

THE UNIVERSITY OF BIRMINGHAM

**AN IMMUNOLOGICAL STUDY OF BRAZILIAN SNAKE VENOM ANTIGENS-
TOWARDS A SPECIES-SPECIFIC DIAGNOSTIC ASSAY**

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SYNOPSIS

Snakebite by venomous snakes is a serious health problem in many Countries of the world. The identification of the offending snake through venom analysis would allow for specific treatment to be given minimising mortality and morbidity rates.

This work focused on developing an immunoassay for the species-specific identification of *Bothrops* and *Lachesis* venoms. Crossreactivity studies showed that the venoms shared many crossreacting epitopes. The use of venom adsorbents yielded antivenoms capable of discriminating the homologous venom from heterologous venoms in 'in vitro' tests both in buffer solutions and when added to normal human serum. In the conditions studied, species-specific venom identification was independent of venom concentration and antivenom dilution. Western blot analysis, revealed in a venom "pool" from a single species, components that could be species-specific markers. These were shown to be present also in venoms from individual snakes of the same species from different geographic regions. Immunoaffinity chromatography of the *B. jararaca* venom suggests a partial purification of its species-specific component.

The work showed the possibility of species-specific identification of Bothropic and Lachetic venoms. This identification is obtained through the demonstration of specific venom components. The application of this methodology to the clinical identification of snakebites could assist in the improvement of treatment of these casualties.

DEDICATION

To my wife Anamaria, for her unquestioned love, dedication and companionship.

To my sons, Felippe and André, for their immense love so vividly shown in their beautiful little faces and for their intriguingly beautiful innocence that makes my everyday existence a meaningful pleasure.

To my parents, Ibrahim and Berenice, my brothers, Paulo Roberto, Gustavo Henrique, and Ricardo Alberto and to my aunt, Berenice (Baixinha) for their constant support.

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List of Abbreviations

2β-Me =	2 beta Mercaptoethanol
2D-IEP=	Two dimensional immunoelectrophoresis
A _{280nm} =	Absorbance at 280 nm
B. alt =	<i>Bothrops alternatus</i>
B. atr =	<i>Bothrops atrox</i>
B. cot =	<i>Bothrops cotiara</i>
B. jar =	<i>Bothrops jararaca</i>
B. jsu =	<i>Bothrops jararacussu</i>
B. moj =	<i>Bothrops moojeni</i>
B. neu =	<i>Bothrops neuwiedi</i>
BSA =	Bovine Serum Albumin
C.d.c. =	<i>Crotalus durissus collineatus</i>
C.d.t. =	<i>Crotalus durissus terrificus</i>
CPM =	Counts per minute
DAB =	3',3' diaminobenzidine
DEAE =	Di-ethyl amino-ethyl
dil. =	dilution
ELISA =	Enzyme-linked immunosorbent assay
FCA =	Freund's Complete Adjuvant
FCS =	Foetal Calf Serum
FIA =	Freund's Incomplete Adjuvant
HAT =	Hypoxantine-Aminopterin-Thymidine
HRP =	Horseradish peroxidase
HRPMI=	Hepes buffered RPMI medium
HT =	Hypoxanthine and Thymidine solution
IC =	Immune complexes
IEP =	Immunoelectrophoresis
IgG =	Immunoglobulin G
L. mut =	<i>Lachesis muta</i>
MW =	Molecular Weight
OD =	Optical Density
OPD =	<i>ortho</i> -phenylenediamine
PAGE =	Polyacrylamide Gel Electrophoresis
PBS =	Phosphate Buffered Saline
PEG =	Polyethylene Glycol
PLA ₂ =	Phospholipase A ₂
RIA =	Radio-immunoassay
RPMI =	RPMI-1640 culture medium with L-glutamine
SDS =	Sodium dodecyl sulphate
TCA =	Trichloracetic acid
WHO =	World Health Organisation

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1. INTRODUCTION

1.1. General Introduction

Through science, man is steadily increasing the understanding of himself and his environment. In Biology this has led to the prevention, treatment and cure of many human diseases. Despite this acquired knowledge, however, there are many problems still to be solved.

Since the Renaissance, the use of scientific observations and methods has been applied to the study of the pharmacological and chemical characterisation of snake venoms. This has brought about a sharp decrease in human mortality following snakebite.

However, in many countries of the world snakebites still pose a threat to man and are regarded by the World Health Organisation as a major health problem. This is true for the majority of the developing countries, usually distributed within the warmer areas of the planet. There, snakes are not only more abundant but also exist in greater variety. The generally poor economies of these countries makes the task of providing an efficient health care service and specific treatment for snakebite a difficult one.

The World Health Organisation sees the use of immunodiagnostic methods as a useful means of improving the epidemiological data on snakebite. These data should in turn provide the necessary knowledge to develop specific treatment strategies such as production of species-specific antivenom, or specific supportive treatment where the use of antivenoms is not advisable. They would also provide tools to further studies of the geographic distribution and genetic variability of snakes.

1.1.1. Relationship between Man and Snakes

The relationship between man and snakes dates back to pre-historic times and its closeness may come as a surprise to many. According to Morris *et al.*, [1965], no other animal form has played such an important or varied role in man's thinking. They have been worshipped, and seen as a symbol of procreation, longevity and immortality. They have embodied the

idea of death, life, temptation, sin, disease and cure. They have been used in medicine, religion, commerce and science.

Venomous snakes have played a major role in this relationship due to their ability to cause fatal or serious injuries to man and his livestock. Since the beginning of civilisation different ways to counteract the toxic effects of snake venoms have been tried. This gradually evolved into a broader and more systematic study of snake venoms, including their biochemical characterisation, modes of action, the pharmacological, physiological and immunological mechanisms, and classification.

1.2. Snake Classification

The wide variety of species of snakes that exist, makes the task of organising this diversity into a systematic classification a difficult one. The lack of abundant snake fossils forces the classification to be based on comparative studies of living species. Most snake taxonomy has been based upon external and anatomical features (dentition, skeleton, muscles, etc.). Recently, comparative studies using serological, biochemical and immunological approaches have produced interesting results. Much of this depends on the interpretation of experts. As the research tools developed, new evidence and hypotheses arose and previous conclusions had to be modified. Thus, changes in the classification occurred, with species becoming subspecies and vice-versa. The lack of agreed criteria and frequent disagreement upon common interpretation by the experts, significantly affects the total estimated number of living snake species. There are between 2,500 to 2,700 species of snakes recognised by the specialists [Underwood, 1979]. Snakes are members of the class Reptilia, Order Squamata, Suborder Serpentes. A proposed classification of Snakes [McDowell, 1987] is summarised below and only the Infraorder Alethinophidia is detailed.

Infraorder Cholophidia
3 Families
Infraorder Scolecophidia
3 Families
Infraorder Alethinophidia
Superfamily Acrochordoidea
Family Nigeropheidae
Family Palaeopheidae
Family Anomalopheidae
Family Acrochordidae
Superfamily Anilioidea
Family Loxocemidae
Family Xenopeltidae
Family Aniliidae
Family Uropeltidae
Superfamily Tropidopheoidea
Family Tropidopheidae
Superfamily Bolyeroidea
Family Bolyeriidae
Superfamily Booidea
Family Pythonidae
Family Boidae
Superfamily Colubroidea
Series Proteroglypha
Family <i>Atractaspididae</i>
Family <i>Elapidae</i>
Series Opisthoglypha
Family <i>Colubridae</i>
Family <i>Viperidae</i>

The “advanced snakes” are placed in the Superfamily Colubroidea and a characteristic of this group, absent in some, is the presence of a venom gland. Certain maxillary teeth deliver these secretions (venom) and are often grooved or caniculate. In the Proteroglypha Series, specimens from the *Family Atractaspididae*, and *Elapidae* have anterior fangs but fixed to the maxillary bone. *Family Colubridae* is characterized by: solid maxillary teeth anterior to fangs (if fangs are present), the fangs with open groove. When venom secretion is present (supralabial venom gland) the secretion is carried by fangs on rear of maxilla. The *Family*

Colubridae has a world-wide distribution. With aquatic, fossorial, terrestrial, and arboreal forms, it contains two-thirds of all living snakes. The majority of colubrid species are believed to be harmless, except when venom gland is present.

In Table 1.1, the Opisthoglypha Series where the *Bothrops* genus is placed, is expanded to the level of genera. Only some examples of genera are shown.

Table 1.1: Classification of Snakes in the Opisthoglypha Series.

Family	Subfamily	Genera (relevant examples)
<i>Colubridae</i>	<i>Pareatinae, Boodontinae,</i> <i>Homalospinae,</i> <i>Calamariinae,</i> <i>Xenoderminae, Natricinae,</i> <i>Xenodontinae, Colubrinae,</i> <i>Pseudoxenodontinae</i>	
<i>Viperidae</i>	<i>Azemiopinae</i>	<i>Azemiops.</i> -----
	<i>Crotalinae</i>	<i>Agkistrodon, Bothrops,</i> <i>Crotalus, Lachesis,</i> <i>Hypnale, Trimelerurus,</i> <i>Sistrurus, Calloselasma,</i> etc. -----
	<i>Viperinae</i>	<i>Vipera, Bitis, Causus,</i> <i>Echis, etc.</i>

The genus *Bothrops* is within the Family *Viperidae*. Some of the characteristics of this Family are: dentition reduced to a pair of fangs with enclosed canal (caniculated) and the presence of a thick-walled venom gland in the mouth homologous to that of Proteroglypha and Colubridae. The fangs are not fixed and are pointed towards the front of the mouth during the strike. The Subfamily *Crotalinae*, where the *Bothrops* species are placed, have a distinct characteristic; that is a deep loreal (heat sensory) pit lodged in a fossa on the prefrontal and maxilla bones. This heat sensory organ is not present in the specimens of the Subfamilies *Azemiops* and *Viperinae*. Classification within the *Bothrops* genus is defined

mainly by morphological characteristics such as: size, skin colour, colour and scale pattern and hemipenes characteristics. The *Bothrops* species are found from Central America (one species, *B. asper*, ranges through Central America to nonthern Mexico) down to most of South America and on the Caribbean islands. In a preliminary account of the Neotropical *Crotalinae*, there were 31 types of *Bothrops* including species and subspecies in Brazil. The species for the genus *Bothrops* are: ***B. megaera***, ***B. albocarinatus***, ***B. alternatus***, ***B. alticulus***, ***B. ammodytoides***, ***B. andianus***, ***B. atrox***, ***B. asper***, ***B. barbouri***, ***B. barnetti***, ***B. bicolor***, ***B. bilineatus bilineatus***, ***B. bilineatus smaragdinus***, ***B. brazili***, ***B. castenaldi***, ***B. caribbaeus***, ***B. cotiara***, ***B. dunni***, ***B. erythromelas***, ***B. fonsecai***, ***B. godmanni***, ***B. hyoprorus***, ***B. iglesiasi***, ***B. insulares***, ***B. itapetiningae***, ***B. jararaca***, ***B. jararacussu***, ***B. lanceolatus***, ***B. lansbergii lansbergii***, ***B. lansbergii annectens***, ***B. lansbergii venezuelensis***, ***B. lateralis***, ***B. lichenosus***, ***B. lojanus***, ***B. marajoensis***, ***B. medusa***, ***B. melanurus***, ***B. microphthalmus microphthalmus***, ***B. microphthalmus colombianus***, ***B. moojeni***, ***B. nasutus***, ***B. neuwiedi neuwiedi***, ***B. neuwiedi bolivianus***, ***B. n. meridionalis***, ***B. n. diporus***, ***B. n. paranaensis***, ***B. n. lutzi***, ***B. n. pauloensis***, ***B. n. piauhyensis***, ***B. n. pubescens***, ***B. n. urutu***, ***B. nigroviridis nigroviridis***, ***B. nigroviridis aurifer***, ***B. nummifer nummifer***, ***B. nummifer mexicanus***, ***B. nummifer occidus***, ***B. oligolepsis***, ***B. ophryomegas***, ***B. peruvians***, ***B. picadoi***, ***B. pictus***, ***B. pirajai***, ***B. pradoi***, ***B. pulcher***, ***B. punctatus***, ***B. roendingeri***, ***B. sanctaecrucis***, ***B. schilegelli***, ***B. sphenphrys***, ***B. supraciliaris***, ***B. undulatus***, ***B. venezuelensis***, ***B. xantogrammus***, ***B. yucatannicus*** [Hoge, 1965]. The species in bold are found in Brazil.

1.3. Geographical Distribution

Snakes are found in all continents of the earth, excluding only a few regions which are entirely free of snakes (Figure 1.1)

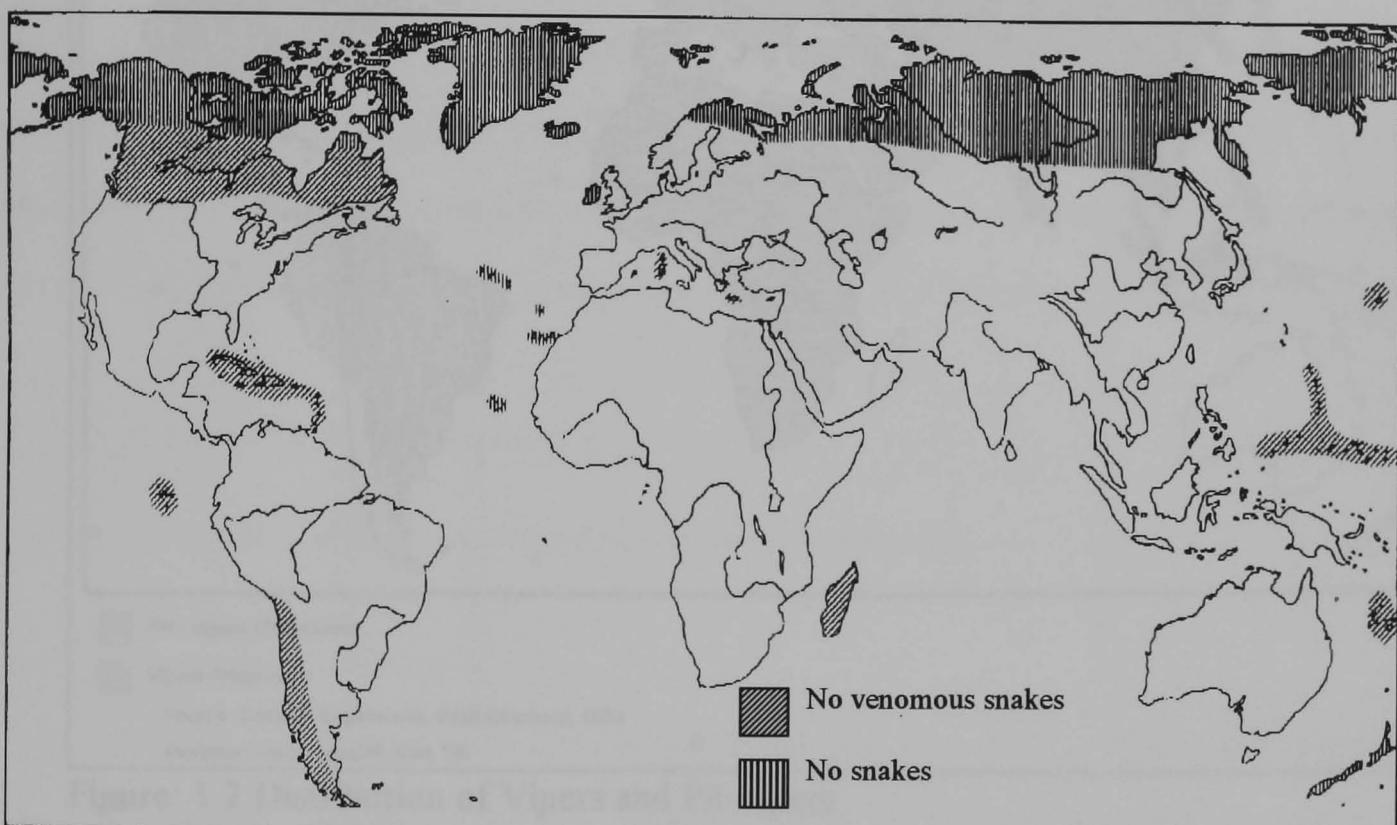


Figure 1.1 World regions without any snakes and without venomous snakes

Source: Snakes, Engelmann, Wolf-Eberhard, 1984

Publisher: Croom Helm Ltd, Kent, UK

Being ectothermic, snakes are more abundant in the warmer regions where the climate suits them. The tropical regions are where they achieve their greatest variety and highest population density. In general, only Northern parts of Europe, Asia and North America are devoid of snakes. The distribution of vipers and pit vipers (which includes the *Bothrops* species) is shown in Figure 1.2

In Brazil the *Bothrops* genera is found in the whole of the Brazilian territory. Many species share the same geographical area. In Fig. 1.3 this can be observed, with examples of the main species of *Bothrops* found in Brazil. A more detailed list showing the distribution of

venomous snakes including the *Bothrops* species in the various states of Brazil is too long to be presented here but is included in Appendix I.

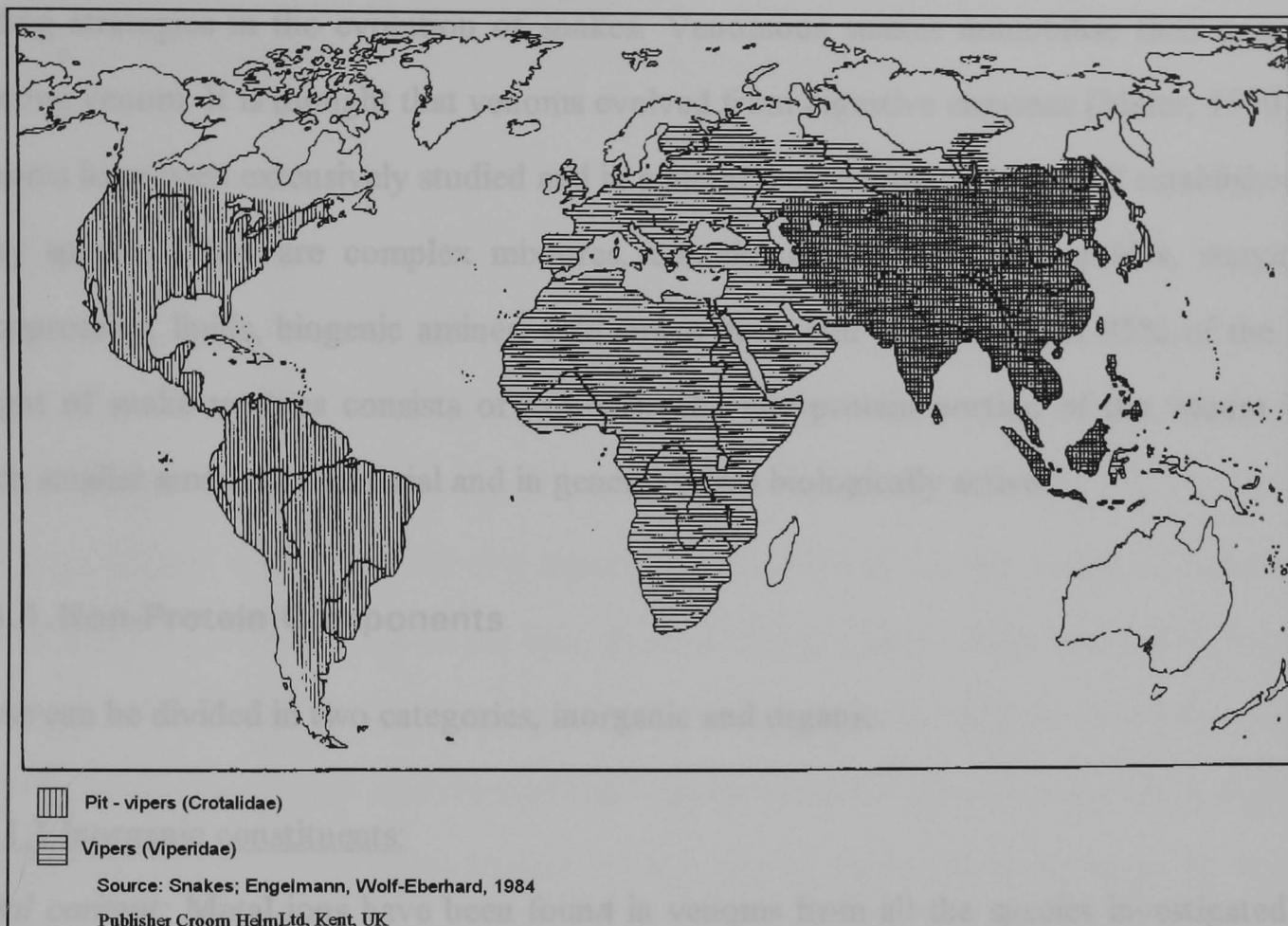
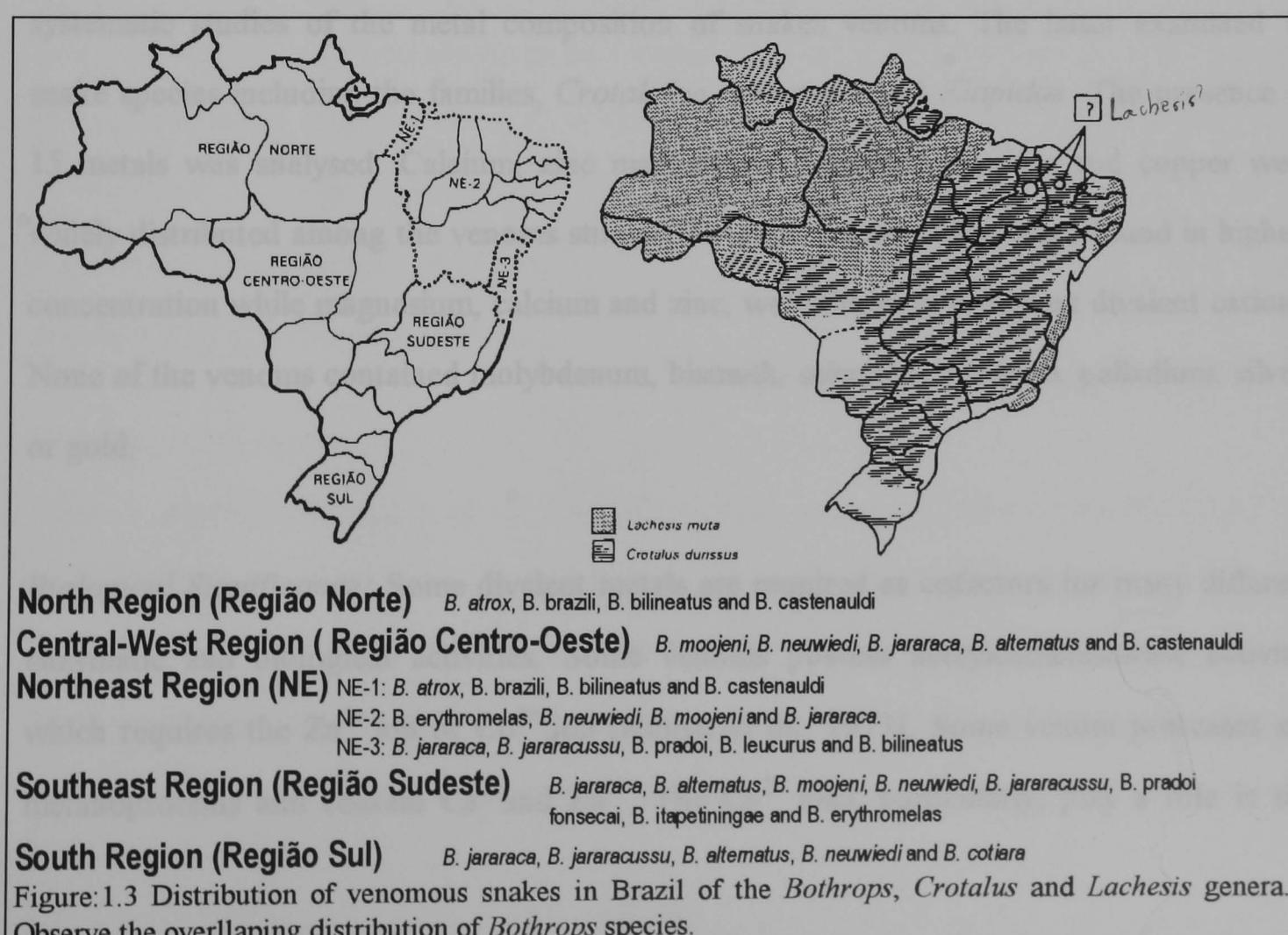


Figure: 1.2 Distribution of Vipers and Pit-vipers



1.4. Snake Venom Components

The absence of limbs and of cutting and grinding teeth forced the development of new feeding strategies in the evolution of snakes. Venomous snakes immobilise their prey by injecting venom. It is thought that venoms evolved from digestive enzymes [Meier, 1990].

Venoms have been extensively studied and in general their composition is well established in many species. They are complex mixtures, containing peptides, polypeptides, enzymes, glycoproteins, lipids, biogenic amines, free amino acids and metals. About 95% of the dry weight of snake venoms consists of proteins. The non-protein portion of the venom is a much smaller amount of material and in general is less biologically active.

1.4.1. Non-Protein Components

These can be divided in two categories, inorganic and organic.

1.4.1.1. Inorganic constituents:

Metal content: Metal ions have been found in venoms from all the species investigated so far. Devi [1968], and more recently Friederich and Tu [1971], have done extensive and systematic studies of the metal composition of snakes venoms. The latter examined 17 snake species including the families, *Crotalidae*, *Viperidae* and *Elapidae*. The presence of 15 metals was analysed. Calcium, zinc magnesium, sodium, potassium and copper were widely distributed among the venoms studied. Potassium and sodium were found in highest concentration while magnesium, calcium and zinc, were the most prevalent divalent cations. None of the venoms contained molybdenum, bismuth, selenium, platinum, palladium, silver, or gold.

Biological Significance: Some divalent metals are required as cofactors for many different enzymatic and biological activities. Some venoms possess acetylcholinesterase activity, which requires the Zn^{2+} ion or Co^{2+} ion [Kumar *et al.*, 1973]. Some venom proteases are metalloproteins and contain Ca^{2+} and Zn^{2+} . The Ca^{2+} ions, particularly, play a role in the

activation of phospholipase A₂. Haemorrhagic and proteolytic activity decreases markedly after dialysis, [Friederich and Tu, 1971], which is partially restored after the addition of magnesium. This suggests a role for metal ions in these activities.

1.4.1.2.Organic Constituents:

1.4.1.2.1.Lipids:

Very little work has been done on snake venom lipids. Ganguly and Malkana., [1936], reported that cholesterol and lecithin were present in cobra venom. Devi [1968], and Bieber [1979] presented results on the lipid content of the venoms from *Naja naja* and *C. d. terrificus*. Kabara and Fischer, [1969] reported that 0.43% of the dry weight of *Naja naja* venom was lipid. The removal of the lipid fraction of *Naja naja* venom resulted in the loss of 40% to 50% of the lethality. Toxicity was not restored by the addition of this fraction to the extracted venom. They also showed that organic acids of snake venoms are present as triglycerides and phospholipids. Moroz *et al.*, [1966], observed a decrease in *Vipera palaestinae* neurotoxin activity with the removal of lipids. This toxicity was restored with venom reconstitution. From the above observations the fraction appears to be maintaining venom toxicity. Citrate was shown to be a major component of snake venoms when venoms of *Bothrops asper*, *Crotalus atrox*, *C. viridis*, *C. adamanteus*, *Sistrurus miliarius barbouri*, *C. horridus horridus*, *Agkistrodon contortrix mokase*, *A. contortrix contortrix* and *A. piscivorus piscivorus* were analysed by gas liquid chromatography and mass spectrometry, [Freitas *et al.*, 1992].

1.4.1.2.2.Carbohydrates:

There is limited information about the carbohydrate content of snake venoms. They are found in the form of glycoprotein rather than free sugar, and are present in many venoms [Basu *et al.*, 1970]. Sialic acid was detected in the venom of *Naja naja*, [Bragança and Patel, 1965] but played no role in toxicity. Oshima and Iwanaga., [1969], investigated many venoms and found that most sugars present were either neutral, aminosugars, or sialic acid.

No mucopolyssacharide was found. Major venom toxins were proved not to be glycoprotein, and the exact biological functions of venom carbohydrates has not yet been established.

1.4.1.2.3.Riboflavin:

The yellow colour of the majority of snake venoms is due to the presence of L-amino acid oxidase, which contains riboflavin as its coenzyme. This enzyme has been found in many venoms of different genera. Zeller [1948] demonstrated a correlation between the intensity of the yellow colour and the L-amino acid activity of snake venom. White venoms do not possess L-amino acid activity. The prosthetic group of L- amino acid oxidase is FAD [Singer and Dearney., 1950]. This is true for all snake venoms except for that of *Trimeresurus flavoviridis* [Inamasu *et al.*, 1974].

1.4.1.2.4.Nucleosides and Nucleotides:

The presence of these compounds in snake venoms has been little studied. Reports showed that the nucleosides adenosine, guanosine and inocene were the most abundant in the tested venoms. Doery [1957], reported the presence of hypoxanthine, a purine base in the venom of *Notechis scutulatus*. Further studies by other authors showed the presence of guanosine, adenosine and inocene, all derivatives of purine, in venoms from various different species. The total amount of purine compounds in the venom dry weight is between 1.4 and 4.3%. The role of these substances in snake venoms is not known and nothing can be said about their wide distribution among different snake species [Bieber, 1979].

1.4.1.2.5.Free Amino Acids and Peptides:

Free amino acids are found only in small amounts in snake venoms. However, many authors have demonstrated free amino acids in venoms of different species of snake. The ninhydrin reaction, paper chromatography and paper electrophoresis have been used to demonstrate their presence. [Sasaki, 1960], showed the presence of glycine, serine, cysteine, threonine, lysine, tyrosine, valine, phenylalanine and leucine in the venom of *Trimeresurus*

musquamatus. Devi [1968]; Hirakawa [1974]; Shipolini *et al.*, [1965], are among the authors that have shown free amino acids in different species of snake. The results suggest that all amino acids found in proteins are detected in free form in snake venoms. However, not all amino acids are found in all venom extracts. In a review [Devi, 1968], the largest number was 5 free amino acids in one venom. These studies do not reveal any physiological or pharmacological role in venom of free amino acids. Nor is it yet determined whether their content is due to their cleavage from proteins through venom protease reactions.

1.4.2. Proteins

Snake venom proteins can be divided into three main groups: enzymes, toxins and nerve growth factors. Enzymes are responsible for the tissue necrosis, blood coagulation or anticoagulation and pain [Iwanaga and Suzuki, 1979]. Toxins act by disrupting nerve transmission or damaging skeletal muscle [Karlsson, 1979].

Enzymes and toxins are sometimes distinguished by the fact that some toxins do not have enzymatic activity and some enzymes are not toxic. Crotoxin, however, a neurotoxin, has phospholipase A₂ activity. Nerve growth factors cause differentiation of sympathetic or sensory neurones and are found in all *Crotalidae*, *Viperidae* and *Elapidae* venoms so far studied [Angeletti *et al.*, 1979; Levi-Montalcini and Angeletti, 1968].

1.4.2.1. Enzymes

There are 26 enzymes so far identified in various snake venoms, of which ten are present in every snake venom, although no single snake contains all of them. Some are characteristic of snake families: Elapidae have acetylcholinesterase which is never found in *Viperidae*. Crotalid and viperid venoms contain endopeptidase, arginine ester hydrolase, thrombin-like enzymes, collagenase, hyaluronidase and prothrombin activator which are not found in other snake venoms so far investigated [Iwanaga and Suzuki, 1979]. Enzymes found in snake venoms can be broadly classified as proteolytic, arginine ester hydrolases, collagenase, hyaluronidase, phospholipase A, phospholipase B, phosphodiesterase, acetylcholinesterase,

5' nucleotidase and L-amino oxidase [Tu, 1977]. The pathophysiological activity of these enzymes can be summarised as follows: local capillary damage and tissue necrosis caused by proteinases, phospholipases, arginine ester hydrolases and hyaluronidases [Kaiser and Michl, 1958; Slotta, 1955; Suzuki and Iwanaga., 1970]; coagulant and anticoagulant effects caused by proteinases and phospholipase A [Meaume, 1966]; acute hypotension and pain due to release of vasoactive peptides by kinin-releasing enzymes (kininogenase) [Suzuki and Iwanaga., 1970].

Snake venom toxins are mainly divided into those acting on muscle cells, capillaries, or skin, (proteins with or without enzymatic activity), those acting on the nervous system, (post-synaptic or pre-synaptic, small peptides or enzymes), and those acting on cell membranes (cardiotoxins).

1.4.2.2.Neurotoxins

1.4.2.2.1.Post synaptic neurotoxins:

These neurotoxins bind to the nicotinic acetylcholine receptors competitively with acetylcholine, producing a non-depolarising neuromuscular block. They are subdivided into two main groups, the short chain neurotoxins (60 to 65 amino acids and four disulphide bridges) and long-chain neurotoxins (71 to 74 amino acids and five disulphide bridges). Molecular weights vary from 7 to 8 kDa [Harris, 1989; Minton, 1990].

1.4.2.2.2.Pre synaptic neurotoxins:

These inhibit the release of acetylcholine at the myoneural junction. These toxins are phospholipases, or have phospholipase as a subunit. Hydrolytic activity is essential for the expression of toxicity. Most have molecular weights of 13 to 22 kDa, with few having multiple subunits and correspondingly higher molecular weight [Harris, 1989; Minton, 1990].

1.4.2.3.Myotoxins

These are characterised as either small basic polypeptides with no enzymatic activity or as large, basic proteins possessing phospholipase A₂ activity. Snake venom polypeptides that induce skeletal muscle contracture or produce myonecrosis or myoglobinuria are characterised as myotoxins [Stocker, 1990a].

1.4.2.3.1.Non-enzymatic myotoxins:

The non-enzymatic myotoxins so far isolated are very similar to each other in amino acid sequence, molecular weight and isoelectric point. Stocker, [1990a] and Mebs, [1985] described seven myotoxic polypeptides consisting of 42-50 amino acid residues and 3 intramolecular disulphide bridges.

A basic dimeric polypeptide with a subunit molecular weight of 16 kDa has been found in the venom of *Bothrops nummifer*. This causes muscle cell damage *in vivo* and *in vitro* and, when injected intramuscularly in mice, leads to the release of creatine kinase. It appears to be devoid of PLA₂ activity. A myotoxin devoid of phospholipase A₂ activity was isolated from *B. jararacussu* snake venom. Called Bothropstoxin or BthTX, it is a single chain peptide with a molecular weight of 13 kDa, with 12 half cysteine residues. It has an isoelectric point of 8.2 [Homsi-Brandenburg *et al.*, 1988].

1.4.2.4.Phospholipase A₂ myotoxins:

These myotoxins are potent neurotoxins, mainly presynaptic. They are classified as B-neurotoxins or phospholipase A₂ myotoxins. Their composition varies, as some consist of only a single amino acid chain and others consist of two, three, or more polypeptide chains non-covalently bound. They are homologous to mammalian phospholipases. In phospholipase A₂ myotoxins with subunits, the acidic subunit does not cause myonecrosis by itself, but only potentiates the myonecrosis caused by the basic subunit, which is myotoxic on its own [Ownby, 1990 ; Rosenberg, 1990]

1.4.2.5.Haemorrhagic toxins

These are proteins which can be basic, neutral or acidic. All haemorrhagic toxins isolated to date have proteolytic activity when tested using casein or dimethylcasein as a substrate. Many toxins have fibrinogenolytic activity. Their structure varies widely, and they can be divided into two groups; low molecular weight (22 to 26 kDa) and high molecular weight (60 to 90 kDa). Haemorrhagins cause disruption of the collagenous basement membrane of the vascular endothelium allowing leakage of blood components [Kamiguti *et al.*, 1991; Ownby, 1990]. Three haemorragic factors have been isolated from the venom of *B.jararaca* and 2 from the venom of *B. neuwiedi*, [Assakura *et al.*, 1986]. Although haemorrhagins are known as locally acting agents, recently, Kamiguti *et al.*, [1991] reported that a haemorrhaging fraction from the venom of *B. jararaca* induces systemic bleeding in rats.

1.4.2.6.Cardiotoxins

These are highly basic polypeptides consisting of about 60-62 aminoacid residues, with four intramolecular disulphide bridges. They are also known as cytotoxins, and direct lytic factor [Harris, 1989]. The pharmacological actions of cardiotoxins comprise haemolysis, cytolysis, depolarisation of muscle membranes, and specific cardiotoxicity. These toxins act by disorganising the membrane of cells, but their mode of action is still unknown.

Sarafotoxins are a group of several cardiotoxic polypeptides which have been isolated from the venom of the burrowing snake, *Atractaspis engaddensis*. The amino acid sequence shows a high degree of homology with that of endothelin, a 21-residue vasoconstrictor peptide found in porcine aortic endothelium [Weiser *et al.*, 1984]

1.4.2.7.Proteinase Inhibitors

Several polypeptides, composed of 52-65 amino acids and cross-linked by 2-3 disulphide bridges, have been isolated from some elapid and viperid venoms. They act either as proteinase inhibitors or represent structural analogues of proteinase inhibitors. Some inhibitor analogues which are devoid of any proteinase inhibitor potency exert an acetylcholine release-facilitating effect on the neuromuscular junction [Stocker, 1990a].

1.4.2.8.Bradykinin-Potentiating Peptides

These are a number of closely related peptides, widely distributed in venoms from many species. They are low molecular weight, proline rich peptides composed of 5 to 12 amino acids residues, that potentiate the depressor activity of bradykinin. This effect is mediated by an inhibition of the carboxypeptidase that converts angiotensin I into angiotensin II. They have been isolated from the venoms of *B. jararaca*, *B. jararacussu*, *Agkistrodon halys blomhoffi* and *A. h. pallas*.

Ferreira [1965] showed for the first time the presence of bradykinin-potentiation activity by peptides in the venom of *Bothrops jararaca*. Kato *et al.*, [1966], isolated two tripeptides, A and B, from venoms of *A. halys blomhoffi*, *C. adamanteus*, *Bothrops jararaca* and *Trimeresurus flavoviridis*. Ferreira *et al.*, [1970] purified nine peptides from the venom of *B. jararaca*. The smallest had five amino acids and the largest 13. Their sequence showed the presence of pyroglutamic acid, in the amino terminal position. This is a characteristic of all snake venom peptides that exhibit bradykinin potentiation and inhibition of the angiotensin I to angiotensin II conversion. Longer peptides also have a common carboxyl terminus. With one exception, all end in isoleucine-(proline)₂. They tend to produce longer physiological effects [Ondetti *et al.*, 1971].

A new Bradykinin-Potentiating peptide (peptide P) has been isolated from the venom of *B. jararacussu*. Its molecular weight is 1,095 Da, as determined by mass spectrometry. Its bradykinin-potentiated activity, tested in the guinea-pig ileum, was stronger than that observed from captopril, bradykinin potentiator B and BPf_{5a}, appearing to be three-fold more potent than captopril [Ferreira *et al.*, 1992].

1.4.2.9.Choline Esterase Inhibitors

A protein of 55 kDa which inhibits also choline esterase has been found in the venom of the many banded krait *Bungarus multicinctus*. It has also been isolated from the venom of *Naja naja atra* [Takeuchi and Tanaka., 1980].

1.4.2.10.Phospholipase Inhibitors

A polypeptide with molecular weight between 1,500 and 5,000, Da as estimated by gel chromatography, with phospholipase A₂ inhibition activity, was isolated from the venom of *Bothrops neuwiedi*. A phospholipase inhibitor was found also in the venom of *Naja naja* and there is evidence for the presence of such inhibitors in the venoms of *B. jararaca*, *B. jararacussu* and *B. atrox*. Vipoxin, a pre synaptic neurotoxin from *Vipera ammodytes ammodytes* venom, has a basic toxic component with weak phospholipase activity and an atoxic acidic component containing the inhibiting activity [Rosenberg, 1990; Stocker, 1990a]

1.4.2.11.Nerve Growth Factors (NGF)

These are snake venom proteins that in tissue culture, produce outgrowth of nerve tissue. This activity has been identified in six species of the subfamily *Viperinae*, nine species from the subfamily *Crotalinae* and five species from the subfamily *Elapidae*. Molecular weights range from 21 to 54 kDa. The molecular structure is varied and some have two subunits, the one from *Crotalus adamanteus* venom is a protein complex of 40 to 54 kDa. *Vipera russelli*'s NGF is a glycoprotein of 37 kDa, the NGF of *Bothrops jararaca* has a molecular weight of 34 kDa and that of *Bothrops atrox* is a dimer with 35 kDa [Angeletti and Bradshaw., 1979; Stocker, 1990a]

1.4.2.12.Lectins

Haemagglutinating activity has been reported in many snake venoms and several lectins have been isolated from them. The natural role of the vast majority of lectins is not known, but they are useful biochemical tools due to their ability to bind specific sequences of

sugars. Five lectins were isolated from the snake venoms of *Agkistrodon contortrix contortrix*, *A. piscivorous leucostoma*, *C. atrox*, *B. atrox* and *Dendroaspis jamesoni*. Thrombolectin from *B. atrox* [Gartner *et al.*, 1980] and lectins from *A. c. contortrix*, *A. p. leucostoma* and *C. atrox* were characterised as disulphide-linked dimers of molecular weight 28 kDa and their lactose-specific red cell agglutinating action was Ca^{2+} dependent. The lectin from *D. jamesoni* was also lactose-specific but not Ca^{2+} dependent. All lectins investigated, except Thrombolectin, exerted a mitogenic action. Recently a galactose-binding lectin from the venom of *B. goodmani* was isolated which did not exert mitogenic activity on human peripheral blood mononuclear cells. It is an acidic protein (pI 4.9), with a subunit of 14 kDa, which exists mostly as a disulphide dimer of 28 kDa [Mastro *et al.*, 1986; Stocker, 1990a].

1.4.2.13. Proteins Affecting Platelet Functions

There are different types of components affecting platelet functions in snake venoms, such as non-coagulant inducers of platelet aggregation, platelet aggregation inhibitors, platelet aggregation inducers. These can be classified as enzymic or non-enzymic. *Echis carinatus* venom contains an inhibitor of collagen-induced platelet aggregation. It is a heat-stable glycopeptide with a molecular weight of 6.8 kDa. Agglutination of formol-fixed platelets in the presence of von Willebrand factor is induced by venom coagglutinins, which are non enzymic proteins found in numerous *Viperidae* and *Crotalidae* snake venoms. The interaction of these coagglutinins with platelets and von Willebrand factor is species-specific. Thrombolectin, a lectin from *B. atrox* venom, may also cause platelet agglutination [Stocker, 1990b].

1.5. Immunological and Biological characterisation of Snake Venoms

The study of snake venom immunology has progressed substantially since its early days at the end of the nineteenth century. Development of knowledge can be characterised by three main steps. First, animals injected with non lethal doses of a venom developed resistance

against it. Second, venoms were shown to be a "mosaic of antigens" and cross-reactivity and cross-neutralisation was shown to exist; the resistance conferred to animals through the neutralisation of the venoms was found to be due to specific immunoglobulins. Third, is the current emphasis on the elucidation of the molecular characteristics of toxic components of venoms by immunological means [Ménez, 1985].

The study of the biological activities of snake venoms developed in parallel with the immunological studies. The identification and isolation of venom components exerting specific biological functions was important in the further characterisation of venoms and their variability. The characterisation of isolated components made possible the use of specific antisera as therapeutic reagents and as research tools.

The literature contains extensive reviews on this subject, e.g. [Boquet, 1979; Hawgood, 1992; Ménez, 1985; Ménez *et al.*, 1990; Russell, 1988; Russell and Lauritzen., 1966] and I will attempt here to highlight only some of the more fundamental work on the immunological and biological characterisation of snake venoms, with emphasis on studies of the *Bothrops* genera.

1.5.1. Historical background

Sewall [1887], was the first to show that pigeons immunised with non-lethal doses of *Sistrurus catenatus* venom, for a period of months, could survive a dose lethal to non immunised pigeons. Experimenting on small rodents, Kaufmann, [1889], Phisalix and Bertrand, [1894], came to the same conclusions using the venom of *Vipera aspis*. Later, Phisalix, Bertrand, Calmette and Fraser, all demonstrated that the serum of immunised animals contained the antitoxic activity [cited by Russell, 1988]. This led directly to the therapeutic use of hyper-immunised animal serum against snake venom poisoning [Calmette, 1894; Fraser, 1895]. Calmette postulated that his antivenom, produced against cobra venom, could neutralise any venom, since all snake venoms possessed the same toxic components, varying only in quantity, but this was soon disproved. It was shown that venoms contained distinct biological properties; some could coagulate the blood, others

inhibited coagulation and others disrupted the nervous system, agglutinated and lysed mammalian erythrocytes, while others behaved as proteolytic substances and some arrested an isolated heart in systole [Boquet, 1979].

Experiments with venom and antivenom demonstrated that some venoms could have more than one effect upon the victim and that venoms belonging to the same family, or even different families, contained toxic substances of a very similar nature. Antivenoms could neutralise or attenuate to various degrees similar toxic properties of venoms other than the ones they were prepared against [Boquet, 1979]. In the light of these observations the term "mosaic of antigens" was used to describe this diversity. Experimenters had the task of characterising and understanding this diversity.

1.5.2. Snake venom characterisation

Immunological techniques, such as gel immunodiffusion, immunoelectrophoresis, ELISA, and also physicochemical approaches were widely used to demonstrate immunological and biological similarities and differences in snake venoms belonging to various genera, families and species.

Although single antivenoms could inhibit toxic effects of venoms belonging to different species, it was also shown that in some cases they could be ineffective against venoms of snakes of the same species but from different geographical regions. Vellard [1939], reported that antivenoms produced against the venom of *Crotalus durissus terrificus* in the south of Brazil, were unable to neutralise the venoms of the same species from Venezuela. Gonçalves and Vieira, [1950], using electrophoretic and toxicity experiments, showed differences in the venom composition of individual snakes of the same species (*Crotalus durissus terrificus*). This difference was responsible for eliciting distinct symptoms in mice. Specimens from southern Brazil were shown to contain a neurotoxic component, "crotamine", which was not present in other *Crotalus durissus terrificus* from the north of

Brazil. The author also refers to a personal communication of Vital Brazil, in which *Crotalidae* venoms from Argentina and Goiás (a Central-West state of Brazil) elicited in rats distinct pharmacological effects. Based on his observation, Gonçalves and Vieira, postulated the importance of producing antivenoms with geographical specificity

1.6. Antivenoms.

Antivenoms were first produced for therapeutic use [Calmette, 1894; Phisalix and Bertrand., 1894], who immunised horses with increasing doses of snake venoms. In 1901, Vital-Brazil, prepared antivenoms against Bothropic and Crotalic venoms [Hawgood, 1992].

Little improvement in the basic processing methodology of hyperimmune serum has occurred through the years. The stages in production of commercial antivenoms involves: courses of immunisation of horses, collection of plasma, ammonium sulphate precipitation of the hyperimmune serum, enzyme digestion of the serum and concentration and packaging [Sullivan, 1987].

The World Health Organisation, [WHO, 1981] published a set of guidelines to help standardisation of antivenoms covering every aspect of antivenom production.

Venoms from snakes of the same species should be collected from different areas, and should include those species causing major health problems. Venoms should be characterised by measuring the lethal, defibrinating, haemorrhagic and necrotising activities. Immunisation schedules can be optimised by the laboratories. Horses should be 5-8 years old, in good health and be vaccinated against tetanus. Antivenom antibodies should be concentrated by any suitable method, and preferably enzyme-refined to become less prone to elicit allergic reactions. No definite method for the assessment of antivenom potency is available; a suggested procedure involves testing the neutralisation of lethality, necrotising, defibrinating and other venom activities, comparing a test antivenom against a reference venom. Generally this test is carried out by mixing and incubating venom and antivenom and injecting the mixture into mice.

1.6.1. Immunisation and venom characteristics

Venoms contain many active components that act locally or systemically. Apart from the damage they can cause to the horses, there is also a concern about the immunogenicity of the components. Small highly toxic components, usually bad immunogens, are sometimes injected with large non-toxic, but good immunogens into horses. Some authorities argue that these large components will divert the immune response away from the small toxic component and that to achieve high titres of antibodies against the small toxic component, large doses would be required. The damage to the animals, even if the doses were to be increased slowly, would still be excessive.

Some venoms are good immunogens as a whole, and high titres are observed at completion of the immunisation schedule. Bothropic venoms are one such example, but prolonged immunisations are deleterious to the animals, usually meaning a short period of productive life for the horse.

Many methods have been tried, with variable degrees of success, to increase antibody titres, while shortening immunisation periods and limiting or abolishing animal damage.

1.6.2. Venom detoxification and adjuvants

Various added substances and pre-treatment processes have been tried, including hypochloride-treatment of venoms and mixtures of soap and venom. Detoxification of venom by photo-oxidation in the presence of Methylene Blue was used with little success. Hydrogen-peroxide has been used in the presence of traces of copper, and bile-detoxified venoms and venoms treated with organic gold. None of these methods was of practical use. Formalin rendered venoms atoxic but also largely non-immunogenic [Christensen, 1979; Sullivan, 1987]. Recently, iodination with non-radioactive iodine rendered snake venom atoxic but with retained immunogenicity [Daniel *et al.*, 1987; Heneine *et al.*, 1988].

Other methods not involving detoxification but adsorption of the venoms onto inert carriers have been successful in many instances. The slow absorption rate of the venom from the

injection site minimises the harmful effects. Prolonged stimulation by the antigen usually elicits a good immune response. Calmette, working on an idea received from Dr. Roux, impregnated chalk with venom and coated it with collodion. Other adsorbents were aluminium phosphate, aluminium hydroxide, bentonite, Freund's adjuvant, olive oil and gum arabic and sodium chloride [Christensen, 1979].

Recently, the use of venom incorporated into liposomes or into osmicated liposomes, has been shown to elicit an effective and sustained immune response with a small number of doses, usually one or two [Freitas *et al.*, 1989; New *et al.*, 1984; Theakston, 1989a]. Venoms can be incorporated into liposomes, whether detoxified or not. Both oral and parenteral immunisations with venom incorporated into liposomes has produced high antibody titres [Laing *et al.*, 1987].

Most of these adjuvants will find supporters and critics. Their success very much depends on how they are prepared and on how the assessment of improvement is made.

1.6.3. Practical Considerations of Antivenom Production

The guidelines published by WHO [1981] are not always followed exactly. Each country has its peculiar modifications and the decision on how best to accommodate local conditions within the WHO guidelines has to be made locally.

The decision of which venom or venoms to use as antigens is normally based on the frequency of bites by different snakes, the severity of the effects and availability. Availability is important for a continuing production programme and can be achieved either by keeping a collection of snakes for venom extraction or by buying venoms from a certified producer. The second option has the problem of not being able to certify the species origin of the venom, as there is not to date an acknowledged method of so doing.

