 0.5M	1.0mL	2.0mL
40% bis acrylamide	350 µL	700 µL
10% APS	30 µL	60 µL
TEMED	5 µL	10 µL

Stacking gel (Table 2.3-2) was then poured on top of the resolving gel, the relevant combs placed into the top, taking great care not to introduce any air bubbles, and left to polymerise. Venom samples at a concentration of 1g/L in PLOB – a carrier buffer containing Tris base, SDS, glycerol and bromophenol blue, to allow visualisation of the sample (see Appendix 2, Buffers) were prepared. If reduced samples were being run, the PLOB contained 15% β-mercaptoethanol. In this instance, the samples were heated at 100°C for 10 minutes to denature the proteins. For non-reduced samples, the PLOB used did not contain β-mercaptoethanol. The glass plates containing the polymerised gels were placed in an electrophoresis tank and 5µL *Promega* broad range protein marker added to a small well. If a 15 well comb was used, 10µL venom sample/ well was added, if a 1 well comb was used, 110µL venom sample was added to the well. The tank was filled with electrophoresis running buffer and the gels run at 200V until the samples had reached the bottom of the gel (around 1 hour). The gels were then either left to stain overnight in Coomassie Blue or immunoblotted onto a nitrocellulose membrane. Gels

which were stained overnight were destained until the bands were visible and the surrounding gel colourless.

2.3.2 Western Blot

Western blotting is a method of immunoblotting in which the SDS-PAGE separated venom proteins are blotted onto a membrane (the solid phase) and the membrane then probed with antibodies to determine which proteins are bound and, more importantly, those which are not. The bound antibodies are visualised with an appropriate enzyme-conjugated secondary antibody which results in a colour change on addition of its substrate. The major drawback of this method is that there is considerable cross-reactivity with heterologous antibodies.

Method:

SDS-PAGE gels were run, as described above, using a 1 well comb. A nitrocellulose membrane was placed on the gel and sandwiched between filter paper and foam, carefully rolling out any air-bubbles. This was placed in an electrophoresis tank containing immunotransfer buffer (Appendix 2), taking precautions to prevent overheating by using an icepack and magnetic stirrer, and run at 100V for one hour. The protein bands on the membrane were visualised temporarily using Ponceau S stain. The membranes were cut and marked according to the experiment, washed with water to remove the stain, and the protein marker bands marked with a ball-point pen, cut off and set aside.

The membranes were blocked with blocking solution (BS) - 5% milk powder in TBST (Appendix 2) - overnight at 4°C. The following morning, the membranes were washed for an hour with at least 3 changes of TBST. Primary antibody was made up in BS and added to the membrane for 3 hours, after which the membranes were again washed as before. The bound antibody was probed using the relevant secondary antibody in BS for 1 hour. After washing, the bands were visualised by adding substrate (Diaminobenzamine solution containing hydrogen peroxide). After a final wash to stop the reaction, the

membranes were dried, photographed or scanned and preserved using a laminator. Visualised bands were compared to the separated homologous venom proteins on the Coomassie Blue-stained gel.

2.3.3 Endpoint ELISA

The ELISA is a binding assay in which the solid phase is bound to the wells of (usually) a 96-well plate. This enables more than one sample to be run on a plate. This technique can be automated and therefore is suitable for large-scale screening of samples. The disadvantage of the technique is that due to the multiple steps involved any error is potentially multiplied, giving the assay a high coefficient of variation.

Method:

A 96 well microtitre plate was coated with 100µL/well of a 2mg/L venom solution in 100mmol bicarbonate buffer, pH 9.6 (0.2µg venom/well). The plate was covered and incubated for 2 hours at 37°C, then overnight at 4°C. The following day the plates were washed 3-5 times with TBST. The plates were blocked with 150µL/well BS, incubated for 30 minutes at 37°C and then emptied and washed twice with TBST. Plates not used immediately were stored at -20°C and defrosted when required. Doubling dilutions of sample (antiserum, antivenom or PIS (pre-immune serum)) in TBST were prepared from a suitable starting dilution (usually 1:1000), each well containing 100µL of diluted sample. The plate was covered and incubated at 37°C for 1 hour, then washed as above with TBST. The appropriate secondary antibody for the sample used was diluted in TBST and 100µL/well added to the plate, which was covered and incubated at 37°C for 1 hour, then washed as above with TBST. One 10mg tablet of ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)) was dissolved in 50mL ELISA citrate buffer, pH 5.0 and 50µL hydrogen peroxide added just prior to use. One hundred µL of this was added to each well and the plate left at room temperature until a green colour had developed (5-10 minutes) then the reaction was stopped with 100µL/well 1% SDS solution. The absorbance was read at 405 nm using an Optimax or Omega Polestar microtitre plate reader. The titre at 50% of maximum binding was calculated: $\{[(A_{max} - A_{min})/2] + A_{min}\}$ and the corresponding dilution read

from the graph (EC_{50}). The end-point titre was calculated by reading the dilution at which the absorbance was the mean of the PIS control absorbances plus 2 x standard deviations.

2.3.4 Avidity ELISA

The avidity ELISA is a variation of the standard ELISA which measures the strength of antigen-antibody binding (avidity) by adding different concentrations of chaotrophic agent and determining the concentration which reduces antigen-antibody binding by 50% (M_{50}).

Method:

The plates were coated with venom and blocked as described above. A 1:1000 dilution of antivenom in BS (100 μ L/well) was added to the wells of Rows 2-9 and BS only to Row 10. The plates were covered and incubated for 1 hour at 37°C then emptied and washed 5x with TBST. Dilutions of ammonium thiocyanate (NH_4SCN) in water (WFI) were prepared as shown in Table 2.3-3, starting with an 8M solution (6.1g/10mL).

Table 2.3-3: Preparation of ammonium thiocyanate

Concentration NH_4SCN	Volume 8M NH_4SCN (μ L)	Volume WFI (μ L)
8M	2000	0
7M	1750	250
6M	1500	500
5M	1250	750
4M	1000	1000
3M	750	1250
2M	500	1500
1M	250	1750
0M	0	2000

Each concentration of NH_4SCN (100 μ L/well) was added to rows 2-10 of the plate (duplicate water-only rows – Rows 9 and 10), covered and incubated for 15 minutes at 37°C. Plates were then emptied and washed 5x with TBST. The assay was completed as for the end-point ELISA from the addition of secondary antibody. The concentration of ammonium thiocyanate which caused a 50% reduction in absorbance was calculated.

2.3.5 Small scale affinity chromatography (SSAC)

Affinity chromatography uses a gel such as sepharose to which is covalently attached the appropriate venom as the solid phase. The antibodies are mixed and incubated with the gel in a column following which any unbound antibodies are washed off. Bound antibodies are then eluted, in this case by reducing the pH, the eluate collected and protein concentration measured. The gel in the column is re-equilibrated and can be used multiple times. The main advantages of this method are that a measurement of specific antibody concentration can be made and expressed as a percentage of initial sample protein concentration and the column can be re-used 40 times or more. The main disadvantage is that there is some non-specific binding to the gel.

This technique can be reversed by coupling antibodies to the gel and collecting the unbound and bound fractions of venom, and by analysing its components identifying which toxic components are bound and which are not. This technique is called 'Antivenomics' (Calvete et al, 2009).

2.3.5.1 Column coupling

A 1mg/mL solution of venom in coupling buffer was made, allowing 5mg venom/1g CNBr-activated sepharose 4 fast flow gel. An extra 1.5mL venom solution was made up for protein measurement by measuring optical density (OD) at 280nm, using an extinction coefficient of 1. A suspension containing 1g sepharose gel in 10mL swelling solution was prepared, covered with parafilm and left to stand for 30 minutes. It was then washed with a further 35mL swelling solution. Washing with coupling buffer activated the gel, which was packed into a 10mL glass chromatography column and 5mg of venom/g gel in coupling buffer added. The column was mixed for 4 hours at room temperature on an end-over-end mixer, then incubated overnight at 4°C before mixing for a further hour at room temperature. The venom solution was drained off and retained for OD measurement at 280nm. The percentage coupling was calculated $\{(1-(\text{OD post}/\text{OD pre})) \times 100\}$. 1M ethanolamine was next added to the column and mixed for a further 2 hours. This step blocked any unbound sites on the activated gel. The column was then washed with a further 5mL of

coupling buffer, followed alternately by 15mL each of sodium acetate buffered saline, pH4.0 and coupling buffer, pH 8.3 for 3 cycles. The coupled gel was re-suspended in 3.5mL PBW before performing a blank run using 10 column volumes (35mL) PBW followed by glycine elution (20mL) and a final wash with PBW containing 1% sodium azide as a preservative during storage. The column was stored at 4°C until required.

2.3.5.2 SSAC

The appropriate sepharose venom-coupled column was placed in a vertical position and allowed to equilibrate to room temperature. The column was washed with at least 10 column volumes (CV) of PBW without sodium azide (35mL for standard columns). A known volume (S) of antibody-containing sample was added to the column (usually 1mL). Antiserum samples were filtered through a 0.2µm syringe filter prior to addition. The column was capped and placed on an end-over-end mixer for 2 hours at room temperature. The column was then replaced in a vertical position and washed with a further 10x CV of PBW. Bound venom-specific antibodies were then eluted using glycine HCl buffer at pH2.5 and the volume collected recorded (V) – this was usually 20mL. The column was equilibrated with a further 10x CV PBW, containing azide if the column was destined to be stored until further use. The OD at 280nm of the eluate was measured and the specific antibody concentration (SAbC) calculated, using an appropriate extinction coefficient for the donor species immunoglobulin or fragment in the sample (E).

$$\text{Formula: SAbC} = \frac{\text{OD} \times \text{V}}{\text{E} \times \text{S}} \text{ g/L}$$

2.4 Cytotoxicity Assays

Venoms contain many toxic components and can target different cell types and therefore exhibit a multiplicity of pathological lesions. For this reason it was decided to investigate whether, by selecting a number of different cell lines to represent the major target organs of venom toxins to form a matrix of cells, the lethality of venom in mice (and other victims of snakebite) could be predicted.

If so, whether the efficacy of antivenom in neutralising these lethal effects could likewise be predicted, thus potentially replacing the *in vivo* pre-clinical assays.

The objectives included developing an assay which was robust, simple to perform and affordable. Initially two cell lines were chosen – VERO cells (green monkey kidney) which are epithelial in origin and SH SY5Y cells (derived from a human neuroblastoma). Viperid venoms are generally cytotoxic and haemotoxic and therefore likely to be cytotoxic to the epithelial cells. VERO cells are adherent, easy to grow and do not have any special requirements for growth.

Neurotoxic elapid venoms act on the cholinergic neuromuscular junctions (NMJs). Primary cell cultures creating a NMJ are available, but require considerable expertise and time to produce and are not available as an established immortalised cell line. The SH SY5Y cells were donated but subsequently obtained from the European collection of authenticated cell cultures (ECACC) – they are adherent dopaminergic cells with some cholinergic properties. They are much slower to grow than the VERO cells and show differentiation into neuronal cells after around 3 days, with formation of dendritic processes.

2.4.1 Cell culture methods

2.4.1.1 Cell growth, passage and harvest.

Cells were added to approximately 30mL of suitable culture medium to support growth of the cells to be used in a T75 culture flask. The flask was placed in an incubator at 37°C, 5% CO₂ and in a humidified atmosphere and the cells grown until 80-100% confluent. To harvest the cells, they were first washed twice with 10mL PBSa then with 1mL Trypsin EDTA. Approximately 2mL Trypsin EDTA was added to the flask which was returned to the incubator until the cells had detached (approximately 10 minutes). The cells were washed off the bottom of the flask with culture medium containing 10% foetal calf serum and the resulting suspension harvested into a fresh tube. An aliquot of 100µL was diluted 1:10 in Trypan blue (a dye excluded by live cells) and a cell count

performed using an Improved Neubauer haemocytometer as follows: Stained cells were pipetted under the coverslip of the haemocytometer and the number of cells counted in the four large corner squares of the grid. The concentration of cells was calculated using the following formula: Cell count = (N x D)/V cells/mL, where N is the mean number of cells/ square counted, D the dilution factor (10) and V the volume of the shoulder square (10^{-4} mL). The cells were diluted in medium to the appropriate seeding density required for the experiment, or the appropriate volume added to a fresh culture flask for later use.

2.4.1.2 Freezing cells for storage in liquid nitrogen

Cells were harvested as described above. The resulting suspension was centrifuged (237 RCF for 5 minutes). The supernatant was discarded, and the pellet re-suspended in 1:1 DMSO: Medium to produce a suspension containing 5×10^6 cells/mL. One mL of this suspension was added to each cryovial. The vials were put into an insulated box in the -80°C freezer, allowing slow freezing of the cells. After 24 hours they were transferred to a liquid nitrogen storage tank. When required a vial of cells was retrieved from storage and rapidly thawed in a waterbath at 37°C before adding to a flask containing pre-warmed medium.

2.4.1.3 Neutral red assay

Neutral red is a dye which is taken up by viable cells into the lysosomes. This assay was chosen because it is simple to perform and relatively inexpensive. A solution containing 20% 0.33g/L neutral red solution was made up in PBSa and 50 μL /well added to the test cell culture plate. After 2 hours incubation, the supernatant was discarded and the plate washed twice with PBSa. De-stain solution, containing 50% ethanol and 1% acetic acid, was added to the plate (150 μL /well) and the plate put on a plate shaker for 10 minutes prior to reading absorbance in an *Optimax* or *Omega Polestar* plate reader at 540nm and 690 nm. The banking absorbance at 690nm was subtracted from that at 540nm and the percentage of mean control cell growth calculated.

2.4.2 Cytotoxicity

A suspension containing 0.75×10^5 VERO cells/mL was prepared from a T75 tissue flask containing VERO cells in log growth phase and 100 μ L/ well used to seed rows B – H of 96 well tissue culture plates (row A contained medium only). The plates were placed in the incubator at 37°C, 5% CO₂ with a humidified atmosphere for 20 - 24 hours. Venom dilutions containing 50 to 5000 ng in 100 μ L DMEM/10/HEPES were prepared and added in triplicate to the appropriate well of the pre-incubated cells. DMEM/10 only was added to the outer wells and the penultimate column as a negative control. After 4 hours' incubation a neutral red assay was performed as previously described. The mean absorbance of Row A was subtracted from those of the test wells. Percentage of control cell growth for each test well was calculated and the mean for each venom dose plotted against venom dose. Confidence intervals of 95% were calculated and added to the curve. The venom dose which corresponded to 50% cell death was read from the graph (LC₅₀).

2.4.3 Cytotoxicity Neutralisation

A suspension containing 0.75×10^5 VERO cells/mL was prepared from a T75 tissue flask containing VERO cells in log growth phase and 100 μ L/ well used to seed rows B – H of 96 well tissue culture plates (row A contained medium only). The plates were placed in the incubator at 37°C, 5% CO₂ with a humidified atmosphere for 20 - 24 hours. Antivenom were dialysed into 0.9% sodium chloride, to remove any preservative, and diluted to a concentration of 25g/L prior to preparing dilutions in medium containing 10% HEPES buffer and filtered using a 0.2 μ m syringe filter.

A volume of 200 μ L/well DMEM/10/HEPES was placed into the outer wells of 96 well dilution plates and 100 μ L/well into wells B-F, 3-11 and G2-11. A further 100 μ L/well was added to wells G8-11 (medium control). A volume of 200 μ L/well diluted antivenom (in triplicate), antiserum or PIS was added to wells B2-F2 and 100 μ L transferred from well to well across the plate to create doubling dilutions. The final 100 μ L was discarded from wells B11-F11. A volume of 100 μ L/well diluted antivenom was added to wells G2-4 (antivenom

control). Venom dilutions containing 2x to 5x LC₅₀/well in 50µL were prepared in DMEM/HEPES from defrosted 10g/L stock solutions and filtered through a 0.2 µm syringe filter. A volume of 100µL/well was transferred to the corresponding well of the dilution plate. [(Rows B-F, columns 2-11, and G5-7 (venom control)].

Table 2.4-1: Plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	Med	Med	Med	Med	Med	Med	Med	Med	Med	Med	Med	Med
B	Med	1 AV Ven	2 AV Ven	4 AV Ven	8 AV Ven	16 AV Ven	32 AV Ven	64 AV Ven	128 AV Ven	256 AV Ven	512 AV Ven	Med
C	Med	1 AV Ven	2 AV Ven	4 AV Ven	8 AV Ven	16 AV Ven	32 AV Ven	64 AV Ven	128 AV Ven	256 AV Ven	512 AV Ven	Med
D	Med	1 AV Ven	2 AV Ven	4 AV Ven	8 AV Ven	16 AV Ven	32 AV Ven	64 AV Ven	128 AV Ven	256 AV Ven	512 AV Ven	Med
E	Med	1 AS Ven	2 AS Ven	4 AS Ven	8 AS Ven	16 AS Ven	32 AS Ven	64 AS Ven	128 AS Ven	256 AS Ven	512 AS Ven	Med
F	Med	1 PIS Ven	2 PIS Ven	4 PIS Ven	8 PIS Ven	16 PIS Ven	32 PIS Ven	64 PIS Ven	128 PIS Ven	256 PIS Ven	512 PIS Ven	Med
G	Med	1 AV Ven	1 AV Ven	1 AV Ven	Med Ven	Med Ven	Med Ven	Med	Med	Med	Med	Med
H	Med	Med	Med	Med	Med	Med	Med	Med	Med	Med	Med	Med

Key: AV = Antivenom, AS = Antiserum, PIS = pre-immune serum, Figures refer to dilution. Ven = venom, Med = DMEM/10/HEPES

The venom/antivenom solutions were incubated for 30 minutes at 37°C. A volume of 100µL was transferred from each well of the dilution plate to the corresponding well of the pre-cultured VERO cells and this plate returned to the incubator for 4 hours before performing a neutral red assay for viable cells, as previously described. Absorbance was read at 540nm and 690nm and the corrected absorbance and EC₅₀ absorbance calculated (see formulae below).

Where A_{540} = absorbance @ 540nm; A_{690} = absorbance @ 690nm:

$$\text{Absorbance} = (A_{540} - A_{690}) - \text{Mean absorbance Row A } (A_{540} - A_{690})$$

Percentage control cell growth was calculated for each well as described for the cytotoxicity assay, and plotted against antivenom dilution. The dilution

which corresponded to 50% control cell growth was read from the graph (EC_{50}).

2.4.1 Real Time Cell Assays: xCELLigence®

Cytotoxicity and neutralisation assays were performed using the xCELLigence system to examine the 'real time' growth of cells. Sixteen-well e-plates were used, which could be run as single, double or triple-plate assays in the three available xCELLigence cradles. The plates have a central strip devoid of electrodes which allows visualisation of growing cells. The system was used for optimisation of assays and not for data collection so duplicate samples were often not used.

The plates were seeded with cells and then allowed to stand for 30 minutes at room temperature to allow the cells to settle prior to insertion of the plate(s) into the xCELLigence cradle(s). Cell index (CI) – a measure of electrical resistance across the bottom of each well - was recorded every 15 minutes until test compound(s) were added. At this time the CI was 'normalised' to a value of 1.0 to allow for variation in cell growth of each well. Immediately after addition of the test substance(s) CI was recorded every 5 minutes for 1 hour, then every 15 minutes thereafter – in some cases, for more than 72 hours. CI reflects the proportion of the bottom of the well covered by cells, but does not differentiate between increased numbers and increased size of cells. The real-time cell assay (RTCA) software was used to calculate 'slope' (rate of change), LC/EC_{50} over the study period and LC/EC_{50} at given time points, which include the R^2 value.

2.5 IgG Purification

Antiserum was thawed in the fridge for 72 hours prior to dilution 1:3 with 0.9% saline. Caprylic acid was added to a concentration of 6% volume/volume of the undiluted serum and mixed thoroughly for 30 minutes, then allowed to stand for 1 hour at room temperature before centrifugation at 4733 RCF for 60 minutes. The suspension was removed and filtered through a GF pre-filter followed by a 0.45 μ m filter and the precipitate discarded. The resulting solution was concentrated and reformulated into sodium citrate buffered saline (SCS),

pH 6.0 using a BioRad mini diafiltration system and 3x filters with a 30kDa cut-off. Protein concentration was measured by OD and adjusted to 25g/L with SCS.

2.6 Other

2.6.1 Protein concentration by optical density (OD)

An appropriate dilution of sample (D), containing less than 1g/L protein was prepared. A spectrophotometer with a UV lamp was set to 280nm wavelength for immunoglobulin samples and also to 260nm wavelength if protein composition was unknown. Approximately 1mL of sample buffer was added to a quartz cuvette, with a 1cm path length and used as a blank reading. The sample was mixed thoroughly and the optical density (OD) measured. Protein concentration (C) was calculated using the formula:

$$C = \frac{OD \times D}{E} \text{ g/L, where E is the extinction coefficient}$$

If the protein composition was unknown, the following formula was used:

$$C = (OD_{280} \times 1.55) - (OD_{260} \times 0.76)$$

2.6.2 FPLC/SEC

Fast phase liquid chromatography (FPLC), also known as size exclusion chromatography (SEC), was used to measure the purity of antibody samples and to assess digestion of IgG into its fragments. An *AKTA Purifier 10/10XT* system was used with a superose 12 HR column and a 100 μ L loop. The running buffer was sodium citrate buffered saline (SCS). Samples were diluted 1:10 in SCS and 1mL loaded into the system. The trace was retrieved after 75 minutes and the area of each peak as a percentage of total peak area recorded (Figure 2.6-1).

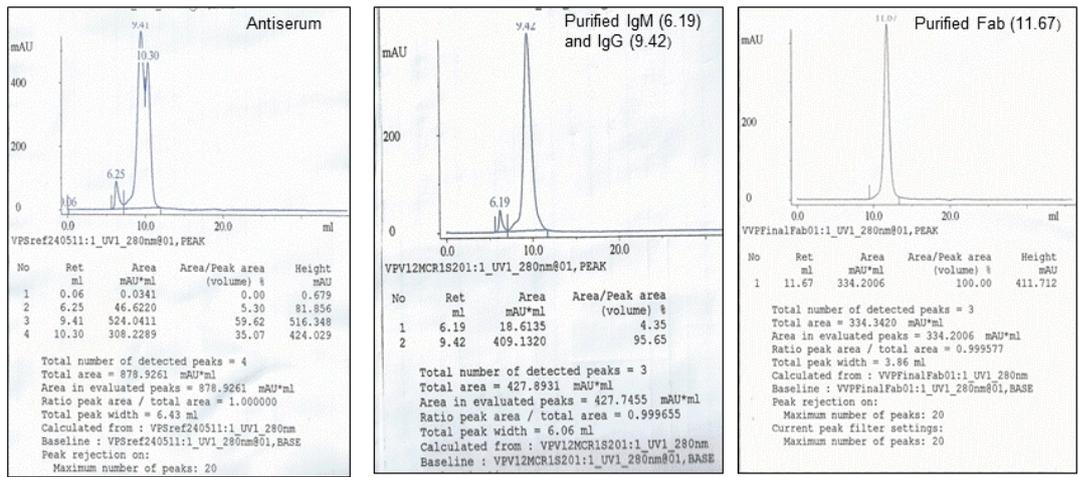


Figure 2.6-1: FPLC outputs showing peaks obtained from starting antiserum, purified IgG (with some IgM) and purified Fab fragments.

Chapter 2A: Refinement Methods

This chapter will cover the methods used to assess pain and suffering in the mice used in the *in vivo* refinement assays to determine the efficacy of analgesia and to establish humane end-points (HEPs). It also covers a novel method of scoring post mortem lesions, adjusted for survival time as a means of reducing the numbers of mice required to compare antivenom efficacy.

2.7 Behavioural observations

Observations were recorded at regular intervals, using Table 2.7-1 shown below, in the form of number/survivors or individual mouse identity, where n was the number of mice showing the behaviour and s the number of surviving mice in the group. Observations were made after the lid of the cage was removed – if the mice were sleeping this roused them and stimulated exploratory behaviour. A list of definitions for observed behaviours is listed below:

Depression: Mouse failed to exhibit normal exploratory behaviour when disturbed.

Slumping: Mouse lying flat with neck extended (Figure 2.7-1).

Hunching: Mouse sitting with back arched in hunched position, with head down. (Figure 2.7-2).



Figure 2.7-1: slumping



Figure 2.7-2: Hunching with staring coat



Figure 2.7-3: Dyspnoea

Staring coat: Fur looks dull and hairs erect (Figure 2.7-2).

Tachypnoea: Rapid, usually shallow, breathing.

Dyspnoea: Increased respiratory effort (Figure 2.7-3).

Paradoxical respiration: An extreme form of dyspnoea where during inspiration the abdominal wall is sucked in and during expiration it bulges out.

Respiratory distress: Mouse propped up in an attempt to breathe, signs of cyanotic (blue) extremities.

Haemorrhage: Epistaxis (blood from nose) and/or haemoptysis (blood from mouth).

Blue tail: Bruising on the tail around the injection site.

Mobility: Mouse active and moving around cage. Hyperactivity often precedes leaping or convulsions.

Twitching: Twitching of limb(s) or body – often progresses to convulsions.

Leaping: Mouse leaps in the air – repetition of this behaviour is an indication of impending death so mouse euthanased.

Convulsions: Self-explanatory. Mouse euthanased

Paralysis: Paralysis of one or more limbs. A neurological examination (see section 2.8) was carried out on mice showing mild paralysis. If generalised or progressive, mouse euthanased.

Lost righting reflex: Mouse unable to right itself when turned on its back (see section 2.8). Mouse euthanased.

Moribund: Mouse unreactive, but still breathing, -euthanased.

Schedule 1: Mouse euthanased.

Death: Mouse found dead.

Table 2.7-1: Behavioural observations template

Observation/Time									
Depression									
Hunching									
Slumping									
Staring coat									
Tachypnoea									
Dyspnoea									
Paradoxical resp									
Resp ^y distress									
Haemorrhage									
Blue tail									
Mobility									
Twitching									
Leaping									
Convulsions									
Paralysis									
Lost righting reflex									
Moribund									
Schedule 1									
Death									

A note was also made of presence or absence of normal behaviours, such as exploration, nesting or fighting.

2.8 Neurological observations

It was noted that envenomed mice often started showing neurological signs such as twitching, leaping or convulsions prior to death, even with non-neurotoxic Viperid venoms. The aim was to see if death in these mice could be predicted prior to being '*in extremis*'.

A simple neurological examination was developed based on a more extensive examination used to assess the neurological function of cats and dogs in veterinary practice. The mice used to develop the examination were regularly handled pet mice, which were not unduly stressed by these tests.

2.8.1 Visual placing test:

The mouse is suspended by the base of its tail and brought towards the edge of a surface (Figure 2.8-1). Its ability to reach forward first with its forelimbs, then its hindlimbs shows that its visual cortex, cerebellum, brainstem and spinal reflexes are all intact.



Figure 2.8-1: Visual placing – mouse held by tail and brought towards a surface. Normally reaches for surface

2.8.2 Ear tickle

The ear is touched gently – the mouse moves its ear away from the stimulus (Figure 2.8-2). This involves cranial nerves V (sensory) and VII (motor).



Figure 2.8-2: Ear tickle – ear twitches in response to light touch

2.8.3 Tail curl

When a finger is placed under the mouse's tail, it will normally wrap around the finger (Figure 2.8-3). This involves both sensory and motor pathways. Loss of this reflex may be an early sign of ascending paralysis.

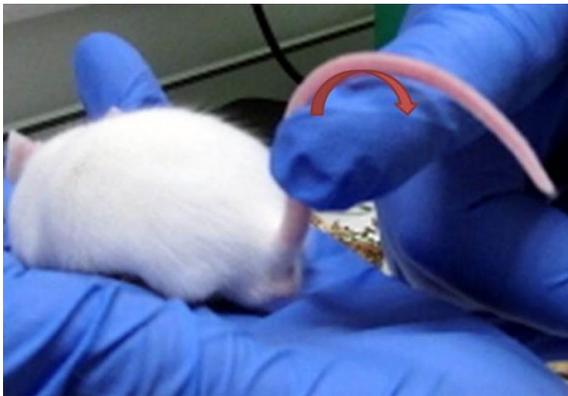


Figure 2.8-3: Tail curl – tail curls around finger

2.8.4 Righting reflex

One of the most distressing positions for a mouse, as shown by ultrasound recordings of distress calls at frequencies inaudible to the human ear, is to be held upside down [personal communication, Miller, Newcastle University, August 2012]. A normal mouse vigorously resists being turned over (Figure 2.8-4) and this reflex is one of the last to be lost. If poor it is a robust humane end point.

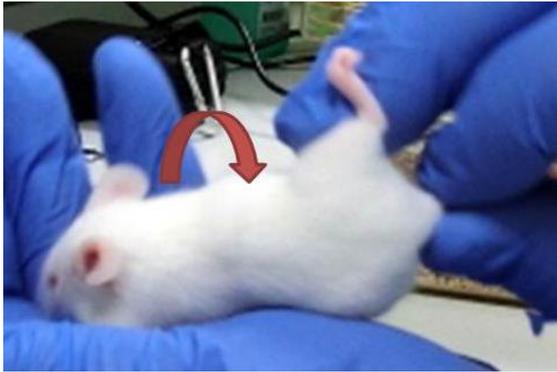


Figure 2.8-4: Righting reflex – mouse resists being turned upside-down

2.8.5 Corneal reflex:

The edge of the cornea is touched (Figure 2.8-5). If this reflex is intact, the mouse will blink– it is the final reflex to be lost and thus any live mouse without this reflex should be euthanased.



Figure 2.8-5: Corneal reflex – mouse blinks when eyelids or cornea is touched

2.9 Physiological observations

Physiological parameters can be affected by many emotions – fear, pain, increased activity and pleasurable anticipation such as food, to name but a few (Flecknell, 1999). The aim here was to investigate whether there was any relationship between objective measurement of temperature, pulse rate, breathing rate, oxygen saturation and pulse distension with the subjective facial grimace scale and activity scores, behavioural and neurological observations.

2.9.1 Surface Temperature measurement



Figure 2.9-1: Surface temperature measurement

Temperature can be measured in a number of ways, including via implanted chips, rectal probes and infrared (IR) thermometer. Implanted chips were too expensive for the number of mice used, rectal probes require mice to be anaesthetised and are therefore unsuitable for this project.

An IR thermometer was obtained and surface temperature measurements made by pointing the beam at the concavity immediately behind the mouse's skull. For a valid reading, the mouse must have all 4 feet on the ground and a consistent height was obtained by holding the beam source level with the edge of the cage. Using this technique repeatable readings were obtained, both by the same and different operators. The technique is illustrated in Figure 2.9-1.

2.9.2 MouseOx System

To measure other physiological parameters a collar allowing conscious monitoring, in conjunction with the relevant software, was obtained (MouseOx®Plus, Starr Life Sciences). Briefly, a collar attached via a wire to a recording device was applied to the neck of one mouse from each group of 5 (MouseOx, Harvard Apparatus) and data recorded (Figure 2.9-2) for 5 minutes prior to intervention, and for 5 minutes every 30 minutes thereafter, using conscious monitoring software. Parameters measured were: 'error', oxygen saturation (%), pulse rate (beats per minute), respiration rate (breaths per minute), pulse distension (mmHg) and the presence (1) or absence of activity (0). When 'Error' was 0 – all parameters were being detected; the greater the error value, the fewer the parameters were being detected. Activity of the mouse reduces the ability of the collar to pick up oxygen saturation, breath rate, pulse distension and finally pulse rate. The traces were analysed by calculating the mean of 8 values measured on the trace. Where possible, readings were taken when the 'error' and 'activity' values were '0'.

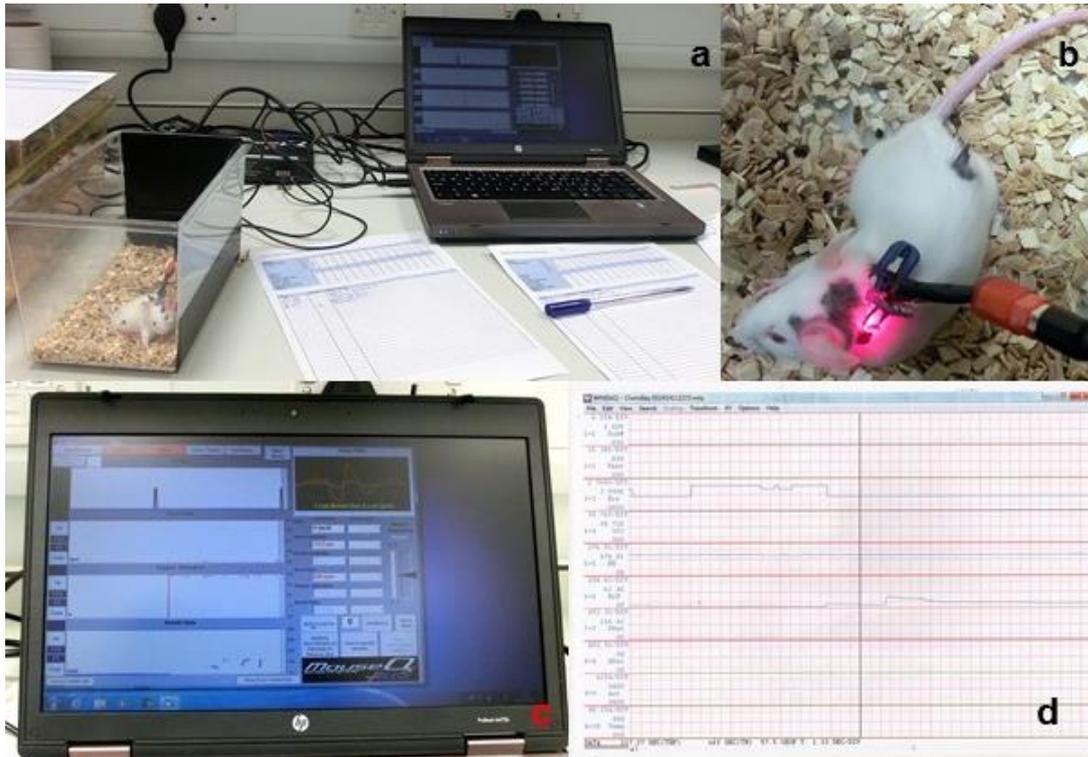


Figure 2.9-2: Conscious monitoring using the MouseOx collar. a. Set-up for recording; b. Collar emits visible red light and non-visible infrared light; c. Data-recording software; d. Trace obtained for analysis.

Understanding the Limitations of MouseOx® Plus in Conscious Unrestrained Applications

The MouseOx® Plus can measure vital signs in mice, rats and other small laboratory animals that are conscious and unrestrained. However, there are some limitations to its use. Oxygen saturation cannot be measured while the animal is moving because the nature of pulse oximetry which allows calculations of oxygen saturation to be obtained from absorption of light measurements requires that the distance between the sensor pads does not change. When the animal moves, or even flexes muscles under the skin without obvious surface movement of the sensor pads, this assumption is violated preventing the measurement of oxygen saturation. Thus, an animal that is conscious and unrestrained must also be still while the saturation measurement is being made. Measurement of pulse rate can be maintained during moderate movement.

2.10 Pain Assessment

2.10.1 Mouse Grimace Scale (MGS) Score

A system of facial expressions described by Mogil (2010) in the MGS Manual was used as a scoring system for pain in the mouse undergoing the lethality assays for preclinical testing of venoms and antivenoms. Mice were placed into a Perspex box with two sides at 90° blacked out. After a short period of acclimatisation photographs were taken in rapid succession on the camera's 'best shot' setting. The photograph which best demonstrated the facial features was selected, cropped and adjusted for optimum intensity, colour and brightness using Picasa 3 software. The photographs were then scored as described below (MGS manual – J. Mogil).

Coding procedures:

All faces were coded for the presence and intensity of the following specific facial Action Units (AU), relative to the status of the region of the baseline prototype.

A. Intensity ratings:

Intensity ratings were coded for each AU.

AU was not present = 0; AU moderately visible = 1; AU severe = 2

An MGS score for each photograph was calculated by averaging intensity ratings for each AU. An MGS difference score (relative to baseline/"no pain") was then calculated for each subject, and averaged across a group. These calculations are described in detail at the end of this section.

B. Action units:

1. Orbital Tightening

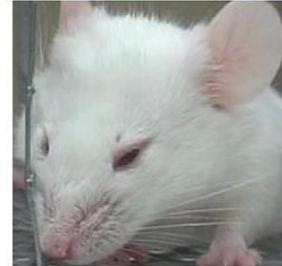
Mouse must display a narrowing of the orbital area, a tightly closed eyelid, or an eye squeeze. An eye squeeze is defined as the orbital muscles around the eyes being contracted. A wrinkle may be visible around the eye. As a guideline, any eye closure that reduces the eye size by more than half should be coded as a "2". Note that sleeping mice display closed eyes, and this may be mistaken for a tightly closed eyelid. Photographs of sleeping mice should therefore not be taken and/or coded.



'0'



'1'

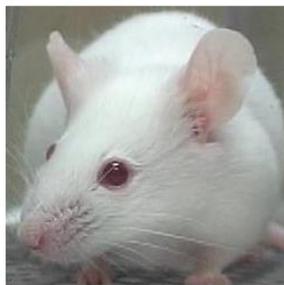


'2'

Figure 2.10-1: Images illustrating the scoring of 'orbital tightening'

2. Nose Bulge

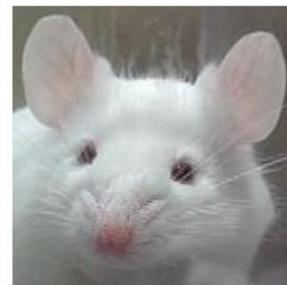
Mouse must display a bulge on top of the nose. The skin and muscles around the nose will be contracted creating a rounded extension of skin visible on the bridge of the nose. A nose bulge may also be coded if a coder sees vertical wrinkles extending down the side of the nose from the bridge. In frontal headshots, a bulge may be seen as a widening of the nose area (i.e., V-shape connecting eyes to nose appears broader). *Note that a nose bulge may also appear when mice are actively exploring (i.e., sniffing). Ideally, these photographs should not be taken and/or coded, as they may inflate baseline MGS scores.



'0'



'1'



'2'

Figure 2.10-2: Images illustrating the scoring of 'nose bulge'

3. Cheek Bulge

The cheek muscle is contracted and extended relative to the baseline condition; it will appear to be convex from its neutral position. Note; the cheek is considered to be the area directly below the eye and extending to the beginning of the whiskers

on the nose (in humans, the infraorbital triangle). The distance from eye to whisker pad may appear shortened relative to baseline.

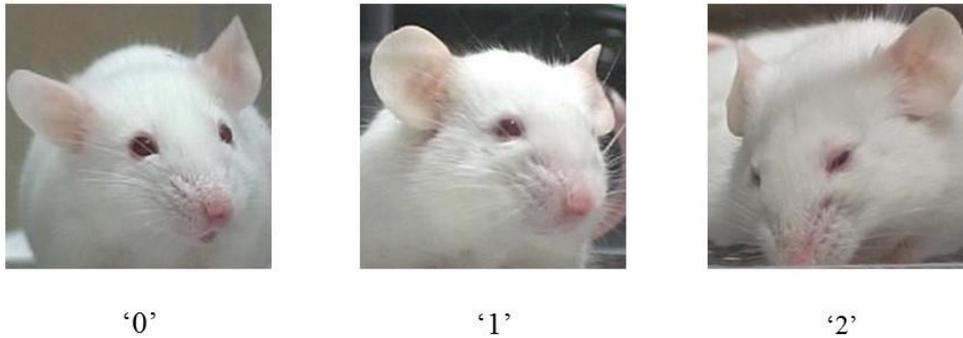


Figure 2.10-3: Images illustrating the scoring of 'cheek bulge'

4. Ear Position

Ears may be pulled back from their baseline position, or may be seen as laid flat against the head. In a typical baseline position ears are roughly perpendicular to the head and are directed forward. In pain, the ears tend to rotate outwards and/or back, away from the face. As a result, the space between the ears may appear wider relative to baseline. Note that mice engaged in exploration or grooming may also pull ears back, but distance between ears tends to narrow rather than widen. In any case, these may cause confusion, and it is advised that photographs of mice actively exploring or grooming not be taken and/or coded.



Figure 2.10-4: Images illustrating the scoring of 'ear position'

5. Whisker Change

Whiskers must have moved from the baseline position. They could either be pulled back to lay flat against the cheek or pulled forward as if to be “standing on end”. Whiskers may also clump together compared to baseline whiskers, which tend to be fairly evenly spaced.



Figure 2.10-5: Images illustrating the scoring of ‘whisker change’

iii. Calculating MGS scores:

- All AUs for each photograph were averaged to give the MGS score. Most of these preliminary calculations were carried out using Excel (see example below):

	A	B	C	D	E	F	G
1	Photo ID#	Orbital	Nose	Cheek	Ears	Whiskers	MGS
2	1	2	1	1	2	1	1.4
3	2	0	0	1	0	1	0.4
4	3	1	2	2	2	1	=AVERAGE(B4:F4)

- After unblinding, data was sorted by subject (e.g., “Mouse”) then by condition. Note that “no pain” photographs were almost always baseline photographs taken prior to noxious stimulation:

	A	B	C	D	E	F	G	H	I
1	Photo ID#	Mouse	Condition	Orbital	Nose	Cheek	Ears	Whiskers	MGS
2	27	A1	no pain	0	0	1	0	1	0.4
3	58	A1	No pain	0	0	0	0	0	0
4	1	A1	Pain	2	1	1	2	1	1.4
5	206	A1	Pain	1	2	2	2	1	1.6

- Mean MGS score for all “no pain” and all “pain” photographs was calculated for each subject and the mean for the “no pain” photographs subtracted from the mean for the “pain” photographs to give an MGS difference score for each subject:

	A	B	C	D
1	Mouse	MGS_no pain	MGS_pain	MGS_diff
2	A1	0.2	1.5	1.3
3	A2	0.4	1	0.6
4	A3	=AVERAGE(no pain)	=AVERAGE(pain)	=MGS_pain-MGS-no pain

- Difference scores were then averaged across subjects to give a mean difference score, which were analyzed for significance using a one-sample Student’s t -test compared to 0.

Iv. Attribution:

Langford et al, 20103

Please address all questions to Dr. Jeffrey S. Mogil at jeffrey.mogil@mcgill.ca.

2.10.2 Activity score

It became apparent that mice experiencing pain were less active, and as they recovered from envenomation, they became more active. To perform an activity score, the lid, food, bedding and nest box were removed from the cage. Using the video setting on a camera, a recording was made for 30 seconds prior to any intervention (analgesia administration or venom injection) and then for 30 seconds every 30 minutes. Each mouse was individually marked for identification. The number of head movements performed over 30 seconds was counted for each mouse. Head movements were selected to differentiate between mice which were completely immobile and those which showed some interest in their surroundings.

2.11 Pathology

Initially post mortem examinations were carried out to try to determine the likely cause of death and target organs of the venom toxins. It was noted that the severity of the pathological lesions appeared to be related to the dose of venom received and the length of time the mouse had been exposed to the venom. Post mortem

examinations were carried out on mice which had died or euthanased during the experiment and at the end any surviving mice were euthanased in a rising concentration of CO₂. If blood samples were required, cardiac puncture was performed prior to performing a gross post mortem examination and blood stored in appropriate sample tubes. Photographs were taken as applicable and samples collected in 10% formalin for histopathology. If relevant, urine samples were collected in Eppendorf tubes.

2.11.1 Gross post mortem examination

Post mortem examinations were performed on mice as soon as possible after death or CO₂ euthanasia. External features were observed for surface haemorrhages, epistaxis, haemoptysis, hyphaema and colour of extremities. Signs of sweating, salivation and voided urine were noted.

A mid-line incision was made from the level of the pubis up the abdomen and thorax, extending to the ramus of the mandible. The skin was reflected, pinned back and a note made of abnormalities in the subcutaneous tissues – pale, congested, jaundiced, or haemorrhagic. Similarly, the musculature of the body wall and hind limbs were examined before making a midline incision into the abdominal cavity. Any intra-abdominal blood, with or without clots, was recorded and the colour of the abdominal organs noted. The size of the bladder and colour of urine were recorded, and a sample of urine collected to be examined for blood, haemoglobin or protein. The testicles were inspected for evidence of haemorrhage.

The gastrointestinal tract (GIT) was removed by severing the oesophagus, where it passes through the diaphragm, and the rectum, by carefully teasing it free from the mesentry. Any discolouration - congestion, haemorrhage, dark green, black or yellow - was noted. The spleen was removed with the GIT. The stomach and sections of the intestines were split longitudinally, washed gently with 10% formalin before placing in a labelled Falcon tube, containing sufficient 10% formalin to cover the contents, to allow later histopathological examination.

The liver was excised, making a note of gall bladder size. The kidneys were left *in situ*, unless required for histopathology. Before opening the thorax, the diaphragm was examined for haemorrhages and signs of haemothorax, which is best visualised through the diaphragm.

The thoracic wall and diaphragm were removed to allow inspection of the thoracic contents; the heart, lungs and thymus (pluck). The pluck was removed by splitting the mandible, dissecting the tongue, larynx, trachea and oesophagus followed by lungs, heart and thymus.

Any abnormality of the forelimbs was noted (muscle or joint haemorrhage) and samples taken for histopathology, before turning the carcass over to examine the cranium. A midline incision was made over the skull and neck and the skin reflected to expose the eyes and cranium, noting any congestion or haemorrhage. Finally, a square of the skull extending from between the eyes to the foramen magnum was removed, to allow examination of the brain. The brain was carefully dissected free from the skull and the ventral surface of the brainstem, mid-brain and hypothalamus examined.

Photographs were taken of representative pathological lesions and of any unusual or unexpected findings.

2.11.3 Blood smears

Thin blood smears were made and stained with Diff-Quick™, then examined using oil immersion microscopy.

2.11.4 Clotting

Samples were collected via cardiac puncture from several mice from the same treatment group so that there was sufficient blood to fill a 1.5mL sodium citrate blood tube. The samples were kept on ice prior to centrifugation to remove the red cells. The serum was frozen prior to dispatch to a specialist laboratory for measurement of clotting factors.

2.11.5 Histopathology

Samples in 10% formal saline were sent to the Department of Veterinary Pathology, University of Liverpool for histopathology examination. The samples were first embedded in paraffin blocks prior to slicing with a microtome. Any samples containing bone were decalcified before processing. Sections were mounted and stained with haematoxylin and eosin. Other stains were used where appropriate.

2.11.6 Results analysis

Presence of congestion, petechial, ecchymotic, haemorrhage, any change in colour or consistency and whether focal or diffuse, was recorded in Post mortem examinations were performed on mice as soon as possible after death or CO₂ euthanasia. External features were observed for surface haemorrhages, epistaxis, haemoptysis, hyphaema and colour of extremities. Signs of sweating, salivation and voided urine were noted.

A mid-line incision was made from the level of the pubis up the abdomen and thorax, extending to the ramus of the mandible. The skin was reflected, pinned back and a note made of abnormalities in the subcutaneous tissues – pale, congested, jaundiced, or haemorrhagic. Similarly, the musculature of the body wall and hind limbs were examined before making a midline incision into the abdominal cavity. Any intra-abdominal blood, with or without clots, was recorded and the colour of the abdominal organs noted. The size of the bladder and colour of urine

were recorded, and a sample of urine collected to be examined for blood, haemoglobin or protein. The testicles were inspected for evidence of haemorrhage.

The gastrointestinal tract (GIT) was removed by severing the oesophagus, where it passes through the diaphragm, and the rectum, by carefully teasing it free from the mesentry. Any discolouration - congestion, haemorrhage, dark green, black or yellow - was noted. The spleen was removed with the GIT. The stomach and sections of the intestines were split longitudinally, washed gently with 10% formalin before placing in a labelled Falcon tube, containing sufficient 10% formalin to cover the contents, to allow later histopathological examination.

The liver was excised, making a note of gall bladder size. The kidneys were left *in situ*, unless required for histopathology. Before opening the thorax, the diaphragm was examined for haemorrhages and signs of haemothorax, which is best visualised through the diaphragm.

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Photographs were taken of representative pathological lesions and of any unusual or unexpected findings.

Table 2.11-1.

Congestion, mild haemorrhage or a change in colour/consistency was assigned a score of '1' and a score of '2' for moderate to severe haemorrhage for each organ affected. The total score was termed the 'pathology score'.

2.11.7 Survival (Mortality) Score

To quantify time taken for lesions to develop the duration of survival was taken into consideration. The majority of experiments ran over 6 hours, so this time was divided into twelve 30 minute periods. Mice which died in the first 29 minutes were assigned a 'survival' score of 12, those which died within 30 to 59 minutes a score of 11, and so on. Those mice which survived for 6 hours were assigned a score of 0. The pathology and survival scores were added together, the sum of which was taken as a measure of the severity of venom-induced lesions and termed the pathology/survival score (PSS).

On reflection, a better term for this score would be 'mortality score', as the highest score is assigned to those individuals which died in the shortest time.

Chapter 3. Reduction: Analysis of the correlation of *in vitro* and *in vivo* preclinical efficacy of multiple antivenom and venom combinations

3.1 Objectives

- To test the hypothesis that the results of *in vitro* assays can accurately predict those of *in vivo* antivenom (AV) efficacy assays.
- On the basis of these results to be able to recommend a reduction in the) number of *in vivo* assays required for preclinical testing of antivenoms.

Lethality is the single most important metric in the preclinical study of snake venom toxicity and in the evaluation of the neutralizing ability of antivenoms (WHO, 2010). *In vivo* preclinical testing was not carried out as part of this study because poor binding profiles and cytotoxicity neutralisation between the majority of heterologous venom/antivenom combinations suggested a poor outcome of neutralisation of venom toxicity. It was therefore considered unethical to proceed to *in vivo* studies when there would be no medical advantage by doing so. Evidence would suggest that the homologous venom/antivenom combinations are effective, both *in vivo*, and clinically- EchiTabG against *E. ocellatus* venom (Casewell et al, 2010); EchiTab Plus- ICP against *B. arietans* and *N. nigricollis* venoms (Segura et al, 2010); and SAIMR polyvalent snake antivenom against *D. angusticeps* venom (Hodgson and Dividson, 1996). However, the deficiencies of this section of the study regarding the correlation of *in vitro* and *in vivo* assays are covered in Chapter 4.

3.2 Introduction

The present WHO ‘gold’ standard *in vivo* preclinical assays of venom toxicity (LD₅₀) and AV efficacy (ED₅₀) (Theakston and Reid, 1983), required before any antivenom may be administered to human snakebite patients, were developed by the Alistair Reid Venom Research Unit (ARVRU). A number of modifications have since been introduced, such as ‘range finding’, to reduce the numbers of mice required to perform these assays. For ‘range finding’ LD₅₀ or ED₅₀ experiments only one or two mice per dose is used and, in this way,

the likely range of doses required to perform the full assay is determined (Meier et al, 1986). At ARVRU, it has been shown that the group size could be reduced from 6 to 5 mice, without affecting the Probit analysis (Finney, 1971), potentially saving at least 4 mice per experiment.

The ARVRU has also pioneered experimental approaches to reduce the use of *in vivo* murine lethality assay including the use of non-sentient fertilised hen's eggs (Sells et al, 1997; Sells et al, 1998; Sells et al, 2001), *in vitro* ELISA (Theakston and Reid, 1979) and a haemolytic test (Theakston and Reid, 1983). Success in achieving a substantial reduction has been hampered by lack of detailed knowledge of the many constituents of venom, which have to be assessed as a whole, rather than individually. The current 'gold standard' tests of venom toxicity and preclinical antivenom efficacy are the *in vivo* LD₅₀ and ED₅₀. New technologies applied to the individual venom proteins show some promise in the development of novel *in vitro* assays to be designed which enable a reduction in the number of animals required (Sells, 2003).

3.2.1 Insensate hens' eggs

Fertile hens' eggs which have been incubated for less than 10 days do not have a complete nervous reflex system and therefore are held not to experience pain. The embryos have a vascularised yolk sac membrane with a normal blood circulation and display a primitive embryonic beating heart, the arrest of which provides a clear endpoint for lethality testing. The results of mouse and egg LD₅₀s showed a strong correlation, as did the ED₅₀s and could therefore substantially reduce both the numbers of experimental animals required and also the considerable costs involved in delivering improved clinical treatment of snake-bite (Sells et al, 1997; Sells et al 1998; Sells et al, 2001; Sells, 2003). However, because of the rudimentary development of both the cardiac and neurological systems of the eggs employed, the embryo lacks key receptors excluding the use of cardiotoxic and neurotoxic venoms from this type of assay. This effectively restricts the use of egg assays to haemorrhagic venoms, making the technique too limited for more general use (Sells, 2003).

3.2.2 ELISA

Endpoint ELISA has been used to screen antisera and AVs for their venom binding potency since all effective AVs will require high ELISA IgG titres. Results using four medically important African venoms, including both Viper and Elapid venoms, showed good correlation with *in vivo* ED₅₀ (Theakston and Reid, 1979). It is rapid, cheap, simple, economical in terms of the amounts of venom used and, most importantly, offers opportunities to reduce the need for live animals (Theakston et al, 1977; Peres et al, 2006; Kulawickrama et al, 2010). One publication (O'Leary et al, 2014) claims that an ELISA to measure venom-antivenom complexes may be used to totally replace *in vivo* testing of antivenom efficacy. However, ELISA results of venom-antivenom interactions do not always (for example, Cook et al 2010) reflect an AVs ability to neutralise the toxic effects of venom *in vivo*, and ELISA is therefore an unreliable replacement for *in vivo* testing, despite its potential to substantially reduce the numbers of animals required.

Avidity ELISA is a variation of the standard ELISA assay in which antibodies bound to antigen are exposed to increasing concentrations of a chaotropic agent, such as ammonium thiocyanate, and the resistance to elution of the antibody by the chaotropic agent is measured (Pullen et al, 1986; MacDonald et al, 1988). This methodology is not applicable to all antibody-antigen systems (Hall et al, 1988) and an invalid result may occur in some instances due to changes induced in binding to the secondary antibody (Gray et al, 1993). It has been shown (Ibrahim and Farid, 2009, Cook et al, 2010; Casewell et al, 2010) that avidity ELISA had a better correlation to *in vivo* lethality tests than the standard ELISA.

3.2.3 In vitro clotting test

Varying venom doses are incubated at 37°C with 200µL of human or mouse plasma and clotting time recorded. The 60-s clotting dose is calculated by plotting clotting time against venom dose and reading the dose at 60s from the resultant curve (Casewell et al, 2014; Theakston and Reid, 1983). Neutralisation of clotting may be assessed using this technique, by incubating

antivenom varying doses with the 60-s clotting dose of venom prior to the addition of plasma. Use of this test is limited to those venoms which contain procoagulant toxins.

3.2.4 Nerve-muscle preparations

Nerve-muscle preparations, such as chick *biventer-cervis* and rat or mouse phrenic nerve-diaphragm preparations are used to assess the neuromuscular effects of neurotoxic venom components (Barfaraz et al, 1994; Bulbring et al, 1946; Harvey et al, 1994; Ginsborg et al, 1960). The chick preparation is more robust and more easily implemented than either rodent nerve-diaphragm model and is therefore suitable to use as a means of assessing whether the neuromuscular effects of snake venoms are neutralised by antivenom (Harvey et al, 1994). However, the WHO guidelines state that neurotoxicity should be determined using *in vivo* LD₅₀ (WHO, 2010).

3.3 Materials and Methods

3.3.1 Materials:

See Appendix II for details of materials used

3.3.1.1 Venoms

Table 3.3-1: Venoms selected showing family, origin, source, batch number (BN) and characteristics

Species	Common name	Family	Origin	Source/BN	Characteristics
<i>Echis ocellatus</i>	African saw-scaled viper	Viper	Nigeria	LSTM/B1L1	Coagulopathic/ Local
<i>Bitis arietans</i>	Puff adder	Viper	Nigeria	LSTM/B1L3	Haemorrhagic/ Local
<i>Vipera berus</i>	European adder	Viper	Russia	Latoxan/ PA480	Haemorrhagic/ Local
<i>Naja nigricollis</i>	Black-necked spitting cobra	Elapid	Nigeria	LSTM/B1L1	Cytotoxic
<i>Dendroaspis angusticeps</i>	Eastern green mamba	Elapid	Tanzania	LSTM/old stock	Neurotoxic

3.3.1.2 Antivenoms

The choice of antivenom was made as shown in Table 3.3-2.

Table 3.3-2: Antivenoms used showing venoms in immunogen, donor, fragment, manufacturer and reason for inclusion

Antivenom	Vs Venom	Donor/ Fragment	g/L diluent	Manufacturer	Comments
EchiTabG	E.oc	Sheep/ IgG	25/ SCS	MicroPharm	Monovalent
ViperaVet	V.ber V.amm V.asp V.lat	Sheep/ IgG	25/ SCS	MicroPharm	Vipera polyvalent
EchiTab plus	E.oc, B.ar N.nig	Horse/ IgG	25/ 0.9%NaCl	Instituto Clodomiro Picado	Pan-African polyvalent
SAIMRp	B.ar B.gab N.ann N.mel N.mos D.pol D.ang D.jam H.haem	Horse/ F(ab') ₂	25/ 0.9%NaCl	South African Vaccine Producers	Pan-African polyvalent
TA	T.alb	Horse/ IgG	12/ 0.9%NaCl	Thai Red Cross	Asian Viper (unrelated)
King Cobra (KC)	O.han	Horse/ IgG	9/ 0.9%NaCl	Thai Red Cross	Asian elapid (unrelated)
Banded Krait (BK)	B.fas	Horse/ IgG	25/ 0.9%NaCl	Thai Red Cross	Asian elapid (unrelated)
Australian polyvalent (CSL)	P.aus N.scu P.text A.ant O.scut	Horse/ IgG	25/ 0.9%NaCl	Commonwealth Serum Laboratories	Australian elapids (unrelated) Haemorrhagic Neurotoxic

Key for Table 3.3-2

E.oc	<i>Echis ocellatus</i>	B.ar	<i>Bitis arietans</i>
V.ber	<i>Vipera berus</i>	V.amm	<i>Vipera ammodytes</i>
V.asp	<i>Vipera aspis</i>	V.lat	<i>Vipera latastei</i>
N.nig	<i>Naja nigricollis</i>	B.gab	<i>Bitis gabonica</i>
N.ann	<i>Naja annulifera</i>	N.mel	<i>Naja melanoleuca</i>
N.mos	<i>Naja mossambica</i>	D.pol	<i>Dendroaspis polylepis</i>
D.ang	<i>Dendroaspis angusticeps</i>	D.jam	<i>Dendroaspis jamesoni</i>
H.haem	<i>Hemachatus haemachatus</i>	T.alb	<i>Trimeresurus (Cryptelytrops) albolabris</i>
O.han	<i>Ophiophagus hannah</i>	B.fas	<i>Bungarus fasciatus</i>
P.aus	<i>Pseudechis australis</i>	N.scu	<i>Notechis scutatus</i>
P.text	<i>Pseudonaja textalis</i>	A.ant	<i>Acanthophis antarcticus</i>
O.scut	<i>Oxyuranus scutellatus</i>		

Note: *Trimeresurus albolabris* has since been reclassified as *Cryptelytrops albolabris*.

Each antivenom was diluted to a concentration of 25g/L, in order that the results of each *in vitro* assay were directly comparable. Antivenoms of unknown formulation and/or containing preservative were dialysed into 0.9% sodium chloride, protein concentration measured by OD, then diluted to 25g/L. Malaysian Green Pit Viper (*Trimeresurus albolabris*) and King Cobra

(*Ophiophagus hannah*) antivenoms had a final protein concentrations of 12g/L and 9g/L respectively; the dilution for these antivenoms was adjusted to provide an equivalent amount of starting protein in each assay.

3.3.1.3 Secondary antibodies

Donkey anti-sheep IgG (H+L)-HRP (Sigma)

Rabbit anti-horse (Fab')₂ (H+L)-HRP (Sigma)

Rabbit anti-horse IgG (H+L)-HRP (Sigma)

3.3.2 Methods

The details of the following assays are described in Chapter 2.

3.3.2.1 Western Blot

The proteins of the five non-reduced test venoms were fractionated using a 15 well comb for subsequent staining with Coomassie Blue (see Section 2.3.1) or a single well comb for subsequent immunoblotting (see Section 2.3.2). The membranes were cut into 9 strips before one strip from each blotted venom was probed with AV or normal sheep/horse serum (PIS), using a dilution of 1:1000, except for *Trimeresurus albolabris* antivenom (12g/L) and king cobra antivenom (9g/L), when dilutions of 1:500 and 1:250 were used, respectively, to compensate for their lower starting concentration as noted above.

3.3.2.2 Endpoint ELISA

Endpoint ELISAs were performed as described in section 2.3.3. A starting concentration of 25mg/L was used for all antivenoms.

3.3.2.3 Avidity ELISA

Avidity ELISAs were performed as described in Section 2.3.4 using the antivenom dilutions employed for the endpoint ELISA (25mg/L).

3.3.2.4 Small Scale Affinity Chromatography (SSAC)

Sepharose Fast Flow gel was coupled to each of the five venoms described in Section 2.3.5.1 and 1mL of AV added to each venom-coupled columns as

described in Section 2.3.5.2. Venom specific antibody (VSAb) concentrations and percentage VSABs were calculated.

3.3.2.5 Immunocytotoxicity (ICT) assays

Immunocytotoxicity refers to the neutralisation of venom cytopathic effect (VCPE) by antibodies.

Cytotoxicity assessment for each venom was performed as described in Section 2.4.2. A 'checkerboard' neutralisation assay was then performed (Section 8.4.2.3), using a 96 well culture plate, with venom concentrations from 0x to 5x LC₅₀ in columns B-G of a 96 well culture plate and serial antivenom dilutions from row 2 through to row 10. The results of these assays allowed the venom concentration and starting antivenom dilution to be selected so that a cytotoxicity neutralisation assay could be performed for each venom/antivenom (V/AV) combination as described in Section 2.4.3.

3.4 Results

3.4.1 Western Blot

An SDS-PAGE was performed to separate the protein components of the five test venoms which were then stained with Coomassie Blue (Figure 3.4-1C).

The Viper venoms contained components of higher molecular weight than the two elapid venoms, which is consistent with the presence of enzymes such as snake venom metalloproteases (SVMPs) and serine proteases (SVSPs), with fewer, less intensely staining low molecular weight compounds. In contrast, the elapid venoms appear to be rich in low molecular weight compounds, consistent with PLA₂s and 3FTxs. The mamba venom was particularly rich in compounds with a molecular weight less than 15kDa. However, both elapid venoms contained proteins of 50kDa or greater. The proteomes of all five venoms are discussed in relation to their venom-induced pathogenesis in Chapter 1.

Single venom gels were blotted onto nitrocellulose membranes and probed with the selection of antivenoms listed in Table 3.3-2. The resulting blots are illustrated in Figure 3.4-1. To assess the degree of crossreactivity the number, position (MW) and intensity of staining of bands is compared to the stained venom gels.

Echis ocellatus (Figure 3.4-1E) Antivenoms '1' (EchiTabG) and '3' (EchiTabPlus) are this venom's homologous antivenoms. EchiTabPlus shows the strongest binding, but EchiTabG shows more low molecular weight bands than the venom gel. There is considerable crossreactivity with ViperaVet, directed against unrelated vipers from a geographically distant continent, although there are a number of protein bands missing from the blot that are present in the venom gel. Bands are visible on all the other antivenom blots, demonstrating some crossreactivity, but there are bands missing and the blots the staining is of lower intensity, indicating binding to fewer venom proteins and to a lesser extent than to its homologous antivenom. There are even a couple of bands visible on the normal horse serum blot, suggesting that there is some non-specific binding present.

Bitis arietans (Figure 3.4-1B). Antivenoms '3' (EchiTabPlus) and '4' (SAIMR polyvalent) are this venom's homologous antivenoms. Once again, the strongest binding is by EchiTabPlus and more bands are visible than on the venom gel. There is a similar pattern of binding by SAIMR polyvalent antivenom, but the staining has a much lower intensity. There is good crossreactivity between this venom and EchiTabG or ViperaVet, the two ovine IgG antivenoms, with a greater staining density than for SAIMR polyvalent; this may be because a different secondary antibody was used, which may have had different affinities for the two antivenom donor proteins. There is some crossreactivity with the unrelated viper antivenom, *Trimeresurus albolabris* (5), but little with the unrelated, geographically distinct King cobra antivenom (6). There is considerable crossreactivity with CSL Australian polyvalent antivenom (7). Normal sheep serum shows little crossreactivity with this venom, with only one distinct band, but there are two distinct bands on the blot

probed with normal horse serum and a few very faint bands, again indicating some non-specific binding.

Vipera berus (Figure 3.4-1V). Antivenom '2' (ViperaVet) is the homologous antivenom for this venom. The higher molecular weight bands are distinct, with merging of the bands of molecular weight less than around 40 kDa. There is good crossreactivity between this antivenom and the others which are directed against Vipers for protein bands greater than 25kDa. There are around six faintly staining bands on the King cobra antivenom (6) blot and some strong high molecular weight bands on the CSL antivenom (7) blot. The normal sheep serum blot shows one strong high molecular weight band and a few very faintly staining bands. There are no visible bands on the normal horse serum blot.

Naja nigricollis (Figure 3.4-1N). EchiTabPlus (3) is the venom's homologous antivenom, and this antivenom's blot shows the most binding. The bands representing components less than 50kDa are not distinct, but there does appear to be binding to lower molecular weight proteins and peptides. SAIMR polyvalent antivenom (4) shows a similar pattern of binding, but staining of this blot is less intense. Other *Naja* species, including other spitting cobras, are included in the immunogen mix of this antivenom. There is crossreactivity with the higher molecular weight venom components and the two viper antivenoms, EchiTabG (1) and ViperaVet (2), but only 2 bands on the *Trimeresurus albolabris* antivenom (5) blot. There are both high and low molecular bands on the other two elapid blots (6 and 7).

Dendroaspis angusticeps (Figure 3.4-1D). SAIMR polyvalent antivenom (4) is the only antivenom to contain antibodies directed specifically against this venom and this blot shows the most intense staining of any of the antivenom blots of this venom. Unlike the venom gel for this species, staining of the lower molecular weight components is faint, suggesting a poor immune response to these compounds. Once again, there is crossreactivity of high molecular weight venom components with antivenoms directed against both Vipers and

Elapids; whilst only the CSL Australian polyvalent antivenom shows any binding to low molecular weight components.

It is thus concluded that there is generally good crossreactivity between high molecular weight venom components and heterologous venoms, whilst binding to lower molecular weight bands is more specific, at least to the same family. Intensity of binding is also more specific for the same family or genus. Lower molecular weight components stained less well than the higher molecular weight components. This may be either because the low molecular weight compounds are less immunogenic, or due to larger molecular weight compounds having more epitopes available to bind to antibodies. It would appear that this assay has a high sensitivity but poor specificity.

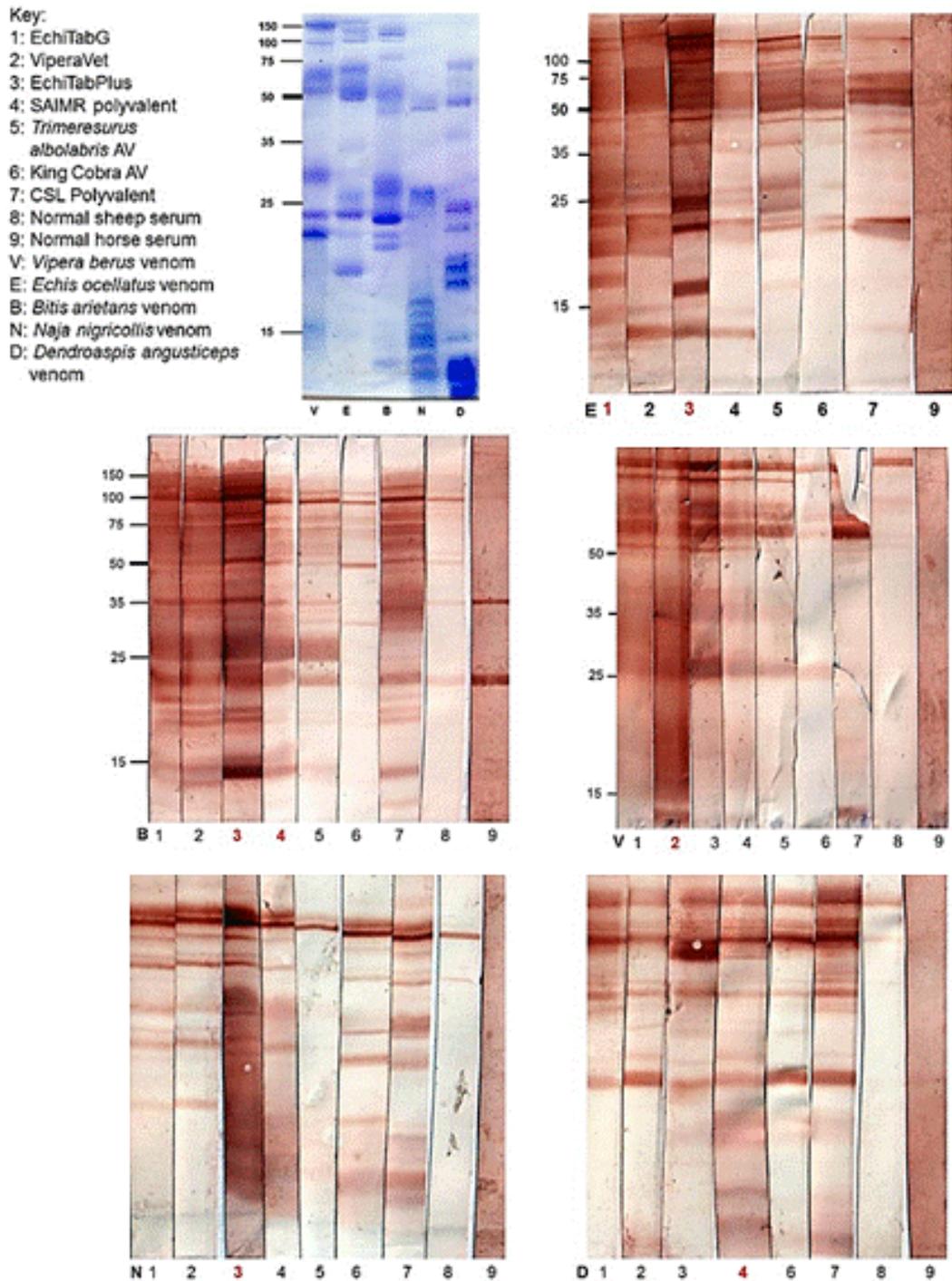


Figure 3.4-1: SDS-PAGE of venoms: C= SDS-PAGE stained with Coomassie Blue, V=*Vipera berus*, E=*Echis ocellatus*, B=*Bitis arietans*, N=*Naja nigricollis*, D=*Dendroaspis angusticeps*; Blots of venoms with a variety of antivenoms to demonstrate crossreactivity.

3.4.2 End-point ELISA

A starting dilution of 25mg/L was used for all antivenoms, the end point titre was defined as the dilution at which the absorbance was equal to the mean absorbance of the control plus two standard deviations (SD).

The results are summarised in Table 3.4-1, for EchiTabG, ViperaVet, EchiTabPlus and SAIMR polyvalent antivenoms in Figure 3.4-2 and for *Trimeresurus albolabris* antivenom, King cobra and CSL Australian polyvalent antivenoms in Figure 3.4-3.

EchiTabG and ViperaVet, the two ovine IgG antivenoms, had the highest binding titre to their homologous venoms, *E. ocellatus* venom and *V. berus* venom respectively. EchiTabG and ViperaVet bound well to *V. berus* venom and *E. ocellatus* venom, respectively and, to a lesser extent, they bound to *B. arietans* venom and the two elapid venoms. The *B. arietans* venom plate achieved nearly the same absorbances as the *V. berus* venom plate, although maximum binding was not achieved at the initial antivenom dilution. However, the absorbances of the elapid venom plates were around half of the homologous venom/antivenom plates; in this case, endpoint titre was a more accurate measure of antivenom binding, and demonstrates that the crossreactivity of EchiTabG for the two heterologous viper venoms, *V. berus* and *B. arietans*, was similar.

Trivalent EchiTabPlus and SAIMR polyvalent antivenoms (Figure 3.4-2) are directed against the majority of medically important snake venoms from Sub-Saharan Africa; both contain antibodies to *B. arietans* venom. EchiTabPlus contains antibodies to *N. nigricollis* venom, whilst SAIMR polyvalent antivenom does not. The latter does however, contain antibodies to the venoms of other cobras (both spitters and non-spitters) of the genus, *Naja*. Of the antivenoms selected, only SAIMR polyvalent antivenom contains antibodies to *D. angusticeps* venom.

Table 3.4-1: ELISA results summary.

Antivenom		EoV	BaV	VbV	NnigV	DaV
VPV	B	0.720	0.240	1.680	0.176	0.240
	E	21,000	70,000	220,000	7,500	12,000
EOG	B	1.680	0.200	0.800	0.248	0.320
	E	1,000,000	250,000	200,000	6,500	28,000
ETP	B	0.560	0.224	0.240	0.320	0.160
	E	70,000	12,000	30,000	50,000	4,000
SAIMRp	B	0.260	0.400	0.400	0.248	0.480
	E	15,000	50,000	60,000	60,000	40,000
TA	B	0.208	0.133	0.250	NE	NE
	E	22,000	2,000	30,000	3,000	NR
KC	B	0.105	0.075	0.125	0.155	0.200
	E	6,000	1,900	5,000	50,000	70,000
CSL	B	NR	NE	0.112	0.160	0.160
	E	NR	NR	2,000	20,000	15,000

D=dilution, B= 50% maximum binding (gV/gAV), E = end-point titre. NR = Not readable, NE = Not effective. Figures in red: Homologous V/AV

The highest binding of EchiTabPlus was to *E. ocellatus* venom, which was greater than that to *N. nigricollis* venom and *B. arietans* venom respectively. Whilst these experiments demonstrated that binding to *V. berus* venom was greater than that to *B. arietans* venom, in another set of experiments the reverse was observed; the results of this second set of experiments can be found in the chapter supplement. Whilst EC₅₀ dilution of EchiTabPlus bound to *D. angusticeps* venom was only slightly lower than to the other venoms, its assay end-point titre was found to be much lower, suggesting that EchiTabPlus would be ineffective at neutralising *D. angusticeps* venom.

SAIMR polyvalent antivenom had the highest binding to *D. angusticeps* venom, followed by *B. arietans* venom / *V. berus* venom, *N. nigricollis* venom and *E. ocellatus* venom in descending order. The endpoint titre of SAIMR polyvalent antivenom binding to *E. ocellatus* venom was four times lower than that of *N. nigricollis* venom. Given that *V. berus* venom is from an unrelated genus and that the snake originates from a different continent, the efficacy of SAIMR polyvalent antivenom at binding to this venom was somewhat unexpected.

Trimeresurus albolabris antivenom, an equine whole IgG formulation (Figure 3.4-3) showed similar binding to the viper venoms but negligible binding to the two elapid venoms.

King Cobra antivenom and CSL Australian polyvalent antivenom showed insignificant binding to any of the viper venoms, there was, however, some binding by King Cobra antivenom to both elapid venoms and by CSL Australian polyvalent antivenom to *N. nigricollis* venom (Figure 3.4-3).

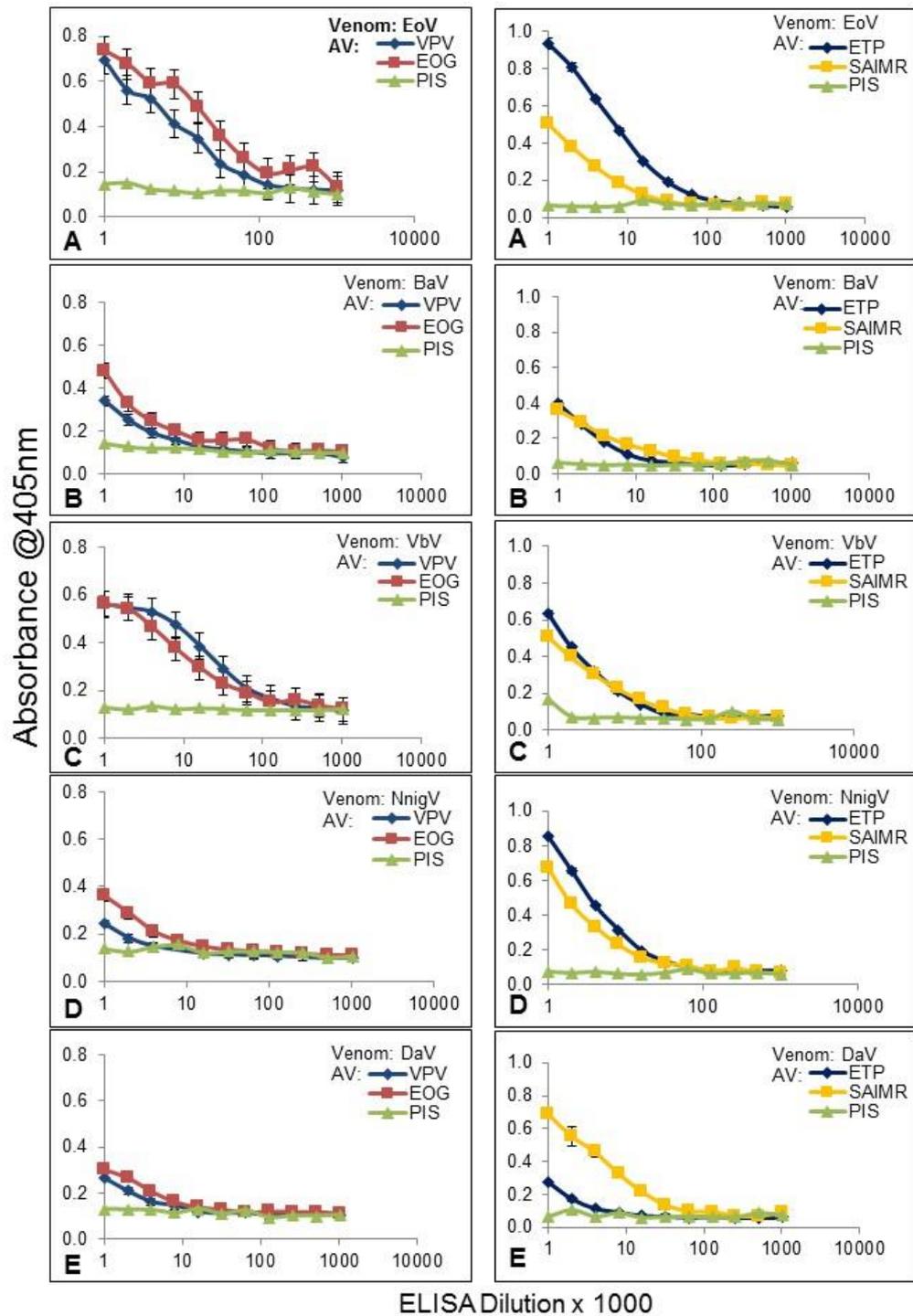


Figure 3.4-2: Binding of ViperaVet (VPV, EchiTabG (EOG) (left), EchiTabPlus (ETP) and SAIMR polyvalent antivenom (right) to *E. ocellatus* venom (A), *B. arietans* venom (B), *V. berus* venom (C), *N. nigricollis* venom (D) and *D. angusticeps* venom (E) by ELISA

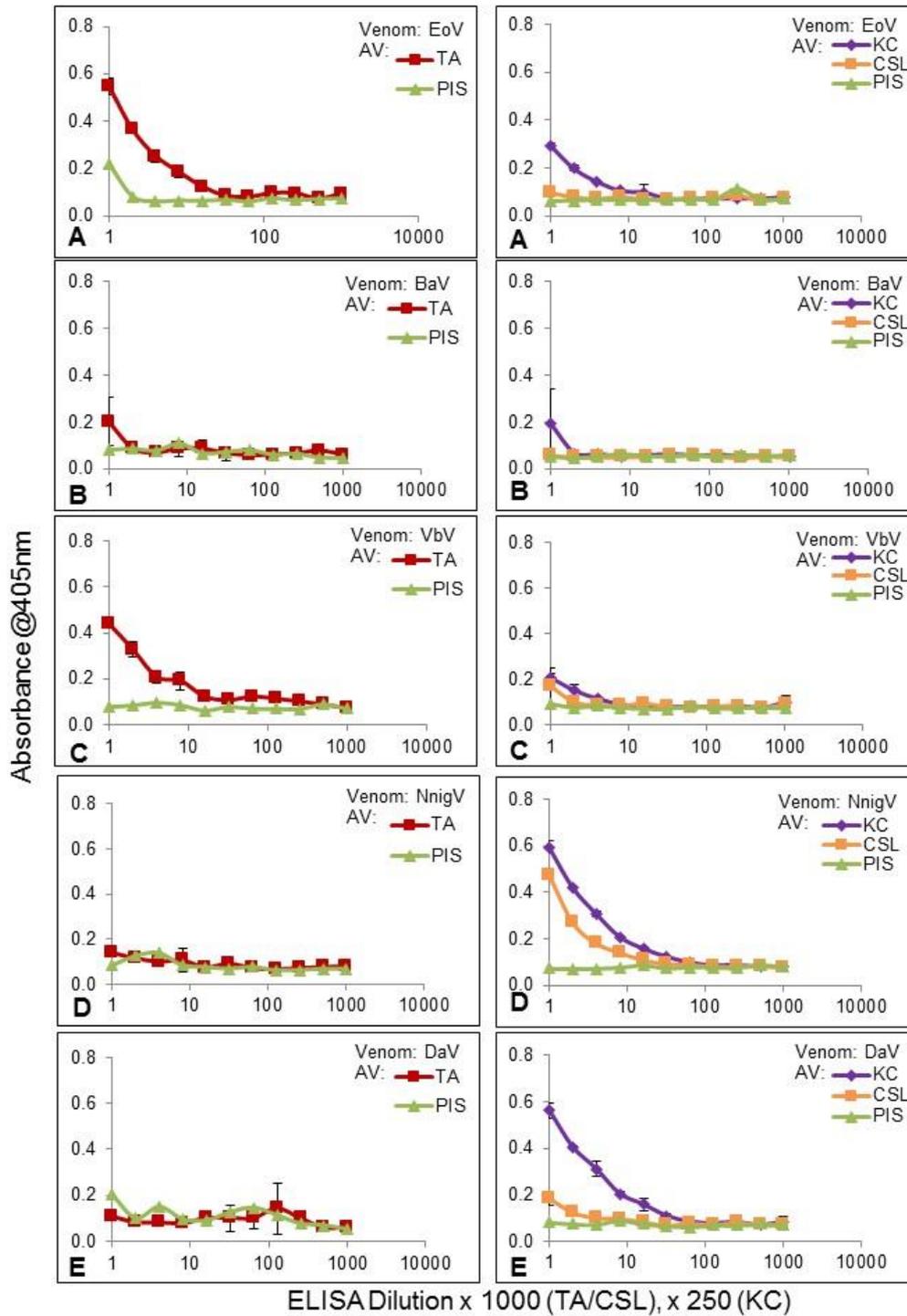


Figure 3.4-3: Binding of *Trimeresurus albolabris* antivenom (left), King Cobra and CSL Australian polyvalent antivenoms (right) to *E. ocellatus* venom (A), *B. arietans* venom (B), *V. berus* venom (C), *N. nigricollis* venom (D) and *D. angusticeps* venom (E) by ELISA

3.4.3 Avidity ELISA

A dilution of 25g/L was used for all antivenoms, and the results showed that antivenoms had a higher avidity for their homologous compared to heterologous venoms. In this assay, the highest avidity observed was 50% disruption of binding by 4.8M ammonium thiocyanate, between SAIMRp and *V. berus* venom, then by 4.4M ammonium thiocyanate, between the two polyspecific antivenoms, ViperaVet and EchiTabPlus, and their homologous venoms, *V. berus* venom and *N. nigricollis* venom, respectively. These results are summarised in Table 3.4-2 and illustrated in Figure 3.4-4, Figure 3.4-5 and Figure 3.4-6. The graphs of endpoint ELISA venom/antivenom binding are almost identical to those illustrating the disruption of venom/antivenom binding by ammonium thiocyanate.

