ASSESSMENT OF THE POTENTIAL OF CAMELID ANTIBODIES TO IMPROVE THE TREATMENT OF SNAKE ENVENOMING.

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

by

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Dedication

This thesis is dedicated to my Mum and Dad, whose hard work, love and support has allowed me to pursue opportunities which were not available to themselves. I could not have achieved this without them and their selflessness has given me a great life. Thank you.

This thesis is also dedicated to Amanda Ball, for showing great belief in me, helping me through the difficult days and also giving me encouragement when I needed it most.

Abstract

Snake envenoming is a significant cause of mortality and morbidity in sub-Saharan Africa. The only effective treatment, antivenom, has been in short supply since the 1990s. Whilst the humanitarian response by some antivenom producers has significantly improved the situation, strategies to ensure the long term stability of antivenom supply are still required. Camelid IgG has been reported to be less immunogenic, less able to activate the complement cascade and more thermostable than IgG from other mammals, and has the ability to bind epitopes that are unreactive with other mammalian IgGs. The aim of this thesis is to investigate whether the attributes of camelid IgG could translate into an antivenom with immunological and venom-neutralising efficacy advantages over conventional equine and ovine antivenoms against the three most medically important snakes of West Africa, *Echis ocellatus*, *Bitis arietans* and *Naja nigricollis*. Camels were immunised with either one of these venoms (to generate monospecific IgG) or all three venoms (to generate polyspecific IgG). Examination of the serological response of immunised camels showed that 7 out of 8 camels showed antibody titres and avidities comparable to, or exceeding that, of commercial equine and ovine antivenoms. When compared using a range of preclinical assays, the *E. ocellatus* monospecific camel IgG and one of the polyspecific camel IgGs were broadly as effective as the two commercial antivenoms against *E. ocellatus* venom effects. The pool of polyspecific IgGs also showed comparable neutralising ability to the ovine antivenom. The *B. arietans* monospecific IgG was also considered effective when compared to a polyspecific commercial antivenom against *B. arietans* venom in preclinical testing. However, the pool of polyspecific IgG proved to be ineffective at neutralising *B. arietans* venom induced lethality and had poor efficacy against the haemorrhagic effects of the venom. Unfortunately, none of the camel IgG preparations were able to neutralise the lethal effects of *N. nigricollis* venom. It had been hoped that the unique properties of camel IgG may have improved neutralisation of the poorly immunogenic toxins typically found in elapid venoms. Although this appeared not to be the case, current antivenoms shown to be effective against this venom typically require greater levels of IgG than permitted in this study. Examination of the efficacy of the three subclasses of camelid IgG showed that heavy chain IgG unique to camelids was capable of neutralising haemorrhagic and coagulopathic effects of *E. ocellatus* venom and displayed greater thermostability than conventional IgG at 80°C. This improved stability did not prevent the formation of aggregates, and aggregation was similar for both camel and sheep IgG after pasteurisation, suggesting that camel IgG offers no extra benefit if an antivenom requires pasteurisation. VHH, the smallest, intact binding fragment extracted from heavy chain IgG using papain, was capable of preventing the lethal, haemorrhagic and coagulopathic effects of *E. ocellatus* venom. This important finding proves the principle that an antivenom derived from or supplemented with recombinant VHH would be capable of treating the major pathological effects of the venom. It is possible that the supplementation of an antivenom with recombinant VHH capable of neutralising the major venom toxins would enable the administration of less protein to a victim of snakebite. This coupled with VHHs low immunogenicity is likely to reduce the incidence of adverse reactions. The small size and rapid tissue ingress of VHH may provide a treatment for the local tissue destructive effects of snake venom, for which there is currently no effective treatment. This study has highlighted the preclinical effectiveness and heat stability of camel-derived antivenoms. Further work is required to examine how the properties of camel IgG and VHH can be best put to use to improve the safety and supply of antivenom whilst reducing the cost of antivenom therapy.

Acknowledgments

I would like to thank my supervisors Dr Rob Harrison and Dr Simon Wagstaff for giving me the opportunity to study such a fascinating area of research and for all of their help, advice and support. I would also like to thank all the members of the venom unit that have worked there over the past four years and who have made my time at the unit an enjoyable and interesting one, especially the MSc students (now MDs) Tim Owen and Chamali Samarasekara. I would like to thank Dr Gavin Laing for his help and advice and allowing me to call on his 20+ years of experience and Paul Rowley for providing venoms and information without requiring me to ever touch a snake. Thanks to Dr Wernery and the team at CVRL for providing the camels and sera that made this project possible.

Thanks To Drs Rajatileka and Lynd, whose experience of the process of submission and also of SPIDER made things much more straight forward for me.

I would like to thank all of the people in other departments of the school who have become my friends, offered me advice and helped me to remain positive throughout the process of writing this thesis. They are too numerous to mention but all are held in high regard.

I acknowledge the financial support of the Medical Research Council and LSTM for this studentship.

Declaration

The research presented in this thesis, entitled 'Assessment of the potential of camelid antibodies to improve the treatment of snake envenoming.' was instigated, designed and collaborations facilitated by my primary supervisor, Dr Rob Harrison. He and Dr Simon Wagstaff (secondary supervisor) provided experimental guidance and supervision of the work, which I performed by myself, except for the following:

All camels were provided by and cared for by the Central Veterinary Reseach Laboratory in Dubai. All immunisations, haematology, blood collections and separation of sera from camels were carried out by Dr Ulrich Wernery and Joerg Kinne and are therefore acknowledged in the four research chapters.

Experimental Work

Research chapter 1: All ELISA and immunoblot protocols were optimised by myself and I examined the immune reactivity of sera on several occasions as batches of camel sera were sent from Dubai. The full examination of immune reactivity was assessed by ELISAs and Immunoblots carried out by Tim Owen, as part of his MSc research project supervised by Dr Harrison. Avidity ELISAs were carried out by myself and all experimental data was processed and converted to publication quality figures by me.

Research Chapter 2: All experiments were performed by myself except for the IgG Titre ELISA used in Figure 1, which was performed by Tim Owen.

Research Chapter 3: All ELISA and immunoblot protocols were optimised by myself. All chromatographic separations of IgG subclasses, MCD, MHD, Heat treatment and turbidity ELISAs were performed by myself. Time Course ELISA, Endpoint titrations, Immunoblots and avidity ELISAs were performed by Chamali Samarasekara, as part of her MSc research project supervised by Dr Harrison. Help and advice programming the AktaPrime chromatography instrument was provided by Dr Wagstaff.

Research Chapter 4: All experiments were conducted by myself.

<u>Written Work</u>

The first draft of chapters 1 and 2 were written by myself, reviewed by Dr Harrison and amended by myself.

The research chapters 3-5 within this thesis are presented in research manuscript format and have been submitted (the first two accepted) for publication to Toxicon. Due to the manuscript format of this thesis, there are necessarily repetitions of the major themes in the introductions to each chapter. This has been kept to a minimum. Each paper/chapter contains its own methods section, although a short methods section has been included in the thesis (chapter 2) to expand on the succinct format required for publication. These chapters closely follow the format of the submitted manuscripts except that the figure legends are on the same page as the relevant figures. I prepared the first draft of each paper and then passed

them to Dr Harrison for editing and then, together we prepared the final manuscript for submission.

I wrote Chapter 6 in the format of a traditional thesis chapter, which Dr Harrison has reviewed, and is currently preparing it for publication.

The first draft of the concluding discussion was shown to Dr Harrison, and then amended as advised.

Abbreviations

BaV	*Bitis arietans* venom
BVDV	Bovine viral diarrhoea virus
CDR	Complementarity determining region
CL	Confidence limits
ddH_2O	Double distilled water
°C	Degrees centigrade
DNA	Deoxyribonucleic acid
ED_{50}	Effective dose that prevents 50% death
ELISA	Enzyme linked immunosorbant assay
EoV	*Echis ocellatus* venom
$F(ab')_2$	Antibody gamma fragment heterodimer
Fab	Antibody gamma fragment
Fc	Antibody gamma fragment crystallisable
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
HCIgG	Heavy chain IgG
i.d.	Intradermal
i.m.	intramuscular
IgG	Immunoglobulin G
kDa	Kilodalton
LD_{50}	Lethal dose that causes 50% death
MCD	Minimum coagulation dose
mg	Milligram(s)

MHD	Minimum haemorrhagic dose
Min	Minute(s)
ml	millilitre(s)
MS	Monospecific
MVM	Minute virus of mice
NnV	Naja nigricollis venom
PAGE	Polyacrylamide gel electrophoresis
PLA_2	Phospholipase A_2
PS	Polyspecific
PVR	Pseudorabies
RPM	Revolutions per minute
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SVMP	Snake venom metalloproteinase
VDS	venom delivery system
VHH	Variable domain of camelid heavy chain antibody

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CHAPTER 1

General Introduction

1. 1 Introduction to snakes

1.1.1 Snakes

Snakes are a member of the very successful order Squamata, which also includes lizards. The remarkable radiation of snakes from lizards during the cretaceous period, about 200 million years ago (Calvete et al., 2007), has left them well adapted for a number of niches, leading to the total number of snake species now numbering approximately 3000 (White, 2000). Their geographical distribution is widespread living on all continents except Antarctica, but being particularly abundant in the tropics, due to the advantages the warm climate offers for cold-blooded animals. Despite this many snakes survive in cold regions, such as the European adder, which is found further north than the Arctic Circle. Within this area snakes inhabit a range of habitats including underground burrows, trees and oceans (invading depths of up to 150 metres) (Encarta, online).

Development of features such as the dry, horny epidermis which contains high amounts of keratin has been key to the snake's success, providing a waterproof seal to limit water loss in harsh arid climates. This is coupled with kidneys that produce uric crystals further reducing water loss. Being ectothermic negates the requirement of regular feeding to maintain body temperature, whereas a mouse might eat its bodyweight each day, some snakes will feed on a rodent only once every week to two weeks. It is some of the snake's other adaptations however, that make them as widely known and feared as they are:

-Despite poor vision and hearing, snakes are good hunters. The skull bone is able to detect vibrations of passing prey and the tongue, combined with

Jacobson's organ, is sensitive to odours allowing trails to be followed to locate prey.

-The flexible jaw bone (due to evolutionary loss of bone in that area) allows prey larger than their own diameter to be consumed

-Threat to people, due to the development of suffocation techniques by boas and pythons or by the ability to inject potent and deadly venom

1.1.2 Evolution of the snake venom delivery system

The fossil record for snake evolution is very limited, most likely due to the small, delicate bones not remaining intact or fossilising successfully. This has led to snake evolution becoming an ever changing story, with different groups putting forward different hypothesis and trying to reconstruct the finer points of the phylogenetic tree. One aspect of this debate is the evolution of the venom delivery system (VDS) which allowed venom toxins to be directed effectively into the target, aiding in the subduing and possibly the digestion of prey and also as a potent defensive weapon.

The first recognisable snakes to evolve from the common ancestor (thought to be a burrowing lizard) are the *Henophidia,* a superfamily commonly called the primitive snakes. This family contains the boa and python species amongst others and it is this design that typifies the early snakes. Primitive heavy skull structure, large muscular bodies with remnants of legs sticking out, left over from the lizard ancestor. It is likely that both the early snakes and the burrowing lizard ancestor had venom proteins but lacked an effective VDS (Fry et al., 2006). The introduction of the VDS is a contentious subject, with recent work undermining a fairly straight forward explanation. Previously

it was thought that there was a group referred to as Colubroidea, which contained one large family of non-vemonous snakes (Colubridae) and two smaller families of venomous snakes Elapidae (cobras and similar species) and the Viperidae (vipers) (Jackson, 2007). In this model the Colubridae were considered ancestors of the more advanced Elapids and Viperids. This would seem to make sense as the Elapids and Viperids have the most advanced VDS and highly potent venoms.

Recent examination of snake fangs from the Oppenheim quarry in Germany showed that despite dating from the Early Miocene (23 mya) the fangs were identical to those of modern day elapids and vipers (Kuch et al., 2006). This finding suggests that these snakes had developed fangs much further back in time than was previously thought.

Some revised phylogenetic trees now feature Viperidae at the basal end nearest to the primitive snakes (Jackson, 2002). It is likely that the successful radiation of coloubroid snakes is more a consequence of the changing ecology during the Paleocene period coupled with a sleeker, more compact frame as compared to primitive snakes, than the actual development of the VDS (Kuch et al., 2006).

1.1.3 Fangs

Yet another area of confusion and debate is the development of tubular fangs which are common to the elapids, viperids and atractaspidids. Whilst the three groups developed the musculature with which to compress the venom gland and excrete the venom independently, and also derived the venom gland from Duvernoy's gland independently, the fangs seem to have most likely come from a common ancestor (Jackson, 2007; Vonk et al., 2008).

However, it is important to note that the fangs length and position differ between the venomous snake groups (Figure 1.1).

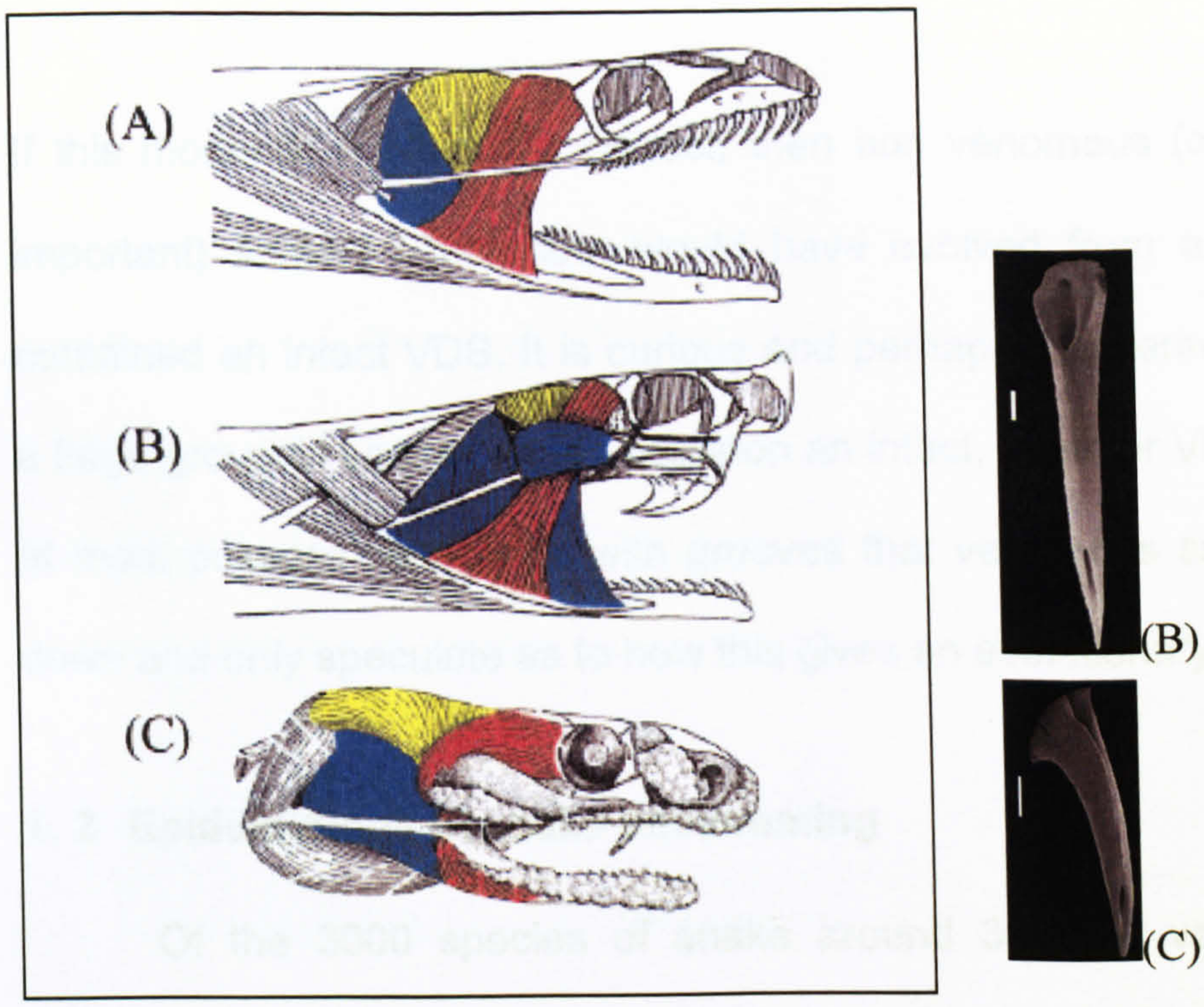

Figure 1.1: Different musculature (from (Jackson, 2002) and fangs (Kuch et al., 2006) of (A) python (B) viper (C) elapid.

Despite supposedly being the most primitive of the venomous snakes, the Viperids do in fact possess the most highly developed VDS in the animal kingdom. The fang is an enlarged maxillary tooth, which is tubular and with a bevelled tip, in resemblance of a hypodermic needle, perfectly adapted for delivery of venom. The fangs of Viperids are more elongated then those of Elapids and require a hinge system to retract them after extension, in order to close the mouth (solenoglyphous). During an attempt to bite, the maxilla can rotate independently, enabling biting with one or both fangs. When preparing to bite the fang can be hyper-extended to vertical to enable a greater reach and penetration.

In comparison Elapids have front fixed fangs that cannot move (proteroglyphous), thus requiring them to be shorter, to enable the mouth to close.

If this model of evolution is correct, then non venomous (or non-medically important) snakes (Colubrids) would have evolved from an ancestor that contained an intact VDS. It is curious and perhaps counterintuitive that such a large group of snakes would abandon an intact, superior VDS altogether or at most possess rear fangs with grooves that venomous saliva may trickle down and only speculate as to how this gives an evolutionary advantage.

1. 2 Epidemiology of snake envenoming

Of the 3000 species of snake around 350 are venomous (White, 2000). An early attempt to seriously estimate the incidence of snakebite recorded global figures of 50,000 deaths per year (Swaroop and Grab, 1954). Whilst the work in this study was laudable and valuable it is expected that these figures represent an underestimation of the total numbers of victims of snakebite, due to the reliance on hospital admissions. The undertaking of more in-depth population studies has highlighted the unreliability of hospital statistics. One study, that examined the underestimation of snakebite mortality by hospital statistics in Monaragala, Sri Lanka, showed that hospital reporting missed 62.5% of deaths, due to snakebite, in that region (Fox et al., 2006). Reasons for such disparity between true number of bites and those recorded by hospitals include the popularity of traditional remedies, where in some regions up to 90% of victims seek a source of ineffective traditional medicine and 89% try two traditional remedies (Newman et al., 1997). This

delay can lead to victims either arriving too late for hospital treatment or dying en route. Other factors that cause the avoidance of hospital treatment include: the cost of treatment (Einterz and Bates, 2003) and the distance that has to be travelled.

As illustrated by Theakston et al., 2003, extrapolating local surveys of snakebite incidence and mortality to provide regional or global statistics is unreliable due to the range of variation between regions and countries. For example mortality rates ranging from 2 to 16 per 100, 000. Accepting these drawbacks the following recent figures (Kasturiratne et al., 2008) represent the best available estimates. (See Table 1.1)

Region	**Current Estimate of Envenomings per Year**	**Current Estimate of Deaths per Year**
Asia	237,379 - 1,184,550	15,385 - 57,636
Australasia	1,460 - 5,895	229 - 520
Europe	3,961 - 9,902	48 - 128
Latin America	81,427 - 137,123	647 - 3,459
North America	2,683 - 3,858	5 - 7
North Africa/Middle East	3,017 - 80,191	43 - 78
Sub-saharan Africa	90,622 - 419,639	3,529 - 32,117
- of which occur in West Africa	*27,999 - 294,700*	*1,590 - 18,654*
Total	**420,549 - 1,841,158**	**19,886 - 93,945**

Table 1.1: Global estimates of snake envenomations and deaths.
Data taken from (Kasturiratne et al., 2008).

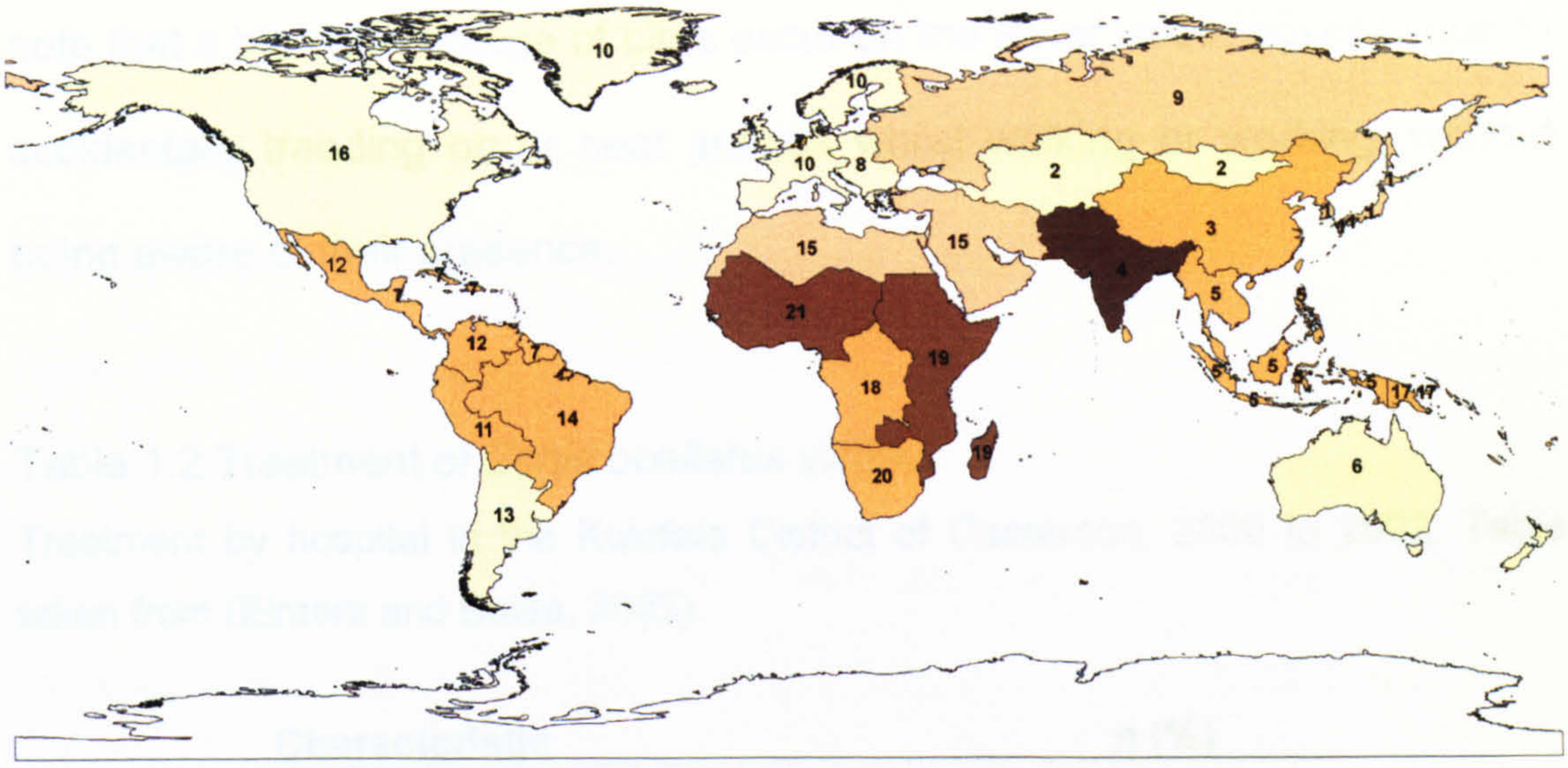

Figure 1.2: Regional distribution of deaths due to snakebite (Low estimates used).

Figure taken from (Kasturiratne et al., 2008)

1.2.1 Victims of envenoming

As the data in Table 1.1 and Figure 1.2 suggests, the majority of deaths from snake envenoming occur in the tropics and subtropics, and is higher in rural areas than in urban settings. This is due to the natural habitat providing greater protection to the snake and the risk of exposure to snakebite being high in communities reliant on agricultural work. Most victims are bitten during routine activities such as whilst working in fields or pastures, herding cattle or walking between the home and place of work (Pugh et al., 1979). The essential nature of these activities negates any behavioural strategies proposed to reduce human / snake contact. Table 1.2 is data from a region of Cameroon, showing an example of common activities of the victim when the bite occurred (Einterz and Bates, 2003). It is interesting to

note that a high percentage of bites occur on the lower limbs, possibly due to accidentally treading on or near snakes whilst walking or working, without being aware of their presence.

Table 1.2 Treatment of *Echis ocellatus* victims

Treatment by hospital in the Kolofata District of Cameroon, 2000 to 2002. Table taken from (Einterz and Bates, 2003).

Characteristic	***n* (%)**
Activity of victim when bitten	
Working in a pasture or field	31 (52.5)
At home	10 (16.9)
Walking on a road	8 (13.6)
Chasing mice	4 (6.8)
Fetching water	5 (8.5)
Unspecified	1 (1.7)
Time bite was received	
During the day	41 (69.5)
During the night	17 (28.8)
Unspecified	1 (1.7)
Site of bite	
Lower extremity	48 (81.4)
Upper extremity	11 (18.6)

With greater contact and risk of being bitten, it is not surprising that snake envenoming is a particular blight of the rural poor. In one study it accounted for 28% of all accidental deaths (Trape et al., 2001). Many people that survive envenoming suffer debilitating long term physiological and psychological sequelae, with about 400,000 amputations each year (Mion and Olive, 1997) due to the local effects of envenoming, which can leave victims unable to work. The socioeconomic cost of snakebite is high in these communities.

1. 3 Africa

With up to an estimated 419,639 envenomations and 32,117 deaths, there is a high human and economic cost due to snakebite in Africa, with West Africa being particularly affected, accounting for up to 18,654 deaths (Kasturiratne et al., 2008). In certain rural areas such as the Benue Valley in Nigeria, bite incidence can be up to 497 per 100 000 population, with a mortality rate of 12.2% (Pugh and Theakston, 1980). In areas of West Africa, during peak times such as harvest, the majority of hospital beds are often occupied by snakebite victims (70% in Ougadougou, Cameroon (Revault, 1996); and 90% in Zamko, Nigeria (RAH, personal observation)). Snakebite therefore is a considerable drain on limited rural health resources. This situation is further exacerbated by the shortage of effective antivenom as brought to the world's attention in the Lancet in 2000 (Theakston and Warrell, 2000)

1.3.1 Snakes of most medical importance in West Africa

In Africa there are about 400 species of snakes, with 93 species that are harmful to humans. Within that number 29 are from the Elapidae family and 42 from Viperidae (Spawls and Branch, 1995).

West Africa may account for over half of the total number of deaths due to snakebite in the whole of Afria (Kasturiratne et al., 2008). The three species considered most medically important in West Africa are the saw-scaled viper (*Echis ocellatus)*, the puff adder (*Bitis arietans)* and the spitting cobra (*Naja nigricollis)* (Warrell and Arnett, 1976)

1.3.2 Echis ocellatus (**Family: Viperidae**)

Echis ocellatus is a small, robust viper ranging from 30 to 50 cm and reaching a maximum of around 65cm (Spawls and Branch, 1995). It is typified by the pear shaped head and thin neck, being grey or brown in colour with dark crossbars of different size along the back (Figure 1.3). To the uninitiated it may appear to be an insignificant threat. Described as irritable, when threatened *E. ocellatus* rubs its coils together in a C-shaped pattern creating a characteristic rasping sound (Pugh et al., 1979; Spawls and Branch, 1995). This snake is also particularly unusual for its aggressive behaviour, and may actually move towards a perceived threat.

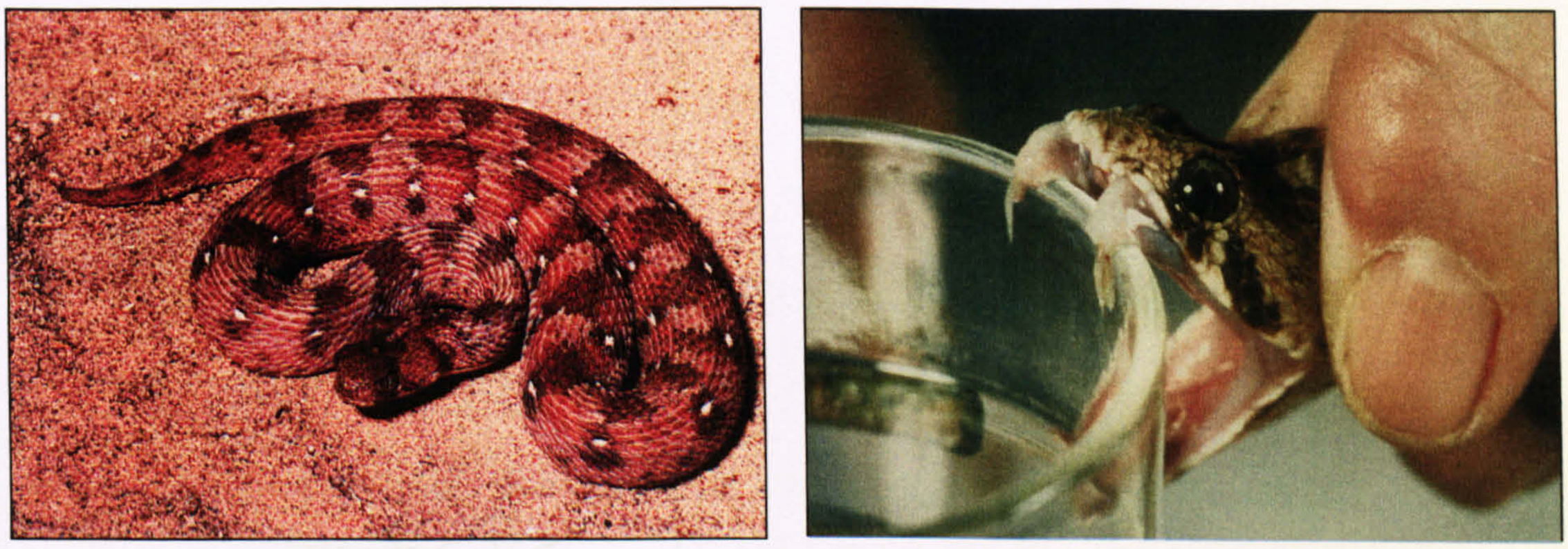

Figure 1.3: *Echis ocellatus*.

Photographs: Left taken from (Spawls and Branch, 1995), Right from ARVRU archive

1.3.2.1 Distribution

Echis ocellatus is found in large numbers throughout West Africa, particularly in Guinea and Sudan savannah region (Spawls and Branch, 1995)

Figure 1.3). In south-eastern Senegal it is the most prevalent venomous species (Trape et al., 2001). It is endemic to regions of Nigeria, such as the

Benue and Niger valley (Warrell and Arnett, 1976; Pugh et al., 1979; Pugh and Theakston, 1980; Trape et al., 2001).

Typically found in grassland but also, unusually, in well wooded areas, whilst it is normally terrestrial it can occasionally seek shelter in low bushes. The limits to *E. ocellatus* distribution to the region shown (Figure 1.4) do not appear to be due to specific environmental conditions, and are perhaps limited by competition from *Bitis arietans*, as both snakes are rarely found in the same area (Pitman, 1973; Warrell and Arnett, 1976).