Which venom or venoms to use in the immunisation schedules is also a problem. The criteria based on the frequency of bites is disputed, as conflicting snakebite statistics exists

and it is impossible to correctly identify the offending snake in the majority of accidents. This makes any data on the subject statistically invalid. In addition the data on frequency of bites is normally biased towards regions and it does not represent the real numbers in the country as a whole. Cardoso and Brando, [1982] reported that 90% of the Bothropic bites in São Paulo State, treated in the Hospital Vital Brazil, were due to *B. jararaca*. Kouyoumdjian *et al.*, [1988] reported that in the region of São José do Rio Preto, in the north of the São Paulo State, *B. moojeni* was responsible for the majority of the bites, (88%) followed by *B. alternatus*, (9.5%) and *B. jararaca*, (1.5%). In a survey of the snakebite accidents in the metropolitan area of the city of Salvador, (Capital of Bahia, a North-Eastern State of Brazil), the sole causal agent was *Bothrops leucurus*. In countries in which there are various species of snakes of medical importance, the composition of the immunising mixture is highly relevant.

Antivenoms can be monovalent, made by immunising with only one venom, or polyvalent, made by immunising with a mixture of venoms based on the frequency of bites. In Brazil the commercial production of antivenoms is based on an arbitrary mixture of fixed proportions of venoms, based on accidents treated in the Hospital Vital Brazil in the city of São Paulo.

The complex nature of venom composition and its variability, raises questions concerning the ability of the antivenom to neutralise the relevant components responsible for the pathophysiological action of the venom as a whole and of components of venoms not present in the mixture [Dias da Silva *et al.*, 1989; Moura da Silva *et al.*, 1990]. This preoccupation dates back to 1955. In cross-neutralisation studies of Bothropic venoms, three monovalent antivenoms were shown to be as potent and polyspecific as the polyvalent antibothropic sera. It was concluded that any one of three venoms could be used alone for immunisation and that the antivenoms produced would each have the polyvalence and potency needed to treat any Bothropic accident of Brazil. The venom of *B. jararaca* was suggested by the author [Schöttler, 1955].

However, recent reports contradict these findings. Experiments on the cross-species neutralisation of ten *Bothrops* venoms by monovalent antivenom sera (raised against a single venom species) revealed that a mixture of venoms of *B. jararaca*, *B. jararacussu*, *B. alternatus*, *B. moojeni* and *B. neuwiedi*, should be in the "pool" of venoms used for immunisation. The antibodies elicited by the mixture were capable of neutralising, to varying degrees, the lethal activities present in all ten venoms [Dias da Silva *et al.*, 1989].

In another set of experiments, extensive cross-reactivity of Bothropic venoms was reported, confirming older data from the literature. The monovalent antivenoms to *B. erythromela* and *B. moojeni* neutralise the venom of *B. jararaca* as efficiently as the homologous antivenom [Moura da Silva *et al.*, 1990].

In a study of the neutralisation of the haemorrhagic activity of Bothropic venoms by the polyvalent anti-Bothropic serum, Domingues *et al.*, [1990a], reported a low neutralising potency against the *B. jararaca* venom, which comprises 50% of the venom "pool". The author argues that the criteria based on the frequency of bites to make the immunising venom "pool" should be revised.

Moura da Silva *et al.*, [1990], performed studies based on observations of structural homology and possible similar biological activities between a myotoxin from *B. jararacussu* venom with PLA₂ activity, and the PLA₂ of *Crotalus durissus terrificus*. The results showed that the anti-Bothropic-Crotalic antivenom was more efficient in neutralising the haemorrhagic, lethal and myonecrotic effects of the *B. jararacussu* venom than the anti-Bothropic antivenom normally used for treatment of poisonings from this snake.

The criteria for testing the neutralisation potency of antivenoms uses one reference venom that should be representative of the venoms in the "pool". The activity presently tested is lethality. The WHO guidelines suggests also the assessment of other venom activities, such as haemorrhagic, necrotising, coagulant and defibrinating properties.

A study was performed on the neutralisation of the haemorrhagic activity of *B. jararaca* reference venom, *B. atrox* venom and *Lachesis muta* venom, by the anti-Bothropic, (FUNED), anti-Bothropic-Lachetic, (FUNED) and anti-Lachetic, (Butantan) antivenoms. The authors concluded that the criteria of accident frequency used to choose the reference venom is not valid and it should be based on quantitative studies of cross-neutralisation [Domingues, *et al.*, 1990b].

These findings probably represent the situation not only in Brazil, but also in other countries where different poisonous snakes exist. They indicate a need for further study on the characterisation of toxicity and biological activities of snake venoms and especially of cross-neutralisation.

1.6.4. Present Situation and Studies on Antivenom Production

The present situation of antivenom production has not evolved much further from earlier practice. The enzyme-purified antivenom probably has been the only improvement; it minimises the chances of allergic reactions. However the fractionation by ammonium sulphate does not adequately purify the product. The remaining non-immunoglobulin content is high and contributes to host adverse reactions such as serum sickness [Sullivan 1987].

Purification of antivenom IgG by affinity chromatography has been performed on antibody with high affinity towards Crotalid venoms. The purified immunoglobulin was mainly IgG(T), [Sullivan, 1987]. Experiments have shown that horse IgG(T) is superior to whole antivenom in lethality protection and protection to toxic effects [Sullivan, 1987]. Recently, Fernandes *et al.*, [1991], compared the neutralising potencies of horse IgG, horse IgG(T) and horse IgG depleted of IgG(T). The results showed that both IgG and IgG(T) had the

same neutralising potency, whereas IgG depleted of IgG(T) had a greatly reduced potency, indicating that the neutralising capacity resides mostly with the IgG(T).

A novel approach for the preparation of antivenom is the use of avian antibodies. Carroll *et al.*, [1992] immunised hens with detoxified venoms and purified antibodies from the egg yolk, (IgY), using PEG-precipitation followed by affinity chromatography on venom adsorbents. On a protein weight basis the two avian antivenoms tested were 6.3 and 2.0 times more effective than the respective reference sera, produced in horses.

Studies on antivenom cross-neutralisation of specific toxic components has provided evidence that some specific components can elicit antivenoms that are more potent than others and have greater polyvalence. Also, some venoms possess larger quantities of these specific toxic components than others. Based on these facts it has been proposed to isolate components accounting for the main toxic effects such as neurotoxicity, coagulation, necrotising activity, haemorrhagins, etc., and to inject them as a mixture to produce more effective antivenoms [Mebs and Doll, 1992; Mebs *et al.*, 1988]. The isolated toxic component(s) can also be used to enrich venom "pools" used in antivenom production.

In a study to investigate the effects of different immunogens on the potency of antivenoms, Li and Ownby, [1992], separated the venoms of *Crotalus atrox*, *C. adamanteus*, *C. d. terrificus* into different molecular weight fractions, small, medium and large. In comparing the antivenoms produced separately to each fraction, with the commercially available Wyeth antivenom, no improvement was observed regarding lethality protection and neutralisation of haemorrhagic activities.

Monoclonal antibodies (Mabs) capable of blocking or neutralising toxic fractions may be a future possibility for providing specific protection. However the mode of action of Mabs is not known and it is critically important to determine whether the antibodies interact at the

toxic or enzymatic site of the molecule. The application of antibodies for treatment of *Viperinae* envenoming is less hopeful, as a multiplicity of toxins are responsible for the systemic signs. A "cocktail" of monoclonal antibodies would be needed [Theakston, 1989b]. Ménez [1985] considered that in the case of elapid bites, one monoclonal antibody could neutralise the main toxin.

Another possibility is that high molecular weight toxic components may have many antigenic determinants and there is concern about the possibility that only one antibody could be able to exert neutralisation [Perez *et al.*, 1984].

In contrast, Iddon *et al.*, [1988] and Pukrittayakamee *et al.*, [1983], have reported on monoclonal antibodies which are capable of significant neutralisation of venom components, factor X activator of Russell's viper and the haemorrhagic factor of the Nigerian *Echis carinatus* respectively.

Some toxic components are known to have isotypes. Crotoxin has 15, [Faure and Bon, 1987], and it is also critical to test specificity, affinity and neutralisation potency of the antibodies against the toxin isotypes.

A study [Middlebrook, 1991], on the neutralisation of pseudexin by monoclonal antibodies has been reported. Three monoclonal antibodies were able to recognise all pseudexin isozymes (isozymes). In some cases antibody binding was essentially equivalent with each pseudexin isozyme. On the other hand, several monoclonal antibodies differed by two to three orders of magnitude in their binding to different pseudexin isozymes. In competition assays to displace rabbit polyclonal IgG, the maximum values observed were 45-50% inhibition. A mixture of monoclonal antibodies produced essentially the same result.

Stiles [1991], tested twelve monoclonal against cobrotoxin from *Naja naja atra* venom for cross-reactivity with eight different snake venoms toxins binding to linear epitopes, and for preventing cobrotoxin binding to acetylcholine receptor (AchR) *in vitro*, and for protection

of mice against cobrotoxin. There was little reactivity with other snake toxins. None of the monoclonal antibodies bound to the reduced cobrotoxin or any of the synthesised regions of cobrotoxin spanning the whole molecule, thus suggesting the recognition of conformational epitopes. The *in vitro* binding of toxin to AchR was competitively inhibited to varying degrees even when the antibodies were pre-incubated prior to the addition of AchR. The inhibition was dependent on the antibody:toxin ratio. An antibody:toxin ratio of 2.5:1, with 6 µg of cobrotoxin, delayed the time of death of mice 3.7-23.8-fold compared with control mice.

1.6.5. Future Contribution of Recombinant DNA Technology

Recombinant DNA technology is just beginning to be applied to the study of snake venoms. The ability to clone toxins, (haemorrhagins, neurotoxins, myotoxins or mutant forms with single or more mutations) will allow the possibility to look into the many aspects governing toxicity, mode of action, immunological reactivity, neutralisation and crossspecific-neutralisation. Mutant toxins with retained immunogenicity but no toxicity could be envisaged as ideal immunising agents. Tamya *et al.*, [1985] has cloned the cDNA encoding a curarimetric precursor with the aim of producing mutant proteins with single mutations by means of site-directed mutagenesis.

1.7. Envenomation

Changes in the physiological equilibrium state of a person is the characteristic result of envenoming. Bites by venomous snakes do not always result in envenoming. The figures reported vary, but about 20% to 40% of bites do not cause any envenoming [Boyden, 1980; Talpers and Bergin., 1985]. However the consequences of snakebite are unpredictable in their early stages, and medical observation is needed to assess the severity of the clinical symptoms.

The severity of snakebite is dependent upon many factors; age and general health of the victim, bite site, amount of venom injected, species involved, sensitivity of the patient to venom and treatment.

The complex nature of snake venom composition and their variability account for widely variable clinical manifestations [Chippaux *et al.*, 1991].

The previously held views of separate properties of cytotoxic and haemotoxic viperid venoms, neurotoxic elapid venoms and myotoxic sea snake venom, is today untenable [Warrell, 1992]. The increased amount of data on snake envenoming has shown that venoms belonging to snakes of different families can induce similar signs and symptoms, and snakes from the same species but from geographically distant places induce different clinical manifestations of envenoming.

Generally the syndromes reported include, oedema, haemolysis, shock, bleeding, pituitary failure, renal failure, myonecrosis, neurotoxicity. and combinations of the above [Nelson, 1989].

In Brazil, data on the epidemiology of ophidic accidents has been scarce but more data is becoming available due to a concerted effort from the Ministry of Health and producers of antivenoms. A recent review gives an update on the information on snakebite and mortality in Brazil. In the beginning of the century the number of ophidic accidents were then evaluated as 19,200 each year, being mainly due to the *Bothrops* species. In the year of 1987, 21,463 cases with 123 deaths were notified to the Department of Health. The incidence for Brazil was calculated as 15.5/100,000 inhabitants and the death rate of 0.6% showed a variation within the geographic macro regions from 0.3% (Southeast) to 2.1% (North). From June 1986 to December 1987, there were 20,884 notifications of accidents with information on the genus of the offending snake. The majority, 88.3%, were caused by *Bothrops*, 8.3% by *Crotalus*, 2.7% by *Lachesis* and 0.7% by *Micrurus*. The number of deaths caused by the *Bothrops* species were greatest followed by *Crotalus*, but this may be due to the frequency of their presence because the deadliness of bothropic accidents (0.5%)

is low compared to the crotalic (3.0%). A similar picture is observed in the State of São Paulo, Brazil, where, for the years of 1986, 1987 and 1988, 6,308 accidents were notified (incidence of 6.8/100,000 inhabitants). The cases (4,685) with reference to the genus of the snake were: 85.3% by *Bothrops*, 13.6% by *Crotalus* and 1.1% by *Micrurus*. Lethality was 0.3%. Bites occur mostly over the hot season when snakes are more active, and within the rural areas where the rural labourer without adequate protective clothing (long-leg boots, puttees and leather gloves) are at high risk. Interestingly the most severe accidents occur with workers using long-leg boots and puttees, usually caused by large snakes [Ribeiro, 1990]. It is noteworthy that the higher death rates occur in regions where the antivenoms used for treatment were not produced with venoms from snakes present in those areas.

1.7.1. Clinical Effects of Envenoming

1.7.1.1. Blood Disorders

In most cases where there is envenoming by pit vipers, the most common systemic disturbance is blood incoagulability and increased coagulation times [Misra and Misra., 1982; Nelson, 1989; Sano-Martins, 1990]. However systemic bleedings are less common. The incoagulability is attributed mainly to fibrinogen consumption induce by thrombin-like fractions. Mild thrombocytopenia is usually observed.

1.7.1.2. Local Effects

1.7.1.2.1. Pain

Usually thought to be the characteristic of viperid venoms, it is seen in elapid bites and is absent in some viperid envenomation [Campbell, 1979; Minton, 1990; Warrell, 1989]. Immediately following the bite there is some pain, which almost always is confined to the area of the bite. In cases of *Vipera russelli* venom it can be extended to the lymph nodes [Warrell, 1989]. Cobra bites almost invariably are painful [Minton, 1990]. It is present in the North American *Crotalus* but rarely reported in bites by the South American *Crotalus*.

1.7.1.2.2.Oedema and Swelling

This is predominantly seen with vasculotoxic venoms such as the viperids. It is present also with cobra venoms, specially the Africa spitting cobras, *Naja nigricollis* and *Naja mossambica* where there is a marked local swelling. It is less evident with Kraits and Mambas, both elapids [Minton, 1990].

Oedema is usually seen around the injured area, within minutes to 2 hours of the bite. Swelling is limited to the subcutaneous tissues. The swollen part is painful to palpation. The swelling may extend to other parts of the body and peak between the second and fourth day.

Local oedema is typical of Bothropic bites. It is probably caused by the direct effect of venom on vessels and release of endogenous mediators like histamine, kinins and prostaglandins, (due to the action of venom components on mast cells), kininogens and phospholipids or a combination of these. In Bothropic snake bites the degree of swelling has prognostic value [Rosenfeld, 1971].

1.7.1.2.3.Echymoses

Echymoses and discoloration of the skin often appear within several hours of the bite. Echymosis may occur along with oedema and without bleb formation. It is not usually confined to the bitten area and can be seen over the entire extremity. Discoloration is sometimes seen with cobra bites, often preceding necrosis [Minton, 1990].

1.7.1.2.4.Necrosis

This is caused mainly by viperid venoms. Contrary to the belief that myonecrosis is caused by a rise in intracompartmental pressure, it is now known to be due to; a) direct action of myotoxins on the skeletal muscle plasma membrane, b) ischemia, thrombosis blocking local blood vessels causing dry gangrene as a consequence of the vascular damage, and haemorrhage and arterial lesions.

1.7.1.3.Shock

The principal cause of shock is the loss of plasma and blood cells into tissues in the area of the bite, as well as into the lungs, peritoneum and other parts of the body. Early shock is common with some vipers and can be very dangerous if it occurs within the first three days. It usually happens between the 6th and 26th hours of envenoming. Tendency to shock is recidivist and ends in death [Misra and Misra., 1982; Rosenfeld, 1971].

1.7.1.4.Neurotoxicity

This is a predominant feature of elapid and hydrophid bites. It is also observed in bites of rattlesnakes from the *Sistrurus* and *Crotalus* genera. Few neurotoxins have been identified in venoms of vipers. Before the onset of muscle paralysis there are pre-paralytic symptoms, common to most Elapid bites: vomiting, the commonest, appears within 5 minutes of the bite and headache within 2 minutes which may last for up to one day. Another main symptom is the development of cranial nerve palsies. These are characteristically manifested as ptosis and ophthalmoplegia with blurred vision or diplopia, difficulty in swallowing with an inability to handle oral secretions, slurred speech, weakness of facial muscles and occasionally loss of the sense of smell and taste. The pupils are usually dilated and have a slow response to light. Drowsiness often accompanies this syndrome, sometimes with mental confusion, which is an important sign of respiratory failure. Flaccid paralysis affects all muscle groups. Breathing becomes shallow and diaphragmatic. The patient becomes cyanosed and paralysed, consciousness is lost, convulsions may occur and eventually there is cardiac arrest. Pre-synaptic neurotoxins tend to have a delayed onset but give prolonged symptoms, whereas post-synaptic toxins account for early paralytic symptoms, such as ptosis and ophthalmoplegia [Campbell, 1979; Minton, 1990].

1.7.1.5.Renal Failure

Renal failure is an important complication of snakebite. It is present with poisoning of *Viperinae*, *Crotalinae* and sea snakes. The pathogenesis is still unclear but a direct nephrotoxic effect, shock, haemolysis, haemoglobinuria, mioglobinuria secondary to

rhabdomyolysis, and haemolytic-uraemic syndrome, may all play a part. The differences are probably due to venom variability, but evidence for all of the above mechanisms has been reported. Oliguric and non-oliguric renal failures have been observed. Proteinuria and gross or microscopic haematuria is consistently found in patients with renal involvement. In patients with renal failure a rise of blood urea, creatinine and potassium is common; acute pulmonary oedema and shock may also be present [George *et al.*, 1987; Nelson, 1989]

1.7.1.6.Pituitary Failure

A few cases have been reported where this symptom is present. It was first described [Wolff, 1958] in a patient bitten 7 years before by a *Bothrops jararacussu*. Russell's viper bites of southern India and Burma account for the majority of reported cases of pituitary failure [Eapen *et al.*, 1976; Majeed and Thomas., 1987; Tun-Pe *et al.*, 1987]. It is the only long term sequel to severe envenoming of Russell's viper [Warrell, 1989]. Brain haemorrhage is also present in some Bothropic accidents [Kouyoumdjian, 1990].

1.8. Treatment

The many signs and symptoms of snake poisoning have contributed to the large number of measures proposed by the layman and physicians for treatment.

Since ancient times a variety of means and methods of treating snakebites has been used. The Roman Pliny (23-79 AD), recommended a draught of the victims own urine and also the snake's liver. In ancient Greece the "*priapus cervis*" was prescribed, a solution in wine of a powder made from the sex organs of the red deer. A famous medicine, "*Theriac*", had snake flesh as a component, which was used to treat snakebite as well as a great number of other illnesses. It is believed to have been discovered by Mithridates, King of Pontus [Engelmann, 1984].

The treatment of snakebite today is based on scientific principles. The clinical measures applied for the various snake poisonings are basically the same. Broadly these measures can be classified as: first aid measures, supportive measures and, the most successful and widely used, antivenom therapy.

1.8.1. First Aid

First aid measures are concerned with delaying the spread of the venom and, still advocated by some physicians, the removal of as much as possible of the venom from the bite site before total diffusion into the patient has occurred.

The first was attempted by the use of a tourniquet [Fairley, 1929], and is today regarded by most authorities as deleterious to the patient's health. It exacerbates the local symptoms of haemorrhage and necrosis, usually leading to the need for amputation of the limb or part of it. The use of firm bandages over the bitten area, and over the length of the limb where possible, is the accepted measure, together with the immobilisation of the limb. Reassurance of the victim and rapid transport to the nearest hospital is important [Sutherland, 1975]. Removal of the venom, implying the need of incision or excision around the bite site, is still proposed, [Stewart *et al.*, 1981]. Studies have shown that these measures can remove up to 35% to 50% of the venom if suction starts within minutes after the bite [McCullough and Gennaro, 1970; Shulov *et al.*, 1969]. Incisions might open the lymph channels facilitating the resorption of venom, [Leopold and Merriam, 1960], and if performed by untrained persons it might lead to nerve and muscle damage to the limb, resulting in severe consequences and even the need for amputation [Glass, 1976]. Cryotherapy was proved to bring no benefit in slowing venom adsorption, and studies showed that prolonged exposure to cold could result in gangrene of the bitten area or limb [Gill, 1970; McCullough and Gennaro, 1963; Moseley, 1966; Russell, 1979]. The effect of local application of heat or cold on the development of tissue necrosis in envenomed rats was recently studied [Cohen *et al.*, 1992]. The application of heat was intended to increase local blood-flow and

accelerate diffusion of venom from the bite area to minimise local reactions. The authors observed no beneficial effect of heat or cold on the development of tissue injury after injecting rats with necrotising venoms. They argue that prompt use of antivenom is the measure of critical importance.

1.8.2. Supportive Measures

The many proposed measures aim to counteract specific venom effects. Intravenous fluids are essential in most cases. Oral fluids, specifically in cases of Elapid envenoming where pharyngeal paralysis can occur, are discouraged [Sutherland, 1975]. Coagulation defects must be looked for and treated according to need. Plasma, cryoprecipitate and platelets, can be used. Burgess and Dart, [1991] points out the risks of opting for this kind of treatment instead of antivenom therapy.

1.8.3. Other Drugs

Many of the toxic effects of snake venoms are produced by the interaction of venom components with the normal physiological systems of the victim, either by blockage, as in the case of neurotoxicity, or by activation, as in the coagulopathy syndromes. Based on this knowledge, it was proposed to counteract the toxic effects by using antagonistic drugs.

Cholinergics have been suggested for treatment of the paralysis of elapid bites. Studies suggest both favourable and unfavourable results may ensue [Bannerjee *et al.*, 1972; Pawar and Singh, 1987; Vieira *et al.*, 1987; Warrell *et al.*, 1983]. Zanin and Coelho, [1990] report the use of neostigmine for the treatment of bites by the *Micrurus* genera in Brazil. Watt *et al.*, [1986] showed that edrophonium improved the patient's visual and pulmonary conditions, following neurotoxic symptoms caused by the bite of *Naja naja philippinensis*. However in electromyography studies with the venom of *Micrurus laticollaris*, edrophonium failed to abolish myasthenia-like patterns [Pettigrew and Glass, 1985]. Pawar and Singh, [1987] concluded that once weakness is established to the point where

assisted ventilation is needed, neither anti-cholinergics nor antivenom will affect the outcome.

Heparin has been considered where disseminated intravascular coagulation is present. The present medical opinion is that when the symptoms are caused directly by the venom, heparin is contraindicated [Sutherland, 1975]. The direct action of brazilian snake venoms on fibrinogen is not inhibited by heparin and its use to correct coagulation disorders should be avoided [Amaral *et al.*, 1991]. The use of intravenous corticosteroids is discouraged by most authorities, the only controlled trial showed no benefit in their use, [Kurecki and Brownlee, 1987; Nelson, 1989]. Antibiotics should be used according to need, and usually of broad spectrum [Amaral *et al.*, 1991; Kurecki and Brownlee, 1987; Nelson, 1989].

Plant extracts are used in popular medicine to treat snakebite. Duke [1985] and. Mors [1991], stated that 578 species of plants have been cited in the literature as having antidotal activity against snake venoms or some of their components. Water-soluble extracts or methanol extracts are used. In Brazil a popular medicine used to treat snakebite, known as "Específico Pessoa", is sold in the North and Northeast of the country. The plant that gives origin to the extract is kept secret. Nakagawa *et al.*, [1982] isolated from the extract two compounds, cabenegrin AI and AII thought to contain the active components. When injected i.v. (1.0 mg/kg) in a beagle 15 minutes prior to the injection of *B. atrox* venom (2.5 mg/kg, i.v.) it restored respiratory function, blood pressure and cardiac symptoms to normal in 60 to 90 minutes. AII had similar effects as AI. Mors *et al.*, [1989] reported the neutralisation of lethal and myotoxic activities of South American rattlesnake venom by extracts of the plant *Eclipta prostrata*. The authors noted that the plant has the same anti-snakebite reputation in China. Of the extracts and compounds tested, 3 extracts and 2 compounds (Active CH₂Cl₂, Wedelolactone) completely abolished mortality and the other 3 greatly reduced it in the experimental conditions used. The crude extract also inhibited the myotoxic activities of the venom both '*in vitro*' and '*in vivo*'. A large number of reports of plants extracts being tested under controlled conditions were recently reviewed [Martz,

1992]. The author also suggests that such components or extracts, if proven effective against snakebite, could be used as first aid measures or as supportive treatment.

1.8.4. Serum Therapy (Antivenoms)

There are many reports in the literature concerning the treatment of snakebite by antivenoms see Amaral *et al.*, 1991; Boyden, 1980; Joseph-Ryan, 1983; Kouyoumdjian and Polizelli, 1988; Kurecki and Brownlee, 1987; Misra and Misra., 1982; Nelson, 1989; Persson and Irestedt, 1981; Pugh and Theakston, 1987; Rosenfeld, 1971; Sherry, 1985; Sutherland, 1990; Wagner and Golladay, 1989]. Broadly, all classify the measures as: First aid and Medical treatment, the latter being supportive measures, and antivenom therapy. The decision on which of these measures to use is based on clinical observation of the patient and laboratory tests.

Since the early work on antivenoms, [Brazil, 1901; Calmette, 1894], the mortality and morbidity due to snakebite has decreased sharply in some countries [Warrell, 1992]. Serum therapy for snakebite is generally accepted as the most effective treatment. However good, the use of antivenom still has its problems and the decision to use it depends on the physician's evaluation of the patient's clinical status, based on the observation of clinical signs of envenoming, local or systemic, and laboratory tests. With few exceptions most snake poisonings are graded as one of the following:

1. No envenoming
2. Minor - local reactions, no systemic signs, normal laboratory results.
3. Moderate - increased local reactions, some systemic signs, abnormal laboratory results.
4. Severe - marked local reaction, severe systemic signs, abnormal laboratory results.

Antivenom should be given intravenously (i.v.), usually diluted in normal saline or other isotonic solution. The subcutaneous or intramuscular routes are not indicated because of the slow adsorption of the antivenom [Campbell, 1979; Sutherland, 1975].

The amount of antivenom to be administered will depend on the severity of the bite and type of venom. The return of the coagulation time to normal and the cessation of gum bleeding are used as signs of treatment efficacy in some types of poisoning. In general there is no definite treatment scheme and the doses vary for each case. A report containing some dose recommendations for various species [Nelson, 1989] shows great variation, from 3-4 vials for all sea snakes. Tu [1987] suggests 10-40 ampoules (100 to 400 ml) for severe Crotalid envenoming. In Brazil the suggested initial doses for Bothropic envenoming are: 4 - 12 ampoules (40 to 120 ml) for minor to severe respectively. For Crotalic bites, all cases are considered moderate to severe and the doses are: 15 - 30 ampoules (150 to 300 ml) or more ampoules respectively. For the Lachetic accidents 8 - 16 ampoules, (80 to 160 ml); and for elapidic enough to neutralise 150 mg of venom [Amaral *et al.*, 1991].

Antivenom should be given as early as possible, in the right dose and through the appropriate route [Chippaux and Goyffon, 1992; Minton, 1990; Rosenfeld, 1971]. To be most effective it should be used within 4 to 24 hours of the bite. Dwivedi *et al.*, [1989] report a case of a 19 year old man who was admitted to hospital 8 days after the bite, showing signs of envenomation (bleeding from multiple sites, echymoses, melenae and frank hematuria). The administration of 40 ml of antivenom diluted in 500 ml of isotonic solution, promptly reversed the course of envenoming. In cases of Coral snake and Krait envenoming, antivenom should be given before signs of envenoming develop, because these venoms are difficult to neutralise after binding to the myoneural junction [Minton, 1990].

Hypersensitivity reactions are a common side effect of serum therapy. Measures to minimise risks of early reactions differ among authorities. Skin and conjunctival tests are proposed by

some [Amaral *et al.*, 1991; Boyden, 1980; George *et al.*, 1987]. In Australia they are regarded as unreliable, delaying urgent therapy. Use of appropriate premedication (adrenaline) before infusion of antivenom is advocated for the prevention of an adverse reaction [Sutherland, 1990].

Antivenom therapy if necessary, should not be withheld even if the patient has a history of horse serum allergy or is positive on the sensitivity test. The benefits far outweigh the adverse reactions and these can be properly controlled in a hospital environment [Burgess and Dart, 1991; Otten and McKimm, 1983]. Early reactions, i.e. anaphylaxis, can be controlled by epinephrine and antihistaminics; late reactions, i.e. serum sickness, can be ameliorated with steroids and antihistaminics [Burgess and Dart, 1991].

Despite its 90 year history, antivenom therapy remains largely empirical [Cardoso, 1990; Ho *et al.*, 1986]. In a controlled study, patients with Bothropic envenoming were divided into two groups: minor and moderate envenoming. Half of the patients with minor envenoming received 4 ampoules and the other half received the same number but with half the protein content per ampoule. The same scheme was adopted for the moderate group but each patient received 8 ampoules of antivenom instead. The results showed no difference in the clinical outcome of the treatment between the groups [Castro *et al.*, 1990].

In another study, patients were separately treated for Bothropic bites with antivenom from the 3 different producers in Brazil. The incidence of early reaction was 87% for the Butantan antivenom, 36.5% and 56% for the Vital-Brazil and FUNED antivenoms respectively [Dias da Silva *et al.*, 1993]. This seems to confirm reports that patients are probably receiving excessive doses of highly efficient antivenom, increasing the incidence of early anaphylactic reactions [Theakston, 1992].

Antivenom is the only specific treatment for snakebite in most parts of the world. They are clinically effective against haemostatic disorders, hypotension and post-synaptic

neurotoxicity, but are much less effective against pre-synaptic neurotoxicity, nephrotoxicity and local necrosis [Warrell, 1992].

Further studies on the kinetics of envenoming and treatment of snakebite, on the immunology of venom toxins, antivenoms and on the mechanisms of toxicity should improve treatment, reduce reactions, and minimise mortality and morbidity.

1.9. Diagnosis of Snake Bites

Snakebite by members of the *Crotalinae* Subfamily are usually medical emergencies requiring immediate attention and the exercise of considerable judgement by the physician. Delayed or inadequate treatment may result in tragic consequences for the patient. Before any treatment is given, it is essential to establish a working diagnosis. It must be remembered that not all snakes are venomous and that not all venomous snakebite result in envenomation. Hyperventilation, weakness, anxiety, and nausea can be mistaken for poisoning. Unnecessary treatment may cause discomfort to the patient and/or provoke serious complications, [Russell, 1979].

To date, the diagnosis of snake venom poisoning is based on the presence of local or systemic signs and fang marks. These are often imprecise and only the positive identification of the offending snake by trained persons can confirm without doubt the diagnosis.

The clinical manifestations, local or systemic, from which a clinical response to snakebite is made is dependent upon a number of factors, mainly; 1) the age and size of the victim; 2) the nature, location and depth of bite; 3) the amount of venom injected; 4) the species and age of snake involved; 5) the victim's sensitivity to the venom. However, some local or systemic signs are more frequently observed within a family or genus of snakes, and can be used as guidelines to the diagnosis and grading of the severity of the envenoming.

The diagnosis of snakebite through clinical symptoms is used in most countries of the world, even in Australia, the only country known to be using a diagnostic kit [Boyden, 1980; George *et al.*, 1987; Joseph-Ryan, 1983; Kurecki and Brownlee, 1987; Misra and Misra., 1982; Nelson, 1989; Pugh and Theakston, 1987; Sherry, 1985; Sutherland, 1975; Talpers and Bergin, 1985; Warrell, 1989]. In countries where there is availability of polyvalent antivenom this proves to be the most successful empirical treatment irrespective of species diagnosis.

In Brazil, a set of basic rules for the clinical diagnosis of snakebite has been laid down by Rosenfeld, [1971] in his extensive review of the “Symptomatology, Pathology and Treatment of Snakebite in South America”. Venoms were classified by their physiopathologic activity, which would elicit specific signs and symptoms. The use of such criteria provides the ability to identify the genus or possibly the species of the offending snake. Snakes from the *Bothrops* and *Lachesis* genera have venoms classified as Proteolytic and Coagulant; from the *Crotalus* genera, Haemolytic and Neurotoxic, and from the *Micrurus* genera, Neurotoxic. Based on this classification a differential diagnosis for envenomation can be made, (Table 1.2):

Table 1.2: Differential Diagnosis of Envenomation by Brazilian Venoms

Venom Type	Local Reaction	Neurotoxic Facies	Methemo-globinuria	Haematuria	Incoagulable blood
Proteolytic & Coagulant	++	-	-	+	++
Haemolytic & Neurotoxic	-	+	++	-	-(+)
Neurotoxic	-	++	-	-	-

The severity of snakebite of different venom types can be classified as follows (Table 1.3):

Table 1.3: Classification of Symptoms in Envenoming by Brazilian Snakes

Venom Type	Mild Cases	Serious cases	Severe cases
Proteolytic + Coagulant	Small local reaction, coagulable blood	Evident local reaction, incoagulable blood	Intensive local reaction, incoagulable blood, haematuria, generalised pain, prostration
Haemolytic + Neurotoxic	Absent neurotoxic facies, normal urine	Neurotoxic facies, methemoglobinuria	Neurotoxic facies, Methemoglobinuria, generalised pain, agitation or torpor
Neurotoxic	Absent neurotoxic facies, local paresthesia	Neurotoxic facies, paresthesia over the entire extremity	Neurotoxic facies, thick salivation, difficulty in swallowing and speaking, prostration, unconsciousness

The local signs for the Proteolytic and Coagulant venoms are: pain, erythematous oedema and echimoses, haemorrhagic phlyctema (possibly) and necrosis. General symptoms are: dizziness, nausea, uneasiness, bilious or bloody vomitus, haematuria and melena, epistaxis or gingival haemorrhage.

For haemolytic and neurotoxic venoms, the local symptoms are: strong pain immediately after bite, local paresthesia and anaesthesia. Oedema rare, frequently no apparent lesion at the site of the bite. General symptoms: visual disturbances (ophthalmoplegia) and blepharoptosis, neurotoxic facies and loss of equilibrium. In serious cases, agitation, bilious vomitus, delayed pupillary reflexes, prostration and coma, mydriasis, methemoglobinuria and progressive anuria may occur.

For neurotoxic venoms, the local symptoms are: pain, absence of oedema or any local reaction, progressive paresthesia and drowsiness. General symptoms: Ophthalmoplegia and neurotoxic facies. Loss of equilibrium, thick salivation, torpor.

These guidelines are still used in Brazil with little modifications. The Department of Health of the São Paulo State issued a manual in 1984 for the treatment of human envenoming by poisonous animals. Apart from the inclusion of snake morphologic characteristics to aid in the identification of snakes and their geographical distribution, the method for the clinical

diagnosis of snakebite are those described by Rosenfeld, [1971]. Similarly the Brazilian Ministry of Health issued in 1991 [Amaral *et al.*, 1991], a Manual for the Diagnosis and Treatment of Snakebite, where the same methods proposed by Rosenfeld are used with minor modifications due to updated information on the snake venom's toxic activities. The diagnostic table for the Brazilian snakebite accidents in the manual is as follows (Table 1.4):

Table 1.4: Diagnostic Table of Brazilian Snakebite Accidents

Snake genus	Venom activities		Immediate signs and symptoms (up to 6 hours after the bite)	Late symptoms hours after the bite)
<i>Bothrops</i>	Proteolytic Coagulant Hemorrhagic	Evident local reactions	Pain, oedema, erythema. Increased blood coagulation time, Haemorrhages. Shock.*	Blisters, echymoses, necrosis, oliguria and anuria (acute renal failure)
<i>Lachesis</i>	Proteolytic Coagulant Hemorrhagic "Neurotoxic"	Evident local reactions	Few studied cases: clinical manifestations similar to <i>Bothrops</i> snakebite, with additional signs of vagal stimulation (Slow heart beat, hypotension and diarrhoea).	
<i>Crotalus</i>	"Neurotoxic"	Discrete or absent local reactions	Neurotoxic facies, Ophtalmoplaxia. Reports of acute respiratory deficiency in severe cases	
	Myotoxic	Discrete or absent local reactions	General muscular pain. Red or brown urine. Discrete oedema on bite site.	Red or brown urine (Hemoglobinuria and mioglobinuria). Oliguria and anuria (acute renal deficiency).
	Haemolytic	Discrete or absent local reactions	Red urine	
	Coagulant	Discrete or absent local reactions	Increased blood coagulation time. Rare haemorrhages.	
<i>Micrurus</i>	Neurotoxic	Discrete or absent local reactions	Neurotoxic facies, diplopia, ophtalmoplegia, sialorrhea, difficulty on swallowing and early acute respiratory deficiency.	

(*) In bites caused by young *Bothrops jararaca*, oedema and pain at bite site may not be present. Coagulant activity is predominant.

The same manual also includes morphologic characteristics of snakes and geographical distribution as an aid to diagnosis.

Numerous authors have described differences in symptomatology after envenoming by snakes belonging to the same species from different geographical localities and also different age, and this is more evident from widely distributed species see [Chippaux *et al.*, 1991; Minton, 1990]. Young *Bothrops jararaca* snake venom has weaker proteolytic activity than the adult snakes. Local reactions may not be present [Amaral *et al.*, 1991; Rosenfeld, 1971].

Studies are being carried out to try to correlate the distance of fang marks in *Bothrops jararaca* bites, and the age of the snake, as this has importance in the prognosis [Jorge and Ribeiro, 1987].

The characteristic clinical symptoms for a given family, genus or species of snake are not always present in the accidents caused by the snakes belonging to them.

Tests for the detection of snake venoms in body fluids or wound aspirates have clear clinical, experimental and medicolegal implications [Cox *et al.*, 1992; Currie *et al.*, 1988; Minton, 1987; Ratanabanangkoon *et al.*, 1987; Theakston, 1989b]. Bioassays and immunological tests have been used to show the presence of venoms or antivenom antibodies in many types of samples, and have been used for the diagnosis of snakebite in patients and post mortem material.

1.9.1.1.Bioassays

Trethewie [1970] tried to identify snake venoms through bioassays of their neurotoxicity, and Okonogi *et al.*, [1976] through the identification of haemorrhagic activity. Both assays proved expensive, time-consuming and non-specific. Eaker [1969] using chromatographic techniques and bioassay, (toxicity), identified cobra neurotoxin from the oedema fluid of a dead victim.

1.9.2.Immunological Assays

1.9.2.1.Gel diffusion and electrophoretic assays

Lamb [1902], using immunoprecipitation techniques, showed that cobra antiserum formed a precipitate with cobra venom but not with the venoms of *Echis carinatus*, a viper, and of *Hoplocephalus curtus* and *Bungarus fasciatus*, both colubrine snakes. This was the first report of the differentiation of snake species by immunological methods. Muelling *et al.*, [1957] used the precipitin test for the first time to elucidate the cause of death of an 18 year old girl. Homogenate from the skin surrounding the bite was mixed with horse anti-cobra

serum and the reaction was positive up to a dilution of 1:100, 000, 000. Yadlowski *et al.*, [1980] used precipitin reactions to detect *Viperidae* venom on the material used by the victim to commit suicide. It was not possible however, to identify the venom's species. Greenwood [1974], used immunodiffusion and counter-current electrophoresis in a study of 101 cases of snakebite and detected venom in 40 patients. The monovalent antivenoms were raised in rabbits and some were made specific by adsorption with appropriate freeze-dried venoms. Blister fluid, urine and sera were analysed. Twenty seven out of seventy four wound aspirates were positive and nine out of thirteen blister aspirates. No venom was detected in the 84 sera tested. Urine samples concentrated fifty-fold to a hundred-fold were employed on counter-current electrophoresis. Nineteen out of seventy six gave positive results. Samples from seven patients known to have had *Naja nigricollis* envenoming showed cross reaction against other *Naja* venoms. In 40 of 44 cases the results were confirmed on both assays. Immunoprecipitation techniques are recognised to lack sensitivity and rely on having the right antigen/antibody ratio to form a precipitate. This results in the need to use a series of dilutions of the reagents, which makes it time consuming and not reliable for routine clinical use.

1.9.2.2. Radioimmunoassay (RIA)

RIA for the detection of snake venoms was developed as a solid phase competitive assay [Coulter *et al.*, 1974]. Monovalent antibody to various snake venoms is adsorbed to a polystyrene tube or plate wells to form the solid phase. Different dilutions of known venom concentrations are labelled with ^{125}I and are added to the solid phase for the production of a standard curve. In the test, samples are incubated with the solid phase and, after washing, the labelled venoms are added. After washing again, the solid phase is counted for radioactivity. If the count is high it indicates that the samples contains no venom, if it is low the sample contains venom that competed for the antivenom, blocking the reaction of the labelled venoms. Radioimmunoassay was used [Sutherland, 1975] to detect venom in human tissue and fluids. Tiger snake venom was detected *post mortem* at the level of

210 ng/ml in the serum of a child and at the levels of 20 ng/ml and 35 ng/ml from sera of two adults bitten by unknown snakes. Sutherland and Coulter, [1977] report 3 cases of suspected snakebite, one *post mortem*, where the competitive RIA detected venoms of Brown snake or Tiger snake in the samples. In two cases the administration of antivenom improved the condition of the patients. One major drawback in the competitive RIA was the variable ^{125}I -labelling of the different crude venoms, and its multiple components, and the effect of the iodination on their antigenic sites. A new solid phase sandwich radioimmunoassay was developed [Coulter *et al.*, 1978], where a range of antibody solutions of different specificity is bound to a solid phase (CH-Sepharose 4B) and reacted against unknown samples of venom. Uptake of venoms is demonstrated by reaction against ^{125}I -Labelled antibodies of the same specificity range as the bound ones. The presence of venom in the samples is measured by higher radioactivity counts than the negative controls. Pukrittayakamee, *et al.*, [1987] using a monoclonal antibody against the factor X activator, detecting Russell's viper venom in body fluids. Quantities of 4 ng/ml in urine and 5 $\mu\text{g}/\text{ml}$ in serum were detected. Radioimmunoassays were found by several workers to be sensitive, accurate and reproducible [Boulain *et al.*, 1982; Grognat *et al.*, 1986 and Mollier, 1987] but require technical expertise, expensive equipment and carry the dangers of handling radioisotopes, thus remaining more of a tool for research and forensic applications, than a routine test system.

1.9.2.3.ELISA

The adaptation of the Enzyme-linked immunosorbent assay to snake venom detection was first performed [Theakston *et al.*, 1977] using a double antibody sandwich assay. Using 14 venoms and 5 antisera, homologous venoms were detected in concentrations of 1 to 5 ng/ml with minor cross-reactions. Venoms inoculated into rabbits at doses of 1 LD₅₀ were detected in their blood collected at periodic bleedings after the venom inoculation. The time of the assay was 3 hours. Coulter [1980], developed a variant of the assay that allowed results to be obtained in 30 to 40 minutes. Monovalent antibodies were purified by Protein

A chromatography and used to coat the plates and to prepare conjugates. Samples were swab eluates from bite sites and had to be diluted 1/2 to 1/100 to overcome cross reactions. Incubation times of the samples could be cut down to as little as 1 minute but to 5 to 30 minute as average. Samples were added to homologous and heterologous antivenom IgG and to normal IgG as a control. Tests were compared with RIA, yielding similar results. The authors attribute the greater specificity and sensitivity to the Protein A-purified IgG.

An enzyme immunoassay system suitable for the diagnostic of snakebite in the field was reported [Hurrell and Chandler, 1982]. Glass capillary tubes were coated with rabbit IgG antibodies against one of the five immunologically distinct snake venoms from Australia. The tubes were joined together by a silicone rubber tubing and connected to a small syringe. The patient's samples, (urine, swab eluate, blood with anticoagulant, and serum diluted 1/2), were drawn into the tubes using a syringe. After an incubation period, washing buffer was drawn up in the same manner three times, and the conjugate was aspirated into the tubes. After another incubation period and wash, substrate was drawn up and the colour reaction observed against a white background. A two year review of the use of this kit gave divided opinions. The authors, Hurrell and Chandler, [1982] supported the usefulness of the test and attributed the conflicting results to the use of blood samples instead of bite swabs. Sutherland [1982], disagreed with the authors, pointing out the unreliability of the test. Jamieson *et al.*, [1989] reported that the kit confirmed the diagnosis of snake bite in only 12.8% of the samples tested, including bite swabs, urine and blood. Theakston [1989b] and Cox *et al.*, [1992] also point to the unreliability of the test and showed cross reactions between venoms of snakes belonging to different genera.

Labrousse *et al.*, [1988] reported an ELISA test for rapid and sensitive detection of venom antigens after an experimental bite of *Vipera ammodytes*. Results could be obtained in 15 minutes, and 5 ng of venom per ml of serum could be detected. However the cross-reactivity observed impaired its use in countries where snakes belonging to different genera coexist in the same areas. Cox *et al.*, [1992], report a novel ELISA method which correctly

identified snake venoms from 5 genera of medically important snakes from Australia. The two-antibody sandwich method detected levels of 10 ng/ml in laboratory trials. Clinical tests are being carried out.

Tan *et al.*, [1992c], used indirect and double-sandwich ELISA procedures for the detection of Malayan cobra (*Naja naja sputatrix*) and monocellate cobra venom. Extensive cross-reaction was observed in both assays, specially against cobra venoms. The authors conclude that there is a need for more specific antibodies for the immunodiagnosis of Asian cobra bites.

In situations where the most medically important snakes, responsible for around 90% of accidents, belong to the same genus [Amaral *et al.*, 1991; Cardoso, 1990; Ribeiro, 1990], and extensive cross-reactivity is present between their venoms [Dias da Silva *et al.* 1989; Moura da Silva *et al.*, 1990; Silles-Villarroel *et al.*, 1974] it has proved more difficult to detect venoms or venom components specifically at species level [Barral-Neto *et al.*, 1990].

To overcome the problems of cross-reactivity, researchers are producing antibodies, (polyclonal or monoclonal) against specific venom toxins. However, reports of such studies show that both polyclonal and monoclonal antibodies raised against purified toxins cross-react with venoms of the same genus, the same family and even between members of different families [Lomonte *et al.*, 1990; Mandelbaum, 1988; Weinstein *et al.*, 1985].

1.10. Vaccination

The idea of becoming immune to the effects of poisons and venoms is not recent. According to Russell [1988], it is not known when man first attempted to experiment with immunising himself or others. There are many accounts of snake venoms being used in various forms or preparations to treat or protect against diseases [Morris and Morris, 1965; Russell, 1988]. Mithridates VII, a Greek ruler and physician is recorded to have tried to immunise himself

by drinking the blood of ducks, which he kept on a ration of one poison or another [Russell, 1988].

The work of Sewall [1887] is the first recorded to have experimented in a controlled manner on active immunisation with snake venoms, originating the studies on vaccination against snake bite. His inoculation of sub lethal and then increasingly lethal doses of the venom of *Sistrurus catenatus catenatus*, diluted in glycerine, into pigeons, resulted in the immunity of the pigeon against lethal doses of the venom. Kaufmann [1889], using the venom of *Vipera berus*, obtained similar results.

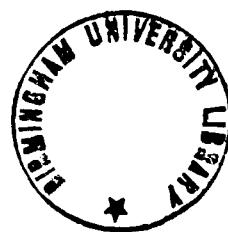
Other workers followed and Haast and Winer, [1955] reported that following a series of injections of venom of the Cape cobra, (*Naja flava*), the subject tolerated an injection of 40 mg of venom with no ill effects. Weiner [1961] reported a successful immunisation of a professional snake handler against the venom of *Notechis scutatus* after a series of injections over a 13 month period. In an interesting experiment, Flowers [1963] immunised himself against the venom of *Naja naja*, injected in sodium alginate as adjuvant. He was later bitten by a *Naja naja* and suffered no systemic symptoms although necrosis developed in the bite site. The first large scale trial in humans was carried out [Sawai and Kawamura, 1969] with 43,446 volunteers receiving the toxoid of *Trimeresurus flavoviridis*. The results suggest that the toxoid was efficient in preventing local lesions. Sawai [1979], reviews the studies on vaccination with snake venom. Many methods for venom detoxification in one way or another were able to confer different levels of immunity to the animals tested, [Christensen, 1979], and some have been proposed as suitable for human vaccination. New *et al.*, 1984, immunised mice with the venom of *Echis carinatus* entrapped into liposomes. One year after the immunisation the mice were challenged with a minimum lethal dose of the venom and the results demonstrated a level of protection acquired by the immunised animals. Later, Daniel *et al.*, [1986], detoxified the venom of the South American rattlesnake, *Crotalus durissus terrificus*, by stepwise iodination of the venom with non-radioactive iodine. The detoxified venom was capable of eliciting an immune response in mice and rabbits, affording

protection against lethal doses of the venom. Heneine *et al.*, [1988], applied the same iodination method to achieve similar results with the venom of *Bothrops jararaca*. Sawai, and Kawamura, [1969] reports on a large scale trial of the active immunisation of monkey and human subjects with the Russell's viper venoid. Immunised monkeys withstood intramuscular challenge with 2 mg/kg of the crude venom often weeks after the immunisation. Sawai and Kawamura, [1992] report on their studies of cobra venom toxoid; the venoms of *Naja naja atra*, *Naja naja kaouthia* and *Naja naja naja*, were detoxified by the formalin method. Rabbits, guinea pigs and mice were immunised with the toxoid. Tests showed an antivenom activity in the serum of the animals and, when challenged with doses of the crude venoms, immunised animals demonstrated a much higher survival rate compared with the control group. In contrast with bacterial and viral infections, where there is an incubation period before the onset of the disease allowing time to mount an efficient immune response, the toxic effects of snake venoms begin in minutes and can kill in hours. It is doubtful whether venom immunisation could elicit an adequate and maintained level of circulating antibodies able to neutralise the venom in the short period of time required [Warrell, 1992]. However, as some authors suggest, the active immunisation of snake venoms could be of value in situations where minor envenoming occurs and also in reducing the volumes of antivenom when treatment is required

1.11. Aims of the study

The initial objective of this study was to identify and try to isolate species-specific antigens in the venoms of *B. jararaca*, *B. jararacussu*, and *B. alternatus*, and to study the possibility of applying these antigens in the generation of species-specific antivenoms capable of identifying their homologous venoms species-specifically. This was extended with the inclusion of the venoms of *B. atrox*, *B. neuwiedi*, *B. cotiara* and *L. muta*.

The second objective was to develop immunoassays for the species-specific identification of the above venoms based upon the detection of the species-specific venom antigens.



2. MATERIAL AND METHODS

2.1. MATERIAL

2.1.1. Animals and Chemicals

All animals were from the animal house of the Department of Immunology of the University of Birmingham. All animals received humanitarian treatment according to the guidelines contained in the Instruction for Contributors found in issue no.1 of all volumes of TOXICON (since 1992). The number of animals was kept to a minimum. Balb/c mice were of both sexes, 2 to 3 months old. For the preparation of antisera, New Zealand rabbits were used at the Liverpool School of Tropical Medicine. I am indebted to Dr. R.G.D. Theakston and Dr. G. D. Laing for the rabbit immunisations.

