

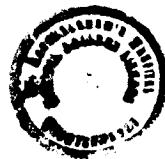
**PRODUCTION AND ASSESSMENT OF OVINE ANTIVENOMS
FOR THE TREATMENT OF SNAKE ENVENOMING IN
SAUDI ARABIA**

BY

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**FOR MY WIFE
F. AL-ASMARI**

AND

**MY CHILDS
SARAH, BAYAN AND YAISR**

ABSTRACT

Venoms from the most poisonous snakes found in Saudi Arabia were assessed for their physical and chemical characteristics and for their enzymatic and biological activities. Venom from *Atractaspis microlepidota* was the most lethal in mice followed by the elapids *Naja haje arabica* and *Walterinnesia aegyptia*. Among the vipers, *Cerastes cerastes* venom was the most lethal whereas the remainder (*Echis pyramidum*, *Echis coloratus* and *Bitis arietans*) showed similar but lower lethality.

Antivenoms were raised in sheep by immunising with a low dose of venom (0.5mg) which was then doubled every four weeks. To optimise the antibody response, groups of sheep were immunised with a low, medium and high dose and the monthly bleeds were assessed by ELISA and small-scale affinity chromatography.

The immunoglobulin fraction was partially purified by sodium sulphate precipitation and digested with either papain, to form Fab fragments, or with pepsin to produce F(ab)₂. The different antivenom fractions produced were characterised and assessed for their ability to neutralise the enzymatic and biological activities of the corresponding venoms. Fab was equally effective as F(ab)₂ in most enzymatic and biological assays but the two fractions were less efficient than IgG. The ovine Fab provided good protection in mice against the lethality of these venoms and effectively neutralised their biological and enzymatic activities.

The commercial antivenoms currently available in Saudi Arabia showed only partial neutralisation of the enzymatic and biological activities of these venoms and showed *in vivo* protection only when using large amounts. They offered no protection against *W. aegyptia* venom. The monospecific ovine Fab raised against *E. pyramidum* and *E. coloratus* venoms were more efficient than the polyclonal Fab raised against a mixture of the two venoms.

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ABBREVIATIONS

ADP	Adenosine diphosphate
BCG	Bromocresol green
BDL	Below detectable level
BET	Bacterial endotoxin test
BSA	Bovine serum albumin
CM	Carboxymethyl
CNBr	Cyanogen bromide
cpm	Counts per minute
CV	Coefficient of variation
DAS	Donkey anti-sheep
DEAE	Diethylaminoethyl
DIC	Disseminated intravascular coagulation
ED ₅₀	Median effective dose of antivenom
EDTA	Ethylenediamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
EU	Endotoxin unit
FAD	Flavin adenine dinucleotide
FPLC	Fast protein liquid chromatography
HPLC	High pressure liquid chromatography
hr	Hour(s)
HRP	Horseradish peroxidase
HYL	Hyaluronidase
IEMA	Immunoenzymometric assay
IgG	Immunoglobulin G
im	Intramuscular
ip	Intraperitoneal
iv	Intravenous
K	Affinity constant
kDa	Kilodalton

LAL	Limulus Amebocyte Lysate
L-AAO	L-Amino acid oxidase
LD ₅₀	Median lethal dose of venom
M	Moles per litre
mCi	Millicurie
MDD	Minimum defibrinogenating dose
MHD	Minimum haemorrhagic dose
min	Minute(s)
MND	Minimum necrotic dose
MWt	Molecular weight
NHS	Normal horse serum
NSB	Non-specific binding
NSS	Normal sheep serum
OD	Optical density
O/N	Overnight
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PDE	Phosphodiesterase
PEG	Polyethylene glycol
PLA ₂	Phospholipase A ₂
PPP	Platelet-poor plasma
PRP	Platelet-rich plasma
RIA	Radioimmunoassay
RPC	Reversed phase chromatography
rpm	Revolutions per minute
rt	Room temperature
s	Second(s)
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error of mean
TRIS	Tris(hydroxymethyl)amino methane
WHO	World Health Organisation

Publication Associated with this Work

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CHAPTER I

INTRODUCTION

1.1 HISTORICAL SUMMARY

From the earliest times, people have been aware of the possible dangers of bites and stings by insects, snakes, fish and other species. The oldest medical writings are the ancient papyri from Egypt, dating from around 1600 B.C. These contain various prescriptions used to treat injuries or disease and, to treat snake bites; the Hearst Medical Papyrus recommends leek, garlic, onion juice, salt, bile, and incense, to be bandaged onto the place where the bite occurred. The best summary of classical Greco-Roman and Islamic medicine is given by Francis Adams (1846) who reported that the general treatment of snake bites included immediate sucking of the bitten area, followed by cupping and, then, as introduced later by the Islamic physicians, by cautery. Various local applications were suggested including onions, wine and hot vinegar, with various internal remedies comprising "theriacs", which were mild stimulating materials.

The ancient fear of mad dogs and their bites was dramatically allayed by the insight of Louis Pasteur (1885). Suspecting an infectious agent, he was successful in devising a protective antibody preparation without being able to isolate or identify the virus. Pasteur's discoveries lead to the establishment of Pasteur Institutes throughout the world for the preparation of antirabies vaccines and for immunological research in general.

The pioneer investigations of Sewall (1887), at the University of Michigan, ushered in a new era in the treatment of snake envenomation. He showed that the experimental species (the pigeon) could be protected by prior immunisation with rattlesnake venom. These studies led to investigations by Calmette (1894a,b, 1895, 1898) who obtained immunity in experimental animals by immunising them with venoms (acquired, active immunity) and showed that the serum of immunised animals could protect other animals against cobra venoms (passive immunity).

Meanwhile, Sir Thomas Fraser, a well-known Edinburgh toxicologist, also investigated immunisation against cobra venom and obtained a specific immune serum (1895). In 1896, Calmette published a small volume on the venom of serpents that provided the background for immunisation and, as a result, several Pasteur Institutes throughout the world undertook to supply "antivenoms, or antivenins". At present, more than 70 institutions world-wide are devoted to the production of antivenoms.

Systematic studies of the biological actions of snake venoms were undertaken by Flexner (1863-1954) and Noguchi (1876-1928) at the Rockefeller Institute in New York. They described in detail the effects of various snake venoms in producing haemolysis and other toxic effects on blood and also showed that snake venoms could cause bacteriolysis. Keyes (1875-1928) investigated cobra venom and showed that lecithin is a compliment for cobra haemolysin. In 1963, Russell characterised venoms from fifteen kinds of poisonous snake and sixteen kinds of venomous marine animal. The venoms from these sources contained many enzymes and nonenzymatic proteins of high molecular weight. Some of these proteins liberated bradykinin and histamine from cells and these substances contribute greatly to local pain and tissue damage at the bite site. In 1938, Slotta and Fraenkel-Conrat isolated "crotoxin" from the venom of *Crotalus terrificus* and reported that it has a molecular weight of 30kDa. Subsequently it has been shown that most snake venoms contain at least a dozen proteins with varying activities.

1.2 VENOMOUS SNAKES

1.2.1 Classification and Geographical Distribution

Snakes (or ophidia/serpents) are members of the class Reptilia (phylum Chordata), which includes other backboned animals such as crocodiles and lizards. They form

an evolutionary link between the amphibians and the warm-blooded birds and mammals. The oldest known snakes date from the late Cretaceous and Eocene period about 120 million years ago. They were often large and similar to modern boas and pythons which are probably their direct descendants (Romer, 1947). Currently there are about 3000 species of snakes in the world separated into 10 or 12 families, of which about 375 species are considered venomous (Russell, 1983). These are members of the following families (1) Colubridae, (2) Elapidae, (3) Hydrophiidae, (4) Viperidae (subfamily Viperinae and subfamily Crotalinae) and (5) Atractaspidinae.

The Colubridae is the largest family and comprises about 70% of all the genera. These snakes are distributed throughout the world in diverse habitats. Thus while most are terrestrial, some are aquatic, others arboreal and some are fossorial. Some colubrids are diurnal and others are nocturnal. All have teeth on both jaws. The aglyphous colubrids have solid teeth which are not grooved and they have no venom glands. The opisthoglyphous colubrids have enlarged grooved maxillary teeth half way along the upper jaw, approximately under the eyes, as well as venom sacs. They are poisonous in varying degrees but the venom is usually of low toxicity to man, and the small volume that can be obtained from individual snakes has enabled very few toxicological studies.

The Elapidae is the second largest family and contains nearly half all known venomous snake species (169 species). They are thought to have evolved from the Colubridae, from which they differ in having a more efficient system for injecting venom. Thus the fangs are effectively tubular and are positioned at the front of the mouth where they may be brought into use more easily. The Elapidae are distributed throughout Asia (cobras and kraits) Australia (death adder and tiger snake), the Americas (coral snakes) and Africa (cobras and mambas). Bites by elapids are

notable for producing minimal to moderate local but prominent neurological manifestations and death, if it occurs, is usually due to respiratory arrest Reid and Theakston (1983).

The Hydrophiidae (sea snakes, 35 species) are sometimes classified as elapids which have adapted to a marine habitat. They inhabit the tropical Indian and the western Pacific Oceans. The snakes are proteroglyphous with fangs present at the front of the upper jaw and these are usually followed, after an interspace, by solid teeth posteriorly on the maxillary bone. There are also teeth on the mandible. Sea snakes are diurnal or nocturnal, depending on surface water temperatures (Gasperetti, 1988). Their venom is extremely toxic, much more so than the most venomous terrestrial snakes and this enables them to capture their agile prey, mainly fish and eels, as it paralyses them very rapidly. Fortunately they are not aggressive and, in the Arabian Gulf, sea snakes are commonly caught in fish traps or nets from which they are casually picked by hand and thrown back into the water.

Members of the subfamily Crotalinae are also known as pit vipers due to differential temperature receptors located in a pit on both sides of the head between the nostril and eye. They have a worldwide distribution and consist of six genera: *Agkistrodon* found in Asia and America, *Trimeresurus* restricted to Asia, and the other four genera *Bothrops*, *Crotalus*, *Sistrurus* and *Lachesis* which are found only in the New World.

The Viperinae subfamily is commonly called the true or Old World vipers and is closely related to the crotalids. Vipers are distributed in Europe, Africa and Asia and some Pacific Islands, but none inhabit the American or Australian continents. There are about 40 species represented by nine genera: *Bitis*, *Causus*, *Cerastes*, *Atheris*, *Adenorhinus*, *Vipera*, *Echis*, *Eristicophis* and *Pseudocerastes*. Vipers are solenoglyphous snakes, with a highly specialised skull structure that allows very

efficient movement of the long, frontal fangs. The double socketed maxilla is short and deep and rotates around the maxillo-prefrontal bone in a parasagittal plane. On its end is one hollow fang, and its replacement fangs.

Family Atractaspididae is a monogeneric family comprising small, venomous fossorial snakes of about 14 species. Kochva (1987) placed these snakes in a separate family with the vernacular designation of "burrowing asps". There is now little doubt that they are related to the Aparallactinae, which they resemble in many external features and their mode of life. Atractaspids have unique dentition, unlike any other snake, with only superficial resemblance to that of the snakes of the family Viperidae (Broadley, 1983).

1.2.2 Snake Venoms and Their Constituents

Snake venoms are complex mixtures consisting mainly of proteins. Thus the dry weight is typically about 10% of the wet weight with approximately 90% of the dry weight comprising proteins. The more lethal fractions of snake venoms appear to be peptides and, perhaps, certain nonenzymatic proteins, although the enzyme components certainly contribute to the overall deleterious effects of the crude venom. Enzymes comprise from 80-95% of the total dry weight of viperid and crotalid venoms and from 25-70% of elapid venoms (Mebs, 1970). There are at least 26 venom enzymes with molecular weights ranging between 11 and 150kDa (Table 1.1). Although no single venom contains all of these, at least 10 enzymes are found in most snake venoms, while the remainder are scattered throughout the venoms of the six families of poisonous snakes. For example elapid venoms are rich in acetylcholinesterase, whereas crotalid and viperid venoms lack this enzyme but are rich in endopeptidase. Colubrid venoms are normally free from clot-promoting enzymes (Eagle, 1937).

Table 1.1 Enzymes in snake venoms.

Enzyme	Molecular Weight (Da)
Phospholipase A ₂	11,000 - 15,000
L-Amino acid oxidase	100,000 -130,000
Phosphodiesterase	115,000
5'-Nucleotidase	100,000
Phosphomonoesterase	100,000
Ribonuclease	15,900
Hyaluronidase	> 60,000
NAD-nucleosidase	100,000
Arylamidase	100,000
Endopeptidase	21,400 - 95,000
Arginine ester hydrolase	27,000 - 30,000
Kininogenase	33,500
Thrombin-like enzyme	28,000 - 33,000
Factor X activator	78,000
Factor V activator	20,000
Prothrombin activator	56,000

Table 1.1 lists the enzymes found most commonly in snake venoms together with their MWt.

Many venoms contain components that affect nerve function, and these include postsynaptic or presynaptic toxins. Curaremimetic postsynaptic neurotoxins, consisting of either 60-62 amino acids ("short") or 70-75 amino acids ("long"), bind to the nicotinic acetylcholine receptors localised at the skeletal muscle endplate and, thereby, prevent the depolarising action of acetylcholine (Dufton and Hider 1983).

To-date about 70 such toxins have been isolated from elapid and sea snake venoms. Presynaptic neurotoxins inhibit or potentiate transmitter release from motor nerve terminals, and have been isolated from elapid (β -bungarotoxin, notexin and taipoxin), crotalid (crototoxin and mojave toxin), and viperid venoms (caudoxin, ammodytoxin A, B and C, and viperotoxin).

Cytotoxins can affect many kinds of cell, causing haemolysis, cytolysis, depolarisation of muscle membranes and specific cardiotoxicity by interacting with membrane phospholipids. Cardiotoxic polypeptides, or sarafotoxins, are low molecular weight (7kDa) basic polypeptides commonly found in venoms from cobras and *Atractaspis microlepidota*. Bradykinin-potentiating peptides comprise only 5 to 12 amino acid residues; they potentiate the depressor activity of bradykinin and this effect is also mediated by inhibition of the carboxypeptidase that converts angiotensin I into angiotensin II. Both these actions will cause a fall in blood pressure.

Snake venom polypeptides that induce skeletal muscle contracture or produce local myonecrosis or myoglobinuria are categorised as myotoxins. Myotoxins contribute to local tissue destruction, affecting skin and muscle layers. In addition to nonenzymatic myotoxins, phospholipase A₂ and haemorrhagic proteinases may also damage muscle cells. Some snake venoms can inhibit and others stimulate platelet aggregation. Nonenzymatic inhibitors (disintegrins) have been isolated from many venoms and various enzymes also have an effect on platelet function. The so-called "cobra venom factor" (CVF) is a nonenzymatic, nontoxic protein of molecular

weight 140kDa found in the venom of cobras which, in the presence of a plasma cofactor, inactivates the human complement factor C3. CVF has been used as a tool in research on complement and illustrates the value of snake venom proteins for investigating physiological processes.

In addition, snake venoms contain inorganic substances, such as sodium, calcium, potassium, magnesium, zinc, and small amounts of iron, cobalt, manganese and nickel. The importance of the metal content is not known, although zinc ions are necessary for anticholinesterase activity, and calcium plays a role in the activation of phospholipase A₂ and the direct haemolytic factor. Some of the proteases are metalloproteins. Most snake venoms also contain small amounts of carbohydrates (glycoproteins), lipids (primarily phospholipids), biogenic amines (serotonin and histamine), nucleotides (adenosine, guanosine and inosine), nucleosides and free amino acids.

1.2.3 Treatment of Snake Bite

First-aid treatment of snakebite should include reassurance and immobilisation of the patient and bandaging the bitten limb to delay systemic absorption of venom during transport to the nearest hospital. Incision and suction have been shown to extract only small amounts of venom and then only if carried out within a few minutes of the bite (Reid, 1972). Medical treatment may include tetanus antitoxin and blood transfusion and, for the first night, analgesics may be necessary.

In systemic snakebite poisoning, specific antivenom is the most important therapeutic agent available. If used correctly, it can be effective, even when its administration is delayed hours or even days after the bite. The rational indication for giving antivenom is clinical evidence of systemic poisoning. For example, neurotoxic signs plus local swelling (not due to a ligature) might indicate the need for cobra antivenom while haemorrhage indicates the requirement for a viperine antivenom. Non-clotting blood can be a sensitive sign of systemic poisoning and a useful means

of differentiating systemic envenomation by different snakes (Reid, 1972). Thus *Echis* poisoning will be accompanied by non-clotting blood, whereas the venom of the puff adder does not affect clotting (Reid, 1972). The circumstances of the bite and generalised myalgia indicate the need for a sea snake antivenom.

Monospecific antivenoms (section 1.4.4) should always be given if available and if the snake species responsible for envenomation is known. Many people urge the development of specific diagnostic tests to elucidate the species of snake involved so as to enable correct choice of a monospecific antivenom. Using a monospecific product is especially important in neurotoxic poisoning because polyvalent antivenoms are usually less potent against elapid than against viper venoms. In many tropical countries these distinctions are academic as regards choice of antivenom because, as in Saudi Arabia, only one a polyspecific antivenom is available. Serum sensitivity may be assessed by injecting 0.2ml, or less, of the antivenom subcutaneously. If a severe reaction occurs within minutes, antivenom may be contraindicated, unless the morbidity and mortality associated with the particular envenoming is high (Reid, 1972). However, the predictive value of skin tests is unreliable as an indicator of hypersensitivity and adrenaline should be available in a syringe for immediate injection if an antivenom reaction occurs despite negative tests for sensitivity.

Antivenom is usually administered by slow intravenous infusion, but it has been shown that the incidence of early anaphylactic reactions is the same when undiluted antivenom is injected as a bolus intravenously. Antivenom should always be given intravenously, if possible, because this is the most effective and safest route (Reid, 1972).. In the future it may be possible to give Fab antivenoms intramuscularly or subcutaneously when medical staff are not available, for example while hiking far from a large town. Thus the small molecular weight of the Fab antibody fragment (section 1.4.2) should readily allow its re-distribution into the general circulation.

In viper envenomation, the effect of the antivenom should be assessed by checking the ability of the blood to clot. This should be done at six hourly intervals after the initial dose and a further dose of antivenom should be administered whenever the blood fails to clot. Deaths from delayed haemorrhages are well known.

Antivenoms currently available in Saudi Arabia are manufactured by Behringwerke AG (Germany) and Pasteur-Mérieux (France). Both are polyspecific equine (Fab)₂ for the Near and Middle East and the Middle East and North Africa respectively. There is no specific antivenom available against *Atractaspis microlepidota* venom (Coppola *et al.*, 1992) and neither of the polyspecific antivenoms is effective against *Walterinnesia aegyptia* bites (Dowling *et al.*, 1968). In the event of severe envenomation by the latter snake, it may be possible to reverse curare-like paralytic effects by administering neostigmine (Naphade *et al.*, 1977) but atropine should be given first to counteract muscarinic activity. Finally, Tiger Snake antivenom from CSL (Australia) is used paraspesifically to treat sea snake envenoming (Baxter and Galichio, 1974).

1.2.4 Laboratory Diagnosis

Laboratory tests are important in helping establish the diagnosis and in assisting management and treatment of snake bites. The initial assessment should include pulse and respiration rate, blood pressure, temperature, skin discolouration, and the presence and extent of oedema. Haematological tests should include blood grouping and cross-matching, clotting and clot retraction times, and complete red and white cell, and platelet counts. The erythrocyte sedimentation rate (ESR), prothrombin time and arterial blood gases are also of importance.

The demonstration of noncoagulating blood is the single most valuable test in *Echis* envenomation and, as stated earlier, the test should be repeated every six hours after the first dose of antivenom until clotting is re-established (Reid, 1972).. Following this the test should be repeated daily for three days to ensure that coagulability is maintained and no further venom has been absorbed from the bite site (Reid, 1972). In severe cases, changes in urine composition (protein, blood, glucose, urea and creatinine) should be monitored as well as any cardiac abnormalities by electrocardiogram and by determining serum levels of creatinine phosphokinase. Plasma electrolyte assays are required and a low bicarbonate concentration reflects the metabolic acidosis that is a feature of severe experimental viper bite poisoning. Potassium levels are sometimes raised.

Specific snake venom antigens can be detected in wound aspirate, swabs or biopsies, serum, urine, cerebrospinal fluid and other body fluids. Enzyme immunoassay has been the most widely used technique (Theakston *et al.*, 1977). In Saudi Arabia there is no commercial test available, but immunoassay has proved a valuable tool in the clinical investigation of snake bite (Warrell, 1995).

1.3 SAUDI ARABIAN VENOMOUS SNAKES

1.3.1 Classification and Geographical Distribution

The Kingdom of Saudi Arabia (KSA) and the surrounding areas are inhabited by a number of venomous snakes that are of potential medical importance. Indeed several of the world's most lethal snakes are found, with the venomous varieties being divided into four families: Elapidae, Hydrophiidae, Viperidae and Atractaspidae (Gasperetti, 1977). Vipers cause most of the snakebites to humans and, of the ten venomous species of land snakes, the sand viper (*Cerastes gasperettii*) is the most frequently observed and carpet vipers (genus *Echis*) are thought to be responsible for most of the serious bites and for most of the occasional deaths.

There are two species of elapids in Saudi Arabia namely *Naja haje arabica*, the arabian cobra (Figure 1.1.1) and *Walterinnesia aegyptia* (Figure 1.1.2). The former located in the mountainous areas of the southern half of the Kingdom (Figure 1.2.3) and the second in the northern and central regions (1.2.7) where they are found close to villages but tend to stay in bushes, rocky areas, and rodent burrows. They prey mostly on reptiles and amphibians but will also eat small mammals and birds.

The Arabian cobra is mainly diurnal and is the largest snake in the region, with a maximum length of 2.5m, accounting for its Arabic name of "hannish thaiban" (snake of snakes). Its colour varies from golden yellow to brown or grey and it was represented on the crown of the Pharaohs. The snake's head is flat and its neck can flare widely to form the characteristic hood.

Walterinnesia aegyptia (Innes cobra or the desert black cobra) has a small, flat head and grows to about 1.2m in length. The snake's scales are glossy and this helps distinguish it from the dull scales of the cobra. It inhabits gardens and irrigated areas, but can also be found on rocky hillsides and in sandy desert regions with little vegetation. It is partly fossorial (Reid, 1972).

There are 10 species of sea snakes (family Hydrophiidae) present in the Arabian Gulf, Gulf of Oman, Arabian Sea and Gulf of Aden but none has been found in the Red Sea. Their venom is highly toxic and induces paralysis and rhabdomyolysis but they are not aggressive and their short fangs make significant envenomation a rarity.

Vipers are the most important snakes in Arabia from the medical standpoint and have a highly sophisticated system to deliver venom. Thus they have hollow fangs situated in the front of the mouth which function like hypodermic needles and are capable of

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penetrating deep into subcutaneous and muscular tissues. The fangs fold into the roof of the mouth at rest but are erectile and become almost perpendicular in the biting mode.

Three species of saw-scaled or carpet vipers (genus *Echis*) are currently recognised on the Arabian peninsula (Gasperetti, 1988; Cherlin, 1990). *Echis carinatus sochureki* (the carpet viper) is distributed as shown in Figure 1.2.1 and is reddish-brown with a pretty white pattern which accounts for its common name of carpet viper (Figure 1.1. 5). *Echis coloratus* (Burton's viper or the saw-scaled viper) (Figure 1.1.4) is found in Egypt, Lebanon, Jordan, Palestine, and the Arabian Peninsula (Figure 1.2.6). Saint-Hilaire carpet viper (*Echis pyramidum*) is also seen in Saudi Arabia (Russell, 1991). When threatened, their bodies characteristically inflate and serrated scales rasp against each other and produce a sound like a saw cutting wood. Loud hissing and a savage strike follow this display. These snakes move with great speed and agility and can inflict the most catastrophic of bites. Their head is broad and distinct from the neck with a white arrow-like marking of four points: three directed posteriorly and one anteriorly. Their colour is tan to olive brown with white spots undulating laterally on the back and they attain a length from 0.4 to 0.8m. *Echis* species are nocturnal in the summer and diurnal in the winter and find shelter in almost anything from rodent burrows to rock fissures.

Bitis arietans, the puff-adder, is found in the mountainous areas of Saudi Arabia as well as throughout Africa (Figure 1.2.4) and has been described as the greatest killer of man (Dowling *et al.*, 1968). Its head is very distinct and the eyes are vertically elliptical (Figure 1.1.7). A diagonal light-coloured band is located from the posterior aspect of the eye to the corner of the mouth. The colour of the snake may vary from yellow to brown or black but alternating dark and light chevrons on the back are unique and help to differentiate this snake from others. It is a fat snake which attains

a length of about 1.5m. The puff-adder is nocturnal, slow-moving and usually inhabits grasslands where they are easily trodden upon. When such an accident occurs, it strikes rapidly, afflicting a most dangerous bite (Russell, 1991).

Cerastes gasperettii, the sand or horned viper, is the commonest venomous snake in the Kingdom (Figure 1.2.5). The head is flattened and distinct from the neck, and the pupils are vertically elliptical (Figure 1.1.6). These snakes escape their predators by sinking into the sand by the use of lateral scales which have serrated angled keels capable of removing sand from under the animal and placing it over them. Upright spines or supraorbital horns serve to keep the eyes free of sand. The snake's colour is yellow to light brown with dark rows on the dorsum and its colour matches the sand of the region. It attains a length of about 0.75m and can be found in arid places, near rocks, or in rodent burrows. It is nocturnal and travels using a side-winding motion.

Saudi Arabia hosts two subspecies belonging to the family Atractaspididae: *Atractaspis microlepidota andersonii* and *Atractaspis microlepidota engaddensis* (Figure 1.1.3). The main taxonomic difference between the two is that the former has a fully divided nasal scale while in *engaddensis* the nasal scale is divided in the lower half only. The scale counts in *engaddensis* are also higher. They are very similar in appearance with a small head, long fangs, tiny round eyes, and a black body and they range in length from 0.5 to 0.6m. They are nocturnal and are found often in inhabited areas rather than in the desert. They have a sophisticated venom delivery system with a unique biting action. Fang erection can occur independently on either side of the mouth and occurs by cranial kinesis of the anterior skull elements, which permits a limited rotation of the fang bearing maxillary upon the prefrontal bone. Atractaspidids are dangerous snakes to handle and must not be picked up by gripping them behind the head, as with other snakes, because they can inflict a venomous bite backwards, downwards or sideways. Branch (1981) suggested that

they should be called "side-biting snakes" whereas Broadley (1983) called them "stiletto snakes" and Kochva *et al.* (1982) suggested "burrowing asp". The Arabs call them "al-aswad", meaning "the black one".

Pseudocerastes persicus (the Persian horned viper) possesses supraorbital horns, composed of several small scales, in contrast to the single upright spine or "horn" of *C. cerastes*. *Vipera lebetina*, or the levantine viper, is sluggish and appears to be unaware of stimuli during the day. At night the animal may strike quickly and savagely and is an important cause of snake bite in the Middle East.

The photographs and geographical distributions of these Saudi Arabian snakes were adapted from Al-Sadoon and Farraj (1992).

1.3.2 Clinical Manifestations

The symptoms, signs and gravity of snake envenomation are dependent upon many factors including (a) the age, size and general health of the victim, (b) the nature, location, depth and number of bites, (c) the amount of venom injected, (d) the species of snake involved, (e) the condition of the fangs and venom glands, (f) the victim's sensitivity to the venom, (g) the pathogens present in the snake's mouth, and finally (h) the speed and type of first aid received initially and the subsequent medical care. It is customary to divide the manifestations of snakebite into local and general. Venom poisoning may also be classified as minimal, moderate or severe.

Following envenoming, spread of the various components depends, to a large extent, on their molecular weights. Thus Reid and Theakston (1983) showed that most of the toxic constituents of viper venoms have a large molecular weight and are absorbed only slowly, via the lymphatic system, to give maximum serum levels after 6 to 24 hours. By this time significant amounts were present in urine. In contrast, elapid

venoms, with their smaller molecular weight components, are absorbed much faster via the capillaries and peak serum levels are attained within 60 minutes (Sutherland *et al.*, 1981).

The main clinical patterns of snakebite poisoning in human victims are often distinctive, with viper venoms being chiefly vasculotoxic, those of elapids neurotoxic, and those of sea snakes myotoxic. Elapid poisoning is severe if neurotoxic signs start within one hour or less of the bite. Although no proven bites have been reported by the Arabian cobra (*N. h. arabica*), the effects of its venom probably resemble those of the Egyptian cobra (*N. haje*). The latter have a powerful neurotoxin in their venom and have killed humans within two hours despite the use of antivenom (Warrell *et al.*, 1976). There is also some local necrosis in cobra bites and occasional cardiotoxicity. The venom of the desert black cobra (*W. aegyptia*) lacks cardiotoxic properties but is extremely neurotoxic and Dowling and colleagues (1968) described local pain and swelling with fever, general weakness, headache, nausea and vomiting. Sea snake bites produce no local pain or swelling but, after a few hours, there is generalised pain and stiffness of muscles, trismus, ptosis, bulbar and respiratory paralysis, myoglobinuria and life threatening hyperkalaemia (Reid, 1979).

The venom of *A. microlepidota* causes mostly local irritation and oedema. However, fever, vomiting, and haematuria are seen in severe cases, and deaths have been reported (Mehrtens, 1987). The sequelae of *A. engaddensis* envenoming are haemorrhagic, neurotoxic and cardiotoxic (Ovadia, 1987) and coronary vasospasm has been reported as cause of death (Lee *et al.*, 1989).

B. arietans venom causes massive local swelling that frequently involves the entire limb, with bruising, blistering, enlarged tender regional nodes and necrosis. Systemic

effects include a mild coagulopathy, thrombocytopenia and spontaneous intra-abdominal bleeding. Shock results from hypovolaemia caused by extravasation of plasma into the bitten limb (Chapman, 1968; Warrell, 1995).

The bite of *C. cerastes* is painful but usually not serious and is rarely fatal. In Saudi Arabia, ARAMCO Medical Department treated 26 cases of presumed *C. cerastes* bites in 10 years; less than half needed hospital admission and there were no fatalities (Warrell, 1993).

Echis species are responsible for many fatalities and untreated envenomation is associated with a 7% to 15% mortality rate (Warrell *et al.*, 1974 and 1976). Transient haemostatic failure, which may include disseminated intravascular coagulation, prolonged clotting time, hypofibrinogenemia and increased fibrin split products, is the hallmark of *Echis* envenomation (Rechnic *et al.*, 1962; Yatziv *et al.*, 1974). Haemostatic failure due to *E. coloratus* venom is less severe than that caused by *E. carinatus* (Fainaru *et al.*, 1974; Mann, 1978). Oedema is the most reliable sign of *Echis* envenoming and may be evident within 20 minutes. In severe envenoming, the oedema tends to spread over the entire extremity and sometimes into adjacent parts.

In severe systemic poisoning, acute renal failure is a serious complication; it may occur with bites by all types of poisonous snakes and may require dialysis. Death is usually most rapid after elapid bites (average time about five hours after the bite), and most protracted after viper envenoming (where the average is two to three days after the bite). Death due to sea snake bites usually occurs 12 to 24 hours after the bite and, as in elapid bite, is mainly due to respiratory failure. Shock and haemorrhage into vital organs are the main causes of death in bites from a viper.

1.3.3 Biological Effects

The composition and toxicity of snake venoms within a species may vary geographically and seasonally and differences may occur in specimens collected "the same day on the same hilltop" or "from under the same rock" (Minton and Minton, 1969). The median lethal dose test (LD_{50}) introduced by Trevan (1927) has gained wide acceptance as a measure of acute toxicity, although its original purpose was to determine and standardise biological potency. The LD_{50} is determined by constructing a curve showing the relationship between dosage and mortality, and the dose that would be expected to kill 50% of the animals is read from this curve. The most lethal Saudi Arabian venom is that of *A. microlepidota*; however the venom yield is low compared with *N. h. arabica* and *B. arietans* (Table 1.2). Various investigators have devised simplified procedures to obtain equally accurate and reproducible results for the LD_{50} with fewer animals (Reed and Muench, 1938; Lichfield and Wilcoxon, 1949; Meir and Theakston, 1986). One problem is the difference in susceptibility of animals to the lethal effect of venom which also varies with the species, sex, age and weight of the tested animal and the route of administration of the venom. The WHO (1981) recommends the iv route.

Generally, for the more common laboratory animals, the order of sensitivity to the lethal toxicity of a snake venom is pigeon > guinea-pig > mouse > rabbit and rat (Brazil, *et al.*, 1966; Chang and Lee, 1977; Glenn and Straight, 1982). Pigeons were used extensively for toxicity testing during earlier investigations (Sewall, 1887), but mice are presently the most common species employed and their use is recommended by the WHO (1981). Male mice are more susceptible than females (Aird and Kaiser, 1985) and 16g mice are more sensitive than 25g mice (Russell, 1983). Generally, mice of one sex with body weights ranging between 18 to 22g are used to determine the mouse LD_{50} of snake venoms and the ED_{50} of antivenoms (WHO, 1981).

Some snakes kill their victim by causing muscular and respiratory paralysis due to the presence of neurotoxic components in their venoms. *In vitro*, these toxins induce

Table 1.2 Comparative Toxicity of the Venomous Snakes Found in Saudi Arabia

Venoms	Venom yield (mg)	Reported Mouse LD ₅₀ (µg)	Injection Route	Estimated LD man (mg/70kg)	Reference
<i>Echis pyramidum</i>	25-35	24	iv	3-5	Minton and Minton, 1969
		19	iv		Theakston and Reid, 1983
		38	iv		
		18	iv		
<i>Echis coloratus</i>	-	9	iv	-	Wieser <i>et al.</i> , 1984
		25	iv		Theakston and Reid, 1983
		5			Ovadia and Kochva, 1977
		12			Gitter <i>et al.</i> , 1962
<i>Bitis arietans</i>	160-200	7	iv	90-100	Minton and Minton, 1969
		20	iv		Theakston and Reid, 1983
<i>Cerastes cerastes</i>	20-45	13	iv	40-50	Minton and Minton, 1969
		5	iv		Theakston and Reid, 1983
		9	iv		Hassan and Hawary, 1975
<i>Walterinnesia aegyptia</i>	25	6	ip	-	Gitter <i>et al.</i> , 1962
<i>Naja haje arabica</i>	175-300	5	ip	15-20	Minton and Minton, 1969 Mohammed <i>et al.</i> , 1973
<i>Atractaspis microlepidota</i>	4-10	2	iv	-	Wieser <i>et al.</i> , 1984

Table 1.2 lists the approximate venom yield obtained by milking each of the seven species of snake studied in this thesis together with the LD₅₀ of the venoms in mice using the intravenous (iv) or intraperitoneal (ip) route of administration.

neurotoxicity and myotoxicity in isolated nerve-muscle preparations with a reduction in contractile response to stimulation. Rat, mouse and guinea pig diaphragms, frog sartorius muscle, guinea pig ileum, and other preparations have been used extensively to demonstrate neurotoxic and myotoxic activities of the snake venoms.

Snake venoms contain two types of haemolytic factors. One is a direct haemolytic factor (DHF) which, although itself only weakly haemolytic, acts synergistically with phospholipase A₂ (Lee, 1971). The other is an indirect factor which lyses red cells only slowly and its lytic activity can be enhanced by the addition of serum or phosphatidylcholine. Some venoms contain both factors (such as cobra venom) while others (such as crotalid venoms) contain only the indirect factor. It was believed initially that the haemolytic activity is due to phospholipase A₂ causing hydrolysis of phospholipids to liberate fatty acids and lyso-derivatives which haemolyse red blood cells.

Snake venoms are referred to as "coagulant" or "anticoagulant" depending upon their primary effect on clotting mechanisms and some venoms contain both activities. Snake venoms have four general types of action (Denson, 1969); (1) activation of Factor X, (2) conversion of prothrombin to thrombin in the presence of Factor V, (3) conversion of prothrombin to thrombin in the absence of Factor V, and (4) direct conversion of fibrinogen to fibrin. The thrombin-like action on fibrinogen depletes plasma fibrinogen with formation of microclots which are different from ordinary fibrin clots and are rapidly lysed by the fibrinolytic action of tissues, resulting in prolonged defibrinogenation and prevention of coagulation (Tu, 1982). Thus, the venoms are coagulants *in vitro* but anticoagulants *in vivo*.

In *Echis* envenoming, fibrinogen levels are severely depleted while fibrin degradation products are increased. Factors II, V, VIII, X and XIII are also depleted (Warrell *et al.*, 1975). In 1988, Kamiguti and colleagues reported that the venom of

E. carinatus pyramidum from Saudi Arabia was a poor activator of prothrombin and contained a calcium-dependent Factor X activator, unlike venom from *E. carinatus* from India, Iran, Oman, Nigeria and Kenya.

The *in vitro* coagulation assay is performed by mixing the venom with plasma or a fibrinogen solution and recording the clotting time. The minimum clotting dose (MCD) can be defined as the least amount of venom which clots a standard human plasma or fibrinogen solution in 60sec at 37°C (Theakston *et al.*, 1982). The defibrinogenating assay is performed *in vivo*, the venom being injected (iv) into mice and the plasma fibrinogen concentration being measured after 60min (Theakston and Reid, 1983). The minimum defibrinogenating dose (MDD) can be defined as the minimum amount of venom that results in a decrease of the fibrinogen level to 10% of the standard plasma level, or as the least amount of venom producing non-clotting blood (Theakston *et al.*, 1982, Theakston and Reid, 1983).

Studies have shown that *Bitis* species venom contains the protein bitistatin which inhibits platelet aggregation and prolongs the bleeding time in human and canine models (Shebuski *et al.*, 1989). *Echis* venom contains echistatin, which is also a powerful inhibitor of platelet aggregation (Gan *et al.*, 1988). Further studies on *Bitis* venom revealed a kinin-releasing enzyme capable of hydrolysing insulin and fibrinogen (Sekoguchi *et al.*, 1986). The venom induces platelet aggregation and contains both coagulant and anticoagulant components (El-Asmar *et al.*, 1986; Soslau *et al.*, 1988).

A. microlepidota venom contains sarafotoxins which have 60% homology with the endothelins, the endogenous vasoconstrictor peptides. In humans, envenoming causes acute anaphylactic symptoms and ECG changes consistent with the coronary vaso-constriction and atrioventricular block produced by sarafotoxins, (Alkan and Sukenik, 1975).

Most of the toxic elements in the Arabian venoms are enzymes which can act in a number of ways; (1) local capillary damage and tissue necrosis is caused by phospholipases, hyaluronidases, and proteolytic enzymes, (2) diverse coagulant and anticoagulant actions result from various proteinases and phospholipases, and (3) acute hypotension and pain are induced by the release of vasoactive peptides by kinin-releasing enzymes. Snake venoms contain many types of proteases, including endopeptidases, peptidases, and proteases with limited specificity (such as thrombin-like enzyme, arginine ester hydrolase, prothrombin activator, Factor X activator, and kininogenases). Venoms that are rich in protease activity produce marked tissue destruction and may cause coagulation, haemorrhage, necrosis and fibrinolysis.

1.4 ANTIVENOMS

Antivenoms have been used for neutralising venoms from snakes, scorpions and spiders for just over a century. In addition, exogenous antibodies have been used to provide passive immunity against infectious agents, as antidotes to toxins, and to prevent the rejection of transplanted tissue. All uses of antibodies in biology and medicine have been complicated by the difficulty of generating large amounts of specific antisera by conventional immunisation; the unpredictability of the immune response; the immunogenicity of minor contaminants; and the heterogeneity of even highly specific antibodies.

1.4.1 Antibody Structure

Antibodies or immunoglobulins are a group of glycoproteins which constitute about 20% of all the proteins present in serum and tissue fluid of mammals. Essentially they are bifunctional; one region (the Fab fragment) recognises foreign cells and macromolecules (antigen) while a different region (the Fc fragment) mediates the activation of complement, and binding to macrophages and white cells, which helps eliminate microrganisms.

There are five general classes of antibodies; IgM, IgG, IgA, IgD and IgE with molecular weights ranging from 150 to 900kDa. IgG comprises about 75-85% of the antibodies of a normal person (Figure 1.3). The immunoglobulins differ from each other in size, charge, amino acid composition and carbohydrate content, but have the same basic structure comprising two identical light and two heavy polypeptide chains held together by non-covalent forces and by interchain disulphide bridges. Each chain consists of one sequence of amino acids and contains one or more intrachain disulphide bond. There is considerable amino acid homology between these various chains and they are all folded into a three-dimensional structure with a distinctive Y shape.

In any one molecule all the light chains are identical as are the heavy chains and the entire molecule is symmetrical about its long axis. The N-terminal domain of each chain shows much more variation in its amino acid sequence than the other domains and is known as the variable region to distinguish it from the relatively invariant constant regions. The variable portion is different for each specific antibody, and it is this portion that attaches specifically to a particular antigen. The constant portions of the antibody determine most of its gross physical and chemical properties and establish such factors as adherence of the antibody to specific structures within the tissues, attachment to the complement complex and the ease with which the antibodies pass through membranes.

1.4.2 Antibody Fragments

Many proteolytic enzymes have been used to cleave immunoglobulins, including pepsin, plasmin, bromelain, chymotrypsin, trypsin, elastase and papain. In 1939 Pope used the proteolytic enzyme pepsin to cleave immunoglobulins into $F(ab)_2$ and Fc. Pepsin is the main proteolytic enzyme involved in gastric digestion in mammals and birds, and its optimal action is at acidic pH. The portion of the polypeptide chain of

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the IgG molecules relatively accessible to proteolytic enzymes is called the hinge region and is situated between the CH1 and CH2 domains of the heavy chain.

Papain is one of the sulphhydryl proteases isolated from the latex of the green fruit of *Carica papaya*. It consists of a single 212 amino acid polypeptide chain and has a MWt of 23.4kDa. It is activated by thiol compounds like cysteine and by metal-binding agents such as EDTA, while its activity can be terminated irreversibly by iodoacetamide. It functions best at neutral pH. Papain cleaves the IgG molecule into three fragments of similar size, namely two Fab fragments and one Fc fragment each of approximately 50kDa. An Fab comprises four separate domains, consisting of 110-120 amino acids. Each domain has one three-stranded and one four-stranded segment joined by a single disulphide bond, while an interchain disulphide bond covalently couples CH1 to CL. The Fc fragment readily crystallises out of solution and is composed of the C-terminal halves of the heavy chains.

If pepsin is used, digestion occurs a little closer to the C-terminus than with papain. Thus the inter heavy-chain disulphide bridges are preserved and a large F(ab)₂ fragment (approximately 100kDa MWt) is released. Being divalent, this fragment will precipitate antigen. Pepsin also extensively degrades the Fc fragment.

The affinity constants (K) of the fragment may decrease with the size of the fragment in the order IgG > F(ab)₂ > Fab (Ferrone *et al.*, 1988). Nevertheless Fab fragments have a number of advantages over IgG. First, they are not associated with type III hypersensitivity reactions because, with only one antigen binding site, they do not form immune complexes. This contrasts with F(ab)₂. Second, lacking the Fc region, they do not bind complement or macrophages which helps eliminate vasoactive amine release (Smith *et al.*, 1979). Third, because of its large molecular weight IgG is cleared by cells of the immune system, whereas some Fab is eliminated via the kidney. Finally, IgG has a volume of distribution similar to the plasma volume, while Fab fragments (because of extracellular space penetration) have a value some eight to

nine times greater (Smith *et al.*, 1979; Sullivan 1987). Owing to their smaller size, Fab fragments also distribute around the body more rapidly; this, in conjunction with their greater distribution volume, helps ensure their importance as therapeutic agents.