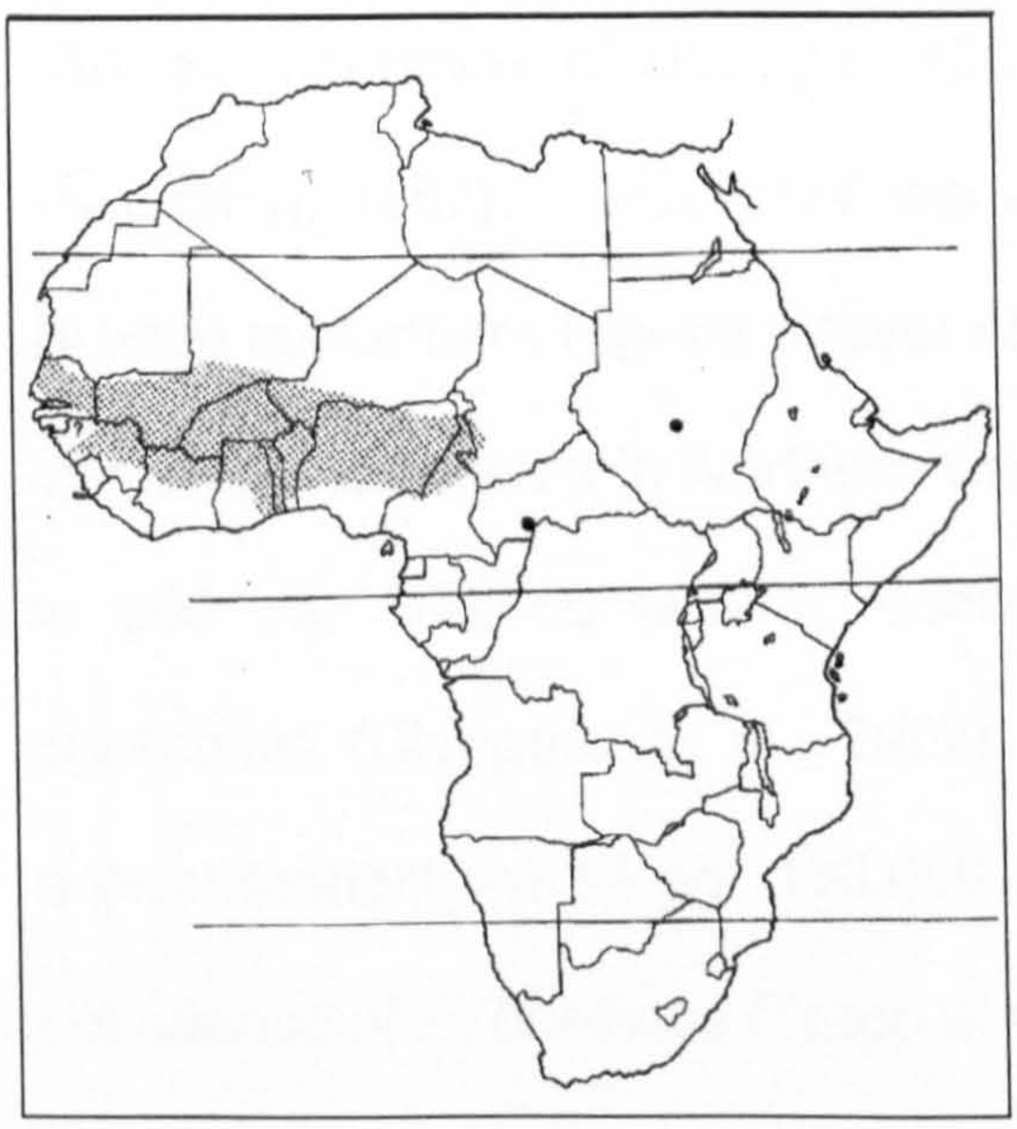

Figure 1.4: Distribution of *E. ocellatus* (shaded area) (Spawls and Branch, 1995)

1.3.2.2 Incidence of envenoming

As previously explained it is hard to provide accurate figures for victims of snake envenomation. It is even more difficult to provide accurate figures of which species of snake is responsible for the bite, as not all victims that make it to primary healthcare arrive with the specimen. Since snakes of the *Echis*

genus are responsible for more deaths worldwide than any other snake (Warrell et al., 1977), there is more epidemiological data available, compared to other species, but it is still not comprehensive. Despite these drawbacks it has been said that this particular species represents the most medically important snake in Africa and could account for up to 70% of all deaths there (Stock et al., 2007).

Early studies in parts of West Africa found the incidence and mortality of bites was as high as 120 and 8, respectively, per 100,000 population per year (Warrell and Arnett, 1976). This figure is superseded by the highest values for bites by *E. ocellatus* in West Africa, found in the Benue and Niger valleys with an incidence of 602 per 100,000 and 12.3% mortality (Pugh and Theakston, 1987). *E. ocellatus* was also found to be responsible for 95% of all bites in Northern Nigeria (Meyer et al., 1997)

In a study conducted in Northern Cameroon envenomations ranged from 50 to 250 per 100,000 with *E. ocellatus* responsible for 85% of identified snakebites (Chippaux et al., 2002). In the Bandafassi area of Senegal an annual mortality of 14 per 100,000 was recorded and attributed to the high prevalence of *E. ocellatus* (Trape et al., 2001).

E. ocellatus is nocturnal, although most bites occur during the day, probably when the snake is disturbed by people stepping on or around it, during their course of work or walking. Hospital statistics from Kolofata, Cameroon, show 69.5% of bites occurring during the day, with 52.5% of the victims bitten whilst working in a field or pasture (Einterz and Bates, 2003).

To summarise: *E. ocellatus* is responsible for most snakebite deaths in the region of West Africa due to being the most prevalent snake in many areas,

coupled with small size and aggressive behaviour. Also adding to the high death rate is the fact that this snake rarely fails to envenom, having a very low 'dry bite' rate of 10% compared with an average dry bite rate of 50% for all venomous snakes (Theakston et al., 2003). *E. ocellatus* is suspected of being responsible for over 70% of deaths due to snakebite in all of Africa (Stock et al., 2007).

1.3.2.3 Effects of *E. ocellatus* venom

Envenoming by *E. ocellatus* leads to a range of clinical manifestations that are commonly seen in viperidae bites, particularly prolongation of the blood clotting time (haemotoxic effects).

Typically following envenoming by *E. ocellatus* there is immediate pain at the bite site, which is severe (Warrell et al., 1977; Rugman et al., 1990). Local bleeding (in 80% of cases) and swelling (100%) rapidly occur, within 30 minutes (Warrell et al., 1977). Other local effects include blistering (13%) and necrosis (11%).

Systemic effects of envenoming include incoagulable blood (93%) and spontaneous haemorrhage, which is the most important clinical effect and is seen in 57% of victims, most commonly from the gingival sulcus (Warrell et al., 1977). The onset of spontaneous bleeding can range from 1 to 30 hours (Warrell et al., 1977). Death is usually a result of haemorrhagic shock, due to intra-abdominal bleeding or intracranial haemorrhage (Warrell and Arnett, 1976).

LD_{50} in mice: 11.5 µg (Gutierrez et al., 2005)

Average yield per venom extraction (dry weight): 10 mg

1.3.3 Bitis arietans (***family: Viperidae***)

Bitis arietans, also known as the puff-adder due to the threat display that involves inflating its body and hissing loudly, is a thick snake that can reach up to 1.5 metres long (Bey, T.A. et al., 1997). Colours vary from yellow-brown to a light orange-brown base colour with dark, backwards facing chevrons (Figure 1.5) (Spawls and Branch, 1995). When threatened, as well as the loud hissing, this snake forms a taught S shape ready to strike; despite its locomotion across the ground being slow, *B. arietans* can strike rapidly.

Whilst most *Bitis* species are not responsible for many human envenomings, *Bitis arietans* is considered belligerent and willing to bite (Spawls and Branch, 1995).

Figure 1.5: *Bitis arietans* (Spawls and Branch, 1995)

1.3.3.1 Distribution

Bitis arietans is the most widely distributed species of venomous snake in Africa (Warrell et al., 1975). Its range encompasses sub-Saharan Africa (except rainforest regions of Central and West Africa) and can be found as

far north as Morocco and as far south as South Africa (Figure 1.6) (Spawls and Branch, 1995).

A nocturnal snake, which relies heavily on camouflage as it is sluggish in movement, *B. arietans* seems to prefer savannah but can be found in a wide range of habitats, being only absent from extreme desert, high montane grassland and certain rainforests. (Spawls and Branch, 1995)

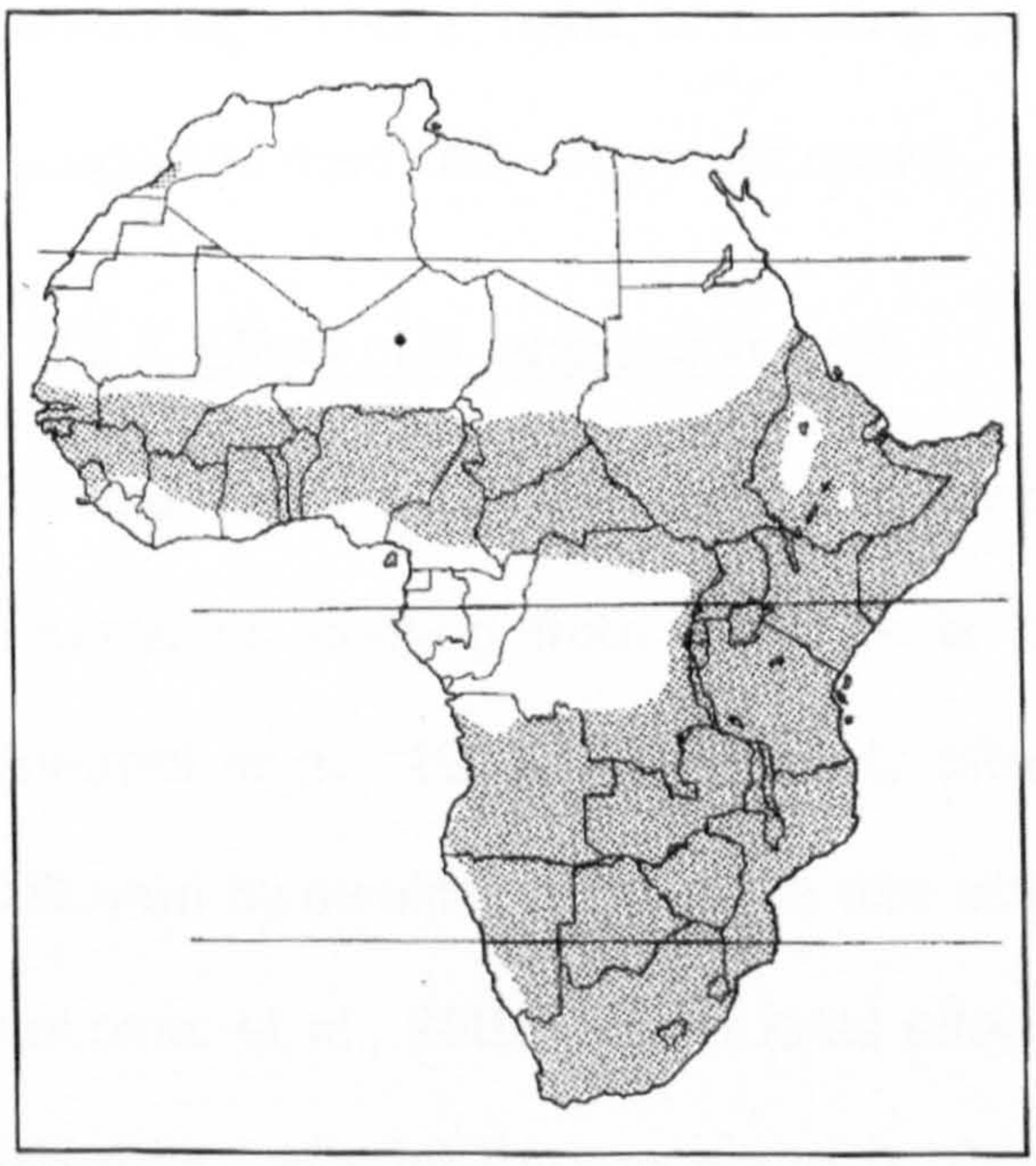

Figure 1.6: Distribution of *B. arietans* (shaded area) (Spawls and Branch, 1995)

1.3.3.2 Incidence of *B. arietans* envenoming

The puff adder can be a significant public health concern in certain regions; in one study it was responsible for three quarters of the bites at Mpilo Central Hospital in Zimbabwe. (Muguti et al., 1994). A 1976 survey in Johannesburg found that the majority of venomous bites were also by this snake (Rippey et al., 1976; Lavonas et al., 2002). Despite being referred to as causing more death to man and domestic animals than all other African snakes put

together (Warrell et al., 1975), the number of human deaths due to *Bitis arietans* envenoming is unlikely to be as high as those from *E. ocellatus* (Stock et al., 2007). Indeed, deaths from puff adder bites are estimated to occur in 5-10% of cases, and serious morbidity is usually only associated with poor care of the patient (Spawls and Branch, 1995). Widespread distribution, willingness to bite, potent venom, long fangs, the use of camouflage and a habit of basking by footpaths has made *B. arietans* a recognised medically important snake.

1.3.3.3 Effect of *B. arietans* venom

Clinical reports of *B. arietans* envenoming are scarce, with the major information coming from 10 patients treated in northern Nigeria in 1975 (Warrell et al., 1975). Immediately after envenomation intense pain is felt, followed by swelling around the bite site within 30 minutes (Bey et al., 1997; Lavonas et al., 2002). Other local effects that may present include swelling, blistering, arterial thrombosis with necrosis developing up to a week later (Warrell et al., 1975). Systemic effects include spontaneous haemorrhage, hypotension, bradycardia and renal damage. One case describes severe coagulopathy, not thought to be common in cases of *B. arietans* poisoning (Lavonas et al., 2002). Death is thought to be due to circulatory collapse or renal failure (Warrell et al., 1975).

LD_{50} in mice: 16.7 µg (Gutierrez et al., 2005)

Average yield per venom extraction (dry weight): 150-250 mg

1.3.4 Naja nigricollis ***(family: Elapidae)***

Known as the black-necked spitting cobra, *Naja nigricollis* is the typical cobra shape, with a broad head, characteristic hood and long cylindrical body (Spawls and Branch, 1995). Typically this species reaches 1-1.5 metres long, with the maximum length being 2 metres. Colours vary widely with geography, including all black, all red, or grey with pink bands, but in West Africa the predominant colour is black or grey-black with red bars on the neck (Figure 1.7) (Spawls and Branch, 1995). Unlike the extendable fangs of vipers, *N. nigricollis,* like all elapids, has shorter, fixed front fangs. When disturbed, *N. nigricollis* will rear up and spread its hood and stand its ground: although it is quick moving and agile and can escape by climbing trees or rocks (Spawls and Branch, 1995).

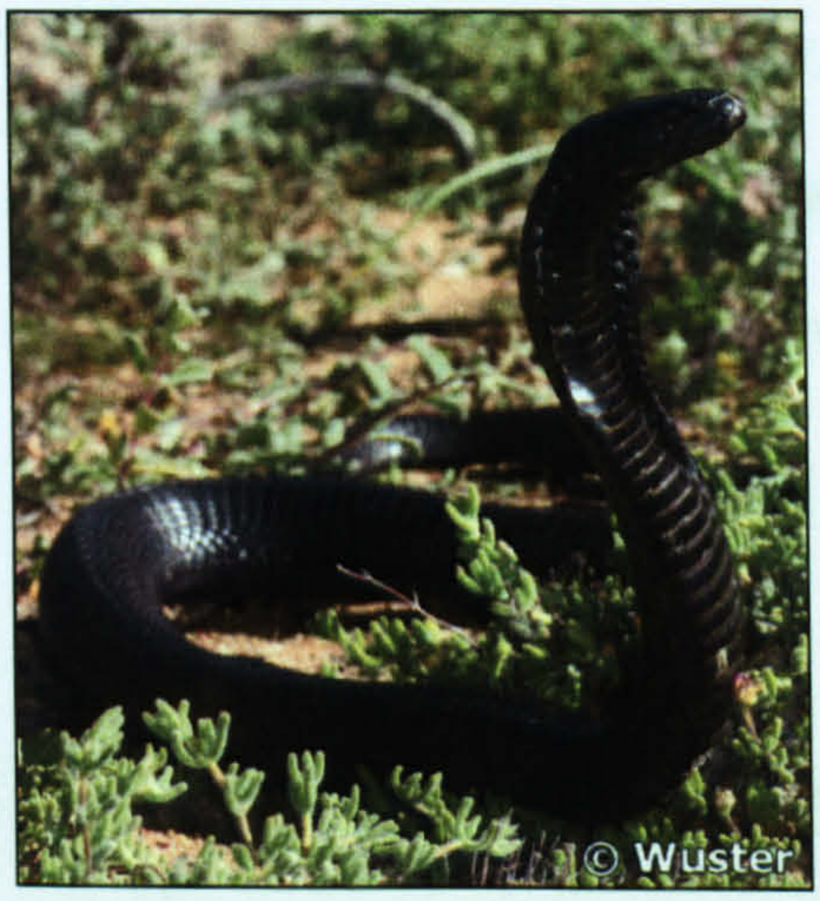

Figure 1.7: *Naja nigricollis* (courtesy of W. Wuster)

1.3.4.1 Distribution

The black colour form is found from Senegal, through to West Africa including southern Sudan and Uganda and as far south as Tanzania and also Kenya. The brown colour form is found in parts of Ethiopia, Somalia, Kenya, Uganda and Tanzania (Spawls and Branch, 1995).

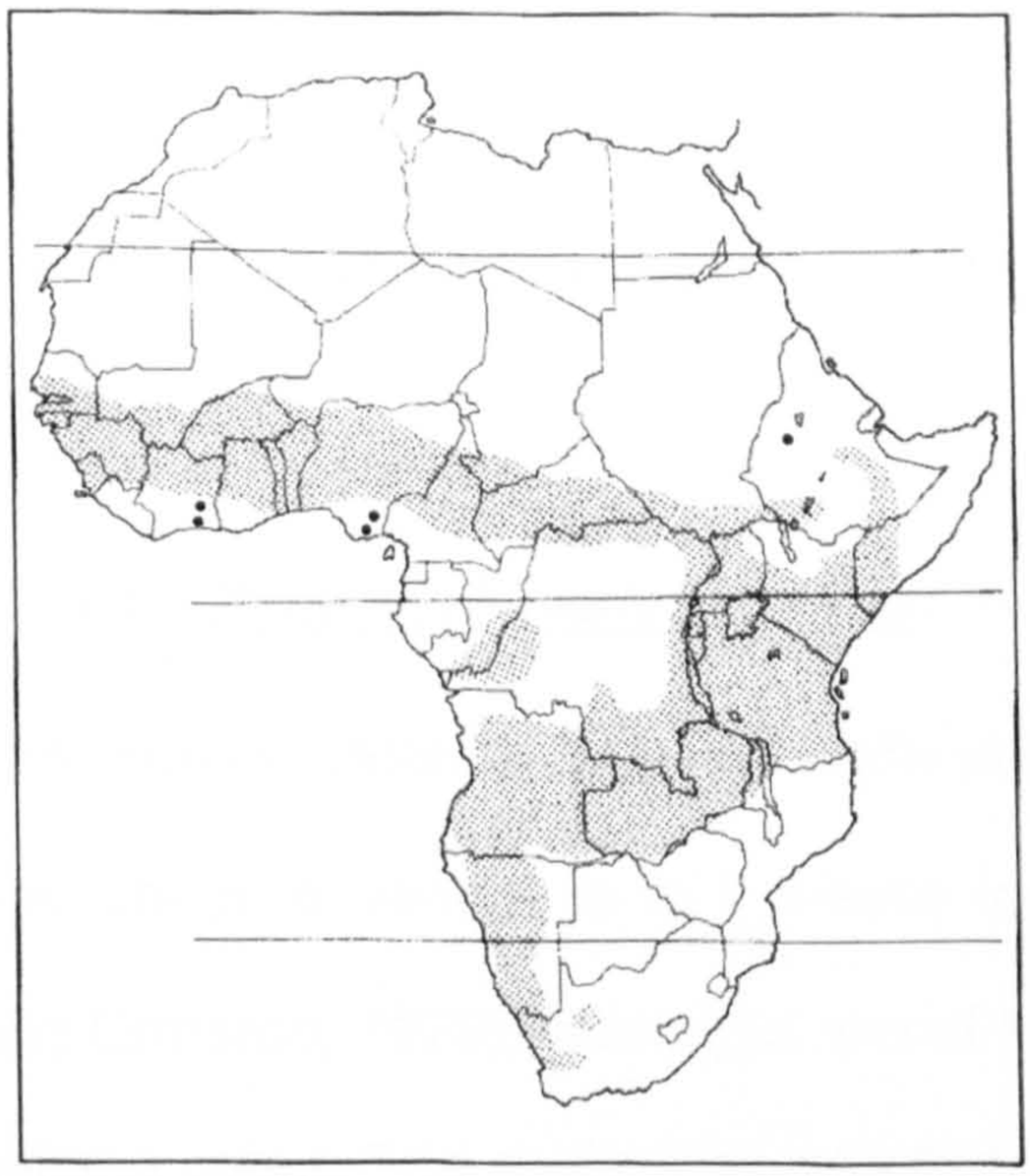

Figure 1.8: Distribution of *N. nigricollis* (shaded area) (Spawls and Branch, 1995)

The adult snake is usually active at night, but can sometimes be seen basking or hunting in the day. Usually considered a savannah dweller, this snake is opportunistic and can be found in a range of habitats including forest, villages and towns: even living in junk piles (Spawls and Branch, 1995).

1.3.4.2 Incidence of *N.nigricollis* envenoming

Despite being described as rarely biting, preferring to spit venom into the eyes of aggressors (Stock et al., 2007), epidemiological data suggests that, in certain regions, *N. nigricollis* is responsible for more cases of snakebite than any other speicies. One study in Malumfashi, northern Nigeria (Pugh et al., 1980) showed this species to be responsible for 106 bites, leading to an estimate of 15-20 bites per 100,000 people per year with a mortality of 5%. This snake can commonly encounter humans when it enters homes looking

for food; this study found 72% of *N. nigricollis* bites occurred in domestic settings at night (Pugh et al., 1980), usually as a result of accidentally rolling onto the snake whilst asleep.

1.3.4.3 Effects of *N. nigricollis* venom

The primary defence *Naja nigricollis* employs when challenged is to eject a fine stream of venom up to 5 metres in to the face of an aggressor (Warrell and Ormerod, 1976), leading to one of the common names for this snake: the spitting cobra. The snake has adapted well to spitting at the face, and is not fooled in to spitting at a moving hand, where the venom would not dissuade the harasser (Westhoff et al., 2005). This cobra has an amazing stamina, able to spit over 30 times in 8 minutes (Westhoff et al., 2005).

The effect of venom on the eye of a victim can vary although intense pain is usually felt. A study undertaken in Nigeria detailed 9 cases of snake venom ophthalmia (Warrell and Ormerod, 1976) in which 5 of the patients had conjunctivitis, some severe, all of which healed with no long-term damage. Of the remaining 4 victims, all had corneal ulceration which led to permanent blindness in two cases. The severity of symptoms is likely to be linked to the quantity of venom affecting the eye (Gruntzig et al., 1985) and whether the victim quickly rinsed the eyes with water.

The effect of envenomation by *N. nigricollis* is unusual for an Elapid, in that the venom is mainly cytotoxic and not particularly known for neurotoxicity (Warrell et al., 1976). In a study of fourteen patients admitted to hospital in Nigeria (Warrell et al., 1976) local swelling was present in all cases and in four cases was considered massive, surrounding the whole limb and some of

the trunk; limb circumference increased by up to 27%. Tender, enlarged lympth nodes and blistering were also commonly seen, with the latter leading on to necrosis of the tissue (Warrell et al., 1976). Of the 106 victims of *N.nigricollis* envenoming from Malumfashi, 40 had obvious scarring due to the necrotic effects of the venom, 20 of which had some lasting deformity (Pugh et al., 1980).

Systemic effects of the venom include spontaneous haemorrhage in a small proportion of victims (Warrell et al., 1976).

LD_{50} in mice: 20.9 µg (Gutierrez et al., 2005)

Average yield per venom extraction (dry weight): 150-350 mg

1. 4 Pathology of snake envenoming

The clinical effects of snake envenoming are typically divided into two categories: local effects and systemic effects.

1.4.1 Local effects of viper envenoming

Severe pain, rapid swelling of the bitten limb (sometimes involving the whole limb and trunk), bruising, blistering and haemorrhage at the site of envenoming are commonly reported (Warrell, 1996). These symptoms are thought to be due to the action of metalloproteinases and phospholipases, which increase vascular permeability and trigger the release of pro-inflammatory mediators. Necrosis of muscle tissue can be present in some cases and is due to the myotoxic and cytotoxic effects of venom, especially phospholipase A_2 and ischemia due to metalloproteinases affecting the local blood supply. The tissue damaging effects of the venom combined with

swelling of the bitten limb can lead to serious and permanent sequelae such as extensive tissue loss and chronic ulceration (Warrell et al., 1975). In some cases necrosis is extensive enough to require amputation (Lavonas et al., 2002).

1.4.2 Systemic effects of viper envenoming

Disruption of haemostasis is a consistent feature of viper envenomations. Typically observed is disruption of normal blood coagulation (coagulopathy) leading to bleeding that occurs distant from the site of envenomation, most often seen in the gums but epitaxis, ecchimosis, petechiae, macrohaematuria, uterine bleeding, haemoptysis and bleeding from old wounds may be other manifestations of coagulopathy (Warrell, 1996). Abdominal, subarachnoid and intracranial haemorrhages may occur and can represent serious threats to a victim's survival. Such clinical symptoms are due to the actions of several toxin groups including SVMPs, disintergrins, C-type lectins, Serine proteases and PLA_2s.

Shock, hypotension and hypovolaemia due to extravasation into the bitten limb (caused by SVMP action on vascular endothelium) are seen in some victims of viper envenomation. Acute renal failure and respiratory distress may also be reported in cases of viper bites. Other symptoms may include: vomiting, sweating, abdominal pain and diarrhoea.

The period of time from viper envenoming to death can range from a few hours to several weeks. For *Echis* species a range of 25 hours to 41 days was reported with the median time to death being 5 days (Warrell and Arnett, 1976).

1.4.3 Local effects of elapid envenoming

Envenoming by many species of Elapid causes minimal local effects and, as in the case of krait bites (*Bungarus* species), may not even wake the victim. However, bites by African spitting cobras (including *N. nigricollis*) and many Asian cobras can cause swelling, blistering and necrosis. Onset of swelling can range from a few minutes to three hours, and in some cases can extend from the bitten limb to the trunk. Affected limbs have been reported to increase in size up to 27 per cent (Warrell et al., 1976). Blisters can appear between a few hours and several days after envenoming and can lead to deeper tissue necrosis. Necrosis in these cases can damage entire areas of muscle and artery and require debridement and skin grafting. In the most serious cases, the victim will be left with lasting reduced mobility of the limb or may require amputation.

1.4.4 Systemic effects of elapid envenoming

The typical systemic feature of elapid envenoming is one of neurotoxicity. Early signs include heaviness of the eyelids, blurred vision, headache, vertigo, vomiting and ptosis which may progress to opthalmoplegia. Paralysis may spread to the face affecting the mouth, jaw and tongue and preventing the victim from speaking, swallowing or protruding their tongue.

Intercostal muscles and the diaphragm undergo paralysis leading to abdominal breathing. Progression of this paralysis causes agitation, sweating, tachycardia, shallow breath, stupor, coma and ultimately death.

Other symptoms may be present such as haemostatic disruption, changes to heat rhythm and renal failure (Australian elapids).

Death from elapid envenoming can be rapid with a mean value ranging from 8.4 – 18 hours for *Bungarus caeruleus* and *Naja naja* respectively (Reid, 1968; Warrell, 1996).

With regard to *N. nigricollis*, the classic neurotoxic effects of other elapids are rarely present. Whilst the most common serious effect of *N. nigricollis* envenoming is local necrosis, disruption to haemostasis causing spontaneous haemorrhage is also a feature. In cases of *N. nigricollis* envenoming, the lack of classic symptoms common to most elapid bites can create difficulty in distinguishing it from viper bites (such as *B. arietans*) and increases the risk of misdiagnosis (Warrell et al., 1976).

1. 5 Composition of and biological effects of venom toxins

Snake venoms are a complex mixture of a variety of biologically active proteins. Attempts are underway to characterise the main constituents of medically important snake venoms, although there is little information regarding the exact composition and functionality of venoms from specific species (Harrison, 2004). Despite containing over a hundred or more constituents, it seems possible that the main causes of pathology seen in cases of envenoming may be due to a relatively small number of toxins (Harrison et al., 2003). Identification of these common toxins could help to develop a toxin-specific antivenom that could be used to treat bites from a

wide range of snake species (Wagstaff, S. C. et al., 2006). An examination of the most medically significant toxins follows.

1.5.1 The main toxins involved in viper envenoming pathology

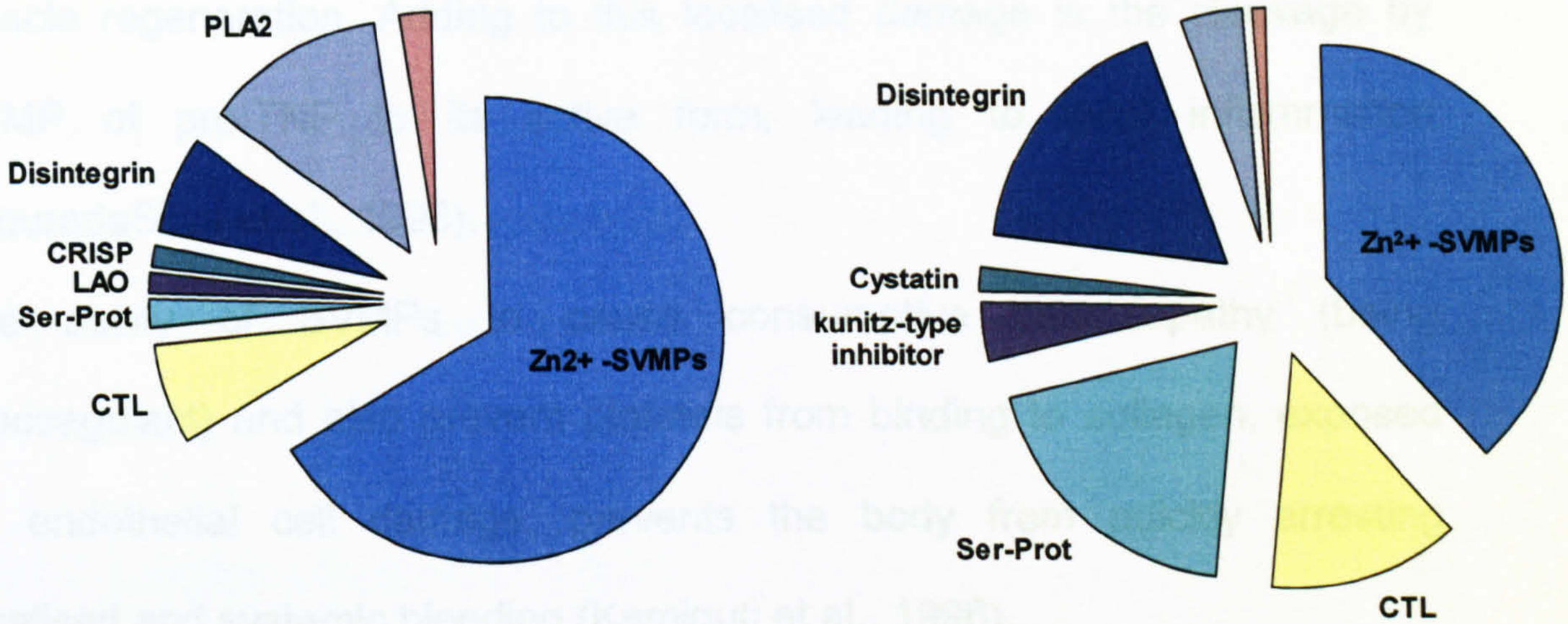

Figure 1.9 Composition of venom from two species of viper.

Composition of venom determined by proteomics for:

A) *E. ocellatus* (Wagstaff et al., 2009)

B) *B. arietans* (Juarez et al., 2006)

Zn^2+-metalloproteinase (SVMPs); LAO, L-amino acid oxidase; PLA2, phospholipase A_2; CRISP, cysteine-rich secretory protein; CTL, C-type lectin-like protein; Ser-Prot, serine proteinase.

1.5.1.1 Snake venom metalloproteinases (SVMPs)

SVMPs are responsible for a wide range of effects seen in victims of *E. ocellatus* bites, from local tissue destructive effects to systemic haemorrhaging (Howes et al., 2007). SVMPs range in size from 20 to 100 kDa and based on the presence of certain domains: have been classified into

four main groups: P-I, P-II P-III and P-IV (Gutierrez and Rucavado, 2000), with P-III being the most haemorrhagic.

Within minutes of a bite SVMPs will begin degrading the extracellular matrix and damaging the integrity of blood vessels through direct cytotoxic effects on endothelial cells (Kamiguti et al., 1996), leading to impairment of skeletal muscle regeneration. Adding to this localised damage is the cleavage by SVMP of pro-TNF to its active form, leading to local inflammation (MouradaSilva et al., 1996).

The ability of SVMPs to cause consumptive coagulopathy (being procoagulant) and also prevent platelets from binding to collagen, exposed by endothelial cell damage, prevents the body from quickly arresting localised and systemic bleeding (Kamiguti et al., 1996).

SVMPs are thought to be largely responsible for the local effects of envenoming, with the destruction of endothelial cells and direct myonecrotic action that can lead to permanent tissue loss (Gutierrez et al., 1995). Indeed, studies have shown that early injection of SVMP inhibitors can reduce the effects of local tissue damage and haemorrhage (Rucavado et al., 2000; Howes et al., 2007); SVMPs are a key component in the local effects present after viper envenoming.

1.5.1.2 Phospholipase A_2 (PLA_2)

Snake venom PLA_2 are small molecules of polypeptides that catalyse the hydrolysis of phopholipids. These enzymes have a wide range of pharmacological effects within the victim, including: cytotoxic, myotoxic, haemolytic, anticoagulant and also tissue damaging effects (Kini, 2005).

Some PLA_2s act on negatively charged Factor Xa inhibiting the formation of the prothrombinase complex, giving an overall anticoagulant effect (Kini, 2005), whilst others hydrolyse phospholipids on platelets, leading to a release of factors that cause aggregation (Perales et al., 2005). The myotoxic action of some PLA_2s is initiated by binding to the muscle sarcolemma, leading to breakdown of the plasma membrane (Villalobos et al., 2007). Inflammation can be triggered by PLA_2, characterised by increased microvascular permeability, leading to oedema formation and release of inflammatory mediators (Koh et al., 2006),

1.5.1.3 L-Amino acid oxidase (LAOs)

LAOs are widely found in snake venoms and are able to induce apoptosis in endothelial cells by the production of H_2O_2 (Sakurai et al., 2001), leading to bleeding from blood vessels at the site of the bite. LAO's function with regard to platelet aggregation is not straightforward, with some LAOs inhibiting aggregation (Takatsuka et al., 2001), whilst others activate aggregation (Stabeli et al., 2004).