A detailed list of chemicals, suppliers and recipes are included in Appendix II. The chemicals used, unless otherwise stated, were from Sigma

2.1.2. Venoms

Venoms used were from Brazilian snakes, either from a pool of venoms from snakes of the same species, or milked from individual snakes, as specified below. Venoms were obtained from the Instituto Butantan (São Paulo, SP) and Fundação Ezequiel Dias (FUNED), Belo Horizonte, MG. The venom of *B. cotiara* was a gift from Prof. Ibrahim F. Heneine, (Federal University of Minas Gerais, Belo Horizonte, MG). Complete addresses of these Institutions is given in Appendix II.

1) Venoms from a pool were:

Genus Bothrops: *B. jararaca*, *B. jararacussu*, *B. alternatus*, *B. atrox*, *B. moojeni*,
B. neuwiedi, *B. cotiara*.

Genus Lachesis: *L. muta*

Genus Crotalus: *C. durissus terrificus* and *C. durissus collineatus*.

2) Venoms from individual species:

Genus Bothrops: B. jararacussu, B. neuwiedi, B. moojeni.

2.1.3. Antivenoms: mouse monovalent and horse polyvalent

Monovalent antivenoms were produced by injecting rabbits with a pool of venoms from a single species, and were kindly supplied by Dr. R. G. D. Theakston, Liverpool School of Tropical Medicine.

The polyvalent antivenoms used were the commercially available antibothropic antivenoms obtained from the Instituto Butantan, SP, Brazil and Fundação Ezequiel Dias, MG, Brazil and were produced in horses by injection of a pool of venoms as described by Theakston *et al.*, [1991].

2.1.4. Preparation of antibody conjugates

Enzyme antibody conjugates can be prepared by covalent coupling of antibodies to enzymes. A large variety of enzymes can be coupled to antibodies allowing many applications in immunohistochemical staining, immunoblotting and enzyme-linked immunosorbent assay (ELISA). Enzyme antibody conjugates have been used for the quantitative or qualitative assay of biological substances via enzyme-substrate reactions that yield a measurable colour [Avrameas, 1969].

2.1.4.1. Anti-Rabbit IgG-HRP conjugate

The method described [Wilson *et al.*, 1978] was used here. Briefly, 7.0 mg of Horseradish peroxidase (HRP) dissolved in 1.0 ml of distilled water, was activated by the addition of 0.2 ml of freshly prepared 0.1 M solution of sodium *m*-periodate in distilled water. The mixture was gently mixed by rotation for 20 min at room temperature (RT) and subsequently dialysed overnight in a large excess (3 litres) of 1.0 mM sodium acetate buffer pH 4.4 at 4 °C. The pH of this mixture was raised to pH 9.0 - 9.5 by addition of 20 µl of 0.2 M sodium carbonate/bicarbonate buffer pH 9.5. To this, 14 mg of Sheep IgG anti-rabbit IgG

(The Binding Site, Birmingham, England), was added immediately, dissolved in 1.0 ml of the carbonate/bicarbonate buffer pH 9.5. After a mixing period of 2 hours at room temperature, 100 µl of freshly prepared sodium borohydride solution, 4.0 mg/ml, in distilled water, was added and the mixture was left to stand for 2 hours at 4 °C. Then the mixture was dialysed overnight in a large excess of 0.1 M borate buffer pH 7.4. It was aliquoted and frozen at -20 °C. The conjugate was titrated and used at 1:20,000 dilution in the ELISA and at 1:5,000 in the immunoblot assays. These dilutions were determined in preliminary trials.

2.1.4.2. Alkaline phosphatase conjugates

These were kindly supplied by Dr. David Theakston. The conjugates were rabbit monovalent antivenom IgG's conjugated to alkaline phosphatase and were used at 1:500 and 1:50 dilutions in the ELISA tests.

2.1.5. Preparation of immunoadsorbents

Many cross-linking agents have been used to prepare water insoluble protein polymers employed as immunoadsorbents. Of these, glutaraldehyde allows the preparation of effective, stable and specific immunoadsorbents. Glutaraldehyde is known to react mainly with ε-amino groups of peptides, specially those on lysine. Optimal insolubilization is achieved around the isoelectric point of each protein and at high concentrations. The antigen or antibody binding capacity is best preserved at pH 5.0. In order to reach high insolubilization rates and preserve immunoreactivity of insolubilized proteins, bovine serum albumin (BSA), with an isoelectric point around 4.9 and many lysine residues, is used to assist in the insolubilization of proteins. At pH 5.0, BSA is insolubilized and acts as the insoluble supporting matrix. Cross-linking agents are also employed for the introduction of active groups into an insoluble matrix which can subsequently be used for the immobilization of proteins. A high concentration of glutaraldehyde allow for the reaction of one of its aldehyde groups to react with free amino residues present in polyacrylamide gels.

The other free aldehyde groups can then combine with the protein of interest, added subsequently. Immunoadsorbents can be used to purify antigens or antibodies [Ternynck *et al.*, 1976].

2.1.5.1.Glutaraldehyde adsorbents

2.1.5.1.1.Adsorbents with equal amounts of venom

Adsorbents were used to remove cross-reacting antibodies in the various antivenoms studied. Thus the venom adsorbent used to remove the cross reacting antibodies out of the anti-*B. jararaca* antivenom, was composed of a mixture of all venoms except the *B. jararaca* venom, and were tested for crossreactivity against the other *Bothrops* venoms studied. The method followed was a variation of that described by Ternynck *et al.*, [1976], developed by Catty *et al.*, [1988]. Venoms (5 mg each) in volumes of 0.1 ml in 0.2 M phosphate buffer, pH 7.0 were mixed together in a small flask. 1.0 ml of 2.5% glutaraldehyde solution was added dropwise with constant stirring. Then, the mixture was left standing at room temperature for 3 hours, as the gel formed. The gel was mashed to fine particles with a metal spatula and washed copiously with water by mixing and centrifuging at 4,000 g for 30 min at 4 °C, followed by 3 washes with 0.2 M phosphate buffer pH 7.2. The gel was mixed with 0.1 M glycine-HCl buffer, pH 3.0 for 15 min, centrifuged as above. The supernatant was removed and the gel washed with PBS until the pH reached 7.4. Reactive sites were quenched with 1.0% (w/v) BSA in PBS (equal volume to gel) for 2 hours at room temperature and the adsorbent was washed as above. The adsorbents were stored at 4 °C in PBS with 0.1% (w/v) sodium azide.

2.1.5.1.2.Adsorbents with optimal amounts of venom

The adsorbents were prepared with optimal amounts of each venom based on a crossreactive index derived from ELISA cross-reactivity studies (see Appendix IV). The insolubilization method used was that of Ternynck *et al.*, [1976]. The total amount of venom was 20 mg and of BSA 80 mg in a volume of 2.0 ml (to keep concentrations

between 40 to 50 mg/ml and a BSA/antigen ratio of 4:1) of 0.2 M sodium acetate buffer pH 5.0. Then, 0.2 ml of a 2.5% glutaraldehyde solution was added dropwise with constant stirring. The flasks were allowed to stand for three hours at room temperature. The gel formed was mashed into small particles with a metal spatula washed three times with PBS by mixing and centrifuging as above. Then the gel was mixed with 0.2 M HCl-glycine pH 2.8 for 10 min. After centrifugation the pH was raised to pH 7.0 with 2.0 ml of 1M K₂HPO₄ and the gel washed three times with distilled water. The remaining reactive sites were blocked with 1 M ethanolamine, pH 7.4, by overnight incubation at 4 °C. It was then washed with PBS as above. The adsorbents were then ready to use.

2.1.6. Polyacrylamide gel immunoadsorbents:

Venom adsorbents were also prepared using activated polyacrylamide gel beads [Ternynck *et al.*, 1976]. The venoms were used in optimal amounts as above. Twenty mg of the venom mixture was insolubilized within 10 ml of activated polyacrylamide beads (Biogel P2, from Biorad). The beads were hydrated for 24 h in distilled water and then washed several times by decantation with the same medium. The beads were activated by mixing 50 ml of sedimented beads, 125 ml of distilled water, 50 ml of 25% glutaraldehyde, 25 ml of 1 M phosphate buffer, pH 7.4, with overnight incubation at 37 °C. Next the beads were washed with distilled water by successive centrifugation at 4,000 g until no odour of glutaraldehyde was detected and the OD_{280nm} reading was zero. Venoms were then added to the "activated" beads in 20 ml of PBS, pH 7.4, and mixed overnight by rotation at room temperature. After successive washes with PBS until the OD_{280nm} reading was less than 0.05, the gel was suspended in an equal volume of 1 M ethanolamine pH 7.4 and incubated for 2 hours at room temperature. The adsorbent was then washed with PBS and treated with 30 ml of 0.1 M Glycine-HCl buffer pH 2.8 for 15 min and neutralised with 30 ml of 1 M K₂HPO₄. After a further wash cycle with PBS, the adsorbents were stored at 4 °C with 0.01% (w/v) sodium azide.

2.1.7. Immunoaffinity column for the purification of *B. jararaca*-specific components

The characteristic of Protein A to bind to the Fc region of the IgG molecule of many species has been explored for the preparation of immunoaffinity matrixes. Antibodies with the desired specificity are bound to immobilised Protein A and a crosslinking agent is used to covalently "cement the IgG to the immobilised Protein A. This leaves the antigen binding portion of the immunoglobulin free to interact with the specific antigen in the mobile phase. The coupling of the purified absorbed rabbit IgG, anti-*B. jararaca* venom to Sepharose CL-4B-Protein-A beads followed the method described in Harlow and Lane, [1988]. Briefly, 2.0 mg of the purified absorbed antivenom IgG was added to 0.5 ml of Sepharose CL-4B-Protein-A beads in PBS and gently rotated for 1 hr at room temperature. The slurry was washed twice with 0.2 M sodium borate, pH 9.0, by centrifugation at 3,000 g for 5 min. The beads were resuspended in the same buffer and dimethylpimelimidate (solid) was added to a final concentration of 20 mM. After 30 min of gentle mixing, the reaction was stopped by one wash with 0.2 M ethanolamine, pH 8.0, followed by 2 hr incubation with gentle mixing at room temperature with 0.2 M ethanolamine as above. After three washing steps with PBS, the gel was ready to use.

2.1.8. Purification of IgG

2.1.8.1. DEAE-Shepadex

The isolation of immunoglobulins by DEAE-matrix is achieved by the principle of ion exchange. Antibodies have a more basic isoelectric point than the majority of other serum proteins and this fact can be explored in two ways for their isolation [Harlow and Lane, 1988]. In the method used here the pH of the medium was kept around the isoelectric point of most antibodies and so it did not bind to the matrix. They can be recovered by chromatography or decantation of the supernatant after centrifugation. To achieve higher purity this method is often used in conjunction with a previous ammonium sulphate precipitation step of the serum immunoglobulins. The two step method was used here. Briefly, rabbit monovalent

antivenom serum (5 ml) was precipitated with 5 ml of saturated ammonium sulphate (SAS), added dropwise with constant stirring. The mixture was then centrifuged at 4,000 g and the pellet resuspended in 1.5 ml of 0.02 M phosphate buffer pH 7.2 and dialysed against the same buffer extensively. The whole of the dialysate was applied to a DEAE column equilibrated with the same buffer, of 19.0 ml column volume and eluted with 0.02 M phosphate buffer pH 7.2 at a flow rate of 20 ml/hour.. The eluate was monitored at $A_{280\text{nm}}$ by an LKB-Uvicord. The first peak was collected in aliquots of 5.0 ml and dialysed against water overnight, lyophilised and resuspended in 600 μl of PBS. Purity was tested by immunoelectrophoresis. The concentration of purified IgG was determined by the absorbance at $A_{280\text{nm}}$ using the extinction coefficient [Harlow and Lane, 1988] for rabbit IgG, given below.

$$E = \frac{1.0 \text{ mg / ml}}{1.0 \text{ cm}} = 1.35 \text{ at } A_{280\text{nm}}.$$

2.1.8.2. Quantitation of rabbit IgG in sera

The radial immunodiffusion (RID) kit supplied by The Binding Site (Birmingham, UK) was used according to instructions for the quantitation of the IgG content in the rabbit antivenom sera. Samples diluted 1:2 with PBS, and controls (supplied in the kit) were applied to an agarose gel containing anti-rabbit IgG and allowed to diffuse for 48 hours into the gel containing anti-rabbit IgG antibodies. Precipitation rings formed and their diameters were read using an illuminated reader from The Binding Site. The concentration of rabbit IgG was obtained from a reference table provided with the kit.

2.1.9. Radiolabelling of sheep anti-mouse IgG

Labelling of proteins with radioactive iodine is an effective method It is used for the assaying and screening of antibodies or antigens and for histochemical techniques. Iodide

solutions are oxidised with Chloramine-T to produce iodine which attacks tyrosil residues mainly. These conjugates have been used in radioimmunoassays for the detection of venoms [Greenwood, *et al.*, 1974]. The chloramine-T method [Greenwood *et al.*, 1963] was used with some modifications. One hundred µg of purified sheep IgG anti-mouse IgG in 100 µl of 0.5 M phosphate buffer pH 7.6 was dispensed into a disposable plastic tube (Alpha Labs Limited) and to this was added 5 µl of ¹²⁵I in the form of NaI (sodium iodide for protein iodination, Amersham Radiochemicals), at 100 mCi/ml, followed by 25 µl of chloramine-T at 2.0 mg/ml in phosphate buffer. The mixture was mixed, and the tube was capped and incubated for 5 minutes at room temperature inside the hood. The reaction was stopped by addition of freshly prepared 50 µl of sodium metabisulphite at 2.5 mg/ml in phosphate buffer, and the mixture was applied to a small (5 ml) Shephadex G-25 column using 3% BSA in 0.05 M Tris/HCl pH 8.0 as the eluting buffer. The first 2 millilitres of eluate were collected and iodine incorporation tested as follows. To 20 µl of the labelled antibody solution in a plastic tube, 1 ml of 10% trichloroacetic acid (TCA) and 100 µl of rabbit serum were added. After 5 minutes incubation at room temperature the tube was centrifuged in an Eppendorf centrifuge, at approximately 10,000 g for 5 minutes and the supernatant was removed. The supernatant and precipitate were then counted for 1.0 minute in a LKB Mini Gamma Counter. From these results the % of bound radiolabel was calculated as follows:

$$\text{Bound counts per minute (precipitate)} = x$$

$$\text{Free counts per minute (supernatant)} = y$$

$$\% \text{ bound label} = \frac{x}{x + y} \times 100$$

These results allowed an estimation of the labelling efficiency.

2.2. METHODS

2.2.1. Analytical Techniques

2.2.1.1. Immunoelectrophoresis

This method combines the electrophoretic migration of antigens in agarose with immunoprecipitation in gel. It is used for the qualitative and/or quantitative assessment of antigens either as single or mixed molecules and is essential for assaying of antisera specificity. Antigens separated by electrophoresis are allowed to diffuse and react with antibodies applied in a trough parallel to the electrophoretic run. Precipitin arcs are seen where antigen and antibodies react. The method [Grabar and Williams, 1953] was used as modified by Catty and Raykundalia, [1988]. Briefly, 9.6 ml of 1.0 % agarose gel with 3.0 % PEG 6,000 in 0.05 M barbital buffer pH 8.6, was poured onto a glass plate of 8 x 8 cm. Venoms, 5.0 µg each, in Barbital buffer, pH 8.6, were added to the wells in volumes of 5.0 µl. The electrophoretic run was performed at 12 mA per plate for 2 hr. Antivenoms were added to troughs, each containing 750 µg of IgG in 70 µl and allowed to diffuse overnight at 4 °C. Gels were washed with four changes of saline, and then four changes of distilled water. They were pressed dry and stained with Coomassie Blue for 1 hour and destained until a clear pattern was observed.

2.2.1.2. Polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gels have been used extensively as solid support for the separation of molecules under an electric field. The possibility of varying the pore sizes of the gel and therefore the sieving effect allows for the separation of mixtures of molecules of different sizes and/or isoelectric point. The use of detergents such as SDS and reducing agents such as β-mercaptoethanol (β-Me) make it possible for the comparison of molecular weights of the molecules and information about number of, and size of, aminoacid chains for each moiety. The anionic detergent SDS is used for an estimation of the molecular weights of the molecules being studied. When used in concentrations already established it confers similar

negative electric charge to all molecules of the sample allowing for their separation by their molecular weight only [Hames and Rickwood, 1990].

2.2.1.2.1.Non-SDS

Without SDS the sample molecules are separated by their electrophoretic mobility. The method [Laemmli, 1970] was used but buffers did not contain either SDS or 2- β Me. Runs were performed in slab gels of 7.5% acrylamide with a 3% stacking gel. 25 μ g of each venom was added in a final volume of 2.5 μ l of sample buffer without SDS. The electrophoresis was run for 1 hr, 50 min on a Pharmacia Mini-Gel apparatus with constant current of 30 mA. Gels were stained with Coomassie blue for 1 hour and destained until a clear pattern was observed. Protein molecular weight markers (SDS-Blue), 4.0 μ l in sample buffer, were used in separate lanes in sample buffer containing SDS (to allow for a good separation of these). These molecular weight standards were used as a reference. In some experiments the gels were not stained and were used in immunoblot experiments (see below 2.2.1.3).

In a second protocol, after the electrophoretic separation of the venoms the gels were not stained. Venom bands were cut out to be analysed for venom components carrying species-specificity. In this experiment the gels were cast with blank combs where the samples are applied in the whole of the surface of the gel. Only one lane was used for the molecular weight standards. Venoms were then added, 450 μ g in 75 μ l of sample buffer. This is equivalent of 30 μ g of venom per lane \times 15 lanes. After the run, the lane containing the molecular weight standards plus a small adjacent area were cut off and stained. These were used as a reference for the removal of the wanted venom bands. The electrophoretic conditions were the same as for the first protocol. For this protocol four gels were prepared for each of the venoms tested in order to have more material for the subsequent analysis. The elution procedure is detailed under the Immunoblot section.

2.2.1.2.2.SDS

The Laemmli [1970] method was used under non-reducing conditions. Venoms were mixed in equal parts with the 2 times concentrated sample buffer and 25 µg of each was applied to the gel in volumes of 2.5 µl. Slab polyacrylamide gels of 10% (w/v) with 5% (w/v) stacking gels were used and the electrophoresis run was carried out for 1.0 hours with 40 mA/2 gels. In separate wells, molecular weight markers SDS-7B and Dalton VII were applied in 4.0 µl/well. Staining for both gels was as described in Section 2.2.1.1. In some runs the gels were not stained but used in immunoblot experiments.

2.2.1.3.Immunoblot

The transfer of molecules to membranes such as nitrocellulose are employed after their separation through electrophoresis. The method is good for the assessment of antigens as single and mixed molecules and also for testing specificity of antisera. It offers advantages over immuno-electrophoresis such as higher sensitivity, better separation of antigens and estimation of molecular weights [Harlow and Lane, 1988; Catty and Raykundalia, 1988]. The venoms separated by PAGE were transferred to nitrocellulose membranes [Towbin *et al.*, 1979] in a wet transfer apparatus Protean II from Bio-Rad. The transfer buffer was the non-SDS PAGE reservoir buffer with 20 % methanol (see appendix). Transfer was done over 50 min to 1 hr with a current of 0.18 A per 9 x 7 (cm) gel. Nitrocellulose membranes were then incubated with washing/blocking buffer, PBS + Tween 0.1% (v/v) overnight at 4 °C. After washing for 1 hr with four changes of the washing buffer the nitrocellulose was cut into strips as appropriate and the antivenoms, absorbed and non absorbed, were added at varying dilutions and incubated for 1 hr in PBS+ 0.1% Tween 20. After washing, as above, the conjugate, sheep anti-rabbit IgG + HRP, was added at 1:5,000 dilution in blocking buffer and incubated at room temperature for 1 hr with mixing. Another washing step followed and the substrate was added, 10 mg of 3'3'-diaminobenzidine (DAB) in 20 ml of 50 mM Tris buffer pH 7.4 with 5 µl of 30 % H₂O₂. Colour was developed for 1 hour or

less, depending on the intensity of the staining. The colour reaction was stopped by washing the nitrocellulose with PBS. The substrate was prepared by dissolving DAB in the substrate buffer and filtering through a filter paper prior to the addition of H₂O₂.

The transfer procedure was also used to elute samples out of polyacrylamide gel strips. For this the gel strips from two gels were eluted in dialysis bags with 2 ml of transfer buffer. The transfer conditions were the same as described above in this section but it was carried out for two hours to ensure that all material had in fact eluted out of the gel strip. Next the pieces of gel were removed, the dialysis bags closed and dialysed overnight against distilled water (3 litres) with constant stirring. After this, the samples were removed from the bags and lyophilised.

2.2.1.4. 2-Dimensional Immunoelectrophoresis

This method combines the electrophoretic separation of antigens in a flat gel as a first step, (first dimension), with the rocket immunoelectrophoresis principle as a second step (second dimension), at an angle of 90° in relation to the first dimension. Separated antigens are electrophoresed into an antibody agarose gel to produce a pattern of rockets [Laurell, 1965]. The method has broad analytical value in examining the complexity of antigens and also for the study of antibody specificity. Single excised precipitin peaks can be used for immunisation, giving access to antigens which are difficult to purify. One of the variations of this method includes an intermediate gel placed between the first dimension gel strip and the second dimension gel, prior to running the second dimension electrophoresis. This gel can contain antigens or antibodies depending on the objectives of the experiment. Applications of the methodology can be found elsewhere [Axelsen, 1983].

2.2.1.4.1. With monovalent rabbit antisera in the second dimension gel

The method [Laurell, 1965] was used as modified [Catty and Raykundalia, 1988]. Due to the small amounts of rabbit monovalent antivenom, this technique was performed in small glass plates, 5 x 4 cm. The venom samples were added at concentrations of 5 µg/5 µl in

barbital buffer 0.05 M, pH 8.6, and electrophoresed in a 1.0 % agarose gel (as described above for the immunoelectrophoresis) for 1 hr and 50 min, at a constant 10 V/cm. Strips of gel containing the separated bands were cut out and placed on another glass plate for the second dimension run. Two ml of agarose containing 5.75 mg of antivenom immunoglobulin at 56 °C was poured over the remaining surface of the glass plate. After allowing the gel to set for 5 min, they were placed in the electrophoresis tank for the second dimension run. Plates were run for 16 hours, (2.5 V/cm) at a perpendicular direction to the first run. Water cooling was used. Gels were washed with four changes of saline and then four of distilled water. They were pressed dried and stained as in Section 2.2.2.1.1.

2.2.1.4.2. With intermediate gel

In this experiment an agarose gel strip containing a mixture of antivenom immunoglobulin, heterologous to the venom separated in the first dimension, was poured between the venom strip separating the first dimension gel and the second dimension gel (reference gel) containing the specific non-absorbed rabbit antivenom IgG. The concentration of each of the immunoglobulins in the intermediate gel varied from 3 times to 10 times greater than that of the specific immunoglobulin in the reference gel. Control gels with saline in the intermediate gels were run simultaneously. The volume of the intermediate gel was 0.8 ml and of the reference gel, 1.2 ml. The conditions of the assay were as above.

2.2.1.4.3. With polyvalent horse antisera

These experiments were carried out in glass plates of 8 x 8 (cm). In the first dimension 9.6 ml of 1% agarose + 3% (w/v) of PEG 6,000 was poured onto the plates. 6 µl of a 50 mg/ml venom solution in barbital buffer was applied into the wells and electrophoresed at 80 V/plate (10 V/cm) for approximately 3 hours. For the second dimension, gel strips were cut out and each one separately placed onto another glass plate, and 8.2 ml of agarose containing 0.5 ml of polyvalent antivenom (Butantan Institute) was poured over the remaining area of the glass plate and allowed to set. The second dimension, perpendicular to

the first one, was run overnight at 20 V/plate (2.5 V/cm). Water cooling was used. Washing and staining were as in Section 2.2.1.1.

2.2.1.4.4. With intermediate gel

In this experiment an agarose gel strip containing a mixture of antivenom immunoglobulin, heterologous to the venom electrophoresed in the first dimension was poured between the venom strip separating the first dimension gel and the second dimension gel (reference gel) containing the specific unabsorbed antivenom. The concentration of each of the immunoglobulins in the intermediate gel varied from 3 times to 10 times greater than that of the specific immunoglobulin in the reference gel. Control gels with saline in the intermediate gels were run simultaneously. The volume of the intermediate gel was 1.2 ml and of the reference gel, 7.6 ml. The conditions of the electrophoresis run were as above.

2.2.1.5. ELISA

Enzyme immunoassays (e.g.: ELISA and Immunoblotting) take advantage of the biological properties of two macromolecules, enzymes and antibodies. Antibodies are able to distinguish closely related compounds, and enzymes, biological catalysts, accelerate specific chemical reactions enormously and can be detected by adding substrate. The principle of enzyme immunoassays is to conjugate an enzyme to one of the immunoreactants, let the immunological reaction take place, add substrate, and determine the substrate-product conversion by the enzyme. The amount of product indicates how much enzyme-labelled immunoreactant is incorporated in the complex, thus, allowing a qualitative or quantitative measure of the immunoreactants. The assay can be performed on a variety of solid supports and many variations of the assay can be performed, such as: direct sandwich or indirect sandwich. The assay can also be competitive or non-competitive. The enzyme immunoassays have many applications and have been used for the study of: infectious diseases, histology, parasitology, virology, and any other field where antigen and antibody specific

antibody can be obtained. (Kurstak, 1986). In snake venom research enzyme immunoassay was first used by Theakston *et al.*, [1977]

2.2.1.5.1.Antigen capture ELISA

Two protocols were performed.

1) Rabbit IgGs that had been absorbed on their respective venom adsorbents, and which showed complete or increased species-specificity were used to coat ELISA plates at 40 µg/ml in coating buffer (0.05 M Carbonate buffer, pH 9.6), in 100 µl/well. The plates were incubated overnight at 4 °C. The plates were then blocked with blocking buffer for 1 hr at 37 °C. Different venom solutions either in PBS or in normal human serum diluted to 1:50, were added in different concentrations to different wells containing the homologous or the heterologous coating IgG. After 1 hr incubation at 37 °C, sheep anti-rabbit IgG conjugated to Alkaline phosphatase was added in blocking buffer (PBS + 5 % (w/v) low fat milk, 100 µl/well was added, followed by another incubation step. Colour was developed with the appropriate substrate solution (10 mg of N-p-nitrophenyl phosphate dissolved to 10 ml of substrate buffer). The optical density at A₄₀₅ nm was read, without stopping the colour reaction, on a Titertek ELISA reader. After the incubation step every addition of reagent was preceded by a washing step of 3 x 5 min with washing buffer and tapped dry.

2) In the other experiment polyvalent anti-Bothropic horse IgG F(ab')₂ was added in coating buffer to ELISA plates at 0.5% (v/v) and incubated overnight at 4 °C. After washing with saline + 0.05% (v/v) Tween 20, 3 times for 5 minutes each, the plates were blocked as above in protocol 1, and the venoms were added in different concentrations to different wells in incubating buffer and incubated for 1 hr at 37 °C. After washing, rabbit absorbed antivenoms were added to wells containing their homologous and heterologous venoms, incubated as above and washed again. Sheep anti-rabbit IgG conjugated to horse-radish peroxidase, at 1:20,000 dilution in blocking buffer, was added to the plate and incubated as above. Following a washing step, substrate solution (10 mg OPD dissolved to

25 ml in 0.15 M citrate buffer pH 5.0 + 5.0 µl of 30% H₂O₂) was added and the colour developed for 30 minutes. The colour reaction was stopped with 50 µl/well of 20 % (v/v) sulphuric acid and the colour measured at A_{492nm} in a Titertek ELISA reader.

2.2.1.5.2.Indirect Antibody ELISA

Two assay protocols were performed.

- 1) Different venom species solutions at graded concentrations (0.01 to 10 µg/ml), diluted in coating buffer, were coated onto ELISA plates, 100 µl/well, and left overnight at 4 °C. After a washing step (see section 2.21.5.1), fixed or varying dilutions of venom-affinity absorbed antivenom sera were added to different wells, 100 µl/well, in incubation buffer containing either the homologous or heterologous venom, and incubated for 1 hr at 37 °C. Sheep anti-rabbit IgG, conjugated to horse radish peroxidase, was added at 1:20,000 dilution in blocking buffer followed by another incubation step. Then substrate solution (10 mg OPD dissolved to 25 ml in 0.15 M citrate buffer pH 5.0 + 5.0 µl of 30% H₂O₂), was added and the colour reaction was stopped with 50 µl of a 20% (v/v) solution of sulphuric acid after 30 min. Optical densities were read at 492 nm using a Titertek ELISA reader. Between incubation steps, plates were washed with 3 changes of 5 min each, with 0.9% (w/v) saline + 0.5% (v/v) Tween 20.
- 2) In the second protocol fixed concentrations of the different venoms (1 µg/ml) were coated separately onto ELISA plates as above. The remaining steps were as described above for the first protocol of this section.

2.2.1.5.3.Inhibition ELISA

Inhibition experiments were performed for each homologous system. Plates were coated overnight with venom of a single species (1.0 µg/ml) in coating buffer, 100 µl/well. The homologous non-absorbed rabbit antivenoms were titrated against the homologous venom by antibody capture ELISA and a dilution yielding an optical density of 1.0 at A_{280nm} was used in this assay. A fixed volume of these antivenoms diluted in diluting buffer (2x final dilution) were separately added to different sets of test tubes which contained varying

concentrations of one of the heterologous venoms or the homologous venom (2x final concentration) in a 1:1 (v/v) ratio. The mixture was vortexed and 100 µl/well was added to the ELISA plate coated with the homologous venom and incubated at 37 °C for 1 hr. Antiglobulin conjugate was added at 1:20,000 dilution and incubated as above. Substrate solution, was added and the colour developed for 30 min and stopped with a 20% (v/v) sulphuric acid solution, 50 µl/well. Absorbance was read at A_{492nm} in a Nunc ELISA reader. Between all the incubations the plates were washed three times for 5 min each with washing buffer and tapped dry.

2.2.1.6. Solid Phase Radioimmunoassay

This method was used for the screening of the murine hybridoma clones for antibody. Microtitration plates with round bottoms were coated with 50 µl/well of a 2.5 µg/ml venom solution overnight and washed three times for five minutes with washing buffer, PBS + 5% Foetal calf serum (FCA), and then blocked with blocking buffer, PBS + 3% BSA for 1 hr at 37 °C. After a washing step, 50 µl of the supernatant of each clone was added per well and the plate incubated for 1 hr at 37 °C. After a second wash the radiolabelled anti-mouse Ig was added at 1:300 dilution and the plate was again incubated as above. Following another washing step the wells were counted for radioactivity in a gamma counter for 1 min each.

2.2.1.7. Affinity absorptions of antivenoms.

Antivenom sera or purified antivenom-IgG, (2 ml) prior to absorption were diluted 1:2 with PBS. The batch method was used and the absorption step was done at room temperature for 2 hr in a rotating mixer. The adsorbents were then centrifuged at 4,000 g at 4 °C, for 30 min. The supernatant was pipetted out and filtered through a 0.45 µm, Mylex filter (Millipore) after each absorption. The absorbed antivenoms were stored at 4 °C until the next adsorption step or until tested for cross-reactivity. Absorbed antisera or (IgG) showing species-specificity was aliquoted in Eppendorf tubes and stored at -20 °C. Adsorbents were washed with 4 ml of 0.1 M Glycine-HCl buffer, pH 2.3, three times for 10 min each. The

pH was raised to pH 7.0 with 4 ml of 1 M K₂HPO₄ and the adsorbents washed three times with PBS again. After the final PBS wash the adsorbents were ready for use. The same procedure was used with the polyacrylamide-gel adsorbents but the antivenom's volumes used for the absorptions were equal to 4 ml.

2.2.1.8.Immunisation of rabbits with precipitin lines

The 2D-IEP gels were analysed by dark-field illumination and the peaks showing species-specificity were cut out of the gel with the help of a scalpel and a magnifying glass. They were placed separately in glass tubes containing 5 ml of PBS and washed repeatedly for 3 days to remove non-precipitated or weakly associated antigens from the precipitate. After the washing step the gel was placed overnight in a freezer at -20 °C. After freezing, the agarose was liquefied by thawing. The volume was measured with the help of a pipette and mixed with the same volume of Freund's Complete Adjuvant (FCA) or Freund's Incomplete Adjuvant (FIA). The emulsions were injected subcutaneously into rabbits in volumes of 200 µl. The first injection was in FCA and the subsequent ones in FIA. The rabbits were injected subcutaneously once every two months and received a final boost 3 days after the final injection. The serum was analysed by ELISA for antivenom titre 10 days after an injection at the dilutions of 1:50, 1:100, 1:500 and 1:1,000 in PBS. The plate was coated with 1µg/ml of the specific venom, 100 µl/well. The alkaline phosphatase anti-rabbit IgG conjugate was used at 1:1,000 dilution. All volumes were of 100 µl. The immunisations and serum assays were performed at the Liverpool School of Tropical Medicine. Peaks 1, 2, 3 and 4 of *B. moojeni*, peaks 2, 3, and 4 of *B. atrox* and peak 1 of *B. neuwiedi* were used.

2.2.1.9.Immune complexes

Immune complexes were formed with the absorbed rabbit antivenom sera. The concentration of species-specific antivenom IgG in the antisera was assumed to be 10% of the total IgG. The venom species-specific component was also assumed to be 10% of the total venom protein and it was used in a four fold excess of the antibody concentration.

Antivenom and venom were mixed in PBS + 8% (w/v) PEG 6,000 to obtain a final concentration of 4% (w/v) PEG. The mixture was incubated for 1 hr at 37 °C and allowed to stand overnight at 4 °C. The solution was centrifuged at 10,000 g at 4 °C and the precipitate washed by 3 additional centrifugation steps with 2 % (w/v) PEG 6,000. After the final wash the precipitate was resuspended in 100 µl of PBS and mixed with 100 µl of either FCA or FIA and injected into mice. Typically, volumes of antivenom sera containing 50 µg of IgG were mixed with 200 µg of venom in a final volume of between 50-80 µl. The mice received four equal doses, intraperitoneally, one per week. The first in FCA and the other in FIA. The animals were tail bled before each injection for titration of the antivenom response by ELISA, using 1µg/ml of venom to coat the plates. Antivenom giving OD readings higher than 1.0 at dilutions \geq 1:1,000 were considered good for spleen removal.

2.2.1.10. Monoclonal Antibodies

The method [Galfré and Milstein, 1981] as described in Brown and Ling, [1989] was used. Positive wells were cloned in HAT medium and some were grown as ascitic tumours in pristane-primed Balb/c mice. Clones were screened by Radio-immunoassay using [¹²⁵I]-Sheep anti-mouse IgG in 5 % foetal calf serum. The fusion protocol and cell culture methods are given in Appendix III.

2.2.1.11. Affinity chromatography isolation of *B. jararaca* specific components

One ml of a solution of *B. jararaca* venom, 10 mg/ml in PBS, was applied to an affinity column (anti-*B. jararaca* absorbed IgG bound to Protein A Sepharose CL-4B) and allowed to pass through by gravity. Column volume was equal to 0.5 ml. The column was then washed with a 10X column volume for each of the buffers: 0.1 M Tris pH 8.0 and 0.01 M Tris, pH 8.0. After the final wash the reading at $A_{280\text{nm}}$ returned to the base line and the bound antigen was eluted with 0.1 M Glycine-HCl pH 2.5. The eluate was collected in 500 µl aliquots in tubes containing 50 µl of 1.0 M Tris pH 8.0. The OD of the eluate was read on a Pharmacia-LKB spectrophotometer at $A_{280\text{nm}}$. Six chromatography runs were

performed with each 1.0 ml of venom being chromatographed twice. The pooled samples, in a final volume of 14 millilitres were dialysed with distilled water and concentrated in an Amicon concentrator using a YM50 membrane with a cut-off of 5.0 kDa, to final volume of 1.0 ml in distilled water. The protein content was estimated by measuring the absorbance at $A_{280\text{nm}}$. One unit of absorbance = 1.0 mg/ml.

2.2.1.12. Statistical Analysis

The calculations were performed with the software SigmaStat, using the method of Student-Newman-Keuls Test for pairwise comparisons at $p < 0.05$.

3. RESULTS

3.1. Introduction

Snake venoms contain a complex mixture of protein components. Experiments were carried out in order to compare and analyse this complexity between the venom pool of each species and their immunoreactivity.

3.1.1. Venom characterisation

Polyacrylamide electrophoresis were performed in order to compare and analyse the venom patterns of all venom pools studied. In Fig. 3.1 (A) the electrophoretic separation was carried out in non-SDS and non-reducing conditions. It can be observed that the venom patterns are different for each specific venom pool. All venoms have components with electrophoresis mobility varying from low (upper part of gel) to high (lower part of gel). Some venom species have clearly identified venom components with the same electrophoretic migration, as can be observed in lanes 1, 3, 4, and 7 regarding the stained band numbered 1. Similarly the venoms in lane 1, 3, 4, 6 and 8 share the protein stained band number 2. In lanes 5 and 6 the protein band number 3 seems to be shared only by the venoms of *B. moojeni* and *B. neuwiedi*. In lane 2 the stained band numbered 4 seem to be present only in the venom of *B. jararacussu* as no other venom show a sharp stained band with same electrophoretic migration. Bands with unique electrophoretic migration are also clearly observed in lanes, 1, 3 and 8 which are bands numbered 5, 7 and 6 respectively. In Fig. 3.1 (B) is shown the SDS-PAGE venom patterns obtained without reducing conditions. Again the complexity and differences between the stained pattern for each venom species is observed. All venoms display components within the range of high molecular weight (above 70 kDa) and low molecular weight (below 24 kDa). There is a band of approximately 48.5 kDa that is shared by all venoms. In lane 5 the *B. moojeni* venom do not show any components with molecular weight higher than 70 kDa.

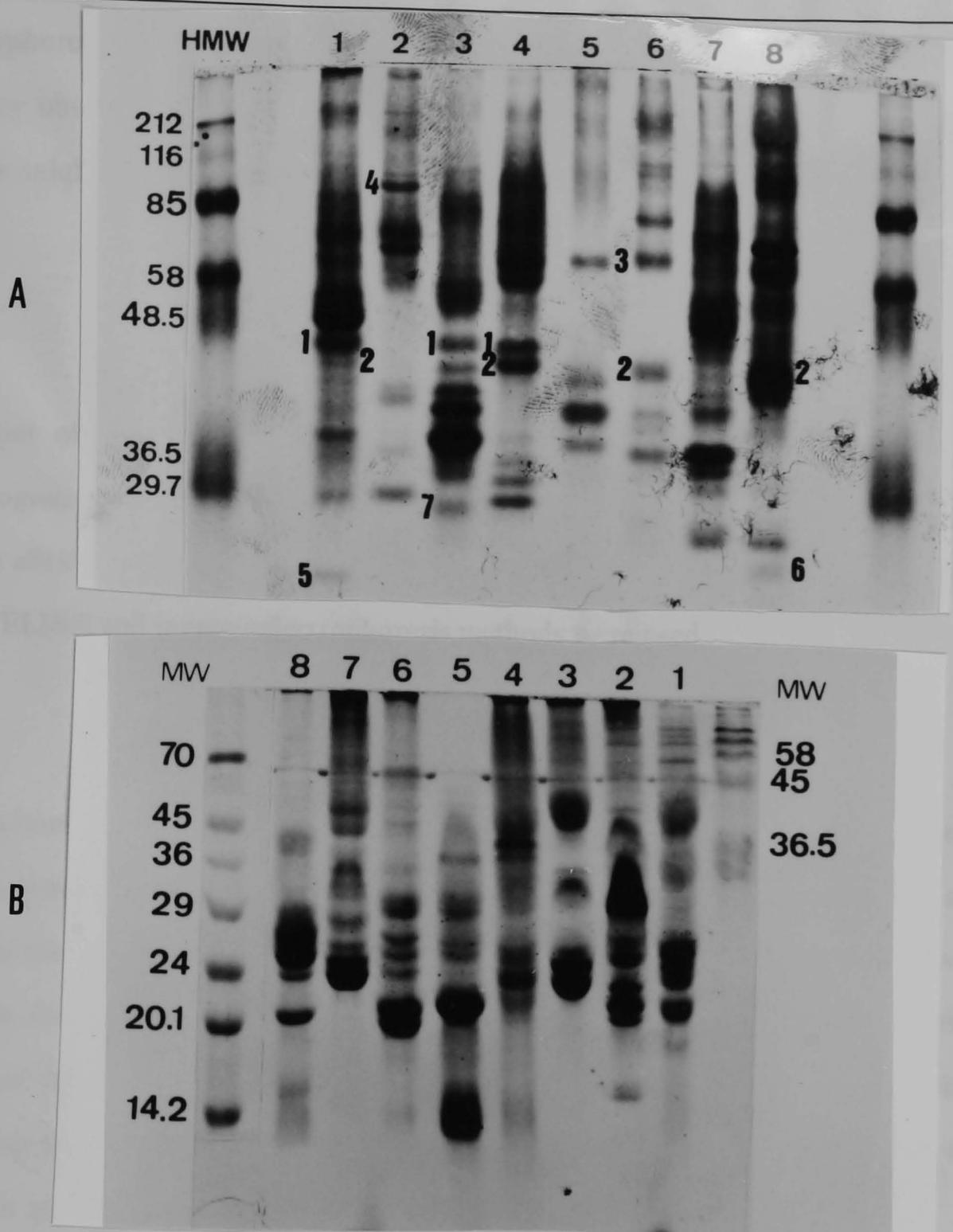


Figure 3.1 (A and B). Polyacrylamide gel electrophoresis of Bothropic venoms **A:** Non-SDS. 25 µg of each venom was fractionated in a 7.5 % gel. **B:** SDS. 25 µg of each venom was fractionated in a 10% gel. Venoms for gels **A** and **B**: 1- *B. jararaca*, 2- *B. jararacussu*, 3- *B. alternatus*, 4- *B. atrox*, 5- *B. moojeni*, 6- *B. neuwiedi*, 7- *B. cotiara*, 8- *L. muta*. MW are molecular weight standards.

The results of both polyacrylamide gel electrophoresis separations show the complexity of Bothropic snake venom composition. The electrophoretic patterns in both experiments are different for each species. Many venom components from different species have the same

electrophoretic mobility and molecular weight. The components with unique electrophoretic mobility observed in some venoms in Fig. 3.1 (A) cannot necessarily be interpreted as species unique products. Similar results were not observed in Fig. 3.1 (B) in the presence of SDS..

3.1.2. Immunoreactivity studies of venoms and antivenoms.

This part of the study was aimed at obtaining qualitative and quantitative data on the homologous reactivity and cross-reactivity of the venoms with monovalent antivenoms, and also to obtain some data on the antigenic similarities between the venoms studied. For this study, ELISA and immunoelectrophoresis methods were used.

3.1.3. Antibody capture ELISA

The reaction of a monovalent antivenom with its homologous venom and with heterologous venoms was used in order to obtain information on the existence of antigenic similarities between the venoms. By looking at Fig. 3.2 (A-D) and 3.3 (A, B) it can be observed that in all cases the antivenoms broadly dilute to give equivalent dilution curves indicating a high degree of cross-reactivity. Some antivenoms react more strongly with heterologous venoms than with the homologous venom. This can be observed with the antivenoms against *B. jararaca* and *B. jararacussu* venoms (Fig. 3.2 (A) and (B) respectively).

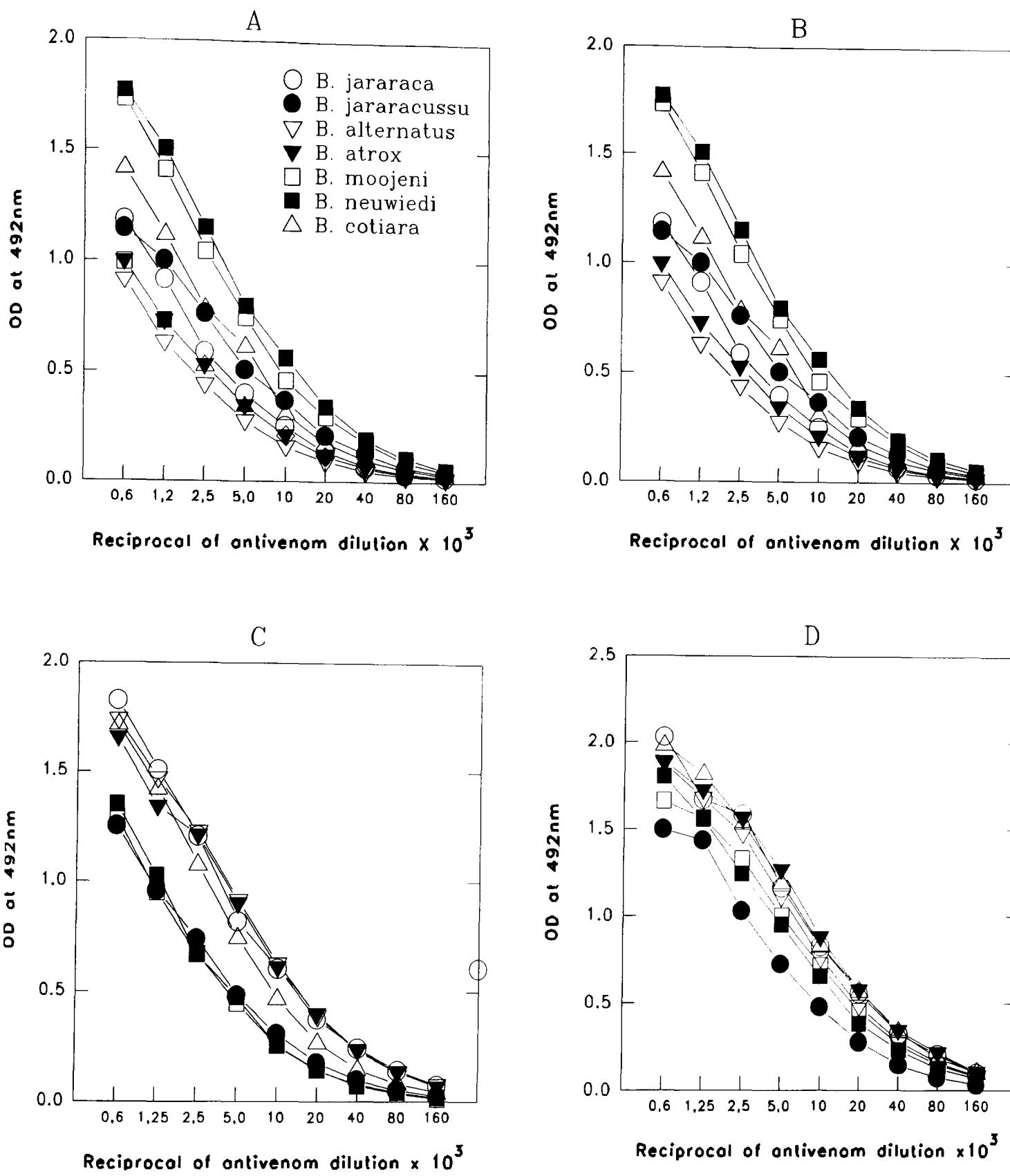


figure 3.2 (A-D). Indirect Antibody ELISA showing the reactivity of non-absorbed rabbit monovalent antivenoms with homologous and heterologous venoms. The IgG concentration for all antivenoms was 7.0 mg/ml. Antivenoms: **A:** anti-*B. jararaca*; **B:** anti-*B. jararacussu*; **C:** anti-*B. alternatus*; **D:** anti-*B. atrox*. Venoms are as listed in plate A.

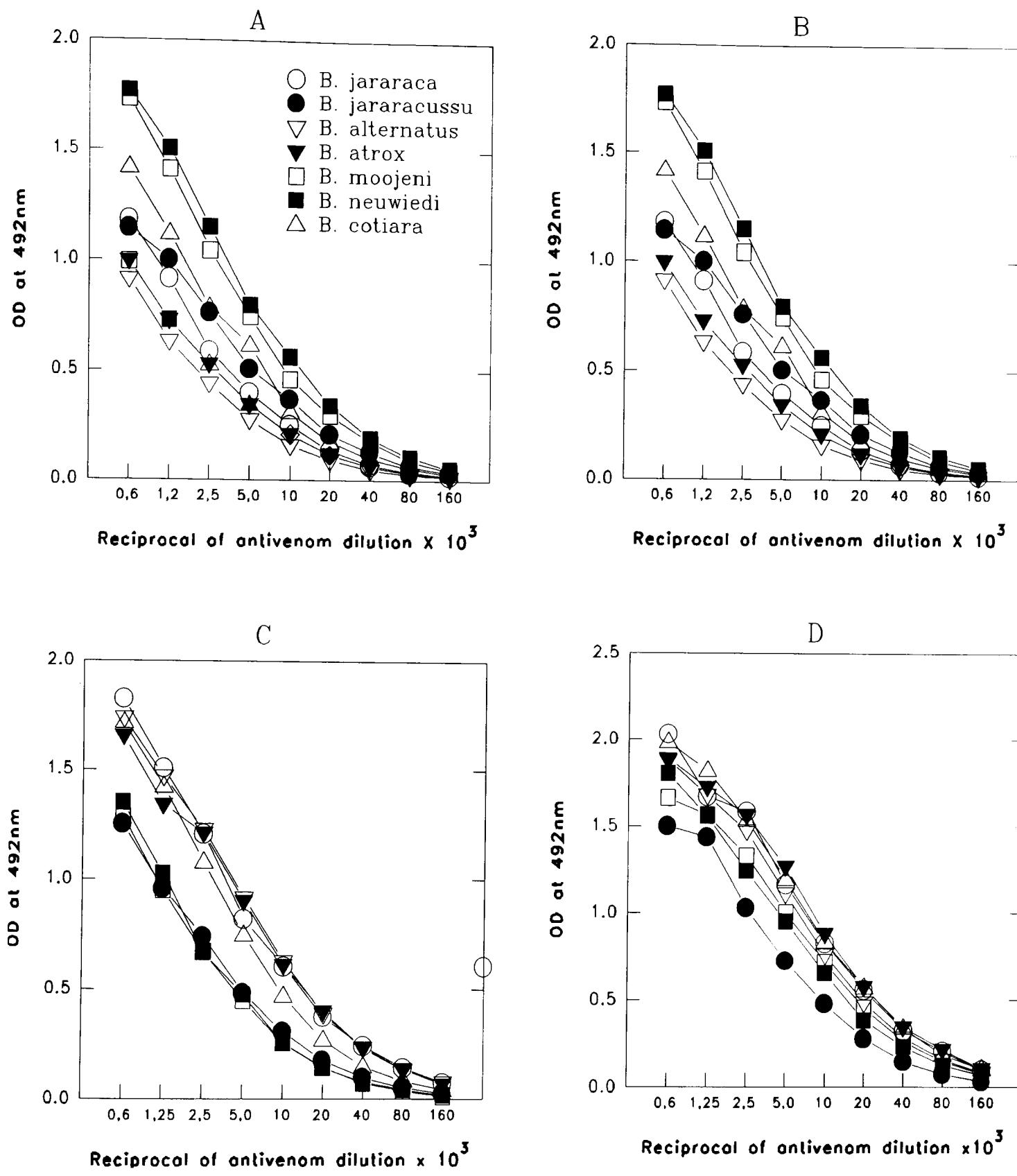


Figure 3.2 (A-D). Indirect Antibody ELISA showing the reactivity of non-absorbed rabbit monovalent antivenoms with homologous and heterologous venoms. The IgG concentration for all antivenoms was 7.0 mg/ml. Antivenoms: **A:** anti-*B. jararaca*; **B:** anti-*B. jararacussu*; **C:** anti-*B. alternatus*; **D:** anti-*B. atrox*. Venoms are as listed in plate A.