Table 3.4-2: Avidity ELISA Summary.

AV/Venom	EoV		BaV		VbV		NnV		DaV	
	M ₅₀	A ₅₀								
VPV	2.2	0.03N	2.7	0.14Y	4.4	0.18Y	2.8	0.06N	2.0	0.04N
EOG	3.0	0.12Y	4.1	0.15Y	4.2	0.13Y	3.2	0.09Y/N	2.8	0.04N
ETP	2.8	0.31Y	2.2	0.23Y	3.7	0.12Y	4.4	0.22Y	NE	0.03N
SAIMRp	2.0	0.03N	3.4	0.18Y	4.8	0.08N	4.0	0.15Y/N	4.0	0.07Y/N
TA	2.6	0.06N	1.0	0.05N	NE	0.03N	2.4	0.04N	NE	0.01N
KC	NE	0.02N	2.8	0.03N	NE	0.01N	3.0	0.09Y/N	NE	0.01N
CSL	NE	0.01N	2.4	0.04N	NE	0.01N	2.7	0.10Y/N	NE	0.01N

M: Molarity of ammonium thiocyanate reducing absorbance by 50%

A₅₀: Absorbance (maximum – minimum)/2

NE: Not effective

Homologous antivenoms highlighted in red

A₅₀ in bold are those results which exceed twice the standard deviation of the PIS (negative control)

Y=effective, N = not effective

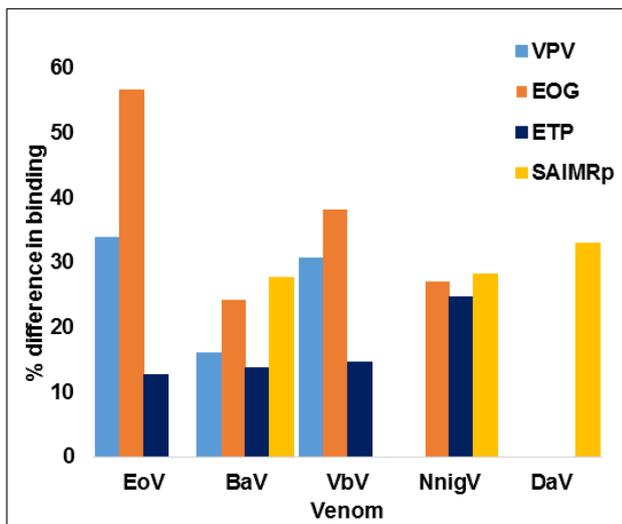


Figure 3.4-4: Difference between binding by addition of 8M ammonium thiocyanate and control:

(0Mabs - 8Mabs) x100

0M[control] abs).

Results where 50% absorbance <LDL were excluded.

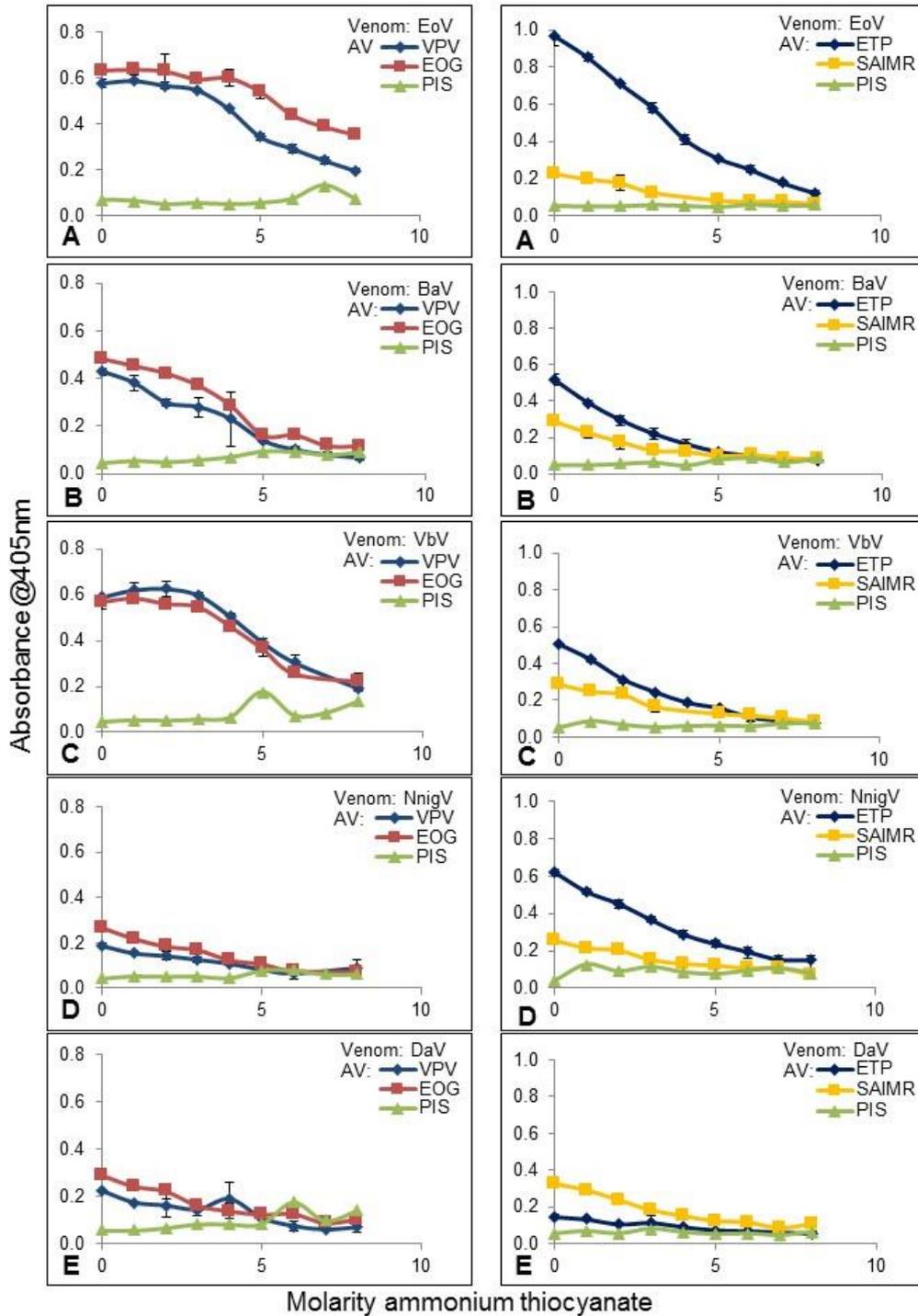


Figure 3.4-5: Disruption of V/AV binding by ammonium thiocyanate – ViperaVet (VPV, EchiTabG (EOG) (left), EchiTabPlus (ETP) and SAIMR polyvalent antivenom (right) to *E. ocellatus* venom (A), *B. arietans* venom (B), *V. berus* venom (C), *N. nigricollis* venom (D) and *D. angusticeps* venom (E)

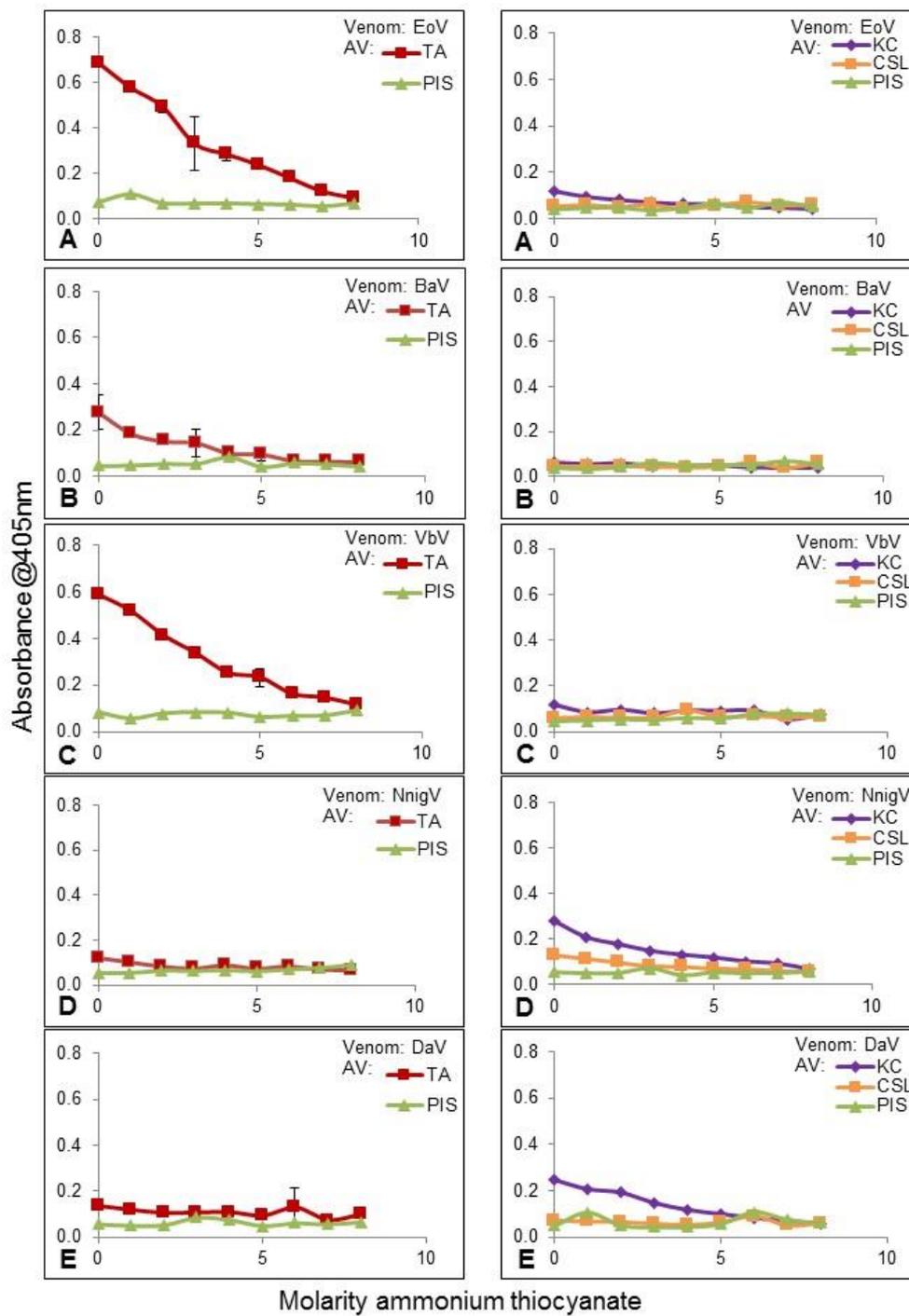


Figure 3.4-6: Disruption of V/AV binding by ammonium thiocyanate - *Trimeresurus albolabris* antivenom (left), King Cobra and CSL Australian polyvalent antivenoms (right) to EoV (A), *B. arietans* venom (B), *V. berus* venom (C), *N. nigricollis* venom (D) and *D. angusticeps* venom (E) by ELISA

3.4.4 Small scale affinity chromatography

Small scale affinity chromatography (SSAC) is used to measure binding of different antivenoms to venom, or combination of venoms, which have been covalently bound to the matrix of a chromatography column; the assay measures the venom-specific antibody (VSAb) content of a sample, regardless of donor species or antibody fragment. The results are summarised in Table 3.4-3; showing that non-specific binding was greater for normal sheep serum (SPIS) than for normal horse serum (HPIS). As for ELISA, results show binding of venom to their homologous antivenom to be greater than for heterologous antivenoms. Binding of antivenom was considered relevant if the VSAb content exceeded that of normal serum from the same donor species. Again, the highest binding occurred between EchiTabG and *E. ocellatus* venom (16.9% VSAb), closely followed by that between ViperaVet and *V. berus* venom (14.6% VSAb). It is interesting that the VSAb content was always less than 20% of total protein, highlighting that more than 80% of the protein in antivenom is ineffective at binding to, or neutralising venom components.

***Echis ocellatus* venom**

EchiTabG had the greatest concentration of VSABs (17% of total IgG) to *E. ocellatus* venom, with EchiTabPlus containing 10% VSABs. Similarly, SAIMRp contained a higher concentration of VSABs to *E. ocellatus* venom than the normal sera, but ViperaVet, *Trimeresurus albolabris*, Banded Krait and CSL antivenoms contained VSABs equivalent to normal sera and therefore did not exhibit significant crossreactivity to *E. ocellatus* venom.

Table 3.4-3: Specific antibody concentrations of a selection of antivenoms determined by SSAC. Homologous antivenoms shown in red.

Venom/ Antivenom	EoV		BaV		VbV		NnigV		DaV	
	VSAb (g/L)	%SAb								
VPV	1.31	5.2	1.18	4.72	3.64	14.6	0.27	1.08	0.36	1.44
EOG	4.23	16.9	1.11	4.44	1.97	7.9	0.87	3.48	0.59	2.36
ETP	2.48	9.9	1.60	6.4	1.54	6.2	2.05	8.2	0.47	1.9
SAIMRp	1.27	5.1	1.59	6.36	1.86	7.4	1.77	7.12	3.00	12.00
TA	0.65	2.6	0.50	2.34	1.00	4.0	0.19	0.89	0.35	1.64
BK	0.66	2.6	1.08	4.32	0.86	3.4	0.78	3.12	0.49	1.96
CSL	0.56	2.2	0.76	3.04	0.71	2.8	1.04	4.16	0.78	3.12
SPIS	1.52	N/A	0.70	N/A	1.54	N/A	1.02	N/A	0.48	N/A
HPIS	0.53	N/A	0.50	N/A	0.81	N/A	0.71	N/A	0.30	N/A

***Bitis arietans* venom**

EchiTabPlus and SAIMRp had the highest concentration of VSABs to *B. arietans* venom, with ViperaVet, EchiTabG and, surprisingly, Banded Krait antivenoms showed levels of specificity higher than normal sera. *Trimeresurus albolabris* antivenom and CSL did not show significant crossreactivity to this venom.

***Vipera berus* venom**

The *V. berus* venom column bound to VSAB in ViperaVet, EchiTabG, SAIMRp and EchiTabPlus at an appreciably higher concentration than the normal sera. *Trimeresurus albolabris* antivenom bound to *V. berus* venom at a VSAB concentration higher than that of non-specific binding, suggesting some crossreactivity, but the two elapid antivenoms (Banded Krait and CSL Australian polyvalent) had VSAB concentrations equivalent to the non-specific binding by HPIS.

***Naja nigricollis* venom**

EchiTabPlus and SAIMRp were the only antivenoms containing significant VSABs to *N. nigricollis* venom, with a slightly higher level of these VSABs in CSL, and EchiTabG antivenoms than in normal sera. There was no crossreactivity of *N. nigricollis* venom with ViperaVet, *Trimeresurus albolabris* or Banded Krait antivenoms.

***Dendroaspis angusticeps* venom**

SAIMRp contained the highest concentration of VSABs directed against *D. angusticeps* venom. Both normal sheep and horse serums had low non-specific binding in this column. CSL antivenom had a VSAB concentration at least twice that of normal sera; EchiTabG contained VSABs slightly higher than that of the normal sera and ViperaVet, whilst *Trimeresurus albolabris* and Banded Krait antivenoms contained VSABs equivalent to those of the normal sera and therefore showed no significant crossreactivity with this venom.

3.4.4.1 Immunocytotoxicity (ICT): EC₅₀

The median cytotoxicity of venoms is shown in Table 3.4-4 and neutralisation of their cytopathic effect (CPE) by different antivenoms in Table 3.4-5. It was observed that the CPE of venoms (Table 3.4-5) was neutralised by their homologous antivenoms.

Table 3.4-4: Median cytotoxicity (LC₅₀) of five snake venoms

Venom	LC ₅₀ (ng/well)
<i>V.berus</i> venom	100
<i>E. ocellatus</i> venom	80
<i>B. arietans</i> venom	200
<i>N. nigricollis</i> venom	400
<i>D. angusticeps</i> venom	50

***Echis ocellatus* venom**

The best neutralisation of the CPE of *E. ocellatus* venom was by its homologous monovalent antivenom, EchiTabG followed by its homologous polyvalent antivenom, EchiTabPlus. ViperaVet and SAIMRp antivenoms afforded some neutralisation, as did *Trimeresurus albolabris* antivenom, but less effectively. There was no effective neutralisation by either of the elapid antivenoms (King Cobra or CSL). The results are illustrated in Figure 3.4-7A and Figure 3.4-8A.

***Bitis arietans* venom**

The CPE of *B. arietans* venom was effectively neutralised by its homologous antivenoms SAIMRp and EchiTabPlus, the latter being the least potent (Table 3.4-5). EchiTabG, a heterologous antivenom, appeared to be remarkably effective at reducing its CPE with *Trimeresurus albolabris* antivenom being nearly as effective as SAIMRp. Once again, the two elapid antivenoms were ineffective at neutralising this venom's CPE. Figure 3.4-7B and Figure 3.4-8B.

***Vipera berus* venom**

The CPE of *V. berus* venom was best neutralised by its homologous antivenom, ViperaVet, but was also neutralised by heterologous antivenoms directed against African vipers (EchiTabG, EchiTabPlus and SAIMRp).

However, it was not neutralised by *Trimeresurus albolabris*, Banded Krait or CSL antivenoms directed against elapid venoms. Figure 3.4-7C and Figure 3.4-8C.

Table 3.4-5: Antivenom neutralisation of venom cytotoxicity by a selection of homologous and heterologous antivenoms (Homologous V/AV in red) showing EC₅₀ concentration (mg/L) and AV potency (median mg V neutralised by 1mg AV) NE = not effective, ND = not done.

Venom/ Antivenom	EoV (400ng/well)		BaV (1000ng/well)		VbV (500ng/well)		NnigV (2000ng/well)		DaV (200ng/well)	
	EC ₅₀	V/AV	EC ₅₀	V/AV	EC ₅₀	V/AV	EC ₅₀	V/AV	EC ₅₀	V/AV
EC ₅₀ (mg/L)/ Potency (V/AV)										
VPV	1250	6.4	NE	NE	109	92	NE	NE	NE	NE
EOG	278	28.8	147	136.0	166	60	NE	NE	NE	NE
ETP	1190	6.7	1389	14.4	357	28	2083	19.2	NE	NE
TA	10250	0.7	410	41.7	NE	NE	NE	NE	NE	NE
SAIMRp	2083	3.8	208	96.0	208	48	625	64.0	278	14.4
CSL	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE
BK	ND	ND	ND	ND	NE	NE	ND	ND	ND	ND
KC	NE	NE	NE	NE	ND	ND	NE	NE	NE	NE

***Naja nigricollis* venom**

The CPE of *N. nigricollis* venom was neutralised by its homologous antivenom, EchiTabPlus and, less effectively, by SAIMRp antivenom, showing crossreactivity with the snake venoms from the same genus, against which is directed. There was no neutralisation by any of the viper (*ViperaVet*, EchiTabG, *Trimeresurus albolabris*) or unrelated elapid antivenoms, King Cobra or CSL (Figure 3.4-7D and Figure 3.4-8D).

***Dendroaspis angusticeps* venom**

D. angusticeps venom was neutralised by its homologous antivenom, SAIMRp. It was not neutralised effectively by any of the other antivenoms. Results are illustrated in Figure 3.4-7E and Figure 3.4-8E.

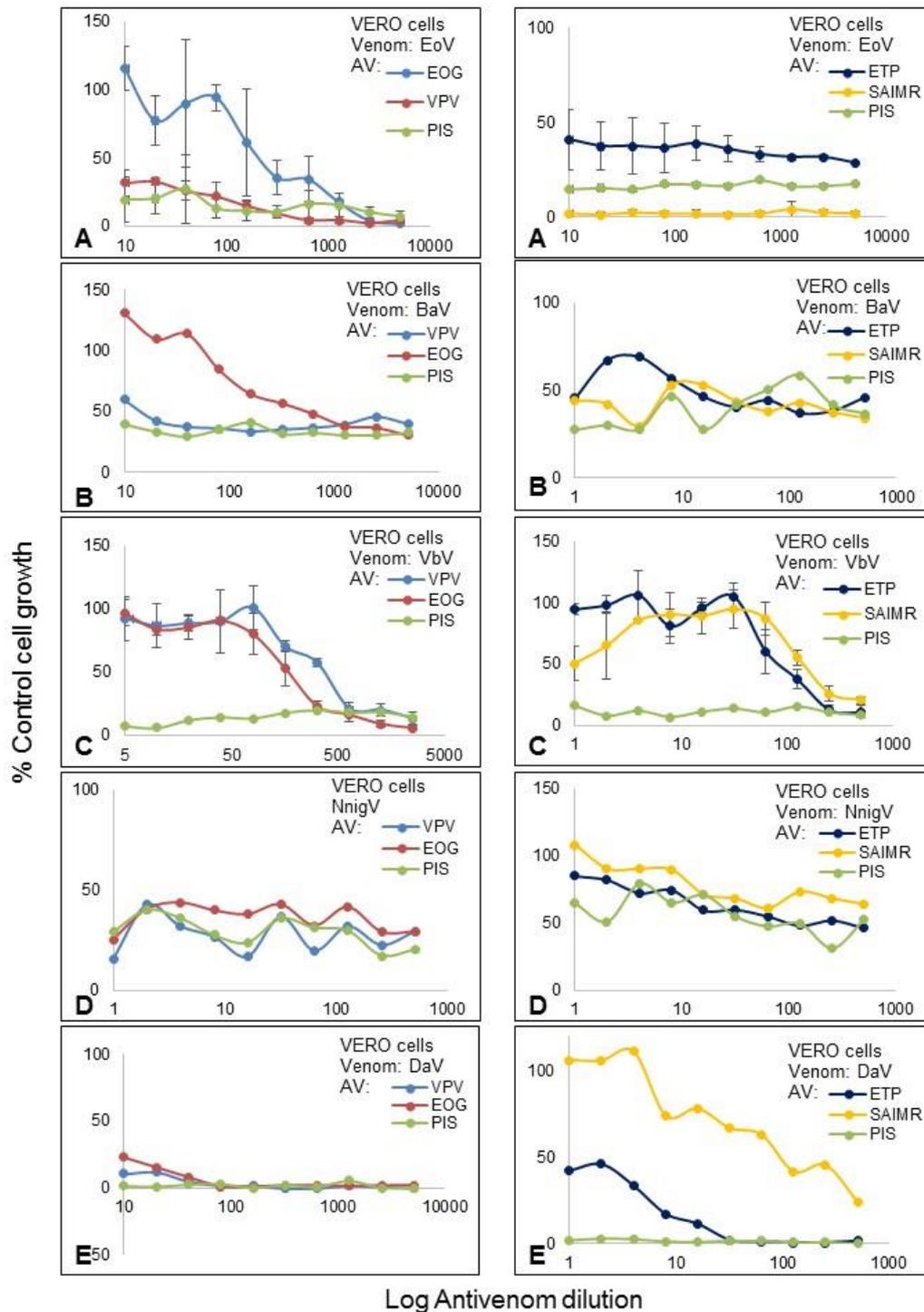


Figure 3.4-7: Neutralisation of CPE of *E. ocellatus* venom (A), *B. arietans* venom (B), *V. berus* venom (C), *N. nigricollis* venom (D) and *D. angusticeps* venom (E) by ViperaVet, EchiTabG (left), EchiTabPlus and SAIMRp (right).

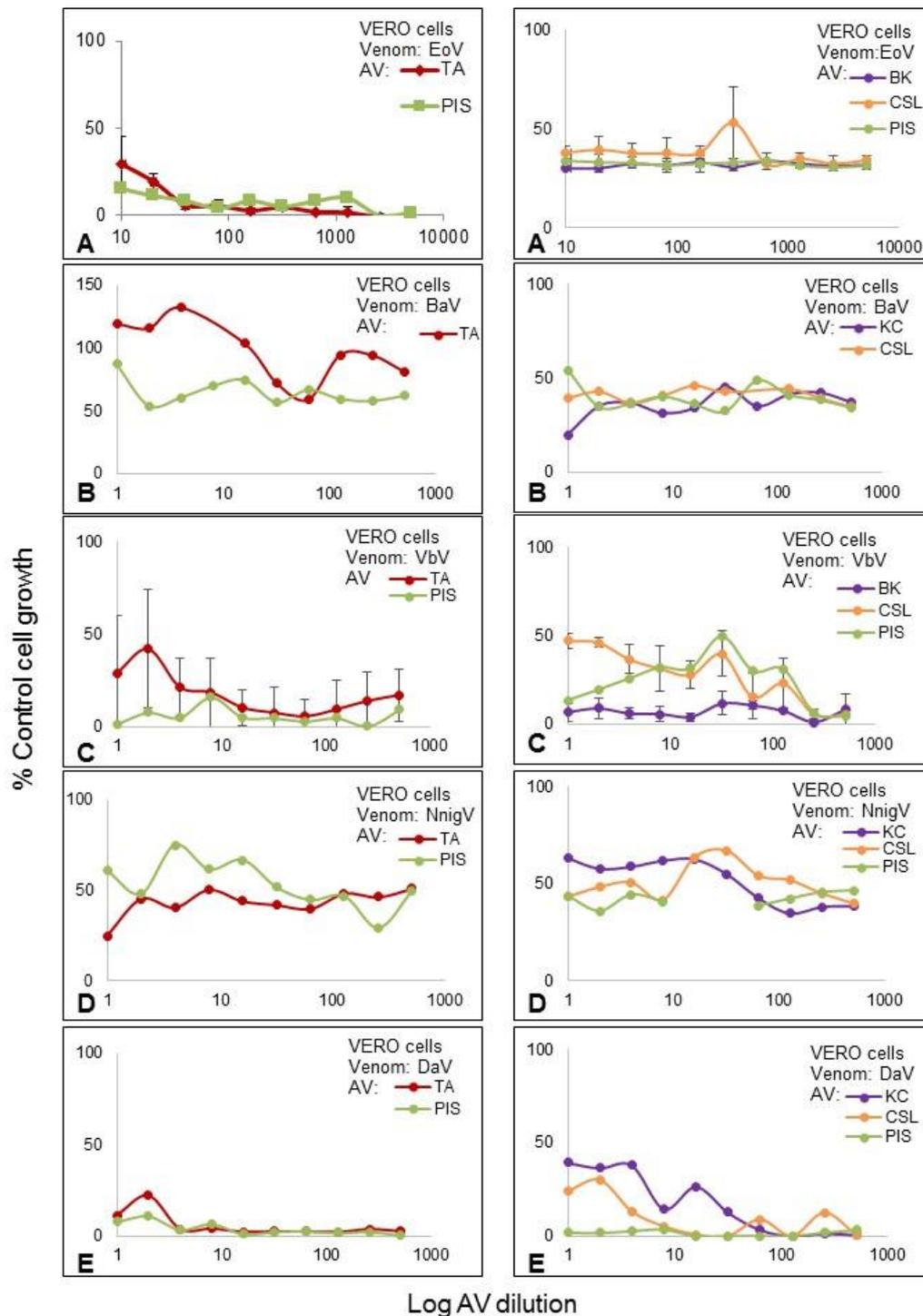


Figure 3.4-8: : Neutralisation of CPE of *E. ocellatus* venom (A), *B. arietans* venom (B), *V. berus* venom (C), *N. nigricollis* venom (D) and *D. angusticeps* venom (E) by *Trimeresurus albolabris* antivenom (left), King cobra and CSL antivenoms (right).

3.4.4.2 Relationship between the results of all *in vitro* assays

The relationships between all the *in vitro* results are described for each venom below. Correlation is expressed as R^2 , a rank correlation coefficient.

***Echis ocellatus* venom**

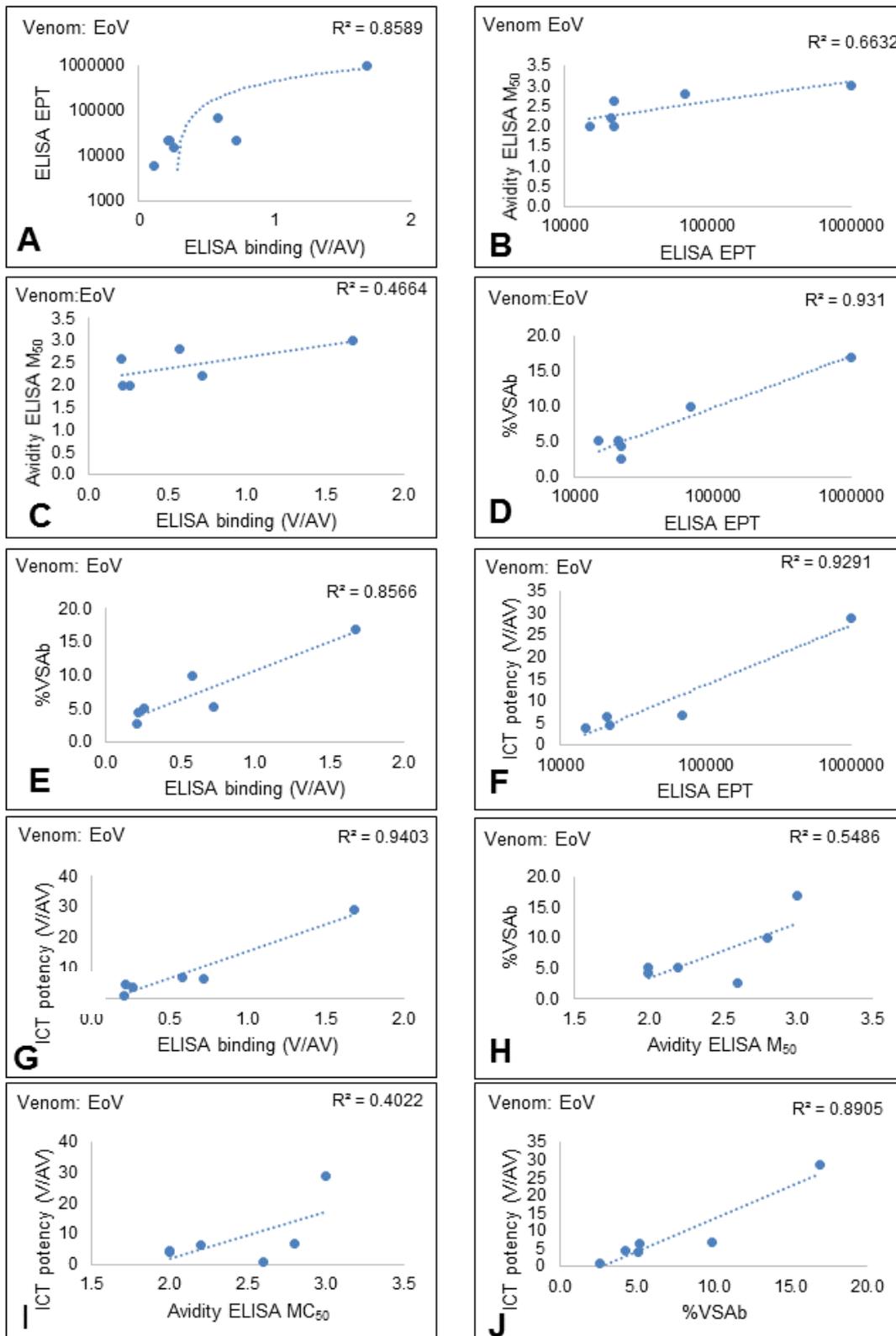


Figure 3.4-9: Linear correlation of results (R^2) between assays - *E. ocellatus* venom

The correlation (R^2) between assays for *E. ocellatus* venom is summarised in Table 3.4-6. There was a strong correlation between ELISA endpoint titre and binding with %VSAb, between ELISA endpoint titre and binding with ICT potency and between %VSAb with ICT potency. However, there was a poorer correlation between Avidity ELISA M_{50} with the results of the other assays.

Table 3.4-6: Linear correlation of results (R^2) between assays - *E. ocellatus* venom

<i>Echis ocellatus</i>	ELISA binding V/AV	ELISA EPT	Avidity M_{50}	%VSAb	ICT potency V/AV
ELISA binding V/AV	-	0.860	0.454	0.857	0.940
ELISA EPT	0.860	-	0.663	0.931	0.929
Avidity M_{50}	0.454	0.663	-	0.549	0.402
%VSAb	0.857	0.931	0.549	-	0.891
ICT potency V/AV	0.940	0.929	0.402	0.891	-

***Bitis arietans* venom**

The correlations (R^2) between assays for *B. arietans* venom are summarised in Table 3.4-7. There was a smaller correlation between assay results for this venom than for *E. ocellatus* venom. There appeared to be a particularly poor correlation between ICT potency and %VSAb (Figure 3.4-10J). The correlations between both ELISA binding and ICT potency (Figure 3.4-10D) and between ELISA binding and avidity M_{50} (Figure 3.4-10B) were also poor. The highest correlation was between ICT potency and ELISA end-point titre (Figure 3.4-10E) or Avidity ELISA M_{50} (Figure 3.4-10 I).

Table 3.4-7: Linear correlation of results (R^2) between assays - *B. arietans* venom

<i>Bitis arietans</i>	ELISA binding V/AV	ELISA EPT	Avidity M_{50}	%VSAb	ICT potency V/AV
ELISA binding V/AV	-	0.366	0.142	0.536	0.102
ELISA EPT	0.366	-	0.605	0.273	0.719
Avidity M_{50}	0.142	0.605	-	0.357	0.705
%VSAb	0.536	0.273	0.357	-	0.150
ICT potency V/AV	0.102	0.719	0.705	0.150	-

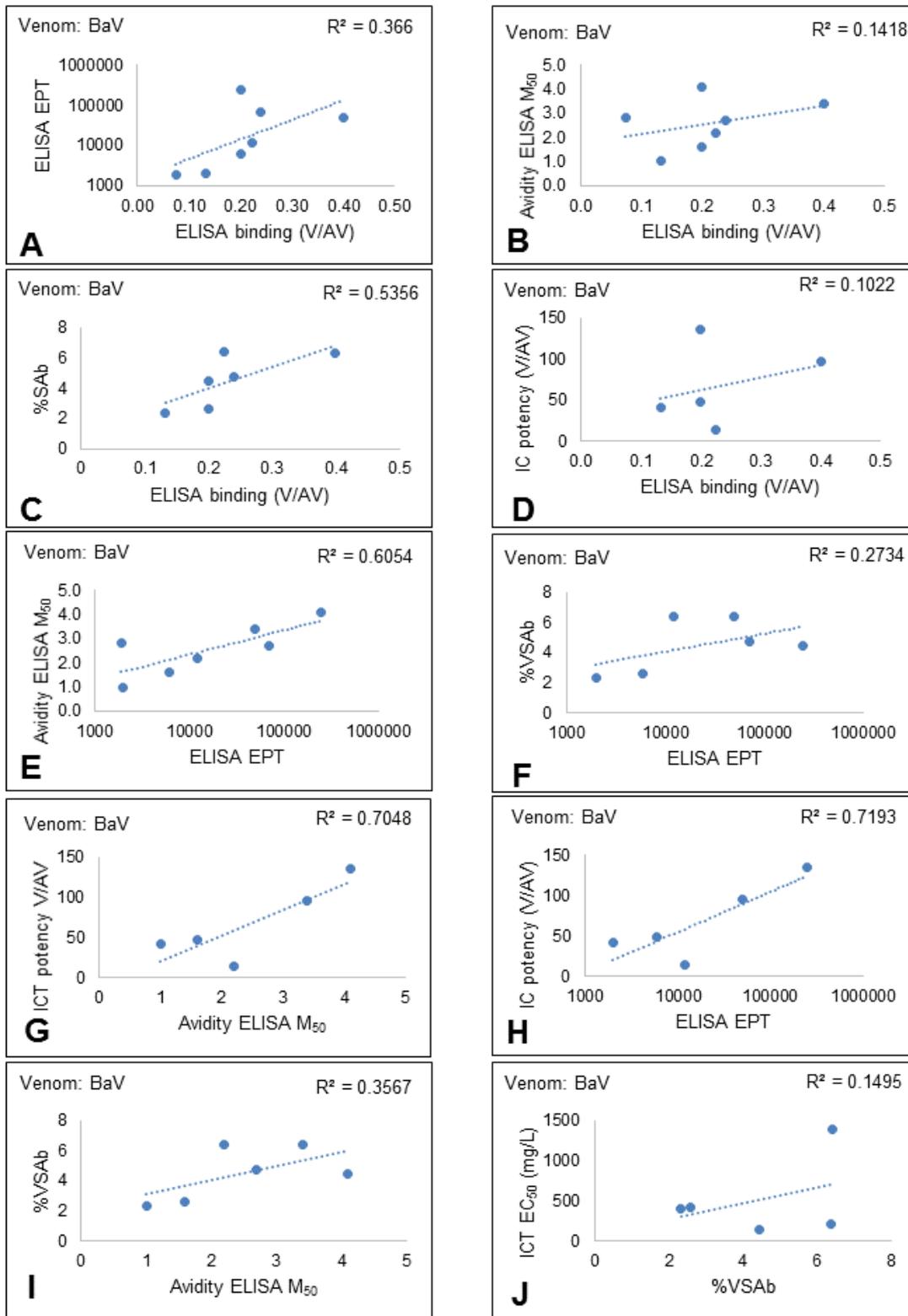


Figure 3.4-10: Linear correlation of results (R^2) between assays - *B. arietans* venom

Vipera berus venom

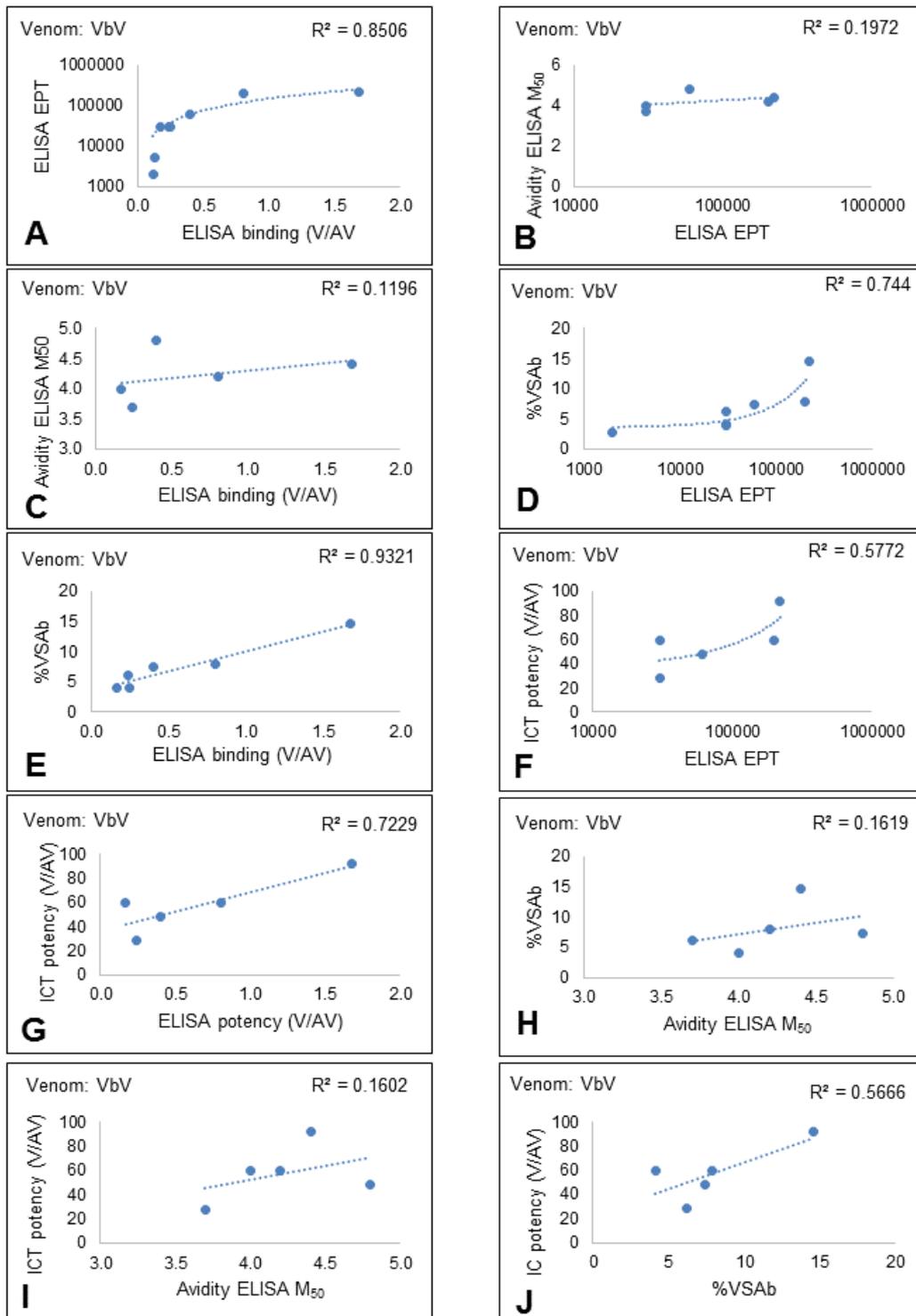


Figure 3.4-11: Linear correlation of results (R^2) between assays – *V. berus* venom

The correlation (R^2) between assays for *V. berus* venom are summarised in Table 3.4-8. There was no correlation between avidity M₅₀ and the other assay results, mainly because the M₅₀ results were similar for all the test antivenoms.

Table 3.4-8: Linear correlation of results (R²) between assays - VbV

<i>Vipera berus</i>	ELISA binding V/AV	ELISA EPT	Avidity M ₅₀	%VSAb	ICT potency V/AV
ELISA binding V/AV	-	0.851	0.120	0.932	0.723
ELISA EPT	0.851	-	0.095	0.744	0.577
Avidity M ₅₀	0.120	0.095	-	0.162	0.160
%VSAb	0.932	0.744	0.162	-	0.567
ICT potency V/AV	0.723	0.577	0.160	0.567	-

***Naja nigricollis* venom**

The best correlation (R²) of results for *N. nigricollis* venom was between %VSAb and ELISA end-point titre. There were also moderately good correlations between ELISA binding and Avidity M₅₀, between %VSAb and Avidity M₅₀ and between ELISA end-point titre and ICT potency. There was a poor correlation between ELISA binding and end-point titre, and also between ICT potency and ELISA binding (Table 3.4-9).

Table 3.4-9: Linear correlation of results (R²) between assays - *N. nigricollis* venom

<i>Naja nigricollis</i>	ELISA binding V/AV	ELISA EPT	Avidity M ₅₀	%VSAb	ICT potency V/AV
ELISA binding V/AV	-	0.177	0.798	0.578	0.111
ELISA EPT	0.177	-	0.528	0.867	0.677
Avidity M ₅₀	0.798	0.528	-	0.677	0.342
%VSAb	0.578	0.867	0.677	-	0.283
ICT potency V/AV	0.111	0.677	0.342	0.283	-

***Dendroaspis angusticeps* venom**

Only two of the antivenoms successfully neutralised the CPE of *D. angusticeps* venom, providing insufficient data for comparison with other assays. Avidity M₅₀ results correlated well with the other assay results (Figure 3.4-13B, D and F). There was a poor correlation between ELISA 50% binding and end-point titre (Figure 3.4-13A). These results are summarised in Table 3.4-10

Table 3.4-10: Linear correlation of results (R²) between assays - *D. angusticeps* venom

<i>Dendroaspis angusticeps</i>	ELISA binding V/AV	ELISA EPT	Avidity M ₅₀	%VSAb
ELISA binding V/AV	-	0.306	0.994	0.639
ELISA EPT	0.306	-	0.962	0.550
Avidity M ₅₀	0.994	0.962	-	0.895
%VSAb	0.639	0.550	0.895	-

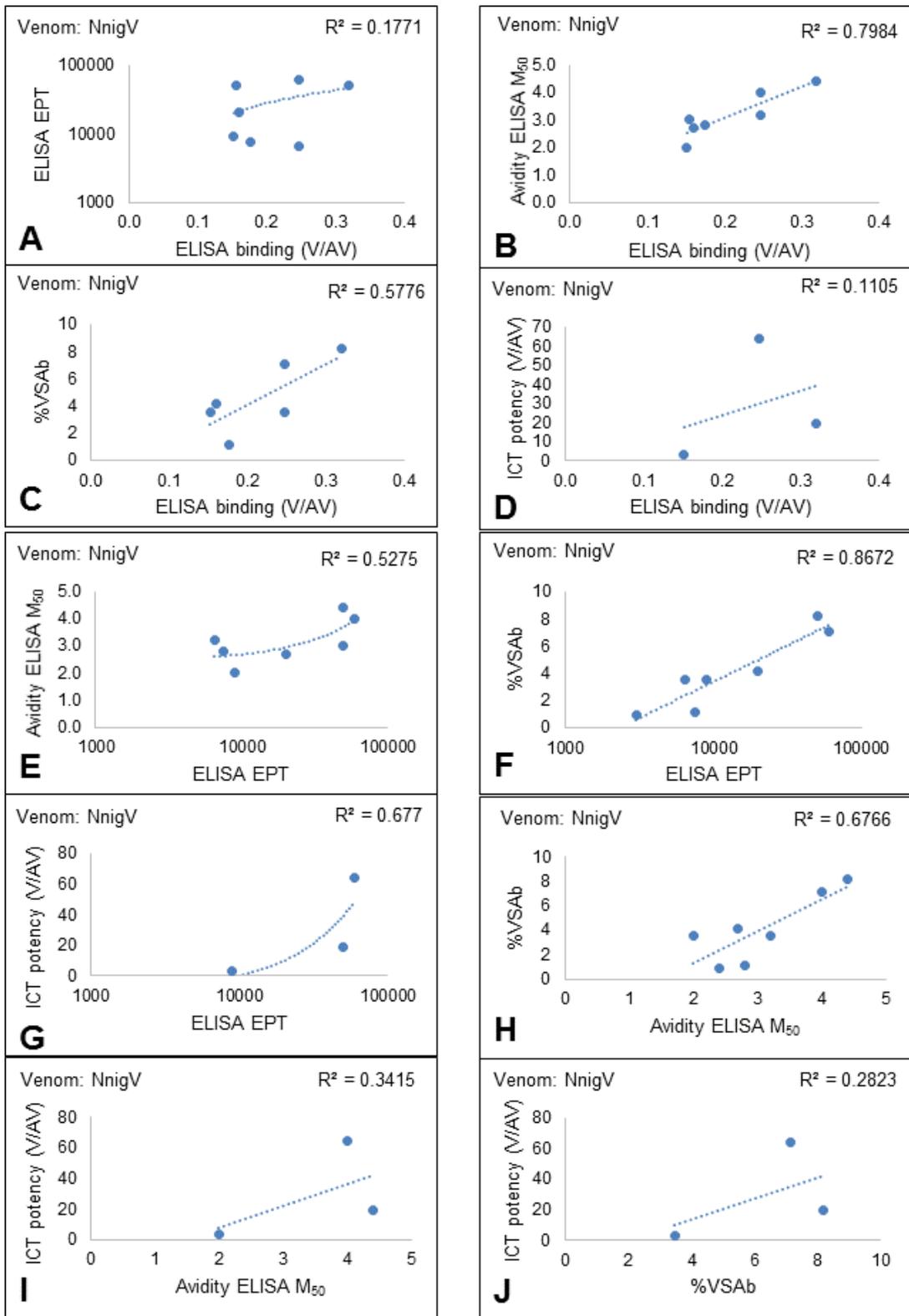


Figure 3.4-12: Linear correlation of results (R^2) between assays - *N. nigricollis* venom

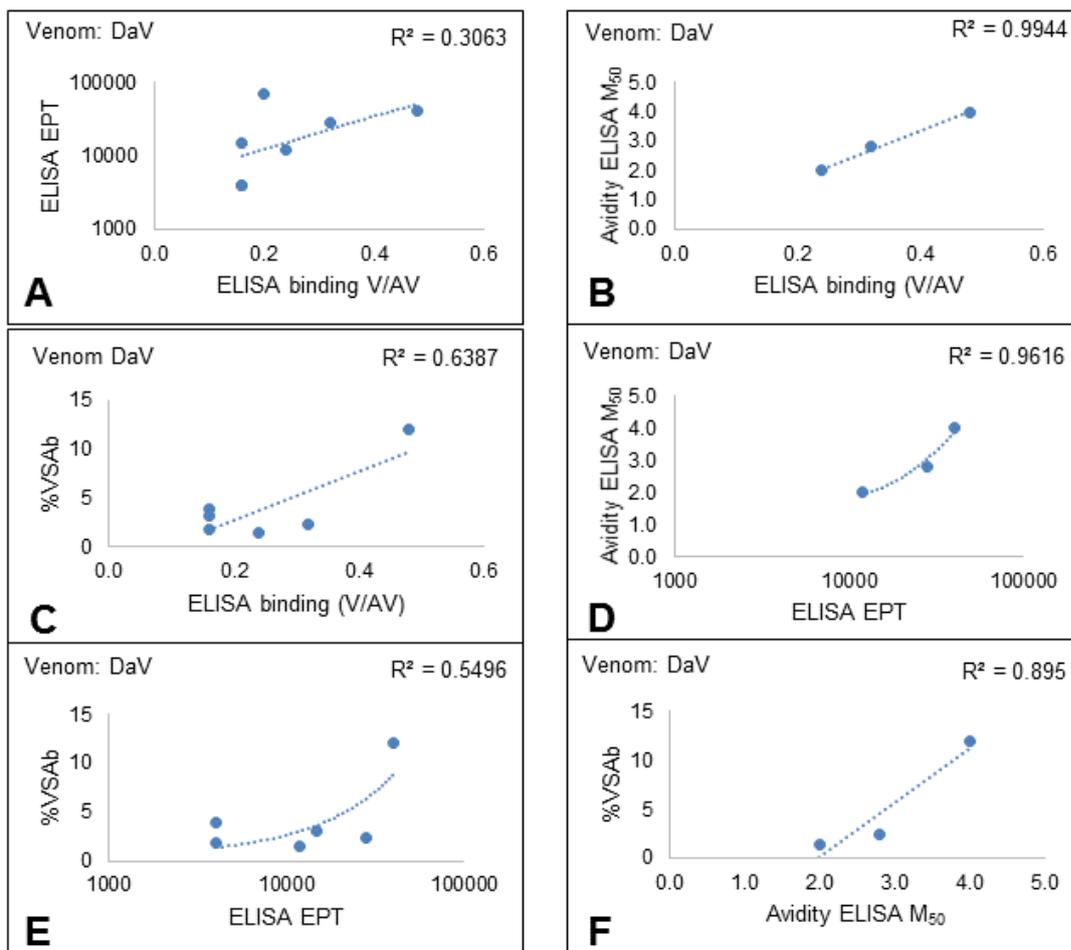


Figure 3.4-13: Linear correlation of results (R^2) between assays – *D. angusticeps* venom

Pooled venoms

When results from all five venoms are pooled, the correlation (R^2) between different assays is poor (Table 3.4-11).

Table 3.4-11: Linear correlation of results (R^2) between assays – pooled venoms

All Venoms	ELISA potency V/AV	Avidity M50	%VSAb	ICT potency V/AV
ELISA potency V/AV	-	0.18	0.33	0.21
Avidity M50	0.18	-	0.29	0.34
%VSAb	0.33	0.29	-	0.39
ICT potency V/AV	0.21	0.34	0.39	-

The results of all the assays performed are summarised in Table 3.4-12.

Table 3.4-12: Summary of *in vitro* assay results. WB = Western blot; ELISA = EC50 potency; V/AV = potency: 50% venom (mg) neutralised by 1mg AV; M = molarity ammonium thiocyanate required to reduce binding by 50%; ICT = immunocytotoxicity EC₅₀ potency; PSS = pathology/survival score. Y = effective, NE = not effective, ND = not done. **Results in red indicate AV binding or neutralisation efficacy.**

AV	Assay	EoV	BaV	VbV	NnigV	DaV
VPV	WB	Y	Y	Y	>25kDa	>25kDa
	ELISA V/AV	0.720 Y	0.240 N	1.680 Y	0.176 N	0.240 N
	Avidity M	2.2 N	2.7 Y	4.4 Y	2.8 N	2.0 N
	SSAC g/L	1.3 N	1.2 Y/N	3.6 Y	0.3 N	0.4 N
	ICT V/AV	6.4	NE	92 Y	NE	NE
EOG	WB	Y	Y	Y	>25kDa	>25kDa
	ELISA V/AV	1.680 Y	0.200 Y	0.800 Y	0.248 N	0.320 N
	Avidity M	3.0 Y	4.1 Y	4.2 Y	3.2 Y/N	2.8 N
	SSAC g/L	4.2 Y	1.1 Y/N	2.0 Y/N	0.9 N	0.6 N
	ICT V/AV	28.8 Y	136.0 Y	60 Y	NE	NE
ETP	WB	Y	Y	Y	Y	>25kDa
	ELISA V/AV	0.560 Y	0.224 Y	0.240 Y	0.320 Y	0.160 Y/N
	Avidity M	2.8 Y	2.2 Y	3.7 Y	4.4 Y	NE
	SSAC g/L	2.5 Y	1.6 Y	1.5 Y	2.1 Y/N	0.5 N
	ICT V/AV	6.7 Y	14.4 Y	28.0 Y	19.2 Y	NE
SAIMRp	WB	Y/N	Y	Y/N	Y	Y
	ELISA V/AV	0.260 Y	0.400 Y	0.400 Y	0.248 Y	0.480 Y
	Avidity M	2.0 N	3.4 Y	4.8 N	4.0 Y/N	4.0 Y/N
	SSAC g/L	1.3 Y/N	1.6 Y	1.9 Y	1.8 Y	3.0 Y
	ICT V/AV	3.8 Y/N	96.0 Y	48.0 Y	64.0 Y/N	2.7 Y
TA	WB	Y	Y	Y/N	N	ND
	ELISA V/AV	0.208 Y	0.133 Y/N	0.250 Y	NE	NE
	Avidity M	2.6 N	1.0 N	NE	2.4 N	NE
	SSAC g/L	0.7 N	0.5 N	1.0 N	0.2 N	0.4 N
	ICT V/AV	0.7 N	41.7 Y	NE	N	N
BK ¹ /KC ²	² WB	2 bands	N	N	Y/N	Y/N
	² ELISA V/AV	0.105 Y/N	0.075 Y/N	0.125 Y/N	0.155 Y	0.200 Y
	² Avidity M	NE	2.8 N	NE	2.7 Y/N	NE
	¹ SSAC g/L	0.7 N	1.1 Y/N	0.9 N	0.8 N	0.5 N
	ICT V/AV	² 0.7 N	² NE	¹ NE	² NE	² NE
CSL	WB	Y/N	Y	2 bands	Y	Y
	ELISA V/AV	NE	NE	0.112 Y/N	0.160 Y	0.160 Y/N
	Avidity M	NE	2.4 N	NE	2.7 Y/N	NE
	SSAC g/L	0.6 N	0.8 N	0.7 N	1.0 Y/N	0.8 Y/N
	ICT V/AV	NE	NE	NE	NE	NE
Sheep PIS	SSAC g/L	1.5	0.7	1.5	1.0	0.4
Horse PIS	SSAC g/L	0.5	0.5	0.8	0.7	0.3

3.5 Discussion

Many studies (Archundia et al, 2011; Bogarin et al, 2000; Casewell et al, 2010; Casewell et al, 2014a; Fernandes et al, 2000; Furtado et al, 2010; Gutierrez et al, 2008; Maduwage et al, 2016; Nalbantsoy et al, 2009; Sanchez et al, 2008; Sanchez et al, 2011; Segura et al, 2012) have been conducted on paraspecificity of antivenoms for closely related snake venom species, usually of the same genus, but none of the studies appear to have looked at the cross-reactivity of such a diverse selection of antivenoms with venoms encompassing two families and five different genera of snake species. The main objective of this study was to examine the ability of each assay at recognising antivenom specificity, and sensitivity in prediction of *in vivo* efficacy. An assay which demonstrates such specificity and sensitivity could be recommended to reduce the need to preclinically test every batch of antivenom manufactured, whose *in vivo* neutralizing efficacy has previously been demonstrated. Ideally the assay should be quick, simple, robust and inexpensive, without the need for costly specialist equipment. To this end immunoblotting, ELISA, avidity ELISA, SSAC and immunocytotoxicity (ICT) were selected. The latter requires specialist facilities, but it is hypothesized that a cell-based assay may give differentiation between binding to, and neutralisation by, antibodies.

Figure 3.5-1 shows the phylogeny of medically important African snakes and demonstrates cross-neutralisation of venoms by existing antivenoms, for example, EchiTab-Plus ICP neutralises its homologous venom, *N. nigricollis* and heterologous venoms *N. mossambica* and *N. pallida*, but not those of *N. katiensis* or *N. nubiae* (Petras et al, 2011). EchiTabG neutralised its homologous *E. ocellatus* venom, and heterologous *E. pyramidum leakeyi* and *E. coloratus* venoms, but not Asian *E. carinatus* venom (Casewell et al, 2010). The failure of cross-neutralisation between *E. ocellatus* and *E. carinatus* is also described by others (Mebs et al, 1981; Visser et al, 2008). Similarly, antivenom directed against *D. polylepis*, *D. viridis*, *B. arietans* and *B. gabonica* failed to cross-neutralise *D. angusticeps* or *B. nasicornis* venoms (Ramos-Cerillo, 2008).

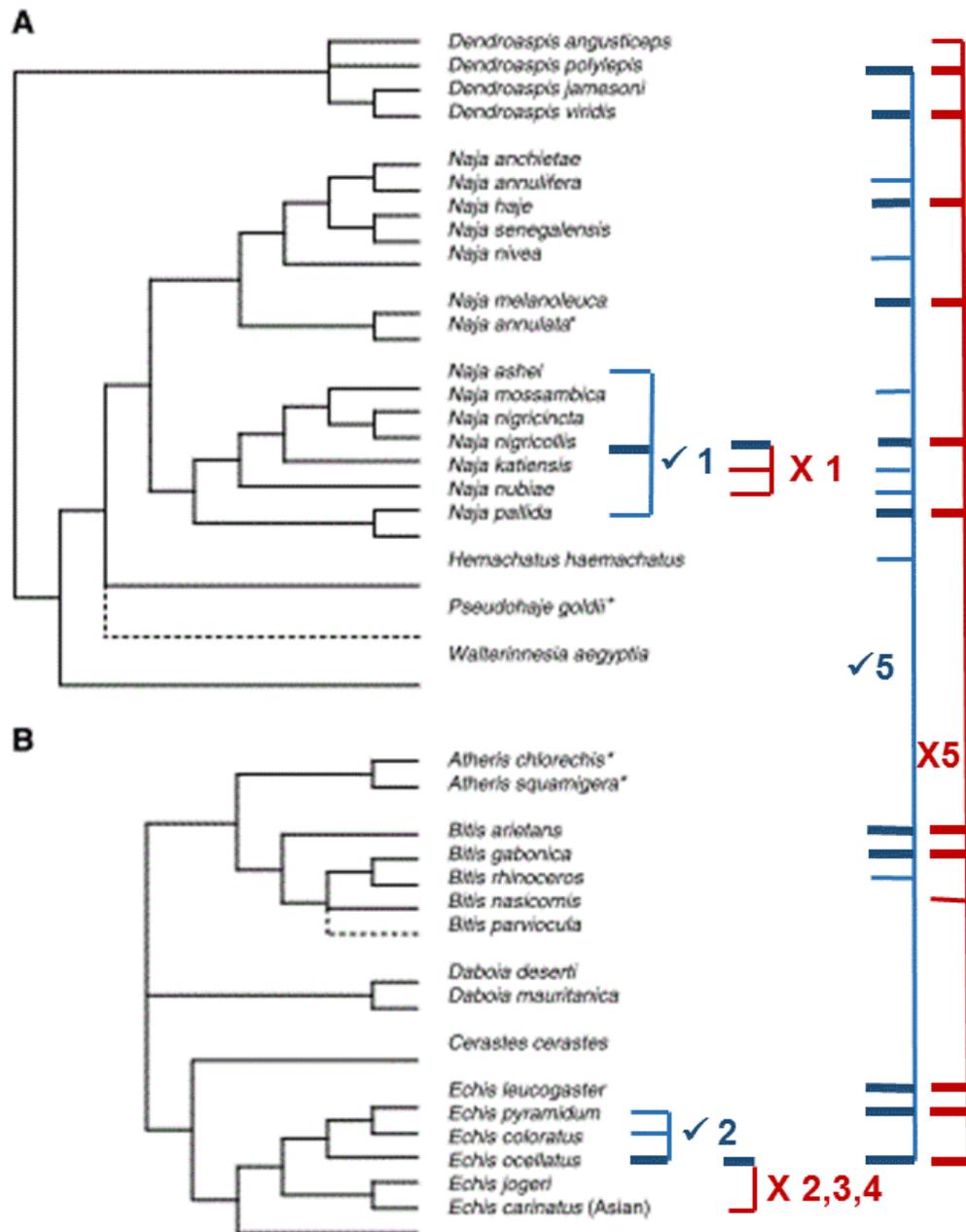


Figure 3.5-1: Phylogeny of (A) medically important African elapids and (B) viperids and reported antivenom cross-neutralisation outcomes. *Bitis parviocula* and *Pseudohaje goldii* have not been included in any comprehensive phylogenetic analyses, and their placement should be regarded as tentative. Brackets on right of tree indicate cross-neutralisation. Bold lines indicate venoms used to raise antivenom, thin lines paraspecific activity, blue with tick mark indicates effective cross-neutralisation, and red with cross mark indicates lack of cross-neutralisation (Williams et al, 2011). 1 (Petras et al, 2011); 2 (Casewell et al, 2010); 3 (Mebis et al, 1981); 4 (Visser et al, 2008); 5 (Ramos-Cerillo et al, 2008)

Immunoblotting demonstrated that heterologous venoms directed against viper venoms bound to components of other viper venoms, but that they also bound to higher MW proteins of the elapid venoms. Generally, the low MW bands (e.g. 3FTxs, PLA₂s, vasoactive peptides) were not bound by heterologous antivenoms, and sometimes poorly by their homologous antivenom. This has been demonstrated by other researchers (Boldini-Franca et al, 2010; Fahmi et al, 2012; Gutierrez et al, 2014; Gutierrez et al, 2013) using immunoblotting and/or antivenomic approaches. Immunoblotting and antivenomics can identify which venom components are not bound and, as information is accrued on the pathological importance of these components, ineffective antivenoms can be rejected prior to *in vivo* testing.

The ELISA results showed cross-reactivity of viper antivenoms to both heterologous viper venoms and elapid antivenoms for heterologous elapid venoms. *B. arietans* venom showed least binding to the test antivenoms, even to its homologous antivenoms (EchiTabPlus and SAIMRp). *E. ocellatus* venom was bound by nearly all the antivenoms, including submaximally by King Cobra antivenom, but not at all to CSL antivenom. Although many of the Australian snake venoms are coagulopathic as well as neurotoxic, the coagulopathic toxins are serine proteases, which are structurally different from the coagulopathic SVMPs found in *E. ocellatus* venom, and therefore induce the production of different Ab populations. EchiTabG antivenom bound well to the two heterologous viper venoms, despite their apparently different pathological profile of envenoming. Antivenoms which did not bind well to venoms produced results which generated incomplete dilution curves, making calculation of 50% binding erroneous; in this case, the ability to bind was better represented by calculating end-point titre. However, these results are an indication that binding between these venom/antivenom combinations is inadequate to afford effective neutralisation of venom toxicity.

Avidity ELISA results are more specific when efficacy is defined as 50% absorption being greater than the lower detectable limit. Very few antivenoms, including ViperaVet and EchiTabG for *B. arietans*, EchiTabG and EchiTabPlus

for *V. berus* venom, but not *Trimeresurus albolabris*, King cobra or CSL antivenoms, showed cross-reactivity for heterologous venoms, showing the assay is more specific for venom species.

Results of the avidity ELISA was expressed as the M_{50} (molarity of ammonium thiocyanate which resulted in 50% disruption of binding). These results did not appear to correlate with the end-point titre ELISA results. Casewell (et al, 2010), describe the results of the avidity ELISA as percentage reduction - the absorbance of 8M ammonium thiocyanate expressed as the percentage of control (0M) absorbance. Figure 3.4-4 shows the results from section 3.4.3 expressed in this way. Results where venom/antivenom binding was considered 'not effective' were excluded.

Despite being a simple, robust and reproducible method, SSAC has not been widely used to measure VSAb concentration of antivenoms. Of all the publications listed, only one (Casewell et al, 2010) used SSAC as a means of comparing antivenom efficacy. However, the reverse, using antivenom as the solid phase, is becoming more widely used as a means of identifying which venom components are bound, and is called 'antivenomics' (Boldini-Franca, 2010; Calvete, 2016; Fahmi, 2012; Gutierrez et al, 2008, Gutierrez et al, 2013a, Gutierrez et al, 2013b, Gutierrez et al, 2014; Jorge et al, 2015; Kurtovik et al, 2014; Petras et al, 2011; Sanchez et al, 2015). The SSAC assay identified homologous antivenoms, with the highest antibody titre, and those heterologous antivenoms directed against venoms of the same genera. VSAb concentrations of antivenoms showing weak cross-reactivity were only slightly higher than those of the normal sera (non-specific binding). A study performed, using aliquots of the same reference sample of *Vipera* antisera, showed a coefficient of variation of 3.5% over 27 runs (data not shown). This adds weight to the argument for using this assay to monitor antivenom quality over time.

The ICT assay is discussed in more detail in Chapter 8. However, in short, *B. arietans* venom and *N. nigricollis* venom had a poor CPE on the selected VERO cell line, which led to incomplete cell death, even at venom

concentrations equivalent to $5 \times LC_{50}$ (The dose of venom resulting in the death of 50% of cells compared to untreated controls). The variable response of cells to venom made this assay difficult to standardize and therefore unsuitable as a QC test.

There was a poor correlation between assays when results from all venoms were pooled, but there was however a correlation between assays when the results from venoms were considered individually. This may be because by analysing results together the power of single venom analyses is lost and reflects the likelihood that one method is unlikely to be useful 'across the board' due to inherent differences in venom composition and toxin modes of action.

Effectiveness, defined as having a 50% reduction in measurable effect greater than the lowest detectable limit, or non-specific binding for VSAb concentration by SSAC, had the highest venom specificity by SSAC and the highest sensitivity by immunoblotting and ELISA.

Antivenomics is increasingly used to identify deficiencies in antivenom efficacy, revealing poor affinity for low molecular weight venom components such as 3FTxs, PLA₂s, disintegrins, CRISPS, vasoactive peptides, serine proteases and P-I SVMPs (Gutierrez et al, 2014). This technique shows only 'binding' of antivenom to venom proteins and therefore must be used in conjunction with 'neutralisation' assays of venom specific activities, such as lethality, defibrinogenation, coagulation, haemorrhage, neurotoxicity and necrosis, to demonstrate antivenom efficacy (Calvete et al, 2016). The quantitative binding assays such as end-point and avidity ELISAs and SSAC measure total binding and may miss failure of antibodies to bind to key venom components, such as those listed above. Binding may also be affected by binding of venom toxins to the solid phase (ELISA plate or Sepharose gel) and the resulting epitopes presented to the antibody added. Similarly, cell based assays may demonstrate neutralisation of venom cytotoxicity, but this may not equate to neutralisation of lethal *in vivo* effects. These factors may explain the

poor correlation of immunological assays when results from all venoms were pooled, and highlights that one method is unlikely to 'fit all'. It would be advisable to use several different assays, which have been tailored to the toxin profile of the venoms against which the antivenom is directed (WHO, 'Guidelines for the production, control and regulation of snake antivenom immunoglobulins' update in progress).

3.5.1 Conclusions

In their present form, none of the assays described here, can replace the *in vivo* lethality assays. Immunoblotting, ELISAs (both end-point and avidity) and SSAC measure binding of antivenom to venom, but this is unlikely to be synonymous with neutralisation (Casewell et al, 2010; Cook et al, 2010c). Cytotoxicity assays may demonstrate neutralisation of toxins which cause a cytopathic effect, but, as discussed more fully in Chapter 8, do not demonstrate neutralisation of intravascular or extracellular venom effects such as disruption of the clotting cascade, defibrinogenation or inflammatory response with the release of inflammatory mediators such as histamine. The main weakness in this study is the lack of *in vivo* comparators because it would be unethical to do so.

The results presented in this chapter highlight the complexity of venom activity and its neutralisation. No one *in vitro* assay can assess the toxicity of all venoms and predict *in vivo* efficacy of antivenom. The selection of assays to test antivenom efficacy for regulatory purposes need to be carefully selected, based on the principal venom toxin characteristics identified.

Chapter 4. Reduction: Correlation of *in vitro* and *in vivo* preclinical assays using variant venom-specific IgG samples

4.1 Introduction

The previous chapter investigated the immune-binding relationship of 35 venom/antivenom combinations. It was considered unethical to run this matrix of *in vivo* experiments (350 mice needed). Instead, in this section, the *in vivo* / *in vitro* assay assessment was performed using sheep antisera with high, medium or low concentrations of antivenom IgG, but all immunised with the same venom mixture. This study was made possible by the unique access to a flock of 25 sheep immunised for antivenom production, from which individual samples had been collected over a period of more than 2 years.

This is believed to be the first study to utilise multiple antiserum samples containing different VSAb concentrations to investigate the correlation between the numerous assays, both *in vitro* and *in vivo*, employed to test antivenom efficacy.

Assays performed on the pre- and post-purified IgG are shown in Table 4.1-1.

Table 4.1-1: Assays performed pre- and post-purification on *Vipera* antiserum samples containing different VSAb concentrations

Assay	Pre-purification	Purified IgG
FPLC (Fast-phase liquid chromatography)	-	+
Western Blot	-	+
SSAC (Small scale affinity chromatography)	+	+
ELISA (Enzyme-linked immunosorbent assay)	+	+
Avidity ELISA	-	+
Immunocytotoxicity EC ₅₀	+	+
ED ₅₀ (<i>in vivo</i> median effective dose)	-	+

4.2 Objective

The objective of this set of experiments was to demonstrate that *in vitro* experiments can predict the *in vivo* efficacy of antivenom, and that both can differentiate between samples of different quality. Given that the majority of *in*

in vitro tests show only binding of antibodies to venom components, and not their ability to neutralise their pathological effects, if neutralisation can be demonstrated the *in vitro* tests might be used to monitor the quality of subsequent batches of AV provided that the following conditions are met:

- the starting material (antiserum) came from the same pool of donors
- no change in immunogen used (e.g. batch of venom)
- consistent VSAb concentration of their pooled antiserum
- robust and fixed manufacturing process.

4.3 Materials and Method

4.3.1 Sheep and antiserum production

A flock of 25 mixed breed female sheep were maintained on pasture during the summer months and indoors, fed commercial concentrates with ad lib hay and water, during the winter. Body condition score (a subjective measure of muscle/fat on the animal's back) was maintained at a score greater than, or equal to, 3 out of a maximum of 5; below this level the sheep were fed individually and/or removed from the flock until health was restored. The sheep were regularly checked by a veterinary surgeon as well as by the stockmen.

The sheep were immunised with a 1:1:1:1 mixture of the four major species of European viper, namely *Vipera ammodytes*, *V. aspis*, *V. berus* and *V. latastei*. Immunisations and blood removal was on a four week cycle, blood being taken two weeks after each immunisation. After bleeding, the red blood cells were removed and serum pooled. The details of production and assessment of antiserum for antivenom production are described in the publication attached as Appendix III of this thesis (Bolton et al, 2014).

Individual samples were obtained from the sheep at weeks 6, 10, 50, 74, 90, 110 and 126 and VSAb concentration measured by small scale affinity chromatography (SSAC). It was from this library of samples that serum for this set of experiments was derived (Table 4.3-1). The samples were divided into those with the lowest VSAb concentration (<4g/L) – α VipL; those with a moderate VSAb concentration (5.7 to 6.3g/L) – α VipM; and those with the highest VSAb concentrations (>7.8g/L) – α VipH.

Table 4.3-1: Antiserum samples selected for study. (Week after primary immunisation, sheep i.d. number (prefixed with CF11xx and VSAb concentration in g/L))

Low VSAb concentration (<4.0g/L)			Med VSAb concentration (5.7-6.3g/L)			High VSAb concentration (> 7.8g/L)			
Week	No	VSAb concentration	Week	No	VSAb concentration	Week	No	VSAb concentration	
6	44	3.46	6	51	6.22	6	50	7.87	
	45	3.53		55	5.97		54	8.14	
	48	3.40		57	5.97		74	46	11.04
	64	2.98		61	6.16			47	7.88
10	44	3.45	10	62	6.14	110	51	10.43	
	48	3.18		68	6.28		52	9.53	
	56	3.02		62	5.84		58	10.39	
	60	3.65	50	49	6.04		46	9.40	
	64	1.82		51	5.91		50	9.07	
	65	2.67		66	6.17		51	12.70	
	67	2.70		74	48		6.08	52	13.68
50	59	3.21	59		6.04	53	8.51		
	60	3.57	62		6.09	58	9.86		
	90	56	3.25	66	5.78	63	11.62		
64		2.92	90	45	6.26	66	7.95		
67		3.40		47	6.48	68	7.82		
110	44	2.89	110	48	5.86	126	52	12.31	
	56	3.02		59	5.98		55	9.55	
	64	3.08	126	54	6.03		58	10.25	
126	57	3.24		66	5.99	59	8.82		
Mean		3.12	Mean		6.06	Mean		9.84	

4.3.2 Immunoglobulin (IgG) purification

Once the samples were pooled, as described above, initial assessments were performed as listed in Table 4.1-1 above and the resulting aliquots frozen prior to purification.

For purification, the three aliquots of *Vipera* antisera were thawed at 4°C for 72 hours, then diluted 1:3 in 0.9% saline and 6% of the volume of the undiluted serum of Caprylic acid was added (Table 4.3-2). It was thoroughly mixed for 30 minutes, on a roller mixer, prior to standing for 1 hour at room temperature. The mixture was then centrifuged at 5000rpm for 60 minutes and the precipitate discarded.

Table 4.3-2: Dilution of antisera and addition of caprylic acid. αVipH = high VSAb, αVipM = medium VSAb, αVipL = Low VSAb.

Aliquot	α-VipH	α-VipM	α-VipL
Volume serum (mL)	54	56	68
Volume 0.9% saline(mL)	108	112	136
Volume Caprylic Acid (mL)	3.25	3.35	3.40

The suspension was removed and filtered through a 0.65µm pre-filter and then a 0.45µm filter. The resulting solutions were concentrated and reformulated into sodium citrate buffered saline (SCS) (APPENDIX II), with pH 6.0, by means of a BioRad mini diafiltration system using three filters with a 30kDa cut-off. The solutions were concentrated down to 50mL and protein concentration measured by OD at 280nm. Each sample was diluted with SCS to produce a solution with a concentration around 25g/L (Table 4.3-3).

Table 4.3-3: Reformulation of final product

Sample	Absorbance @ 280nm (1:100)	Conc (g/L)	Sample volume (mL)	Volume Sodium citrate buffer added (mL)	Final volume (mL)	Final concentration IgG (g/L)
α-VipL	0.3564	23.8	35	0	35	23.8
α-VipM	0.5906	39.4	30	17	47	24.1
α-VipH	0.5291	35.3	40	16	56	23.5

4.3.3 *In vitro* assessment

The following assays were performed to assess antiserum and purified IgG:

- Fast phase liquid chromatography (FPLC)
- ELISA
- Avidity ELISA
- Small scale affinity chromatography (SSAC)
- Immunocytotoxicity (ICT)

The methodology of these assays is described in detail in Chapters 2 and 3.

4.3.4 *In vivo* murine lethality neutralisation assays

Murine lethality assays were performed as described in Section 2.2.2, with some modifications. Purified IgG/AV at a volume equivalent to 1x ViperaVet (see Appendix II, section 2.3.2) ED₅₀ (44µL/mouse) was pre-incubated with 2.5 x *V. berus* venom LD₅₀ (18.2µg/mouse) at 37°C for 30 minutes. Two groups per test sample, comprising of five CD1 male mice weighing 18-22g, were injected, via the tail vein, with 200µL venom/IgG mixture. A commercially purified ovine IgG antivenom, ViperaVet (VPV), was used as the control. Regular behavioural, physiological and neurological observations were made (see Chapter 2A). Humane end-points were employed to euthanase those

mice deemed terminally affected, using CO₂ anaesthesia. Post mortem examinations were carried out on all mice and a pathology, survival and pathology/survival score assigned as described in Chapter 2A. This was performed as a randomized double blind experiment.

4.4 Results

4.4.1 FPLC

To assess the efficacy of caprylic acid to remove non-IgG serum components, mainly albumin, FPLC was performed on the purified IgG pools. The purity of the three IgG samples was similar (all greater than 95%) and not related to VSAb concentration (Figure 4.4-1). Similarly, the percentage of the peak area of the IgM peak –the small peak to the left of the large peak - did not appear to be related to VSAb concentration. The FPLC chromatograms are illustrated in Figure 4.4-1. A summary of the results are shown in Table 4 of the tables section of the supplement for this chapter.

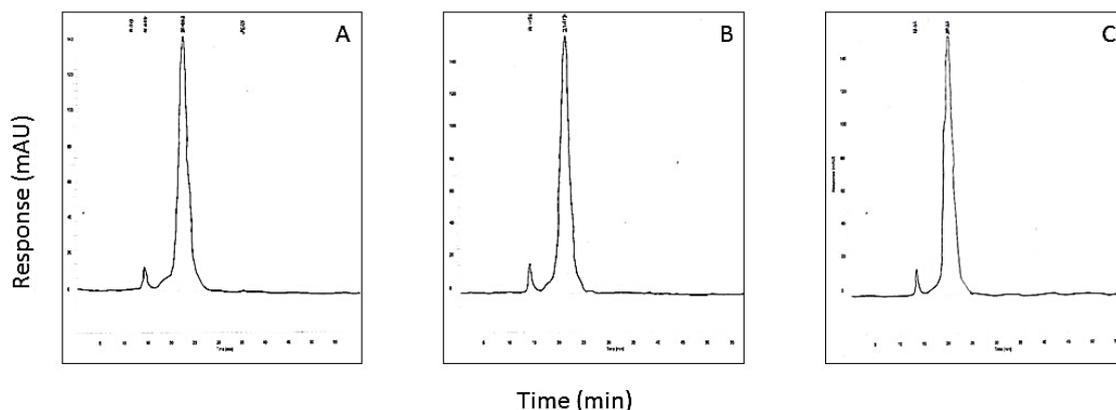


Figure 4.4-1: Chromatogram of purified Vipera antiserum containing: A- α -VipH; B- α -VipM and C- α -VipL. The small peak is IgM and the large peak IgG.

4.4.2 Western Blot

The four *Vipera* venoms against which antiserum was raised, were separated by SDS-PAGE (Panel A, Figure 4.4-2) and blotted onto nitrocellulose membranes (Panels B, C and D, Figure 4.4-2). The membranes were then probed with α -VipH, M and L IgG.

The blots demonstrated that antibodies from all three IgG samples bound to all the venom protein bands, albeit with slightly different intensities. This would suggest that antibody response was quantitative, not qualitative.