1.5.1.4 C-type lectins

C-type lectins (CTL) are a class of proteins that bind sugar moities in the presence of calcium ions, although many snake venom C-type lectins can in fact bind to proteins without the need to recognise specific carbohydrates (Lu et al., 2005). The action of CTL is to disrupt haemostasis; the mechanism by which disruption occurs is varied. Botrocetin and bitiscetin bind to von

Willebrand factor allowing binding to GPIb on the platelet, resulting in aggregation (Jennings et al., 2005). Other CTLs (e.g. echicetin) bind to GPIb itself, therefore preventing binding of von Willebrand factor and inhibiting aggregation. Another method by which venom CTLs inhibit coagulation is by binding to clotting factors IX and X, preventing platelet aggregation.

1.5.1.5 Hyaluronidase

Hyaluronidase cleaves the internal glycosidic bonds of some acid mucopolysaccharides in animal connective tissue, such as hyaluronic acid and chondritin sulphate (Yingprasertchai et al., 2003). This action reduces the viscosity of the hyaluronic acid, making the extracellular matrix more flexible and permeable (Harrison et al., 2007). This allows greater penetration of other toxins within the venom and leads to hyaluronidase being known as a 'spreading agent.' Inhibition of hyaluronidase in whole venom leads to a significant reduction in local events such as oedema, myonecrosis and leads to prolonged survival in mice (Yingprasertchai et al., 2003).

1.5.1.6 Serine proteases

Many serine proteases are fibrinogenolytic; resembling, in some part of their structure, that of thrombin. These proteases lead to the clotting of fibrin (Castro et al., 2004), thus using up important resources needed for coagulation. Serine proteases can also show fibrinolytic properties, breaking up fibrin clots and destroying lasting prevention of bleeding from damaged blood vessels (Swenson and Markland, 2005). A further process that serine

proteases use to disrupt the coagulation system is by converting plasminogen to its active form (plasmin), the main function of which is also to degrade fibrin clots (Zhang et al., 1995).

1.5.1.7 Disintegrins

These low molecular weight proteins have a high affinity for the platelet receptor GP IIb-IIIa, inhibiting the binding of fibrinogen to this glycoprotein and preventing fibrinogen-dependant platelet aggregation (Smith et al., 2002). Disintegrins can also block the binding of HUVEC to the extracellular matrix, preventing the repair of damaged blood vessels (Lu, Q. et al., 2005).

From these brief descriptions it can be seen that not only are there many pathways by which a toxin family act to cause pathology, but that many different toxins may effect the same system, requiring any treatment to be targeted against more than one family of toxins if it is to be successful.

1. 6 Treatment of snakebite in Africa

1.6.1 Traditional treatment.

Traditional treatments of snakebite remain popular, with 90% of victims seeking one traditional remedy and almost all of these people seeking a second traditional remedy (Newman et al., 1997) in some areas. At a study in rural Kenya, 68% of victims first sought a traditional remedy (Snow et al., 1994) and the lowest incidence of seeking medical aid occurred in *Naja nigricollis* victims: 8.5% (Pugh et al., 1980). Such traditional methods include

drinking a herbal emetic, addition of plant material to the wound, use of a tourniquet and local incisions. Traditional remedies remain very popular despite the fact that there is little or no proven efficacy. Indeed, it has been shown that the application of burnt bone commonly referred to as the black stone has no beneficial effect (Chippaux et al., 2007). Although several plant studies have shown that extracts of plants can inhibit the effects of venom in animal studies (Pithayanukul et al., 2005; Ode and Asuzu, 2006), none have shown that topical application of plant compounds after a snakebite is an effective treatment.

The popularity of traditional remedies may also be due to the fact that in cases of snakebite, 50% of cases may result in a 'dry' bite (Theakston et al., 2003): no venom is injected. Treatment of such dry bites by traditional methods may lead to the conclusion that the intervention has prevented symptoms of envenoming from developing, when in reality there was no venom injected into the victim. Untreated mortality figures due to snakebite can be 10-20% (Pugh and Theakston, 1980; Laing et al., 1995), so again any survival might mistakenly be attributed to the effects of traditional treatments. The only effective treatment for snake bite is the use of antivenom, reducing mortality from greater than 50% to less than 5% (Theakston et al., 2003).

1.6.2 History of antivenom

In 1887, Harry Sewall developed the idea of the first antivenom by demonstrating that increasing doses of venom administered to pigeons could protect against the effects of rattlesnake venom (Sewall, 1887). By 1895 Albert Calmette had shown that serum from an immunised horse could be

used to treat cobra envenoming in a human (Calmette, 1895; Lalloo and Theakston, 2003), leading to his production of the first commercial antivenom in 1898. Whilst these initial antivenoms used whole serum, in the 1930s Pope used ammonium sulphate precipitation to separate IgG from the remaining serum proteins.

Newer techniques of extracting IgG by caprylic acid fractionation appear to offer advantages over ammonium sulphate precipitation, by producing less non-IgG proteins, few aggregates and activating compliment to a lesser degree. This improved purity results in fewer adverse reactions to the administration of the antivenom: in one study preparation of antivenom by ammonium sulphate caused twice as many adverse reactions than preparation by caprylic acid (52% vs 25%) (Otero et al., 1999).

The use of papain or pepsin to produce fragments of the IgG molecule, Fab and $F(ab')_2$ respectively can also reduce the incidence of both early and late adverse reactions (Leon et al., 2001). Early reactions, anaphylactic (rare) or anaphylactoid, including bronchospasm and hypotension (Chippaux and Goyffon, 1998; Lalloo and Theakston, 2003) are thought to be due to the activation of complement and the effects of aggregates including the Fc portion. The removal of the Fc portion of IgG reduces complement activation (Leon et al., 2001) but does not prevent it entirely as the Fc portion and aggregates of this fragment may remain, since they can de difficult to remove completely after enzymatic treatments.

Late reactions, commonly called 'serum sickness' occur 1 to 3 weeks after antivenom administration and are typified by symptoms such as fever, urticaria, adenopathy, nephropathy and proteinuria (Chippaux and Goyffon,

1998). These develop when the patient produces antibodies directed against the antivenom IgG and although uncomfortable, such reactions are rarely considered serious. IgG fragments also produce lower anti-immunoglobulin titres in the patient than intact IgG (Leon et al., 2001) and are therefore expected to reduce the incidence of late adverse reactions. However, the removal of the Fc portion effects the pharmacokinetics of an antivenom with the clearance time of each of the different molecules effecting an antivenom's effectiveness. Elimination half-life of IgG, $F(ab')_2$ and Fab are 60-190 hours 18 hours and 4 hours respctively (Meyer et al., 1997). Rapid removal of Fab fragments from the circulation requires the use of repeated doses of Fab antivenoms to successfully treat snake envenoming (Ariaratnam et al., 1999; Boyer et al., 1999; Dart et al., 2001).

Removal of the Fc portion, whilst affecting an antivenom's elimination half-life does not guarantee reduction in the incidence of adverse reactions. Incidence of such reactions can range widely depending on the purification process of antivenom preparation irrespective of whether IgG, $F(ab')_2$ or Fab is used, leading some to conclude that properly purified IgG causes very few reactions other than mild discomfort (Stock et al., 2007). An antivenom of high purity and lacking the Fc portion, causes mild reactions to occur in less than 5% of all cases (Chippaux and Goyffon, 1998).

The importance of good quality antivenom was highlighted in the 1970s when supplies of the effective South African Institute for Medical Research (SAIMR) antivenom were limited and replacement with an ineffective Iranian antivenom lead to a fall in hospital attendances and a rise in snakebite mortality (Pugh et al., 1979). A steady supply of an effective and safe

antivenom is crucial to gaining confidence in the local communities affected by snakebite.

Whilst the technology to improve the safety and efficacy of antivenoms has improved the supply has dwindled from around 250,000 vials per year to less than 20,000 vials for the whole of Africa. The number of manufacturers reduced from three (Behringwerke, Aventis-Pasteur and South African Vaccine Producers (SAVP), formerly SAIMR) to just one (SAVP) which was unable to meet the continents estimated requirement of 1.5-2 million doses per year (Theakston and Warrell, 2000; Stock et al., 2007). Better quality of antivenoms leads to higher manufacturing costs, and with the rural poor unable to afford the equivalent of 4 months earnings for one ampoule of antivenom (Einterz and Bates, 2003), the attraction for multinationals and biotech companies to invest in production is very limited indeed.

Response to this crisis and the appeal to manufacturers, to use any spare capacity to produce antivenoms for this area, has been encouraging and three experimental antivenoms are currently undergoing trials in Nigeria. This should hopefully lead to a much needed increase in the supply of antivenom.

1.6.3 Local effects of envenoming

Despite antivenom being the only effective treatment for snakebite, it is not wholly effective at protecting against every aspect of damage induced by toxins in the venom. As a consequence, victims of snakebite may recover fully from the systemic effects of envenoming but suffer permanent disfigurement or disability. For every death due to snakebite, it is likely that several more survivors will suffer permanent damage, loss of mobility and even the requirement of removal of necrotic tissue or amputation (Theakston

et al., 2003; Stock et al., 2007). Local effects are typified by oedema, myonecrosis and local haemorrhage (figure 8) (Rucavado et al., 2000). Exact numbers of people who suffer permanent local damage are unknown, and the efficacy of treatments tends to be anecdotal.

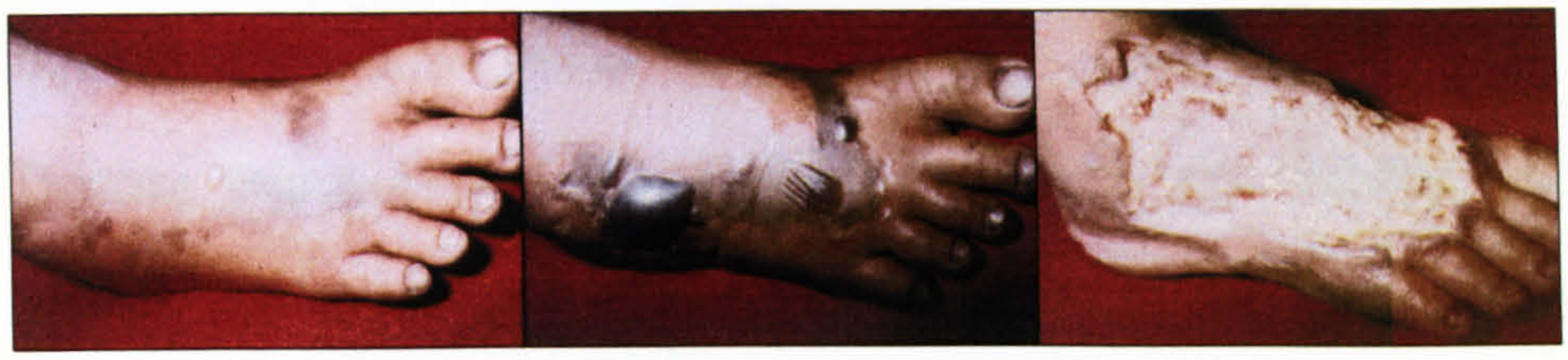

Figure 1.10 Local effects of envenoming:
oedema, blistering and finally removal of necrotic tissue

Although evidence is sparse, it is obvious from the literature that the treatment with antivenom does not completely prevent the local effects of envenoming (Warrell et al., 1976; Gutierrez et al., 1998) and seems unlikely to significantly reduce these effects, unless administered immediately after envenoming. It was hoped that the use of $F(ab')_2$ and Fab fragments would improve neutralisation, as the smaller size of the fragments would allow more rapid and greater penetration into tissues where the toxins were located. It has been shown that Fab fragments are indeed distributed more rapidly and with greater concentration into the extravascular spaces than $F(ab')_2$ and IgG when injected intramuscularly (Hammoudi-Triki et al., 2007), but have been shown to be either no more effective (Leon et al., 1997; Leon et al., 2000) or only slightly more effective (Latif et al., 2003) than IgG at neutralising local effects when injected intramuscularly in animal models. It has been shown that the pharmacokinetics of the venom of scorpions and snakes are significantly different than those for Fab (Ismail et al., 1998; Hammoudi-Triki et al., 2007). One study showed that after intramuscular injection, desert

cobra venom reached its maximum concentration in the tissue (t_{max}) within 60 minutes. For Fab fragments it took over 4 times longer to reach t_{max} (Ismail et al., 1998). It appears that despite the smaller size of the Fab fragment compared to intact IgG, they are still unable to diffuse quickly enough to neutralise the local effects of venom.

Despite this apparent failure, it is possible to significantly reduce the local effects of snakebite via an intramuscular injection. This has been demonstrated with the use of various different inhibitors and chelating agents such as; batimastat, EDTA (Rucavado et al., 2000) and other inhibitors directed against the action of SVMPs (Perales et al., 2005; Howes et al., 2007); or those directed against hyaluronidase (Yingprasertchai et al., 2003; Girish and Kemparaju, 2006). These experiments show that prompt intramuscular injection of inhibitors of a low molecular weight can significantly reduce the localised effects of envenoming, at least in animal models.

One unusual suggestion on reducing local effects of envenoming was the simple action of moving the bitten limb around (Blaylock, 2002), in mice this was found to significantly reduce these effects, probably due to the lymphatic or venous removal of toxins from the bite site. Whilst it is unclear whether this would accelerate the systemic effects of envenoming, it does at least indicate that the use of tourniquets would be deleterious.

1. 7 Camelid antibodies

In 1989, a project student working at free university in Belgium for a team led by Raymond Hamers, was looking at how camels fight off parasites. In one of the tests it was noticed that the camel seemed to have a class of antibodies

that were smaller than conventional IgG. By 1993 the preliminary investigations were completed and the results published: Camelids posses a unique class of IgG lacking light chains (Hamers-casterman et al., 1993) (Figure 1.11 Camelid IgG)

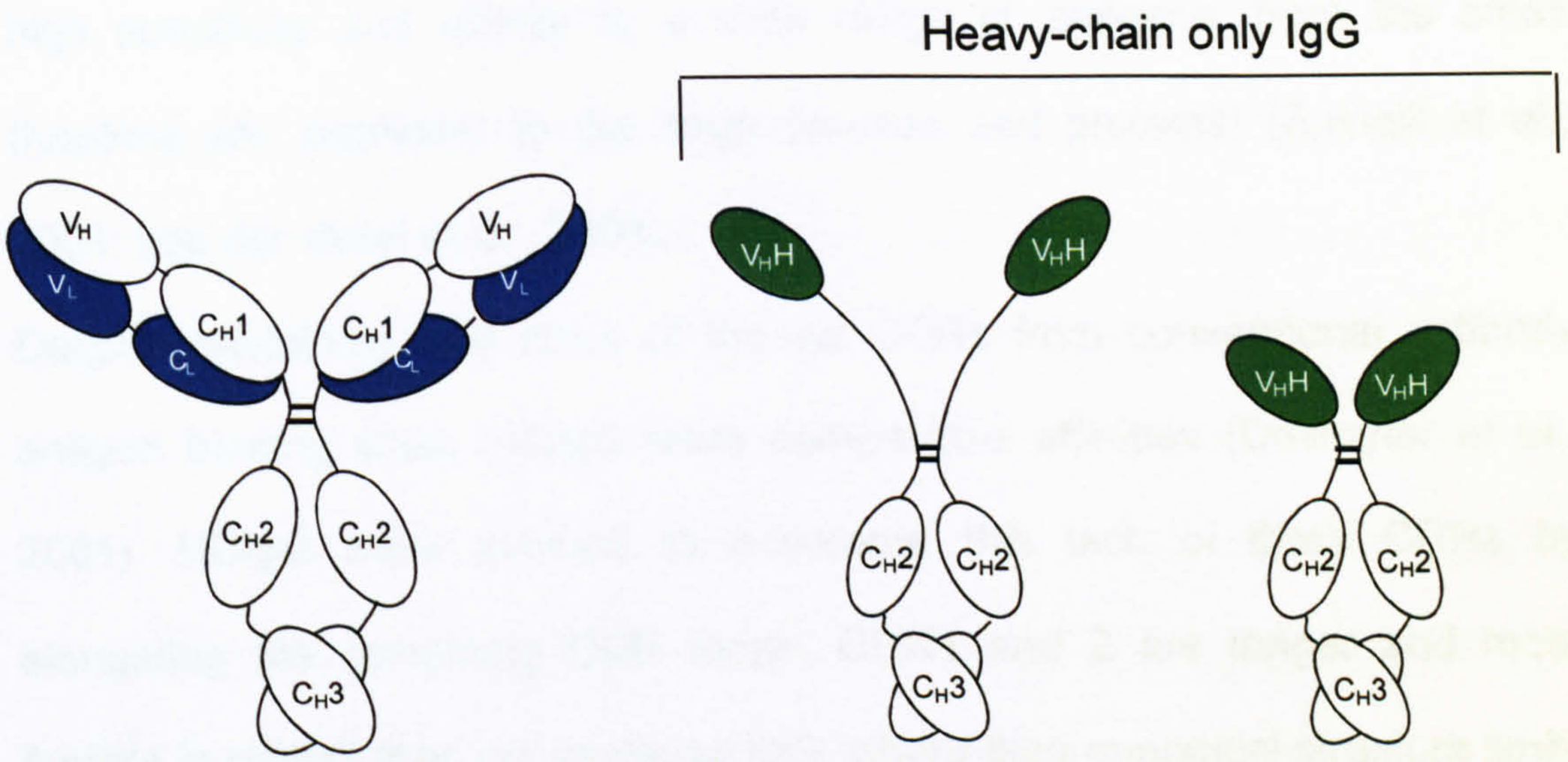

Figure 1.11 Camelid IgG

[adapted from (Hamers-casterman et al., 1993; Daley et al., 2005)]

Conventional antibodies are produced by B cells, with each cell producing antibodies specific for one epitope. Individual specificity is due to variations in the residues that form the loops within both the heavy and light regions towards the tip of each arm of the antibody, shown as V_L or V_H in Figure 1.11. These regions, known as complimentarity-determining regions (CDRs), are responsible for forming the unique shape that will bind a particular epitope. However, since the CDRs from both the V_L and V_H form the antigen binding site, then both heavy and light chains together are involved in binding, rather than individually.

From this, it would be expected that, since the unique camelid antibodies known as heavy-chain only IgG (HCIgG) lack the light chain, they would have a poorer ability to bind to epitopes.

This is in fact not the case, and it has been shown that HCIgGs can bind with high specificity and affinity to a wide range of antigens; from the small (haptens and peptides) to the large (viruses and proteins) (Spinelli et al., 2001; van der Vaart et al., 2006).

Despite containing only three of the six CDRs from conventional antibody antigen binding sites, HCIgG have comparable affinities (Desmyter et al., 2001). HCIgG have evolved to overcome this lack of three CDRs by elongating the remaining CDR loops. CDR1 and 2 are longer and more flexible in HCIgG than conventional IgG, where their canonical structure limits the number of possible conformations (De Genst et al., 2006) and CDR3 is also extended, providing a greater area for antigen binding and allowing for the recognition of a greater array of antigens. (Desmyter et al., 2001).

Conventional antibodies have an antigen binding surface that is either flat or concave, requiring flat or concave antigens. The CDR3 loop of the HCIgGs can protrude past the surface, enabling binding with clefts and cavities which are inaccessible to conventional antibodies. Such clefts and cavities are of biological significance, often creating the site of interactions such as those between enzymes and substrates or between ligands and receptors. It has been shown that the CDR3 loop can be shaped similarly enough to mimic the substrate and enable the VHH to become a potent competitive inhibitor of the relevant enzyme (Lauwereys et al., 1998; Revets et al., 2005).

The protruding binding site of the HCIgG also may allow recognition of epitopes that are densely packed and that conventional IgG would be unable to access. Both qualities are of interest when hoping to neutralise the effects of the many proteins and enzymes found in snake venom.

HCIgG has successfully been isolated from camels, Llamas, and most recently Alpacas (Maass et al., 2007). The ratio of conventional IgG to heavy chain IgG varies between the three species (Table 1.3) with Camels containing the greatest proportion of HCIgG.

Table 1.3 Percentage of conventional and heavy chain IgG typically found in camelids
[data from (Hamers-casterman et al., 1993; van der Linden et al., 2000; Maass et al., 2007)]

	Camel	*Llama*	*Alpaca*
Conventional	~25	55-75	~50
Heavy-Chain only	~75	25-45	~50

Why these animals have evolved this unique class of antibodies remains a mystery, and whilst immune responses generate antigen specific antibodies of both classes, it is likely that the HCIgG is able to recognise different epitopes to the conventional IgG. Indeed, studies show that the two classes of antibody can respond with different affinites to the same protein. It is likely therefore, that the HCIgG broadens the ability of camelids to recognise a greater range of epitopes and inhibit a greater number of enzymes (Lauwereys et al., 1998; van der Linden et al., 2000).

1.7.1 VHH

Most of the research surrounding camelid antibodies has not focused on the whole intact molecule of HCIgG but instead on the binding region referred to as VHH. This is due to the fact that VHH have many unique properties which may make them ideal for use in a wide range of applications.

VHH are the smallest known intact antigen binding unit. At 15 kDa they are smaller than Fab fragments (50 KDa) and ten times smaller than conventional IgG. This small size may allow for greater penetration into tissues and organs. Since VHH have evolved not to require attachment to light chains they are naturally more stable, being soluble and much less likely to form aggregates than the VH domains of conventional antibodies or single chain variable fragments (ScFv). VH from conventional IgG uses hydrophobic areas to interact with the CH1 and VL domains. When the VH is separated from these groups, the hydrophobic areas cause the fragment to become sticky, making VH much less effective (Revets et al., 2005).

This structural stability of VHH also extends to extremes of temperature and pH. VHH clones retained binding affinity and specificity during and after heating to 90°C (van der Linden et al., 1999). Similarly, a VHH clone has been developed to treat *E. coli* infection in pigs via oral application and has been shown to survive the harsh effects of low pH and proteolytic enzymes (Harmsen et al., 2006).

The simple, sturdy structure of VHH combined with the ease of production of recombinant forms has opened up the potential for improvements in many fields including: cancer screening (Cortez-Retamozo et al., 2002), cancer

treatment (Cortez-Retamozo et al., 2004), affinity chromatography (Klooster et al., 2007) and as an antiviral (Pant et al., 2006).

It is hoped that some of these properties may also aid the treatment of snake envenoming

1.7.2 Rationale for development of camelid antivenoms

Most of the research into camelid HCIgG has focused on the properties of recombinant VHH. However, there may be properties of the intact HCIgG that are favourable to developers of antivenom against snake envenoming:

1.7.2.1 Lower immunogenicity

Camel IgG (containing both conventional and HCIgG) was compared with IgG prepared from horses and sheep in a number of experiments to determine immunogenicity (Herrera et al., 2005). To test the likelihood of causing early adverse reactions (EAR) upon the administration of either one of these antivenoms, each species' IgG was incubated with human plasma in order to determine the level of complement activation (thought to play a key role in EAR). Both the horse and the sheep IgG caused complement activation whilst the camel IgG did not.

Another mechanism proposed to initiate EAR is the sensitisation of humans to various animals, through close contact, which allows the person to develop antibodies against the animal. When human IgG was added to ELISA plates containing the antivenoms, horse and sheep IgG again showed a much greater response than camel IgG.

In order to examine late onset reactions known as serum sickness, researchers injected the three antivenoms into mice and measured the production of mouse IgG that reacted with the foreign IgG. The results showed that camel IgG generated a significantly smaller response than both horse and sheep IgG.

These results show that camel IgG has the potential to reduce the incidence of both early and late adverse reactions in patients administered antivenom.

1.7.2.2 Heat stability

The heat stability of recombinant VHH is very impressive (Goldman et al., 2006); (Omidfar et al., 2007), retaining the ability to bind when heated to 90°C (van der Linden et al., 1999). However, the ability of the intact native HCIgG to withstand heat treatment is unknown. This is an important area that requires investigation, because a heat-stable antivenom would confer two advantages:

1) Safety

There is a theoretical risk of zoonotic transfer of diseases from the immunised animal to the patient. Whilst many viruses are destroyed by caprylic acid precipitation of serum during production (Burnouf et al., 2004), the ability to heat treat HCIgG (coupled with low pH) would greatly improve the safety of the antivenom. Such a step may also reduce the need for further, more complex, purifications and may help to reduce the price of treatment.

2) Distribution and shelf life

Certain clones of camel VHH have displayed one hundred percent binding affinity after incubation for one week at 37°C (Ghahroudi et al., 1997). It is worthwhile investigating if such a property exists in the native HCIgG as this could lead to the possibility of an antivenom that requires neither refrigeration or lyophilisation. A room-temperature stable antivenom would allow greater distribution to clinics with no refrigeration, making the medicine more available to the rural areas where snakebite is more common, thus reducing the travel time for patients (which can sometimes be days) and improving their prognosis.

It has already been shown that immunisation of camels with a small, weakly antigenic but potent scorpion neurotoxin, enabled the isolation of immunoglobulins that offered good neutralising abilities (Meddeb-Mouelhi et al., 2003). It is therefore hoped that such a strategy would also provide an effective camel derived antivenom for treatment of envenoming by the three most medically important species of snake in West Africa. It has already been demonstrated that camels raise an immune response against *E. ocellatus* venom and that IgG extracted from this offers a protective effect in animal models (Harrison et al., 2006). Furthermore, following the good response to a poor immunogen in the scorpion study, it is of interest as to whether a camel could mount a better response to *N. nigricollis* venom, which has historically produced poor antibody titres in horses. Developing an antivenom which is obtainable from camels may one day allow antivenom production to occur in countries affected by snakebite, which also have easy access to these animals.

1.7.2.3 Treating the local effects of envenoming

The administration of certain inhibitors or chelators soon after injection of venom in animal models reduces the local effects of envenoming (Rucavado et al., 2000; Yingprasertchai et al., 2003; Perales et al., 2005; Girish and Kemparaju, 2006; Howes et al., 2007). Such agents are indiscriminate in their action, and may cause further problems to the patient. Specific antigen binding antibodies that neutralises the range of toxins would be a safer approach that would recognise the many different toxins involved in causing local pathology. The failure of Fab fragments to penetrate tissue rapidly enough to be effective would seem to have ended such a hope, until the discovery of VHH.

VHH are smaller than Fab fragments (by a factor of three) and have even been shown to penetrate the blood-brain barrier (Muruganandam et al., 2002). This raises the potential that administration of VHHs may be able to penetrate tissues quickly enough to neutralise specific snake venom toxins and the ability of VHH to bind to the active sites of enzymes (De Genst, Erwin et al., 2006) may also expedite the process. If a suitable method of transdermal delivery can be found, toxin specific VHH may be capable of neutralising the causative agents of local pathology and perhaps also the toxins responsible for the systemic effects of envenoming, enabling the patient more time to travel to a clinic and receive the proper antivenom. It is also likely that side effects would be minimal with one study showing that when used as a therapy in mice, the mice did not generate a specific IgG response to the VHH (Coppieters, 2006).

1. 8 Aims of this study

It is the aim of this study to:

(i) Examine the immune response of camels immunised with either a single venom or multiple venoms from the three most medically important snakes of West Africa in a range of serological assays.

(ii) Assess the neutralisation of snake venom pathology by camel IgG and compare the effectiveness to current, clinically effective antivenoms.

(iii) Determine which assays are predictive of the likely preclinical *in vivo* performance of IgG from individual animals and therefore discover how to detect poor responding animals before their sera is used for antivenom production, without using the large numbers of mice required for *in vivo* assessment.

(iv) Investigate the role of IgG subclasses in the immune response of camels to venom immunisation and discover whether a particular subclass is responsible for neutralising a certain venom pathology

(v) Elucidate the thermostability of each IgG subclass and assess whether this may permit the pasteurisation of a camel-derived antivenom without loss of activity or formation of aggregates

(vi) Generate VHH and examine its venom neutralising abilities in preclinical assays as a first step in validating that VHH may prove useful in the treatment of the local effects of envenoming.

CHAPTER 2

Materials and methods

Methods specific to a chapter are detailed in the materials and methods section of that particular chapter. However, because of the publication manuscript format of this thesis, more detailed methodologies, which could not be permitted in specific chapters, are described in this chapter.

2. 1 General ELISA protocol

For one 96 well plate: 10 µl of 1 mg/ml solution of specific venom was added to 10 ml of 0.05 M carbonate coating buffer. 100 µl of this solution was added to each well of a 96 well ELISA plate (Immunosorb, Nunc, UK), giving a final venom concentration of 100 ng/well and incubated overnight at 4^0C. Wells were washed with 6 changes of TBST [0.01 M Tris-HCl, pH 8.5; 0.15 M NaCl; 0.1% Tween 20] then blocking solution was prepared by adding TBST to 0.5g of fat-free dried milk (Marvel, UK), to a final volume of 10 ml. 100 µl per well was added and incubated at room temperature for 3 hours. Non-bound material was removed by washing with 6 changes of TBST. 10 µl of test sera or primary antibody (usually at 10 mg/ml) was added to 10 ml of blocking solution (5% dried milk in TBST) giving a 1:1000 dilution: 100 µl of which was added per well and incubated at 4^0C overnight. Plates were washed with 6 changes of TBST. 20 µl of rabbit anti-camel IgG (in house preparation, at 1:100 dilution) was added to 10 ml of TBST (final dilution = 1:5000), 100 µl was added per well and incubated for 2 hours* at room temperature. Again, 6 changes of TBST removed unbound material, then 5 µl of horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma, UK) was added to 10 ml (1:2000 dilution) and 100 µl added to each well before incubating at room temperature for 2 hours*. After washing with 6 changes of TBST, the results

were visualised by adding substrate (0.2% 2,2-azino-bis (2-ethylbenzthiazoline-6-sulphonic acid), in phosphate-citrate buffer pH 4 with 0.0015% hydrogen peroxide; Sigma, UK) and absorbance read at 405nm in an ELISA plate reader.

*For ELISAs using IgG as the primary antibody, the 2 hour incubations were reduced to 1 hour.

2. 2 Chaotropic ELISA

The general ELISA protocol was undertaken but with the following additional step after TBST washing of overnight test sera / primary antibody incubation: ammonium thiocyanate solution was prepared at 1, 2, 4, 6, and 8 M in PBS. Wells in row 2 of the ELISA plate contained 100 µl of TBST as a control. The various concentrations of ammonium thiocyanate were added quickly to the plate, one dilution per row and 100 µl per well. The plate was incubated for 15 minutes at room temperature before removal of thiocyanate by 6 changes of TBST. The secondary antibody was then added and the remainder of the general ELISA protocol was followed. Relative avidity was determined as the concentration of ammonium thiocyanate required to reduce the absorbance to 50%.

2. 3 Immunoblotting protocol

110 µg of the relevant venom was prepared with 2x protein loading buffer (0.15 M Tris-HCl pH 6.8; 1.2% SDS; 30% Glycerol; 15% β-mercaptoethanol) to a final volume of 110 µl and boiled for 10 minutes. This was added to a

single preparative well* 15% SDS-PAGE gel under reducing conditions and run for ~50 minutes at 200 mA 400 mV 100 W. Proteins were transferred for 1 hour with constant stirring (25 mM Tris Base; 190 mM Glycine; 20% Methanol) to nitrocellulose paper, the filters were reversibly stained with Ponceau S (2% Ponceau S; 30% trichloroacetic acid; 30% Sulfosalicylic acid) to confirm successful transfer of the proteins from the gel onto the paper, the nitrocellulose paper was cut into equal strips and incubated in blocking buffer (2.5 g dried-milk in 50 ml TBST) overnight at 4°C with gentle agitation. After washing for 1 hour with 3 changes of TBST, the strips were incubated in the relevant sera or IgG preparation (10 µl in 10 ml of blocking buffer giving a 1:1000 dilution) overnight at 4°C with gentle agitation. Buffer was carefully discarded and strips washed for 1 hour with 3 changes of TBST. The strips were then incubated with rabbit anti-camel IgG (non-commercial preparation, 100 µl in 50 ml of TBST giving a final dilution of 1:5000) for two hours at room temperature. Strips were washed for one hour with 3 changes of TBST before incubation for 1 hour with goat anti-rabbit IgG (25 µl in 50 ml TBST giving 1:2000 dilution) at room temperature. After washing with TBST (3 changes) strips were developed using DAB (50 mg 3,3-diaminobenzidine, 100 ml PBS and 0.024% hydrogen peroxide). Water was used to stop the reaction after sufficient development.

*For immunoblots of IgG preparations 7µg of *E. ocellatus* venom in reduced protein loading buffer was added to each well of 15% SDS-PAGE (BioRad Protean II) with the final well containing the molecular weight marker (Broad range molecular weight protein markers, Promega).