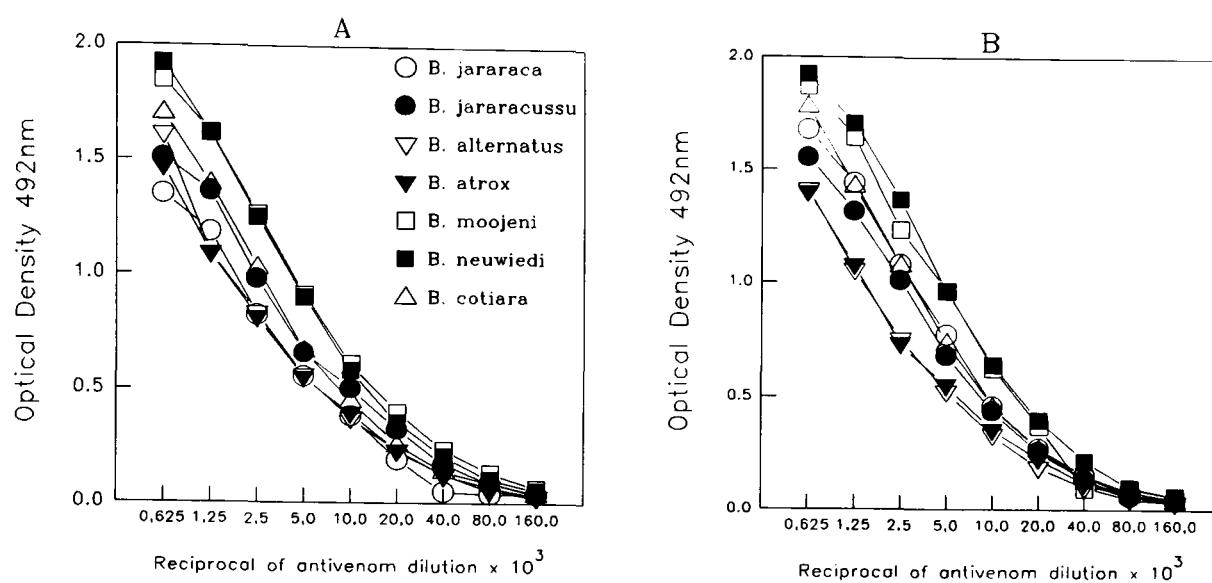


Figure 3.3 (A and B). Indirect Antibody ELISA showing the reactivity of non-absorbed rabbit monovalent antivenom with homologous and heterologous venoms. The IgG concentration for all antivenoms was 7.0 mg/ml. Antivenoms: **A:** anti-*B. moojeni*; **B:** anti-*B. neuwiedi*. Venoms as listed in plate A.

In Fig. 3.4 (A-F), the linear regressions for the dilution curves above show parallel, near parallel, and even superimposing curves, indicating high degrees of cross-reaction between venoms. Table 3.1 shows the slope values for each individual antivenom linear regression for the above titration curves. Identical slopes are found for some antivenoms when reacted against homologous and heterologous venoms. These results indicate the existence of crossreacting epitopes on all venoms studied.

Table 3.1. Slopes of linear regression from antivenom titration reactivity curves.

Antivenoms	Venoms						
	<i>B. jararaca</i>	<i>B. alternatus</i>	<i>B. atrox</i>	<i>B. cotiara</i>	<i>B. moojeni</i>	<i>B. neuwiedi</i>	<i>B. jararacussu</i>
anti- <i>B. jararaca</i>	0.22	0.20	0.22	0.28	0.27	0.26	0.15
anti- <i>B. alternatus</i>	0.22	0.22	0.21	0.22	0.15	0.16	0.15
anti- <i>B. atrox</i>	0.25	0.25	0.25	0.26	0.22	0.23	0.20
anti- <i>B. moojeni</i>	0.18	0.18	0.17	0.21	0.24	0.24	0.19
anti- <i>B. neuwiedi</i>	0.22	0.17	0.17	0.22	0.24	0.25	0.20
anti- <i>B. jararacussu</i>	0.14	0.11	0.12	0.12	0.21	0.24	0.15

The point of intersection of two lines drawn from the (vertical) venom species and (horizontal) monovalent antivenoms indicate the slope value for that particular venom/antivenom reaction. Venoms and antivenoms are grouped together according to similar slope values. Results are the mean of two experiments.

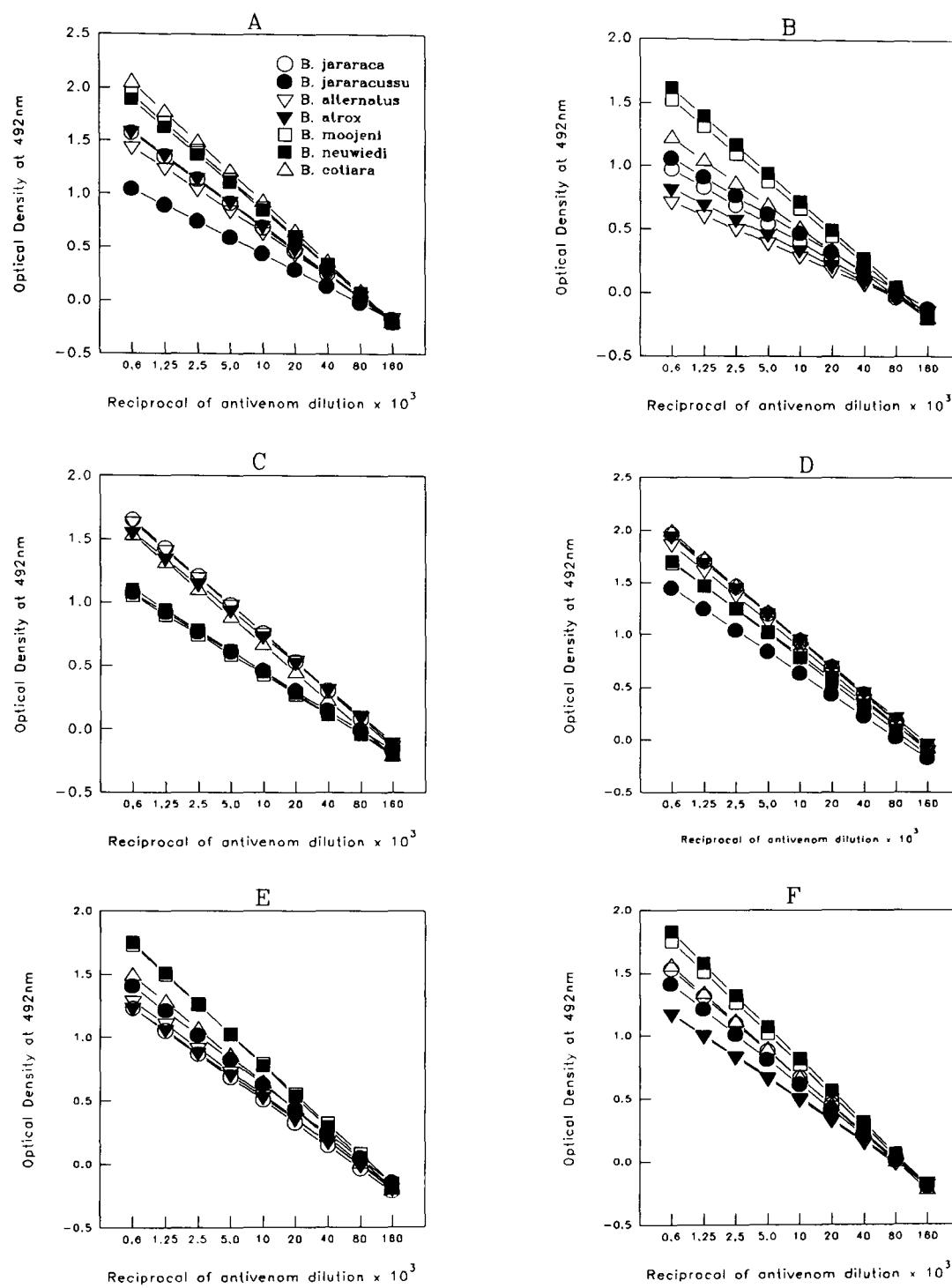


Figure 3.4 (A-F) Shows the linear regressions calculated for all the antivenom curves in the antibody-capture assay. Antivenoms: A: anti-*B. jararaca*; B: anti-*B. jararacussu*; C, anti-*B. alternatus*; D, anti-*B. atrox*; E, anti-*B. neuwiedi*; F, anti-*B. moojeni*. Venoms are as listed in plate A. All values represent the mean of duplicate experiments. The symbols in the linear regression curves are used for the identification of each venom.

3.1.4. Inhibition ELISA

These experiments were performed to obtain additional information on the cross-reactivities shown in the antibody ELISA. This was done by analysing the degree of inhibition of the homologous reaction between a venom and its specific antivenom achieved by the heterologous venoms. The results in Fig 3.5 (A-D) and Fig. 3.6 (A, B), show the linear regression curves of the inhibition assays. It can be observed that all antivenoms were inhibited to some degree by the heterologous venoms. There are no superimposed curves and most individual inhibition curves are non-parallel.

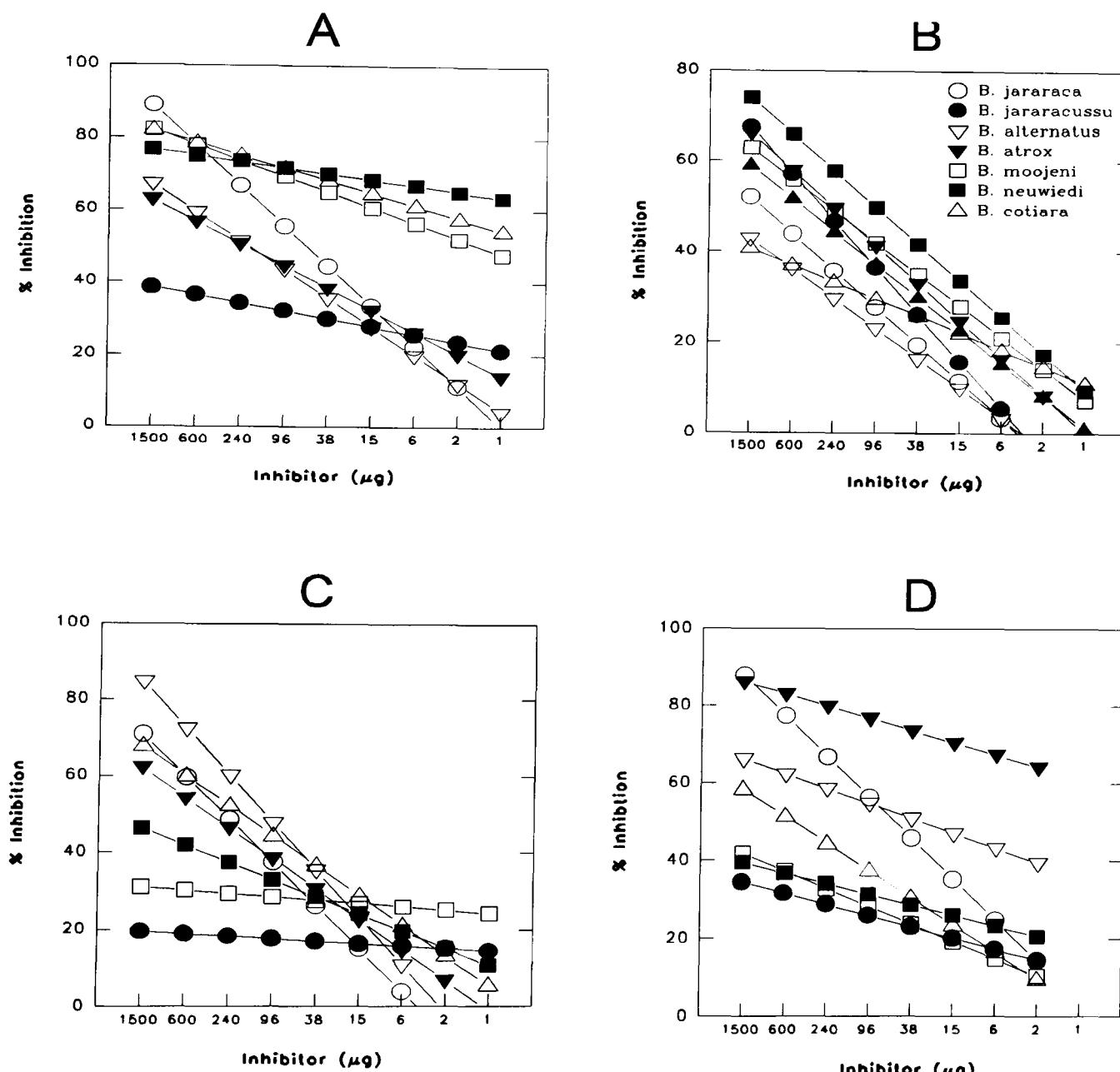


Figure 3.5 (A-D). Linear regression curves showing inhibition ELISA results. Non-absorbed monovalent antivenoms were inhibited by the homologous and heterologous venoms. Plates were coated with 1 μ g of a single specific venom as: A: *B. jararaca*; B: *B. jararacussu*; C: *B. alternatus*; D: *B. atrox*; Antivenoms: A: Anti-*B. jararaca*; B: anti-*B. jararacussu*; C: anti-*B. alternatus*; D: anti-*B. atrox*. Inhibiting venoms are as listed in the key of 3.5 B. Concentration of IgG for all antivenoms was equal to 7.0 mg/ml.

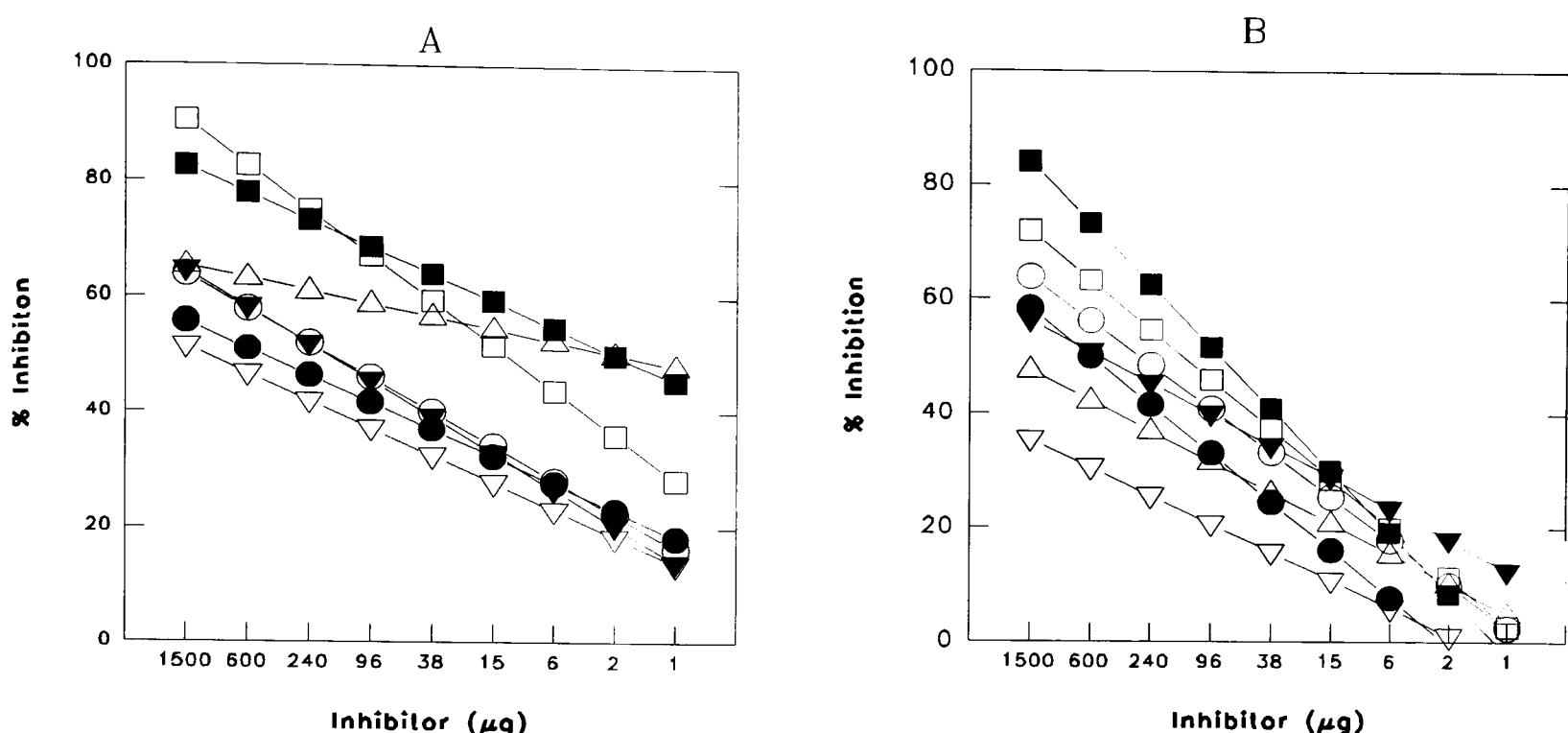


Figure 3.6 (A and B). Linear regression curves showing inhibition ELISA results. Non-absorbed monovalent antivenoms were inhibited by the homologous and heterologous venoms. Plates were coated with a single specific venom, 1 µg/ml, 100 µl/well. Venoms: A; *B. moojeni*, B; *B. neuwiedi*. Antivenoms: A; anti-*B. moojeni*, B; anti-*B. neuwiedi*. Venoms are symbols as in key of Figure 3.5 (B). Concentration of antivenom IgG as in Figure 3.5.

This can be more clearly observed in Table 3.2 where the slopes for each individual inhibition curve are shown. Note the non-concordance of slope values for antivenoms with the homologous and heterologous venoms.

Table 3.2. Slopes of linear regression from antibody inhibition curves

Antivenoms	Venoms						
	<i>B. jararaca</i>	<i>B. jararacussu</i>	<i>B. alternatus</i>	<i>B. atrox</i>	<i>B. moojeni</i>	<i>B. neuwiedi</i>	<i>B. cotiara</i>
anti- <i>B. jararaca</i>	11.1	2.2	8.0	6.1	4.3	1.6	3.5
anti- <i>B. jararacussu</i>	8.13	10.3	6.6	8.27	6.95	8.1	3.7
anti- <i>B. alternatus</i>	11.2	0.6	12.3	7.9	0.8	4.5	7.8
anti- <i>B. atrox</i>	10.5	2.8	3.8	3.1	4.5	2.7	0.45
anti- <i>B. moojeni</i>	5.9	4.6	4.7	6.3	7.8	4.6	2.2
anti- <i>B. neuwiedi</i>	7.7	8.4	5.0	6.0	8.7	10.8	5.3

The point of intersection of two lines drawn from the (vertical) venom species and (horizontal) monovalent antivenoms indicate the slope value for that particular venom/antivenom reaction. The bordered cells show the slope for the homologous venom/antivenom reaction. The results are the mean of two experiments.

The results confirm the existence of cross-reacting epitopes in the Bothropic venoms studied. They also show that there are differences in these cross-reactivities as some venoms show higher degree of inhibition of some antivenoms than others. These differences could be indicating the presence of different concentrations of similar epitopes in the venoms (and possibly similar venom components) or the presence of cross-reacting epitopes with varied degrees of reactivity towards the heterologous antibodies, or both. The unique slope values observed for the homologous reaction could be indicative of species-specific venom epitope(s) or components.

The inhibition analysis allowed for a venom reactivity index to be calculated. For this the total amount of venom needed to inhibit by 50% the homologous reaction of an antivenom was used as a measure. Table 3.3 shows the amount of each venom needed to cause a 50% inhibition. Values greater than 1500 µg were used as 1500 µg and smaller than 1.0 µg as 1.0 µg. In Table 3.4 the venoms are classified according to their reactivity index.

Table 3.3 Amount of venom producing 50% inhibition of heterologous and homologous antivenoms

Antivenom	Venoms (µg)						
	<i>B. jararaca</i>	<i>B. jararacussu</i>	<i>B. alternatus</i>	<i>B. atrox</i>	<i>B. moojeni</i>	<i>B. neuwiedi</i>	<i>B. cotiara</i>
anti- <i>B. jar</i>	59.6	>1500	198.0	215.0	1.5	<1.0	<1.0
anti- <i>B. jsu</i>	171.0	312.0	>1,500	242.0	280.0	97.0	>1,500
anti- <i>B. alt</i>	280.0	>1500	110.0	372.0	>1,500	>1,500	193.0
anti- <i>B. atr</i>	55.0	>1500	31.0	<1.0	>1,500	>1,500	525.0
anti- <i>B. moj</i>	171.0	508.0	1100	171.0	13.0	2.4	2.4
anti- <i>B. neu</i>	304.0	605.0	>1,500	508.0	149.0	82.0	>1500

The intercepting lines drawn from the (vertical) venoms columns and (horizontal) antivenom columns indicate the value in µg of venom needed to inhibit by 50% the respective antivenom. Bordered cells show values for a 50% inhibition in the homologous venom/antivenom system.

Table 3.4 Classification of immunoreactivity of venoms according to their antivenom inhibition activity

Reactivity grading	Venom	Amount of venom (µg)
1st	<i>B. atrox</i>	1509
2nd	<i>B. jararaca</i>	2040
3rd	<i>B. neuwiedi</i>	3182
4th	<i>B. moojeni</i>	3560
5th	<i>B. cotiara</i>	3721
6th	<i>B. alternatus</i>	4439
7th	<i>B. jararacussu</i>	6750

Venoms were graded according to their immunoreactivity based on the total amount needed for each one to inhibit the specific reaction of all antivenoms tested by 50%.

The results of Table 3.4 indicate that the venom from *B. atrox* is the most reactive, as a smaller amount of the venom is needed to obtain a 50% inhibition of the antivenoms.

3.1.5. Immunoelectrophoresis of Venoms

The ELISA results showed the existence of cross-reactivity between the Bothropic venoms studied. However no information is given as to whether the crossreacting antigenic determinants are present in only one venom component or more. In order to visualise the crossreacting component(s) or any possible species-specific component, immunoelectrophoresis tests were performed and the monovalent antivenoms were reacted with the electrophoresed homologous and heterologous venoms. In Fig. 3.7 (A-D) and Fig. 3.8 (A, B) it can be observed that there is an extensive cross-reaction between the monovalent antivenoms and the heterologous venoms.

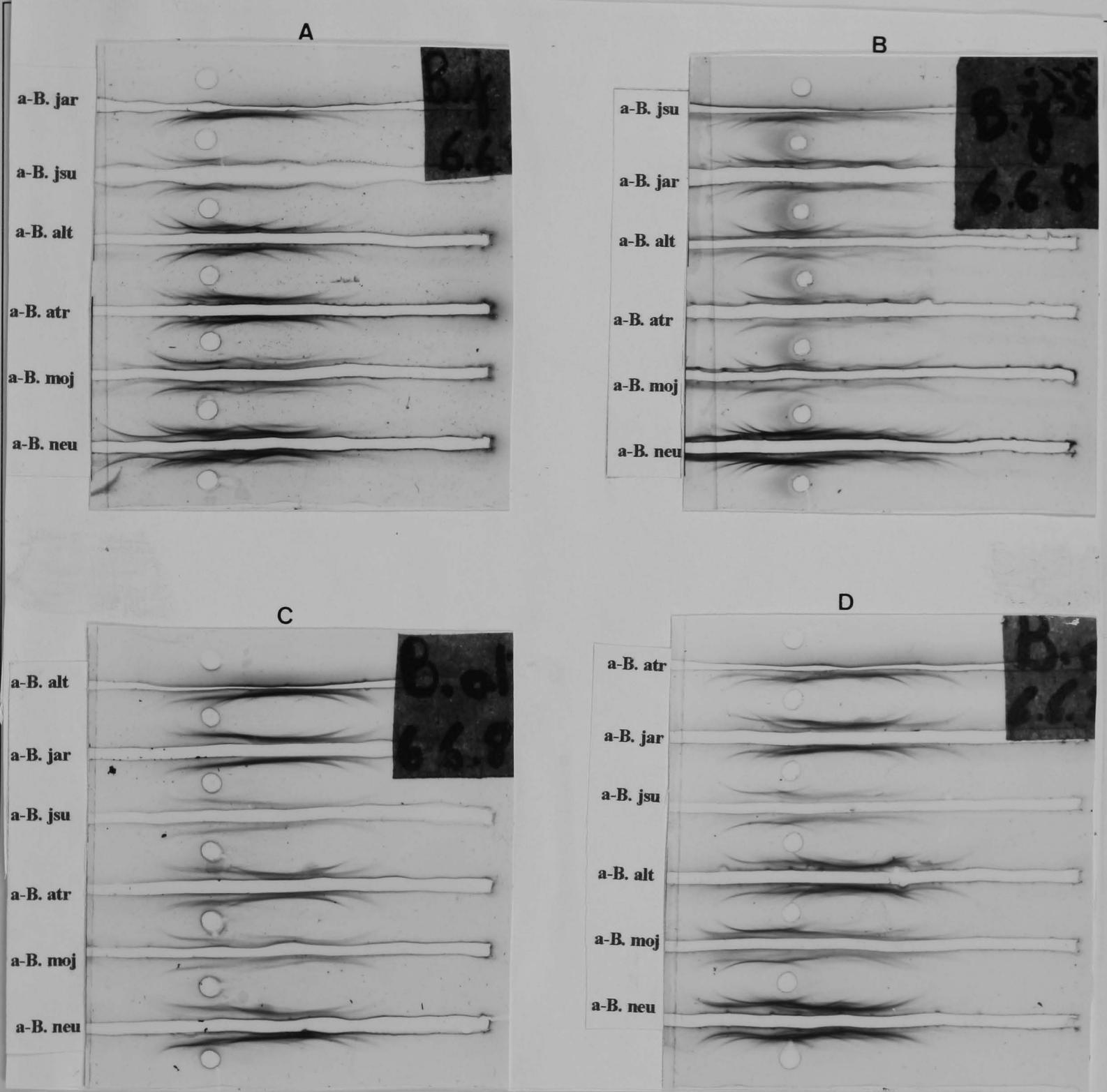


Figure 3.7 (A-D). Immunoelectrophoresis of Bothropic venoms against homologous and heterologous rabbit monovalent antivenom showing extensive cross-reactivity. Each plate contains 5.0 µg of a single specific venom applied to the wells in a volume of 1 µl. Homologous and heterologous antivenoms were added to the troughs as indicated in volumes of 70 µl containing 740 µg of IgG. Venoms: **A:** *B. jararaca*; **B:** *B. jararacussu*; **C:** *B. alternatus*; **D:** *B. atrox*; Antivenoms: anti-*B. jararaca* (a-B. jar); anti-*B. jararacussu* (a-B. jsu); anti-*B. alternatus* (a-B. alt); anti-*B. atrox* (a-B. atr); anti-*B. moojeni* (a-B. moj); anti-*B. neuwiedi* (a-B. neu).

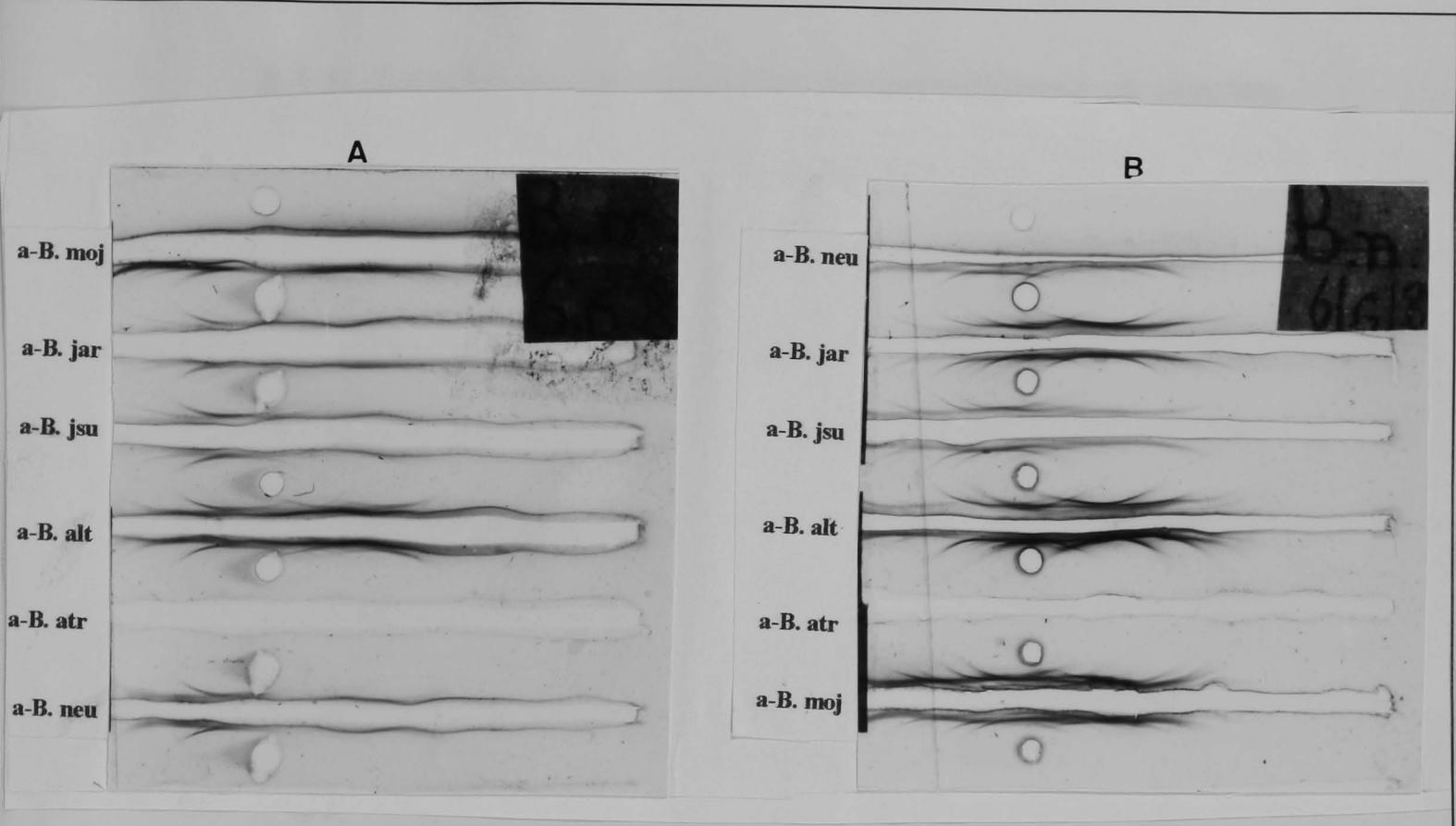


Figure 3.8 (A and B). Immunoelectrophoresis of Bothropic venoms reacted with homologous and heterologous rabbit monovalent antivenom showing extensive cross-reactivity. Each plate contains 5.0 µg of a single specific venom applied to the wells in a volume of 1 µl. Homologous and heterologous antivenoms were added to the troughs as indicated in volumes of 70 µl containing 740 µg of IgG. Venoms: **A:** *B. moojeni*; **B:** *B. neuwiedi*. Antivenoms: anti-*B. jararaca*, (a-B. jar); anti-*B. jararacussu*, (a-B. jsu); anti-*B. alternatus*, (a-B. alt); anti-*B. atrox*, (a-B. atr); anti-*B. moojeni*, (a-B. moj); anti-*B. neuwiedi*, (a-B. neu).

Some antivenoms react to more venom components in an heterologous venom than the heterologous venom's own homologous antivenom. This is observed with the anti-*B. neuwiedi* antivenom against the venoms of *B. jararaca*, *B. jararacussu* and *B. atrox*, Fig. 3.7 (A, B, and D) respectively, and with the anti-*B. alternatus* and anti-*B. moojeni* against the venom of *B. neuwiedi*, Fig. 3.8 (B). The results show clearly that more than one venom component is responsible for the crossreactivities observed in all cases shown. No precipitin line specific for any of the venom species studied could be observed.

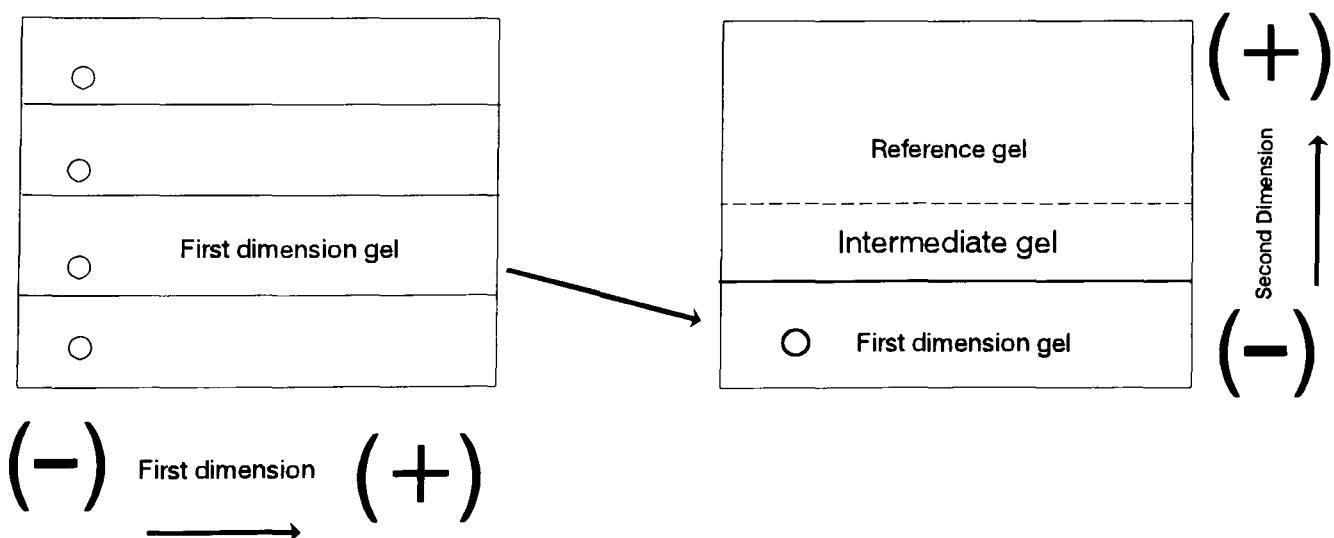
3.1.6. 2-Dimensional gel immunoelectrophoresis of venoms

The better resolution power of this technique was used in order to show the existence or not of species-specific venom components in the Bothropic venoms used in this study. For this the variation of the technique which places an intermediate gel between the first and second dimension gels was used. The intermediate gel containing a mixture of heterologous antivenoms IgG. This was devised to precipitate the crossreacting components of the electrophoresed venom, allowing a visualisation of any specific component(s) for that venom that would precipitate in the second dimension gel (reference gel) containing the homologous antivenom.

3.1.7. 2-Dimensional gel immunoelectrophoresis with monovalent rabbit antivenom in the reference gel.

Before setting up the experiment with the intermediate gel the technique was standardised without any intermediate gel. The patterns shown in Fig. 3.9 (A-D) and Fig. 3.10 (A, B) were obtained with rabbit monovalent antivenom IgG in the reference gel.

Drawing showing how the 2D-IEP is performed. The samples migrate from the negative side to the positive side. If an intermediate gel is used it is placed between the First dimension gel and the reference gel. 2D-IEP Figures in the Results section are representative of the second dimension electrophoresis run.



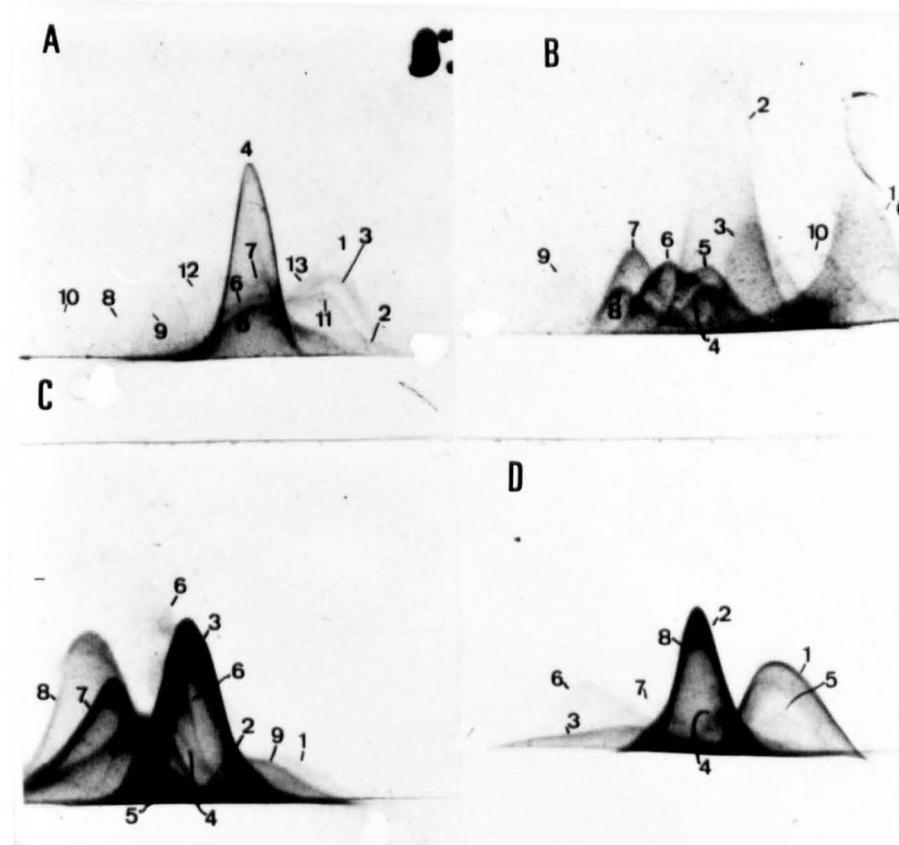


Figure 3.9 (A-D). 2-Dimensional immunoelectrophoresis of Bothropic venoms. Venom components were separated in the first dimension and then reacted with their specific non-absorbed monovalent antivenom by electrophoresis in the second dimension in agarose gel. Venoms were 5.0 µg in all cases in 5.0 µl. Monovalent specific antivenom IgG was applied at 2.5 mg/ml in 2.0 ml for all cases studied. **A:** *B. jararaca* venom/antivenom system, **B:** *B. jararacussu* venom/antivenom system, **C:** *B. alternatus* system, **D:** *B. atrox* system. Visible peaks for each system are numbered. First dimension electrophoresis, 10 V/cm, 1 hr, 30 min. Second dimension 2.5 V/cm for 16 hr.

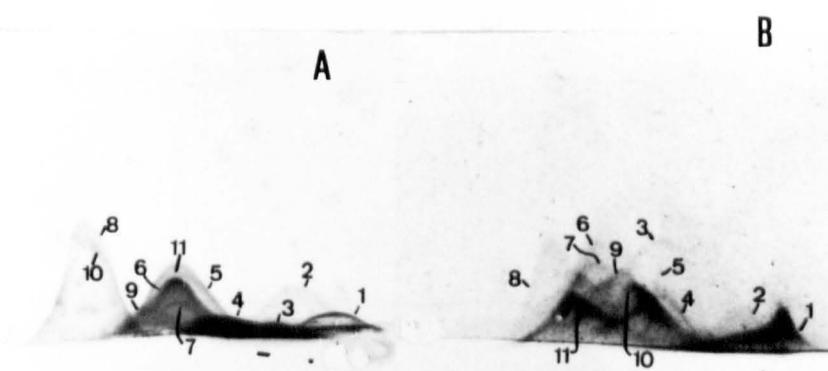


Figure 3.10 (A, B) 2-Dimensional immunoelectrophoresis of Bothropic venoms. Venom components were separated in the first dimension and then reacted with their specific non-absorbed monovalent antivenom by electrophoresis in the second dimension in agarose gel. Venoms were 5.0 µg in all cases in 5.0 µl. Monovalent specific antivenom IgG was applied at 2.5 mg/ml in 2.0 ml for all cases studied in the reference gel of second dimension. Experimental conditions as in Figure 3.9. Venom/antivenom system, **A**: *B. moojeni*, **B**: *B. neuwiedi*

These results were obtained with an IgG/venom ratio of 500:1. Multiple precipitin arcs are observed for all the venoms studied and the most clearly identified were numbered. Again there is evidence of the multicomponent nature of Bothropic snake venoms. The precipitin patterns are different for each venom species used. The results shown here are the results of great efforts to ensure that the technique was standardised.

3.1.8. 2-Dimensional gel immunoelectrophoresis of venoms with intermediate gel.

In these experiments the IgG/venom ratio was maintained at 500:1 and the same batches of antivenom IgG as in the previous 2D-IEP experiments were used. The intermediate gel contained equal amounts of the heterologous antivenom IgGs in final concentrations higher than that used for the homologous IgG in the reference gel. This was done in order to try to ensure that all, or most, crossreacting components would be precipitated in the intermediate gel. Fig. 3.11 shows the results for the *B. moojeni* venom. In plates (B) and (C) it is evident that a precipitin arc shows reactivity only in the reference gel.

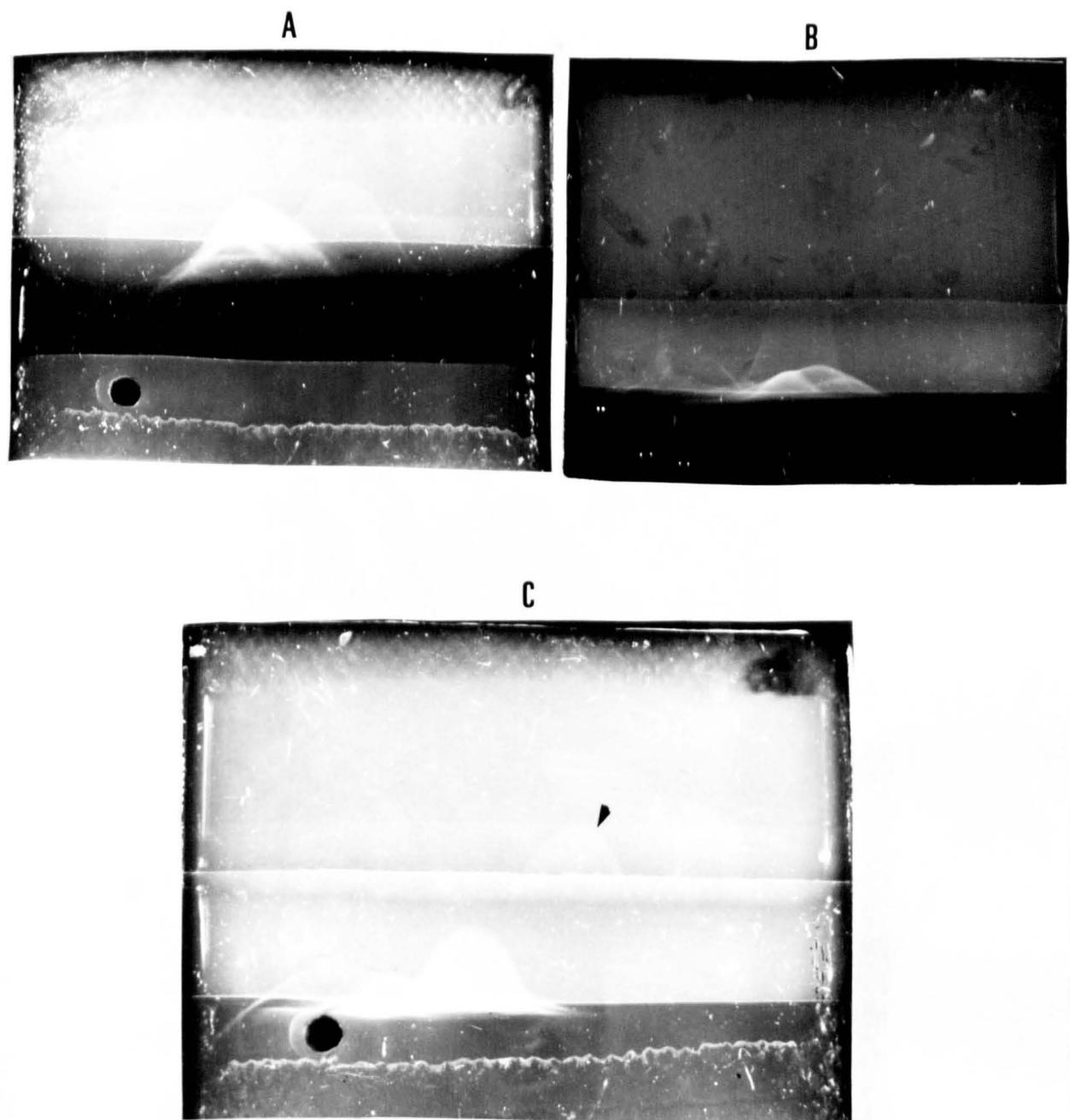


Figure 3.11 2-Dimensional immunoelectrophoresis of *B. moojeni* venom with intermediate gel containing a mixture of heterologous antivenom IgG's. Experimental conditions as in Figure 3.9. **A:** Control with saline in the intermediate gel, **B:** Intermediate gel containing 3 times concentration of IgG as in reference gel. **C:** Intermediate gel containing 5 times the concentration of IgG. Antivenoms IgG in intermediate gel: anti- *B. jararaca*, anti-*B. jararacussu*, anti- *B. alternatus*, anti-*B. atrox* and anti-*B. neuwiedi* added in equal amounts. Arrow in reference gel indicates a peak with species specificity.

In Fig. 3.12 (A, B) the results for the *B. atrox* venom is shown. In plate B, a precipitin arc is observed showing no reaction with the intermediate gel.

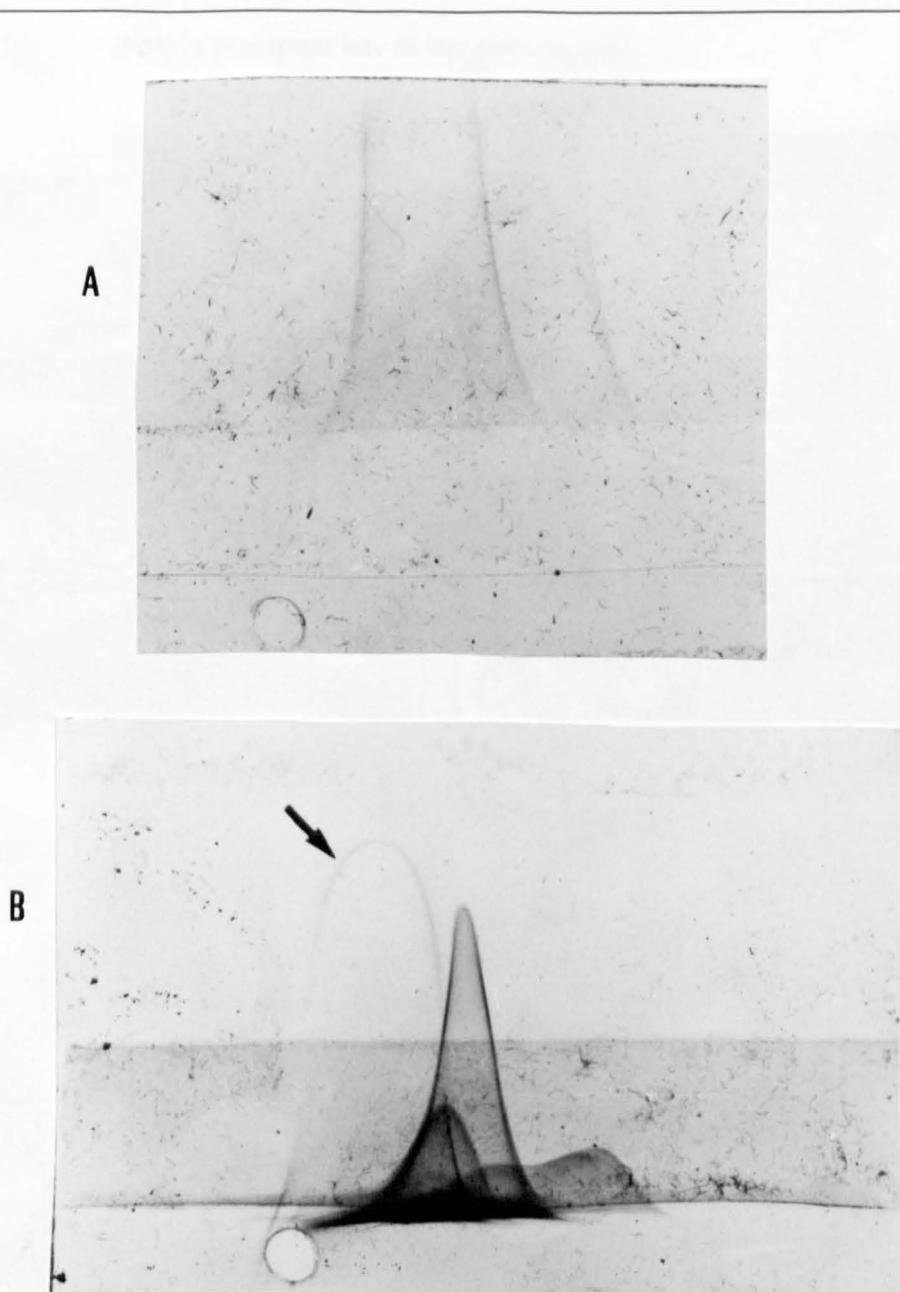


Figure 3.12 2- Dimensional immunoelectrophoresis of *B. atrox* venom. Conditions as in Figure 3.9. **A**- Control gel with saline in intermediate gel. **B**. Intermediate gel containing mixture of heterologous antivenoms IgGs as: anti-*B. jararaca*, anti-*B. jararacussu*, anti-*B. alternatus*, anti-*B. moojeni* and anti-*B. neuwiedi* in equal amounts 5 times more concentrated than IgG in reference gel. Arrow in reference gel indicates a peak with species specificity.

The result for the *B. alternatus* venom is shown in Fig. 3.12 (A-C). In plate C two precipitin arcs can be observed in the reference gel. Both show also reactivity with the intermediate denoted by the extension of their 'feet' into this gel. One of them indicated by the arrow shows a sharper precipitin line in the reference gel.