1.4.3 Monoclonal and Polyclonal Antibodies

A polyclonal antiserum is the conventional product of an immunised animal. After immunisation a venom antigen will, according to the clonal selection theory of immunity (Burnet, 1959), bind with the best fitting antibody structures, found as cell membrane receptors on precursor lymphocytes. Binding to its receptor stimulates each particular lymphocyte to multiply and give rise to a clone of memory and of antibody-secreting cells. Venom generally consists of a large number of components. Each component may have many epitopes and new epitopes may be created through their metabolism by the host animal. The specificity of an antivenom results from the selection of the B-lymphocyte repertoire by the venom antigens. Thus a polyclonal antivenom contains the products of many responding clones of cells and is, in consequence, heterogeneous at many levels, including the classes and subclasses (isotypes) of the antibodies produced and their specificity, titre and affinity. The significance of such heterogeneity within and between polyclonal antisera in practice means that each antivenom is unique in its specific antibody composition. In general, polyclonal antibodies are easy and simple to produce and they have high affinity for antigen.

In 1975 Köhler and Milstein developed an alternative method for antibody production which yields monoclonal antibodies. These are derived from a single antibody producing cell clone, immortalised by fusion to a tumour cell line to form a hybridoma. The secreted antibody is homogeneous in specificity, affinity and isotype and each monoclonal product is specific to a single antigenic determinant (epitope) of the immunogen. In principle each monoclonal antibody can be prepared in unlimited amounts. One major disadvantage of monoclonal antibodies is that, due to their monospecificity, they lack or have only poor antigen-precipitation and

neutralisation properties. They also require considerable investment in time and equipment. At present, monoclonal antibodies are unsuitable for elapids antivenom production because venoms contain such a large number of constituents. Some workers have suggested that the best interim arrangement is to develop defined polyclonal antibodies using mixtures of monoclonal antibodies, thus recognising all the circulating isoforms until their unique roles can be determined. As mentioned above most of the very high affinity antibodies that have been produced have been polyclonal. This situation is changing with improved fusion and selection protocols but the high cost of monoclonal antibodies represents a real problem, especially for the developing world where envenoming claims most of its victims.

1.4.4 Monospecific and Polyspecific Antivenoms

When only one species of venomous snake occurs in a particular area, the choice of antivenom is simple and animals may be immunised with one venom to produce a monospecific antivenom. When more than one species of venomous snake exist a mixture of two, or more, of the different venoms may be used for immunisation to provide a polyspecific antivenom ("polyvalent" in USA). A monospecific antivenom is greatly to be preferred when the offending snake is known or its venom has been identified by a specific diagnostic test. Polyspecific products are useful when the offending snake is not known and this is common in countries with several poisonous snakes, which includes most tropical countries.

The victim may not be able to identify accurately the snake and it is for this reason that the majority of antivenoms are polyspecific. They provide a broad but reduced cross-reactivity against a number of snakes and difficult choices are avoided. However, there are a number of arguments against this approach. With the ability to produce only a limited number of antibodies in any one host animal, combining complex mixtures, such as snake venoms, may result in some components failing to elicit an immune response or, conversely, all antigens eliciting only a weak response. Also, because many venom components share elements of secondary structure, those

of low molecular weight that are weakly immunogenic or in low concentration may be selected against, in favour of the larger more immunogenic components. Finally, the large number of possible epitopes may also severely immunocompromise the host animal since so many antibodies may be directed against the venoms that only a few may remain to provide protection against environmental pathogens.

If four venoms of equal immunogenicity were mixed in equal amounts to form the immunogen, the resulting antivenom should in theory provide only 25% of the protection afforded by a similar monospecific antivenom against any one snake. However, because the venoms used usually show a high degree of cross-neutralisation, this figure is often much higher (Theakston and Smith, 1995). A second approach, which we adopt, is to mix monospecific antisera, each produced in separate animals. This method maximises the possible number of available antibody-producing cells and, in theory, should provide a wider range of specific antibodies. Nevertheless, because these antisera are then mixed, a dilutional effect is produced, the magnitude of which is dependent on the number of monospecific antisera included in the product. Such dilution should, in theory, reduce the effectiveness of the product but, again because many venoms are highly cross-reactive, the dilution effects are often much reduced. Studies comparing the ability of polyspecific, monospecific and mixed monospecific antivenoms to neutralise the activity of various venoms have shown that, as anticipated, monospecific products offer the best protection, and mixed monospecific antivenoms are, in general, better than polyspecific antivenoms (Smith *et al.*, 1992).

1.5 REQUIREMENTS FOR PRODUCTION OF ANTIVENOMS

1.5.1 Animal

Antisera against snake venoms intended for therapeutic use have occasionally been raised in goats (Cohen and Seligmann, 1966; Mohammed *et al.*, 1966; Russell *et al.*, 1970; Kochalaty *et al.*, 1971). However, historically all commercial snake antivenoms are prepared in horses (Minton, 1974). The use of horses for the

production of therapeutic sera was first described by Roux and Martin (1894) and they were the natural animal to choose at the turn of the century when they were available in vast numbers. Horses are also easy to handle, thrive in most climates, yield large volumes of serum, and methods of purifying and processing horse serum are well-developed. Big horses are obviously preferable because of their larger blood volume, but breed and age are not important. Tetanus is a common disease among horses, and they should be immunised prophylactically against the relevant organism and/or toxin.

A risk of some untoward reaction is attached to the injection of serum from any animal, and horse serum has gained a particularly bad reputation in this respect. This is, in part, probably because it has been more widely used than sera from other animals. The incidence of the delayed type of reaction, serum sickness, has been drastically reduced by the introduction of pepsin-digested antibodies lacking Fc but horses have an immunoglobulin class, termed IgG_T, which increases considerably following immunisation (Fernandes *et al.*, 1991) and is particularly immunogenic.

In this study antivenoms were raised in sheep. There are both practical and theoretical reasons for considering sheep as a suitable replacement for horses. Sheep are widely available, inexpensive and easy to handle. They are more resistant to disease, have a rapid immune response and usually attain high circulating levels of antibodies. Because the immune response in sheep is predominantly of the humoral type (antibody producing), immunisation with Freund's complete and incomplete adjuvants does not induce a severe local reaction (Sjöström *et al.*, 1994). In addition, sheep immunoglobulin is easy to cleave by pepsin and by papain, producing less allergic and less immunogenic F(ab)₂ and Fab fragments respectively.

1.5.2 Immunogen

Substances which can induce an immune response (humoral, cellular, or mixed T and B cells) when introduced into an animal are called immunogens. Certain conditions

must be satisfied in order for a molecule to be immunogenic. The molecules should be foreign to the host. As a general rule, molecules smaller than 1kDa are only weakly immunogenic and macromolecular proteins with MWt greater than 100kDa are the most potent immunogens. A molecule must possess a certain degree of complexity and immunogenicity increases with structural complexity. Finally, the ability to respond to a particular immunogen varies with the genetic constitution of the animal being immunised.

The quality of an antivenom depends, in part, on the initial venom pool used as immunogen. Thus the composition of a venom varies with respect to age of the snake, its geographical distribution and the season. Ideally the venom should be a pool from the whole distribution area, collected at different times of the year, and from snakes of mixed age and sex. Venoms contain many components and the best immunogens are those present in high concentrations and of large molecular weight. It is sometimes stated that antigen competition may result in a low antibody response to some components. In the case of important low molecular weight toxins, for example in elapid venoms, it may be necessary to isolate the toxin for use either to supplement the venom used as immunogen or to produce separate antibodies directly towards the toxin to supplement the final antivenom (Sells *et al.*, 1994).

1.5.3 Adjuvant

Adjuvants are agents used to potentiate the immune response and, customarily, they are administered with the antigen but this is not always the case. Theoretically adjuvants may act by one or more of the following mechanisms: (1) directly increasing the number of cells involved in antibody formation, (2) ensuring a more efficient processing of the antigen, (3) prolonging the duration for which the antigen is maintained in the immunised animal, and (4) increasing the synthesis and release of antibody from the antibody-forming cells.

Some of the different adjuvants used in the past for antivenom production include chalk, mineral oils, lipopolysaccharides from gram-negative bacteria, saponin, yeast,

aluminium phosphate, aluminium hydroxide gels, sodium alginate, bentonite and Freund's adjuvants. Freund's complete (a suspension of killed *Mycobacterium* in a mixture of paraffin oil and an emulsifying agent) and incomplete (lacking the *Mycobacterium*) adjuvants used in this study are classic examples of water-in-oil emulsion adjuvants.

Venom has also been incorporated into liposomes and New and colleagues (1985) showed that a single injection of *E. carinatus* venom incorporated into sphingomyelin-cholesterol liposomes could produce a high antibody response in mice, rabbits and sheep. They also suggested that oral ingestion of liposome entrapped venom could be used for active immunisation in areas with a high incidence of snake bites.

1.5.4 Immunisation Protocol

Hyperimmunisation has been the procedure most commonly employed for producing antivenoms for the last 100 years since antivenoms were first produced. It is based on the historical findings of Sewall (1887) who demonstrated active immunisation of pigeons using increasing amounts of a rattlesnake venom injected over the course of a month. This demonstration had a profound influence on the thinking of von Behring and Kitasato (1890) and Calmette (1894a,b), and they used a similar hyperimmunisation technique to produce immune sera in horses against diphtheria and tetanus toxins and against cobra venom, for therapeutic use.

The procedure involves immunising a host with repeated injections of increasing doses of immunogen given at weekly or still shorter intervals. It is possible that this procedure may be necessary to induce an immune response to the smaller and less immunogenic components of a venom or those present at very low concentrations especially in the period before adjuvants were developed. However, it is expensive in terms of venom and the amounts given might be sufficient to induce tolerance.

In this work, a low dosage immunisation procedure was used for producing antibodies to the venoms collected from Saudi Arabian snakes. To find the optimal immunisation dose for each venom, dose-response studies were performed. The sheep were initially immunised with 0.5mg of venom and the dose was then doubled every four weeks for some of the sheep so that 1mg or 4mg were being injected until the maximum response was obtained.

1.5.5 Antiserum Collection and Processing

Blood is collected under aseptic conditions and allowed to clot. Cellular components are separated by centrifugation and the serum is collected and may be heated for 30min at 56°C to destroy complement and fibrinogen. The serum may then be salt precipitated to yield an IgG-rich fraction largely devoid of albumin. Ammonium sulphate is commonly used for this purpose. However in this work sodium sulphate was employed because it results in a pellet that is more easily reconstituted containing lower levels of albumin, and avoids the addition of potentially toxic ammonium ions. It has also been suggested that ammonium sulphate irreversibly denatures immunoglobulin, reducing the yield of viable antibodies (Sullivan, 1987).

Some producers of antivenom still use intact IgG or even unprocessed serum or plasma for the treatment (Theakston and Warrell, 1991). However, to overcome hypersensitivity reactions, most antivenom manufacturers digest immunoglobulin using pepsin to produce bivalent F(ab)₂ fragments which lack the C terminal Fc domains (Latifi, 1978). A more recent development in immunotherapy is the use of Fab fragments produced by digesting IgG with the plant enzyme papain.

The total immunoglobulin fraction of any immune serum will, by definition, contain antibodies that are directed against molecules other than the immunogen. These antibodies comprise the background complement of immunoglobulins acquired by the animal during its lifetime and, as such, are of no therapeutic benefit to the patient. Sheep antisera typically contain about 25g of immunoglobulin per litre, of which

only a quarter to a third can be expected to be specific to the immunogen (Smith *et al.*, 1992). Thus if the total immunoglobulin fraction is administered, the non-effective molecules simply increase the burden of foreign protein given to the envenomed patient and the possible risk of side-effects. It is for this reason that affinity chromatography techniques have been applied in recent years to enable the isolation of antibodies specific only to the venom components.

Such procedures are based on antigens (in this case a venom) bound to an inert matrix over which the antivenom is passed. Specific antibodies or fragments bind to the venom on the matrix while non-specific components pass through. The matrix may then be washed and the specific antibodies or fragments eluted. Although affinity purification has long been used as a means of isolating antibodies to specific venom components, it was Russell and colleagues (1985) who first pointed to this technique as a means of reducing antivenom doses. Using polyacrylamide as a support matrix they showed that material produced in this way was more effective in neutralising lethal, haemorrhagic, coagulant, platelet-aggregating, necrotising and neurotoxic activities than the starting material.

1.6 ASSESSMENT OF ANTIVENOMS

The primary role of any antivenom is to have a significant effect in reducing morbidity and mortality in humans. The general techniques applied to antivenoms include physicochemical assessment of purity, measurement of binding activity and assessment of ability to neutralise the biological and enzymatic venom activities. The concentration and purity of antivenoms can be assessed by methods such as FPLC, reversed radial immunodiffusion and SDS-PAGE. As venoms are complex mixtures of toxins with different pharmacological and pathological activities, the antivenom should ideally be titrated against each important activity.

1.6.1 Binding Assays

These are a group of closely related *in vitro* assays which involve the non-covalent, high affinity and reversible binding of a ligand by a specific binding protein of biological origin. Such assays are subdivided primarily according to the binding protein employed. Thus immunoassays employ specific antibodies, while binding protein assays use naturally occurring plasma proteins, and receptor binding assays employ proteins that are usually extracted from the organ (s) which is targeted by a drug or toxin (Chard, 1990).

Immunoassays are the most common form of binding assay and are based on the non-covalent reversible binding of an antigen by a specific antibody. They have been introduced for the detection and determination of a variety of macromolecules and haptens in biological fluids. They can be categorised into those in which no labelled reactant is required and those employing labelled antigen or antibody. In non-labelled immunoassay, the antibody-antigen complexes are detected and measured directly. Precipitation immunoassays are the most commonly employed of these, and depend upon the antibodies precipitating antigens from solution by formation of a lattice of cross-linked antibody-antigen molecules. This precipitate can be measured directly in solution, or after precipitation in agar or agarose. Such procedures are widely used to assess antivenoms, with double immunodiffusion being one of the simplest and earliest techniques used. (Russell, 1967; Greenwood *et al.*, 1974). However, these assays are relatively insensitive and time consuming.

Labelled immunoassays employ either labelled antigen or antibody and can be categorised into isotopic and nonisotopic immunoassays. The isotopic assays employ gamma or beta emitting isotopes as labels and can be further subdivided into radioimmunoassays (RIA) in which labelled antigens are employed and immunoradiometric assays (IRMA) which require isotopically labelled antibodies. Non-isotopic immunoassays employ either antigens or antibodies labelled with a nonisotopic substance such as an enzyme or a fluorescent molecule. Another example is passive haemagglutination, using reagent labelled with erythrocytes,

which was used by Boche and Russell (1968) to assess both venom and antivenom; however the assay has disadvantages including the instability of the labelled reagents and imprecise end-point determination (Theakston, 1983).

RIA was first developed by Yalow and Berson (1960) for the quantitation of insulin. In 1974, Coulter and his colleagues developed a solid phase RIA using ^{125}I -labelled venom, for the detection of Australian snake venoms in biological samples. This technique has been used by many investigators to detect venoms or venom toxins in biological samples (Greenwood *et al.*, 1974; Sutherland *et al.*, 1975). A sandwich IRMA, using ^{125}I -labelled antibody raised against snake venom, has been used by other investigators (Sutherland and Coulter, 1977a,b; Coulter *et al.*, 1978). Both RIA and IRMA are reproducible, sensitive, and can be used to detect and assess both venom and antivenom levels in biological samples. Their disadvantages include the short shelf life of the isotopically labelled reagent and the expensive counting equipment required. The techniques also require considerable technical expertise.

Enzyme-labelled immunoassays employ enzyme-labelled reagents. Those which employ labelled antigens are called enzymeimmunoassays (EIA) while those employing labelled antibodies are called immunoenzymometric assays (IEMA) (Smith *et al.*, 1981). Traditionally, both EIA and IEMA performed in microtitre plates are called "Enzyme-linked immunosorbent assays" (ELISA). The type of ELISA introduced by Theakston and his colleagues, in 1977, is the most commonly used method in snake venom and antivenom research and involves the use of coated microtitre plates or tubes. There are two systems. The double-sandwich ELISA is performed by adding venom to antibody coated plates ("capture antibodies"), washing, and then adding a specific antibody directed against the venom and conjugated to an enzyme, such as horseradish peroxidase or alkaline phosphatase ("labelled antibodies") followed by a substrate. The second approach involves coating the plates with venom and adding antivenom followed by a species-specific conjugated second antibody. The former methods have a great clinical potential for detecting and identifying snake venoms, while the later are of great value in detecting and identifying assessing and standardising antivenoms (Theakston and Reid, 1979).

Both systems are sensitive, reproducible and employ inexpensive and stable reagents. Theakston and Reid (1979) showed good correlation of *in vitro* ELISA titres and the *in vivo* ED₅₀ in mice. However, some snake venoms possess peroxidase and alkaline phosphatase activity which may interfere with endpoint detection to give false positive results. Also, some preservatives inhibit the enzymatic activity of the conjugate and the assays are affected by temperature.

The binding capacity of an antivenom depends upon its antibody titre and affinity. The titre is defined as the dilution which binds 50% of the label and indicates the relative abundance of relevant antibodies in a preparation. The higher the titre, the more potent and economical will be the use of the antiserum. The affinity (equilibrium) constant is the definitive thermodynamic characteristic of a binding site. The term affinity is used to describe the strength of binding between an antibody and an antigen. Thus high affinity can be seen as binding involving a lot of energy or lasting a long time.

1.6.2 Neutralisation of Biological Activity

The potency of an antivenom may be assessed by determining its ability to inhibit or neutralise the biological effect(s) of the venom using animals, organs from an animal or cells. Ideally, its lethality, neurotoxicity, myotoxicity and haemolytic and coagulation effects should be assessed since these are the most important toxic effects that may be induced by the venom. Potency of an antivenom is demonstrated by means of its ability to protect experimental animals from the lethal effects of the venom. The median effective dose (ED₅₀) can be defined as the amount of antivenom required to protect 50% of tested animals from the lethal effect of a given amount of venom, usually two or five times the LD₅₀. Results are expressed as mg of venom neutralised per ml of antivenom or by the number of LD₅₀ neutralised by a given amount of antivenom.

1.6.3 Neutralisation of Enzymatic Activity

Numerous assays exist to assess the enzymatic activities of venoms. However, there have been few detailed studies of the neutralisation of these activities by antivenoms and this is another aim of the present study. Enzyme assays are simple and quick and can be subdivided into colorimetric and turbidometric assays. Colorimetric assays are based on the ability of enzymes or venoms to hydrolyse synthetic substrates either to liberate a coloured product (which can be measured spectrophotometrically) or to change the acidity of the solution (leading to change in a pH indicator). The former approach is the more common and is used to assess phosphodiesterase (Ballario *et al.*, 1977) and L-amino acid oxidase (Johnson *et al.*, 1987; Ueda *et al.*, 1988). The latter has been used to assay phospholipase A₂ which hydrolyses a substrate, phosphatidylcholine, to lysophosphatidylcholine and fatty acids (Canziani *et al.*, 1982; Johnson *et al.*, 1987).

Turbidometric assays have been used widely to measure the activity of enzymes. They can be divided into those which act to inhibit the formation of a colloid and those which break it down once formed. For example, hyaluronidase inhibits colloid formation by stopping the cross linking of a substrate reagent comprising hyaluronic acid and cetyltrimethylammonium bromide (Xu *et al.*, 1982; Genge *et al.*, 1985), while phospholipase A₂ clears the turbidity of a suspension of egg yolk (Vidal and Stoppani, 1971).

1.7 AIM OF STUDY

To improve the production and assessment of antivenoms for use in Saudi Arabia where the existing antivenoms are produced by techniques which have hardly changed during the past 50 years.

Antivenoms will be produced for the following species of snake: *Cerastes cerastes*, *Walterinnesia aegyptia*, *Echis coloratus*, *Echis carinatus*, *Bitis arietans*, *Naja haje arabica* and *Atractaspis microlepidota*.

CHAPTER II

MATERIALS, INSTRUMENTATION AND METHODS

2.1 MATERIALS

2.1.1 Venom Collection

The venoms used in this study were collected from snakes of many different regions of Saudi Arabia, which were housed and milked by members of the Department of Zoology, King Saud University, Riyadh. Their geographical distribution and the yields obtained by milking are listed in Table 2.1. The snakes were milked every three months by applying pressure to the venom glands and collecting the venom in a small beaker. The fresh venom was filtered and then lyophilised and stored in the dark at 4 °C. Most venoms were yellow in colour but venom from *B. arietans* was white and that from *N. h. arabica* only very slightly yellow.

2.1.2 Antivenoms and Immunochemicals

Pasteur-Mérieux (Middle-East and North Africa) and Behringwerke AG (Near and Middle East) antivenoms were bought from their manufacturers. Normal horse and sheep sera, and donkey anti-sheep and sheep anti-horse sera were obtained from Polyclonal Antibodies Ltd (Dyfed, UK). Freund's Complete and Incomplete adjuvants were from Sigma (Poole, Dorset, UK).

2.1.3 General Chemicals and Materials

L-amino acid oxidase, ammonium acetate, adenosine diphosphate (ADP), ammonium persulphate, L-ascorbic acid, N- α -benzoyl-L-arginine-p-nitroanilide (L-BAPNA), albumin, bromophenol blue, 4-chloro-1-naphthol, Coomassie brilliant blue, cresol red, L-cysteine, o-dianisidine, N,N-dimethyl casein, ethylenediamine tetra-acetic acid (EDTA), fibrinogen, D-glucose, hexadecyl-trimethylammonium bromide (CTMBr), horseradish peroxidase, hyaluronic acid, iodoacetamide, leucine, mercaptoethanol, 3-(N-morpholino)propane sulfonic acid (MOPS), pepsin, o-phenylene-diamine dihydrochloride, L- α -phosphatidylcholine, p-nitrophenyl thymidine-5-phosphate, potassium carbonate, sodium chloride, sodium hydroxide, sodium periodate, tris(hydroxymethyl) methylamine and tetramethylenediamine (TEMED) were bought from Sigma (Poole, Dorset, UK).

Table 2.1 Venom Yield and Geographical Distribution of Saudi Arabian Snakes

N	Northern region
S	Southern ""
E	Eastern ""
W	Western ""
C	Central ""

Family name	Species	Venom yield per milking (mg) (Gasperetti, 1988)	Geographical distribution
Elapidae	<i>N. h. arabica</i>	170-300	SW
	<i>W. aegyptia</i>	25	C, NE and N
Viperidae	<i>E. pyramidum</i>	20-35	C, SW
	<i>E. coloratus</i>	10-40	SW, C, E and N
	<i>C. cerastes</i>	20-40	Throughout all the country
	<i>B. arietans</i>	160-200	SW
Atractaspididae	<i>A. microlepidota</i>	4-8	C, S and N

Table 2.1 summarizes the amount of venom (in mg) obtained per milking and the geographical distribution of Saudi Arabian snakes.

Acetic acid, agarose, acetonitrile, ammonium hydrogen carbonate, barbital buffer, bromocresol green, dimethyl sulfoxide, ethanolamine, gelatin, glycerol, glycine, hydrochloric acid, hydrogen peroxidase solution (30% v/v), methanol, potassium dihydrogen orthophosphate, potassium chloride, sodium acetate, sodium azide, sodium carbonate, sodium citrate, sodium dihydrogen orthophosphate, sodium dodecyl sulphate (SDS), sodium hydrogen carbonate, sodium nitrate, sodium sulphate, sodium tetraborate, sulphuric acid, thiomersal, trichloroacetic acid, 2,4,6-trinitrobenzene sulphonic acid, Triton X-100 and Tween 20 were all purchased from BDH (Poole, Dorset, UK).

N,N-methylene-bis-acrylamide, magnesium sulphate, magnesium chloride, calcium chloride and urea were obtained from Aldrich (Gillingham, Dorset, UK). CNBr activated Sepharose 4B, electrophoresis calibration kit, electrophoresis film, filter paper and Superose 12 (HR 10/30) were obtained from Pharmacia LKB (Uppsala, Sweden). Standard endotoxin, pyrogen free water and Limulus Amebocyte Lysate were from Biowhittaker (Walkersville, USA). Papain was bought from Merck (Darmstadt, Germany), microtitre plates (Nunc) from Gibco BRL (Renfrewshire, UK), Cut-off dialysis tubing (MWt 12 kDa) and nitrocellulose membranes from Sartorius (Belmont, Surrey, UK).

2.2 LIST OF GENERAL INSTRUMENTATION

Blotting apparatus; model II-S Sartoblot, Sartorius (Belmont, Surrey, UK)

Centrifuge; MSE Mistral 6000 and 3000 Mistral (Sussex, UK)

Cuvettes; 10mm ultra-UV disposable cuvettes from Elkay (Basingstoke, Hampshire, UK)

Electrophoresis unit; model SE600 (standard size) and Mighty Small II, model SE250, Hoefer Scientific Instruments (San Francisco, USA)

ELISA plate reader; Titertek multiscan plus MK II, Flow Laboratories (Lugano, Switzerland)

ELISA plate washer; model S8/12, Flow Laboratories (Lugano, Switzerland)

FPLC; comprising two P-500 pumps and an LCC-500 plus controller unit, Pharmacia LKB (Uppsala, Sweden)

Freeze-drier; Hetosic, Heto Lab Equipment (Birkerod, Denmark)

HPLC; High pressure liquid chromatography system, Shimadzu SPD-10A (Kyoto, Japan)

Incubator; model 6A2N from LEEC Ltd (Colwick, Nottingham, UK)

pH meter; AGB-75, Labtech Instruments (Wrexham, Wales, UK)

Platelet aggregometer; Lumi-Aggregation Module, series 1000B, Payton Scientific (Milano, Italia)

Power supply; model 400, Shandon, Southern Products Ltd (Runcorn, Cheshire, UK)

Spectrophotometer; model 4050 Ultrospec II from LKB (LKB Biochrome, Cambridge, UK)

Vortex mixer; maxi-mix II from Clandon Scientific Ltd (Aldershot, Hampshire, UK)

2.3 GENERAL METHODS

2.3.1 Immunoglobulin Fractionation

Serum (aproximately 1500ml) was first filtered through glass wool to remove solid and lipid material, then mixed with an equal volume of sodium sulphate (36% w/v) stirred for 60 min and centrifuged at 2500g and 25°C for 30min. The precipitate was twice re-suspended in sodium sulphate (18% w/v) and then redissolved to its original volume (aproximately 1500ml) in PBS (50mM sodium phosphate buffer, pH 7.4, containing NaCl 0.8% (w/v) and KCl 0.02% (w/v) and dialysed to remove any trapped salt at 4°C for 48hr.

2.3.2 Papain Digestion

Proteolytic cleavage of immunoglobulins with papain was carried out as described by Porter (1959). The pH of approximately 500ml of the immunoglobulin fraction was adjusted to 7.0 and after adding EDTA (0.8mg/ml of solution), papain (16% w/w of the total IgG weight) and L-cysteine (160mg/ml to activate papain) the reaction mixture was incubated at 37°C for 24hr to ensure that most of the IgG had been digested. The reaction was stopped by adding iodoacetamide (2.8mg/ml) and the reaction mixture was dialysed (using dialysing tube with 12kDa MWt cut-off) against PBS to remove the low molecular weight reagents and finally centrifuged at 2500g and 25°C for 30min to sediment the Fc fraction.

2.3.3 Pepsin Digestion

Digestion with pepsin was performed as described by Pope (1939). Approximately 500ml of the IgG fraction was dialysed against acetate buffer (sodium acetate, 0.1M, NaCl, 10mM, pH 3.5) for 18hr at 4°C or until the pH was 3.5. Pepsin (2% w/w of the total weight of IgG to be digested) was added, dissolved in a minimum amount of acetate buffer, and the reaction was allowed to proceed for 5hr at 25°C with gentle shaking. The reaction was terminated by adding a saturated Tris free base solution until the pH reached 7.5 after which the digest was dialysed against saline (NaCl, 9g/l) and centrifuged at 2500g and 25°C for 30min to sediment the Fc fragment. The supernatant was further purified by salt precipitation using sodium sulphate (section 2.3.1).

2.3.4 Protein Concentration

The protein concentration of immunoglobulin and its fragments as well as the venom solutions was assessed by the absorbance at 280nm using an extinction coefficient (1g/l solution, 1cm light path) of 1.5 for IgG, 1.45 for F(ab)₂, 1.4 for Fab and 1 for venom.

2.3.5 Albumin Concentration

Ovine albumin concentrations were kindly measured by Robert Bush (Therapeutic Antibodies Ltd, London) using a separation fluoroimmunoassay. Briefly, known concentrations of ovine albumin competed with a fixed amount of fluorescein-labelled ovine albumin for a limited and constant amount of specific antibody to obtain the standard curve. The sample containing an unknown amount of albumin was then run in the same way and the results read off the standard curve. The amount of albumin was expressed as a percentage of the weight of the protein.

2.3.6 Fc Concentration

Ovine Fc levels were kindly measured by Robert Bush (Therapeutic Antibodies Ltd, London) using a separation fluoroimmunoassay. A standard curve was constructed by allowing unlabelled ovine Fc to compete with a fixed amount of fluorescein-labelled Fc

for a limited and constant amount of specific antibody. The amount of Fc in the sample was expressed as a percentage of the weight of protein in the samples.

2.3.7 Immunoelectrophoresis

Immunolectrophoresis (IEP) was performed as described by Graber and William (1953). This technique separates molecules according to their charge, size and shape, followed by an immundiffusion step where the separated samples are brought into contact with antisera, to form an antigen-antibody complex which gives a precipitin line. Sample and standards ($3\mu\text{l}$, 2.5g/l) were separated electrophoretically for 90min at 150V in an agarose gel (2%w/v in barbital buffer, 8.6g/l , pH 8.6). Donkey anti-sheep IgG serum was then applied into the troughs and allowed to diffuse into the gel overnight. After washing with saline, proteins were visualised by staining with Coomassie brilliant blue 1% (w/v). The IEP was used semi-quantitatively for measuring Fc since a precipitation band was visible at a concentration of 80mg/l but not at 40mg/l .

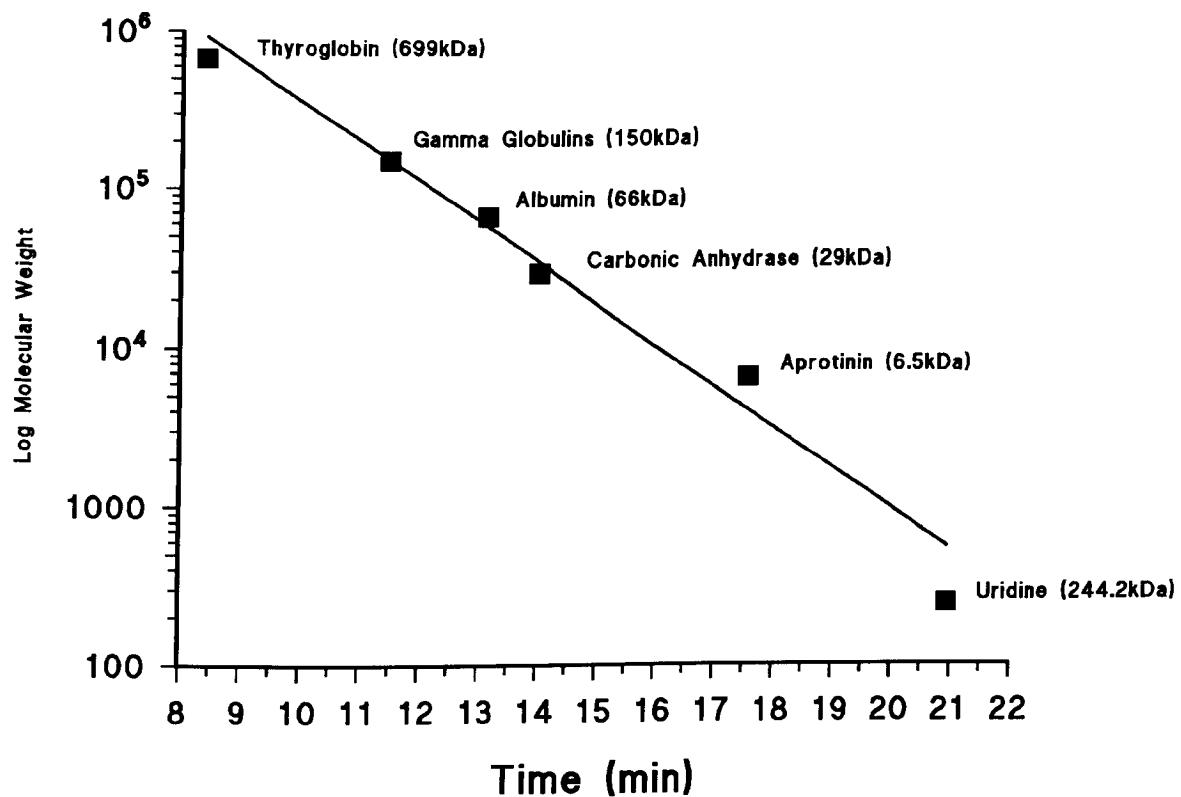
2.3.8 Fast Protein Liquid Chromatography

FPLC, employing medium pressure (1-1.5 mPa) and a cross linked agarose medium (Superose 12 HR 10/30, 15cm length and 10mm diameter column, volume 25ml), ensured optimal separation of components with MWt between 1 and 300 kDa. All the buffers were de-gassed and the sample (1g/l) venom was dissolved in saline and centrifuged to remove any solid material. Approximately $200\mu\text{g}$ was applied to the column and the appropriate sensitivity levels were set for measuring the eluted proteins with 154 mM NaCl at pH 5.5, flow rat at 0.5ml/min and the absorbance read at 280nm . To assess the molecular weight of eluted components, proteins of known size were applied to the column and their retention time plotted against the molecular weight (Figure 2.1).

2.3.9 Reversed Phase High Pressure Liquid Chromatography

Saudi Arabian venoms were analysed by HPLC reversed phase chromatography. Approximately $200\mu\text{g}$ of 1mg/ml venom was applied to the column (Supelco RP LC-8-

Figure 2.1 Calibration Curve for the Superose 12 FPLC Gel Filtration Column



A calibration curve for the FPLC Superose 12 column was obtained by running proteins of known molecular weight. Their retention times were measured and plotted against their MW_t and the best curve was fitted using linear regression.

DB, 15cm length, 4.6mm diameter and 5nm beads), which was equilibrated with 5% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid (TFA). The components were eluted with a gradient of 90% (v/v) acetonitrile in 0.1% (v/v) TFA at a flow rate of 0.5ml/min and the absorbance read at 280nm.

2.3.10 SDS-PAGE

SDS-PAGE was performed according to the method of Laemmli (1970) using a water cooled, discontinuous, vertical slab gel system comprising a stacking gel (10% v/v acrylamide) and a resolving gel (10% v/v acrylamide). Either a standard size or a mini gel system was used; the advantage of the latter was that it gave a faster running time (1hr compared with 3-4hr) and used less protein (10 μ g protein per well compared with 40 μ g). The resolving gel was prepared by mixing the stock solutions (Table 2.2) and adding TEMED immediately before pouring the gel followed by methanol on top to exclude oxygen. After polymerisation (20min) the methanol was replaced with the stacking gel (Table 2.2) and an appropriate comb was inserted. The sample was mixed with an equal volume of either non-reducing sample buffer (Tris 25mM, pH 6.8, containing SDS 2% w/v, glycerol 16% v/v and bromophenol blue 0.01% w/v) or reducing sample buffer (containing mercaptoethanol 4% v/v). Electrophoresis was performed in running buffer (Tris 25mM, pH 8.3, containing glycine 0.2M and SDS 0.1% w/v) using 20mA/gel until the tracking dye had entered the resolving gel and then 40mA. The gels were used either for Western blotting (section 2.4.1.3) or stained with Coomassie brilliant blue (1 % w/v in destaining solution) for 1hr, destained (methanol : acetic acid : water, 4:1:5 v/v) until the background was clear, and then dried and photographed.

2.3.11 Sterility

Sterility was assessed by inoculating 1ml of antivenom into thioglycolate and tryptic soya broths (20ml) which encourage and support the growth of anaerobic, aerobic and facultative bacteria, yeasts and moulds. The thioglycolate broth was incubated at 32.5 ± 2.5°C and the tripticase soya broth at 22.5 ± 2.5°C for 14 days and both were observed for growth indicated by the presence of turbidity.

Table 2.2 Mixing of Reagents for SDS-PAGE

RESOLVING GEL:

REAGENTS	VOLUME stand. system	VOLUME mini system
Tris buffer (1M, pH 8.8)	11.25 ml	1.9 ml
SDS solution (10% w/v)	0.3 ml	50 µl
Acrylamide/bis-acrylamide,(30%, 29.15g acrylamide + 0.85g N,N'-methylene-bis-acrylamide per 100ml)	10 ml	1.66 ml
Water	7.75 ml	1.3 ml
Ammonium Persulphate (1.5% w/v)	0.7 ml	116 µl
TEMED (N,N,N',N'-tetramethylenediamine)	30 µl	5 µl

STACKING GEL:

REAGENTS	VOLUME Stand. System	VOLUME Mini System
Tris buffer (1M, pH 6.8)	1.2 ml	360 µl
SDS solution (10% w/v)	0.1 ml	30 µl
Acrylamide/bis-acrylamide, stock solution (30%)	1.5 ml	450 µl
Water	6.7 ml	2 ml
Ammonium persulphate (1.5% w/v)	0.5 ml	150 µl
TEMED	20 µl	5 µl

Table 2.2 lists the reagents used for preparing SDS-PAGE with the top table showing the reagents used for preparing the resolving gel and the bottom table those used for preparing the stacking gel.

2.3.12 Bacterial Endotoxin Test

A semi quantitative test for gram-negative bacterial endotoxin was used. The LAL reagent (Limulus amebocyte lysate) was reconstituted with low endotoxin water and mixed in equal parts (10 μ l) with doubling dilutions of the sample. After incubation for 1hr, the presence of endotoxin causes coagulation. The lowest dilution displaying coagulation was compared with standards and expressed in EU/ml. Controls are needed because, if the sample causes inhibition of gel-formation, the test is not valid.

2.4 ASSESSMENT METHODS

2.4.1 Binding Assays

2.4.1.1 Enzyme Linked Immunosorbent Assay

To assess antibody titres, plates were coated with venom (2 μ g/ml, 100 μ l/well) dissolved in coating buffer (NaHCO₃, 0.1M, pH 9.6 containing 0.1% w/v thiomersal) for 2hr at 37°C. Plates were then washed three times with 300 μ l/well of washing buffer (sodium phosphate 10mM, NaCl 0.8% w/v, KCl 0.02% w/v, thiomersal 0.01% w/v and Tween 20, 0.1% v/v, pH 7.4) using an automatic plate washer. Two-fold serial dilutions (which will be referred to as doubling dilutions) of the antiserum (100 μ l/well) dissolved in washing buffer were incubated in the venom-coated wells for 1hr at 37°C and, after washing, a second antibody directed against the first (viz donkey anti-sheep or sheep anti-horse as appropriate) and coupled to horseradish peroxidase was added (100 μ l/well, diluted 1:500 in washing buffer). After being incubated for 1hr at 37°C and washing, enzyme substrate was added (100 μ l/well of o-phenylenediamine, 0.01% w/v, dissolved in sodium citrate buffer, 0.07M, pH 5.0 containing H₂O₂, 20 μ l/100 ml of a 30% v/v solution). The reaction was stopped after 10min with sulphuric acid (3M, 50 μ l/well) and the optical density read at 492nm using an automatic ELISA plate reader. The antibody titre, defined as the dilution of antivenom which gave 50% of maximum binding, was calculated after correcting for non-specific binding by normal sheep serum (usually 4%). To minimise assay variation, a control sample for each antivenom was included and all samples were corrected for any variation. The between assay variation (CV) was 8% for *E. pyramidum* antivenom when performed on seven separate occasions.

2.4.1.2 Small Scale Affinity Purification

To isolate immunoglobulins specific to venom components, venom was coupled to Sepharose 4B activated with cyanogen bromide. The gel was first washed for 15 min on a sintered glass filter with dilute hydrochloric acid (1mM) to remove preserving agents and swell the beads. After briefly washing with the coupling buffer (NaHCO_3 , 0.1M, pH 8.3, containing 0.5M NaCl), the gel was incubated with the venom (5mg/g of dry gel) in a stoppered vessel overnight at 4°C and any remaining active groups blocked with glycine (0.2M, pH 8.0). Non-bound venom was removed by washing with three cycles, each consisting of a wash with acetate buffer (0.1M, pH 4, containing 0.5M NaCl) followed by a wash with Tris buffer (0.1M, pH 8, containing 0.5M NaCl). The affinity matrix was finally equilibrated with saline and stored at 4°C. All the eluants were checked for protein by $\text{OD}_{280\text{nm}}$ to ensure that the venom components had bound covalently. To determine its maximum binding capacity, the column was incubated with different amounts of immunoglobulin for two hr at rt and any unbound material was removed by washing with PBS. The bound antibody was then eluted with glycine (10mM, pH 2.5) and dialysed against saline (0.9% NaCl w/v) overnight to raise the pH and remove glycine (Figure 2.2).

2.4.1.3 Western Blotting

Venom was studied as described by Towbin and colleagues (1979). Venom was separated on 10% SDS-PAGE gel (0.35mg protein on a mini system, 1.2mg on a standard system) using non-reducing sample buffer. Part of the gel was stained with Coomassie blue (as reference) and the remainder of the proteins was transferred to nitrocellulose paper (0.45mm pore size) with a horizontal semi-dry blotting system using 0.8mA/cm^2 for 2hr. During transference the gel and nitrocellulose paper were surrounded by filter paper soaked in blotting buffer (Tris 25mM, pH 8.3 containing glycine 0.2M and 20% v/v methanol). Staining the SDS-PAGE gel with Coomassie blue 1% (w/v) after transference showed that the low MWt bands had been displaced but some of the high MWt components remained.

Figure 2.2 Small Scale Affinity Purification of Equine Antivenom (Pasteur)

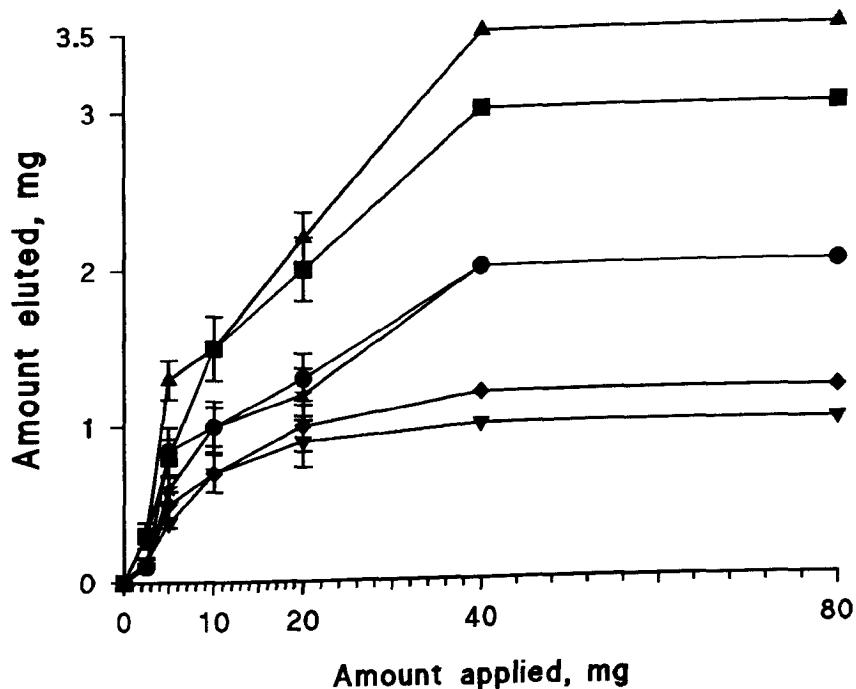


Figure 2.2 shows the binding capacity of increasing amounts of the Pasteur antivenom for venoms from *E. pyramidum* (▲), *E. coloratus* (■), *W. aegyptia* (▼), *N. h. arabica* (●), *C. cerastes* (★) and *B. arietans* (◆) coupled to a solid phase matrix. The bound antibodies were eluted with glycine (10mM, pH 2.5) and the protein concentration read at 280nm. The Pasteur antivenom contained the highest concentration of specific antibodies against *E. pyramidum* and the lowest against *W. aegyptia* venom.