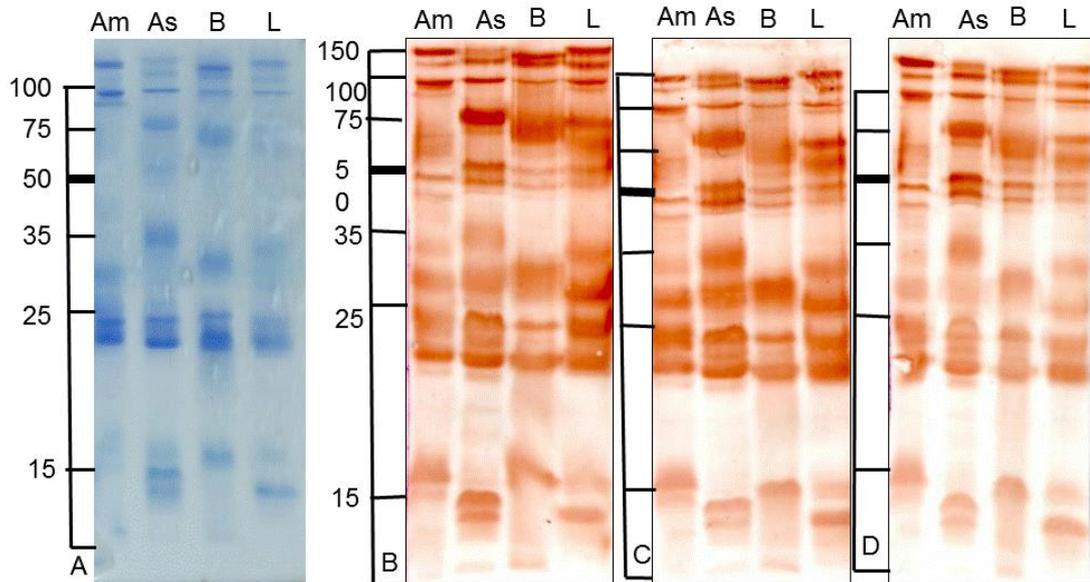


Figure 4.4-2: A: SDS PAGE of *Vipera* venoms; Blot of venoms probed with B: α -VipH; C: α -VipM and D: α -VipL. MW marker to left, thick line = MW 50; Am = *V. ammodytes*; As = *V. aspis*; B = *V. berus*; L = *V. latastei*

4.4.3 ELISA

ELISA results can be affected by laboratory environmental conditions. To overcome this problem aliquots of a reference antiserum were used to correct for these inherent variations. The EC₅₀ and endpoint titres of the three IgG samples reflected their calculated VSAb concentration. The binding potency was calculated by dividing the amount of venom/well by the amount of IgG/well, giving the results shown in Table 4.4-1 and Figure 4.4-3.

Table 4.4-1: ELISA EC₅₀ titre pre- and post-purification and endpoint titre post-purification.

Sample	EC ₅₀ dilution	Endpoint titre dilution	EC ₅₀ IgG/well (ug)	EC ₅₀ Potency V/AV
α -VipL	19,090	80,000	0.12	1.54
α -VipM	32,727	200,000	0.07	2.63
α -VipH	52,727	300,000	0.04	5.45

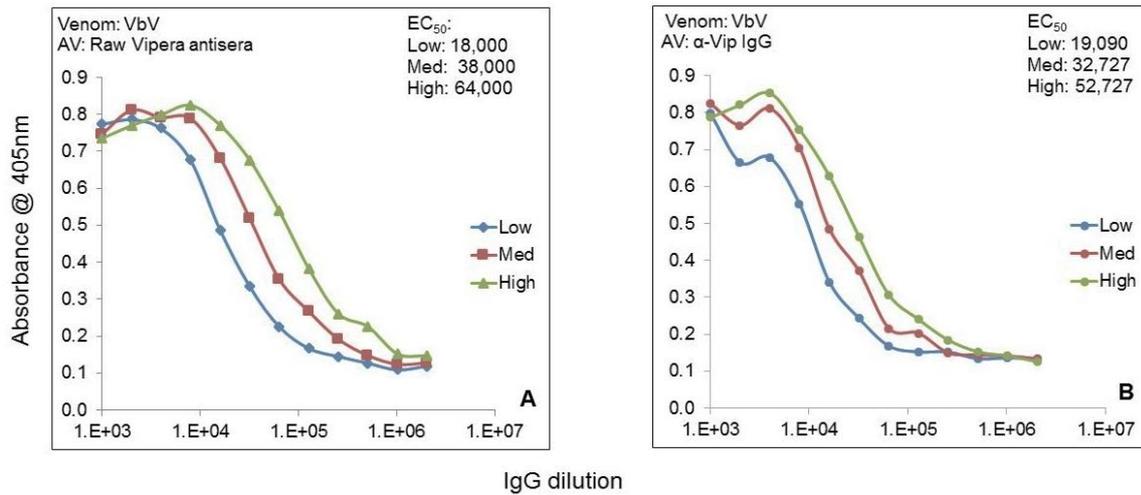


Figure 4.4-3: Binding of ViperaVet antisera (A) and purified *Vipera* antisera (B) to *Vipera berus* venom measured by ELISA

4.4.4 Avidity ELISA

An avidity ELISA was performed on the α-Vip IgG samples and a GMP manufactured sample of antivenom (ViperaVet). The 'high' VSAb concentration sample had antibodies with the greatest avidity, having the same avidity as antibodies from unpurified reference antiserum. ViperaVet, manufactured under GMP conditions, had an antibody avidity mid-way between the 'low' and 'medium' VSAb concentration samples. The results are summarised in Table 4.4-2 and Figure 4.4-4

Table 4.4-2: Avidity ELISA – molarity of sodium thiocyanate resulting in 50% disruption of *V. berus* venom binding (M_{50}) to α -VipH, M and L IgG and ViperaVet antivenom. PC = protein concentration

Sample	PC (g/L)	M_{50}
α -VipL	23.8	4.5
α -VipM	24.1	6.0
α -VipH	23.5	7.8
VPV	25.0	5.4

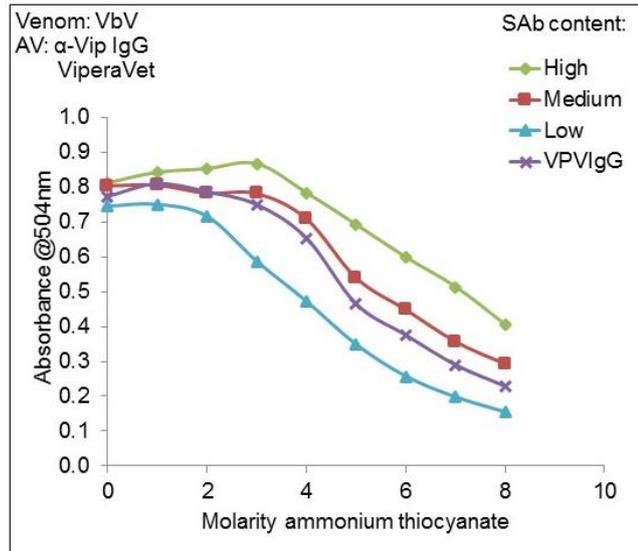


Figure 4.4-4: Disruption of *V. berus* venom binding to α -VipH, M and L IgG and ViperaVet antivenom by the chaotrophic agent, ammonium thiocyanate.

4.4.5 SSAC

SSAC was performed using a column conjugated to the same 1:1:1:1 mix of *Vipera* venoms used to immunise the donors, in order to select samples with the high, medium and low VSAb concentration for each group of pooled antisera. The results, using an SSAC column coupled to *V. berus* venom alone, are summarised in Table 4.4-3. A sample of normal sheep serum (PIS) showed non-specific binding of 1.0g/L

Table 4.4-3: Summary of VSABs to *V. berus* venom measured by SSAC.

Sample	VSAb (g/L)	Total IgG(g/L)	% VSAb
α -VipL	3.9	23.8	16.4
α -VipM	5.1	24.1	21.2
α -VipH	6.5	23.5	27.7

The results demonstrate the same pattern as the ELISA results in that the VSAb concentration and the percentage VSAb followed the trend 'low' to 'high'. SSAC was also performed post-purification using the 1:1:1:1 *Vipera* mix SSAC column (Table 4.4-4), which followed the same trend.

Table 4.4-4: Summary of VSABs to a mix of four *Vipera* venoms measured by SSAC after purification.

Sample	VSAB (g/L)	Concentration (g/L)	% VSAB
α -VipL	2.4	23.8	10.1
α -VipM	5.2	24.1	21.6
α -VipH	7.0	23.5	29.8

4.4.6 Immuno-cytotoxicity (ICT)

Neutralisation of the cytopathic effect (CPE) of *V. berus* venom was performed using the α -VipH, M and L IgG pools both pre- and post-purification. This assay was used to demonstrate not only binding by, but also neutralisation of venom toxicity of the different α -*Vipera* IgG samples. All samples, pre- and post-purification, effectively neutralised the venom CPE and in both instances, the EC₅₀ dilution increased with increasing VSAB concentration (Table 4.4-5). The full data set can be viewed in the chapter supplement.

Table 4.4-5: Summary of immunocytotoxicity assays. Neutralisation of VbV cytotoxicity with three different VSAB concentration *Vipera* antisera pre- and post-IgG purification

	Pre-EC ₅₀ dilution	Purified EC ₅₀ dilution	EC ₅₀ IgG/well (ug)	Potency mgV/gAV
α -VipL	120	250	5.00	40
α -VipM	420	380	3.29	61
α -VipH	700	1000	1.25	160

The purified 'low' and 'high' samples required less antivenom to neutralise the venom CPE than their pre-purified antisera, but the difference between pre-purified and purified 'medium' sample was minimal. The results are illustrated in Figure 4.4-5. The cells treated with purified 'low' sample only achieved around 70% of control cell growth and the pre-purified sample around 80% maximum neutralisation. The initial 1:10 dilution of the pre-purified 'medium' sample, incubated with *V. berus* venom, appeared to have a growth enhancing effect on the VERO cells. This observation is discussed in more detail in Chapter 8.

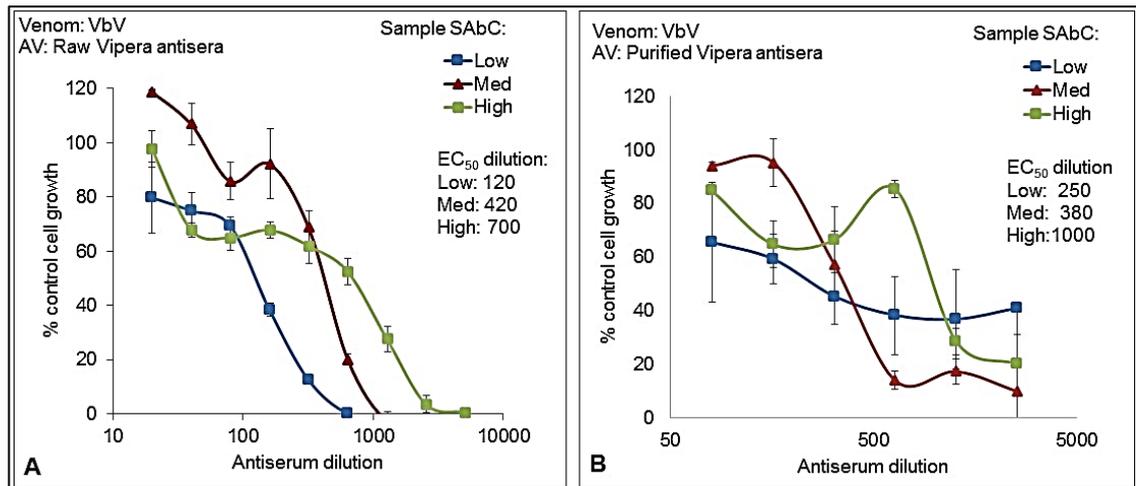


Figure 4.4-5: Neutralisation of VbV cytotoxicity with three different VSAb concentration Vipera antisera pre- (A) and post (B) IgG purification.

4.4.7 *In vivo* modified ED₅₀

An ED₅₀ assay was performed in which only one dose of venom and AV was used (10 mice per group). The efficacy of the α -VipH, M and L IgG samples and ViperaVet (VPV), preincubated with *V. berus* venom, was compared using the pathology/survival score (PSS) described in Chapter 2A; the dose groups were double-blinded. In the α -VipM IgG group mice numbers 6 and 10 were misinjected, so their data has not been included (hence only 8 mice are reported in this group).

A 'cage-side' MGS scoring was performed at each time point, without using photography to determine the score of each facial feature; however no significant differences between the groups was observed. No analgesia was given and the mean MGS scores in all groups remained high throughout the experiment. A table detailing, and a figure illustrating, these results are located in the chapter supplement. Pathology and survival times are summarised in Table 4.4-6.

Table 4.4-6: Effect of antivenom VSAb concentration on pathological lesions of mice envenomed with *V. berus* venom. Only 8 mice in α -VipM IgG group due to mis-injection of 2 mice

Venom	<i>Vipera berus</i> 2.5x LD ₅₀ : 36.5 μ g/mouse			
	α -VipH	α -VipM	α -VipL	VPV
Lung C	9/10	3/8	7/10	10/10
Myocardial H - V	2/10	6/8	6/10	2/10
Myocardial H - A	9/10	8/8	10/10	10/10
Organ (eg liver, kidney) C	7/10	7/8	10/10	6/10
Duodenal H	1/10	0/8	0/10	0/10
Mid SI H	2/10	0/8	4/10	2/10
Dist SI/LI H	1/10	0/8	0/10	0/10
Stomach H	2/10	0/8	3/10	0/10
Cranial C	8/10	7/8	10/10	10/10
Orbital H	3/10	4/8	5/10	2/10
Brain C	6/10	2/8	10/10	4/10
Other H	1/10	0/8	0/10	0/10
Mean pathology score (P)	9.7	8.8	12.6	9.7
Deaths in 6h	3/10	6/8	7/10	2/10
Mean time to death (min)	294.3	106.9	136.2	307.7
Survival score (S)	2.3	8.8	7.8	1.9
PS score (PSS)	12.0	17.6	20.4	11.6
95% CI for PSS	\pm 4.2	\pm 3.5	\pm 3.0	\pm 4.2

Key: H = haemorrhage C = congestion
V = ventricles A = atria
SI = small intestines LI = large intestines
P = pathology score S = survival score
PSS = pathology/survival score

The GMP manufactured VPV gave a superior neutralisation over all the laboratory purified IgG samples, despite having an intermediate VSAb concentration. Of the three test samples the mean pathology scores, survival scores and therefore PSSs showed an inverse relationship to the VSAb concentration, except for the α -VipH group which had a slightly higher pathology score than the α -VipM group. In general, more mice in the α -VipL group exhibited the pathological lesions listed in Table 4.4-6. One mouse in the α -VipH group, during post mortem examination, was found to have haemorrhages throughout its body, including throughout the entire intestinal tract, which is unusual for *V. berus* venom (see Chapter 7). It was found dead at the end of the experiment, the likely cause of death being an intracerebral haemorrhage; this mouse had a high pathology score of 15 (See Chapter 2A for scoring system), which may account for this group's greater than expected PSS (11.5 \pm 4.6) if said mouse's score is omitted).

4.4.8 Correlating results of the *in vitro* and *in vivo* assays

Results of all four *in vitro* and *in vivo* assays performed are shown in Table 4.4-7. The results of the GMP, clean-room manufactured ViperaVet are included as a control. ViperaVet was manufactured from a pool of antiserum derived from the same flock, but taken at a single point in time. The VSAb concentration by SSAC, ELISA binding, Avidity ELISA M₅₀ (Molarity of ammonium thiocyanate resulting in disruption of 50% of maximum binding), ICT potencies and *in vivo* results appropriately reflect the VSAb concentration of the α -VipH, M and L IgG samples. Despite having a lower VSAb concentration than any of the laboratory purified samples, ViperaVet had ELISA binding, Avidity ELISA M₅₀ and ICT potencies similar to the ‘medium’ sample and *in vivo* results better than the ‘high’ sample.

Table 4.4-7: Summary of analysis of α -VipH, M and L IgG samples

Assay	α -VipH	α -VipM	α -VipL	VPV
VSAb concentration (g/L)	6.5	5.1	3.9	3.6
ELISA potency (V/IgG)	5.45	2.63	1.54	2.27
Avidity Elisa M ₅₀	7.8	6.0	4.5	5.4
ICT potency (V/IgG)	160	61	40	48
<i>In vivo</i> (Deaths)	3/10	6/8	7/10	2/10
<i>In vivo</i> PSS	12.0	15.7	20.4	11.6

4.4.9 Statistical Analysis

Scatter plots were created to investigate the correlation, if any, of the assay results.

Table 4.4-8: Correlation of assay results – R squared values

R ²	ELISA V/IgG	Avidity M ₅₀	SSAC	ICT V/IgG	PSS
ELISA V/IgG	-	0.9635	0.8467	0.9107	0.9964
Avidity M ₅₀	0.9635	-	0.8164	0.8357	0.9812
SSAC	0.8467	0.8614	-	0.9294	0.9789
ICT V/IgG	0.9107	0.8357	0.9294	-	0.9717
PSS	0.9964	0.9812	0.9789	0.9717	-

Table 4.4-8 shows R² values of a linear relationship between assay results of purified IgG samples and VPV. ViperaVet results were not included when comparing *in vitro* assays with *in vivo* PSS scores; the reason for this will be discussed in the next section. There was an extremely good correlation, R² >

0.8, between all results, notably between PSSs and all four *in vitro* assays results ($R^2 > 0.97$).

4.5 Discussion

This, as far as is known, is the first study to compare results of *in vitro* and *in vivo* assays for samples derived from the same pool of donors, which contain different VSAb concentrations. Each pool of raw antisera was purified using the same small-scale manufacturing process, and was carried out in as sterile a manner as possible in laboratory conditions.

FPLC showed that all samples had a purity of >95%, each with a small higher MW peak, most likely to be IgM and/or aggregates. Further identification of this peak is not within the scope of this project.

Immunoblots showed that VSAb composition was similar in all three samples, demonstrating a quantitative rather than qualitative immune response.

As expected, ELISA and SSAC demonstrated different VSAb concentrations of the samples.

Avidity ELISA showed that the higher the VSAb concentration, the greater the molarity of ammonium thiocyanate required to disrupt 50% of venom/antivenom binding, thus demonstrating that a higher VSAb response also produces antibodies with greater avidity.

Immunocytotoxicity assays also demonstrated that the CPE of *V. berus* venom was effectively neutralised by all three samples. The α -VipL IgG sample only demonstrated a maximum neutralisation of 70% (at a 1:10 dilution), but its raw antiserum did achieve 100% neutralisation. The raw antisera and purified α -VipM and α -VipH samples demonstrated fluctuation of CPE at the lowest dilutions; briefly, it represents the summation of growth promoting effects and lethal CPE of low venom concentrations. Possible reasons for this are discussed in more detail in Chapter 8.

The WHO recommended *in vivo* murine lethality test – median effective dose (ED₅₀) - is considered the ‘gold standard’ assay used to demonstrate antivenom efficacy (WHO, 2010). The aim of this project is to implement the 3Rs into these assays. To perform full ED₅₀s on the three purified samples plus the GMP manufactured ViperaVet would have required a minimum of 80 mice, in reality, more than 100. Our modified ED₅₀ required 40 mice, a saving of greater than 50%. The dose of IgG selected was 1xED₅₀ of ViperaVet, the value of this batch having been demonstrated in an earlier assay. Previous experience has established that aggregates tend to form using ovine IgG products at higher antivenom and venom concentrations and for this reason a dose of 2.5 times the *V. berus* venom LD₅₀s was used.

The combination of survival and pathology scores as a measure of severity of envenomation is well illustrated in this study. The α-VipM group had a relatively low pathology score, but relatively high survival score. The combination of scores differentiated between α-VipH, α-VipM and α-VipL VSAbs concentration samples. The ViperaVet control performed better than any of the laboratory purified samples, having lower survival and pathology scores as well as death rate. These results were not included in the comparison with other *in vitro* assays because manufacturing processes were not identical. It would appear to be very likely that the laboratory manufactured samples contained some endotoxins, which may have produced an adverse result. It is a salutary lesson in the importance of GMP in antivenom production, and the presence of endotoxins may well explain the high proportion of adverse reactions seen after administration of some AVs. It would have been interesting to have tested the laboratory samples for endotoxins using the *Limulus* amoebocyte lysate (LAL) test (WHO, 2010a).

Two ‘misinjections’ occurred, when the mice failed to receive their full dose of venom/antivenom. When the identity of the mice was revealed it became apparent that both mice were from the α-VipM group, and the data from these mice was not included. This study has shown that mice which die rapidly after venom/antivenom injection generally show signs of ventricular myocardial

haemorrhage, with a greater proportion of mice in the α -VipL groups suffering myocardial haemorrhage. Mice surviving longer demonstrated signs of cardiac failure, such as congestion of the lungs and other major organs. Mice surviving for several hours also suffered intestinal haemorrhage.

In summary, all five assays performed were able to demonstrate binding or neutralisation of *V. berus* venom, and to differentiate between samples containing different VSAb concentrations. The most significant finding of this study is the high correlation of results between different assays, most importantly between *in vitro* and *in vivo* assays. There were only three different VSAb concentrations, so only three points on the comparison between purified IgG *in vitro* vs *in vivo*.

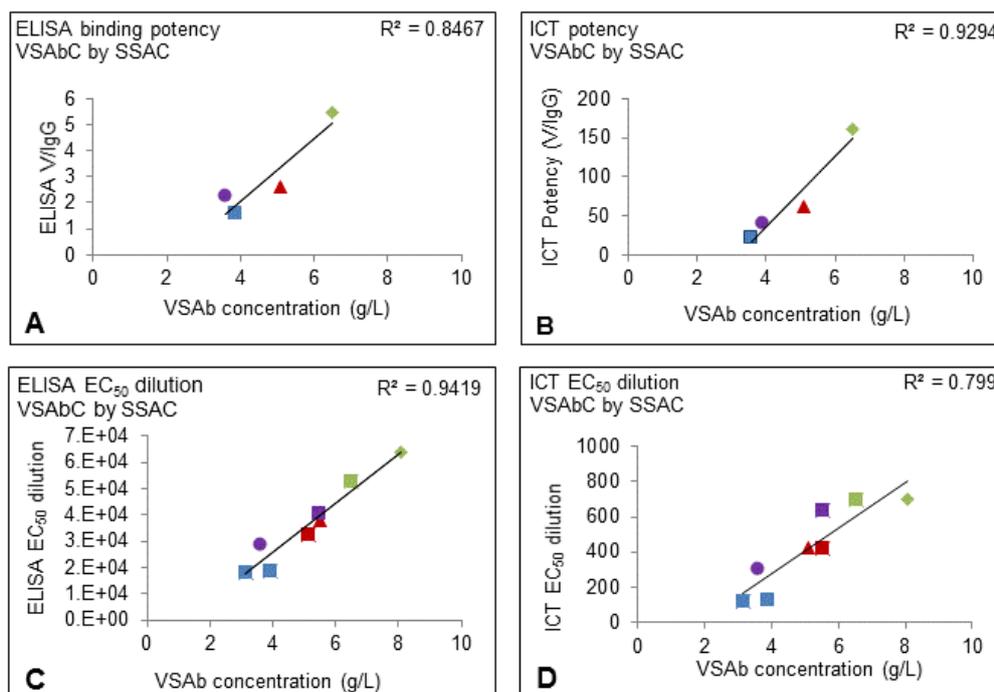


Figure 4.5-1: Correlation between VSAb concentration by SSAC and ELISA potency (A), ICT potency (B), ELISA EC₅₀ dilution (C) and ICT EC₅₀ dilution (D). See Figure 4.5-2 for key.

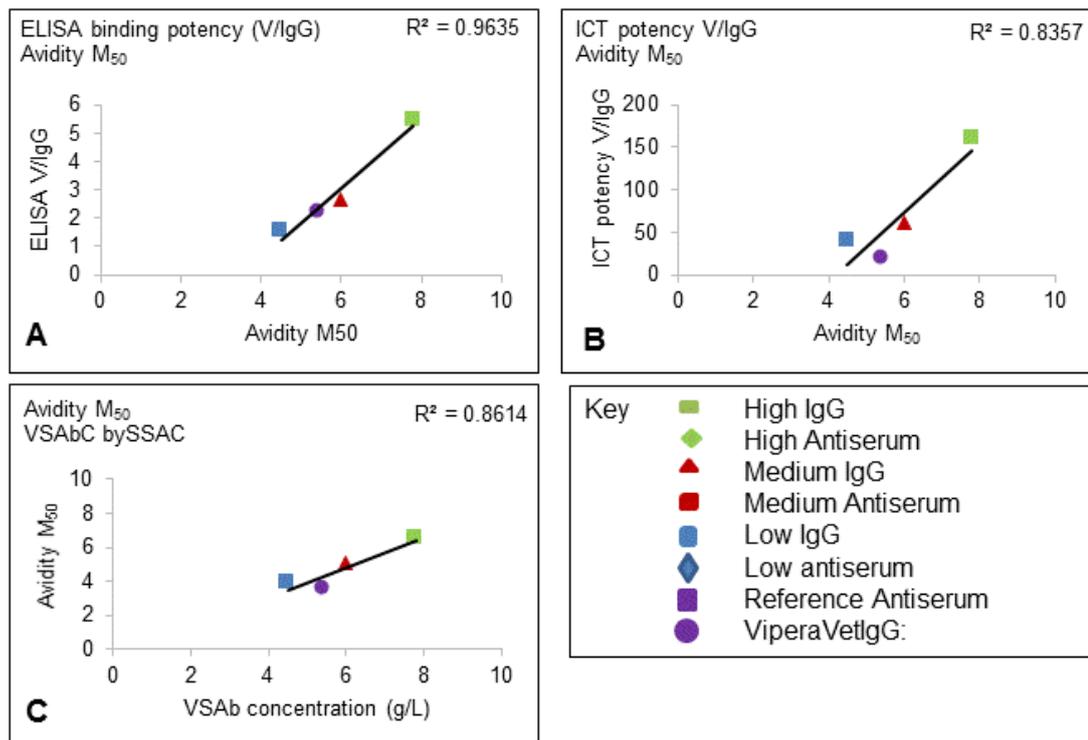


Figure 4.5-2: Correlation between molarity of ammonium thiocyanate required to disrupt 50% venom-IgG binding (M_{50}) and ELISA binding potency, VSAb concentration by SSAC and ICT neutralising potency

Figure 4.5-1 illustrates the correlation between ELISA and ICT EC_{50} – to provide more data-points, results of pre-purified antisera are included in the charts where dilution is plotted against VSAb concentration.

Figure 4.5-2 shows the correlation between Avidity M_{50} and ELISA binding potency (panel A), ICT potency (panel B) and VSAb concentration by SSAC (panel C). The key for Figures 1.5-1 to 1.5-3 is also shown in this figure.

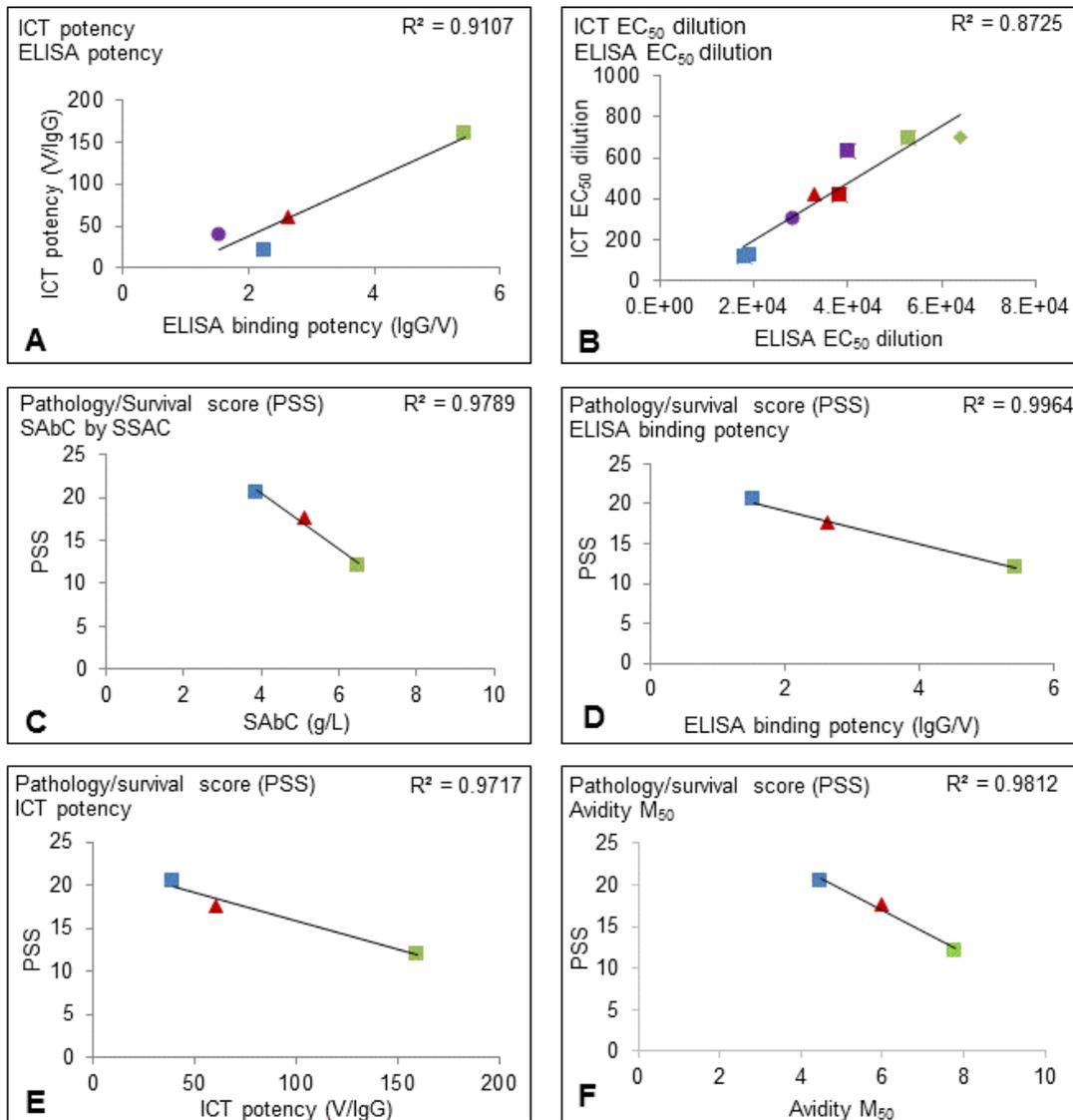


Figure 4.5-3: Correlation between ELISA binding and ICT neutralising potencies (A), ELISA and ICT EC₅₀ dilutions (B) ; *in vivo* PSS and *in vitro* VSAb concentration (C), ELISA binding potency (D), ICT neutralising potency (E) and Avidity M₅₀ (F).

Figure 4.5-3 demonstrates the correlation between ELISA with ICT assays (A and B) and *in vivo* pathology-survival score (PSS) with the four *in vitro* assays (C, D, E & F). All four *in vivo* vs *in vitro* assays showed a convincing linear correlation.

ELISA is widely used to measure antibodies (as well as antigen or antigen/AB complexes). It can be used to screen large numbers of samples, and the process can be automated. However, it involves multiple steps, which may be temperature sensitive, and therefore potentially a problem in the tropics or

laboratories which are not climatically controlled, resulting in a coefficient of variation in excess of 10% in all but the most experienced operators (MicroPharm data). The secondary antibody used needs to be specific for the antibody fragment and species from which it is derived.

SSAC produces a result in g/L, is robust and reproducible (MicroPharm, unpublished data and Harrison, 2004), with a coefficient of variation of 3.5% (MicroPharm data). The chromatography column can be used to detect antibodies and their fragments from any snake species, without the need for a specific secondary antibody, and can be re-used multiple times (over 80 runs – Harrison, 2004). However, the assay, which can be mechanized, is not as suitable as ELISA for screening multiple samples, although several columns can be run simultaneously.

Immunocytotoxicity assays produced considerable variation within and between assays (See chapter 8). Cells are living entities and may therefore be an indicator of neutralizing efficacy of antibodies to toxins which exert their effect via receptors or by damaging the cell membrane. Cell culture facilities are expensive to set up and maintain, and considerable expertise is required to successfully maintain cells in culture.

4.5.1 Conclusions

The results of this section of the project achieved the objective of demonstrating a good correlation between *in vitro* and *in vivo* results, but these results only apply to the venom studied and the antibodies raised against it.

Several other studies have already demonstrated such a correlation between ED₅₀s and ELISA (Theakston et al, 1979), insensate hen egg assay-haemorrhagic viper venoms only (Sells 2001), VSAb concentration by SSAC (MicroPharm, unpublished data) and ICT assays (Nalbantsoy et al, 2012). In conclusion, these results suggest that each batch of antivenom manufactured need not undergo *in vivo* lethality testing, provided that *in vivo* efficacy is unequivocally established, and that the criteria listed in the introduction are

met, i.e. consistent starting material (antiserum from the same pool of individuals) and a robust, fixed manufacturing process.

On the basis of these results, the recommendation is that a validated *in vitro* assay is performed on a sample of each batch of antivenom to monitor VSAb concentration. If there is any deviation from the normal range, any change in starting material or manufacturing process, then an ED₅₀ should be performed to confirm or refute *in vivo* antivenom efficacy of that batch.

Chapter 5. Refinement: Analgesia

5.1 Objectives

Refinement refers to the reduction of pain, suffering and distress experienced by the subjects of the WHO-recommended protocols for murine lethality assays of snake venom toxicity and antivenom efficacy. In this chapter I present the results of work undertaken to establish whether the introduction of effective analgesia to alleviate pain is an effective refinement that does not affect the biological or statistical validity of the assay.

The final objective is to inform antivenom manufacturers and researchers of the results and, if the first objective is achieved, to encourage incorporation of analgesia into routine venom toxicity and antivenom efficacy testing.

This chapter will be confined to the alleviation of pain – Chapter 6 will address HEPs and other refinements to these assays.

5.2 Introduction

Pain can be defined as an unpleasant sensation occurring in varying degrees of severity as a consequence of injury, disease or emotional disorder. Pain is a major symptom of many envenomed snakebite victims, both human and animal, a notable exception being those bitten by kraits (Ariaratnam et al, 2008). Treatment protocols for snakebite include analgesics, such as paracetamol or mild opiates (Menon et al, 2016; WHO 2010b, Warrell, 1999). Despite this, protocols for the murine lethality tests of snake venom characterisation and antivenom efficacy do not include routine use of analgesia. Here, the objective is to implement this refinement into these assays and to validate these modifications.

Achieving this objective requires a system to measure the alleviation of pain using analgesics. However, pain response in animals is not a simple reflex and the resulting behaviours are species-specific. In order to assess analgesic efficacy, accurate methods of measuring pain and distress need to be employed. When assessing pain and distress in test subjects a researcher

must have an in-depth knowledge of normal species-specific behaviours, as well as the observational skills required to appreciate when these behaviours deviate from the norm.

5.2.1 Pain assessment

The assessment of pain in both animals and humans tends to be subjective and, in the case of animals often based on anthropomorphic assumptions. There is therefore a need to have a robust and reproducible method of pain assessment in laboratory animals to ensure that claimed refinements can accurately be evaluated (Flecknell, 1994). Historically, the efficacy of analgesia has been tested by inducing pain using mechanical, thermal or chemical stimuli and measuring a drug's ability to suppress the test animal's reflex response; for example the writhing test (Botting et al, 2005) and licking a hind paw injected with an irritant (Hunskar et al, 1985). Other methods examine responses involving conscious perception of pain *via* the thalamus and cortex (Bianchi et al, 1954).

5.2.1.1 Facial Expressions:

The mouse grimace scale (MGS) score was recently developed (Langford et al, 2010; Mogil, 2010), based on a facial scoring system, to assess pain in children too young to verbally express their suffering (Lee, 2002). Facial grimace has since been adopted both by researchers and clinicians to assess pain in laboratory and domestic animals (Leach et al, 2012; Matsumiya et al, 2012; Sotocinal et al, 2011; Holden et al, 2014; Dalla Costa et al, 2014) and it has been adopted for the purposes of this work to incorporate analgesia into venom LD₅₀s and antivenom ED₅₀s. The MGS score is described in detail in Section 2.10.1 (Chapter 2A).

5.2.1.2 Behavioural analysis:

The absence of certain behaviours such as nest building, grooming, exploration and rearing, or the presence of abnormal behaviours, such as abdominal press, limping or chewing at the painful area, may be used to assess pain.

Perception of pain in mice can be affected by genotype, sex, stress (stress induced analgesia), behaviour immediately prior to receiving a noxious stimulus and social interactions (Mogil, 2007), whilst another study revealed that pain and analgesia are heritable traits in both humans and mice (Lariviere et al, 2010). To reduce variables in preclinical tests all mice are male and of the same (CD1) strain.

5.2.1.3 Physiological parameters:

The identification of biochemical or physiological indications of pain and distress would allow quantitative evaluation of methods employed to alleviate suffering (Flecknell, 1994). However, the large number of factors influencing these physiological parameters render them unreliable as objective measures of pain (Flecknell, 1999) at this time.

5.2.2 Analgesia

For the purposes of this study, analgesics can be divided into two broad groups, namely non-opiates, including non-steroidal anti-inflammatory drugs (NSAIDs), and opiates. Paracetamol is the drug of choice for the relief of pain in human snakebite victims. For severe pain opiates may be administered with the caveat that, in humans, they may cause respiratory depression and must therefore be used with caution if neurotoxic envenoming is suspected.

Progress in the use of analgesia in experimental animals has been made over the past few years (Stokes et al, 2009). Buprenorphine is the most commonly-reported analgesic used post-operatively in rats and mice, and the use of NSAIDs has increased since 2001 (Stokes et al, 2009). Although reported administration of analgesics has increased and there has been some refinement in the selection of anaesthetic agent used, the findings of a literature review (Richardson and Flecknell, 2005) suggested that there is still significant scope for improvement with respect to pain relief in laboratory rodents (Stokes et al, 2009). A number of studies have shown that pre-emptive use of analgesia was more effective than waiting until pain had become established (Woolf, 1986, Lascelles 1995).

5.2.2.1 Opiates

5.2.2.1.1 Buprenorphine

The efficacy of Buprenorphine in laboratory animals is well documented (Matsumiya et al, 2012; Liles et al, 1992a; Liles et al 1992b). Buprenorphine is an opioid analgesic, with equivalent analgesic activity in humans of 0.3mg to 10mg morphine sulphate. It is metabolised in the liver and clearance is related to hepatic blood flow. It exerts its effect by high affinity binding to mu (μ) subclass opioid receptors in the CNS. Although it is classed as a partial agonist, it behaves in a similar manner to classical μ -agonists such as morphine, with the unusual property of a very slow rate of dissociation from its receptor; which could account for its longer duration of action than morphine, the unpredictability of its reversal by opioid antagonists, and its low level of manifest clinical dependence (Reckitt Benckiser Drug Reference Encyclopaedia).

Buprenorphine behaves as a full μ -opioid agonist for analgesia, with no ceiling effect, but there is a ceiling effect for respiratory depression. The effects of buprenorphine can be completely reversed by naloxone. No problems are encountered when switching between or combining buprenorphine and other opioids. Buprenorphine exhibits a pronounced anti-hyperalgesic effect that might indicate potential advantages in the treatment of neuropathic pain. It is suggested that buprenorphine's opioid action is mainly at the level of the spinal cord, not the brain, and is different from those of fentanyl and morphine (Pergolizzi et al, 2010). The pronounced anti-hyperalgesic effect of buprenorphine is thought to be because of its κ -receptor antagonism; and its metabolism is largely unaffected by renal insufficiency. (Pergolizzi et al, 2010). Severe pain in laboratory rodents is effectively treated using buprenorphine, and in combination with its longer duration of action (Gopal et al, 2002; Gades et al, 2000; Foley et al, 200; Wereszczyfiska-Siemiakowska et al, 1987; Liles et al, 1993a) led to its selection as the drug of choice for this study.

5.2.2.1.2 Morphine

Morphine sulphate (hereafter referred to as 'morphine') is a pure opioid agonist and is relatively selective for the μ - receptor. It binds to the receptor producing changes in both neurotransmitter release and calcium uptake. Receptors are located throughout the brain, spinal cord and gastrointestinal tract (GIT) (Jaffe, 1990).

In humans the principal effect of morphine is analgesia, but it also acts centrally to produce anxiolysis, euphoria and feelings of relaxation. It inhibits the respiratory centre (brainstem), cough centre (medulla) and stimulates the chemoreceptor trigger zone (CTZ), resulting in respiratory depression, cough suppression and centrally-induced nausea and vomiting respectively (mice are incapable of vomiting). It also causes miosis (pupillary constriction), even in the dark (Jaffe, 1990).

In the GIT, gastric, biliary and pancreatic secretions are reduced, smooth muscle spasm and compartmentalisation are increased but the propulsive peristaltic waves are inhibited. As a result increased transit time of digesta, combined with increased absorption of water, contribute to the development of constipation. Spasm of the sphincter of Oddi may result in increased biliary tract pressure and there may also be spasm of the urinary bladder sphincter muscle (Jaffe, 1990). Peripheral vasodilation resulting in orthostatic hypotension, compounded by histamine release, can occur, which may, in turn, cause pruritus, flushing, red eyes and sweating (Jaffe, 1990). Potentially this could exacerbate venom toxin-induced hypotension.

In humans, when morphine is administered orally around two thirds is absorbed, with maximum analgesic effect after 60 minutes. Bioavailability is less than 40% due to a significant first pass hepatic metabolism. Once absorbed it is distributed to skeletal muscle, kidney, liver, GIT, lungs, spleen and CNS, although only small quantities cross the blood-brain barrier. Around 20-25% is reversibly bound to plasma proteins (Jaffe, 1990).

Morphine is conjugated to glucuronide in the liver to Morphine-3- glucuronide (M3G) (inactive) and Morphine-6-glucuronide (M6G) (active, but which is not produced in mice). The majority is excreted as M3G in urine, but there is minor enterohepatic circulation with 7-10% excreted in faeces (Handal et al, 2002). In mice subcutaneous or intramuscular injection of morphine results in their tails going into spasm in dorsiflexion such that it is almost parallel to the spinal column, the 'Straub reaction' (Straub, 1911; Bilbey et al, 1960). This is accompanied by restlessness, excitability extensor rigidity of the hind-limbs, forcible voidance of faeces and prominence of the perineum, all of which arise from direct stimulation of the spinal cord.

Morphine is undoubtedly an effective analgesic in mice, however its half-life is less than 2 hours and therefore frequent administration is required. In mice receiving potentially haemotoxic venoms repeated injections are contraindicated, which makes, if viable, the use of oral morphine an attractive alternative. There is a dearth of information on the pharmacokinetics of oral morphine in mice, and for the purposes of this study it was therefore necessary to extrapolate from human data. There are apparently no references to dose of morphine given orally to mice so, given that in man approximately 50% of the ingested dose is absorbed, the highest published injectable dose-rate for mice was given (10mg/kg), with the assumption that a dose of 5mg/kg would be absorbed. We hoped that by giving the equivalent of half of the maximum injectable dose that we would avoid inducing the hyperactivity described in the literature (Straub, 1911; Bilbey et al, 1960). Oral morphine is available in liquid form as a syrup, which the mice appeared to find palatable, therefore making repeated dosing practical. Oral morphine was therefore the second analgesic to be investigated here.

5.2.2.2 Non-opiates

5.2.2.2.1 Paracetamol/acetaminophen

Analgesia and hypothermia induced by paracetamol are mediated by inhibition of a cyclo-oxygenase, designated COX-3 (Botting et al, 2005). It is ineffective in the relief of post-operative pain in mice (Matsumiya et al, 2012; Miller et al,

2011), such that given the apparent severity of pain induced by envenoming, it would appear to be an inappropriate choice of analgesic despite being administered in the case of human envenoming (WHO, 2010b).

5.2.2.2.2 Non-steroidal anti-inflammatory drugs (NSAIDs)

NSAIDs are generally effective analgesics for mild to moderate pain in laboratory animals (Wright-Williams et al, 2007; Arras, 2007). They can be divided into the cyclo-oxygenase (COX) inhibitors, which provide pain relief by inhibiting production of inflammatory mediators such as prostaglandins, prostacyclins and thromboxanes, and the centrally-acting, aniline-derived analgesics such as paracetamol (see above). The former lead to a smaller inflammatory response and thus analgesia, whilst the latter have few anti-inflammatory properties (Liles et al, 1992b).

NSAIDs are potentially a very useful group of analgesics and should always be considered when attempting to provide pain relief in laboratory animals. They may be used in combination with opiates, their inhibition on clotting make these drugs unsuitable for use in assays involving potentially anticoagulant venom toxins. For this reason the use of this class of drugs was not investigated in this study.

5.3 Materials and Methods

5.3.1 Venoms and antivenoms:

The five venoms selected were:

- *Echis ocellatus* (EoV)
- *Bitis arietans* (BaV)
- *Naja nigricollis* (NnigV)
- *Dendroaspis angusticeps* (DaV)
- *Vipera berus* (VbV)

See Appendix II, section II.3.1 for venom details

Their homologous antivenoms were used as listed below:

- EchiTabG (MicroPharm) (EOG) was used to neutralise EoV
- EchiTabPlus-ICP (ETP) was used to neutralise BaV and NnigV

- SAIMR polyvalent snake antivenom (South African Vaccine Producers) was used to neutralise DaV
- ViperaTab (MicroPharm) (VPT) was used to neutralise VbV

See Appendix II, section II.3.2 for product details.

5.3.2 Analgesics:

Analgesics used are listed in Table 5.3-1

Table 5.3-1: Analgesics investigated for efficacy in envenomed mice. i.p. = intraperitoneal.

Drug	Route	Class	Mode of Action
Buprenorphine 0.3mg/mL	i.p.	Opiate	Partial mu agonist
Oramorph® 2mg/mL	Oral	Opiate	Oral morphine. Primarily mu agonist, but also κ- and δ- agonist.
Morphine Sulphate 10mg/mL for injection	i.p.	Opiate	Primarily mu agonist, but also κ- and δ- agonist.

5.3.3 Mice

See Appendix II, section II.3.7

5.3.4 Refinement observations to assess pain and analgesia efficacy

- MGS
- Activity score
- Behaviour
- Physiological
- Neurological
- Pathology/Survival time

Details of these observations can be found in the Chapter 2A (Refinement methods).

5.3.5 Analgesia LD₅₀

These experiments were conducted as described in Chapter 2 for the median lethal dose, analgesia being administered 30 minutes prior to venom injection. Initially 1x LD₅₀ dose of venom was given, but deaths reduced the number of results available for statistical analysis therefore the dose was reduced to

0.75x LD₅₀ dose of venom. Experiments performed are summarised in Table 5.3-2.

Table 5.3-2: Analgesic LD₅₀s performed

Key: Tx = number of mice treated, C = number of control mice

Venom	xLD ₅₀	Buprenorphine			Morphine		
		mg/kg	Tx	C	mg/kg	Tx	C
EoV	1	1.5	10	10	-	-	-
	0.75	1.5	7	7	10	10	10
1.0		7	-		-	-	
BaV	1	1.5	10	10	10	10	10
	0.75	1.5	7	7	-	-	-
1.0		7	-		-	-	
NnigV	1	1.5	10	10	-	-	-
	0.75		-	-	10	10	10
DaV	1	1.5	10	10	-	-	-
	0.75		-	-	10	10	10
VbV	1	1.5	10	10	-	-	-
	0.75		5	5	10	30	20

5.3.6 Analgesia ED₅₀

These experiments were conducted as described in Chapter 2 for the median effective dose. Analgesia was administered 30 minutes prior to venom/antivenom injection. The experiments are summarised in Table 5.3-3.

Table 5.3-3 Analgesic ED₅₀s performed

Key: Tx = number of mice treated, C = number of control mice

Venom	xLD ₅₀	Antivenom	xED ₅₀	Buprenorphine			Morphine		
				mg/kg	Tx	C	mg/kg	Tx	C
EoV	2.5	EOG	1.5	-	-	-	10	10	10
BaV	5	ETP	1.0	-	-	-	10	10	10
NnigV	1	ETP	1.0	-	-	-	10	10	10
DaV	1	SAIMR	150µl	-	-	-	10	10	10
VbV	5	VPT	1.5	1.5	10	10	10	20	20

Only one ED₅₀ experiment was performed using buprenorphine analgesia because it had become apparent that buprenorphine induced hyperactivity of the mice, with an associated increase in death rate. The ED₅₀ of EchiTabPlus required to neutralise 2.5x LD₅₀ of *N. nigricollis* venom was greater than the maximum allowable injection volume, therefore 1x LD₅₀ of venom was used with the equivalent reduction in antivenom dose.

5.3.7 Dose response studies

5.3.7.1 Buprenorphine

An initial dose response study was performed to establish the effect of buprenorphine alone on the mice. The experimental design is summarised in Table 5.3-4. On Day 1 the mice were injected with one of 3 doses of buprenorphine or an equivalent volume of saline, and observations performed every 30 minutes for 2 hours. The experiment was repeated on Day 2 to ascertain whether heating the mice affected their response to buprenorphine. The mice were injected with buprenorphine, a set of background observations performed and then their cage was placed under an infrared heat lamp. Observations were then performed as for Day 1.

Table 5.3-4: Buprenorphine dose response – no venom

Buprenorphine dose (mg/kg)	Number of mice
2.0	2
1.5	2
1.0	2
Control	4

A second dose response study was performed using 45 mice in total (six groups of seven mice and three control mice with no interventions). The experimental design is summarised in Table 5.3-5. Observations were made as described previously and post mortem examinations performed at the end of the experiment.

Table 5.3-5: Buprenorphine dose response in envenomed mice.

Buprenorphine dose (mg/kg)	1.5	1.0	Control
EoV	7	7	7
BaV	7	7	7
Control	-	-	3

5.3.7.2 Morphine Sulfate

A dose response study was conducted using two doses of morphine analgesia on mice envenomed with 0.75 x LD₅₀ *N. nigricollis* venom and an equal number of non-envenomed mice. Each mouse was injected with a standard dose of morphine sulphate (5mg/kg) intraperitoneally 30 minutes prior to i.v. injection of venom or an equal volume of PBS. Three hours after the initial morphine

dose the mice were given one of two additional doses of oral morphine. Routine observations were made, including post mortem examinations.

The rationale for this experimental design was to investigate whether additional dosing with oral morphine affected outcome of envenomed mice or behaviour of non-envenomed mice. There was a particular concern that higher doses of morphine may cause hyperactivity, as described in the literature (Straub, 1911; Bilbey et al, 1960). An elapid venom was used because the neurotoxic components of its venom was more likely to interact with a centrally acting opiate than a non-neurotoxic viper venom. In this experiment, the initial dose of morphine was injected to ensure that each mouse received an initial dose of morphine equivalent to the selected oral dose. Although the mice were generally willing to take their medication, there were some which did not swallow all of their computed dose.

5.3.8 Venom dose response

This experiment was designed to investigate analgesic efficacy at different doses of venom. An analgesic LD₅₀, using three different doses of *V. berus* venom, was performed on mice with and without buprenorphine analgesia (Table 5.3-6). This experiment was performed to see if venom dose affected the efficacy of buprenorphine analgesia.

Table 5.3-6: Venom dose response in mice dosed with buprenorphine analgesia (Bup)

Venom dose (µg)	Number of mice	
	Bup	Control
6.5	5	5
5.0	5	5
3.0	5	5

5.3.9 Statistical analysis

Mean MGS, activity and pathology-survival scores were calculated and plotted with error bars showing 95% confidence intervals (CI), and a Student t-test employed to determine statistical significance. Kaplan-Meier (KM) survival analysis was performed to determine whether administration of analgesia

affected survival rates. To maximise the power of the statistical analyses performed on the resulting data, the results were pooled for all venoms.

5.4 Results

5.4.1 Baseline effects of analgesia (no venom)

Buprenorphine

Mice were given analgesia without venom to identify whether analgesia induced behaviours which might be confused with those behaviours resulting from envenomation. As the dose rate for mice quoted in the literature varies quite considerably, different doses were administered (1.0 to 2.0mg/kg) to establish the optimum dose exhibiting maximum analgesic effect without inducing adverse behavioural effects. Activity was measured by counting the number of 'behaviours' (e.g. exploring, grooming, eating, rearing) in 30 seconds.

The saline treated mice became less active with time, probably due to becoming familiar with their environment. Mice are nocturnal animals and prefer to sleep during the day. The reduction in activity of the buprenorphine treated mice was significantly less than the controls (student t-test, $p < 0.05$). There was no change in activity when the buprenorphine-treated mice were heated. The results are illustrated in Figure 5.4-1

The buprenorphine treated mice exhibited almost manic feeding behaviour and seemed more active than the saline treated mice. In the second buprenorphine experiment the mice were given a dose of 2mg/kg on Day 1 (no venom). The mice appeared hyperactive therefore were given the lower dose of 1.5mg/kg on Day 2 prior to venom injection.

The statistical analysis performed (student t-test, unless otherwise stated) on the results presented in this chapter can be found in the chapter supplement.

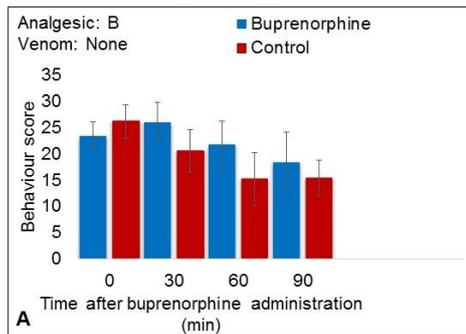


Figure 5.4-1: The effect of analgesia on number of behaviours performed by mice treated with buprenorphine compared to those treated with saline. Error bars = 95%CI

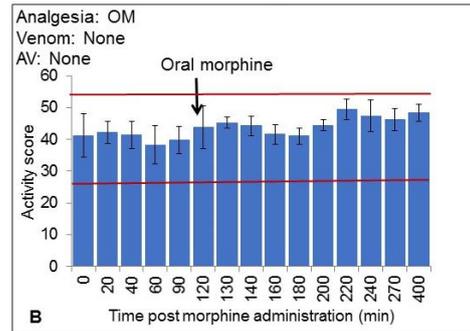


Figure 5.4-2: The effect of morphine on the number of distinct head movements performed by mice given oral morphine, followed by a second dose after 2 hours. Red lines indicate mean activity of untreated mice \pm 2x standard deviations. Error bars = 95%CI.

Morphine

At this later stage in the project, activity measurements were changed to count the number of distinct head movements made by each mouse in 30 seconds. It was noted that some mice were stationary, but looked around, taking an interest in their surroundings, whilst the worst affected mice did not even move their heads. Counting head movements differentiated the severity of suffering between these two groups of mice. Mice treated with oral morphine remained active throughout the study period and their activity score remained within the mean and 2x standard deviation of activity score prior to administration of morphine. A second dose was given two hours after the first, and the activity score remained within normal limits (Figure 5.4-2).

5.4.2 Analgesia dose response studies

5.4.2.1 Buprenorphine

Two different doses of buprenorphine were given to mice envenomed with two different viper venoms that exhibit distinct pathological lesions to determine whether analgesia was equally effective at controlling pain under these differing conditions. As higher dose rates were being used, it is possible that a 'ceiling effect' had been attained at the lower dose rate. Again, the same

mice were used on consecutive days with the same doses of buprenorphine thus acting as their own non-venomated controls.

MGS scores were significantly lower at all time points in mice that had been venomated with *E. ocellatus* venom and dosed with 1.0 mg/kg buprenorphine than the control mice. Those mice which had received 1.5mg/kg buprenorphine and *E. ocellatus* venom only had an MGS score significantly lower than controls at 90 and 120 minutes (Figure 5.4-3A). Mice venomated with *B. arietans* venom had MGS scores significantly lower (student t-test, $p < 0.05$) than control mice at both doses of buprenorphine, 1.5mg/kg being more effective than 1.0mg/kg (Figure 5.4-3B).

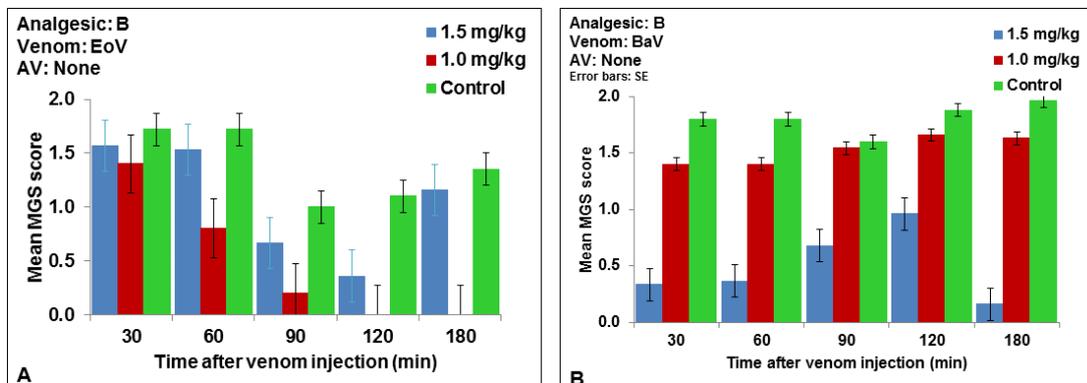


Figure 5.4-3: The effect of buprenorphine (1.5 or 1mg/kg) on mean MGS score of mice injected with *E. ocellatus* venom (A) or *B. arietans* venom (B)

Activity score did not appear to be dose-responsive in mice that had received no venom or those which had received either intravenous *E. ocellatus* venom or *B. arietans* venom. However, there was a statistically significant (student t-test, $p < 0.05$) increase in activity score at 30, 60 and 90 minutes post buprenorphine injection in both non-venomated (Figure 5.4-4A and Figure 5.4-5A) and venomated mice (Figure 5.4-4B and Figure 5.4-5B).

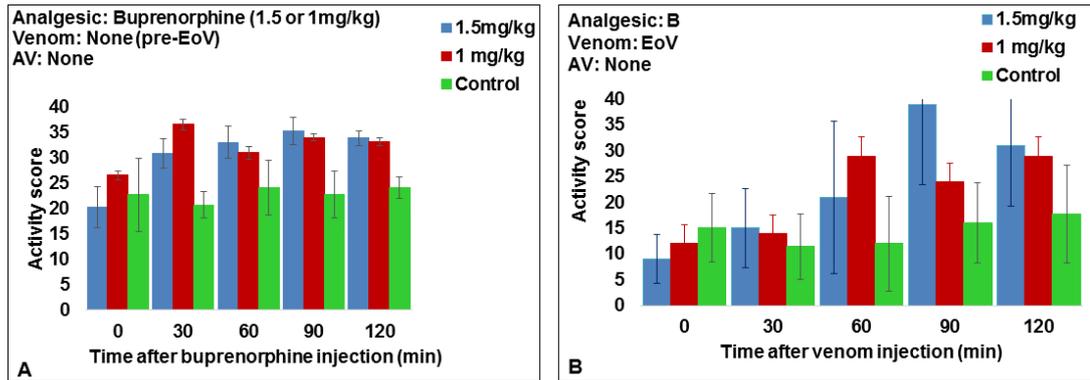


Figure 5.4-4: The effect of buprenorphine (1.5 or 1mg/kg) on the activity of mice injected with *E. ocellatus* venom (B) or no venom (PBS) (A)

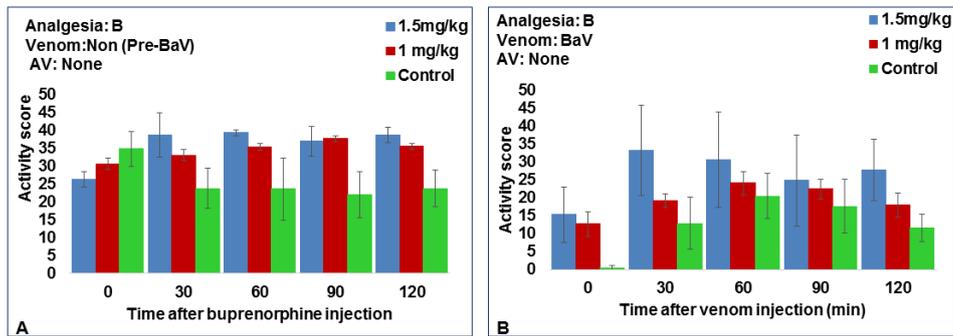


Figure 5.4-5: The effect of buprenorphine (1.5 or 1mg/kg) on the activity of mice injected with *B. arietans* venom (B) or no venom (PBS) (A)

The relationship between activity and MGS scores was examined using a scatterplot. When the data from all the mice was pooled, R^2 was 0.45 (see supplement; slide 5), but when only buprenorphine-treated envenomed mice were included, R^2 was 0.77 (Figure 5.4-6). The anomaly in these results probably reflects the number of other variables, in particular, the fact that MGS score can only be between 0 and 2, and that activity score cannot be less than zero and has to have a maximum value.

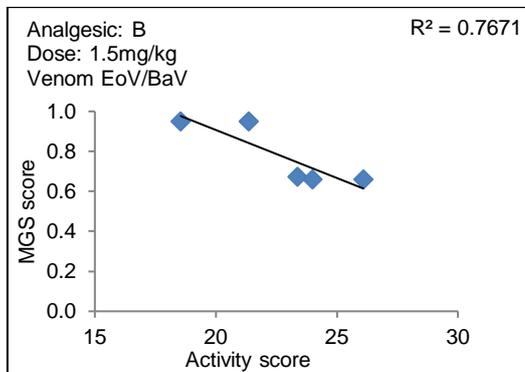


Figure 5.4-6: Relationship between mean activity and mean MGS scores in buprenorphine-treated mice envenomed with *B. arietans* venom or *E. ocellatus* venom. Each point represents the mean value of activity or MGS score of all mice (n=14) receiving 1.5mg/kg buprenorphine at each time point (n=5).

There did not appear to be any significant difference in pathology-survival score between mice given buprenorphine, at either dose, and untreated mice injected with either venom (Figure 5.4-7).

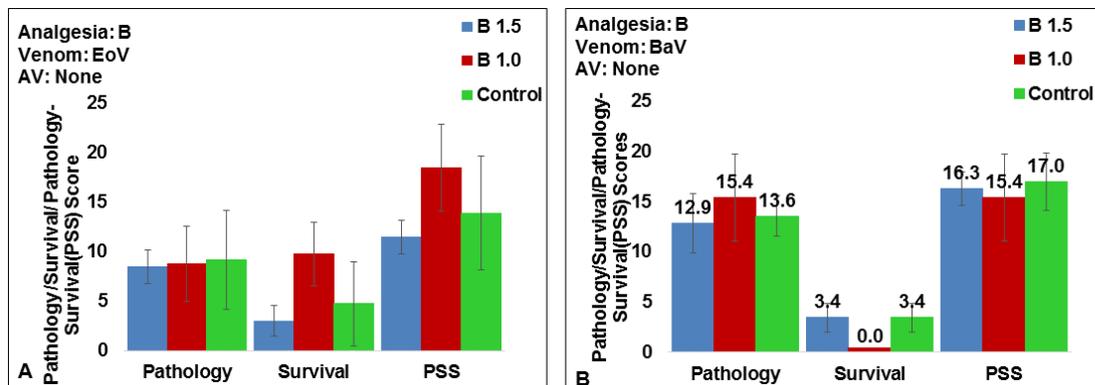


Figure 5.4-7: The effect of buprenorphine (1.5 or 1mg/Kg) on ampunt of pathology in and survival of mice injected with *E. ocellatus* venom (A) and *B. arietans* venom (B).

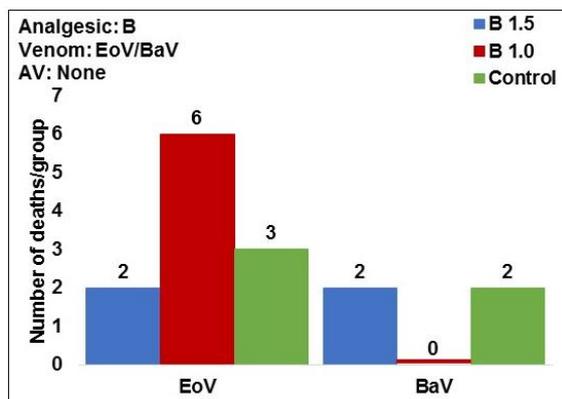


Figure 5.4-8: The effect of buprenorphine on death rate of mice injected with *E. ocellatus* venom or *B. arietans* venom.

There were, however, twice as many deaths in the group of mice injected with *E. ocellatus* venom and 1.0mg/kg buprenorphine (Figure 5.4-8), but no observable difference between death rate for buprenorphine treated and untreated mice in mice injected with *B. arietans* venom.

Morphine

The dose response study for morphine analgesia was designed to identify possible toxic effects of subsequent dosing with oral morphine. To ensure all mice got their initial dose of morphine, it was injected intraperitoneally prior to envenomation or by i.v. injection of PBS (controls). Three hours after the initial morphine dose, the mice were 'topped up' with one of 2 doses of oral morphine and monitored for a further 150 minutes. In this experiment a neurotoxic (for

mice) elapid venom was used because interaction between opioid analgesics and neurotoxins is more likely than with non-neurotoxic viper venoms. The experimental design is shown in Table 5.4-1

Table 5.4-1: Morphine dose response study—numbers of mice in each treatment group

MS = Morphine sulphate injection prior to venom injection; OM = oral morphine given 3h after morphine injection
NnigV = *Naja nigricollis* venom; PBS = Phosphate buffered saline

Venom	Number of mice		
	MS	OM dose (mg/kg) 3h	
	5mg/kg	10mg	15mg
NnigV	20	10	10
PBS	20	10	10

The results are shown in Figure 5.4-9 and Figure 5.4-10 and summarised in the 'Tables' supplement for this chapter.

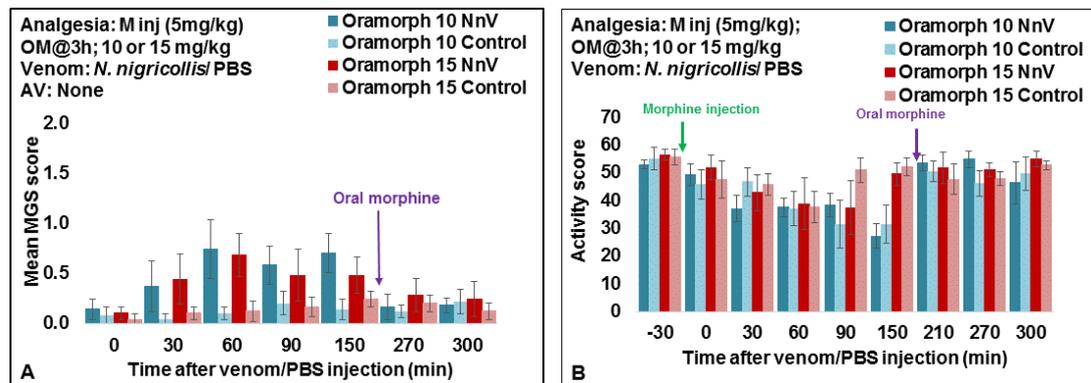


Figure 5.4-9: Effect of morphine injected i.p. (5mg/kg) followed by oral morphine (10 or 15 mg/kg) 3 hours later on MGS (A) and activity (B) scores of mice injected with either *N. nigricollis* venom or PBS (controls).

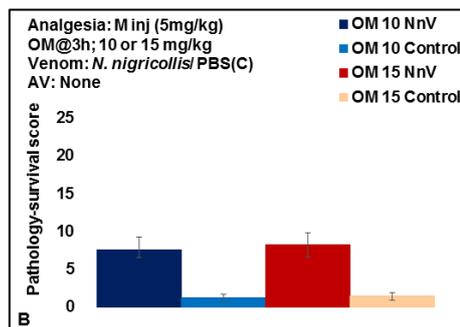


Figure 5.4-10: The effect of morphine injected i.p. (5mg/kg) followed by oral morphine (10 or 15 mg/kg) 3 hours later on PSS of mice injected with *N. nigricollis* venom or PBS (controls).

5.4.3 Analgesia in LD₅₀ assays

5.4.3.1 Buprenorphine

Two groups of 10 mice were assigned to each treatment group, one receiving buprenorphine intraperitoneally, and the controls given an equal volume of normal saline by the same route, approximately 30 minutes prior to i.v. injection of one of the 5 test venoms and observations were regularly performed. After 3 hours, the surviving mice were recovering and there was no discernable difference between groups.

Results are shown in Table 5.4-2. In all tables in this chapter, n indicates the number of mice from which data was obtained. Deaths before the end of the experiment resulted in a reduction of numbers from which data could be obtained (i.e. where n=<10).

Table 5.4-2: Summary of the effect of buprenorphine on MGS score and pathology/survival of envenomed mice. () = number of mice. 95% CI in green. B = buprenorphine treated, C = control.

Venom		EoV		BaV		VbV		NnigV		DaV	
Analgesia											
Time (h)	Observation	B	C	B	C	B	C	B	C	B	C
0	Mean MGS Score 95% CI (n)	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.12	0.00	0.00
		0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.11	0.00	0.00
(10)		(10)	(10)	(10)	(10)	(10)	(10)	(10)	(10)	(10)	(9)
1		1.24	1.87	1.28	1.95	1.15	1.98	1.80	1.74	1.93	2.00
		0.54	0.33	0.38	0.06	0.44	0.04	0.00	0.25	0.12	0.00
2		(10)	(9)	(8)	(8)	(8)	(10)	(6)	(7)	(9)	(9)
		1.11	2.00	1.16	1.88	1.43	1.88	1.70	1.13	2.00	2.00
3		0.56	0.00	0.19	0.10	0.39	0.20	0.58	1.17	0.00	0.00
		(9)	(8)	(5)	(5)	(8)	(10)	(2)	(3)	(9)	(9)
Pathology Score (95% CI)		1.04	2.00	-	-	1.28	1.76	2.00	0.67	2.00	1.87
		0.52	0.00	-	-	0.61	0.29	0.00	1.30	0.00	0.29
Survival time (95% CI)		(9)	(8)	(0)	(0)	(8)	(10)	(2)	(3)	(9)	(9)
	3.7	3.8	7.6	6.2	2.4	2.6	6.4	4.9	4.9	3.6	
PSS (95% CI)	0.9	1.4	1.4	1.2	0.5	0.7	3.0	2.5	1.6	1.5	
	330	295	70	77	286	360	126	126	319	360	
Deaths	59	86	27	28	88	0	78	84	4	0	
	4.7	6.0	17.6	16.0	5.4	2.6	14.7	11.7	6.4	3.6	
Deaths	2.2	3.8	1.1	0.7	3.1	0.7	5.4	5.2	3.2	3.2	
	1/10	2/10	10/10	10/10	3/10	0/10	8/10	8/10	4/10	0/9	

Buprenorphine administration effectively reduced MGS scores in mice envenomed with viper venoms (example Figure 5.4-11A), but not those envenomed with elapid venoms (example Figure 5.4-12A).

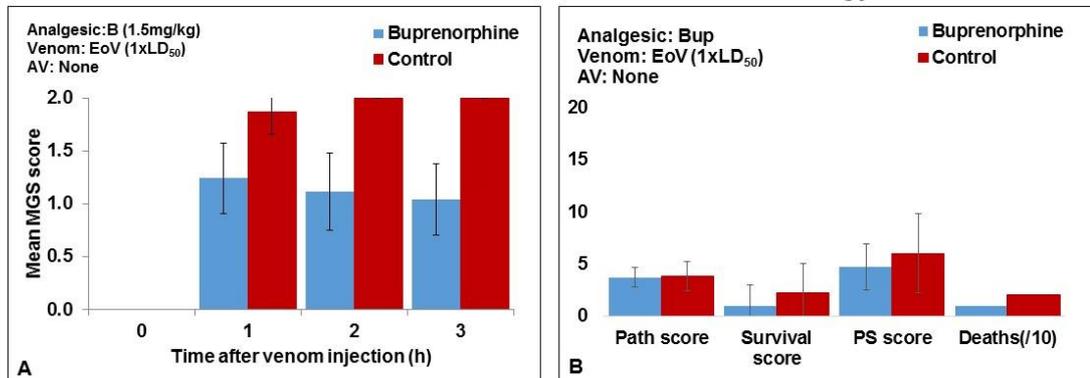


Figure 5.4-11: The effect of buprenorphine (1.5mg/Kg) on MGS score and pathology/survival of mice injected with *E. ocellatus* venom, selected as an example of a viper. The results for mice envenomed with the other viper venoms studied can be found in the Chapter supplement.

PS scores of control mice were lower than those of buprenorphine treated mice except for those injected with *E. ocellatus* venom (Figure 5.4-11B).

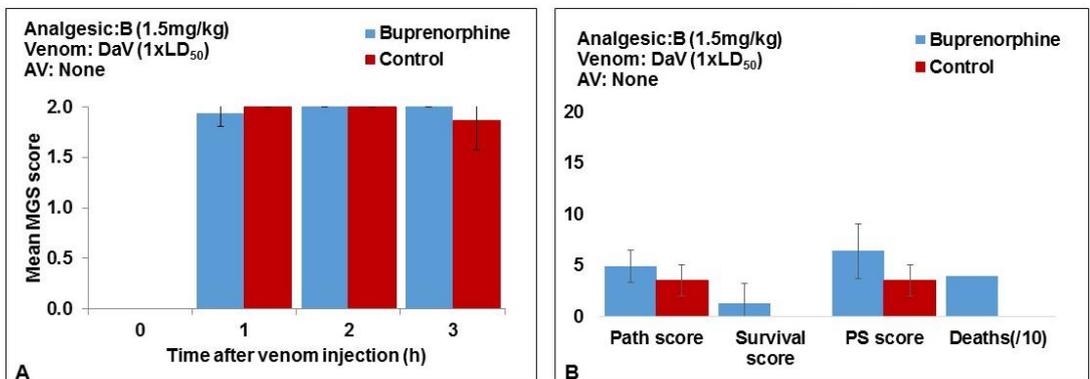


Figure 5.4-12: The effect of buprenorphine (1.5mg/Kg) on MGS score and pathology/survival of mice injected with *D. angusticeps* venom, selected as an example of an elapid. The results for mice envenomed with the other elapid venom studied can be found in the Chapter supplement.

5.4.3.2 Kaplan-Meier survival analysis

The survival time data was pooled from all the venoms studied and analysed using Kaplan-Meier (KM) survival analysis; this non-parametric statistical analysis estimates the probability of death and effectiveness of medication.

Here the KM survival analysis is used to determine any statistically significant differences in death rates between analgesia-treated and control mice.

In the case of buprenorphine, there was a statistically higher probability ($p < 0.05$) of death in treated vs untreated mice (Table 5.4-3 and Figure 5.4-4).

Buprenorphine: Means and Medians for Survival Time				
Analgesia	Mean ^a			
	Estimate	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	289.393	15.957	258.117	320.669
1	253.883	18.049	218.506	289.260
Overall	271.501	12.189	247.611	295.391

a. Estimation is limited to the largest survival time if it is censored.

Table 5.4-3: KM survival analysis of envenomed mice treated with buprenorphine. 0 = no analgesia; 1 = buprenorphine analgesia.

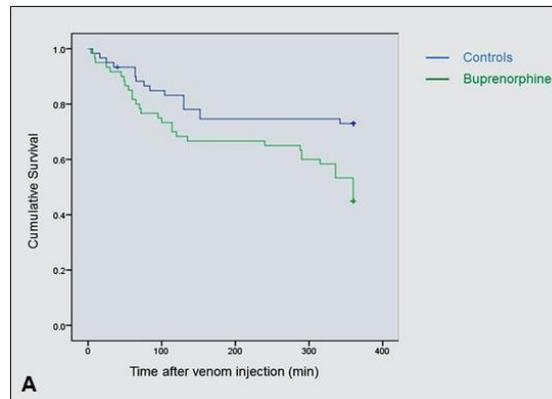


Figure 5.4-13: KM survival analysis of envenomed mice treated with buprenorphine. As deaths occur, cumulative survival falls.

5.4.3.3 Morphine

Experiments were carried out as above, except the treated mice were given oral morphine prior to venom injection. Mice were ‘topped up’ after 3 hours if their MGS score and/or behaviour indicated undue suffering. In reality, most of the mice had started to recover or had completely recovered after 3 hours. The effect of oral morphine on MGS score is shown in Table 5.4-4, Figure 5.4-14A and Figure 5.4-15A; on activity score in Table 5.4-5, Figure 5.4-14B and Figure 5.4-15B.

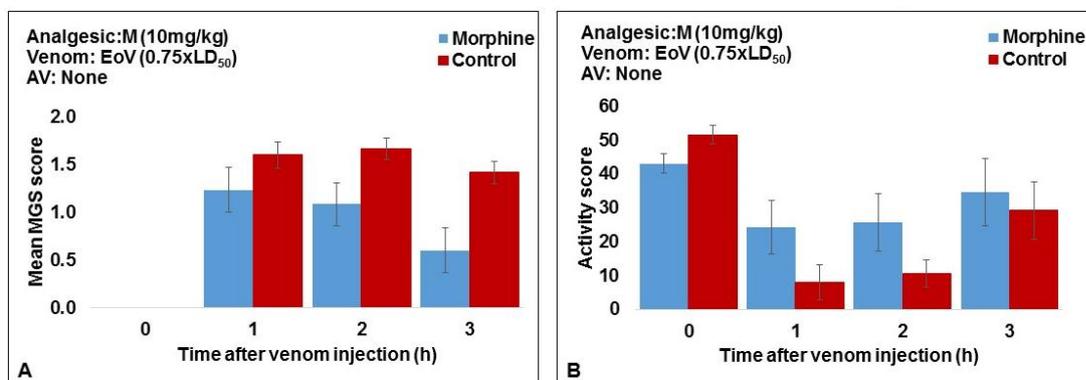


Figure 5.4-14: The effect of oral morphine (10mg/Kg) on MGS and activity scores of mice injected with *E. ocellatus* venom (viper).

Mice envenomed with any of the five venoms studied showed a significant reduction of MGS scores, increased activity scores but, no significant difference in pathology/survival score. Examples of the results are illustrated for a viper (*E. ocellatus* venom - Figure 5.4-14) and an elapid (*D. angusticeps* venom - Figure 5.4-15), the complete result set can be found in the chapter supplement.

Table 5.4-4: The effect of oral morphine on MGS score of envenomed mice. () = number of mice. 95% CI in green. M = oral morphine, C = control.

Venom		EoV		BaV		VbV		NnigV		DaV	
Analgesia		M	C	M	C	M	C	M	C	M	C
Pre-venom	Mean MGS Score ()=num 95% CI	0.00 (20) #	0.00 (20) #	0.00 (10) #	0.00 (10) #	0.03 (30) 0.02	0.00 (20) #	0.06 (10) 0.06	0.00 (10) #	0.08 (10) 0.09	0.00 (10) 0.00
		1.23 (12) 0.24	1.60 (13) 0.14	0.61 (10) 0.22	0.99 (8) 0.25	0.95 (28) 0.19	1.63 (14) 0.20	1.70 (8) 0.26	1.90 (8) 0.10	1.43 (7) 0.27	1.84 (9) 0.12
		1.08 (12) 0.22	1.66 (13) 0.11	0.70 (10) 0.36	0.95 (8) 0.37	0.78 (26) 0.21	0.57 (14) 0.32	1.14 (7) 0.47	1.88 (5) 0.24	1.26 (7) 0.30	1.80 (9) 0.12
		0.60 (11) 0.24	1.42 (13) 0.12	0.46 (10) 0.23	0.85 (8) 0.35	0.48 (18) 0.21	0.39 (14) 0.49	0.37 (7) 0.25	1.92 (5) 0.10	0.89 (7) 0.35	1.84 (9) 0.08

Table 5.4-5: The effect of oral morphine on activity score of envenomed mice. () = number of mice. 95% CI in green. M = oral morphine, C = control.

Venom		EoV		BaV		VbV		NnigV		DaV	
Analgesia		M	C	M	C	M	C	M	C	M	C
Pre-venom	Activity Score ()=num 95% CI	43.1 (20) 2.8	51.6 (20) 2.7	40.0 (10) 3.0	24.4 (10) 14.3	50.1 (30) 3.1	58.3 (20) 2.5	55.2 (10) 2.3	59.2 (10) 2.3	59.3 (10) 3.0	63.8 (10) 1.6
		24.2 (12) 7.9	8.0 (13) 5.1	32.5 (10) 3.7	23.6 (8) 8.9	33.6 (28) 6.0	23.1 (14) 5.3	11.3 (7) 5.3	1.3 (8) 0.3	13.6 (7) 7.2	5.3 (9) 4.0
		25.8 (12) 8.6	10.6 (13) 4.0	42.7 (10) 2.9	26.9 (8) 6.6	34.7 (26) 5.9	34.7 (14) 5.1	27.4 (7) 10.0	16.2 (5) 17.2	15.1 (7) 8.5	5.6 (9) 3.1
		34.6 (12) 9.9	29.3 (13) 8.5	35.1 (10) 7.1	26.1 (8) 3.2	50.3 (18) 2.9	47.6 (14) 6.0	36.1 (7) 9.4	5.8 (5) 5.1	21.9 (7) 10.8	8.3 (9) 4.8

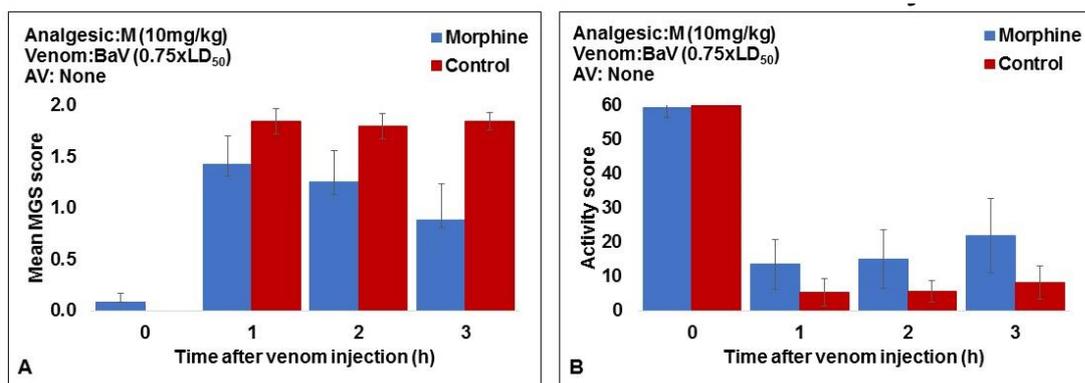


Figure 5.4-15: The effect of oral morphine (10mg/Kg) on MGS and activity scores of mice injected with *D. angusticeps* venom (elapid).