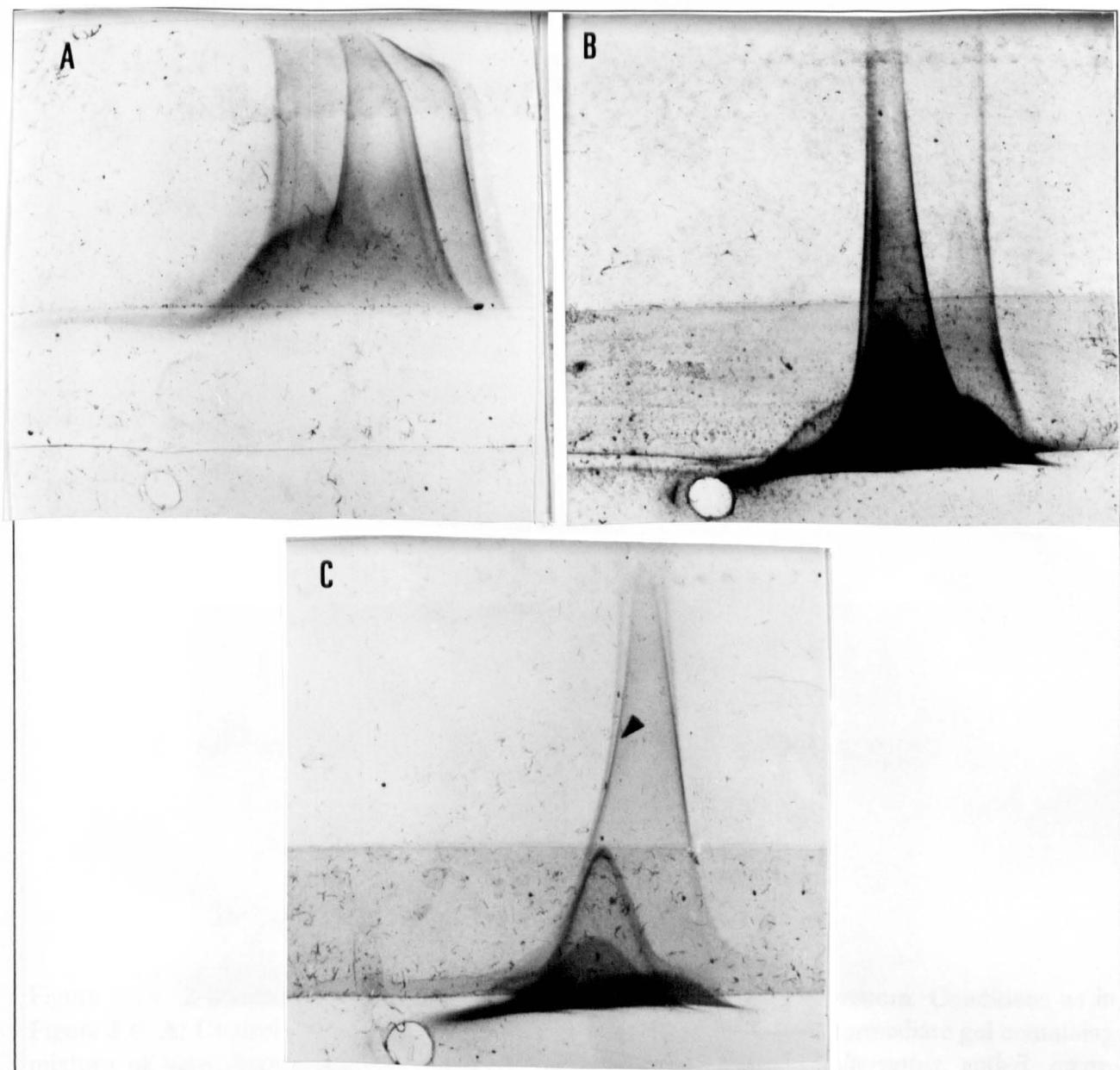


Figure 3.13 2-Dimensional immunoelectrophoresis of *B. alternatus* venom. Conditions as in Figure 3.9. **A**- Control gel with saline in intermediate gel. **B**. Intermediate gel containing mixture of heterologous antivenoms IgGs as: anti-*B. jararaca*, anti-*B. jararacussu*, anti-*B. atrox*, anti-*B. moojeni* and anti-*B. neuwiedi* in equal amounts 5 times more concentrated than IgG in the reference gel. Arrow in reference gel indicates a peak with species specificity.

In Fig. 3.14 (A-C) are shown the result for the *B. jararacussu* venom

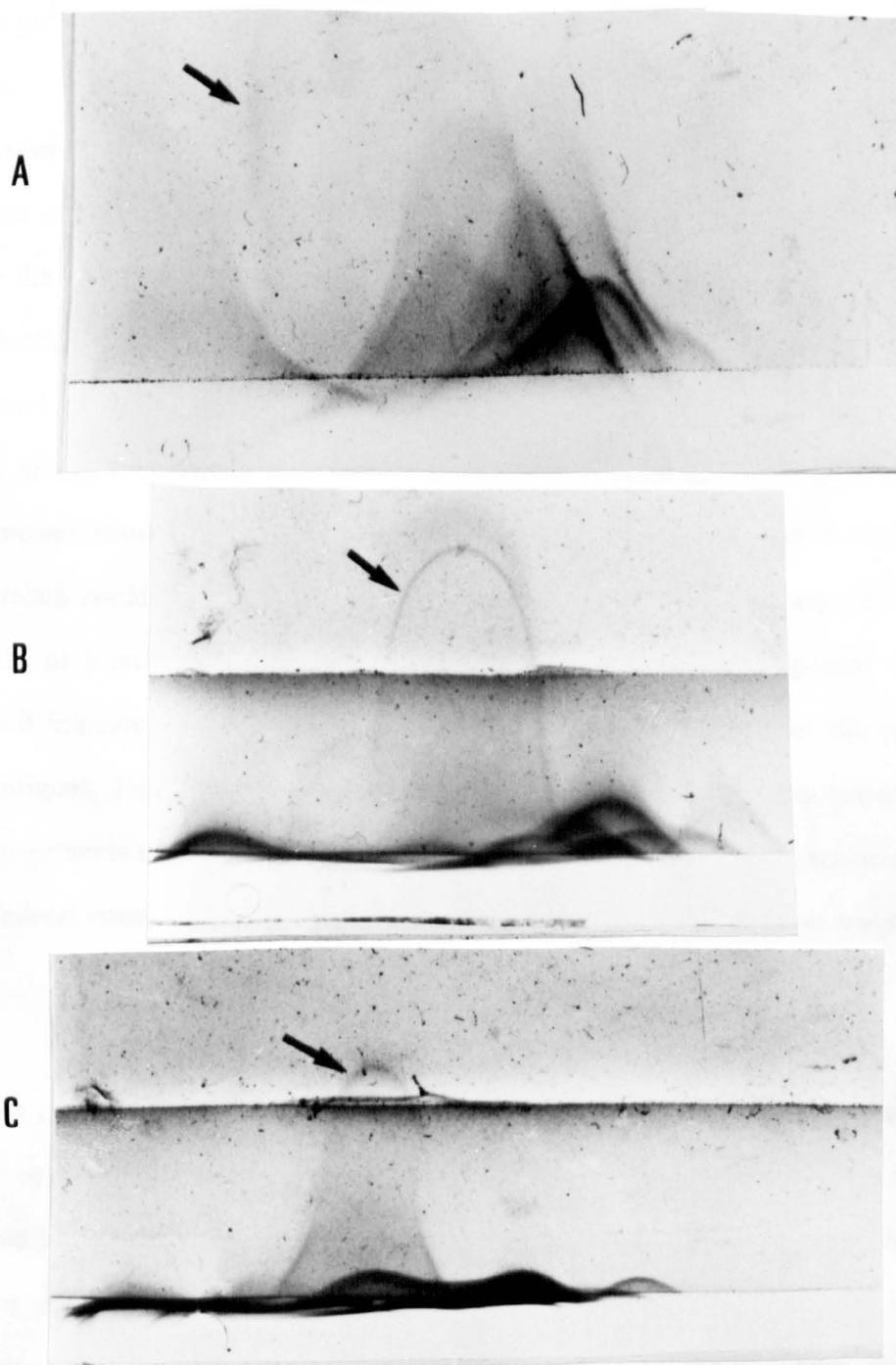


Figure 3.14 2-Dimensional immunoelectrophoresis of *B. jararacussu* venom. Conditions as in Figure 3.9. **A:** Control gel with saline in intermediate gel. **B:** Gel with intermediate gel containing mixture of heterologous antivenoms IgGs: anti-*B. jararaca*, anti-*B. alternatus*, anti-*B. atrox*, anti-*B. moojeni* and anti-*B. neuwiedi* in equal amounts, 5 times more concentrated than IgG in the reference gel. Arrow in reference gel indicates a peak with species-specificity.

It can be observed in plates (B) and (C), of Figure 3.14 that only one precipitin arc showing reactivity in the reference and intermediate gel, and none show reactivity only in the

reference gel. In plate C, where the concentration of the IgG in the intermediate gel is 10 X that of the reference gel, only the upper part of the peak reacts with the reference gel. The *B. moojeni* result, (Fig. 3.11), seems to indicate the existence of a species-specific component in that venom. This is probably also true for the venom of *B. atrox* (Fig. 3.12). However the results for the venoms of *B. alternatus* and *B. jararacussu* failed to show any clear indication of such specific venom components. For the *B. alternatus* venom (Fig. 3.13) in plate C, the sharper precipitin line of the peak identified (arrow) in the reference gel is indicative of higher reactivity with the homologous antivenom IgG. It is possible that using higher concentrations of IgG in the intermediate gel could have resolved the two peaks and a clearer result could be obtained. The failure to observe any precipitin arcs in the reference gel in some of these results does not confirm the absence of species-specific components. Nor does it indicate where such arcs are observed, that they represent the only species-specific antigens. The results do however, show that the precipitin peaks obtained with the venom components of *B. moojeni* and *B. atrox* with species-specific characteristics could be used for animal immunisation to try to generate species-specific antivenom reagents.

3.1.9. Production of species-specific antivenoms

The results obtained in the 2D-IEP with an intermediate gel (Section 3.1.8) indicated the existence of species-specific venom components for some of the studied venoms. As pointed out before it would be worthwhile to inject such components into animals for the production of species-specific antivenoms. It was decided then to repeat the 2D-IEP experiments on larger gel plates, cut out from the agarose gel the precipitin peaks showing species-specificity and use them for the immunisation of rabbits.

3.1.9.1.2D-IEP with polyvalent antivenom in the reference gel

The purpose of this experiment was to standardise the technique, before setting up the experiment with the intermediate gel. The glass plates used were 8 X 8 cm. The antivenom used in the reference gel was a commercial preparation of a polyvalent antithropic horse antivenom $F(ab')_2$. They were in large supply, contrary to the short supplies of monovalent antivenom. As the commercial antivenoms are obtained mostly from long term immunised horses they should contain antivenom antibodies with higher affinity yielding better resolved precipitin patterns. Fig. 3.15 (A-H) shows the precipitin patterns obtained with 0.5 ml of the polyvalent antithropic antivenom in the reference gel and 300 μ g of venom separated in the first dimension electrophoresis.

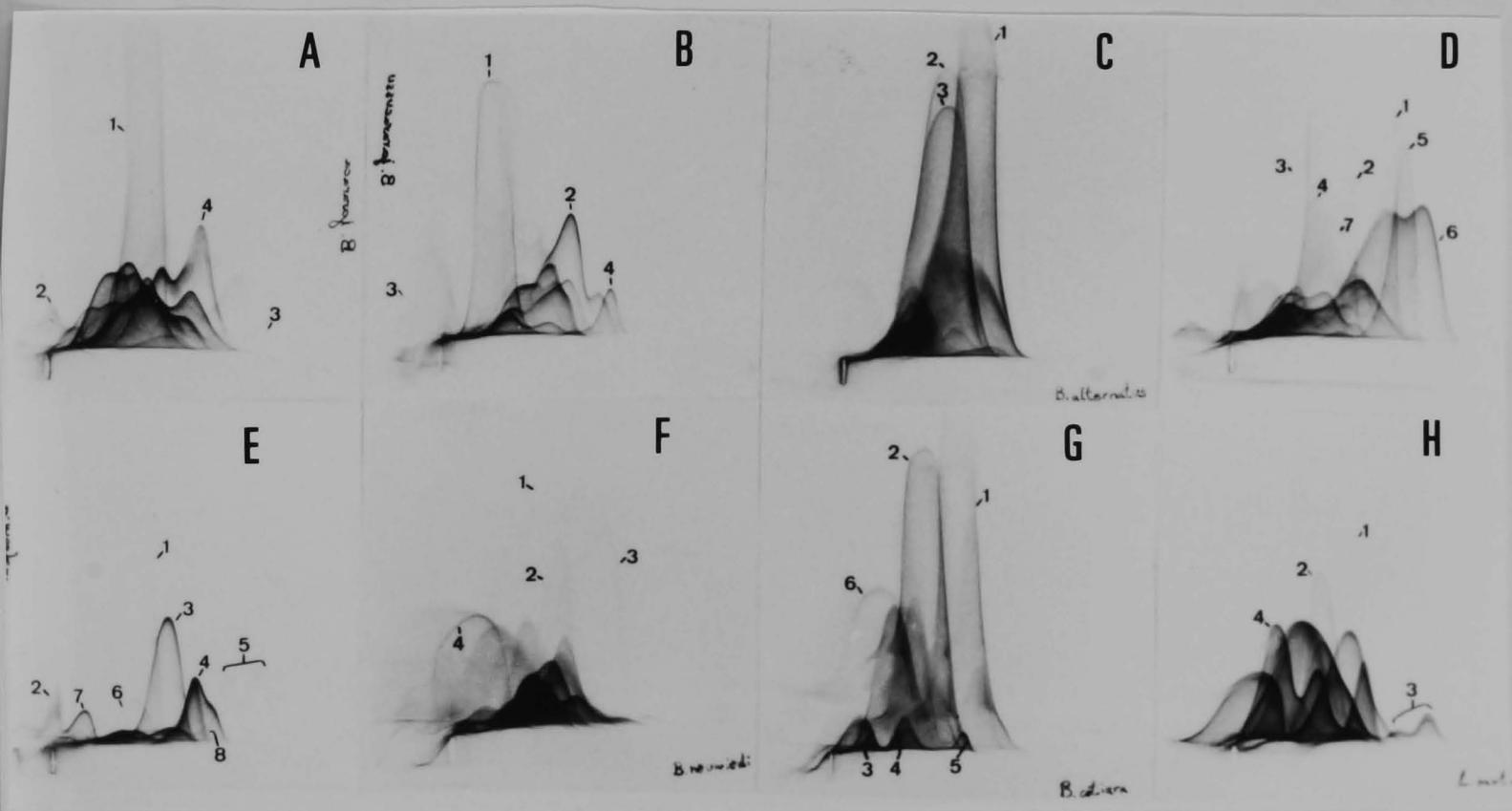


Figure 3.15 2-Dimensional immunoelectrophoresis of venoms using polyvalent antithropic horse $F(ab')_2$ in the reference gel. 300 μ g of each venom was applied separately in each plate in 6 μ l. Reference gels contains 0.5 ml of polyvalent antivenom in all cases. Electrophoresis conditions as in Figure 3.9. First dimension run for 3.0 hr and second for 16 hr. Visible peaks are numbered. Venoms: **A**: *B. jararaca*, **B**: *B. jararacussu*, **C**: *B. alternatus*, **D**: *B. atrox*, **E**: *B. moojeni*, **F**: *B. neuwiedi*, **G**: *B. cotiara*, **H**: *L. muta*.

In all cases there is a good resolution of the precipitin peaks and the precipitin patterns are different for each of the species of venom used. When compared to the similar experiment

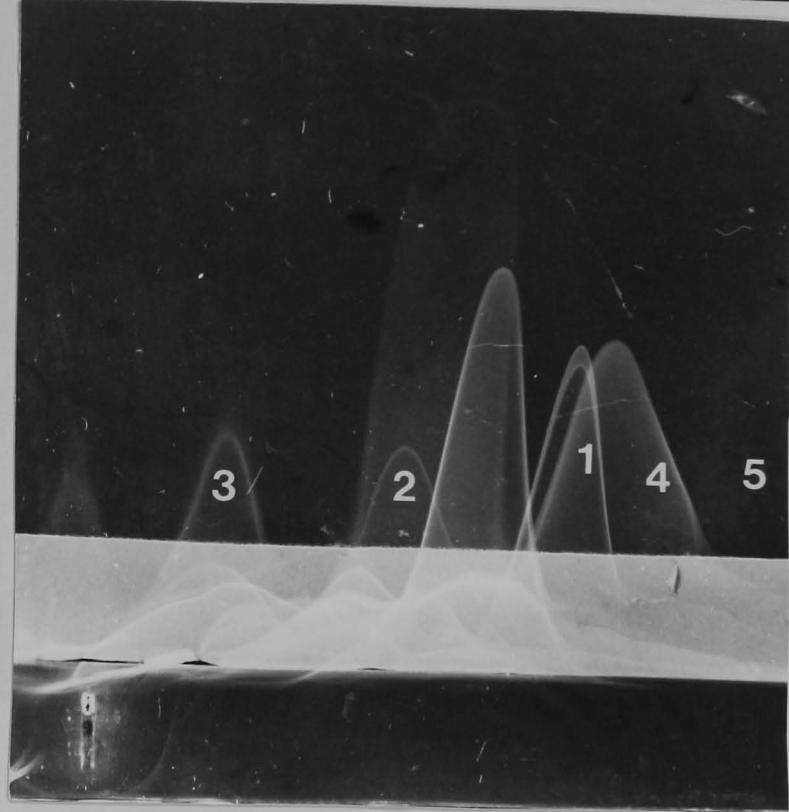
in Section 3.1.7, performed with the monovalent antivenom in the reference gel, the precipitin peaks obtained here with the polyvalent antivenom are better resolved. The precipitin patterns are different for each venom species. The results were considered satisfactory and the conditions as standardised.

3.1.9.2. 2D-IEP with intermediate gel and polyvalent antivenom in the reference gel.

The objective of this experiment was to produce specific single antigens to be used in immunisation schemes to generate species-specific antivenoms. The use of an intermediate gel containing a mixture of monovalent antivenom IgGs, heterologous to the venom separated in the first dimension, had the same purpose stated in Section 3.1.8; that is, absorb the crossreacting venom components allowing the visualisation of species-specific component(s) as precipitin peaks in the reference gel. The precipitin peaks with the species-specific characteristics would be excised from the agarose gel and after a washing procedure immunised into rabbits. The intermediate gel antivenom mixture contained equal amounts of each heterologous IgG making a total of 20 mg. In Fig. 3.16 (A) is the pattern observed for the *B. moojeni* venom. There are five peaks showing species-specificity. Peak no.5 is not clearly visible in the picture. In plate (B) is a similar *B. moojeni* experiment stained with Coomassie Blue, after the excision of the specific peaks. In Fig. 3.17 (A, B) is the result for the *B. atrox* venom. Four peaks are observed with species-specific characteristics. Peak no.4 is not clearly visible in the picture. Peaks 2 and 3 seem to be composed of more than one component as their forward feet show lines reacting with the intermediate gel and lines not reacting. For this reason they were also excised. In (B) another *B. atrox* plate shows the excised peaks and is stained as in Fig. 3.17(B). Fig. 3.18 (A, B) shows the *B. neuwiedi* results. Only two peaks (not clearly visible in the plate (A)) were observed with species-specific characteristics. In plate (B) the excised peaks are shown after protein staining, as in (Fig. 3.17 (B)). In all cases the results in plates (A) were analysed with the aid of an illuminating box and a magnifying glass in a dark room for better accuracy in determining

the specific peaks and excision of these. Not all crossreacting components were precipitated in the intermediate gel, this is particularly true for the *B. neuwiedi* venom Fig. 3.18(A, B). However it was possible to excise peaks with the desired specificity.

A



B

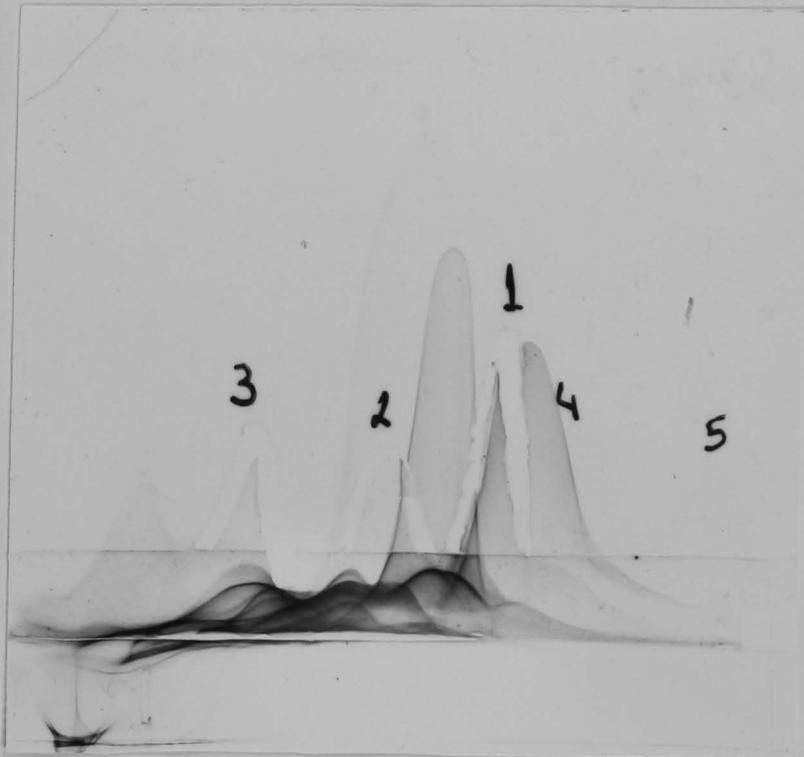


Figure 3.16 (A and B) 2-Dimensional immunoelectrophoresis of *Bothrops moojeni* with intermediate gel, using polyvalent antithrombic horse F(ab')₂ in the reference gel. Intermediate gel contains a mixture of anti-*B. jararaca*, anti-*B. alternatus*, anti-*B. atrox*, anti-*B. moojeni* and anti-*B. neuwiedi* IgG added in equal amounts in a total concentration of 20 mg/ml. Electrophoresis conditions as in Figure 3.9. First dimension run for 3 hours and second dimension run for 16 hours. Peaks showing species-specificity are numbered. Plate B shows the peaks excised from agarose gel for rabbit immunisation.

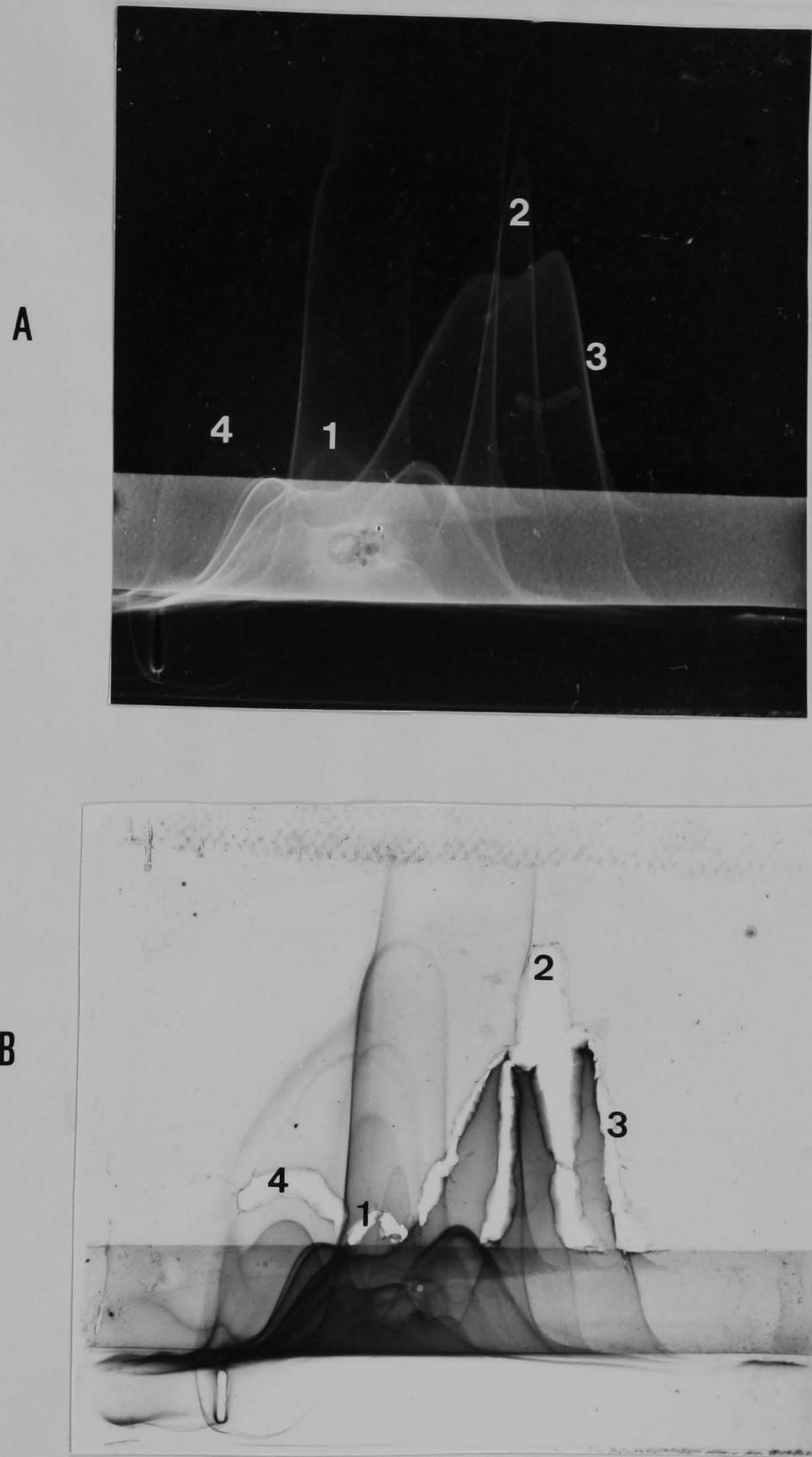


Figure 3.17 (A and B) 2-Dimensional immunoelectrophoresis of *Bothrops atrox* with intermediate gel, using polyvalent antithropic horse F(ab')₂ in the reference gel. Intermediate gel contains a mixture of anti-*B. jararaca*, anti-*B. jararacussu*, anti-*B. alternatus*, anti-*B. moojeni* and anti-*B. neuwiedi* IgG added in equal amounts in a total concentration of 20 mg/ml. Electrophoresis conditions as in Figure 3.9. First dimension run for 3 hours and second dimension run for 16 hours. Peaks showing species-specificity are numbered. Plate B shows the peaks excised from agarose gel for rabbit immunisation.

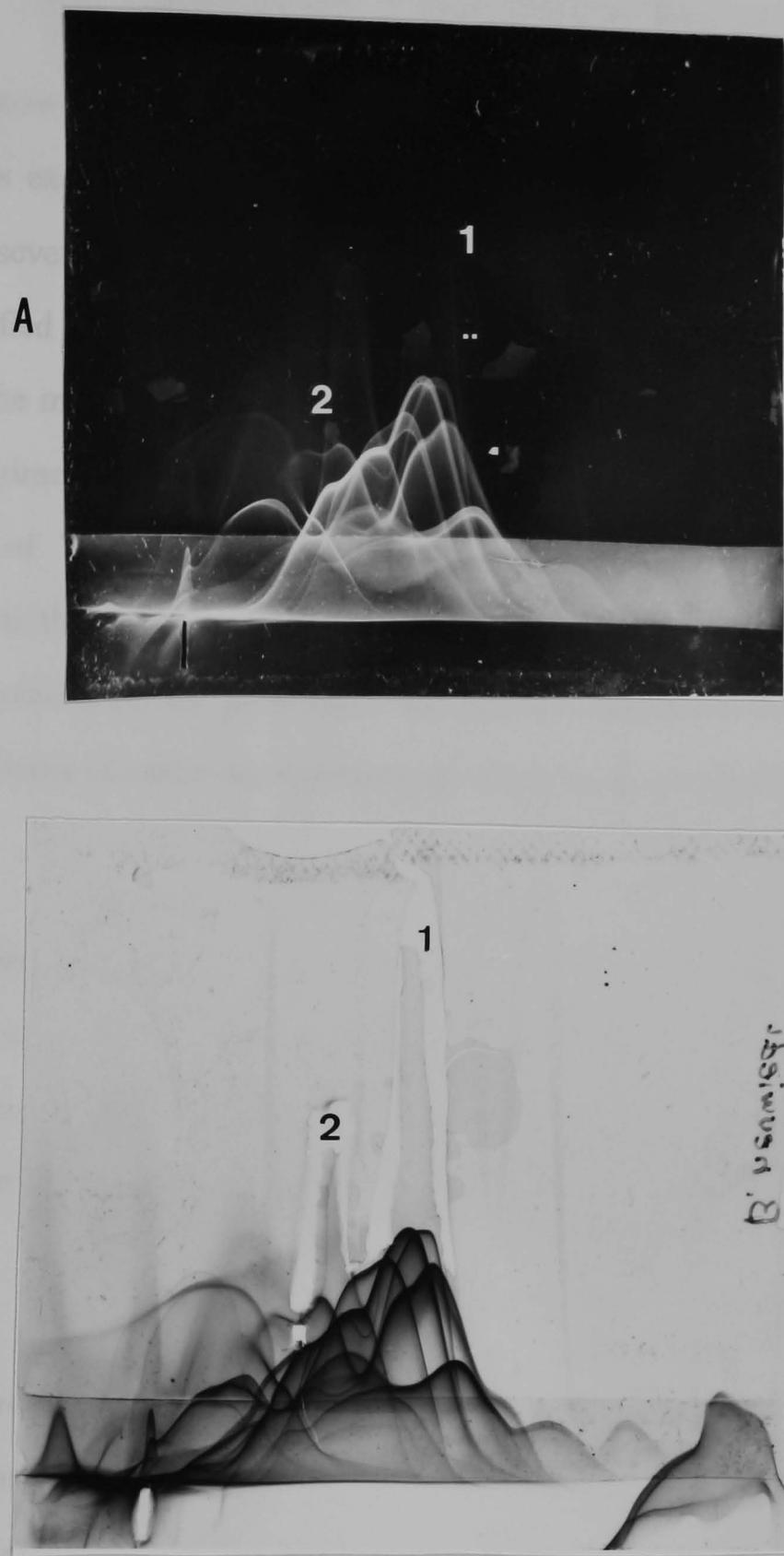


Figure 3.18 (A and B) 2-Dimensional immunoelectrophoresis of *Bothrops neuwiedi* with intermediate gel, using polyvalent antithropic horse Fab₂ in the reference gel. Intermediate gel contains a mixture of anti-*B. jararaca*, anti-*B. jararacussu*, anti-*B. alternatus*, anti-*B. atrox* and anti-*B. moojeni* IgG added in equal amounts in a total concentration of 20 mg/ml. Electrophoresis conditions as in Figure 3.9. First dimension run for 3 hours and second dimension run for 16 hours. Peaks showing species-specificity are numbered. Plate B shows the peaks excised from agarose gel for rabbit immunisation

3.1.9.3. Immunisation of rabbits with venom immune complexes in agarose gels.

In order to raise species-specific antivenoms against some of the Bothropic venoms studied, the precipitin peaks excised from the agarose gels in the experiments of Section 3.1.9.2, were washed with several changes of 0.1 M PBS, pH 7.4, to remove any non-precipitated material and emulsified with FCA for the first dose and FIA for the subsequent doses. Each dose consisted of the material from two identical precipitin peaks for each venom, excised from different experiments. The immunisations were carried out at the Venom Unit at the Liverpool School of Tropical Medicine by Dr. G.D.R. Laing. After the 16 week immunisation scheme there was no detectable antivenom activity in any of the immunised rabbits sera. This procedure for production of specific antivenom was discontinued. It demanded large amounts of monovalent antivenom which were not available.

3.1.9.4. Venom affinity adsorption of monovalent antivenom.

Based on the previous results in which the 2D-IEP assays had strongly indicated the presence of species-specific venom components, a second approach was used to generate species-specific antivenoms. It consisted in the absorption out of the cross-reacting antibodies in the antivenoms by affinity adsorption onto venom adsorbents.

3.1.9.4.1. Adsorption on venom adsorbents containing a mixture of heterologous venoms in equal amounts

The antivenoms were added to the adsorbents containing the heterologous venoms and mixed for a period of time. After the adsorption step(s) it was expected that the cross-reacting antivenom antibodies would have bound to the venom adsorbent leaving the specific antibodies in the supernatant. Prior to testing the absorbed antivenoms for loss of cross-reactivity they were tested first for the maintenance of the specific reactivity. In Fig. 3.19 (A-D) it can be observed that the absorbed antivenoms display a decrease in the intensity of the homologous reaction when compared to the unabsorbed antivenom but maintain the specific reactivity, in this case, after two adsorption steps. It can also be observed that some antivenoms show a sharper decrease in the specific reactivity than

others, (Fig. 3.19 (D)). The decrease in the specific reactivity was shown later to be followed by a decrease in the IgG content of the absorbed antivenoms after the adsorption steps (see Appendix IV). The absorbed antivenoms were then tested for cross-reactivity with the heterologous Bothropic venoms.

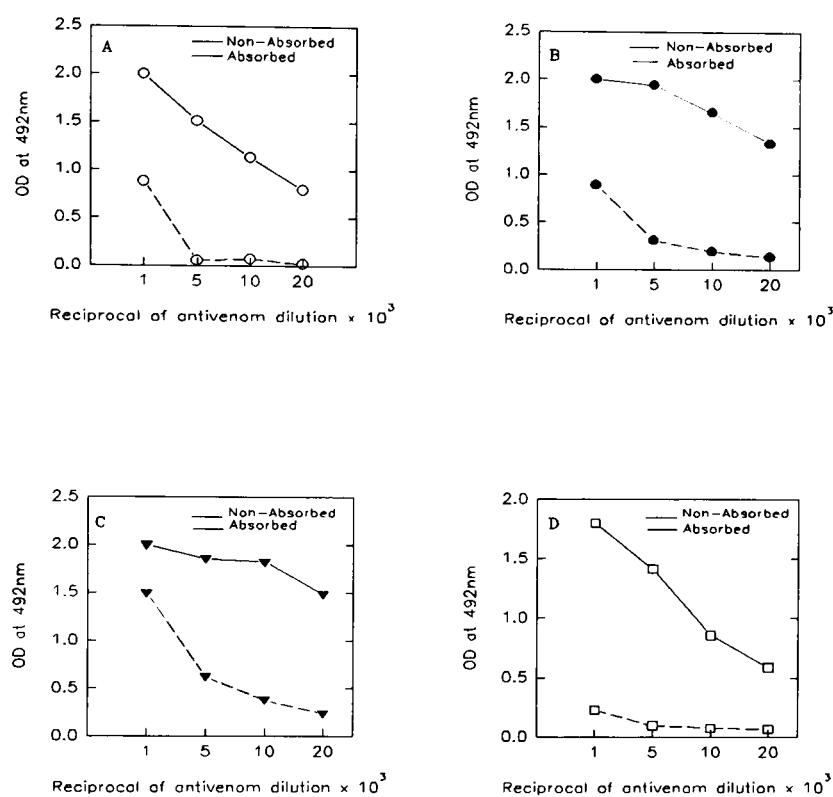


Figure 3.19 ELISA results comparing the specific reaction of unabsorbed and absorbed antivenoms with their homologous venom. Plates coated with a single venom at $1\mu\text{g}/\text{ml}$, $100\mu\text{l}/\text{well}$. Absorbed and unabsorbed antivenoms were reacted separately with their homologous venoms at the same dilutions. A: *B. jararaca* venom and homologous antivenoms; B: *B. jararacussu* venom and homologous antivenom; C: *B. alternatus* venom and homologous antivenom; D: *B. moojeni* venom and homologous antivenom.

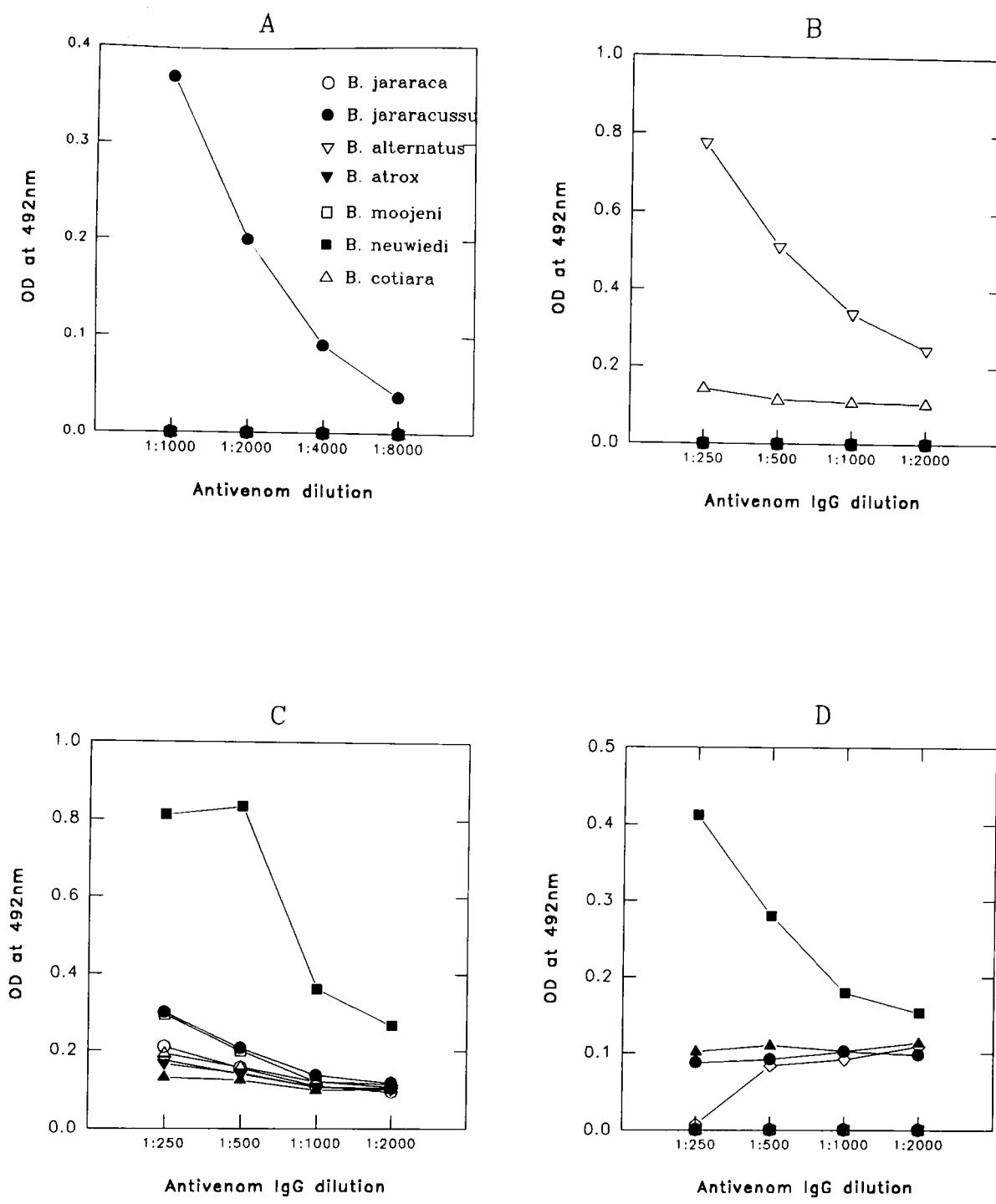


Figure 3.20 (A-D) ELISA showing species-specificity of absorbed antivenoms. Different dilutions of absorbed monovalent antivenoms were reacted with their homologous and heterologous venoms. **A:** anti-*B. jararacussu*, **B:** anti-*B. neuwiedi*, **C:** anti-*B. neuwiedi*, **D:** anti-*B. moojeni*. Venoms are as listed in **A** and were coated onto the plate at 1 µg/ml, 100 µl/well.

In Fig. 3.20 (A-D) the same batch of absorbed antivenoms used for the results in Fig. 3.19 were used, except for the anti-*B. jararacussu*. Note the increased species-specificity of all absorbed antivenoms. Anti-*B. jararacussu* in Fig. 3.20 (A) was used at higher dilution and shows no cross-reactivity at all. The other antivenoms were used at lower dilution.

Anti-*B. alternatus*, Fig. 3.20 (B), anti-*B. neuwiedi*, Fig. 3.20 (C), and anti-*B. moojeni*, Fig. 3.20 (D), show some residual cross-reactivity.

Fig. 3.21 shows a picture of an ELISA plate of a similar experiment using the same batch of absorbed antivenoms as in Fig. 3.20 (A-D). Observe the increased species-specificity for the absorbed antivenoms. Again the absorbed anti-*B. neuwiedi* displays higher cross-reactivity.

Note that the absorbed anti-*B. jararacussu*, Fig. 3.21 (A), maintains its increased species-specificity even when used at the lower dilution of 1:250. Another example of the increased

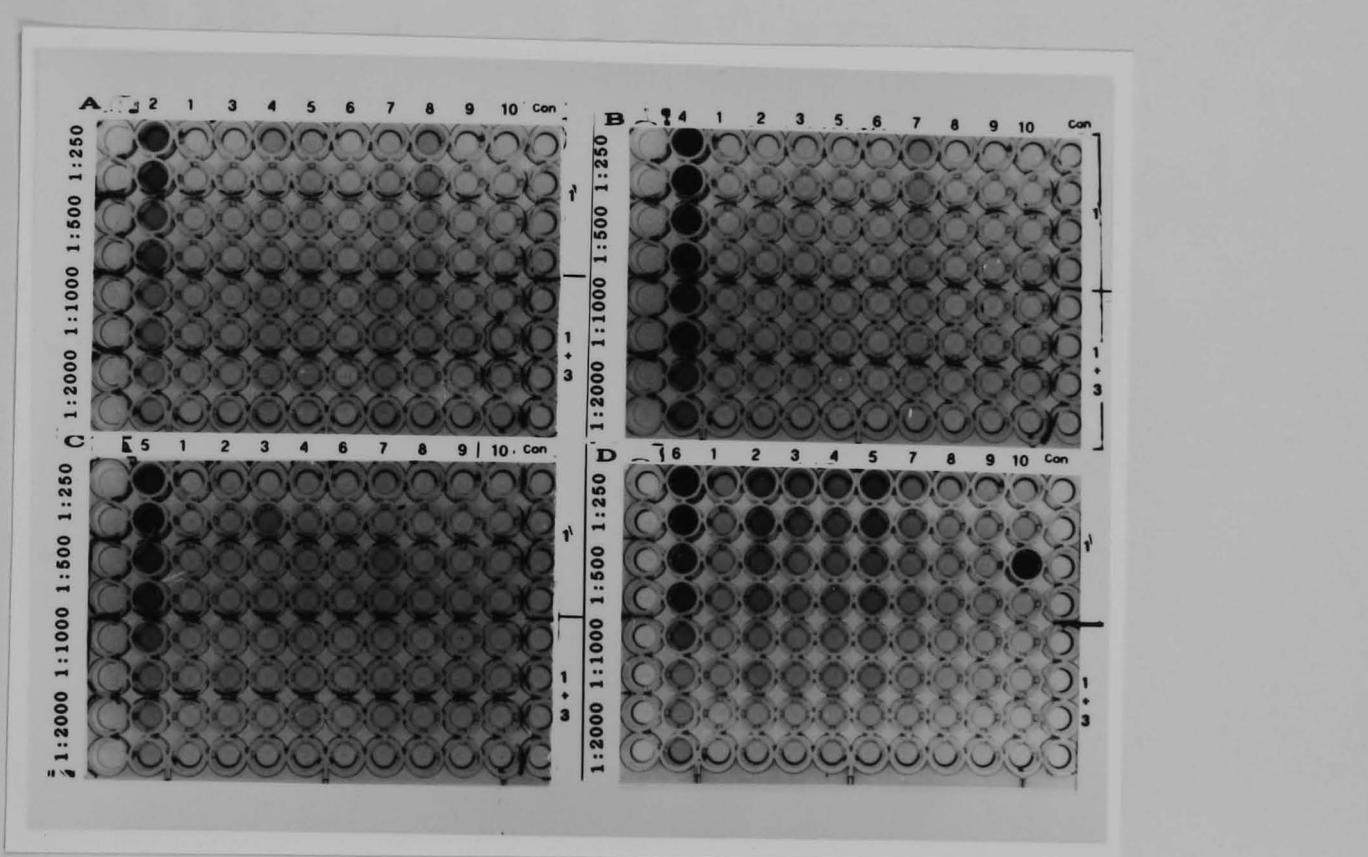


Figure 3.21 (A-D) ELISA plates showing the reaction of 10 venoms against the absorbed antivenoms. **A:** anti-*B. jararacussu*, **B:** anti-*B. alternatus*, **C:** anti-*B. moojeni*, **D:** anti-*B. neuwiedi*. Venoms coated at 1.0 µg/ml, 100 µl/well and labelled as: **1:** *B. jararaca*, **2:** *B. jararacussu*, **3:** *B. atrox*, **4:** *B. alternatus*, **5:** *B. moojeni*, **6:** *B. neuwiedi*, **7:** *B. cotiara*, **8:** *L. muta*, **9:** *Crotalus d. collineatus*, **10:** *Crotalus d. terrificus*. Antivenoms dilution's as shown on the left of each plate. **1+3** = Venom + conjugate; **1'** Conjugate alone. Conjugate, Sheep anti-rabbit IgG-HRP, used at 1:20,000 dilution.

species-specificity after absorption steps are shown in Fig. 3.22(A-D), where a new batch of absorbed antivenoms was used. Fig. 3.22(A) shows the 3X absorbed anti-*B. jararaca*, used at 1:100 dil.. Note the remaining minor cross-reactivity against some heterologous venoms.

In (B) the 2 X anti-*B. jararacussu* at 1:100 dil. also displays some cross-reactivity, more

accentuated against the venoms of *B. moojeni* and *B. neuwiedi*. In (C) the 3X absorbed anti-*B. moojeni* (1:200 dil.) showing a more significant cross-reactivity against the venom of *B. neuwiedi*. In (D) the quasi complete species-specificity of the 3X absorbed anti-*B. neuwiedi* used at 1:200 dil. and in (E) the species-specific reaction of 3X absorbed anti-*L. muta* (1:200 dil.). These experiments show that the absorption of antivenoms on venom affinity adsorbents yielded reagents with increased species-specificity as tested by ELISA.

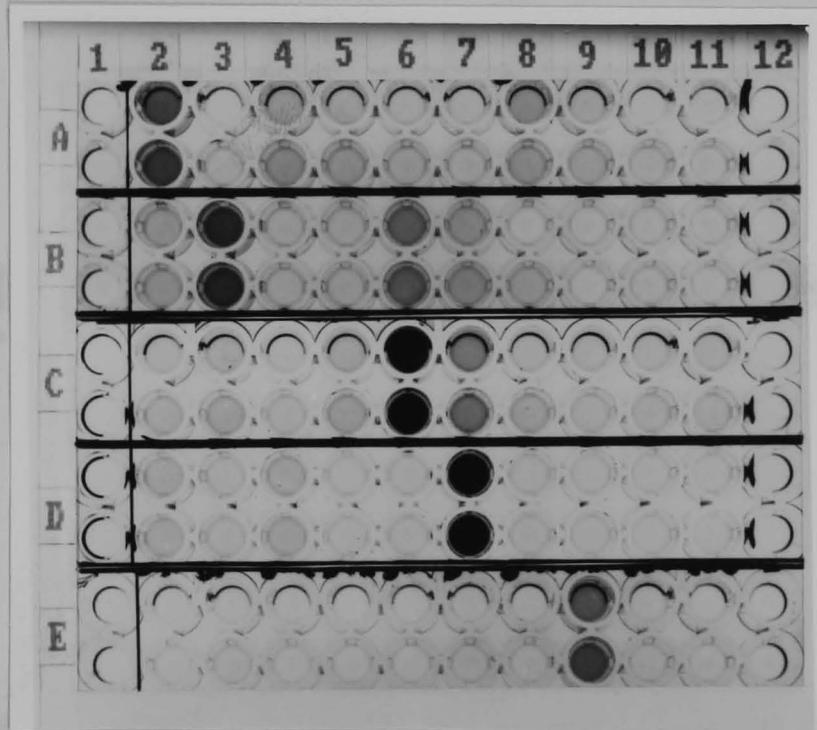


Figure 3.22 (A-D) ELISA plates showing the reaction of ten venoms against the absorbed antivenoms. **A:** anti-*B. jararaca*, 1:100 dilution (dil.); **B:** anti-*B. jararacussu*, 1:100 dil.; **C:** anti-*B. moojeni*, 1:200 dil.; **D:** anti-*B. neuwiedi*, 1:200 dil.; **E:** anti *L. muta* venoms, 1:200 dil. ^{Venoms} 2: *B. jararaca*, 3: *B. jararacussu*, 4: *B. alternatus*, 5: *B. atrox*, 6: *B. moojeni*, 7: *B. neuwiedi*, 8: *B. cotiara*, 9: *L. muta*, 10: *Crotalus d. terrificus*, 11: *Crotalus d. collineatus*. Column 1, buffer alone; Column 12, venom + conjugate. Conjugate, sheep anti-rabbit IgG-HRP, used at 1:20,000 dilution.

In order to increase further the species-specificity of the absorbed antivenoms, specifically those with some remaining cross-reactivity, venom adsorbents containing a single venom were prepared. These single venom adsorbents were used to "fine tune" the species-specificity of absorbed antivenoms by specifically absorbing out any remaining cross-reactivity against a specific venom. Fig. 3.23 (A) shows the results of such fine tuning for anti-*B. jararaca* antivenom, and Fig. 3.23 (B) for anti-*B. jararacussu* antivenom.