After transference, non-specific sites on the nitrocellulose paper were blocked with gelatin for 30min (Tris 50mM, gelatin 0.4% w/v, Tween 20, 0.05% v/v, pH 7.5). and, after washing (Tris 50mM, Tween 20, 0.05% v/v, pH 7.5), the paper was cut into strips and incubated with antivenom (200mg/l) for 1hr at rt. After washing, the strips were incubated for 1hr at rt with the appropriate second antibody coupled to horseradish peroxidase, diluted 1:500 in washing buffer and the strips were then incubated with substrate (4-chloro-1-naphthol 0.02% (w/v) dissolved in citrate buffer 0.07M, pH 5, containing 0.05% of a 30% v/v H₂O₂) until the bands were well developed (about 10min) when they were finally washed in water and photographed.

2.4.2 Enzymatic Assays

2.4.2.1 Phospholipase A₂ (PLA₂) Activity

A cresol red method was used (Canzaini *et al.*, 1982) in which the substrate solution was prepared by dissolving L- α -phosphatidylcholine extracted from egg yolk (0.4% w/v, initially 120mg dissolved in 1ml methanol), Triton X-100 (1% v/v) and cresol red (0.1% w/v) in a salt solution (NaCl 100mM, KCl 100mM and CaCl₂ 10mM, adjusted to pH 8.5). This solution (2.8ml) was mixed with venom (100 μ l) and either buffer or antivenom (100 μ l) and the decrease in absorbance due to the released fatty acid was followed at 573nm. The initial reaction rate was proportional to the venom concentration and the amount of venom used (25-0.25 μ g) was adjusted so that the decrease in absorbance could be followed easily. There was some spontaneous hydrolysis of the substrate which was assessed and subtracted from the readings and PLA₂ activity was expressed as the decrease in absorbance per min per mg venom. The within assay variation (CV) measured 10 times using *E. pyramidum* venom was 6% and the between assay variation, performed on 10 different occasions, was 8%.

2.4.2.2 Hyaluronidase (HYL) Activity

HYL was assayed by the method of Di Ferrante (1956). Hyaluronic acid in the presence of cetyltrimethylammonium bromide (CTMB), precipitates to form a turbid solution. However when hyaluronate has been depolymerised no turbidity develops when CTMB is added.

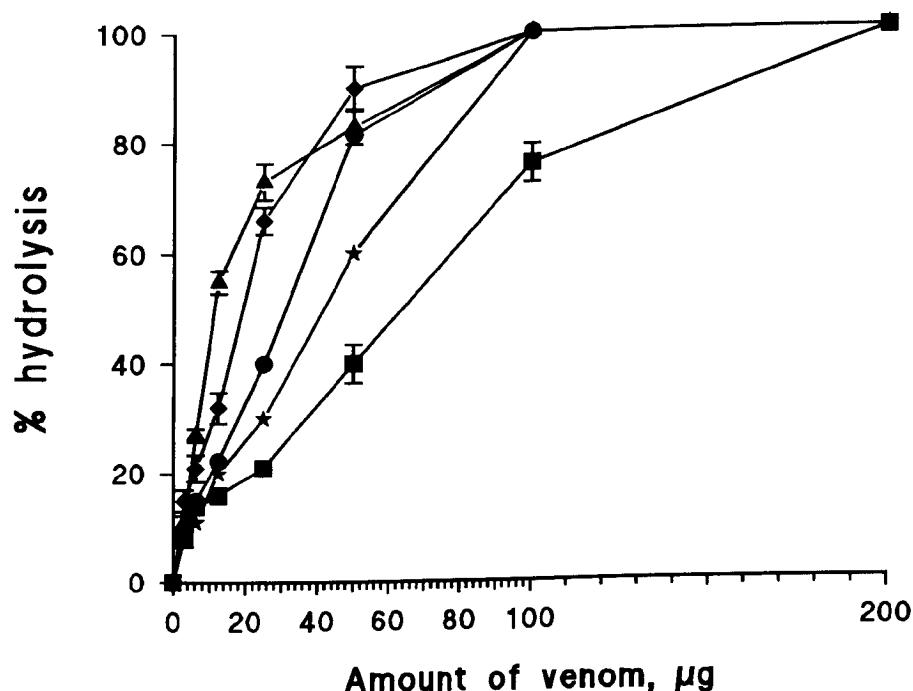
Different concentrations of venom (50 μ l) were mixed on microtitre plates with hyaluronic acid (50 μ l, 0.05% w/v) and assay buffer (50 μ l of 0.2M sodium acetate, pH 6, containing 0.15M NaCl) or antivenom and incubated for 30min at 37°C. The reaction was stopped by adding CTMBr (150 μ l, 2.5% w/v dissolved in 2% w/v NaOH) and the turbidity was read within 10min at 405nm using an automatic ELISA plate reader. Hyaluronidase activity was expressed as the amount of venom required to reduce the initial turbidity by 50% (Figure 2.3). For neutralisation experiments doubling dilutions of the equine and ovine antivenoms (50 μ l) were pre-mixed for 30min at 37°C with the amount of venom which reduced 50% of the absorbance at 405 nm.

2.4.2.3 Proteolytic Activity

2.4.2.3.1 Casein assay

This was performed according to the method of Lin and colleagues (1969). Different concentrations of venom (20 μ l, in saline) were added to dimethyl casein (1ml, 0.1% w/v in -3(N-morpholino propane sulphonic acid {MOPS} buffer, 5mM, pH 7.2) and incubated in a water bath at 38°C for 30min and the reaction was stopped by immersing the test tubes in a boiling water bath for 5min. To detect the released amino acids, sodium bicarbonate (1ml, 4% w/v solution, pH 8.5) and 2,4,6-trinitrobenzene sulphonic acid (1ml of a 0.1% v/v aqueous solution) were added and incubated in a waterbath at 50°C for 30min. The reaction was stopped by adding hydrochloric acid (0.5ml, 1M) and SDS (1ml, 10% w/v) and the absorbance was read at 340nm. All samples were assayed in duplicate. The proteolytic activity was expressed as mole of amino terminals released per g venom and was determined by dividing the absorbance at 340nm by 10^4 , the extinction coefficient for most trinitrophenyl amino acids. The between assay variance (CV) for *E. pyramidum* venom performed on seven different occasions was 7%. There was no increase in absorbance if *E. pyramidum* venom was incubated without casein, showing that there were not enough amino acids in the venom to contribute to the reading. It was not possible to study the neutralisation of proteolytic activity using casein as substrate since the antivenom interfered with the assay.

Figure 2.3 Hyaluronidase Activity of Saudi Arabian Venoms.



The hyaluronidase activities of increasing concentrations of venoms from *E. pyramidum* (■), *E. coloratus* (▲), *N. h. arabica* (◆), *C. cerastes* (★) and *B. arietans* (●) were measured using a hyaluronic acid substrate in the presence of CTMBR. Activity is expressed in the terms of % reduction in initial turbidity at 405nm. At 50% hydrolysis *E. coloratus* venom had the highest activity and *E. pyramidum* venom the lowest.

2.4.2.3.2 L-BAPNA assay

Proteolytic activity was also measured using L-BAPNA (N- α -benzoyl-L-arginine-p-nitroanilide). The substrate (5mg/ml, 100 μ l) was mixed on a microtitre plate with assay buffer (100 μ l, 0.1M sodium phosphate, containing 0.1M KCl, pH 6.8) or antivenom and venom solution (100 μ l), incubated for 60min at 37°C and the absorbance at 405nm was recorded using an ELISA plate reader. The activity was expressed as the increase in absorbance at 405nm per mg venom.

2.4.2.4 L-Amino Acid Oxidase (L-AAO) Activity

L-AAO was measured by a method (Ueda *et al.*, 1988) in which riboflavin is oxidised, producing H₂O₂ that activates horseradish peroxidase to enable the conversion of o-dianisidine to oxo-dianisidine with an absorbance maximum at 530nm. L-Leucine (0.1ml, 0.1M), the substrate for L-AAO, was incubated with o-dianisidine (0.1ml, 0.25% w/v dissolved in dimethyl sulphoxide), horseradish peroxidase (0.1ml, 0.004% w/v), venom solution (0.1ml) and assay buffer (2.6ml, 0.1 M sodium phosphate, pH 6.8) or antivenom for 1hr at 37°C. The reaction was terminated by adding sulphuric acid (2ml, 50% v/v) and the samples were left standing for 30min before the absorbance was recorded. Only freshly dissolved venom samples were used, as L-AAO is known to be very unstable. L-AAO activity was expressed as the increase in absorbance at 530nm per mg venom. For neutralisation experiments, doubling dilutions of the equine or ovine antivenoms were pre-mixed for 30min at 37°C with venom.

2.4.2.4 Phosphodiesterase Activity

Phosphodiesterase activity was measured as described by Ballario and colleagues (1977). The synthetic substrate p-nitrophenyl thymidine-5'-phosphate (PNPT-5'-P) is hydrolysed with the liberation of 4-nitrophenyl phosphate which gives a yellow colour. The assay was performed in disposable polystyrene cuvettes by incubating venom solution (100 μ l), assay buffer (100 μ l, Tris buffer, pH 8.5, 0.1 M, containing MgCl₂, 2 mM and Tween-20, 1ml/l) and substrate (1.3ml, PNPT-5'-P, 0.07 g/l) for 30 min at at

37°C. The reaction was stopped (0.5ml, sodium hydroxide, 0.2M) and, after 10 min, the absorbance at 400nm was recorded (Figure 2.4). To measure inhibition, mixtures of venom and antivenom were assessed as described above and the percentage inhibition plotted against the amount of antivenom used.

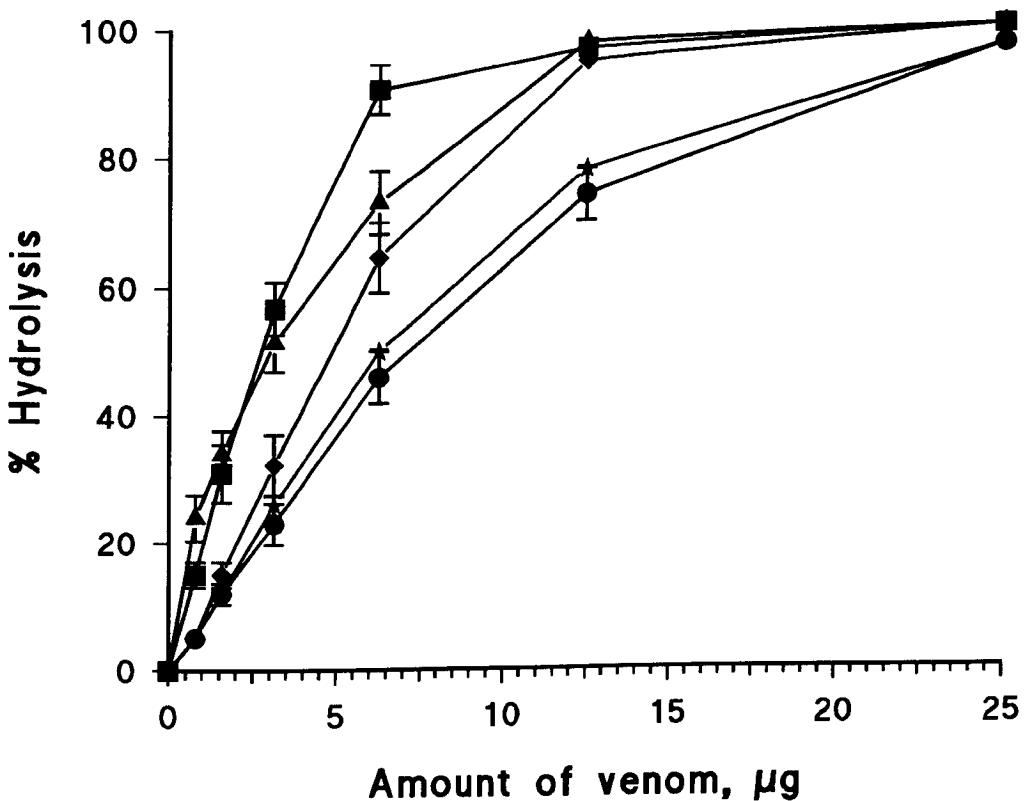
2.4.3 Biological Assays

2.4.3.1 Indirect Haemolytic Activity

Indirect haemolytic activity was assessed according to the method of Al-Abdulla and colleagues (1991) using human erythrocytes and phosphatidylcholine as substrate for venom phospholipases. The erythrocyte suspension was prepared from human blood (9ml:1ml 3.8% w/v sodium citrate) by centrifugation for 10min at 2000g and 25°C, after washing the erythrocytes three times in buffer (Tris 1mM, pH 7.5, containing sodium citrate 0.38% w/v, NaCl 100mM, KCl 100mM and CaCl₂ 10mM), they were resuspended in buffer (2.5% v/v). This preparation was used the same day it was prepared. The erythrocyte suspension (1ml) was pre-incubated with assay buffer (2.9ml, Tris 1mM, pH 7.5, containing NaCl 100mM, KCl 100mM and CaCl₂ 10mM) and L- α -phosphatidylcholine (0.1ml of a 1% w/v solution) for 30min at 37°C with constant shaking to prevent cells from sedimenting. Venom solution was added (1ml) and, after a further 30min incubation, the tubes were centrifuged at 25°C and 2000g for 15min, and the absorbance of the released haemoglobin in the supernatant was read at 540nm. The indirect haemolytic activity was expressed as percentage haemolysis against mg venom. The within assay variance (CV) for seven samples of *E. pyramidum* venom was 8%. There was no direct haemolysis of human erythrocytes with any of these venoms (up to 1g/l) if the lipid substrate was omitted.

For neutralisation experiments the amount of venom inducing 100% haemolysis was incubated with doubling dilutions of the antivenom (0.5ml) for 30min at 37°C and then mixed with the erythrocyte suspension.

Figure 2.4 Phosphodiesterase Activity of Saudi Arabian Venoms.



The phosphodiesterase activity of increasing concentrations of venoms from *E. pyramidum* (■), *E. coloratus* (▲), *N. h. arabica* (◆), *C. cerastes* (★) and *B. arietans* (●) was measured using the substrate p-nitrophenyl thymidine-5-phosphate (PNPT-5-P). Activity is proportional to the yellow colour produced due to the liberation of 4-nitrophenyl phosphate and is measured spectrophotometrically at 400nm. *E. pyramidum* venom showed the highest activity whereas venom from *C. cerastes* and *B. arietans* showed the lowest.

2.4.3.2 Platelet Aggregation

2.4.3.2.1 Preparation of human PRP and PPP

Platelet-rich plasma (PRP) was prepared from whole blood collected by venepuncture (9ml:1ml 3.8% w/v sodium citrate) by centrifugation at 250g for 10min and 25°C which precipitated the white and red cells while leaving virtually all the platelets in the supernatant (PRP). Platelet-poor plasma (PPP), used for blanking the aggregometer, was prepared from the remaining blood by re-centrifugation at 1500g for 10min and 25°C. The PRP was left standing for 30min before use and all experiments were completed within 3hr after collection using plastic tubes and pipettes because glass (unless silanised) can induce aggregation. Blood samples were not taken if aspirin had been consumed during the previous 10 days.

2.4.3.2.2 Measurement of platelet aggregation

The aggregometer measured light transmission at 600nm which increases as platelets aggregate. Initially the base-lines were set using PRP (10% light transmission) and PPP (90% light transmission), with a chart speed of 2cm/min. All samples were pre-heated to 37°C, with magnetic stirring at 500rpm. The PRP (450µl) was equilibrated until the baseline was stable after which the venom (50µl) was added followed by ADP (20µl, 0.1M) to induce aggregation. The between assay coefficient of variance for *C. cerastes* venom, performed on six different occasions, was 6%. For neutralisation experiments different amounts of the antivenoms raised against *C. cerastes* and *B. arietans* venoms were pre-incubated at 37°C for 30min with venom before being added to the PRP.

2.4.3.3 Coagulant Activity

The minimum coagulant dose (MCD), defined as the least amount of venom that clots human plasma (MCD-P) or bovine fibrinogen (MCD-F) in 60 sec at 37°C, was estimated as described by Theakston and Reid (1983). Venom (50µl) and assay buffer (50µl, Tris, 0.1M, pH 7.5 containing NaCl, 9g/L) were mixed and 50µl of this mixture was added to pre-warmed (37°C) citrated human plasma or fibrinogen (200µl, 2g/l) and

the clotting time measured and plotted against the concentration of venom used. For neutralisation experiments, venom (50 μ l) was mixed with increasing amounts of antivenom and the volume made up to 100 μ l with assay buffer. Then 50 μ l of this mixture was added to the pre-warmed human plasma and the clotting time measured as above. Complete inhibition was assumed when no clot formation occurred within 2hr, and zero inhibition when the clotting time was identical to that using venom alone.

2.4.3.4 Minimum Haemorrhagic Dose (MHD)

Haemorrhagic, necrotising and defibrinogenating activities were measured using the facilities of the Liverpool School of Tropical Medicine (Liverpool, UK). MHD was measured as described by Theakston and Reid (1983) and is defined as the least amount of venom which, when injected intradermally into rats, results in a haemorrhagic lesion of 10mm diameter 24 hours later. Different concentrations of venoms (0.1ml in saline) were injected into the shaved dorsal skin of rats (male Sprague Dawley, 180-250g) under light halothane and oxygen anaesthesia. After 24 hours the animals were killed, the dorsal skin removed, and the diameter of the lesion measured on the inner surface of the skin, in two directions at right angles, using calipers and background illumination. Care was taken not to stretch the skin. The mean diameter of the haemorrhagic lesion was calculated for each venom dose and the MHD was estimated by plotting the mean lesion diameter against venom dose and reading off the dose corresponding to a 10mm lesion. For neutralisation a mixture of antivenom and the MHD of the venom was pre-incubated at 37°C for 30min before being injected intradermally into rats. The haemorrhagic lesion was measured 24hr later.

2.4.3.5 Minimum Necrotizing Dose (MND)

The MND was measured as described by Theakston and Reid (1983) and is defined as the least amount of venom which, when injected intradermally into rats, results in a necrotic lesion of 5mm diameter three days later. The method used was the same as that for the MHD, except that the dorsal skin was removed three days after intradermal injection of venom. The MND was calculated by plotting the mean lesion diameter against the dose of

venom and reading off the dose corresponding to a diameter of 5mm. For neutralisation studies different amounts of antivenom and the MND of the relevant venom were pre-incubated at 37°C for 30min before being injected intradermally into rats as described above.

2.4.3.6 Minimum Defibrinogenating Dose (MDD)

The MDD, using whole blood, was defined as the minimum dose of venom that produced non-clotting blood within 60min of its intravenous injection and was measured as described by Theakston and Reid (1983). A wide dose-range of *E. pyramidum*, *E. coloratus*, *E. ocellatus* and *W. aegyptia* venoms (100 µl in saline, pH 5.5) were injected intravenously into 4 adult male mice (Swiss Albino) weighing 20-25g. One hour after injection, the mice were anaesthetised with halothane and oxygen and bled by cardiac puncture. The blood was pooled and placed in a clotting tube and left at room temperature for 1hr where the clot quality was recorded using a 1-5 grading system (Figure 2.5) according to Reid (1967). For neutralisation studies, different amounts of antivenom and the MDD of the relevant venom were pre-incubated at 37°C for 30min before being injected into mice.

2.4.3.7 Neurotoxicity and Myotoxicity

The mouse hemidiaphragm preparation was used to determine the neurotoxic and myotoxic activities of *N. h. arabica* and *W. aegyptia* venoms. Venom alone or pre-mixed with antivenom, was added to such a preparation and the contractile activity of the muscle recorded after either indirect (nerve) or direct (muscle) stimulation.

2.4.3.7.1 Nerve muscle preparation

This was prepared as described by Bülbüling (1946). Adult male mice (Swiss Albino) weighing 25-30g were sacrificed by stunning, followed by exsanguination, and the left and right phrenic nerves located, dissected out and freed from connective tissue (Figure 2.6). The nerves and diaphragm, together with a rib and a piece of vertebra, were transferred to

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a petri dish containing continuously oxygenated Krebs' buffer (containing NaCl 6.9g/l, KCl 0.3g/l, sodium bicarbonate 2.1g/l, potassium dihydrogen phosphate 0.2g/l, magnesium sulphate 0.3g/l, calcium chloride 0.3g/l and glucose 2g/l, pH 7.4). The diaphragm was then cut into two segments and each was carefully arranged on a diaphragm holder, with the costal margin attached to the lower electrode on the holder with a piece of string attached to the central tendon. The phrenic nerves were carefully wrapped around two platinum wire electrodes and the preparation was then transferred to a 100ml organ bath containing Krebs' buffer maintained at 37°C (\pm 1°C) and equilibrated with a gas mixture of O₂ (95%) and CO₂ (5%). Contractions of the muscle were elicited either by stimulation of the nerve with a pulse of 1-10V for 0.1ms duration at a rate of 15/min or by direct stimulation of muscle with a pulse of 50V for 0.5ms duration at 15/min. The tension was recorded isometrically by means of a force displacement transducer coupled to a Devices MX2 amplifier and recorder.

2.4.3.7.2 Effects on the nerve-muscle preparation

The neuromuscular blocking activity was determined by adding Krebs' buffer (60ml) containing venom and recording the contractions after stimulating the nerve (indirect stimulation). After 30min the venom was washed out and replaced by fresh Krebs' buffer every 30min. Each experiment was conducted for 240min, and all the values reported are means \pm SEM of four preparations. Control experiments (venom-free Krebs' buffer) showed that contractions fell by a maximum of 10% in 240min. For neutralisation experiments venom was pre-incubated with different concentrations of antivenom at 37°C for 30min before being added to the nerve-muscle preparation. For control experiments, normal sheep serum was used.

2.4.3.8 Lethal Toxicity

Traditionally the toxicity of a venom is assessed *in vivo* using mice. To determine the 50% intravenous lethal dose (ivLD₅₀) of a venom, groups of mice receive increasing doses of venom dissolved in saline (total volume 200μl) via the tail vein. Normally five groups of five male TFW mice (18-20g) were used and the number of survivors in each

group were counted after 24hr. The doses were adjusted so the highest dose gave 100% mortality while all the mice survived at the lowest dose. To establish 50% of the protective amount of an antivenom (ED_{50}), different amounts of the antivenom were pre-mixed for 30min at 37°C with 5 times the ivLD₅₀ followed by the same procedure as described for LD₅₀. Control animals were injected with only venom or antivenom (5 times the highest amount used). The analysis of the slopes of the dose-response regressions for the LD₅₀ and ED₅₀ was performed by the probit method (Finney, 1971).

CHAPTER III

CHARACTERISATION OF SAUDI ARABIAN VENOMS

3.1 SUMMARY

Saudi Arabian venoms were assessed for their physicochemical, enzymatic and biological activities. Each produced 8 to 14 bands on SDS-PAGE, using non-reducing conditions, with molecular weights ranging from 23 to above 94kDa. All the venoms showed high phospholipase A₂ activity which decreased in the order *E. coloratus* > *E. pyramidum* = *N. h. arabica* > *C. cerastes* = *B. arietans* venom. All the venoms tested contained L-amino acid oxidase and *E. pyramidum* and *E. coloratus* venoms had the highest activity. Most also showed high hyaluronidase activity, decreasing in the order *N. h. arabica* = *C. cerastes* > *E. coloratus* = *B. arietans* > *E. pyramidum*. High proteolytic activity was found in *E. pyramidum* venom using both dimethylcasein and L-BAPNA as substrate. *C. cerastes* and *B. arietans* venoms showed low phosphodiesterase activity whereas the other venoms were highly active in this respect.

Studies of the biological effects of the venoms revealed that *E. coloratus* venom had the highest indirect haemolytic activity, whereas *B. arietans* venom showed very little activity. *C. cerastes* and *B. arietans* venoms initiated platelet aggregation using human platelet-rich plasma. The coagulant activity of two *Echis* species (*E. coloratus* and *E. pyramidum*) venom assessed showed high activity. Both *Echis* species also had high haemorrhagic, necrotic and defibrinogenating activities. Using a mouse hemidiaphragm preparation, venom from *N. h. arabica* inhibited nerve stimulated muscle contraction but had no direct effect on the muscles. Lethality (iv LD₅₀) in mice decreased in the order *A. microlepidota* > *N. h. arabica* > *C. cerastes* > *B. arietans* > *E. coloratus* > *E. pyramidum*.

W. aegyptia venom activities are described in Chapter VI.

3.2 PHYSICOCHEMICAL ASSESSMENT

3.2.1 Reversed Phase Chromatography

The results of reverse-phase HPLC of venoms from the four viper species found in Saudi Arabia are shown in Figure 3.1. *E. pyramidum* venom showed 10 peaks with retention times ranging from 14.3 to 31.3min, while, *E. coloratus* venom had 11 peaks with retention times from 14.3 to 25.8min. Different venom batches showed the same profile. *C. cerastes* venom showed 9 peaks with retention time ranging from 14.4 to 26.3min and the first peak represented about 20% of the total components. Finally *B. arietans* venom had 11 peaks with retention times ranging from 5.1 to 27.4min and the total component found in the first peak represented about 72% of the total.

3.2.2 Fast Protein Liquid Chromatography

The results of fast flow chromatography of 200 μ g of each of the five venoms are shown in Figure 3.2. The molecular weights of the peaks were estimated from the calibration curve. *E. pyramidum* and *E. coloratus* venoms showed peaks with MWt ranging from 158 to 0.16kDa and 123 to 0.2kDa respectively. *C. cerastes* venom showed seven peaks with MWt ranging from 184 to 0.3kDa and a further five peaks with MWt below 0.3kDa. *B. arietans* venom showed seven peaks with MWt ranging from 133 to 0.25kDa. Finally *N. h. arabica* venom showed six peaks with MWt that ranged from 158 to 5kDa.

3.2.3 Non-reducing SDS-PAGE

Venoms from Saudi Arabian species produced from 8 to 14 bands on 10% SDS-PAGE gels under non-reducing conditions, with molecular weights ranging from 20 to above 94kDa (Figure 3.3). Unfortunately many bands of MWt below 23kDa could not be visualised. *E. pyramidum* and *C. cerastes* venoms showed a similar bands as did the *E. coloratus* and *B. arietans* venoms. All the venoms had several intense bands with MWt of under 30kDa and many less intense bands of MWt from 43 to 94kDa. *E. coloratus*

Figure 3.1 RP- HPLC of Saudi Arabian Viper Venom

mAbs 280nm

E.pyramidum

Peak no.	% of venom	Retention time (mins)
1	14.4	14.3
2	2.5	15.5
3	0.6	17.8
4	12.9	18.9
5	15.2	20.3
6	7.2	21.7
7	11.5	23.3
8	24.9	24.2
9	6.6	30.4
10	4.2	31.3

E.coloratus

Peak no.	% of venom	Retention time (mins)
1	11.3	14.3
2	1.1	14.8
3	0.7	15.2
4	1.2	16.2
5	6	18.8
6	13.4	20
7	12	20.5
8	12.5	21.2
9	15	21.8
10	17.5	23.6
11	9.6	25.8

C.cerastes

Peak no.	% of venom	Retention time (mins)
1	20.9	14.4
2	8.5	18.5
3	6.3	19
4	11	19.5
5	10.4	20.3
6	9.4	21.2
7	15	22.8
8	9	23.7
9	0	26.3

B.arietans

Peak no.	% of venom	Retention time (mins)
1	72	5.1
2	0.5	14.4
3	1.6	17.2
4	1.4	17.7
5	0.7	18.7
6	0.3	19.4
7	6.7	20.7
8	8.2	22.2
9	4.2	24
10	0.5	25.3
11	3.9	27.4

Retention time (min)

Retention time (min)

Figure 3.1 shows the profile of Saudi Arabian venoms from *E. pyramidum*, *E. coloratus*, *C. cerastes* and *B. arietans* when analysed by RP HPLC. Approximately 200µg from (1mg/ml) of each venom was applied to the column (Supelco RP LC-8-DB, 15cm length, 4.6mm diamter and 5nm beads), which was equilibrated with 5% acetonitrile in 0.1% trifluoroacetic acid (TFA) and the protein eluted with a gradient of 90% acetonitrile in 0.1% TFA at flow rate of 0.5ml/min and the absorbance read at 280nm.

Figure 3.2 FPLC Gel Filtration of Venoms from Saudi Arabian Vipers

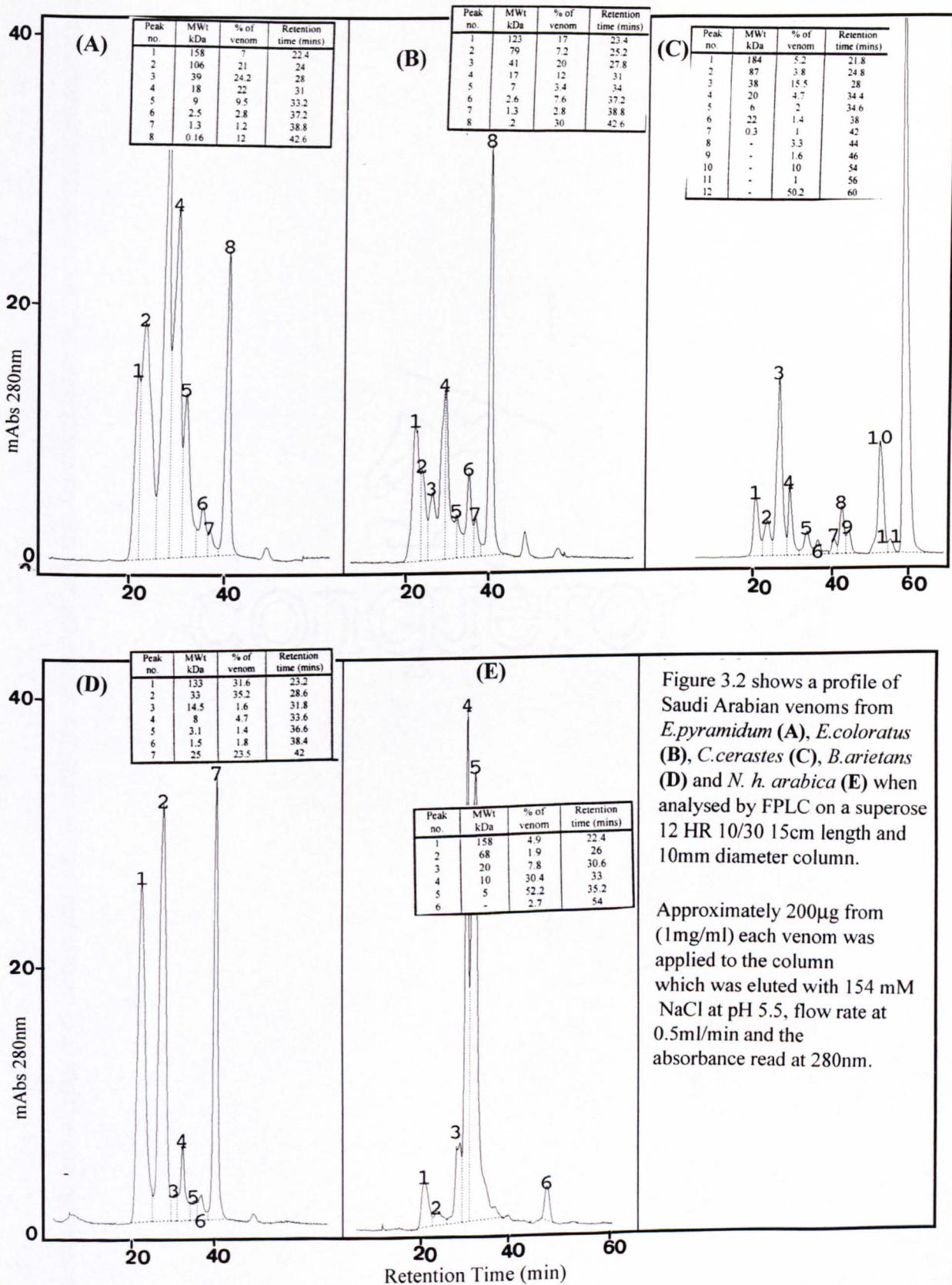
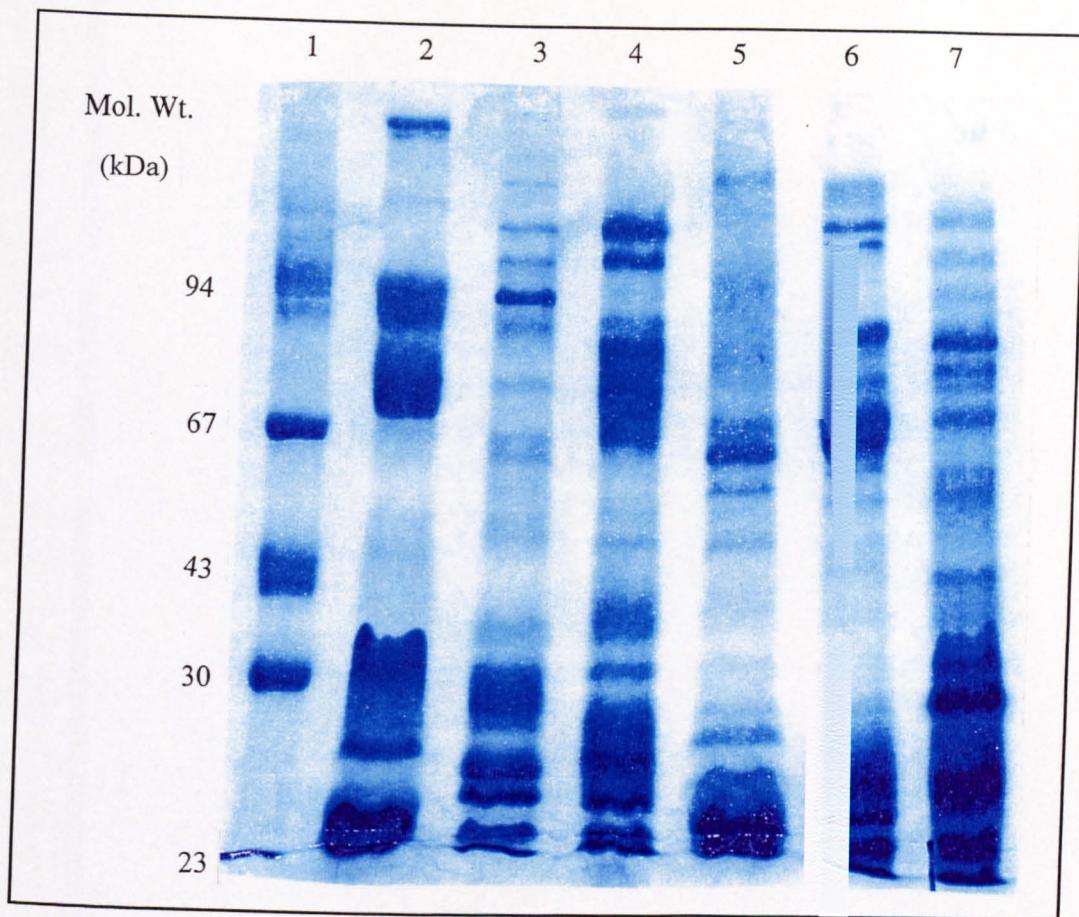


Figure 3.2 shows a profile of Saudi Arabian venoms from *E.pyramidum* (A), *E.coloratus* (B), *C.cerastes* (C), *B.arietans* (D) and *N.h.arabica* (E) when analysed by FPLC on a superose 12 HR 10/30 15cm length and 10mm diameter column.

Approximately 200µg from (1mg/ml) each venom was applied to the column which was eluted with 154 mM NaCl at pH 5.5, flow rate at 0.5ml/min and the absorbance read at 280nm.

Figure 3.3 SDS-PAGE of Saudi Arabian Venoms



Venoms from *W. aegyptia* (2), *B. arietans* (3), *C. cerastes* (4), *N. h. arabica* (5), *E. pyramidum* (6) and *E. coloratus* (7) were resolved by SDS-PAGE using 10%, non-reducing gels. All venoms produced between 8 to 14 bands with MWt ranging from 20 to 94 kDa. *E. pyramidum* and *C. cerastes* venoms shared many bands as did the *E. coloratus* and *B. arietans* venom. *E. coloratus* venom contained the most bands (14) including those with the highest and lowest molecular weights. *N. h. arabica* venom lacked many of the bands found in *W. aegyptia* venom. Lane 1 contained MWt markers.

venom contained the most bands (14) including those with the highest and lowest molecular weights. *N. h. arabica* venom lacked many of the bands found in *W. aegyptia* venom.

3.3 ENZYMATIC ACTVITIES

All methods were described in Chapter II and venom activity is expressed as the amount of venom required to produce 50% activity.

3.3.1 Phospholipase A₂

E. coloratus venom showed the highest PLA₂ activity (0.5μg), although *E. pyramidum* and *N.h. arabica* venoms were only slightly less potent (1μg). Similar activities were produced by *C. cerastes* and *B. arietans* venoms (4μg) (Table 3.1).

3.3.2 Phosphodiesterase

About double the activity was found in the venoms from the *Echis* species (*E. pyramidum* and *E. coloratus*) than in *N. h. arabica*, *C. cerastes* and *B. arietans* venoms.

3.3.3 Hyaluronidase

The venom from *E. coloratus* and *N. h. arabica* showed the highest hyaluronidase activity followed by those from *B. arietans* and *C. cerastes* venoms while *E. pyramidum* venom showed the least effect.

3.3.4 L-Amino-Acid Oxidase

E. pyramidum and *E. coloratus* venoms showed the highest activity, followed by *C. cerastes* venom while *N. h. arabica* venom was slightly less potent. The activity of *B. arietans* venom was the lowest as suggested by its white colour, since this enzyme is the cause of the yellow colour of many venoms.

Table 3.1 Enzymatic Activities of Saudi Arabian Venoms

Species	Amount of venom (μg) required to produce 50% activity					
	PLA ₂	PDE	HYL	L-AAO	Proteolytic	
					L-BAPNA	dimethyl casein
<i>E.pyramidum</i>	1	3	62	7	20	10
<i>E.coloratus</i>	0.5	3	14	7	20	20
<i>C.cerastes</i>	4	6	40	12	8	15
<i>B.arietans</i>	4	7	30	50	35	35
<i>N.h.arabica</i>	1	5	20	20	60 ¹	60 ¹

Table 3.1 lists the amount of venom (in μg) from *E. pyramidum*, *E. coloratus*, *C. cerastes*, *B. arietans* and *N. h. arabica* that was required to produce 50% phospholipase A₂, phosphodiesterase, hyaluronidase, L-amino acid oxidase and proteolytic activity using L-BAPNA and dimethyl casein as substrates.

¹The maximum was 60% activity.

3.3.5 Proteolytic Activity

Proteolytic activity was assessed using dimethyl casein as substrate. Venom from *E. pyramidum* showed the highest activity while *N. h. arabica* venom had the lowest effect using this substrate (100 μ g produced 60% of maximum activity). Findings were similar when using L-BAPNA as substrate (Table 3.1).

3.4 BIOLOGICAL ASSESSMENT

All methods were performed as described in Chapter II.

3.4.1 Indirect Haemolysis

E. coloratus venom showed the highest indirect haemolytic activity with 25 μ g causing 100% haemolysis (Table 3.2). *E. pyramidum*, *N. h. arabica* and *A. microlepidota* venoms showed medium activity (50 μ g) whereas *C. cerastes* and *B. arietans* venoms were less active (100 μ g).

3.4.2 Coagulation

The minimum coagulant dose for human plasma (MCD-P) by venom from Saudi Arabian snakes of medical importance is given in Table 3.2. *E. coloratus* venom was more effective (3 μ g) than *E. pyramidum* venom (4 μ g). Both *Echis* species had negligible action on bovine fibrinogen. The venoms from *C. cerastes*, *B. arietans* and *N. h. arabica* had no detectable procoagulant activity using either human plasma or bovine fibrinogen and venom amounts up to 1mg.

3.4.3 Platelet Aggregation

Neither of the two Saudi Arabian elapid venoms (*N. h. arabica* and *W. aegyptia*) tested induced platelet aggregation when added to PRP. Addition of ADP showed complete aggregation which indicates that there were no inhibitors present in the two venoms. The

Table 3.2 Biological Activities of Saudi Arabian Venoms (Maximum Venom Activity in µg)

Species	Coagulation	Indirect haemolysis	Platelet aggregation	Haemorrhage	Necrotic	Defibrino -genation
<i>E. pyramidum</i>	4	50	-	8	20	7
<i>E. coloratus</i>	3	25	-	16	20	12
<i>C. cerastes</i>	NA	100	1	-	-	-
<i>B. arietans</i>	NA	*100	10	-	-	-
<i>N. h. arabica</i>	NA	50	-	-	-	-
<i>A. microlepidota</i>	-	50	-	-	-	-

Table 3.3 lists the biological activities recorded as the minimum amount of venom (in µg) from *E. pyramidum*, *E. coloratus*, *C. cerastes*, *B. arietans* and *N. h. arabica*, required to produce maximum coagulant, indirect haemolytic, platelet aggregation, haemorrhagic, necrotic and defibrinogenating activities.

NA: no venom activity up to 1mg,

-: not tested and

*: 30% activity only

viperid venoms tested initiated platelet aggregation, although to very different degrees (Figure 3.4). Just 1 μ g of *C. cerastes* venom was sufficient whereas it required a higher amount of *B. arietans* venom (10 μ g). It was not possible to study the effects of the venom from *Echis* species as they caused coagulation.

3.4.4 Neurotoxicity and Myotoxicity

The neurotoxic and myotoxic activities of *N. h. arabica* venom were studied using the mouse phrenic nerve hemidiaphragm preparation. The venom progressively depressed and finally abolished muscle contractions caused by indirect stimulation, while the response to direct stimulation was not affected as compared to the control. Venom at concentrations of 5.0 and 2.5mg/l produced complete inhibition in 20 ± 2 and 100 ± 8 minutes respectively, while a concentration of 1.25mg/l took 180 ± 14 minutes (Figure 3.5). To determine if this effect was dose dependent, the time taken to reach 80% paralysis was measured at three different concentrations (1.25, 2.5 and 5mg/l) and values of 35, 20 and 10 minutes respectively were found (Figure 3.6). The muscle did not recover after repeated washing every 30 minutes with Krebs' solution.

3.4.5 Haemorrhagic Activity

E. pyramidum venom showed higher haemorrhagic activity than *E. coloratus* venom (Figure 3.7).

3.4.6 Necrotic Activity

Both *E. pyramidum* and *E. coloratus* venoms showed similar necrotic activity producing a 5mm² lesion on the dorsal skin of rats (Figure 3.8).

Figure 3.4 Effect of *C.cerastes* Venom on Aggregation of Human Platelets.