There were some discrepancies in death rate with different venoms, more deaths occurring in morphine treated mice envenomed with *E. ocellatus* venom and *D. angusticeps* venom, but fewer deaths in those envenomed with *B. arietans* venom, *V. berus* venom and *N. nigricollis* venom (Table 5.4-6, Figure 5.4-16 and Figure 5.4-17). This led to concerns whether administration of morphine may affect the assay results.

Table 5.4-6: The effect of oral morphine on pathology/survival of envenomed mice. () = number of mice. 95% CI in green

Venom	EoV		BaV		VbV		NnigV		DaV	
	M	C	M	C	M	C	M	C	M	C
Analgesia										
Pathology Score	10.2 (20) 2.3	9.6 (20) 2.0	10.7 (10) 1.7	11.9 (10) 0.9	8.7 (20) 1.2	8.9 (15) 1.1	7.1 (10) 1.9	9.6 (10) 1.8	7.6 (10) 2.4	8.5 (10) 0.9
Mean survival time (min)	209 (20) 76	243 (20) 72	360 (10) #	288 (10) 94	283 (30) 23	224 (20) 52	273 (10) 87	213 (10) 97	235 (10) 104	325 (10) 69
Survival Score	5.2 (20) 2.6	4.1 (20) 2.5	0 (10) #	2.4 (10) 3.1	1.0 (30) 0.9	2.7 (20) 1.9	3 (10) 3.0	5.1 (10) 3.4	4.3 (10) 3.6	1.2 (10) 2.4
Pathology/ Survival score	15.9 (20) 5.0	13.7 (20) 4.3	10.7 (10) 1.7	14.3 (10) 3.4	9.6 (20) 1.8	12.5 (15) 2.8	10.1 (10) 4.7	14.7 (10) 4.8	11.9 (10) 5.6	9.7 (10) 2.8
Deaths	9/20	7/20	0/10	2/10	4/30	6/20	3/10	5/10	4/10	1/10

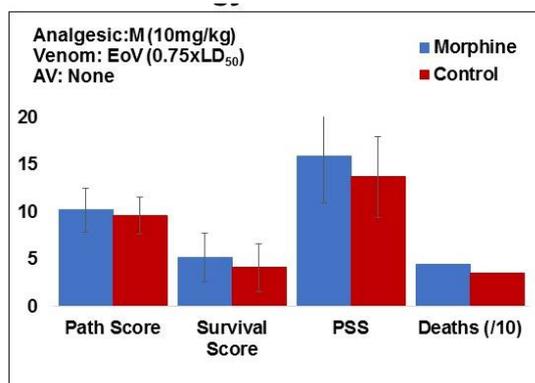


Figure 5.4-16: The effect of oral morphine (10mg/Kg) on pathology/survival of mice injected with *E. ocellatus* venom (viper).

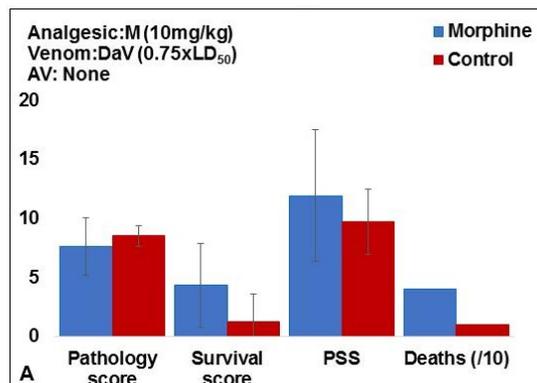


Figure 5.4-17: The effect of oral morphine (10mg/Kg) on pathology/survival of mice injected with *D. angusticeps* venom (elapid).

5.4.3.4 Kaplan-Meier survival analysis

Kaplan-Meier survival analysis was performed on results pooled from all venoms, as described above. In this case, the results showed that predicted survival times were not significantly different ($p < 0.05$) in morphine-treated or control mice. The results are illustrated in Table 5.4-7 and Figure 5.4-18.

Table 5.4-7: Kaplan Meier survival analysis of envenomed mice treated with or oral morphine. 0 = no analgesia; 1 = oral morphine analgesia.

Morphine: Means and Medians for Survival Time				
Analgesia	Mean ^a			
	Estimate	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	300.018	12.170	276.165	323.872
1	295.567	11.640	272.753	318.381
Overall	297.686	8.413	281.196	314.175

a. Estimation is limited to the largest survival time if it is censored.

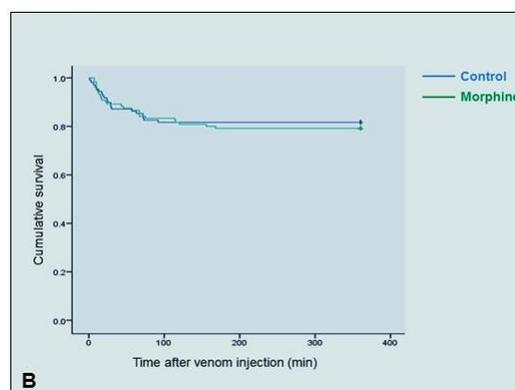


Figure 5.4-18: Kaplan Meier survival analysis of envenomed mice treated with or oral morphine.

Considered collectively, these results showed no difference in death rate when the experimental results using all venoms studied were pooled; ten mice per group being insufficient to perform the KM survival analysis for individual

venoms. Therefore the number of mice employed in experiments using venoms for which there were discrepancies in death rates between morphine treated and untreated control mice was increased. The analgesia LD₅₀ experiments using *E. ocellatus* venom, *B. arietans* venom, *V. berus* venom and *D. angusticeps* venom, using 30 mice per experimental group, were repeated.

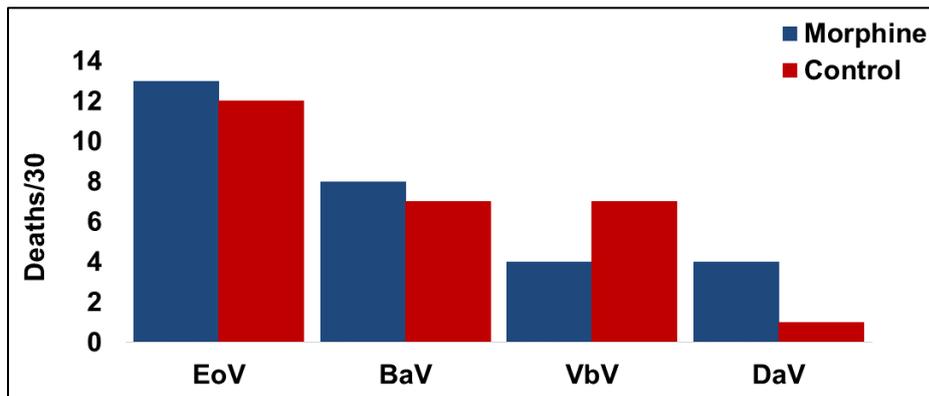


Figure 5.4-19: The effect of morphine analgesia on death rate of mice envenomed with *E. ocellatus* venom (EoV), *B. arietans* venom (BaV), *V. berus* venom (VbV) or *D. angusticeps* venom (DaV)

The results show that there were fewer deaths in the morphine-treated than in control mice envenomed with *V. berus* venom, and more deaths in morphine-treated than control mice, envenomed with *D. angusticeps* venom. KM analysis revealed that there was no significant difference ($p < 0.05$) between survival of morphine-treated or control mice envenomed with *E. ocellatus* or *B. arietans* venoms (Table 5.4-8 A and B).

Table 5.4-8: Kaplan Meier survival analysis of mice envenomed with *E. ocellatus* (A); *B. arietans* (B); *V. berus* (C) or *D. angusticeps* (D) venoms comparing morphine analgesia with untreated subjects. There were 30 mice in each treatment group.

A	Mean ^a				
	Analgesia	Estimate	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
Control	225.133	30.189	165.964	284.303	
Morphine	220.233	29.900	161.629	278.838	
Overall	222.683	21.247	181.039	264.328	

a. Estimation is limited to the largest survival time if it is censored.

B	Mean ^a				
	Analgesia	Estimate	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
Control	276.700	27.568	222.667	330.733	
Morphine	266.300	28.369	210.696	321.904	
Overall	271.500	19.790	232.711	310.289	

a. Estimation is limited to the largest survival time if it is censored.

C	Mean ^a				
	Analgesia	Estimate	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
Control	285.833	24.585	237.647	334.020	
Morphine	324.767	16.644	292.143	357.390	
Overall	305.300	15.056	275.790	334.810	

a. Estimation is limited to the largest survival time if it is censored.

D	Mean ^a				
	Analgesia	Estimate	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
Control	348.333	11.471	325.851	370.816	
Morphine	318.400	19.907	279.382	357.418	
Overall	333.367	11.649	310.535	356.199	

a. Estimation is limited to the largest survival time if it is censored.

However, the mean survival time for morphine-treated mice envenomed with *V. berus* venom was within the 95% confidence intervals (CI) of the control mice, but that of the control mice was lower than the lower 95% CI value for morphine-treated mice (Table 5.4-8C). The mean survival time for morphine-treated mice envenomed with *D. angusticeps* venom was lower than the lower 95% CI of control mice, but that of the control mice was within the 95% CIs of the morphine-treated mice (Table 5.4-8D); i.e. the results were inconclusive where $p < 0.05$. The KM analysis plots are illustrated in Figure 5.4-20

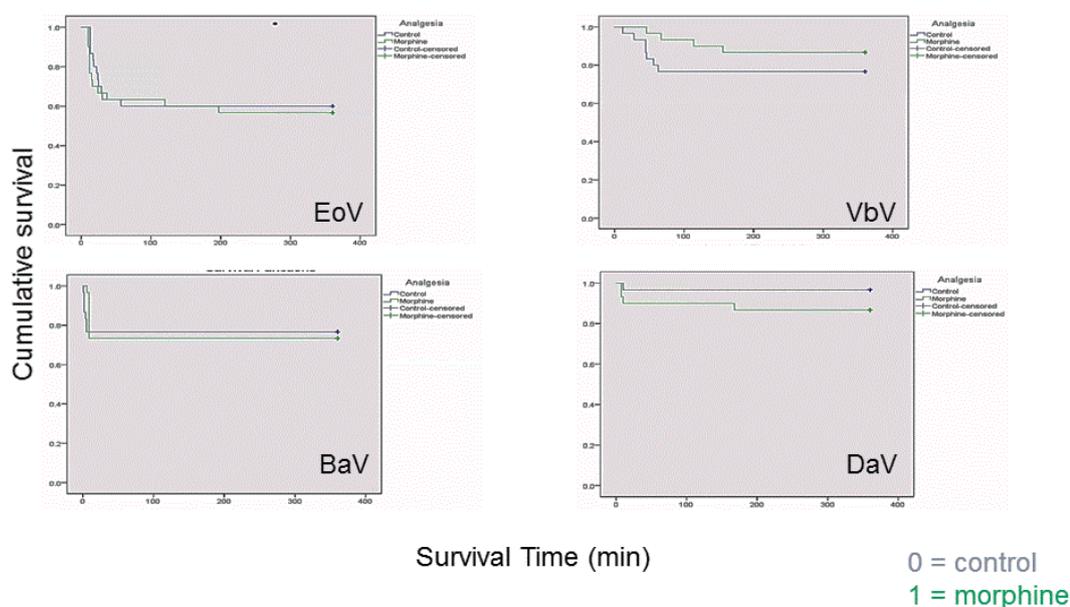


Figure 5.4-20: Kaplan Meier survival analysis plots for analgesic venom LD₅₀s comparing survival of morphine-treated versus control mice. N=30 for each experimental group.

5.4.3.5 Analgesia in LD₅₀ assays – the effect of variant venom doses on the analgesic benefits of buprenorphine

An experiment was conducted to determine the effectiveness of buprenorphine at different venom doses. Regular preclinical testing of antivenoms directed against *V. berus* venom is carried out by the ARVRU, therefore to minimise the number of mice required, this experiment was carried out in conjunction with a routine LD₅₀ for this venom. Three different doses of venom were chosen and the experiment conducted as for the other analgesia LD₅₀s described above.

The MGS score was less in mice treated with buprenorphine compared to untreated controls at all three venom doses (Figure 5.4-21). PSS was significantly higher in the buprenorphine-treated mice (at 90%CI), as was the death rate in treated groups (Figure 5.4-22). Interestingly, deaths at the lower venom doses were HEPs due to hyphaema, enlargement of the eye(s) as the result of haemorrhage into the orbit(s), and occurred several hours after envenomation.

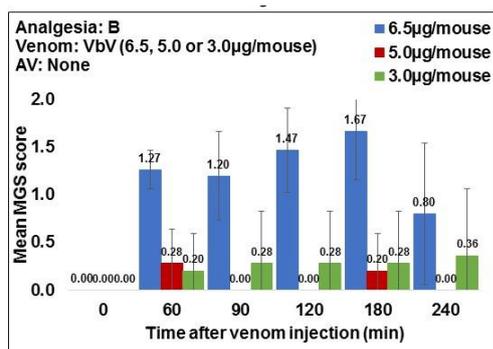


Figure 5.4-21: The effect of buprenorphine dose on mice envenomed with different doses of VbV.

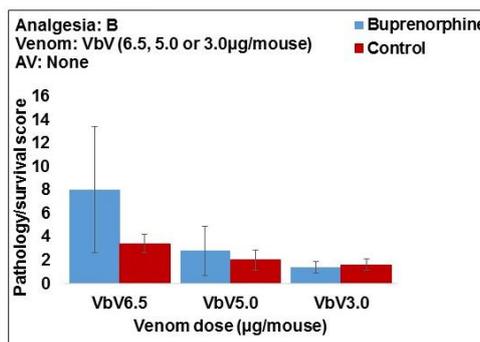


Figure 5.4-22: The effect of buprenorphine dose on pathology/survival of mice envenomed with different doses of VbV.

5.4.4 Analgesia in ED₅₀ assays

These experiments were performed to determine:

- the pain-relieving benefits of analgesia in ED₅₀ assays
- whether administration of analgesia affected the metric of the ED₅₀ results

5.4.4.1 Buprenorphine

Only one venom/antivenom combination, *V. berus* venom with ViperaTab antivenom, was performed using buprenorphine analgesia for the reasons stated in the experiment of Section 1.4.3.5. The experiment was not repeated using the other venom/antivenom combinations because, by this time, it had become obvious that administration of buprenorphine was affecting the metric of the LD₅₀ assays, with the buprenorphine-treated groups having a significantly higher death rate ($p < 0.05$) than the untreated controls, using KM

survival analysis. In addition, buprenorphine appeared to be ineffective at relieving the pain and suffering associated with elapid envenomation.

In the ED₅₀ experiment that was performed, it was shown that buprenorphine administration reduced the mean MGS score (Figure 5.4-23A) whilst activity scores (Figure 5.4-23B) were higher than in control mice, thus demonstrating that analgesia is effective. There were no deaths in either group and the PSS was not significantly different (Figure 5.4-24). The full set of results can be found in the chapter supplement in Table 5. These results did not show the increased lethality in the buprenorphine treated group, but it would appear that the antivenom in this experiment had completely neutralised the lethal effects of the venom. The buprenorphine treated mice were more active prior to venom injection than the controls ($p < 0.05$), potentially resulting in a higher death rates in groups where the antivenom dose did not completely neutralise venom lethality.

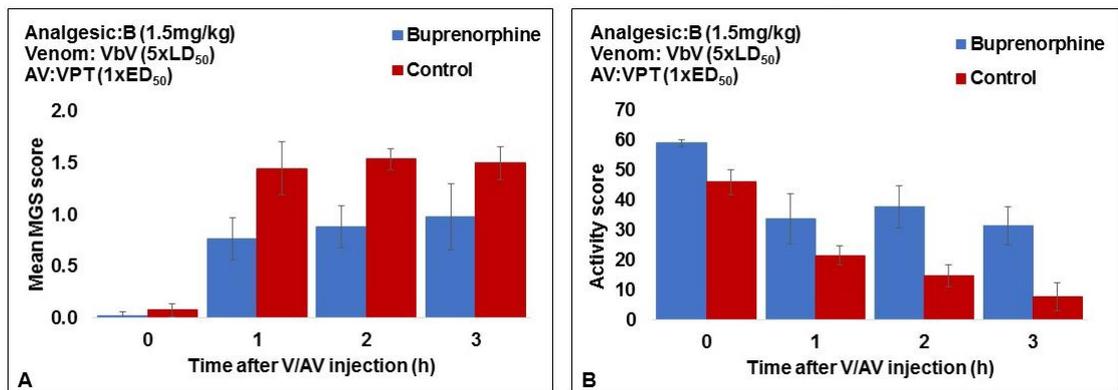


Figure 5.4-23: The effect of buprenorphine (1.5mg/Kg) on MGS (A) and activity (B) scores of mice injected with VbV incubated with VPT antivenom

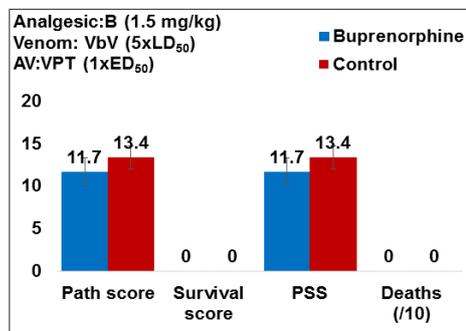


Figure 5.4-24: The effect of buprenorphine (1.5mg/Kg) on pathology/survival of mice injected with *V. berus* venom incubated with ViperaTab antivenom.

5.4.4.2 Morphine

Having determined that buprenorphine did not satisfy the criteria for an acceptable analgesic to use in the preclinical assays of venom toxicity and antivenom efficacy, and having shown morphine to be an acceptable alternative in LD₅₀ assays, ED₅₀s were performed on all five of our selected venom/antivenom combinations using this analgesic.

All the venom/antivenom combinations showed a reduction in mean MGS score (Table 5.4-9), examples are illustrated in Figure 5.4-25A and Figure 5.4-26A. There was an inverse relationship between MGS and activity scores, the morphine treated mice showing greater activity than the controls at each time point (Table 5.4-10), examples are given in Figure 5.4-25B and Figure 5.4-26B. One viper venom and one elapid venom and their homologous antivenoms were selected here; the two venoms which showed differences in estimated survival times using KM survival analysis.

All the graphs illustrating these results can be found in the chapter supplement.

Table 5.4-9: The effect of oral morphine (10mg/Kg) on MGS of mice injected with venom incubated with their homologous antivenom. M= morphine treated, C= controls

Venom		EoV		BaV		VbV		NnigV		DaV	
Antivenom		EchiTabG		EchiTabPlus		ViperaTab		EchiTabPlus		SAIMRp	
Analgesia		M	C	M	C	M	C	M	C	M	C
Pre-venom	Mean MGS score (n) 95% CI	0.00 (10) #	0.00 (10) #	0.18 (10) 0.04	0.00 (10) #	0.07 (20) 0.04	0.00 (20) #	0.14 (10) 0.08	0.00 (9) #	0.14 (10) 0.08	0.00 (10) #
1h		1.71 (7) 0.20	1.86 (7) 0.14	0.44 (10) 0.10	0.91 (9) 0.19	1.17 (18) 0.30	1.65 (20) 0.16	0.48 (10) 0.31	0.98 (9) 0.21	0.22 (10) 0.14	0.89 (10) 0.15
2h		1.61 (5) 0.19	1.86 (7) 0.12	0.14 (10) 0.14	0.62 (9) 0.14	0.92 (18) 0.31	1.42 (20) 0.20	0.54 (10) 0.30	1.16 (9) 0.17	0.12 (10) 0.09	0.98 (10) 0.27
3h		1.60 (5) 0.18	1.71 (7) 0.17	0.06 (10) 0.06	0.27 (9) 0.26	0.32 (18) 0.19	0.52 (20) 0.26	0.28 (10) 0.12	1.00 (9) 0.27	0.12 (10) 0.06	0.57 (10) 0.21

Table 5.4-10: The effect of oral morphine (10mg/Kg) on activity of mice injected with venom incubated with their homologous antivenom.

Venom		EoV		BaV		VbV		NnigV		DaV	
Antivenom		EchiTabG		EchiTabPlus		ViperaTab		EchiTabPlus		SAIMRp	
Analgesia		M	C	M	C	M	C	M	C	M	C
Pre-venom	Mean activity score (n) 95% CI	50.1 (30) 3.1	58.3 (20) 2.5	43.1 (20) 2.8	51.6 (20) 2.7	40.0 (10) 3.0	24.4 (10) 14.3	55.2 (10) 2.3	59.2 (10) 2.3	59.3 (10) 3.0	63.8 (10) 1.6
		33.6 (28) 6.0	23.1 (14) 5.3	24.2 (12) 7.9	8.0 (13) 5.1	32.5 (10) 3.7	23.6 (8) 8.9	11.3 (7) 5.3	1.3 (8) 0.3	13.6 (7) 7.2	5.3 (9) 4.0
		34.7 (26) 5.9	34.7 (14) 5.1	25.8 (12) 8.6	10.6 (13) 4.0	42.7 (10) 2.9	26.9 (8) 6.6	27.4 (7) 10.0	16.2 (5) 17.2	15.1 (7) 8.5	5.6 (9) 3.1
		50.3 (18) 2.9	47.6 (14) 6.0	34.6 (12) 9.9	29.3 (13) 8.5	35.1 (10) 7.1	26.1 (8) 3.2	36.1 (7) 9.4	5.8 (5) 5.1	21.9 (7) 10.8	8.3 (9) 4.8

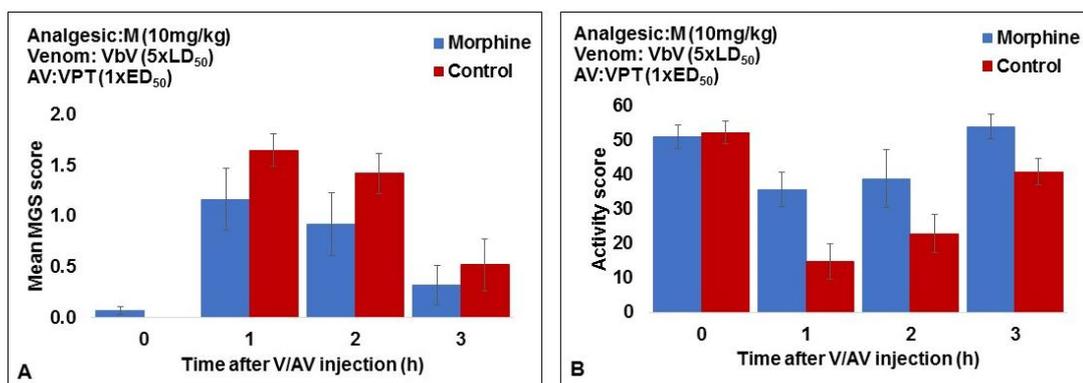


Figure 5.4-25: The effect of oral morphine (10mg/Kg) on MGS (A) and activity (B) scores of mice injected with *V. berus* venom incubated with VPT antivenom

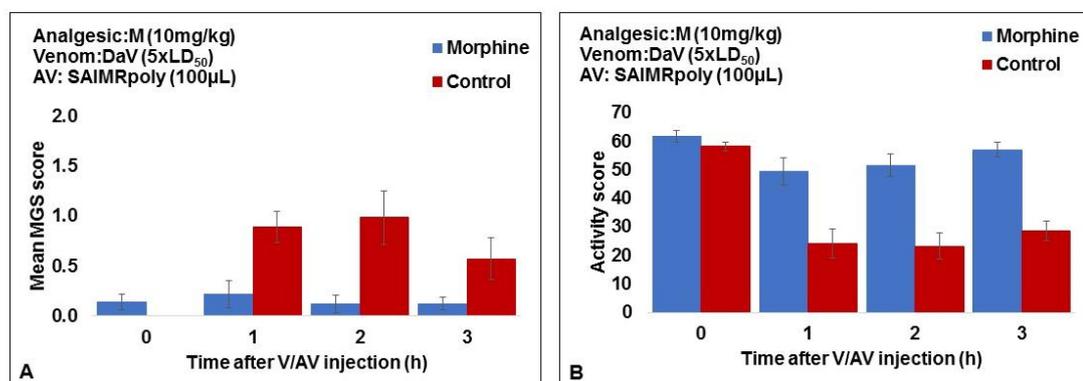


Figure 5.4-26: The effect of oral morphine (10mg/Kg) on MGS (A) and activity (B) scores of mice injected with *D. angusticeps* venom incubated with SAIMRp antivenom.

There was no significant difference in PSS between morphine treated and control mice (Table 5.4-12); Kaplan Meier survival analysis showed no significant difference ($p < 0.05$) in estimated survival time (Table 5.4-11).

Table 5.4-11: Kaplan Meier survival analysis analgesia ED₅₀s

Analgesia	Mean			
	Estimate	Std. Error	95% confidence intervals	
			Lower Bound	Upper Bound
Control	337.200	11.020	315.602	358.798
Morphine	323.917	12.894	298.645	349.189
Overall	330.558	8.502	313.894	347.223

Examples are shown in Figure 5.4-27 and Figure 5.4-28.

Table 5.4-12: The effect of oral morphine (10mg/Kg) on pathology/survival of envenomed mice. Numbers in brackets = number of subjects, numbers in green = 95% CI

Venom	EoV		BaV		VbV		NnigV		DaV	
AV	EchiTabG		EchiTabPlus		ViperaTab		EchiTabPlus		SAIMRp	
Analgesia	M	C	M	C	M	C	M	C	M	C
Pathology Score	13.4 (10) 2.5	12.3 (10) 2.0	4.3 (10) 0.7	6.8 (10) 1.0	6.0 (20) 1.3	6.2 (20) 1.1	4.9 (10) 0.9	6.8 (9) 1.4	6.2 (10) 1.3	7.0 (10) 1.0
Mean survival time (min)	205 (10) 103	259 (10) 101	360 (10) #	325 (10) 69	329 (20) 34	360 (20) #	360 (10) #	360 (9) #	360 (10) #	360 (10) #
Survival Score	5.5 (10) 3.6	3.6 (10) 3.6	0 (10) #	1.2 (10) 2.4	1.1 (20) 1.4	0 (20) #	0 (10) #	0 (9) #	0 (10) #	0 (10) #
PSS	19.9 (10) 6.5	15.9 (10) 5.2	4.3 (10) 0.7	8.0 (10) 3.0	7.0 (20) 2.0	6.2 (20) 1.1	4.9 (10) 0.9	6.8 (9) 1.4	6.2 (10) 1.3	7.0 (10) 1.0
Deaths	5/10	3/10	0/10	1/10	2/20	0/20	0/10	0/9	0/10	0/10

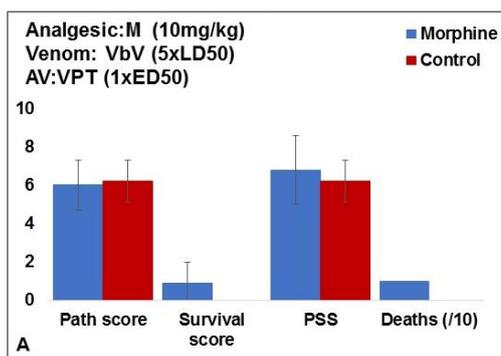


Figure 5.4-27: The effect of oral morphine (10mg/Kg) on pathology/survival of mice injected with *V. berus* venom incubated with ViperaTab antivenom.

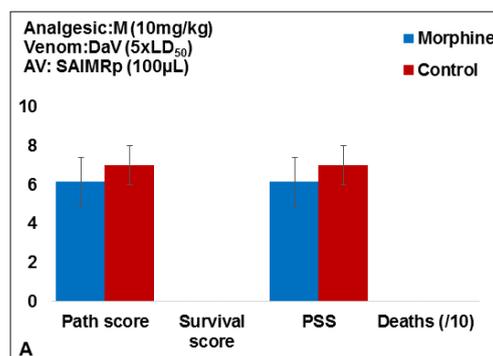


Figure 5.4-28: The effect of oral morphine (10mg/Kg) on pathology/survival of mice injected with *D. angusticeps* venom incubated with SAIMRp antivenom.

5.4.5 Preclinical testing – A retrospective analysis of outcome of full LD₅₀/ED₅₀ assays between morphine-treated and untreated mice

Previous analgesic LD₅₀s and ED₅₀s used only one dose of venom and antivenom, but preclinical assays of antivenom efficacy (PCT) use a variety of venom/antivenom doses, including one group of ‘all deaths’; it was necessary to ensure that giving analgesia did not affect the overall outcome of the experiment. Experimental PCT data from the ARVRU archives were compared with recent data when analgesia was implemented into the routine preclinical testing (PCT) of ViperaTab (VPT) antivenom. This involved both full *V. berus* venom LD₅₀ (Table 5.4-13) as well as the antivenom ED₅₀ (Table 5.4-14).

Kaplan Meier survival analysis was performed and showed no significant difference in predicted survival time for the LD₅₀s (Figure 5.4-29A), but significantly longer survival and fewer deaths in the morphine-treated ED₅₀ groups (Figure 5.4-29B). This may have been due to the mismatch of venom dose given to the mice (Table 5.4-14). However, the results of the two ED₅₀s were well within the normal range for ViperaTab antivenom (MicroPharm, unpublished data).

Table 5.4-13: Deaths in routine *V. berus* venom PCT with and without morphine

Venom Dose (μg)	Deaths (/5)	
	Morphine	No analgesia
2	0	0
5	1	1
6	2	1
10	5	4

Table 5.4-14: Deaths in routine PCT of ViperaTab antivenom with and without morphine

Antivenom Dose (μl)	Deaths (/10)	
	Morphine	No analgesia
3	8	10
3.5	3	9
5	1	4
10	0	0
Venom dose (μg)	30.25	36.5

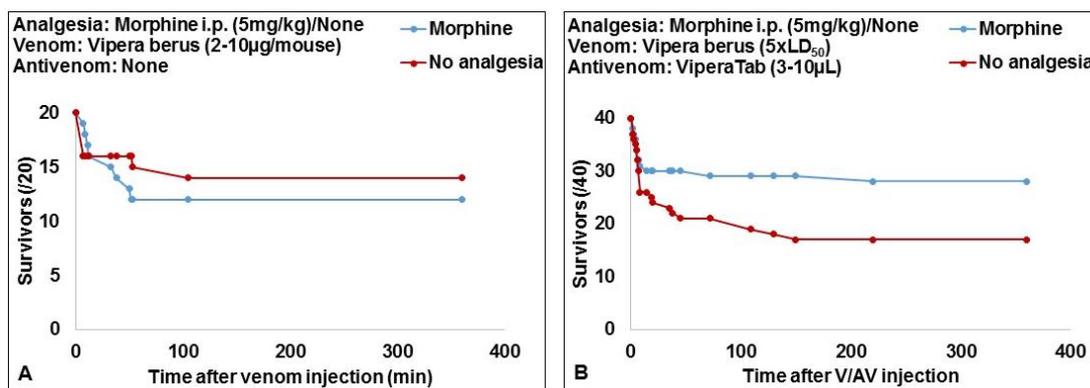


Figure 5.4-29: Survival of mice in routine preclinical testing of *V. berus* venom (A) and ViperaTab antivenom (B) with and without morphine analgesia (5mg/kg i.p. prior to venom injection followed by 10mg/kg orally, q3h, if required)

The results, using retrospective comparison of morphine analgesia versus no analgesia in the PCT of ViperaTab antivenom, caused concern that the use of analgesia affected the outcome of the assay. To address this concern, venom/antivenom combinations representing two vipers and an elapid venom family were selected, and subject numbers increased to 30 per group, as described in Section 5.4.3.3. It was shown that there was no significant difference between death rate of morphine-treated groups and the untreated controls (Figure 5.4-30).

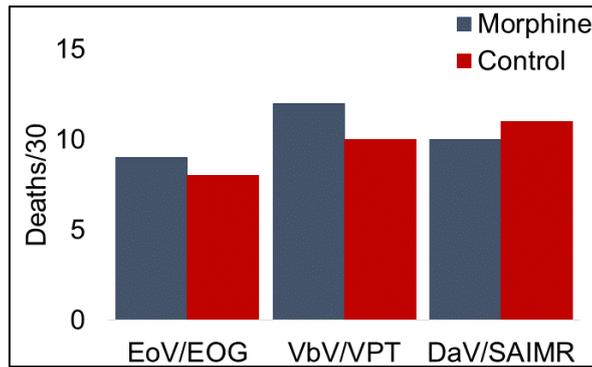


Figure 5.4-30: Analgesia in ED₅₀s: *E. ocellatus* venom (EoV)/ EchiTabG antivenom (EOG); *V. berus* venom (VbV)/ ViperaTab antivenom (VPT); *D. angusticeps* venom/ SAIMR polyvalent antivenom. Number of deaths/30 for morphine-treated vs control mice.

This was confirmed using Kaplan Meier survival analysis (Figure 5.4-31).

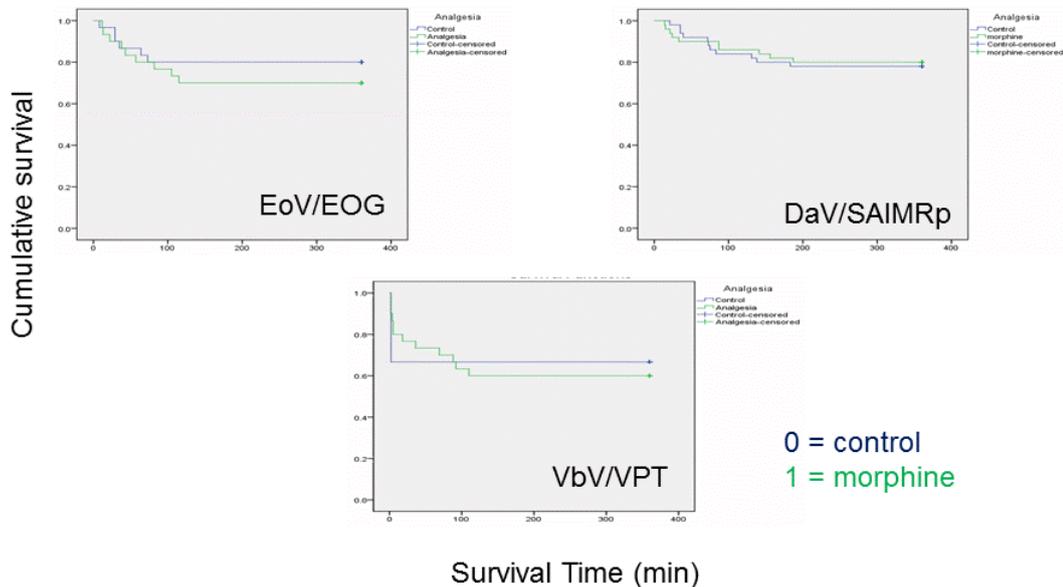


Figure 5.4-31: Kaplan Meier survival analysis plots for analgesic venom/antivenom ED₅₀s comparing survival of morphine-treated versus control mice. N=30 for each experimental group. (Abbreviations as Figure 5.4-30)

5.5 Discussion

The WHO recommended murine tests of venom LD₅₀ and antivenom ED₅₀ inflict considerable pain, distress and suffering on their subjects. As stated in Chapter 1, it is estimated that in excess of 30,000 mice are used annually in these assays. Testing antivenom efficacy is essential to avoid clinical failure of antivenom where mortality due to snakebite increased from 1.8% to 12.1% after the introduction of an ineffective antivenom (Visser et al., 2008). This is believed to be the first project in which the major focus has been to minimise the pain, distress and suffering inflicted on the subjects of these assays,

although use of analgesia has been included in assay protocol, but its efficacy was not recorded (Chacon et al, 2015)

5.5.1 Pain assessment

5.5.1.1 MGS score

The first hurdle was a reproducible, objective method of assessing pain in mice, therefore allowing the benefit, or otherwise, of analgesic intervention to be assessed. A system of scoring facial grimace was adopted by Mogil, 2010 (MGS Manual) and by Langford et al., 2010. Photographs were taken, cropped so that only the face was visible and then scored according to the manual. Scoring was performed by the author throughout the study. To assess MGS consistency, a selection of 20 photos were circulated amongst both experienced animal technicians and other *in vivo* researchers for scoring. As expected, there was considerable variation in scoring, but there was agreement as to which mice were experiencing most pain and those experiencing the least discomfort.

5.5.1.2 Activity

Initial experiments relied on MGS scoring alone, but when it became apparent that buprenorphine analgesia induced hyperactivity (see below) a system of activity scoring was developed. A video recording of 30 seconds duration was made and the number of head movements of each individual during that period was counted – this differentiated between mice taking no interest in their surroundings and those which remained immobile but which took an interest in their surroundings. It was subsequently observed that activity score showed an inverse relationship to MGS score, which might reasonably be expected, given that the less pain experienced, the more normal activity is displayed. Subsequently a combination of MGS and activity scores were used as a more objective/ less subjective measurement of pain.

5.5.2 Analgesics

5.5.2.1 Buprenorphine

Buprenorphine has been shown to be effective at reducing severe pain in laboratory rodents (Gopal et al, 2002; Gades et al, 2000; Foley et al, 2001; Wereszczyfiska-Siemiakowska et al, 1987; Liles and Flecknell, 1993a) and has a half-life sufficient for effective analgesia to last for a 6 hour assay. However, it was noticed that mice treated with doses sufficient to relieve the pain of envenomation induced hyperactivity, including manic feeding behaviour. Analysis of results using Kaplan-Meier survival showed a significant increase in death rate of treated compared to control mice, when the results from all venoms studied were pooled. In addition, although buprenorphine appeared to be effective in reducing MGS score in mice envenomed with viper venoms, there was no reduction in MGS score in those envenomed with elapid venoms. It was therefore concluded that buprenorphine was not a suitable analgesic to use in these assays.

5.5.2.2 Morphine

Morphine is regarded as the 'gold standard' opiate for pain relief, but is rarely used in laboratory animals due to its short half-life (less than 2 hours). Repeated injections are to be avoided in mice envenomed with coagulopathic venoms as this could potentially induce haemorrhage and therefore impact on the results of the experiment. The previous professional veterinary experience of the author of using liquid oral morphine in rodents indicated that it was effective, and could be administered with the minimum of restraint. No reference to the use of oral morphine in laboratory animals could be found, so the maximum recommended injectable dose (10mg/kg) was decided upon. Mice were 'topped up' with a further dose (10mg/kg) every 2 hours or as required. Generally the mice appeared to find oral morphine palatable, but there were a small number of mice who resisted attempts to dose them – these were usually the mice who subsequently had a higher MGS score and required further dosing later in the experiment. Palatability could also be improved by adding fruit juice or undiluted squash, such as Ribena® to the morphine solution. Mice given oral morphine alone showed no change in activity despite

reports in the literature of hyperactivity and a characteristic dorsiflexion of their tails – the Straub phenomenon - following injection of morphine sulphate (Straub, 1911; Bilbey et al, 1960).

Potential side-effects of opiates include hyperactivity with the Straub reaction, hypotension, respiratory depression, drowsiness, GIT stasis with constipation and spasm of both the sphincters of Oddi and the bladder. Hyperactivity was apparent in the buprenorphine treated mice, but not in those dosed with oral morphine or injected with 5mg/kg morphine sulphate. Some buprenorphine-treated mice demonstrated the Straub phenomenon. It was postulated that the higher death rate in buprenorphine treated mice was related to hyperactivity and consequently more rapid distribution of venom toxins or the enhancement of venom-toxin induced hypotension. This was particularly noticeable following *B. arietans* envenomation, after which all the mice underwent a period of slumping, presumably due to profound hypotension, not reversed by antivenom. After about 30 minutes the surviving mice began to recover with few subsequent deaths and complete recovery after 3 hours. Post mortem examination showed little difference between opiate treated mice and controls except that, particularly in buprenorphine treated mice, there was a higher incidence of dilated bladders. Both the morphine and buprenorphine treated mice tended to have somewhat more congested organs than control mice, but this was a pathological feature of many of the venoms studied and therefore it was difficult to quantify the difference between treated and untreated mice.

5.5.3 Dose Response

Having established that oral morphine was largely effective, with the caveat that a minority of mice did not receive their full dose and therefore had insufficient analgesia, it was decided to give the initial dose by injection. In the dose response study, an injected dose of 5mg/kg was chosen, assuming bioavailability of 40-50% of the oral dose. The Straub effect was not observed in any of the mice, and activity did not appear to be affected by the selected dose. A second dose of morphine was administered orally 3 hours after the

first dose and observations for any signs of hyperactivity or toxicity performed. In the control mice, which were injected with PBS instead of venom, there was no significant difference in MGS score, activity score or surface temperature, nor in pathology-survival score (PSS), although there was slight but insignificant trend upwards with increasing doses of Oramorph. In the venom treated mice there was a noticeable reduction in MGS score but no significant change in activity score or surface temperature with increasing doses of Oramorph. There was no difference in PSS between the 10 and 15 mg/kg dose groups. From this experiment it can be concluded that a dose of 5mg/kg morphine sulphate can be injected into mice without causing a significant increase in activity and that this dose can be 'topped up' with up to 15 mg/kg oral morphine without adverse effects.

5.5.4 Analgesic LD₅₀s and ED₅₀s

Results of the LD₅₀ and ED₅₀ experiments showed that MGS scores were significantly reduced ($P < 0.05$) and activity scores significantly increased ($P < 0.05$) in morphine-treated mice when compared to controls in all five venoms studied, as well as those pre-incubated with their homologous antivenoms. Unlike buprenorphine, oral morphine appeared to be effective at reducing MGS scores in mice envenomed with *D. angusticeps* venom, despite the ptosis observed in these subjects.

There was a significantly smaller reduction in activity following envenomation of morphine treated mice, compared to controls, without the hyperactivity and manic feeding behaviour observed in mice treated with buprenorphine.

There was no significant difference in pathology-survival score (PSS) between morphine-treated and untreated mice. When group sizes were increased, there were small, but significant differences ($P < 0.05$) between estimated survival time for some venom groups, but not for any of the venom/antivenom combinations studied.

These results would suggest that oral morphine is effective at reducing pain in mice envenomed with a variety of venoms, with or without their antivenoms

and that there are only marginally significant effects on the outcome of the experiment in terms of pathology or deaths. These results highlight the potential differences in interaction of any additional substances (analgesics) administered with variable venom toxin profiles, and the need to define these prior to incorporation into the LD₅₀ and ED₅₀ assays.

5.5.5 Preclinical testing of antivenoms

Validation of commercial regulatory assays would require duplicate experiments to be performed in which doses administered to analgesia-treated groups were matched exactly with untreated control groups. This experiment has been performed using Tramadol analgesia in mice envenomed intraperitoneally with *Bothrops asper* venom (Chacon et al, 2015). There was no significant difference between the ED₅₀s of the two groups of mice, but no attempt was made to confirm the efficacy of the analgesia administered.

As the result of the outcome of this project, the ARVRU has introduced morphine analgesia into routine preclinical testing of venom toxicity and antivenom efficacy. Ideally duplicate experiments should have been run simultaneously, but to reduce the numbers of mice required, a retrospective comparison was performed, as described in Section 5.4.5.

5.5.6 Recommended Analgesia

It can be concluded that, although effective at reducing pain scores in mice envenomed with a variety of Viperid venoms, Buprenorphine administration appears to be ineffective at reducing pain scores in mice envenomed with elapid venom from three species belonging to two different genera. In addition, the increased PSS, possibly induced by hyperactivity, makes this particular opioid analgesic unsuitable to be recommended for use in commercial preclinical assays.

In contrast, oral morphine was effective at reducing pain scores without adversely affecting the lethality assay results in all five venoms studied, with or without their homologous antivenom. The disadvantage of its short half-life can be overcome by repeat dosing, as required, using minimal restraint

consequent to its apparent palatability. Injection of 5mg/mL intraperitoneal morphine, prior to venom administration, could be used to ensure all mice are given the correct initial dose; individuals can then be 'topped up' with liquid oral morphine thereafter on the basis of their MGS and activity scores.

Another consideration is the availability of opiate analgesia and the regulations for their use in different parts of the world. It may be that other effective analgesics, such as Tramadol (Chacon et al, 2015), at relieving the pain associated with envenomation in mice need to be identified.

The current WHO guidelines (2010a) do not include use of analgesia in the protocols for the LD₅₀ and ED₅₀ assays of venom toxicity and antivenom efficacy. On the basis of these results it is possible to recommend that the use of analgesia be considered. It has been shown that morphine analgesia is effective in envenomed mice. However, there may be some subtle effects on assay outcome, and therefore this possibility should be investigated prior to incorporation into subsequent assays.

Chapter 6. Refinement using Humane Endpoints offers the potential to Reduce numbers of mice required for Preclinical Testing of Antivenoms

6.1 Objectives

Refinement is the reduction of pain, suffering and distress experienced by *in vivo* experimental subjects, in this case, those used in WHO-recommended assays of venom toxicity and antivenom efficacy. The Refinement objective was to implement effective analgesia and to identify humane end-points (HEPs) and, in so doing, reduce the duration and intensity of pain, suffering and distress inflicted during these assays. In addition, any changes to the WHO recommendations had to be shown not to affect the outcome of HEP determination.

6.2 Introduction

This chapter covers the identification of HEPs and their use to reduce the duration and degree of pain and distress suffered by the experimental mice. The present recommended assay duration is 24 hours and the experimental metric is death (WHO, 2010). A method, 'staging', resulting in a reduction of the numbers of mice required for an LD₅₀ or ED₅₀ assay is described.

6.2.1 Refinement: Humane End Points

A HEP is defined by the Institutional Animal Care and Use Committee (IACUC) as "the earliest scientifically justified point at which pain or distress in an experimental animal can be prevented, terminated, or relieved, whilst meeting the scientific aims and objectives of the study". The HEP can be defined by physiological or behavioural signs and may be alleviated by administration of a treatment, such as analgesia, or termination of the experiment by withdrawal of the noxious agent or euthanasia.

Adoption of humane end points is therefore critical, particularly in studies where significant pain is anticipated.

6.2.2 Principles for the Establishment of Humane End points

Principles for the establishment of HEPs were defined by Demers et al, 2006 as follows:

- I. *There is strong evidence that animals experience pain and distress in situations comparable to those that cause pain and distress in humans*
- II. *Death or severe pain and distress should be avoided as end points*
- III. *The earliest possible end point should be used that is consistent with the scientific objectives*
- IV. *Studies should be designed to minimise any pain or distress likely to be experienced by the animals, whilst meeting the scientific objectives.*
- V. *The duration of studies involving pain and distress should be kept to a minimum*
- VI. *Pilot studies should be encouraged as a means of determining morbidity, time course of effects, and frequency of observation required to set an earlier end point.*
- VII. *Before commencing an experiment, agreement should be reached on (i) appropriate points for the study and (ii) the persons to be responsible for making the judgement that the end point has been reached.*
- VIII. *A team approach should be used, employing the professional judgement of scientist, veterinarian, animal care staff, and ethics committee to agree on the appropriate end point for the study.*
- IX. *Research and animal care staff must be adequately trained and competent in recognition of species-specific behaviour and, in particular, species-specific signs of pain, distress, and moribundity.*

- X. *Animals should be monitored by means of behavioural, physiological, and/or clinical signs at an appropriate frequency to permit timely termination of the experiment once the end point has been reached.*

6.3 Assessment of suffering

Assessment of pain, using the MGS and activity scores is discussed in Chapter 5 - Analgesia. These parameters are not used as HEPs in isolation, but are considered when making a decision to euthanase an experimental subject.

6.3.1 Behavioural and Neurological Observations

6.3.2 Physiological parameters

The key physiological parameters of arterial oxygen saturation (O₂), carotid pulse rate (PR), respiration rate (RR) and 'pulse distension (PD)' [volume of blood passing through the carotid artery during systole] of conscious mice were measured using a MouseOX® Plus system (Starr Life Sciences). This consists of a collar applied to the neck of a mouse from which two different wavelengths of light are shone through the mouse's neck, which are detected and analysed using software supplied by the manufacturer (See Chapter 2A, section 2A.3.2). The best readings were obtained when the mice were immobile and to this end gentle restraint of the mouse, by holding the base of its tail on a Vetbed® (synthetic sheepskin), resulted in improved tolerance of the collar and consequently better readings (Figure 6.3-1), with less distress to the subject.



Figure 6.3-1: Mouse-Ox recording

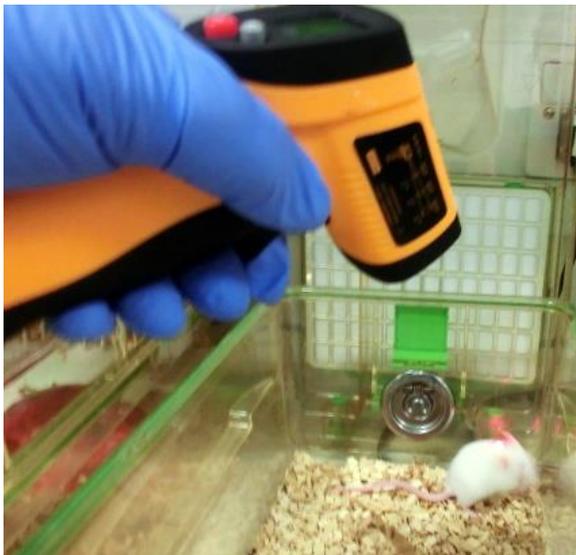
6.3.3 Body Temperature

It has been reported (Newsom et al, 2004; Kort et al, 1998 and Warn et al, 2003) that in other murine disease models that body temperature is a reliable indicator of impending death. It was therefore decided to investigate the use of body temperature as a HEP indicator in envenomed mice.

Temperature is often recorded in anaesthetised mice, using a rectal or oesophageal probe, by using a subcutaneously or intraperitoneally surgically implanted microchip. The results correlated well in the mouse, but subcutaneous temperature in the rat fell before intraperitoneal temperature (Lavoie et al, 2003).

Analysis of results from a microchip transponder implanted in rats experimentally infected with *Klebsiella pneumoniae* showed that the statistical analysis was not altered when a drop in body temperature below 36°C was used as an endpoint, rather than time of death, thus sparing animals from further suffering (Kort et al, 1998).

Rectal temperature probes can only be used in adequately restrained or



anaesthetised mice and implantation of telemetric transponders for routine assays would be prohibitively expensive and time consuming, due to animal numbers. An alternative approach is the use of an infrared (IR) thermometer, which measures surface body temperature. When the sensor was aimed just behind the cranium in mice standing on all

Figure 6.3-2: Recording surface body temperature using an infrared thermometer

four feet and from a constant height, reproducible results were

obtained (Figure 6.3-2); however, results were less reliable for very active

mice. Use of an IR thermometer, with the sensor pointed at the sternum of mice, produced results which correlated well with readings from both rectal probes and intraperitoneal telemetric chips in hypothermic mice, however the correlation was less convincing in pyrexia mice (Newsom et al, 2004).

A study which monitored mice with an experimental fungal infection using an IR thermometer calibrated to an implanted telemetric chip, caused little distress to the mice and demonstrated that mice rarely recovered if their body temperature dropped below 33.3°C. Adoption of this end point in fungal sepsis experiments significantly reduced suffering in the terminal stages of this model of infection (Warn et al, 2003).

6.3.4 Staging

'Staging' is the term adopted for a method developed at LSTM that reduces the number of mice required for standard preclinical testing of venom toxicity and antivenom efficacy. In staging experiments, results from previous experiments are used to estimate the likely range of venom or V/AV doses required to result in an 'all deaths' group, an 'all survivors' group and two intermediate survival groups.

6.4 Methods

Experiments were performed as described in Chapter 2, section 2.2, and 2A. The behavioural, physiological, clinical and neurological observations performed are described in detail in Chapter 2A.

6.5 Results

6.5.1 Humane End-Points: Clinical Observations

Clinical signs considered when defining HEPs are listed for each venom in Table 6.5-1.

The following observations were identified, in their own right, as HEPs:

a. Convulsions: Convulsions were observed to be an indicator of inevitable death and were often preceded by 'leaping' where the mouse 'stargazes' then launches itself into the air without apparent motive. Not all mice which exhibited 'leaping' went on to develop convulsions, but presence of this behaviour warranted close observation of the affected individual.

b. Epistaxis (Figure 6.5-1): Epistaxis (bleeding from the nose) occurs when there is severe haemorrhage into the lungs. Prior to implementation as a HEP no mouse was observed to survive this degree of pulmonary haemorrhage.



Figure 6.5-1: Epistaxis

c. Paradoxical respirations: Paradoxical respirations occur when there is severe dyspnoea (difficulty breathing) and the effort of breathing is so great that, as the chest wall expands the abdomen is pulled in, and as the individual attempts to breathe out the abdomen is pushed out. This looks like a 'see-saw' effect between the thorax and abdomen. The severity of pathological lesions resulting in this phenomenon is not only extremely distressing for the individual, but very unlikely to be reversible without intervention.

d. Respiratory distress with cyanosis (Figure 6.5-2): Affected mice either show increased effort to breath or breaths are slow and very shallow, the latter being associated with paralysis of the respiratory muscles by neurotoxic venom components. Affected individuals had a bluish tinge to their muzzle and tails (cyanosis) indicating lack of circulating oxygen.



Figure 6.5-2: Respiratory distress

- e. Paralysis of one or more limbs (Figure 6.5-3): Observations of mice which developed paralysis determined that the paralysis invariably progressed, and was eventually fatal



Figure 6.5-3: Hind limb paralysis

- f. Loss of righting reflex: When turned upside down the mouse is unable to turn back over. One of the most distressing things for a mouse is to be turned onto its back (Leach et al, personal communication). Loss of righting reflex in a conscious mouse is an indication of impending death. However, in these experiments it was noted that mice envenomed with BaV underwent an immediate and profound slump, and that a few individuals went on to recover completely even after having suffered a loss of righting reflex.
- g. Opisthotonus: Extensor rigidity of the entire body – if conscious, this is excruciatingly painful, and is almost inevitably a sign of imminent death and should therefore be used as an indication for euthanasia.
- h. Rapid deterioration of neurological function: The neurological examination was generally only performed on mice which were immobile or had obvious neurological deficits. Loss of sensation in the ear or tail shortly followed by loss of visual placing reflex invariably progressed to loss of righting reflex and death.

- i. Hyphaema (Figure 6.5-4): This is haemorrhage into the anterior chamber of the eye. In itself, it would not necessarily be fatal, but the pain associated with it made it ethically unreasonable not to euthanase the affected individual. Postmortem revealed that these individuals had also haemorrhaged into the orbital sinus behind their eyes.



Figure 6.5-4: Hyphaema

Table 6.5-1: Clinical signs observed which may be considered when defining HEPs

HEP	MOR	LRR	CON	OPI	EPI	PR	RD	CYA	PAR	DNF	LEA	HYP
EoV	+	+	+		+	+	+	+	+	+	+	
BaV	+	+	+	+			+		+	+	+	
VbV	+	+	+				+		+	+	+	+
NnigV	+	+	+	+			+	+	+	+	+	
DaV	+	+	+	+		+	+		+	+		
CrV		+	+	+								
CcV		+	+		+	+				+		
DtV		+	+		+		+					
NnubV	+	+										
NhajV	+	+				+	+		+	+		
NpalV	+	+	+			+	+		+	+	+	
NnivV	+	+					+		+	+		
EpIV	+	+	+		+	+	+	+				
EcsV			+		+	+	+	+				
EcolV	+	+			+		+				+	
AsV		+					+		+	+		
DpV	+	+	+			+	+		+	+		

Key:	HEP	Humane Endpoint	EoV	<i>Echis ocellatus</i> venom
	MOR	Moribund	BaV	<i>Bitis arietans</i> venom
	LRR	Lost righting reflex	VbV	<i>Vipera berus</i> venom
	CON	Convulsions	NnigV	<i>Naja nigricollis</i> venom
	OPI	Opisthotonus	DaV	<i>Dendroaspis angusticeps</i> venom
	EPI	Epistaxis	CrV	<i>Calloselasma rhodostoma</i> venom
	PR	Paradoxical respirations	CcV	<i>Cerastes cerastes</i> venom
	RD	Respiratory distress	DtV	<i>Dispholidus typus</i> venom
	CYA	Cyanosis	NnubV	<i>Naja nubiae</i> venom
	PAR	Paralysis	NhajV	<i>Naja haje</i> venom
	DNF	Deteriorating neuro function	NpalV	<i>Naja pallida</i> venom
	LEA	Leaping	NnivV	<i>Naja nivea</i> venom
	HYP	Hyphaema	EpIV	<i>Echis pyramidum leakeyi</i> venom
			EcsV	<i>Echis carinatus sochurekei</i> venom
			EcolV	<i>Echis coloratus</i> venom
			AsV	<i>Aspidelaps scutatus</i> venom
			DpV	<i>Dendroaspis polylepis</i> venom

6.5.2 MouseOx

Use of the MouseOx was limited to a maximum of 4 mice in any one day, especially when other time-consuming observations such as MGS and activity scores were also being made in the same experimental sequence. Typical MouseOx results are shown in Figure 6.5-5 and show an initial fall in pulse and respiratory rates with a fall in arterial oxygen saturation. In some mice the oxygen saturation recovered as the mouse recovered clinically. The correlation between the physiological parameters and our MGS and activity scores was investigated using a scatter plot, which gave the results shown in Figure 6.5-6.

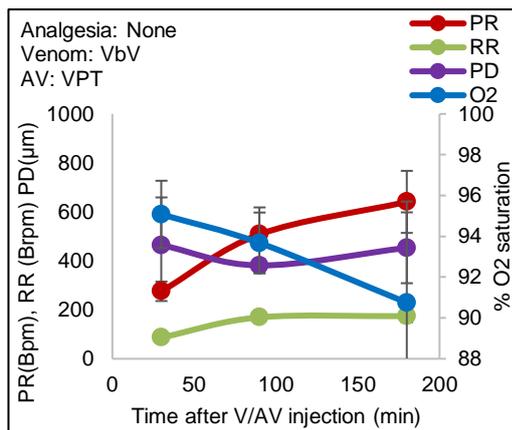


Figure 6.5-5: Physiological changes following injection with *V. berus* venom/ViperaTab AV.

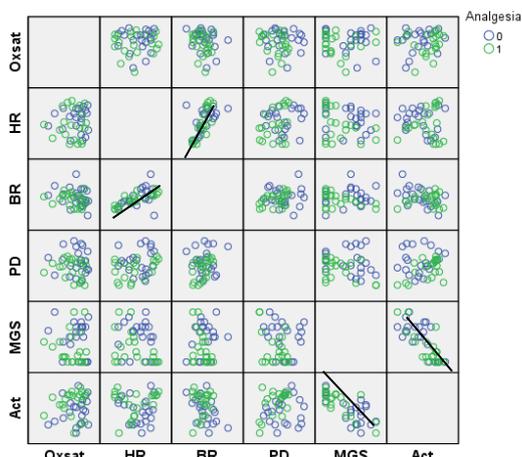


Figure 6.5-6: Scatterplots showing correlation between physiological parameters, MGS and activity (results pooled from all venoms). Analgesia: 0=none, 1= morphine

There would appear to be, as illustrated by the lines of ‘best fit’, a relationship between MGS and activity scores, and between pulse rate (‘heart’ rare – HR) and respiration rate (‘breathing’ rate- BR) but not between any of the other parameters. For example, no correlation was found between subjective physiological parameters and objective pain measurements.

6.5.3 Temperature

The infrared probe used provided surface temperature readings that are obviously lower than core temperatures, but, importantly, it does detect variations in temperature. It was found that subsequent death or development of other HEPs resulting in euthanasia was not associated with a particular ‘cut-off’ surface temperature reading. However, a fall in surface temperature was associated with mice that were patently unwell, and often the first signs of their recovery was a rise in surface temperature. To illustrate this Table 6.5-2 shows one group taken from the many LD₅₀ experiments performed. It demonstrates a steady fall in the body temperature of ‘Mouse 4’ prior to its euthanasia. ‘Mouse 1’ shows a fall in body temperature, a recovery, and a final decline in surface temperature; although this subject survived to the end of the

experiment, it was moribund throughout, including at the final time point. 'Mouse 2', another 'survivor' showed a fall in surface temperature, followed by a rise towards the end of the experiment, associated with a full recovery.

Table 6.5-2: Example of surface temperature readings following envenomation

Time	Survival time (min)	Temperature °C							
		0930	1028	1055	1125	1200	1243	1420	
Mouse	1	Survived	30.3	25.4	25.7	29.0	27.1	26.7	24.5
	2	Survived	30.1	28.9	26.5	26.1	27.5	27.2	29.1
	3	43	29.8	28.2	-	-	-	-	-
	4	115	27.1	26.3	25.9	25.1	-	-	-
	5	13	30.3	-	-	-	-	-	-

Surface temperatures and health status of survivors at the end of experiments were recorded. There was a significant difference ($p < 0.05$) between those that had recovered and those that were ill, and also between those that were 'ill' but improving, and those that were 'ill' and deteriorating (Figure 6.5-7).

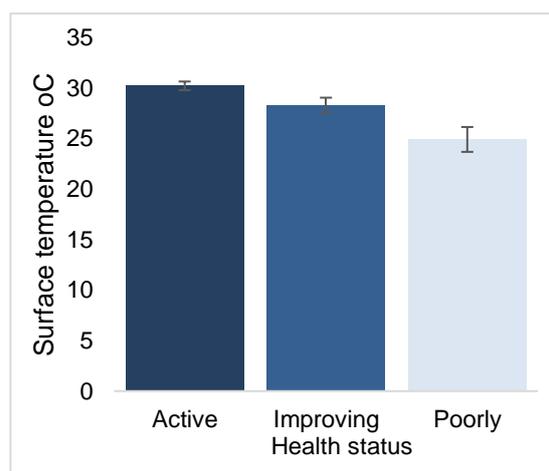


Figure 6.5-7: Relationship between surface temperature and health status of survivors at the end of LD₅₀/ED₅₀ experiment

6.5.4 Outcome of using HEPs

Archived experimental protocols from the last 10 years were searched and analysed according to survival time and numbers of survivors. The results of these analyses were compared assays which lasted for 24 hours, and those which lasted less than 9 hours.

6.5.4.1 *Vipera berus* venom

Over 75% of all mice died or were euthanased within the first hour in all *V. berus* venom assays (Figure 6.5-8). In the 24 hour assays (n=127), there were no deaths after 6 hours and, in the shorter assays (n=1156), only 2 deaths occurred between 6 and 9 hours. Only one mouse that was ‘well’ at the end of the working day became ‘ill’ overnight in the 24 hour assays and, of the surviving mice, over 95% were ‘improving’ at the end of the shorter assays.

Status	Number	%	Data source	
Total mice	127	100	All mice in 24h assays	
Survivors	Total	78	Total survivors for 24h	
	Health 9h	77	98.7	
	Ill	1	1.3	
	Health EOE	76	97.4	
	Ill	2	2.6	
			% recorded survivors' status	
Died	Total	49	Total deaths in 24h	
	Found dead	Total	7	14.3
		0-1h	5	71.4
		1-2h	2	28.6
		2-3h	0	0
		3-6h	0	0
		6-9h	0	0
	Schedule 1	Total	42	85.7
		0-1h	36	85.7
		1-2h	4	9.5
		2-3h	0	0
		3-6h	2	4.8
		6-9h	0	0
	Total deaths	Total	49	100
		0-1h	41	83.7
1-2h		6	12.2	
2-3h		0	0	
3-6h		2	4.1	
6-7h		0	0	
			% total deaths recorded	

Status	Number	%	Data source		
Total mice	1156	100	All mice in assays <9h		
Survivors	Total	664	57.4		
	Well	445	83.0		
	Ill	Total	91	17.0	
		Better	68	95.8	
			Worse	3	4.2
			% recorded survivors' status		
Died	Total	492	42.6		
	Found dead	Total	22	5.6	
		0-1h	12	54.5	
		1-2h	4	18.2	
		2-3h	5	22.7	
		3-6h	1	4.5	
		6-9h	0	0	
	Schedule 1	Total	368	94.4	
		0-1h	282	76.6	
		1-2h	30	8.2	
		2-3h	44	12.0	
		3-6h	10	2.7	
		6-7h	2	0.5	
	Total deaths	Total	390	100	
		0-1h	294	75.4	
1-2h		34	8.7		
2-3h		49	12.6		
3-6h		11	2.8		
6-7h		2	0.5		
			% total deaths recorded		

Figure 6.5-8: Comparison of survival of mice between *V. berus* venom LD₅₀s/ED₅₀s for 24 hours (left) and less than 9 hours (right)

6.5.4.2 *Echis ocellatus* venom

Deaths in mice envenomed with *E. ocellatus* venom were more prolonged, with over 25% of deaths occurring overnight in the 24 hour assays (n=162) – 87% of deaths were discovered early the next day (Figure 6.5-9). A larger percentage of mice envenomed with *E. ocellatus* venom than those subjected to *V. berus* venom were ‘ill’ at the end of the short assays (n=1042) and of these only 63% were improving, compared to >95% of *V. berus* mice.

Status	Number	%	Data source	
Total mice	162	100	All mice in 24h assays	
Survivors	Total	79	48.8	
	Health 9h	Well	80	77.7
		Ill	23	22.3
	Health EOE	Well	72	91.1
Ill		7	8.9	
Died	Total	83	51.2	
	Found dead	Total	22	28.2
		0-1h	1	4.5
		1-2h	0	0
		2-3h	1	4.5
		3-6h	0	0
		6-9h	1	4.5
		9-24h	19	86.5
		Total	56	71.8
	Schedule 1	0-1h	25	44.6
		1-2h	16	28.6
		2-3h	3	5.4
		3-6h	7	12.5
		6-9h	2	3.6
		9-24h	3	5.4
		Total	78	100
Total deaths		26	33.3	
0-1h	16	20.5		
1-2h	4	5.1		
2-3h	7	9.0		
3-6h	3	3.8		
6-7h	2	2.5		
9-24h	22	28.2		

Status	Number	%	Data source	
Total mice	1042	100	All mice in assays <9h	
Survivors	Total	609	58.4	
	Well	Total	188	78.3
		Better	52	21.7
	Ill	Better	19	63.3
Worse		11	36.7	
Died	Total	433	41.6	
	Found dead	Total	26	14.9
		0-1h	11	42.3
		1-2h	3	11.5
		2-3h	6	23.1
		3-6h	6	23.1
		6-7h	0	0
		Total	148	85.1
		Schedule 1	0-1h	109
	1-2h		15	10.1
	2-3h		11	7.4
	3-6h		11	7.4
	6-7h		2	1.4
	Total		174	100
	Total deaths		120	69.0
	0-1h		18	10.3
1-2h	17	9.8		
2-3h	17	9.8		
3-6h	17	9.8		
6-7h	2	1.1		

Figure 6.5-9: Comparison of survival of mice between *E. ocellatus* venom LD₅₀/ED₅₀ for 24 hours (left) and less than 9 hours (right)

6.5.4.3 *Bitis arietans* venom

Mice envenomed with *B. arietans* venom showed a survival pattern similar to *V. berus* venom in that over 75% of deaths in both 24h (n=205) and shorter assays (n=213) occurred within the first hour of the assay (Figure 6.5-10). In the 24h assays, 87% of mice were 'well' at the end of the assay, and all mice that survived overnight had completely recovered by the next morning. Of the mice surviving at 9h, 9 were 'ill' and 10 mice died overnight. Unfortunately, in these experiments, mice were not individually identified, making following clinical progression of individual mice unreliable. Of the mice that died overnight, 9 were in ED₅₀ experiments.

Status	Number	%	Data source	
Total mice	205	100	All mice in 24h assays	
Survivors	Total	109	53.2	
	Health 9h	Well	62	87.3
		Ill	9	12.7
	Health EOE	Well	82	100
Ill		0	0	
Died	Total	96	46.8	
	Found dead	Total	31	32.3
		0-1h	14	45.2
		1-2h	1	3.2
		2-3h	1	3.2
		3-6h	4	12.9
		6-9h	1	3.2
		9-24h	10	32.3
		Total	64	66.7
	Schedule 1	0-1h	59	92.2
		1-2h	2	3.1
		2-3h	1	1.6
		3-6h	1	1.6
		6-9h	1	1.6
		9-24h	0	0
		Total	95	100
Total deaths		73	76.8	
0-1h	3	3.2		
1-2h	2	2.1		
2-3h	5	5.3		
3-6h	2	2.1		
6-7h	2	2.1		
9-24h	10	10.5		

Status	Number	%	Data source		
Total mice	213	100	All mice in assays <9h		
Survivors	Total	104	48.8		
	Well	Total	80	76.9	
		Better	24	23.1	
	Ill	Better	19	18.3	
Worse		5	4.8		
Died	Total	109	51.2		
	Found dead	Total	4	3.7	
		0-1h	0	0	
		1-2h	4	100	
		2-3h	0	0	
		3-6h	0	0	
		Total	105	96.3	
		Schedule 1	0-1h	89	84.8
			1-2h	7	6.7
	2-3h		3	2.9	
	3-6h		6	5.7	
	6-7h		0	0	
	Total		109	100	
	Total deaths		89	81.7	
	0-1h		11	10.1	
	1-2h	3	2.8		
2-3h	6	5.5			
3-6h	0	0			
6-7h	0	0			

Figure 6.5-10: Comparison of survival of mice between *B. arietans* venom LD₅₀/ED₅₀ for 24 hours (left) and less than 9 hours (right)

6.5.4.4 *Naja nigricollis* venom

Mice envenomed with *N. nigricollis* venom (Figure 6.5-11) showed a relatively short clinical progression (less than 6 hours) – only 1 of 66 animals died overnight and over 90% of mice had completely recovered from the venom effects at the end of the working day in both 24h and shorter assays (n=211).

Status	Number		%	Data source	
Total mice	66		100	All mice in 24h assays	
	Total	30	45.5	Total survivors for 24h	
Survivors	Health 9h	Well	28	90.3	% recorded survivors' status
		Ill	3	9.7	
	Health EOE	Well	28	93.3	
		Ill	2	6.7	
Died	Total	36	54.5	Total deaths in 24h	
	Found dead	Total	13	36.1	% total deaths (time recorded)
		0-1h	8	61.5	% total found dead
		1-2h	3	23.1	
		2-3h	1	7.7	
		3-6h	0	0	
		6-9h	0	0	
	9-24h	1	7.7		
	Schedule 1	Total	23	63.9	% total deaths (time recorded)
		0-1h	18	78.3	% total Schedule 1 deaths
		1-2h	4	17.4	
		2-3h	1	4.3	
		3-6h	0	0	
		6-9h	0	0	
	9-24h	0	0		
	Total deaths	Total	36	100	Total deaths (time recorded)
0-1h		26	72.2	% total deaths recorded	
1-2h		7	19.4		
2-3h		2	5.6		
3-6h		0	0		
6-7h		0	0		
9-24h	1	2.8			

Status	Number		%	Data source	
Total mice	211		100	All mice in assays <9h	
	Total	124	58.8	Total survivors in assays <9h	
Survivors	Well	Total	115	92.7	% recorded survivors' status
		Ill	9	7.3	
	III	Better	8	6.5	
		Worse	1	0.8	
Died	Total	87	41.2	Total died in assays <9h	
	Found dead	Total	18	28.7	% total deaths time recorded
		0-1h	13	72.2	% total found dead
		1-2h	5	27.8	
		2-3h	0	0	
		3-6h	0	0	
		6-7h	0	0	
	Schedule 1	Total	69	79.3	
		0-1h	52	75.4	% total Schedule 1
		1-2h	13	18.8	
		2-3h	3	4.3	
		3-6h	1	1.4	
		6-7h	0	0	
	Total deaths	Total	87	100	
		0-1h	70	80.5	% total deaths recorded
		1-2h	18	20.7	
2-3h		8	9.2		
3-6h		1	1.1		
6-7h		0	0		

Figure 6.5-11: Comparison of survival of mice between *N. nigricollis* venom LD₅₀s/ED₅₀s for 24 hours (left) and less than 9 hours (right)

6.5.4.5 Other venoms

Status	Number		%	Data source	
Total mice	48		100	All mice in 24h assays	
	Total	22	45.8	Total survivors for 24h	
Survivors	Health 9h	Well	19	67.9	% recorded survivors' status
		Ill	9	32.1	
	Health EOE	Well	20	90.9	
		Ill	2	9.1	
Died	Total	26	54.2	Total deaths in 24h	
	Found dead	Total	6	23.1	% total deaths (time recorded)
		0-1h	0	0	% total found dead
		1-2h	0	0	
		2-3h	0	0	
		3-6h	2	33.3	
		6-9h	0	0	
	9-24h	4	66.7		
	Schedule 1	Total	20	76.9	% total deaths (time recorded)
		0-1h	18	90.0	% total Schedule 1 deaths
		1-2h	0	0	
		2-3h	0	0	
		3-6h	0	0	
		6-9h	0	0	
	9-24h	2	10.0		
	Total deaths	Total	26	100	Total deaths (time recorded)
0-1h		18	69.2	% total deaths recorded	
1-2h		0	0		
2-3h		0	0		
3-6h		2	7.7		
6-7h		0	0		
9-24h	6	23.1			

Status	Number		%	Data source	
Total mice	1053		100	All mice in assays <9h	
	Total	567	53.8	Total survivors in assays <9h	
Survivors	Well	Total	432	91.5	% recorded survivors' status
		Ill	40	8.5	
	III	Improving	29	6.1	
		Deteriorating	4	0.8	
Died	Total	486	46.2	Total died in assays <9h	
	Found dead	Total	39	8.6	% total deaths time recorded
		0-1h	31	6.8	% total found dead
		1-2h	1	0.2	
		2-3h	1	0.2	
		3-6h	6	1.3	
		6-7h	0	0	
	Schedule 1	Total	417	91.4	
		0-1h	324	71.1	% total Schedule 1
		1-2h	54	11.8	
		2-3h	10	2.2	
		3-6h	28	3.1	
		6-7h	1	0.2	
	Total deaths	Total	456	100	
		0-1h	363	79.6	% total deaths recorded
		1-2h	55	12.1	
2-3h		11	2.4		
3-6h		34	7.5		
6-7h		1	0.2		

Figure 6.5-12: Comparison of survival of mice between other venom LD₅₀s/ED₅₀s for 24 hours (left) and less than 9 hours (right)

Figure 6.5-12 shows pooled survival data for all other venoms studied, namely other *Echis* spp., *Vipera* spp., *Naja* spp., *Cerastes cerastes*, *Dendroaspis polylepis*, *Aspidelaps* spp. and *Dyspholidus typus*. This shows that more than

70% of deaths occurred in the first hour in both 24h and shorter duration assays, however 23% of mice died overnight in the 24 hour assays. It is not easy to analyse this pooled data any further because of the wide variety of venoms used and their unique clinical courses.

6.5.4.6 All venoms- pooled results

Similarly, Figure 6.5-13 shows the analysis of pooled results from all assays, demonstrating that ~70% of mice die or are euthanased in the first hour of the experiment, and that over 80% of the remaining mice have fully recovered within 6 hours. The number of mice which were ‘ill’ at the end of a working day corresponded closely to the number of deaths overnight; of 45 mice which were ‘ill’ at the end of the day 39 died overnight, a close correspondence.

Status		Number	%	Data source	
Total mice		608	100	All mice in 24h assays	
	Total	318	52.3	Total survivors for 24h	
Survivors	Health 9h	Well	266	85.5	% recorded survivors' status
		Ill	45	14.5	
	Health EOE	Well	278	87.4	
		Ill	13	4.1	
Total	290	47.7	Total deaths in 24h		
Died	Total	Total	79	27	% total deaths (time recorded)
		0-1h	28	37	% total found dead
	Found dead	1-2h	6	8	
		2-3h	3	4	
		3-6h	6	8	
		6-9h	2	3	
		9-24h	34	43	
		Total	205	71	% total deaths (time recorded)
	Schedule 1	0-1h	156	76	% total Schedule 1 deaths
		1-2h	26	13	
		2-3h	5	2	
		3-6h	10	5	
		6-9h	3	1	
		9-24h	5	2	
	Total	284	100	Total deaths (time recorded)	
	Total deaths	0-1h	190	67	% total deaths recorded
		1-2h	32	11	
		2-3h	8	3	
		3-6h	16	6	
6-7h		5	2		
9-24h		39	14		

Status		Number	%	Data source		
Total mice		1156	100	All mice in assays <9h		
	Total	664	57.4	Total survivors in assays <9h		
Survivors	Well	Total	91	17.0	% recorded survivors' status	
		Ill	68	95.8		
	Ill	Improving	3	4.2		
		Deteriorating	3	4.2		
Total	492	42.6	Total died in assays <9h			
Died	Total	Total	22	5.6	% total deaths time recorded	
		0-1h	12	54.5	% total found dead	
	Found dead	1-2h	4	18.2		
		2-3h	5	22.7		
		3-6h	1	4.5		
		Total	368	94.4		% total deaths (time recorded)
		Schedule 1	0-1h	282		76.6
			1-2h	30	8.2	
	2-3h		44	12.0		
	3-6h		10	2.7		
	6-7h		2	0.5		
	Total		390	100	Total deaths (time recorded)	
	Total deaths	0-1h	294	75.4	% total deaths recorded	
		1-2h	34	8.7		
		2-3h	49	12.6		
		3-6h	11	2.8		
		6-7h	2	0.5		

Figure 6.5-13: Comparison of survival of mice between all venom LD₅₀s/ED₅₀s for 24 hours (left) and less than 9 hours (right)

Archived data was used to perform these analyses. However, the 24 hour assays lacked the recorded detail which was initiated during this project. Mice were not individually identified, so there was no certainty that the mice which were ill were the same ones which subsequently died. Humane end-points were not used – many of the mice which were ill at the end of the working day may well have fulfilled our criteria for euthanasia and therefore there would likely have been fewer deaths overnight.

To perform assays to validate the use of HEPs unequivocally for each venom studied, would require the consumption of large numbers of mice, which is ethically contrary to the objectives of this project.

6.5.5 Staging

The use of ‘staging to reduce the numbers of mice used to perform LD₅₀ and ED₅₀ experiments is illustrated in Table 6.5-3. Nine venom doses from 2 - 25µL/mouse were prepared and of these, only 5 doses were used. In the past all nine doses would have been used and the groups dosed sequentially without waiting to see the potential outcome, unless deaths were within minutes of injection.

Table 6.5-3: Illustration of ‘staging’ using an example taken from an LD₅₀ experimental sheet. Groups not used are crossed out and in red.

Doses per mouse (ul)			Gp/# mice	Preparation of injection (8 doses)			Time:			
AV	Diluent	Venom ul		AV	Diluent	Venom	Injection	F - Dead	Sch - 1	# deaths
-	98	2	A5	-	784	16	-	-	-	-
-	96	4	B5	-	768	32	-	-	-	-
-	95	5	C5	-	760	40	-	-	-	-
-	94	6	D5	-	752	48	10.00	0	0	0/5
-	92	8	E5	-	736	64	10.05	0	0	0/5
-	90	10	F5	-	720	80	09.55	0	1	1/5
-	88	12	G5	-	704	96	-	-	-	-
-	85	15	H5	-	850	150	10.15	0	1	1/5
-	75	25	I5	-	600	200	11.12	0	5	5/5

Time of injection illustrates the initial selection of doses, which was based on the results of previous LD₅₀ experiments performed using the same venom. Injected mice were observed for an hour after 4 groups had been injected and the number of likely deaths estimated. A venom dose of 15µL/mouse did not appear to be likely to result in ‘all deaths’ therefore a higher dose of 25µL/mouse was selected for the final dose. In this way, the LD₅₀ was completed in a single day using only 25 rather than 45 mice, had all the prepared venom doses been used. It is important to note that this protocol can only be used by employing all of the observations described to identify the likely outcome of each mouse.

Of 39 LD₅₀/ED₅₀ assays which employed this method, the planned number of mice was 1,800; the actual number of mice used was 1,019, saving 781 mice, 43.3% of the planned number.

6.6 Discussion

As stated in Chapter 1, there are 45 antivenom manufacturers listed on the WHO website <http://apps.who.int/bloodproducts/snakeantivenoms/database/>, making in excess of 120 different snake antivenoms. Assuming that, on average, at least 2 batches of antivenom are manufactured per annum, and that every batch is tested *in vivo* as recommended by the WHO (WHO, 2010), this equates to more than 25,000 mice used for regulatory testing alone, and does not include those used for development of new antivenoms or research into venom toxicity. The objective was to reduce the duration of pain, harm and distress suffered by the subjects of the *in vivo* WHO-recommended venom LD₅₀ and antivenom ED₅₀ assays by identifying HEPs. However, the prerequisite for adoption of these HEPs is that the methods have been adequately validated and that they have been demonstrated to produce results which are equivalent to, or generate better information than the existing method, which uses death as the metric (Stokes, 2002). In this way, these changes would not compromise the validity of the results and therefore public health, and would ensure that animals do not suffer needless pain, harm or distress (Sass, 2000). The incorporation of HEPs is being encouraged by regulatory authorities, such as the European Pharmacopoeia (Ph.Eur, 2014), but the research required to validate these changes is extensive (Castle, 1999), which makes implementation of the 3Rs into testing of Biologicals frustratingly slow (Hendriksen, 2002).