2. 4 Protein G chromatography

The IgG sample was diluted to 5 mg/ml using sodium phosphate buffer (pH 7.4) and passed through a 0.2 nm filter to remove any particulates. 5 ml of IgG solution was loaded on to the 5 ml loop of the Akta prime plus (GE healthcare). Sodium phosphate buffer (pH 7.4) was primed at 40 ml/min for 35 ml before equilibrating the 16/20 column containing 6 ml of protein G Sepharose (GE healthcare) at 2 ml/min for 60 ml. After equilibration, sample was injected on to the column at 2 ml/min with 60 ml. During this process, 5 ml fractions were collected after 5 ml for a total of 25 ml and contained IgG2. Elution buffer pH 3.5 (0.15 M NaCl, 0.58% acetic acid) was primed at 40 ml/min with 35 ml before using 30 ml at 2 ml/min to elute IgG3 from the column and collect in 5 ml fractions. 300 µl of 2.5 M Tris (pH 9) was added to neutralise each fraction. 15 ml of Sodium phosphate buffer was primed and the column was washed with 30 ml at 2 ml/min. A second elution buffer (0.1 M glycine-HCl, pH 2.7) was primed at 40 ml/min with 35 ml, then IgG1 was eluted with 30 ml at 2 ml/min. Collected 5 ml fractions were neutralised using 150 µl of 2.5 M Tris (pH 9). Finally, sodium phosphate buffer was primed as previously described and 60 ml at 2 ml/min was used to equilibrate the protein G sepharose column. 20% ethanol was used as a preservative if the column was not to be used immediately again.

2. 5 Protein A chromatography

Exactly the same method was used for Protein A chromatography as previously described for Protein G chromatography except that only one elution was performed using pH 2.7 elution buffer.

2. 6 Buffers and solutions

ELISA

Carbonate coating buffer: 1.59 g Na_2CO_3

2.93 g Na_2HCO_3

Up to 1 litre with ddH_20

Citrate buffer: 525 mg Citric acid

Make up to 50 ml ddH_2O

pH adjusted with 10 M NaOH to pH 4

Substrate buffer: 1 tablet of ABTS dissolved in 670 µl ddH_2O

200 µl ABTS solution

10 ml Citrate buffer

10 µl H_2O_2

SDS-PAGE

15% separating gel: 3.75 ml ddH_2O

2.5 ml Tris pH 8.8

3.75 ml 40% bis-acrylamide

100 µl 10% SDS

60 µl 10% Ammonium persulphate (APS)

7 µl Temed

Stacking gel: 5 ml ddH_2O

2 ml Tris pH 6.8

750 µl 40% bis-acrylamide

100 µl 10% SDS

60 µl 10% Ammonium persulphate (APS)

10 µl Temed

2x PLOB: 10 ml 1.5 M Tris (pH 6.8)
6 ml 20% SDS
30 ml glycerol
1.8 mg bromophenol blue
Make up to 100 ml with ddH_2O

For reducing PLOB: add 15 µl β-mercaptoethanol to 85 µl of the above recipe

SDS-PAGE running buffer: 151 g Tris base
720 g Glycine
50 g SDS
Make up to 10 litres with ddH_2O

Coomassie blue: 2 mg CBB-R250
450 ml Methanol
100 ml Acetic acid
Make up to 1 litre with ddH_2O

Destain: 4.5 L Methanol
1 L Acetic acid
Make up to 10 litres with ddH_2O

<u>Immunoblotting</u>

Transfer buffer: 6 g Tris base
28.8 g Glycine
400 ml Methanol
Make up to 2 litres with ddH_2O

Ponceau S solution: 2 g Ponceau S

30 g Trichloroacetic acid

30 g Sulfosalicylic acid

Make up to 100 ml with ddH_2O

TBST: 10 ml Tween 20

50 ml 2 M Tris base (pH 8.5)

300 ml 5 M NaCl

Make up to 10 litres with ddH_2O

10% Blocking buffer: 10 g skimmed milk powder (marvel)

Made up to 100 ml with TBST

DAB: 50 mg DAB

100 ml PBS

25 µl H_2O_2

Chromatography

Sodium phosphate buffer: 19 ml 0.2 M NaH_2PO_4-$1H_20$

(pH 7.4) 81 ml 0.2 M Na_2HPO_4-$7H_20$

Make up to 1 litre with ddH_2O

Elution buffer pH 3.5: 8.76 g NaCl

5.8 ml of 100% Acetic acid

Make up to 1 litre with ddH_2O

Elution buffer pH 2.7: 7.51 g of Glycine

Adjust pH with 10 M HCl

Make up to 1 litre with ddH_2O

CHAPTER 3

Title: Analysis of camelid IgG for antivenom development: Serological responses of venom-immunised camels to prepare either monospecific or polyspecific antivenoms for West Africa.

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Abbreviated title: Camelid IgG for antivenom development

Key words: Snake Antivenom, Camelid IgG, *Echis ocellatus, Bitis arietans, Naja nigricollis*

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3. 1 Abstract

Snake envenoming is a significant cause of mortality and morbidity in sub-Saharan Africa. The only effective treatment, antivenom, has been in short supply since the 1990s. Whilst the humanitarian response by some antivenom producers has significantly improved the situation, strategies to ensure the long term stability of antivenom supply are still necessary. We are investigating whether the potential safety and logistic advantages of camel IgG antivenom can be exploited to improve antivenom provision in many countries where snakebite is endemic. This study assessed the IgG titre, specificity and avidity of camels immunised with either individual venom or a mixture of venoms from the three most medically important snakes of West Africa, the saw-scale viper (*Echis ocellatus*), the puff adder (*Bitis arietans*) and the spitting cobra (*Naja nigricollis*).

Seven of the eight immunised camels generated IgG titres and avidities comparable to, or exceeding, that of commercial equine and ovine antivenoms that are highly effective in envenomed patients. In this the first of a series of reports on the potential utility of camelid IgG antivenom, we describe an immunisation protocol that induced potent, sustained serological response of very high antibody avidity. These attributes suggest, from an immunological perspective, that camel IgG antivenoms should be as efficacious as current equine and ovine antivenoms.

3. 2 Introduction

Snake envenoming is a significant cause of global mortality and morbidity, and a particular burden on the rural poor communities of Asia and Africa. The situation in sub-Saharan Africa, with an estimated maximum of 420,000 envenomings and 32,000 deaths every year, indicates the scale of the problem (Kasturiratne et al., 2008). In West Africa the annual incidence of snake envenoming is high, with reports of snakebite incidence rates as high as 497 per 100,000 people in the Benue Valley, Nigeria, (Warrell and Arnett, 1976) and possibly accounts for more than half of the number of total snakebite deaths in sub-Saharan Africa (Kasturiratne et al., 2008). Agricultural workers, because of their non-mechanised farming methods, are most affected by envenoming, which presents a daily occupational hazard. In periods of peak snakebite incidence, the majority of hospital beds are often occupied by snakebite victims (70% in Ougadougou, Cameroon (Revault, 1996); and 90% in Zamko, Nigeria (RAH, personal observation)). Many of those that survive envenoming suffer permanent physical, and perhaps psychological, impairment which reduces their ability to work. This, in turn, has a negative economic effect upon the immediate family and the wider community and also poses a significant fiscal burden on regional health centres.

The only effective treatment of systemic snake envenoming is antivenom. However, because of the combined effects of Africa's reliance upon commercial antivenom production, the high cost of antivenom manufacture, weak governmental demand and consequent poor financial incentives for commercial production, Africa suffered a crisis in antivenom supply in the

1990s (Theakston and Warrell, 2000; Stock et al., 2007). In response to the consequent increase in snakebite mortality and morbidity (Theakston and Warrell, 2000) antivenom producers in Costa Rica, Columbia and Wales agreed to use their spare manufacturing capacity to provide antivenoms for Africa (Laing, G. D. et al., 2003; Theakston et al., 2003; Gutierrez et al., 2005). This important humanitarian response to a medical crisis in Africa emphasises the need to ensure a long term, stable supply of antivenom to those countries where snake envenoming is endemic.

Antivenoms currently manufactured for Africa are derived from horses or sheep, animals that are not well-suited to the arid climatic conditions of much of sub-Saharan Africa. From a logistical perspective camels represent an attractive alternative for antivenom production in these regions: they are well adapted and hence probably cheaper to maintain under arid conditions than either horses or sheep; they are as facile to handle, immunise and bleed as horses; the yield of blood is similar to that from a horse. From a scientific perspective, the unique physicochemical properties of camelid IgG offer intriguing possibilities to improve the clinical effectiveness of antivenom treatment (Harrison and Wernery, 2007). Camel IgG is less immunogenic and less likely to activate the complement cascade than ovine or equine IgG (Herrera et al., 2005), suggesting that patients treated with camelid IgG antivenom will suffer less from the anaphalactoid and serum sickness adverse effects that are often associated with current antivenom treatment. It is also possible that the unusual thermostability of camelid IgG (Omidfar et al., 2007) can be exploited to prepare antivenom that remains efficacious after room temperature storage – an attribute which would significantly

extend the supply chain of snakebite treatment in areas of Africa remote from the provision of electricity. We are also examining whether the 15 kDa antigen binding domain (VHH) of the unique heavy chain-only IgG subclasses (Lauwereys et al., 1998; van der Linden et al., 1999) can be exploited to develop, for the first time, a treatment of the tissue-destructive effects of snake envenoming (Harrison and Wernery, 2007).

To experimentally assess the potential of camelid IgG antivenom to improve efficacy and logistics of snakebite treatment, we first conducted a study to determine whether there was any immunological reason to select one species of camelid over another for snake antivenom production. We determined that the IgG response to immunisation with venom (*Echis ocellatus*, the saw-scale viper) and ability to neutralise venom-induced pathology was very similar in dromedary camels and llamas (Harrison et al., 2006). Other reports of the venom-neutralising efficacy of IgG from camels immunised with scorpion toxins (Meddeb-Mouelhi et al., 2003; Hmila et al., 2008), llamas immunised with South American snake venoms [Gutierrez, personal communication] and camels immunised with Indian viper venoms [Tanwar, personal communication] indicate that there is a growing awareness of the potential advantages of camelid IgG antivenom.

Encouraged by the results of our 2006 study, we sequentially present here the results of three studies examining the (i) IgG titre, and avidity, (ii) IgG neutralisation of venom-induced pathology and (iii) camel IgG subclass responses of dromedary camels immunised with either a mixture of venom from the most medically-important snake species in Nigeria; (*E. ocellatus*, *Bitis arietans* (puff adder) and *Naja nigricollis* (spitting cobra)) to generate a

polyspecific antivenom appropriate to West Africa, or immunised with individual venoms from these snakes to generate three monospecific antivenoms.

3. 3 Materials and methods

3.3.1 Animals

Snakes and snake venom: specimens of *E. ocellatus*, *B. arietans* and *N. nigricollis* of mixed age and sex collected from Nigeria were maintained in the herpetarium of the Alistair Reid Venom Research Unit at the Liverpool School of Tropical Medicine. Venom was extracted from the snakes, frozen, lyophilised and stored at 4°C as a powder.

Dromedary camels: animals of mixed age and sex with no previous history of disease or immunisation were maintained in the Central Veterinary Research Laboratory, Dubai for this project. The health of the camels was monitored daily and blood chemistry analyses performed as a routine. Blood taken from the jugular vein was left at room temperature to clot and sera collected by centrifugation and stored at -20°C.

3.3.2 Venom immunisation protocol and sera collection

Five camels were immunised with a mixture of venom from Nigerian *E. ocellatus, B. arietans* and *N. nigricollis* to prepare a polyspecific antivenom. These camels were termed polyspecific (PS) and numbered 1-5. Three additional camels were immunised with venom from a single snake species to prepare three distinct monospecific antivenoms: camel Eo-MS was immunised with *E. ocellatus* venom only; camel Ba-MS was immunised with *B. arietans* venom only, and camel Nn-MS was immunised with *N. nigricollis* venom only. Venom powder of the required amount (either mixed in a 1:1:1 ratio for the PS camels or prepared as single venom samples) was re-suspended in 1ml sterile PBS, an equal volume of adjuvant added (and

manually emulsified in case of the Freund's adjuvants) and administered in four equal volumes, by subcutaneous injection, into two sites either side at the base of the neck; locations selected on the basis of their proximity to major local draining lymph nodes.

In attempt to avoid the local inflammatory reactions associated with the Freund's adjuvants (Angulo et al., 1997), the first 3 immunisations were administered with GERBU a water based adjuvant (GERBU Biochemicals GmbH, Gaiberg, Germany) that does not form an emulsion. However, owing to signs of medical distress following the 2nd and 3rd immunisations that we assumed were associated with the rapid in situ distribution of venom, all subsequent immunisations were administered as slow-releasing emulsions in Freund's adjuvants. The amount and timing of venom immunisation and the adjuvant used for each immunisation is described in Table 3.1. Blood samples were taken from the jugular vein of each camel 2 weeks after each immunisation. The blood was allowed to clot at room temperature (RT), centrifuged and sera collected and stored at -20°C.

3.3.3 Immunoassays

3.3.3.1 General ELISA procedure

100 ng/well of specific venom was prepared in coating buffer (formulation) and incubated overnight at 4°C in 96 well plates (Immunosorb, Nunc, UK). 100 µl blocking buffer (5g fat-free dried milk (Marvel, UK) in 100 ml TBST [0.01 M Tris-HCl, pH 8.5; 0.15 M NaCl; 0.1% Tween 20]) was added to the wells for 3 hours at RT and non-bound material removed by washing with 6

changes of TBST. Test sera (100µl at 1:1000 dilution in blocking buffer) were added to each well and incubated overnight at 4°C. After washing in TBST, plates were incubated with rabbit anti-camel IgG (in-house preparation) at 1:5000 for 2 hours. The plates were washed in TBST and then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma, UK) at 1:2000 for 2 hours. After washing, the results were visualised by adding substrate (0.2% 2,2-azino-bis (2-ethylbenzthiazoline-6-sulphonic acid), in phosphate-citrate buffer pH 4 with 0.0015% hydrogen peroxide; Sigma, UK) and absorbance read at 405nm in an ELISA plate reader.

3.3.3.2 Time course ELISA

The IgG response of each camel during the course of the venom immunisation was assessed by ELISA, with each assay being performed in triplicate against the three venoms using the general ELISA procedure.

Final IgG titre to venom: The IgG titre of each camel was assessed by an end point titration ELISA performed on sera collected at week 64. The general ELISA procedure was used on sequentially diluted (1:5) camel serum starting at a 1:250 dilution in blocking buffer. The titre is described as the dilution at which absorbance matched that of the negative control (un-immunised camel) plus 2 standard deviations.

3.3.3.3 Chaotropic ELISA

The strength of the antibody-antigen interactions was determined using a chaotropic ELISA method (MacDonald et al., 1988), whereby camel sera (1:1000 in blocking buffer) from week 64 was incubated in venom-coated

ELISA plates overnight at 4°C. After washing, ammonium thiocyanate was added to the wells in a range of concentrations (from 0 to 8 M) for 15 minutes. Plates were washed, and all subsequent steps were the same as the general ELISA. Relative avidities are calculated as the concentration of ammonium thiocyanate required to remove 50% of the bound antibody.

3.3.3.4 Time course Immunoblotting

The specificity of the IgG response of individual camels to individual venom proteins throughout the course of the 64 week immunisation period was assessed by immunoblotting. 110 µg of the relevant venom, prepared in 2x protein loading buffer (0.15 M Tris-HCl pH 6.8; 1.2% SDS; 30% Glycerol; 15% β-mercaptoethanol) was boiled and fractionated using a single preparative well 15% SDS-PAGE gel under reducing conditions. After transfer (25 mM Tris Base; 190 mM Glycine; 20% Methanol) to nitrocellulose paper, the filters were reversibly stained with Ponceau S (2% Ponceau S; 30% trichloroacetic acid; 30% Sulfosalicylic acid) to confirm successful transfer of the proteins from the gel onto the paper, the nitrocellulose paper was cut into strips and incubated in blocking buffer overnight. After washing in TBST, the strips were incubated in the relevant sera (1:1000 dilution in blocking buffer) overnight at 4°C with gentle agitation. All the washing and antibody incubation steps were identical to the ELISA protocol. The substrate used in this case was DAB (50 mg 3,3-diaminobenzidine, 100 ml PBS and 0.024% hydrogen peroxide).

Controls for the single and multiple venom-immunised camels were EchiTAb G (an ovine IgG antivenom monospecific to *E. ocellatus*; MicroPharm, UK

[Landon, personal communication]) and EchiTAb-Plus-ICP (an equine IgG polyspecific antivenom prepared against *E. ocellatus, B arietans* and *N nigricollis*; Instituto Clodomiro, University of Costa Rica; (Gutierrez et al., 2005)). The secondary antibodies employed with these African antivenoms were horseradish peroxidise conjugated donkey anti-sheep and rabbit anti-horse antibodies (Sigma, UK), respectively. These assays therefore required one antibody detection step compared to the two step procedure required for the camel assays. Sera or IgG from *E. ocellatus* venom-immunised camels or llamas from our preliminary study (Harrison et al., 2006) were also used to compare the camelid responses of the previous and present experiments. Sera or IgG from a non-immunised camel was employed throughout as a negative control.

3. 4 Results

The camels remained in good health throughout the 64 week study, with the exception of the brief period associated with the 3rd immunisation with the non-emulsion GERBU adjuvant. The local inflammatory reaction to immunisation with Freund's Complete and Incomplete adjuvant were moderate-substantial and therefore broadly similar to that observed in horses used for antivenom production (Angulo et al., 1997).

Table 3.1 Camel immunisation protocol detailing the amount of venom administered and the adjuvant used.

		Venom for immunisation (mg)				
Week	**Imm'n no.**	**Mix**	**Eo**	**Ba**	**Nn**	**Adjuvant**
0	1º	1.5	0.5	0.5	0.5	GERBU
3	2º	1.5	0.5	0.5	0.5	GERBU
6	3º	6.0	2.0	2.0	2.0	GERBU
11	4º	1.5	0.5	0.5	0.5	FCA
14	5º	1.5	0.5	0.5	0.5	FIA
17	6º	6.0	2.0	2.0	2.0	FIA
20	7º	12.0	4.0	4.0	4.0	FIA
23	8º	12.0	4.0	4.0	4.0	FIA
26	9º	12.0	4.0	4.0	4.0	FIA
35	10º	12.0	4.0	4.0	4.0	FIA
43	11º	12.0	4.0	4.0	4.0	FIA
47						
51	12º	12.0	4.0	4.0	4.0	FIA
56						
60	13º	12.0	4.0	4.0	4.0	FIA
64						

FCA = Freund's complete adjuvant
FIA = Freund's incomplete adjuvant

3.4.1 IgG titre and specificity analysis

Time Course Assays: The time course ELISA (Figure 3.1) and immunoblotting (Figure 3.2) demonstrate that the single and multiple venom-immunised camels responded to immunisation with satisfactorily high IgG

titres and broad venom protein-specificity. The *E. ocellatus* immunised camel (Eo-MS) showed the most rapid response to venom-immunisation, a result consistent with our previous study (Harrison et al., 2006). The two other single venom-immunised camels (Ba-MS and Nn-MS) also generated high titre responses to respective immunisation with *B arietans* and *N nigricollis* venom, albeit with a slightly delayed profile in comparison with *E. ocellatus* venom immunisation. There was evidence of extensive immunological cross-reactivity in the viper venom-immunised camels; thus in both the ELISA and immunoblotting time course assays Eo-MS sera showed substantial reactivity to *B. arietans* venom as did Ba-MS sera to *E. ocellatus* venom. The peak in Ba-MS reactivity to *N. nigricollis* venom is considered a technical anomaly and not a true cross-reactivity between the viper and elapid venoms. Nn-MS showed no reactivity to either *E. ocellatus* or *B. arietans* venom above that of background (negative control ELISA and immunoblot – data not shown).

The time course ELISA response of PS1-5 to each of the three venoms was similar, and also similar to the response of the single venom-immunised camels to the respective venom. The only notable difference between the multiple and single venom-immunised animals was that the titre of PS1-5 sera to *E. ocellatus* venom took longer to reach a plateau than sera from Eo-MS. The maturation of the IgG titre to *B. arietans* and *N. nigricollis* venoms was indistinguishable between sera of PS1-5 and Ba-MS and Nn-MS.

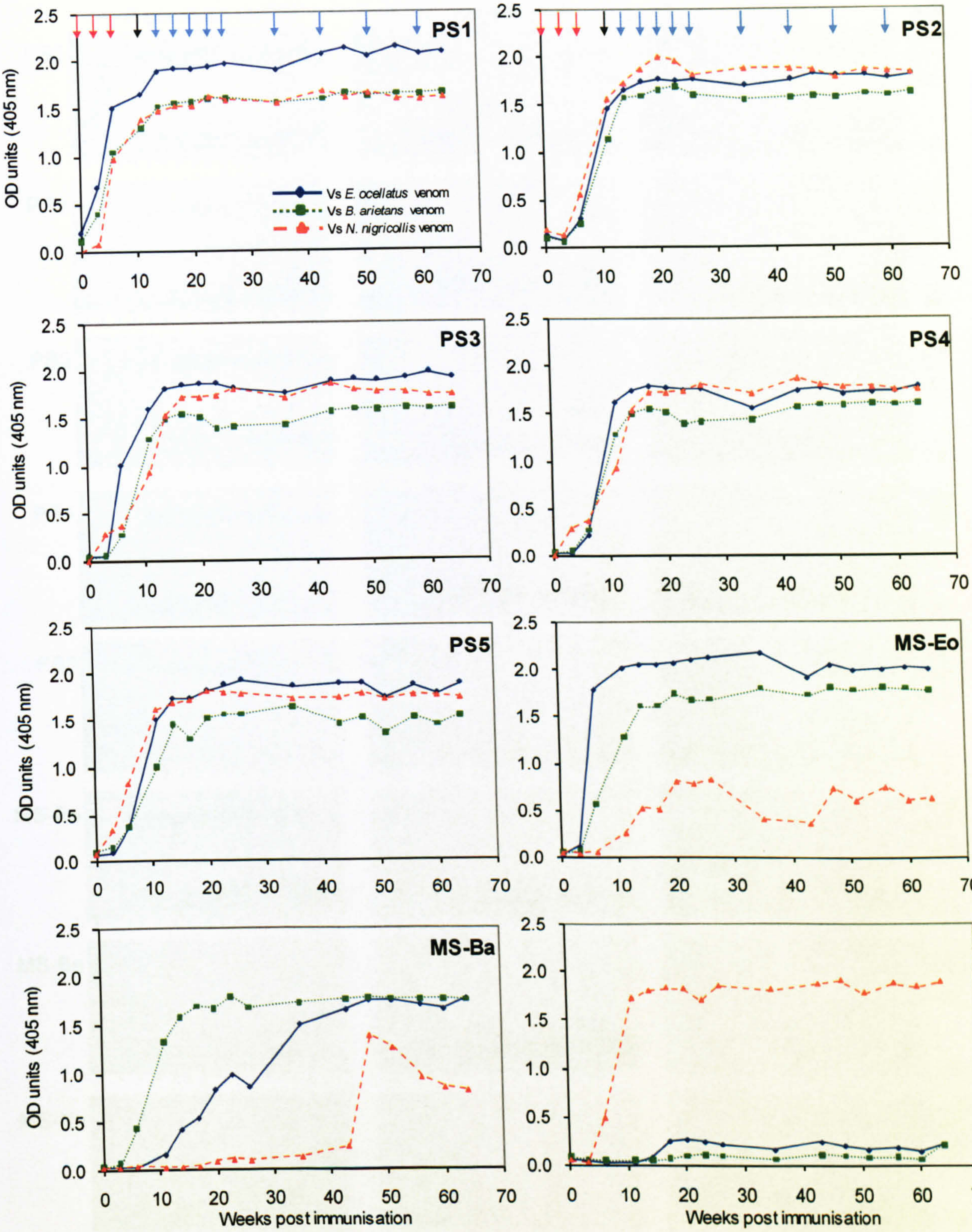

Figure 3.1 Immune reactivity of camel sera to snake venoms.

Immune reactivity (Time Course ELISA) to *E. ocellatus* (solid line, diamonds), *B. arietans* (dotted line, squares) and *N. nigricollis* (dashed line, triangles) venoms of sera (diluted 1:1000) from camels immunised with all three venoms (PS1-5) or with venom from one snake species; *E. ocellatus* (Eo-MS), *B arietans* (Ba-MS) or *N nigricollis* (Nn-MS). The arrows indicate immunisation events: see Table 3.1 for details of immunisation protocol.

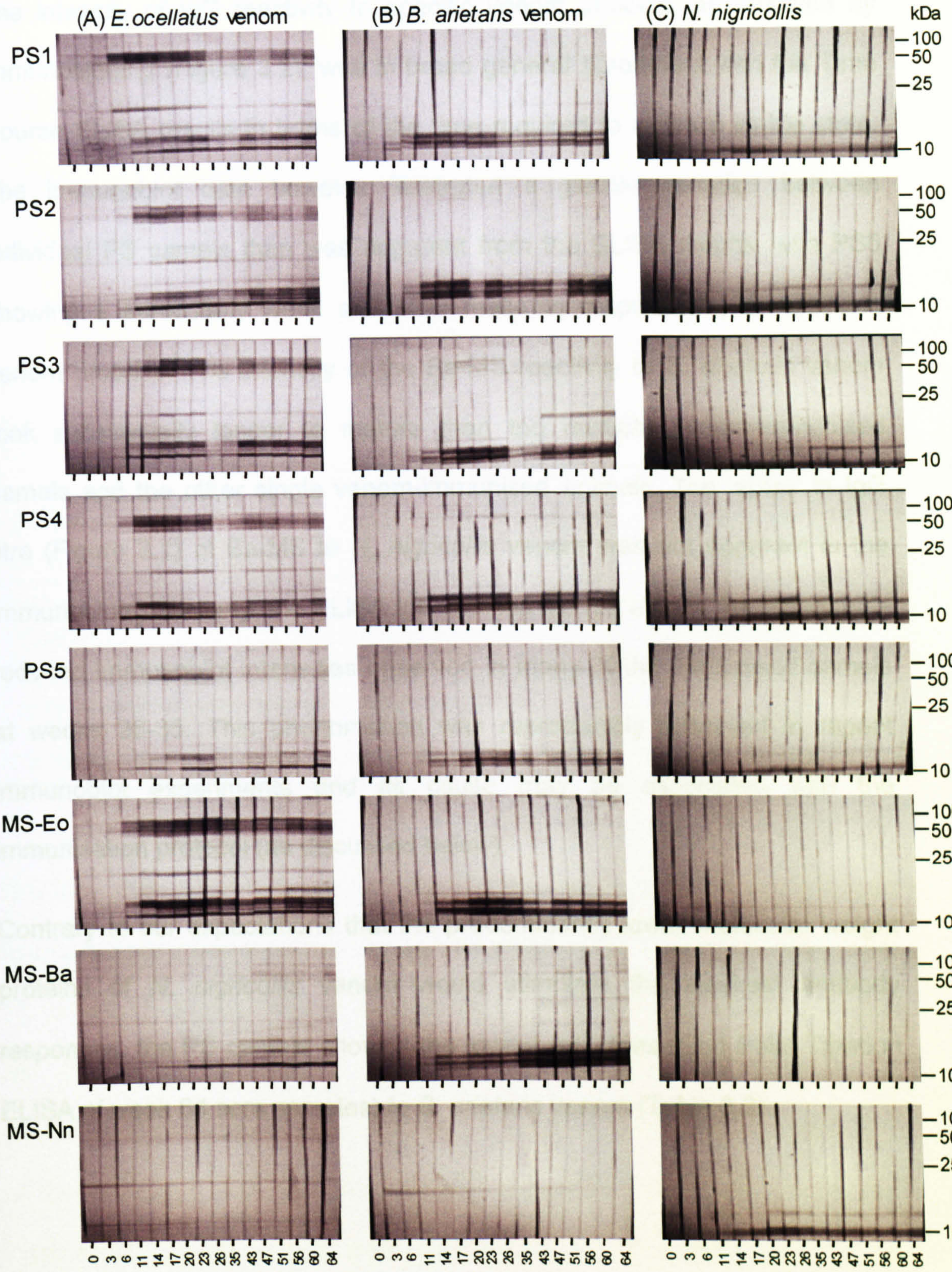

Figure 3.2 Immune reactivity to individual venom proteins

Immune reactivity to individual venom proteins of (A) *E. ocellatus* (E.o.), (B) *B. arietans* (B.a.) and (C) *N. nigricollis* (N.n.) of sera from camels immunised with all three venoms (PS1-5) or with single venoms (Eo-MS, Ba-MS, Nn-MS) revealed by immunoblotting. Numerals on the horizontal axis refer to weeks after immunisation and, on the vertical axis to the molecular mass of the venom proteins.

The intensity of IgG reactivity to specific venom proteins, as revealed by immunoblotting (Figure 3.2), was in broad general agreement with the Time Course ELISA results in terms of the time required to reach a stable state. The immunoblot data however illustrated a greater variation between individual PS camels than was apparent from the ELISA results; with PS5 showing a particularly weak and slow maturing response to *E. ocellatus* venom proteins. The intensity of the Ba-MS reactivity to *B. arietans* venom took substantially longer to mature than the multiple venom-immunised camels and the other single venom-immunised animals. The 'spike' in IgG titre (Figure 3.1) of Ba-MS to *N. nigricollis* venom was not apparent in the immunoblots. Similarly, the ELISA IgG profiles did not exhibit the temporarily reduced immunoblot intensities observed in many of the immunised camels at weeks 26-35. This phenomenon was reproducibly observed in repeat immunoblot experiments and its cause may be associated with the immunisation protocol (as discussed below).

Contrary to our expectations that the predominantly lower molecular weight proteins of *N. nigricollis* venom would stimulate the weakest antibody responses, the PS camels showed the lowest IgG titres (End Point Titration ELISA of week 64 sera samples) to *B. arietans* venom (Table 3.2).

Table 3.2 Comparison of camel IgG titres after immunisation with *E. ocellatus, B. arietans and N. nigricollis* venoms. (ELISA end point titration; 64 week sera samples).

Camel	IgG titres to the following venoms:		
	E. ocellatus	***B. arietans***	***N. nigricollis***
PS1	1.94×10^6	3.24×10^5	1.94×10^6
PS2	3.24×10^5	3.24×10^5	1.94×10^6
PS3	3.24×10^5	3.24×10^5	1.94×10^6
PS4	3.24×10^5	3.24×10^5	3.24×10^5
PS5	5.40×10^4	5.40×10^4	3.24×10^5
MS-Eo	1.94×10^6	3.24×10^5	1.50×10^3
MS-Ba	5.40×10^4	1.94×10^6	1.50×10^3
MS-Nn	$<2.50 \times 10^2$	2.50×10^2	3.24×10^5
Camelid			
*B1 (camel, Yemen)	3.24×10^5	9.00×10^3	$<2.50 \times 10^2$
*8418 (llama, Netherlands)	1.94×10^6	3.24×10^5	9.00×10^3
Antivenom (IgG)			
**EchiTAbG®	1.94×10^6	1.94×10^6	5.40×10^4
***EchiTAb-Plus-ICP	5.40×10^4	5.40×10^4	5.40×10^4

* terminal serum from Harrison et al., 2006

** EchiTabG®, MicroPharm Ltd, UK.

***EchiTAb-Plus-ICP, Instituto Clodomiro, University of Costa Rica (formerly Pan African polyspecific, Gutierrez et al., 2005)

This may have been a function of the multiple venom-immunisation regimen because Ba-MS had a higher titre against *B. arietans* venom than any of the PS camels. The same pattern is noted for Eo-MS against *E. ocellatus* venom with the exception of PS1, which showed the same IgG titre. For *N. nigricollis,* Nn-MS has a lower titre than three PS camels and the same titre as the remaining two. Notably, the IgG titre of PS5 was nearly a log-fold lower against all the venoms than the other PS camels; a result matching the immunblot, but not the Time Course ELISA, results. Consistent with our previous experiment, the IgG titre to *E. ocellatus* venom of PS1-4 and Eo-MS were similar to the *E. ocellatus* venom-immunised camels and llamas (Harrison et al., 2006). Significantly, the IgG titre against all three venoms of

the PS camels equalled or exceeded that of the commercial polyspecific antivenom, EchiTAb-Plus-ICP, which has proven to be a highly, and equally, effective therapeutically in Nigerian patients envenomed by either *E. ocellatus, B. arietans* or *N. nigricollis* (Nasidi, personal communication). Similarly, the IgG titre to *E. ocellatus* venom of Eo-MS matched that of the highly effective (Nasidi, personal communication) ovine monospecific EchiTAbG® antivenom.

3.4.2 IgG avidity analysis

In view of the therapeutic intent of the polyspecific and monospecific camel antivenoms, we felt it important to determine the binding strength (avidity) of the venom/antivenom interactions, in addition to IgG titre and specificity. To achieve this we employed a chaotropic ELISA method (MacDonald et al., 1988) that measures the amount of ammonium thiocyanate required to reduce the IgG binding to venom by 50% (by ELISA OD units). The measurable outcome is termed the Relative Avidity Index, a measure that lacks the precision of the dissociation constant (K_d (Friguet et al., 1985) used to measure the interaction between a monoclonal antibody and a single antigen) which, unlike others (Raweerith and Ratanabanangkoon, 2005), we could not employ because venom consists of numerous proteins and the antisera of the immunised camels were consequently polyclonal. We therefore utilised the chaotropic ELISA to compare the relative maturation of IgG titre and IgG avidity of PS1-5 and Eo-MS to *E. ocellatus* venom over the course of the 64 week immunisation period (Figure 3.3).