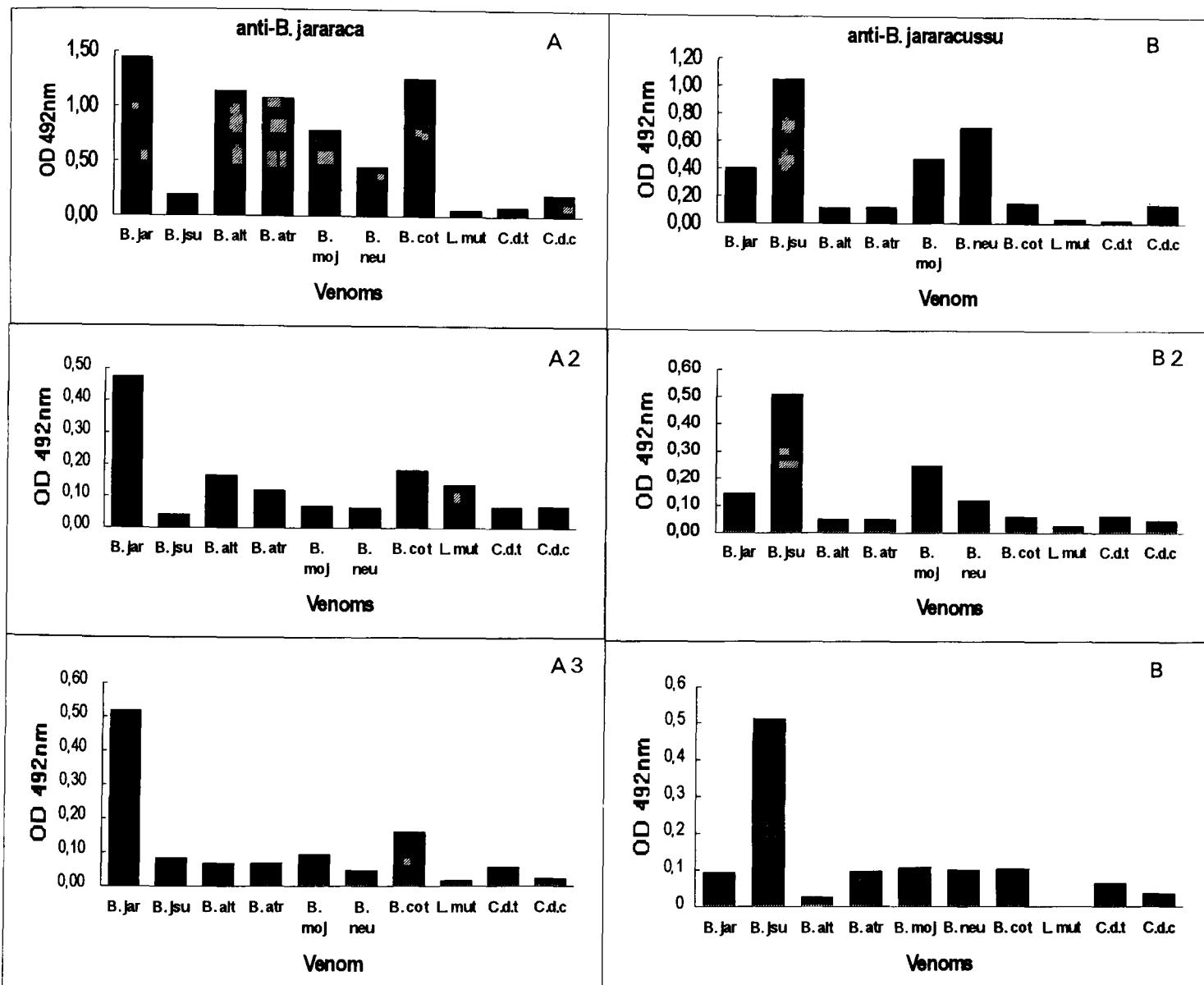


Figure 3.23 (A-B) ELISA results showing increase in the species-specificity of monovalent antivenoms after absorption in venom adsorbents containing a single venom.

Plate A. A1: anti-*B. jararaca* after two absorption in a venom adsorbent prepared with a mixture of venoms in equal amounts each. A2: Same antivenom after separate absorption of 30 min in a *B. alternatus* venom adsorbent, 20 min in a *B. mojeni* adsorbent, 20 min in a *B. cotiara* adsorbent. A3: Same as in A2 but in venom adsorbents of *B. alternatus* venom, 20 min, *B. cotiara* venom 20 min and *L. muta* 20 min. Antivenoms: A1 and A2 diluted 1:100, A3 diluted 1:75. Plate B. B1: Same as in A1 but in anti-*B. jararacussu* adsorbent. B2: Same as A2 but in *B. jararaca* adsorbent 20 min, *B. neuwiedi*, 20 min, B3: Same as in B2 but in *B. jararaca* adsorbent 20 min and *B. mojeni* 20 min. Antivenoms: B1 and B2 diluted 1:100, B3 diluted 1:75. Venom are labelled as: B. jar = *B. jararaca*; B. jsu = *B. jararacussu*; B. alt = *B. alternatus*; B. atr = *B. atrox*; B. moj = *B. mojeni*; B. neu = *B. neuwiedi*; B. cot = *B. cotiara*; L. mut = *L. muta*; C.d.c = *Crotalus durissus collineatus*; C.d.t = *Crotalus durissus terrificus*.

There is an increase in the specificity of both antivenoms. Note in Fig. 3.23 (A2) that the serial absorption on single venom adsorbents of *B. alternatus*, *B. moojeni* and *B. cotiara*, besides decreasing cross-reaction against these venoms also decreased the cross-reaction against the venoms of *B. atrox* and *B. neuwiedi*, which suggests the existence of common cross-reacting components between these venoms. Similar results are shown in Fig. 3.24 (A) for the anti-*B. moojeni*.

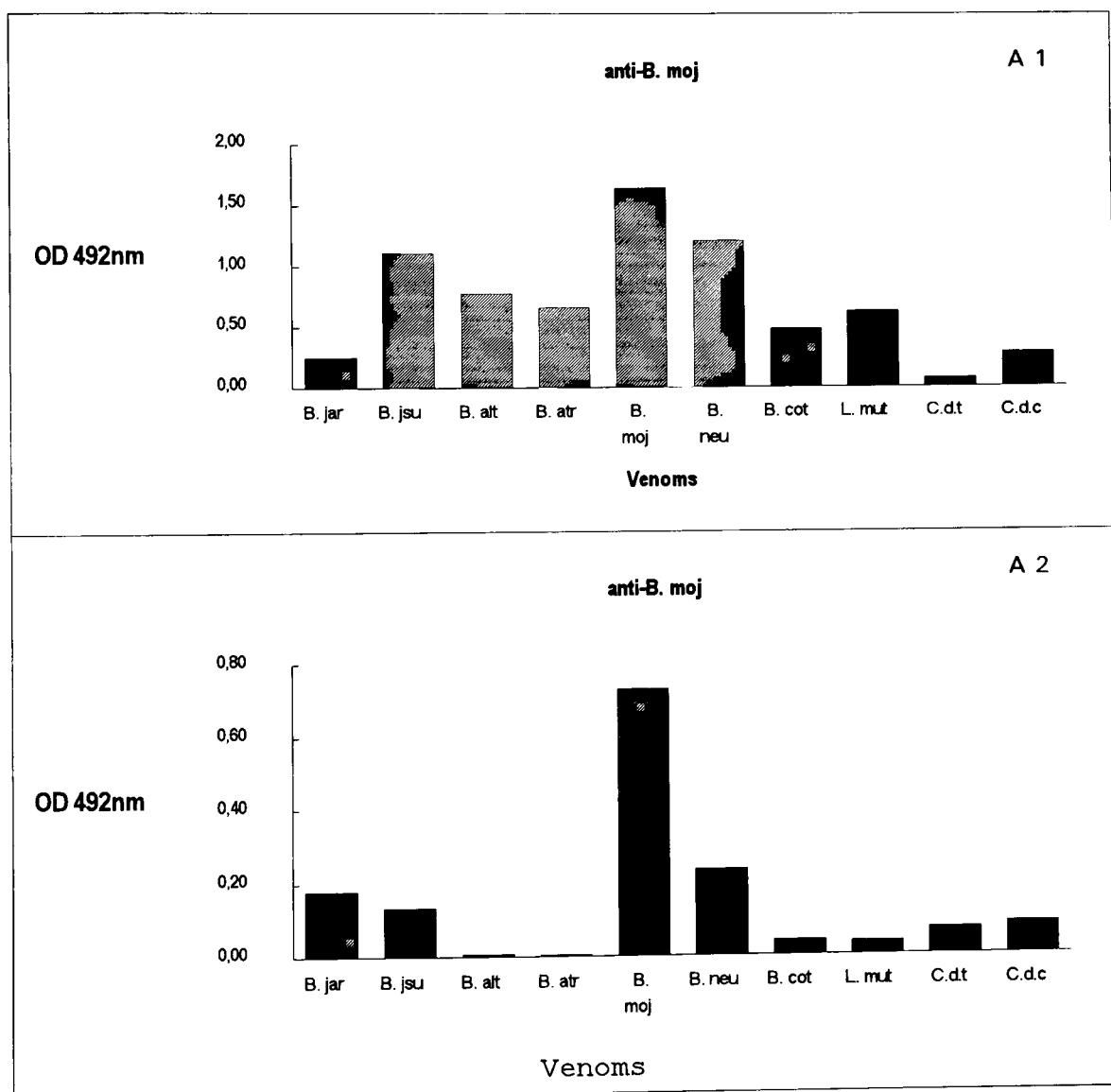


Figure 3.24. Plate A: A1: Same as in A1, in Figure 3.23, but absorbed in anti-*B. moojeni* adsorbent. A2 Same as in A2 in Fig. 3.23 but absorbed in *B. alternatus* adsorbent 20 min, *B. neuwiedi* adsorbent 20 min and *B. jararacussu*. Venoms labelled as in Figure 3.23.

These results confirm that the absorption of monovalent antivenoms in venom affinity adsorbents increases the species-specificity of the antivenoms, and that this increase in specificity is accompanied by a decrease in the intensity of the specific reaction observed as a drop in the OD values.

3.1.9.4.2. Absorption of antivenoms in polyacrylamide venom adsorbents.

Although the absorption procedures shown above were efficient in increasing species-specificity of antivenoms they were time consuming and laborious. Another procedure was tried by preparing polyacrylamide venom adsorbents with proportional amounts of the crossreacting venoms. Based on cross-reactivity results of the antivenoms absorbed two times in the venom adsorbents containing mixtures of equal amounts of venoms, a cross-reactivity index was calculated (see Appendix IV). This index provided guidance on different venom amounts, proportional to their cross-reactivity towards a single antivenom. The venoms were then insolubilized on the polyacrylamide beads in proportional amounts to their reactivity towards the antivenom to be absorbed. The method of protein insolubilization using polyacrylamide beads, retains up to 95% of the antigens antigenicity contrary to 25% when insolubilized with glutaraldehyde only [Ternynck *et al.*, 1976]. Therefore they should work better as immunoadsorbents. Venom adsorbents were prepared in volumes of 20 ml each. With these adsorbents it was expected that for a given antivenom, after a number of absorption steps, the decrease in the cross-reactivity against the heterologous venoms present in the adsorbent would be similar for all, that is, the difference between the specific and heterologous reactions should be nearly the same for all venoms. In Figures 3.25 (A-D) and 3.26 (A, B) the results of the absorbed antivenoms after two adsorption steps in the polyacrylamide venom adsorbents are shown. In Fig 3.25 (A-D) it is observed that only anti-*B. jararacussu* (B) and anti-*B. alternatus* (C) showed any increase in specificity against some of the venoms. In (A) and (D) there was a loss of the specific reactivity, with the continuance of the cross-reactivity.

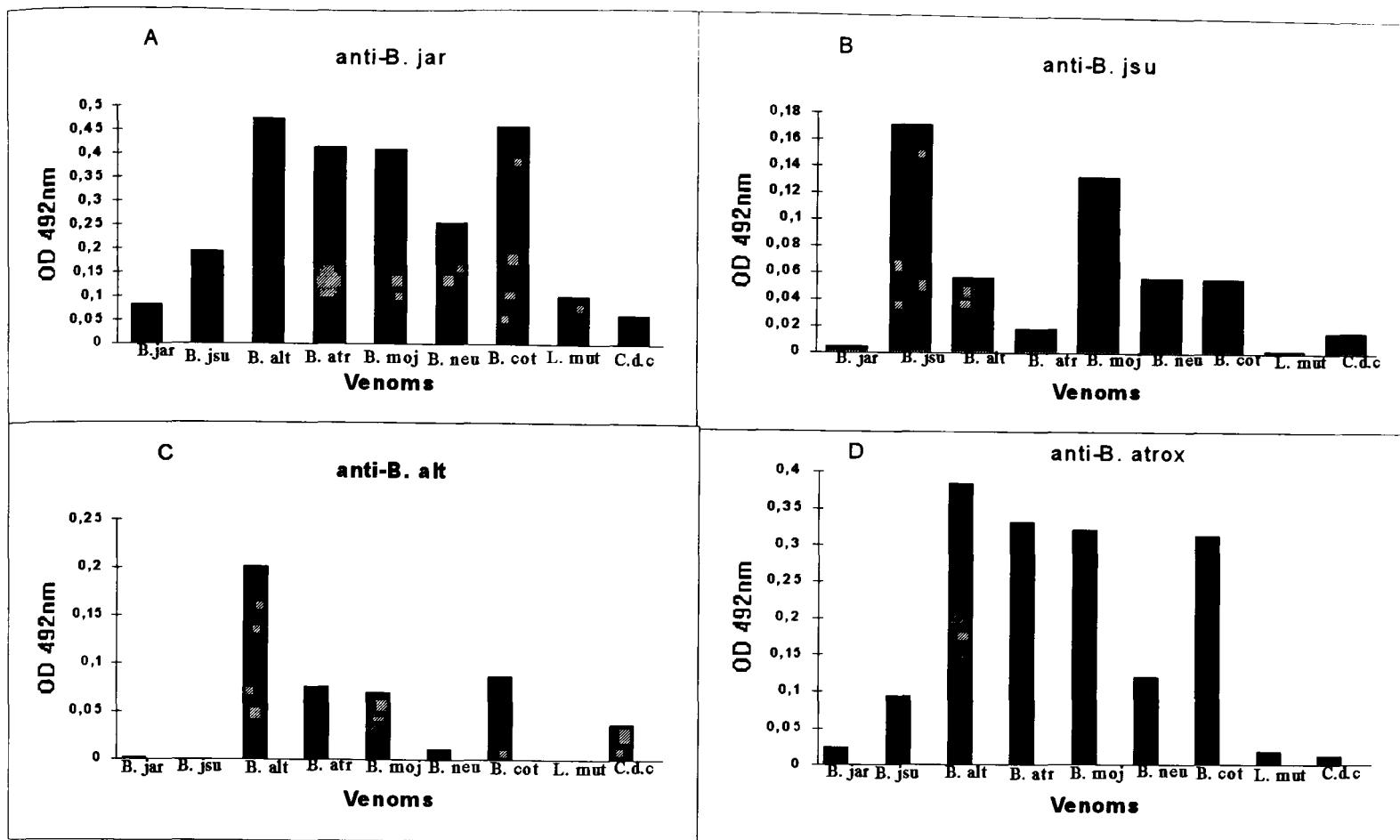


Figure 3.25 ELISA (A-D) showing the cross-reactivity of monovalent antivenoms after 2 absorption steps in a polyacrylamide gel venom adsorbent containing a mixture of heterologous venoms in proportional amounts. Antivenoms are as indicated in the charts. Venoms coating plates: 1 μ g/ml, 100 μ l/well and labelled as in Figure 3.23.

In Fig. 3.26 (A and B) again there was no marked increase in the species-specificity of any of the antivenoms tested against all of the heterologous venoms.

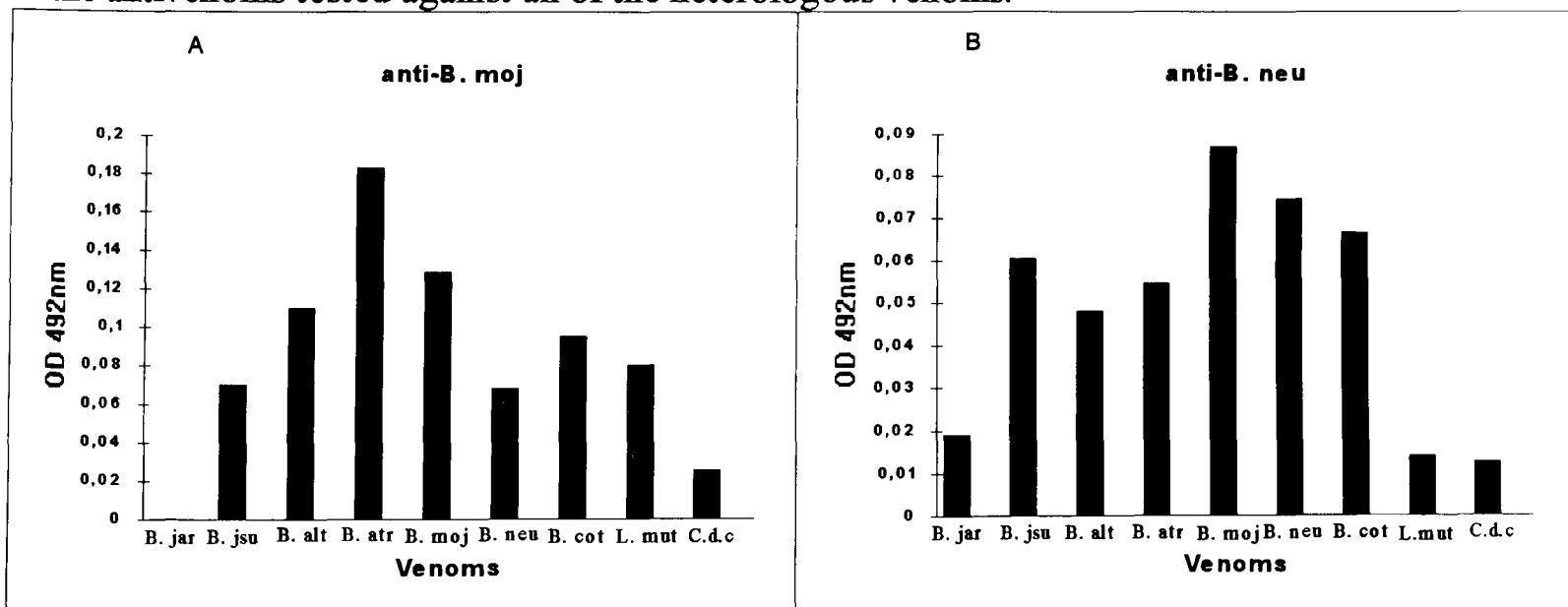


Figure 3.26 (A and B) ELISA showing the cross-reactivity of monovalent antivenoms after 2 absorption steps in a polyacrylamide gel venom adsorbent containing a mixture of heterologous venoms. Antivenoms are as indicated in the charts. Venoms coating plates: 1 μ g/ml, 100 μ l/well and labelled as in Fig. 3.23.

The results indicate that the use of polyacrylamide adsorbents did not improve the procedure for obtaining species-specific antivenoms. In fact the absorptions resulted in loss of the specific reactivity for some antivenoms, Fig. 3.25 (A).

3.1.9.4.3. Absorption of antivenoms in glutaraldehyde venom adsorbents with proportional amounts of venoms.

As the polyacrylamide venom adsorbents did not show any improvement on the absorption procedure, it was decided to prepare new venom adsorbents with the proportional amounts of venoms, but this time using only glutaraldehyde as the insolubilization medium. The same cross-reactive index was used for selection of the amounts of venoms and the results are shown in Figures 3.27 (A-D) and Fig. 3.28 (A, C). All antivenoms were absorbed four times and it can be observed that in all cases there was a clear increase in the species-specificity. In all cases shown the specific reaction is at least two times greater than the non-specific reaction. The results showed that the absorption of antivenoms in venom adsorbents with proportionally-added venoms yielded antivenoms with species-specificity. Also in most cases the difference between the remaining cross-reactivities and the specific reaction are somewhat constant suggesting that the proportional amounts of venoms added to the adsorbents were near optimal.

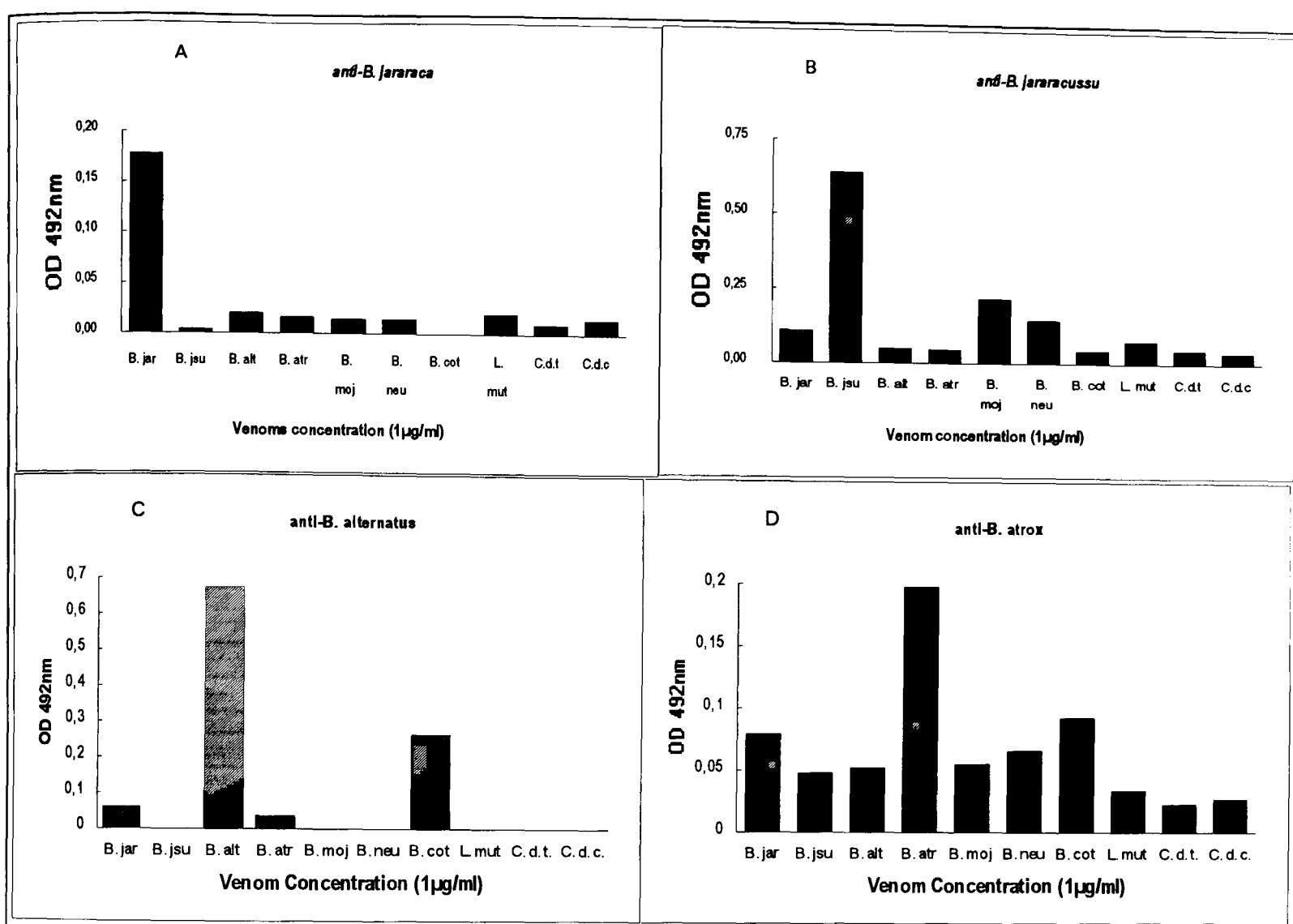


Figure 3.27 (A-D) ELISA showing the cross-reactivity of monovalent antivenoms after 4 absorptions in glutaraldehyde venom adsorbents containing proportional (optimal) amounts of heterologous venoms. A: anti-*B. jararaca* diluted, 1:100; B: anti-*B. jararacussu* diluted 1:100; C: anti-*B. alternatus* diluted 1: 100; D: anti-*B. atrox* diluted 1:100. Venom labelled as in Fig. 3.23.

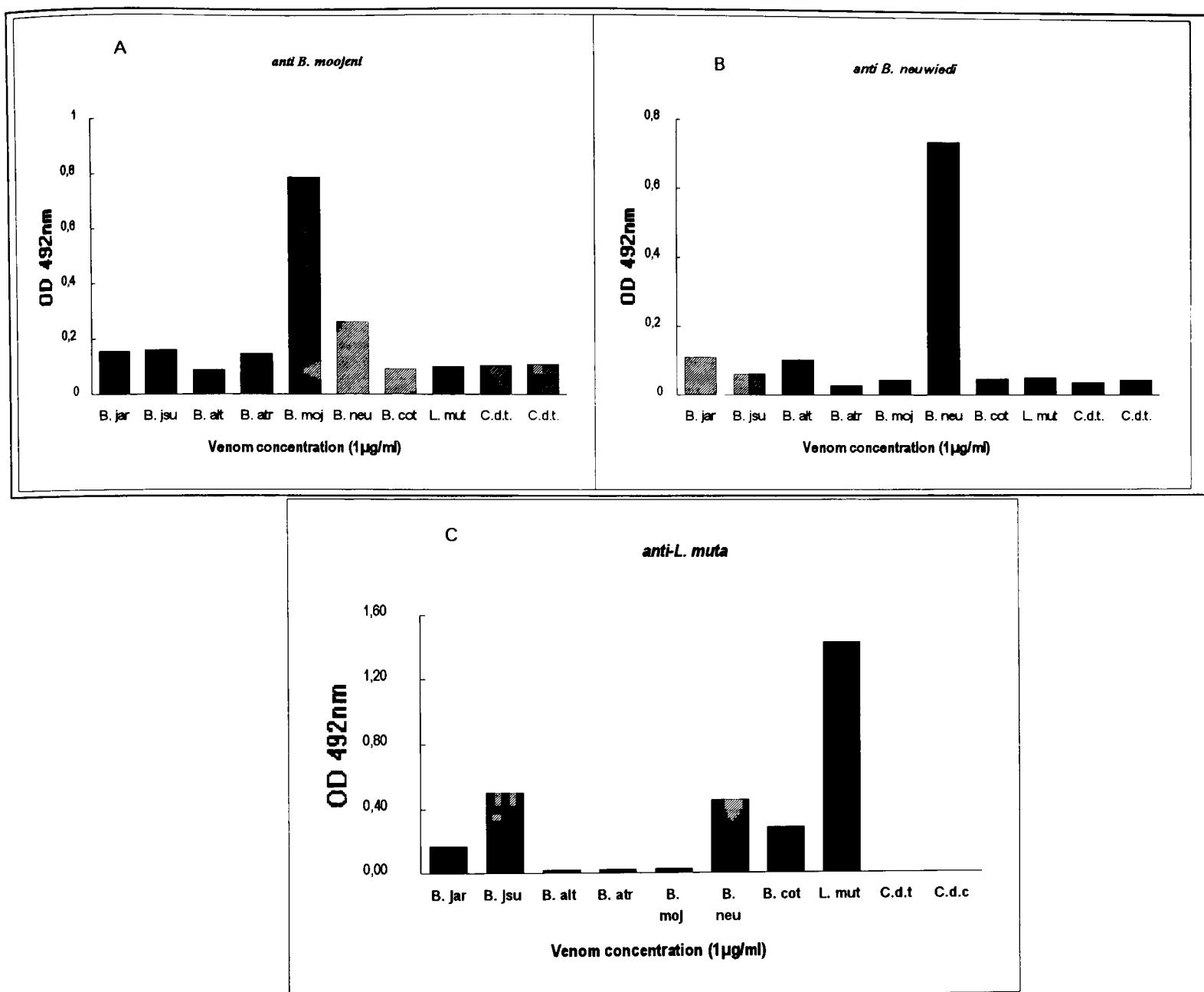


Figure 3.28 (A-C) ELISA showing the cross-reactivity of monovalent antivenoms after 4 absorptions in glutaraldehyde venom adsorbents containing proportional (optimal) amounts of heterologous venoms. **A:** anti-*B. moojeni* diluted 1:100; **B:** anti-*B. neuwiedi* diluted 1:100; **C:** anti-*L. muta*, diluted 1:400.

3.1.10.Efficiency of Antibody [^{125}I] Conjugation

The efficiency of the labelling was tested by solid-phase radioimmunoassay as described in Materials and Methods. Below is a typical result of the labelling procedure.

<u>Counts per minute</u>		
TCA precipitate	(X)	485,561.6
Supernatant	(Y)	16,156.6
Total		501,718.2

$$\% \text{ efficiency} = \frac{485561.6}{501718.2} \times 100 = 96.7$$

$\approx 97.0\%$ efficiency

The result show that the labelling was efficient as more than 95% of the radioactive ^{125}I was incorporated onto the antibody molecules in the solution. All radio-labelled conjugates prepared had labelling efficiency between 93% and 99%.

3.1.11.Production of species-specific monoclonal antibodies

The species-specific reagents produced by affinity absorption were used to precipitate in the homologous venom the component(s) responsible for the specific reactivity. These immune complexes (IC) were then used to immunise mice in order to remove their spleen for the production of monoclonal antibodies. The absorbed antivenoms used were partly from the same batch responsible for the results in Section 3.1.9.4.1 above and also from other batches prepared in the same manner; that is, after four absorption steps and showing specific reaction at least two times greater than the non-specific reaction. For the

production of IC the absorbed antivenoms against *B. alternatus*, *B. atrox*, *B. moojeni* and *B. neuwiedi* were used due to their larger stocks. In Fig. 3.29 it can be observed that all mice antisera displayed some significant cross-reactivity. The mice immunised with the *B. atrox* venom IC had higher non-specific reactivity than specific reactivity.

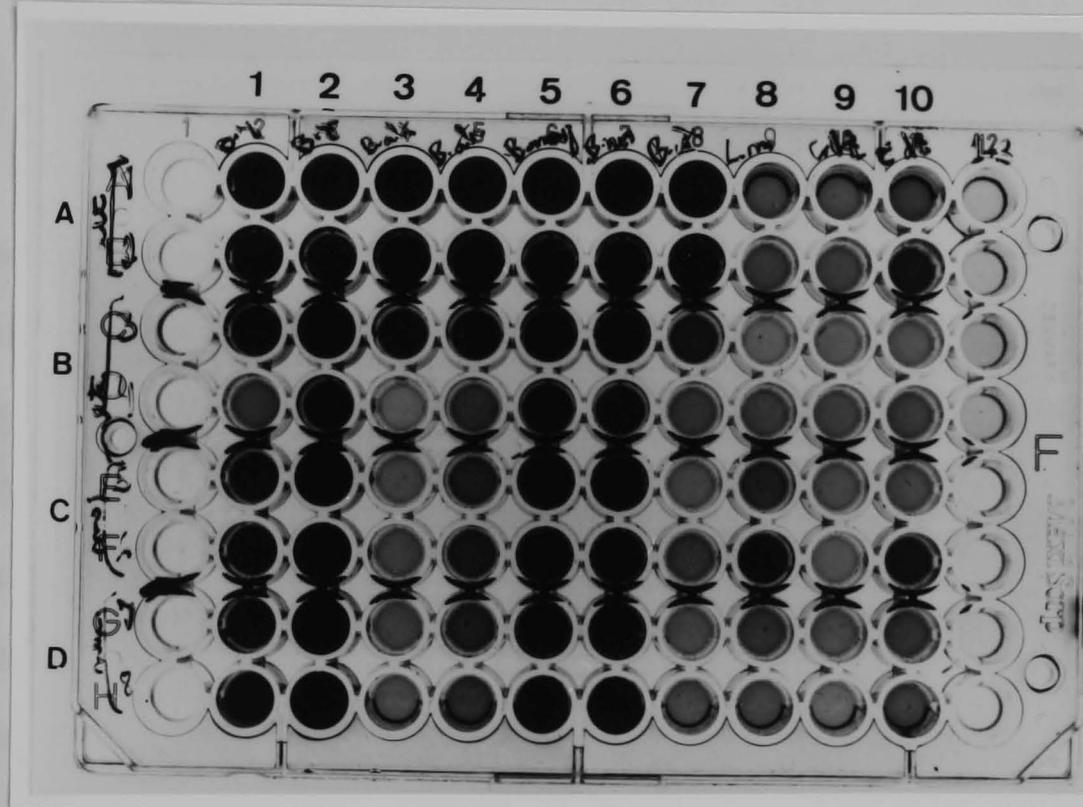


Figure 3.29 ELISA plate showing reactivity of mice serum immunised with species-specific venom immune complexes. Plates were coated with different venom solutions at 1 µg/ml, 100 µl/well. Mice serum at 1:1,000 dilution was added to wells containing the homologous or heterologous venom. Sheep anti-mouse IgG conjugated to horse-radish peroxidase was used as conjugate at 1:8,000 dilution. Venoms: 1. *B. jararaca*; 2, *B. jararacussu*; 3, *B. alternatus*; 4, *B. atrox*; 5, *B. moojeni*; 6, *B. neuwiedi*; 7, *B. cotiara*; 8, *L. muta*; 9, *Crotalus durissus terrificus*; 10, *Crotalus d. collineatus*. Antivenoms: **A**, anti- *B. alternatus*; **B**, anti-*B. atrox*; **C**, anti-*B. moojeni*; **D**, anti-*B. neuwiedi*; **C**, Control: venom + conjugate.

The mice immunised with the IC of the *B. moojeni* venom was killed and its spleen removed for fusion. Of the 256 clones obtained 176 displayed reactivity towards the specific *B. moojeni* venom in solid phase radioimmunoassay (Appendix VI). The high responders were

cultured further and later tested for species-specificity by solid phase radioimmunoassay. Some clones were used for the production of ascitic fluid in mice and the ascitic fluid tested for specificity. None of the clone supernatants or ascitic fluids tested had any antivenom reactivity (some results are shown in the Appendix VI) and were discarded. The results show that although clones were obtained with some anti-*B. moojeni* activity, none maintained this antivenom activity.

3.1.12. Production of species-specific antivenom using polyacrylamide gel slices containing antigens as immunising agent.

This was another attempt to produce species-specific antivenoms in large volumes to allow the preparation of assays for the specific identification of venoms in fluids. To identify the venom components responsible for species-specificity Western blots were developed with a single absorbed antivenom. For this a single absorbed antivenom was reacted in immunoblot assay with its homologous and heterologous venoms. Fig. 3.30 (A-C) shows the immunostaining patterns obtained with the 2X absorbed antivenoms of *B. jararacussu*, (A); *B. alternatus*, (B) and *B. moojeni* (C) and the numbered bands, specifically stained by them, that were chosen for the immunisation procedure. The absorbed antivenoms for *B. jararacussu*, Fig. 3.30 (A), and for *B. alternatus*, Fig. 3.30 (B) stained only bands in their homologous venoms. In Fig. 3.30 (C) the absorbed anti-*B. moojeni* stained two homologous bands and bands on the heterologous venom of *B. neuwiedi*. Before immunising animals with the bands stained by the homologous absorbed antivenoms, they were eluted from polyacrylamide gels and tested for cross-reactivity by ELISA, against the heterologous Bothropic antivenoms. For this, 400 µg of each venom in 40 µl of sample buffer was applied to a blank comb and separated in a non-SDS/PAGE, 7.5% gel. The gel strips to be cut out were identified by their position in relation to the molecular weights, as measure in the immunoblot assays. The eluted material for each venom was obtained from 4 gels and after lyophilisation it was resuspended in a final volume of 500 µl of saline. The

amount of protein was measured by the absorbance at A_{280nm}. One unit of absorbance = 1 mg and the results are shown below:

Venom	Venom bands	Protein content (μg)
<i>B. jararacussu</i>	1	126.5
	2	125.4
<i>B. alternatus</i>	1	130.9
	2	123.8
	3	136.4
<i>B. moojeni</i>	1	132.0
	2	121.1

Venom bands were diluted in coating buffer to make up 1.0 ml and ELISA plates were coated with 2 μg/well of each. In Fig. 3.31 (A-C) the results of the crossreactive assays are shown. All eluted venom bands exhibited a cross-reaction against the heterologous antivenoms tested. In Fig. 3.31 (A) the band n°.1 from *B. jararacussu* venom exhibited a higher reactivity against the absorbed homologous antivenom compared to the reactivity against the non-absorbed homologous and heterologous antivenoms. *B. jararacussu* venom band n°.2 Fig. 3.31 (A) displayed similar results except against the non-absorbed anti-*B. alternatus* antivenom.

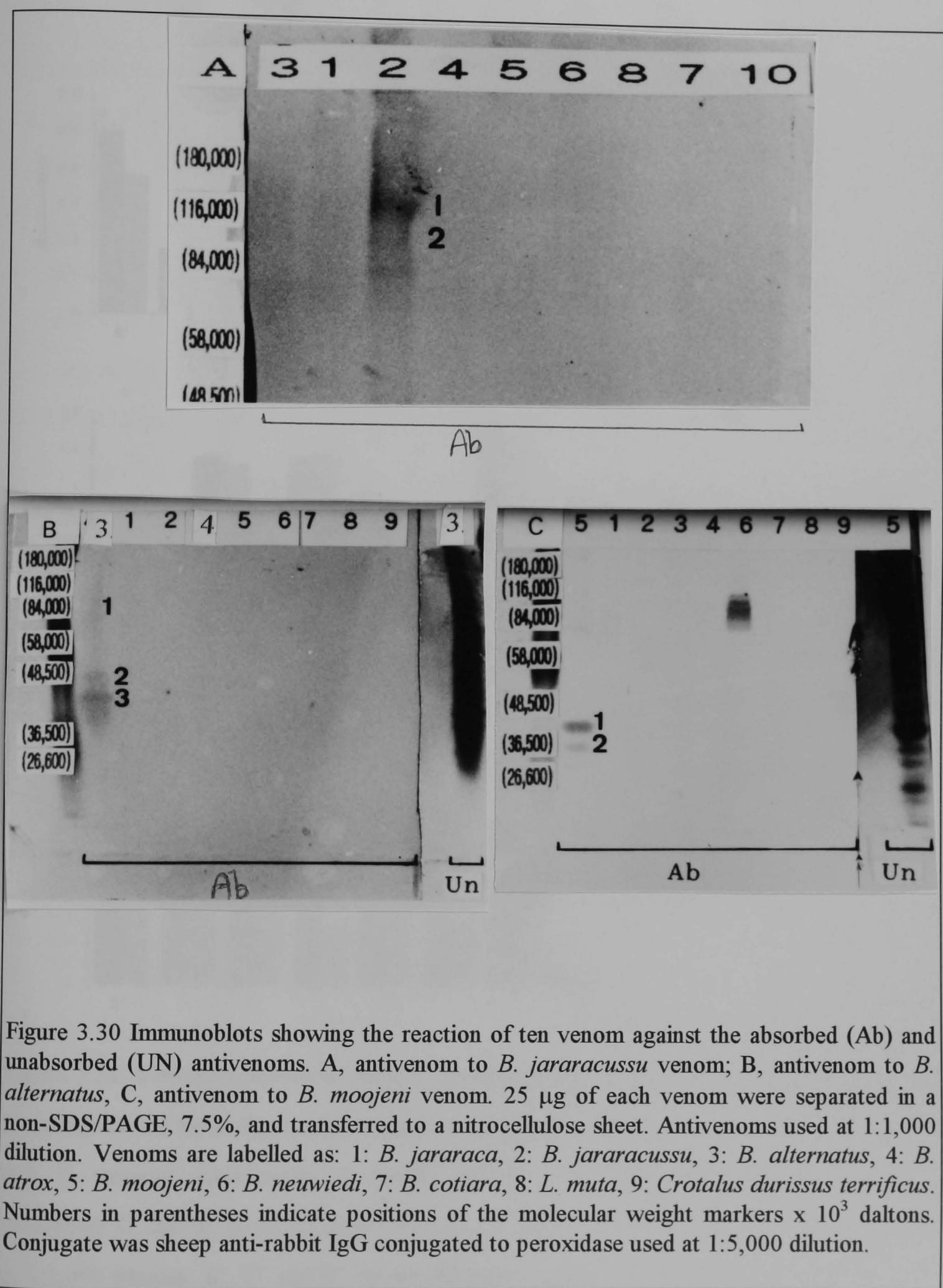


Figure 3.30 Immunoblots showing the reaction of ten venom against the absorbed (Ab) and unabsorbed (UN) antivenoms. A, antivenom to *B. jararacussu* venom; B, antivenom to *B. alternatus*, C, antivenom to *B. moojeni* venom. 25 µg of each venom were separated in a non-SDS/PAGE, 7.5%, and transferred to a nitrocellulose sheet. Antivenoms used at 1:1,000 dilution. Venoms are labelled as: 1: *B. jararaca*, 2: *B. jararacussu*, 3: *B. alternatus*, 4: *B. atrox*, 5: *B. moojeni*, 6: *B. neuwiedi*, 7: *B. cotiara*, 8: *L. muta*, 9: *Crotalus durissus terrificus*. Numbers in parentheses indicate positions of the molecular weight markers $\times 10^3$ daltons. Conjugate was sheep anti-rabbit IgG conjugated to peroxidase used at 1:5,000 dilution.

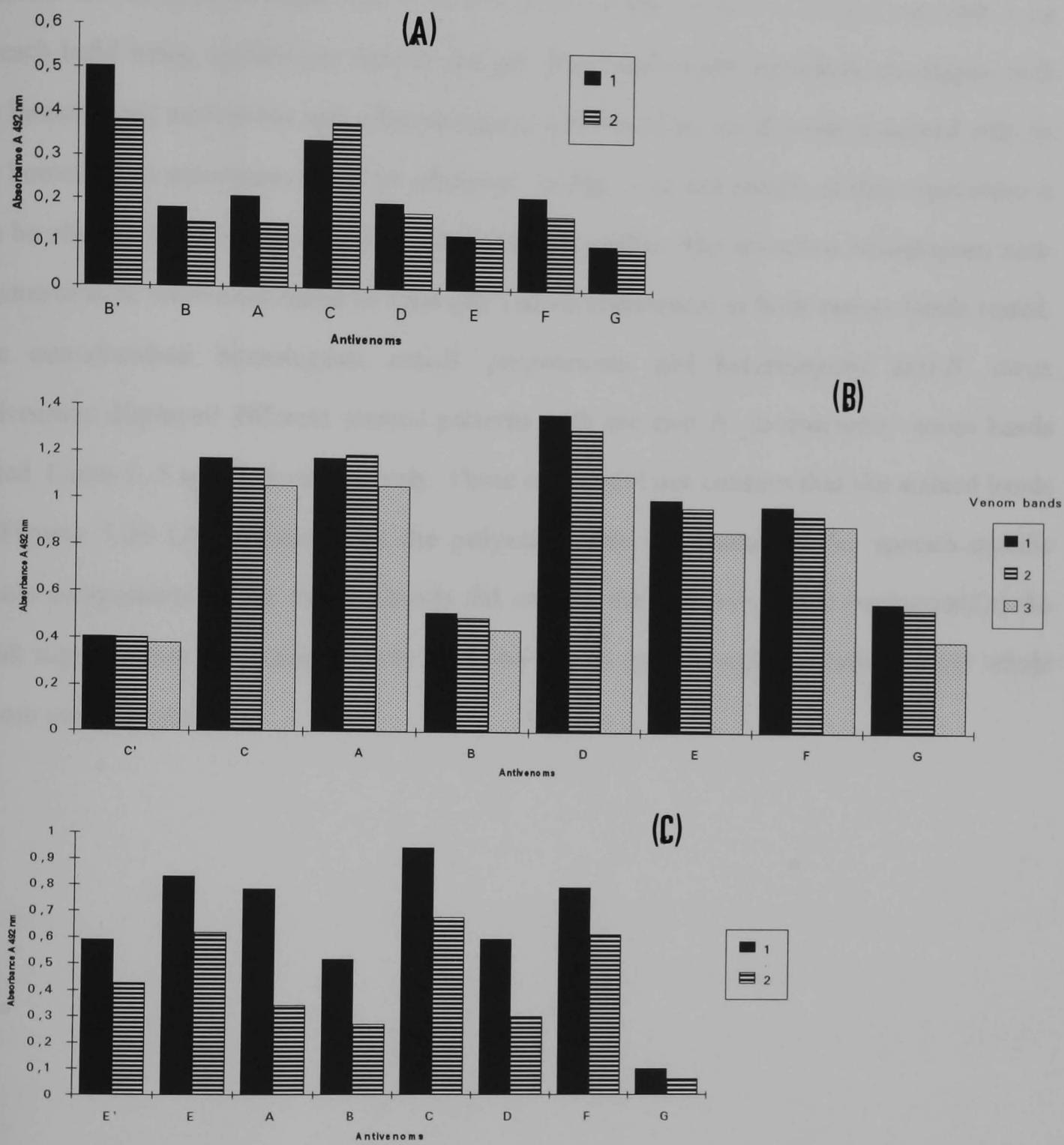


Figure 3.31. Venom components eluted from polyacrylamide gels were reacted against their homologous and heterologous antivenoms. Venoms: (A); *B. jararacussu*; (B), *B. alternatus*; (C), *B. moojeni*. Antivenoms. A, anti-*B. jararaca*; B, anti-*B. jararacussu*, C, anti-*B. alternatus*; D, anti-*B. atrox*; E, anti-*B. moojeni*; F, anti-*B. neuwiedi*; G, anti-*B. cotiara* used at 1:1,000 dilution. B', C' and E' are the absorbed antivenoms to venoms (A), (B) and (C) respectively. (B') was used at 1:100 dil., and (C' and E') at 1:250. Venom bands are labelled as in (B).

Western blot analysis of these two *B. jararacussu* venom bands was carried out with 1 µg of each band being applied per lane in the gel. The bands were separately developed with the homologous antivenom and a heterologous antivenom to see if band(s) stained only by the homologous antivenom could be observed. In Fig. 3.32 the results of this experiment it can be observed and no specifically stained band is visible. The absorbed homologous anti-*B. jararacussu* antivenom failed to stain any venom component in both venom bands tested. The non-absorbed homologous anti-*B. jararacussu* and heterologous anti-*B. atrox* antivenoms displayed different stained patterns with the two *B. jararacussu* venom bands tested. Lanes 2, 5 and 3, 6 respectively. These results did not confirm that the stained bands of Figures 3.30 (A) excised from the polyacrylamide gel contained the species-specific venom components. If the excised bands did contain the species-specific component(s) the result suggests that species-specificity could be due to specific epitopes only and not whole venom components.

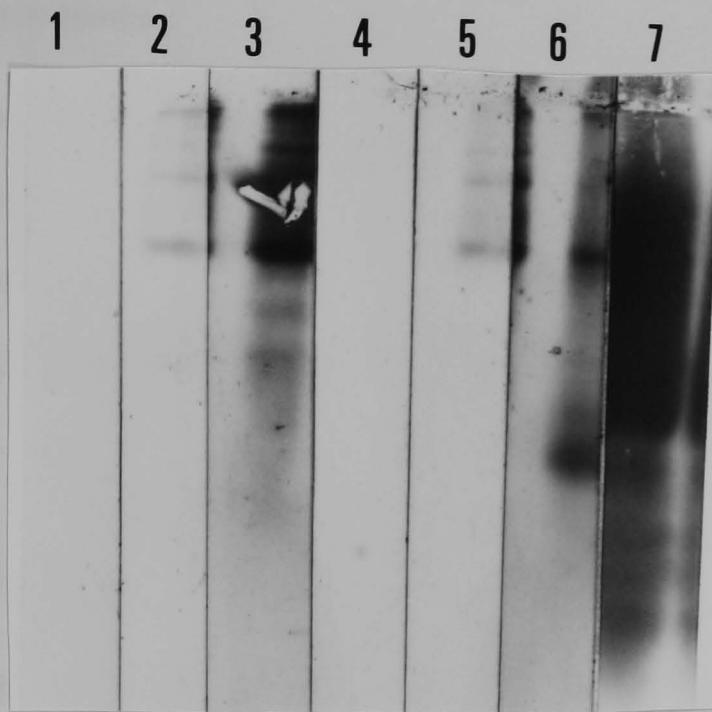


Figure 3.32 Immunoblot analysis of *B. jararacussu* venom components eluted from a non-SDS 7.5% polyacrylamide gel. Venom bands stained by the absorbed antivenom, in Figure 3.30, (A) were eluted and tested for venom specificity. Lanes 1, 2, 3: Band 1 as in Figure 3.30 (A) and 4, 5, 6 Band 2. Antivenoms: lanes 1 and 4, 2X absorbed anti-*B. jararacussu*, 1:100 dilution; 2 and 5, un-absorbed anti-*B. jararacussu*, 1:1,000 dil.; 3 and 6, unabsorbed anti-*B. atrox*, 1:1,000; Lane 7, *B. jararacussu* venom stained by the unabsorbed anti-*B. jararacussu*, 1:1,000 dil. Conjugate as in Fig.3.30

The failure of the absorbed anti-*B. jararacussu* to stain venom component(s) on both eluted venom bands in the Western blot analysis could indicate that: the excised bands did not contain the species-specific component(s), or that either the concentration of the specific venom component(s) was not sufficiently high or the specific epitopes were somehow denatured during the electro-elution process. Based on this set of results, showing the existence of cross-reacting venom components in the eluted bands, the immunisation of animals using the gel slices was not carried out.

3.1.13.Cross-reactivity studies with antivenoms absorbed in glutaraldehyde venom adsorbents with proportional venom amounts.

None of the approaches tried so far to isolate the species-specific venom component(s) for immunisation purposes were successful. Therefore it was decided to prepare larger volumes of the absorbed antivenoms by absorption on venom affinity adsorbents. These would be used for cross-reactivity studies and to identify specific venom components by ELISA and Western blots.

3.1.14.Purification of IgG by DEAE

The purification of the antivenom IgG from the absorbed antivenoms was carried out to allow the development of antigen capture assays using the absorbed IgGs as the capturing reagents. The protein concentration yielded for each purified IgG from the initial 5 ml of serum varied and a typical result is shown: anti-*B. jararaca*, 9.3 mg; anti-*B. jararacussu*, 10.1 mg; anti-*B. alternatus*, 7.6 mg; anti-*B. atrox*, 12.0 mg; anti-*B. moojeni*, 11.3 mg; anti-*B. neuwiedi*, 19.3 mg in final volumes of 600 µl. Fig. 3.33 shows the purity of the IgG tested by immunoelectrophoresis. The reaction with the anti-whole rabbit serum did not reveal any other serum proteins but only a reaction similar to the anti-rabbit IgG antiserum. The results show that there was a loss of IgG during the purification process as 5 ml of hyperimmunised rabbit serum contains around 50 mg of IgG, and that an adequate degree of purity was achieved for the different antivenom IgGs.