Animal research is an invaluable tool in the investigation of venom toxicity and antivenom efficacy, being the only 'holistic' platform available at this time. The use of biomarkers to predict death of an experimental subject at the earliest opportunity is to be encouraged (Franco et al, 2012), rather than allowing these animals to suffer the terminal effects of envenomation, or to wait until

they become moribund before, being humanely dispatched. These biomarkers can be behavioural, other clinical signs, as well as more objective measurements, such as weight or body temperature changes (Hankenson et al, 2013).

Recognition of clinical signs relating to impending death requires continuous monitoring by a skilled observer, who has an in depth knowledge of the normal behaviour of the test species, as well as the likely pathological lesions resulting from the venom being studied (Morton, 2000). The study of venom toxicity is extremely complex due to the large number of enzymes, toxins and non-toxic proteins contained within. Humane endpoints will need to be tailored to each different venom, or family of venoms, based on knowledge of the pathophysiology of the venom toxins and close observation of early assays to establish the clinical progression of envenomation by each species.

Table 6.5-1 in the results section of this chapter summarises the severe abnormal clinical findings observed, not only in mice envenomed with one of the five venoms selected for this project, but also those envenomed with all other venoms studied by the ARVRU during the project. It was apparent that each venom produced a unique clinical progression but that venoms from closely related species produced similar clinical signs.

Mice envenomed with *E. ocellatus* venom, and the other *Echis* species demonstrated clinical signs associated with severe haemorrhage. High doses of venom resulted in convulsions and loss of righting reflex, whilst less severely affected mice showed an initial slump, followed by inactivity, hunching, grimacing and a staring coat. Leaping in the air for no apparent reason was a common observation, and was often a prelude to convulsions with or without epistaxis, which was always associated with significant pulmonary haemorrhage. 'Leaping' was not, in itself, a HEP, but warranted close observation of the individual. It was often followed by hyperactivity which progressed to convulsions. Mice which survived the initial slump often became dyspnoeic and adopted postures to facilitate breathing, such as propping

themselves up. Distressed mice often exhibited paradoxical respirations and instead of exhibiting the orbital tightening of pain, described in the previous chapter, it was observed that they became wide-eyed and terminally they were gulping for air. It was observed that mice envenomed with venoms containing toxins which caused a consumptive coagulopathy showed a more prolonged clinical course than haemotoxic venoms, such as *B. arietans* venom, which target platelets rather than the coagulation cascade (Calvete et al, 2007). This can be explained by the 'Russian roulette' nature of coagulopathy in that the site of haemorrhage can dictate whether it is fatal or not which could potentially invalidate the use of HEPs to reduce the assay duration from 24 hours to 6 hours. If HEPs are used to determine when a mouse is euthanased, cardiac puncture can be performed and the blood collected subjected to a 20 minute whole blood clotting test (WBCT), which is performed by placing the blood in a clean glass tube and examining it for the presence of clots after 20 minutes (See Chapter 7 – Pathology). The 20min WBCT may also be performed on survivors after they are euthanased at the end of the experiment. By doing this more mice could be saved by eliminating the need to perform other *in vivo* assays, such as the MHD (minimum haemorrhagic dose) and MDD (minimum defibrinogenating dose), and results are likely to be more reproducible than waiting for a lethal haemorrhage to occur.

Many of the venoms investigated were cardiotoxic (see Chapter 7 – Pathology) and produced rapid death, preceded by convulsions or loss of righting reflex and total unresponsiveness. However, *B. arietans* venom produced a profound slump, resulting in mice that were almost completely unresponsive and virtually unable to right themselves. These mice retained some muscle tone, unlike those suffering from flaccid paralysis. Some of these mice went on to recover completely and it was therefore concluded that, for this venom, 'loss of righting reflex' was not a valid HEP. It is possible that this slump is the result of low molecular weight vasoactive adenosine (Aird, 2002), or other hypotensive venom components, causing a profound drop in blood pressure and this effect did not appear to be neutralised by any of the AVs tested. Mice surviving initial cardiac injury went on to develop signs of congestive heart

failure (CHF) such as dyspnoea due to pulmonary congestion, or neurological signs due to either cerebral congestion or hypoxia affecting the CNS. Mice have a large venous sinus behind their eyes, and, particularly in mice that had received *V. berus* venom, this contained excessive amounts of blood.

Neurotoxic venoms, such as *Naja* spp. and *Dendroaspis* spp. showed similar signs in the acute stages of envenomation to the haemotoxic viper venoms, namely, slumping, immobility, loss of righting reflex and convulsions. With lower neurotoxic venom doses ptosis was commonly seen in mice which had received mamba venom, but rarely in those which had received cobra venoms; sweating, paralysis and respiratory distress with slow, shallow, often imperceptible breathing and cyanosis was also observed with neurotoxic venoms. Mice which had received *D. angusticeps* venom, but not *D. polylepis* venom, showed muscle fasciculations, which, in some cases, were difficult to differentiate from paradoxical respirations. On post mortem examination these mice had collapsed lungs, which were often congested and/or consolidated (See Chapter 7), these pathological lesions being irreversible without intervention such as chest drains or positive pressure ventilation. It can be concluded that respiratory distress in these animals is a valid HEP.

Deterioration in neurological function in mice envenomed with both neurotoxic and non-neurotoxic venoms was a strong indicator of impending death. Paralysis of one hind limb was frequently seen in mice which had been injected with *V. berus* venom, it was observed that the paralysis in these mice was progressive and inevitably resulted in death. Therefore the observation of unilateral hind-limb paralysis was adopted as a HEP in *V. berus* venom assays. Clinical signs adopted as HEPs for each of the 'core' venoms studied are summarised in Table 6.6-1

It had been hoped that physiological parameters could be used as an objective measurement to predict impending death and/or pain. To this end a MouseOx collar was used to monitor conscious mice. Unfortunately it was

only possible to monitor one mouse at a time and the best measurements were obtained by gently restraining the mouse on a fleecy bed. Generally, the pulse and respiration rates fell immediately after envenomation and increased as they recovered. Oxygen saturation also fell after venom was injected, but this measurement was unreliable and affected by even small movements of the subject. Pulse distension is a measure of the difference between diastolic and systolic blood pressure, but could not differentiate between a low diastolic pressure and a high systolic pressure. Scatter plots of these parameters and the subjective pain measurements, MGS and activity scores, showed that there was a relationship between pulse rate and respiratory rate, and an inverse relationship between MGS and activity scores.

However there is no apparent relationship between physiological parameters and pain scores. Perhaps this is not surprising because, although pain can cause increased pulse rate and respiratory rate, increased activity is associated with increased pulse rate and respiratory rate and added to this, fear, stress of handling and effects of venom toxins will also have an impact on physiological parameters. The results are interesting, but there are too many uncontrollable variables to provide meaningful results, and the equipment is too complex and expensive for use other than as a research tool. Measurements could only be made on one mouse at a time, making it impractical for collecting sufficient data for statistical analysis. Results can be found in the chapter supplement.

Surface body temperature readings, taken with the sensor of an IR thermometer pointing at the back of the mouse, were several degrees (centigrade) lower than those taken from the base of the sternum (Newsom et al, 2004), and, in agreement with the observation that readings from active mice were unreliable. Taking readings from the sternum requires restraint of the mouse and is therefore stressful and as readings were performed hourly and, sometimes more frequently, on envenomed mice, the method of minimal

intervention was adopted; the assumption was made that if a mouse was too active to obtain a valid reading, it was not under consideration for euthanasia.

Table 6.6-1: HEPs adopted for each of the five ‘core’ venoms

Venom	HEPs
<i>E. ocellatus</i>	Epistaxis Convulsions Loss of righting reflex Repeated ‘leaping’ Respiratory distress, cyanosis and paradoxical respirations
<i>B. arietans</i>	Opisthotonus Convulsions Respiratory distress Deteriorating neurological function, once recovered from initial slump
<i>V. berus</i>	Convulsions Loss of righting reflex Respiratory distress Deterioration of neurological function Hind limb paralysis Hyphaema
<i>N. nigricollis</i>	Convulsions/opisthotonus Respiratory distress-imperceptible breathing and cyanosis Paralysis/ deterioration of neurological function Loss of righting reflex
<i>D. angusticeps</i>	Convulsions/ opisthotonus Respiratory distress-imperceptible breathing and cyanosis Paralysis/ deterioration of neurological function Loss of righting reflex

Our findings revealed that, in envenomed mice, the more poorly the animal, the lower its body temperature. However, many mice died before body temperature had fallen significantly, meaning that no ‘cut off’ temperature could be identified. However, the trend was much more significant; a rise in surface temperature often being the first sign of recovery whilst conversely a protracted fall in surface temperature indicated an increasing likelihood of death/euthanasia.

Analysis of the archived data provided information on survival patterns in animals over 24 hours compared to those in assays less than 9 hours which could be completed in a working day. One of the drivers for reducing assay duration, apart from reduction in duration of pain, harm and distress was the Home Office requirement to observe mice regularly overnight in order to avoid unnecessary suffering of mice undergoing the terminal stages of envenomation. The analysis of pooled data demonstrated that the vast majority of mice died within the first hour after venom injection, and that most of those surviving to the end of the working day had completely recovered. In 24 hour assays, the number of mice which died overnight tallied with the number of mice which were ill at the end of the previous day, although mice were not individually identified, so it is not certain that these were the same mice.

6.7 Conclusions

Our findings suggest that assay duration could be reduced from 24 hours to 6 hours by using predefined, venom-specific HEPs, although the archive data would suggest that a significant number of deaths occur between 6 and 24 hours in mice subjected to coagulopathic venoms, such as *E. ocellatus*. The use of the 20 minute whole blood clotting test for coagulopathic venoms and post mortem pathological lesions to confirm antivenom neutralisation of potentially lethal venom pathological lesions, including haemorrhage, in surviving mice, could be used to provide more robust assay results than the present lethality assays in addition to substantially reducing pain, harm and suffering. The potential of post mortem observations to replace other *in vivo* assays of venom toxicity will be discussed further in Chapter 7. Before implementation into regulatory testing of venom toxicity and antivenom efficacy, these changes would have to be validated for each venom and venom/antivenom combination.

'Staging' can be used to substantially reduce the numbers of mice in LD₅₀/ED₅₀ assays, but requires experienced observers and an in depth knowledge of the

clinical progression of envenomation in mice for the venom or venom/antivenom being assessed.

It is imperative that the present severity of the murine lethality assays used to test venom toxicity and antivenom efficacy is reduced, not only for ethical reasons, but to satisfy public acceptance of these assays.

6.8 Further Work

- The clinical progression of envenomation with a wide range of venoms and venom/antivenom combinations must be established before HEPs can be defined.
- Ideally, 24 hour assays need to be documented with details of the clinical progression of each individual mouse, to confirm that it is indeed the mice that are ill at the end of the assay that die overnight.
- As part of the validation process, LD₅₀/ED₅₀ assays should be performed in which the time at which a HEP intervention could or should ethically be made is recorded. The animal should then be left to determine the survival outcome, and results compared.

Chapter 7. Reduction: Pathology

7.1 Introduction

Snakebites are a major, but neglected, public health problem in the rural tropics of Sub-Saharan Africa, Asia and South America. The resulting pathological lesions are responsible for causing significant permanent disability as well as mortality in the victims of envenoming (Alam et al, 2015; Baruah et al, 2004; Habib et al, 2013; Habib et al, 2015; Maduwage et al, 2013; McNally and Reitz, 1987; Parathakan and Govindarajan, 2016; Rahman et al, 2010; Wongtongkam et al, 2002). Morbidity results from amputation of digits or limbs (Abubakar et al, 2010), chronic pain and scarring at the bite site, disfigurement, abortion and foetal death (Habib, 2008 et al), neurological disabilities, blindness and psychological effects (Williams et al, 2011).

The chronic *sequelae* resulting from snakebite affect both human and animal victims (Anlen, 2008; Hoffman, 1993; Reid, 1975; Spiller and Bosse, 2003). Mice are the preferred experimental model for snake venom studies. There is, however, a paucity of published data on the pathogenesis of systemic envenoming in mice, and little published on post mortem findings of mice and, indeed, of envenomed humans and domestic animals. Most of the data on snake venom/venom toxin pathogenesis is derived from experiments examining the mode of action of specific venom toxins in mice and rats, for example: Baldo et al, 2010 (haemorrhagic snake venom metalloproteinases (SVMPs)); Gutierrez et al, 2009 (local tissue damage); Hernandez et al, 2011 (skeletal muscle necrosis) and Herrera et al 2015 (extracellular matrix degradation).

Much of the human pathological data is extrapolated from clinical data collected from snakebite victims attending hospital, the vast majority of whom survived envenomation, albeit some with temporary or permanent physical or psychological disabilities. In many of the areas in which snakebite is common, post mortem (PM) examination is considered to be a desecration of the body, making PM examination unethical. Even in animals there are only a few publications on the post mortem findings of snakebite victims (Mitchell, 1915;

Banga, 2009; Jacoby-Alner, 2011). The preclinical testing experiments of this research provided unique opportunities to examine venom induced pathology in the murine model of envenoming.

7.2 Objectives

- The objective of performing PM examinations on experimentally envenomed mice was to establish a 'pathological profile' for as many different venoms as possible. PM examinations were only performed on mice subjected to envenomation as part of another experiment.
- Secondly, to compare venom pathological profiles of mice that did (ED₅₀) and did not (LD₅₀) receive antivenom to identify the extent to which an antivenom neutralised venom-induced pathological lesions.
- The third objective was to compare venom pathogenesis in experimentally envenomed mice with that of human snakebite victims.
- The final objective was to create a simple, objective scoring system for ante- and post-mortem pathological lesions, which could be used to compare efficacy of antivenoms against the same venom(s). In this way, a full ED₅₀ assay need not be performed, potentially substantially reducing the numbers of mice required.

7.3 Materials and Methods

A summary of the *in vivo* experiments, detailing venom and antivenom doses and numbers of mice used, from which data was used for this chapter can be found in Appendix IV.

7.3.1 Venoms:

7.3.1.1 Viper species

Echis ocellatus, *E. coloratus*, *E. pyramidum leakeyi*, *E. carinatus sochureki*

Bitis arietans

Vipera berus

Cerastes cerastes

Callesolasma rhodostoma

7.3.1.2 Elapid species

Naja nigricollis, *N. nubiae*, *N. pallida*

N. haje, *N. nivea*

Dendroaspis angusticeps, *D. polylepis*

Aspidelaps scutatus

7.3.1.3 Colubrid species

Dispholidus typus

'Core' venoms in bold

7.3.2 Antivenoms

Table 7.3-1: Antivenoms used in Pathology section. See below for key to numbers

Antivenom	Manufacturer	Directed against:
EchiTabG	MicroPharm Ltd	1
ViperaTab		5
ViperaVet		5, 21, 22, 23
European, Viper venom antiserum	Institute of Immunology, Croatia	22
EchiTabPlus-ICP	Instituto Clodomiro Picado	1,4,6
SAIMR polyvalent	South African Vaccine and Serum Producers (formerly South Africa Institute of Medical Research)	4, 8, 9, 10, 11, 13, 18, 14, 15, 19
SAIMR monovalent		1
SAIMR boomslang		12
FAV Afrique	Sanofi	1, 16, 4, 13, 14, 11, 17, 8, 15, 6, 20
Inoserp Panafrica	Inosan Biopharma	6, 11, 1, 4, 16, 3, 2, 13, 10, 14, 17, 8, 7, 15
VINS Africa	VINS bioproducts Ltd	1, 16, 4, 13, 8, 6, 15, 11, 14, 17
PSV Polyvalent snake antiserum -Africa	Premium Serum & Vaccines Pvt Ltd, India	1, 2, 16, 4, 13, 6, 8, 15, 10, 11, 14, 17

Key for Table 7.3-1

1	<i>Echis ocellatus</i>	13	<i>Bitis gabonica</i>
2	<i>Echis coloratus</i>	14	<i>Dendroaspis jamesoni</i>
3	<i>E. pyramidum leakeyi</i>	15	<i>Naja melanoleuca</i>
4	<i>Bitis arietans</i>	16	<i>Echis leucogaster</i>
5	<i>Vipera berus</i>	17	<i>Dendroaspis viridis</i>
6	<i>Naja nigricollis</i>	18	<i>Hemachatus haemachatus</i>
7	<i>Naja pallida</i>	19	<i>N. mosambica</i>
8	<i>Naja haje</i>	20	<i>N. senegalensis</i>
9	<i>Naja nivea</i>	21	<i>V. aspis</i>
10	<i>Dendroaspis angusticeps</i>	22	<i>V. ammodytes</i>
11	<i>Dendroaspis polylepis</i>	23	<i>V. latastei</i>
12	<i>Dispholidus typus</i>		

7.3.3 Post mortem examinations

Post mortem examinations were performed on mice as soon as possible after death or CO₂ euthanasia. Some experimental objectives required a cardiac puncture to be performed, to obtain larger blood samples than could be obtained from a tail snip, immediately after death. The blood was then placed in an appropriate serum, heparin, EDTA or citrate tube, a thin blood smear made and a sample (~0.5mL) put in a glass tube for a 20 minute whole blood clotting test (20WBCT).

External features were observed for surface haemorrhages, epistaxis, haemoptysis, hyphaema and colour of extremities. Signs of sweating, salivation and voided urine were noted.

A mid-line incision was made from the level of the pubis up the abdomen and thorax, extending to the ramus of the mandible. The skin was reflected, pinned back and a note made of abnormalities in the subcutaneous tissues – pale, congested, jaundiced, or haemorrhagic. Similarly, the musculature of the body wall and hind limbs were examined before making a midline incision into the abdominal cavity. Any intra-abdominal blood, with or without clots, was recorded and the colour of the abdominal organs noted. The size of the bladder and colour of urine were recorded, and a sample of urine collected to be examined for blood, haemoglobin or protein. The testicles were inspected for evidence of haemorrhage.

The gastrointestinal tract (GIT) was removed by severing the oesophagus, where it passes through the diaphragm, and the rectum, by carefully teasing it free from the mesentery. Any discolouration - congestion, haemorrhage, dark green, black or yellow - was noted. The spleen was removed with the GIT. The stomach and sections of the intestines were split longitudinally, washed gently with 10% formalin before placing in a labelled Falcon tube, containing sufficient 10% formalin to cover the contents, to allow later histopathological examination.

The liver was excised, making a note of gall bladder size. The kidneys were left *in situ*, unless required for histopathology. Before opening the thorax, the diaphragm was examined for haemorrhages and signs of haemothorax, which is best visualised through the diaphragm.

Table 7.3-2: Template for recording post mortem findings, pathology, survival and pathology/survival score (PSS)

Date		Venom/dose μg			Analgesia/dose	
Group:		AV/dose μL				
Mouse		1	2	3	4	5
Epistaxis/haemoptysis						
Incoagulable blood						
Tongue						
Ears						
Body wall						
Muscle						
Haemoperitoneum						
Haematuria						
Bladder						
Liver						
Kidneys						
Spleen						
Stomach						
Intestines						
Haemothorax						
Right lung						
Left lung						
Atria						
Left ventricle						
Right ventricle						
Cranium						
Orbits						
Brain						
Score						
Survival time/score						
PSS						
Comments						

Key: C = congestion P = petechiation V = ventricles
 H = haemorrhage SI = small intestines A = atria
 E = ecchymoses LI = large intestines M = multiple
 D = diffuse F = focal SEH = sub-epicardial haemorrhage

Score: 0/NAD = absent 1 = mild (congested, pale or minor haemorrhage)

2 = severe (diffuse haemorrhage, collapsed or consolidated lungs)

The thoracic wall and diaphragm were removed to allow inspection of the thoracic contents; the heart, lungs and thymus (pluck). The pluck was

removed by splitting the mandible, dissecting the tongue, larynx, trachea and oesophagus followed by lungs, heart and thymus.

Any abnormality of the forelimbs was noted (muscle or joint haemorrhage) and samples taken for histopathology, before turning the carcass over to examine the cranium. A midline incision was made over the skull and neck and the skin reflected to expose the eyes and cranium, noting any congestion or haemorrhage. Finally, a square of the skull extending from between the eyes to the foramen magnum was removed, to allow examination of the brain. The brain was carefully dissected free from the skull and the ventral surface of the brainstem, mid-brain and hypothalamus examined.

Photographs were taken of representative pathological lesions and of any unusual or unexpected findings. Table 7.3-2 shows the template used to record post mortem findings.

7.3.4 Pathology-survival score

All pathological lesions were scored as follows: 0 (absent); 1 (mild – congestion, pale or small, focal haemorrhages); 2 (severe – significant haemorrhage, extensive lung collapse or consolidation). Figures 7.4-1 to 7.4-12 illustrate examples of the range of severity of pathological lesions observed and how they were scored to obtain the ‘pathology score’

The survival time after venom injection was noted and assigned a score 0-12. The 6 hour assay was divided into 12 half hour periods and a score of ‘12’ assigned to those which died in the first 30 minutes, ‘11’ for those which died 31-60 minutes, and so on, so that those which survived to the end of the experiment scored ‘0’.

7.3.5 Histopathology

The following organs were placed in 10% formalin for subsequent histopathology: GIT, spleen, liver, kidneys, diaphragm, heart, lungs, skeletal muscle, thymus and brain. Selected samples were processed by Dr Gail Leeming from the Veterinary Pathology Department, Liverpool University.

Briefly, after the tissues were embedded and sliced with a microtome, the sections were stained with haematoxylin and eosin (H&E), periodic acid-Schiff (PAS) – a stain for carbohydrate, or Perl's Prussian blue (PPB) – a stain for iron which is used to detect haemosiderin.

7.3.6 20 minute whole blood clotting test (20WBCT)

Blood collected via cardiac puncture was placed in a small, glass test tube, which was left to stand and was examined periodically for the presence or absence of a clot. If there was no clot formation after 20 minutes, the blood was deemed 'incoagulable'.

7.3.7 Blood smears

Thin blood smears were performed and stained with 'Diff-Quik' (Fisher Scientific), which is a modified Giemsa stain. The smears were examined microscopically for white blood cell, platelet and red blood cell morphology.

7.4 Results

7.4.1 Mouse pathology

The observed pathological lesions of systemic envenoming reflected the generalised assumption that viper venoms are haemorrhagic and elapid venoms neurotoxic. The result of the five 'core' venoms and other venoms studied (for other projects) are summarised in Table 7.4-1 (viper) and Table 7.4-2 (elapid and colubrid). The haemorrhagic viper venoms could be further subdivided into those which were coagulopathic (*Echis* spp, *Cerastes cerastes* and *Calloselasma rhodostoma*) and those which were not (*Bitis arietans* and *Vipera berus*). The one Colubrid venom studied, *Dispholidus typus* (Boomslang) was coagulopathic. Many of the mice envenomed with Viper venoms had terminal convulsions, 'leaping' or paralysis of one or both hind limbs (See Chapter 6, HEPs). Post mortem examination of these individuals rarely revealed gross changes to the brain, but more frequently myocardial haemorrhage (Figure 7.4-7C), usually accompanied by signs of congestive heart failure (Figure 7.4-6C), or severe lung pathology. The assumption was made that the cause of death in these animals was due to cerebral anoxia

arising from inadequate oxygenation in severely compromised pulmonary tissue or poor circulation resulting from heart failure.

Table 7.4-1: Viper venom pathological lesions

Venom	EoV	BaV	VbV	CrV	CcV	EpIV	EcolV	EcsV	N	M
Body wall	H/E	P	-	C	C	-	-	-	-	-
Sweating	-	-	-	-	-	-	-	-	-	-
Epistaxis	+	-	(+)	-	+	+	+	+	-	-
Incoagulable	+	-	-	+	+	+	+	+	-	-
Tongue/SG	H	P(SGs)	-	-	-	-	-	-	-	-
Eyes	H (face)	-	H	-	-	-	-	-	-	-
Ears	H	-	-	-	-	-	-	-	-	-
Muscle	H	H	-	-	-	-	-	-	-	-
Muscle fasc.	-	-	-	-	-	-	-	-	-	-
Haemothorax	+	±clot	±clot	±	-	+	+	+	-	-
Pulmonary H	H/E	P/E/H	M _{ottled}	+	+	H/P/E	E	H/E	-	-
Pulmonary C	+	+	+	+	M _{ottled}	-	-	-	±	-
Lung collapse	-	+	+	+	+	-	-	-	-	-
Lung consol	-	+	+	+	-	-	-	-	-	-
Atrium	H	H	H/C	H/C	H	H	-	H	-	±C
Ventricles	H	H/E/P	H/SEH/C	C	H/SEH	H/SEH	-	H	-	-
Microcardia	+	+	-	-	-	+	-	+	-	-
Diaphragm	H	P	-	-	-	-	-	-	-	-
Liver	C/Pale	C	C	C	C/Pale	C/Pale	-	-	C	C
Kidney	C/Pale	C/s _{wollen}	C/s _{wollen}	C	C/Pale	C/Pale	-	-	-	-
Spleen	C/Pale	C	C	C	C/Pale	C/Pale	-	-	-	-
Stomach	Bk	H/Bk/P	H/Bk	-	Bk	-	-	-	-	-
Duodenum	G/Bk/H	H/P/G	-	-	H/G	H/G	-	H	-	-
Mid SI	G/Bk/H	G	H/G/C	-	G	H/G	-	H	-	-
Distal SI/LI	G/Bk/H	G	H _{colon}	-	G	H/G	-	H _{gas}	-	-
Mesentery	-	P	-	-	-	-	-	-	-	-
Haematuria/Hburia	-	-	-	-	-	-	-	-	-	-
HaemoP	-	-	-	±	-	-	-	±	-	-
Cranium	C/H	P/H/C	C	C/P	C	-	-	-	-	-
Orbital sinus	H	H	H/C	H	H	-	-	-	-	-
Brain	C	C/H/P/E	C	C	-	-	-	-	-	-
Tail	H	-	B _{ruised}	-	-	-	-	B _{ruised}	-	-
Testicles	H	P/H	-	-	-	-	-	-	-	-
Feet	-	-	-	-	-	-	-	-	-	-

Key:

EoV	<i>Echis ocellatus</i> venom	SI	Small intestines
BaV	<i>Bitis arietans</i> venom	LI	Large intestines
VbV	<i>Vipera berus</i> venom	HaemoP	Haemoperitoneum
CrV	<i>Calloselasma rhodostoma</i> venom	SEH	Subepicardial haemorrhage
CcV	<i>Cerastes cerastes</i> venom	G	Green
EpIV	<i>Echis pyramidum leakeyi</i> venom	Bk	Black
EcolV	<i>E. coloratus</i> venom	Fascic	Fasciculations
EcsV	<i>E. carinatus sochurekei</i> venom	Hburia	Haemoglobinuria
H	Haemorrhage	Y	Yellow
C	Congestion	+	Present
E	Ecchymoses	±	Mild
P	Petechiae	-	Absent
Consol	Consolidation		

The majority of the venoms studied produced pathology of the gastrointestinal tract (Figure 7.4-3). These changes were observed in animals which survived for several hours after venom injection, and tended to occur in a venom-specific site in the intestines. In the mid small intestines through to the large intestines, pathology in different animals showed progression from an

increasingly intense green colour (Figure 7.4-3C and D) prior to frank haemorrhage (seen as a red-tinged tarry colour – Figure 7.4-3 E, G and H). A similar progression from dark discolouration to obvious haemorrhage of the stomach wall was seen, which may have been due to ulceration of the stomach lining, but was not confirmed histologically.

Table 7.4-2: Elapid venom/*colubrid venom pathological lesions

Venom	NnigV	DaV	NnubV	NhajV	NpalV	DpV	AsV	DtV*
Body wall	C	-	Y	-	C	C	-	P _{ale} /Y
Sweating	-	+	-	+	+			-
Epistaxis	-	-	-	-	-	-	-	+
Incoagulable blood	-	-	-	-	-	-	+	+
Eyes	-	-	-	-	-	-	-	O _{paque}
Ears	-	-	-	-	-	-	-	-
Muscle	-	-	-	-	C	-	-	+
Muscle fascic	-	+	-	-	-	-	-	-
Haemothorax	-	-	-	-	-	-	-	+
Pulmonary H	-	-	-	-	-	-	M _{ottled}	H/E/P
Pulmonary C	+	+	-	+	+	+		+
Lung collapse	+	+				+	+	+
Lung consolidation	+	+		+				
Atrium	C/>	H	H	C/>	H	C/>	H	H/C
Ventricles	H/SEH/C	H/SEH/C		SEH/C	H	H/SEH/C	SEH	H/SEH/C
Liver	C	+/-C	G _{allbladder}	C	C	C		C/Pale
Kidney	C	+/-C	C	C	C	C		C/Pale
Spleen	C	+/-C	C	C	C	C		C/Pale
Stomach	H/Bk	H/Bk	Y	-	-	-		H
Duodenum	H/C	H/G	Y	-	-	-		H
Mid SI	H/C	H/G	Y	-	-	-		H/G
Distal SI/LI	H	H/G	Y	-	-	-		H/G
Haematuria/Hburia	+	-	+	-	-	-		-
Haemoperitoneum	-	+/-	+	-	-	-	+	+
Cranium	C	C	C	-	C	C	C	C/H
Orbital sinus	C	C		-	-	-	C	C
Brain	P/C	C	C	-	C	C		C
Tail	-	-	-	-	C	-		-
Testicles	-	H	-	-	-	-		-
Feet	-	-	-	-	C	-	-	-

Key:

NnigV = *Naja nigricollis* venom
 DaV = *Dendroaspis angusticeps* venom
 NnubV = *Naja nubiae* venom
 NhajV = *Naja haje* venom
 NpalV = *Naja pallida* venom
 DpV = *Dendroaspis polylepis* venom
 AsV = *Aspidelaps scutatus* venom
 DtV = *Dispholidus typus* venom
 + = frequently observed
 +/- = observed occasionally

H = haemorrhage
 C = congestion
 SEH = subepicardial haemorrhage
 G = green
 Y = yellow
 Bk = black
 Hburia = haemoglobinuria
 SI = small intestines
 LI = large intestines
 Fascic = fasciculations
 > = enlarged

The elapid venoms showed evidence of inducing neurotoxicity at post mortem examination in the form of pulmonary congestion, consolidation and collapse (Figure 7.4-8B and C), although these observations were less frequent in mice envenomed with spitting cobra venoms (*Naja nigricollis* and *N. nubiae*). All the elapid venoms showed evidence of inducing myocardial

damage, either as myocardial haemorrhage (Figure 7.4-7 or C, E, F and G) signs suggestive of cardiac insufficiency (congested organs).

The following figures (Figure 7.4-1 to 7.4-12) show the pathological lesions observed and how they were scored.

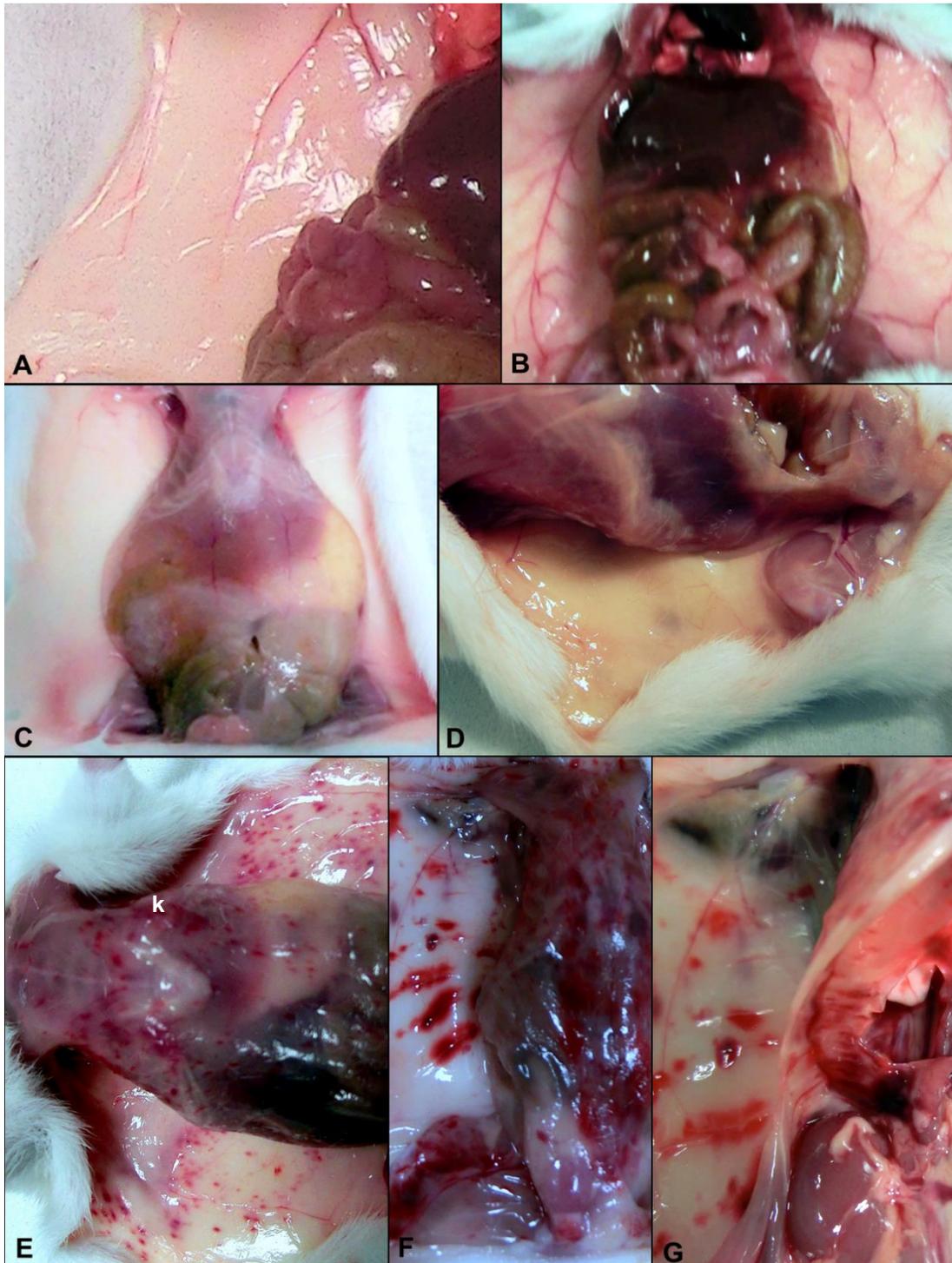


Figure 7.4-1: Post mortem lesions of the body wall of envenomed mice. A: Normal, score=0; B: Congestion/hyperaemia, score=1; C: Pale, score=1; D: Jaundiced/yellow,

score=1; E: petechial haemorrhages in subcutaneous body wall and abdominal and thoracic body wall musculature, score=2; F and G: Similar distribution of ecchymotic 'paint splash' haemorrhages, score=2.



Figure 7.4-2: Post mortem lesions of the abdominal cavity of envenomed mice. A: normal liver (score=0) with pale green intestines (score=0); B: congested liver – score=1- and spleen, score=1; C: Pale liver (score=1), spleen (score=1) and kidney (score=1); D: Haemoperitoneum – arrow shows accumulation of blood between intestinal loops (score=2); E: Diaphragmatic haemorrhages (score=2). Normal lung tissue (arrow) can be seen through the diaphragm.

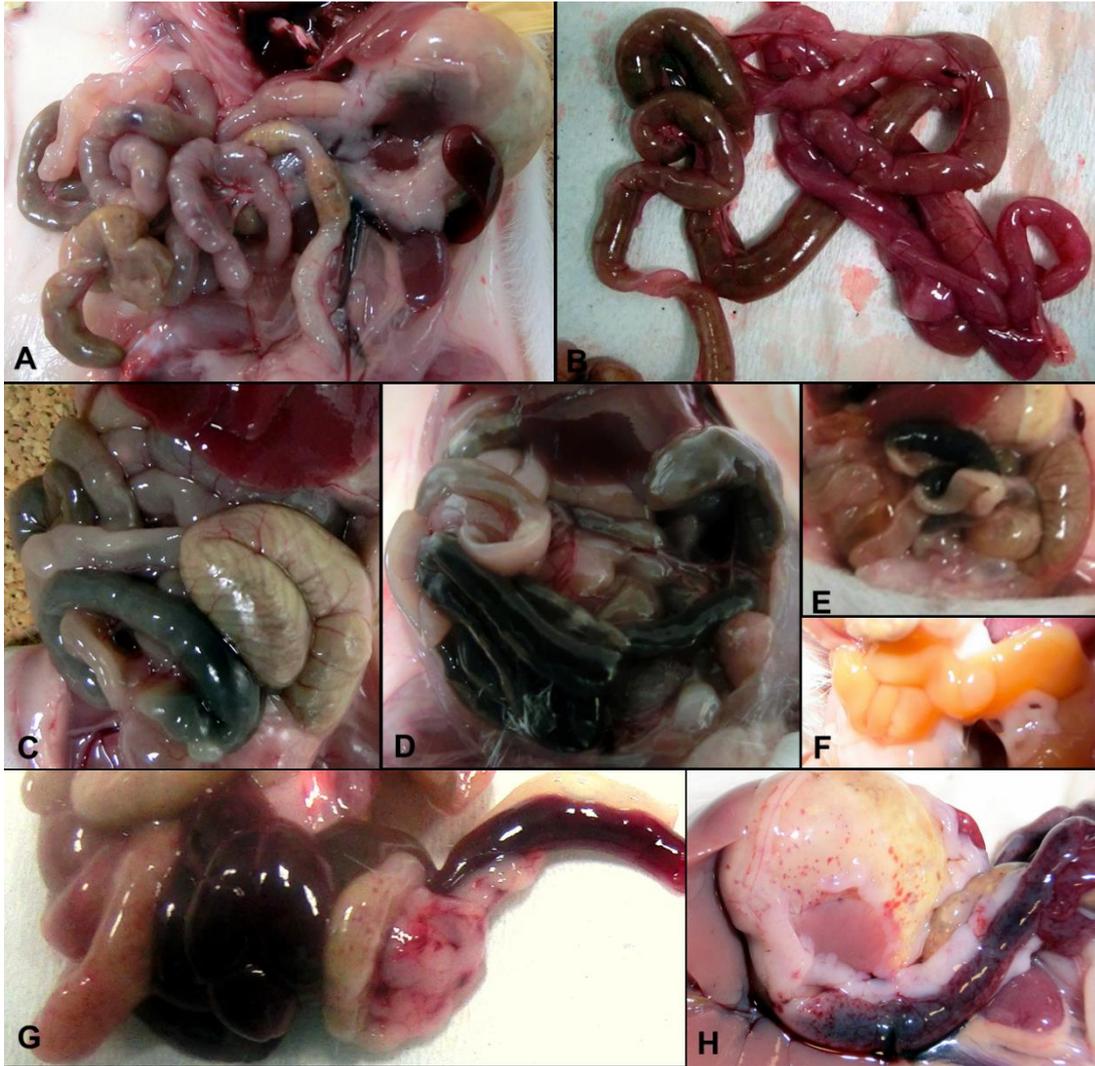


Figure 7.4-3: Post mortem lesions of the intestines of envenomed mice. A: normal intestines (score=0); B: congested intestines (score=1); C: Loops of pale green to darker green intestines, ?pre-haemorrhagic change, (score 0); D: dark green intestines (score=1); E focal loop of black intestines (score=1); F Loop of yellow intestines (score=1); G intestinal haemorrhage (score=2); H: duodenal haemorrhage (score=2) and petechiation of mesentry (score=1) and serosa of stomach(score=1).

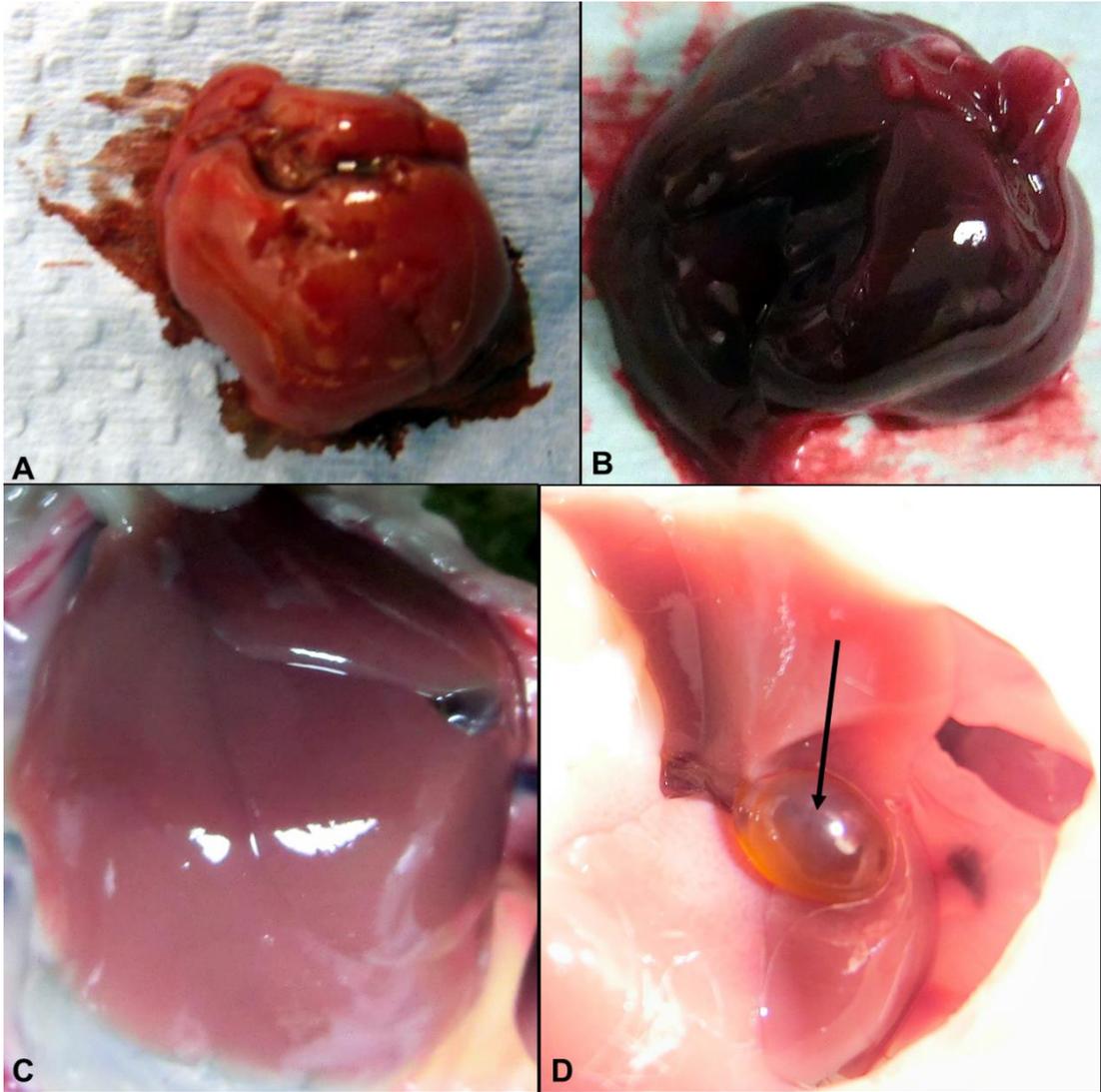


Figure 7.4-4: Post mortem lesions of livers of envenomed mice. A: normal liver (score=0); B: congested liver (score=1); C: pale liver (score=1); D: pale liver with large, distended gall bladder (score=1).

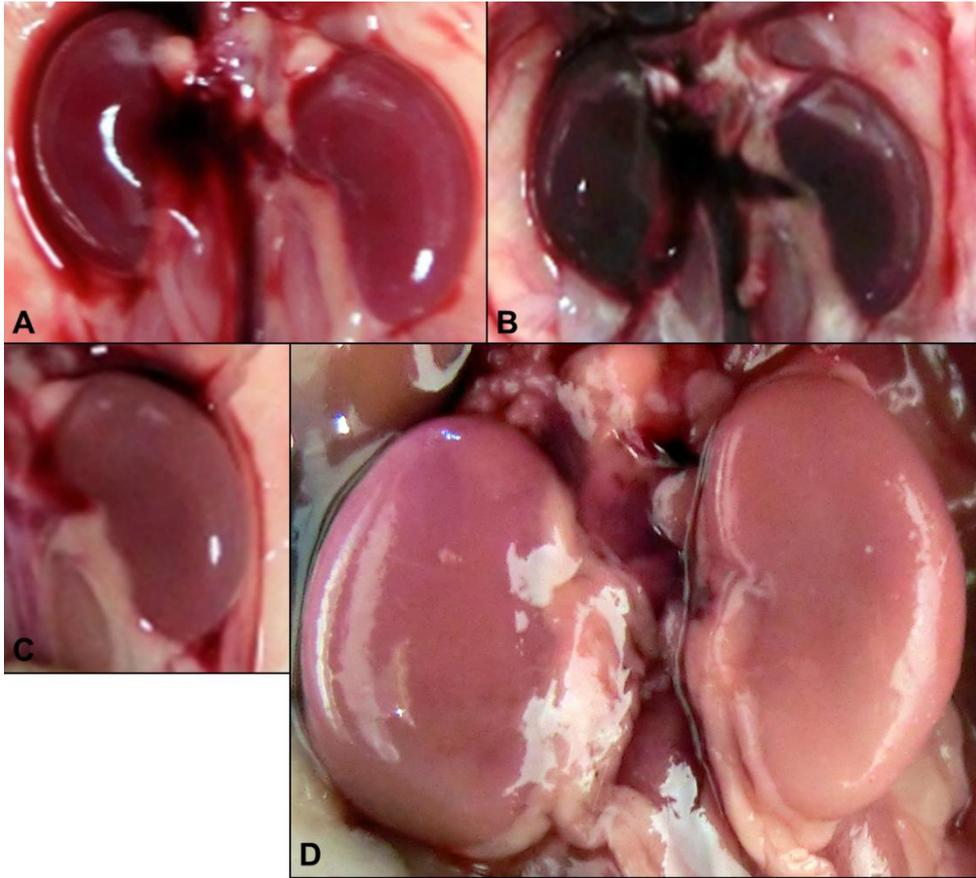


Figure 7.4-5: Post mortem lesions of kidneys of envenomed mice. A: Normal kidneys (score=0); B: congested kidneys (score=1); C: pale kidneys (score=1); pale and swollen kidneys (score=1).

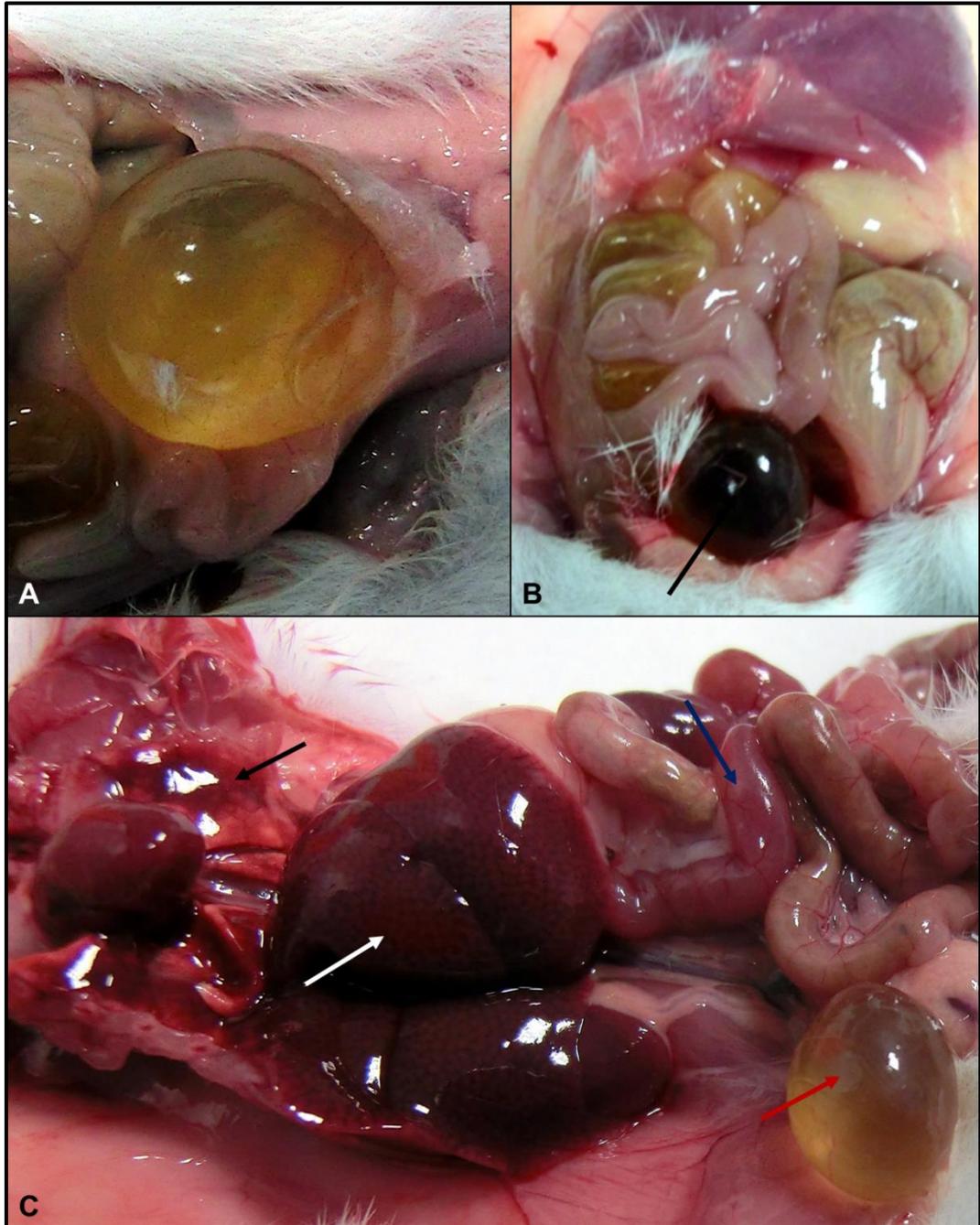


Figure 7.4-6: Post mortem observations of bladders in envenomed mice. A: Distended bladder (score=0); B: Bladder containing dark urine indicative of haematuria, haemoglobinuria or myoglobinuria (score=2); C congested organs – lungs (black arrow, liver (white arrow), intestines (blue arrow) and distended bladder (red arrow).

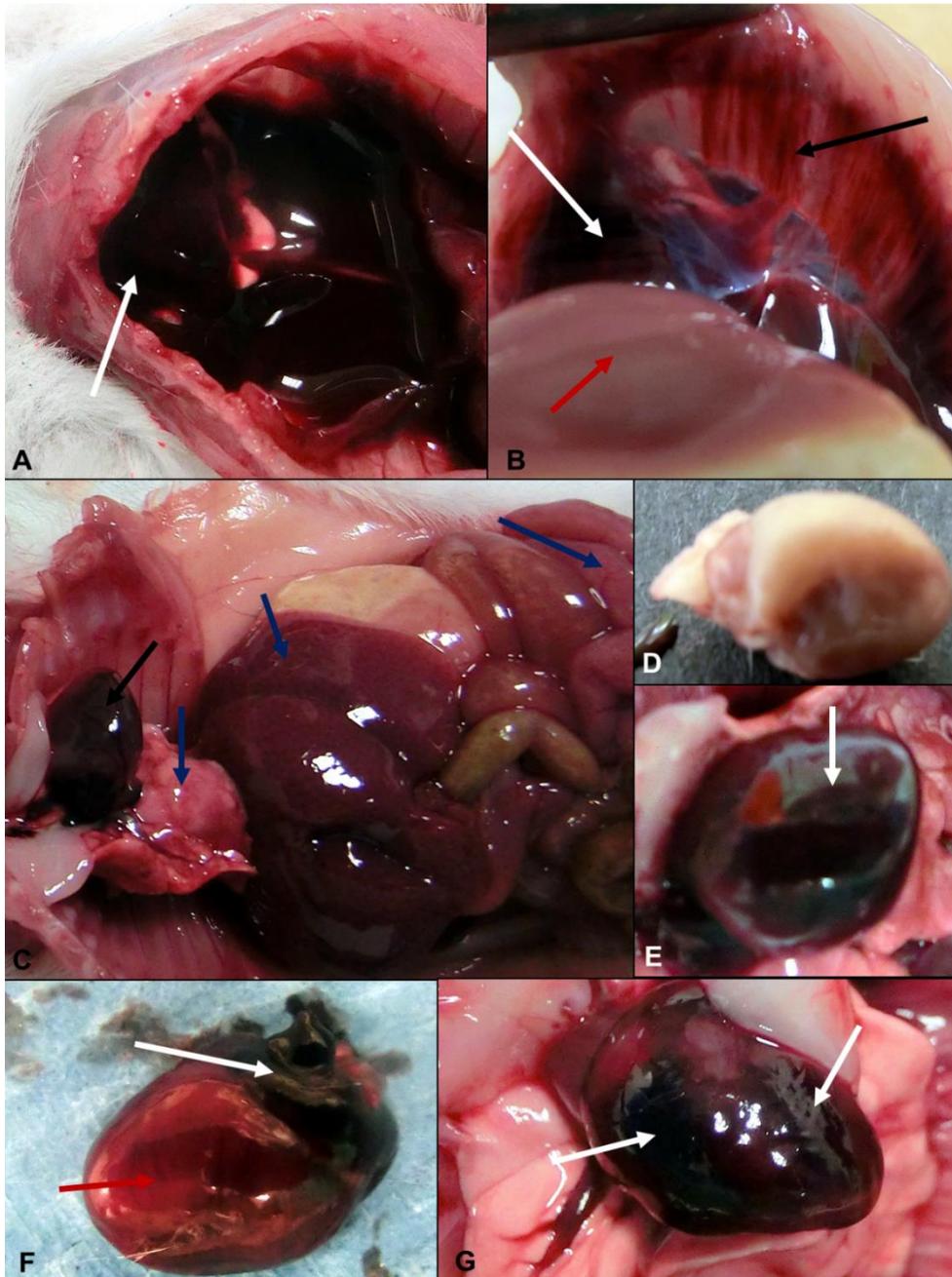


Figure 7.4-7: Post mortem observations of thoracic organs in envenomed mice. A: Haemothorax – free blood in the thoracic cavity (white arrow-score=2); **B:** Haemothorax viewed through the diaphragm (white arrow), diaphragmatic haemorrhage (black arrow, score=2 for each ventricle), pale liver (red arrow – score=1); **C:** Ventricular myocardial haemorrhage (black arrow-score=2) with congested organs (blue arrows – score=1 for each organ); **D:** Fixed heart of normal mouse, showing no gross evidence of haemorrhage (score=0); **E:** right ventricular haemorrhage (white arrow-Score=2); **F:** fresh post mortem specimen of heart showing atrial haemorrhage (white arrow – score=2) and congestion/hyperaemia of the ventricular myocardium (red arrow-score=1 for each ventricle); **G:** Large haemorrhages of ventricular myocardium (white arrows – score=2 for each ventricle).

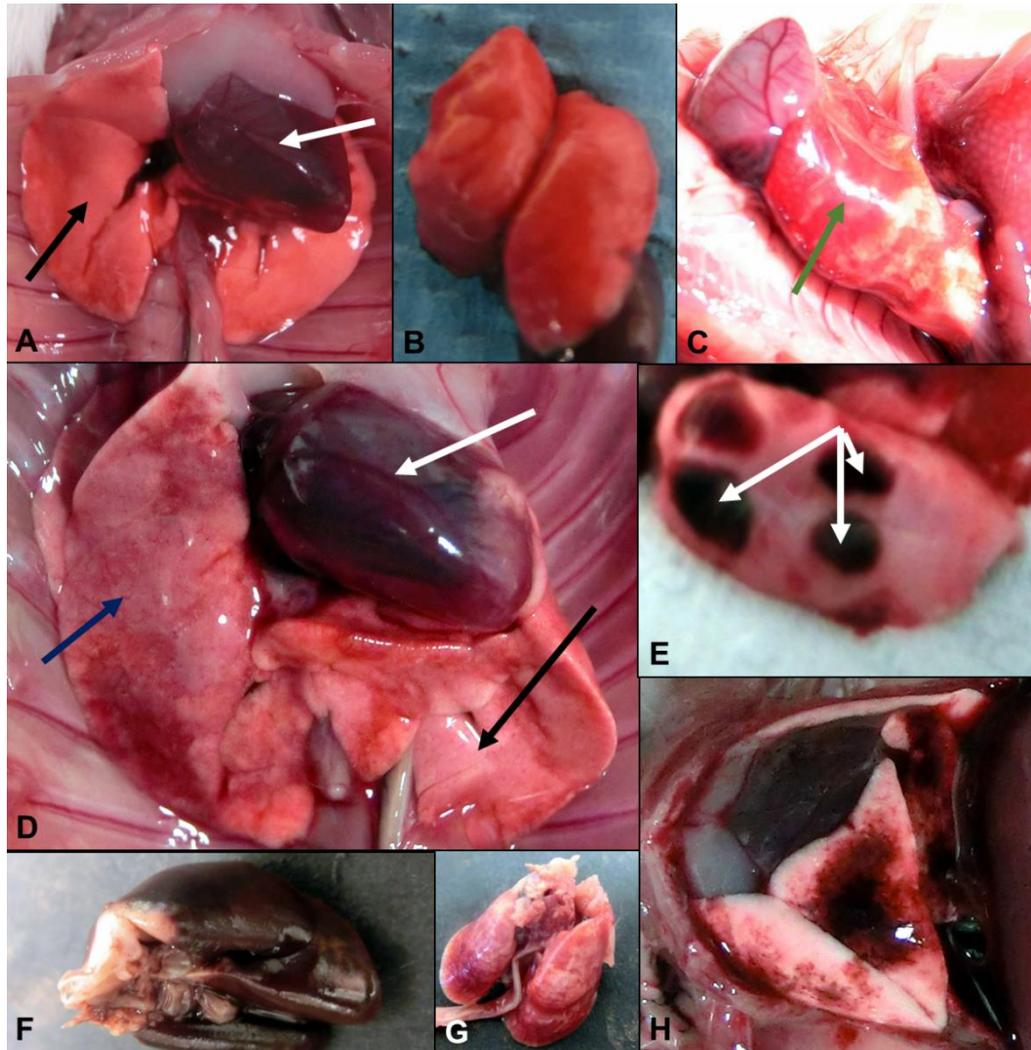


Figure 7.4-8: Post mortem observations of lungs in envenomed mice. A: Normal lungs (black arrow, score =0) and right ventricular haemorrhage (white arrow, score =2); B: Congested lungs (score =1); C: collapsed and consolidated lungs (green arrow, score=2); D: Mottled, collapsed right lung (score=1), normal lung (score=0) and right ventricular subepicardial haemorrhage (score=2); E: multiple focal pulmonary haemorrhages (white arrows, score=2); F: fixed specimen showing extensive pulmonary haemorrhage (score=2 for each lung); G: mottled, bilateral, pulmonary congestion (score=1 for each lung); H: Mottled pulmonary haemorrhage (score =2).

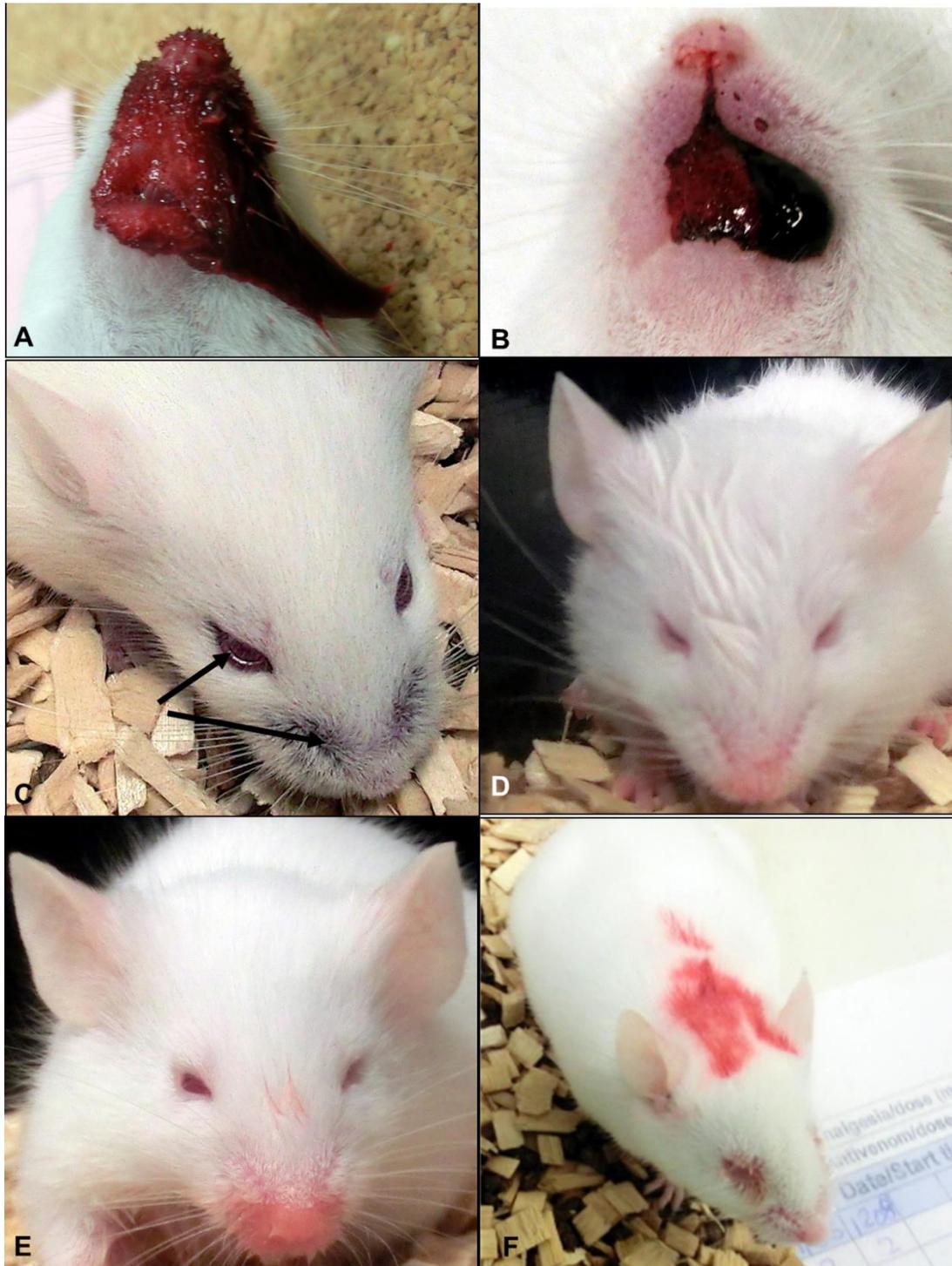


Figure 7.4-9: Post mortem and antemortem observations of facial features of envenomed mice. A: Epistaxis – blood from the nose (score=2); B: Haematemesis – blood from the mouth (score=2); C: Subcutaneous haemorrhage seen around the eyelids and whisker pad (arrows-score=2); D: Ptosis and sweating; E: salivation and ptosis; F: mucoid ocular secretions.

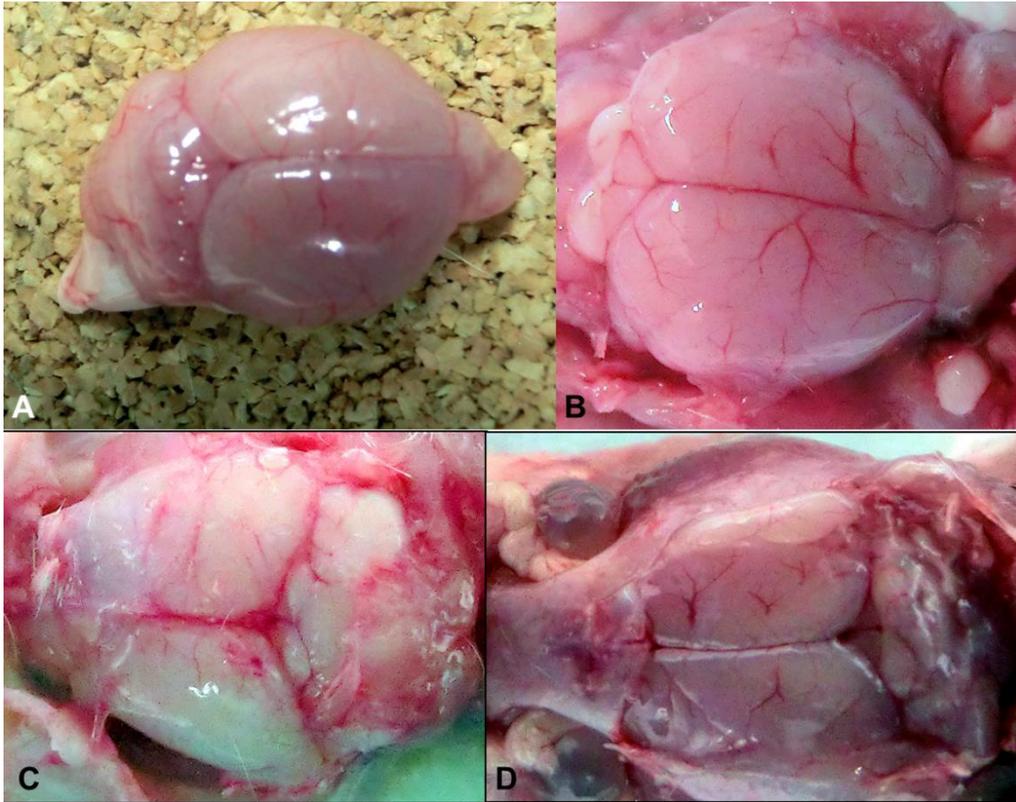


Figure 7.4-10: Post mortem lesions observed in the brains of envenomed mice. A: normal brain (score=0); B: hyperaemia (score=0); C: hyperaemia/congestion (score=1); D: hyperaemia/congestion of brain *in situ*.

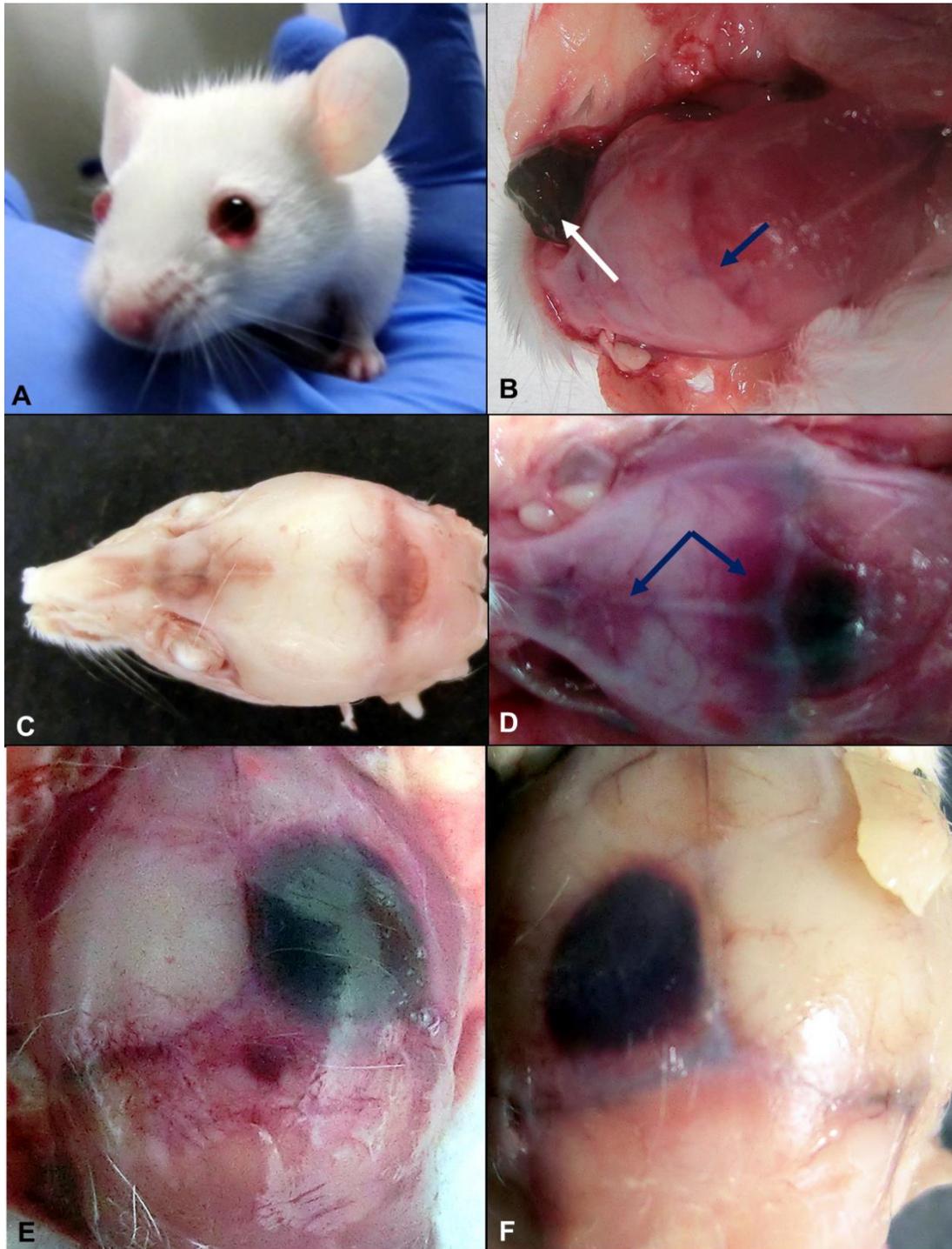


Figure 7.4-11: Post mortem and antemortem lesions of the eyes, skulls and brains of envenomed mice. A: Hyphaema – haemorrhage into the chambers of the eye; B: Haemorrhage into the orbital sinus of the skull, associated with hyphaema. Blue arrow shows hyperaemia/ congestion of cranium; C: Fixed specimen of skull of a normal CD1 mouse; D: Intracranial haemorrhage with surrounding contusions (blue arrows); E and F: Intracranial haemorrhages. The fresh specimen (E) shows hyperaemia around the suture lines of the cranium.

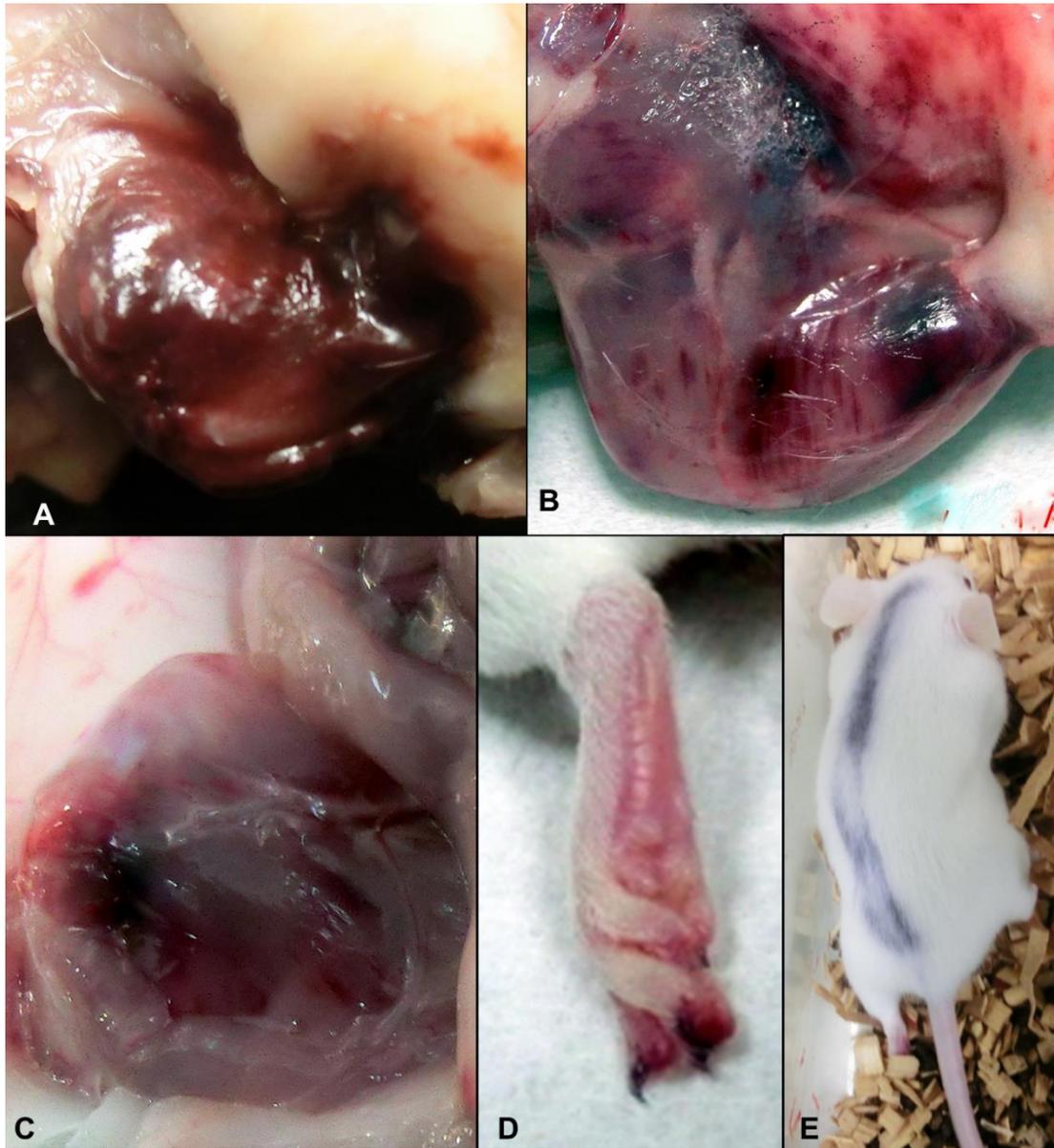


Figure 7.4-12: Postmortem and antemortem observations of the limbs of envenomed mice. A: Fixed specimen showing haemorrhage of the muscles of the forelimb (score=2); B:Haemorrhagic lesions in the muscles of the hind limb (score=2); C: Haemorrhagic lesion over the stifle joint (score = 2); Congestion of phalangeal pads of the hind limb (score=1); Antemortem left hind limb paralysis (not scored).

7.4.1.1 *Echis* species

The most striking feature of the pathological lesions induced by the venom of *Echis spp* was incoagulable blood with widespread haemorrhage. Most commonly, there was extensive pulmonary haemorrhage (Figure 7.4-8E and F) and haemothorax (free blood within the thoracic cavity – Figure 7.4-7A and

B). Epistaxis was frequently seen shortly before death, and was associated with severe pulmonary haemorrhage.

The post mortem findings of mice envenomed with *Echis ocellatus*, *E. carinatus sochureki* or *E. coloratus* venoms and those with a pre-incubated mixture of *E. ocellatus* venom with EchiTabG antivenom (EOG) or South African Vaccine Producers monovalent Boomslang antivenom (Sb) are listed in Table 7.4-3. Some of the mice which had received venom/antivenom containing aggregates had congested or oedematous organs (Figure 7.4-5D), indicated in the table as 'Aggregate pathology' to differentiate from those with haemorrhagic lesions associated with envenomation by this species.

Mice treated with venom and homologous antivenom still exhibited incoagulable blood and haemorrhages, but with a significant reduction in the number of mice which had 'pale organs' (Figures 7.4-2C, 7.4-4C, 7.4-5C) suggesting that the haemorrhages were less severe. There was also an increase in the proportion of mice showing intestinal haemorrhage (Figure 7.4-3), which suggested that the mice had survived longer. The data was taken from all dose groups of all lethality experiments performed from 2012 until 2016, using this family of venoms.

Mice injected with *E. ocellatus* venom preincubated with Boomslang antivenom showed a completely different pathological profile and will be considered below in Section 7.5.3

Table 7.4-3: Post mortem (PM) examination observations of mice subjected to different doses of *Echis ocellatus* venom alone (green) or in combination with different antivenoms (blue) – (2.5 x venom LD50 + different doses of EOG or 150µL/mouse of Sb), showing occurrence of pathological lesions as a percentage of the total number of PM examinations performed (n). ‘Aggregate pathology’ refers to pathological lesions which differs from that seen with venom alone

Pathological lesions	Venom	EoV			EcsV	EcoIV
	Antivenom	None	EOG	Sb	None	None
Body wall H		E:4	0	P:80	0	0
Body wall C		1	9	0	0	0
Incoagulable blood		85	73	100	100	90
Epistaxis		41	15	40	0	0
Hyphaema/Eye H		0	1	0	0	0
Ptosis		0	0	0	0	0
Ears H		0	0	0	0	0
Muscle H		3	0	60	0	0
Muscle fasciculation		0	0	0	0	0
Haemothorax		54	61	0	100	90
Pulmonary H		86	72	80	70	50
Pulmonary C		4	15	0	0	0
Lung collapse		3	0	0	0	0
Lung consolidation		0	0	0	0	0
Atrium H		54	32	80	60	0
R ventricle H		52	48	100	60	0
L ventricle H		8	4	80	60	0
Congested organs		25	24	0	0	0
Pale organs		27	12	60	0	0
Stomach H		25	4	H:60P:20	0	0
Intestinal H		27	59	20	10	10
Mesentery H		0	0	100	0	0
Haemoperitoneum		5	1	0	0	0
Haematuria/Hburia		0	1	0	0	0
Cranium – C or H		58	23	20	0	0
Orbital sinus H		6	10	0	0	0
Brain C		6	18	40	0	0
Tail H		8	1	0	0	0
Other H		5	0	0	50	0
PMs performed (n)		79	82	5	10	10
Aggregate path (%)		N/A	7	0	N/A	N/A

Key EoV: *Echis ocellatus* venom
EcoIV: *Echis coloratus* venom
Sb: SAIMR boomslang antivenom
E: Ecchymoses
C: Congestion
PM: Post mortem examination

EcsV: *Echis carinatus sochureki* venom
EOG: EchiTabG antivenom
H: Haemorrhage
P: Petechial haemorrhage
Hburia: Haemoglobinuria

Table 7.4-4: Post mortem (PM) examination observations of mice subjected to different doses of *Echis pyramidum leakeyi* venom alone (green) or 5x venom LD₅₀ in combination with different antivenoms, equivalent to 1 or 2 x ED50 dose of SAIMR monovalent *Echis* antivenom (blue), showing occurrence of lesions observed as a percentage of the total number of PMs performed (n). *indicates 2/4 aggregate related deaths

Pathological lesions	Venom Antivenom	<i>Echis pyramidum leakeyi</i> venom					
		None	Sm	FA	VINS	Ino	PSV
Body wall H		0	0	0	0	0	0
Body wall C		0	0	0	25	57	0
Incoagulable blood		67	100	100	100	71	100
Epistaxis		8	0	57	0	14	0
Hyphaema/Eye H		0	0	0	0	0	0
Ptosis		0	0	0	0	0	0
Ears H		0	0	0	0	0	0
Muscle H		0	0	0	0	0	0
Muscle fasciculation		0	0	0	0	0	0
Haemothorax		67	0	86	0	0	50
Pulmonary H		92	0	71	0	57	25
Pulmonary C		0	0	57	100	43	50
Lung collapse		0	0	0	50	0	0
Lung consolidation		0	0	0	0	0	0
Atrium H		0	100	100	100	100	50
R ventricle H		17	100	57	100	43	25
L ventricle H		8	0	14	100	0	0
Congested organs		8	0	86	0	43	100
Pale organs		8	50	71	50	57	0
Stomach H		0	0	29	0	43	50
Intestinal H		25	50	29	0	57	100
Mesentery H		0	0	0	0	0	0
Haemoperitoneum		0	0	0	0	0	0
Haematuria/Hburia		0	0	0	0	0	0
Cranium – C or H		0	0	0	25	57	0
Orbital sinus H		0	0	0	0	0	0
Brain C		0	0	0	0	0	0
Tail H		0	0	0	0	0	0
Other H		0	0	0	0	0	0
PMs performed (n)		12	2	7	4	7	4*

Sm SAIMR polyvalent antivenom FA FAV Afrique antivenom
VINS VINS African Ino Inoserp African antivenom
PSV Premium Serum and Vaccine – H Haemorrhage
Pan African E Ecchymotic haemorrhages
C Congestion Hburia Haemoglobinuria
P Petechial haemorrhages n Number of mice
PM Post mortem examination

Pathological lesions in mice induced by *E. pyramidum leakeyi* venom alone, or pre-incubated with one of five antivenoms, is listed in

Table 7.4-4. Four of these AVs (FAV Afrique, Inoserp Pan African, VINS African and Premium Serum and Vaccine Institute, India - Pan African [PSV]) were selected to demonstrate their Neutralising efficacy relative to the fifth 'gold standard' South African Vaccine producers' monovalent *Echis* antivenom (SAIMRm), is listed in Table 7.4-4. In terms of number of deaths and pathology score, Inoserp and Premium Serum & Vaccines (PSV) antivenoms performed better than FAV Afrique or VINS antivenoms, but not as well as the SAIMRm antivenom. None of the antivenoms completely neutralised the venom coagulopathy at the doses used, which was also observed for mice subjected to a combination of *E. ocellatus* venom and EchiTabG.