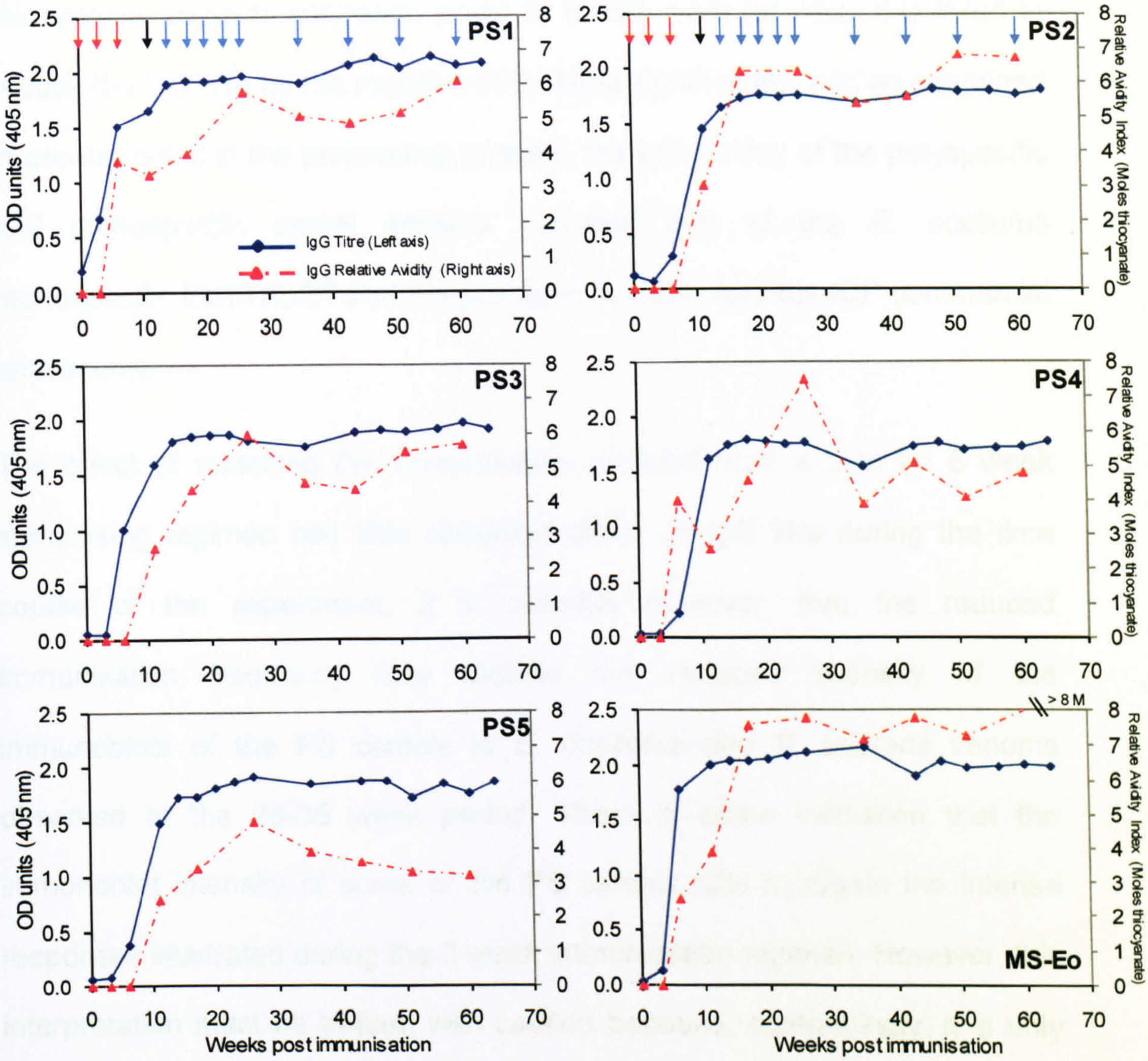

Figure 3.3 Immune reactivity and relative avidity to *E. ocellatus* venom

Immune reactivity (ELISA) and Relative Avidity (Chaotropic ELISA) to *E. ocellatus* venom of sera from camels immunised with all three venoms (PS1-5) or with single venom (Eo-MS). Left axis: IgG titre (solid line, diamonds). Right axis: Relative avidity index (dashed line, triangles)

The most notable outcome of this analysis was that, in all the camel sera examined, IgG titre reached a plateau several weeks before IgG avidity had reached maximal levels. The sera from Eo-MS showed a substantially greater Relative Avidity Index than the PS camels, despite the IgG titre profiles being very similar. It is important to note that the camel sera showed very high Relative Avidity Indices, such that, the addition of 8 M ammonium

thiocyanate (close to saturation point) to Eo-MS sera (at week 64) failed to reduce the IgG titre by the required 50%. Most significantly, and as examined in greater detail in the proceeding chapter, the IgG avidity of the polyspecific and monospecific camel antisera matched that of the *E. ocellatus* monospecific EchiTAbG® and the polyspecific EchiTAb-Plus-ICP commercial antivenoms.

The effect of switching the immunisation protocol from a 3 to an 8 week immunising regimen had little observed effect on IgG titre during the time course of the experiment. It is possible however, that the reduced immunisation frequency may explain the reduced intensity of the immunoblots of the PS camels to *E. ocellatus* and *B. arietans* venoms observed at the 26-35 week period. There is some indication that the immunoblot intensity of some of the PS camels fails to regain the intense responses illustrated during the 3 week immunisation regimen. However, this interpretation must be treated with caution because, contrastingly, it is only after the switch to the 8 week immunisation protocol that the intensity of Ba-MS and PS3 to *B. arietans* venom reached maximal levels. There was also some suggestion that the IgG avidity profiles in PS3-5 fell after the immunisation protocol changed from a 3 to 8 weekly regimen. More importantly, it is clear that, contrary to our expectations, the prolonged interval between immunisation failed to markedly improve IgG avidity.

3. 5 Discussion

This research program was instigated in response to the need for research to improve the supply of safe, affordable and effective antivenom to the rural poor communities that still suffer from very high levels of snakebite-induced death (Gutierrez et al., 2006; Kasturiratne et al., 2008; Williams et al., 2010). In terms of safety, we believe camelid IgG is an attractive candidate to meet these objectives because of its reported lower potential to induce adverse effects (Herrera et al., 2005). Owing to both the desert-adapted nature of camels and the reported thermostability of its IgG (Omidfar et al., 2007), a camelid IgG antivenom should also offer commercial incentives to its local manufacture, and hence supply to the arid regions of Africa and Asia where the risk of snakebite is often high (eg North Nigeria). While the accompanying study reports on the preclinical effectiveness of our experimental camelid IgG antivenoms, the purpose of this study was to provide information important to the future manufacture of camelid IgG antivenom by conducting detailed immunological analyses of the IgG responses of camels to venom immunisation.

The proprietary nature of the majority of the commercial antivenom manufacturing protocols has resulted in a paucity of publicly-available detail on effective venom-immunisation protocols and the basis for deciding when, during the immunisation process, to initiate harvesting of IgG for antivenom formulation. This consideration, together with the novelty of camel immunisation, prompted us here to examine the maturation of IgG titre, specificity and avidity of camels immunised with venoms from the most medically-important snakes of West Africa. For this project we adapted the

'low-dose, low-volume, multi-site' venom immunisation protocol (Pratanaphon et al., 1997; Chotwiwatthanakun et al., 2001) that showed highly encouraging preclinical effectiveness against Asian elapid venoms that are, because of the low molecular weight venom toxins, generally considered to be weakly immunogenic. The initial 3 week- (for the first 6 immunisations) and then 8 week-interval between immunisation protocol, induced venom-specific IgG responses that rapidly rose to a peak and then remained at that plateau for the remainder of the study. The attempt to reduce inflammation associated with Freund's Adjuvants (used most frequently in antivenom production) by using GERBU adjuvant was abandoned after the third immunisation in favour of Freund's because of clinical indications that the animals were suffering mild reactions to venom immunisation – presumably because the non-emulsion GERBU adjuvant does not have the 'slow-release' properties of the water-in-oil adjuvants. An earlier report observed similar problems using a sodium alginate adjuvant (Angulo et al., 1997). It is difficult to judge the effect that changing the adjuvant after the third immunisation had on the camels' immune response, but it is perhaps pertinent that the *E. ocellatus* venom/Freund's adjuvant-immunised camels and llamas of our preliminary study (Harrison et al., 2006) reached an IgG titre plateau between 4-6 weeks (2-3 immunisations). In this study Eo-MS reached the same point after 11 weeks (3 immunisations).

We examined the maturation of the IgG response of the venom-immunised camels in terms of IgG titre, venom protein specificity and, because of the toxin-neutralising requirement of antivenom, avidity. With very minor individual variation, the PS camels showed a rapid rise and sustained IgG

titre to each venom used in the immunising mixture. The same pattern of IgG titre maturation was evident in the three single venom-immunised camels, with Eo-MS showing the most rapid response, indicating perhaps that *E. ocellatus* is more immunogenic than the other viper and elapid venoms. The time course immunoblots showed much the same IgG maturation profile, albeit slightly later than the IgG titre analysis. The most significant observation was that the maturation of IgG avidity was markedly delayed behind that of the IgG titre and specificity. The importance of this in deciding when to initiate harvesting of IgG for antivenom formulation is dependent upon a positive association between IgG avidity and the ability of IgG to reverse venom-induced pathology – the subject of the accompanying report.

The 3 then 8 week venom-immunisation regimen induced a stable plateau of venom-specific IgG titre; the decline in IgG titre noted in other studies involving a prolonged interval between the 'establishing' immunisation period and the next boosting immunisation (17 weeks; (Chotwiwatthanakun et al., 2001)) was not observed. We did note that there was some indication that IgG avidity of the immunised camels appeared more sensitive to the prolonged intervals between immunisations. This was a surprise because we had expected that the avidity of IgG generated after initiating the 8 week boosting immunisation regimen would be higher than that achieved during the initial 3 week immunisation regimen. The avidity of IgG from memory B cells activated by boosting immunisations is considered to be substantially greater than that from primary activated B cells (Neuberger et al., 2000).

The fact that the IgG titre to venom from the elapid, *N nigricollis*, was as high as that to the viper venoms, in both the PS1-5 and Nn-MS was very

encouraging since the low molecular weight elapid neurotoxins are considered immunoloigally problematic (Sunthornandh et al., 1992) - one of the primary reasons for the establishment of the 'low volume, multi site' immunisation protocol (Pratanaphon et al., 1997; Chotwiwatthanakun et al., 2001). The publication of this highly effective equine venom immunisation protocol, has proved very beneficial in our camel-immunisation study: a potent demonstration of the wide-applicability of the immunisation regimen and the benefit gained from making it publicly available.

There was some indication that the immunoblot intensity, but surprisingly not ELISA-determined titre, of IgG from the *B arietans*-immunised camel to *B arietans* venom increased only after initiation of the 8 week booster immunisation regimen. This phenomenon was not observed with the other single venom-immunised camels and a causal explanation is not obvious.

The immunoblot analysis also suggested that multiple-venom immunisation induced a wider spectrum of IgG specificities than single venom-immunisation, perhaps indicating that cross-reactive proteins induced a synergistic immunological outcome (Angulo et al., 1997). Thus, the number of venom proteins, particularly the viper venom proteins, reactive with sera from some of PS1-5 was greater than that of sera from Eo-MS and Ba-MS. The potential of this cross-reactivity of viper venom proteins to generate an antivenom of enhanced venom-neutralising capability needs to be balanced by the observations that the intensity (as assessed by SDS-PAGE, data not shown) and antibody cross-reactivity of 'cross-reactive' proteins suggest they are quantitatively minor venom components.

The combination of the end point titration and immunoblot analyses demonstrate that, as expected for outbred animals, there was discernible individual variation in immune responses of the PS camels to venom immunisation. From an antivenom-manufacturing perspective, these variant analyses provide an immunological justification for perhaps excluding PS-5 from the pool of IgG collected for antivenom preparation. This study therefore corroborates the need for several animals for antivenom preparation.

The final, end point titration analysis indicated that IgG titres of the multiple and single venom-immunised camels to *B. arietans* venom was a log fold lower than that to *E. ocellatus* or *N. nigricollis* venoms. Perhaps the most notable result was that all the camels, whether multiple or single venom-immunised, responded to venom immunisation with IgG titres that matched or exceeded that of commercially-produced antivenoms that are highly effective in treating human victims of envenoming by these three snake species (Habib et al., in preparation). This study has therefore demonstrated, using four IgG-analysis tools, that venom immunisation of camels provokes a potent, sustained serological response and confirms from an immunological perspective that camelid IgG antivenoms should be as efficacious as conventional equine and ovine antivenoms – the subject of the accompanying report.

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CHAPTER 4

Title: Analysis of camelid antibodies for antivenom development: Neutralisation of venom-induced pathology

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Abbreviated title: Camelid IgG for antivenom development

Key words: Snake Antivenom, Camelid IgG, Venom, *Echis ocellatus, Bitis arietans, Naja nigricollis* preclinical assays, ED_{50}

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4. 1 Abstract

Camelid IgG has been reported to be less immunogenic, less able to activate the complement cascade and more thermostable than IgG from other mammals, and has the ability to bind antigens that are unreactive with other mammalian IgGs. We are investigating whether these attributes of camelid IgG translate into antivenom with immunological and venom-neutralising efficacy advantages over conventional equine and ovine antivenoms. The objective of this study was to determine the preclinical venom-neutralising effectiveness of IgG from camels immunised with venoms, individually or in combination, of the saw-scaled viper, *Echis ocellatus*, the puff adder, *Bitis arietans* and the spitting cobra, *Naja nigricollis*- the most medically important snake species in West Africa. Neutralisation of pathological effects of venoms from *E. ocellatus, B. arietans and N. negricollis* by IgG from the venom-immunised camels, or commercial antivenom, was compared using assays of venom lethality (ED_{50}), haemorrhage (MHD) and coagulopathy (MCD). The *E. ocellatus* venom ED_{50}, MHD and MCD results of the *E. ocellatus* monospecific camel IgG antivenom were broadly equivalent to comparable ovine (EchiTAbG®, MicroPharm Ltd, Wales) and equine (SAIMR Echis, South African Vaccine Producer, South Africa) antivenoms, although the equine antivenom required half the amount of IgG. The *B. arietans* monospecific camel IgG neutralised the lethal effects of *B. arietans* venom at one fourth the concentration of the SAIMR polyspecific antivenom (a monospecific *B. arietans* antivenom is not available). The *N. nigricollis* camel IgG antivenom was ineffective (at the maximum permitted dose, 100 µl) against the lethal effects of *N. nigricollis* venom. All the equine polyspecific antivenoms required more than 100 µl to be effective against this venom. The polyspecific camel IgG antivenom, prepared from five camels, was effective against the venom-induced effects of *E. ocellatus* but not against that of *B. arietans* and *N. nigricollis* venoms. No direct correlation was seen between camel IgG relative avidity and titre and the effectiveness of venom neutralisation in preclinical assays.

4. 2 Introduction

Snake envenoming is a significant cause of mortality and morbidity for the rural poor communities of sub-Saharan Africa (Kasturiratne et al., 2008); Harrison et al., 2009). However, because of a variety of complex fiscal reasons associated with commercial antivenom production and government demand, antivenom for sub-Saharan Africa has been in short supply since the 1990s (Theakston and Warrell, 2000). New strategies to ensure the long term stability of antivenom supply are therefore necessary. We are investigating whether the distinct attributes of camel IgG (see preceding paper) can be exploited to improve the efficacy, safety and logistics of antivenom provision in many countries where snakebite is endemic. Camelid IgG can bind epitopes that are non-reactive with other mammalian IgGs (Lauwereys et al., 1998), a feature we hope can be exploited to overcome the weakly immunogenic nature of many of the most pathologenic elapid neurotoxins. Camel IgG is also less immunogenic and less capable of activating complement than equine IgG (Herrera et al., 2005) indicating that intravenous administration of a camelid antivenom might be less likely to induce the serum sickness-like and anaphylactoid adverse reactions associated with current equine and ovine antivenom treatment. In the preceding paper we described the serological responses of camels immunised with venoms from the three most medically-important snake species in West Africa (the saw-scaled viper, *Echis ocellatus*; the puff adder, *Bitis arietans* and the spitting cobra, *Naja nigricollis*) to produce three monospecific antivenoms (one for each snake species) and a polyspecific antivenom against all three snake species. We demonstrated that venom

immunisation of camels stimulated IgG titres, antigen-specificities and relative avidities that matched that of conventional equine and ovine antivenoms currently used in West Africa.

Whilst these results are very encouraging, the 'gold standard' of preclinical assessment of antivenom efficacy is the use of venom-neutralisation assays recommended by the WHO (WHO, 1981; Theakston and Reid, 1983; Pharmacopoeia) and in particular the ED_{50} assay. The median effective dose (ED_{50}) is the least amount of antivenom required to prevent death in 50% of mice challenged with five times the venom LD_{50} dose (1 venom LD_{50} is the amount of venom that kills 50% of the injected mice). ED_{50} results provide a basis for selecting antivenoms for human clinical trials and have been used to indicate the appropriate dose range for initial phase 1 dose-finding human clinical trials (Laing et al., 1995; Theakston et al., 1995; Abubakar et al. 2010). Although not a representation of envenoming and treatment of a patient (the assay involves incubating a mixture of venom and antivenom at 37°C for 30 minutes before intravenous administration into mice), the results of ED_{50} assays usually provide a reasonably satisfactory correlation with the clinical efficacy of an antivenom (Warrell et al., 1986; Laing et al., 1992).

Envenoming by *E. ocellatus* is the predominant cause of snakebite morbidity and mortality throughout most of West Africa, including regions in Senegal (Trape et al., 2001), Cameroon (Einterz and Bates, 2003) and particularly areas in Nigeria, where the number of deaths due to *E. ocellatus* envenoming has been reported as high as 60 per 100, 000 population per year (Pugh and Theakston, 1980). The potency of *E. ocellatus* venom and a typically low

incidence (less than 10%) of 'dry bites' (victims being bitten without being envenomed) leads to an untreated mortality rate in envenomed victims of 10-20% (Warrell et al., 1977). The pathology subsequent to *E. ocellatus* envenoming is characterised by haemorrhage, oedema, blistering and necrosis close to the bite site (local effects) and incoagulable blood and spontaneous bleeding from areas distant from the site of envenoming (systemic effects) (Warrell et al., 1977). The systemic effects can lead to death by intra-abdominal or intracranial bleeding (Warrell and Arnett, 1976). Snake venom metalloproteinases (SVMPs) are primarily responsible for both localised and systemic haemorrhage (Kamiguti et al., 1998) whilst venom prothrombin activators cause consumptive coagulopathy (Warrell et al., 1977). The local manifestations of *B. arietans* envenoming include oedema, blistering, haemorrhage and necrosis and systemic effects include haemorrhage and hypotension but, unlike *E, ocellatus* envenoming, no effects on blood clotting (Warrell et al., 1975). Unusually for African elapid species, human envenoming by *N. nigricollis* is not associated with neurotoxicity but with extensive tissue necrosis, and occasionally with systemic haemorrhage and complement depletion (Warrell et al., 1976).

To assess the performance of the experimental camel IgG antivenoms we used assays that mimic, as closely as possible, the clinical effects of *E. ocellatus, B. arietans* and *N. nigricollis* venom. Therefore, in addition to the ED_{50} assays, we examined the ability of the camel monospecific and polyspecific IgG antivenoms to reverse (i) *E. ocellatus* and *B. arietans* venom-induced haemorrhage by determining the minimum amount of

antivenom required to neutralise the minimum haemorrhagic dose (MHD) (Theakston and Reid, 1983) and (ii) *E. ocellatus* venom-induced consumptive coagulopathy by finding the minimum dose of antivenom that prevents the minimum coagulant dose (MCD) of venom inducing coagulation of a standard solution of citrated human plasma (Theakston and Reid, 1983). The efficacy of the camel IgG antivenoms to reverse the effects of *N. nigricollis* envenoming was performed using the venom lethality ED_{50} assay, and not a specific assay of neurotoxicity, because human *N. nigricollis* envenoming is not associated with neurotoxicity. The results from the camel IgG antivenom assays are compared with that of equine and ovine antivenoms currently used in Africa.

4. 3 Materials and Methods

4.3.1 Animals

Snakes and snake venom: specimens of *E. ocellatus*, *B. arietans* and *N. nigricollis* of mixed age and sex collected from Nigeria were maintained in the herpetarium of the Alistair Reid Venom Research Unit at the Liverpool School of Tropical Medicine. Venom was extracted from the snakes, frozen, lyophilised and stored at 4°C as a powder.

Dromedary camels: animals of mixed age and sex with no previous history of disease or immunisation were maintained in the Central Veterinary Research Laboratory, Dubai for this project. The health of the camels was monitored daily and blood chemistry analyses performed as a routine. Blood taken from the jugular vein was left at room temperature to clot and sera collected by centrifugation and stored at -20°C

The five camels immunised with venom from all three snake species to generate the polyspecific antivenom are identified as PS 1-5. The camel immunised with only *E. ocellatus* venom to generate the monospecific antivenom is identified as Eo-MS and the camels immunised with venom from either *B. arietans* or *N. nigricollis* to generate the respective monospecific antivenoms are identified as Ba-MS and Nn-MS. For details of the venom immunisation and bleeding regimen, we refer the reader to the preceding paper accompanying this report (Chapter 3).

4.3.2 Antivenom

IgG was extracted from sera of the eight venom-immunised camels 60 weeks after the first immunisation by adding caprylic acid (Sigma, UK) to a final concentration of 5%, stirring vigorously for 2 hours at room temperature to precipitate non-IgG proteins and the mixture centrifuged at 13,000 RPM for 25 mins. The IgG was dialysed overnight in sodium phosphate buffer (pH 7.4) to buffer the IgG and remove the caprylic acid. IgG was concentrated (using 30 kDa cut-off spin columns, Vivaspin, UK) to 100 mg/ml for use in the preclinical assays. IgG from the multiple venom-immunised camels was examined at the individual level (PS1) or as a pool of PS1-4 (PS-Pool) to reduce the numbers of mice. IgG from camel PS5 was not used because our initial immunological (see preceding report: chapter 3) and preclinical assays indicated that this animal had failed to mount satisfactory antibody responses to venom immunisation.

The South African Vaccine Producers (SAVP, formerly the South African Institute for Medical research, SAIMR) *Echis* monospecific antivenom, a mainstay for therapy of *Echis* envenoming in Africa, and the SAVP polyspecific antivenom were used as controls. Both are $F(ab')_2$ IgG antivenoms. EchiTAbG® an intact IgG ovine *Echis* monospecific antivenom recently introduced therapeutically in Nigeria was included as a control.

We also utilised IgG from two *E. ocellatus* venom-immunised camels from an earlier preliminary study (Harrison et al., 2006) as additional comparison controls.

4.3.3 Preclinical assays:

4.3.3.1 Antivenom Neutralisation of Venom Lethality

The LD_{50} of *E. ocellatus* venom was determined by injecting the tail vein of 18-20 g male CD-1 mice (Charles River, UK) with varying doses of venom in 0.1 ml of PBS. Five mice were used per group and the number of deaths occurring within 7 hours after injection was recorded. The *E. ocellatus* venom LD_{50} and 95% confidence intervals were calculated by probit analysis (Finney, 1971). Assessment of the potency of IgG to neutralise the venom lethal effects (ED_{50}) was conducted as outlined by (Laing et al., 1992) with adaptations. Briefly, different concentrations of the relevant IgG were premixed with 5 x 1 venom LD_{50} at 37°C for 30 minutes then injected into the tail vein of CD-1 mice. After 7 hours, deaths were recorded and the ED_{50} was established using probit analysis (Finney, 1971). The 7 hour time frame for the LD_{50} and ED_{50} assays was used instead of the more conventional 24 hour period as a result of numerous earlier assays which revealed that over 98% mice succumbed to *E. ocellatus* envenoming within 7 hours of injecting the venom or venom/antivenom mixture (data not shown). These observations, with this venom, permitted us to make this humane reduction in the duration of the experiments without risking invalidating the results. The same protocol was followed to assess the venom LD_{50} and antivenom ED_{50} for *B. arietans* and *N. nigricollis* venoms, except that the original 24 hour time frame was used instead of 7 hours.

4.3.3.2 Antivenom Neutralisation of Venom-induced Hemorrhage

The minimum amount of *E. ocellatus* or *B. arietans* venom inducing an 8 mm haemorrhagic lesion 2 hours after intradermal injection into the shaved dorsal skin of CD1 mice was used to determine one venom MHD. Varying doses of IgG were incubated with the 1 venom MHD at 37°C for 30 mins and the mixture injected intradermally (max volume of 50 µl) into groups of 3 mice per IgG dose. The size of the haemorrhagic lesion was measured under background illumination and the minimum amount of IgG (µl) required to completely prevent haemorrhage was determined. The 2 hour, rather than the conventional 24 hour time frame was used subsequent to preliminary experiments which demonstrated that the size of the *E. ocellatus* venom-induced haemorrhagic lesion, and the minimum amount of antivenom required to prevent haemorrhage, differed little in animals assessed 2, 7 and 24 hours after injection. We demonstrated that the 8mm lesion observed 2 hours after injection proceeded in other injected animals into a 10 mm lesion 24 hours after injection. This permitted us to make a humane reduction in the duration of the experiments without risking invalidating the results.

4.3.3.3 Antivenom Neutralisation of Venom-induced Procoagulant Effects

The minimum amount of *E. ocellatus* venom required to clot citrated human plasma in 60 seconds at 37°C (MCD-P) was determined by preparing a range of venom concentrations in physiological saline solution (PSS) to a final volume of 50 µl. This was added to 0.2 ml of citrated plasma at 37°C and the time taken to clot the plasma was recorded (Theakston and Reid, 1983). Various amounts of the SAVP antivenom (max volume 25 µl in PSS)

was added to 0.2 ml of citrated plasma, and then one venom MCD-P added and the minimum amount of IgG required to completely prevent clotting for a period of 5 mins at 37°C was determined.

4.3.3.4 Modified MCD-P procedure

To examine the ability of camel sera to inhibit the procoagulant effects of *E. ocellatus* venom the standard MCD-P assay was modified in an effort to standardise the venom/antivenom preparations for all the preclinical assays. The standard assay (as above) was followed except venom and *serum* was pre-incubated at 37°C for 30 mins then cooled on Ice before adding to the plasma. As with the standard assay, prevention of coagulation was determined by incubation at 37°C for 5 mins. The pre-incubation allowed the assessment of neutralisation of serum which has a lower concentration of IgG than standard antivenoms.

4.3.4 Immunological assays.

The experimental protocols used here for the Time-course ELISA and chaotropic IgG Avidity assays were identical to those used in the preceding paper accompanying this report (Chapter 3).

4. 4 Results

4.4.1 Preclinical activity of E. ocellatus, B. arietans and N. nigricollis venoms

The results of the assays to determine venom-induced (i) lethality (LD_{50}), (ii) haemorrhage (MHD) and (iii) coagulant effects (MCD) are described in Table 4.1 and are little different from previous studies examining these venoms in these assays (Theakston and Reid, 1983; Laing, G. D. et al., 2003; Gutierrez et al., 2005; Segura et al., In Press).

Table 4.1 Activity of *E. ocellatus*, *B. arietans* and *N. nigricollis* venom

Assay	*Echis ocellatus*	*Bitis arietans*	*Naja nigricollis*
LD_{50} (µg/mouse)	14.85 (12.65- 20.15)	33.80 (29.68-45.18)	17.70 (11.80-26.14)
MHD (µg/mouse)	10	6	NA
MCD (µg)	0.125	NA	NA

Values in parentheses represent 95% Confidence Limits; MHD = Mass of venom required to produce a 10 mm lesion after 24 hours when injected intradermally (mean of 3 mice); MCD = Mass of venom required to clot a standard volume of human citrated plasma in 60s (mean of 2 assays); NA = venom lacks activity required for the assay.

4.4.2 Preclinical assessments of camel IgG and commercial antivenoms against E. ocellatus venom

The ED_{50} of the SAVP *Echis* monospecific antivenom was 535 µg (95% confidence limits, 417-628 µg) (Table 4.2). The ED_{50} (1129 µg (447-2267) for the comparable monospecific camel IgG, Eo-MS was less effective than the SAVP *Echis* antivenom (but statistically no different because of the high 95% confidence limits that are typical in these assays) but more effective than the ovine *E. ocellatus* monospecific IgG antivenom, EchiTAbG® (MicroPharm, Wales). The camel polyspecific antivenom was marginally less effective (an ED50 of 1407 µg/mouse (781-2037)) than the camel *E. ocellatus*

monospecific antivenom and as effective as EchiTAbG® which has proven highly efficacious in a recent human clinical trial (Theakston, personal communication). IgG from one of the polyspecifically-immunised camels, PS1, proved to be the most effective (an ED_{50} of 448 µg (117-992)) antivenom at neutralising *E. ocellatus* venom-induced lethality.

Table 4.2 Comparative neutralising efficacy of *E. ocellatus* venom by experimental camel IgG and conventional antivenoms

	Neutralisation of *E. ocellatus* venom effects		
Preparation	**ED_{50} (µg/mouse)**	**MHD (µg)**	**MCD (µg)**
PS-Pool	1407 (781 - 2037)	500	600
PS1 alone	448 (117 - 992)	600	200
Eo-MS	1129 (447 - 2267)	400	750
B1+B2	2321 (1143 - 3067)	*	1000
EchiTAbG®	1461 (882 - 2273)	-	-
SAVP (SAIMR) (ms)	535 (417 - 628)	300	480

* Camels B1 and B2 partially neutralised haemorrhage at 210 µg and 175 µg respectively (see Harrison et al., 2006)

ED_{50} measured against $5xLD_{50}$; Values in parentheses represent 95% Confidence Limits; µg = Mass of IgG required to neutralise venom effect; B1+B2= pooled camel IgG extracted from terminal sera from Harrison et al., 2006; EchiTAbG® = *E. ocellatus* monospecific IgG antivenom, Micropharm Ltd, UK; SAVP (SAIMR) (ms) = *Echis* antivenom, South African Vaccine Producers

With regards *E. ocellatus* venom-induced haemorrhage, the SAVP *Echis* monospecific antivenom (300 µg) was marginally more effective than the *E. ocellatus* monospecific camel IgG antivenom (Eo-MS, 400 µg) and the camel polyspecific antivenom (PS-Pool, 500 µg) in the MHD neutralisation assay (Table 4.2). In this instance, IgG from the polyspecifically-immunised camel, PS1, was least effective (600 µg) in neutralising venom-induced haemorrhage. Conversely, PS1 IgG was the most effective at reversing *E.*

ocellatus venom-induced coagulation (200 µg in the MCD neutralisation assay).

IgG pooled from two camels of an earlier study (Harrison et al., 2006) which had been immunised with the *E. ocellatus* venom on six occasions and exhibited satisfactory preclinical neutralisation of venom-induced haemorrhage (210 µg and 175 µg respectively in a MHD assay) proved much less effective in the present ED_{50} assay than the camels of this study which had been subjected to a considerably longer venom immunisation protocol.

4.4.3 Preclinical assessments of camel IgG and a commercial antivenom against B. arietans venom

The monospecific camel IgG generated against *B. arietans* venom, Ba-MS was almost four times more effective at preventing venom-induced death than the SAVP polyspecific antivenom (ED_{50} results of 2352 µg and 9272 µg respectively), although this difference is not significant because of the wide range of the 95% confidence limits, Table 4.3). IgG from camel Ba-MS was also more effective at preventing *B. arietans* venom-induced haemorrhage in the MHD assay than the SAVP polyspecific antivenom.

Table 4.3 Comparative neutralising efficacy of *B. arietans* venom by experimental camel IgG and conventional antivenoms

	Neutralisation of *B.arietans* venom effects	
Preparation	**ED_{50} (µg/mouse)**	**MHD (µg)**
PS-Pool	NE	1900
PS1 alone	NE	1900
Ba-MS	2352 (1687 - 2760)	350
SAVP (SAIMR) (ps)	9272 (2637 - 13951)	450

ED_{50} measured against 5xLD_{50}; Values in parentheses represent 95% Confidence Limits; µg = Mass of IgG required to neutralise venom effect; NE = Ineffective at maximum volume allowed (100 µl); SAVP (SAIMR) (ps) = polyvalent antivenom, South African Vaccine Producers

IgG from either the PS-Pool or from PS1 was unsuccessful at preventing *B. arietans* venom-induced lethality at the maximum volume (100 µl) permitted by the UK Home office for the ED_{50} assay. This prevented the calculation of the ED_{50} for either of these samples. From the MHD assay results it is clear that IgG from both PS-Pool and PS1 were less effective than the SAVP antivenom at neutralising *B. arietans* venom-induced haemorrhage.