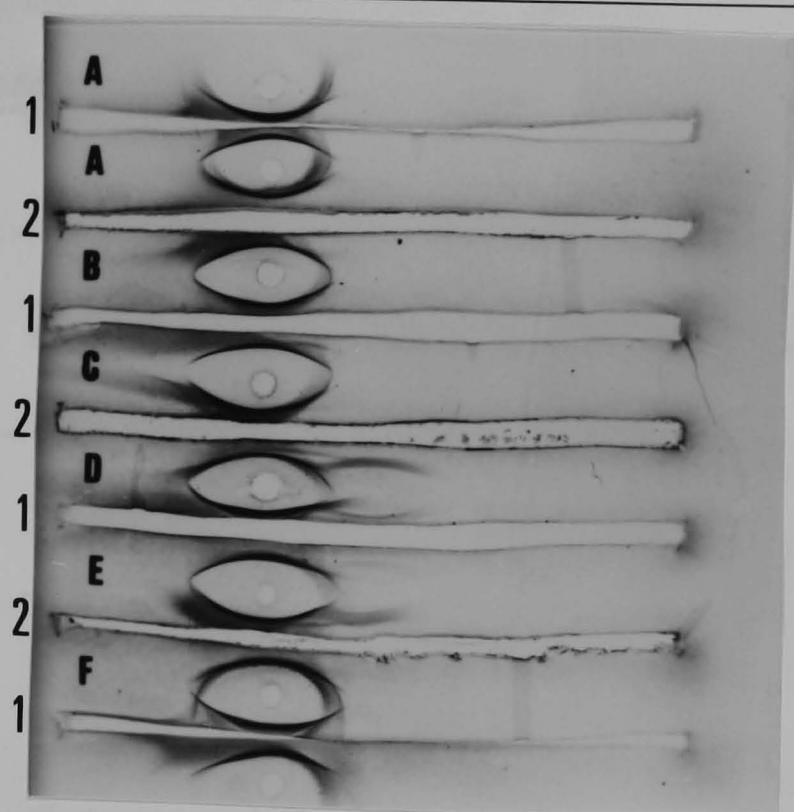


Figure 3.33. Purity of rabbit antivenom IgG isolated by DEAE column chromatography tested by immunoelectrophoresis against sheep anti-whole rabbit serum (1) and anti-rabbit IgG (2). Antivenoms are labelled as: A, anti-*B. jararaca*; B, anti-*B. alternatus*; C, anti-*B. jararacussu*; D, anti-*B. atrox*; E, anti-*B. moojeni*; F, anti-*B. newwiedi*. Antisera were applied in 70 µl. Antivenoms 4.5 µg

3.1.15. Reproducibility of the absorption procedure

To demonstrate that the absorption procedure could produce species-specific antivenom sera or antivenom IgG reliably, 2 batches of monovalent antivenom IgG were separately absorbed four times each in venom adsorbents with equal amounts of venom. They were then tested for their level of cross-reactivity. Table 3.5 shows the difference between the homologous and heterologous reactions as a percentage of the OD readings. The smallest difference is found in the *B. atrox* antivenom against the *B. alternatus* venom, (46%). Generally the minimum difference between the specific and the non-specific reaction is about two-fold.

Table 3.5. Summary of the reactivity (as percentage of OD reading) between the species-specific absorbed antivenoms and the homologous and heterologous venoms.

Antivenoms	Venoms							
	<i>B. jar</i>	<i>B. jsu</i>	<i>B. alt</i>	<i>B. atr</i>	<i>B. moj</i>	<i>B. neu</i>	<i>B. cot</i>	<i>L. mut</i>
<u>against</u>								
<i>B. jararaca</i>	100%	29 (14)	50 (5)	50 (7.5)	38 (2)	30 (2)	50 (3.5)	21 (5.8)
<i>B. jararacussu</i>	20 (5.5)	100%	12 (2)	14 (1.6)	47 (3)	30 (3)	1	18
<i>B. atrox</i>	50 (3.5)	38 (8)	54 (2)	100%	50 (7)	50 (14)	50 (20)	24 (14)
<i>B. moojeni</i>	19 (1.7)	25 (8)	9 (2)	20 (3)	100%	41 (13)	11 (1)	8 (1.8)
<i>B. neuwiedi</i>	17 (4)	10 (4)	16 (3)	3.8	6.8 (1)	100%	6	8 (2)
<i>L. muta</i>	16 (9)	30 (5)	14	1.6	1.8	30 (15)	15 (5)	100%

Homologous reaction is 100%. Numbers in brackets are standard deviation of two experiments. Venoms are: *B. jar*, *B. jararaca*; *B. jsu*, *B. jararacussu*; *B. alt*, *B. alternatus*; *B. moj*, *B. moojeni*; *B. neu*, *B. neuwiedi*; *B. cot*, *B. cotiara*; *L. mut*, *L. muta*.

The degree of specificity achieved by the antivenoms are different from each other, as some absorbed antivenom, retain higher cross-reactivity against the heterologous venoms than the others. Absorbed anti-*B. atrox* displays the smallest overall specificity and the absorbed anti-*B. neuwiedi* the highest overall species-specificity. The results indicate that the absorption of the antivenoms yielded in an reproducible manner antivenoms with species-specificity.

3.1.16.Cross-reactivity studies with the absorbed antivenoms.

As shown in Table 3.5 the absorption of monovalent antivenoms onto venom adsorbents yielded antivenoms with reproducible species-specificity. New batches of monovalent antivenom IgG were absorbed 4X each in adsorbents with proportional amounts of venom to test their suitability for use in diagnostic assays.

3.1.16.1.Indirect Antibody ELISA

ELISA plates were coated with different concentrations of the Bothropic venoms. Then, to each plate was added only one absorbed antivenom and its reactivity against the homologous and heterologous venoms compared. In Fig. 3.34 (A) it can be observed that the absorbed anti-*B. jararaca* can discriminate the homologous from the heterologous venoms. The specific reaction is three times higher than the non-specific ones. In Fig. 3.34 (B) the absorbed anti-*B. jararacussu* also discriminates the specific from the non-specific venoms and the specific reaction is also three times higher compared to most non-specific reaction except for the *B. moojeni* cross-reaction. In Fig. 3.34 (C) the absorbed anti-*B. alternatus* also shows higher reactivity towards the specific venom throughout the range of venom concentration. A higher cross-reactivity is observed only against the venom of *B. crotiara* but it is nearly constant throughout the range of venom concentration. In (D) the absorbed anti-*B. moojeni* shows similar results with specific reactivity three times higher than the non-specific ones except for the *B. neuwiedi* venom. In (E) the absorbed anti-*B. neuwiedi* antivenom shows almost complete species-specificity throughout the range of venom concentration with only a minor cross-reaction against the venom of *B. alternatus* that becomes more evident at the 10 µg/ml venom concentration. In Fig. 3.34 (F-G) separate cross-reactivity patterns are seen for the anti-*B. atrox* and anti-*L. muta*. They were tested only against each other due to their more specific geographical distribution. In (F) the absorbed anti-*B. atrox* can discriminate the specific venom from the non-specific throughout the range of venom concentrations and regardless of antivenom dilution. Similar results are

observed in (G) for the absorbed anti-*L. muta* that also specifically identifies the *L. muta* venom with no cross-reaction at all with the *B. atrox* venom.

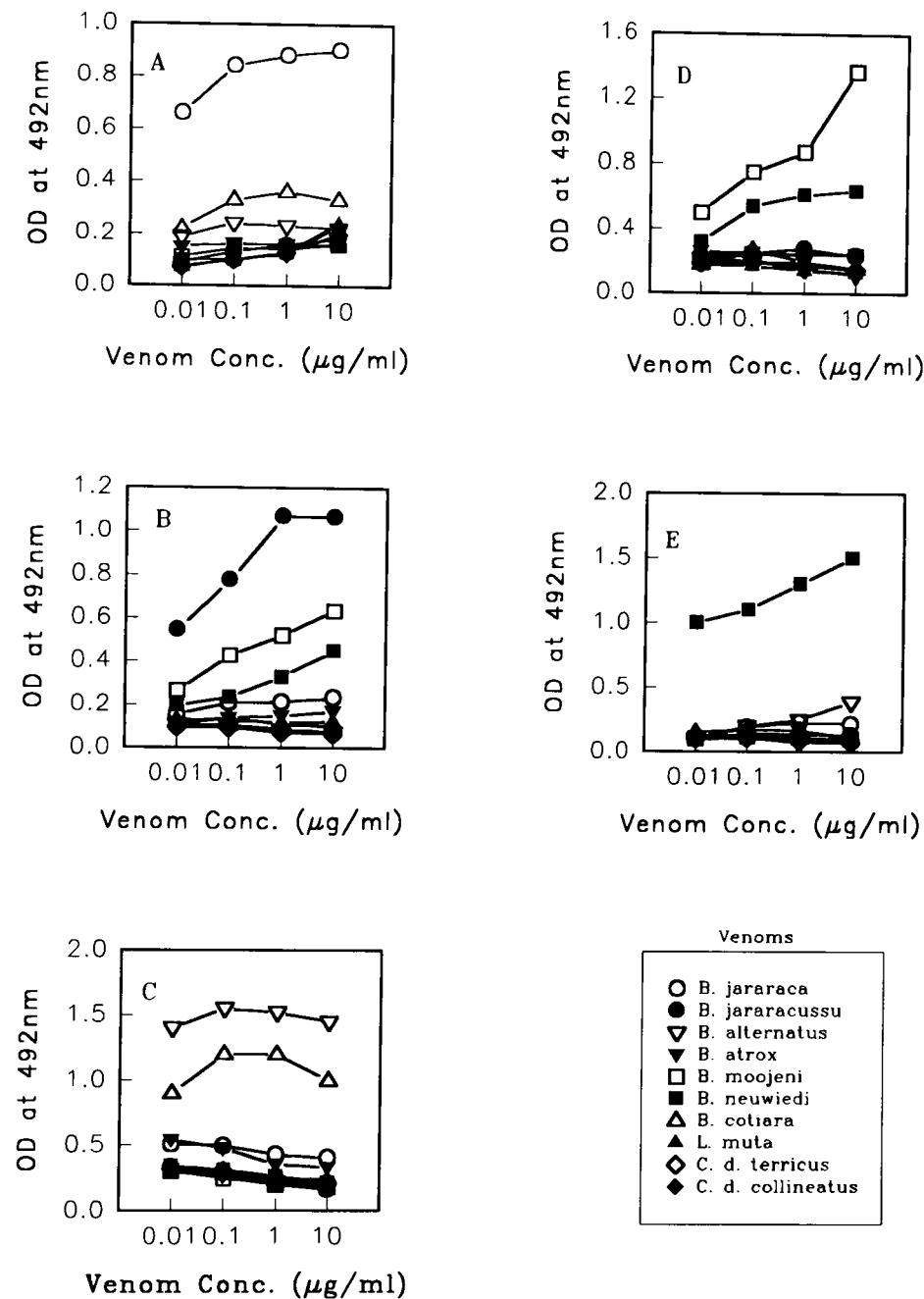


Figure 3.34 (A-E) ELISA reactivity of absorbed antivenoms against increasing concentrations of homologous and heterologous venoms. Different venom solutions were coated onto the plate in different wells. Absorbed antivenoms were separately added to wells containing the homologous or heterologous venom. **A:** anti-*B. jararaca* at 1:100 dil, **B:** anti-*B. jararacussu* at 1:100 dil, **C:** anti-*B. alternatus* at 1:100 dil, **D:** anti-*B. moojeni* at 1:200 dil, **E:** anti-*B. neuwiedi* at 1:200 dil.

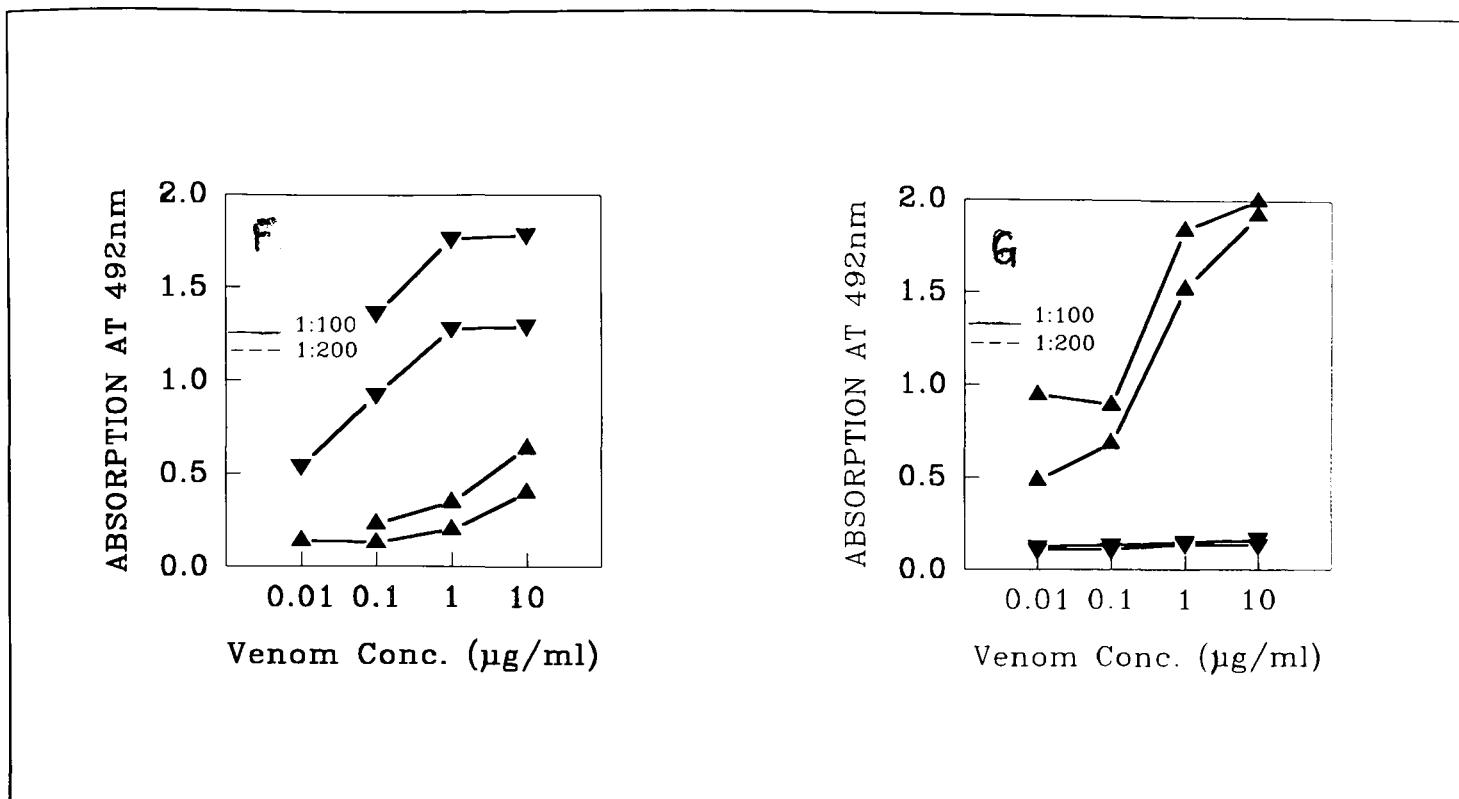


Figure 3.34 (F-G) Reactivity of absorbed anti-*B. atrox* (F) and anti-*L. muta* (G) against homologous and heterologous venoms by indirect antibody ELISA. Different venom solutions were separately added to different wells containing the homologous and heterologous venoms. F, Absorbed anti-*B. atrox* at 1:100 and 1:200 dilution. G, Absorbed anti-*L. muta* at 1:100 and 1:200 dilution. Venoms: *B. atrox* ▼; *L. muta* ▲.

These results together indicate that the absorption of monovalent antivenoms increases the species-specificity and they were then able to discriminate between the specific and non-specific venom independently of venom concentration. The results of Fig. 3.34 (F, G) also indicate that the species-specificity of the absorbed antivenom is independent of antivenom dilution, confirming the previous results in Fig. 3.20.

3.1.16.2. Antigen capture ELISA

These experiments were carried out to see if the increased specificity towards the homologous venom observed in the indirect antibody ELISA could be maintained in this type of assay. The same set of absorbed antivenoms IgG used for the indirect antibody assay of Section 3.1.16.1 was used here to coat ELISA plates at 40 $\mu\text{g}/\text{ml}$. The absorbed

antivenoms against the *B. atrox* and *L. muta* venoms were not used. The conjugates used were separately prepared for each snake venom species and were monovalent non-absorbed rabbit antivenom IgG fractions conjugated to alkaline phosphatase. These were supplied by Dr. RDG Theakston from the Venom Unit at the Liverpool School of Tropical Medicine, UK. In Fig. 3.35 (A-E) it can be observed that in all cases there is an increased species-specific detection of the homologous venom.

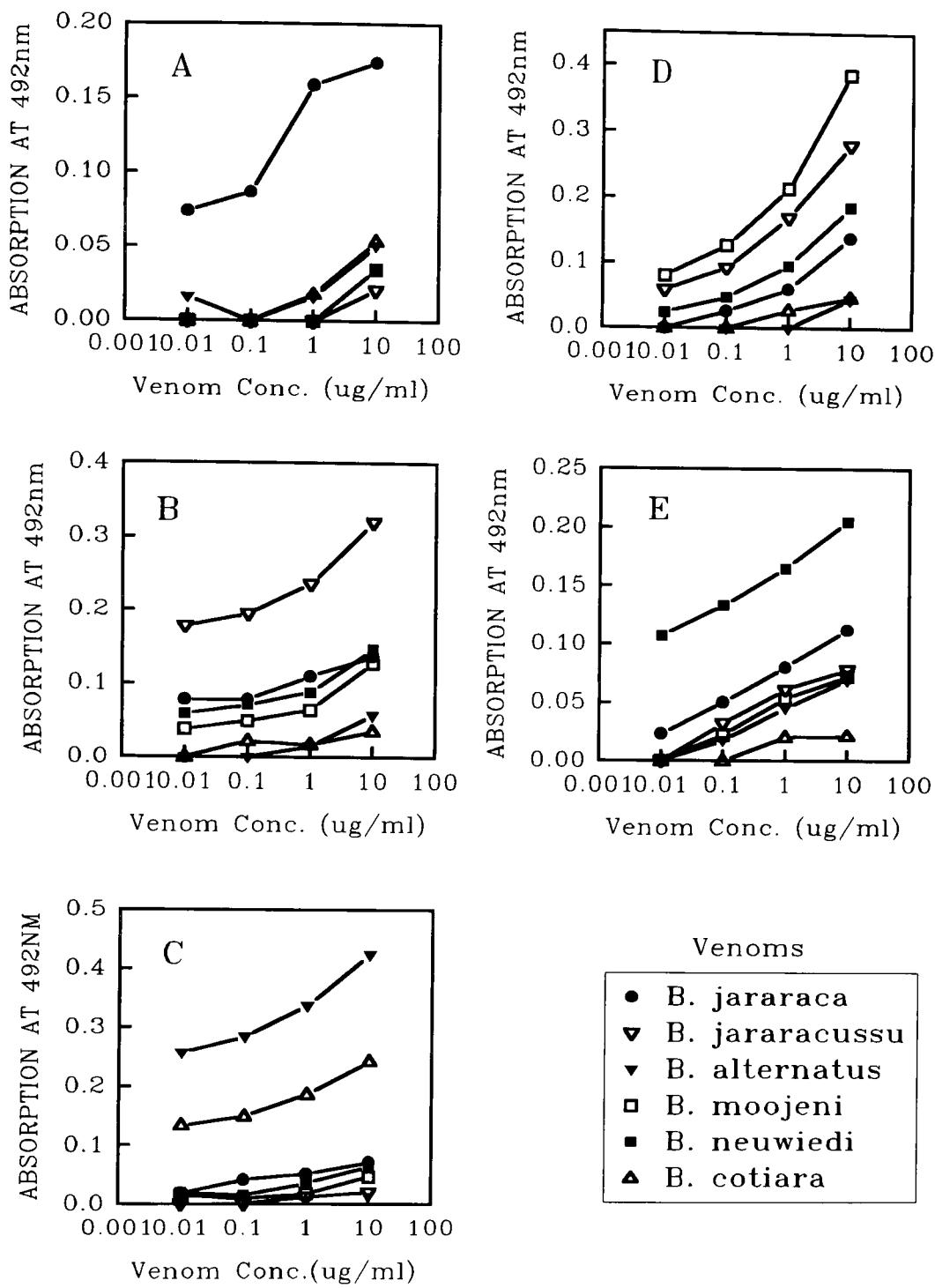


Figure 3.35 (A-E) Antigen-capture assay (ELISA) for homologous and heterologous venoms. Absorbed antivenom IgGs were coated onto plates at 40 $\mu\text{g}/\text{ml}$. Different venom solutions were added to different wells containing the homologous or heterologous antivenom IgG. Unabsorbed monovalent rabbit antivenom IgG, conjugated to alkaline phosphatase was used as conjugate at 1:500. Antivenoms: **A:** anti-*B. jararaca*, **B:** anti-*B. jararacussu*, **C:** anti-*B. alternatus*, **D:** anti-*B. moojeni*, **E:** anti-*B. neuwiedi*.

These results are similar to those obtained in the indirect antibody ELISA and shown in Fig. 3.34 (A-E). The cross-reactivities observed in Fig. 3.35 (A-E) maintain a nearly constant

ratio to the specific reaction throughout the range of venom concentrations. It is clear that a degree of species-specificity was achieved and that this was not dependent on venom concentration. It suggests the existence of species-specific venom components or epitopes that would be responsible for the species-specificity. The conjugate was a monovalent non-absorbed IgG, which indicates that the absorbed antivenoms were responsible for the species-specificity and these were capturing mainly, venom components containing species-specific epitopes. The results demonstrate the potential of these absorbed reagents as tools in the specific detection of venoms for the diagnosis of snake bites.

In order to increase the sensitivity of the assay, a variation of it was tested where only homologous reactions were assayed. The plates were coated with a 0.5% (v/v) solution of a commercial preparation of a polyvalent antibothropic horse F(ab')₂ (Butantan Institute, Material and Methods). A range of venom concentrations (0.001 to 10 µg/ml) was added as a second layer. The same set of absorbed rabbit antivenom IgGs used in the previous antigen capture assays of this section was added at two different dilutions. The results show (Fig. 3.36 (A-D)) that in all cases it was possible to easily detect venom concentrations of 0.001 µg /ml at both antivenom dilutions used. At the antivenom dilution of 1:30, even lower venom concentrations could probably have been detected, judging by the absorbance values observed.

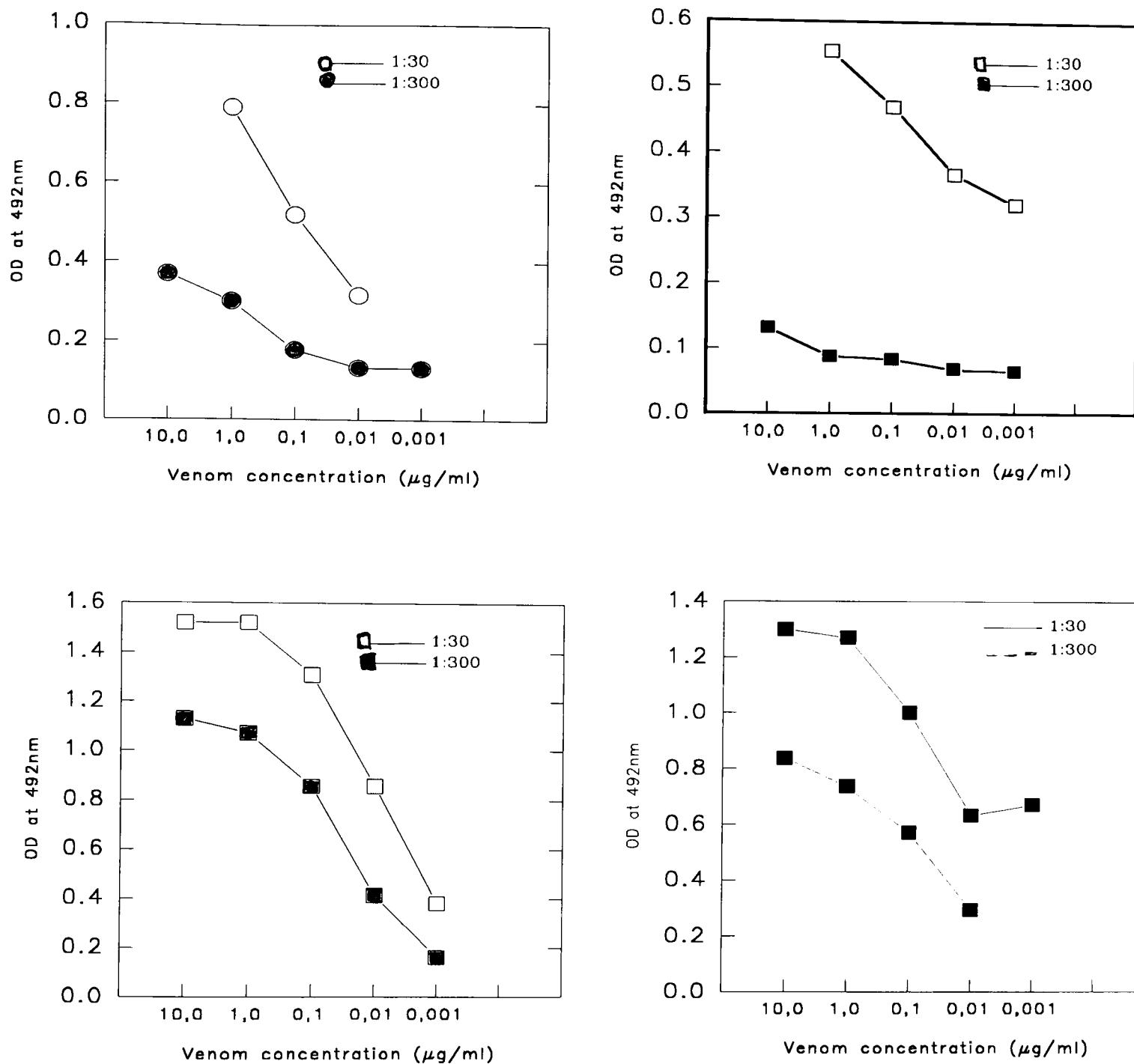


Figure 3.36 (A-D) Antigen-capture assay (ELISA) of homologous venom. Plates were coated with 5% (v/v) of polyvalent horse anticoagulant F(ab')₂. Venoms were added in different concentrations to wells containing the antibody. Absorbed rabbit antivenoms were added to wells containing their homologous venoms. Sheep anti-rabbit IgG conjugated to horse-radish peroxidase was used as conjugate at 1:20,000 dil. **A:** anti-*B. jararaca*, **B:** anti-*B. jararacussu*, **C:** anti-*B. moojeni*, **D:** anti-*B. neuwiedi*.

3.1.17. Specific detection of venom antigens in normal human serum

During the work, snakebite patients sera was not available to be assayed for the specific detection of venom. An alternative was to add venom to normal human serum to mimic a snake bite situation and then to assay the serum by antigen capture ELISA. The same set of absorbed antivenom IgGs used in the results of Fig. 3.34 was used here to coat the ELISA plates at the same 40 µg/ml concentration. Again the conjugate was the monovalent rabbit antivenom conjugated to alkaline phosphatase from the same batch used above. Fig. 3.37 shows the homologous detection of Bothropic venoms down to the concentration of 0.01 µg/ml for all of the specificities tested.

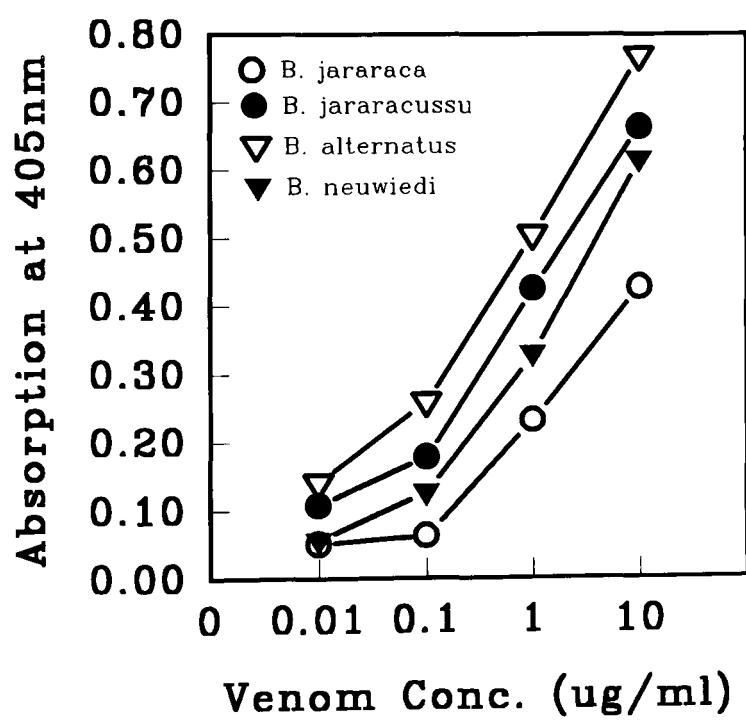


Figure 3.37 Antigen-capture assay (ELISA) showing the homologous detection of venoms added to normal human serum diluted at 1:50. Absorbed antivenom IgG samples were coated onto the plates at 40 µg/ml. Different venom concentration (0.01 to 10 µg/ml) were added to different wells containing the homologous absorbed antivenom IgG. Unabsorbed antivenom rabbit IgG conjugated to alkaline phosphatase was used as conjugate at 1:50 dilution. Results are the means of three experiments.

To test the specificity of this assay, plates were similarly coated with the same set of absorbed IgGs. Plates were coated with antivenom IgGs from only one specificity and a single concentration of the homologous and heterologous venoms added to the plates. The same batch of alkaline phosphatase conjugates were added to the plates containing the coating IgG with the same specificity. In Fig. 3.38 (A-D) it can be observed that the *B. jararaca* venom (A) could be specifically detected in human serum. The same was observed for the venoms of *B. jararacussu*, (B); *B. alternatus* (C) and *B. neuwiedi*, (D). The venom of *B. alternatus* (C) displayed cross-reactivity only with the venom of *B. cotiara* of about 30%. The venom of *B. neuwiedi* also displayed a more significant cross-reactivity against the venom of *B. jararaca*, up to about 30%. The other venoms exhibit lower levels of cross-reactivity against all of the heterologous venoms. The venoms tested could be species-specifically detected in normal human serum suggesting the possibility of using these absorbed reagents for diagnostic assays.

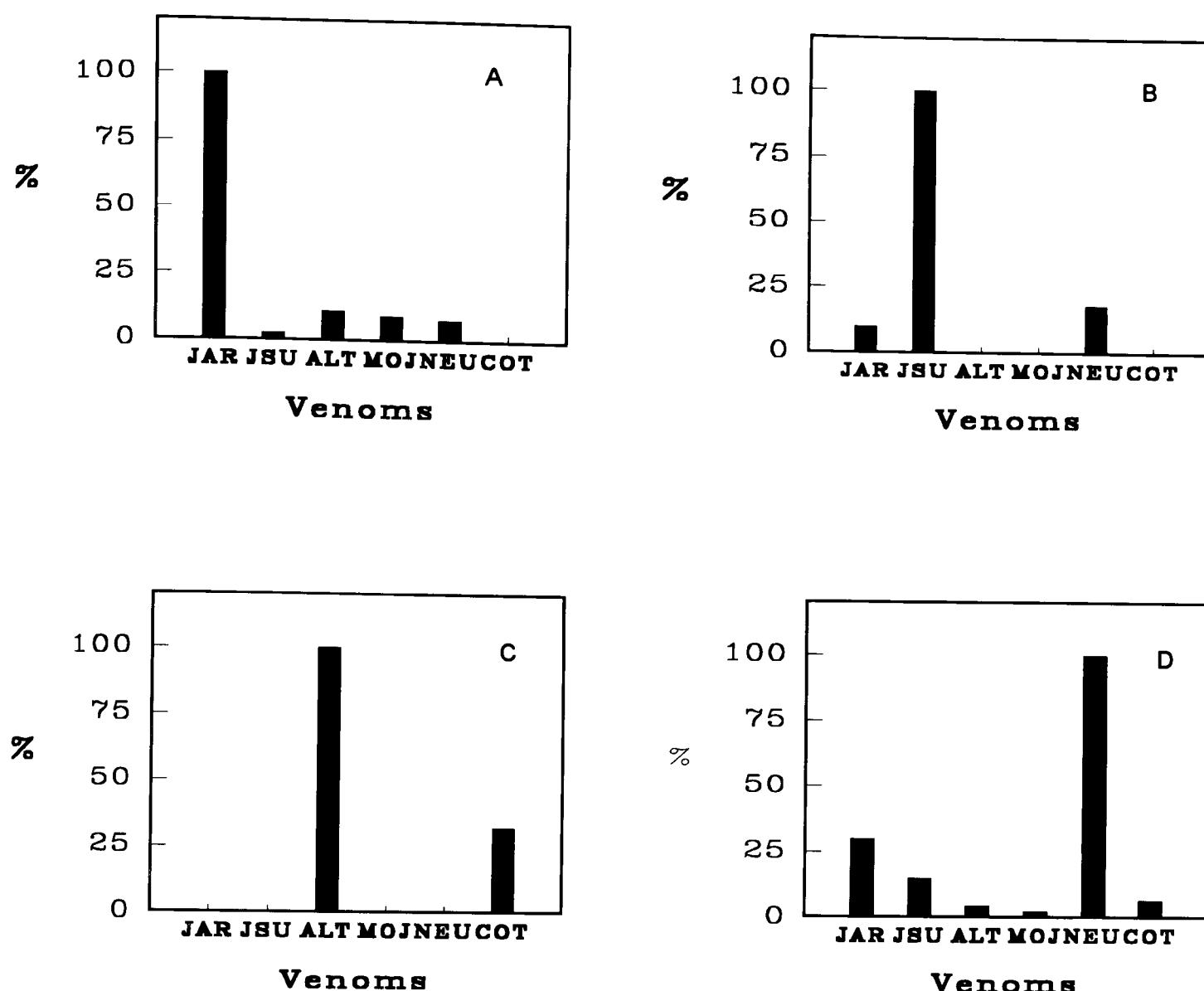


Figure 3.38 (A-D) Species-specific venom detection (ELISA). Different venom solutions at $1\mu\text{g/ml}$ in normal human serum diluted 1:50 were added to plates coated with a single absorbed antivenom IgG at $40\ \mu\text{g/ml}$. Un-absorbed antivenom IgG conjugated to alkaline phosphatase was used as conjugate at 1:100 dilution. Antivenom: A: anti-*B. jararaca*, B: anti-*B. jararacussu*, C: anti-*B. moojeni*, D: anti-*B. neuwiedi*. Results are means of two experiments.

3.2. Immunoblot analyses of venom species-specific components

It was shown previously in Section 3.1.12 that the absorbed antivenoms stained specific venom components on the homologous venoms in Western blot assays. This was shown for the *B. jararacussu*, *B. alternatus* and *B. moojeni* venoms. Here this analysis is extended to other venoms that were electrophoresed in non-SDS gels and SDS gels prior to the transfer to the nitro-cellulose sheets. Pools of venoms and venoms from individual snakes from

different geographic locations were used in order to verify if the specifically stained components were truly a species trait, or not.

3.2.1. Western blot analysis of Brazilian venoms separated by non-SDS and SDS/PAGE

The objective of these assays was to visualise specific stained bands by the absorbed antivenoms in the homologous venoms. The non-SDS gels were used to avoid possible denaturation of any venom epitope(s) responsible for species-specificity. It was shown before (Section 3.1.12) that there was no loss of reactivity in the non-SDS conditions for three venoms tested. Nonetheless the non-SDS assays were carried out before the SDS assays for all venoms. In Fig. 3.39 (A) the absorbed anti-*B. jararaca* antivenom was reacted to the homologous and heterologous venoms separated in a non-SDS gel. Two stained bands were observed in the homologous venom stained by the 3X absorbed anti-*B. jararaca* antivenom and there was no staining of any heterologous venom component. Plate (B) is a similar experiment to (A) but the venoms were electrophoresed in a SDS gel and the nitro-cellulose was immunostained with the 4X absorbed anti-*B. jararaca* IgG from the same batch used in Fig. 3.34. Two strongly stained bands can be seen in the homologous venom with approximate molecular masses of 60 kDa and 22 kDa with no corresponding stained bands in the heterologous venom. Other bands stained in the homologous venom show corresponding stained bands with same molecular mass in some heterologous venoms. Analysing the results of (A) and (B) it can be concluded that one of the specifically stained venom components could be a small basic protein because in the non-SDS gel both bands migrated to the upper part of the gel, characteristic of proteins with a basic isoelectric point and in the SDS gel one of them shows a small molecular mass of about 18 kDa. The heterologous stained bands observed in (B) could be explained by an incomplete degree of species-specificity achieved by the 4X absorbed antivenom IgG or by SDS conditions exposing some epitopes with lower affinity that react with the still present cross-reacting antibodies in the absorbed antivenom IgG. These results strongly suggest the existence of species-specific component(s) or epitopes in the venom of *B. jararaca*.

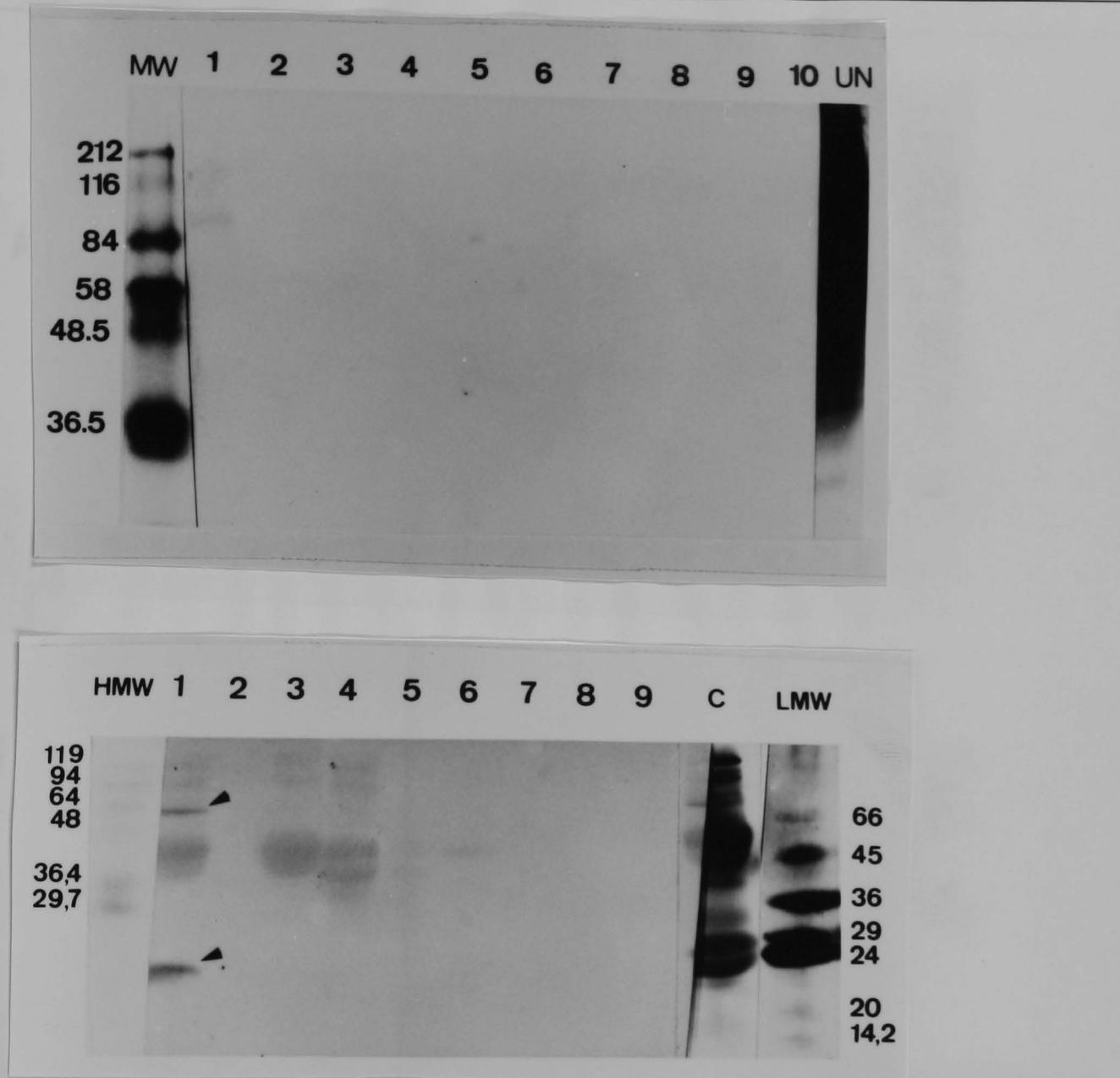


Figure 3.39 Immunoblot analysis showing species-specific components of *Bothrops jararaca* venom. A: Venoms were separated in a non-SDS PAGE 7.5% gel and transferred to a nitrocellulose membrane. Absorbed antivenom was added to NC lanes numbered from 1 to 10 at 1:800 dil. Unabsorbed anti-*B. jararaca* was added to the homologous venom at 1:2,000 as control (UN). MW: Molecular weight standards. B: Venoms were separated in a SDS/PAGE 10% gel and transferred to NC. Absorbed antivenom IgG was added to NC lanes numbered 1 to 9 at 1:800 dil.. Un-absorbed anti-*B. jararaca* was added to *B. jararaca* venom at 1:2,000 dil. as control (C). Venoms in A and B are labelled as: 1: *B. jararaca*; 2: *B. jararacussu*; 3: *B. alternatus*; 4: *B. atrox*; 5: *B. moojeni*; 6: *B. neuwiedi*; 7: *B. cotiara*; 8: *L. muta*; 9 *C. durissus terrificus*; 10: *C. durissus collineatus*. LMW and HMW, low and high molecular weight standards.

Fig. 3.40 (A, B) show the results for the *B. jararacussu* venom. A different batch of absorbed antivenom from the one used in Fig. 3.30 was prepared and used here in (A). A single diffuse band was stained in the homologous *B. jararacussu* venom by the absorbed anti-*B. jararacussu* antivenom. No heterologous venom bands were observed.

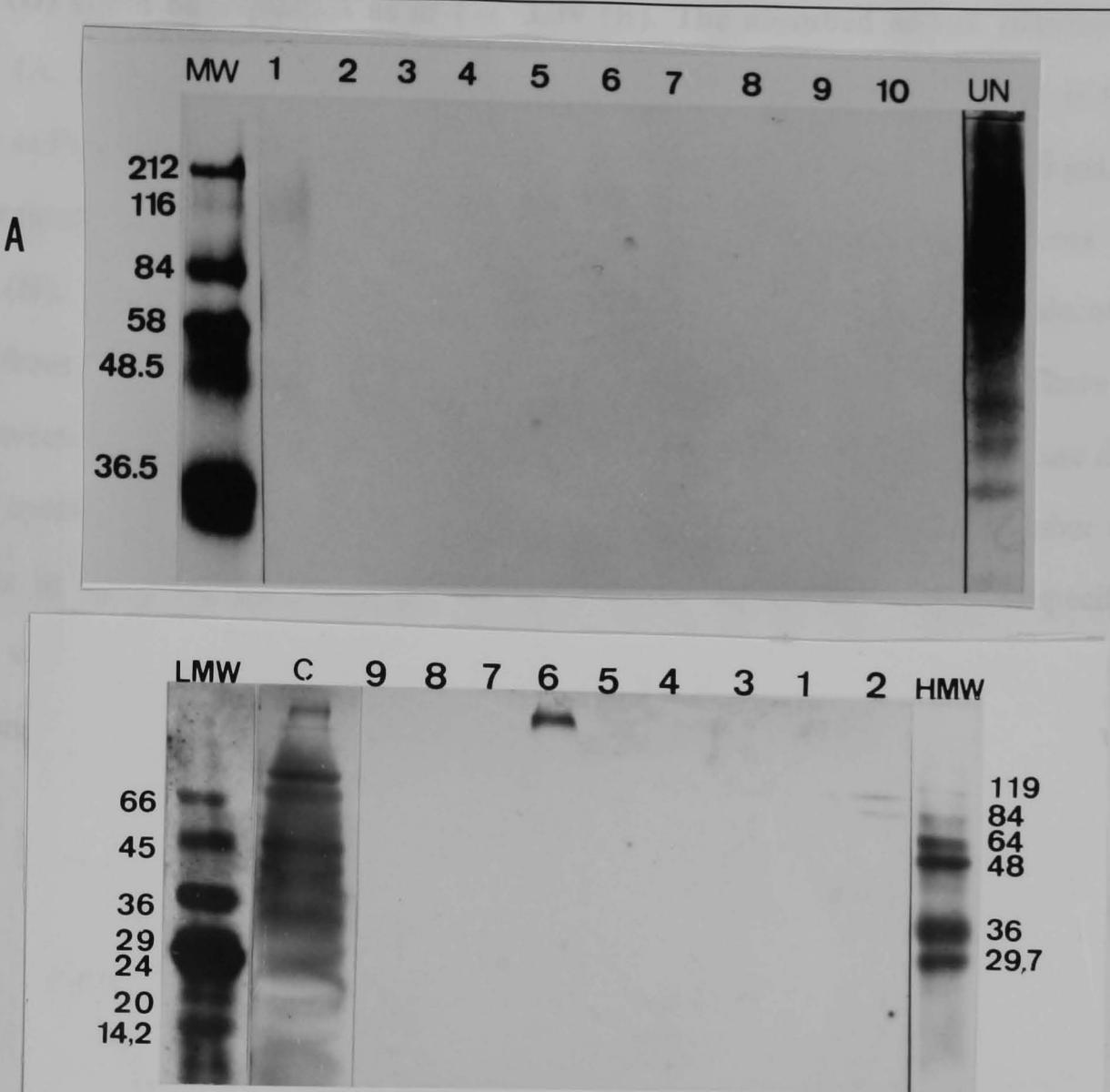


Figure 3.40 Immunoblot analysis showing *Bothrops jararacussu* species-specific components. **A:** Venoms were separated in a non-SDS PAGE 7.5% gel and transferred to a nitro-cellulose membrane. Absorbed anti-*B. jararacussu* antivenom was added to lanes numbered from 1 to 10 at 1:600 dilution. Unabsorbed anti-*B. jararacussu* antivenom was added to the homologous venom at 1:2,000 as control (UN). MW: Molecular weight standards. **B:** Venoms were separated in a SDS/PAGE 10% gel and transferred to nitrocellulose. Absorbed antivenom IgG was added to lanes numbered 1 to 9 at 1:800 dil. Unabsorbed anti-*B. jararacussu* was added to *B. jararacussu* venom at 1:2,000 dil. as control (C). Venoms in A and B are labelled as: 1: *B. jararaca*; 2: *B. jararacussu*; 3: *B. alternatus*; 4: *B. atrox*; 5: *B. moojeni*; 6: *B. neuwiedi*; 7: *B. cotiara*; 8: *L. muta*; 9: *C. durissus terrificus*; 10: *C. durissus collineatus*. LMW and HMW, low and high molecular weight standards in kDa.

In (B) the venom separation was carried out in a SDS gel and the nitro-cellulose was immunostained with 4X absorbed anti-*B. jararacussu* IgG (same used in the results of Fig. 3.34). Two specific stained bands were seen in the homologous venom of approximate molecular mass of 84 kDa and 116 kDa and a heterologous stained band was seen in the venom of *B. neuwiedi* with molecular mass above 119 kDa. The heterologous stained band

in Fig. 3.40 (B) could be explained as in Fig. 3.39 (B). The absorbed anti-*B. alternatus* (Figure 3.41 (A, B)) stains bands only in the homologous venom. Figure 341 (A), is the same pattern as Fig. 3.30 (B) and the venom separation was carried out in a non-SDS gel. 3 specifically stained bands were seen with no stained band with the heterologous venoms. In Figure 3.41 (B), 5 specifically stained bands are visualised. The approximate molecular masses vary from 24 kDa for one band, 43 kDa and 45 kDa for two other bands. There is one band between 66 and 84 kDa and one in the range of 119 kDa. The results indicate the existence of species-specific venom components of *B. alternatus*. The higher number of stained bands in the SDS gel could be due to a better separation of venom-specific components, with comparable charge-mass ratio but different molecular masses. Also the SDS conditions might have exposed epitopes in the specific venom .

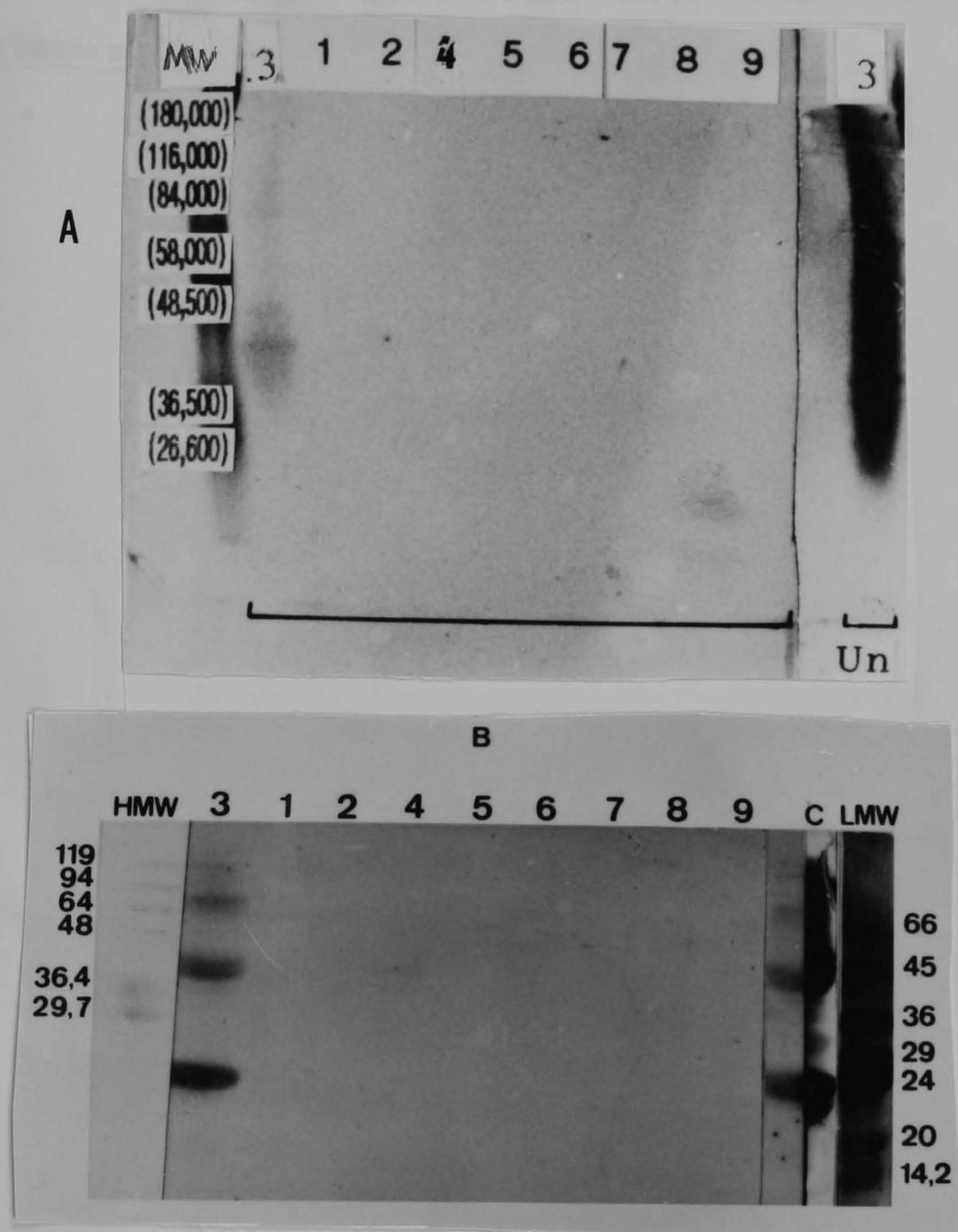


Figure 3.41 Immunoblot analysis showing *Bothrops alternatus* species-specific components. **A:** Venoms were separated in a non-SDS PAGE 7.5% gel and transferred to a nitro-cellulose membrane. Absorbed anti-*B. alternatus* antivenom was added to lanes numbered from 1 to 10 at 1:1,000 dilution (dil). Unabsorbed anti-*B. alternatus* antivenom was added to the homologous venom at 1:2,000 dil. as control (UN). MW: Molecular weight standards. **B:** Venoms were separated in a SDS/PAGE 10% gel and transferred to nitrocellulose. Absorbed antivenom IgG was added to lanes numbered 1 to 9 at 1:800 dil. Unabsorbed anti-*B. alternatus* added to *B. alternatus* venom at 1:2,000 dil. as control (C) . Venoms in A and B are labelled as: 1: *B. jararaca*; 2: *B. jararacussu*; 3: *B. alternatus*; 4: *B. atrox*; 5: *B. moojeni*; 6: *B. neuwiedi*; 7: *B. cotiara*; 8: *L. muta*; 9: *C. durissus terrificus*; 10: *C. durissus collineatus*. LMW and HMW, low and high molecular weight standards in kDa.