7.4.1.2 *Bitis arietans* venom

The major pathological lesions observed due to the venom of this species were widespread petechial haemorrhages and those related to myocardial injury. Duodenal haemorrhage was observed in mice who had survived for more than an hour (Figure 7.4-3H). Cranial congestion (Figures 7.4-10D and 7.4-11D/E) or haemorrhage and muscle haemorrhage (Figure 7.4-12A and B) was also a frequent finding. These findings are listed in Table 7.4-5. This venom was also used in the study comparing efficacy of a number of polyvalent antivenoms directed against African snake venoms.

The data for venom only pathological lesions is taken from different dose groups of venom, ranging from rapidly lethal to doses of venom from which all the mice survived. The venom/antivenom data was derived from mice which had received 5x the venom LD₅₀ pre-incubated with different antivenom doses.

It should be noted, however, that when using comparable doses of antivenom, the majority of deaths resulted from myocardial injury (Figure 7.4-7C, E, F and G), manifested by myocardial haemorrhage. None of the four antivenoms studied was as effective as SAIMRp at neutralising the lethal pathological lesions of *B. arietans* venom. The results of this study are to be reported by Harrison et al (paper in progress).

Table 7.4-5: Post mortem (PM) examination observations of mice subjected to different doses of *Bitis arietans* venom alone (green) or 5x venom LD₅₀ in combination with different antivenom doses(blue), showing occurrence of lesions observed as a percentage of the total number of PMs performed (n).

Pathological lesions	Venom AV	<i>B. arietans</i> venom							
		-	EP	EG	S	FA	V	I	PSV
Body wall P		31	0	0	7	67	92	54	62
Body wall C		15	0	20	0	0	0	15	8
Incoagulable blood		0	0	0	0	0	0	0	0
Epistaxis		0	0	0	7	0	0	0	0
Hyphaema/Eye H		0	0	0	0	0	0	0	0
Ptosis		0	0	0	0	0	0	0	0
Ears H		0	0	0	0	0	0	0	0
Muscle H		24	5	0	0	17	58	15	69
Muscle fasciculation		0	0	0	0	0	0	0	0
Haemothorax		0	0	0	14	8	25	0	8
Pulmonary H/P		24	0	40	29	42	33	62	31
Pulmonary C		30	25	20	86	17	75	31	31
Lung collapse		2	50	0	0	0	0	0	0
Lung consolidation		3	10	0	0	0	0	0	0
Atrium H		100	0	100	93	100	100	92	92
R ventricle H		88	100	100	50	92	100	92	100
L ventricle H		41	85	0	0	58	100	62	46
Congested organs		68	0	60	14	17	0	0	15
Pale organs		3	55	20	0	0	42	31	23
Stomach H		11	0	20	7	58	33	62	31
Intestinal H		25	0	80	21	58	25	46	46
Mesentery H		0	70	0	0	0	8	0	15
Haemoperitoneum		2	0	0	0	0	0	0	0
Haematuria/Hburia		0	0	0	0	0	0	0	0
Cranium – C or H		87	0	80	21	50	83	38	77
Orbital sinus		11	50	0	0	8	0	8	0
Brain C		23	30	0	7	8	17	15	15
Tail H		0	0	0	0	0	0	0	0
Other H		18	10	0	0	0	0	0	0
PMs performed (n)		120	20	5	14	12	12	13	13

EP	EchiTabPlus-ICP	EG	EchiTabG
S	SAIMR polyvalent	FA	FAV Afrique antivenom
V	antivenom VINS African	I	Inoserp African antivenom
PSV	Premium Serum and Vaccine – Pan African	H	Haemorrhage
C	Congestion	E	Ecchymotic haemorrhages
P	Petechial haemorrhages	Hburia	Haemoglobinuria
PM	Post mortem examination	n	Number of mice

7.4.1.3 *Vipera berus* venom

Vipera berus venom also induces myocardial injury and the pathological lesions of envenomed mice reflect this; with myocardial haemorrhage (Figure 7.4-5 E, F and G) and congested organs (Figure 7.4-5B – lungs, Figure 7.4-4B - liver, Figure 7.4-3B- intestines) being the most frequent observations. The venom is likewise haemorrhagic, although not coagulopathic – clots

were often visible in haemorrhages into the abdomen or thorax. Cranial congestion was often seen, with or without congestion of the brain (Figure 7.4-5 and D). One lesion that seemed unique to *Vipera berus* envenoming was hyphaema (haemorrhage into the eye) (Figure 7.4-5), accompanied by haemorrhage into the orbital sinus (Figure 7.4-5). The latter was a common finding, even in the absence of hyphaema. The pathological observations are listed in Table 7.4-6.

Once again, because of varying venom and AV doses, the frequency of pathological lesions in mice that received a mixture of venom and AV is not a true reflection of those which have failed to be neutralised by the AV. This is considered further in the discussion section of this chapter.

Table 7.4-6: Post mortem (PM) examination observations of mice subjected to different doses of *Vipera berus* venom alone (green) or 5x venom LD₅₀ in combination with different antivenom doses (blue), showing occurrence of lesions observed as a percentage of the total number of PMs performed (n).

Pathological lesions	Venom Antivenom	<i>V. berus</i> venom					
		-	VPT	VVF	VVG	Zag	EG
Body wall H		0	1	0	0	0	0
Body wall C		2	27	73	0	18	0
Incoagulable blood		0	1	0	0	0	0
Epistaxis		0	1	0	0	0	0
Hyphaema		1	2	0	0	0	0
Ptosis		0	0	0	0	0	0
Ears H		0	0	0	0	0	0
Muscle C		0	4	0	0	0	0
Muscle fasciculation		0	0	0	0	0	0
Haemothorax		0	1	7	0	3	0
Pulmonary H		3	10	13	0	5	0
Pulmonary C		37	43	7	73	43	0
Lung collapse		2	0	0	0	0	0
Lung consolidation		2	0	7	0	5	0
Atrium H		87	99	100	98	80	100
R ventricle H		64	57	73	45	42	100
L ventricle H		32	1	27	0	5	0
Congested organs		77	88	20	80	67	100
Pale organs		0	1	0	0	0	0
Stomach H		8	4	20	13	0	0
Intestinal H		13	15	80	20	15	0
Mesentry H		0	0	0	0	0	0
Haemoperitoneum		0	1	0	0	0	0
Haematuria/Hburia		0	0	0	0	0	0
Cranium – C or H		46	86	27	93	25	0
Orbital sinus H		21	51	0	38	0	0
Brain C		12	26	27	55	2	0
Tail H		0	0	0	0	0	0
Other H		12	0	0	0	0	0
PMs performed (n)		90	136	15	40	60	3
Aggregate path (n)		N/A	0	0	30	0	0

VPT	ViperaTab antivenom	VVF	ViperaVet Fab
VVG	ViperaVet IgG	Zag	Zagreb European Viper venom antiserum
EG	EchiTabG	H	Haemorrhage
C	Congestion	E	Ecchymotic haemorrhages
P	Petechial haemorrhages	n	Number of mice
Hburia	Haemoglobinuria	PM	Post mortem examination

7.4.1.4 *Naja* species

The pathological effects of the venom of three species of spitting cobras (*N. nigricollis*, *N. nubiæ* and *N. pallida*) plus two non-spitting cobras (*N. haje*, and *N. nivea*) were investigated. All of the cobra venoms induced myocardial injury (Figure 7.4-7C, E, F and G) with pathological evidence of congested

organs (Figure 7.4-2B), except for *N. nivea*. All venom pathological profiles showed evidence of respiratory paralysis, with respiratory distress ante-mortem and post mortem examination revealing lung consolidation and collapse (Figure 7.4-8C). Interestingly, two of the spitting cobra venoms induced jaundice (Figure 7.4-1D) in some of their victims (*N. nubiae* and *N. pallida*) and the third species of spitting cobra, *N. nigricollis*, induced haemoglobinuria (identified using a standard urine dipstick test) in some mice (Figure 7.4-6B). It is possible that the observed jaundice resulted from haemolysis. This could have been confirmed by examining serum or plasma for free haemoglobin.

The major pathological lesions that were observed are listed in Table 7.4-7 (*N. nigricollis*), Table 7.4-8 (*N. haje*), Table 7.4-9 (*N. pallida*) and Table 7.4-10 (*N. nubiae* and *N. nivea*) venoms with or without antivenom. The primary post mortem examination findings in mice envenomed with *N. nigricollis* venom was myocardial haemorrhage (Figure 7.4-7C), congested organs (Figures 7.4-1B, 7.4-2B) and consolidated lungs (Figure 7.4-8C). That observed in mice envenomed with *N. nubiae* venom was jaundice (Figure 7.4-1D), yellow intestines (Figure 7.4-3F) and an enlarged gall bladder (Figure 7.4-6D) being the most salient features.

Table 7.4-7: Post mortem (PM) examination observations of mice subjected to different doses of *Naja nigricollis* venom alone (green) or 5x venom LD₅₀ in combination with different antivenom doses(blue), showing occurrence of lesions observed as a percentage of the total number of PMs performed (n).

Pathological lesions	Venom Antivenom	<i>Naja nigricollis</i> venom						
		-	EP	S	F	V	I	PSV
Body wall H		0	0	0	0	0	0	0
Body wall C		39	100	40	50	14	0	100
Incoagulable blood		0	0	0	0	0	0	0
Epistaxis		0	0	0	0	0	0	0
Hyphaema/Eye H		0	0	0	0	0	0	0
Ptosis		0	0	0	0	0	0	0
Ears H		0	0	0	0	0	0	0
Muscle H		0	0	0	0	0	0	0
Muscle fasciculation		0	0	0	0	0	0	0
Haemothorax		0	0	0	0	0	0	0
Pulmonary H		3	0	0	0	0	0	0
Pulmonary C		39	84	50	100	100	100	100
Lung collapse		36	0	0	0	43	14	25
Lung consolidation		64	21	20	25	0	86	50
Atrium H		67	89	50	100	100	100	100
R ventricle H		76	68	10	0	100	100	75
L ventricle H		27	0	0	0	43	29	0
Congested organs		76	100	20	100	100	86	100
Pale organs		0	0	0	0	0	0	0
Stomach H		9	5	0	0	43	0	25
Intestinal H		12	32	0	0	29	0	0
Mesentry H		0	0	0	0	0	0	0
Haemoperitoneum		0	5	0	0	0	0	0
Haematuria/Hburia		3	0	0	0	0	0	25
Cranium – C or H		76	47	10	0	71	29	0
Orbital sinus H		6	16	10	0	0	0	0
Brain C		42	5	0	0	0	0	0
Tail H		0	0	0	0	0	0	0
Other H		0	0	0	0	0	0	0
PMs performed (n)		33	19	10	4	7	7	4

S SAIMR polyvalent antivenom F FAV Afrique antivenom
V VINS African I Inoserp African antivenom
PSV Premium Serum and Vaccine – Pan African EP EchiTabPlus-ICP
C Congestion H Haemorrhage
P Petechial haemorrhages E Ecchymotic haemorrhages
Hburia Haemoglobinuria n Number of mice
PM Post mortem examination

Post mortem (PM) examination observations of mice subjected to different doses of *Naja haje* venom alone (green) or 5x venom LD₅₀ in combination with different antivenom doses(blue), showing occurrence of lesions observed as a percentage of the total number of PMs performed (n).

Pathological lesions	Venom	<i>Naja haje</i> venom					
	Antivenom	-	S	F	V	I	PSV
Body wall H		0	0	0	0	0	0
Body wall C		0	Y:100	0	0	0	0
Incoagulable blood		0	0	0	0	0	0
Epistaxis		0	0	0	0	0	0
Hyphaema/Eye H		0	0	0	0	0	0
Ptosis		0	0	0	0	0	0
Ears H		0	0	0	0	0	0
Muscle H		0	0	0	0	0	0
Muscle fasciculation		0	0	0	0	0	0
Haemothorax		0	0	0	0	0	0
Pulmonary H		0	100	0	0	0	0
Pulmonary C		50	100	100	100	75	100
Lung collapse		0	0	50	25	25	100
Lung consolidation		100	0	50	0	25	50
Atrium H		100	100	100	100	100	100
R ventricle H		50	100	100	25	50	100
L ventricle H		0	0	0	0	0	0
Congested organs		100	100	100	100	100	100
Pale organs		0	0	0	0	0	0
Stomach H		0	100	0	0	25	0
Intestinal H		0	100	0	0	50	25
Mesentery H		0	0	0	0	0	0
Haemoperitoneum		0	0	25	0	25	0
Haematuria/Hburia		0	0	0	0	0	0
Cranium – C or H		50	0	0	0	0	0
Orbital sinus H		0	0	0	0	0	0
Brain C		0	0	0	0	0	0
Tail H		0	0	0	0	0	0
Other H		0	0	0	0	0	0
PMs performed (n)		2	1	4	4	4	4

S	SAIMR polyvalent antivenom	F	FAV Afrique antivenom
V	VINS African	I	Inoserp African antivenom
PSV	Premium Serum and Vaccine – Pan African	H	Haemorrhage
C	Congestion	E	Ecchymotic haemorrhages
P	Petechial haemorrhages	Hburia	Haemoglobinuria
PM	Post mortem examination	n	Number of mice

Table 7.4-8: Post mortem (PM) examination observations of mice subjected to different doses of *Naja pallida* venom alone (green) or 5x venom LD₅₀ in combination with different antivenom doses(blue), showing occurrence of lesions observed as a percentage of the total number of PMs performed (n).

Pathological lesions	Venom Antivenom	<i>Naja pallida</i> venom					
		-	S	F	V	I	PSV
Body wall Y		0	50	50	0	100	100
Body wall C		100	100	0	100	14	0
Incoagulable blood		0	0	0	0	0	0
Epistaxis		0	0	0	0	0	0
Hyphaema/Eye H		0	0	0	0	0	0
Ptosis		0	0	0	0	0	0
Ears H		0	0	0	0	0	0
Muscle H		0	0	0	0	0	0
Muscle fasciculation		0	0	0	0	0	0
Haemothorax		0	0	0	0	0	0
Pulmonary H		0	0	0	0	0	0
Pulmonary C		0	0	0	0	0	0
Lung collapse		0	100	0	30	57	75
Lung consolidation		0	0	50	40	14	0
Atrium H		100	0	100	100	100	75
R ventricle H		100	50	100	90	71	25
L ventricle H		100	0	0	20	29	0
Congested organs		100	100	100	100	100	100
Pale organs		0	0	0	0	14	0
Jaundice		0	50	50	100	100	100
Stomach H		0	50	0	Y50	29	0
Intestinal Y/G		0	100	0	100	0	0
Mesentry H		0	0	0	0	0	0
Haemoperitoneum		0	0	0	10	0	0
Haematuria/Hburia		0	50	0	10	0	0
Cranium – C or H		100	0	0	0	0	0
Orbital sinus H		0	0	0	0	0	0
Brain C		100	0	0	0	0	0
Tail		0	0	0	0	0	0
Other H		0	0	0	0	0	0
PMs performed (n)		2	2	4	10	7	4

S SAIMR polyvalent AV
V VINS African
PSV Premium Serum and Vaccine – Pan African
F FAV Afrique antivenom
C Congestion
G Green
I Inoserp African antivenom
H Haemorrhage
Y Yellow/jaundiced
Hburia Haemoglobinuria
n Number of mice
PM Post mortem examination

Table 7.4-9: Post mortem (PM) examination observations of mice subjected to different doses of *Naja nubiae* or *Naja nivea* venoms (green) showing occurrence of lesions observed as a percentage of the total number of PMs performed (n).

Pathological lesions	Venom	NnubV	NnivV	S	SAIMR polyvalent AV
Body wall H		0	0	V	VINS African
Body wall C		0	0	PSV	Premium Serum and Vaccine – Pan African
Incoagulable blood		0	0	F	FAV Afrique antivenom
Epistaxis		0	0	C	Congestion
Hyphaema/Eye H		0	0	G	Green
Ptosis		0	0	I	Inoserp African antivenom
Ears H		0	0	H	Haemorrhage
Muscle H		0	0	Y	Yellow/jaundiced
Muscle fasciculation		0	0	Hburia	Haemoglobinuria
Haemothorax		0	0	n	Number of mice
Pulmonary H		0	0	PM	Post mortem examination
Pulmonary C		0	0		
Lung collapse		5	26		
Lung consolidation		40	63		
Atrium H		10	0		
R ventricle H		10	0		
L ventricle H		20	0		
Congested organs		0	0		
Pale organs		0	0		
Jaundice		55	0		
Enlarged gall bladder		55	0		
Stomach H		0	0		
Intestinal H		Y75	79		
Mesentery H		0	0		
Haemoperitoneum		0	0		
Haematuria/Hburia		0	0		
Cranium – C or H		45	16		
Orbital sinus H		0	0		
Brain C		25	5		
Tail H		0	0		
Other H		0	0		
PMs performed (n)		20	19		

7.4.1.5 *Dendroaspis* species

Venoms from both *Dendroaspis* species studied showed evidence of myocardial injury (Figure 7.4-7C, E, F, G) with congested organs (Figures 7.4-2B, 7.4-3B, 7.4-4B, 7.4-5B, 7.4-6C, 7.4-8B, 7.4-10B-D). Mice envenomed with *D. angusticeps* (Table 7.4-10) showed autonomic nervous signs, such as sweating (Figure 7.4-9D) and salivation (Figure 7.4-9E), as well as muscle fasciculations, respiratory distress and the paralysis ante-mortem, also seen with *D. polylepis* envenomation (Table 7.4-11). Interestingly, the muscle fasciculations continued after death (defined by cardiac arrest). A similar

pathological profile was seen for the venom of both species, namely myocardial haemorrhage of both atria and ventricles (Figure 7.4-7F and E), congested tissues (Figures 7.4-2B, 7.4-3B, 7.4-4B, 7.4-5B, 7.4-6C, 7.4-8B, 7.4-10B-D), lung collapse and consolidation (Figure 7.4-8C), distal intestinal haemorrhage (Figure 7.4-3D) and cranial and cerebral congestion (Figure 7.4-10B-D).

Table 7.4-10: Post mortem (PM) examination observations of mice subjected to different doses of *Dendroaspis angusticeps* venom (DaV) alone (green) or in combination with SAIMR polyvalent antivenom (blue), showing occurrence of lesions observed as a percentage of the total number of PMs performed (n). *total n=67; **total n=56

Pathological lesions	Venom Antivenom	DaV		SAIMRp	SAIMR polyvalent antivenom
		-	SAIMRp		
Body wall H		0	0	C	Congestion
Body wall C		3	4	H	Haemorrhage
Sweating		68	37*	R	Right
Incoagulable blood		0	0	L	Left
Epistaxis		0	0	NR	Not recorded
Hyphaema/Eye H		0	0	Hburia	Haemoglobinuria
Ptosis		NR	NR	PM	Post mortem examination
Ears H		0	0		
Muscle H		0	0		
Muscle fasciculation		NR	93**		
Haemothorax		0	0		
Pulmonary H		0	0		
Pulmonary C		10	81		
Lung collapse		19	35		
Lung consolidation		8	33		
Atrium H		58	80		
R ventricle H		53	69		
L ventricle H		0	2		
Congested organs		44	73		
Pale organs		0	4		
Enlarged gall bladder		0	4		
Jaundice		0	8		
Stomach H		10	0		
Intestinal H		34	0		
Mesentery H		0	0		
Haemoperitoneum		2	6		
Haematuria/Hburia		0	0		
Cranium – C or H		25	79		
Orbital sinus C or H		5	19		
Brain C		3	40		
Tail H		0	0		
Other H		0	0		
PMs performed (n)		59	48		

Table 7.4-11: Post mortem (PM) examination observations of mice subjected to different doses of *Dendroaspis polylepis* venom alone (green) or in combination (5x venom LD₅₀) with varying doses of different antivenoms (blue), showing the occurrence of lesions observed as a percentage of the total number of PMs performed (n). *Aggregate related pathology

		% total PMs					
Pathological lesions	Venom	Dendroaspis polylepis venom					
	None	-	S	F	V	I	PSV
Body wall H		0	0	0	0	0	0
Body wall C		67	0	22	0	50	11*
Incoagulable blood		0	0	0	0	0	0
Epistaxis		0	0	0	0	0	0
Hyphaema/Eye H		0	0	0	0	0	0
Ptosis		0	0	0	0	0	0
Ears H		0	0	0	0	0	0
Muscle H		0	0	0	0	0	0
Muscle fasciculation		0	0	0	0	0	0
Haemothorax		0	0	0	0	30	0
Pulmonary H		0	0	0	0	0	0
Pulmonary C		100	0	78	75	70	78
Lung collapse		0	0	67	100	60	89
Lung consolidation		67	100	22	25	60	0
Atrium H		100	100	100	100	100	89
R ventricle H		100	100	44	100	90	100
L ventricle H		33	0	11	50	20	22
Congested organs		100	0	0	25	0	0
Pale organs		0	0	0	0	0	0
Stomach H		0	0	33	0	20	33
Intestinal H		0	50	11	0	10	0
Mesentery H		0	0	0	0	0	0
Haemoperitoneum		0	0	0	0	0	11*
Haematuria/Hburia		0	0	0	0	0	0
Cranium – C or H		33	0	11	50	50	11*
Orbital sinus H		0	0	11	0	0	0
Brain C		33	0	11	25	0	11*
Tail H		0	0	0	0	0	0
Other H		0	0	0	0	0	0
PMs performed (n)		3	2	9	4	10	9
Aggregate pathology (n)		N/A	0	0	0	0	1(11%)*

S SAIMR polyvalent antivenom F FAV Afrique antivenom
V VINS African I Inoserp African antivenom
PSV Premium Serum and Vaccine – H Haemorrhage
 Pan African Hburia Haemoglobinuria
C Congestion n Number of mice
PM Post mortem examination

Other venoms

Boomslang (*Dispholidus typus*) is a colubrid snake with venom that causes a coagulopathy similar to that induced by *E. ocellatus* venom. Indeed, an *in vitro* coagulation test, using human plasma, is positive for both venoms (data not included). EchiTabG antivenom inhibits *in vitro* coagulation of plasma by

Boomslang venom but it does not completely neutralise the coagulopathy *in vivo*. However, it does significantly increase survival times and markers for thrombin generation, thus showing that there is some cross-protection afforded (Casewell et al, publication in progress).

Table 7.4-12: Post mortem (PM) examination observations of mice subjected to different doses of *Dispholidus typus* venom alone (green) or in combination with different antivenoms (blue), showing occurrence of pathological lesions as a percentage of the total number of PMs performed (n).

		% total PMs			
Pathological lesions	Venom	<i>Dispholidus typus</i> venom			
	None	-	Sb	EG	αE
Body wall H		0	0	0	0
Body wall C		0	0	0	140
Incoagulable blood		100	0	100	80
Epistaxis		14	0	20	20
Hyphaema/Eye H		0	0	0	0
Ptosis		0	0	0	0
Ears H		0	0	0	0
Muscle H		0	0	0	0
Muscle fasciculation		0	0	0	0
Haemothorax		76	0	20	0
Pulmonary H		57	0	80	60
Pulmonary C		33	0	0	40
Lung collapse		5	0	20	20
Lung consolidation		0	0	0	0
Atrium H		43	0	0	0
R ventricle H		71	0	100	40
L ventricle H		14	0	0	0
Congested organs		43	0	0	0
Pale organs		48	0	40	60
Jaundice		5	0	0	0
Stomach H		5	0	0	20
Intestinal H		24	0	40	60
Mesentery H		0	0	0	0
Haemoperitoneum		14	0	0	0
Haematuria/Hburia		0	0	0	0
Cranium – C or H		90	0	100	60
Orbital sinus H		43	0	0	0
Brain C		10	0	0	20
Tail H		0	0	0	0
Other H		0	0	0	0
PMs performed (n)		21	2	5	5

Sb SAIMR polyvalent antivenom EG EchiTabG
αE α-Ecarin I Inoserp African antivenom
C Congestion EP EchiTabPlus-ICP
Hburia Haemoglobinuria H Haemorrhage
n Number of mice PM Post mortem examination

The pathological profile of this venom (Table 7.4-13) is similar to that induced by *Echis* species (Table 7.4-12 and 7.4-4), most commonly demonstrating

incoagulable blood, epistaxis (Figure 7.4-9A and B)), haemothorax (Figure 7.4-7A and B), pulmonary (Figure 7.4-8E-H) and other organ haemorrhages (Figure 7.4-12A-C). Myocardial haemorrhage (Figure 7.4-7C, E, F, G) with congested organs, possibly indicating antemortem heart failure (Figures 7.4-2B, 7.4-3B, 7.4-4B, 7.4-5B, 7.4-6C, 7.4-8B, 7.4-10B-D), was also observed.

It is interesting to note that venom-induced pathological lesions was completely neutralised when 150µL/mouse SAIMR boomslang antivenom was administered with 2.5x LD₅₀ Boomslang venom (30µg/mouse).

The desert horned viper (*Cerastes cerastes*) produces another haemorrhagic venom, whose pathological profile includes incoagulable blood, epistaxis, haemothorax, pulmonary haemorrhage, myocardial injury and congested or pale organs (depending on severity of haemorrhages). These findings are listed in Table 7.4-14, which also includes those seen in *Calloselasma rhodostoma* (Malaysian pit viper) and *Aspidelaps scutatus* (shield-nosed cobra) envenomations.

C. rhodostoma is a viper from the Asian subcontinent and has a coagulopathic venom. The pathological lesions observed are listed in Table 7.4-14, which were primarily myocardial haemorrhage, congested organs and intestinal haemorrhage.

Aspidelaps scutatus is an elapid with neurotoxic and haemorrhagic venom (Table 7.4-14). Some of the mice developed a mucoid coating of their corneas (Figure 7.4-9F). This may either have been due to a failure of secretion of the watery component of tears or because the mouse was unable to blink properly. Paralysis was observed in mice which had received higher doses of venom, but those which had received a lower doses showed signs of a 'myaesthesia gravis'-like phenomenon. Thus, when these mice were disturbed, they were initially active, but gradually slowed down until they slumped, then slept until the next observation period (half an hour later), when the activity described was repeated. Eventually these mice recovered completely, unlike those which were completely paralysed and succumbed, if not euthanased.

Table 7.4-13: Post mortem (PM) examination observations of mice subjected to different doses of *Cerastes cerastes*, *Calloselasma rhodostoma* or *Aspidelaps scutatus* venoms alone (green) or in combination with antivenom (blue), showing occurrence of pathological lesions as a percentage of the total number of PMs performed (n).

Pathological lesions	% total PMs				
	Venom	CcV		CrV	AsV
	None	-	EG	-	-
Body wall H		0	0	0	0
Body wall C		28	100	0	0
Incoagulable blood		100	0	100	50
Epistaxis		4	0	0	0
Hyphaema/Eye H		0	0	0	0
Ptosis		0	0	0	0
Ears H		0	0	0	0
Muscle H		0	0	0	0
Muscle fasciculation		0	0	0	0
Haemothorax		28	0	0	17
Pulmonary H		16	0	44	17
Pulmonary C		40	0	0	0
Lung collapse		36	0	0	83
Lung consolidation		0	0	0	0
Atrium H		100	100	100	100
R ventricle H		88	0	44	67
L ventricle H		36	0	44	0
Congested organs		80	0	0	0
Pale organs		28	0	0	0
Stomach H		0	0	0	0
Intestinal H		12	0	0	0
Mesentery H		0	0	0	0
Haemoperitoneum		0	0	0	33
Haematuria/Hburia		0	0	0	0
Cranium – C or H		84	0	78	67
Orbital sinus H		0	0	0	0
Brain C		44	0	33	17
Tail H		0	0	0	0
Other H		0	0	0	0
PMs performed (n)		25	2	9	6

CcV *Cerastes cerastes* venom
CrV *Calloselasma rhodostoma* venom
AsV *Aspidelaps scutatus* venom
EG EchiTabG antivenom
H Haemorrhage
C Congestion
Hb Haemoglobin
n number of mice
PM Post mortem examination

7.4.2 Blood films

Blood smears were performed using samples from mice envenomed with *Echis ocellatus* venom. Cell morphologies observed are listed in Table 7.4-14. The most striking observation was collections of white blood cells at the margins of the smears the most prominent cell type being ‘toxic’ neutrophils (neutrophils with cytoplasmic vacuolation and basophilic cytoplasm) (Figure 7.4-13A and D), although in some films mononuclear cells were the dominant cell type (Figure 7.4-13B). Many of the films showed damaged cells or cell

debris, with few recognizable cells (Figure 7.4-13C). One blood film taken from a mouse which had survived for 3 hours showed evidence of immature red blood cells (Figure 7.4-13A) having a more eosinophilic cytoplasm with basophilic stippling.

In conclusion, blood films showed evidence of cytotoxicity, cell damage and destruction of white blood cells. This concurs with findings in human snakebite patients, who exhibit leukocytosis and thrombocytopenia (Warrell et al, 1977).

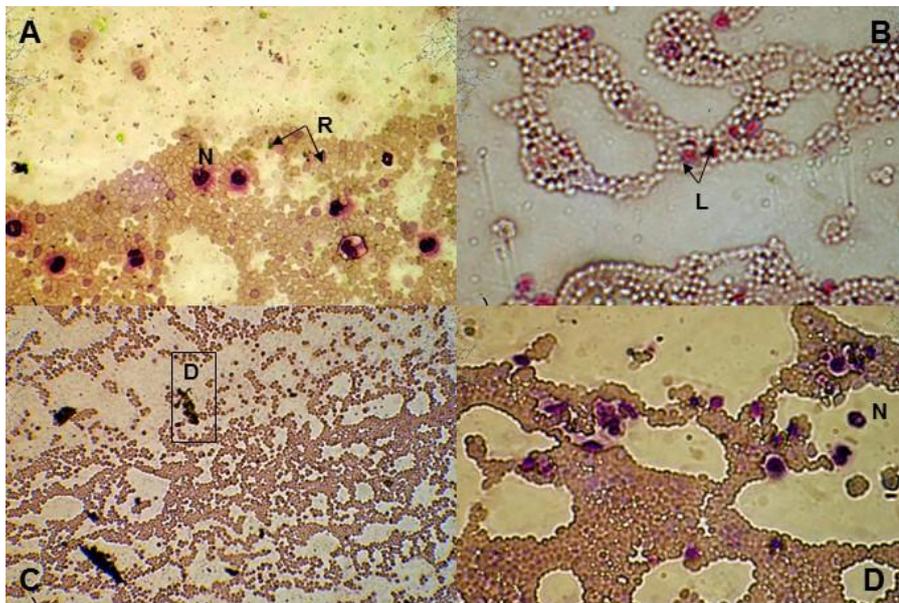


Figure 7.4-13: Blood smears from mice envenomed with *Echis ocellatus* venom: A 400x Survived 525 minutes, received 8 μ L venom; B: 400x Survived 348 minutes, received 32 μ L venom; C: 100x Suffered terminal convulsions, received 32 μ L venom; D: 400x. Survived 238 minutes, received 48 μ L venom. N= toxic neutrophil; R=immature red blood cell; L=lymphocyte; D=cell debris.

Dose ul	Survival time	RBC	WBC	Neutrophils	Morphology	Lymphocytes	Monocytes	Eosinophils	Basophils	Platelets	Comments
8	Survivor		++	++	Mature	+	+/-	-	-	+++	1x macrophage seen
8	post iv,		++	+	Toxic ●/ multi-lobed	++	-	-	-	+++	
8	Survivor		+++	+++	Toxic	+++	+	-	-	+++	
8	Survivor		++	++	Toxic	+++	+	-	-	+++	Cell debris
8	Survivor		+	+	Toxic	+		-	-	+++	Poor quality slide
8	525 min		+++	++	Toxic	+++	++	++	-	?	Cell debris, macrophages +++
16	Survivor		+++++	++	Toxic	+++++	+++	-	+	Not seen	Basophilic clumps of cells ?microclots?
16		+++	++	Toxic- multi-lobed	+++	++	-	-	++	Platelets clumped	+++
24	8 min		+	+	Mature	+	-	-	-	Clumped	Platelets appear clumped in clumps of RBCs ?micro clots
32	20 min		+	+/-	Toxic	+	-	-	-		Post convulsion Cell debris +++++
32	8 min		++++	≈/-	●	++++	++++	-	-	None seen	
32	8 min		+	None seen	-	+	-	-	-	None seen	Cell debris +++++
32	8 min		+/-	-	-	+/-	-	-	-	Not seen	Cell debris ++
32	352 min		++++	+	Toxic	++++	++++	-	-	+/-	Cell debris +++
40	503 min		++++	++	Toxic	++++	++++	-	-	+/-	Cell debris +++
40	113 min		+++	+	Toxic	++++	+++	-	+	+/- clumped	Cell debris/ damaged cells
40	37 min		++++	+	Toxic	++++	++	-	-	+/-	Huge clumps of ? lymphocytes
48	166 min		++++	+	Toxic	++++	++++	+	-	None seen	Damaged cells
48	166 min	?retics	++++	+	●	++++	+++	-	-	None seen	Damaged cells
48	Survivor		++	+/-	-	++	-	-	-	None seen	Cells with large, granular, eosinophilic nuclei
48	238 min		+++	+	Toxic	+++	++++	-	-	+/- clumped	2 clumps seen
48	4 min										Lung tissue also present. Few recognisable cells. Debris++++
48	4 min		+++			+++	-	-	-	-	Cell debris+++++
48	188 min		++++	+	Toxic	+++	++++	-	-	Not seen	Cell debris ++

Table 7.4-14: analysis of blood smears from mice injected with Echis ocellatus venom. ● = toxic neutrophil with 'doughnut' nucleus. References for identification: Linden et al, 2012; Torrance, 2000.

Key RBC = red blood cells

- + = a few cells present
- +++ = many cells present
- +/- = one or two cells seen
- Retics = reticulocytes

WBC = white blood cells

- ++ = moderate number of cells present
- ++++ = large population of cells
- = no cells seen/identified

'Survival time' refers to length of survival, experiment number (MVxxx) and mouse number (A/Bn and whether the mouse suffered terminal convulsions)

7.4.3 Histopathology

7.4.3.1 *Echis ocellatus* venom

The liver showed vacuolation of hepatocytes and was negative for Periodic acid-Shiff and so this was therefore likely to be hydropic change. The sinusoids contained few erythrocytes in one sample, but mild to moderate congestion with inflammatory foci in another.

The lungs showed increasingly extensive, acute haemorrhagic foci involving alveoli, bronchioles and perivascular tissue, with increasing doses of venom. This is illustrated in Figure 7.4-14 and Figure 7.4-16A.

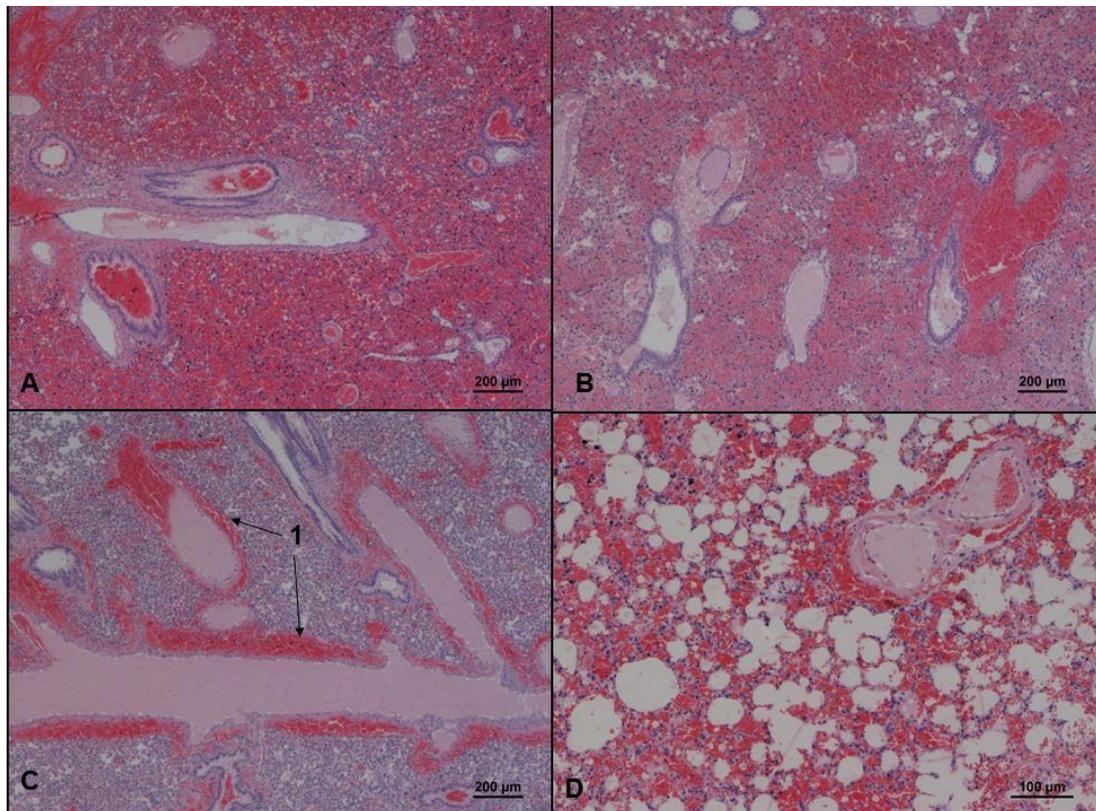


Figure 7.4-14: Histopathology of mouse lung envenomed with *E. ocellatus* venom. A: 4x – showing intra-alveolar haemorrhage; B: 4x – showing extensive haemorrhage; C: 4x – showing perivascular haemorrhage (arrows, ‘1’); D: 10x – showing intra-alveolar and perivascular haemorrhage

Both sub-epicardial and sub-endocardial haemorrhage were seen in atrial and ventricular myocardium (Figure 7.4-15 B, C and D). Haemorrhage into the brown fat adjacent to the atria was observed in one sample (Figure 7.4-15A).

The thymus exhibited extensive haemorrhage, with loss of existing tissue, especially within the cortex. Remaining cells frequently exhibited necrosis or morphology consistent with apoptosis (Figure 7.4-16B).

Skeletal muscle and diaphragm showed areas of lysis in which the myocytes appeared shrunken, angular, had eosinophilic sarcoplasm, loss of striation and centralised nuclei (Figure 7.4-16C).

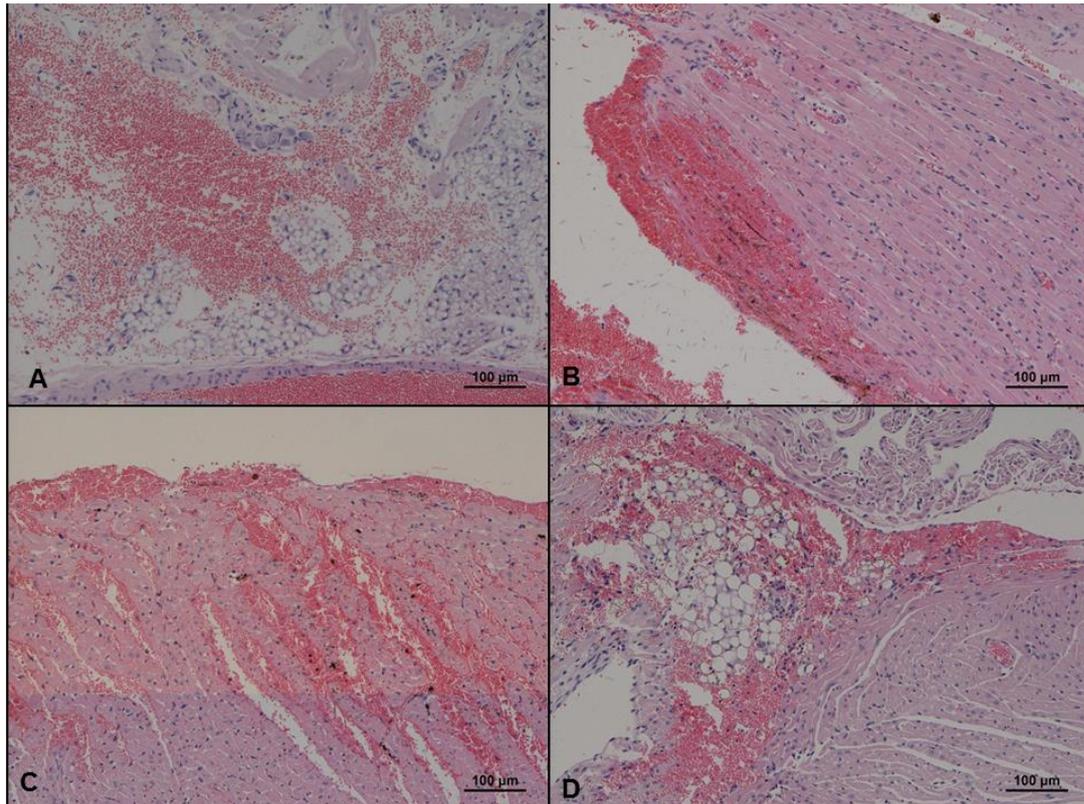


Figure 7.4-15: Histopathology of mouse heart envenomed with *E. ocellatus* venom. A: 10x - haemorrhage into pericardial brown fat; B: 10x – sub-endocardial haemorrhage, ventricle; C: 10x – sub-epicardial haemorrhage, ventricle ; D: 10x – subepicardial haemorrhage, atrium

The kidneys were pale, but showed no histological abnormality. The stomach and intestines showed focal red areas of mucosa, with a few free erythrocytes within the lumen. There were areas of neutrophil infiltration, which was also seen in the adjacent mesentery/omentum. The brain exhibited mild to moderate hyperaemia.

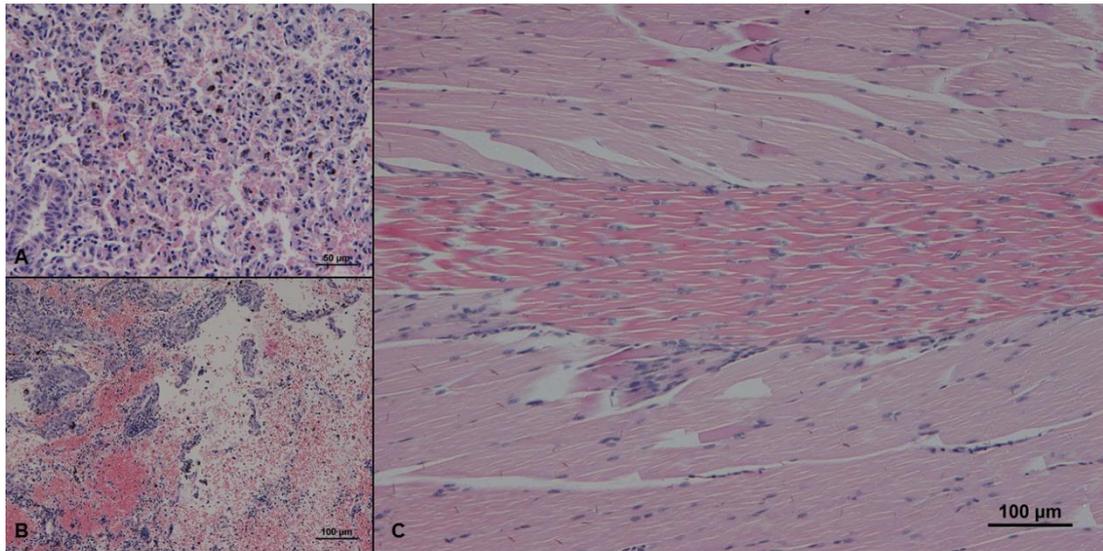


Figure 7.4-16: Histopathology of mouse tissues envenomed with *E. ocellatus* venom. A: 20x lung – showing perivascular and intra-alveolar haemorrhage; B: 10x thymus – showing haemorrhage, apoptosis and necrosis; C: 10x muscle – showing lysis of muscle fibril.

7.4.3.2 *Vipera berus* venom pre-incubated with ViperaVet antivenom

The overall histological picture was one of hyperaemia/congestion. A particularly interesting observation was the increase of mitotic cells plus anisokaryosis (variation in size of nuclei) within the liver (Figure 7.4-17). The phenomenon of increased cell growth in low venom concentrations was seen in the cytotoxicity experiments.

The question arises whether this is an *in vivo* example of this effect. The stomach and intestines exhibited infiltration of the lamina propria with inflammatory cells and hyperaemia. The kidneys and lungs showed hyperaemia. There was also some focal intra-alveolar haemorrhage in the lungs (Figure 7.4-18A and B). Focal haemorrhages were also seen in the diaphragm (Figure 7.4-18D) and thymus. In the heart, the atria were dilated, as was the right ventricle. Hyperaemia was noted in the myocardium as well as multifocal, sub-endocardial haemorrhage (Figure 7.4-18C).

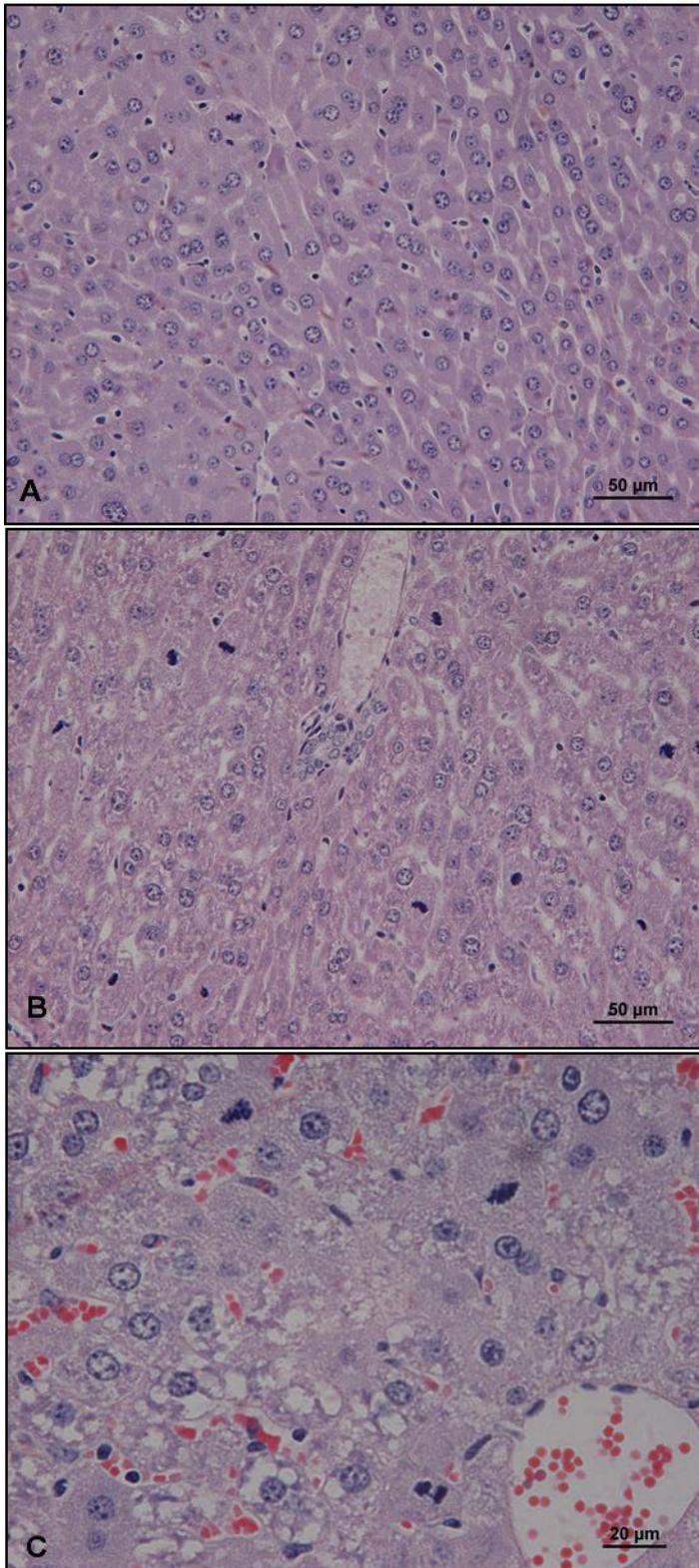


Figure 7.4-17: Histopathology of mouse liver envenomed with 5xLD₅₀ *V. berus* venom pre-incubated with different doses of ViperaVet antivenom (VPV) showing increased numbers of mitotic cells. A: 40μL VPV, 20x; B: 35μL VPV,20x; C: 35μL VPV, 40x

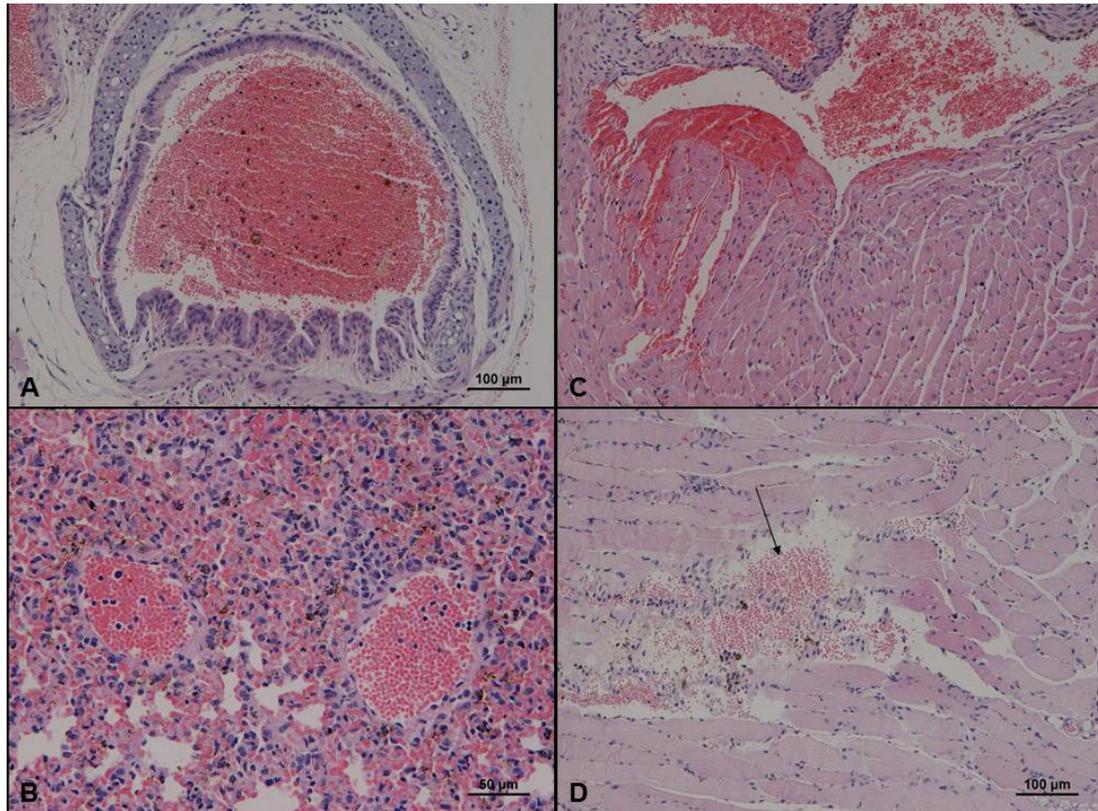


Figure 7.4-18: Histopathology of mouse tissues envenomed with 5xLD₅₀ *V. berus* venom pre-incubated with different doses of ViperaVet antivenom (VPV). A: 35µL VPV, 10x showing bronchus full of blood; B: 40µL VPV, showing hyperaemia/congestion of lungs and intra-alveolar haemorrhage; C: 35µL VPV, 10x showing sub-endocardial ventricular myocardial haemorrhage; D: 40µL VPV, 20x focal haemorrhages in the diaphragm (arrow).

7.5 Discussion

As far as is known, this is the only study to look at mouse pathological lesions induced by such a wide range of snake venoms, encompassing not only different genera and families, but also originating from diverse geographical habitats.

The most interesting finding was the realization that each venom studied had its own unique set of pathological lesions, the severity of which was dose and time dependent. This observation is perhaps not surprising, given that each venom has its own unique toxin profile.

7.5.1 Comparative pathology of envenomed humans and mice

A review of the literature on snakebite was undertaken to ascertain the extent to which venom-induced pathological lesions in the mouse model reflects those observed in human victims of envenoming. This is important because the validity of the murine preclinical assays has been criticised as not sufficiently reflecting the human condition (Maduwage et al, 2016).

7.5.1.1 *Echis* species venom: comparative pathology

Human envenoming by *Echis* spp. are characterised by incoagulable blood and haemorrhage, and the small amount of post mortem data (Professor David Warrell, personal communication) demonstrates many of the post mortem observations in mice. A comparison of envenoming by *Echis* spp. is listed in Table 7.5-1 and examples shown in Figure 7.5-1 (subcutaneous haemorrhages); Figure 7.5-2 (incoagulable blood); Figure 7.5-3 (intra-oral haemorrhage); Figure 7.5-4 (Pale tissues); Figure 7.5-5 (pulmonary haemorrhage); Figure 7.5-6 (muscle injury/haemorrhage) and Figure 7.5-7 (intracerebral haemorrhage).



Figure 7.5-1: *Echis* spp. induced ecchymotic haemorrhages of the body wall. A: human (Kochar et al, 2007); B: mouse

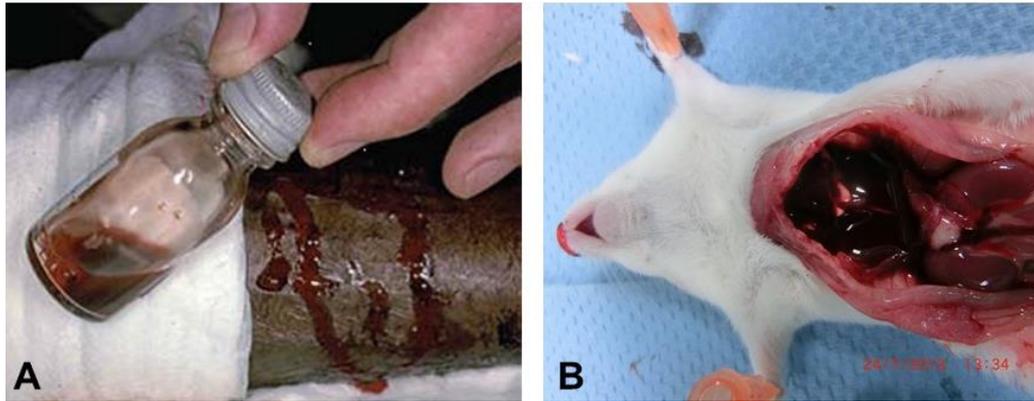


Figure 7.5-2: *Echis ocellatus* induced incoagulable blood A: human (Warrell, 1977); B: mouse.

Table 7.5-1: Comparative pathology – *Echis ocellatus* envenoming. References: (1)-Warrell et al, 1977; (2)-Karaye et al, 2012

Pathological lesions	Mouse	Human
Coagulopathy Haemorrhagic	Incoagulable blood	Consumptive coagulopathy (↓Fs V, VII, II & XIII and fibrinogen) and thrombocytopenia (1)
	Haemothorax	Extrapleural haemorrhage (1)
	Pulmonary haemorrhage, epistaxis and haemoptysis	Pulmonary haemorrhage, epistaxis and haemoptysis (1)
	Intestinal haemorrhage	Haematemesis, melaena (1)
	Microcardia	Hypotension and hypovolaemia (1)
	Myocardial haemorrhage	ECG abnormalities Rarely myocardial infarction (2)
	Other haemorrhages – oral, injection site	Other haemorrhages – oral, bite site, old wounds



Figure 7.5-3: *Echis ocellatus* induced oral haemorrhage A: human (Warrell, 1977); B: mouse

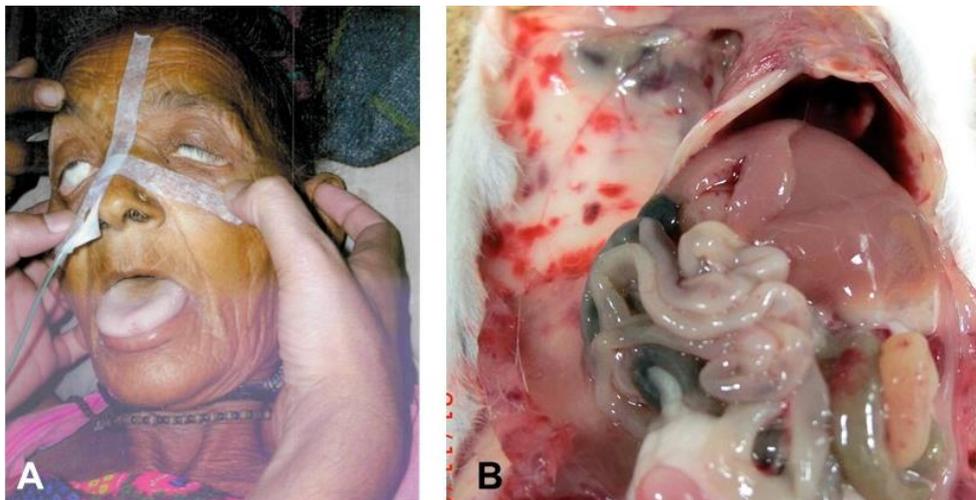


Figure 7.5-4: *Echis* spp. induced anaemia A: pale extremities in a human patient (Kochar, 2007) B: pale organs in an experimental mouse.

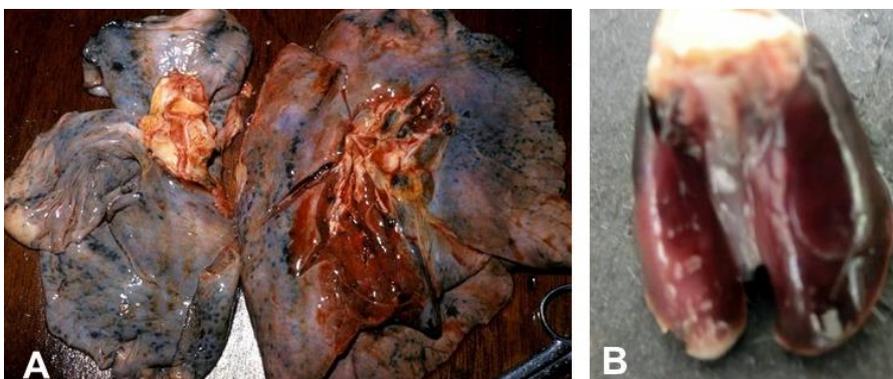


Figure 7.5-5: *Echis ocellatus* induced pulmonary haemorrhage at post mortem examination A: human (Warrell, personal communication); B: mouse

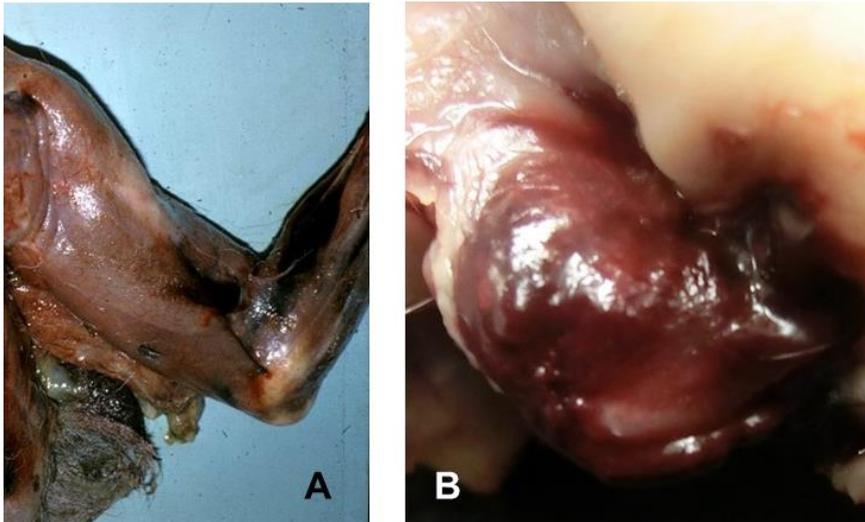


Figure 7.5-6: *Echis ocellatus* induced haemorrhage into the forearm muscles at post mortem examination A: monkey (Warrell, personal communication); B: mouse

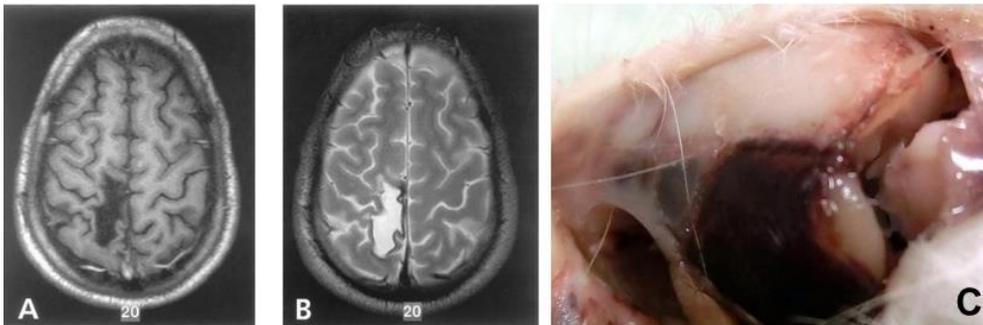


Figure 7.5-7: *Echis* spp. induced intracerebral haemorrhage. A: T1 weighted MRI image of human patient; B: T2 weighted MRI image of human patient (Bartholdi et al, 2004); C: post mortem examination of mouse brain showing an intracranial haemorrhage.

7.5.1.2 *Bitis arietans* venom: comparative pathology

Systemic envenoming by *Bitis arietans* induces thrombocytopenia, with its characteristic petechial haemorrhages. Profound hypotension is seen in humans (Figure 7.5-8); in mice there is a slump, which may be so severe that the individual loses its righting reflex (refer to Chapter 6: Humane Endpoints). Muscle injury is apparent in both humans and mice after envenoming, which may be so severe in human victims that amputation is necessary (Figure 7.5-9). Table 7.5-2 lists the pathological lesions observed in envenomed mice and humans.

Table 7.5-2: Comparative pathology – *Bitis arietans* envenoming. References: (3)-Warrell et al, 1975; (4) Lavonas et al, 2002; (5) Firth et al, 2011; (6) WHO, 2010; (7) West et al, 2014.

Pathological lesions	Mouse	Human
Haemorrhagic – petechiae (1-2mm haemorrhages)	Widespread petechiation – body wall, serosal surfaces, lungs	Petechiation of serosal surfaces
Thrombocytopenia	Thrombocytopenia	Thrombocytopenia and spontaneous bleeding(3,4)
Cardiovascular	Myocardial haemorrhage	Bradycardia Hypotension (3,4) Arrhythmias
Musculoskeletal	Muscle haemorrhage and necrosis	Tissue and muscle necrosis (3,4,5, 6)
Gastrointestinal	Duodenal haemorrhage	Vomiting (6)
Neurological	Intra cranial haemorrhage	Neurotoxic effects (7)



Figure 7.5-8: *B. arietans* envenomation showing A: hypotension in human patient (Warrell, 1975); B: profound slump seen immediately after venom injection in a mouse

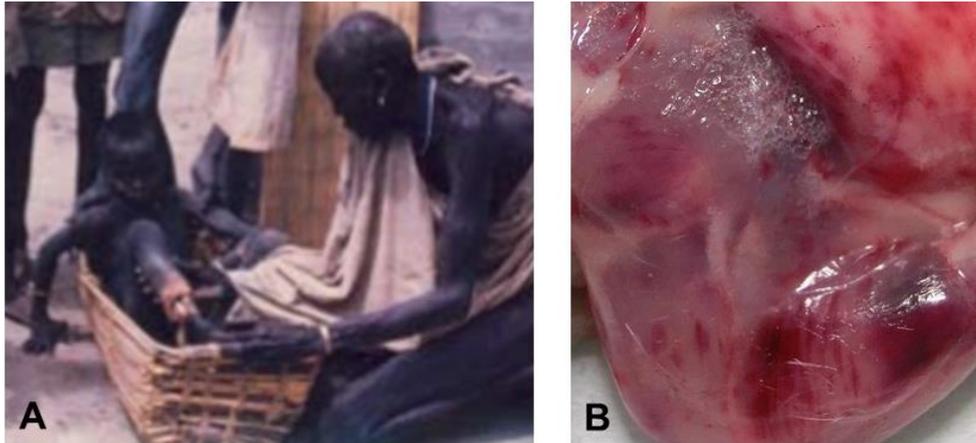


Figure 7.5-9: *B. arietans* envenomation showing A: tissue destruction and muscle necrosis in a child (West et al 2014); B: muscle haemorrhage in a mouse observed at post mortem examination.

7.5.1.3 *Vipera berus* venom: comparative pathology

The European Adder, *Vipera berus*, is the only venomous snake native to the UK and there are relatively few reported bites in humans; only 32 bites in 2016 required treatment with antivenom (*ViperaTab* sales, MicroPharm Ltd, personal communication). There are, however, considerably more bites in dogs and other domestic animals.

Table 7.5-3: Comparative pathology – *Vipera berus* envenoming. References: *: Personal communication – veterinary case (8) –Anlen, 2008 (horse); (9) – Bart et al, 2016 (human); (10)- Peleander et al, 2010 (dog); (11) – Hoffman et al, 1993 (horse); (12) Audebert et al, 1992 (human); (13) – Malina et al, 2008 (human); (14) – Karlson-Stiber et al, 1994 (human); (15) Reid, 1976 (human).

Pathological lesions	Mouse	Human/ Dog/ Horse
Cardiovascular	Myocardial haemorrhage Subendocardial haemorrhage	Hypotension (12) Arrhythmias* (8,14,15) Myocardial injury (10,11) Subendocardial haemorrhage (15)
	Congested organs	Pulmonary congestion (15)
Coagulation	Mild haemorrhage	Mild haemorrhage (12,14,15) Disseminated intravascular coagulation (DIC)* Pulmonary embolism (9)
Pulmonary haemorrhage	Mottled lungs	Pulmonary haemorrhage (15)
Gastrointestinal tract	Intestinal haemorrhage	Vomiting and/or diarrhoea (12,14,15)
Neurotoxicity	Cranial congestion Hyphaema Hind limb paralysis	Neurotoxicity (13) CNS depression (14)
Leucocytosis	Present	Present (14,15)

The clinical presentation has similarities in all species, namely severe oedema spreading from the bite site. This localised oedema is not seen in the intravenously venom-injected mouse model, which shows only signs of systemic envenoming.

Cardiovascular signs occur in all envenomed species, as listed in Table 7.4-18. Figure 7.5-10A shows the physiological consequence of myocardial injury in a dog bitten by an adder. The resulting tissue damage is irreversible with antivenom. Figure 7.5-10B shows venom induced myocardial haemorrhage, which is associated with muscle necrosis, as demonstrated histopathologically. A 45 year old man who was intravenously envenomed by *Vipera aspis* died within minutes; autopsy showed massive myocardial injury (de Haro et al, 2009) – exactly that which was observed in our mice.

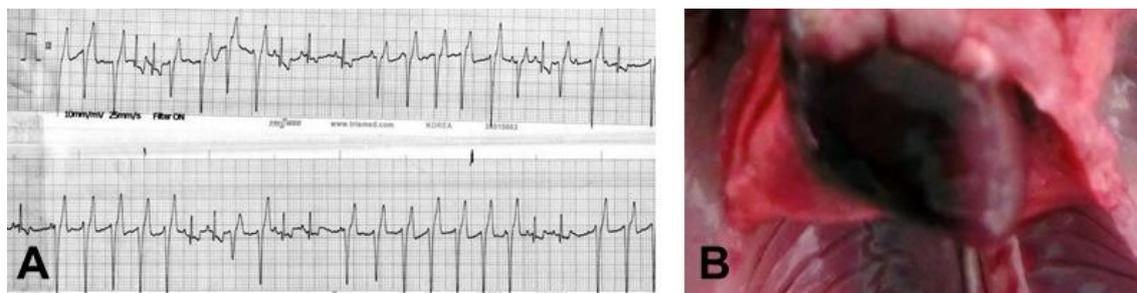


Figure 7.5-10: *V. berus* envenomation resulting in myocardial injury as denoted by A: multiple ventricular premature complexes on an ECG of a canine patient (personal communication) and B: haemorrhage of the right ventricular myocardium of a mouse.

Coagulation disturbances have been noted in human cases of systemic envenoming by this species (Bart et al, 2016; Boels et al, 2012; Moser and Roeggia, 2009; for example). In the mouse model, free blood was often observed in the thorax, and occasionally in the abdomen; clots were nearly always present. In humans, vomiting and/or diarrhoea are common in systemic envenoming; mice are unable to vomit, but at post-mortem intestinal haemorrhage was frequently observed.

Although neurological signs are rarely seen in human *V. berus* snakebite patients, it has been reported that certain populations of *V. berus* in Hungary (Malina et al, 2008) and particularly *V. aspis* (Ferquel et al, 2007) and *V. ammodytes* (Latinovik et al, 2016) appear to have venom which contains a

neurotoxin, the signs of which are restricted to diplopia, dizziness and drowsiness. Whilst comatose patients have been described, these are thought to be due to profound hypotension (Malina et al, 2008). In mice, hyperaemia and congestion of the brain was observed as well as ante mortem hind limb paralysis (Chapter 6). It is unlikely that this was attributable to hypotension as it was an observation made exclusively after recovery from the initial 'slump'. Possibly it was the result of cerebral anoxia or localised brain lesions, such as ischaemia due to microthrombi or small intracerebral haemorrhages. Alternatively, it may result from spinal cord pathology – a peripheral neuropathy is an unlikely explanation as, if left, the ascending paralysis was progressive. The presence of an unknown presynaptic neurotoxin (PLA₂) is also a possible explanation for this observation.

7.5.1.4 Other viper venoms: comparative pathology

Myocardial injury is frequently observed in mice envenomed by any viper species, and this is, indeed, the case in naturally envenomed humans and animals. Myocardial injury has been identified in humans (Moser et al, 2009), dogs (Pelander et al, 2010), horses (Anlen, 2008) and at post mortem examination in a cow (Figure 7.5-11A) as well as in the mouse model (Figure 7.5-11B). Figure 7.5-12: A- mouse and B- horse shows the histopathology of venom-induced myocardial injury.

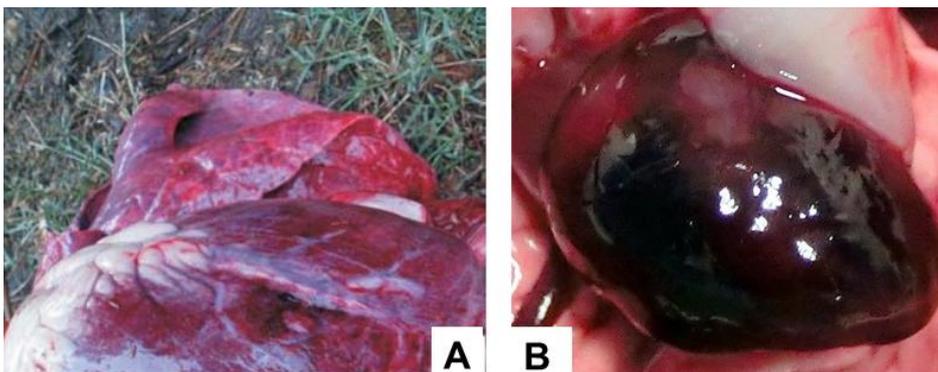


Figure 7.5-11: Viper envenomation resulting in ventricular myocardial haemorrhage A: cow (Banga et al, 2009); B: mouse.

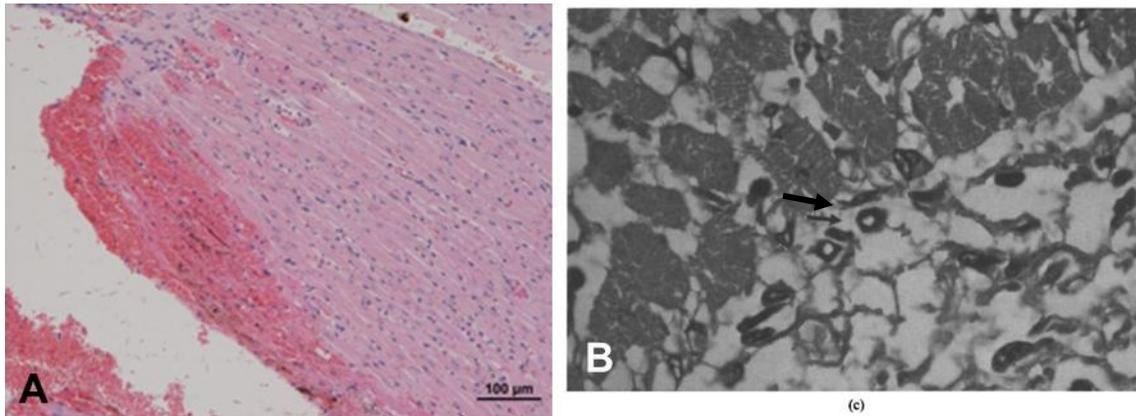


Figure 7.5-12: Viper envenomation showing myocardial damage. A: sub-endocardial haemorrhage and myocardial necrosis in a mouse; B: 200x right ventricular myocardium of a horse – fibre necrosis is characterized by cytoplasmic vacuolation and pyknotic nuclei (arrow). On the left are normal fibres (Hoffman et al, 1993).

Muscle injury commonly occurs following viper bites (*Crotalus* spp. – Azevedo-Marques et al, 1985), *Bitis arietans* (Warrell, 1975), and an unknown Indian viper (possibly Russell’s viper) in a cow (Banga et al, 2009). Figure 7.5-13 A and B show electron micrographs of muscle biopsies from the contralateral limb to that which was bitten, panel C shows a section of muscle from a mouse injected with *E. ocellatus* venom. Both show evidence of muscle injury, with fibres which have undergone apoptosis and necrosis.

Pulmonary haemorrhage resulting in epistaxis or haemoptysis is seen in victims envenomed with coagulopathic viper venoms. Figure 7.5-14 demonstrates haemoptysis resulting from *Callesolasma rhodostoma* venom in a human patient (A) (Warrell, 1999) and the mouse model (B).

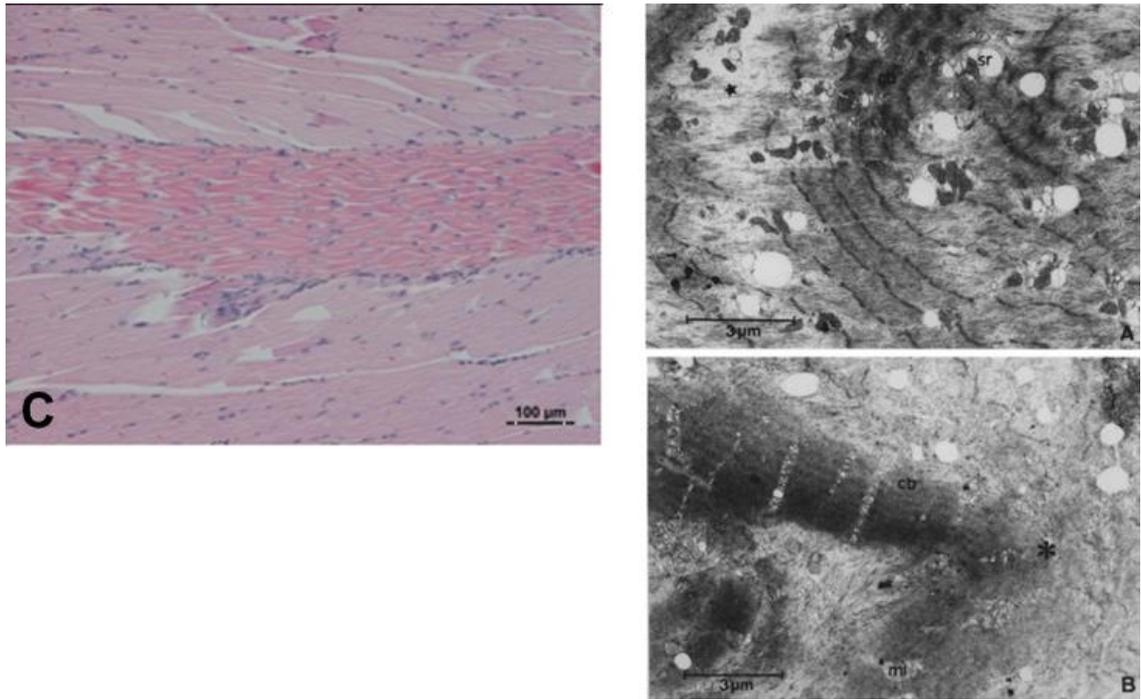


Figure 7.5-13: Viper envenomation showing myonecrosis A: Human patient electron micrograph – sample from the contralateral limb shows disorganization and lysis of myofilaments and B: shows myofibrillar disintegration with loss of cross striations, contraction band formation and swollen mitochondria (Azevedo-Marques et al, 1985). C: shows myolysis in a mouse envenomed with *E. ocellatus* venom. (Section 7.4.3.1, Figure 7.4-16C).

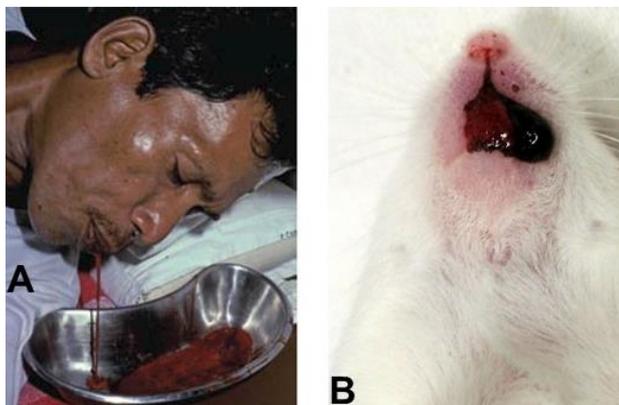


Figure 7.5-14: Haemoptysis following *Calloselasma rhodostoma* envenomation in A: human patient with concurrent TB (Warrell, 1999) and B: in a mouse.

7.5.1.5 *Naja nigricollis* venom: comparative pathology

In humans, the effects of *N. nigricollis* envenoming is largely localised, resulting in swelling and tissue necrosis; the classical signs of elapid neurotoxicity are not seen (Warrell, 1976). Spontaneous haemorrhage, including haematuria and subarachnoid haemorrhage may occur and these

were observed in the mouse model. The major difference between mouse and human envenoming is that mice exhibit paralysis, dyspnoea with post mortem examination evidence of respiratory failure (lung collapse and consolidation). Whilst in humans cardiotoxicity is rarely observed, myocardial haemorrhage is a commonly observed lesion in the mouse model.

Table 7.5-4: Comparative pathology – *Naja nigricollis* envenoming. References: (16) – Warrell, 1976

Pathological lesions	Mouse	Human (16)
Cardiovascular	Myocardial haemorrhage Pale organs	Occasional
Haematological	Haematuria Congestion of cranium and brain Subarachnoid haemorrhage	Defective clot retraction Subarachnoid haemorrhage Complement depletion
Neurological	Collapse and consolidation of lungs (respiratory paralysis)	Drowsiness in children
Autonomic	Urinary retention	Vomiting, frequent bowel movements

7.5.1.6 *Dendroaspis* species (mamba) venom: comparative pathology

There are relatively few humans bitten by *D. angusticeps* due to their shy nature and arboreal habitat, reflected in the near absence of published accounts of envenomation by this species. In contrast, there are many more humans who fall prey to the notoriously aggressive black mamba, *D. polylepis*; but there is a paucity of published pathological observations of victims.

Mamba venom is primarily neurotoxic and envenoming is potentially fatal unless antivenom is administered promptly and/or mechanical ventilation is available. Venom toxins cause descending paralysis and death from respiratory failure. This occurs both in human snakebite victims and in the mouse model. In both cases, the onset of paralysis is heralded by ptosis – paralysis of the facial muscles responsible for opening the eyes; shown in Figure 7.5-15, which also shows sweating due to the effects of venom toxins on the autonomic nervous system.

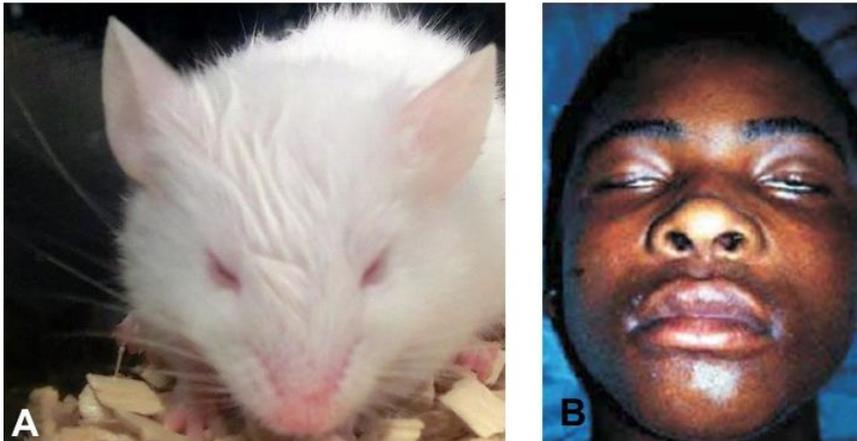


Figure 7.5-15: Ptosis and sweating following envenomation with *Dendroaspis* spp venom A: human patient (photo by John Thirsk, WHO, 2010) and B: mouse.

Table 7.4-20 lists the common pathological lesions observed in human snakebite victims and in the mouse model. *D. angusticeps* venom contains a unique toxin – fasciculin, which inhibits acetylcholinesterase and induces muscle fasciculations (see section **Error! Reference source not found.**). This species of venom can also produce a pronounced local reaction as well as mild haemostatic disturbances (WHO, 2010).

Cardiotoxicity has been reported following mamba bites (Blaylock, 1982); in the mouse model myocardial haemorrhage is not an infrequent post mortem observation.