4.4.4 Preclinical assessments of camel IgG and a commercial antivenom against N. nigricollis venom.

None of the camel IgG preparations were effective at the maximum permitted dose in reversing *N. nigricollis* venom-induced lethality. Tests on an equine polyspecific antivenom (EchiTAb-Plus-ICP, Instituto Clodomiro Picado) and an experimental equine ,anti-African cobra antiserum (Casasola et al., 2009) were efficacious, but, required considerably more antivenom/antisera than the 100 µl permitted under our licence to prevent 50% venom-induced lethality in mice (Gutierrez et al., 2005).

4.4.5 The relationship between IgG titre, avidity and effectiveness against E. ocellatus venom

To determine whether results from the immunological assays could be used to indicate the efficacy of an antivenom, we compared (i) the titre, (ii) the relative avidity and (iii) the neutralisation of *E. ocellatus* venom-induced coagulation of IgG from individual camels over the course of the 64-week immunisation period (Figure 4.1). IgG titres reached a plateau before that of IgG relative avidity (with minor individual variation).

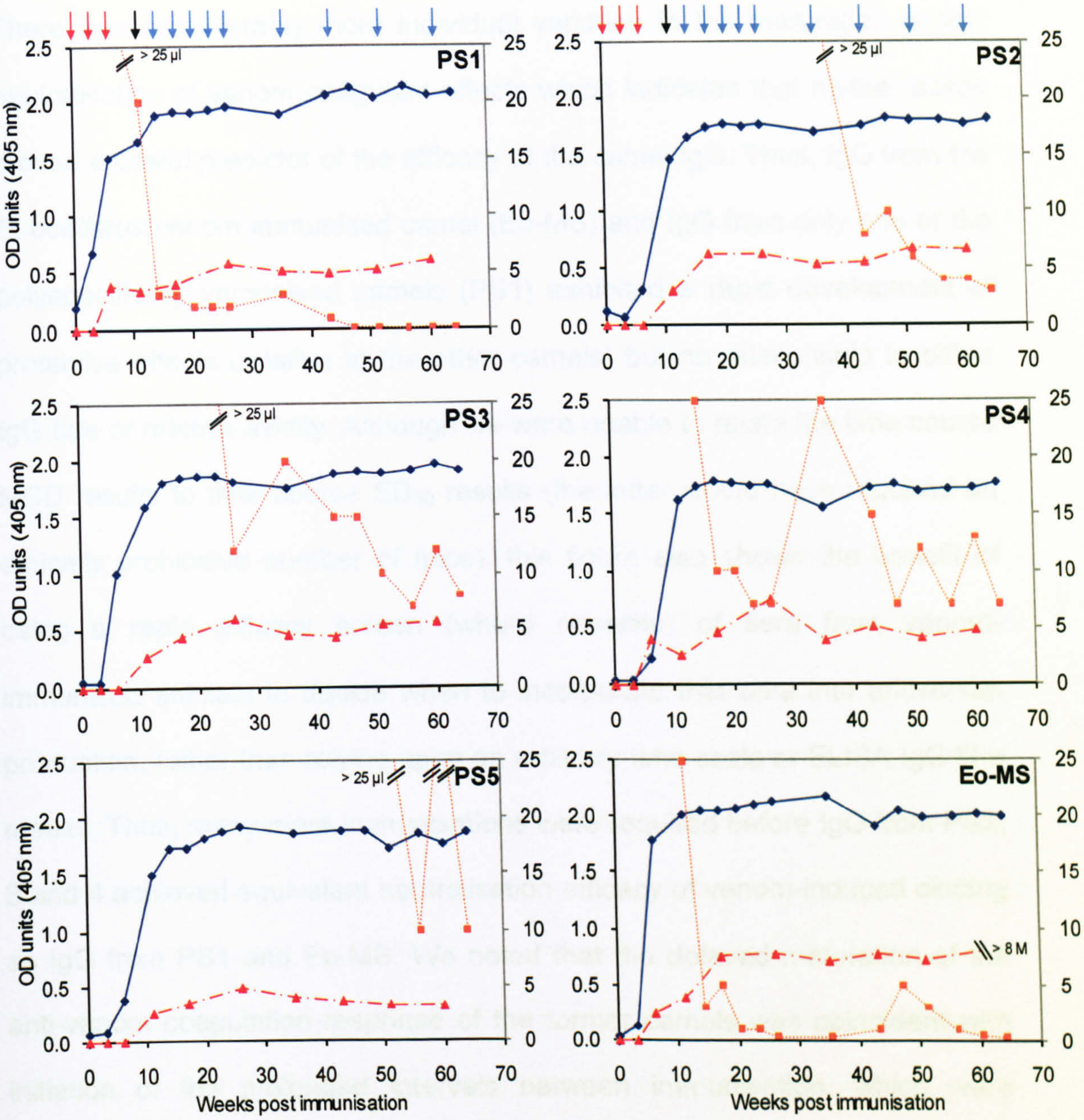

Figure 4.1 Serological responses to *E. ocellatus* venom of venom-immunised camels.

The titre, (ELISA: Left axis, solid line, diamonds), relative avidity (Chaotropic ELISA: Right axis, dashed line, triangles, units = moles of thiocyanate,) and venom-neutralising ability (modified MCD neutralisation of *E. ocellatus* venom, Right axis, dotted line, squares, units = µl) of sera from camels immunised with all three venoms (PS1-5) or with *E. ocellatus* venom (Eo-MS). Arrows represent time-points when immunisations were administered.

There was considerably more individual variation in the maturation of IgG neutralisation of venom coagulant effects which indicates that neither assay proved a useful predictor of the efficacy of the camel IgG. Thus, IgG from the *E. ocellatus* venom immunised camel (Eo-MS) and IgG from only one of the polyspecifically immunised camels (PS1) exhibited a rapid development of protective effects (relative to the other camels) but no relationship to either IgG titre or relative avidity. Although we were unable to relate the time course MCD results to time course ED_{50} results (the latter would have required an ethically prohibitive number of mice), this figure also shows the benefit of using a rapid efficacy screen (where possible) of sera from venom-immunised animals to decide when to incorporate that sera into antivenom production, rather than relying upon an arbitrary time scale or ELISA IgG titre results. Thus, many more immunisations were required before IgG from PS2, 3 and 4 achieved equivalent neutralisation efficacy of venom-induced clotting as IgG from PS1 and Eo-MS. We noted that the delayed maturation of the anti-venom coagulation response of the former camels was coincident with initiation of the prolonged intervals between immunisation, which were (apparently unsuccessfully) introduced to enhance IgG avidity. We caution against inferring much immunological significance to this observation because the anti-venom coagulation response of IgG from PS1 and Eo-MS had fully matured during the initial immunisation phase, which utilised a 3 week interval between immunisations.

4. 5 Discussion

This is the first demonstration that IgG from *E. ocellatus* venom-immunised camels has a comparable neutralising efficacy as conventional monospecific equine and ovine antivenoms against the lethal, haemorrhagic and coagulopathic effects of *E. ocellatus* venom. Furthermore, the equivalent preclinical effectiveness of the camel IgG antivenom and the SAVP antivenom; a pepsin-digested $F(ab')_2$ preparation, indicates potential savings in antivenom manufacturing costs of a whole camelid IgG antivenom. The *B. arietans* monospecific camel IgG antivenom (Ba-MS) showed excellent neutralisation of the lethal and haemorrhagic effects of *B. arietans* venom. To our knowledge, the IgG from camel Ba-MS is the first monospecific antivenom prepared against *B. arietans* venom and thus we were unable to conduct comparative efficacy experiments with other monospecific control antivenoms.

The polyspecific camel IgG (PS-Pool) proved less effective at neutralising *E. ocellatus* venom than both the monospecific camel-derived IgG and the commercial equine antivenom, but was as effective as EchiTAbG® an ovine IgG preparation being utilised effectively in Nigerian victims of *E. ocellatus* envenoming. Less encouragingly, the PS-Pool IgG was unable to reverse the lethal effects of *B. arietans* envenoming and was less effective at preventing haemorrhage induced by venom from this viper; results that do not compare favourably with the equine SAVP and a Costa Rican polyspecific antivenom (Gutierrez et al., 2005). Since the polyspecific camels were immunised with equal amounts of all three venoms, we interpret these results as indicating

that *E. ocellatus* venom is more immunogenic than *B. arietans* venom, and that no benefit was gained, in this study, from any boosting of IgG induction via stimulation by cross-reactive viper venom proteins. However, this interpretation is inconsistent with our observation that IgG from Eo-MS and Ba-MS had identical ELISA end point titres to their respective venoms. We are therefore unable to explain why IgG from the PS camels (immunised with the mixture of venoms) proved efficacious against *E. ocellatus*, but not against *B. arietans*.

Neither IgG from the polyspecific (PS-Pool) nor from the *N. nigricollis*-monospecific (Nn-MS) camels proved effective, at the maximum permitted dose, in neutralising the lethal effects of *N. nigricollis* envenoming. The superficially unfavourable comparison of the *N. nigricollis* ED_{50} results of our camelid IgGs with equine antivenoms needs to be viewed with caution because the latter required 2-3 fold the amount of antivenom permitted in our preclinical assays (Gutierrez et al., 2005; Casasola et al., 2009). The well recognised difficulties in preparing effective antivenom against cobra venoms is thought to be a consequence of the weak immunogenicity of cobra venom proteins (Chinonavanig et al., 1988; Sunthornandh et al., 1992). We had hoped that the natural ability of the unique camelid immune system to react with epitopes that fail to stimulate antibody responses in other mammals (Lauwereys et al., 1998) would translate, in this study, into the development of potent IgG specificities in the *N. nigricollis*-immunised camels, particularly the monospecific camel, Nn-MS. Initially this seemed the case because results from the immunological assays of Nn-MS and the PS1-4 camels

indicated that immunisation with *N. nigricollis* venom had stimulated venom-specific IgGs whose titre and relative avidity equalled those stimulated by the viper venoms (Chapter 3). It was therefore very disappointing to observe here that the highly encouraging venom-neutralising efficacy of the *E. ocellatus* and *B. arietans* camel monospecific antivenoms was not matched by IgG from Nn-MS or from the PS-Pool. It is clear therefore that preparing effective antivenoms against elapid venoms, whether in camelids or other large mammals, may benefit from utilising venom-immunisation protocols distinct from the one used here. Our venom-immunisation protocol was adapted from the low dose, low volume, multi-site protocol used in Thailand to dramatically improve the efficacy of experimental antivenoms against Asian elapids (Pratanaphon et al., 1997; Chotwiwatthanakun et al., 2001). Nevertheless, in our hands, this adapted protocol failed to engender satisfactorily effective IgG neutralisation of the lethal effects of *N. nigricollis* venom.

We targeted four major draining lymph nodes in the neck of the camel for immunisation. The frequency of the immunisations was designed to (i) quickly establish a serological response to the venom proteins (3 week intervals between the first 6 immunisations) and (ii) then augment the avidity of the IgG/venom protein interactions by encouraging IgG production from memory B cells by introducing 3 month intervals between all subsequent booster immunisations. We predicted that an improved relative avidity would equate to improved neutralisation of the effects of venom toxins. Our immunisation protocol did stimulate rapid IgG response to all the venoms,

including that of *N. nigricollis* (see Table 3.1 and Figure 3.1, the time course ELISA profiles in chapter 3) and generated IgGs with high avidities (the relative avidity indices ranged from 3 M to over 8 M), but the relative avidity profiles did not show an enhancement subsequent to implementing the 3 month interval immunisation protocol. While avidity ELISA is a useful tool for examining the effectiveness of immunisations in humans (Borrow et al., 2001; Goldblatt et al., 2002), we are forced to conclude that neither the relative avidity nor the antigen specificity (gleaned from venom immunoblots) assays provide an adequately accurate prediction of the venom-neutralising efficacy of an antivenom. The observation that the most preclinically effective camel IgGs of this study (Eo-MS, Ba-MS and PS1) had the highest IgG end point titres agrees with an early report showing a statistical association [r= 0.83 ($p<0.005$)] between ELISA end point titres and antivenom efficacy (Rungsiwongse and Ratanabanangkoon, 1991). However, the important lack of correlation between the results of the immunological and preclinical assays of Nn-MS and PS1-4, indicate that ELISA end point titre results should also not be used to predict the efficacy of an antivenom. Regrettably therefore, we can not recommend that the severe in vivo rodent preclinical assays be replaced by any of the above immunological assays.

Nevertheless immunological assays play an important, albeit early, role in antivenom preparation, particularly in a study such as this where an animal is assessed as a potentially new species for antivenom production. Thus, the results of the immunological assays, in combination with the modified MCD assay, were important in enabling us to evaluate the responses of camels to

venom immunisation and to assess which camels, and at what stage of the immunisation schedule, produced IgG that appeared promising. For example, we were able to quickly identify that the antibody response of PS5 was inadequate and recommend against inclusion of its IgG from the pool of IgGs for antivenom preparation. By contrast, the IgG response of PS1 was exceptionally promising.

In conclusion, we have demonstrated that camels immunised with snake venom are capable of producing IgG with similar venom-neutralising capabilities as a commercial $F(ab')_2$ equine and ovine IgG antivenoms. In some geographical areas camelids may be cheaper and easier to maintain than horses and sheep, which may translate into potential benefits for antivenom producers (improved cost-effectiveness) and thereby make treatment more affordable for the envenomed victim. It remains a serious concern however, that camels were no better than horses in responding to elapid venom immunisation with venom-neutralising IgG. While most antivenom manufacturers utilise Freund's complete adjuvant for the primary immunisation followed by less inflammatory adjuvant systems, a recent report (Waghmare et al., 2009) indicates that other immune-adjuvants hold promise in antivenom development. Here, to avoid the inflammation induced by Freund's complete adjuvant, we used GARBU in the initial immunisations but this was discontinued in favour of the Freund's adjuvant system because the camels suffered venom like effects – presumably because the non-emulsifying formulation of the GARBU/venom immunising mixture permitted rapid distribution of the venom. It is very important that the toxinological

community resolves the problems of generating potent anti-elapid antivenoms and independent assessments of immunisation protocols is urgently required. Ideally, this effort should be coordinated by a global body such as the World Health Organisation.

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CHAPTER 5

Title: Analysis of camelid IgG for antivenom development: Immunoreactivity and preclinical neutralisation of venom pathology by IgG subclasses, and the effect of heat treatment.

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5. 1 Abstract

Antivenom is the most effective treatment of snake envenoming, and is manufactured from the IgG of venom-immunised horses and sheep. Over 50% of camelid IgG is, uniquely amongst mammals, lack light chains and have been reported to be less immunogenic and less likely to activate complement than equine or ovine IgG. Camelid IgG therefore potentially offers safety advantages over conventional IgGs used for antivenom manufacture. The reported thermostability of camelid IgG also holds promise in the inclusion of a relatively inexpensive anti-microbial heat step in antivenom manufacture. However, these potential benefits of camelid IgG would be nullified if any one of the three camel IgG subclasses dominated, or under-performed, the serological response of camels to venom immunisation because of the prohibitive manufacturing costs of having to purify, or exclude, one or more IgG subclasses. This study compared the titre, antigen-specificity, relative avidity and ability to neutralise the haemorrhagic and coagulopathic effects of *Echis ocellatus* venom of each IgG subclass purified from camels immunised with venom from either *E.ocellatus* alone or a mixture of venoms. The results demonstrated that no one IgG subclass consistently out-performed or under-performed the others in their immunereactivity to venom proteins and ability to neutralise venom-induced pathologies. We concluded therefore that there was no immunological or immunotherapeutic reason to recommend costly production steps to isolate, or exclude, a particular IgG subclass for the manufacture of camelid antivenoms. In contrast, the immunoreactivity of the heavy and light chain

IgG1 subclass was markedly more vulnerable to extreme heat treatment than the heavy chain-only IgG2 and IgG3 subclasses. We therefore felt unable to recommend the inclusion of a cost-attractive anti-microbial heat step into the manufacturing process of camelid antivenoms.

5. 2 Introduction

Snakebite is a serious, often life-threatening, daily hazard in large tracts of Africa and Asia (Kasturiratne et al., 2008), which primarily affects the rural poor (Harrison et al, 2009). Antivenom therapy represents the only effective treatment for snake envenoming but because of the complex interplay between diverse fiscal, epidemiological and clinical issues, including (i) high costs of manufacture that are often passed on to governments and patients, (ii) a general paucity on burden data for specific regions, (iii) a lack of political recognition of regional snakebite problems and (iv) inadequate advocacy and funding, some regions, particularly in Africa, suffer from a severe shortage of life-saving antivenom (Theakston and Warrell, 2000). The toxinological community has responded to this situation with publications highlighting the problem (Gutierrez et al., 2006; Williams et al., 2010) and, in Latin America, by the formulation of an 'alliance' (CYTED – Ciencia y Technologia para el Desarrollo) to coordinate disparate activities to improve antivenom delivery and effectiveness in the region (Gutierrez et al., 2007; Gutierrez et al., 2009) and by the inclusion of snakebite in the World Health Organisation list of Neglected Tropical Diseases (WHO, 2009). One of the common recommendations is that research is needed to make antivenoms more effective, safer and less expensive {Gutierrez et al., 2006; WHO, 2010; Harrison et al., 2009; Williams et al., 2010).

With that objective we have embarked on a research project to determine whether antivenoms prepared from IgG of venom-immunised camels offer benefits in terms of effectiveness, safety and cost of manufacture (Harrison et al., 2006; Harrison et al., 2007). Camelid IgG is unique amongst mammals

in that over 50% of the IgG lack light chains (Hamers-casterman et al., 1993), and perhaps because of its unique heavy chain-only IgG subclasses, offers exciting possibilities to improve antivenom in four main areas. (a) The report that camelid IgG proved less immunogenic and less prone to activate complement (Herrera et al., 2005) suggested the possibility that camel IgG antivenom might reduce the incidence of adverse anaphylactic and serum sickness associated with many equine antivenoms. (b) Reports that camelid IgG binds some epitopes that are not bound by IgG of other mammalian species (Lauwereys et al., 1998; De Genst, E. et al., 2006) suggested that camelid IgG antivenom might show an improved binding to the low molecular weight elapid neurotoxins that are typically weakly immunogenic in venom-immunised horses (Chinonavanig et al., 1988; Pratanaphon et al., 1997). (c) The much reported thermostability of camelid IgG (Omidfar et al., 2007) indicated that liquid camel IgG antivenom could be stored at ambient temperature storage without the expense required to maintain a cold chain, and that a cost-benefit antimicrobial heat step could be introduced into the manufacturing process. (d) Camels might be very attractive to the manufacture of antivenom in countries where the climatic conditions are not favourable for the maintenance of horses or sheep.

The studies performed in Tunisia describing the experimental venom-neutralising effectiveness of intact IgG or heavy chain-only IgG (Meddeb-Mouelhi et al., 2003) indicate that camels hold promise in the development of antivenoms to treat scorpion envenoming. We demonstrated that camels and llamas immunised with *Echis ocellatus* venom responded with virtually indistinguishable IgG reactivities and preclinical venom-neutralising

effectiveness (Harrison et al., 2006), which indicated that either camelid species could be harnessed into antivenom production. Encouraged by these results, we immunised dromedary camels with venoms from the most medically important snakes of West Africa (the saw-scaled viper, *Echis ocellatus*; the puff adder, *Bitis arietans*; the spitting cobra, *Naja nigricollis*), either with a single venom to prepare monospecific antivenoms to each snake species, or with the three venoms combined to prepare a polyspecific antivenom for West Africa. The monospecific and polyspecific camel antisera (chapter 3) responded to venom immunisation with equally impressive venom species-specific IgG titres (ELISA), specificity (immunoblotting) and antigen binding (relative avidity ELISA). We next demonstrated that the *E. ocellatus*- and *B. arietans*-monospecific antivenoms (but not the *N.nigricollis*-monospecific antivenom) were as effective as the nearest comparable antivenoms in current clinical use in Africa, based on preclinical assays of venom-induced lethality, haemorrhage and coagulopathy (Chapter 4).

These results indicated the potential of IgG from venom-immunised camels for antivenom production. Camel IgG consists of three subclasses, IgG1, a conventional heavy and light chain molecule and IgG2 and IgG3 that lack light chains. A recent report showed that the serological response of nematode-infected llamas was dominated by the IgG1 subclass (Daley, L.P. et al., 2005). We were consequently concerned that the immunological reactivity and preclinical efficacy of the IgG2 and IgG3 subclasses from venom-immunised camels might differ from the conventional IgG subclass. The costs of antivenom production to the manufacturer and hence to the

patient/hospital are a paramount concern. We felt it was important to ascertain whether there were strong immunological and/or venom-neutralising reasons to select, or exclude, a specific IgG subclass for the development of camelid IgG antivenoms. Such a step in the antivenom manufacture protocol would be prohibitively expensive and cease further interest in the development of camel antivenoms, irrespective of the potential clinical gains.

5. 3 Materials and methods

5.3.1 Venom

Specimens of *E. ocellatus* of mixed age and sex collected from Nigeria were maintained in the herpetarium of the Alistair Reid Venom Research Unit at the Liverpool School of Tropical Medicine. Venom was extracted from the snakes, frozen, lyophilised and stored at 4°C as a powder.

5.3.2 Preparation of IgG

One camel (MS-Eo) was immunised with *E. ocellatus* venom, to generate monospecific antibodies and 2 other camels (PS1 and PS2) were immunised with *E. ocellatus*, *Bitis arietans* and *Naja nigricollis* venom to produce polyspecific antibodies (see Chapter 3 for details of the venom immunisation protocol). Sera collected at 7 time-points was used in this study and IgG extracted using 5% caprylic acid (Sigma), stirred vigorously at room temperature for 2 hours, before centrifuging at 13, 000 RPM for 25 minutes. The supernatant was dialysed with three changes of 20 mM sodium phosphate buffer (pH 7.4).

5.3.3 Separation of IgG subclasses by protein G chromatography

5 ml of IgG at 5 mg/ml was passed through a 0.2 nm filter and injected onto a 16/20 chromatography column containing 6 ml of Protein G sepharose (GE Healthcare) at 0.2 ml/min using an Akta Prime Plus instrument (GE Healthcare). The 'flow through' fractions (IgG2 subclass) were collected and labelled. The IgG3 subclass was then eluted from the Protein G matrix using 0.15 M NaCl, 0.58% acetic acid at pH 3.5. Finally, the IgG1 subclass was eluted from the column with 0.1 M glycine-HCl, pH 2.7. Eluted fractions were

neutralised using 2.5 M Tris-HCl, pH 9.0 and dialysed against 20mM sodium phosphate buffer pH 7.4. IgG fractions were stored at -20°C.

5.3.4 Time-course subclass ELISA

96 well ELISA plates (Nunc) were coated with 100 ng of *E. ocellatus* venom in carbonate buffer, pH 9.6 and incubated at 4°C overnight. Plates were washed after each stage, using 6 changes, with TBST (0.01 M Tris-HCl, pH 8.5; 0.15 M NaCl; 1% Tween 20). Next, plates were incubated at room temperature for 3 hours with 5% milk in PBS to 'block' non-specific reactivity. The primary antibodies comprised total IgG (natural levels of all subclasses after caprylic acid fractionation) and IgG subclasses from MS-Eo, PS1 and PS2 diluted 1:1000 of 10 mg/ml (TBST with 5% milk) from each time point. These were added to the wells in duplicate and left overnight at 4°C. Rabbit anti-camel IgG (non-commercial preparation, 1:5000) was added for 1 hour at room temperature. The plates were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma, UK) diluted 1:2000 for 1 hour. The results were visualised by adding substrate (0.2% 2,2/-azino-bis (2-ethylbenzthiazoline-6-sulphonic acid), in phosphate-citrate buffer pH 4 with 0.0015% hydrogen peroxide; Sigma, UK) and read at 405 nm. Results are the means of the duplicates.

5.3.5 End point titration ELISA

The method was identical to the time-course ELISA, with the following exception; the primary antibodies were the total IgG and the three IgG subclasses purified from sera collected from MS-Eo, PS1 and PS2 sixty weeks after immunisation and the IgG samples were serially diluted from

1:200 of 10 mg/ml (5% milk / TBST) by a factor of 6 across the plates. The titre is described as the dilution at which absorbance was greater than that of the negative control (un-immunised camel) plus 2 standard deviations.

5.3.6 Chaotropic ELISA

Plates were coated with *E. ocellatus* venom (see time-course ELISA) and incubated overnight at 4°C with total IgG and IgG subclasses from EoMS, PS1 and PS2 (extracted from week 60 sera and diluted 1:1000 from a starting concentration of 10 mg/ml in 5% milk/TBST). After washing, ammonium thiocyanate was added to the wells in a range of concentrations (0-8 M) for 15 minutes. Plates were washed, and all subsequent steps were the same as the time-course ELISA. Relative avidity was determined as the concentration of ammonium thiocyanate required to reduce the ELISA OD reading by 50%.

5.3.7 Immunoblotting

7µg of *E. ocellatus* venom in reduced protein loading buffer was added to each well of 15% SDS-PAGE (BioRad Protean II) with the final well containing the molecular weight marker (Broad range molecular weight protein markers, Promega). After separation, proteins were transferred to nitrocellulose paper, cut into strips, blocked with 5% milk/TBST (3 hours, room temperature) and incubated in the IgG subclasses and total IgG from MS-Eo, PS1, PS2 (1:1000 of 10 mg/ml in 5% milk/TBST) at 4°C overnight. Strips were washed (3 changes of TBST over an hour) and placed in rabbit anti-camel IgG (non-commercial preparation, 1:5000) for two hours at room temperature. After washing, strips were added to goat anti-rabbit IgG 1:2000

for an hour at room temperature. Strips were washed and then developed using DAB (50 mg 3,3-diaminobenzidine, 100 ml PBS and 0.024% hydrogen peroxide).

5.3.8 Neutralisation assays

Total IgG and IgG subclasses from MS-Eo and PS1 were concentrated to 25 mg / ml using spin columns (Sartorius) for use in the following assays.

5.3.8.1 Neutralisation of the procoagulant effects of *E. ocellatus* venom:

The minimum dose of *E. ocellatus* venom required to clot citrated human plasma in 60 seconds at 37°C (MCD-P) was determined by preparing a range of venom concentrations in physiological saline solution (PSS) to a final volume of 50 μl. This was added to 0.2 ml of citrated plasma at 37°C and the time taken to clot was recorded (Theakston and Reid, 1983). Various amounts of the relevant total IgG or IgG subclass (final volume 25 µl in PSS) were added to 0.2 ml of citrated plasma then the MCD-P was added and the minimum amount of total IgG or IgG subclass required to completely prevent clotting for a period of 5 mins at 37°C was determined. The assay was repeated to ensure accuracy.

5.3.8.2 Neutralisation of the haemorrhagic effects of *E. ocellatus* venom:

The minimum amount of *E. ocellatus* venom required to cause a haemorrhagic lesion of 10 mm after 24 hours when injected intradermally into the shaved dorsal area of CD1 mice (18-20g, Charles River) (MHD) was determined. Various amounts of total IgG and IgG subclasses were

incubated with the one venom MHD at 37°C for 30 mins. The mixture (50 µl) was injected intradermally into groups of 3 mice per IgG dose. After 2 hours, the mice were killed by CO_2 inhalation, the dorsal skin removed and the minimum amount of total IgG or IgG subclass (in microlitres) required to completely prevent haemorrhage in all three mice was assessed.

5.3.8.3 Heat stability assessment

MS-Eo IgG subclasses obtained by protein G separation at a concentration of 5 mg/ml were heated to: (a) 60°C for 10 hours and (b) 80°C for 5 minutes using a hot block (Grant Bio). These conditions were respectively selected based on protocols for pasteurisation of human plasma (Mannucci, 1993) and the protocol previously used on the antibody-binding fragments (V_{HH}) of camelid IgG2 and IgG3 subclasses (ref to VHH heat treatment). The immune reactivity of these heated IgG samples were compared to non-heated samples (control) using end point titration ELISA. End point titration was performed as previously outlined except the starting dilution of the IgG was 1:250 of 5 mg / ml. Turbidity of MS-Eo and sheep IgG was assessed by heating samples (50 mg/ml) at either: (a) 60°C for 10 hours or (b) 80°C for 5 minutes. Absorbance at 590 nm was measured and compared to untreated samples (Rojas et al., 1994; Otero et al., 2006).

5. 4 Results

5.4.1 Reactivity of camelid IgG subclasses to E. ocellatus venom.

The time course ELISA results (Figure 5.1) indicated that the heavy chain-only IgG2 and IgG3 subclasses were somewhat slower to reach a plateau titre that the heavy and light chain IgG1 subclass. However, once the plateau was attained (approximately 30 weeks after immunisation) the titres of the three subclasses were very similar, albeit with slight variation between individual camel IgG titres to venom immunisation. A result confirmed by the results of the End Point ELISA assays performed on IgG taken at the end of the experiment – 60 weeks after immunisation (Table 5.1). In contrast, the time course immunoblotting results (Figure 5.1) showed that the IgG subclass specificity to *E. ocellatus* venom proteins was more varied. It was apparent that that the protein-specificity of IgG1 in all three camels was more comprehensive and a better representation of the un-fractionated IgG (IgG total) than either the IgG2 or the IgG3 subclasses.

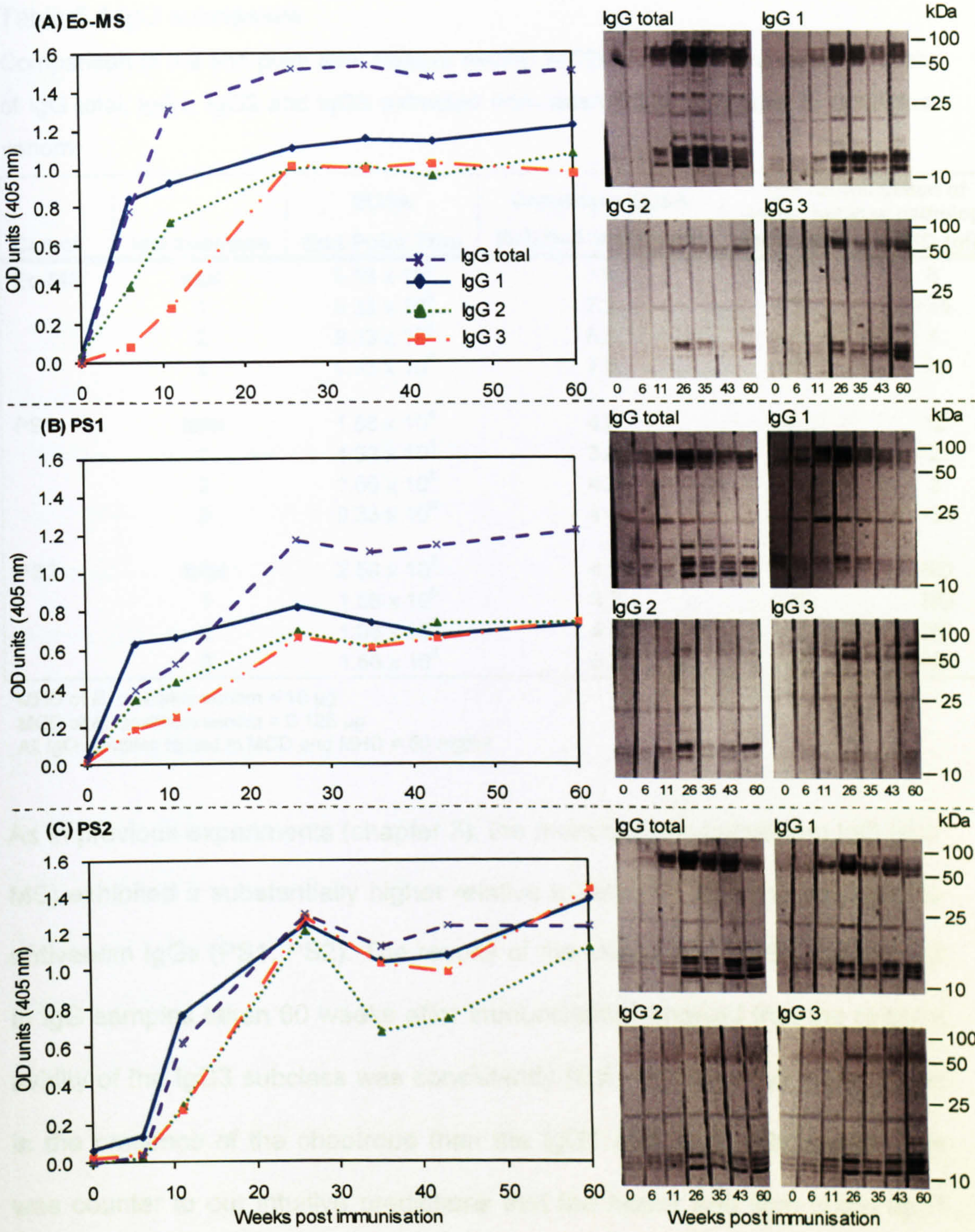

Figure 5.1 Immune reactivity (time course ELISA) to *E. ocellatus* venom by Total IgG (dashed lines, crosses), IgG1 (solid line, diamonds), IgG2 (dotted line, triangle) and IgG3 (solid line and two dots, squares) at seven time-points over a 60 week period (x axis) from camels: A – monospecific camel (Eo-MS), B – Polyspecific camel 1 (PS1) and C – Polyspecific camel 2 (PS2). Immunoblots showing the response of Total IgG, IgG1, IgG2 and IgG3 against *E. ocellatus* venom (under reducing conditions) for the same time-points as the ELISA are shown on the right for each camel. Numerals on the horizontal axis refer to weeks after immunisation and, on the vertical axis to the molecular mass of the venom proteins.