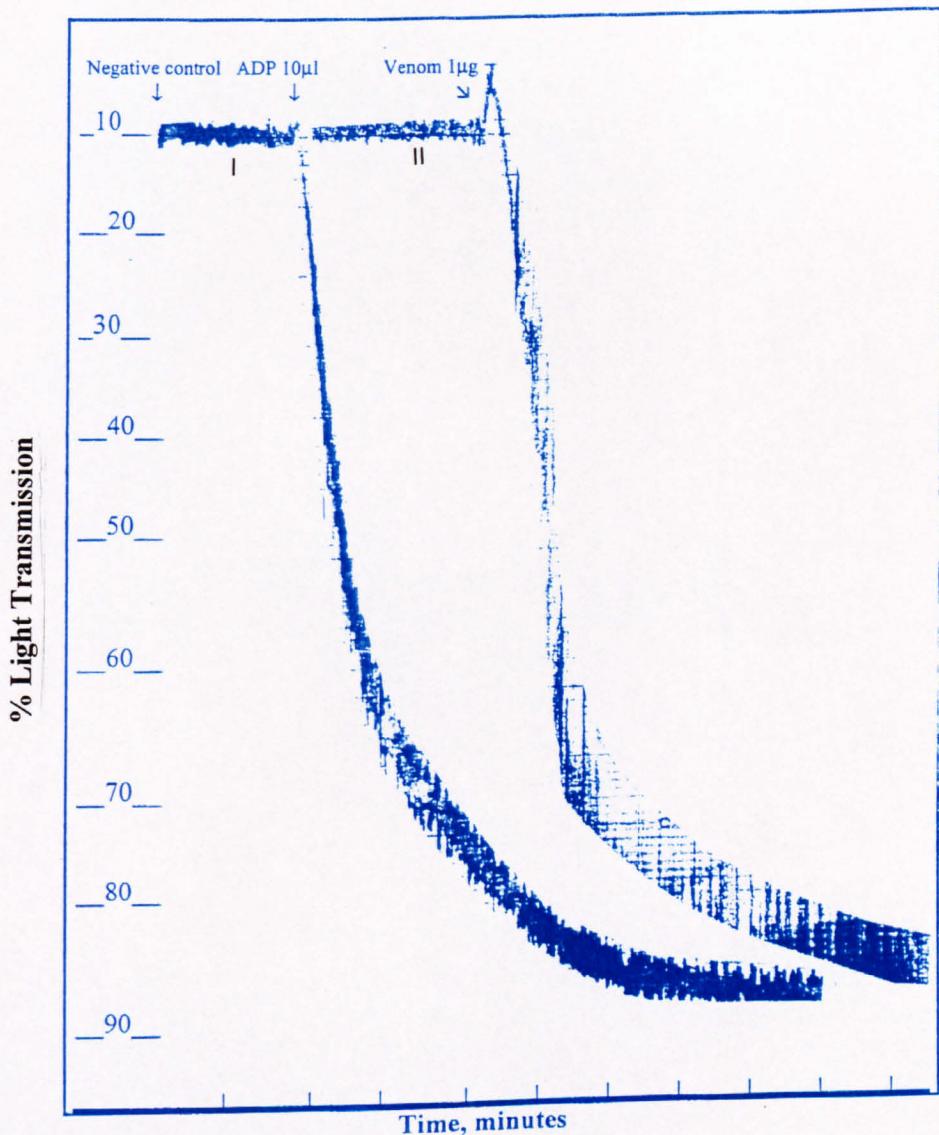
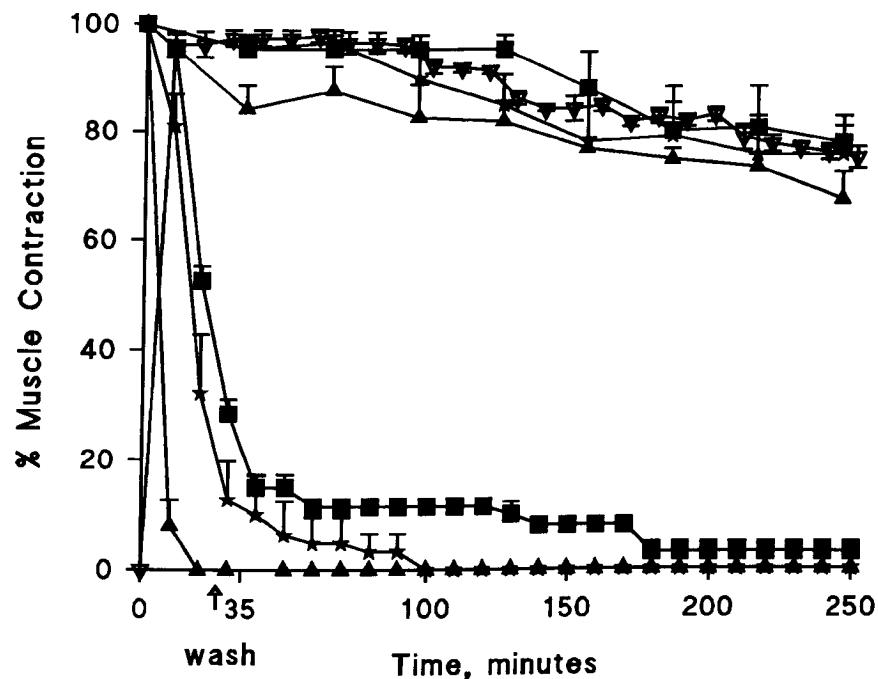


Figure 3.4 shows the aggregating effect of venom from *C. cerastes* on platelet-rich plasma (PRP). Initially base-lines were set using PRP (10% light transmission) and platelet-poor plasma (90% light transmission), using a chart speed of 2cm/min. The aggregometer measures light transmission at 600nm which increases as platelets aggregate. Platelet aggregation was initiated with ADP (I) or with 1 μ g *C. cerastes* venom (II).

Figure 3.5 Neurotoxicity and Myotoxicity of *N. h. arabica* Venom (Using The Mouse Phrenic Nerve Hemidiaphragm)



A mouse hemi-diaphragm preparation was incubated with 1.25 (■), 2.5 (★) or 5 (▲)mg/l of *N. h. arabica* venom for 30min. No change in muscle contraction recorded after direct (muscle) stimulation (solid lines), whereas a marked change in muscle contraction was recorded after indirect (nerve) stimulation (dashed lines). Results are expressed as the mean \pm SEM of four preparations. Preparation was washed every 30 min.

Figure 3.6 Dose Response Curve for Venom of *N. h. arabica* Using Indirect (Nerve) Stimulation

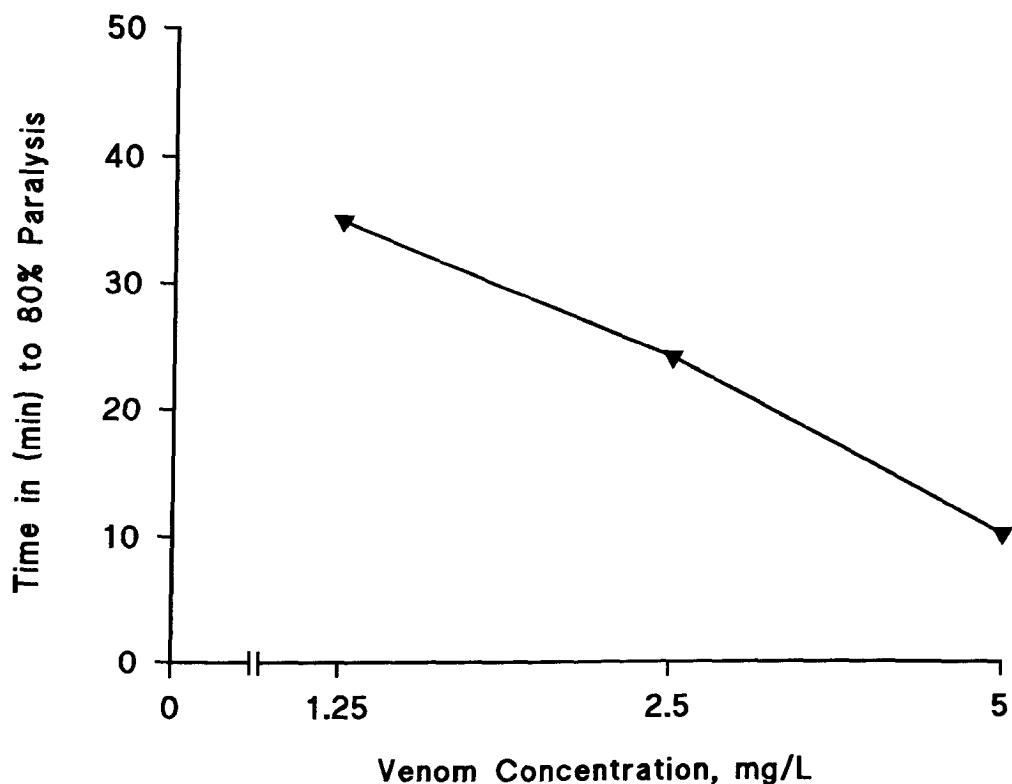


Figure 3.6 shows the dose dependent, neurotoxic activity of *N. h. arabica* venom. 1.25, 2.5 and 5mg/l caused 80% of total paralysis after 35, 25 and 10 minutes respectively.

Figure 3.7 Haemorrhagic Activity of *E.pyramidum*(a) and *E. coloratus*(b) Venoms

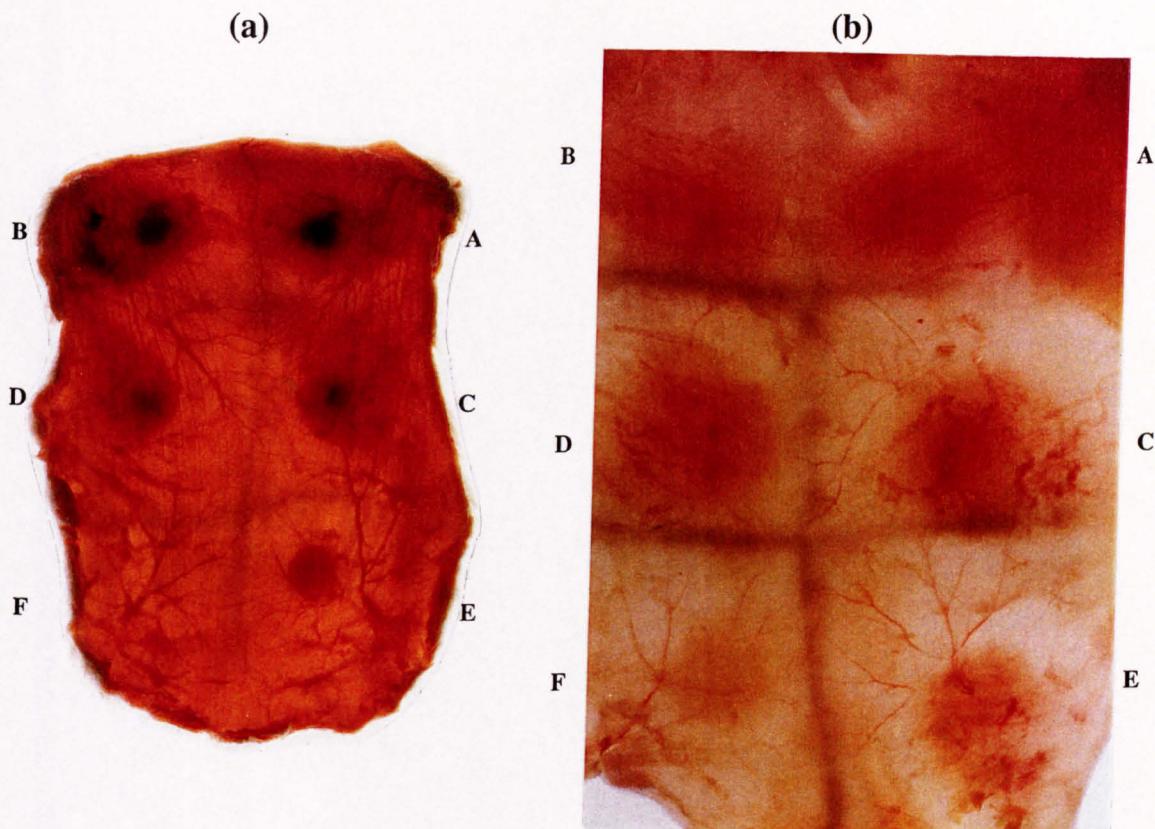


Figure 3.7 shows the haemorrhagic lesions produced when $40\mu\text{g}$ (A), $20\mu\text{g}$ (B), $10\mu\text{g}$ (C), $5\mu\text{g}$ (D), $2.5\mu\text{g}$ (E) and $1.25\mu\text{g}$ (F) of *E. pyramidum* (a) and *E. coloratus* (b) venoms were injected into the dorsal skin of rats. After 24 hours the animals were killed, the skin removed, and the diameter of the lesions measured on the inner surface of the skin, in two directions at right angles, using calipers and background illumination. The mean diameter of the haemorrhagic lesion was calculated for each venom dose and the MHD estimated by plotting the mean diameter against dose and reading off the dose corresponding to a 10mm lesion.

Figure 3.8

Necrotic Activity of *E.pyramidum* (a) and *E. coloratus* (b) Venoms

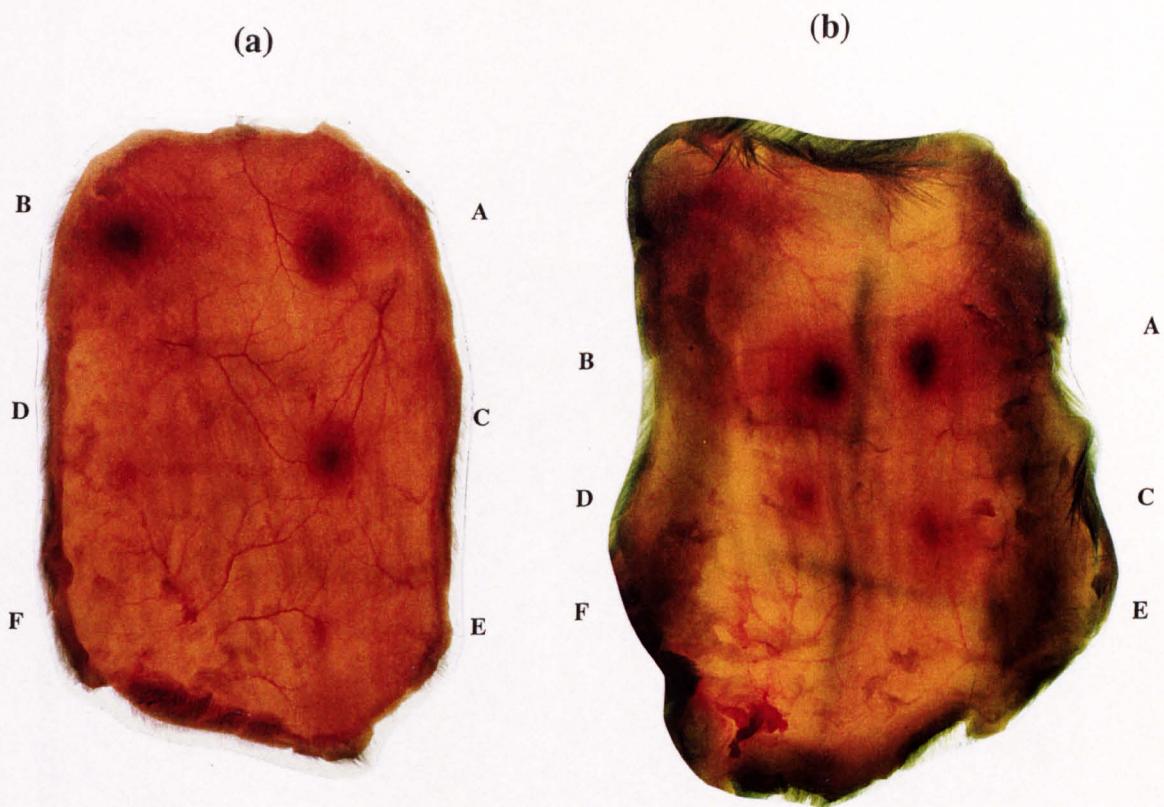


Figure 3.8 shows the necrotic lesions produced when different concentrations of *E. pyramidum* (a) and *E. coloratus* (b) venoms 40 μg (A), 20 μg (B), 10 μg (C), 5 μg (D), 2.5 μg (E) and 1.25 μg (F) were injected into the dorsal skin of rats. After three days the animals were killed, the dorsal skin removed, and the diameter of the lesions measured on the inner surface of the skin, in two directions at right angles, using calipers and background illumination. The mean diameter of the necrotic lesion was calculated for each venom dose and the MND estimated by plotting the mean lesion diameter against dose and reading off the dose corresponding to a 5mm lesion.

3.4.7 Defibrinogenating Activity

The minimum defibrinogenating dose (MDD) of venom from *Echis* species that produced non-clotting blood within 60 minutes of intravenous injection in mice are given in Table 3.2. *E. pyramidum* venom showed higher activity than *E. coloratus* venom (7 μ g and 12 μ g respectively).

3.4.8 Lethality

Very small amounts of two venoms were required for the intravenous LD₅₀ in mice; *A. microlepidota* (0.2 μ g/g) and *N. h. arabica* (0.3 μ g/g) (Table 3.3). Slightly more venom was required for *C. cerastes* (0.6 μ g/g) and about twice again for *B. arietans* (1.05 μ g/g), *E. coloratus* (1.1 μ g/g) and *E. pyramidum* (1.4 μ g/g) venom.

3.5 DISCUSSION

Of the Saudi Arabian venoms tested, those from *A. microlepidota* and *N. h. arabica* were the most lethal. *A. microlepidota* had a low milking yield and several of the snakes did not survive long in captivity. *N. h. arabica* venom was slightly less toxic but this snake may inject a large amount of venom, so that death may occur in a few minutes. The lethality of *E. coloratus* and *B. arietans* venoms agreed with published values (Theakston and Reid, 1983) and they were less toxic weight for weight than the two elapid venoms.

PLA₂ enzymes are major constituents of snake venoms. In this study all the venoms tested contained PLA₂ with *E. coloratus*, *E. pyramidum* and *N. h. arabica* venoms showing the highest activity. PLA₂ can cause disruption of lipid membranes *in vivo* and, *in vitro*, the lyssolecithin formed may cause further damage, such as lysis of erythrocytes. However, haemolysis does not occur *in vivo* as lyssolecithin is bound by albumin. As there were no direct haemolytic factors, indirect haemolytic activity should correspond with PLA₂ activity. Snake venom PLA₂ show a wide variety of toxicities and induce

Table 3.3 Lethal Toxicity (iv LD₅₀) of Venoms from Saudi Arabian Snakes

Species	LD ₅₀ (µg venom /g)	95% Confidence limit/ 20g
<i>Epyramidum</i>	1.4	24-33
<i>E. coloratus</i>	1.1	18-29
<i>C. cerastes</i>	0.6	8-17
<i>B. arietans</i>	1.05	14-26
<i>N. h. arabica</i>	0.3	3-9
<i>A. microlepidota</i>	0.2	3-5

Table 3.3 lists the lethal dose (ivLD₅₀) of venom from *E. pyramidum*, *E. coloratus*, *C. cerastes*, *B. arietans*, *N. h. arabica* and *A. microlepidota*. Mice received increasing doses of venom dissolved in saline (total volume 200µl) via the tail vein. Five groups of five male TFW mice (18-20g) were used and the survivors in each group were counted after 24hr. The doses were adjusted so the highest dose gave 100% mortality while all the mice survived at the lowest dose. The analysis of the slope of the dose-response regressions for the LD₅₀ was performed by the probit method.

several pathological symptoms (Kini and Evans, 1989). Most venoms contain different PLA₂ and the basic PLA₂ are thought to cause more biological damage such as myonecrosis and increased capillary permeability as compared to their acidic counterparts (Rosenberg, 1979).

Hyaluronidases or "spreading factors" specifically depolymerise hyaluronic acid and, thereby, dissolve the gel surrounding normal cells and facilitate the diffusion of other venom components through the tissues (Boquet *et al.*, 1968). Their action is also associated with the oedema and swelling that may be observed at the bite site. Elapids generally exhibit low hyaluronidase activity but this was not the situation in the present study in which *N. h. arabica* venom showed considerable activity. Most viperid species exhibit moderate to high hyaluronidase activity (Tan and Ponndurai, 1990) and, in the present study, *E. pyramidum* venom showed the lowest hyaluronidase activity. As might be expected, oedema is the most common clinical symptom following moderate or severe envenoming by this snake.

L-AAO is generally thought to be of low toxicity but may contribute to the local tissue damage caused by venoms. Characteristic of L-AAO is that it colours venom yellow, which gradually disappears from lyophilized venom. Some have speculated that this loss of colour is due to a reversible limited conformational change, whereas Ueda (1988) reported that the L-AAO isolated from *Trimeresurus mucrosquamatus* venom was irreversibly inactivated by lyophilisation and freezing. *E. pyramidum* and *E. coloratus* venoms were both deeply yellow and also had the highest L-AAO activity. Yan and colleagues (1994) reported that L-AAO also induces platelet aggregation.

Snake venoms, especially those from Viperidae, contain many proteolytic enzymes (Kocholaty *et al.*, 1971; Seegers and Ouyang, 1979) causing coagulation, haemorrhage, necrosis and fibrinogenolysis (Tu *et al.*, 1969). Venom proteases have different enzyme

activities on different substrates. Most of the venoms tested in the present study hydrolysed dimethyl casein and L-BAPNA.

Coagulation disorders and haemorrhage are among the most prominent features of bites of vipers (Jiménez-Porras, 1970). Many snake venoms contain factors which either promote or inhibit blood coagulation. Thus blood coagulation can be affected by venom proteins which are enzymes (proteases or PLA₂) and, in some cases, by non-enzymatic proteins (Ouyang and Teng, 1972; Teng and Seegers, 1981). Venom PLA₂s act as anticoagulants by inactivating the procoagulant activity of the phospholipids which provide an activating surface for blood clotting factors (Ouyang *et al.*, 1978), while proteases cause cleavage of clotting factors which leads to acceleration or retardation of coagulation. The venom of all species of *Echis* causes hemostatic failure through the combined effect of its procoagulant, fibrinolytic, antiplatelet and haemorrhagic components (Cheymol *et al.*, 1973). In the present study both Saudi Arabian *Echis* species produced considerable coagulant activity. *Echis* species venoms contain a protein that initiates intravascular coagulation by direct activation of prothrombin (Weiss *et al.*, 1973; Kornalik and Blombak, 1975). In this study there was no coagulant activity by Saudi Arabian *C. cerastes* venom, however Abdulla and associates (1992) reported that this venom showed activity at low concentrations and had anticoagulant effects at higher concentrations. Labib and his colleagues (1981) also found that venom from *C. cerastes* snakes disturb the coagulation mechanism.

Clinical effects of puff-adder venom on whole blood, plasma and serum include a decreased platelet count (Takahashi and Tu, 1970) and prolonged prothrombin time (Philips *et al.*, 1973). *B. arietans* venom has been reported to have either coagulant or anticoagulant properties (Visser and Chapman, 1978) while, in this study, the venom did not show any coagulant activity.

Snake venoms affect platelets in various ways. For example, some venoms induce while others inhibit platelet aggregation and both these activities may exist in the same venom. During such aggregation, nucleotides, serotonin and other platelet factors or constituents may be released. Two of the mechanisms by which a venom initiates platelet aggregation are (1) to affect directly the platelet membrane or (2) to affect the platelets indirectly through the release of platelet constituents (especially ADP) (Seegers and Ouyang, 1979). Both *C. cerastes* and *B. arietans* venoms showed a dose-dependent initiation of platelet aggregation. Two fractions isolated from *B. arietans* venom (bitistatin and arietin) inhibited platelet aggregation through interference with fibrinogen binding to fibrinogen receptors on platelet surfaces (Shebuski *et al.*, 1989; Huang *et al.*, 1991). Farid and his colleagues (1990) isolated cerastobin (a thrombin-like enzyme) from *C. cerastes* venom that initiated platelet aggregation. Shebuski and associates (1990) reported that echistatin, a fraction isolated from *E. pyramidum* venom, is an effective antithrombotic agent which inhibits both platelet aggregation *in vivo* in the coronary artery as well as *ex vivo* with a concomitant increase in the bleeding time. Huang and colleagues (1991) isolated a fraction from *Agkistrodon halys* venom (halysin) which has a similar inhibitory effect as echistatin on platelets.

Enhancement of platelet aggregation can be replicated by L-AAO or peroxide and inhibited by catalase and it has been suggested that venom impairs platelet aggregation by generating hydrogen peroxide (Nathan *et al.*, 1982).

General haemorrhage is one of the characteristic symptoms associated with envenomation by snakes from the family Viperidae and especially *Echis* species, as shown in the present study. This has been ascribed to the presence of metalloproteinases known as haemorrhagins. Haemorrhage associated with intravascular coagulation is also a prominent feature of *E. coloratus* envenomation (Rechnic *et al.*, 1962).

All the *Echis* venoms possessed the ability to cause local necrosis when introduced intradermally. The MDD of both *Echis* species were less than their LD₅₀ and the value agreed with those of Theakston and Reid (1983). Certain snake venoms with direct prothrombin converting enzymes can cause defibrination *in vivo* and include those derived from *E. coloratus* (Gitter *et al.*, 1960) and *E. pyramidum* (Kornalik and Pudlak, 1971).

The decrease in muscle contraction observed with *N. h. arabica* venom, using the mouse hemi-diaphragm preparation, could either be due to venom component(s) blocking nerve conduction or direct effects on the muscle. The fact that direct stimulation was not affected, indicated the presence of a component that affects the nerve. Neurotoxic affects have been described for cobra venoms, while the neurotoxic properties of *N. h. arabica* venom are less well established.

CHAPTER IV

ASSESSMENT OF OVINE ANTIVENOMS FOR TREATING ENVENOMATION BY SAUDI ARABIAN VENOMOUS SNAKES

4.1 SUMMARY

Antivenoms were raised in individual groups of sheep against venom from *E. pyramidum*, *E. coloratus*, *C. cerastes*, *B. arietans* and *N. h. arabica*. To optimise the antibody response, groups of three sheep were immunised with increasing venom amounts and the monthly serum samples were assessed by ELISA and small scale affinity purification. After doubling the immunisation dose, there was a marked increase in ELISA titres and specific antibody concentrations. When the humoral response had been optimised, IgG fractions were prepared and digested with papain to form Fab fragments. Purity of the ovine IgG and its Fab fragment was at least 95%. Western blotting showed that antibody populations bound most of the venom components.

The Fab antivenoms neutralised the enzymatic and biological effects and protected mice against the lethal effects of the above venoms. ED₅₀ values were in the order: *E. pyramidum* (11g/g) < *E. coloratus* (13g/g) < *B. arietans* (30g/g) < *C. cerastes* (60g/g) < *N. h. arabica* (143g Fab/g venom). Various methods were used to assess cross-neutralisation of closely related venoms and the highest cross-reactivity was obtained with *E. pyramidum* and *E. coloratus* venoms.

A. microlepidota was not included in this study because of low milking yield of venom.

4.2 PRODUCTION OF OVINE ANTIVENOMS

4.2.1 Immunisation and Bleeding

Groups of three sheep were immunised with different doses of each venom. The venom was dissolved in saline (1.3ml/sheep), sterile filtered, and mixed with Brij 35 (0.2ml/sheep) and adjuvant (3.25ml/sheep) to form a stable water-in-oil emulsion. Freund's complete adjuvant was used for the primary immunisation, and Freund's incomplete adjuvant for all subsequent re-immunisations. Half-Breed Welsh ewes were

immunised at six sites: intramuscularly in the forelimbs and hindlimbs and in two subcutaneous sites on either side of the neck. The sheep were immunised at monthly intervals and aseptically bled (500ml) or sampled (5ml) via the jugular vein two weeks after each immunisation. The blood samples were rolled slowly for 2hr at room temperature to enhance clotting, centrifuged at 4500g and the serum aspirated and stored frozen until used.

4.2.2 Optimisation of Immunisation Dose

For primary immunisation, groups of sheep were injected with 0.5mg of *C. cerastes*, *B. arietans* or *N. h. arabica* venom. Three sheep in each group (the low dose group) were continued at 0.5mg of venom monthly while the dose was doubled in the remainder every month until a maximum of 2 or 4mg was being injected in the medium and high dose group respectively (Table 4.1). Serum samples from individual sheep were assessed by ELISA and small scale affinity chromatography as described in Chapter II. The data for sheep immunised with *E. pyramidum* and *E. coloratus* venoms are described in Chapter V.

4.2.2.1 Enzyme-linked Immunosorbent Assay

The antibody levels rose rapidly and by 22 weeks after the primary immunisation, most sheep had achieved a maximum response (Figure 4.1). The three sheep immunised with only the low dose of *N. h. arabica* venom (0.5mg) achieved relatively the lowest titres (average 0.8×10^6) and increasing the dose to 2mg doubled the response, with average ELISA titres of 1.5×10^6 for sheep S5 and S6, although sheep S4 showed a lower response. The maximum antibody response for *N. h. arabica* venom was achieved using the high venom dose (4mg), with titres averaging 2.4×10^6 throughout the initial 70 week study period.

Table 4. 1 Immunisation and Sampling Schedule

Immunogen Dose (mg)				
Week No.	High dose (sheep 1-3)	Medium dose (sheep 4-6)	Low dose (sheep 7-9)	Sample/Bleed
0	0.5	0.5	0.5	
4	1	1	0.5	
6				Sample
8	2	2	0.5	
10				Sample
12	4	2	0.5	
14				Sample
16	4	2	0.5	
18				Sample
20	4	2	0.5	
From 70 weeks				
72	4	4	1	
74				Bleed
76	4	4	2	
78				Bleed
80	4	4	4	

Groups of three sheep were immunised with different doses of each venom. For primary immunisation, sheep were injected with 0.5mg of *C. cerastes*, *B. arietans* or *N. h. arabica* venom in Freund's complete adjuvant; all further immunisations were in Freund's incomplete adjuvant. The low dose groups continued to receive 0.5mg of venom monthly while the dose was doubled in the remaining sheep every month to a maximum of 2 and 4mg for the medium and high dose groups respectively. At week 72 immunising dose for sheep in the low and medium dose groups were doubled each month to a maximum of 4mg.

Figure 4.1 Monthly ELISA Antibody Titres for Serum Samples from Sheep Immunised with Saudi Arabian Venoms

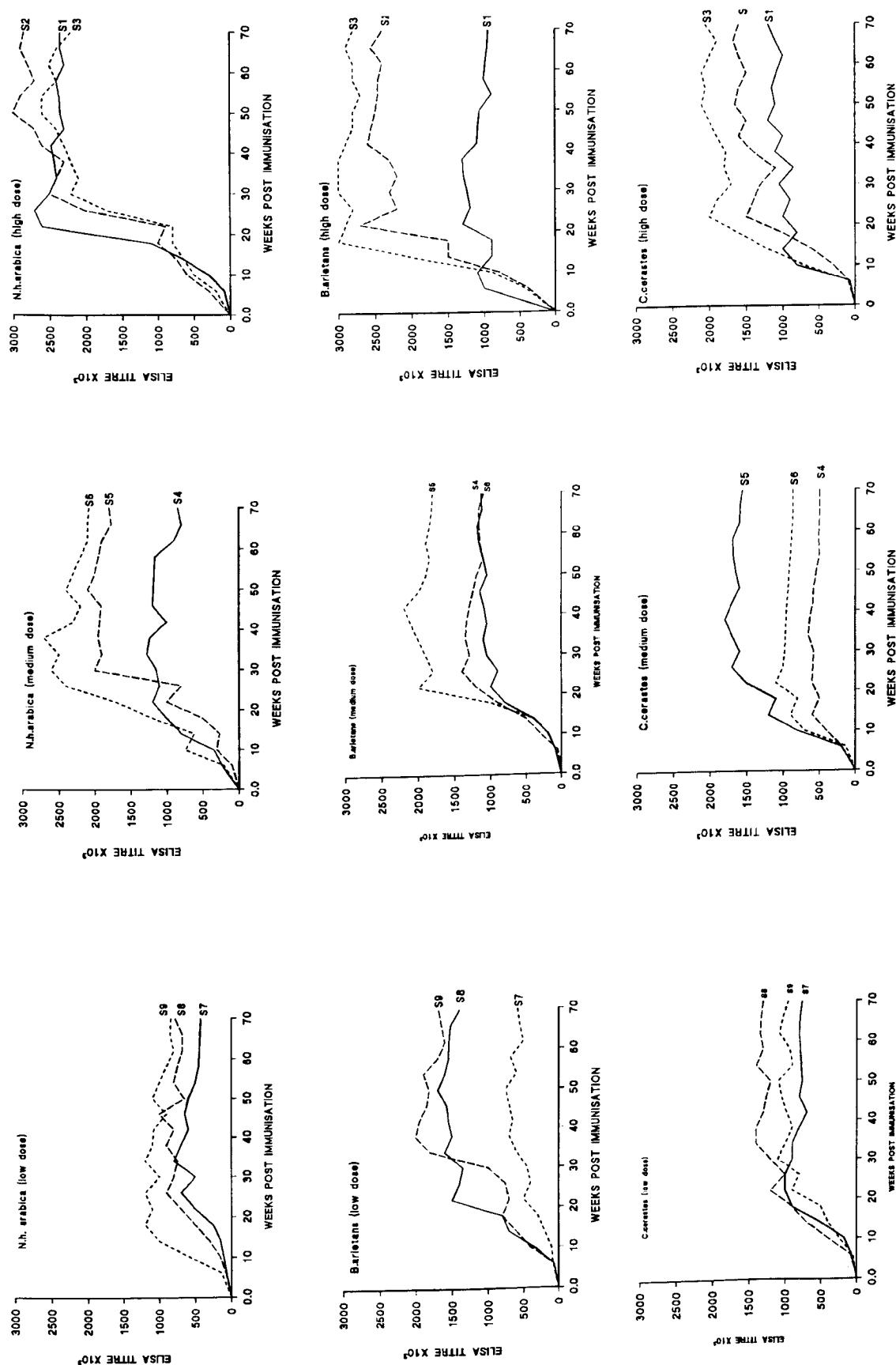


Figure 4.1 shows the monthly antibody titres produced by groups of three sheep immunised with 0.5, 2 or 4mg of venom from *N. h. arabica*, *B. arietans* and *C. cerasodes*. The sheep were bled two weeks after each immunisation and their sera assessed by ELISA. Each point represents the mean for three sheep.

The dose responses against *B. arietans* and *C. cerastes* venoms were, in general, similar to the response to *N. h. arabica* venom. As the dose was doubled titres also increased and the highest response was found using 4mg venom. ELISA titres in the highest dose group immunised with *N. h. arabica* and *B. arietans* venoms were higher than those from sheep immunised with *C. cerastes* venom (Figure 4.2).

After 70 weeks the immunisation dose was increased to 4mg for all the sheep including those in the low and medium dose groups and the antisera were then pooled for each group of nine sheep. There was no further marked increase in ELISA titres with their average values being in the order $N. h. arabica$ 1.6×10^6 = $B. arietans$ $1.6 \times 10^6 > C. cerastes$ 1.3×10^6 .

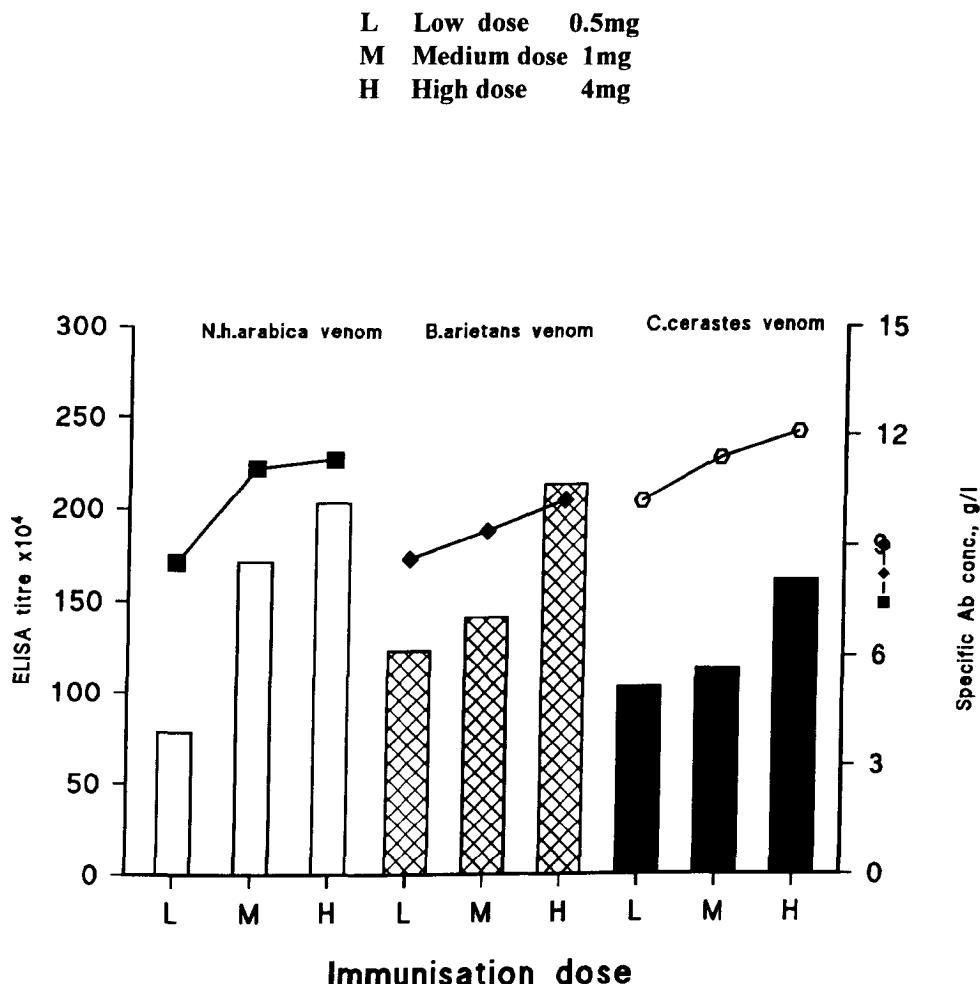
4.2.2.2 Small Scale Affinity Chromatography

N. h. arabica, *B. arietans* and *C. cerastes* venoms coupled to Sepharose 4B were incubated with a serum sample (0.5ml) and the specific antibody concentration determined as described in Chapter II.

Specific immunoglobulin values in the antivenoms raised against *N. h. arabica* venom ranged from 6 to 16g/l throughout the initial 70 weeks of study with the concentrations in the groups receiving the two largest doses being higher than in those receiving the lowest venom dose (Figure 4.3). This is in accord with the ELISA findings. The specific antibody concentrations in the three groups immunised with *B. arietans* venom showed similar levels ranging from about 6 to 11g/l.

The specific immunoglobulin concentrations in the sera of sheep immunised with *C. cerastes* venom were slightly higher and ranged from 9 to 14g/l. Individual sheep showed some variance but the responses assessed using specific antibody levels were more homogeneous than that measured by ELISA. In general, the specific antibody concentrations increased with dose of immunogen (Figure 4.2) but levels were similar in the sheep immunised with medium and high doses of *N. h. arabica* venom.

Figure 4.2 Titres and Specific Antibody Concentrations of Antivenom Produced by Sheep Immunised with Low, Medium and High Dose of Venom



Antibody titres (assessed by ELISA) and specific antibody concentrations (assessed by small scale affinity chromatography) of antiserum obtained from sheep immunised with low, medium and high doses of *B. arietans*, *C. cerastes* and *N. h. arabica* venoms were compared. In all cases both titres and specific antibody concentrations increased with dose. Values are expressed as the mean of three sheep.

Figure 4.3 Monthly Specific Antiserum Concentrations for Serum Samples from Sheep Immunised with Saudi Arabian Venoms

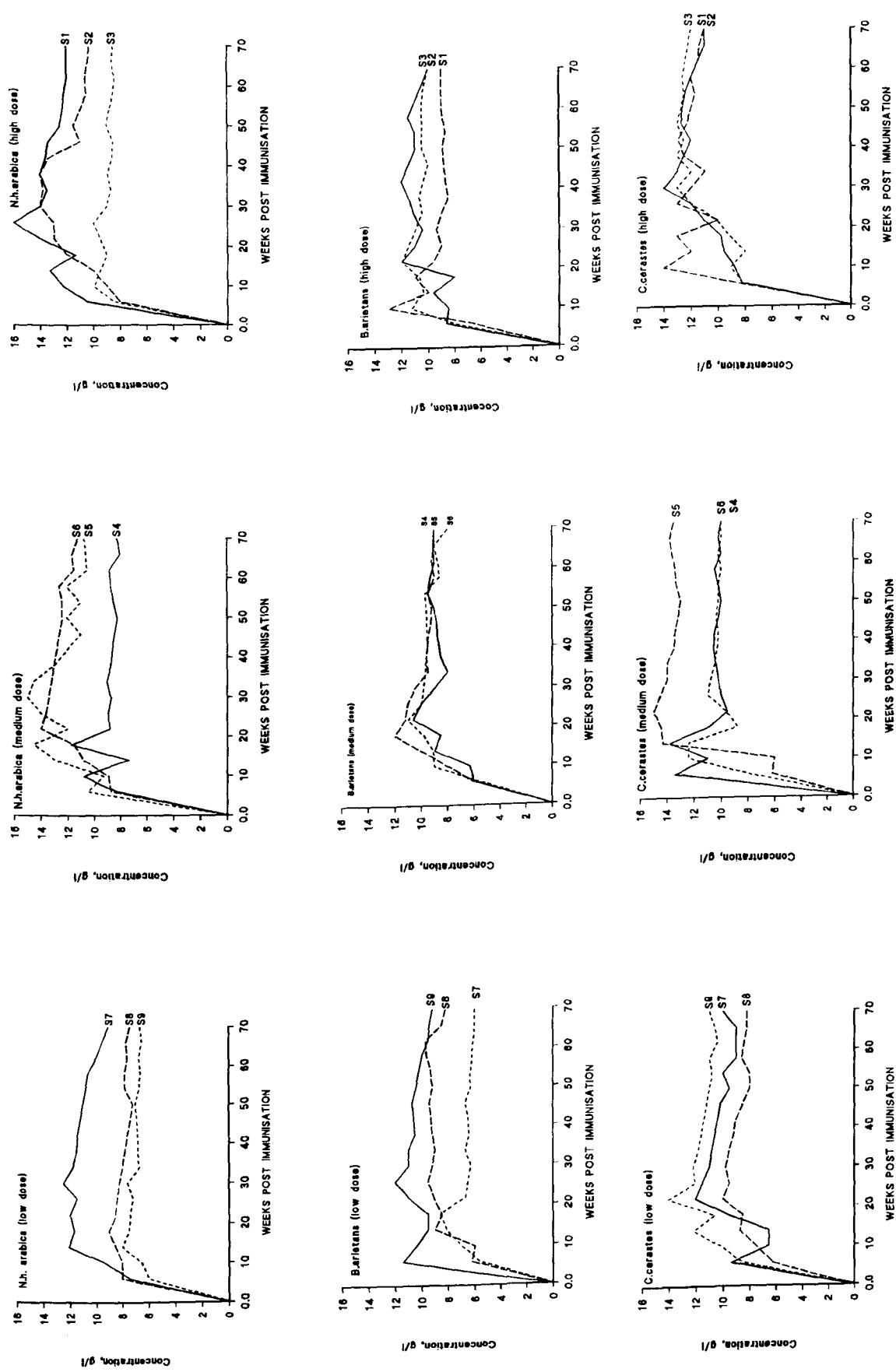


Figure 4.3 shows the results obtained from the small scale affinity chromatography of serum from groups of three sheep receiving monthly immunisation with 0.5, 2 or 4mg of venom from *N. h. arabica*, *B. arietans* and *C. cerastes*. The sheep were bled two weeks after each immunisation and each point represents the mean for three sheep.