Table 7.5-5: Comparative pathology – *Dendroaspis* spp envenoming. References: (17) Hodgson and Davidson, 1996

Pathological lesions	Mouse	Human (17)
Ptosis	✓	✓
Autonomic	Salivation Sweating	Salivation Sweating
Muscle fasciculations	✓	✓
Paralysis	✓	✓
Respiratory paralysis	Collapsed and consolidated lungs	✓
Cardiovascular	Myocardial haemorrhage (atria)	Cardiotoxicity
Gastrointestinal	Intestinal haemorrhage	Vomiting, diarrhoea, abdominal pain

7.5.2 The mouse as a model for human envenoming

The results suggest that the mouse may be a better model of human envenoming than previously thought, with many examples of parallel pathological lesions, particularly those associated with haemorrhage. In the case of coagulopathic venoms, fatality in the mouse is a somewhat 'Russian roulette' event in that whether the mouse dies within the duration of the assay depends on when, if and where (anatomically) haemorrhage occurs. Perhaps doing a 20 minute whole blood clotting test at the end of an assay, or immediately after a mouse dies, would be a more accurate measure of venom toxicity and antivenom efficacy.

There is much debate about the validity of the mouse model for characterising procoagulant venoms of Australian elapids. It was shown that different species showed different susceptibilities to the procoagulant effects of snake venom (Maduwage et al, 2016).

Of the venoms studied, only the spitting cobras, *Naja nigricollis* and *N. pallida* showed widely differing lethal effects in the mouse compared to other species, namely neurotoxicity, with mice subjected to these venoms succumbing to flaccid paralysis and post mortem findings consistent with respiratory paralysis. In envenomed humans, neurotoxicity is rare, and when described is generally confined to somnolence and visual disturbances. This dichotomy may be explained in terms of the mouse being a prey species of these snakes with venom toxins specialised to immobilise and kill its food.

7.5.3 Post mortem examination and pathology/survival score to profile venom-induced pathology by snake species

Post mortem examination has proved to be an exceedingly useful tool in quantifying the systemic pathological effects of venom. It was observed that the number of lesions observed and/or organs affected increased with time. To adjust for this increase in observed post mortem lesions with time, a scoring system was developed in which the longer the survival time, the lower the 'survival score'; the sum of these scores is the 'pathology-survival score'

(PSS). A better term for the survival score would be the 'mortality score' (see Chapter 2A).

Using this system for non-commercial assays, we were able to compare the effect of different antivenoms, using only ten mice, compared to a minimum of twenty for a full ED₅₀. In this way, we achieved a considerable reduction in the numbers of mice required for this comparison of antivenoms.

However, high doses of venom often produced rapid death, with insufficient time for gross lesions to develop, so the PSS had a value of 12 (survival score) plus the pathology score, whereas a mouse surviving until the end of a 6 hour assay would score 0 for the survival score, but, in the case of a haemorrhagic venom for example, may have a pathology score of more than 20. The survival scoring needs to be revised – perhaps using a logarithmic scale. The maximum survival score should also reflect the likely upper range of pathology score for the particular venom being studied.

The 'Pathology score' could be modified to weight those pathological lesions which are potentially lethal, such as myocardial haemorrhage, more highly than those which are not lethal, or merely indicate a greater severity of a pathogenic process, such as testicular haemorrhage. Antemortem observations, such as respiratory distress or paralysis could also be added to the score. In this way, the effect of neurotoxins, which produce minimal gross lesions, could be accounted for in the 'pathology score'. The present system is a greater measure of the severity of envenomation by the enzyme-rich viper venoms than by the neurotoxin-rich elapid venoms.

Of the venoms studied, *Echis species* were haemorrhagic, with incoagulable blood. Another venom which produces a similar pathological profile was the Boomslang (*Dispholidus typus*). When Boomslang antivenom was pre-incubated with *E. ocellatus* venom, the resulting pathology profile was very similar to that of *B. arietans*. It would seem that the Boomslang antivenom had neutralised the procoagulant toxins in the *E. ocellatus* venom, but not those which affected platelet function, nor those which were myotoxic. This was

rather like peeling an onion – by neutralising the foremost pathological effect of the venom, more minor pathological lesions were revealed.

The histopathology of samples from mice envenomed with *E. ocellatus* venom generally confirmed what was observed on gross PM examination – widespread haemorrhages. It also revealed microscopic evidence of myolysis and areas of myocardial injury.

Bitis arietans venom is also haemorrhagic, but the pathognomic feature is widespread petechiation. This would suggest that the primary toxin target is the platelet, and indeed, in clinical envenomings, thrombocytopenia is frequently noted (Warrell et al, 1975). Muscle and cardiac injury were also common and the duodenum appeared to be the most commonly targeted area of the gastrointestinal tract. The specificity of venom toxins for different areas of the gastrointestinal tract is an area for future research.

Vipera berus venom is also haemorrhagic, although results suggest that the blood is not incoagulable given that clots were frequently observed. In the mouse model, cardiotoxicity and signs of congestive heart failure were the most common observation with gastrointestinal pathology most commonly seen in the distal small and in the large intestines.

Histopathology of *V. berus* venom pre-incubated with ViperaVet antivenom showed an increased number of mitotic figures. Whether this is a feature of the cell growth promoting effect described in Chapter 8, or an effect of the ovine Fab AV is unclear. To investigate this phenomenon further, the histopathology of livers of mice which had received low doses of venom or antivenom only could be performed.

Naja nigricollis venom produced signs of neurotoxicity in the mouse model, with ante-mortem respiratory distress and terminal paralysis in high venom dose groups. The venom proteome contains a very small amount of an α -neurotoxin (0.4%) – whether this is sufficient to explain the neurotoxic signs seen in mice, or whether there is some other toxin responsible is open to

debate. The vast majority of toxins in this species' arsenal are consistent with its tissue destructive signs of local envenoming (Petras et al, 2011). The venom was found to contain a cardiotoxin (Petras et al, 2011), which is thought to contribute to local tissue damage, but could also explain the myocardial injury observed in the mouse pathological profile.

Dendroaspis angusticeps venom in the mouse model shows many of the neurotoxic signs of envenoming seen in human victims of bites by this species. Notably sweating, salivation, muscle fasciculations, ptosis with descending paralysis and respiratory distress. The pathological profile also included myocardial injury with signs of heart failure.

The jaundice and/or haemoglobinuria seen in the pathological profile of the spitting cobras is somewhat perplexing. The lesions observed suggests a haemolytic anaemia. This observation warrants further explanatory investigation.

7.5.4 Antivenom efficacy

In Section 7.4.1: Mouse pathological lesions, tables show the percentage of mice post mortem examinations exhibiting each pathological lesion listed. As detailed above, these percentages are not comparable between venom and antivenom or between antivenoms because of the dose discrepancies. The exception is between the five polyvalent African antivenoms studied (South African Vaccine Producers, formerly known as 'SAIMR' – as 'gold standard', FAV Afrique, VINS, Inoserp and Premium Serum and Vaccines Institute, India) in which standard doses of venom and antivenom were used.

A more appropriate method of establishing antivenom efficacy would be to look at which venom-induced pathological lesions are not neutralised at the lowest 'all survivors' antivenom dose. This information could then be augmented using antivenomics to identify unbound toxins. If the unbound toxin's activity correlates to observed pathological lesion it is strong evidence for a deficiency

in antivenom efficacy. The significance of the pathological lesions determines whether the deficiency needs to be addressed.

Another important finding was the ability to differentiate between aggregate-induced and venom-induced pathological lesions. Aggregate-induced pathological lesions showed swollen and congested organs, with marked hyperaemia and congestion of extremities, but without the obvious myocardial haemorrhage observed in congestive heart failure associated with venom-induced pathological lesions. Some of the IgG antivenoms produced aggregates when mixed with high doses of venom – probably due to cross-linkage of antibody-toxin complexes. The pre-incubation of venom with antivenom is an artificial construct because, in the clinical situation, venom is ‘injected’ subcutaneously, or at worst, intramuscularly (although a case of intravenous envenomation has been described: De Haro et al, 2009). Antivenom is then administered, usually intravenously, hours to days later. The probability of sufficient concentrations of venom and antivenom occurring intravascularly is negligible. At present, ED₅₀ assay results can be severely compromised by aggregate deaths. Centrifugation to remove the aggregates may remove potentially active toxins from the mix and result in an erroneously low ED₅₀ value.

7.6 Conclusions

Post mortem examination of envenomed mice has greatly enriched our knowledge and understanding of snake venom induced pathological lesions, the intention being to publish these findings in the near future. The unique ‘pathology profile’ of each venom studied could be explained in most cases by the venom’s proteome, although there are still major gaps in understanding of the mode of action and toxic effect of all venom constituents.

When post mortem pathological lesions are considered, the mouse is a better model for human envenoming than it has previously been given credit for. One major exception is the spitting cobras, particularly with reference to *N. nigricollis*.

The use of toxicovenomics to predict induced pathological lesions is an exciting and interesting area of study. The use of venom pathological profile, in combination with antivenomics, is likely to become a valuable tool in assessing antivenom neutralising efficacy and identification of antivenom shortcomings.

The pathology-survival score (PSS) has the potential to become a valuable means of refining and reducing the present mouse LD₅₀/ED₅₀ assays. Refinement: the use of PM observations allows termination of assays without using death as their endpoint. Reduction: using the PSS as a means to compare the efficacy of AVs to a particular venom, rather than full ED₅₀s for each test antivenom can reduce the numbers of mice required by more than half (>100%).

Chapter 8. Replacement

8.1 Objectives

The objective of this section of the project was to develop a cell based assay comprising a matrix of cell types, which reflect the most relevant cell targets in clinical envenoming by a variety of medically relevant snake venoms. The assay should demonstrate a measurable cytopathic effect (CPE) corresponding in severity to the *in vivo* murine median lethal dose and neutralisation proportional to the murine ED₅₀. The selected cell lines must require minimal intervention for maintenance and the CPE detection system should be simple and robust. This will ensure that the assay can be performed globally without the need for highly specialized and expensive equipment.

8.2 Introduction

The complexity and variety of snake venoms and subsequent pathological lesions of envenomation provides an immense challenge to replace the 'gold standard' WHO *in vivo* tests with an *in vitro* alternative. Many of the available *in vitro* tests show binding, but not necessarily neutralisation, of venom components by antibodies (cf Cook et al, 2010). In order to replace *in vivo* testing, consideration needs to be given to the cost of performing the assay, as well as the expertise and specialist equipment to perform such an assay. Ideally, the assay should be simple, robust even in tropical conditions, and inexpensive so that efficacious, safe and affordable antivenoms (AVs) can be supplied to those most in need.

Cell assays are increasingly used to assess the cytotoxic and metabolic effects of snake venoms as an alternative to *in vivo* assays. They are used as a means to elucidate the mechanism of action of venom components (Lomonte et al 1994a,b,c; Rigoni et al 2004) and to investigate the possible therapeutic use of snake venom toxins (Bradshaw et al, 2014, Conlon et al, 2013). The assays may also be used to assess the efficacy of AV in neutralising these cytotoxic effects. So far, a relationship between cytotoxicity and *in vivo* LD₅₀ has not been demonstrated (Oliviera et al, 2001). The degree of cytotoxicity varies

between the cell line used and the species of snake venom or toxin added (Giron et al, 2005).

8.2.1 Detection methods

There are a number of ways in which the effect of venom on cells can be measured. The simplest is visual assessment of cell morphology by light microscopy. However, this is subjective and prone to considerable variation between operators.

Colorimetric assays measure the uptake or exclusion of dyes such as Trypan Blue, which is excluded by viable cells, but not by dead cells. The disadvantage of this method is that it does not include those cells which are lysed by venom. A sensitive and reproducible assay for cell viability uses Neutral Red, a weakly cationic dye, which is believed to enter cells by non-ionic diffusion through the cell membrane, after which it accumulates in lysosomes (Borenfreund et al, 1985; Oliviera et al, 2001). Another set of assays for viable cells using MTS, a tetrazolium salt, requires the presence of active mitochondria and has the advantage of no washing steps. It can also be used to measure proliferative lymphokines, mitogen stimulations and complement-mediated lysis (Mosman, 1983; Konstantakopoulos et al, 2008; Kalam et al, 2011). However, using MTS assay medium, serum albumin and fatty acids can be a major distorting factor (Huang et al, 2004).

Other available assays include: a luminescence assay to measure ATP and thus cell viability (Promega; CellTiter-Glo®), lactate dehydrogenase (LDH), which is released from damaged cells (Promega; Cyto Tox-ONE™ membrane integrity assay) and a range of assays which detect metabolic changes such as oxidative stress, cell cycle and proliferation analysis (Millipore). These assays have the potential to elucidate the mechanism of action of venom components, but are too complex and expensive to be of use in the screening and assessment of antivenoms.

8.2.2 Cell lines

A wide variety of cell lines have been used in venom cytotoxicity assays, including VERO (Oliviera et al, 2001), mouse kidney (Giron et al, 2005), myoblasts (Angulo et al, 2005; Bustillo et al, 2008; Cintra-Francischinelli et al, 2009, Omran et al, 2004); endothelial cells from a number of anatomical structures (Lomonte et al, 1994, Gremski et al, 2007), cardiomyocytes (Mbuga et al, 1988, Gowda et al, 1993), epithelial cells, fibroblasts, kidney cells, neuroblastoma and many others. Cells may be derived directly from parent tissue (primary cell culture) or may be established cell lines and may originate from different species. When using an established cell line, continual passaging can change the characteristics, therefore a limit to the number of passages used for experimental work should be set, and cells discarded once this limit has been reached.

8.2.2.1 VERO cells

VERO cells are derived from African green monkey kidneys, are morphologically fibroblasts, and form a monolayer when cultured. They have a doubling time of 18 hours during exponential growth, but at lower seed rates can be left for a week or more without the need for a media change. The rapid doubling time makes this cell line suitable for use in a screening assay entailing a rapid turnover. They require a basic medium for optimum growth – (Dulbecco's modified Eagle's medium [DMEM] containing high glucose with added foetal bovine serum, glutamine and non-essential amino acid). This cell line was selected as first choice for this project.

8.2.2.2 SH SY5Y cells

SH SY5Y cells were originally established from a human neuroblastoma derived from a metastasis in bone marrow. The cells propagate by mitosis and differentiate by extending neurites. They can convert glutamate to GABA, are acetylcholinergic, glutaminergic and adenosinergic. They are more slowly dividing than VERO cells, having a doubling time of more than 55 hours, and have more specific media requirements (DMEM-Ham-F12, plus glutamine, non-essential amino acids, foetal bovine serum and sodium pyruvate). Snake

venom neurotoxins target acetylcholinergic neuro-muscular junctions so it was hypothesized that they may produce a CPE in this line by targeting the cell's acetylcholinergic receptors.

Characteristics of cell lines reference: ECACC product information.

8.3 Methods

Cell culture facilities were developed 'from scratch' at the beginning of this project, and entailed optimization of cell culture techniques in the new facility. General cell culture materials and methods are described in Chapter 2, section 2.4.1.

8.3.1 Assay optimization

Firstly the cytopathic effect of venom was established by incubation of VERO cells (1.5×10^5 cells/mL) in medium (DMEM) containing different concentrations of venom (0.1-100mg/L), for varying lengths of time (0.5, 1, 2, 4 & 6 hours). Control cells were incubated in medium alone. The cells were then washed by suspending in PBS, centrifuging and re-suspending the pellet in medium and used to seed a culture plate. Cell growth was assessed using a neutral red assay as described in Chapter 2. The experiment can be found in its entirety in the chapter supplement (vct10).

8.3.1.1 Detection methods

8.3.1.1.1 Visual inspection

An inverted microscope was used to examine the cells in the culture flask or culture plate. The percentage cover of the field was estimated and changes in morphology noted.

8.3.1.1.2 Trypan blue

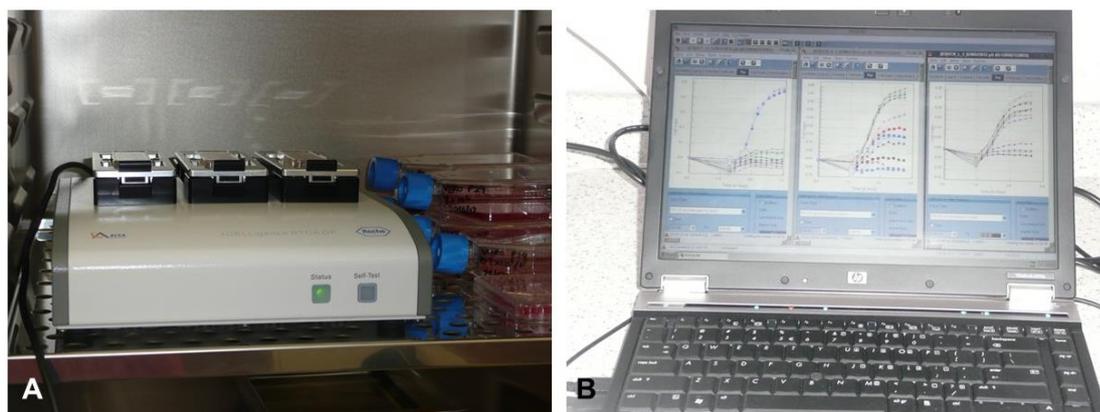
Trypan blue was used to differentiate live from dead cells. It is excluded by live cells, which appear white in a blue background. It was used when cell counts were performed.

8.3.1.1.3 Neutral Red

See Chapter 2, section 2.4.1.

8.3.1.1.4 xCELLigence

Cytotoxicity and neutralisation assays were performed using the xCELLigence system to examine the 'real time' growth of cells. Sixteen-well e-plates were used, which could be run as single, double or triple-plate assays in the three available xCELLigence cradles (Figure 8.3-1).



.Figure 8.3-1: xCELLigence cradle inside incubator (A) and display showing all three cradles in use (B)

The e-plates have a central strip devoid of electrodes which allows visualisation of growing cells. The system was used for optimisation of assays and not for data collection, so duplicate samples were often not used.

The plates were seeded with the appropriate cell count for the experiment being performed (e.g. 1.5×10^4 cells/ well for VERO cells grown in venom) and then allowed to stand for 30 minutes at room temperature to allow the cells to settle prior to insertion of the plate(s) into the xCELLigence cradle(s). Cell index (CI) – a measure of electrical resistance across the bottom of each well - was recorded every 15 minutes until test compound(s) were added. At this time the CI was 'normalised' to a value of 1.0 to allow for variation in cell growth of each well. Immediately after addition of the test substance(s) CI was recorded every 5 minutes for 1 hour, then every 15 minutes thereafter. CI reflects the proportion of the bottom of the well covered by cells, but does not

differentiate between increased numbers and increased size of cells. The real-time cell assay (RTCA) software was used to calculate 'slope' (rate of change), LC₅₀ or EC₅₀ over the study period and LC₅₀ (50% death of cells from venom toxicity) or EC₅₀ (50% neutralisation of venom toxicity) at given time points, which include the R² value.

8.4 Results

8.4.1 VERO Cells

All the venoms tested produced a CPE on VERO cells. Figure 8.4-1A shows normal VERO cell growth. The cells start to round and holes appear in the monolayer (Figure 8.4-1B). The holes enlarge, the cells clump and cell debris can be seen. Eventually only cell debris is visible (Figure 8.4-1C).

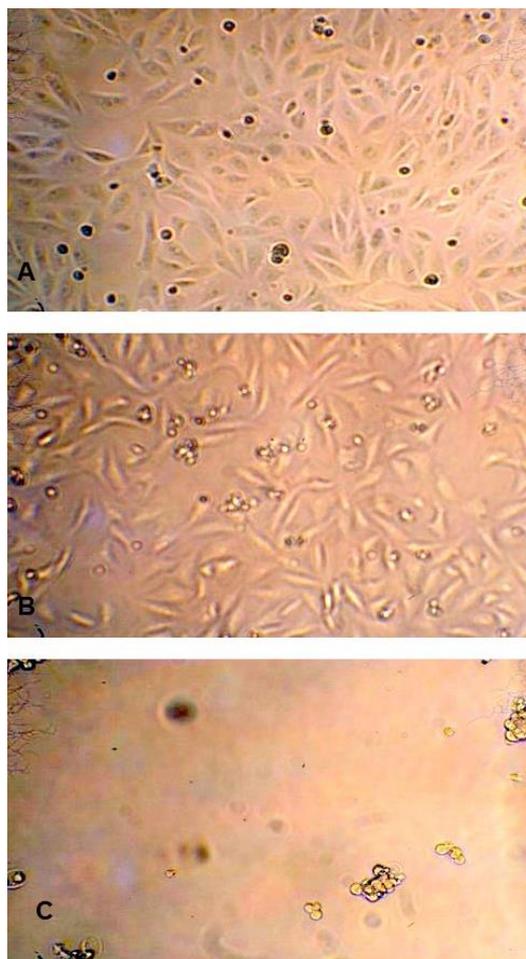


Figure 8.4-1: CPE of VbV on VERO cell
A: normal cells, B: early CPE, C: destruction of the cell monolayer

8.4.1.1 Assay optimization

8.4.1.1.1 Cell growth time ('growth') and assay end-point (EP)

The optimization of cell growth time prior to venom addition and assay end-point were carried out using *E. ocellatus* venom by means of the xCELLigence

system. A complete set of results can be found in the chapter supplement (vctEOG-1). The results are summarised in Figure 8.4-2 and Table 8.4-1.

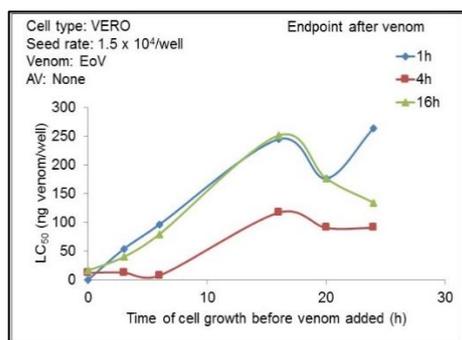


Figure 8.4-2: Venom LC₅₀ of EoV at different growth and end-point times

Table 8.4-1: Venom LC₅₀ of EoV (ng/well). VERO cells, 1.5x10⁴ cells/well; Growth: 0,3,6,16,20 or 24h; EndPoint: Real Time (1,4,16h); detection: xCELLigence

Time (h) Growth/EP	1	4	16
0	-	12.0	15.7
3	53.8	12.6	39.3
6	96.5	7.3	79.0
16	2451.4	117.1	251.1
20	176.7	90.7	176.7
24	264.2	90.7	134.1

The optimum time to add venom, using a seeding rate of 1.5 x 10⁴/well, is after 6 hours of growth, and optimum end-point is 4 hours. However, given that the neutral red has to be left on the cells for 2-3 hours, this regime would not fit into a normal working day. Next the optimal seeding rate for an assay where cells were added to venom and had an end-point of 16h or for venom addition to cells after 16h (overnight) growth, with an end-point of 4 hours, was determined.

8.4.1.1.2 Seed rate

This experiment was performed by growing different numbers of cells in venom using an end-point of 16h. The results are illustrated in Table 8.4-2 and Figure 8.4-3. The complete results are illustrated in the chapter supplement (vct29). A seed rate of 1.5x10⁴ was selected for future experiments using cells grown in venom and an end-point of 16h. The optimum end-point using this seed rate of cells grown in venom was examined using the xCELLigence system with two different venoms (*Echis ocellatus* and *Vipera berus*)

Table 8.4-2: Optimisation of seed rate. VERO cells-growth: 0h; End-point:16h; detection: NR; Venom: VbV

Cells/well	LC50 (ng/well)
2,000	400
5,000	280
10,000	320
15,000	280
20,000	300
50,000	280

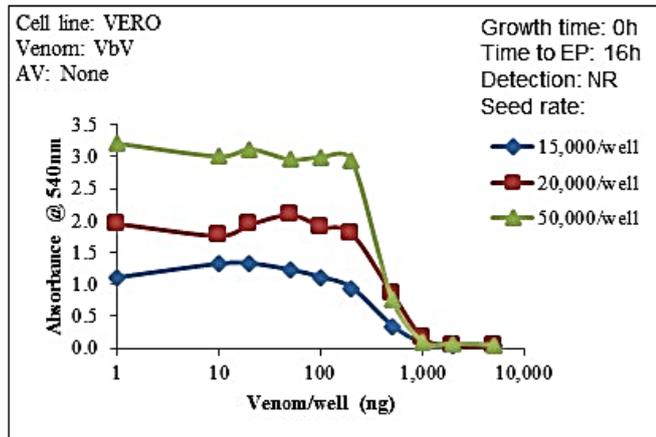


Figure 8.4-3: Optimisation of seed rate

8.4.1.1.3 Assay End-point – VERO cells (1.5×10^4 /well) grown in Viper venoms

Both venoms studied demonstrated an effect on the growth of VERO cells, which was dose dependant. It was possible to calculate the LC₅₀ (The dose which inhibits 50% of maximum growth) at specific time-points after venom addition. The LC₅₀ remained constant from 8 until 14 hours, when it started to rise. An end-point of 16 hours was chosen for performance of neutral red assay to allow each step of the experiment to be performed within normal working hours.

The real-time (RT) growth of VERO cells in different concentrations of *E. ocellatus* venom is shown in Figure 8.4-4A and LC₅₀ plotted from 8-22 hours in Figure 8.4-4B. Similar plots showing VERO cell growth in *V. berus* venom are shown in Figure 8.4-5A and B. The LC₅₀ of *E. ocellatus* venom was 60ng/well and that of *V. berus* venom was 100ng/well after 16 hours growth.

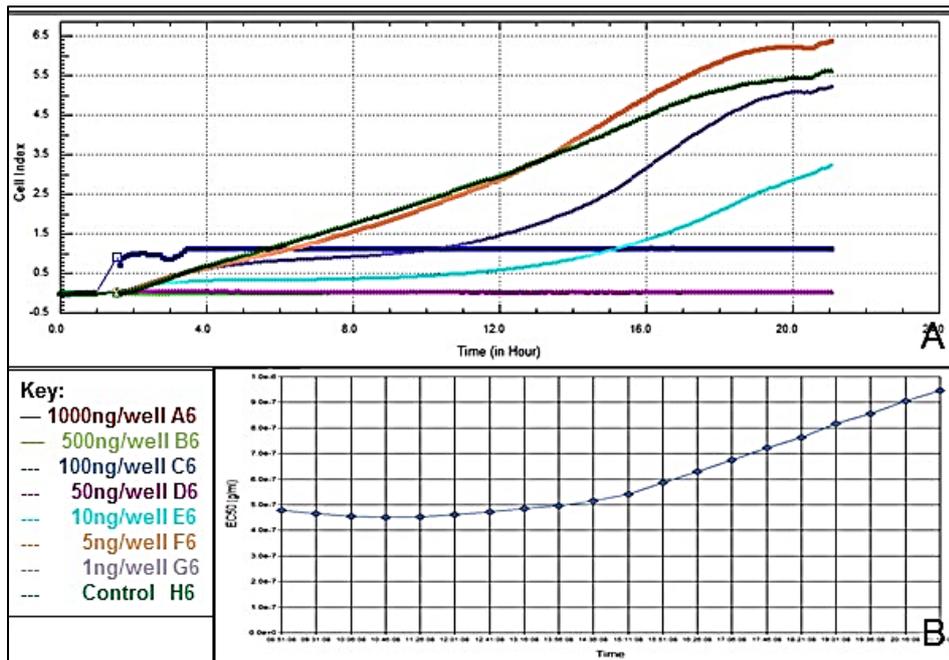


Figure 8.4-4: A – Real time xCELLigence plot of VERO cells grown in different concentrations of *E. ocellatus* venom; B – LC₅₀ 8 – 22hours

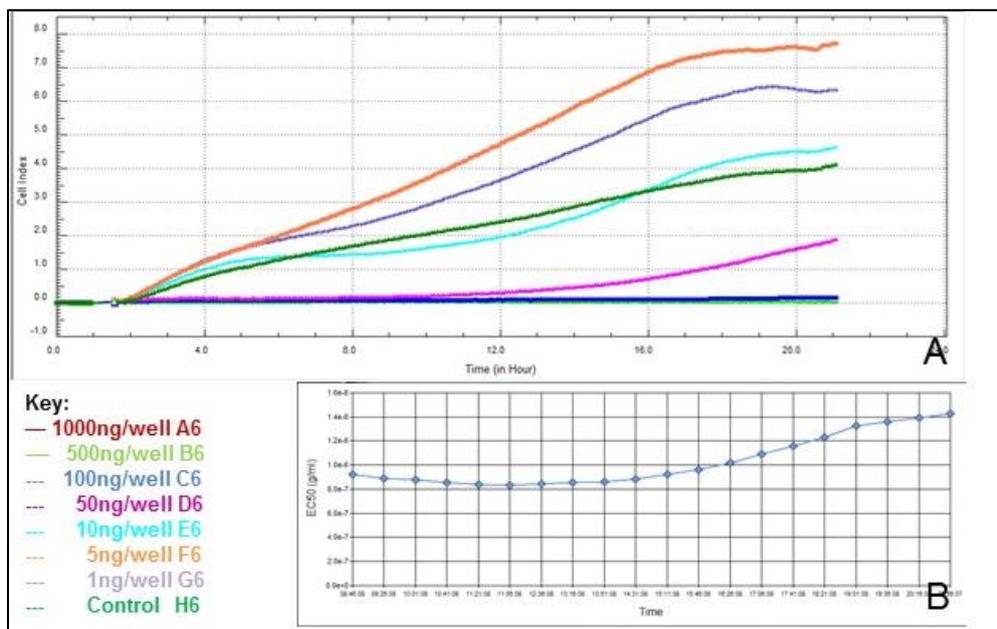


Figure 8.4-5: A: Real time xCELLigence plot of VERO cells grown in different concentrations of *V. berus* venom; B: LC₅₀ 8 – 22hours

8.4.1.1.4 Optimisation of venom cytotoxicity neutralisation

VERO cells (1.5×10^4 /well) were grown in *E. ocellatus* venom, which had been pre-incubated with different dilutions of EchiTabG antivenom (EOG) for 30 minutes at 37°C. The xCELLigence was used to monitor cell growth. The optimum end-point was 16h, with an EC₅₀ dilution of 1:109 at 16h. The results are summarised in Table 8.4-3. and illustrated in Figure 8.4-6.

Table 8.4-3: Summary of antivenom EC₅₀. VERO cells: 1.5×10^4 cells/well; growth: 0h, Venom: EoV 250ng/well; AV: EOG; end-point: RT; detection: xCELLigence

End-Point (h)	EC ₅₀ dilution	R squared
4	75	0.996
8	78	0.998
12	91	0.996
16	109	0.995
20	75	0.995
24	72	0.992

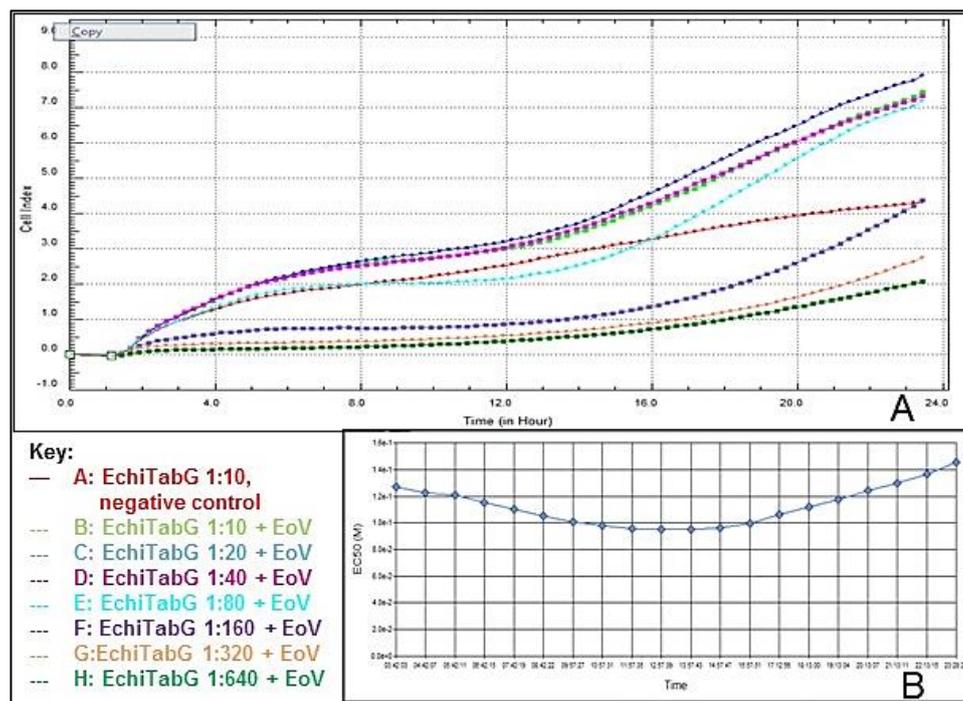


Figure 8.4-6: A: Real time xCELLigence plot of VERO cells grown in different dilutions of EOG, pre-incubated with 250 ng/well EoV; B: EC₅₀ 3 – 24hours

8.4.1.1.5 Assay variation

Once the assay was optimised the repeatability was examined by either growing VERO cells (1.5×10^4 /well) in *E. ocellatus* venom (20-500 ng/well) or

V. berus venom (50 – 1000ng/well), or adding venom to the cells after 8 hours growth. End-point was after 16h and a neutral red assay was used to detect the percentage of surviving cells. The results are summarised in Table 8.4-4, which shows both between and within assay variation for the two venoms and cells at two different growth points. Graphical illustration of these results can be found in the chapter supplement (vcnVP4 and vcnEOG6). Mean LC₅₀ of both venoms was lower when cells were grown for 8h prior to venom addition compared to when the cells were grown in the venom. Mean LC₅₀ of *E. ocellatus* venom was lower than that of *V. berus* venom. The 95% confidence intervals (CI₉₅), and consequently coefficient of variation (CV) were higher for *V. berus* venom than for *E. ocellatus* venom. The CV was greater than 30% for cells grown in *V. berus* venom both for within and between assay variations. This CV is greater than for other *in vitro* assays (SSAC<10%, ELISA 15-20%).

Table 8.4-4 Venom LC₅₀ (ng venom/well) – assay variation. VERO cells, 1.5x10⁴ cells/well; Growth: 0h or 8h; End-point: 16h; Detection: NR. SD = standard deviation, CI₉₅ = confidence intervals, CoV = coefficient of variation.

Assay	Between				Within			
	EoV		VbV		EoV		VbV	
Growth	0h	8h	0h	8h	0h	8h	0h	8h
Replicate 1	180	120	400	340	120	73	350	255
Replicate 2	210	120	750	350	120	110	250	250
Replicate 3	200	150	550	600	150	120	500	205
Mean	193	130	567	497	130	101	367	237
SD	15	17	176	133	17	25	126	49
CI₉₅	17	19	199	151	19	28	143	55
CV (%)	7.8	13	31	27	13	25	34	21

8.4.1.2 Venom cytotoxicity

Table 8.4-5: Venom LC₅₀ - VERO cells, 1.5x10⁴ cells/well; Growth: 0h; End-point: 16h; Detection: NR

Venom	LC ₅₀ (ng/well)
EoV	250
BaV	220
VbV	110
NnigV	900
DaV	600

Table 8.4-6: Venom LC₅₀. VERO cells: 0.75x10⁴ cells/well; Growth: 16h; End-point: 4h; detection: NR

Venom	LC ₅₀ (ng/well)
EoV	90
BaV	200
VbV	80
NnigV	2000
DaV	50

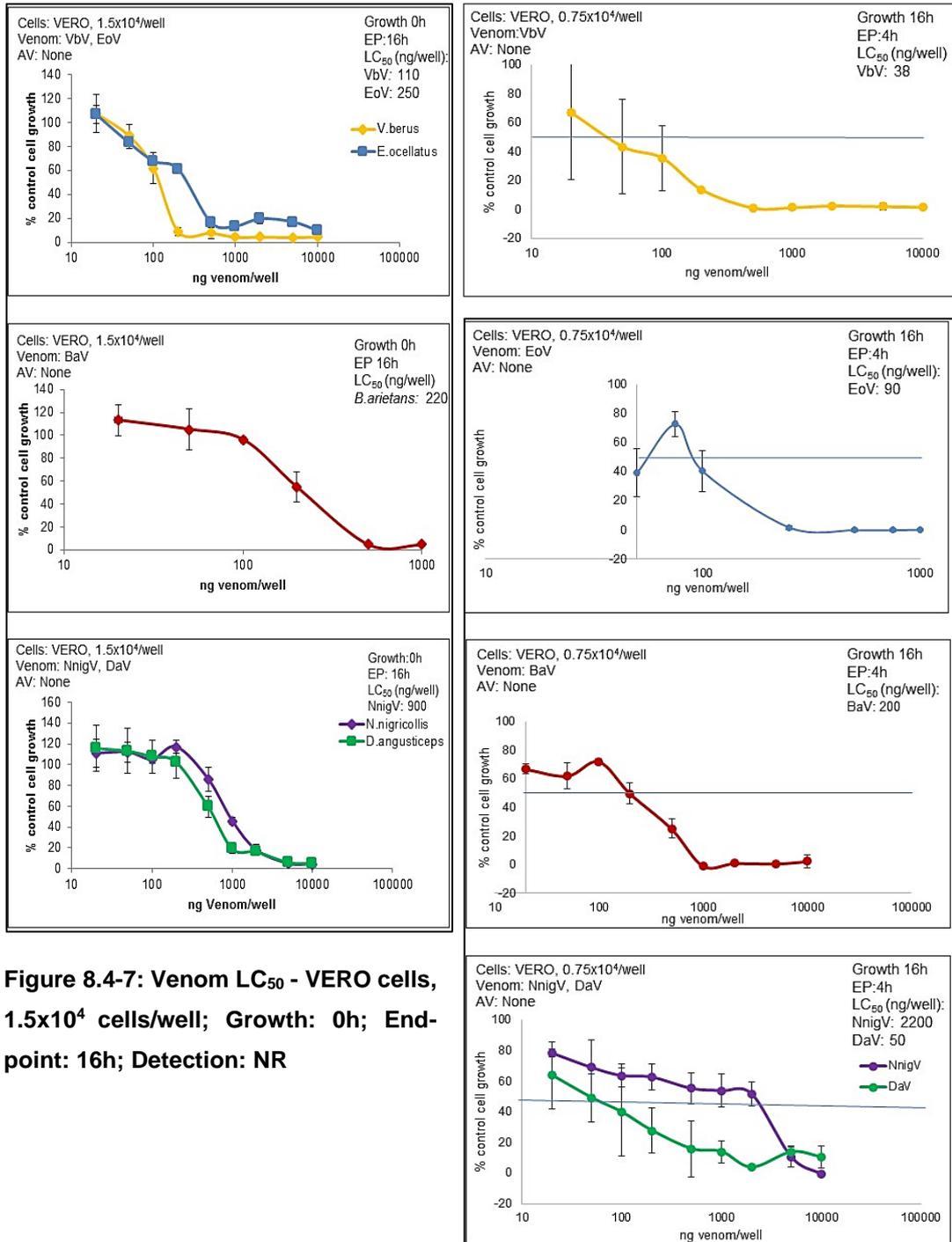


Figure 8.4-7: Venom LC₅₀ - VERO cells, 1.5x10⁴ cells/well; Growth: 0h; End-point: 16h; Detection: NR

Figure 8.4-8: Venom LC₅₀. VERO cells: 0.75x10⁴ cells/well; Growth: 16h; End-point: 4h; detection: NR

Results for the study's five selected venoms are summarised in Table 8.4-5 and Table 8.4-6 and illustrated in Figure 8.4-7 and Figure 8.4-8. Growing the cells for 16h before adding venom is a more sensitive assay than growing the cells in venom, but does add an extra intervention step to the assay. When

cells are grown in venom, they are more sensitive to the effects of the viper venoms than to those of the elapids.

However, *D. angusticeps* venom has a greater cytopathic effect when added to cells which have been grown overnight. The cytopathic effect of *B. arietans* venom is similar when the cells are grown in the venom or when the venom is added to the grown cells.

8.4.1.3 Venom cytotoxicity neutralisation

Venom cytotoxicity neutralisation assays were performed using VERO cells (0.75×10^4 /well) grown for 16h before venom/antivenom addition. A neutral red assay was performed 4h later. In order to optimize the venom concentration and starting AV dilution a 'checkerboard' assay was performed in which one row (B-G) each of a 96 well culture plate contained 0-5x venom LC₅₀. This experiment equates to the *in vivo* range-finding experiments. An example of the results can be seen in section 8.5, Figure 8.5-3.

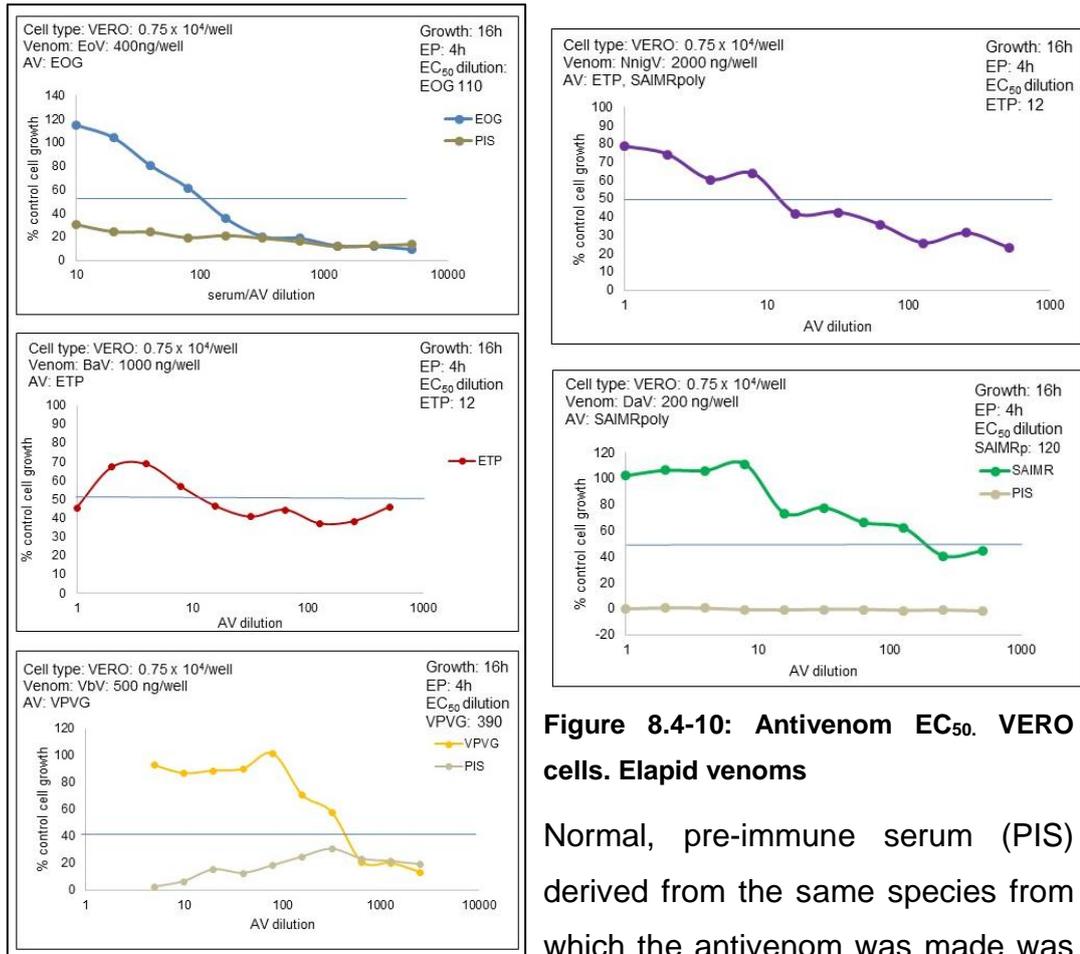


Figure 8.4-9: Antivenom EC₅₀. VERO cells, Viperid venoms

Figure 8.4-10: Antivenom EC₅₀. VERO cells. Elapid venoms

Normal, pre-immune serum (PIS) derived from the same species from which the antivenom was made was used as a negative control.

The results of the full EC₅₀ experiments are summarised in Table 8.4-7. All five venoms were neutralised by their homologous antivenoms, although not all treated cells attained 100% of control cell growth and others outgrew the control cells at higher antivenom dilutions. This is illustrated in Figures 8.4-9 and 8.4-10.

Table 8.4-7: Antivenom EC₅₀. VERO cells: 0.75x10⁴ cells/well; Growth: 16h; End-point: 4h; Detection: NR

Venom	AV	x LC ₅₀	Max neut %	EC ₅₀ dilution	EC ₅₀ (ng/well)	ng venom/well	AV/V (potency)
EoV	EOG	4	93	90	27800	400	35.0
BaV	ETP	5	70	18	69450	1000	69.4
VbV	VPVG	5	102	230	545	500	1.1
NnigV	ETP	2	75	12	208300	4000	52.0
DaV	SAIMRp	5	125	90	278	200	69.5

8.4.2 SH SY5Y Cells

SH SY5Y cells grew as a monolayer, but their cell cycle is longer than VERO cells. They grow with spikey projections (Figure 8.4-11A). If left for a week or more, the cells appeared to 'pile up'. When venom was added, as with the VERO cells, holes started to appear in the monolayer (Figure 8.4-11B). The cells clumped in balls (Figure 8.4-11C) until only cell debris was visible.

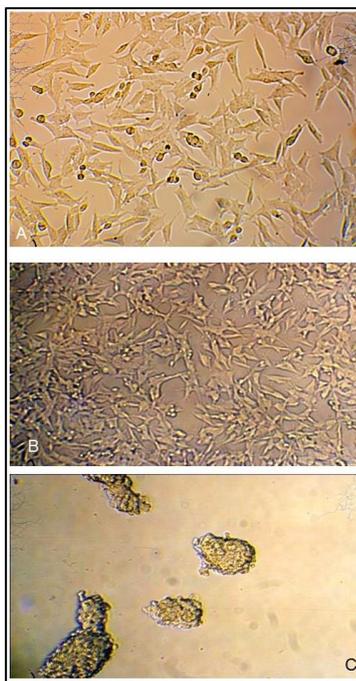


Figure 8.4-11: CPE of VbV on SHSY5Y cells. A: Normal growth; B: Mild CPE; C. Destruction of the monolayer.

8.4.2.1 Assay optimization

The xCELLigence system was used to optimize SH SY5Y cell growth, seed rate and assay end-point, as previously described for VERO cells.

8.4.2.1.1 Seed Rate

The assay was most sensitive at all seed rates for venom addition after between 16 and 24 hours growth, which also had the highest R^2 values. The plots revealed that the maximum effect of venom on the cells occurred within the first 4 hours. After this time, cells which had been treated with lower concentrations of venom had started to regrow. Before making a decision on what time to use as an end-point for the assay it was decided to perform the assay using a variety of other venoms. As the result of this experiment a seed rate of 30,000 cells/well was chosen.

Table 8.4-8 Venom LC₅₀ (ng *V. berus* venom/well). SHSY5Y; Growth: 24h; End-point: RT; Detection: xCELLigence

Seed rate: (cells/well)	10,000		15,000		20,000		25,000		30,000		40,000	
Time (h)	LC ₅₀	R ²										
4	1988	0.77	1535	0.94	2999	0.911	1102	0.97	928	0.96	917	0.96
6	1704	0.84	1291	0.96	1112	0.94	880	0.98	722	0.97	733	0.96
8	1357	0.87	1022	0.98	838	0.96	739	0.99	630	0.97	656	0.96
16	898	0.93	748	0.98	602	0.97	577	0.99	499	0.98	407	0.98
24	882	0.93	732	0.99	593	0.98	551	0.99	479	0.98	394	0.99

8.4.2.1.2 End Point

SH SY5Y cells seeded at 30,000 cells/well and grown for 24 hours before venom addition were sensitive to the cytopathic effects of all four venoms tested.

Table 8.4-9: Venom LC₅₀ of *E. ocellatus*, *B. arietans*, *N. nigricollis* and *D. angusticeps* venoms (ng /well). SHSY5Y: 3x10⁴cells/well; growth: 24hours; End-point: real-time; detection: xCELLigence. Selected end-point highlighted in red. (vct48)

Venom	EoV		BaV		NnigV		DaV	
	LC ₅₀	R ²						
2	462	0.99	1379	0.99	1201	0.99	1998	0.99
4	294	0.99	669	0.99	1097	0.99	940	0.99
6	251	0.99	512	0.99	1007	0.99	703	0.99
8	210	0.99	454	0.99	951	0.99	703	0.99
16	177	0.99	336	0.99	913	0.99	253	0.99
20	175	0.99	334	0.99	893	0.99	224	0.99
24	172	0.99	332	0.99	875	0.99	203	0.99

There appears to be a two-phase effect on this cell line – immediate death of cells followed by a more prolonged effect, the latter being best assessed after 16-20 hours incubation. Three of the four venoms showed a growth stimulating effect at low venom concentrations. The cell line was most sensitive to *E. ocellatus* venom and least sensitive to *N. nigricollis* venom, under these conditions, in this experiment.

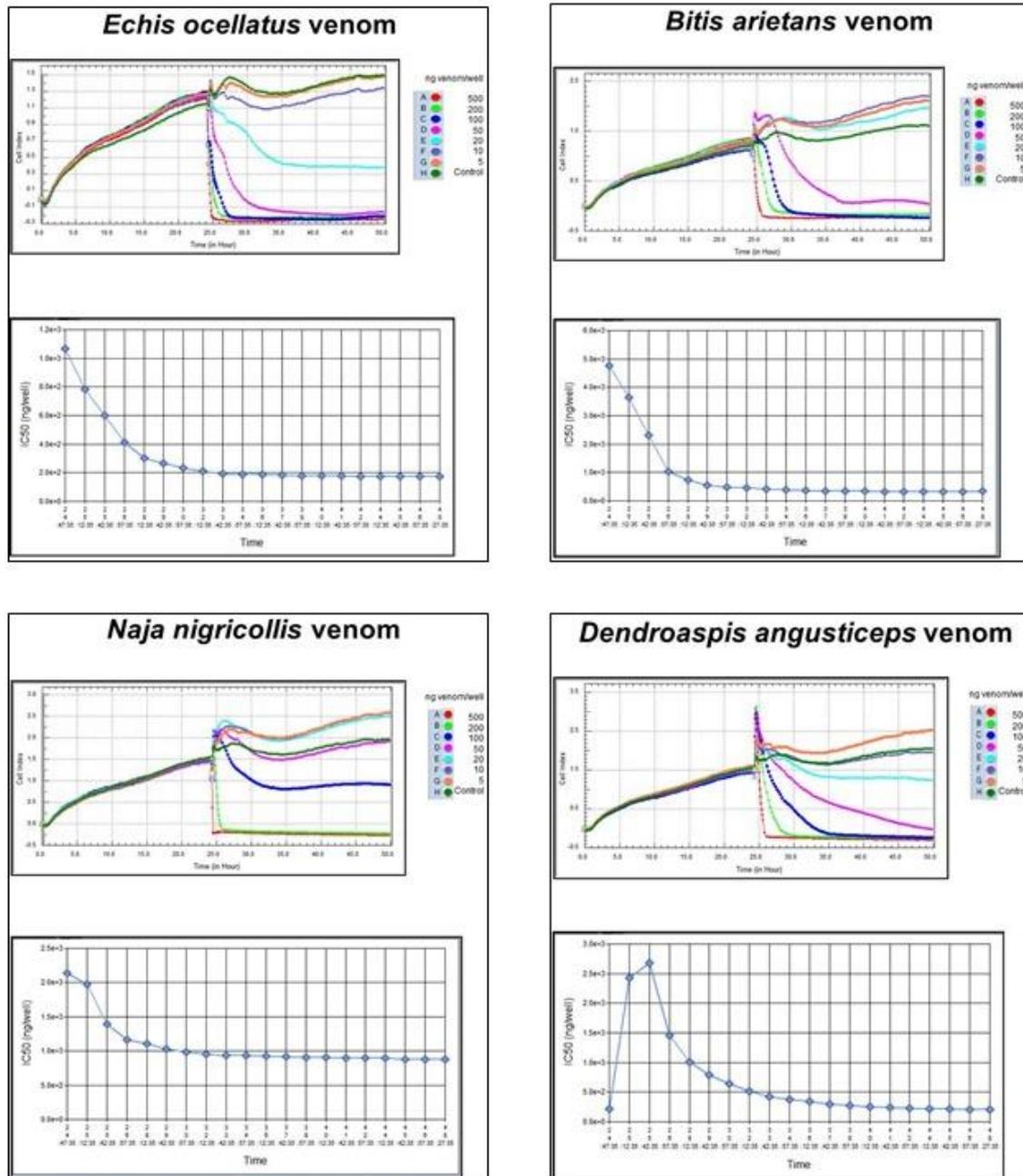


Figure 8.4-12 Venom LC₅₀ of *E. ocellatus*, *B. arietans*, *N. nigricollis* and *D. angusticeps* venoms. SHSY5Y: 3x10⁴cells/well; growth: 24hours; End-point: RT; Detection: xCELLigence. Plots showing venom CPE and LC₅₀ 0-24h after venom addition.

8.4.2.2 Venom cytotoxicity

In this set of experiments (Table 8.4-10), which were repeated (Table 8.4-11 and Figure 8.4-13), this cell line appears to be most sensitive to *D. angusticeps* venom (LC₅₀ of 75ng/well). The results appear to be highly variable, even 'within' assay, shown by the wide CI₉₅ bars in Figure 8.4-13

Table 8.4-10 Venom LC₅₀. SH SY5Ycells-3x10⁴/well; growth: 24h; End-point: 20h; detection: NR

Venom	LC ₅₀ (ng/well)
EoV	120
BaV	140
VbV	600
NnigV	600
DaV	75

Table 8.4-11: Venom LC₅₀. SH SY5Ycells-3x10⁴/well; growth: 24h, End-point: 20h; detection: NR.

Venom	LC ₅₀ (ng/well)
EoV	320
BaV	600
VbV	190
NnigV	500
DaV	180

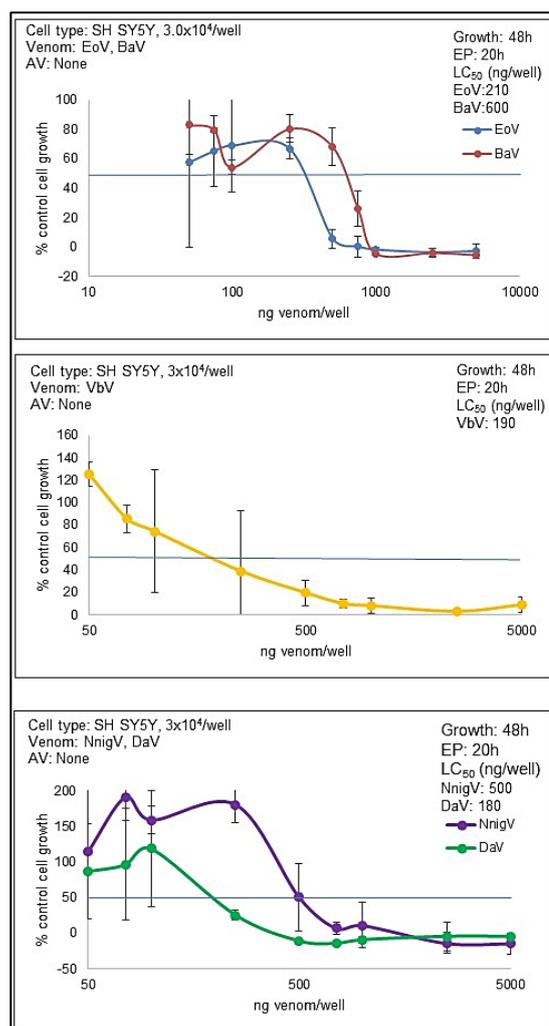


Figure 8.4-13: Venom LC₅₀. SH SY5Ycells-3x10⁴/well; growth: 24h, END-POINT: 20h; detection: NR.

8.4.2.3 Venom neutralisation

As with VERO cells, checkerboard assays were used to optimise venom concentration for the full assays (Table 8.4-12). Because of the wide variation in LC₅₀ it was difficult to pick a suitable venom concentration for neutralisation assays. Neutral red was often, but not consistently, poorly absorbed by the cells. Use of xCELLigence and visual inspection of plates before addition of NR showed that the cells were growing.

Table 8.4-12 Checkerboard AV EC₅₀ to optimise venom concentration and starting dilution of AV. SHSY5Y – 3x10⁴ cells/well; growth: 48h; End-point: 20h; Detection: NR. Selected venom concentrations in red

Venom	Antivenom	LC ₅₀ (ng/well)	x LC50	ng venom /well	EC50 dilution
<i>E. ocellatus</i>	EOG	120	5	600	18
			4	480	320
			3	360	1100
			2	240	2500
			1	120	>2500
			No venom	0	Control
<i>B. arietans</i>	ETP	140	5	700	180
			4	560	180
			3	420	220
			2	280	250
			1	140	>250
			No venom	0	Control
<i>V. berus</i>	VPV	600	5	3000	NE
			4	2400	NE
			3	1800	NE
			2	1200	NE
			1	600	120
			No venom	0	Control
<i>N nigricollis</i>	ETP	600	5	3000	7
			4	2400	8
			3	1800	14
			2	1200	190
			1	600	240
			No venom	0	Control
<i>D. angusticeps</i>	SAIMRp	120	5	600	>250
			4	480	>250
			3	360	>250
			2	240	>250
			1	120	>250
			No venom	0	Control

Table 8.4-13 shows results based on low absorbances. These results were consistent with visual inspection of the plates. There did not appear to be any neutralisation of the CPE of *V. berus* venom by ViperaVet in this cell line, and neutralisation of *B. arietans* venom by EchiTabPlus was only just over 50%. The other venoms were adequately neutralised by their antivenoms, but none achieved 100% of the control cell growth. The results are illustrated in Figure 8.4-14 and Figure 8.4-15.

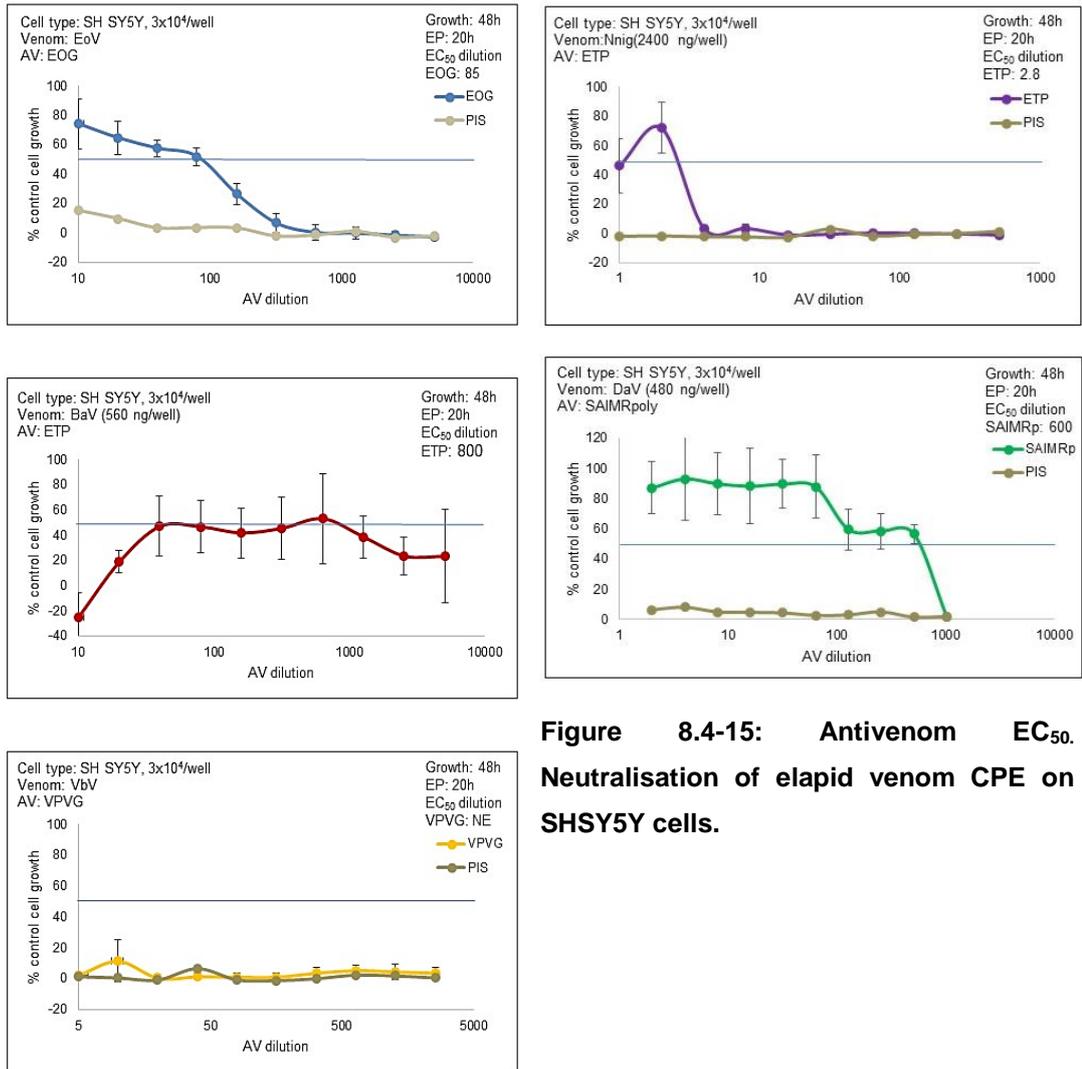


Figure 8.4-14: Antivenom EC₅₀. Neutralisation of viper venom CPE on SHSY5Y cells.

Figure 8.4-15: Antivenom EC₅₀. Neutralisation of elapid venom CPE on SHSY5Y cells.

Table 8.4-13 AV EC₅₀. SHSY5Ycells: 3x10⁴/well; growth: 24h; End-point: 20h; detection: NR.

Venom	Antivenom	LC ₅₀ (ng/well)	x LC ₅₀	Max neut %	EC ₅₀ dilution	EC ₅₀ (ng/well)	ng venom/well	AV/V (potency)
EoV	EOG	120	4	74.0	85	14705	480	30.6
BaV	ETP	140	4	53.0	800	1562	560	2.8
VbV	VPV	600	4	11.3	NE	NE	600	NE
NnigV	ETP	600	4	72.3	2.8	446428	24000	18.6
DaV	SAIMRp	120	4	93.1	600	275083	480	4.3

8.4.3 Co-culture venom cytotoxicity assays

Venom cytotoxicity experiments were set up so that half of a 96 well plate was seeded with SH SY5Y cells and the other half with VERO cells. The purpose

of this experiment was to establish if there was a difference in the CPE of venoms on the two cell lines, and if this difference reflected the toxin composition of the venom.

As well as the five venoms selected for this project other medically important venoms were tested. These included cobra (*Naja* spp.) venoms, some of which were from spitting cobras (*N. nigricollis* and *N. nubiae*) and others from cobras with purely neurotoxic venom (*N. nivea* and *N. haje*). The results are summarised in Figure 8.4-16 and Figure 8.4-17.

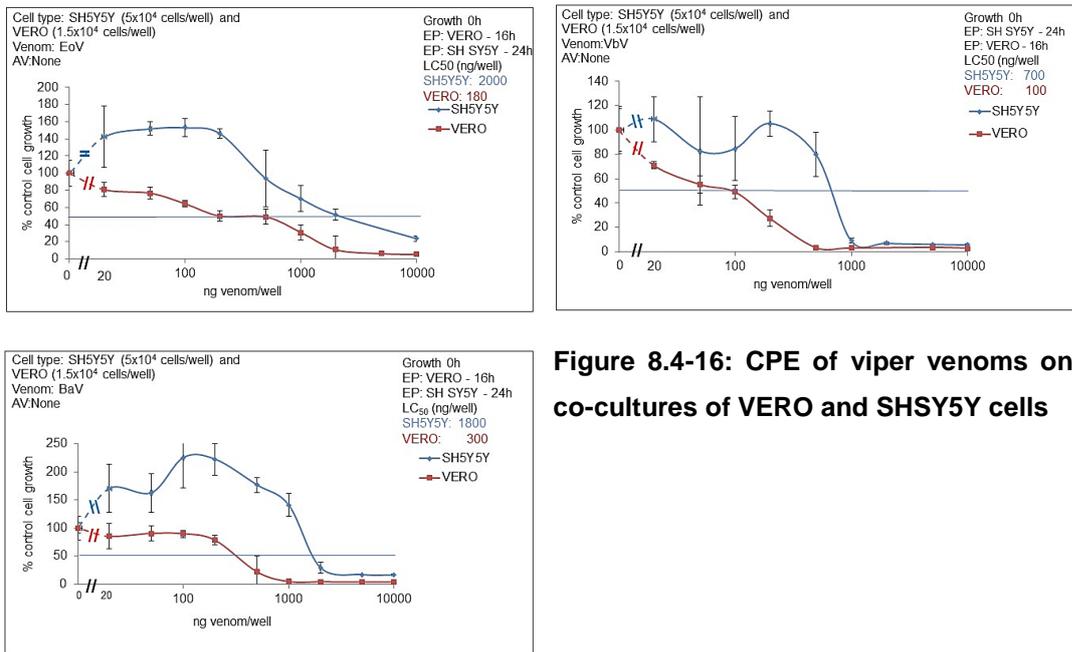


Figure 8.4-16: CPE of viper venoms on co-cultures of VERO and SH5Y5Y cells

Table 8.4-14: LC₅₀ of venoms added to a co-culture of VERO cells (1.5x10⁴ cells/well, growth 0h, End-point 16h) and SH SY5Y cells (5x10⁴ cells/well, growth 0h, End-point 24h). Figures in red are results from repeated experiments

Venom	LC ₅₀ (ng/well)	
	VERO	SH-5Y5Y
<i>Echis ocellatus</i>	180	2000
<i>Bitis arietans</i>	300	1800
<i>Cerastes cerastes</i>	<20	320
<i>Vipera berus</i>	100	700
<i>Naja nigricollis</i>	1200/1200	600/600
<i>Naja nubiae</i>	1000/1500	1100/750
<i>Naja haje</i>	800/1000	2700/1000
<i>Naja nivea</i>	3000/2000	3000/1000
<i>Dendroaspis angusticeps</i>	1100/1100	300/80

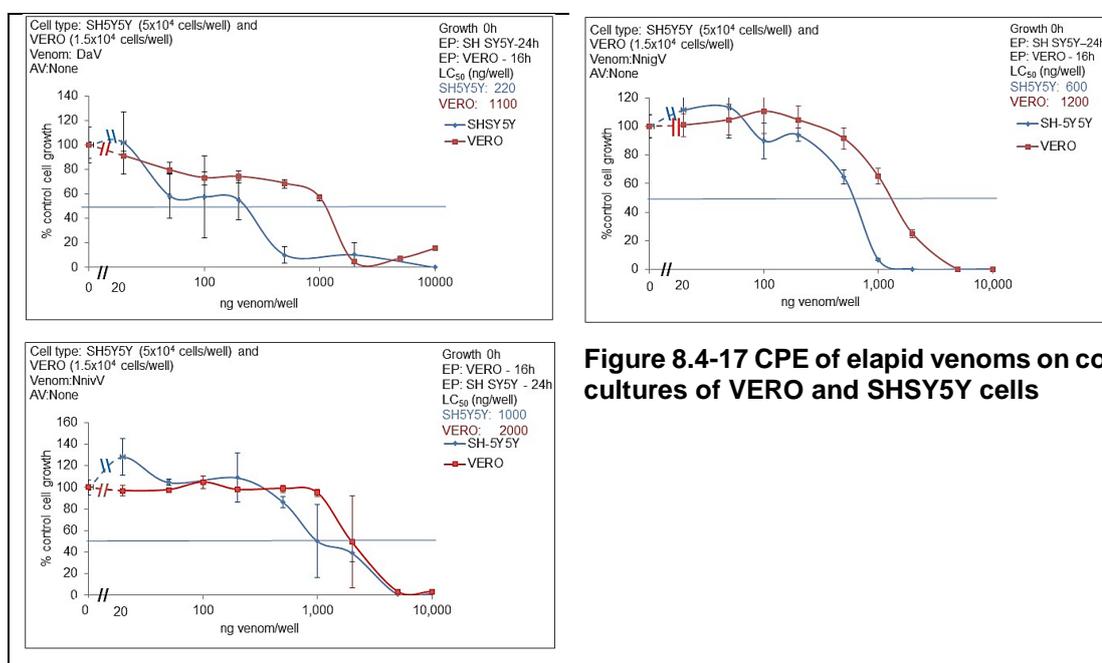


Figure 8.4-17 CPE of elapid venoms on co-cultures of VERO and SHSY5Y cells

The results were variable between assays, particularly those for the SH SY5Y cells. The VERO cells were more sensitive to the CPE of viper venoms than SH SY5Y cells. Generally the converse was true of elapid venoms, although there was little difference between the minimal CPE of neurotoxic cobra venoms on the two cell lines. A complete set of results can be found in the Chapter supplement (vcn39-43).

8.5 Venom-induced cellular overgrowth

During early experiments it was observed that the percentage control cell growth increased beyond 100% initially, with increasing venom concentrations, followed by the expected fall associated with CPE. Often there was a 'hump' or oscillation of the curve before the maximum slope (e.g. and Figure 8.4-17). To begin with, this was attributed to experimental error, but when the xCELLigence was first used the overgrowth of cells in low venom concentrations, compared to control cells grown in medium only, was confirmed. Figure 8.5-1 shows a *V. berus* venom (and *V. latastei* venom) LC₅₀, using neutral red to detect VERO cell viability after 16h. This experiment was run concurrently with an xCELLigence experiment, using the same cell suspension and venom dilutions. The plot showing real-time growth of cells is shown in Figure 8.5-2 The overgrowth occurred with both cell lines studied and with different venoms with or without their antivenoms.

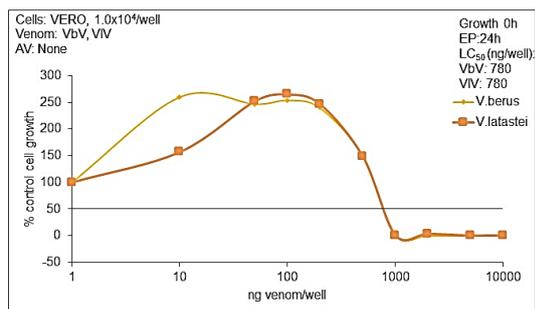


Figure 8.5-1: *Vipera berus* and *V. latastei* venom LC₅₀: VERO cells-1.5x10⁴/well; growth 0h, End-point: 16h; Detection: NR.

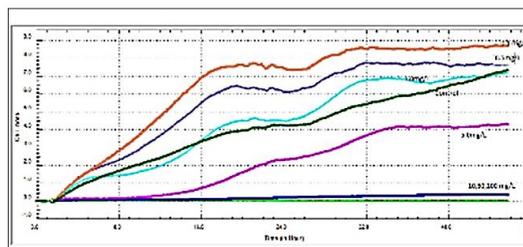


Figure 8.5-2: VbV LC₅₀: VERO cells-1.5x10⁴/well; growth 0h, End-point: RT; Detection: xCELLigence. Control cells in dark green. Venom concentration – cyan<purple<orange<cerise.

Figure 8.5-3 shows checkerboard antivenom EC₅₀s using SH-SY5Y cells grown for 48h with an end-point of 20h. Three of the four venoms studied showed maximum growth at twice their LC₅₀ dose. It is likely that the LC₅₀ selected for *D. angusticeps* venom was too high. The oscillation of the curve prior to its linear maximum slope is also illustrated.

The experiments which yielded the results illustrated here were performed for other reasons- these were merely incidental observations.

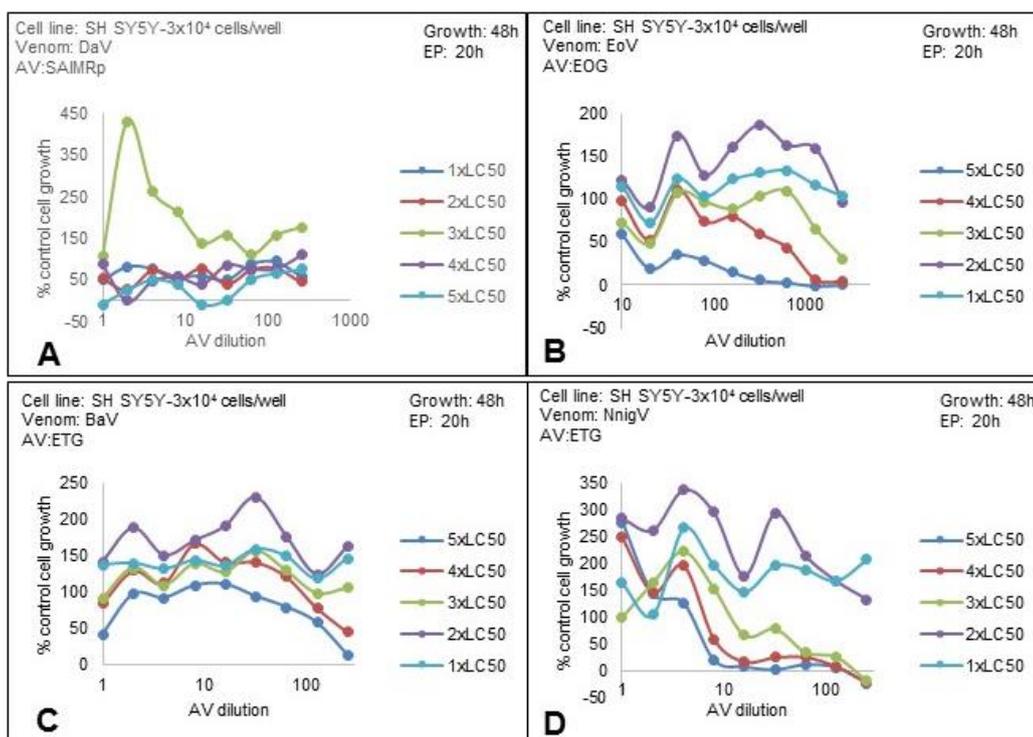


Figure 8.5-3: Checkerboard antivenom EC₅₀. SHSY5Y cells-3x10⁴/well; growth 48h, End-point: 20h; detection: NR

8.6 Discussion

There is considerable diversity in venom composition, each species having a unique profile of toxins, enzymes and non-toxic proteins. There is also variation in venom composition between individuals from different localities and within individuals according to diet and age (Currier, 2012; Fry et al, 2009; Nunez et al, 2009). Each toxin has a specific target organ or chemical reaction (e.g. activation of clotting factors) through which it exerts its pathological effect. To date, the best means to determine if treatment (antivenom) effectively neutralises venom toxicity is using an *in vivo* model (Theakston et al, 2003). Antibodies may bind to their target, but they may not necessarily neutralise its pathological effect (Cook et al, 2010). Cells are living entities, so by selecting a matrix of cell lines which are targeted by a venom's pathologically significant toxins, the hypothesis was that this could provide an *in vitro* alternative to *in vivo* testing of venom toxicity and antivenom efficacy. This is the first project to look at the cytotoxicity of such a diverse selection of snake venoms on multiple cell types. A number of studies have studied cytotoxicity of venoms on multiple cell types (Butron et al; 1993; Gowda et al 1993; Gremski et al,

2007) but this is thought to be the first study to investigate venom CPE using co-cultures of radically different cell lines.

Treating cells with toxins can result in a number of outcomes, including cytolysis either via direct action on the cell membrane or secondary to cell necrosis; reduction of cell viability due to disruption of mitosis or metabolic pathways; or activation of genetically controlled cell death mechanisms (apoptosis) (Riss et al, 2004). Sensitivity to a toxin is partially dependent on the stage of the cell cycle (Riss et al, 2004), which may explain the time-dependent variation in LC₅₀ noted, particularly in cells grown in venom. VERO cells would appear to be more sensitive to venom toxins during their most rapid (log-phase) growth. The two-phase response noted in the SH SY5Y cells could be explained by venom toxins causing immediate cell death by cytolysis, and other toxins switching on programmed cell death (apoptosis) via other mechanisms (Bradbury et al, 2000).

Using alternative detection systems such as measurement of lactate dehydrogenase (LDH) would differentiate between detachment and lysis of cells and there are kits available to detect apoptosis. However, these methods are relatively expensive compared to neutral red and were therefore not used, but would be worth further investigation, particularly as an end point for the SH SY5Y cells.

8.6.1 VERO cells

All the venoms that were studied had a lethal CPE on this cell line, but much higher doses of *N. nigricollis* venom were required to produce its CPE. The cells could either be grown in venom or venom added after 8-16h growth. Adding cells to the venom dilutions was easier than growing the cells to an 80-90% confluent monolayer before venom addition. However, variation of venom LC₅₀, at a given time point, was much greater in assays when cells were added to venom. For this reason it was difficult to select a suitable venom LC₅₀ for neutralisation assays – either the selected venom did not prevent cell

growth at all or there was no growth in any of the venom/antivenom treated wells.

The assay selected to demonstrate neutralisation of venom CPE used a seed rate that produced an 80-90% confluent monolayer after 16h to allow overnight growth prior to venom or venom/antivenom addition. An end-point of 4h was selected, and viable cells were measured using the neutral red assay. LC₅₀ was selected as the measure of venom lethality on which venom dose for neutralisation assays was based to reflect the method used in *in vivo* lethality assays. It may have been better to have calculated the LC₉₀ (90% cell death) as the dose for neutralisation assays.

There may be a number of reasons why there was such a large variation in neutralisation of venom CPE. Cells growing in flasks were harvested at different stages of growth for use in the assays, depending on availability. If possible, cells were harvested when the flasks were <100% confluent and therefore in log phase growth. Sometimes flasks were slightly overgrown and these cells took longer to grow than those harvested when in log phase growth. This may explain why there was more variation in assays where cells were added to venom – cells allowed to grow overnight had time to ‘switch on’ mitosis before venom was added. Venom is composed of multiple toxins and it may be that these toxins affect cells more or less at different phases of growth depending on their mechanism of action. Within and between assay variations were acceptable in experiments performed on the same day, using the same harvested cells, the same venom and AV dilutions. It may be that what is required is more rigorous control of cell growth, cell density and passage number – when using eternal cell lines, cell characteristics can change with increasing numbers of cell cycles, presumably due to mutations.

8.6.2 SH SY5Y cells

SH SY5Y cells are neuroblastoma cells, which are, amongst other properties, acetylcholinergic. They were sensitive to the CPE of viper venoms and to *D. angusticeps* venom, but less so to that of the cobra venoms. *D. angusticeps*

venom contains fasciculins, which are only found in mamba venoms and bind to muscarinic acetylcholine (ACh) receptors (Lauridsden, 2016). It would be interesting to see if these toxins bind to SH SY5Y ACh receptors to produce the observed cytopathic effect. Neurotoxic cobra venoms contain α -neurotoxins, which exert their effect by binding to the postsynaptic ACh receptors, blocking activation and muscle contraction by endogenous ACh. The mechanism of action of cobra venoms on these cells was not elucidated, but is unlikely to have involved ACh receptors. The CPE of all the cobra venoms observed, in both cell lines studied, was much less than that of the viper or *D. angusticeps* venoms. Similar concentrations of *Naja haje* venom were used in a study in which the mechanism of action of venom induced cell death was examined; necrosis was detected by measuring lactate dehydrogenase and creatine kinase, and apoptosis by measuring a fluorescent apoptosis detection system in myoblasts and human embryonic kidney cells (Omran et al, 2004). It was found that cell necrosis was acute, time and dose dependent, in both cell lines and that apoptosis may be dependent on concentration and/or time of exposure to snake venom (Omran et al, 2004)

The CPE of all venoms, apart from *V. berus* venom, were neutralised by their homologous antivenom. This would suggest that ViperaVet did not neutralise the *V. berus* venom toxin(s) responsible for cell death in this cell line. ViperaVet did neutralise the effect of *V. berus* venom in VERO cells, which would suggest that the mechanism of cell death in the two cell lines is different and/or caused by a different toxin. It is also possible to hypothesise that the toxin responsible for cell death of SH SY5Y cells is poorly immunogenic since it is not neutralised by its homologous antivenom, possibly a low MW toxin such as a PLA₂.

Using the xCELLigence system, it was observed that there was a dual phase of cell death – an immediate drop in cell index (CI) followed by a more prolonged effect. It is likely that the immediate effect is enzymatic (e.g. lysis of the cell membrane by PLA₂s) and the more prolonged effect non-enzymatic

(i.e. receptor-mediated). A dual phase of CPE was also observed using L929 (fibroblast) cells and *Vipera (Macrovipera) lebetina lebetina* venom. In this study, the cell medium was refreshed 2 hours after venom addition, then the cell viability examined after 48 hours, using MTT. This generated reproducible results which correlated with *in vivo* LD₅₀ (Nalbantsoy et al, 2012).

Results from these neuroblastoma cells were even more variable than for VERO cells, including within assay variation. One problem encountered was that the cells clumped, making performing an accurate cell count and subsequent homogenous seeding of plate wells difficult. Cells which had been grown sufficiently to differentiate were those which had the greatest tendency to clump. Discussion with other researchers who have worked with these cells confirms this observation. Cells which had differentiated were very slow to start dividing, but cells which were in log phase growth produced a sparse harvest which was insufficient for screening antivenoms. Viable cells had a poor uptake of neutral red dye, but the low absorbance results (% control cell growth) appeared to reflect their microscopic appearance.