Table 5.1 IgG subclasses

Comparison of the end point titre, relative avidity, MCD and MHD neutralising doses of IgG total, IgG1, IgG2 and IgG3 extracted from week 60 sera against *E. ocellatus* venom

Camel	IgG subclass	ELISA End Point Titre	Chaotropic ELISA Relative avidity (M)	IgG neutralisation of venom induced pathology MCD (µl)	MHD (µl)
Eo-MS	total	9.33×10^6	>8	15	8
	1	9.33×10^6	7.2	4.5	>25
	2	9.33×10^6	6.3	>25	4
	3	9.33×10^6	7.8	>25	14
PS1	total	1.56×10^6	4.9	4	12
	1	4.32×10^4	3.4	>25	>25
	2	1.56×10^6	4.2	1	3
	3	9.33×10^6	4.4	4.5	>25
PS2	total	2.59×10^5	4.9	>25	ND
	1	1.56×10^6	3.7	>25	ND
	2	1.56×10^6	4.5	>25	ND
	3	1.56×10^6	5.2	>25	ND

MHD of *E. ocellatus* venom = 10 µg
MCD of *E. ocellatus* venom = 0.125 µg
All IgG samples tested in MCD and MHD = 50 mg/ml

As in previous experiments (chapter 3), the monospecific antivenom IgG (Eo-MS) exhibited a substantially higher relative avidity (M) than the polyspecific antivenom IgGs (PS1, PS2). The results of the chaotropic ELISA (performed in IgG samples taken 60 weeks after immunisation) showed that the relative avidity of the IgG3 subclass was consistently (but not markedly) more robust in the presence of the chaotrope than the IgG1 and IgG2 subclasses. This was counter to our intuitive predictions that the heavy and light chain IgG1 subclass would exhibit stronger antigen-binding activity. We have already reported that results of the relative avidity ELISA can not be used as an accurate predictor of antivenom effectiveness (Chapter 4), despite the fact that all the most effective antiveoms showed high relative avidity levels. We therefore feel that the enhanced performance of IgG3 in this assay is

insufficient justification to recommend the selection of this IgG subclass for antivenom production.

5.4.2 Preclinical neutralisation of E. ocellatus venom-induced pathology by camelid IgG subclasses

It was apparent from the preclinical results described in Table 5.1 that while the un-fractionated IgG of camel PS1 was effective in neutralising both *E. ocellatus* venom-induced haemorrhage (MHD assay) and coagulopathy (MCD assay), that of Eo-MS was only effective in the MHD assay and the un-fractionated IgG of PS2 was ineffective in both assays at the maximum volume (20 µl) permitted in these assays. No one IgG subclass from PS1 or Eo-MS showed a consistently enhanced performance in the MCD assay. In both PS1 and Eo-MS the IgG2 subclass was more effective in the MHD assay than IgG1 or IgG3 subclasses. However, because this result was from only two animals, we are reluctant to assign significance to this observation. We did not conduct assays assessing IgG subclass neutralisation of venom lethality (ED50 assay; Theakston and Reid., 1983) because we were unable to satisfactorily justify the use of 120+ mice for an experiment where it seemed unlikely that one IgG subclass would dominate the result.

Taken in conjunction, we interpret the results of the immunological and preclinical assays as indicating that no one IgG subclass showed a consistently higher titre, more comprehensive venom-protein specificity or venom-neutralising efficacy than the un-fractionated IgG.

5.4.3 Immunological performance of IgG subclasses after heat treatment

This analysis was conducted with IgG samples from camel Eo-MS. While there was no discernable difference in the End Point ELISA titres to *E. ocellatus* venom before treatment, the immunoreactivity of the heavy chain IgG2 and IgG3 subclasses was markedly less effected by incubation under pasteurisation conditions of 60°C for 10 hours (Figure 5.2) than the heavy and light chain IgG1 subclass. Under the shorter but more extreme heat conditions previously used to examine the thermotolerance of recombinant camelid antigen binding domains (VHH; (van der Linden et al., 1999), the immunoreactivity of the IgG3 subclass was substantially more robust than the IgG1 or IgG2 subclasses.

The improved heat stability of camel IgG was also seen in the turbidity assay after heating to 80°C for 5 minutes (Figure 5.3). Camel IgG after this heating step retained a liquid appearance and an absorbance level similar to that of the camel IgG sample heated at 60°C for 10 hours, whilst heating sheep IgG at 80°C for 5 minutes caused the formation of a gel like substance with an absorbance value eleven times greater than camel IgG. Camel and sheep IgG had a comparable absorbance when heated at 60°C for 10 hours.

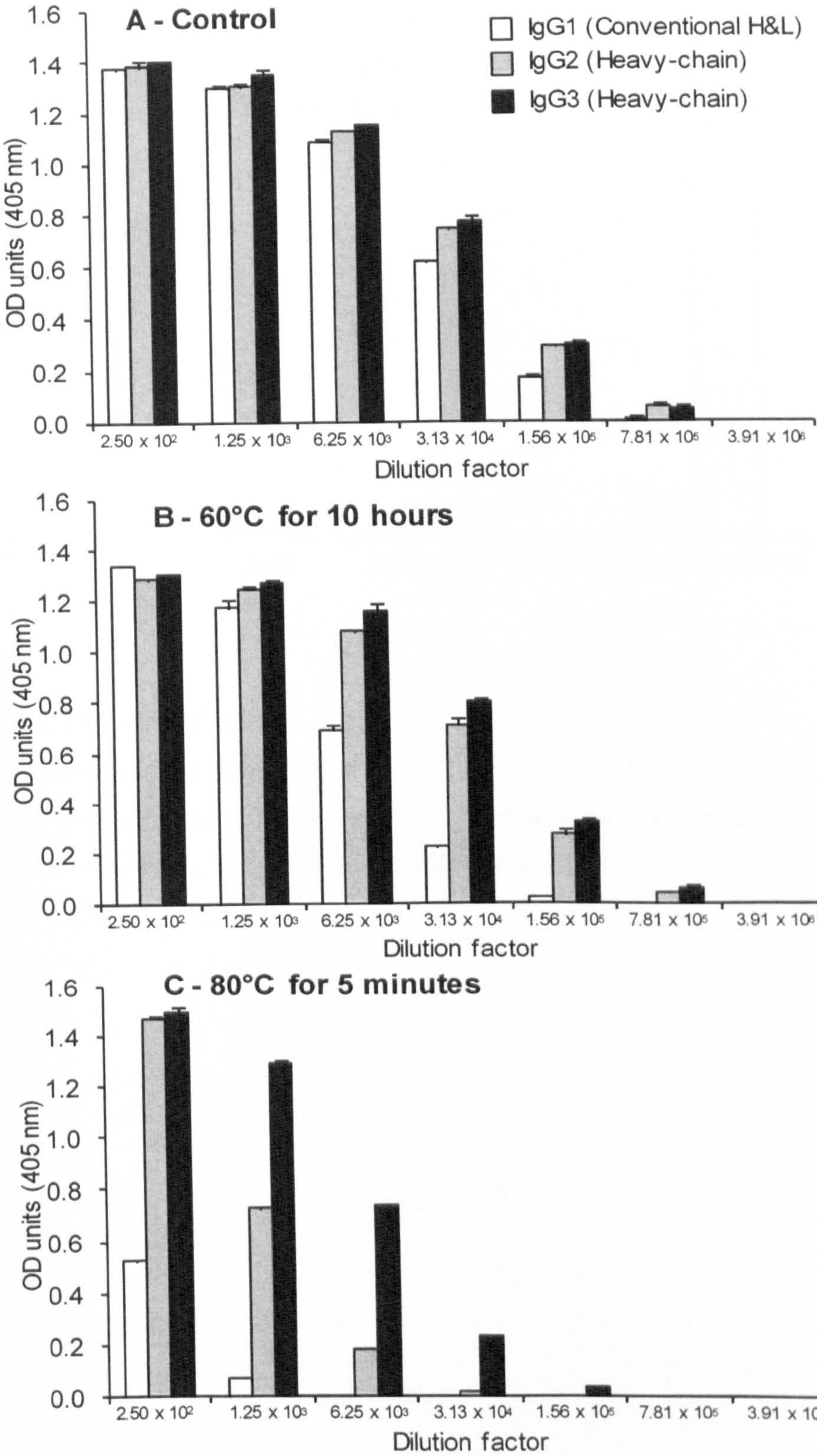

Figure 5.2 The effect of heat treatment on the end point titre ELISA

IgG1 (white columns), IgG2 (Grey columns) and IgG3 (black columns) subclasses of camel IgG against *E. ocellatus* venom after: A - no treatment, B - heated at 60°C for 10 hours and C - heated at 80°C for 5 minutes. Error bars represent S.E.M (n = 3).

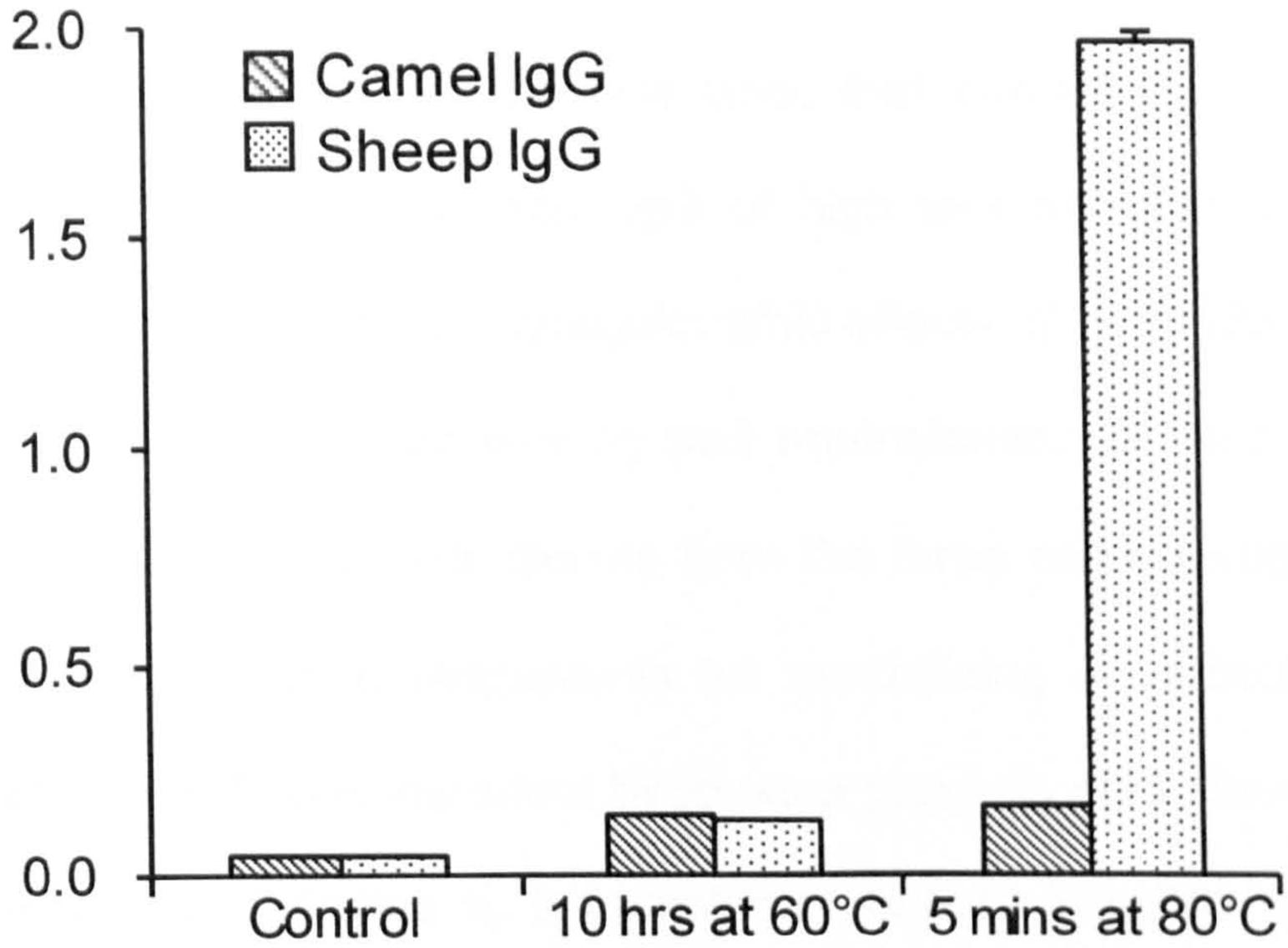

Figure 5.3 Turbidity of camel and sheep IgG

Turbidity (assessed by absorbance at 590 nm) of camel IgG (diagonal lines) and sheep IgG (dots) prepared by caprylic acid fractionation either subjected to heating at: 60°C for 10 hours, 80°C for 5 minutes or without heat treatment. Error bars represent S.E.M (n = 3).

5. 5 Discussion

This report demonstrates, for the first time, that camels immunised with snake venom produce heavy-chain IgG of high titre and potent ability to neutralise the haemorrhagic and coagulopathic effects of *E. ocellatus* venom. The variability in venom toxin binding and neutralisation of venom-induced pathology by IgG, and IgG subclasses from the three camels suggests that no single IgG subclass is responsible for neutralising a particular venom induced pathology. This is important because a previous study found that the antibody response of llamas to a parasitic nematode infection was almost solely IgG1 (Daley et al., 2005) and over 50% of camel IgG comprises the IgG 1 and 2 subclasses. This study showed that the heavy chain IgG subclasses were as capable as IgG1 in binding venom proteins and neutralising their pathological effects. Indeed, no single IgG subclass dominates the serological response to *E. ocellatus* venom. This means that IgG taken from a pool of immunised camels could be processed into antivenom without requiring the implementation of expensive chromatographic separations to select, or indeed to exclude, a specific IgG subclass.

We considered that the much reported (van der Linden et al., 1999; Omidfar et al., 2007; Goldman et al., 2008) thermal stability of recombinant VHH (the antibody-binding fragment of heavy chain-only IgG subclasses) held promise for the inclusion of a pasteurisation step into the manufacture of camelid antivenoms. Pasteurisation represents a cheap and effective anti-microbial system as it does not require additional steps such as the removal of the solvent-detergent, neutralisation of acid or costly equipment such as filters

(Burnouf and Radosevich, 2000) and has an excellent safety record in manufacture of human plasma products (Mannucci, 1993; Chandra et al., 2002). Pasteurisation typically involves treating samples at 60°C for 10 hours and has been shown to inactivate a range of viruses including enveloped (HVBV, HIV, Hepatitis B virus (HBV) and Hepatitis C virus (HCV)) and non-enveloped (Hepatitis A virus (HAV) porcine enterovirus 4 and poliovirus) (Hilfenhaus and Weidmann, 1986; Mannucci, 1993; Nowak et al., 1993). At least one antivenom has been produced using a pasteurisation step to inactivate viruses (Grandgeorge et al., 1996). The sequential treatment of horse serum with caprylic acid and pasteurisation generated an IgG solution with a ≥9 $\log_{10}$ reduction in enveloped viruses and a 4 $\log_{10}$ reduction in non-enveloped viruses including minute virus of mice (MVM) which was used as a model for porcine and equine viruses with the highest acid and heat resistance (Mpandi et al., 2007). Viral inactivation steps that reduce virus titre by the order of 4 $\log_{10}$ or greater are considered effective (EMEA, 1996). However, heating IgG to high temperatures or for long periods can cause irreversible denaturation/aggregation leading to the formation of an insoluble gel substance (Aghaie et al., 2008). Pasteurisation of IgG at concentrations of 25 g L^{-1} and above (concentrations used in antivenom production) required the use of two stabilisers to protect the native structure of the IgG (Aghaie et al., 2008). Without additional stabilisers, pasteurisation of equine IgG increased aggregation from 0.76 to 29% (Mpandi et al., 2007), above the allowed 5% limit for intravenous administration of IgG preparations (European pharmacopeia, 2002). Our results demonstrate, in terms of IgG turbidity, that an ovine IgG antivenom tolerated pasteurisation conditions as

well as our camel IgG antivenoms (Figure 5.3). However, under much more extreme heat conditions (80°C, 5 mins) the camel IgG but not ovine IgG retained its native clarity. This is an important result because it demonstrates that camel IgG is as thermotolerant as the recombinant VHH. This suggested that a heating step, following the virucidal benefit (Steinbuch and Audran, 1969) of using caprylic acid to precipitate IgG from serum, could be introduced as a cheap anti-microbial step in antivenom manufacture. Caprylic acid does not inactivate non-enveloped viruses (Mpandi et al., 2007) and the WHO guidelines and the European Medicines Agency (EMEA) state that using two complimentary viral inactivation steps is preferable (WHO) (EMEA, 1996). Our results demonstrate that the immunoreactivity of camel IgG shows subclass-distinct vulnerability to heat treatment. Under pasteurisation conditions, the immune reactivity of the heavy and light chain IgG1 subclass was markedly lower than the heavy chain-only IgG2 and IgG3 subclasses. Furthermore, the immune reactivity of both IgG1 and IgG2 was severely reduced after being subjected to 80°C for 5 minutes; only the IgG3 subclass retained a reasonable End Point ELISA titre to *E. ocellatus* venom proteins (1.5×10^5). Since the results of the immunological and preclinical efficacy assays indicated the importance of retaining all three subclasses for the production of camelid antivenom, we would therefore not recommend the inclusion of an anti-microbial heat step in the manufacturing protocol.

The generation of a thermostable antivenom that does not require refrigeration would enable the distribution of effective snakebite treatment closer to the areas where it is most needed but that are lacking the

necessary 'cold chain.' Heavy chain-only IgGs retain 88% of their maximal binding capacity after 100 hours at 37°C, compared to ~30% for conventional IgG (Omidfar et al., 2007). If the improved heat stability of camel IgG shown by Omidfar et al and by this paper translates to heat stability over longer periods of time, it may be possible to produce a camel-derived antivenom that is stable at room temperature. The addition of 2 M sorbitol has been shown to improve the long term stability of equine IgG antivenoms (Segura et al., 2009). If camel IgG exhibits improved thermal stability over greater periods of time at room temperature this may reduce the necessity for the addition of stabilisers or the addition of stabilisers to a camel-derived antivenom may extend the shelf-life beyond that of antivenoms derived from equine or ovine sources. Further work is needed to clarify the validity of these potential benefits.

In conclusion, this study demonstrated that no one class of camelid IgG dominates either the serological response of camels to snake venom or the ability of total IgG to neutralise venom-induced pathologies. Therefore, despite the very different biological configuration of the heavy and light chain and heavy chain-only IgG subclasses, we do not feel that there is a compelling immunological or immunotherapeutic reason to justify the expense of chromatographically selecting one IgG subclass for antivenom production. This fiscally-attractive outcome of the study is however balanced by the demonstration that the immune reactivity of IgG1 was particularly vulnerable to heat treatment – even pasteurisation conditions. In

consequence, we would not recommend the inclusion of an inexpensive anti-microbial heat step in the manufacturing protocol of camelid IgG antivenom.

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CHAPTER 6

Title: Preclinical assessment of native VHH neutralising efficacy against *Echis ocellatus* venom.

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6. 1 Abstract

Envenoming by snakes remains a serious but neglected public health problem in many parts of the world. The administration of antivenom remains the 'first line' treatment for snake envenoming. However, the administration of large amounts of foreign protein can have adverse consequences for the patient, either as a serious early hypersensitivity reaction (anaphylaxis) or later development of antibody response against the foreign IgG (serum sickness). Although, effective at treating the systemic effects of envenoming, antivenom is ineffective against the local effects including necrosis which may lead to amputation. Advances in DNA technology and the discovery of a unique antigen binding fragment of camelid IgG may offer a solution to both the problem of adverse reactions and lack of efficacy in treating the local effects of envenoming. Camel sera contains a significant number of IgG that is devoid of light chains, the antigen binding domain of which (VHH) displays low immunogenicity, excellent tissue penetration, excellent stability and can be produced relatively cheaply in large quantities in recombinant form. All of which makes VHH an attractive tool for the treatment of snakebite.

The focus of this work was to investigate the potential of native VHH to neutralise the pathological effects of venom from the most medically important snake in Africa, the saw-scaled viper (*Echis ocellatus*).

Native VHH neutralised the lethal, haemorrhagic and procoagulant effects of *E. ocellatus* venom. When compared by mass, VHH was around three times more effective at preventing the lethal and haemorrhagic effects of the venom and four times more effective at preventing coagulopathy than the SAVP *Echis* monospecific antivenom.

These findings illustrate that an antivenom derived solely of VHH would be capable of neutralising the major pathological effects of *E. ocellatus*. This adds weight to the theory that the production of specially tailored toxin-specific recombinant VHH has the potential to treat both the local and systemic effects of snake envenoming, whilst reducing the incidence of adverse reactions and lowering the cost of treatment.

6. 2 Introduction

Envenoming by snakes remains a serious but neglected public health problem in many parts of the world (Gutierrez et al., 2006; Williams et al., 2010), especially in tropical and sub-tropical regions, where the rural poor are particularly affected (Harrsion et al., 2009). Estimates of the global number of envenomings ranges from at least 420,000 to over 1,800,000 per year, leading to between 20,000 and 94,000 deaths (Kasturiratne et al., 2008). Many that survive envenoming suffer debilitating long term physiological and perhaps psychological sequelae, and it has been estimated that about 400,000 amputations are conducted each year to treat the necrotic consequences of snakebite (Mion and Olive, 1997). Victims are often young and from economically productive roles (e.g. agricultural workers) (Trape et al., 2001; Einterz and Bates, 2003) and the loss or debilitation of these people means the socioeconomic cost of snake envenoming is also predicted to be high.

The administration of antivenom remains the 'first line' treatment for snake envenoming. Since its development at the end of the 1800s (Calmette, 1895), the method of antivenom production has not altered radically. Conventional antivenoms are still produced by immunisation of animals (typically horses or sheep) with venoms then fractionation of sera to isolate antibodies (Lalloo and Theakston, 2003; Gutierrez et al., 2005). The IgG (150 kDa) in current antivenoms is formulated either in complete form or digested by pepsin to form $F(ab')_2$ (100 kDa) or by papain to form Fab (50 kDa) fragments. The final product contains a heterologous mix of polyclonal antibodies or antibody fragments. The parenteral administration of large

amounts (typically 20 – 200 ml) of this foreign protein can have adverse consequences for the patient (Leon et al., 2001) either as a serious early hypersensitivity reaction (anaphylaxis) or later development of antibody response against the foreign IgG (serum sickness). Increasing the purity of an antivenom (e.g. exclusion of pyrogens, removal of aggregates) (Otero et al., 1999), reducing the antibody fragment size and reducing the total amount of foreign protein are steps in antivenom production used to reduce the risk of these adverse reactions (Chippaux and Goyffon, 1998; Dart and McNally, 2001). However, the added manufacturing steps required to generate Fab antivenoms of high purity may increase the cost of a treatment that is already unaffordable to many victims (Theakston and Warrell, 2000).

Whilst these innovations are reducing the adverse effects of antivenom treatment of systemic envenoming, there has been no convincing evidence that their use reduces the incidence or severity of the local effects of envenoming (Dart et al., 2001) for which there still remains no effective medicinal treatment. Such local effects, including: oedema, haemorrhage, ischaemia blistering and necrosis at the site of the bite, can require tissue removal or even amputation. Developing an effective treatment for the local effects of envenoming remains an urgent research priority.

Advances in DNA technology and the discovery of a unique binding fragment in camel IgG may offer a solution to the problem of adverse reactions and have the potential to treat the deleterious local effects of envenoming. More than 50% of camel IgG lack light chains (Hamers-casterman et al., 1993). The antigen binding region (VHH) of these heavy-chain antibodies (HCIgG) has been evolutionarily adapted to function in the absence of the light chain

domain (Desmyter et al., 1996) and is regarded as the smallest intact, single-domain antibody fragment capable of antigen recognition. Their small size (~15kDa) allows rapid and deep tissue ingress and the extended CDR3 loop is capable of potent enzyme / toxin neutralisation (Lauwereys et al., 1998; Abderrazek et al., 2009). For these reasons, prompt administration of toxin specific VHHs after snake envenoming has the potential to prevent or limit the extent of local venom effects.

Recombinant VHH shows low immunogenicity, with no detectable host immune response when injected into mice (Coppieters et al., 2006) or pigs (Harmsen et al., 2009). In a single dose application in humans, no dose-limiting toxicities or serious adverse reactions were reported (Dec 2007 report from www.ablynx.com). The tandem cloning of two VHH connected by a flexible linker or a recombinant Fc portion facilitates the generation of bivalent VHH or bispecific constructs (Conrath et al., 2001) which can further improve binding affinity to the toxin (Hmila et al., 2008) whilst still remaining undetectable by the host's immune system (Harmsen et al., 2009). Such gene-tailored serological constructs directed against specific snake venom toxins responsible for the pathological effects of snake venom has the potential to effectively treat the victims of snake envenoming whilst reducing the overall amount of foreign protein administered. This, coupled with the low immunogenicity of recombinant VHH could help to reduce the incidence of adverse reactions of an antivenom. The Pasteur Group in Tunisia has reported very encouraging results of the use of intact IgG (Meddeb-Mouelhi et al., 2003), and recombinant VHH-derivatives (Hmila et al., 2008; Abderrazek et al., 2009) to treat scorpion envenoming.

We recently demonstrated that intact IgG (chapter 4) and each of the three subclasses (chapter 5) from snake venom-immunised camels were as effective as conventional equine and ovine antivenoms in preclinical assays of snake envenoming. To progress this camelid antivenom project, the objective of this study was to examine the potential of native VHH to neutralise the pathological effects of snake envenoming. The focus of this study was on the most medically important snake in Africa, the saw-scaled viper, *Echis ocellatus,* and the most lethal systemic and local effects of its venom – haemorrhage and incoagulable blood.

6. 3 Materials and method

6.3.1 Venom

Specimens of *E. ocellatus* of mixed age and sex collected from Nigeria were maintained in the herpetarium of the Alistair Reid Venom Research Unit at the Liverpool School of Tropical Medicine. Venom was extracted from the snakes, frozen, lyophilised and stored at 4°C as a powder.

6.3.2 Preparation of IgG

Five camels received immunisations with a mixture of venom from *E. ocellatus*, *Bitis arietans* and *Naja nigricollis* (PS1-5) and one camel (Eo-MS) was immunised with *E. ocellatus* venom only (see chapter 3 for details of the immunisation schedule). From our previous study (chapter 4) IgG2 from Camel PS1 was identified as being able to neutralise both coagulopathy and haemorrhage and was therefore chosen for this study. IgG from PS1 was extracted from sera taken at week 60 using 5% caprylic acid (Sigma), stirred vigorously at room temperature for 2 hours, before centrifuging at 13, 000 RPM for 25 minutes. The supernatant was dialysed with three changes of 20 mM sodium phosphate buffer (pH 7.4).

6.3.3 Protein G separation of IgG subclasses

IgG subclasses were separated as previously described by (Hamers-casterman et al., 1993). Briefly, 5 ml of IgG at 5 mg / ml was passed through a 0.2 nm filter and injected on to a 16/20 chromatography column containing 6 ml of Protein G sepharose (GE Healthcare) at 0.2 ml / min using an Akta Prime Plus (GE Healthcare). The flow through was collected and labelled as IgG2. IgG3 was eluted using 0.15 M NaCl, 0.58% acetic acid at pH 3.5 and

IgG1 with 0.1 M glycine-HCl, pH 2.7. Eluted fractions were neutralised using 2.5 M Tris-HCl, pH 9.0 and dialysed against 20mM sodium phosphate buffer pH 7.4. IgG isotypes were stored at -20°C.

6.3.4 Generation of IgG fragments

PS1 IgG2 was concentrated to 50 mg/ml (Vivaspin, 30 kDa cut off) and 1ml was added to 2 ml of digestion buffer (20 mM sodium phosphate, 10 mM EDTA, 20 mM cysteine.HCl, pH 7.0). The sample was then added to 5 ml of equilibrated immobilised papain slurry (50%) (Fisher / Thermo Scientific Pierce, UK) and incubated at 37°C with constant mixing. After 2 hours, 1.5 ml of 10 mM Tris-HCl, pH 7.5 was added and the tube was centrifuged at 4000 RPM for 2 minutes before removing the supernatant that contained VHH and Fc fragments.

2 ml of the papain-digested solution was injected on to a 16/20 column containing 5 ml of Protein A sepharose (GE Healthcare) at 0.2 ml / min using an Akta Prime Plus (GE Healthcare). The flow through was collected and contained VHH. The bound fraction was eluted using 0.1 M glycine-HCl, pH 2.7 and contained the Fc portion, confirmed by size of fragment after running on SDS-PAGE and staining with coomassie blue. Both fractions were dialysed against 20mM sodium phosphate buffer pH 7.4 and concentrated to 25 mg/ml (vivaspin, 5 kDa cut off)

6.3.5 VHH neutralisation of Venom Lethality

The LD_{50} of *E. ocellatus* venom was determined as described in Chapter 4. Briefly, several groups of five mice were injected with a range of venom

doses and number of deaths per group recorded after 7 hours. LD_{50} and 95% confidence intervals were calculated by probit analysis (Finney, 1971). To determine whether VHH could neutralise the lethal venom effects, two different doses (10 µl and 50 µl of VHH at 25 mg/ml per mouse) were incubated with 5 x LD_{50} of venom (total volume 200 µl) at 37°C for 30 minutes then injected into the tail vein of CD-1 mice (5 mice per group). After 7 hours, the number of deaths was recorded. A full ED_{50} was not possible due to the limited amount of VHH generated. For comparison, the dose required to prevent death in all five mice was taken from the data used to generate the ED_{50} values of the South African Vaccine Producers (SAVP) *Echis* monospecific antivenom and from the polyspecific camel whole IgG PS1 (chapter 4).

6.3.6 VHH neutralisation of haemorrhage

The minimum amount of *E. ocellatus* venom required to cause a haemorrhagic lesion of 10 mm after 24 hours when injected intradermally into the shaved dorsal area of mice (MHD) was determined. Various amounts of VHH at 25 mg/ml were incubated with 1 MHD of venom at 37°C for 30 minutes and the 50 µl mixture injected intradermally into groups of 3 mice per VHH dose. After 2 hours mice were killed by CO_2 inhalation, the dorsal skin removed and the minimum amount of IgG (in microlitres) required to completely prevent haemorrhage was assessed, under background illumination. This was compared to the values determined for whole PS1 IgG, PS1 IgG2 and SAVP *Echis* antivenom

6.3.7 VHH neutralisation of procoagulant effects

The minimum dose of *E. ocellatus* venom required to clot citrated human plasma in 60 seconds at 37°C (MCD-P) was determined by preparing a range of venom concentrations in physiological saline solution (PSS) to a final volume of 50 μl. This was added to 0.2 ml of citrated plasma at 37°C and the time taken to clot the plasma was recorded (Theakston and Reid, 1983). Various amounts of VHH at 25 mg/ml (final volume 25 µl in PSS) were added to 0.2 ml of citrated plasma, and then one venom MCD-P added and the minimum amount of VHH required to completely prevent clotting for a period of 5 minutes at 37°C was determined. This was compared to the values determined for whole PS1 IgG, PS1 IgG2 and SAVP *Echis* antivenom

6.3.8 Heat treatment of VHH

To asses the thermostability of the immune functioning of VHH, we subjected samples of VHH from PS1 (25 mg/ml) to heat at 80°C for 5 minutes in a hot block (Grant Bio); after which the samples were rapidly cooled on ice. The ability of these heat-treated samples to neutralise the haemorrhagic and procoagulant effects of venom was determined as previously described. SAVP antivenom was also subjected to heat treatment.

6. 4 Results

6.4.1 Processing of IgG to obtain VHH

IgG2 was separated from the other IgG subclasses using a protein G column and digested using immobilised papain. The VHH was successfully isolated from the Fc domain by using the Fc binding properties of protein A chromatography (Figure 6.1). The band indicated as VHH had previously been identified by immunoblotting of papain digestion products during optimisation, using an anti-VHH antibody (gift from S. Muyldermans, Vrei Universitaat, Brussels). During the attempts to separate VHH, this small molecular weight fragment has always appeared as a diffuse, faint band (Figure 6.1 B, lane 7). The presence of other bands in the VHH sample could be due to digestion of aggregates present in the IgG2 sample or contaminants from the immobilised papain digest. Since the IgG2 sample (Figure 6.1 A, lane 5) contains very little IgG1, as judged by the lack of a readily detectable band at around 150 kDa, and the IgG1 fraction has no venom neutralising ability as judged by MCD and MHD (see chapter 4), we conclude that the neutralising effects of the VHH fraction is unlikely to be due to contamination by $F(ab')_2$. It is expected that the protein A column binds intact HCIgG, thus removing any undigested IgG2 from the VHH fraction, therefore any neutralisation of venom effects by the VHH fraction must be due solely to the presence of VHH.