4.3 PREPARATION OF IMMUNOGLOBULIN AND Fab FRACTIONS

Bleeds taken 32 weeks after primary immunisation from groups of three sheep immunised with *E. pyramidum* or *E. coloratus* venoms were pooled, filtered and the immunoglobulin fraction purified by salt precipitation as described in Chapter II. The total protein concentration of the serum pools varied from 59 to 65g/l and, of this, about 40% was removed in the supernatant and about 60% remained in the immunoglobulin fraction (Table 4.2). SDS-PAGE showed that salt precipitation removed most of the albumin, while most of the immunoglobulins remained in the precipitated fraction (Figure 4.4) and losses of IgG were negligible. Two fractions were obtained when the IgG fraction was run using FPLC (Figure 4.5). The first peak comprising only 6% of the applied protein, had a retention time of 16min and a MWt of about 747kDa. This fraction is probably aggregated IgG. The second peak had a MWt of 143kDa, a retention time of 23min and represented 94% of applied protein. ELISA titres of the serum and immunoglobulin fractions were similar.

Immunoglobulin fractions (500ml) at concentrations of 35 and 38g/l, were then cleaved with papain (final concentration 16% w/w of the total IgG) at rt (Table 4.2). To optimise the time of digestion, the immunoglobulin fraction was mixed with papain and aliquots were removed at regular intervals and analysed by immunoelectrophoresis as described in Chapter II. After one hour of digestion, Fc and Fab bands had appeared as precipitating arcs together with intact IgG (Figure 4.6). The Fc concentration increased to a maximum level after 5hr and then, with further digestion, the Fc precipitate arc disappeared. All IgG had disappeared completely after 47 hr when only Fab was present.

The yield of Fab varied from 78 to 83% (Table 4.2) and SDS-PAGE showed that the immunoglobulins, and many other components present in smaller quantities in the IgG fraction, had disappeared after papain digestion leaving only one dominant band (Figure 4.4). Two fractions were obtained when subjecting the Fab fraction to FPLC but the first was an extremely small peak with a retention time of 24.7mins. It had a MWt of about 89kDa and this fraction may be dimeric Fab. The second peak had a MWt of 45kDa, a retention time of 27.4min and represented 98% of the total (Figure 4.5).

Table 4. 2 Preparation of Immunoglobulin and Fab Fractions for Two Antivenoms

IgG PRECIPITATION

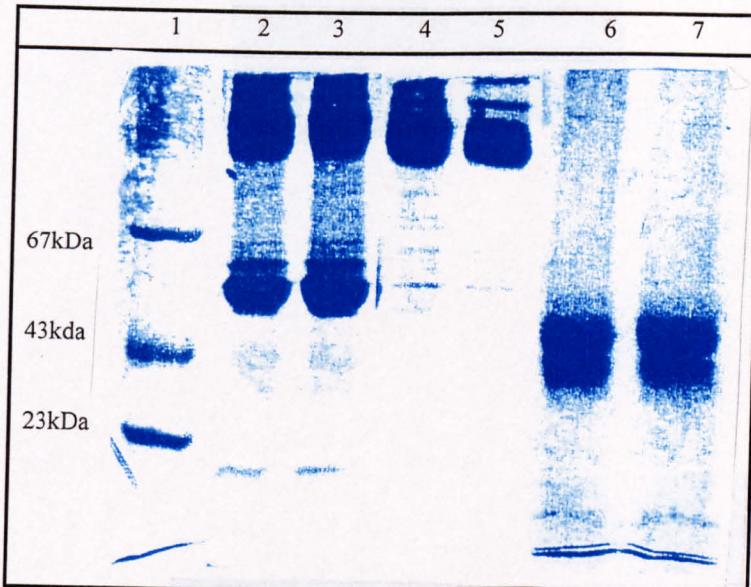
		<i>E. pyramidum</i> antivenom	<i>E. coloratus</i> antivenom
Serum pool	Volume, ml	500	500
	Protein, g/l	59	62
	Specific antibody, g/l	13	14
	ELISA antibody titre	875,000	895,000
IgG fraction	Volume, ml	580	550
	Protein, g/l	35	38
	Specific antibody, g/l	11	12
	ELISA antibody titre	750,000	820,000
	Albumin, mg/l	1.2	5

PAPAIN DIGESTION:

	Volume, ml	630	580
Fab fraction	Protein, g/l	22	24
	Specific antibody, g/l	7	8
	ELISA antibody titre	128,000	124,000
	Albumin, mg/l	0.008	0.008

E. pyramidum and *E. coloratus* antivenoms were subjected to salt fractionation and papain digestion. At each stage of processing total protein concentrations (g/l), specific antibody concentrations (g/l), ELISA titres and albumin concentrations were estimated, the latter by fluoroimmunoassay.

Figure 4.4 SDS-PAGE of Papain Digestion

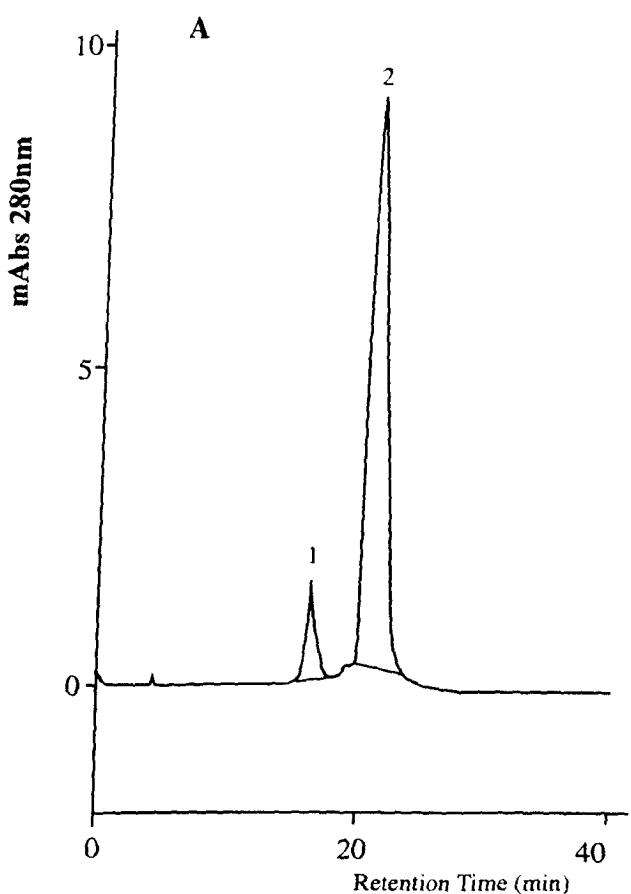


Antisera raised against the venoms from *E. pyramidum* and *E. coloratus* were resolved using 10%, non reducing SDS-PAGE. Serum (lanes 1 and 2) showed two major bands corresponding to IgG and albumin, whereas after salt precipitation only the IgG band remained visable (lanes 4 and 5). After papain digestion one major protein was present (lanes 6 and 7).

Figure 4.5 FPLC of Ovine IgG and Fab Antivenom

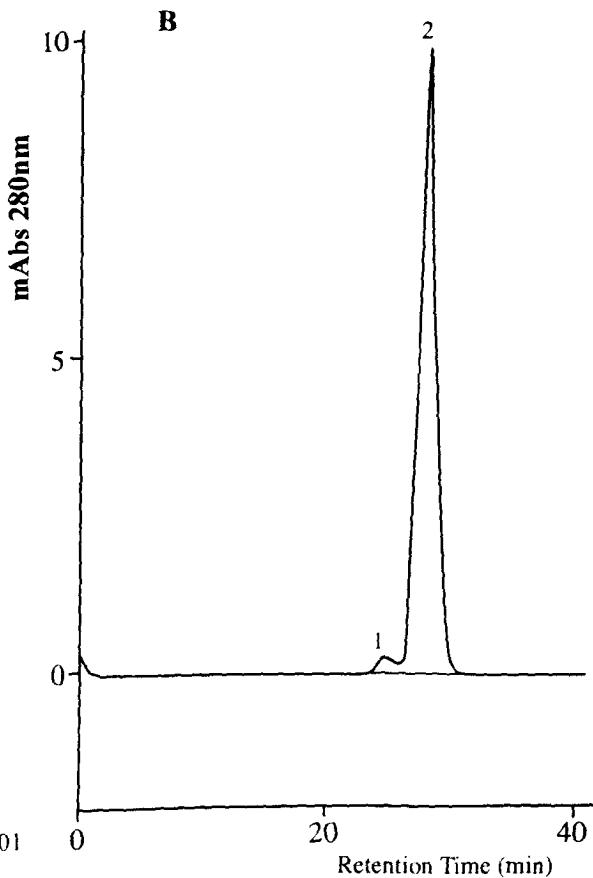
(A) IgG

Peak no.	MWt kDa	% of total protein	retention time (min)
1	747	6	16.5
2	143	94	22.8



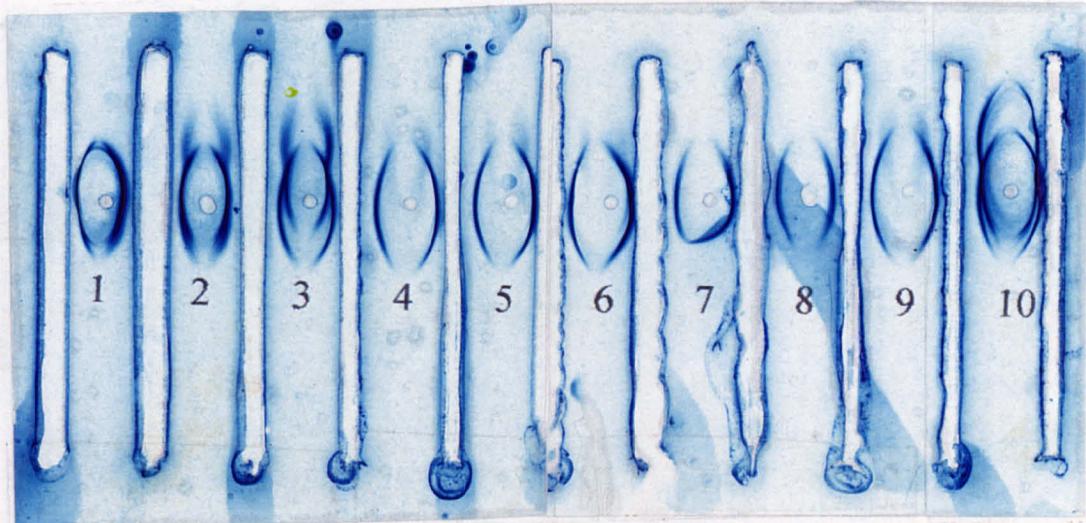
(B) Fab

Peak no.	MWt kDa	% of total protein	retention time (min)
1	89	2	24.7
2	45	98	27.4



200µg of ovine IgG (A) and Fab (B) antivenom raised against venom from *E. pyramidum* were analysed by FPLC on Superose 12 HR column. Proteins were eluted with 154 mM NaCl at pH 5.5.

Figure 4.6 Immunelectrophoresis of Samples from Papain Digestion of Antiserum



Samples taken during the papain digestion of antivenom were assessed by immuno-electrophoresis. After one hour of digestion, Fc and Fab bands appeared as precipitating arcs together with intact IgG. The Fc concentration increased to a maximum level after 5hrs and then disappeared with further digestion. All IgG had disappeared completely after 21hrs. {Lane 1 before digestion, lane 2 after 1hr of digestion, lane 3 after 5hr of digestion, lane 4 after 21hr of digestion, lane 5 after 23hr of digestion, lane 6 after 29hr digestion, lane 7 after 47hr digestion, lane 8 after 91hr digestion, lane 9 after 24hr digestion and 48hr of dialysis, lane 10 Reference (IgG +Fab + Fc)}.

Albumin concentrations were measured by a specific fluoroimmunoassay as described in Chapter II. The two immunoglobulin precipitates had albumin concentrations of 1.2 and 5mg/l respectively which were reduced after digestion with papain to values of only 0.008 mg/l.

The ELISA antibody titre of the Fab fractions appeared to fall markedly compared to the IgG fractions with apparent losses of 81% to 89% (Table 4.2). However, this is an artifact since detection of the antibodies and antibody fragments bound to venom, depends, in the assay used, on the binding of a second antiserum raised against intact ovine IgG. Clearly this will contain many more antibody populations directed against intact IgG (with its retained Fc component) than against Fab and this largely explains the lower apparent titres.

4.4 NEUTRALISATION OF ENZYMATIC ACTIVITIES

Neutralisation of enzymatic activities was assessed as described in Chapter II and the result expressed as the amount of antivenom (g) required per g venom to produce maximum neutralisation.

The specific Fab antivenoms completely neutralised the PLA₂ activity of their five respective venoms (Figure 4.7) with the *C. cerastes* and *B. arietans* antivenoms being the most effective (Table 4.3). The Fab fraction of all five ovine antivenoms also completely neutralised the phosphodiesterase activity of all venoms and again the *C. cerastes* and *B. arietans* antivenoms were the best. The Fab fractions were particularly effective in completely neutralising the hyaluronidase activity of venoms from the five Saudi Arabian venomous snakes, requiring only from 10 to 20g per g of venom and the *C. cerastes* and *B. arietans* antivenoms were, in this instance, no better than the other three products.

Figure 4.7 Neutralisation of Phospholipase A₂ Activity Using Ovine Fab Antivenom

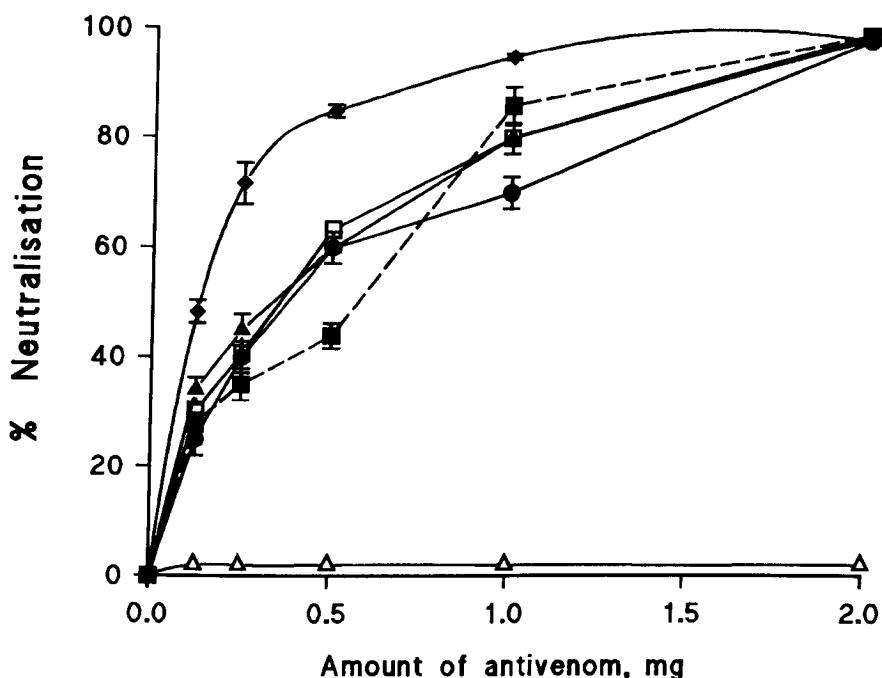


Figure 4.7 shows the efficacy of monospecific ovine Fab in protecting against the phospholipase A₂ activity of venoms from *E. pyramidum* (■), *E. coloratus* (▲), *N. h. arabica* (●), *C. cerastes* (◆) and *B. arietans* (□). The % neutralisation was measured using the substrate phosphatidylcholine and is expressed as the increase in OD at 400nm. Normal sheep serum (▽).

Table 4.3 The Efficacy of Monospecific Ovine Fab in the Inhibition of Venom Enzymatic Activities.

Antivenom raised against venom from	Phospholipase A ₂	Phosphodiesterase	Hyaluronidase
<i>E. pyramidum</i>	500	320	10
<i>E. coloratus</i>	1000	320	13
<i>C. cerastes</i>	125	80	20
<i>B. arietans</i>	125	160	13
<i>N. h. arabica</i>	500	320	20

The efficacy of monospecific ovine Fab fractions in inhibition the phospholipase A₂, phosphodiesterase and hyaluronidase activities of *E. pyramidum*, *E. coloratus*, *C. cerastes*, *B. arietans* and *N. h. arabica* venoms was assessed. Values are expressed in terms of g antivenom/ g venom.

4.5 NEUTRALISATION OF BIOLOGICAL ACTIVITIES

Neutralisation of biological activities was measured as described in Chapter II.

4.5.1 Indirect Haemolytic Activity

The Fab fraction completely neutralised the indirect haemolytic activity of *E. pyramidum*, *E. coloratus* and *C. cerastes* venoms with the antivenom directed against the latter being the most effective (Table 4.4). However, the monospecific antivenom raised against *N. h. arabica* venom neutralised only 90% of the indirect haemolytic activity. The IgG fraction from normal sheep serum showed no inhibition of indirect haemolytic activity.

4.5.2 Coagulant Activity

Ovine Fab raised against *E. pyramidum* and *E. coloratus* venom provided excellent protection against the coagulant activity of the respective venoms using 125 and 167 μ g antivenom respectively (Table 4.4).

4.5.3 Platelet Aggregation

The highest amount of the antivenom raised against *C. cerastes* venom (250 μ g) completely inhibited initiation of platelet aggregation by the venom (Figure 4.8). However, lower amounts (125 and 62 μ g) showed inhibition of the ADP induced initiation. A small amount (50 μ g) of the antivenom raised against *B. arietans* venom also completely inhibited platelet initiation whereas the IgG fraction obtained from normal sheep serum showed no effect.

4.5.4 Haemorrhagic Activity

The ovine antivenoms raised against *E. pyramidum* and *E. coloratus* venoms both provided excellent protection against the haemorrhagic lesion induced by the two venoms (Figure 4.9). However the minimum neutralising dose of specific ovine Fab against *E. coloratus* venom (8g/g) was lower than for the *E. pyramidum* antivenom (16g/g).

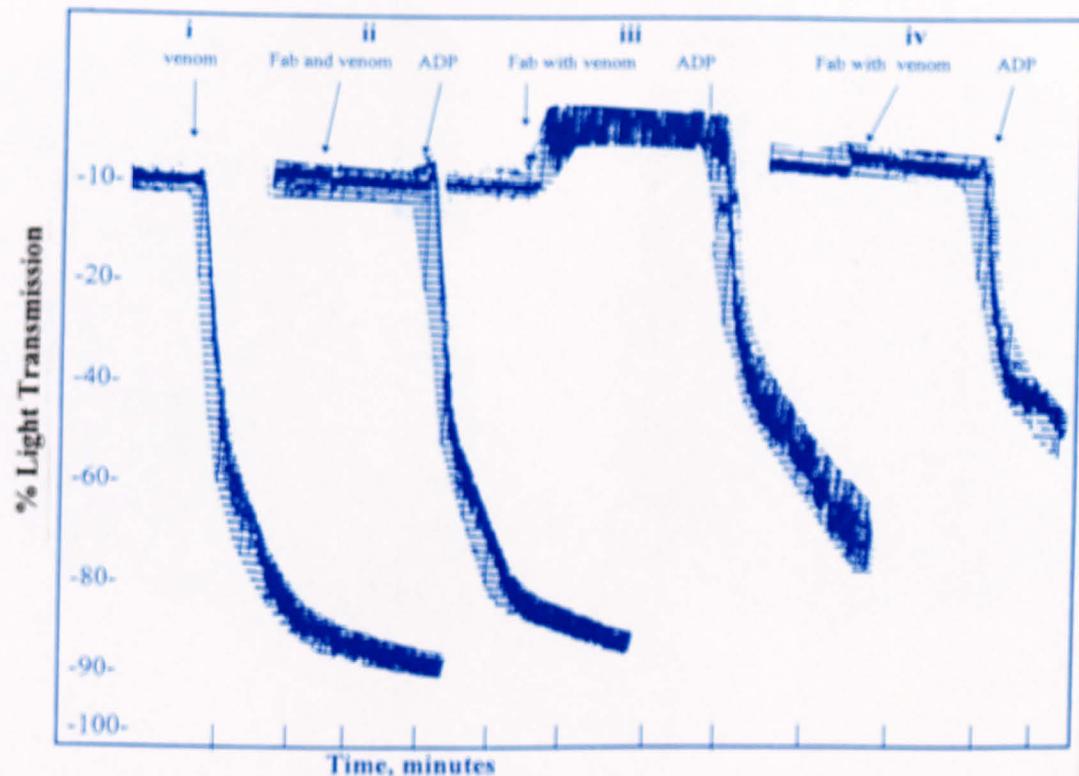
Table 4. 4 The Efficacy of Monospecific Ovine Fab Fractions in the Inhibition of Venom Biological Activities

Antivenom raised against:	Indirect haemolysis	Coagulation	Initiation of platelet aggregation	Haemorrhage	Necrosis	Defibrinogenation
<i>E. pyramidum</i>	100	125	-	16	3	143
<i>E. coloratus</i>	200	167	-	8	6	83
<i>C. cerastes</i>	50	NA	250	-	-	-
<i>B. arietans</i>	NA	NA	50	-	-	-
<i>N. h. arabica</i>	100*	NA	NA	-	-	-

The efficacy of monospecific ovine Fab fractions in inhibiting coagulant, indirect haemolytic, platelet aggregating, haemorrhagic, necrotic and defibrinogenating activities of venom from *E. pyramidum*, *E. coloratus*, *C. cerastes*, *B. arietans* and *N. h. arabica*. Values are expressed in terms of g antivenom/g venom.

-: Not tested, *: Maximum inhibition 90% and NA: No venom activity

Figure 4.8 Neutralising Efficacy of Ovine Fab Against Initiation of Platelet Aggregation by *C.cerastes* Venom



1 μ g *C.cerastes* venom (i) was premixed with 250 μ g (ii) 125 μ g (iii) or 62 μ g (iv) ovine Fab followed by ADP and the degree of inhibition of platelet aggregation measured, using platelet-rich plasma (PRP). An aggregometer was used to measure the activity by light transmission at 600nm.

Figure 4.9 Neutralising Efficacy of Specific Ovine Fab Against Haemorrhagic Activity of *E. carius* (a) and *E. coloratus* (b) Venoms

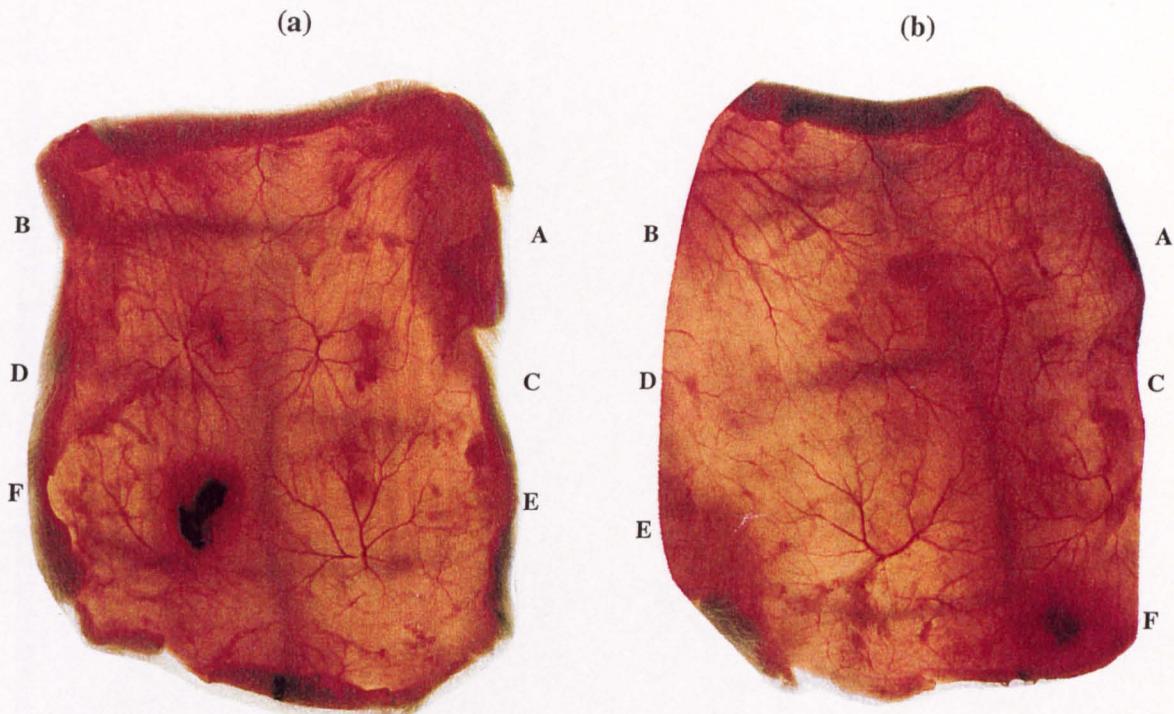


Figure 4.9 shows neutralisation of the haemorrhagic activity caused by 8 μ g *E. pyramidum* venom (a) and 16 μ g *E. coloratus* venom (b) by premixing with antivenoms (A 2000 μ g, B 1000 μ g, C 500 μ g, D 250 μ g, E 125 μ g). Venom (F) and venom/antivenom mixes were injected into the dorsal skin of rats. After 24 hours the animals were killed, the dorsal skin removed, and the diameter of the lesions measured on the inner surface of the skin. Haemorrhagic activity was neutralised at all antivenom concentrations as evidenced by the absence of lesions.

4.5.5 Necrotic Activity

Both monospecific ovine antivenoms raised against *E. pyramidum* and *E. coloratus* venoms showed a marked protection against the necrotic lesion of these venoms (Figure 4.10). Again the minimum neutralising dose of monospecific ovine Fab raised against *E. pyramidum* venom was lower than that raised against *E. coloratus* venom (3 and 12g/g respectively).

4.5.6 Defibrinogenating Activity

Both ovine antivenoms raised against *E. pyramidum* and *E. coloratus* venoms provided a marked protection against the defibrinogenating effect of their respective venoms (Table 4.4) with the minimum neutralising dose of antivenom raised against *E. coloratus* being slightly lower (83g/g) than that raised against *E. pyramidum* venom (143g/g).

4.5.7 Lethal Toxicity

The ED₅₀ of the ovine Fab fractions indicated that the more lethal the venom, the more antivenom was required to protect the mice (Table 4.5). Expressed as the amount of specific Fab required for 50% protection (g Fab/g venom) the protection produced was in the order: *E. pyramidum* (11g/g) < *E. coloratus* (13g/g) < *B. arietans* (30g/g) < *C. cerastes* (60g/g) < *N. h. arabica* (143g/g).

4.6 CROSS-NEUTRALISATION STUDIES

Cross-neutralisation was assessed using Western blotting, ELISA and small scale affinity chromatography as described in Chapter II.

4.6.1 Western Blotting

Antibody populations in each of the five antivenoms bound most of the venom components in the venoms used for immunisation. The antivenom raised against *E. pyramidum* and *E. coloratus* venoms showed more than eight bands on Western blotting (Figure 4.11). *C. cerastes* venom showed nine bands and *B. arietans* venom eight bands.

Figure 4.10 Neutralising Efficacy of Specific Ovine Fab Against Necrotic Activity of *E. carinatus* (a) and *E. coloratus* (b) Venoms

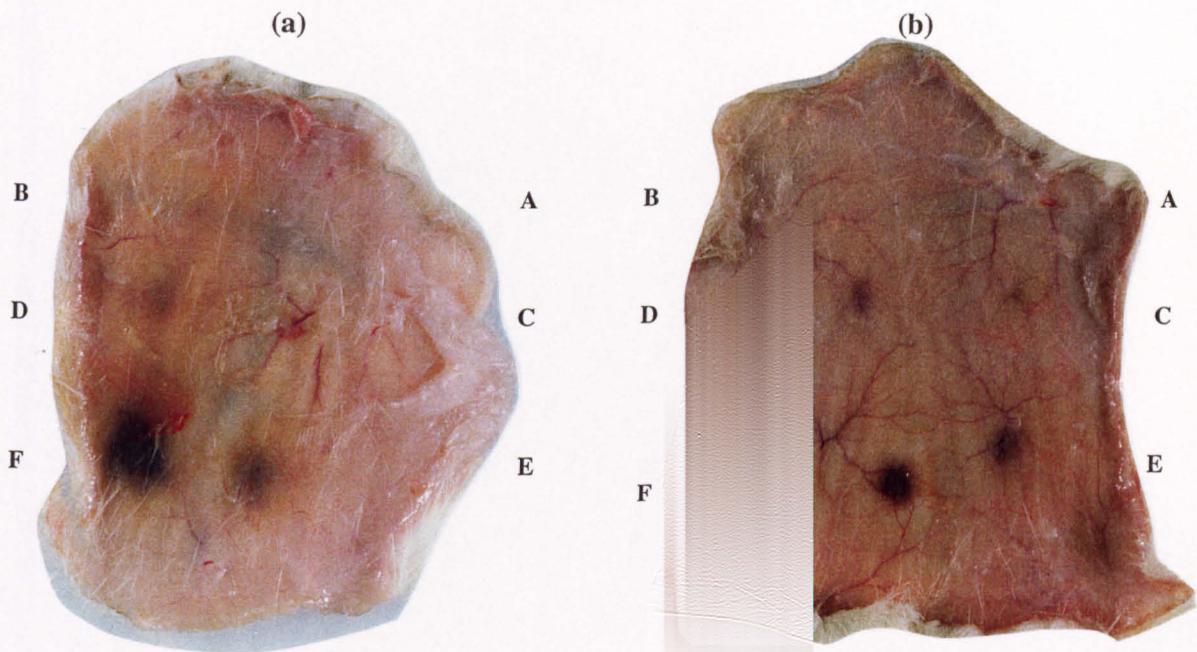


Figure 4.10 shows neutralisation of the necrosis caused by 20 μ g *E. pyramidum* venom (a) and 20 μ g *E. coloratus* venom (b) when pre-mixed with antivenoms (A 500 μ g, B 250 μ g, C 125 μ g, D 62 μ g, E 31 μ g). Venom (F) and venom/antivenom mixes were injected into the dorsal skin of rats. After 72 hours the animals were killed, the dorsal skin removed, and the diameter of the lesions measured on the inner surface of the skin. Lesions were absent at concentrations of Fab of 125 μ g and above.

Table 4.5 Murine ED₅₀ of Ovine Fab Antivenoms Tested Against the Venom Used for their Production.

Antivenom	µg Fab/mouse	µgFab/g (mouse)	g Fab/g venom
<i>E. pyramidum</i>	1500 (1000-2000)*	75	11
<i>E. coloratus</i>	1400 (900-2600)	70	13
<i>C. cerastes</i>	3600 (3000-4000)	180	60
<i>B. arietans</i>	3200 (2600-3700)	160	30
<i>N. h. arabica</i>	4300 (4000-4700)	215	143

The effective dose of monospecific ovine Fab required to protect 50% of mice against five times the lethal dose (ivLD₅₀) of venom from *E. pyramidum*, *E. coloratus*, *C. cerastes*, *B. arietans*, *N. h. arabica* and *A. microlepidota* was assessed. Mice received increasing doses of antivenom and the number of survivors in each group were counted after 24hr. The analysis of the slope of the dose-response for the ED₅₀ was performed by the probit method.

* 95% confidence limits

Figure 4.11 Western Blotting

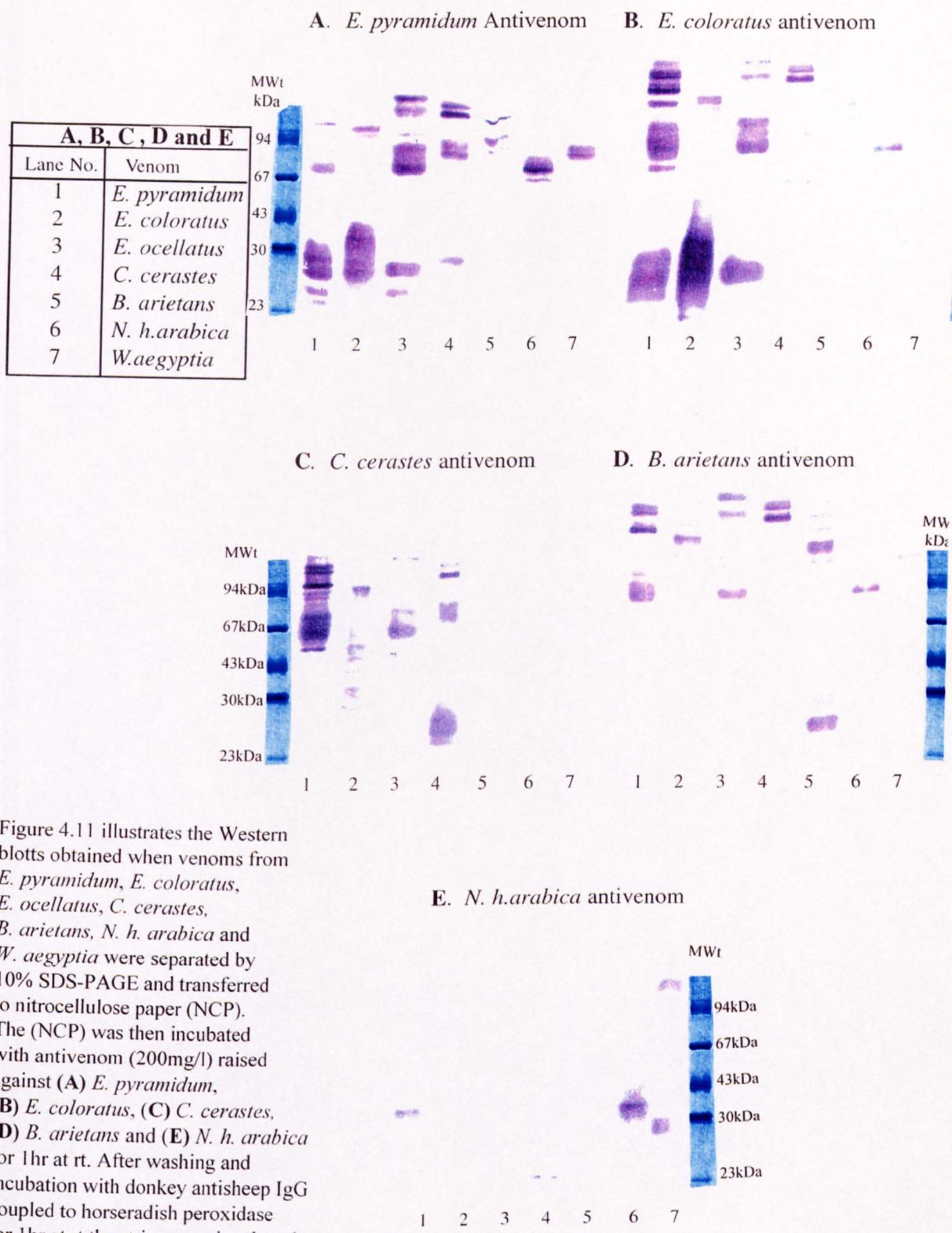


Figure 4.11 illustrates the Western blots obtained when venoms from *E. pyramidum*, *E. coloratus*, *E. ocellatus*, *C. cerastes*, *B. arietans*, *N. h. arabica* and *W. aegyptia* were separated by 10% SDS-PAGE and transferred to nitrocellulose paper (NCP). The (NCP) was then incubated with antivenom (200mg/l) raised against (A) *E. pyramidum*, (B) *E. coloratus*, (C) *C. cerastes*, (D) *B. arietans* and (E) *N. h. arabica* for 1hr at rt. After washing and incubation with donkey antisheep IgG coupled to horseradish peroxidase for 1hr at rt the strips were incubated with substrate until the bands were well developed.

However only five bands were detected by the *N. h. arabica* antivenom while normal sheep serum did not bind to any of the venom components. The problem with using this method was that although clear bands developed, the background gradually deteriorated, often before a photograph had been taken. Unfortunately fewer bands were detected by SDS-PAGE.

Cross-neutralisation was also assessed by incubating each antivenom with seven venoms, separated by SDS-PAGE and transferred to a nitrocellulose membrane. The antibodies raised against the viper venoms in this study bound only two of the elapid venom bands (*N. h. arabica* and *W. aegyptia*). The antibodies raised against *E. pyramidum* venom bound most of the low MWt components found in *E. coloratus* venom and most of the high MWt components of *C. cerastes* and *B. arietans* venoms. Antibodies raised against *E. coloratus* venom bound most of the components found in *E. pyramidum* venom and also six bands of *C. cerastes* venom and three bands of *B. arietans* venom. *C. cerastes* antibodies bound most of the high MWt components found in *E. pyramidum* venom and more than nine bands with *E. coloratus* venom. Antibodies raised against *B. arietans* venom bound most of high MWt components found in *E. pyramidum*, *E. coloratus* and *C. cerastes* venoms whereas only two bands were produced with the *N. h. arabica* and *W. aegyptia* venoms. The *N. h. arabica* antivenoms bound four low MWt bands of *E. pyramidum* venom and only two bands of the other viper venoms, whereas more bands were bound with *W. aegyptia* venom (Figure 4.11).

4.6.2 Enzyme-linked Immunosorbent Assay

Microtitre plates were coated with the different venoms and then incubated with the IgG fraction of the various ovine antivenoms. Cross-neutralisation was expressed as a percentage of the titre obtained with the venom used for immunisation.

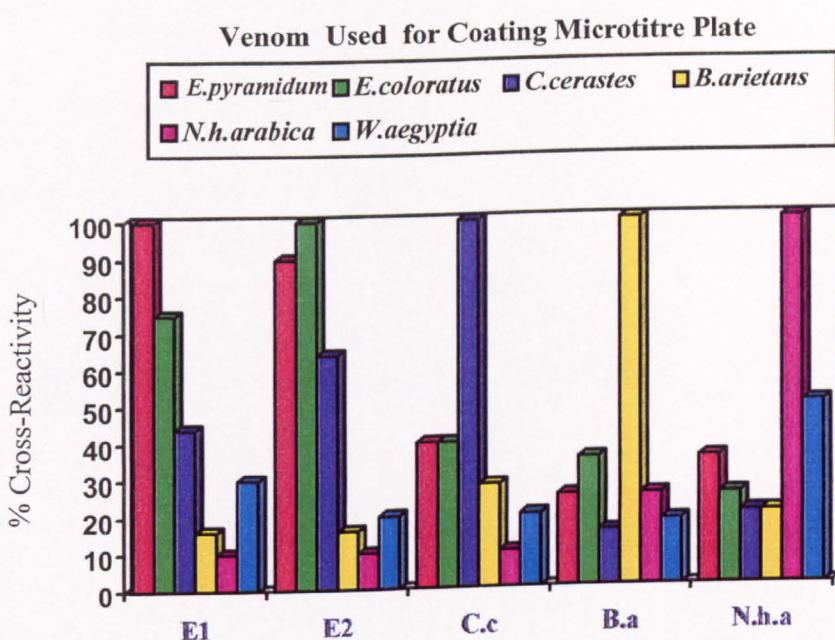
The IgG fraction of the antivenom raised against *E. pyramidum* venom showed the highest cross-reactivity with *E. coloratus* venom, a lower values with *C. cerastes* venom and less than 20% cross-reactivity with venom from *B. arietans*, *N. h. arabica* and *W. aegyptia* (Figure 4.12). Antivenom raised against *E. coloratus* venom showed the highest titre with *E. pyramidum* venom and similar values to the *E. pyramidum* antivenom with the remaining venoms. The antivenoms raised against *C. cerastes* and *B. arietans* venoms showed a maximum cross-reactivity of only 40% with both *Echis* venoms and less than 10% against *N. h. arabica* and *W. aegyptia* venoms. The antivenom raised against *N. h. arabica* venom showed maximum cross-neutralisation with *W. aegyptia* venom followed by 35% with *E. pyramidum* venom and less with other viper venoms (20%).

4.6.3 Small Scale Affinity Chromatography

Small scale affinity chromatography columns were prepared by coupling venoms to Sepharose 4B and these were treated with fixed amounts of the IgG fractions raised against different venoms. Cross-reactivity was expressed as a percentage of the protein recovered from the column relative to that obtained from the homologous antivenom.

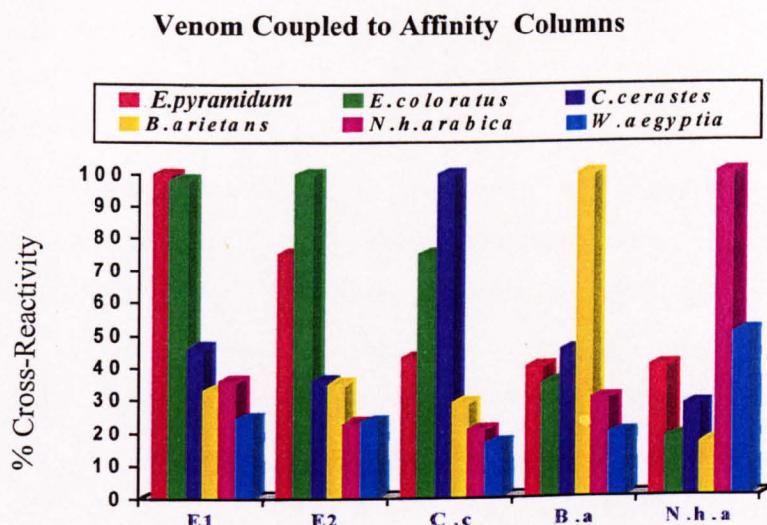
The IgG fraction raised agianst *E. pyramidum* venom bound best to the column coupled with *E. coloratus* venom and showed only low cross-reactivity (22-45%) with all remaining venoms (Figure 4.13). The IgG fraction raised against *E. coloratus* venom showed the highest cross-reactivity with the column of *E. pyramidum* venom (80%) and less (20-30%) with the remainder. The *C. cerastes* antivenom showed the highest percentage recovery from the column of *E. coloratus* venom (70%), followed by *E. pyramidum* venom and lower with the remainder. Maximum cross-reactivity for the *B. arietans* antivenom was with *C. cerastes* venom (40%), followed by *E. pyramidum* venom. Finally the antivenom raised against *N. h. arabica* venom showed a reasonable cross-reactivity with *W. aegyptia* venom (60%) but lower values (15-30%) with viper venoms.

Figure 4.12 Percentage Cross-Reactivity of Ovine Antivenoms with Venoms from Saudi Arabian Venomous Snakes.



Microtitre plates were coated with the different venoms (200ng/well) and then incubated with the IgG fraction of the various ovine antivenoms raised against *E. pyramidum* (E1), *E. coloratus* (E2), *C. cerastes* (C.c), *B. arietans* (B.a) and *N. h. arabica* (N.h.a) venoms. Cross-reactivity was expressed as a percentage of the titre obtained with the venom used for immunisation. Antivenom raised against *E. pyramidum* venom showed the highest cross-reactivity with *E. coloratus* venom and antivenom raised against *E. coloratus* venom likewise showed the highest cross-reactivity with *E. pyramidum* venom. The antivenom raised against *N. h. arabica* venom showed greatest cross-reactivity with *W. aegyptia* venom.

Figure 4. 13 Percentage Cross-Reactivity of Ovine Antivenoms with Venoms from Saudi Arabian Venomous Snakes.