8.6.3 Co-culture experiments

Generally VERO cells were more sensitive to viper venom toxicity and SH SY5Y cells more sensitive to elapid venom toxicity. Neurotoxic cobra venoms (*N. haje* and *N. nivea*) were not very toxic to either cell line. *N. nigricollis* venom was more toxic for SH SY5Y cells than for VERO cells, but had a relatively high LC₅₀ compared to *D. angusticeps* venom. Clinically, *N. nigricollis* venom is cytotoxic, causing tissue necrosis around the bite site, but this was not reflected in its CPE on either cell line studied.

8.6.4 Venom-induced cellular overgrowth

Many of the venoms studied promote cell growth in excess of control cells. This was also observed when venom was incubated with lower dilutions of antivenom (more antivenom/well) – effectively mimicking low venom concentration. In the checkerboard antivenom EC₅₀ experiments, the effect appeared to be maximal at 2x venom LC₅₀.

It was also noted that at low venom concentrations the growth curve oscillated before the linear fall in cell growth. Mathematically this reflects the conflict between stimulation of cell growth and lethal CPE of other venom toxins (Dr D R Bolton, personal communication).

Venoms frequently contain low levels of growth factors – vascular endothelial growth factor (VEGF) (Takahashi et al, 2004) and nerve growth factor (NGF) (Angeletti, 1970). It is possible that these are responsible for stimulation of cell growth, but other venom components could also play a part.

8.6.5 Implementation of cell-based assays into QC testing of AV

Assays for assessment of antivenom EC_{50} using VERO cells were optimised for quality assessment of two antivenoms, namely ViperaVet (directed against four *Vipera* spp., including *V. berus* venom) and EchiTabG (directed against *E. ocellatus* venom). However, the coefficient of variation of assays performed at different time points was in excess of 50% (results not shown), so the assays have not been adopted as part of routine quality control (QC) testing of these antivenom.

One study showed that when the medium on the cells was refreshed 2 hours after venom addition and cell viability examined after 48 hours reproducible results were generated, which correlated with *in vivo* LD_{50} results (Nalbantsoy et al, 2012).

8.7 Conclusions

All the venoms studied produced a cytopathic effect on the selected two cell lines, and showed selectivity that may be expected according to their toxin composition. However, the assays require further optimization to improve their repeatability before they could be recommended for use as part of QC testing of antivenoms. This would include optimisation of cell growth to minimise variation due to using cells at different stages of the cell cycle. It may be possible at this stage to use cell based assays to confirm neutralisation of lethal venom toxins and thus reduce the need for *in vivo* testing.

The neuroblastoma cells were too difficult to grow and handle for general use as a screen for antivenom efficacy, but have proved useful in differentiating between the effects of neurotoxic venoms and those which do not contain neurotoxins.

Cell based assays only respond to toxins with target receptors on the cell line used, or damage the cell membrane, leading to lysis and cell death (e.g. PLA₂s may hydrolyse the membrane phospholipids). Cell based assays are unable to detect the venom toxins which promote or inhibit enzymatic reactions occurring in extracellular compartments. Examples of such reactions include the clotting cascade, hydrolysis of vascular endothelial basement membranes or connective tissue components.

I believe that it is unlikely that, even using a matrix of cell types, an *in vivo* model could be completely replaced by cell-based assays but that they could be used to further reduce the need for *in vivo* testing by demonstrating neutralisation of the venom CPE.

Chapter 9. Discussion and Conclusions

9.1 Introduction

Snakebite poses a challenge as a tropical medical emergency because the treatment is complicated by the severe and diverse pathological lesions consequent to envenoming by snakes whose venom composition varies taxonomically, ontogenetically and geographically.

The present 'gold standard' tests of antivenom efficacy are the WHO-recommended LD₅₀ and ED₅₀ murine assays. Their duration is 24 to 48 hours, depending upon the route of administration, and the metric is death. These assays are of the highest severity in terms of pain, distress and harm inflicted on their subjects. In an age of increasing concern for animal welfare, it is imperative that methods of assessing antivenom efficacy are found which reduce the severity of these assays. It is equally imperative that '3R-modified' preclinical antivenom efficacy protocols remain sufficiently robust to detect ineffective antivenoms and avoid disasters such as those previously described (Visser et al, 2008; Alirol et al, 2015),

In that report, Visser described that their clinical success in managing snakebite victims using FAV Afrique antivenom declined dramatically when they switched to using Asna C antivenom, manufactured in India and that retailed at one tenth the price of FAV Afrique. Visser's team were alarmed at the increased case fatality (from 1.2% to 12.5%) of patients treated with Asna C, despite administering many more vials. The immunogen for the Indian Asna C was the Asian saw-scaled viper, *Echis carinatus*, and not the West African saw-scaled viper, *E. ocellatus*. Our own preclinical antivenom-efficacy analyses demonstrated that an antivenom manufactured for the African *E. ocellatus* saw-scaled viper was ineffective at neutralising the lethal effects of venom from the Asian saw-scaled viper *E. carinatus sochureki* (Casewell et al, 2010). This indicates that preclinical analysis would have likely identified the venom-neutralising inefficacy of Asna C and used as evidence to reject its use in human victims.

The work presented in this thesis has addressed the need to reduce pain and suffering inflicted on the experimental mice used to determine venom toxicity and antivenom efficacy by testing and implementing several 3R interventions into these assays.

To illustrate the 3R benefit of these modified preclinical antivenom efficacy protocols, I have devised a hypothetical 'test case' wherein antivenoms locally available in Chad are tested for their ability to neutralise the lethal effects of venoms from the country's most medically-important snakes. In this 'test case' I compare the hypothetical cost to mice of the current WHO and the '3R-modified LSTM' preclinical protocols.

9.2 Hypothetical testing of the efficacy of antivenom to treat envenoming in a sub-Saharan African country – Chad

9.2.1 Determination of venomous snake 'threat' in Chad

The most medically important snakes in Chad are described by the 'WHO' and 'Women's and Children's Hospital (WCH) Clinical Toxinology' websites²² as:

Vipers: *Bitis arietans* (Puff Adder), *Echis ocellatus* (West African saw-scaled or carpet viper) and *Cerastes cerastes* (Horned Viper),

Elapids: *Naja nigricollis* (black-necked spitting cobra), *Naja haje* (Egyptian cobra), *Naja pallida* (Red spitting cobra), *Naja nubiae* (nubian spitting cobra) and *Naja katiensis* (West African brown spitting cobra)

Colubrids: *Dispholidus typus* (Boomslang)

9.2.2 Hypothetical antivenoms available

Antivenoms likely to neutralise the 9 venoms listed above were identified by consulting the WHO, WCH toxinology and MAVIN¹ (Poisons centre, Munich) websites in Table 9.2-1.

²² Checked 02.02.2017

Table 9.2-1: Antivenoms available which are potentially effective against the snake venoms found in Chad. Numbers 1-6, in green, selected for further investigation

Venoms	Family	Description	1	2	3	4	5	6	7	8	9.
<i>E. ocellatus</i>	Vipers	Procoagulant	b	✓	✓	**	✓	a b*	✓	✓	
<i>B. arietans</i>		Haemorrhagic	a	✓	✓	✓	✓	a b	✓		b
<i>C. cerastes</i>		Haemorrhagic						b			b
<i>N. nigricollis</i>	Elapids	Cytotoxic spitters	a*	✓	✓	✓	✓	a b	✓		
<i>N. nubiae</i>			a*	*	*	*	*	b	*		
<i>N. pallida</i>			a*	*	*	*	*	a b	*		a
<i>N. katiensis</i>			a*	*	*	*	*	*	*		a
<i>N. haje</i>		Neurotoxic	a	✓	✓	✓		a b	✓		b
<i>D. typus</i>	Colubrid	Procoagulant	c								

Key:

- 1a. SAIMR polyvalent South African Vaccine Producers
- 1b. SAIMR Echis
- 1c. SAIMR boomslang
- 2. VINS, Central African antivenom VINS BioProducts Ltd, India
- 3. PSV, Central African Premium serum and Vaccines, India
- 4. Asna C antivenom Bharat Serums and vaccines Ltd
- 5. EchiTabPlus-ICP Instituto Clodomiro Picado, Costa Rica
- 6a. Inoserp sub-Saharan Inosan Biopharma, Spain
- 6b. Inoserp MENA
- 7. FAV Afrique Sanofi-Pasteur
- 8. EchiTabG MicroPharm Ltd
- 9a. NAVPC bivalent Naja/Walterinesia National Antivenom and Vaccine
- 9b. NAVPC polyvalent Production Centre, Saudi Arabia
- * Immunised with venom from same genus
- ** Immunised with Asian saw scaled viper, *E. carinatus*

From these antivenoms, those which are potentially effective at neutralising the effects of the most dangerous (WHO category 1) snakes found in Chad, namely *E. ocellatus*, *B. arietans*, *N. nigricollis* and *N. haje*, are selected. Hypothetically, SAIMR polyvalent/Echis, VINS central African, Serum Institute India central African, Asna antivenom C, EchiTabPlus-ICP and Inoserp sub-Saharan will be considered (Highlighted in green in Table 9.2-1).

9.2.3 Establishing *in vivo* venom toxicity (LD₅₀) for the most medically important snakes in Chad

The first stage of this hypothetical study is to characterise venom toxicity. Literature searching may provide LD₅₀ data for some species, but not for the Chad geographical variants. Since interspecific venom toxicity can vary by geographical origin of the snakes, it is vital that national venom standards are established as a priority (Harrison & Gutierrez, 2016).

The WHO-recommended tests of venom toxicity start with preliminary ‘range-finding’ assays with 2 mice/venom dose group (Meier et al, 1986). The LSTM protocol includes the use of analgesia (Chapter 5) and humane endpoints (HEPs) (Chapter 6), allowing a substantial reduction in assay duration from 24 to 7 hours. Post mortem examination, the pathology-survival score (PSS) (Chapter 7), in combination with HEPs and intensive pre mortem observations allows the use of ‘staging’ (Chapter 6), thus reducing the number of doses in the range finding assay from 10 to 6 and in the full LD₅₀ from 6 to 5, for each venom – a saving of 13 mice/venom and 941 ‘mouse hours’ (number of mice x duration of assay) of pain, distress and suffering (Table 9.2-2). Results shown in Chapters 5 and 6 demonstrate that these changes may be implemented without vitiating statistical validity.

Table 9.2-2: WHO tests of venom toxicity for the 9 hypothetical Chad venoms showing 3R interventions in WHO protocols in comparison to modified LSTM protocols. 3Rs = number of Refinement, Reduction or Replacement interventions; n = total number of mice used; x1000h - 1/1000 number of hours pain and suffering endured = n x duration of experiment (h).

	WHO protocol				3R-modified LSTM protocol			
	Intervention	3Rs	n	x1000 h	Intervention	3Rs	n	x1000 h
1a Venom toxicity estimation	Range finding LD ₅₀ 10 doses/venom n = 2/dose 9 venoms	1	180	4.32	Range finding LD ₅₀ +Analgesia; HEPs +Staging and PM examination 6 doses venom n = 2/dose 9 venoms	5	108	0.76
1b Venom toxicity measurement	LD ₅₀ 6 doses/venom n = 5/dose 9 venoms	0	270	6.48	LD ₅₀ +Analgesia; HEPs +Staging and PM examination 5 doses/venom n = 5/dose 9 venoms	4	225	1.57
OUTPUT	Toxicity measurement	1	450	10.80	Toxicity measurement +Pathology score/venom - live(HEPs) - PM examination +Pain reduction	9	333	2.33
Total Rs	Reduction x1				Refinement x4; Reduction x 5			

9.2.4 *In vitro* assessment of antivenom binding

Snake venom proteins include enzymes, toxins and non-toxic proteins, some of which act synergistically (Petras et al, 2016). When adding antivenom to this toxic mix, binding does not always equate to neutralisation of venom toxicity (Cook et al, 2010c), which gives rise to a conundrum when attempting to devise a ‘one-fits-all’ method to assess preclinical venom toxicity and antivenom efficacy. However, poor binding is synonymous with failure of neutralisation, and therefore binding assays are an important step in rejecting ineffective antivenoms prior to testing *in vivo* efficacy. In Chapter 4, a close correlation between *in vitro* binding by and *in vivo* efficacy of an antivenom is demonstrated, adding credence to the hypothesis that an antivenom which effectively neutralises venom pathogenesis will also bind well to that venom. Chapter 3 highlights the variation in interactions between venoms and antivenoms and that there is a poor correlation between *in vitro* assays when all venom/antivenom combinations were considered together, but that there was a more significant, but venom-specific correlation between these assays when each venom was considered separately.

In this test case, a routine ELISA was performed to assess the IgG titre of each antivenom to each snake venom. The IgG titre results of the SAIMR polyvalent were used to set the ‘approve/reject’ decision making for subsequent ED₅₀ assays of antivenom efficacy.

Table 9.2-3: WHO tests of venom/antivenom binding – these tests may be used on all possible antivenom candidates, here 6 antivenoms are selected for hypothetical testing.

	WHO protocol				3R-modified LSTM protocol			
	Intervention	3Rs	n	x1000h	Intervention	3Rs	n	x1000h
2 AV binding	ELISA (9x6)	1	0	0	ELISA (9x6) SSAC	1	0	0
OUTPUT	Rejection of AVs which fail to bind to all venoms	1	0	0	Rejection of AVs which fail to bind to all venoms	1	0	0
Total Rs	Reduction x1				Reduction x1			

In the test case, the ELISA results indicated that one of the six antivenoms showed considerably less binding to one or more of the 'WHO category 1' snakes and is therefore rejected, leaving 5 antivenoms to assess for '*in vivo*' potency (ED₅₀).

9.2.5 WHO tests of antivenom efficacy, ED₅₀

Range finding experiments are strongly recommended and, like the LD₅₀ experiments, using 2 animals per antivenom dose, thus reducing the number of mice required for the full ED₅₀. The WHO protocol instructs that 2x to 5x the venom LD₅₀ dose is used, where possible, the latter dose being preferable; the range finding experiment provides evidence that the selected venom dose is sufficient to kill all the mice but is not overwhelmingly lethal, even at the highest dose of antivenom.

As well as a reduction in the numbers of mice required for the full ED₅₀, the LSTM protocols include measures which result in a further reduction in numbers of mice required, as well as refinements which reduce suffering endured by the mice (Table 9.2-4), as detailed in Section 9.2.3. In this case, there is a total saving of 585 mice and a reduction of 40,010 'mouse hours' of pain and suffering endured.

Once the ED₅₀s have been completed, the most effective antivenoms can be selected for pre-clinical dose finding and safety studies (Abubakar, S.B et al, 2010) followed by a clinical trial (Abubakar I.S. et al, 2010).

Table 9.2-4: WHO tests of antivenom efficacy for 5 antivenoms at neutralising the lethality of 9 hypothetical Chad venoms showing 3R interventions in WHO protocols compared to modified LSTM protocols. 3Rs = number of Refinement, Reduction or Replacement interventions; n = total number of mice used; x10³h - 1/1000 number of ‘mouse hours’ of pain and suffering endured = n x duration of experiment (h).

	WHO protocol				3R-modified LSTM protocol			
	Intervention	3Rs	n	x10 ³ h	Intervention	3Rs	n	x10 ³ h
3a AV efficacy estimation	5x venom LD ₅₀ ED ₅₀ range finding 10 doses/AV n = 2/dose (20) 9 venoms (180) 5 antivenoms	1	900	21.60	5x venom LD ₅₀ ED ₅₀ range finding 6 doses/AV n = 2/dose (12) 9 venoms (108) 5 antivenoms + Analgesia + HEPs + Staging + PM	5	540	3.78
3b AV efficacy measurement	ED ₅₀ 6 doses/AV n = 5/dose (30) 9 venoms (270) 5 antivenoms	0	1350	32.40	ED ₅₀ 5 doses/AV n = 5/dose (25) 9 venoms (225) 5 antivenoms + Analgesia + HEPs + Staging + PM	4	1125	7.88
OUTPUT	Dose AV which neutralises each venom lethality	1	2250	54.00	Dose AV which neutralises each venom lethality + Reduction: venom-specific path + Pain reduction	9	1665	11.66
Total Rs	Reduction x1				Refinement x4; Reduction x 5			

The overall benefit of the 3R interventions used in this scenario are shown in Table 9.2-5 and illustrate that venom standards can be established at a much reduced cost to mice. This protocol has been used by LSTM to test efficacy of antivenoms against venoms from Kenya, the results of which are included in a publication in progress (Harrison et al).

Table 9.2-5: Benefits of 3R interventions on cost to mice using the modified LSTM protocol. 3Rs = number of Refinement, Reduction or Replacement interventions; n = total number of mice used; x10³h - 1/1000 number of ‘mouse hours’ of pain and suffering endured = n x duration of experiment (h).

	WHO protocol				3R-modified LSTM protocol			
	Intervention	3Rs	n	x10 ³ h	Intervention	3Rs	n	x10 ³ h
Total mice	Most effective AV for Chad identified	3	2700	64.7	Most effective AV for Chad identified +Analgesia +HEPs +Staging +PM	19	1998	13.99
Total Rs	Reduction x 3				Refinement x8; Reduction x 11			

9.2.6 Significance of Output of new LSTM ‘Gold Standard’ preclinical antivenom testing protocol

The approach illustrated in this hypothetical scenario would define the most preclinically effective available antivenom, providing the government with the critical data required to reject importation of ineffective antivenoms and therefore avoid situations such as that which occurred in Ghana (Visser et al, 2008).

Because this approach has identified the most effective antivenom for Chad, the LSTM ‘gold standard’ approach can be used to test all new antivenoms, at a much reduced consumption of and suffering in mice. A summary of the 3Rs output and savings in terms of percentage reductions, both in the use of and suffering endured by mice, of LSTM preclinical testing protocols is shown in Table 9.2-6.

The WHO seeks to establish venom standards from numerous regions in Africa (Harrison & Gutierrez, 2016). Using the modified LSTM 3Rs protocol, and assuming that venom standards are to be established in two countries in each of West, Central, East and South Africa and that there are 9 medically important snakes in each of these countries, a total of 92 venoms, this would equate to a reduction of 8,424 mice and 610 x 10³ ‘mouse hours’ of suffering, as well as the degree of suffering being reduced by the use of analgesia from

'severe' to 'moderate'. When antivenom efficacy for each of these countries is also assessed, as described, this equates to a further saving of 42,120 mice (a total of 50,544 mice) and 3,041 x 10³ 'mouse hours' of suffering (a total of 3,651 x 10³). This figure is in excess of the annual estimated usage (>30,000) of mice to routinely batch test existing antivenoms.

Table 9.2-6: Summary of the 3Rs output of preclinical testing of antivenoms for Chad, using the new LSTM 'gold standard' protocol. 3Rs = number of Refinement or Reduction interventions; Saving = % reduction compared to WHO protocol where n = number of mice used, h = mouse hours of suffering (=number of mice x duration of experiment)

	Intervention	Saving		
		3Rs	%n	%h
1a Venom toxicity measurement	Range finding LD ₅₀ +Analgesia; HEPs +Staging and PM examination 6 doses venom n = 2/dose 9 venoms	5	40	82.4
1b Venom toxicity measurement	LD ₅₀ +Analgesia; HEPs +Staging and PM examination 5 doses/venom n = 5/dose 9 venoms	4	17	75.8
OUTPUT	Toxicity measurement +Pathology score/venom - live (HEPs) - PM examination +Pain reduction	9	26	78.4
AV binding	ELISA (9x14) SSAC	1	100	100
OUTPUT	Rejection of AVs which fail to bind to all venoms		100	100
AV efficacy	5x venom LD ₅₀ + Analgesia ED ₅₀ range finding + HEPs 6 doses/antivenom + Staging n = 2/dose (12) + PM examination 9 venoms (108) 5 antivenoms	5	40	82.5
	ED ₅₀ + Analgesia 5 doses/antivenom + HEPs n = 5/dose (25) + Staging 9 venoms (225) + PM examination 5 antivenoms	4	17	75.8
OUTPUT	Dose AV which neutralises each venom lethality + Reduction: venom-specific path + Pain reduction	9	26	78.4
Total 'Rs'	Refinement x8; Reduction x 11			

Further reductions in the consumption of mice required to ensure that subsequent batches of the most effective antivenom retain their activity can be made by using a binding assay to monitor the quality. Any change in binding, antiserum source, manufacturing process would indicate the need for an *in vivo* ED₅₀ (Chapter 4).

9.3 Interventions used in the LSTM ‘Gold Standard’ preclinical antivenom testing protocol

The results presented in Chapter 5 would suggest that opiate analgesia may be incorporated into murine lethality testing of venom toxicity and antivenom efficacy. We have shown that morphine is effective at alleviating pain in these assays, and can be successfully administered, if required, as an oral ‘top up’ dose, without adversely affecting assay results. There are, however, important caveats to be considered:

- There is some evidence that there may be discrepancies in assay results for some venoms, and therefore the incorporation of analgesia into LD₅₀ and ED₅₀ protocols must be investigated and validated for each venom studied
- The use of analgesia in regulatory preclinical testing of antivenoms must be validated and new acceptance criteria established
- Morphine is not universally freely available for research purposes, therefore there is a need to investigate the use of other opiate analgesics such as Tramadol (Chacon et al, 2016)

This work has successfully established humane endpoints for assays involving the venoms and antivenoms studied, requiring skilled observation, an in depth knowledge and/or experience of the likely venom pathological lesions of the species being studied in the experimental subjects used (Chapter 6). Pilot studies should be used to link observations which would allow a humane endpoint and death of that individual to be linked in a small number of animals; post mortem pathological lesions would corroborate the validity of such an endpoint.

Observations defined to establish humane endpoints can also be used to predict survival of individuals in each dose group and therefore allow selection of the next most appropriate dose group to complete the LD₅₀ or ED₅₀ assay, once again reducing the number of mice used.

As discussed in Chapter 7, post mortem pathological lesions is a means of defining toxicity of each venom and is a means of ensuring that potentially lethal or harmful pathological lesions are neutralised.

Each *in vitro* method has a place in defining venom-antivenom interaction: Western blotting demonstrating which venom toxins are bound by antivenom; end-point or 50% binding ELISA for screening large numbers of samples; avidity ELISA identifying the strength of venom-antivenom binding; small scale affinity chromatography (SSAC) yielding a result directly comparable with the sample total protein concentration; the reverse of SSAC, antivenomics identifying which venom components fail to bind to antivenom.

Cytotoxicity assays were investigated as a possible replacement for *in vivo* assays (Chapter 8), the hypothesis being that cytotoxicity neutralisation is synonymous with neutralisation of the lethal effects of venom (Nalbantsoy et al, 2012). However, a number of problems were encountered: cell lines were selected to reflect venom toxin target tissues and, indeed, all five venoms (and many more – results not shown) studied produced a cytopathic effect (CPE) in both cell lines selected, and initial co-culture experiments demonstrated a predilection of neurotoxic venoms for neural cells and of viper venoms for epithelial-type cells. The cytopathic effect was very variable between experiments, making optimisation of neutralisation assays problematic. Variable results were compounded by the unexpected growth-promoting effects of low venom concentrations and by the ‘sticky’ nature of the neural cells used. In addition, the CPE of these simple cytotoxicity assays did not demonstrate the physiologically induced consequences of venom toxins on host systems such as the coagulation cascade, destruction of structural extracellular matrix proteins, or inflammatory mediator release. It was

concluded that, in their present form, cell-based assays cannot replicate the complexities of a living organism and the multiplicity of extracellular and intravascular cascade reactions which, once started, cannot be reversed by administration of antivenom. However, it is possible that future optimisation of cell-based assays could be used to substantially reduce the number of animals required for *in vivo* efficacy testing.

9.4 Reductions arising from post-mortem observations

The WHO guidelines state that venom toxicity and its neutralisation should extend beyond that of lethality to include local tissue necrosis, haemorrhage, clotting, defibrinogenation and myolysis. The use of additional animals to perform these assays could be obviated by the use of post mortem examinations and collection of relevant samples from mice used in lethality assays. Cardiac puncture immediately following euthanasia can be used to identify coagulopathies; a 20 minute whole blood clotting test should be performed, as a minimum, progressing to measurement of fibrin/fibrinogen, clotting factors and blood counts. Gross muscle damage can be identified and further characterised histologically. Similarly, renal or other organ biopsies may be examined histologically. These examinations could be further enhanced by location of venom or venom/antivenom complexes using secondary antibodies on tissue biopsies. Pathological examination has the potential to greatly enhance and maximise the information obtained from each experiment.

In conclusion, LSTM's Alistair Reid Venom Research (ARVRU) was instrumental in establishing a system for measuring venom toxicity and antivenom efficacy. This system was quickly adopted by the WHO and numerous national and regional pharmacopeias to ensure that only effective antivenoms are approved for treating human victims.

Cognisant of the cost to mice, ARVRU has a history of researching *in vitro* alternatives (Sells et al) to the *in vivo* assays. The results of this thesis illustrates ARVRU's continued commitment to maximising the important

outputs of preclinical analysis of antivenom efficacy whilst minimising the cost to mice.

Chapter 10. Further Work

10.1 Dissemination of 3Rs impact

The 3Rs benefit of the results presented here are clear, and their implementation by others desirable. To disseminate of the results of this work to improve the 3Rs uptake by others working in the field a series of publications is proposed, the subjects of which are listed below:

- Analgesia
- Other Refinements of LD₅₀/ED₅₀ protocols
- *In vitro* alternatives to reduce the need for *in vivo* assays
- Illustration of how the above output would impact on mouse requirement to test antivenoms for a new region such as Chad

Further awareness of these 3R interventions may be achieved by means of presentations at seminars and conferences.

10.2 Further analysis of samples

The second major area of work to be done is the further analysis of samples and data collected in addition to that presented here.

There is a library of fixed samples collected from mice subjected to a variety of venoms, the gross pathology of which is described in Chapter 7, which are available for histopathology. The use of special staining techniques, including labelled antibodies to venom, antivenom or complexes, or products of pathological processes would contribute greatly to the understanding of the pathogenesis of envenomation, and the pharmacokinetics of systemic envenoming and its treatment

The sheer volume of data generated during this project has been overwhelming and therefore there is scope for further analysis, particularly of data relating to the pathological profile of mice subjected to venom/antivenom compared to that of venom alone. Used in conjunction with antivenomics, this could lead to identification of relevant antivenom deficiencies, and ultimately to the production of more effective products.

10.3 Expansion of the data base

Expansion of the data base to include more venoms and venom/antivenom combinations to strengthen the validity of conclusions drawn from the presented results.

10.4 Cytotoxicity assays

Results of cytotoxicity assays showed an unacceptable variability as a means of replacing the present *in vivo* LD₅₀ and ED₅₀. However, this platform has the potential to reduce the need for *in vivo* testing, both for systemic and localised envenoming. One possibility that warrants further investigation is an assay to assess haemorrhage due to the destruction of basement membrane structural proteins using plate well inserts lined with a collagen membrane, having a similar structure to the blood vessel basement membrane, with or without endothelial cells. Indicator cells are grown in the well below the insert. The indicator cells are bathed in medium alone, and venom added to the medium of the insert. The cultures are incubated and the CPE of 'indicator cells' assessed – destruction of the *in vitro* blood vessel would indicate the haemorrhagic potential of the venom.

The use of 3D skin (Chapman et al, 2014) or corneal cells potentially provide a model for the investigation of localised envenoming. The 3D skin model may be used to assess damage to inter-cellular connections and the formation of vesicles. Corneal cells are presently being used to compare the cytotoxic effects of venom from spitting and non-spitting cobras (LSTM, work in progress).

10.5 Growth enhancing effect of venom

One finding in the cytotoxicity experiments which merits further investigation is that of venom-induced growth promotion. The presence of increased mitotic figures in the livers of mice, which had effectively been subjected to low doses of venom (venom plus antivenom), poses the question as to whether this latter observation is related to the former.

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APPENDIX I: EQUIPMENT

0.2µm syringe filters (Pall)
0.45µm syringe filters
0.8µm syringe filters
16-well xCELLigence view-plates
96 well dilution plates
96 well Falcon cell culture plates
96 well microtitre plates (Immulon 4BX)
Akta FPLC system with Superose 12 HR column
Analytical balances
Bio-Rad chromatography columns (10mL)
Bio-Rad gel system for SDS-PAGE (glass plates, frames, electrophoresis tanks)
Bio-Rad mini diafiltration system
Camera (Casio/EX-ZR100) with macro, rapid shot and video settings
Centrifuges
Citrate blood tubes (clotting factors)
Class II Safety cabinet
CO₂ anaesthesia/culling boxes
CO₂ incubator (Binder)
Cool cell
Counter
Cryoboxes
End over end mixer
Filter paper
Freezers: -80°C and -25°C
Fridge: 2-8°C
Haemocytometer
Hannah pH meter
Heat lamp
Inverted microscope
Liquid nitrogen vapour phase storage tank and dewars
Microscope slides

Microscope with oil immersion lens (x 1000)
MouseOx Plus pulse oximeter comprising collar, monitor and conscious monitoring software (Starr Life Sciences Corp/ Harvard Apparatus)
Nitrocellulose membrane
Omega Polestar microtitre plate reader
Optimax microtitre plate reader
Perspex box approximately 15 x 25cm with a long and a short side blacked out.
Plate shaker
Plate washer
Power pack
Quartz cuvette with 1cm path length
Restraint for mice whilst being injected
Rocker
Selection of laboratory glassware and disposables
Selection of pipettes (0.2 - 5000 μ L), including 12-channel pipette (50 - 300 μ L)
Serological pipettors
Spectrophotometer (Beckmann Du®)
T75 and T25 tissue culture flasks
Vision FPLC system with Superose 12 column.
xCELLigence system (Roche/ACEA) for real-time cell assays

APPENDIX II: REAGENTS

II.1. General reagents

II.1.1: Chemicals

Chemical	Formula	MW	Supplier
Water for Irrigation (sterile) (WFI)	H ₂ O	18.02	Baxter
Sodium chloride	NaCl	58.44	Merck
Sodium hydroxide	NaOH	40.00	Merck
Di-sodium hydrogen phosphate.anhydrous	Na ₂ HPO ₄	141.96	Merck
Sodium dihydrogen phosphate.monohydrate	NaH ₂ PO ₄ .H ₂ O	137.91	Merck
Tri-sodium citrate.dihydrate	Na ₃ C ₆ H ₅ O ₇ .2H ₂ O	294.10	Merck
Citric acid monohydrate	C ₆ H ₈ O ₇ .H ₂ O	210.1	Merck
Sodium acetate	CH ₃ COONa	82.03	Merck
Glacial acetic acid	CH ₃ COOH	60.05	Sigma
Hydrochloric acid	HCl	36.46	BDH
Tris base	C ₄ H ₁₁ NO ₃	121.1	Sigma
Glycine	NH ₂ CH ₂ COOH	75	Sigma
Sodium hydrogen carbonate	NaHCO ₃	84.01	Sigma
Sodium carbonate	Na ₂ CO ₃	105.99	Sigma
Sodium azide	NaN ₃	65.00	BDH
40% bis acrylamide	C ₇ H ₁₀ N ₂ O ₂	154.17	Sigma
Sodium dodecyl sulphate (SDS)	NaC ₁₂ H ₂₅ SO ₄	288.37	Sigma
Tetramethylethylenediamine TEMED	C ₆ H ₁₆ N ₂	116.20	Merck
Ammonium persulphate (APS)	(NH ₄) ₂ S ₂ O ₈	228.18	Sigma
Glycerol	C ₃ H ₈ O ₃	92.09	Sigma
Bromophenol Blue	C ₁₉ H ₁₀ Br ₄ O ₅ S	669.96	Sigma
Methanol	CH ₃ OH	32.04	Sigma
Ethanol	C ₂ H ₅ OH	46.07	Sigma
β-mercaptoethanol	C ₂ H ₆ SO	78.13	Sigma
3,3'-diaminobenzidine (DAB)	C ₁₂ H ₁₄ N ₄	214.27	Sigma
2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS)	C ₁₈ H ₁₈ N ₄ O ₆ S ₄	514.62	Sigma
Hydrogen peroxide 30% w/w	H ₂ O ₂	34.01	Sigma
Ethanolamine	NH ₂ CH ₂ CH ₂ OH	61.08	Sigma
Ammonium Thiocyanate	NH ₄ SCN	76.12	Sigma

Chemical	Formula	MW	Supplier
Tween 20	lauric acid, ≥40% (balance primarily myristic, palmitic, and stearic acids)	~1228	Sigma
Caprylic (Octanoic) Acid	C ₈ H ₁₆ O ₂	144.21	Merck
Neutral Red	C ₁₅ H ₁₇ ClN ₄	288.8	Sigma
Trypan Blue solution 0.4%	C ₃₄ H ₂₄ N ₆ Na ₄ O ₁₄ S ₄	960.8	Sigma
Ponceau stain	C ₂₂ H ₁₂ N ₄ Na ₄ O ₁₃ S ₄	760.57	Sigma

II.1.2: Compound reagents:

Reagent	Abbreviation	Formulation	Supplier
Dulbecco's Phosphate buffered saline (without calcium and magnesium ions)	PBSa	DPBS is a balanced salt solution (BSS) used for the handling and culturing of mammalian cells. Phosphate buffering maintains the pH in the physiological range.	Sigma (D8537)
Dulbecco's modified Eagles medium – high glucose	DMEM	DMEM is a modification of Basal Medium Eagle (BME) that contains a four-fold higher concentration of amino acids and vitamins, as well as additional supplementary components.	Sigma (D5671)
Dulbecco's modified Eagles/ Ham's F-12 medium	DMEM/Ham	Originally formulated for rat neuroblastoma cells and MDCK cells. The mixture is extremely nutritious and supports growth of a wide variety of cells including certain epithelial, endothelial and granulosa cells.	Sigma
HEPES buffer	HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid	Sigma (H3375)

Reagent	Abbreviation	Formulation	Supplier
Trypsin EDTA	T/EDTA	sterile-filtered, BioReagent, suitable for cell culture, 0.5 g porcine trypsin and 0.2 g EDTA • 4Na per liter of Hanks' Balanced Salt Solution with phenol red	Sigma (T3924)
Foetal Calf Serum	FCS (HI = heat at 56°C for 30 minutes to inactivate complement)	Certified foetal bovine serum, US origin	Invitrogen Gibco (16000044)
DMEM/10/HEPES	DMEM, FCS and HEPES	DMEM containing 10% FCS and 10% HEPES buffer	In house
L-Glutamine	L-Glu	L-glutamine 200mM, sterile filtered	Sigma (G7513)

Reagent	Abbreviation	Formulation	Supplier
Non-essential amino acids	NEAA	MEM Non-essential Amino Acid Solution (100x)	Sigma (M7145)
Skimmed Milk powder			Marvel
CNBr-activated Sepharose 4 Fast-flow gel			GE Healthcare
Formal saline		40% formaldehyde: 100 mL Sodium chloride: 9 g Deionised water: 900 mL	Sigma
Haematoxylin and Eosin stain	H&E	Haematoxylin (basophilic)-blue and eosin (acidophilic) - pink	
Diff Quik™		Romanowsky stain for blood films	Polysciences Inc.
Ponceau S stain	10mL WFI 0.3mL glacial acetic acid 0.033g Ponceau stain Make up to 30mL with WFI	Used for detection of proteins on cellulose acetate, PVDF, and nitrocellulose membranes.	In house

II.2: Buffers

Buffer	pH	Reagents (concentration: g/L)	Molarity (mMol)
0.9 % Sodium chloride		NaCl (9)	153
Phosphate Buffered Saline (PBS)	7.5	NaCl (9) Na ₂ HPO ₄ .anh (1.2) NaH ₂ PO ₄ .H ₂ O (0.2)	10 (NaPi) 153 NaCl
TBST	8.5	NaCl (8.76) Tris base (1.21) Tween 20 (0.1% v/v)	10 (Tris)
Tris buffer (2.0M)	8.5	Tris base (242.28) Fuming HCl as required	2000
Tris buffer (0.5M)	6.8	Tris base (60.57) Fuming HCl as required	500
TGS SDS running buffer		Tris base (3.02) Glycine (14.4) SDS (1.0)	210
Immunotransfer buffer		Tris base (3.0) Glycine (14.4) Methanol (40%v/v)	210
Coating buffer	9.6	NaHCO ₃ (6.3) Na ₂ CO ₃ (2.7)	100
Assay washing buffer (AWB)	7.5	NaCl (9) Na ₂ HPO ₄ .anh (1.2) NaH ₂ PO ₄ .H ₂ O (0.2) Tween 20 (0.1% v/v)	10 (NaPi) 153 (NaCl)
ELISA citrate buffer	5.0	Trisodium citrate (20.6) Fuming HCl as required	70
Sodium citrate buffered saline (SCS)	6.0	NaCl (9) Tri-sodium citrate.2H ₂ O (5.4) Citric acid. H ₂ O (0.35)	20
Sodium acetate buffered saline (ACS)	4.0	NaCl (9) Sodium acetate (0.33) Glacial acetic acid (0.96)	20
Phosphate washing buffer (PBW)	7.5	NaCl (29.2) Na ₂ HPO ₄ .anh (1.2) NaH ₂ PO ₄ .H ₂ O (0.2) Azide (0.1% w/v) for storage only	500mM NaCl 10mM NaPi
Glycine HCL	2.5	Glycine (7.5) Fuming HCl (3.3mL/L)	100
Coupling buffer	8.3	NaHCO ₃ (8.4) Na ₂ CO ₃ (0.1)	100
Swelling solution		Fuming HCL (86µL/L)	1
Ethanolamine		Ethanolamine (61)	1M

Buffer	pH	Reagents (concentration: g/L)	Molarity (mMol)
SDS Sample buffer (2x PLOB)		H ₂ O (3.55mL) Tris, pH6.8, 0.5M (1.25 mL) 20% Glycerol (2.5mL) 10% SDS (2.0mL) 0.02% Bromophenol blue (1.5mL of 5%)	
Coomassie Blue Destain		Methanol (450mL) Glacial acetic acid (100mL) Water to 1L	

II.3: Specific materials:

II.3.1: Venoms

Species	Origin	Source	Batch no:	Lot no:
<i>Vipera berus</i>	Europe	Latoxan/MicroPharm	PA480	041017
<i>Echis ocellatus</i>	Nigeria	LSTM	#1	#1
<i>Bitis arietans</i>	Nigeria	LSTM	#1	#3
<i>Naja nigricollis</i>	Nigeria	LSTM	#1	#1
<i>Dendroaspis angusticeps</i>	Tanzania	LSTM	Old stock	

II.3.2: Antivenoms

Antivenom	Manufacturer	Target	Donor Species	Formulation
ViperaVetG	MicroPharm	<i>Vipera berus</i> <i>V. ammodytes</i> <i>V. aspis</i> <i>V. latastei</i>	Sheep	IgG
EchiTabG	MicroPharm	<i>Echis ocellatus</i>	Sheep	IgG
SAIMR polyvalent	South African vaccine producers (SAVP)	<i>Bitis arietans</i> <i>B.gabonica</i> <i>Dendroaspis angusticeps</i> <i>D. jamesoni</i> <i>Hemacatus haemacatus</i> <i>Naja nivea</i> <i>N. melanoleuca</i> <i>N.haje annulifera</i> <i>N.mossambica</i>	Horse	F(ab') ₂
EchiTabPlus-ICP	Instituto Clodomiro Picado	<i>E. ocellatus</i> <i>B. arietans</i> <i>N. nigricollis</i>	Horse	IgG

Antivenom	Manufacturer	Target	Donor Species	Formulation
Australian-Papua New Guinea Polyvalent	CSL	Brown snake (<i>Pseudoechis australis</i>) Tiger snake (<i>Notechis scutalis</i>) Death adder (<i>Pseudonaja textalis</i>) Taipan (<i>Oxyuranus scutellatus</i>) Black snake (<i>Acanthophis antarcticus</i>)	Horse	IgG
Green Pit Viper	Thai Cross	Red <i>Trimesurus albolabris</i>	Horse	IgG
Banded Krait	Thai Cross	Red <i>Bungarus fasciatus</i>	Horse	IgG
King Cobra	Thai Cross	Red <i>Ophiophagus Hannah</i>	Horse	IgG

II.3.3: Antisera

Antisera	Species	Common name	Class	Source
Antivipera	<i>Vipera ammodytes</i> <i>Vipera aspis</i> <i>Vipera berus</i> <i>Vipera latastei</i>	Adders	Polyvalent	IgInnovations/ MicroPharm
Antiechis	<i>Echis ocellatus</i>	African saw-scaled viper	Monovalent	MicroPharm
Pre-Immune Serum (PIS)	Normal sheep serum			MicroPharm

II.3.4: Secondary antibodies

Source	Donor	Target species	Target molecule	Enzyme conjugate
MicroPharm	Donkey	Sheep	IgG (whole)	HRP
MicroPharm	Rabbit	Sheep	F(ab') ₂	HRP
Sigma	Rabbit	Horse	IgG(H+L)F(ab') ₂	HRP
Sigma	Donkey	Sheep	IgG(H+L)Fab	HRP
Sigma	Rabbit	Horse	IgG(whole) AP	HRP

HRP: Horseradish peroxidase

H+L: Heavy and light chain

AP: Affinity purified

II.3.5 Cell lines

Cell Line	Source	Morphology	Medium	Additives	Derivation
VERO	ECACC	Epithelial	DMEM/10	Glutamine	African green monkey kidney
SH-SY5Y	Ryan Cameron, University of Glasgow/ ECACC	Neural (dopaminergic)	DMEM/Ham/10	Glutamine, NEAAs	Human neuroblastoma

ECACC: European Collection of Authenticated Cell cultures

II.3.6: Analgesics

Drug	Class	Mode of Action
Buprenorphine 0.3mg/mL	Opiate	Partial mu agonist
Oramorph® 2mg/mL	Opiate	Oral morphine. Primarily mu agonist, but also κ- and δ- agonist.
Morphine Sulphate 10mg/mL for injection	Opiate	Primarily mu agonist, but also κ- and δ- agonist.

II.3.7: Mice

Male CD1 mice (Charles River) weighing 18-22g were housed in groups of five in climatically controlled cages. Water was filtered and a commercial diet available *ad lib*. Woodchip substrate and paper-strip bedding was used. Environmental enrichment was supplied in the form of a raised nest box and a red plastic dome. Mice were generally used from 24 hours to 2 weeks after

arrival in the facility. All mice were weighed and individually marked for easy identification prior to use.

II.3.8: Other

Promega broad range protein marker (SDS-PAGE) MWs: 10, 15, 25, 35, **50**, 75, 100, 150, 225 kDa (50kDa highlighted)

APPENDIX III

Production and Assessment of Ovine Antisera for the Manufacture of a Veterinary Adder Antivenom

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Abstract

Medically important venomous snakes in Western Europe are *Vipera ammodytes*, *V. aspis*, *V. berus* and *V. latastei*. Envenomation of dogs and other animals by these snakes receives limited attention despite the relative frequency and potential mortality and morbidity. This reflects, in part, the lack of a dedicated veterinary antivenom. Successful antivenoms are derived from antisera containing high levels of specific polyclonal antibodies that bind to, and neutralise, all the toxins present. This requires a careful choice of immunogen, animals and immunisation schedule. We detected proteomic variation in the venoms of *V. ammodytes*, *V. aspis*, *V. berus* and *V. latastei* by SDS-PAGE gel electrophoresis. Consequently, we used a mixture containing equal amounts of venom from these species to immunise a flock of sheep. We demonstrate that immunisation resulted in antisera containing high levels of specific antibodies directed against the majority of toxic components found in all four snake venoms using immunoblotting, enzyme-linked immunosorbent assay (ELISA) and small scale affinity chromatography assays. The latter shows that all 25 sheep responded quickly and maintained high levels of specific antibodies throughout the 2 year period of study. This ensures a consistent starting material for the manufacture of a reproducible veterinary antivenom, ViperaVet™. Our next objectives are to purify the antibodies from our antisera and demonstrate their pre-clinical neutralising efficacy in murine animal studies prior to undertaking a clinical trial in envenomed patients.

Introduction

The medically-important venomous European snakes belong predominantly to the genus *Vipera* (Serpentes: Viperidae) and are often referred to as adders. Whilst only *V. berus* is found in the United Kingdom and the Nordic countries, *V. aspis* and *V. ammodytes* have a wide distribution throughout Central and/or Western Europe (Persson, 1995; Garrigues, and others, 2005; World Health Organisation, 2010). An additional species, *V. latastei* is distributed throughout Spain and Portugal (Saz-Parkinson, and others, 2012). Whilst these snakes are relatively small in size they are of medical importance to both humans and animals due to their toxic venom, which exhibit proteolytic, fibrinolytic, anticoagulant and other effects (Ferquel, and others, 2007; Georgieva, and others, 2008; Sanz, and others, 2008). In the event of systemic envenoming, these venom toxins can induce severe hypotension, and myonecrotic, haemorrhagic, cardiotoxic and/or neurotoxic pathological lesions (Calderon, and others, 1993; Persson, 1995; Warrell, 2005; Ferquel, and others, 2007; Luksic, and others, 2008).

The incidence of human envenomation by European adders ranges from 4,000 to 20,000 cases per year with an estimated 50 – 130 deaths (Gonzalez, 1991; Kastaturiratne, and others, 2008; Chippaux, 2012). The incidence in animals is potentially thirty times higher (Mirtschin, and others, 1998) with a significantly higher mortality rate of 3.5 to 14% (Kangstrom, 1989; Aroch & Harrus, 1999; Segev, and others, 2004; Anlen, 2008). The primary victims are dogs, which are generally bitten on the face or legs (Aroch & Harrus, 1999; Campbell, 2000; Willey & Schaer, 2005; Lervic, and others, 2010; Sutton, and others, 2012). The UK Veterinary Poisons Information Service (VPIS) strongly recommends that veterinarians administer human antivenom to animals with systemic envenoming since no veterinary product is currently available (Campbell, 2000; Sutton, and others, 2011, Bates & Warrell, 2013).

The ideal antivenom, whether for humans or animals, should be safe, effective, exhibit batch reproducibility and be accessible and affordable. To address the need for a veterinary antivenom in Europe (Campbell, 2008), MicroPharm Ltd has begun the development of an ovine-derived product for canine use. A key requirement is the availability of large volumes of antisera which contain

substantial amounts of high avidity polyclonal antibodies that bind to and neutralise the main toxic components of the relevant venoms. To this end, sheep were immunised with a mixture comprising equal amounts of four *Vipera* snake venoms (*V. ammodytes*, *V. aspis*, *V. berus*, and *V. latastei*) to account for any potential inter-species variation in venom composition (Casewell, and others, 2009). In this paper we demonstrate that the ovine flock produced antisera which binds the major toxic components of all four venoms, and that the antisera contain high levels of specific antibodies from six weeks post-immunisation throughout the two year period of study. A large number of consistent pools of antisera have been collected from which an antivenom, ViperaVet™, may be manufactured.

Materials and Methods

Ethical approval

All work involving the use of live animals was carried out under licensed approval by the UK Home Office and approved by an internal ethical committee.

Immunisation

A flock of 25 crossbred sheep were immunised with a preparation containing a 1:1:1:1 (by weight) mixture of *V. ammodytes*, *V. aspis*, *V. berus* and *V. latastei* venoms (Latoxan, France). The lyophilised venoms were reconstituted to a concentration of 2g/L in 0.9% saline before being mechanically mixed at a ratio of 1:5 (v/v) with either Freund's complete (primary immunisation) or Freund's incomplete (all subsequent immunisations) adjuvant until a stable emulsion was formed. Each sheep was immunised with a total of 5mL of the solution (2mg venom/sheep) into six different sites by subcutaneous injection. The injection sites were chosen for their proximity to the axillary, inguinal and prescapular drainage lymph nodes. Immunisations were carried out every four weeks for a period of 108 weeks, and blood was collected two weeks after each immunisation, beginning six weeks after the primary immunisation.

Approximately 10mL of blood per kg body weight was collected via the jugular vein into a sterile, pyrogen-free 1L bag, which was weighed to allow the

volume of blood to be estimated. The bags were rotated for 30 minutes to accelerate clotting then centrifuged at 4733g for 20 minutes. The serum was removed, pooled, samples taken for assessment and then frozen for storage at -20°C.

Assessment of antisera

Aliquots of pooled antisera were assessed using the following methods:

SDS-PAGE and Immunoblotting

Lyophilised *V. ammodytes*, *V. aspis*, *V. berus* and *V. latastei* venoms used were reconstituted to 1g/L in a non-reduced protein loading buffer (PLOB). Then 10µg of each was subjected to non-reducing SDS-PAGE gel electrophoresis using a 15% gel with a 200V potential difference across the plate. The resultant proteins were visualised using Coomassie Blue R-250 stain and photographed using a BioRad gel imager.

To assess the binding specificity of antibodies to the different venom components, the four venoms were run separately by gel electrophoresis and the proteins electro-blotted onto a 0.45 µm nitrocellulose membrane (BioRad) using the manufacturer's protocol and a 100V potential difference applied across the plate; confirmation of transfer was visualised by reversible staining with Ponceau S. Subsequently the membrane was blocked by incubation overnight at 4°C using 5% non-fat milk in TBST (0.01M Tris-HCl, pH 8.5; 0.15M NaCl; 1% Tween 20). The membranes were then washed with TBST four times over 60 minutes and incubated for three hours with antisera, which had been diluted 1:2000 (v/v) in blocking buffer. Pre-immune serum (PIS) diluted 1:2000 (v/v) was used as a negative control. After incubation, the membranes were washed again with TBST and incubated for two hours with a 1:5000 (v/v) dilution of secondary antibody (donkey anti-sheep IgG conjugated to horseradish peroxidase, (Sigma)) in blocking buffer. After a final washing step with TBST, the bands were visualised by adding DAB substrate (50mg 3,3-diaminobenzidine, 100ml PBS and 0.024% hydrogen peroxide: (Sigma)).

Indirect ELISA

Ninety six well ELISA plates (Immulon 4BX, Thermo-Fisher) were coated with 2mg/L of *Vipera* venom (either a 1:1:1:1 mixture or individual venoms) in 0.1M carbonate buffer, pH 9.6 and incubated at 37°C for 2 hours and then at 4°C overnight. The plates were then washed three times with assay washing buffer (AWB - 0.01M sodium hydrogen phosphate, pH 7.5; 0.15M NaCl; 0.01% Thiomersol w/v and 0.1% v/v Tween 20). The antisera, a reference antisera sample (positive control) and PIS (negative control) were diluted 1:1000 (v/v) in AWB, added to the first row on the plate followed by subsequent serial doubling dilutions. The plates were then incubated at 37°C for one hour and washed with AWB. The secondary antibody (donkey anti-sheep IgG HRP) was diluted to 1:500 (v/v) in AWB and added to the plate before incubation at 37°C for one hour. Following washing the results were visualised by the addition of substrate (10mg o-phenylene diamine dihydrochloride (OPD) in 100mL citrate buffer - containing 0.015% hydrogen peroxide- at pH 5.0) and the optical density (OD) measured at 492nm using an Optimax micro-plate reader. The EC₅₀ is described as the dilution of antisera resulting in 50% of maximum binding. Inter-assay variation was compensated for by comparison to the reference antisera sample. The reference sample showed inter-assay variation with a mean EC₅₀ dilution of 43,000 (standard deviation (SD), ±22,000; coefficient of variation, 50%). This variation was compensated for by using a reference pool of antisera and adjusting the dilution titre of 50% maximum binding, using an EC₅₀ dilution titre of 40,000 for the reference sample and the formula:

$$S_c = (R_c \times S)/R$$

Where S_c is the corrected EC₅₀ sample dilution, R_c, the corrected EC₅₀ reference dilution (40,000), S is the measured EC₅₀ sample dilution and R the measured EC₅₀ reference dilution. *Small scale affinity purification (SSACSSAC)*

A technique based on affinity chromatography was used to measure the specific antibody concentration (Smith, and others, 1992; Casewell, and others, 2010). *Vipera* venom (5mg of either the 1:1:1:1 mixture or individual venoms) was first coupled to 1g of CNBr-activated 4 Fast Flow sepharose gel (BioRad, UK) and placed in a small chromatography column. The matrix was

washed with 10 column volumes (35mL) of PBW (0.01M sodium hydrogen phosphate; 0.5M NaCl, pH 7.5). One mL of antisera was added to the column, mixed with the gel and left to incubate at room temperature on an end over end mixer for 2 hours. The unbound fraction was subsequently washed off with 35mL of PBW. The bound antibodies were eluted with 20mL 0.1M glycine HCl, pH 2.5 and collected for the measurement of optical density (OD) at 280nm, using an ultra violet lamp. Finally, the column was re-equilibrated with 35mL of PBW and stored at 4°C until further use. The specific antibody concentration (SAbC) was calculated using the formula:

$$\text{SAbC} = (A \times V) / (S \times E)$$

Where A is the OD, V is the volume of the eluate, S is the sample volume and E is the extinction co-efficient for ovine IgG (1.5) (Curd, and others, 1971).

Results

A flock of 25 sheep was immunised four weekly, as described, for a period of two years. Blood was harvested two weeks after every immunisation, resulting in a total volume of more than 300L of antisera. The specific antibody content of each pool of serum, as well as samples from individual sheep at 6, 10, 18, 50, 74, 90 and 110 weeks post primary immunisation, were assessed by ELISA (from week 18) and SSAC.

The non-reduced SDS-PAGE venom profiles reveal inter-specific variation in both molecular mass and quantitative representation (intensity of staining) of the proteins present in the venom of the four *Vipera* species (Figure 1A). The immunoblot shows that the *Vipera* antisera binds to all the major fractions from all four species of snake (Figure 1B), but that normal sheep serum shows no detectable binding to any of the venom proteins (Figure 1C).

Assessment of antisera by ELISA showed that there was considerable variation between the antisera raised in individual sheep but the mean antibody levels remained surprisingly steady throughout the 110 weeks of the study (Figure 2). Prior to the final bleed, the five poorest responding sheep were removed from the flock, thus improving the specific antibody levels in the

resulting pooled serum, as evidenced by the rise in the final data point at 110 weeks (Figure 2). Importantly we demonstrate that the *Vipera* antisera exhibit equally high levels of binding to the four venoms and the 1:1:1:1 mixture (Table 1).

The mean specific antibody concentration (SAbC) of the *Vipera* antisera measured by SSACSSAC, was 6.2g/L, (SD, ± 0.47 g/L; coefficient of variation, $\leq 10\%$). The SAbC of individual sheep samples varied considerably over the study period, exhibiting a range of 1.82 -13.68 g/L. Despite this, the SAbC of the pooled antisera collected from the whole flock remained remarkably constant with a range of 4.02 - 7.81 g/L; (mean, 5.80 g/L; SD ± 1.12 g/L) (Figures 2 and 3). At 14 weeks after primary immunisation there were high levels of specific antibodies to all four individual *Vipera* venoms as well as to the mix of venoms.

Discussion

The four major medically-important snakes found throughout Western Europe belong to the genus *Vipera*, and include the only venomous snake found in the UK, the European adder (*V. berus*). Although mortality rates in both man and animals are low, envenoming by these snakes can result in significant morbidity and prolonged recovery. The British VPIS receive in excess of 100 snake-bite-related enquiries per annum (Sutton, and others, 2011). However, personal discussions with veterinarians suggest that the incidence of snakebite envenoming in the UK is likely to be much greater, (perhaps as high as eight times this figure). The only specific treatment for snake-bite envenomation is antivenom, but recently, the ability of UK veterinarians to purchase human European adder antivenom has become increasingly difficult, leading MicroPharm Ltd to decide to introduce a veterinary antivenom for the treatment of dogs envenomed by *Vipera spp.* throughout Europe.

As mentioned previously, the ideal antivenom should be safe, effective, stable, affordable and reproducible from batch to batch. The key to achieving these targets is the production of antisera containing high levels of specific

antibodies, which bind to and neutralise all the toxic components in the venoms of the target species of snake. The first critical step in the production of antisera is the choice of immunogen against which the antibodies will be directed. Venom composition varies between individual snakes, and also in the same individual depending on its age, diet and other environmental factors (Chippaux, and others, 1991). It is vital to use venom sourced from accurately identified snakes of medically-relevant species, captured from areas within which victims of envenomation are likely to require treatment. The husbandry and health of these individuals should be of a high standard to enable regular milking of venom, which may be pooled and lyophilised for antivenom production (World Health Organisation, 2010).

Horses have traditionally been the serum donor of choice due to the large volume of serum that can be obtained from an individual at any one time. However, in the UK sheep are generally used because they are widely available and relatively inexpensive to purchase and maintain, and their serum has a much less pronounced localised reaction at the injection sites than horses (Landon & Smith, 2003). Ovine immunoglobulin (IgG) is also less glycosylated than equine IgG and it has been postulated that this is therefore less likely to produce a hypersensitivity reaction in the recipient (Sjostrom, and others, 1994). In addition, sheep are a more acceptable choice in the UK where animal welfare is an emotive issue, especially concerning companion animals.

The results of our immunisation strategy, which incorporates a methodology refined over many years of antivenom production to stimulate maximum antibody responses (Landon & Smith, 2003), are illustrated by the results of our immunological assays. Immunoblotting (Figure 1) demonstrated that all major toxic proteins in the four snake venoms used to immunise the flock of sheep were bound by the resultant antisera, but not by normal sheep serum. This result was reflected in the high level of binding measured by ELISA and of specific antibody concentrations measured by SSAC in both individual

venoms and the mixture (Table 1). Moreover, the level of binding shown by all three assays was comparable across the four species of venom.

We were also able to demonstrate that, despite considerable variation in the specific antibody levels of individual sheep, the pooled flock's serum remained remarkably constant (Figure 2). One experiment showed that serum pooled from any ten sheep in the flock had a similar SAbC to that of the whole flock (data not shown), indicating that using multiple individuals has a buffering effect on individual fluctuations of antibody production and thus provides a consistent starting material for antivenom manufacture. This is an additional benefit to utilising ovines for antisera production – typically fewer animals are immunised during the production of equine antivenoms so that any individual variation in antibody response is likely to be more pronounced.

Importantly, the results of our SSAC assay with *Vipera* antisera are comparable to those of highly efficacious and safe human antivenoms. A flock of sheep immunised with *V. berus* venom had a pooled serum SAbC which ranged from 7.6 – 15.6 g/L over a period of 54 weeks (Smith, and others, 1992). The resultant affinity-purified antivenom manufactured from this antiserum for human use showed highly satisfactory results in preclinical *in vivo* murine ED₅₀ assays (Smith, and others, 1992) and clinical trials showed that this antivenom was safe and effective in counteracting the effects of *V. berus* envenoming in human victims (Karlson-Stiber, 1997). Antisera from sheep immunised with *Echis ocellatus* (African Saw-scaled viper) venom showed a similar rapid response, demonstrated by ELISA and SSAC. After 6 weeks the pooled antisera from the flock showed a consistently high SAbC, ranging from 11-15g/L, over the 16 month period of study (Laing, and others, 1995). A later study showed that preclinical testing of purified IgG from this antisera neutralised the toxic effects of *E. ocellatus* venom in mice (Casewell, and others, 2010) and subsequent clinical trials confirmed its safety and efficacy in human victims (Abubakar, and others, 2010). The successful translation of these antisera into safe and effective antivenoms would predict that our antisera shows great promise as a starting material for an antivenom which is both safe and effective. However, it is imperative that the pre-clinical

efficacy of an antivenom is demonstrated prior to use in clinical patients, as successful immunological cross-reactivity does not necessarily equate to neutralisation of the pathological effects of venom *in vivo*. At present the 'gold standard' test of antivenom efficacy is the World Health Organisation approved murine 'effective dose 50' (ED₅₀) assay. Therefore prior to marketing approval, the safety and efficacy of ViperaVet must be demonstrated in a clinical trial. Consequently, the next stage of development for this antivenom is to demonstrate that these encouraging immunoassay results translate into neutralisation of the pathological effects of envenomation in *in vivo* murine ED₅₀ assays, prior to embarking on canine clinical trials.

Our results show that the antisera produced here contains consistently high levels of specific antibodies directed against the majority of toxic components present in the four venoms used to immunise a flock of sheep, suggesting that it can be used for the manufacture of an antivenom directed against the venom of European adders. The collection of large volumes of high quality antisera, which is stable at -20°C for at least 13 years (MicroPharm, unpublished data), provides an excellent basis for the future manufacture of an antivenom that is reproducible from batch to batch. In the near future we will provide such an antivenom, which we have named ViperaVet™, for use to treat canine victims that have been envenomed by European adders.

Acknowledgements:

MicroPharm acknowledges the kind collaboration of the Alistair Reid Venom Research Unit (ARVRU) in this study- FB is a PhD student and NRC an Associate Researcher of the ARVRU at the Liverpool School of Tropical Medicine.

MicroPharm would like to thank Robert Price-Jones, MRCVS for his care of the Vipera flock and his invaluable contribution to the development of ViperaVet™.

This work was privately funded by MicroPharm Ltd.

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Tables

Table 1: Binding of *Vipera* antisera to either individual venoms or a 1:1:1:1 mixture used for immunisation as measured by ELISA or SSAC.

Venom	ELISA EC ₅₀ ⁱ	SAbC by SSAC (g/L)
<i>V. ammodytes</i>	45,000	4.18
<i>V. aspis</i>	50,000	4.47
<i>V. berus</i>	55,000	4.93
<i>V. latastei</i>	52,000	4.88
1:1:1:1 mixture	45,000	5.13

¹ EC₅₀ is the dilution of antiserum which results in 50% maximum binding

Figures

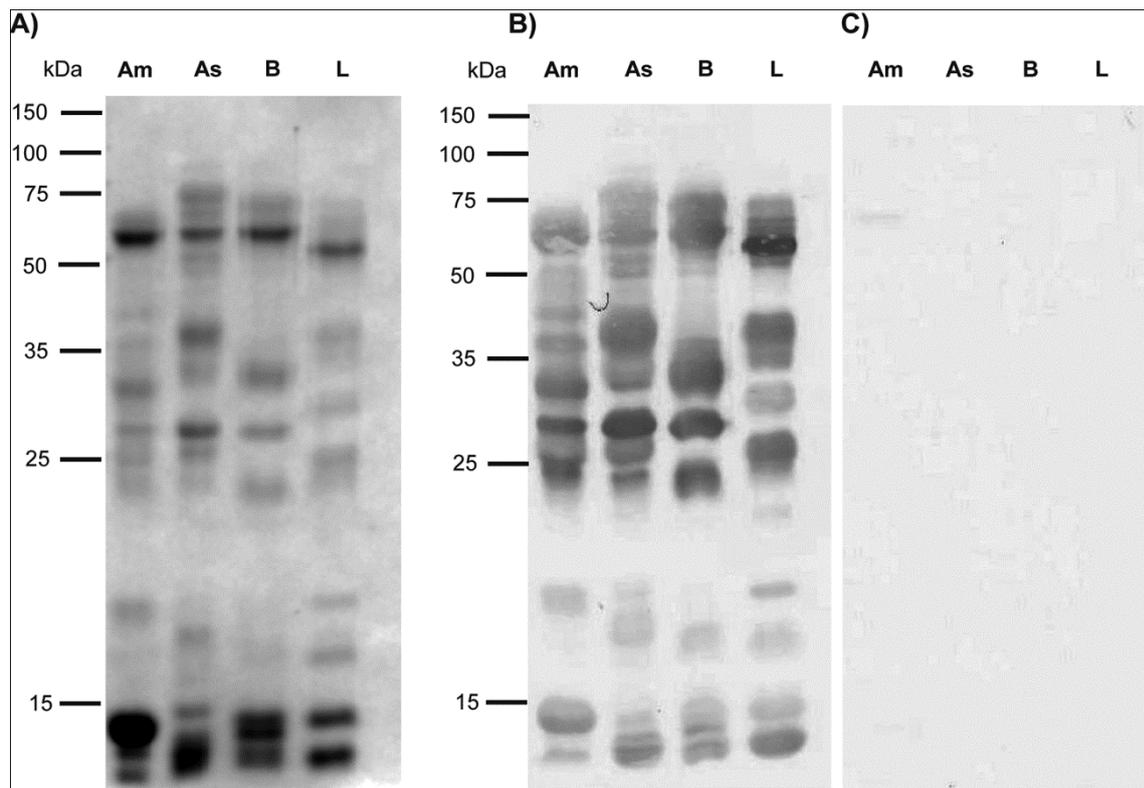


Figure 10.5-1: *Vipera* antisera exhibits binding to the majority of proteins present in *Vipera* venom. The venom proteins of *V. ammodytes* (Am), *V. aspis* (As), *V. berus* (B) and *V. latastei* (L) visualised using non-reduced SDS-PAGE (15% acrylamide gel) (A), after immunoblotting with a 1:2000 dilution of *Vipera* antisera (B) and normal sheep serum (C). Molecular weights were visualised using a protein marker.

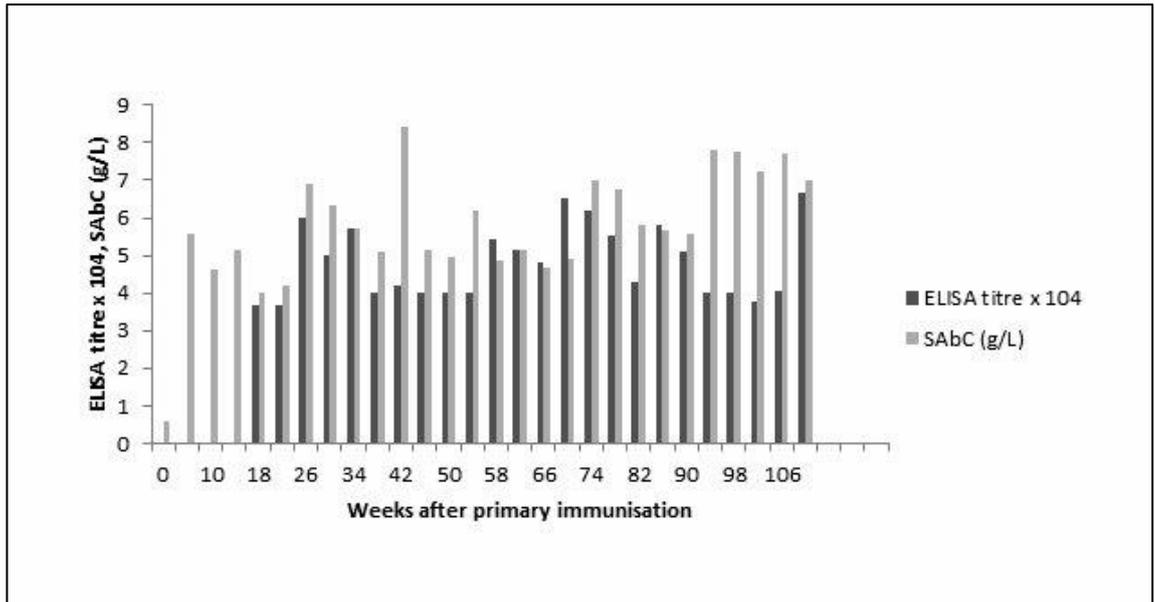


Figure 2: Time course ELISA EC₅₀ titres and SAbC by SSAC of *Vipera* antisera raised in a flock of sheep immunised over 110 weeks.

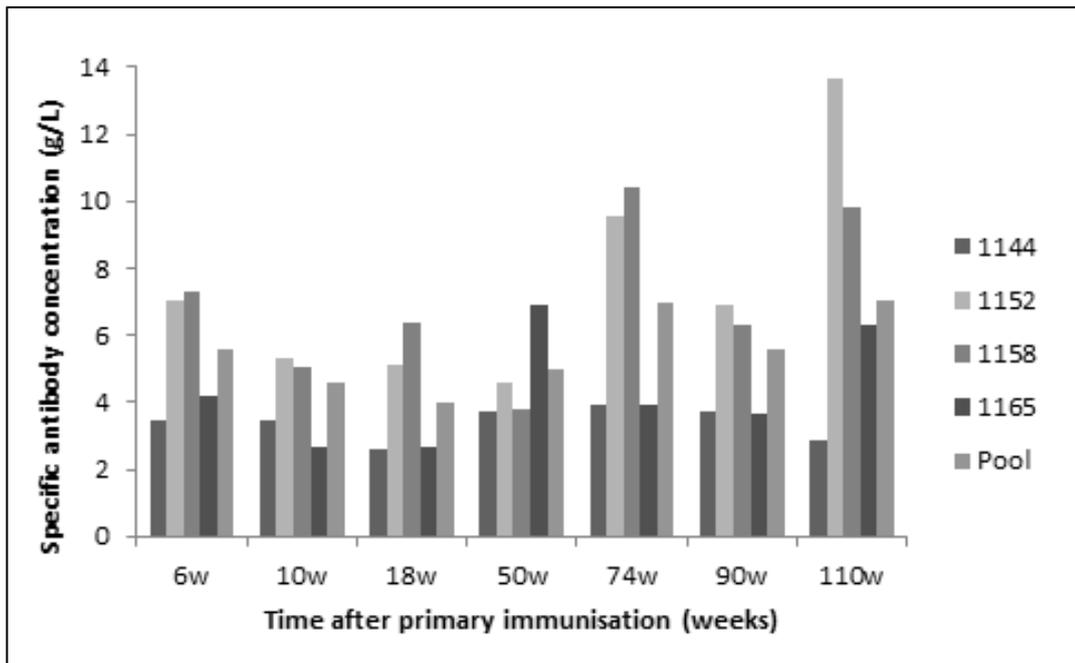


Figure 3: Variation in specific antibody concentration by SSAC over time, in antisera from four individual sheep and the pool of 25 sheep.

APPENDIX IV: *In Vivo* Experimental Summary

Table IV-1: Experimental summary of experiments included in Chapter 7 (Pathology) results – Commercial venom and antivenom testing experiments.

Venom	Dose (µg/mouse)	Antivenom	Dose µL/mouse	Analgesic	Dose	Number of mice
VbV	2	None	N/A	None	N/A	10
	4					10
	5					15
	6					20
	8					15
	10					15
	12					5
	15					10
	25					5
VbV	2	None	N/A	Morphine	5mg/kg ip 10mg/kgpo	5
	5					5
	6					5
	7					5
	10					5
VbV	36.5	Zagreb	7.5	None	N/A	5
			10			6
			15			6
			20			6
			25			5
			30			6
EoV	28	None	N/A	None	N/A	5
	56					5
EoV	70	EchiTabG	20	None	N/A	5
			30			5
			40			10
			50			5
			55			5
			60			5
			65			5
			70			5
VbV	36.5	ViperaTab	3	None	10	10
			4			10
			5			10
			6			10
			7			5
			8			5
			VbV			36.5
13	5					
15	5					
17	5					
20	5					
25	5					

Table IV-1 continued

Venom	Dose (µg/mouse)	Antivenom	Dose µL/mouse	Analgesic	Dose	Number of mice
VbV	79	ViperaTab	4	None	10	10
			5			5
			7			10
			8			5
			10			10
			12			5
			12.5			5
			13.5			5
			14			5
			16			5
			20			5
VbV	27	ViperaTab	2	None	N/A	15
			3			15
			4			15
			5			10
			6			5
			10			10
VbV	35	ViperaTab	3	None	N/A	5
			6			10
			8			5
			10			5
			15			5
VbV	28.5	ViperaTab	3	None	N/A	5
			5			5
			7			5
			10			5
			15			5
VbV	28.5	ViperaTab	1	Morphine	5mg/kg ip 10mg/kgpo	5
			3			10
			3.5			10
			5			10
			7			5
			10			10

Table IV-2: Experimental summary of experiments included in Chapter 7 (Pathology) results – Analgesia experiments (Chapter 5)

Venom	Dose (µg/mouse)	Antivenom	Dose µL/mouse	Analgesic	Dose	Number of mice
EoV	11.4	None	N/A	None	N/A	10
EoV	11.4	None	N/A	Buprenorphine	1.5mg/kg	10
EplV	13.6	None	N/A	None	N/A	5
EplV	13.6	None	N/A	Buprenorphine	1.5mg/kg	5
EcolV	9.8	None	N/A	None	N/A	5
EcolV	9.8	None	N/A	Buprenorphine	1.5mg/kg	5
EcsV	15.1	None	N/A	None	N/A	5
EcsV	15.1	None	N/A	Buprenorphine	1.5mg/kg	5
VbV	7.3	None	N/A	None	N/A	5
VbV	7.3	None	N/A	Buprenorphine	1.5mg/kg	5
VbV	6.5	None	N/A	None	N/A	10

Venom	Dose (µg/mouse)	Antivenom	Dose µL/mouse	Analgesic	Dose	Number of mice
VbV	6.5	None	N/A	Buprenorphine	1.5mg/kg	10
VbV	5.0	None	N/A	None	N/A	5
VbV	5.0	None	N/A	Buprenorphine	1.5mg/kg	5
VbV	3.0	None	N/A	None	N/A	5
VbV	3.0	None	N/A	Buprenorphine	1.5mg/kg	5
BaV	34	None	N/A	None	N/A	10
BaV	34	None	N/A	Buprenorphine	1.5mg/kg	10
NnigV	18	None	N/A	None	N/A	10
NnigV	18	None	N/A	Buprenorphine	1.5mg/kg	10
NnubV	10	None	N/A	None	N/A	10
NnubV	10	None	N/A	Buprenorphine	1.5mg/kg	10
DaV	26	None	N/A	None	N/A	10
DaV	26	None	N/A	Buprenorphine	1.5mg/kg	10
EoV	21	None	N/A	Buprenorphine	1.5mg/kg	7
EoV	21	None	N/A	Buprenorphine	1.0mg/kg	7
EoV	21	None	N/A	None	N/A	7
BaV	25	None	N/A	Buprenorphine	1.5mg/kg	7
BaV	25	None	N/A	Buprenorphine	1.0mg/kg	7
BaV	25	None	N/A	None	N/A	7
Control	None	None	N/A	None	N/A	3
CrV	1.65	None	N/A	Buprenorphine	1.5mg/kg	5
CrV	0.83	None	N/A	None	N/A	5
VbV	79	ViperaTab	16	Buprenorphine	1.5mg/kg	10
				None	N/A	10
BaV	4.25	None	N/A	Buprenorphine	1.5mg/kg	5
				Oral morphine	10mg/kg	20
BaV	2.12	None	N/A	None	N/A	20
				Oral morphine	10mg/kg	10
VbV	4.1	None	N/A	None	N/A	10
				Oral morphine	10mg/kg	30
EoV	14	None	N/A	None	N/A	20
				Oral morphine	10mg/kg	20
EoV	12	None	N/A	Oral morphine	10mg/kg	10
				None	N/A	10
NnigV	13.5	None	N/A	Oral morphine	10mg/kg	10
				None	N/A	10
DaV	19.5	None	N/A	Oral morphine	10mg/kg	30
				None	N/A	30
VbV	27	ViperaTab	4	Oral morphine	10mg/kg	20
				None	N/A	20
EoV	60	EchiTabG	50	Oral morphine	10mg/kg	30
				None	N/A	30
BaV	22.5	EchiTab Plus- ICP	5.5	Oral morphine	10mg/kg	10
				None	N/A	10
NnigV	18	EchiTab Plus- ICP	146	Oral morphine	10mg/kg	10
				None	N/A	10
DaV	65	SAIMRp	150	Oral morphine	10mg/kg	30
				None	N/A	30
DaV	95	SAIMRp	6	Oral morphine	10mg/kg	5
				None	N/A	5
DaV	95	SAIMRp	12	Oral morphine	10mg/kg	5
				None	N/A	5
DaV	95	SAIMRp	18	Oral morphine	10mg/kg	5
				None	N/A	5

Venom	Dose (µg/mouse)	Antivenom	Dose µL/mouse	Analgesic	Dose	Number of mice
DaV	95	SAIMRp	30	Oral morphine	10mg/kg	5
				None	N/A	5
NnigV	13.5	None	N/A	Oral morphine	10mg/kg	10
Control	N/A	None	N/A	Oral morphine	10mg/kg	10
NnigV	13.5	None	N/A	Oral morphine	15mg/kg	10
Control	N/A	None	N/A	Oral morphine	15mg/kg	10

Table IV-3: Experimental summary of experiments included in Chapter 7 (Pathology) results –

Venom	Dose (µg/mouse)	Antivenom	Dose µL/mouse	Analgesic	Dose	Number of mice
VbV	18.2	ViperaVet	44	None	N/A	10
VbV	18.2	Vipera antiserum-High	44	None	N/A	10
VbV	18.2	Vipera antiserum Medium	44	None	N/A	10
VbV	18.2	Vipera antiserum Low	44	None	N/A	10
CcV	2	None	N/A	None	N/A	5
	5					5
	7					5
	10					5
	15					5
BaV	2	None	N/A	None	N/A	5
	4					5
	5					5
	7					5
	20					5
	30					5
	50					5
CcV	38.5	EchiTabG	150	None	N/A	2
BaV	21.5					5
VbV	79					3
AsV	2	None	N/A	None	N/A	5
	3					5
	5					5
	6					5
	7					5
	15					5
DtV	1	None	N/A	None	N/A	5
	6					5
	10					5
	20					5
	30					5
	50					5
EoV	70	SAIMRb	200	None	N/A	5
DtV	30	α-Ecarin	150			5
		EchiTabG	150			5
		SAIMRb	200			5
		SAIMRb	200			5
None	N/A	SAIMRb	200			5

Venom	Dose (µg/mouse)	Antivenom	Dose µL/mouse	Analgesic	Dose	Number of mice
BaV	48.75	None	N/A	None	N/A	2
EpIV	40					2
NnigV	61					2
NnigV	122					1
NhajV	20.38					2
DpV	15.42					3
NpaIV	23.22					2
BaV	48.75	SAIMRp	10	Morphine	5mg ip, 10mg/kg po	5
			30			5
	97.8		10			5
			20			5
			25			5
			30			5
NnigV	61	SAIMRp	10	Morphine	5mg ip, 10mg/kg po	5
			30			5
	122		5			5
			50			5
	61		100			5
			30			5
			40			5
			50			5
			65			5
			70			5
			75			5
			80			5
			100			5
DpV	30.85	SAIMRp	40	Morphine	5mg ip, 10mg/kg po	5
			100			5
			10			5
			12			5
			15			5
			17			5
20	5					
NpaIV	46.5	SAIMRp	50	Morphine	5mg ip, 10mg/kg po	5
			70			5
			80			5
			100			5
NhajV	40.8	SAIMRp	40	Morphine	5mg ip, 10mg/kg po	5
			60			5
			70			5
			72			5
			75			5
			80			5
100	5					
EpIV	80	SAIMRm	15	Morphine	5mg ip, 10mg/kg po	5
			18			5
			20			5
			23			5
			25			5
BaV	97.8	FAV Afrique (FAV)	21.1	Morphine	5mg ip, 10mg/kg po	5
EpIV	80		42.2			5
			87.8			5
			35.1			5
NpaIV	46.5		74			5
		148	5			

Venom	Dose (µg/mouse)	Antivenom	Dose µL/mouse	Analgesic	Dose	Number of mice
NhajV	40.8	FAV Afrique (FAV)	71	Morphine	5mg ip, 10mg/kg po	5
			142			5
DpV	30.85		13.8			5
			27.6			5
NnigV	61		69.2			5
		138.4	5			
BaV	97.8	VINS	21.1	Morphine	5mg ip, 10mg/kg po	5
			42.2			5
EplV	80		87.8			5
			35.1			5
NpalV	46.5		74			5
			148			5
NhajV	40.8		71			5
			142			5
DpV	30.85		13.8			5
			27.6			5
NnigV	61	69.2	5			
		138.4	5			
BaV	97.8	Inoserp	21.1	Morphine	5mg ip, 10mg/kg po	5
			42.2			5
EplV	80		87.8			5
			35.1			5
NpalV	46.5		74			5
			148			5
NhajV	40.8		71			5
			142			5
DpV	30.85		13.8			5
			27.6			5
NnigV	61	69.2	5			
		138.4	5			
BaV	97.8	PSV polyvalent snake antiserum – Africa (PSV)	21.1	Morphine	5mg ip, 10mg/kg po	5
			42.2			5
EplV	80		87.8			5
			35.1			5
NpalV	46.5		74			5
			148			5
NhajV	40.8		71			5
			142			5
DpV	30.85	13.8	5			
		27.6	5			
NnigV	61	69.2	5			
		138.4	5			
BaV	97.8	FAV	105	Morphine	5mg ip, 10mg/kg po	5
		VINS				5
		Inoserp				5
		PSV				5
DpV	30.85	FAV	67	Morphine	5mg ip, 10mg/kg po	5
		VINS				5
		Inoserp				5
		PSV				5

Key:

VbV	<i>Vipera berus</i> venom	N/A	Not applicable
ip	Intraperitoneally	po	<i>Per os</i> (by mouth)
Zagreb	European Viper Venom Antiserum	EoV	<i>Echis ocellatus</i> venom
EpIV	<i>E. pyramidum leakeyi</i> venom	EcolV	<i>E. coloratus</i> venom
EcsV	<i>E. carinatus sochureki</i> venom	BaV	<i>Bitis arietans</i> venom
NnigV	<i>Naja nigricollis</i> venom	NnubV	<i>Naja nubiae</i> venom
DaV	<i>Dendroaspis angusticeps</i> venom	CrV	<i>Calloselasma rhodostomum</i> venom
CcV	<i>Cerastes cerastes</i> venom	AsV	<i>Aspidelaps scutatus</i> venom
DtV	<i>Dispholidus typus</i> venom	SAIMRp	SAIMR polyvalent antivenom
SAIMRb	SAIMR boomslang antivenom	SAIMRm	SAIMR monovalent Echis antivenom
NpalV	<i>Naja pallidavenom</i>	NhajV	<i>Naja haje</i> venom
DpV	<i>Dendroaspis polylepis</i> venom	FAV	FAV Afrique antivenom
VINS	VINS bioproducts African antivenom	Inoserp	Inoserp Panafrica – Inoserp Biopharma
PSV	Premium serums & Vaccines Pvt Ltd, India		