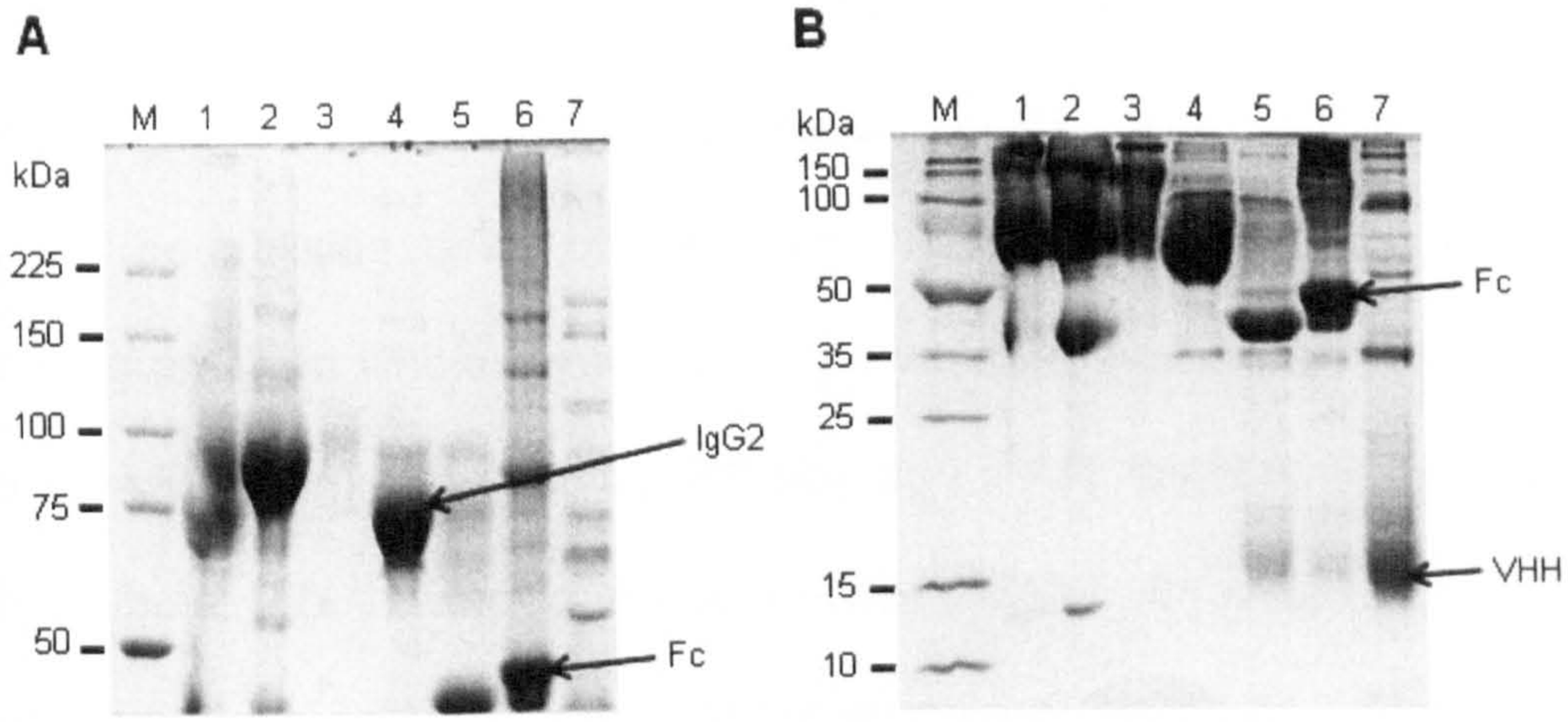


Figure 6.1 SDS-PAGE analysis of the fractionation, protein G separation and papain cleavage of camel IgG.

Proteins separated using (A) 7.5% or (B) 15% SDS-PAGE under non-reducing conditions were visualised by coomassie stain. Lanes correspond to each subsequent step in the IgG separation process as follows: intact IgG after caprylic acid fractionation (lane 1); run through (IgG2, lane 2) and eluted fraction (IgG1 and IgG3, lane 3) from protein G separation; dialysed IgG2 for papain digestion (lane 4); papain digestion products (lane 5); removal of Fc portion (lane 6) and flow through (containing VHH, lane 7) after protein A separation. Lane M: protein standard ladder.

6.4.2 Neutralisation of venom pathology by VHH

VHH neutralised the lethal, haemorrhagic and procoagulant effects of *E. ocellatus* venom (Table 6.1). Prevention of lethality as determined by the dose required to save all five mice when mixed with 5xLD_{50} showed VHH to be nearly three times more effective than the SAVP commercial antivenom (neutralising dose = 720 µg) which is an $F(ab')_2$ antivenom. The two neutralising units of an $F(ab')_2$ fragment have a molecular mass of 100 kDa, approximately three times larger than the mass of two VHH (~30 kDa) so it is perhaps unsurprising that VHH should be nearly three times as effective per microgram than the SAVP antivenom. The VHH fraction was also four times

more potent at neutralising haemorrhage and over four times more effective at preventing venom induced coagulation than the SAVP antivenom.

VHH (neutralising dose of =250 µg) was five times more effective per microgram than whole camel IgG from which it was generated (neutralising dose = 1250 µg) at preventing lethality again this result may be explained by the whole IgG having a mass between 6-10 times greater than VHH. However, VHH required a third of the amount of the intact HCIgG2 to prevent haemorrhage (75 µg vs 150 µg), but double the mass of VHH was required compared to HCIgG to prevent coagulation.

Table 6.1 Comparative neutralising efficacy of *E. ocellatus* venom by an experimental camel antivenom, heavy-chain IgG, VHH and a commercial antivenom

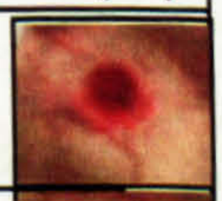
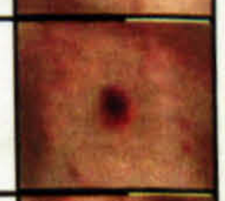
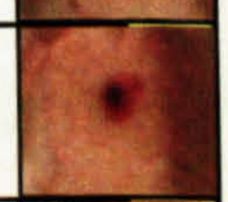
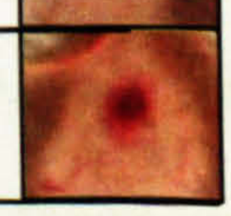
IgG type:	Neutralisation of venom induced: **Lethality (µg)**	**Haemorrhage (µg)**	**Coagulation (µg)**
Camel (PS1)			
Whole IgG	1250	600	200
IgG2 (HcIgG)	ND	150	50
VHH	250	75	100
Horse			
SAVP {F(ab')$_2$}	720	300	480

Lethality = The mass of IgG or fragment that protected 100% of mice (*n*=5) against 5xLD_{50} (74.25 µg of *E. ocellatus* venom); Haemorrhage = Mass required to completely neutralise 1 MHD in all 3 mice (MHD = 10 µg of *E. ocellatus* venom); Coagulation = Mass required to neutralise 1 MCD (0.125 µg of *E. ocellatus* venom); SAVP (ms) = *Echis* antivenom, South African Vaccine Producers; ND = test not performed

6.4.3 Heat stability of VHH

After heating at 80°C for 5 minutes, the VHH fraction was able to prevent venom induced coagulation of plasma and reduced haemorrhage by ~70%. To prevent coagulation, the amount of VHH had to be increased by over six-fold to 625 µg compared to 100 µg for the control (non-heated VHH). Using 250 µg of VHH reduced the size of the haemorrhagic lesion by ~70%. Further increases in the amount of VHH (up to 1000 µg) caused no further reduction in lesion size (Table 6.2). It was not possible to test the SAVP antivenom because it formed a white gel-like substance when heated at 80°C for 5 minutes, even when diluted to 25 mg/ml, the same concentration as the VHH sample.

Table 6.2 Effect of heat treatment on the ability of VHH to neutralise venom-induced haemorrhage.

VHH (µg)	Reduction in mean haemorrhagic lesion size (%)	
0	0	
250	69.8	
375	66.8	
1000	69.4	

Result of neutralisation illustrated by one of the three mouse skins used per group; all doses tested against 1 MHD (MHD = 10 µg of *E. ocellatus* venom); result is the mean of 3 mice.

6. 5 Discussion

Native VHH was successfully extracted, using protein G, immobilised papain and protein A, from the sera of a camel immunised with *E. ocellatus* venom. This VHH fraction alone was shown to have high venom neutralising potency compared to a commercial antivenom and prevented death in five mice when pre-incubated with 5 x venom LD_{50} before i.v. administration. Neutralisation of the haemorrhagic and procoagulant effects of the venom, as well as the lethal effects, illustrates that an antivenom derived solely of VHH would be capable of neutralising the major pathological effects of *E. ocellatus* venom and could therefore be an effective therapeutic. This is an important finding since it has previously been reported that the IgG1 subclass dominated the serological response of llamas to a parasitic nematode infection llamas (Daley, L.P. et al., 2005). Such a response against any of the major venom toxins responsible for the pathology of *E. ocellatus* envenoming would produce a VHH fraction that was at best only partially protective.

It has previously been reported that VHH has a high level of stability, being resistant to heat, proteolysis and extremes of pH (van der Linden et al., 1999; Dumoulin et al., 2002; Harmsen et al., 2006). With regards to heat stability VHH can tolerate 85°C for an hour without any loss of antigen binding activity (Goldman et al., 2008). However, all of these examples are of recombinant VHH which were selected precisely on the basis of their stability. Other reports show that VHH clones are more variable in their susceptibility to denaturation and loss of binding activity (Harmsen et al., 2005; Omidfar et al., 2007). The native VHH extracted in our study showed impressive heat

stability, remaining as a clear liquid when heated to 80°C compared to the commercial antivenom which formed a white gel-like substance. Despite this, heating did diminish the performance of the VHH fraction at inhibiting the action of venom compared to the unheated sample, requiring six times the quantity of VHH to prevent venom-induced coagulation and only partially neutralising haemorrhage. The inability to neutralise haemorrhage beyond 70% of the control, even when greater amounts of VHH were used, may indicate that whilst most VHH showed high heat stability, certain VHH clones responsible for neutralising one or more haemorrhagic toxins were rendered ineffective, thus 30% of haemorrhage cannot be inhibited irrespective of the VHH concentration. The susceptibility of some VHH clones to proteolysis may also explain why, after papain digestion, it requires twice the amount of VHH to prevent coagulation in the MCD assay compared to the intact HCIgG from which the VHH was isolated.

The future of VHH as a treatment of snake envenoming is likely to be in recombinant form since the production of native VHH is laborious and costly, requiring many stages of processing to produce a low yield (in this study 240 mg of whole IgG was processed to produce 20.2 mg of VHH). In contrast to this, recombinant VHH can be expressed relatively cheaply in large quantities (up to 100 mg per litre of culture) using yeast (Frenken et al., 2000). Improvement of the heat tolerance seen in native VHH could be ensured by selection of heat stable recombinant VHH or the grafting of loops from less stable VHH on to a stable universal loop acceptor VHH domain. This procedure should ensure greater resistance to both heat denaturation and

proteolysis (Saerens et al., 2005), potentially allowing a VHH derived antivenom to be pasteurised or stored at room temperature for considerable time without loss of activity. This would both ensure the safety of the antivenom and also allow distribution to areas where constant refrigeration may not be possible. Reducing the time between envenoming and antivenom administration can be key to a victim's survival (Warrell et al., 1977; Pugh and Theakston, 1987) and improved access to antivenoms for people in rural areas where snakebite incidence is high could reduce the delay caused by a long journey to the nearest (but distant) treatment centre, thus improving the geographical application of antivenom therapy.

The onset of local tissue damage after snake envenomation is rapid (Gutiérrez et al., 1984) and the failure of either IgG or Fab antivenoms to prevent the development of the local effects (León et al., 2000) means that there is still no effective treatment. Despite this failure, the injection of metalloproteinase inhibitors shortly after venom administration (Rucavado et al., 2000) confirms that prompt administration of a specific toxin inhibitor can greatly reduce the local venom induced damage. The small size of VHH (15 kDa) facilitates rapid and extensive tissue penetration (Cortez-Retamozo et al., 2002; Muruganandam et al., 2002) greater than that of conventional IgG (Perruchini et al., 2009) and therefore may have the potential to rapidly neutralise the relevant toxins. VHH are of similar size to phospholipase A_2s (14 kDa) and smaller than snake venom metalloproteinases or SVMPs (20-100 kDa), two toxin families responsible for the local pathology seen after viper bites (Gutierrez and Rucavado, 2000; Gutierrez and Ownby, 2003;

Laing, G.D. et al., 2003), which may mean prompt transdermal or intramuscular injection of VHH will have a similar or greater volume and speed of distribution as the relevant pathological toxins and therefore may succeed in reducing local tissue damage where Fab administration has failed. If the optimum transdermal delivery system can be determined, then a first-aid treatment comprising of toxin-specific VHH, to be administered immediately after envenomation, may reduce local tissue damage and reduce systemic venom effects for a short period allowing the victim time to seek the administration of antivenom at the nearest medical centre.

An antivenom derived from VHH of different constructs could offer a more effective treatment than current antivenoms as it could more closely match the toxin biodistibution. For example monomeric and bivalent VHH with rapid tissue penetration and large volumes of distribution could neutralise the effects of the rapidly absorbed smaller molecular weight toxins such as PLA_2s before they exert irreversible damage. The predicted rapid renal clearance of 15 kDa VHH would, at first consideration, seem to negate the utility of an i.v. administered VHH antivenom to treat the systemic effects of envenoming. Indeed, a major problem with Fab antivenoms (e.g. CroFab) is the need for multiple i.v. deliveries because of the rapid i.v. clearance, and consequent recurrence of venom-induced pathology. To address this therapeutic weakness, groups have genetically engineered pentamerised VHH or bispecific VHH linked to an albumin binding VHH. To ensure the maintenance of high levels of antibodies in the vascular compartment providing (i) adequate cycling through interstitial fluid (ii) neutralisation of

toxin components that act on plasma constituents and (iii) remain in circulation for an extended period of time to ensure the neutralisation of large, slowly migrating toxins.

Although current antivenoms contain antibodies to the clinically important toxins in snake venom, they also contain antibodies directed against non-toxic proteins found in venom (Theakston and Reid, 1983). This reduction in dose-efficiency is further compounded by the uneven recognition of toxins, with many clinically important toxins being poorly immunogenic (Chinonavanig et al., 1988; Mandelbaum and Assakura, 1988; Sunthornandh et al., 1992). The development of toxin-specific VHH (and VHH constructs) would improve the dose-efficiency of an antivenom, reducing the risk of adverse reactions and requiring less administration of antivenom per patient, thus improving the supply and possibly lowering the cost of treatment. In the short-term, toxin specific VHH constructs could be used to supplement current antivenoms by boosting the level of antibodies directed against the most pathological toxins. Attempts are under way to generate toxin-specific IgGs capable of neutralising venom toxins responsible for serious pathological effects and have demonstrated promise (Harrison, 2004; Wagstaff, S.C. et al., 2006; Azofeifa-Cordero et al., 2008; Arce-Estrada et al., 2009).

In this chapter it has been demonstrated that VHH are capable of neutralising the lethal, hamorrhagic and coagulopathic effects of *E. ocellatus* venom. This finding proves the principle that VHH can neutralise the damaging effects of the venom, without requiring conventional IgG or its fragments. The

development of VHH-derived treatments for the local effects of envenoming may be possible using toxin-specific recombinant VHH and an antivenom made from, or supplemented with specific VHH (and VHH constructs), this may: (i) reduce the cost of antivenom, (ii) increase the distribution beyond the 'cold chain' and (iii) improve the safety of antivenom therapy.

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Chapter 7

Concluding discussion

The overall aim of this project was to evaluate the effectiveness of camel-derived antibodies as a treatment of snake envenoming by the three most medically important snakes of West Africa. Studies previously performed within the unit showed the potential of IgG from llamas and camels to neutralise *E. ocellatus* venom induced haemorrhage (Harrison et al., 2006). For this study, camels were selected ahead of llamas due to the higher percentage of heavy chain IgG found in their sera which, having unique properties, may have improved the performance of a camel-derived antivenom. Although in the original study the camels generate high titres of *E. ocellatus* specific antibodies, the purpose of this study was to examine this monospecific response in greater detail and also compare this to the immunological response of camels immunised with three venoms, to produce a polyspecific antivenom. To achieve this, camels were immunised to generate either monospecific of polyspecific IgG. GERBU, a water based adjuvant was combined with the venom in the hope that this would reduce the formation of local inflammatory reactions at the sites of injection and thereby improve the welfare of the animals. Unfortunately, by the 2nd and 3rd immunisations some animals showed signs of distress, probably due to the rapid release and distribution of venom from the adjuvant. It is unclear whether this event had a marked effect on the potential of the immunisation protocol to generate toxin neutralising IgG, but highlights the need for trials of both adjuvants and immunisation protocols for use in camel immunisations, to determine the best combination to achieve (i) the greatest number of effective responders to venom immunisation (ii) the generation of the most effective IgG and (iii) reduce suffering to the animals. Similar studies have

been conducted in horses, leading to both an improvement in the immunisation protocol (Pratanaphon et al., 1997; Chotwiwatthanakun et al., 2001) and recently, the choice of adjuvant (Waghmare et al., 2009).

Despite concerns over the adjuvant used for the initial immunisations, the serological responses (as assessed in chapter 3) showed seven of the eight camels had antibody titres and avidity indicies comparable to current equine and ovine antivenoms. Four out of the five camels immunised to generate polyspecific IgG responded to all three snake venoms with high titre and showed good recognition of venom proteins in immunoblotting. Only one camel (PS5) displayed a poor response to venom immunisation as indicated by a reduction in end point titre, avidity and reduced immunoblot intensity and was excluded from further studies.

The indication from the serological analysis that camel antibodies may have similar properties to current equine and ovine antivenoms was largely confirmed for *E. ocellatus* venom by preclinical testing (chapter 4). IgG from two camels (Eo-MS and PS1) and a pool of the polyspecific camel IgGs (PS-pool) offered comparable protection to EchiTAbG® against *E. ocellatus* venom in the ED_{50} assay. PS1IgG had a lower ED_{50} than the SAVP antivenom, the current 'gold standard' in *Echis* antivenom. The *Bitis arietans* monospecific camel IgG was three times more effective than the SAVP polyspecific antivenom. Although comparison of monospecific IgG and a polyspecific antivenom is not ideal, this represented the best available choice since no commercial monospecific *B. arietans* antivenom exists. It is

expected that an effective monospecific IgG would be more efficacious than polyspecific IgG in the ED_{50} assay, and the order of magnitude indicates that the camel-derived *B. arietans* monospecific IgG is highly effective. However, despite PS1 and the PS-pool IgG proving effective against *E. ocellatus* venom, neither could prevent *B. arietans* venom induced death in mice and both were poor at neutralising the haemorrhagic pathology of this venom. It is difficult to explain how IgG from camels immunised with equal amounts of three venoms could display great efficacy at neutralising the lethal and haemorrhagic effects of one viper venom but not to another viper from a similar geographical region. A lack of efficacy was seen for all camel IgGs when tested against *N. nigricollis* venom. This may be explained by stipulation in the Home Office licence that the venom/antivenom volume may not exceed 200 µl, whereas similar antivenoms have required much greater doses to enable the calculation of the ED_{50} (Gutierrez et al., 2005). Had time permitted, other assays examining the neutralisation of venom pathologies such as necrosis or myotoxicity may have enabled a direct comparison between the camel-derived IgG and current antivenoms for neutralisation of *N. nigricollis* venom toxins. However, performance in such assays has never been related to the clinical effectiveness of an antivenom against this particular venom, and therefore would allow only limited conclusions to be drawn.

The good serological response but poor neutralising efficacy of some of the camel IgGs (particularly when tested against *N. nigricollis*) offers a cautionary lesson on the use of serological assays. Whilst assays such as end point titre

and avidity ELISA may indicate good seroconversion by an animal, it does not necessarily follow that IgG from this animal will be effective at neutralising venom pathology. Indeed, the most effective IgG from this study (PS1) does not possess the greatest avidity and the most effective IgG subclass (PS1 IgG2, Chapter 5) has an end point titre and avidity index almost identical to an IgG subclass which is ineffective at the highest dose (PS2 IgG2). The success of the modified-MCD assay at predicting the most effective and least effective camel IgGs indicates that simple, inexpensive functional assays may prove beneficial to antivenom producers wishing to exclude animals producing ineffective IgG from the final product and also enable the monitoring of an individual animal's response over the course of the immunisation schedule, without sacrificing large numbers of mice in ED_{50} assays. Although the use of the MCD assay is limited to venom with procoagulant action such as that from *E. ocellatus*, similar assays may be developed for other species of snake, for example the neutralisation of the haemorrhagic and lethal effects can be assessed using fertile hens eggs (Sells et al., 1997; Sells et al., 2001). Given the number and range of assays employed in this thesis, time did not permit evaluation of all the possible assays that could be used and improving the assessment of sera from immunised animals was not the primary aim.

An extensive investigation of the response of the individual subclasses of Camel IgG was undertaken (chapter 5) to discover (i) if an individual class dominates the immune response to *E. ocellatus* venom, and (ii) whether HCIgG was capable of neutralising the major pathological effects of the

venom. If one class was responsible for neutralising the venom effects, it may have necessitated an expensive chromatographic step during the production of a camel-derived antivenom. Reassuringly, all three IgG subclasses contribute to neutralisation of the pathological effects of *E. ocellatus*. The reported greater thermostability of HCIgG compared to conventional IgG (Omidfar et al., 2007) may have enabled pasteurisation of camel-derived antivenoms without significant loss of activity or the excessive formation of aggregates (above the allowed 5% limit), thus improving the viral safety of an antivenom. Unfortunately, the comparison of camel and sheep IgG after pasteurisation showed similar increases in turbidity and loss of activity when pasteurised. This indicates that despite the improved thermostability of HCIgG (especially when heated at 80°C) camel-derived antivenoms may not offer advantages over conventional antivenoms when pasteurised. Investigations into the long term stability of camel-derived antivenoms at room temperature are required to discover whether the improved thermostability of HCIgG can aid the development of an antivenom that does not require refrigeration.

From the MHD and MCD testing of HCIgG it was determined that the PS1 IgG2 subclass was capable of preventing both venom-induced coagulopathy and haemorrhage, and on this basis was selected as the best candidate for the generation of VHH. Separation of VHH from the Fc portion was successfully achieved using papain cleavage and protein A purification. The VHH fragment was examined using three preclinical assays and, when compared by weight, was nearly three times more effective at preventing

lethality and four times more effective at preventing haemorrhage and coagulopathy than the SAVP *Echis* monospecific antivenom. This excellent result confirms that VHH alone is capable of neutralising the major pathological effects of *E. ocellatus* venom. Unfortunately it was not possible to perform a full ED_{50} assay due to the time consuming efforts required to produce even a small amount of native VHH. The level of processing required to generate native VHH indicates that the cost/benefit of producing this type of antivenom is unfeasibly high. The future of VHH is likely to be in recombinant form, this being cheaper and it is easier to produce large quantities than native VHH and enabling the tailoring of VHH to the requirements of the antivenom (e.g. heat stable, selection of clones to particular toxins). Since VHH can be rapidly distributed in the tissue compartment and, as this thesis demonstrates, are capable of neutralising toxins responsible for the major pathological effects of *E. ocellatus* venom, then VHH may have the potential to treat the local effects of envenoming.

The production of a toxin-specific VHH antivenom or the addition of toxin specific VHH to a conventional antivenom may improve the dose efficacy of an antivenom, requiring less total protein to be administered to the patient and therefore reducing the incidence and severity of adverse reactions. Indeed the low immunogenicity of VHH should greatly improve the safety of antivenom treatment.

The small size of VHH may be one of the reasons for the low immunogenicity reported with no detectable anti-VHH immune response when injected into

mice (Coppieters et al., 2006) or pigs (Harmsen et al., 2009). However, although the small size of VHH contributes to low immunogenicity and deep tissue penetration, it also allows VHH to be rapidly cleared via the glomerular filter in the kidneys, giving VHH a short elimination half-life of 2 hours (Cortez-Retamozo et al., 2002; Harmsen et al., 2005). Viper venoms contain high molecular mass toxins: the intramuscular administration of which (simulating a bite) can lead to continuous absorption into the bloodstream for up to 72 hours (Audebert et al., 1994). Clearly, VHH would be eliminated from the patient before all of the venom toxins have entered the vascular compartment. Clinical observations show that such recrudescence of venom toxins and thus recurrence of the symptoms of envenoming, can occur after antivenom administration, and are more prevalent for Fab antivenoms (than those comprised of IgG or $F(ab')_2$), requiring repeated administrations of antivenom to ensure venom neutralisation (Ariaratnam et al., 1999; Boyer et al., 1999; Dart et al., 2001). The half life of Fab antivneoms range from 4 to 28 hours (Meyer et al., 1997; Ariaratnam et al., 1999) compared to 2 hours for VHH. Therefore, the use of a VHH derived antivenom for snakebite treatment would require the administration of frequent doses over a considerable period of time and would lack practicality.

The use of recombinant technology has overcome the problem of rapid elimination of VHH in several ways by:

A) Joining two distinct VHH domains by a peptide linker. When the VHH targeting *E. coli* F4 fimbriae was linked to a VHH that bound the host animal's IgG the terminal half-life was increased from 2 hours to up to 227 hours (Harmsen et al., 2005).

B) Linking two VHH (forming a bivalent structure) to a third VHH that binds to serum albumin. This extended the half-life to 53 hours Coppieters, 2006 #130}. This method of using bivalent VHH had the added advantage of increasing neutralisation of the target molecule by 500-fold. Despite the larger size of the constructs, no host anti-VHH IgG could be detected even after multiple injections.

C) Fusing bivalent VHH construct to a host's Fc portion, thus forming a chimeric HCIgG. This increased the blockade of the target enzyme from 6 hours to greater than 7 days in mice (Wesolowski et al., 2009). The bivalent nature of this type of construct can also increase the affinity for the antigen (50-fold compared to monovalent VHH)(Hmila et al., 2008).

D) Forming pentamerised VHH. Constructs have a similar molecular mass to conventional IgG but with five binding sites. This is likely to improve both the affinity for the antigen and the serum half-life (Stewart et al., 2007).

Current antivenoms often have pharmacokinetic profiles that do not match that of the target toxins (Gutierrez et al., 2003). The difference in biodistribution between antibody and toxin can result in poor neutralisation and a reduction in the formation of antibody-toxin complexes (Seifert and Boyer, 2001). The use of IgG, IgG fragments or VHH that more closely match the size of the target toxin can improve treatment outcomes. For example, the use of Fab fragments, with their more rapid tissue distribution reverses the effects of digoxin poisoning more rapidly than intact IgG possibly because Fab antibodies arrive at the myocardial interstitial space and sequester digoxin (a small molecule) as it dissociates from the receptor with greater

expedience than the intact IgG molecule (Lloyd and Smith, 1978). For this reason, VHH are being investigated as a treatment for scorpion envenoming (the major toxins of which are ~7 kDa) (Hmila et al., 2008; Abderrazek et al., 2009). The current $F(ab')_2$ antivenoms have shown low efficacy, in part due to the large difference in pharmacokinetics between the antivenom and toxins (Pépin-Covatta et al., 1996) which may be overcome by the smaller size of the VHH molecule. Attempts to discover anti- α-cobrotoxin (an elapid neurotoxin) VHH have been made (Stewart et al., 2007) to improve the treatment of elapid envenoming, where the low immunogenicity of toxins has hindered the generation of conventional antivenoms (Chinonavanig et al., 1988; Sunthornandh et al., 1992).

Although the production of a tailored toxin-specific antivenom that matches the pharmacokinetics of venom toxins has the potential to greatly improve the safety, supply and distribution of antivenom, The feasibility of producing such an antivenom is uncertain. The requirement of expensive, time consuming research and development added to the unknown cost of producing VHH is likely to be a major stumbling block for the future of such recombinant constructs. The switching from animal immunisation to recombinant protein production as a source of antivenom is unattractive to antivenom producers and rightly so, as their primary concern is to produce the cheapest, safe and effective antivenom possible. The communities affected by snake bite are some of the poorest on Earth and therefore ensuring the supply of current antivenoms (production of which may still not meet demand) is of primary concern, as opposed to improvements to an already effective treatment. In the short to medium term, research to improve the response of animals to

immunisation (by improving the effectiveness of adjuvants and immunisation protocols) and examining the use of camel-derived antivenoms is likely to be much more beneficial at reducing the cost and improving safety and supply of antivenom than developing a wholly recombinant antivenom.

The work undertaken in this thesis has helped to elucidate whether the perceived advantages of camelid IgG over IgG from other species are likely to improve current antivenoms. Before undertaking this work, the idea of using the thermostability of camel IgG to allow for a pasteurisation step during manufacture, thus improving the viral safety of an antivenom, was attractive. However, given the results presented here, it is unlikely that camel IgG could offer this advantage. Similarly, the recognition of unique epitopes by HCIgG may have greatly improved the effectiveness of an antivenom against the effects of small molecular weight toxins found in Elapid venoms. Again, from the results presented here, any improvement in the efficacy against such toxins is likely to be modest at best, if there is any improvement at all. The reported lower immunogenicity of camel IgG compared to that from sheep or horses offers a potential improvement by reducing the number of patients experiencing adverse reactions. Therefore it was important to provide information about the effectiveness of camel IgG at neutralising the deleterious effects of venom. The work in this thesis clearly shows that camel IgG is capable of matching the efficacy of current antivenoms in a range of preclinical assays and that therefore further investigations into the development of a camel-derived antivenom is warranted. Caution is urged however, since the neutralisation of *B. arietans* and *N. nigricollis* was not as

effective as expected. The next stage in the development of a camel-derived antivenom will be to assess the effectiveness of different adjuvants and immunisation protocols, to hopefully improve the immune response against venom from the three snakes used here. Assessment to confirm the expected improved safety of camelid antivenom over current equine or ovine antivenoms will require a large clinical trial. The findings from this and information about the cost of producing camel-derived antivenoms will determine whether camels offer an attractive alternative to horses or sheep.

Whilst improvement of the currently available treatments of snake envenoming is important, the development of a treatment against the local effects of envenoming (for which none currently exist) may be where camel IgG, in the form of VHH, offers the most therapeutic benefit. The demonstration in this thesis that VHH are able to neutralise the lethal, coagulopathic and haemorrhagic effects of *E. ocellatus* venom is an important first step in proving the principle that VHH could be an effective at preventing the development of local pathology.

The methods used in this thesis to generate native VHH demonstrate that this process is time consuming and produces little yield. It is therefore most probable that the next stage in the development of a VHH derived treatment for local venom effects will require recombinant VHH. In this regard, the most likely stumbling block is not the lack of technical ability to clone VHH but identifying and separating the most relevant toxins from the complex cocktail of proteins found in snake venom. Panning for effective VHH against one target is time consuming so it is important to reduce the number of targeted

toxins to the absolute minimum required whilst enabling the generation of a combination of VHH clones capable of preventing snake venom induced lethality or local pathology. This approach has been successfully employed by the Pasteur group in Tunisia and has shown that AaHII-neutralising VHH is capable of providing partial protection against the lethal effects of scorpion venom in mice (Abderrazek et al., 2009). Whilst identifying the relevant toxins may seem daunting, some studies have shown a high percentage reduction in particular crude venom induced pathologies when using antibodies generated against a single toxin (Lomonte et al., 1987; Lomonte et al., 1990; Rucavado et al., 1995) or small number of epitopes (Wagstaff et al., 2006). Such protection may be present even if tested against the venom of a different species of snake (Rucavado et al., 1995). Previous research within this group by Dr Howes (unpublished) indicated that IgG generated against one SVMP toxin fraction was able to completely neutralise the haemorrhagic effects of *E. ocellatus* venom. If similar toxin candidates responsible for coagulopathy, inflammation, and myonecrosis can be isolated, the identification of the relevant VHH will be made less difficult. In turn, isolation of specific VHH effective at neutralising particular pathologies may allow identification of the most clinically important toxin epitopes, further improving novel methods of generating antivenom such as DNA immunisation (Wagstaff et al., 2006).

Assuming that generation of large quantities of specific VHH can be achieved, the next challenge will be to prove that transdermal delivery of these can reduce or eliminate the local effects. Using the pig as an experimental model for the study of local necrosis (Imkhieo et al., 2009)

could allow the efficacy of VHH to be tested. This may represent the closest model to the clinical situation in humans and successful neutralisation of local effects in this model would provide convincing evidence that VHH could treat the local effects in humans. This model may also provide further information regarding how prompt VHH therapy would have to be administered after envenoming to be effective.

It was the original intention of this project to generate toxin specific recombinant VHH and assess their potential. Unfortunately, due to circumstances beyond our control, this was not possible. Despite this set back, the work presented here offers an in-depth analysis of the immunological response of camels to both monospecific and polyspecific immunisation, confirmation that camel IgG is as potent as conventional antivenoms at neutralising venom from *E. ocellatus* and *B. arietans* and the generation of native VHH capable of neutralising the major pathological effects of *E. ocellatus* venom.

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