Small scale affinity chromatography columns were prepared by coupling each of the venoms to Sepharose 4B. These were then incubated with a fixed amount of an IgG fraction raised against the venom from *E. pyramidum* (E1), *E. coloratus* (E2), *C. cerastes* (C.c), *B. arietans* (B.a) and *N. h. arabica* (N.h.a). Cross-reactivity was expressed as a percentage of the value obtained using the homologous antivenom. *E. pyramidum* antivenom showed the highest degree of binding to the column coupled with *E. coloratus* venom and *E. coloratus* antivenom likewise showed the highest degree of binding to the column coupled with *E. pyramidum* venom. *C. cerastes* antivenom bound best the column coupled with *E. coloratus* venom and *N. h. arabica* antivenom showed a moderate degree of binding to the column coupled with *W. aegyptia* venom.

4.7 DISCUSSION

A lack of availability and the poor neutralising potency of commercial antivenoms has preventing effective treatment of the majority of patients subjected to snakebite in Saudi Arabia. In this study specific antibodies were raised in sheep against venoms from five of the most venomous snakes found in Saudi Arabia. The resultant antivenoms were designed to alleviate the toxicity of the envenoming, while minimising possible side effects.

Conventional immunisation of horses to produce antivenom follows a hyperimmunisation schedule in which venom is given repeatedly at short intervals in rapidly increasing doses of up to 1200mg per injection (Russell, 1988). Initially, antibody levels increase progressively in the horse, but this may be followed by a decline in titre after further injections of large venom doses (Christensen, 1966; Bouquet, 1979). The necessity for large amounts of venom (which are almost certainly detrimental to the condition of the animal) is, in part, due to the intolerance of horses to such oil-based adjuvants as Freund's complete and incomplete adjuvants. Sheep, in which the immune response is predominantly humoral, do not demonstrate the severe local reactions manifested in horses when such adjuvants are used and, therefore, have an enhanced immune response with much higher circulating antibody levels than found in horses.

In the present study every sheep immunised with a low dose (0.5mg) of any of the venoms responded well as assessed by ELISA titres and small scale affinity chromatography. Increasing the dose up to 4mg resulted in a marked improvement in the response to *N. h. arabica*, *B. arietans* and *C. cerastes* venoms. The usefulness of an antiserum will depend on its antibody titre, its specificity and the affinity with which the antibody populations bind the toxic components of the appropriate venom. Affinity purification should theoretically serve to remove all contaminants and all the non-specific immunoglobulins leaving only specific antibody populations which

comprise some 20-25% of the total immunoglobulin pool. ELISA is presently the most popular technique employed to assess antibody titres. However, it is not certain that all venom components are equally well absorbed to the microtitre plates employed and, therefore, that such titres are an accurate reflection of the presence of therapeutically important antibodies. Thus while ELISA confirms an antibody response it gives no detailed information as to how broad has been the spectrum of the response. This may also vary between an antivenom raised against a complex viperid venom or against an elapid venom containing fewer components.

All the antivenom fractions prepared, as shown by SDS-PAGE and FPLC gel filtration, were at least 95% pure with only minor contaminants present. The major protein bands detected by SDS-PAGE also showed as peaks by gel filtration, but minor bands visible by SDS-PAGE were not always apparent by FPLC. Ammonium sulphate is commonly used to precipitate immunoglobulin but we prefer the sodium salt because it results in a pellet that is more easily reconstituted; the procedure is carried out at room temperature rather than 4°C; the product contains lower levels of albumin; and the addition of potentially toxic ammonium ions is avoided. It has also been suggested that ammonium sulphate may irreversibly denature immunoglobulin and reduce the yield of viable antivenom (Sullivan, 1987). Possible contaminants of Fab are albumin, haem, Fc, IgG and reagents used in the proccesing of antiserum, such as papain. The Fc region of the immunoglobulin molecule is responsible for effector functions such as complement binding and macrophage activation and may, therefore, be responsible for many of the hypersensitivity reactions.

The antivenoms raised in this study completely neutralised the PLA₂ and phosphodiesterase activities of their respective venoms. This is important since the PLA'2s are expected to cause most of the damage that follows envenoming. Less antivenom was required to neutralise the indirect haemolytic activity of the venoms, as

only some of the PLA₂'s are haemolytic. Kemparaju and colleagues (1994) reported that rabbit polyclonal antibodies prepared against PLA₂ obtained from Indian *E. pyramidum* venom did not neutralise the lethality of the PLA₂ isolated from *E. pyramidum* venom. Hyaluronidase activity was neutralised by the ovine antivenoms and this would, hopefully, retard the oedema and other local manifestations.

The monospecific antivenoms completely inhibited initiation of platelet aggregation induced by *C. cerastes* and *B. arietans* venoms. However, the antivenom raised against *C. cerastes* venom did not neutralise the factors inhibiting the ADP activity. *Echis* specific Fab provided good protection, both *in vivo* and *in vitro*, against procoagulant, haemorrhagic, necrotising and defibrinogenating effects. It has been claimed that haemorrhagic factors have several antigenic determinants and that it is unlikely that a single antibody could neutralise the haemorrhagic activity completely (Perez *et al.*, 1984). However, isolation of antibodies from antivenoms using various venom factors such as toxins, haemorrhagins or myotoxic PLA₂ (Lomonte *et al.*, 1985) is a useful procedure for analysing the role that a specific venom component plays in envenomation. The conventional way to assess the effectiveness of an antivenom is to determine its ability to protect mice against set amounts of venom. The ED₅₀ of specific Fab fragments in mice suggests that each antivenom contains Fab populations which bind to and neutralise the most toxic venom components. The large amount of antivenom required to protect mice against *N. h. arabica* venom may reflect the fact that elapid venom contains highly toxic, small MWt neurotoxins, which are of low immunogenicity.

Western blotting showed that most venom components detected on SDS-PAGE were bound by antibody populations present in the antivenom. Venoms consist of many different components, and the closer the evolutionary relationship between species the more common components are found. Western blotting of the antivenoms raised against venom from the two *Echis* species showed a broad range of antibody populations

directed against most of each venom's components. Western blotting also showed that all the viper antivenoms bound to a reasonable extent the five venoms used in this study. However, binding does not necessarily equate to neutralisation of the pharmacological effects of the venom. The cross-reactivity studies undertaken by ELISA gave similar results to those from small scale affinity chromatography. Thus two antivenoms raised against *Echis* species venoms showed good cross-reactivity with each other while the *N. h. arabica* antivenom showed less binding with viper venoms. Mohammed and colleagues (1980) reported that both the monospecific and bispecific equine antivenoms raised against *C. cerastes* and *C. viper* venoms were equally efficient in neutralising their lethality to mice.

In conclusion, we have shown the suitability of sheep as an alternative to horses for antivenom production and by means of a dose response study, the appropriate dose of each venom to employ. The resultant monospecific antivenoms were effective in neutralising enzymatic and biological effects and protected mice in LD₅₀ studies. Finally no specific antivenoms exist for some of venomous snakes found in Saudi Arabia and it is anticipated that the ovine Fab products will be several times more effective (and much safer) than the existing antivenoms, which are of equine origin.

CHAPTER V

COMPARISON OF OVINE AND EQUINE ANTIVENOMS

5.1 SUMMARY

To compare the efficacy of antibody fragments with that of intact IgG, the immunoglobulin fraction of antivenom was partially purified by sodium sulphate precipitation and digested either with papain to form Fab fragments or with pepsin to produce F(ab)₂. All antivenom products raised against the five venoms used in this study neutralised the enzymatic and biological activities of the venoms. However, the molar amounts of the F(ab)₂ and Fab fractions required exceeded that of IgG in most *in vitro* assays. Many antivenoms are raised against a mixture of venoms and two monospecific and one polyspecific antivenom were raised against *E. pyramidum* and *E. coloratus* venoms. The polyspecific ovine Fab adequately neutralised the enzymatic and biological effects and protected mice against lethal amounts of both venoms but was less efficient than the monospecific antivenoms.

Two commercial polyspecific equine F(ab)₂ antivenoms currently available in Saudi Arabia are produced by Pasteur-Mérieux (France) and Behringwerke AG (Germany) and these were compared with the ovine antivenoms. Affinity purification of the Behringwerke and Pasteur products showed that 3 to 13% of the F(ab)₂ were directed against venom components. They partially neutralised the enzymatic and biological activities of the venom but large amounts were required to protect mice against a lethal dose.

5.2 COMPARISON OF IgG, F(ab)₂ AND Fab FRACTIONS

The production and assessment of IgG and Fab fractions were described in Chapter IV.

5.2.1 Pepsin Digestion of Ovine IgG

To prepare ovine F(ab)₂, bleeds were pooled and salt precipitated. The precipitate (mainly IgG) was digested with pepsin and purified by salt precipitation as described in

Chapter II. Digestion was monitored by SDS-PAGE, FPLC and ELISA. SDS-PAGE showed one major band and faint bands at a MWt under 67 kDa could be seen in both commercial and specific ovine F(ab)₂ (Figure 5.1) whereas FPLC gel filtration showed two peaks of which the first had a MWt above 669kDa and the second, representing 95% of applied protein, a MWt of 98kDa (Figure 5.2). Only small amounts of albumin were still present in the final products (0.01 to 0.6mg/l) whereas up to 44mg/l of Fc was found (Table 5.1).

Small scale affinity columns comprising the venoms coupled to Sepharose 4B were incubated with 0.5ml of the various ovine F(ab)₂ fractions and the total concentrations eluted varied from 5 to 7g/l (Table 5.1). Measured by ELISA, the F(ab)₂ fractions showed antibody titres ranging from 170 to 300 x10³ against the five venoms (Table 5.1). The ovine F(ab)₂ was finally concentrated by freeze-drying and resuspended in PBS.

5.2.2 Neutralisation of Enzymatic Activities

The ovine IgG antivenoms and their F(ab)₂ and Fab fractions completely neutralised the PLA₂ activity of the various venoms (Table 5.2). Whereas the IgG and F(ab)₂ fractions of the antivenoms raised against *E. pyramidum* and *E. coloratus* venoms were equally efficient, more F(ab)₂ and Fab than IgG was required to neutralise the remaining antivenoms. All fractions completely inhibited the phosphodiesterase and hyaluronidase activity of the five venoms used in this study and, in general, less IgG than F(ab)₂ and Fab was required to obtain maximum neutralisation. For most antivenoms, the F(ab)₂ and Fab were equally effective.

5.1 SDS- PAGE of Equine and Ovine F(ab)₂ Antivenoms

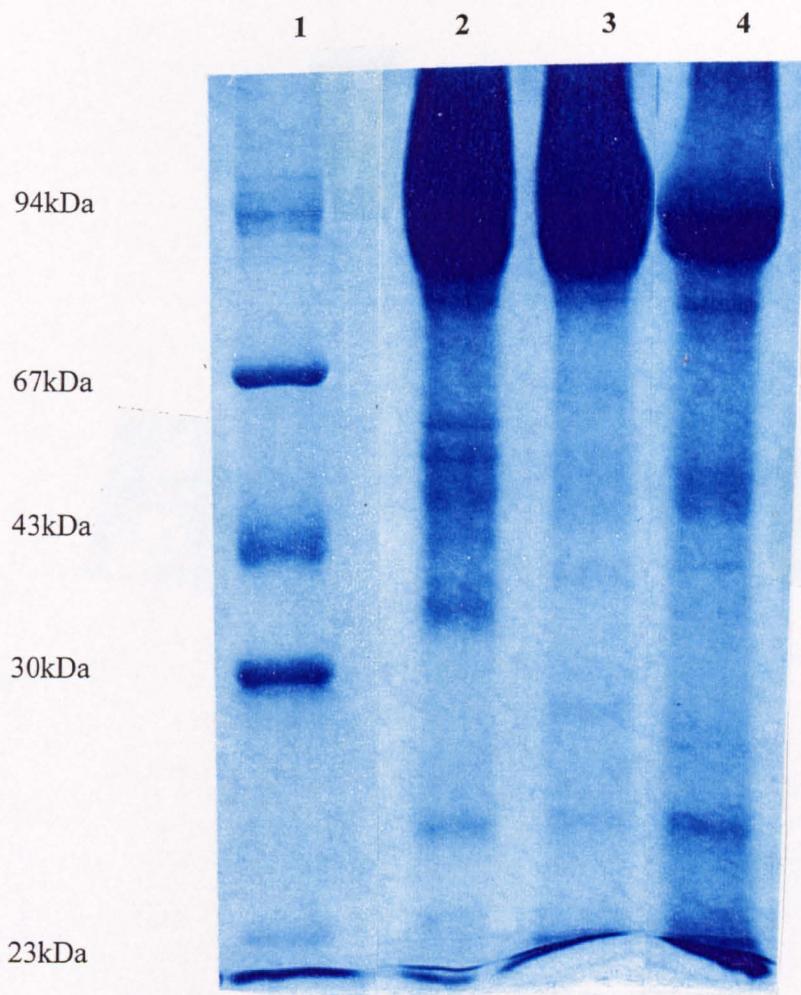
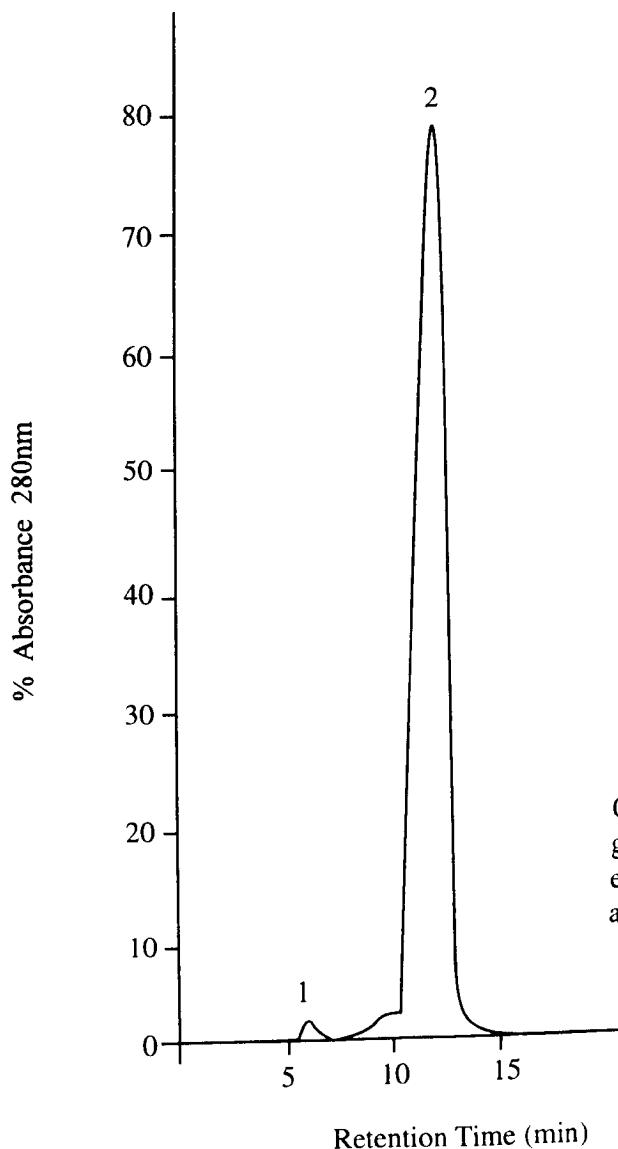


Figure 5.1 Compares the profiles of equine F(ab)₂ produced by Beheringwerke, Pasteur and ovine F(ab)₂ (lane 2, 3 and 4) respectively as resolved by 10% SDS-PAGE under non-reducing conditions. 15 μ l of each product (4 μ g/ μ l) were loaded. Lane 1 shows the MWt marker. Faint bands at a MWt of under 67KDa could be seen in both commercial and ovine antivenoms.

Figure 5.2 FPLC Gel Filtration of Ovine F(ab)₂ Antivenom

Peak no.	MWt. kDa	% of total Protein	Retention time (min)
1	>669	5	7
2	98	95	12.1



Ovine F(ab)₂ (200µg) was analysed by FPLC gel filtration on a Superose 12 HR column, equalibrated and run in 154 mM NaCl at pH 5.5 and the absorbance read at 280nm.

Table 5.1 Preparation of F(ab)₂ Fractions of Ovine Antivenoms

		<i>E.pyramidum</i>	<i>E.coloratus</i>	<i>C.cerasurus</i>	<i>B.arietans</i>	<i>N.h.arabica</i>
IgG fraction	Protein, g/l	34	38	40	36	42
	Specific antibody concentration, g/l	13	14	12	11	13
	ELISA titre	750,000	710,000	800,000	1,000,000	1,200,000
F(ab)₂ fraction	Albumin, mg/l	1.2	5	7	3	9
	Protein, g/l	25	24	25	24	27
	Specific antibody concentration, g/l	6	6	7	5	6
	ELISA titre	250,000	230,000	260,000	170,000	300,000
	Albumin, mg/l	0.01	0.07	0.08	0.04	0.6
	Fc, mg/l	44	<20	>20	30	-

Antivenoms were subjected to salt fractionation followed by pepsin digestion. The protein concentration (g/l), specific antibody concentration (g/l), albumin concentration (mg/l) and ELISA titre of the IgG and F(ab)₂ fractions were measured. The Fc concentration (in mg/l) of the F(ab)₂ fractions were measured using a fluoroimmunoassay.

-: not tested

Table 5.2 The Efficacy of IgG and its F(ab)₂ and Fab Fragments in Inhibiting Enzymatic Activities Expressed as g Antivenom/g Venom.

Antivenom raised against	Phospholipase A ₂			Phosphodiesterase			Hyaluronidase		
	IgG	F(ab) ₂	Fab	IgG	F(ab) ₂	Fab	IgG	F(ab) ₂	Fab
<i>E.pyramidum</i>	250	250	500	160	320	320	10	10	10
<i>E.coloratus</i>	250	250	500	160	320	320	10	13	13
<i>C.cerastes</i>	63	125	125	40	80	80	15	20	20
<i>B.arietans</i>	63	125	125	80	160	160	10	13	13
<i>N.h.arabica</i>	250	500	500	160	320	320	10	15	20

Table 5.2 compares the efficacy of monospecific ovine IgG, F(ab)₂ and Fab fractions to inhibit the phospholipase A₂, phosphodiesterase and hyaluronidase activities of venom from *E. pyramidum*, *E. coloratus*, *C. cerastes*, *B. arietans* and *N. h. arabica*. These values were corrected for values obtained from normal sheep serum.

5.2.3 Neutralisation of Biological Activities

Equal amounts of IgG, F(ab)₂ and Fab offered complete protection against coagulation caused by *E. pyramidum* venom whereas more F(ab)₂ and Fab than IgG was required to inhibit coagulation caused by *E. coloratus* venom (Table 5.3). To neutralise indirect haemolysis the three fractions of the antivenom raised against *C. cerastes* venom were equally efficient, whereas double the amount of Fab and F(ab)₂ compared with IgG were required for the remaining antivenoms.

5.3 COMPARISON OF OVINE MONOSPECIFIC AND POLYSPECIFIC ANTIVENOMS RAISED AGAINST *E. PYRAMIDUM* AND *E. COLORATUS* VENOMS

5.3.1 Production and Assessment of Antivenoms

For primary immunisation groups of three sheep were injected with 0.5mg venom from *E. pyramidum* or *E. coloratus* or with 0.5mg of an equal mixture of the two venoms. This was doubled after four weeks to 1mg and the sheep continued to be immunised at monthly intervals with this amount. Serum samples from individual sheep were assessed by ELISA and small scale affinity chromatography two weeks after each immunisation, as described in Chapter II.

The antibody levels rose rapidly and, by 22 weeks after the primary immunisation, most sheep had reached a maximum (Figure 5.3). The average ELISA titre for the *E. pyramidum* antivenom was 1.7×10^6 and for the *E. coloratus* antivenom 2.1×10^6 . The polyspecific *Echis* antivenom showed very similar values with average titres of 1.6×10^6 against *E. pyramidum* venom and 2.1×10^6 against *E. coloratus* venom. Specific immunoglobulin concentrations rose to a maximum quicker than ELISA titres (Figure 5.4). The average concentration throughout the 70 week study period for the *E. pyramidum* antivenom was 12g/l and for the *E. coloratus* venom 11.8g/l. The mixed

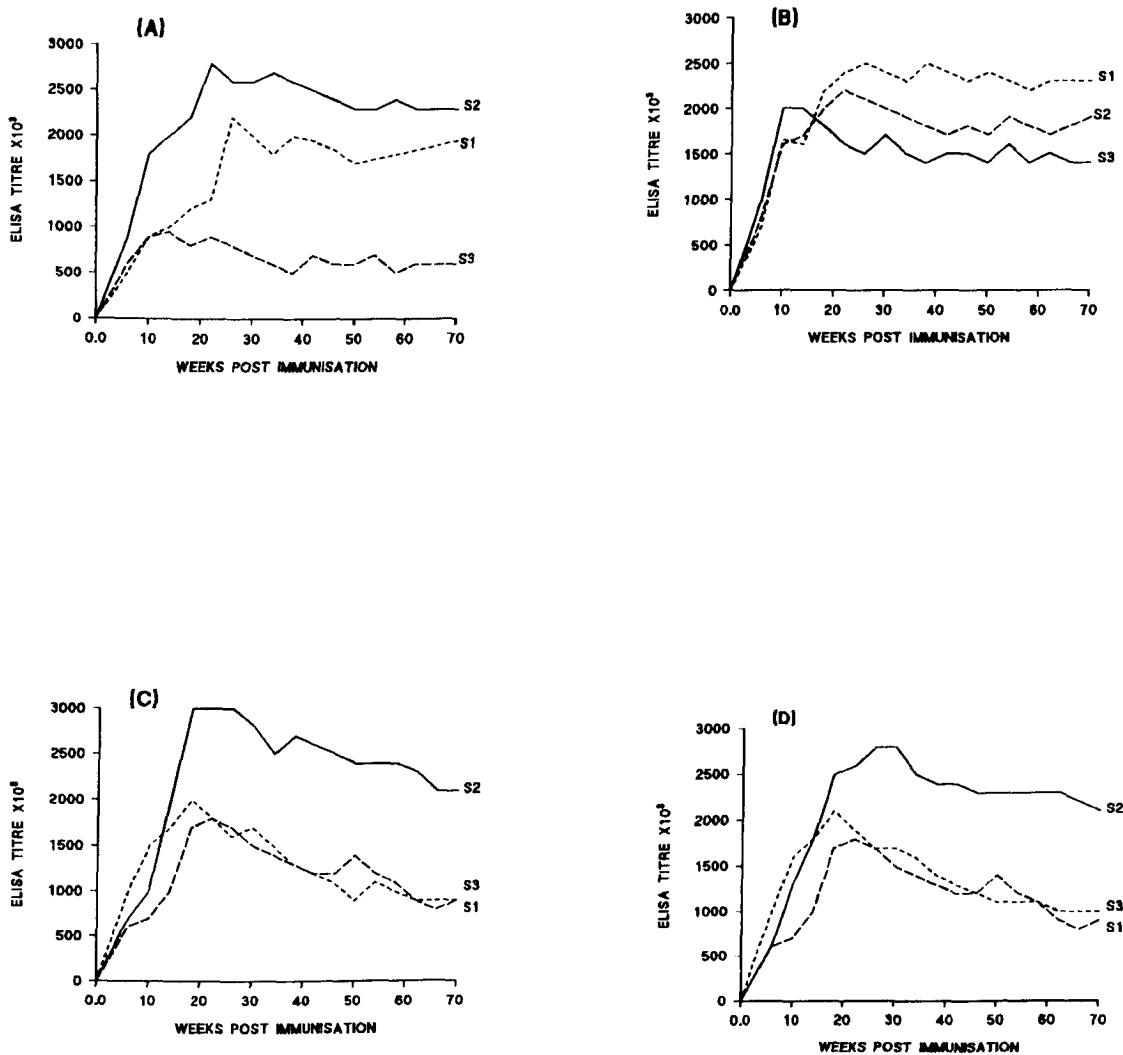
Table 5.3 The Efficacy of Immunoglobulin and Their Intact F(ab)₂ and Fab Fraction to Neutralise Biological Activities (g/g Venom).

Antivenom raised against:	Coagulation			Indirect haemolysis		
	IgG	F(ab) ₂	Fab	IgG	F(ab) ₂	Fab
<i>E. pyramidum</i>	125	125	125	50	100	100
<i>E. coloratus</i>	100	167	167	100	200	200
<i>C. cerastes</i>	NA	NA	NA	50	50	50
<i>N.h.arabica</i>	NA	NA	NA	50	100	100*

Table 5.3 compares the efficacy of monospecific ovine IgG, F(ab)₂ and Fab fractions to inhibit coagulant and indirect haemolytic activities of venom from *E. pyramidum*, *E. coloratus*, *C. cerastes* and *N. h. arabica*.

*: Maximum inhibition 90% and NA: No venom activity

Figure 5.3 Monthly ELISA Titres for Serum Samples from Sheep Immunised with
 (A) *E.pyramidum*, (B) *E.coloratus*, (C) Mixed *Echis* and Tested Against
E.pyramidum and (D) Mixed *Echis* and Tested Against *E.coloratus* Venom.



Serum samples were assessed by ELISA from groups of three sheep immunised monthly with 1mg of venom from *E. pyramidum*, *E. coloratus* and mixed *Echis*. The sheep were bled two weeks after each immunisation and each point represents the mean for three sheep.

Echis antivenom again showed similar values with average specific immunoglobulin concentrations of 11.5g/l against *E. pyramidum* venom and 11.4g/l against *E. coloratus* venom. After 32 weeks bleeds of each antivenom were pooled, the immunoglobulin fraction isolated and Fab fragments prepared as described in Chapter II.

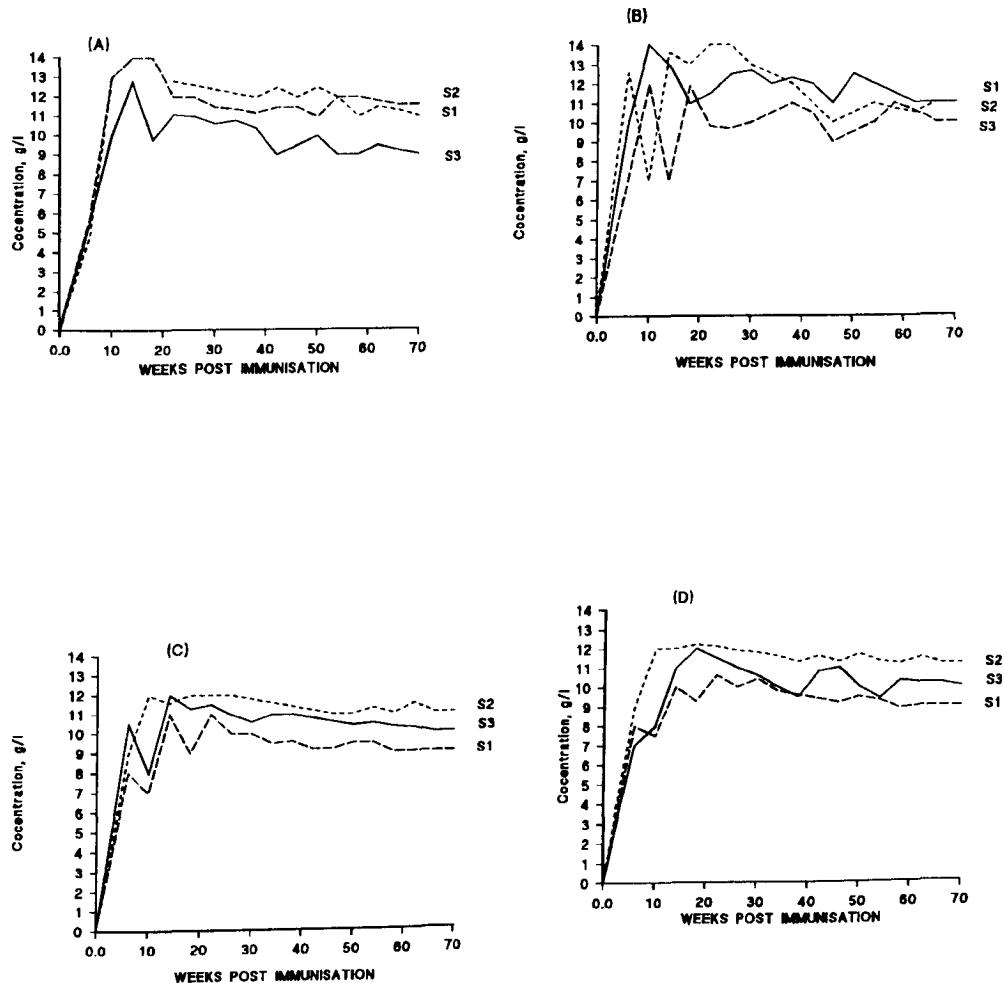
5.3.2 Neutralisation of Enzymatic and Biological Activities

The polyspecific Fab antivenom completely neutralised the PLA₂, PDE and HYL activities of both *E. pyramidum* and *E. coloratus* venoms (Table 5.4). However, considerably less (30-70%) was required of the monospecific antivenoms. The polyspecific ovine Fab also neutralised the coagulant and indirect haemolytic activities of both *Echis* venoms. To protect mice against lethal doses of the venoms less of the monospecific antivenoms compared with the polyspecific antivenom were required.

5.3.3 Neutralisation of Nigerian *E. ocellatus* Venom Activities

Antivenoms raised against *Echis* species are reported to show variable neutralisation of venoms from the same species of snake but which have been collected from different geographical locations. To study this, the biological and enzymatic activities of venoms from Saudi Arabian *E. pyramidum* and Nigerian *E. ocellatus* were compared and the neutralisation of these activities by the monospecific ovine antivenom raised against *E. pyramidum* venom was assessed. Both venoms showed similar PDE and HYL activity but *E. pyramidum* venom had four times higher PLA₂ activity (Table 5.5). Haemorrhagic, necrotic and defibrinogenating activities were similar but the coagulant activity of *E. ocellatus* venom was four times higher. *E. ocellatus* venom also had a slightly lower LD₅₀ than *E. pyramidum* venom (1 compared with 1.4µg/g of mouse). The ovine Fab antivenom raised against *E. pyramidum* venom showed equal neutralisation of PDE and HYL activity but was less effective against PLA₂ activity in the Nigerian venom (Table 5.5). Of particular interest the antivenom in amounts up to 1000mg, did not provide any protection against the potent procoagulant activity of Nigerian *E. ocellatus* venom.

Figure 5.4 Monthly Specific Antibody Concentrations for Serum Samples from Sheep Immunised with (A) *E.pyramidum*, (B) *E.coloratus*, (C) Mixed *Echis* and Tested Against *E.pyramidum* and (D) Mixed *Echis* and Tested Against *E.coloratus* Venom.



Serum samples were assessed by small scale affinity chromatography from groups of three sheep each immunised monthly with 1mg of venom from *E. pyramidum*, *E. coloratus* and mixed *Echis*. The sheep were bled two weeks after each immunisation and each point represents the mean for three sheep.

Table 5.4 The Efficacy of Ovine Polyspecific and Monospecific Fab to Protect Against *E.pyramidum* and *E.coloratus* Venom Activities (g/g).

Venom activities	Polyspecific antivenom		Monospecific antivenoms	
	<i>E.pyramidum</i>	<i>E.coloratus</i>	<i>E.pyramidum</i>	<i>E.coloratus</i>
Phospholipase A ₂	750	750	500	500
Phosphodiesterase	400	400	320	320
Hyaluronidase	20	20	10	13
Coagulation	250	250	125	167
Indirect haemolysis	300	300	100	200
ED ₅₀	16 (13-18)*	24 (18-26)	11 (7-14)	13 (8-24)

Table 5.4 compares the efficacy of monospecific and polyspecific ovine Fab fraction to neutralise lethality and inhibit the phospholipase A₂, phosphodiesterase, hyaluronidase, coagulant and indirect haemolytic activities of *E. pyramidum* and *E. coloratus* venoms.

*: 95% confidence limits

Table 5.5 Comparison of *E.pyramidum* and *E.ocellatus* Venom Activities and their Neutralisation by Ovine Fab Raised Against *E.pyramidum* Venom

Venom activities	<i>E.pyramidum</i> venom (µg)	<i>E.ocellatus</i> venom (µg)	Neutralisation by <i>E.pyramidum</i> Fab g/g of <i>E.pyramidum</i> venom <i>E.ocellatus</i> venom	
Phospholipase A ₂	1	4	500	1500
Phosphodiesterase	3	3	320	320
Hyaluronidase	62	62	10	10
Coagulation	4	1	125	NA (at 1000)
Haemorrhagic	8	9	62	-
Necrotic	20	30	3	-
Defibrinogenating	7	4	143	-
LD ₅₀	1.4 (24-33)*	1 (19-27)*	11 (7-14)	-

Table 5.5 compares the Saudi Arabian *E. pyramidum* and Nigerian *E. ocellatus* venom activities of phospholipase A₂, phosphodiesterase, hyaluronidase, coagulant, haemorrhagic, necrotic, defibrinogenating and LD₅₀. It shows also the efficacy of ovine Fab fraction raised against *E. pyramidum* venom to neutralise some venom activities of *E. ocellatus*.

NA: No antivenom activity

-: Not tested

*: 95% confidence limits per 20g mouse weight

5.4 COMPARISON OF COMMERCIAL ANTIVENOMS

The two commercial antivenoms available in Saudi Arabia and used in this study are polyspecific F(ab)₂ preparations of equine origin. That from Pasteur-Mérieux (France) was raised by immunising horses with a mixture of venoms from *Echis pyramidum*, *Bitis arietans* and *Naja haje haje*. That from Behringwerke AG (Germany) contained antibodies directed against a mixture of venoms for *Echis pyramidum*, *Cerastes cerastes* and *Naja haje haje*. The efficacy of the commercial antivenoms was compared with the ovine Fab and F(ab)₂ produced in this study.

5.4.1 Physicochemical Characterisation

All methods used for characterisation were described in Chapter II.

5.4.1.1 Protein Concentration

Protein concentrations were measured by optical density and the Behringwerke product had a value of 40g/l and that from Pasteur of 20g/l. After dialysis against phosphate buffered saline, to remove their preservatives, the antivenoms were freeze-dried and then reconstituted at a concentration of 100g/l.

5.4.1.2 Sterility and Endotoxin Levels

The commercial antivenoms showed no bacterial growth. The Pasteur antivenom showed no detectable endotoxin (<0.125EU/ml) and no inhibition of gel formation while the Behringwerke product had a low endotoxin concentration of 4 to 16EU/ml with no inhibition of clotting.

5.4.1.3 SDS-PAGE and FPLC

SDS-PAGE of the Pasteur and Behringwerke antivenoms showed one major intense band (Figure 5.1) corresponding to $F(ab)_2$. There is also a faint band in both corresponding to pepsin (33kDa) and two further bands with MWt of 43 and 67kDa. FPLC gel filtration of both antivenoms showed one major peak (1) representing equine $F(ab)_2$ (Figure 5.5). The MWt for the Pasteur product was 97 ± 5 kDa and represented 94% of total protein and that for Behringwerke was 100 ± 4 kDa and represented 96% of total protein.

5.4.1.4 Small Scale Affinity Purification

The specific antibody concentrations in the Behringwerke was ranging from 3 to 9% and Pasteur products ranging from 13 to 8% (Table 5.6). Pasteur showed a lower concentration as compared to Behringwerke against all venoms.

5.4.1.5 Enzyme-linked Immunosorbent Assay

Microtitre plates were coated with different venoms and then incubated with the commercial antivenoms. The antibody titres of the Pasteur antivenom against the five venoms ranged from 80 to 150×10^3 . The Behringwerke product had slightly higher titres ranging from 90 to 170×10^3 (Table 5.6).

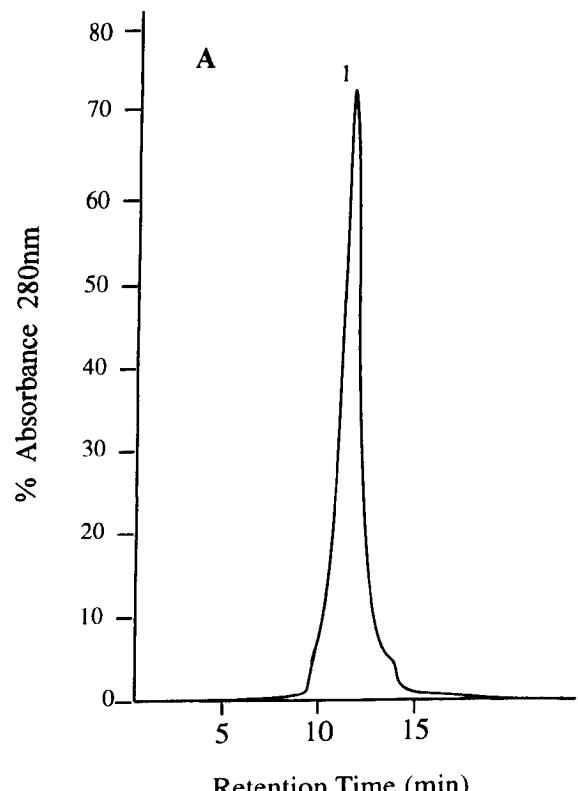
5.4.2 Neutralisation of Enzymatic Activities

Neither the Behringwerke or Pasteur antivenoms completely neutralised PLA₂, PDE or HYL activity of the venoms used in this study (Table 5.7). Both products were most effective against HYL activity, moderately so against PLA₂ and least effective against PDE activity. Equal amounts were required for maximum protection but Behringwerke generally showed higher inhibition. Smaller amounts of ovine $F(ab)_2$ and Fab completely neutralised these enzymatic activities (Table 5.2).

Figure 5.5 FPLC Gel Filtration of Equine F(ab)₂ Antivenoms

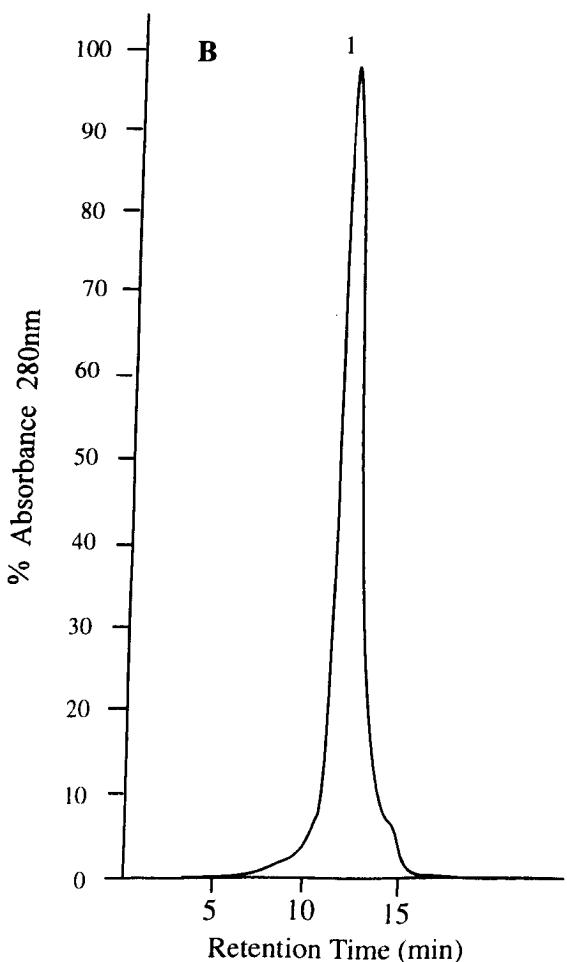
(A) Beheringwerke Antivenom

Peak no.	MWt. kDa	% of total protein	Retention time (min)
1	100 ± 4	96	12



(B) Pasteur Antivenom

Peak no.	MWt. kDa	% of total protein	Retention time (min)
1	97 ± 5	94	12.2



200µg of Beheringwerke (A) equine F(ab)₂ and Pasteur (B) equine F(ab)₂ were analysed by FPLC gel filtration on a superose 12 HR column. Proteins were eluted with 154 mM NaCl at pH 5.5 and the absorbance read at 280nm. Mean ± SEM of five reading.

Table 5.6 Concentrations and Binding Activities of Antibody Populations in the Two Polyspecific Equine F(ab)₂ Antivenoms.

Pasteur (20g/l)	Specific antibody , g/l	<i>E.pyramidum</i>	<i>E.coloratus</i>	<i>C.cerastes</i>	<i>B.arietans</i>	<i>N.h.arabica</i>
		3.5 (9%)	3.0 (8%)	2.0 (5%)	1.2 (3%)	2 (5%)
	ELISA titre	150,000	80,000	130,000	110,000	90,000
Behring- werke (40g/l)	Specific antibody , g/l	5 (13%)	4 (10%)	3 (8%)	4 (10%)	4.5 (11%)
	ELISA titre	170,000	90,000	110,000	150,000	100,000

Table 5.6 shows the binding activity of commercial equine antivenoms with Saudi Arabian venoms. It shows protein and specific antibody concentrations (g/l) and ELISA antibody titres of the equine F(ab)₂ Pasteur and Behringwerke respectively when tested against venom from *E. pyramidum*, *E. coloratus*, *C. cerastes*, *B. arietans* and *N. h. arabica*.

Table 5.7 The Efficacy of Commercial Antivenoms to Inhibit Enzymatic Activities Expressed as g Antivenom/g Venom. Percentage of Maximum Neutralisation Obtained is Given in Brackets.

Venom	Pasteur F(ab) ₂			Behringwerke F(ab) ₂		
	PLA ₂	PDE	HYL	PLA ₂	PDE	HYL
<i>E.pramidum</i>	750 (70%)	320 (30%)	10 (70%)	750 (90%)	320 (50%)	10 (80%)
<i>E.coloratus</i>	1000 (30%)	320 (20%)	13 (90%)	100 (40%)	320 (40%)	13 (80%)
<i>C.cerastes</i>	125 (40%)	160 (20%)	20 (70%)	125 (50%)	160 (30%)	20 (80%)
<i>B.arietans</i>	125 10%)	160 (20%)	13 (70%)	125 (70%)	160 (40%)	13 (80%)
<i>N.h.arabica</i>	500 (50%)	320 (10%)	20 (60%)	500 (90%)	320 (30%)	20 (80%)

Table 5.7 compares the efficacy of equine F(ab)₂ Pasteur and Behringwerke to inhibit the phospholipase A₂ (PLA₂), phosphodiesterase (PDE) and hyaluronidase (HYL) activities of venom from *E. pyramidum*, *E. coloratus*, *C. cerastes*, *B. arietans* and *N. h. arabica*.

5.4.3 Neutralisation of Biological Activities

Neutralisation of biological activities was measured as described in Chapter II.

5.4.3.1 Coagulant Activity

The same amount of both equine antivenoms offered complete protection against the coagulant activities of *E. pyramidum* and *E. coloratus* venoms (Table 5.8). Both were slightly more potent against *E. pyramidum* venom than the ovine Fab antivenom (125 compared with 200g/g) but slightly less against *E. coloratus* venom (200 compared with 167g Fab/g).

5.4.3.2 Indirect Haemolytic Activity

Slightly more Pasteur than Behringwerke was required (Table 5.8) and both only partially neutralised the activity of *N. h. arabica* venom. Ovine F(ab)₂ and Fab antivenoms were significantly more effective except against *E. pyramidum* venom (Table 5.3).

5.4.3.3 Initiation of Platelet Aggregation

Both antivenoms showed very little protection (10%) against the platelet aggregating activity of *C. cerastes* venom (Figure 5.6) and the Pasteur product was surprisingly poor in this respect since it had been raised against this venom. Behringwerke showed 70% inhibition of the effects induced by *B. arietans* venom whereas Pasteur only showed 5% protection. The ovine Fab antivenoms showed complete neutralisation of platelet aggregation by both *C. cerastes* and *B. arietans* venoms using 250 and 50g Fab/g venom respectively.

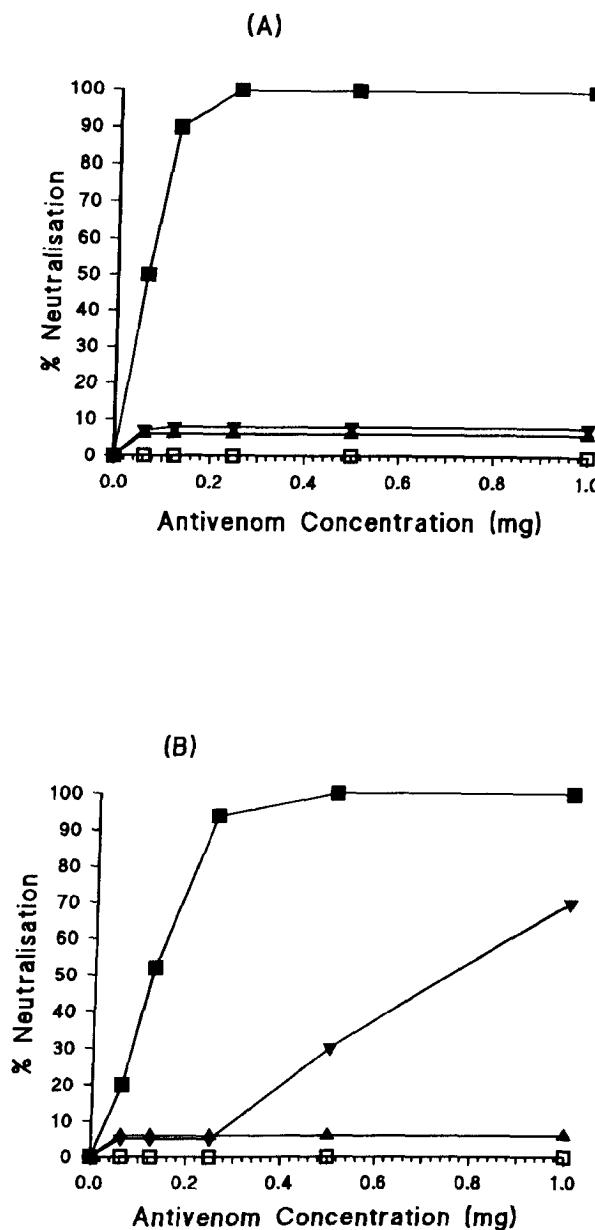
Table 5.8 The Efficacy of Commercial Antivenoms to Inhibit Biological Activities, Expressed as g Antivenom/g Venom. Percentage of Maximum Neutralisation is Given in Brackets.

Venom	Coagulation		Indirect haemolysis		Initiation of platelet aggregation		Haemorrhage		Necrotic		Defibrinogenation	
	P	B	P	B	P	B	P	B	P	B	P	B
<i>E. pyramidum</i>	125	125	100	50	-	-	125	125	12.5 (10%)	12.5 (80%)	142	142
<i>E. coloratus</i>	200	200	400	200	-	-	125 (N)	125 (80%)	500 (N)	500 (N)	500 (N)	500 (N)
<i>C. cerastes</i>	NA	NA	50	50	1000 (10%)	1000 (10%)	-	-	-	-	-	-
<i>B. arietans</i>	NA	NA	NA	NA	1000 (5%)	100 (70%)	-	-	-	-	-	-
<i>N. h. arabica</i>	NA	NA	100 (90%)	100 (90%)	NA	NA	-	-	-	-	-	-

Table 5.8 shows the efficacy of commercial equine Pasteur and Beheringwerke antivenoms tested against the coagulant, indirect haemolytic, platelet aggregation, haemorrhagic, necrotic and defibrinogenating activities of venom from *E. pyramidum*, *E. coloratus*, *C. cerastes*, *B. arietans* and *N. h. arabica*.

P : Pasteur, B: Beheringwerke :- Not tested, NA: No venom activity, (N) No efficacy at the highest antivenom concentration indicated.

Figure 5.6 Neutralising Activity of Ovine and Equine Antivenoms Against Initiation of Platelet Aggregation By (A) *C. cerastes* and (B) *B. arietans*



The efficacy of increasing concentration of Beheringwerke equine F(ab)₂ (▼), Pasteur F(ab)₂ (▲) and ovine Fab (■) in inhibiting *C. cerastes* (A) and *B. arietans* (B) venom induced platelet aggregation were compared using platelet-rich plasma (PRP). Normal sheep serum (□) used as negative control.

5.4.3.4 Haemorrhagic Activity

Both commercial antivenoms protected against the haemorrhagic activity of *E. pyramidum* venom (Figure 5.7). Behringwerke also gave partial protection (80%) against *E. coloratus* venom while the Pasteur product, in amounts up to 1000 g/g, showed no protection. Ovine Fab completely neutralised the activity of both venoms.

5.4.3.5 Necrotic Activity

Beheringwerke antivenom partially neutralised (80%) the necrotic activity of *E. pyramidum* venom, whereas Pasteur showed little protection (10%). Increasing the amount of both antivenoms up to 500 g/g did not give any further improvement. Neither antivenom inhibited the necrotic activity of *E. coloratus* venom. Conversely ovine Fab provided complete protection against *E. pyramidum* and *E. coloratus* venoms at levels of only 3 and 6 Fab g/g respectively.

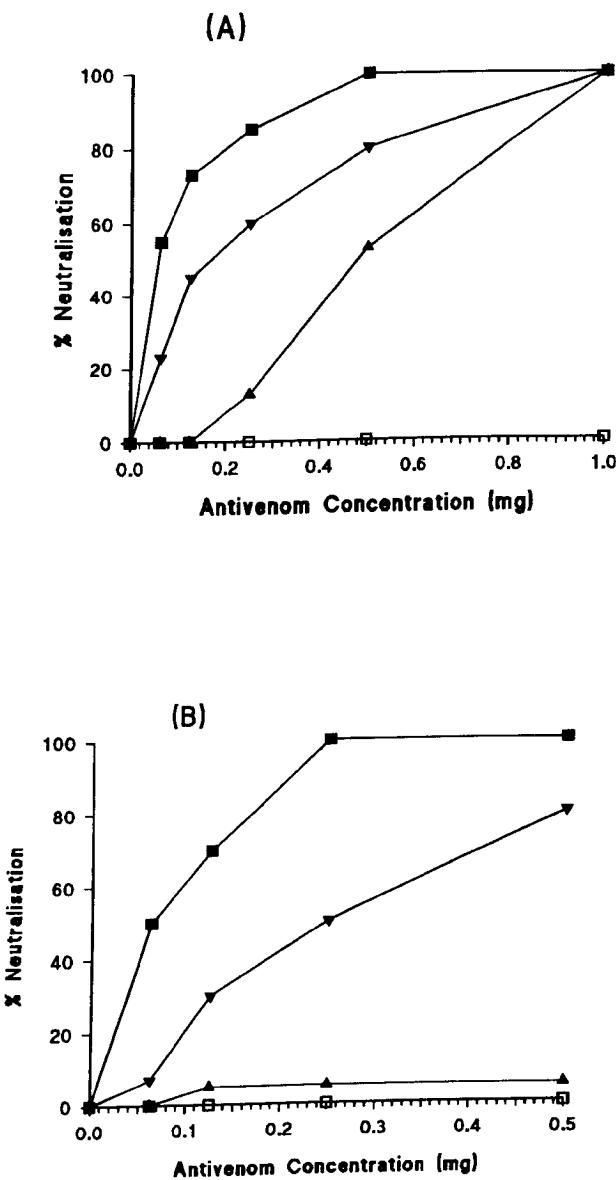
5.4.3.6 Defibrinogenating Activity

Both commercial antivenoms gave complete protection against the defibrinogenating activity of *E. pyramidum* venom whereas neither protected against *E. coloratus* venom even in large amounts. (Table 5.8).

5.4.3.7 Lethal Toxicity

Behringwerke had an ED₅₀ against five times the ivLD₅₀ dose of venoms in the order *E. pyramidum* (8g/g) < *B. arietans* (16g/g) < *E. colorarus* (45g/g) < *C. cerastes* (80g/g) < *N. h. arabica* (187g/g). The Pasteur antivenom had ED₅₀ values that ranged in the order *E. pyramidum* (15g/g) < *B. arietans* (42g/g) < *E. colorarus* (64g/g) < *C. cerastes* (65g/g) < *N. h. arabica* (193g/g) (Table 5.9). The Behringwerke antivenom was more potent than the Pasteur product against all the venoms studied except that from *C. cerastes*. Ovine Fab was considerably more effective than both commercial antivenoms, except against *E. pyramidum* venom, for which its activity was similar to that of Behringwerke (Table 5.3).

Figure 5.7 The Efficacy of Ovine and Equine Antivenoms Against the Haemorrhagic Activity of Venoms from Saudi Arabian (A) *E. pyramidum* (B) *E. coloratus*



The efficacy of increasing concentrations of Beheringwerke equine F(ab)₂ (▼), Pasteur F(ab)₂ (▲) and ovine Fab (■) in inhibiting the haemorrhagic activity of venom from *E. pyramidum* (A) and *E. coloratus* (B) were compared. Venom and antivenom mixes were injected into the dorsal skin of rats. After 24 hours the animals were killed, the dorsal skin removed, and the diameter of the lesions measured on the inner surface of the skin. Normal sheep serum (□) used as negative control.

Table 5.9 ED₅₀ of the Commercial Antivenoms Tested Against Venoms from Saudi Arabian Venomous Snakes

Venom of	Pasteur F(ab) ₂		Behringwerke F(ab) ₂	
	μg F(ab) ₂ /g (mouse)	g F(ab) ₂ /g venom	μg F(ab) ₂ /mouse	g F(ab) ₂ /g venom
<i>E.pyramidum</i>	105 (1400-3100)*	15	1100 (800-600)*	8
<i>E.coloratus</i>	350 (5800-7900)	64	5000 (3800-6300)	45
<i>C.cerastes</i>	195 (3200-4300)	65	4800 (3500-5600)	80
<i>B.arietans</i>	220 (2900-5300)	42	1700 (1100-2500)	16
<i>N.h.arabica</i>	290 (4500-6600)	193	5600 (300-6300)	187

Table 5.9 lists the effective dose of the commercial F(ab)₂ Pasteur and Beheringwerke against five times the lethal dose (ivLD₅₀) of venom from *E. pyramidum*, *E. coloratus*, *C. cerastes*, *B. arietans* and *N. h. arabica*. Mice receive increasing doses of antivenom and the number of survivors in each group were counted after 24hr. The analysis of the slope of the dose-response regressions for the ED₅₀ was performed by the probit method.

* 95% confidence limits per 20g (mouse weight)

5.7 DISCUSSION

The commercial antivenoms available in Saudi Arabia are produced by Pasteur-Mérieux (France) and Behringwerke AG (Germany) by immunising horses. The antisera are then subjected to pepsin digestion of the immunoglobulins and purified and concentrated by salt precipitation to produce bivalent F(ab)₂ fragments which lack the C terminal Fc domains (Latifi, 1978). It is this region of the IgG molecule that is responsible for cell and complement binding and through which many of the hypersensitivity reactions are mediated. The prevalence of horses as a source of immune serum is largely historical since serotherapy developed at the start of this century when horses were plentiful. In this study, sheep were employed.

There are many variables in the production of an antivenom such as differences in the venom pools, the immunisation and bleeding schedules and the processing techniques. The ovine antivenoms were raised using a low-dose, infrequent immunisation schedule whereas conventional hyperimmunisation schedules were used for the equine products. The purity of the final antivenom depends on removing other serum proteins, ensuring that none of the reagents used throughout the processing remain in the final product, and producing a sterile and pyrogen-free product. The technique suggested by the WHO (1981) for purification and cleavage of antivenoms by pepsin digestion and salt precipitation originated with Pope (1939) and has only been modified slightly since then.

The ovine F(ab)₂ was produced on an analytical scale for this study whereas the equine products were prepared on a large scale. Ovine F(ab)₂ was salt precipitated using sodium sulphate whereas the equine fragments were precipitated with ammonium sulphate.

All the equine and ovine F(ab)₂ fragments, as shown by SDS-PAGE and FPLC gel filtration, were at least 95% pure with only minor contaminants present. Both commercial antivenoms were sterile but the Behringwerke product contained low

levels of endotoxin. The ovine antivenoms were not produced under sterile conditions. Small scale affinity purification showed that the percentage of specific antibodies directed against venom components in the equine F(ab)₂ was lower (5 to 13%) than in the ovine F(ab)₂ (19 to 25%). This is probably due to the sheep producing more specific immunoglobulins but might reflect the different venom pools used as immunogen. Thus the venom used as ligand for affinity purification was also used as the ovine immunogen. The equine antivenoms were also polyspecific which is likely to reduce the number of antibody populations against individual venoms.

Most currently available antivenoms consist of IgG or F(ab)₂, whereas Fab may offer certain advantages. Many studies (Russell and Wainschel 1973; Petkovic *et al.*, 1991) have shown that the longer the period between envenoming and antivenom administration, the greater the toxic effects and the more difficult it is to neutralise venom components. Fab, because of its small molecular weight, rapidly distributes from the intravascular compartment into the interstitial space thereby gaining access both to venom components in the general circulation and those localised in tissue and at the bite site. Other advantages of Fab fragments, compared to intact IgG and F(ab)₂, are that they may be less immunogenic and, since they possess only one binding site, do not form immune complexes. They are also partially excreted by the kidneys, whereas IgG is cleared by cells of the immune system (Smith *et al.*, 1979).

Comparing the efficacy of ovine IgG, F(ab)₂ and Fab showed that all completely neutralised the *in vitro* venom activities. However, on a weight and molar ratio, more Fab and F(ab)₂ than IgG were required to neutralise enzymatic and biological activities. Even though IgG may have a higher binding affinity, it is not recommended for therapeutic purposes due to the hypersensitivity and other reactions that may be caused by its Fc component. The ovine Fab neutralised the lethal effect of all venoms in mice suggesting that they contain populations which bind the most toxic venom components.

Both the mono and polyspecific ovine antivenoms raised against *E. pyramidum* and *E. coloratus* venoms protected mice in ED₅₀ studies and neutralised the *in vitro* effects of the venoms. However, the monospecific antivenoms were more effective against their respective venoms. These findings agree with those of Kornalik and Taborska (1989) who reported that monospecific antivenoms provided superior protection as compared to polyspecific products against the lethal effects of *Bothrops* and *E. pyramidum* venoms. Laing and colleagues (1995) also showed that monospecific antivenoms (ovine Fab and South African Institute of Medical Research equine F(ab)₂{SAIMR}) were significantly better than polyspecific antivenoms (Pasteur and Behringwerke) when tested against *E. ocellatus* venom while Mohammed and colleagues (1973) found that the potency of polyspecific antivenoms against the Egyptian venoms of *N. haje* and *N. nigricollis* was comparable for monospecific products.

The antivenom against Saudi Arabian *E. pyramidum* provides good neutralisation of the enzymatic activities of Nigerian *E. ocellatus* venom but did not protect against its potent coagulant activity. Antigenic differences in venom obtained from different geographical areas over the enormous range that these snakes are found might explain the variable potency of particular specific *Echis* antivenoms. Kornalik and Taborska (1973) even reported significant qualitative differences in venoms from individual specimens of *E. pyramidum* caught in the same area. Latifi (1973) found the antivenom raised against the venom of *E. pyramidum* from one country might have little effect in neutralising the venom of *E. pyramidum* from another country. Iddon and colleagues (1985) showed that monoclonal antibodies against a haemorrhagin from one *E. pyramidum* venom was very specific for this particular venom, and no reactivity was observed with *E. pyramidum* from other areas.

The commercial antivenoms showed similar binding activity with venom components from closely related viperid species as well as from some elapid species. Both bound all the venoms tested, but progressively more antivenom was required in the order *E. pyramidum* < *B. arietans* < *C. cerastes* < *E. coloratus* < *N. h. arabica* for the

Behringwerke whereas Pasteur was in the order *E. pyramidum* < *C. cerastes* < *B. arietans* < *E. coloratus* < *N. h. arabica* venoms. Similar results were obtained from small scale affinity chromatography. Both antivenoms showed similar trends apart from *C. cerastes* venom (used as immunogen for Pasteur) and *B. arietans* venom (immunogen for Behringwerke). Behringewerke showed better protection in mice than the Pasteur antivenom. The commercial equine F(ab)₂ showed partial neutralisation against the enzymatic activities of the five venoms used in this study and complete neutralisation of the coagulant and indirect haemolytic activities. However, they did not neutralise initiation of platelet aggregation induced by *C. cerastes* and *B. arietans* venoms (except partial neutralisation by Behringwerke against *B. arietans* venom). Both protected against haemorrhagic activity of *E. pyramidum* venom using a high amount of antivenom.

The ovine Fab and F(ab)₂ preparations compared well with the equine F(ab)₂ products in the lethal and other important *in vivo* and *in vitro* assays and was more effective on a weight-for-weight basis. This emphasizes the importance of immunising with venom which corresponds to the species for which the antivenom is intended.

CHAPTER VI

**ASSESSMENT OF OVINE ANTIVENOM AGAINST DESERT BLACK COBRA
(*WALTERINNESIA AEGYPTIA*) VENOM**

6.1 SUMMARY

The desert black cobra (*Walterinnesia aegyptia*) is an elapid widely distributed throughout the deserts of Saudi Arabia for which currently available antivenoms are ineffective in the treatment of its envenoming. *W. aegyptia* venom was assessed for several of its physicochemical, enzymatic and biological characteristics. Lethality in mice (iv LD₅₀) was high (0.4µg/g of mouse) and the venom showed marked neurotoxicity and haemorrhagic, haemolytic, PLA₂, L-AAO and PDE activity. However, it had only moderate HYL activity, weak proteolytic effects and no coagulant, necrotic or defibrinogenating actions.

Sheep immunised with *W. aegyptia* venom showed a good antibody response as assessed by ELISA and small scale affinity chromatography. Western blotting showed that antibody populations bound most of the venom components. The Fab antivenom adequately neutralised the enzymatic and biological effects and protected mice against lethal amounts of *W. aegyptia* venom. Fractions of ovine antibodies (F(ab)₂ and Fab) were prepared and compared with intact ovine IgG for their neutralising capacity. The amounts required exceeded that of IgG in most *in vitro* assays. Various cross-reactivity methods were used to assess neutralisation by the ovine antivenom of closely related venoms and the highest cross-reactivity was obtained with that from *N. h. arabica*.

The ovine Fab antivenom was compared with two commercial polyspecific equine F(ab)₂ antivenoms (Behringwerke and Pasteur-Mériex) currently available in Saudi Arabia. Affinity purification of these products showed that they comprised 3 and 2 g/l of specific antibody fragments respectively but they only neutralised 35% of the PLA₂ activity and about 50% of both the indirect haemolytic and PDE activity. HYL activity was equally well neutralised by the equine products. The commercial antivenoms showed only a weak cross-reactivity with *W. aegyptia* venom using ELISA and small scale affinity chromatography and did not protect mice against its lethal effects.

6.2 CHARACTERISATION OF *W. AEGYPTIA* VENOM

6.2.1 Physicochemical Assessment

Reverse-phase HPLC showed 13 protein peaks with retention times ranging from 4.6 to 30.8min (Figure 6.1a). In comparison, *N. h. arabica* venom showed 12 protein peaks with retention times ranging from 5.5 to 47.2min (Figure 6.1b) Each venom showed the same profile of different batches. The results of gel filtration chromatography of *W. aegyptia* venom are shown in Figure 6.2. The venom was resolved into five fractions (peaks 1, 2, 3, 4, and 5) with MWt of 150, 40, 20, 10, and less than 10kDa as calculated from the calibration curve. The percentage of each peak were 15, 23, 7, 43 and 0.2% respectively. The protein composition of venom from *W. aegyptia* was also analysed on 10% SDS-PAGE under non-reducing conditions and showed 12 dominant bands with molecular weights ranging from below 23 to above 94 kDa (see Figure 3.3 p73).

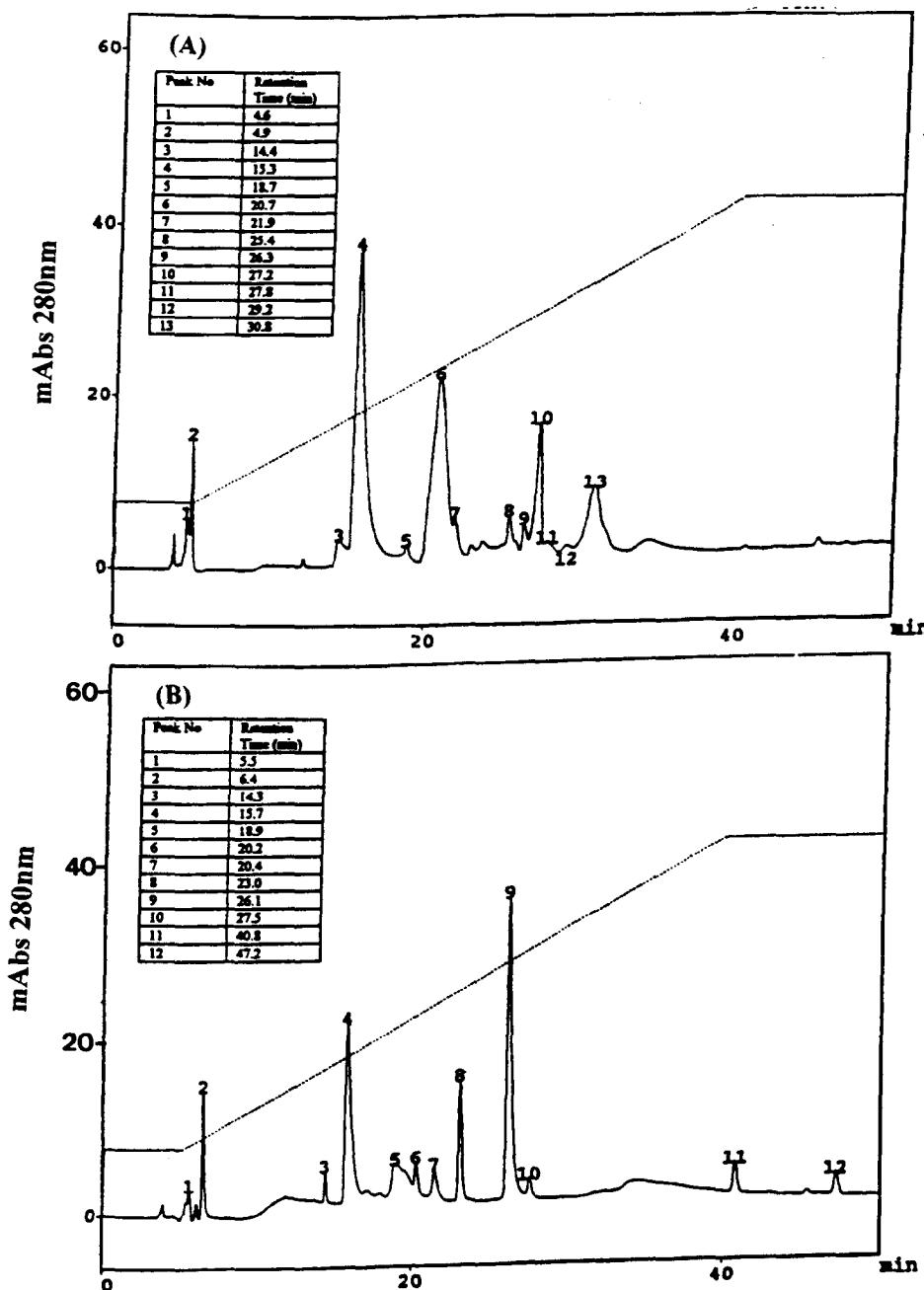
6.2.2 Enzymatic Activities

W. aegyptia venom showed very potent PLA₂ activity and 1µg produced a complete colour change of the pH indicator (Table 6.1). *W. aegyptia* venom had marked PDE activity and also considerable L-AAO effects. However, it had only minimal hyaluronidase and proteolytic activity. For example, using L-BAPNA or dimethyl casein as substrate, maximum proteolytic activity was 40% with 100µg venom.

6.2.3 Biological Activities

All biological assays were performed as described in Chapter II and biological activities of the venom were expressed as the amount required to produce 100% activity.

Figure 6.1 RP-HPLC of (A) *W. aegyptia* and (B) *N. h. Arabica* venoms



The venoms (200 μ g) from *W. aegyptia* (A) and *N. h. arabica* (B) were analysed by reverse phase HPLC. The column (Supelco RP LC-8-DB) was equilibrated with 5% acetonitrile in 0.1% trifluoroacetic acid (TFA) and the protein eluted with gradient of 90% acetonitrile in 0.1% TFA at a flow rate of 0.5ml/min and the absorbance read at 280nm.

Figure 6.2 FPLC Gel Filtration of *W.aegyptia* Venom

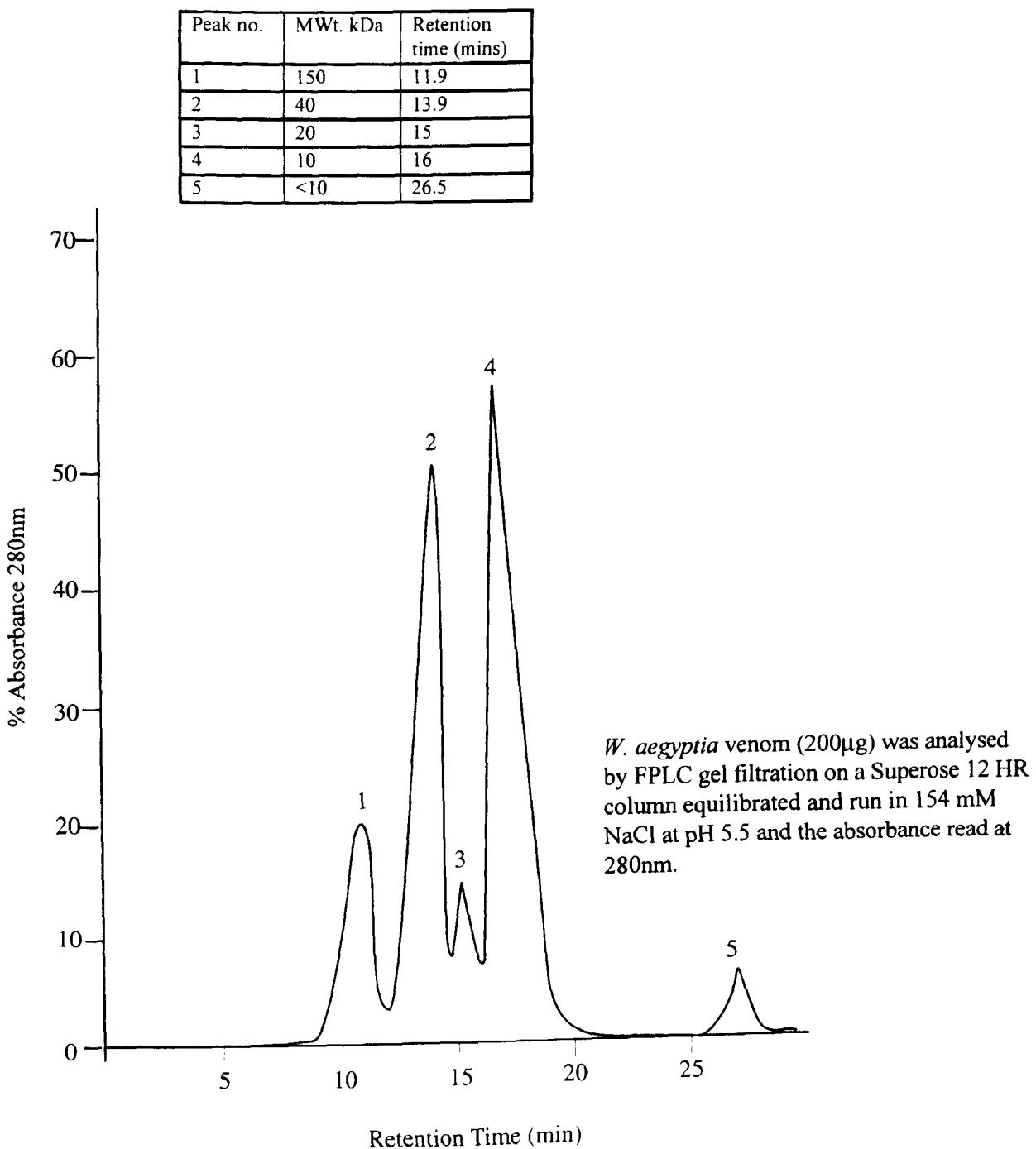


Table 6.1 Enzymatic and Biological Activity of *W.aegyptia* Venom (Venom Activity in µg)

Type of Activity	Venom Activity	Minimum Effective Dose (µg)
Enzymatic	Phospholipase A ₂	0.5 ^a
	Phosphodiesterase	2 ^a
	L-amino acid oxidase	6 ^a
	Hyaluronidase	18 ^a
	Proteolytic activity	100 ³
Biological	Neurotoxicity	300 ¹
	Myotoxicity	300*
	Coagulation	100*
	Indirect hemolysis	25 ¹
	Haemorrhagic	12 ¹
	Defibrinogenating	10*
	Necrotic	12*
	Lethality (iv LD ₅₀)/g	0.4

Table 6.1 lists the minimum amount of *W.aegyptia* venom that is required (in µg) to produce maximum phospholipase A₂, phosphodiesterase, hyaluronidase, L-amino acid oxidase and proteolytic activities using L-BAPNA and dimethyl casein substrate. It also shows its, neurotoxicity, myotoxicity, coagulation, indirect haemolytic, haemorrhagic, necrotic, defibrinogenating and lethal activity.

^aThe minimum amount of venom required to produce 50% activity where ¹= 100%, ³=40% and * = no activity.

6.2.3.1 Coagulation and haemolytic activities

W. aegyptia venom in amount up to 100 μ g had no coagulant activity as assessed using both human plasma and bovine fibrinogen. The venom had powerful indirect haemolytic activity, when using human erythrocytes and phosphatidylcholine as substrate, with 25 μ g inducing 100% haemolysis (Table 6.1). However, in the absence of substrate, 100 μ g of venom caused only 20% direct haemolysis.

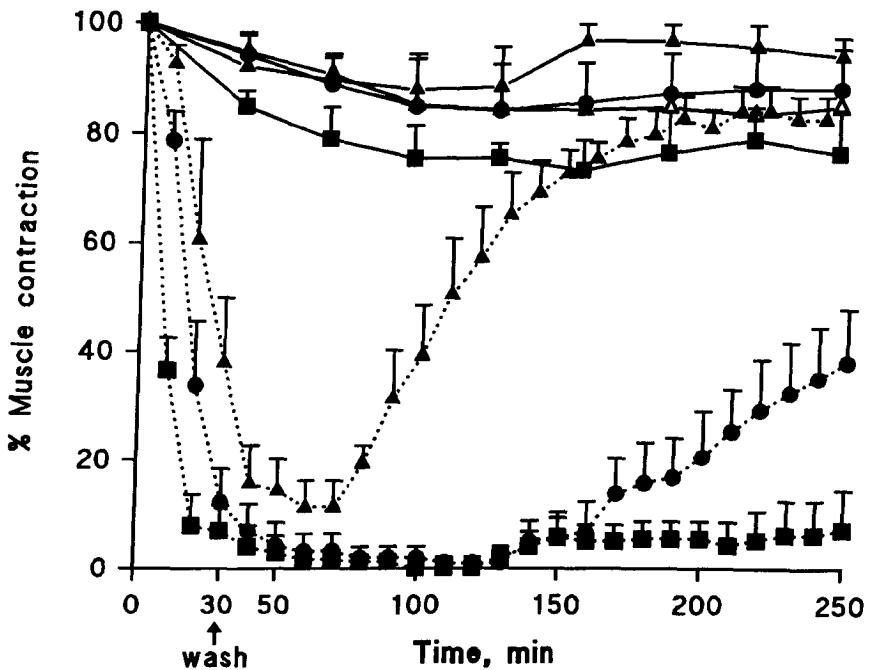
6.2.3.2 Neurotoxicity and myotoxicity

Neurotoxicity and myotoxicity were studied using the mouse phrenic nerve hemidiaphragm preparation. The venom was shown to progressively depress and finally abolish muscle contractions caused by indirect stimulation, while the response to direct stimulation was not affected. Concentrations of 2.5 and 5mg/l produced complete inhibition of muscle contraction in 100 ± 7 and 60 ± 5min respectively, while 1.25mg/l inhibited 90% of muscle contraction in 70 ± 10min (Figure 6.3). Repeated washing every 30min with Krebs' solution resulted in a partial recovery of 40% and 80% respectively to indirect stimulation of the muscle previously treated with 2.5mg/l and 1.25mg/l of *W. aegyptia* venom (Figure 6.4). Neurotoxic activity was dose dependent, as shown by the time taken to reach 80% paralysis at three different concentrations (1.25, 2.5 and 5mg/l) of venom, with results of 45, 30 and 20min respectively (Figure 6.4).

6.2.3.3 Haemorrhagic, necrotic and defibrinogenating activities

W. aegyptia venom showed high and dose dependent *in vivo* haemorrhagic activity and 12 μ g (minimum haemorrhagic dose) produced a lesion of 10mm diameter on the dorsal skin of rats 24hr later (Figure 6.5). It had no necrotic activity when injected intradermally to rats in amounts up to 50 μ g, when checked three days later. At a higher venom concentration the rats died within 4hrs probably due to the neurotoxicity of the venom. *W. aegyptia* venom had no significant effect on defibrinogenating activity, after intravenous injection of up to 12 μ g.

Figure 6.3 Neurotoxicity and Myotoxicity of *W.aegyptia* Venom



A mouse phrenic nerve hemi-diaphragm incubated with 5 (■), 2.5 (●) and 1.25 (▲) mg/l of *W. aegyptia* venom for 30 min. No change in muscle contraction was recorded after direct (muscle) stimulation (solid line) whereas a marked change in muscle contraction was recorded after indirect (nerve) stimulation (broken line). Results are expressed as the mean \pm SEM of four preparations. Preparation was washed every 30min.

Figure 6.4 Dose Response Curve for Venom Using Indirect (Nerve) Stimulation

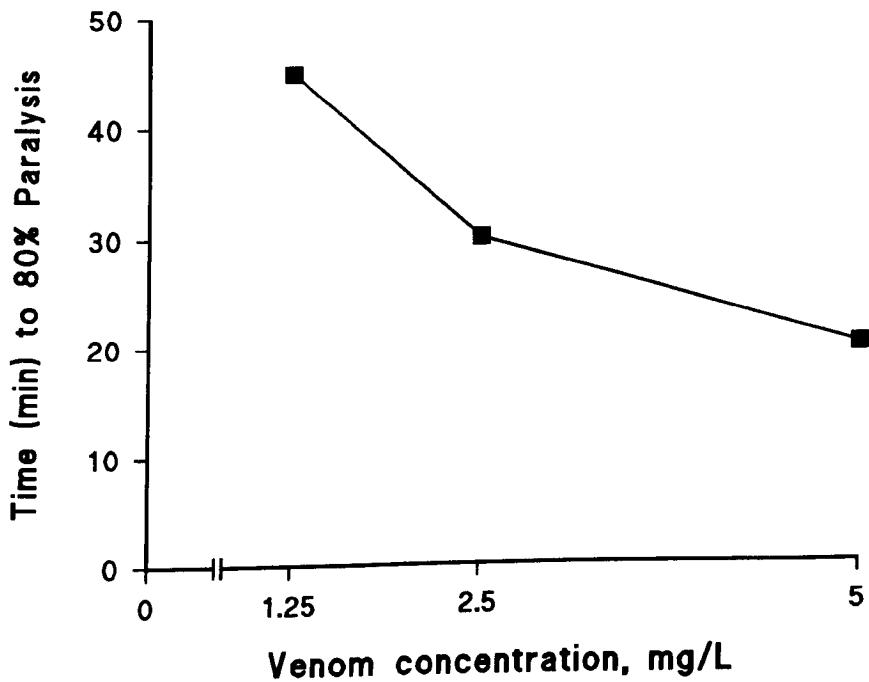


Figure 6.4 shows the dose dependent, neurotoxic activity of *W. aegyptia* venom. 1.25, 2.5 and 5mg/l venom caused 80% of total paralysis after 45, 25 and 20 minutes respectively.

Figure 6.5 Haemorrhagic Activity of *W.aegyptia* Venom

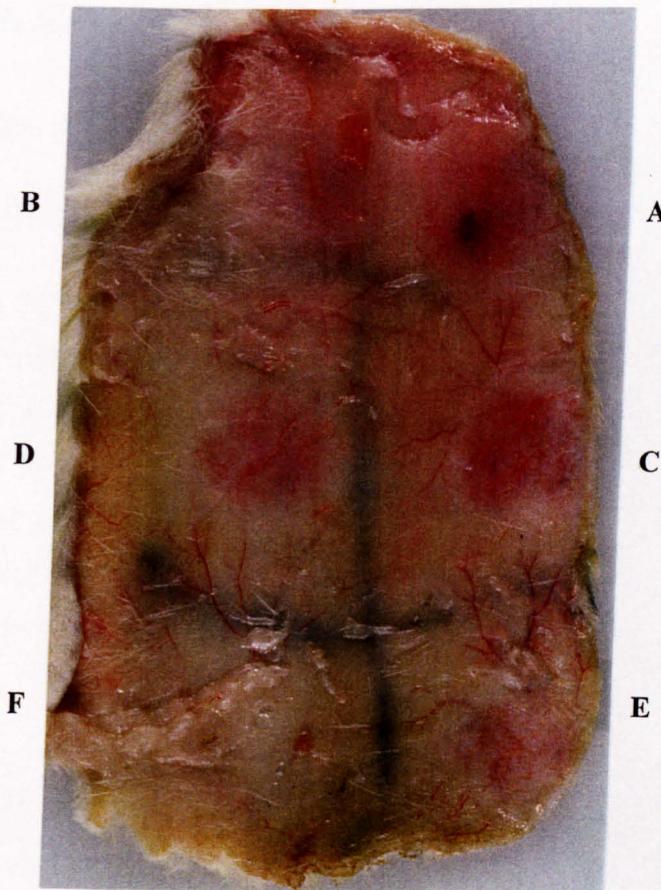


Figure 6.5 shows the haemorrhagic lesions produced when different concentrations of *W. aegyptia* venom (A 12 μ g, B 6 μ g, C 3 μ g, D 1.5 μ g, E 0.75 μ g and F 0.375 μ g) were injected into the dorsal skin of rats. After 24 hours the animals were killed, the dorsal skin removed, and the diameter of the lesions measured on the inner surface of the skin, in two directions at right angles, using calipers and background illumination. The mean diameter of the haemorrhagic lesion was calculated for each venom dose and the MHD was estimated by plotting the mean diameter against dose and reading off the dose corresponding to a 10mm lesion.

6.2.3.4 Lethality

The *W. aegyptia* venom was very toxic to mice with an iv LD₅₀ of 8µg/mouse (0.4µg/g) and 95% confidence limits of 4 to 11µg. The symptoms produced included flaccid paralysis of the extremities and the animals died from respiratory distress within two hr, which are typical findings with an elapid venom containing a powerful neurotoxin(s).

6.3 OVINE ANTIVENOM

All the methods were described in Chapter II.

6.3.1 Characterisation of Ovine Antivenom

Bloods taken 32 weeks after primary immunisation from three sheep immunised with *W. aegyptia* venom were pooled, filtered and the immunoglobulin fraction purified by salt precipitation (Chapter II). The total protein concentration of the serum pools was 60g/l and, of this, about 40% was removed in the supernatant leaving 60% in the immunoglobulin fraction (Table 6.2). SDS-PAGE showed that salt precipitation removed most of the albumin, while virtually all the immunoglobulins appeared in the precipitate. The serum IgG fraction eluted as single peak on FPLC.

The immunoglobulin fraction (520ml) was then digested with papain, to give a product that was 96% Fab as assessed by gel filtration FPLC. SDS-PAGE showed that the immunoglobulins, and many other components present in smaller quantities in the IgG fraction, had disappeared after papain digestion leaving only one band of approximately 46kDa MWt.

To prepare the ovine F(ab)₂, bleeds from sheep immunised with *W. aegyptia* venom were pooled, salt precipitated and the precipitate (mainly IgG) digested with pepsin (20mg/g IgG) and purified by salt precipitation as described in Chapter II. The ovine F(ab)₂ was finally concentrated by freeze-drying and resuspended in PBS (Table 6.2).

Table 6.2 Preparation of Immunoglobulins and their Fractions from *W.aegyptia* Antivenom

	Serum pool	IgG fraction	Fab fraction	F(ab) ₂ fraction
Protein, g/l	60	34	16	18
Specific antibody, g/l	9.6	9	6	6
ELISA titre	150,000	130,000	35,000	75,000

Table 6.2 lists the protein and specific antibody concentrations (g/l) and ELISA antibody titres of the serum, IgG, F(ab)₂ and Fab fractions raised against *W. aegyptia* venom. The immunoglobulin fraction was purified by salt precipitation then cleaved with papain to produce Fab or pepsin to produce F(ab)₂.

6.3.2 Binding Activity

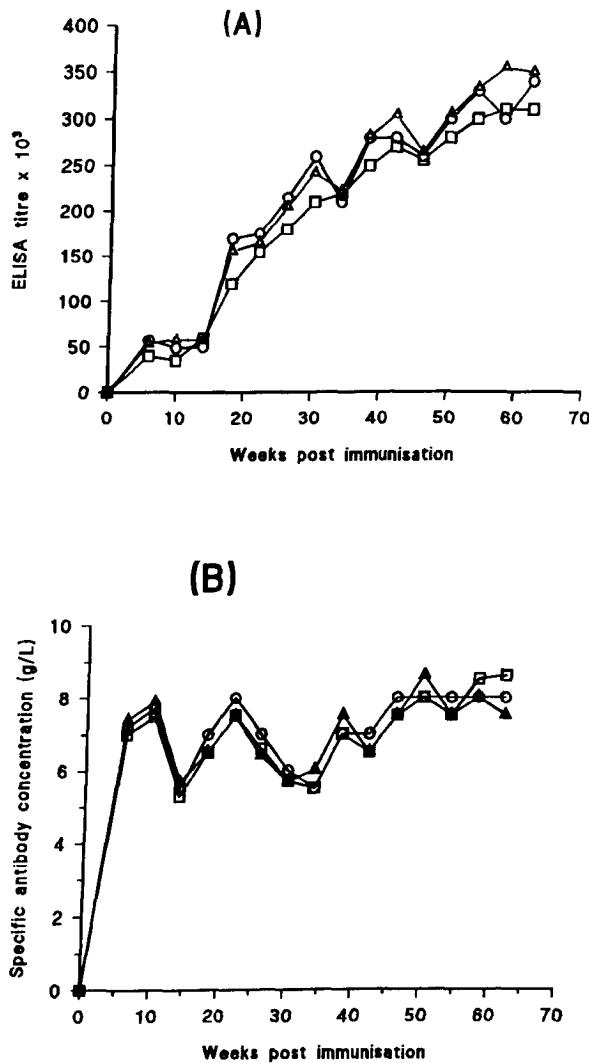
Three Welsh half breed ewes were immunised with 0.5mg of *W. aegyptia* venom at monthly intervals. The antibody titres rose to high levels within 16 weeks of the primary immunisation with all sheep showing similar ELISA titres (1.3, 1.1 and 1.5×10^5). The highest titre for the pooled serum collections throughout the two years of study was 3.5×10^5 (Figure 6.6) and the specific antibody concentration ranged between 7 and 9g/l as determined by small-scale affinity purification. Gradually increasing the dose of immunogen to 4mg/sheep/month after 40 weeks did not significantly improve either the antiserum titre or specific antibody concentration.

The ELISA antibody titre of the Fab fractions appeared to fall markedly compared to the IgG fractions with apparent losses of 70% (Table 6.2). As discussed previously, this loss is apparent rather than real since detection of the antibodies and antibody fragments bound to venom depends on the binding by a second enzyme-labelled antibody reagent raised against intact ovine IgG. This will, therefore, contain many antibody populations directed against the Fc fraction present in intact IgG but not in $F(ab)_2$ or Fab.

6.3.4 Neutralisation of Enzymatic Activities

Neutralisation of enzyme activities was assessed as described in Chapter II by determining the minimum amount of antivenom in mg required to neutralise completely the enzymatic activities induced by 1mg of venom (Table 6.3). Five hundred mg of the specific ovine Fab completely neutralised the PLA₂ activity of *W. aegyptia* venom while 40mg neutralised 80% of its HYL activity. Complete neutralisation was obtained with 62mg of Fab fragment against PDE activity of the *W. aegyptia* venom.

Figure 6.6 Immune Response of Three Sheep Immunised With Desert Black Cobra Venom.



Groups of three sheep were immunised with 0.5mg of *W. aegyptia* venom per month and bled two weeks after each immunisation. Monthly bleeds were assessed by ELISA (A) and small scale affinity chromatography (B) (as described in section 2.4.1.2).

Table 6.3 Comparison of Ovine and Equine Antivenoms

Venom Activity	Minimum Neutralisation Dose*	
	Ovine Fab	Equine F(ab) ₂
Phospholipase A ₂	500	1000 ²
Phosphodiesterase	62	300 ³
Hyaluronidase	40 ¹	25
ED ₁₀₀	250	No protection
Neurotoxicity	100	-
Indirect haemolysis	8	160 ⁴
Haemorrhagic	10	No protection

Table 6.3 compares the efficacy of ovine Fab and the commercial equine F(ab)₂ fractions to inhibit phospholipase A₂, phosphodiesterase, hyaluronidase, indirect haemolytic and haemorrhagic activities of *W. aegyptia* venom.

* The minimum amount of antivenom in mg required to neutralise completely (100%) the effect induced by 1mg of venom, 1: Maximum neutralisation 80%, 2: Maximum neutralisation 35%, 3: Maximum neutralisation 50% and 4: Maximum neutralisation 50%. -: not tested

6.3.4 Neutralisation of Biological Activities

6.3.4.1 Haemolytic activity

The amount of antivenom required to neutralise 100% of the indirect haemolytic activity of venom was 8mg of Fab per mg venom. A control experiment using the IgG fraction of normal sheep serum showed no inhibition.

6.3.4.2 Neurotoxicity

The neutralising efficacy of specific ovine Fab antivenom against the neurotoxic effect of *W. aegyptia* venom was studied *in vitro* using the mouse phrenic nerve hemidiaphragm. Complete protection, as assessed using indirect stimulation, required 100mg of the ovine Fab fraction per mg venom (Figure 6.7).

6.3.4.3 Haemorrhagic activity

The ovine Fab effectively protected against the haemorrhagic lesions normally induced by *W. aegyptia* venom (Figure 6.8) with 10mg giving complete protection against the haemorrhagic activity of 1mg venom (Table 6.3).

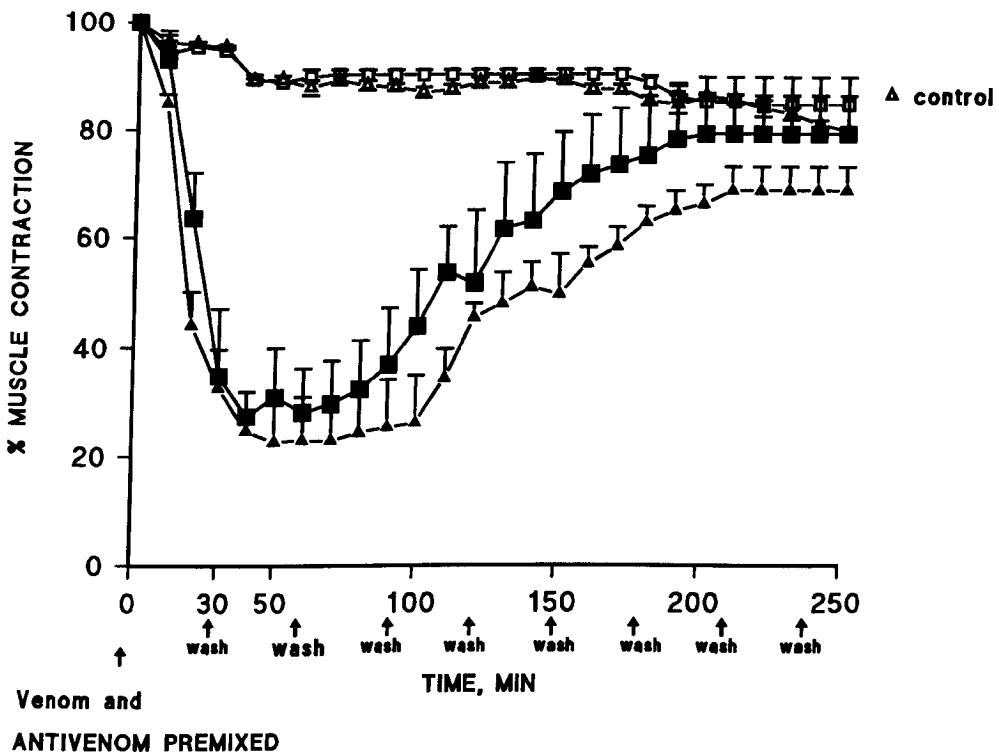
6.3.4.4 Lethality

The antivenom showed very potent protection with an ED₅₀ for the Fab fraction of 5mg/mouse and narrow confidence limits (viz 4.5-6mg/mouse). The total amount of the Fab fraction required for 100% protection was 250mg per mg venom.

6.3.5 Cross-Neutralisation Studies

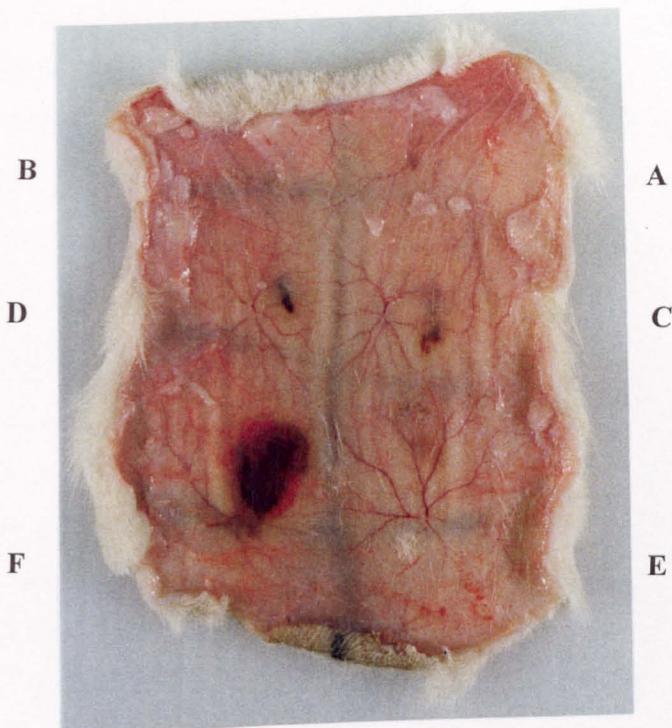
Six venoms (from *W. aegyptia*, *N. h. arabica*, *E. pyramidum*, *E. coloratus*, *C. cerastes* and *B. arietans*) were separated on SDS-PAGE, transferred to nitrocellulose paper and

Figure 6.7 Neutralisation of Indirect Stimulation of *W. aegyptia* venom, Using Specific Ovine Fab Antivenom



A mouse phrenic nerve hemi-diaphragm preparation was incubated with a premixed solution of 5mg/l *W. aegyptia* venom and 500 (□), 250 (■) or 125mg/l (▲) of specific ovine Fab antivenom for 30min. Results are expressed as the mean ± SEM of four preparations. Preparation was washed every 30min.

Figure 6.8 Neutralisation of Haemorrhagic Activity of *W.aegyptia* Venom Using Specific Ovine Fab



Specific ovine Fab (A 500 μ g, B 250 μ g, C 125 μ g, D 62 μ g and E 31 μ g) was premixed with 12 μ g of *W. aegyptia* venom and injected into the dorsal skin of rats. After 24 hours the animals were killed, the dorsal skin removed, and the diameter of the lesions measured on the inner surface of the skin. The only lesion observed was with venom alone (F). Haemorrhagic activity was neutralised at all antivenom concentrations as evidenced by the absence of lesions.

incubated with the IgG fraction of the antivenom raised against *W. aegyptia* venom. Antibodies raised against *W. aegyptia* venom bound most of its own components and a few of the higher MWt components found in each of the other venoms (Figure 6.9). It produced most bands with *N. h. arabica* venom. Normal sheep serum did not bind to any of the venom components.

The IgG fraction of the antivenom raised against *W. aegyptia* venom showed the highest cross-reactivity in an ELISA with *N. h. arabica* venom (60%), followed by *B. arietans* (42%), *C. cerastes* (36%), *E. coloratus* (34%) and *E. pyramidum* (25%) venoms (Figure 6.10).

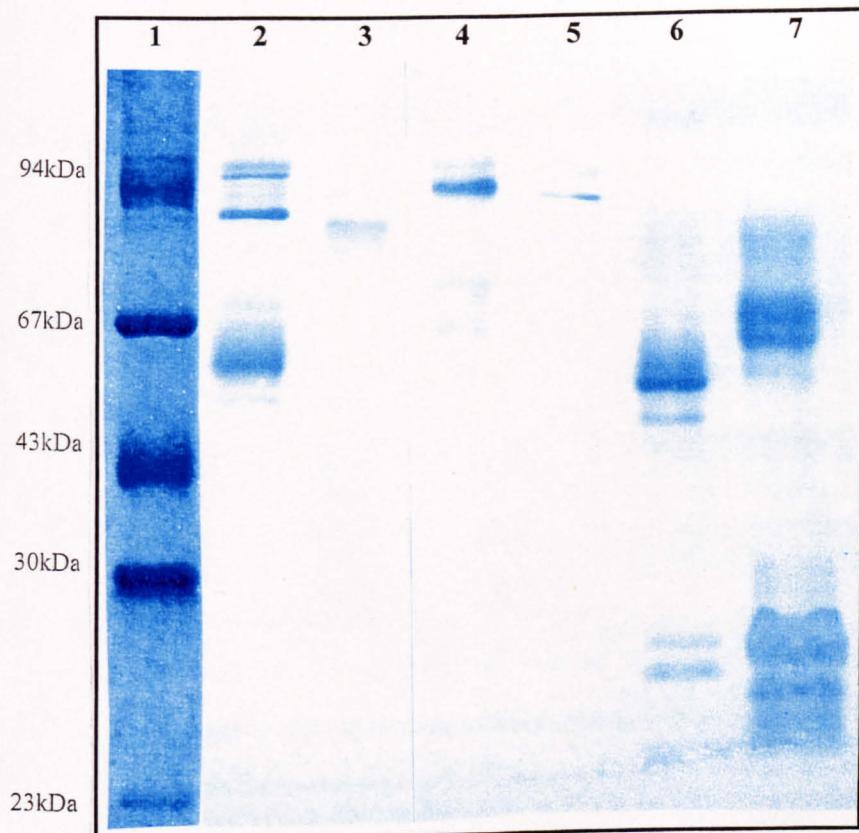
Small scale affinity purification columns were prepared using venoms from *W. aegyptia*, *N. arabica*, *E. pyramidum*, *E. coloratus*, *C. cerastes* and *B. arietans* coupled to Sepharose 4B. These were incubated with a fixed amount of the IgG fraction raised against *W. aegyptia* venom and cross-reactivity was expressed as a percentage of the protein obtained from the antivenom bound to each of different venoms. The highest percentage concentrations were obtained from the column of *N. h. arabica* venom (62%), followed by *B. arietans* (40%), *C. cerastes* (44%), *E. coloratus* (30%) and *E. pyramidum* (30%) venom.

6.4 COMPARISON OF IgG, F(ab)₂ AND Fab FRACTIONS

6.4.1 Binding Activity

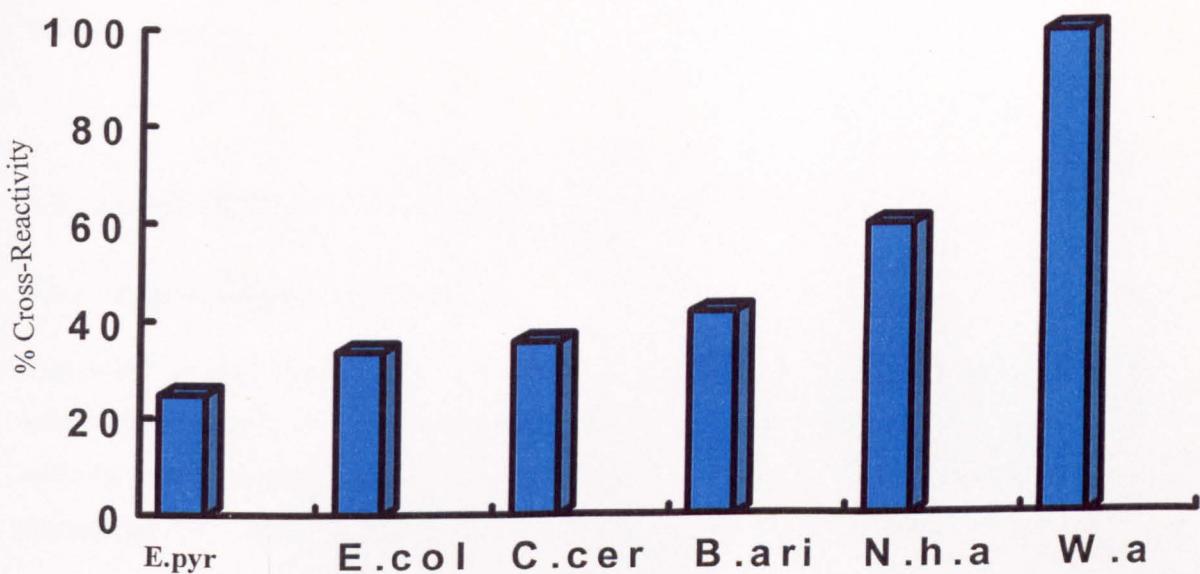
A small scale affinity column comprising *W. aegyptia* venom coupled to Sepharose 4B was incubated with different amounts of antivenom fractions raised against *W. aegyptia* venom. The total concentrations eluted were 9g/l from serum and the IgG fraction whereas 7g/l were obtained for the F(ab)₂ and Fab fractions. The ELISA titres were 320, 75 and 35×10^3 for IgG, F(ab)₂ and Fab respectively but the technique does not enable an accurate comparison (as noted above).

Figure 6.9 Western Blotting of *W. aegyptia* Ovine IgG Antivenom Against Saudi Arabian Elapid and Viper Venoms.



Venoms from *E. pyramidum* (lane 2), *E. coloratus* (lane 3), *C. cerastes* (lane 4), *B. arietans* (lane 5), *N. h. arabica* (lane 6) and *W. aegyptia* (lane 7) were separated on 10% SDS-PAGE gel, then transferred to nitrocellulose paper and incubated with antivenom (200mg/l) raised against *W. aegyptia* venom. After washing, the strips were incubated with donkey anti-sheep IgG coupled to horseradish peroxidase followed by incubation with substrate until the bands were well developed (about 10min). The strips were then washed in water and photographed. Lane 1 contained MWt markers.

Figure 6.10 Percentage ELISA Cross-Reactivity of IgG Ovine Antivenom Raised Against *W.aegyptia* Venom.



Microtitre plates were coated with the different venoms (200ng/well) *E.pyramidum*, *E.coloratus*, *C.cerastes*, *B.arietans*, *N.h.arabica* and *W.aegyptia* and then incubated with the IgG fraction of the ovine antivenom raised against *W.aegyptia* venom. Cross-neutralisation was expressed as a percentage of the titre obtained with *W.aegyptia* venom. The greatest degree of cross-reactivity occurred with *N.h.arabica* venom (60%).

6.4.2 Neutralisation of Enzymatic and Biological Activities

Ovine IgG antivenom and its F(ab)₂ and Fab fractions completely neutralised the PLA₂ activity induced by 1µg of *W. aegyptia* venom at doses of 250µg, 500µg and 500µg respectively. The IgG fraction of antivenom showed higher neutralising efficacy (90%) against the HYL activity than ovine F(ab)₂ and Fab (80%). Similar amounts (250µg) of IgG and its F(ab)₂ and Fab fractions completely neutralised the PDE activity induced by 8µg of *W. aegyptia* venom. The total amounts required to neutralise 100% of the indirect haemolytic activity of 25µg of venom were 100µg of IgG and 200µg of both F(ab)₂ and Fab (Figure 6.11).

6.5 COMPARISON OF OVINE AND EQUINE ANTIVENOMS

6.5.1 Physicochemical Assessment

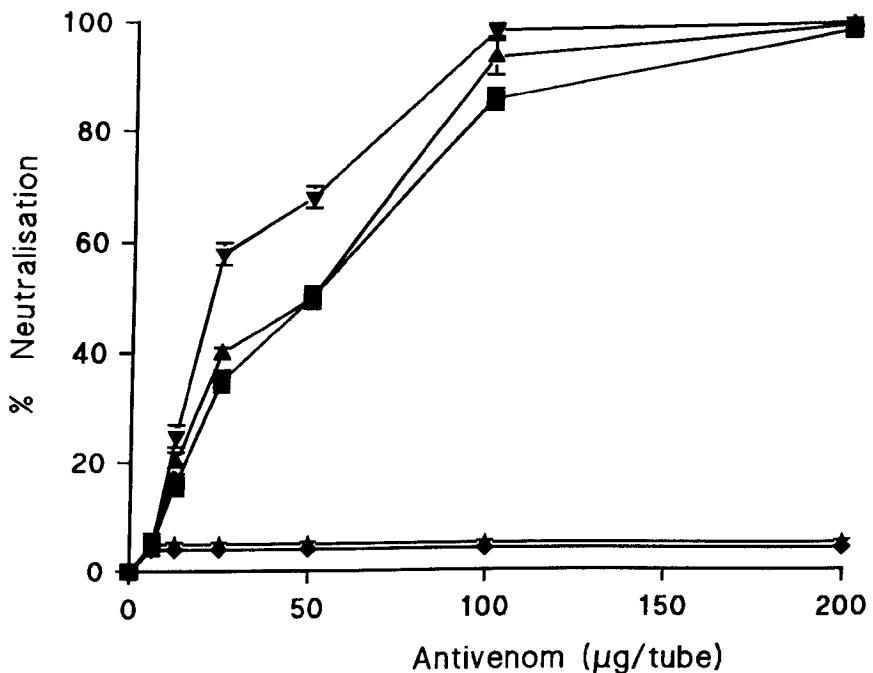
The total protein concentration of ovine F(ab)₂ was 18g/l as assessed using spectrophotometry (Table 6.2) and those of the Behringwerke and Pasteur products were 40 and 20g/l respectively. The antivenoms were dialysed against PBS to remove contaminants then lyophilised and the concentrations of all three adjusted to 100g/l.

Purities were assessed individually by SDS-PAGE and showed a dominant band of F(ab)₂ with a molecular weight of about 90kDa. There was another faint band in all the products with a MWt less than 50kDa. FPLC of the ovine and the commercial antivenoms showed one major peak representing F(ab)₂ and comprising 95% of the total protein applied. Its MWt was about 90kDa.

6.5.2 Binding Assays

Antibody titres were assessed using ELISA and gave a value of 75×10^3 for the ovine F(ab)₂, 20×10^3 for the Behringwerke product and 17×10^3 for the Pasteur antivenom. The specific antibody concentration of the ovine F(ab)₂, using small scale affinity chromatography, was 6g/l as compared with values for the Behringwerke and Pasteur products of 3 and 2g/l respectively.

Figure 6.11 Neutralisation of Indirect Haemolytic Activity of *W. aegyptia* Venom Using Specific Ovine Antivenom.



The efficacy of increasing concentrations of ovine IgG (▼), F(ab)₂ (▲) and Fab (■) in neutralising the haemolytic activity of *W. aegyptia* venom was compared, using phosphatidylcholine as a substrate and human erythrocytes. The activity is expressed as the increase in OD at 375nm. {Normal sheep IgG (◆) and Fab (★)}

6.5.3 Neutralisation of Enzymatic Activities

Both commercial antivenoms could neutralise only 35% of PLA₂ activity of *W. aegyptia* venom (Figure 6.12) as compared with complete neutralisation with the ovine F(ab)₂. However both the equine F(ab)₂ products were more potent against HYL activity with 25mg completely neutralising one mg venom (Table 6.3). The Pasteur and Behringwerke F(ab)₂ could only partially (50%) neutralise PDE venom activity, even when using 300mg antivenom/mg venom (Table 6.3). This compared with the complete neutralisation obtained with 62mg ovine F(ab)₂.

6.5.4 Neutralisation of Biological Activities

The two polyspecific equine antivenoms did not neutralise the haemorrhagic activity of *W. aegyptia* venom but produced 50% neutralisation of indirect haemolytic activity (Table 6.3). Of particular note, neither offered protection against five times the iv LD₅₀ dose of *W. aegyptia* venom (40µg/mouse) even when used in amounts up to 10mg.

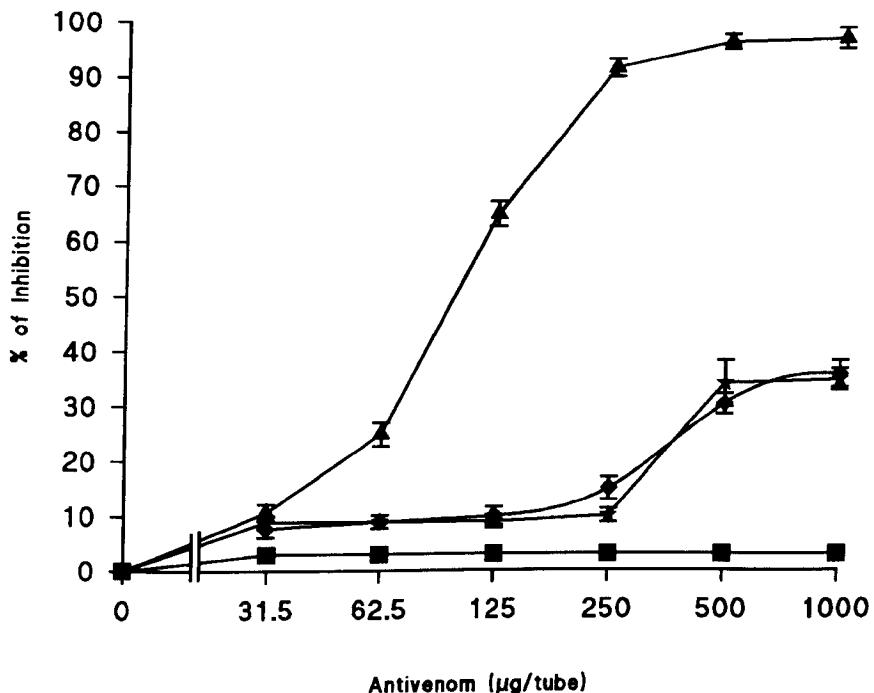
6.5.5 Cross-Neutralisation Studies

Cross-reactivities of the two polyspecific equine antivenoms were assessed using ELISA and small scale affinity purification for comparison with the specific ovine antivenom. Behringwerke antivenom showed 18% cross-reactivity and the Pasteur product 14% as assessed by ELISA. Affinity purification showed that 8% of Behringwerke and 10% of Pasteur F(ab)₂ bound to components of *W. aegyptia* venom.

6.6 DISCUSSION

W. aegyptia is an extremely poisonous snake found in Saudi Arabia, and its envenoming is always serious and can be fatal. In the present study the iv LD₅₀ of *W. aegyptia* venom was 8µg/mouse which agrees with the value of 6µg reported by Gitter

Figure 6.12 Antivenom Neutralised Phospholipase A₂ Activity of *W.aegyptia* Venom Using Specific ovine Antivenom



The efficacy of increasing concentrations of equine F(ab)₂ Beheringwerke (★), Pasteur (◆) and ovine F(ab)₂ (▲) in neutralising the phospholipase A₂ activity of *W. aegyptia* venom was compared. The activity was measured using the substrate phosphatidylcholine and the activity expressed as the increase in OD at 375nm caused by different amounts of each antivenom. Normal sheep IgG was the control (■).

et al. (1962), and of 13 μ g by Hassan and Saddik (1980). Mice that received a lethal dose showed paralysis of the hind legs, impairment of respiration and increasing muscle weakness. They often had convulsions and died within two hours. Venoms from elapid snakes are characterised by their neurotoxic activity and *W. aegyptia* venom showed a dose-dependent inhibition of nerve stimulated muscle contractions using the mouse hemi-diaphragm preparation. The venom of *W. aegyptia* has been fractionated and characterised (Lee *et al.* 1976) with particular reference to the pharmacological properties of the short chain post-synaptic neurotoxins. *W. aegyptia* venom, unlike cobra and some other elapid venoms, appears to be devoid of cardiotoxic components (Lee, 1972).

In this study, *W. aegyptia* venom showed marked haemorrhagic but little proteolytic activity. The haemorrhagic lesions produced at the site of snakebites have been ascribed to proteolytic enzymes (Houssay, 1930; Zeller, 1948) but fractionation of *W. aegyptia* venom yielded a haemorrhagin containing fraction (fraction III) which had no proteolytic activity when tested on gelatine, casein and denatured haemoglobin (Gitter *et al.*, 1962). It seems, therefore, that the action of snake venom haemorrhagins can not be attributed exclusively to proteolytic enzymes. The venom did not exhibit coagulant, necrotic, defibrinogenating or myotoxic activity and its direct haemolytic activity was low (20%). The venom has high PLA₂ activity. Most venoms contain different PLA₂'s and the basic PLA₂'s are thought to cause more biological damage than their acidic counterparts (Rosenberg, 1979). They hydrolyse membrane bound phospholipids and are responsible for many adverse effects directly or indirectly through the release of fatty acids and lysolecithin (e.g. lysis of erythrocytes and leukocytes, neurotoxicity, cardiotoxicity, cytotoxicity and local myonecrotic effects).

In this study *W. aegyptia* venom was found to have high L-AAO activity. This enzyme is generally thought to be of low toxicity but may contribute to the local damage caused by this venom. PDE is one of the well known enzymes contained in snake venoms and hydrolyses both DNA and RNA (Miller *et al.*, 1970) and cAMP (Suzuki *et al.*, 1960). Little is known about its pharmacological effects (Sugino, 1957).

Sheep immunised with a low dose (0.5mg) of *W. aegyptia* venom responded well as assessed by ELISA titres and small scale affinity chromatography. Increasing the venom dose further did not improve antibody levels. The antivenom raised against *W. aegyptia* venom completely neutralised the PLA₂ and PDE activity of the venom and was particularly effective in protecting against indirect haemolytic activity. HYL activity was partially neutralised and this would, hopefully, retard the venom's distribution from the bite site. Ovine Fab also completely neutralised the neurotoxicity of *W. aegyptia* venom as assessed by the mouse hemidiaphragm preparation and a Fab based antivenom should distribute more rapidly and penetrate faster to the neuromuscular junction than intact IgG and F(ab)₂ products due to its smaller size. The ovine Fab antivenom was markedly effective at neutralising the overall lethal effects of *W. aegyptia* venom suggesting that Fab populations bind most venom components.

Western blotting showed that most components in *W. aegyptia* venom which were detected on SDS-PAGE were also bound by antibody populations present in the antivenom. However, it should be noted than binding does not necessarily equate to neutralisation of the pharmacological effects of the venom. Western blotting also showed that this antivenom bound several components in *N. h. arabica* venom and may offer some protection against this species whereas there was less binding against other species tested. *W. aegyptia* and *N. h. arabica* belong to the same family and may have a similar venom composition.

On a weight ratio more Fab and F(ab)₂ than IgG was required to neutralise PLA₂ and indirect haemolytic activity and IgG was more efficient in neutralisation studies. More Fab than F(ab)₂ was required to neutralise PLA₂ and HYL activity.

The equine antivenoms currently available in Saudi Arabia are all polyspecific and subjected to pepsin digestion and salt precipitation. There are many variables in the production of an antivenom such as differences in the immunisation and bleeding schedules. The ovine antivenom was raised using a low-dosage immunisation schedule whereas conventional hyperimmunisation schedules were used for the equine products.

Ovine F(ab)₂ was produced only on a small scale for this study whereas the equine were large scale products. As shown by SDS-PAGE and FPLC, all were at least 95% pure with only minor contaminants present. The major protein peaks detected by SDS-PAGE also showed as peaks by gel filtration, but minor bands shown by SDS-PAGE were not visible by gel filtration.

Small scale affinity purification showed that the proportion of antibodies present in the equine antivenoms and directed against *W. aegyptia* venom components was relatively small. The universal method used to test effectiveness is the ED₅₀ in mice, and both commercial antivenoms were ineffective in this respect although they neutralised hyaluronidase activity more efficiently than the ovine fragments. Both equine F(ab)₂ antivenoms also showed only weak neutralisation of PLA₂, indirect haemolytic activity and phosphodiesterase activity. Antivenoms raised against *W. aegyptia* venom are known to cross-neutralise with other closely related elapid species. However cross-reactivity studies with both commercial equine F(ab)₂ products using ELISA and small scale affinity chromatography showed very weak binding to *W. aegyptia* venom.

CHAPTER VII

FINAL DISCUSSION

FINAL DISCUSSION

The Kingdom of Saudi Arabia and the surrounding areas are inhabited by a number of venomous snakes that are of potential danger. Indeed several of the world's most lethal snakes are found in this area of the Middle East with the venomous varieties being divided into four families: Elapidae, Hydrophidae, Viperidae and Atractaspidae. Of the ten venomous species of land snakes found, vipers cause most of the snakebites. Thus the sand viper (*Cerastes cerastes*) is the most frequently observed whereas carpet vipers (*E. pyramidum* and *E. coloratus*) are responsible for most of the bites and deaths. *Bitis arietans*, the puff-adder, has been described as the greatest killer of man (Dowling *et al.*, 1968) while the venoms from the two elapid species found in Saudi Arabia (*Naja haje arabica* and *Walterinnesia aegyptia*) are extremely poisonous due to their neurotoxicity. Finally, venom from the burrowing asps (*Atractaspis microlepidota andersonii* and *A. m. engaddensis*) are both neurotoxic and cardiotoxic.

There are numbers of deaths attributed to these species annually with rural farmers, Bedouin nomads and members of the armed forces being particularly at risk. Thus it is unfortunate that the Kingdom of Saudi Arabia is one of the least investigated countries concerning envenoming by snakes. The information available is sparse and fragmentary with a number of single reports (Annobil, 1993; Kingston, 1981) and small retrospective studies. As a result, it is not possible to estimate accurately the total number of bites and the resultant mortality (Al-Sadoon, 1989).

The clinical and pharmacological effects that follow envenoming depend primarily on the venom's toxicity and the quantity of venom injected. Toxicity is related to the pharmacological and enzymatic activities of a venom's components and snake venoms are complex mixtures of enzymes and various toxins and non-toxins that may induce both local and systemic effects. Physical, chemical and biological methods were established to measure the *in vivo* and *in vitro* effects of these venoms and it was found

that while the venoms shared many features, there were also differences. For example, all the venoms tested had PLA₂ activity with those from *E. coloratus*, *E. pyramidum* and *N. h. arabica* being the most active. Elapid venoms generally have low hyaluronidase activity but this was not the situation with *N. h. arabica* venom. *C. cerastes* venom showed even higher HYL activity which may contribute to localised swelling, blistering and necrosis. L-amino acid oxidase (L-AAO) is generally thought to be of low toxicity but may also contribute to the local damage. Viperid venoms contain many proteolytic enzymes (Kocholaty *et al.*, 1971; Iwanaga and Suzuki, 1979; Seegers and Ouyang, 1979) that cause coagulation, haemorrhage, necrosis and fibrinogenolysis (Tu *et al.*, 1969; Ewart *et al.*, 1970; Ouyang and Huang 1976) and most of the venoms also hydrolysed the two synthetic substrates studied, namely dimethyl casein and L-BAPNA. General haemorrhage is one of the characteristic clinical manifestations which follows viper envenomation and especially that by *Echis* species as seen in the present study. Both *E. coloratus* and *E. pyramidum* venoms also caused local necrosis when injected intradermally and defibrination *in vivo*. Venoms from both *Echis* species also had considerable coagulant activity with that from *E. ocellatus* venom being the more powerful in this respect. *Echis* species venoms contain proteins that initiate intravascular coagulation.

W. aegyptia venom, unlike cobra and some other elapid venoms, appears to be devoid of cardiotoxic components (Lee, 1972) and, in this study, showed marked haemorrhagic but only minimal proteolytic activity. The venom did not exhibit coagulant, necrotic, defibrinogenating or myotoxic activities. *C. cerastes* venom also showed no coagulant activity and, while *B. arietans* venom has been reported to have either coagulant or anticoagulant properties (Labib *et al.*, 1981; Visser and Chapman, 1978), in this study the venom did not show any coagulant activity. Snake venoms can effect platelets in various ways; for example some venoms induce while others inhibit platelet aggregation and both these activities may exist in the same venom (Shebuski *et al.*, 1989). In this study *C. cerastes* and *B. arietans* venoms showed a dose-dependent initiation of platelet aggregation.

The decrease in muscle contraction observed with *N. h. arabica* and *W. aegyptia* venoms, when using the mouse hemidiaphragm preparation, is due to post-synaptic neurotoxin(s) blocking nerve conduction and neither venom was myotoxic. Venoms from *A. microlepidota*, *W. aegyptia* and *N. h. arabiac* were the most lethal (4-8 μ g/mouse), and were much more toxic to mice than the viperid venoms tested. Whereas *A. microlepidota* and *W. aegyptia* inject only a small amount of venom, *N. h. arabica* injects a large amount, so that death may occur in a few minutes. *E. pyramidum*, *E. coloratus*, *E. ocellatus*, *C. cerastes* and *B. arietans* venoms all showed similar lethality (12-28 μ g/mouse).

It should be stressed that the *in vitro* measurement of enzyme and biological activities made in this study may not relate to the complex interactions that occur in living tissues. Nonetheless, the object of this study was to raise improved antivenoms to these venoms and, to achieve this, it was important to define some suitable *in vitro* effects of the venoms in order to determine if the antivenom could neutralise them.

Most clinicians agree that an appropriate antivenom provides the only specific treatment available for severe envenoming. Many of the techniques used to produce antivenoms in this study differ from conventional methods. The use of the horse for antivenom production dates from over a hundred years ago Roux and Martin (1894) and this species still remains the animal of choice by most groups. The reason is that the horse is docile and, therefore, easy to handle, requires little care and yields a large volume of blood. Methods have also been established for purification of equine blood antibodies. Nonetheless, there are several important reasons to consider their replacement. Horses are no longer widely available and are expensive both to purchase and maintain; they have a powerful cell-mediated immune response which may result in the formation of large abscesses at the injection site; administration of equine immunoglobulin concentrates or F(ab)₂ often causes a Type III immune-complex mediated hypersensitivity reaction and, most importantly, many patients have been sensitised to

horse proteins due to previous exposure and are, therefore, at risk of experiencing a potentially life-threatening Type I anaphylactic hypersensitivity reaction if given equine antivenom. Unfortunately, a negative skin test does not exclude the latter.

In the present study sheep were used. Excellent methods have been developed for immunising, bleeding and processing ovine serum and sheep are relatively inexpensive and widely available in many countries. They may prove more cost-effective than horses for large scale antivenom production especially since, as their immune response is predominantly humoral, they do not demonstrate the severe local reactions manifested by horses when adjuvants are used. The resultant ability to employ adjuvants, such as that introduced by Freund, serves to reduce the toxicity of the venom and greatly enhances the antibody response by only gradually releasing the venom into the host (Christensen 1955; Latifi and Manhouri 1966; Sawai *et al.*, 1972). Thus higher circulating levels of specific antibodies usually result.

Conventional immunisation of horses to produce an antivenom follows a hyperimmunisation schedule in which large amounts of venom are given in rapidly increasing sequential doses of up to 1200 mg per injection (Russell, 1988). Such protocols do not result in maximal or regular increases in serum antibody levels. Thus initially, antibody levels rise progressively but this may be followed by a decline in titre after further stimulation even with large venom doses (Christensen, 1966; Bouquet, 1979). In contrast all sheep showed a good antibody response using a low venom dose (0.5mg) given at infrequent (monthly) intervals. Increasing the dose up to 4mg resulted in a further marked improvement in the response to *N. h. arabica*, *B. arietans* and *C. cerastes* venoms.

The effectiveness of an antiserum will depend on the titre and specificity of its component populations of antibodies and the affinity (strength) with which they bind the many toxic components of the appropriate venom. The total immunoglobulin fraction of any immune serum will, of necessity, also contain antibodies that are

directed against molecules other than those present in the venom. These antibodies comprise the background complement of immunoglobulins acquired by the animal during its lifetime and, as such, are of no therapeutic benefit to the patient. In this study sheep antisera contained 25-30g of immunoglobulin per litre, of which about a quarter to a third were specific to antigens in the venom. Fortunately the ovine antivenoms all contained a broad range of antibody populations directed against most of the venom components, as shown by binding assays. Immunoassays are useful for initial assessment and for making comparisons of antivenoms.

In the first years of the century all the antivenoms given comprised unrefined equine serum or plasma. Then a salt precipitate was used and later pepsin digestion was introduced. In nearly all instances, ammonium sulphate is used to yield an IgG-rich fraction. However, use of the sodium salt results in a pellet that is more easily reconstituted, contains lower levels of albumin and avoids the addition of potentially toxic ammonium ions. While the risk of allergic reactions has been reduced by pepsin digestion the resultant $F(ab)_2$ with its two binding sites can form immune complexes and there still remains some reluctance to administer antivenoms. Consequently there is a need for safer and more efficient products.

A recent development in immunotherapy is the use of Fab fragments produced by digesting IgG with the plant enzyme, papain. This enzyme, a thiol protease from the papaya plant, removes the Fc portion of the IgG molecules to yield two monovalent antibody binding sites (Fab). Fab fragments have a number of advantages over both IgG and $F(ab)_2$. First, they are not associated with type III hypersensitivity reactions because with only one antigen binding site, they do not form immune complexes. Second, lacking the Fc region, they do not bind and activate complement or macrophages thereby avoiding vasoactive amine release. Third, because of its small size, Fab is quickly re-distributed from the general circulation into the extracellular tissue fluids. Venom components in the general circulation are quickly and efficiently bound and neutralised by current antivenoms but many high molecular weight venom components

remain at the bite site causing local effects such as oedema, haemorrhage and necrosis. Current antivenoms do not resolve these effects well since they fail to reach the bite site in large amounts and a Fab antivenom may be more efficient. Many venoms cause severe and permanent local damage and the local infiltration of Fab as well as its intravenous administration might alleviate these effects and will be the subject of further study. Finally Fab, because of its small size, may penetrate more rapidly into the neuromuscular junction and hence, reverse neurotoxicity. However, its small size also means that Fab is excreted partially via the kidneys which may be a disadvantage if its clearance is too rapid. Conversely, antivenom remaining in the body causes a humoral immune response and Fab, because of its faster elimination, may have a lower incidence of this.

The ovine Fab antivenoms produced in this study completely neutralised the enzymatic activities of the venoms. They were particularly effective in protecting against the indirect haemolytic, coagulation, platelet aggregation, haemorrhagic, necrotic and defibrinogenating activities of the various venom components. The Fab antivenoms were extremely effective at neutralising the overall lethal effects of venoms strongly suggesting that Fab populations bind the most toxic venom components. Comparison of ovine IgG with the F(ab)₂ and Fab fragments showed similar and complete protection when using the *in vitro* assays but IgG was more effective than the antibody fragments on a weight basis.

The mono- and polyspecific ovine antivenoms raised against *E. pyramidum* and *E. coloratus* venoms protected mice in ED₅₀ studies and neutralised the *in vitro* effects of the venoms, with the monospecific products being considerably more effective. Monospecific antivenoms should be raised to produce antibodies directed against each of the different venoms and these can then, at a later stage, be mixed to give a polyspecific product suitable to treat envenomings by any of the various snakes found in a particular location. It was of considerable interest that the monospecific *E. pyramidum* antivenom provided good neutralisation of many of the enzymatic activities of Nigerian

E. ocellatus venom but did not protect against its potent coagulant activity. The antigenic differences in *E. pyramidum* venom obtained from snakes in different parts of the enormous geographical areas where they are found may explain the variable potency of particular *Echis* antivenoms and emphasises the importance of immunising with venom from the species of snake collected from the locations for which the antivenom is intended.

Comparison of the ovine Fab and the commercial antivenoms used in Saudi Arabia showed the former to be more effective. The commercial equine F(ab)₂ products showed partial neutralisation of the enzymatic and biological effects of the venoms used in this study and completely neutralised their coagulant and indirect haemolytic activities. Both also protected against the haemorrhagic activity of *E. pyramidum* venom when sufficient amounts of antivenom were used. Behringwerke showed better protection in mice than the Pasteur antivenom for most of the venoms tested but neither provided protection against *W. aegyptia* venom.

Many quality control criteria must be satisfied when producing effective and safe antivenoms. Firstly, venoms are complex biological secretions with large variations in composition and the quality of the antivenom depends, in part, on the venom used as immunogen. Each venom batch should be tested for lethality and characterised by some physical separation and other appropriate method(s). The antivenom should be produced under sterile conditions following the strict regulations which are laid down by many organisations such as FDA in the USA and the Medicines Control Agency (MCA) in the UK. The antivenom should be produced in a controlled, fully validated, optimised and well equipped environment. Specially trained and highly qualified staff are required to adhere to Good Manufacturing Practices (GMP) with all procedures optimised and documented in suitable Standard Operating Procedures (SOPs). The product should be tested to ensure quality, safety and efficacy using suitable quality control techniques. The product must also be free from microbes (as shown by suitable microbiological tests) and pyrogens (as shown using both LAL and the rabbit pyrogen test). Purity of the product can be assessed by FPLC, HPLC, SDS-PAGE and immunoelectrophoresis.

Ion exchange chromatography and, finally, affinity chromatography should be used to remove all contaminating serum proteins and chemicals used in the processing to minimise the amount of foreign protein required and to reduce the risk of anaphylactic reactions. Efficacy of the final product is traditionally tested using ED₅₀ but hopefully, in the future, *in vitro* assays may reduce such testing in mice.

To conclude, the antivenoms produced against Saudi Arabian snake venoms in sheep demonstrated good protection and compared favourably with currently available commercial antivenoms. No specific antivenom has previously been produced against Saudi Arabian *W. aegyptia*, *E. coloratus*, *N. h. arabica* and *A. microlepidota*. The monospecific antivenoms were more efficient than a polyclonal antivenom and the purified antivenoms could be combined to form mixed monospecific products. The only truly realistic assessment of these antivenoms, however, is to perform a properly controlled clinical trial in Saudi Arabia.

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