Figure 3.42 (A) shows the immunoblot results for the *B. moojeni* venom in the non-SDS gel. Two bands are observed in the homologous venom.

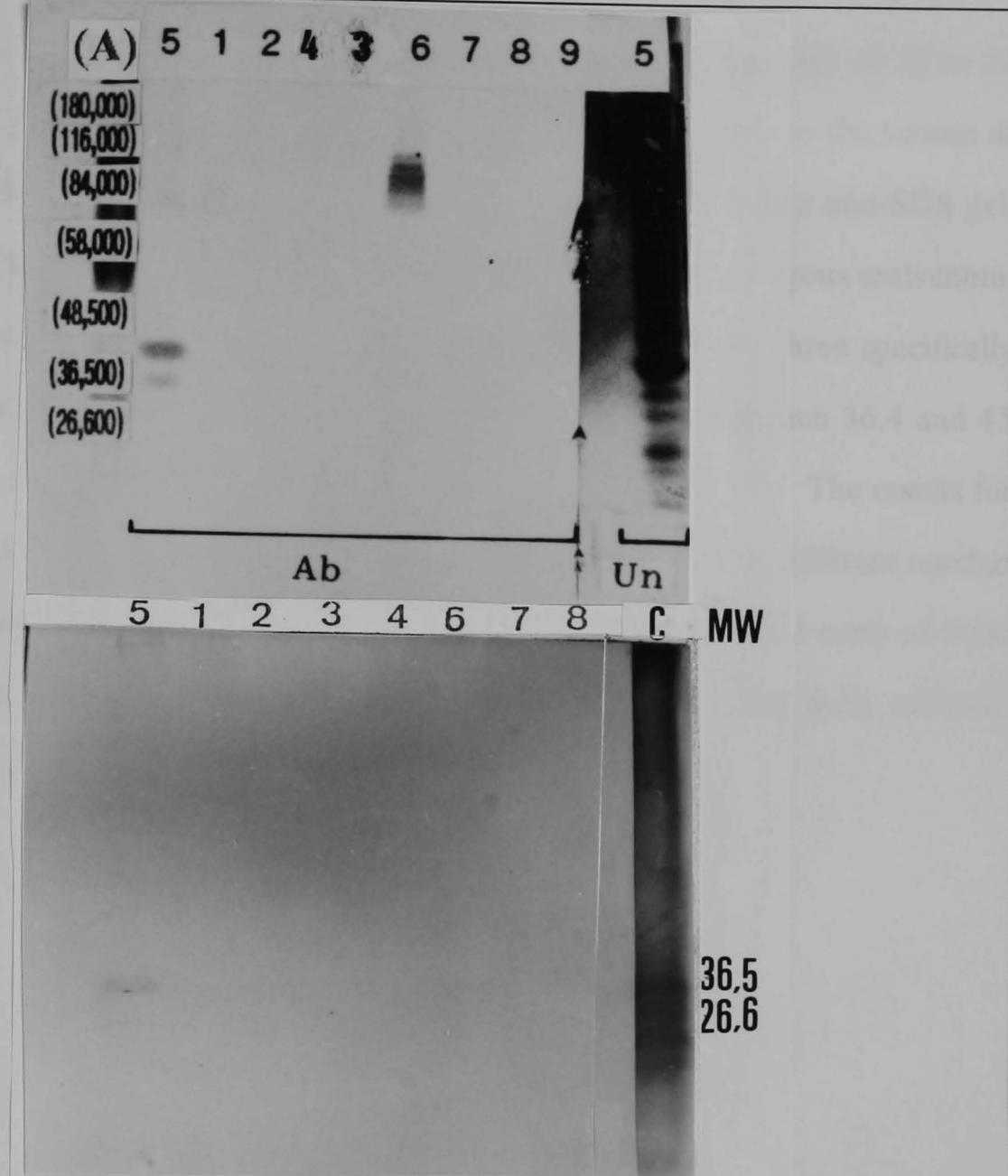


Figure 3.42 Immunoblot analysis showing *Bothrops moojeni* species-specific components. **A:** Venoms were separated in a non-SDS PAGE 7.5% gel and transferred to a nitro-cellulose membrane. Absorbed anti-*B. moojeni* antivenom was added to lanes numbered from 1 to 9 at 1:1,000 dilution. Unabsorbed anti-*B. moojeni* antivenom was added to the homologous venom at 1:2,000 as a control (UN). **B:** Venoms were separated in a SDS/PAGE 10% gel and transferred to nitrocellulose. Absorbed anti-*B. moojeni* venom IgG was added to lanes numbered 1 to 8 at 1:800 dil. Un-absorbed anti-*B. moojeni* was added to *B. moojeni* venom at 1:2,000 dil. as control (C). Venoms in A and B are labelled as: 1: *B. jararaca*; 2: *B. jararacussu*; 3: *B. alternatus*; 4: *B. atrox*; 5: *B. moojeni*; 6: *B. neuwiedi*; 7: *B. cotiara*; 8: *L. muta*; 9: *C. durissus terrificus*. Numbers in brackets and MW, are molecular weight standards in kDa.

Cross-reactive stained bands were seen in the venom of *B. neuwiedi*. In the SDS gel Fig. 3.42 (B) there is one single stained band in the homologous venom with no cross-reactive stained band in the heterologous venoms. Its molecular mass was in the range of 30 to 36 kDa. The result indicates that specific venom components are also present in the venom of *B. moojeni*. The results for the *B. neuwiedi* venom are in Figure 3.43. In the non-SDS gel, Figure 3.43 (A), one band was specifically stained by the absorbed homologous antivenom. There is not non-specific staining observed. In the SDS/PAGE plate (B) three specifically stained protein bands are seen. The first has a molecular mass (MW) between 36.4 and 45 kDa. The second has a MW of 36.4 kDa and the third has a MW of 24 kDa. The results for all venoms tested indicated the existence of species-specific epitopes. The different number of stained bands between the SDS and non-SDS gels could also indicate that some of these venom components bearing the specific epitopes could be oligomers and their sub-units were separated in the SDS treatment.

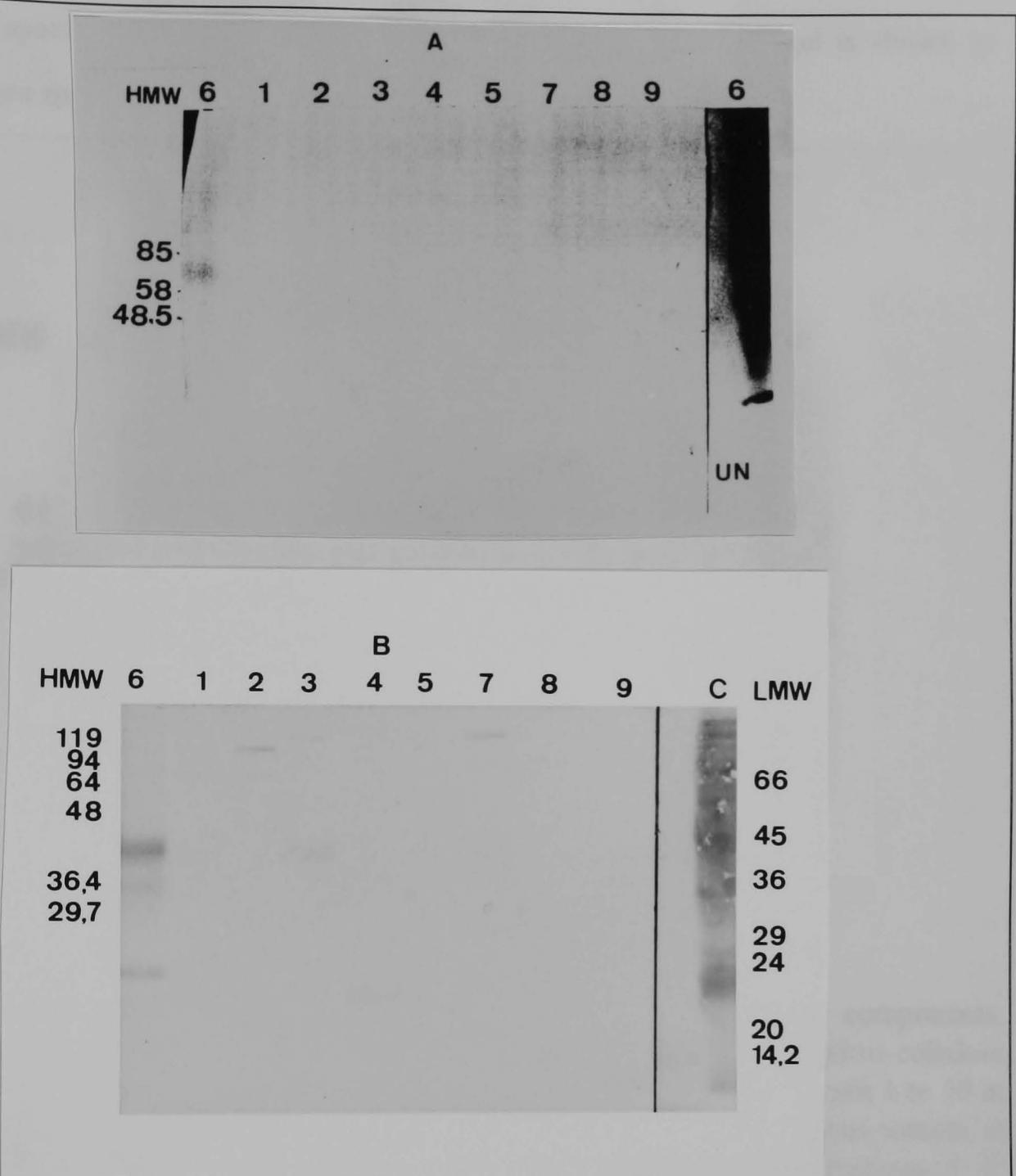


Figure 3.43 Immunoblot analysis showing *Bothrops neuwiedi* species-specific components. **A:** Venoms were separated in a non-SDS PAGE 7.5% gel and transferred to a nitrocellulose membrane. Absorbed anti-*B. neuwiedi* antivenom was added to lanes numbered from 1 to 9 at 1:1,000 dilution. Unabsorbed anti-*B. neuwiedi* antivenom was added to the homologous venom at 1:2,000 as control (UN). MW: Molecular weight standards. **B:** Venoms were separated in a SDS/PAGE 10% gel and transferred to nitrocellulose. Absorbed antivenom IgG was added to lanes numbered 1 to 9 at 1:1,000 dil. Un-absorbed anti-*B. neuwiedi* was added to *B. neuwiedi* venom at 1:2,000 dil. as a control (C). Venoms in A and B are labelled as: 1: *B. jararaca*; 2: *B. jararacussu*; 3: *B. alternatus*; 4: *B. atrox*; 5: *B. moojeni*; 6: *B. neuwiedi*; 7: *B. cotiara*; 8: *L. muta*; 9: *C. durissus terrificus*; 10: *C. durissus collineatus*. LMW and HMW, low and high molecular weight standards in kDa.

The pattern of specific bands for the *L. muta* venom, for the non-SDS gel is shown in Figure 3.44 where specific stained bands can be seen.

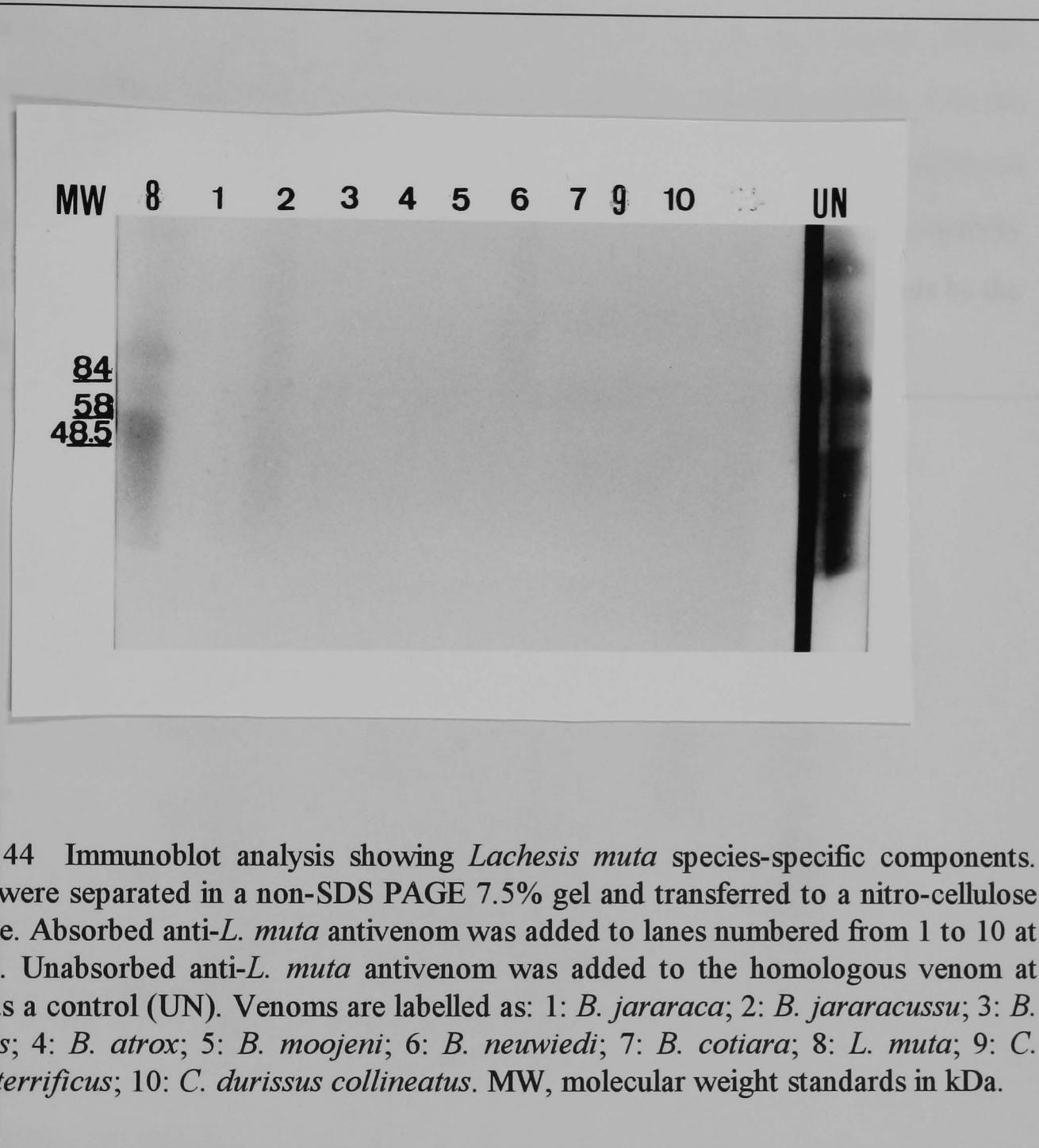


Figure 3.44 Immunoblot analysis showing *Lachesis muta* species-specific components. Venoms were separated in a non-SDS PAGE 7.5% gel and transferred to a nitro-cellulose membrane. Absorbed anti-*L. muta* antivenom was added to lanes numbered from 1 to 10 at 1:800 dil. Unabsorbed anti-*L. muta* antivenom was added to the homologous venom at 1:2,000 as a control (UN). Venoms are labelled as: 1: *B. jararaca*; 2: *B. jararacussu*; 3: *B. alternatus*; 4: *B. atrox*; 5: *B. moojeni*; 6: *B. neuwiedi*; 7: *B. cotiara*; 8: *L. muta*; 9: *C. durissus terrificus*; 10: *C. durissus collineatus*. MW, molecular weight standards in kDa.

Figure 3.45 is the immunoblot result, (non-SDS) of the panel of venoms stained by their homologous absorbed antivenoms. The staining pattern very much resembles the ones described above in Figures 3.39 (A) to 3.44 (A). Not shown before and included here in this assay are the results for the venoms of *B. atrox* and *B. cotiara*. The *B. atrox* pattern (Figure 3.45, n°.4) show two clearly identified visible broad stained bands. Lane 7 of this same Figure show the results for the *B. cotiara* venom. Three bands are visualised. Lane 6 has the

results for the *B. neuwiedi* venom and this time a broad and diffuse band is seen in contrast with the ones in Figure 3.43 (A). This pattern tended to appear in some non-SDS/PAGE immunoblot assays of *B. neuwiedi*, although no non-specific band was ever stained. All the absorbed antivenoms used in the assay of Figure 3.45 were used at 1:500. In Fig. 3.46 the results show an immunoblot study of the venoms of *B. atrox* and *L. muta*. It is observed that the absorption of the monovalent specific antivenoms abolishes the cross-reactivity between the two venoms indicated here by the absence of heterologous stained bands by the two absorbed antivenoms.

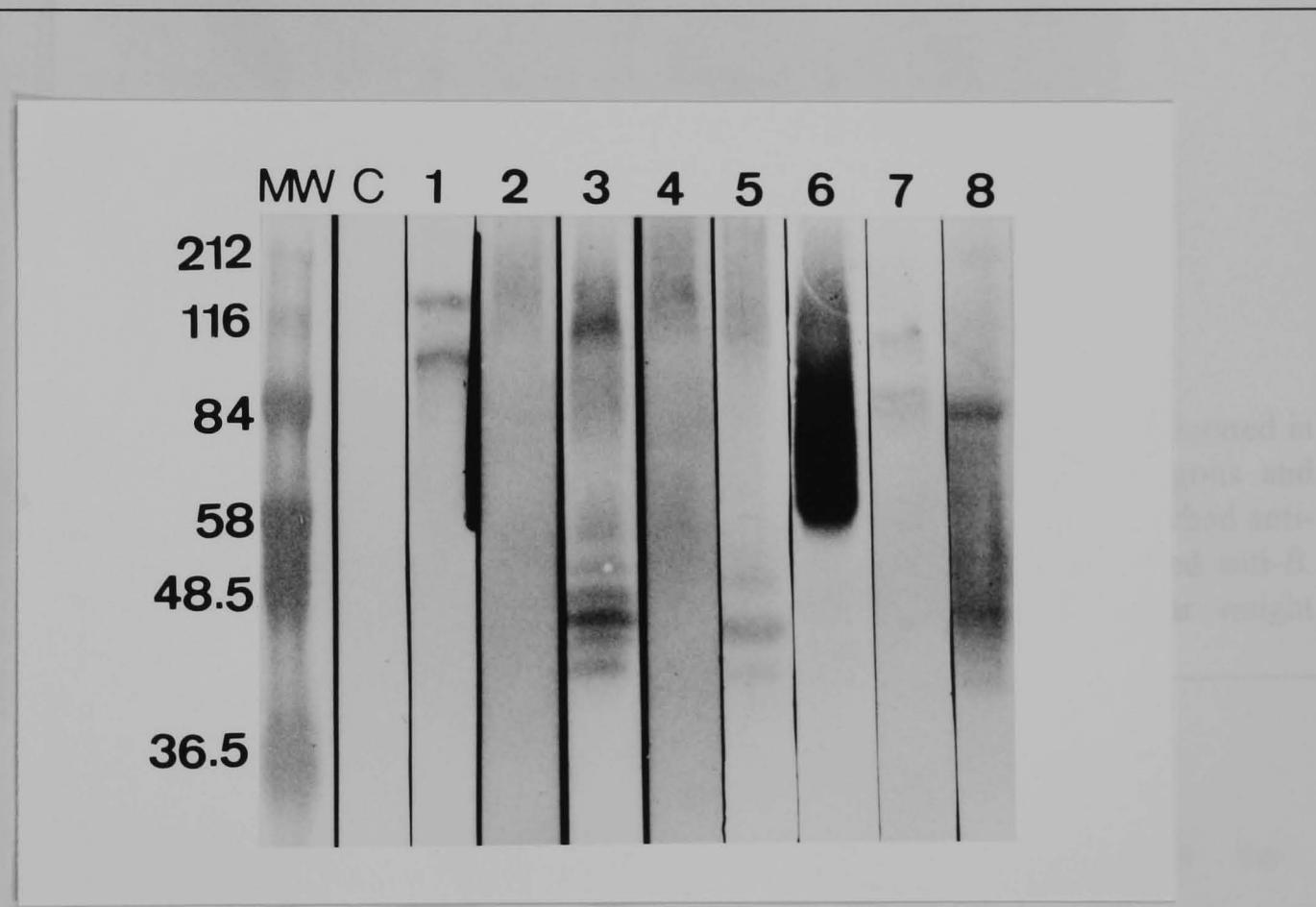


Figure 3.45 Immunoblot of the panel of venoms showing the specific bands stained by their homologous absorbed antivenoms. Venoms were separated in a non-SDS/PAGE, 7.5% gel and transferred to a nitro-cellulose membrane. Venoms and antivenoms: 1: *B. jararaca*, 2: *B. jararacussu*, 3: *B. alternatus*, 4: *B. atrox*, 5: *B. moojeni*, 6: *B. neuwiedi*, 7: *B. cotiara*, 8: *L. muta*. All antivenoms were diluted 1:500. C: control, conjugate alone. MW: molecular weight standards in kDa.

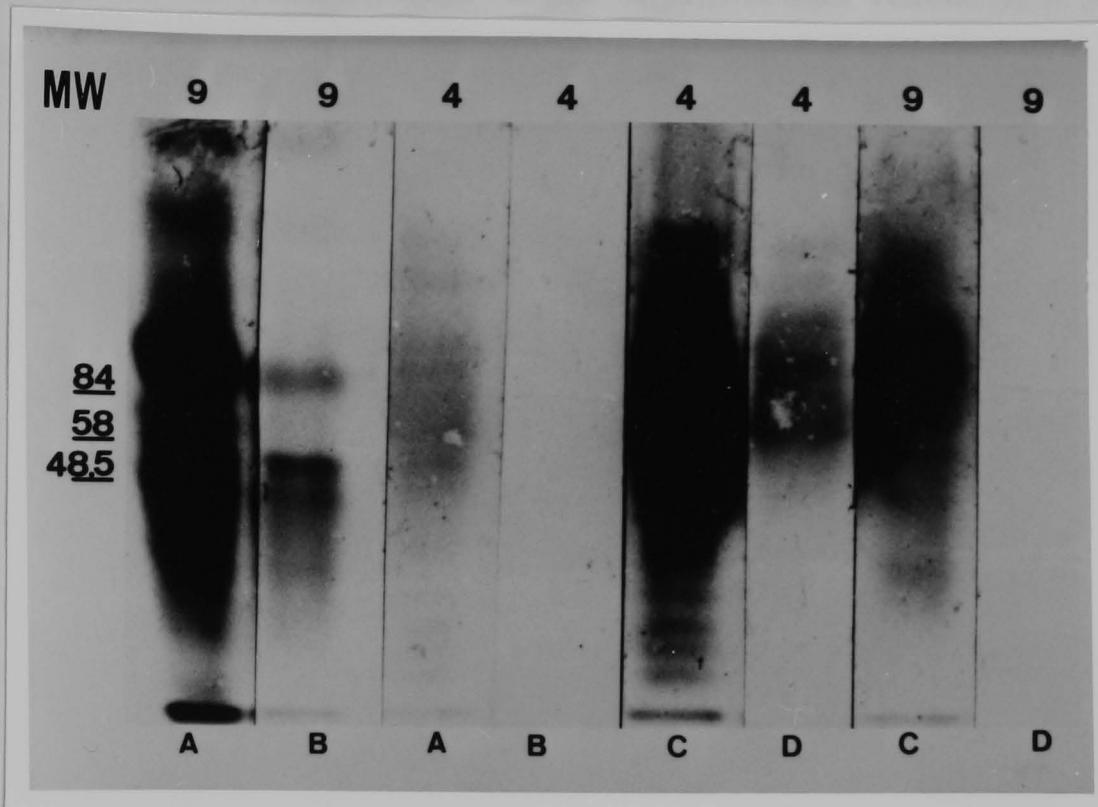


Figure 3.46 Immunoblot analysis of *B. atrox* and *L. muta* venoms. Venoms were separated in a non-SDS gel and transferred to nitrocellulose and reacted with their homologous and heterologous antivenoms. Venoms: 4, *B. atrox*; 9, *L. muta*. Antivenoms: A, Un-absorbed anti-*L. muta* at 1:2,000 dilution. B, absorbed anti-*L. muta* at 1:1,000 dil. C, Un-absorbed anti-*B. atrox* at 1:2,000 dil.. D, absorbed anti-*B. atrox* at 1:1,000 dil. MW: molecular weight standards in kDa.

3.2.2. Identification of the species-specific components on venoms from individual snakes of the same species

Venoms individually extracted from single specimens of the same species were analysed by immunoblots for the presence of the species-specific components shown to exist in the venom pools. If present, the results would strongly corroborate the indication of our previous results of the existence of species-specific venom epitopes. It would also indicate that the specific staining patterns obtained with the venom pools were not due to reactivity with any contaminating epitope(s) from a single venom in the “pool” used. The same batch of absorbed antivenoms used in the previous section to stain the immunoblots carried out in

SDS conditions was employed in these experiments. In Figure 3.47 all of the five venom specimens contain the two bands specific for the *B. jararacussu* venom. It is possible to observe differences in the proportional quantities of the specifically stained venom bands in some of the venoms. Venoms n° 4 and 5 are from the same geographical area and show similar staining patterns by the absorbed and unabsorbed antivenoms.

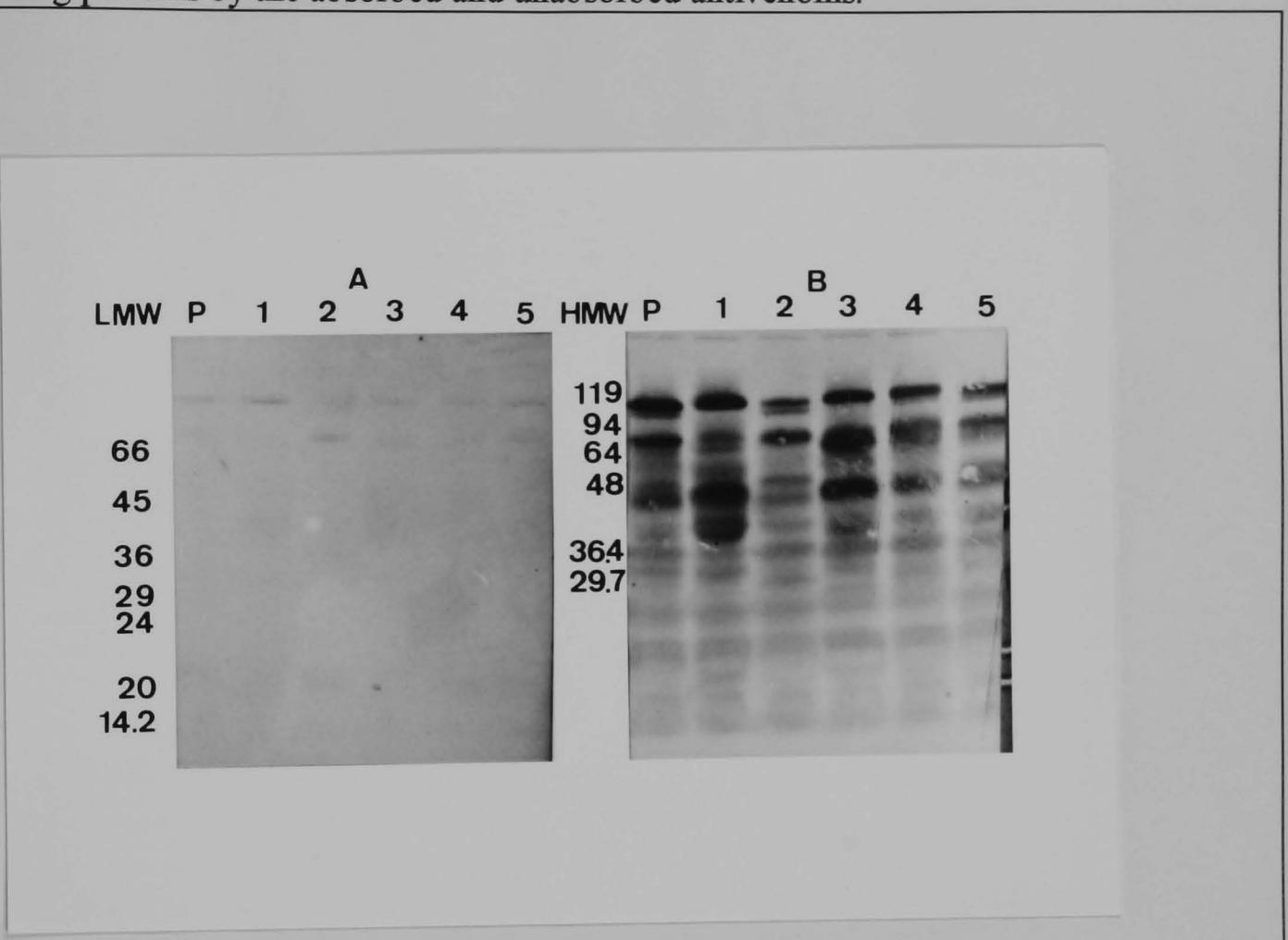


Figure 3.47 Immunoblot of analysis of Bothropic venoms from individual snakes. Venoms from individual snakes of the *Bothrops jararacussu* species and from different geographic regions were separated in a 10% SDS/PAGE gel, and transferred to nitrocellulose. **A:** Venoms were reacted with their homologous absorbed antivenom at 1:800 dil. **B:** Venoms were reacted with their homologous unabsorbed antivenom at 1:2,000 dil. Venoms in A and B are labelled as: 1: "Ipatinga"; 2: "Guaraciaba"; 3: "Ponte Nova"; 4: "Ponte Nova"; 5: "Sabinópolis". HMW and LMW are high and low molecular weight standards, respectively in kDa.

In Figure 3.48 similar results are observed for the *B. moojeni* venom. All venoms showed a strongly stained band with molecular weight of 36 kDa present also in all venom patterns stained by the unabsorbed specific antivenom.

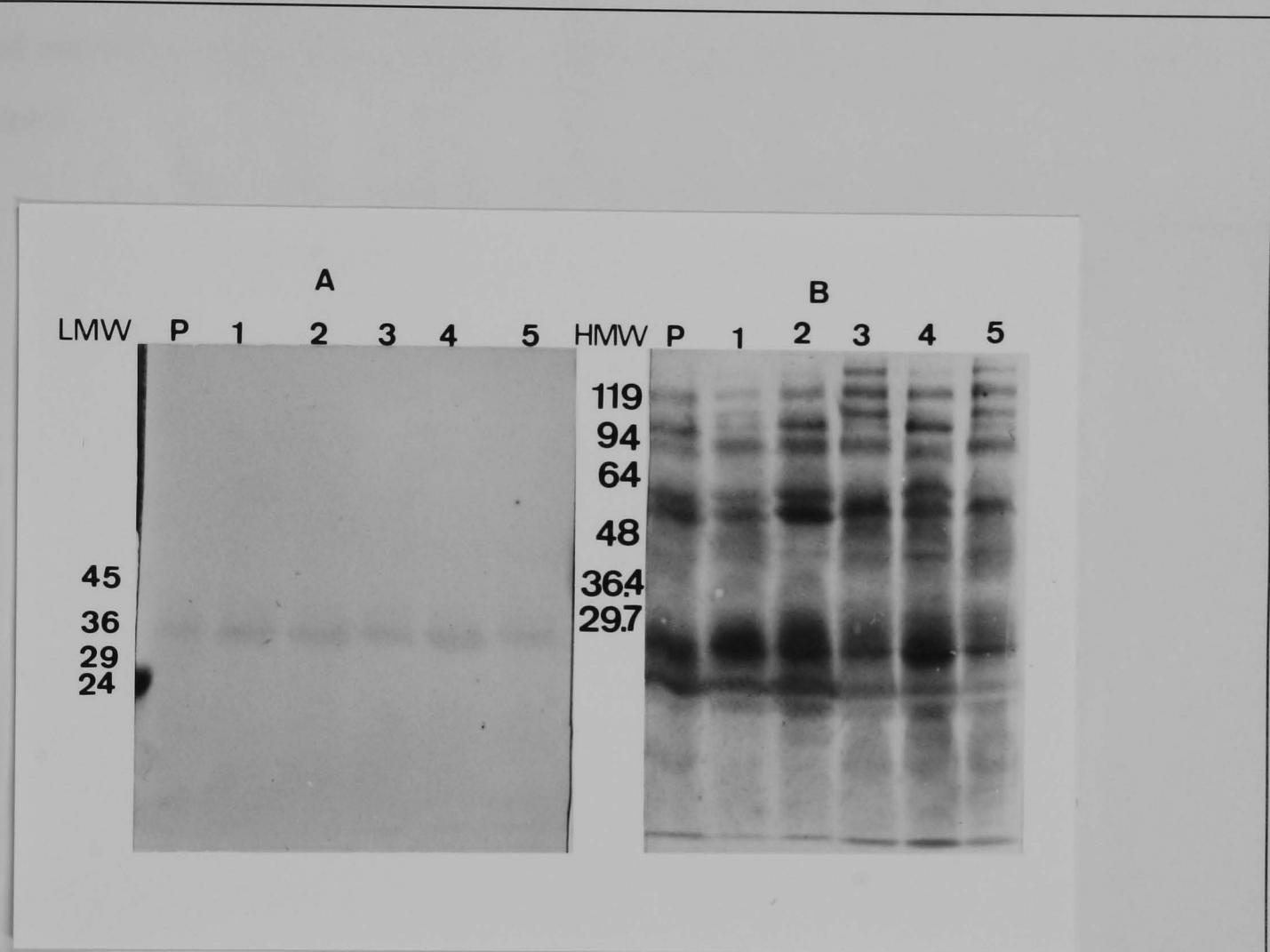


Figure 3.48 Immunoblot of analysis of Bothropic venoms from individual snakes. Venoms from individual snakes of the *Bothrops moojeni* species and from different geographic regions were separated in a 10% SDS/PAGE gel, and transferred to nitro-cellulose. **A:** Venoms were reacted with their homologous absorbed antivenom 1:1,000 dilution. **B:** Venoms were reacted with their homologous un-absorbed antivenom at 1: 2,000 dilution. Venoms in A and B are labelled as: 1: “Araxá”; 2: “Bom Despacho”; 3: “Catalão”; 4: “Caparaó”; 5: “Nova Ponte”. HMW and LMW are high and low molecular weight standards, respectively, in kDa.

In Figure 3.49 the results are shown for *B. neuwiedi* venoms and again the specific components are present in all venoms tested. All venoms have the bands with molecular

weight of around 36.5 and 45 kDa and a band of 24 kDa. Here too differences in the proportional quantities of these bands for each individual snake venom are observed. Venom n° 1 from Araxá and n° 5 from Nova Ponte do not show the band with molecular weight of 36.4 kDa. There is a greater variability within the venom patterns stained by the absorbed anti-*B. neuwiedi* antivenom than seen with the venoms of *B. moojeni* and *B. jararacussu*.

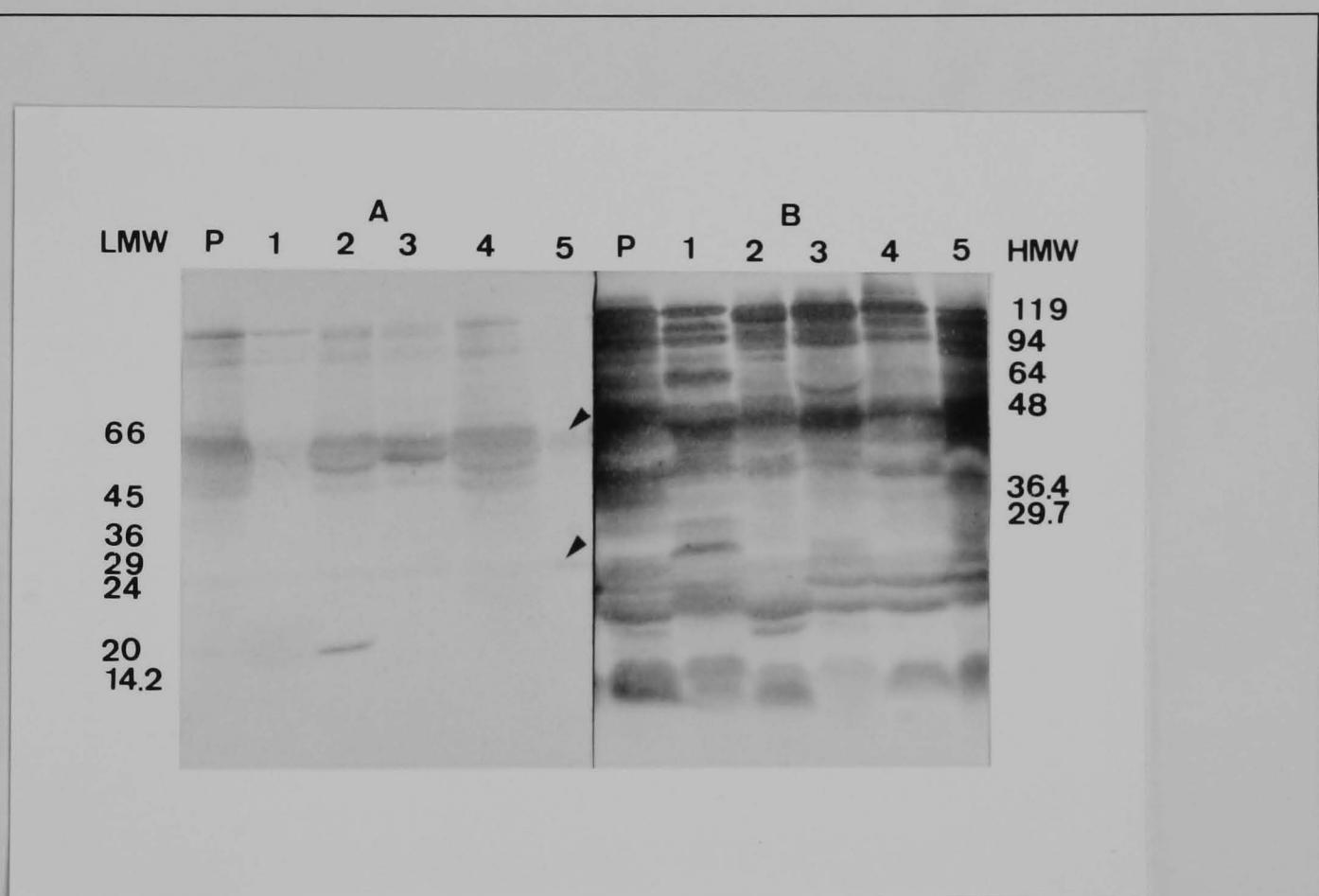
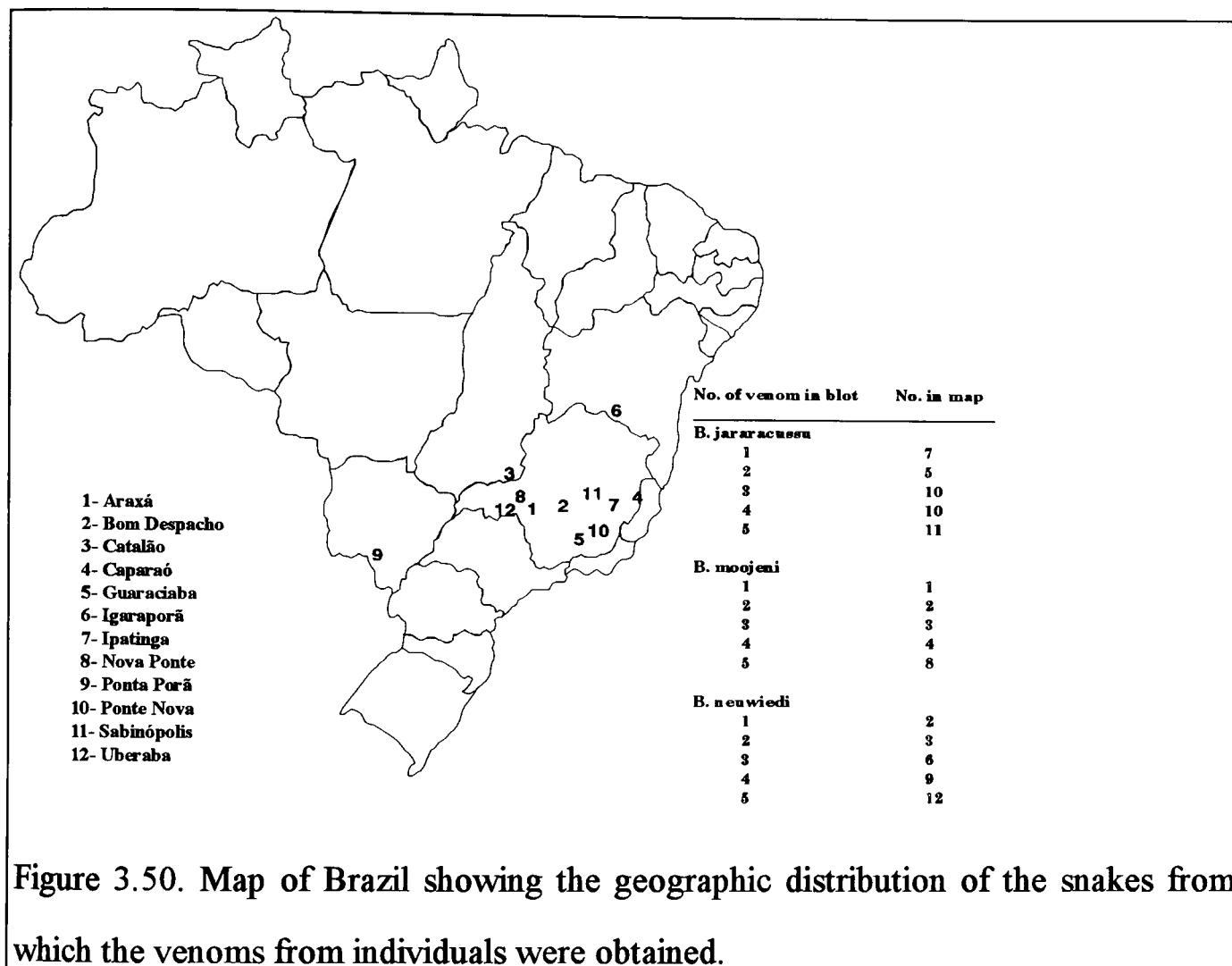


Figure 3.49 Immunoblot of analysis of Bothropic venoms from individual snakes. Venoms from individual snakes of the *Bothrops neuwiedi* species and from different geographic regions were separated in a 10% SDS/PAGE gel and transferred to nitrocellulose. A: Venoms were reacted with their homologous absorbed antivenom at 1:1,000 dilution. B: Venoms were reacted with their homologous unabsorbed antivenom 1:2,000. Venoms in A and B are labelled as: 1: “Bom Despacho”; 2: “Catalão”; 3: “Igaraporã”; 4: “Ponta Porã”; 5: “Uberaba”. HMW and LMW are high and low molecular weight standards, respectively in kDa.

These Western blots results show that the specifically stained venom components present in the venom pool of a single species of snake are also present in the individual venom from specimens of the same species and from different geographical regions. This strongly

suggests that there are venom components bearing epitopes that could be genetic markers of species of snakes.

In Figure 3.50 is shown the geographical distribution of the snakes in Brazil from which the individual snakes venoms were obtained.



3.3. Purification of *B. jararaca* species-specific component

Based on immunoblots results where the absorbed anti-*B. jararaca* antivenom stained two venom components species-specifically, a purification of these venom component(s) was attempted by affinity chromatography. Previous attempts to isolate specific components of *B. jararacussu* by elution from a polyacrylamide gel slice had no success. Here the purification of these components was tried by affinity chromatography. A batch of anti-*B.*

jararaca IgG, absorbed 3X, was insolubilized onto Protein-A Sepharose-CL-4B. *B. jararaca* venom was added to the column and the specific component eluted (see Material and Methods). The eluates yielded a total of 421 µg of protein per 500 µl. Eighty five µl of the eluate was mixed with 43 µl of 2X sample buffer. From this solution, 16 µl (8.9 µg) was applied to each lane of a 10% SDS-PAGE gel. The purification was tested by immunoblot assay using the non-absorbed monovalent antivenom to the other Bothropic venoms studied to test for any cross-reactivity with the eluted sample. In the results (Fig. 3.51) the 4X absorbed anti-*B. jararaca* (same batch used in Figure 3.44(A)) reacted with venom components of molecular weight around 48,500 kDa; 36,500 kDa, and only slightly with venom components at an approximate molecular weight of 24 and 22 kDa (the last three reactions not clearly visible in the photograph). The non-absorbed anti-*B. jararaca* reacted with 4 venom components at the same approximate molecular weight as the absorbed anti-*B. jararaca*. The component with approximate molecular weight (22 kDa) is probably one of the specific components of *B. jararaca* (see Fig. 3.39 (B)). There is very little cross-reaction of the heterologous non-absorbed antivenoms with this venom component. The heterologous antivenoms did however react strongly with the other venom components eluted from the *B. jararaca* venom. The anti-*B. neuwiedi* antivenom showed very little cross-reaction, and with only one of the *B. jararaca* venom components. In Fig. 3.39 (B), the other specifically stained venom component has an approximate molecular weight of 50 kDa, here, all antivenoms tested (except for the non-absorbed anti-*B. neuwiedi*) show a strong reaction against a venom component at the same approximate molecular weight. This could be corroborating our previous suggestion that, either the species-specific components share a same molecular weight to non-specific components or that specificity might be related to specific epitopes and not to whole venom components. The results suggest that species-specific components for the *B. jararaca* venom could have been partially purified, although further studies are needed for a confirmation of this result.

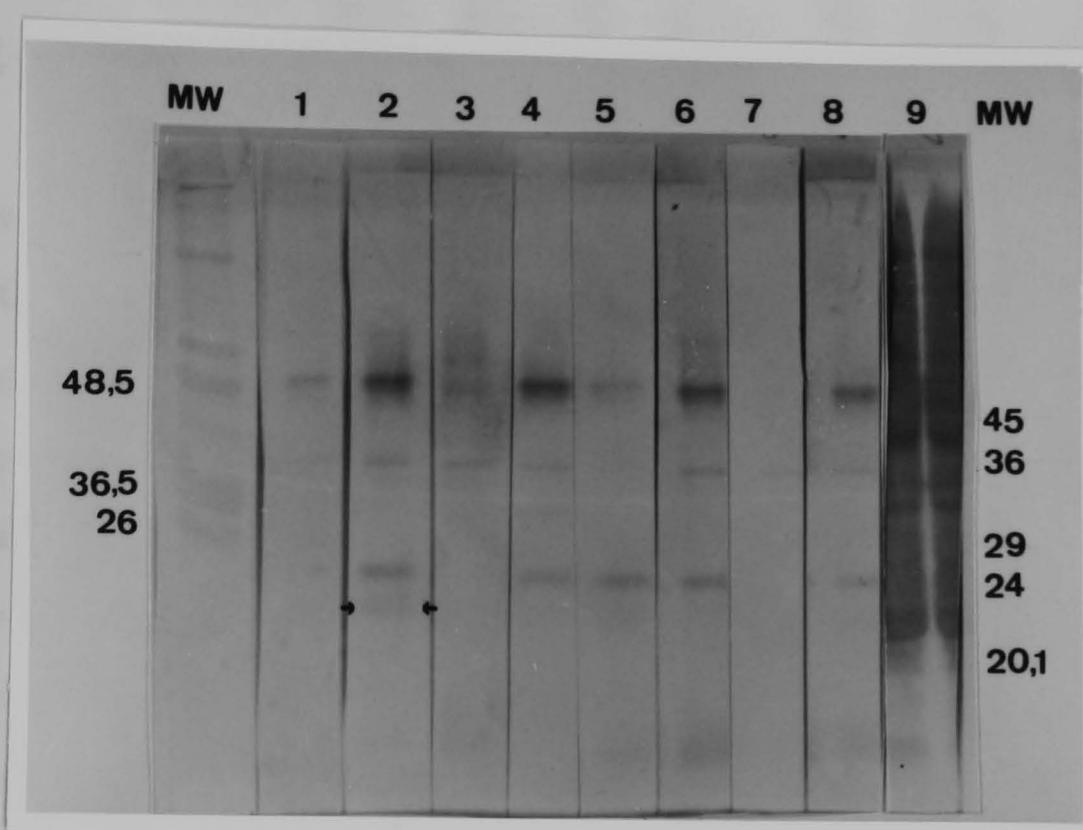


Figure 3.51 Immunoblot of affinity purified *B. jararaca* venom components. Purified antigen was separated in 10%, SDS/PAGE gel, without reducing conditions. After the transfer the nitrocellulose was cut into strips containing the isolated venom components. Each strip was then reacted with one of the antivenoms. Antivenoms: 1, 4X absorbed anti-*B. jararaca*; 2, Non-absorbed anti-*B. jararaca*; 3 non-absorbed anti-*B. jararacussu*; 4, non-absorbed anti-*B. alternatus*; 5, non-absorbed anti-*B. atrox*; 6, non-absorbed anti-*B. moojeni*; 7, Non-absorbed anti-*B. neuwiedi*; 8, non-absorbed anti-*B. cotiara*; Control: 9, whole venom reacted with the non-absorbed anti-*B. jararaca*. The absorbed antivenom was used at 1:500 dilution and others were used at 1:1,000 dil. MW: molecular weight standards in kDa.

4. DISCUSSION

4.1. The search for species-specific antigens in *Bothrops* venoms

As noted in the Introduction, snakebite is a medical problem in many countries [Warrell 1992; WHO 1981] and although mortality has fallen in many places, morbidity statistics remain high. The diagnosis of snakebite is not possible without the capture and identification of the offending snake by experts. Immunological methods have been regarded as the possible way to achieve the identification of snake venoms in body fluids of patients. Within individual snake species, venom antigenic variability has posed a problem for the development of specific diagnostic assays for snakebite. The identification of species-specific markers in the venoms is seen as the solution for the development of such specific assays and for reliable and specific studies on the kinetic of envenoming to be carried out.

In the first part of this study we focused on the immunological characterisation of the venoms in order to be able to define the best strategies that would allow the visualisation of species-specific markers for each of the venoms, if they existed.

PAGE has been used before by a number of research groups to study the antigenic characteristics of venoms, including the genus *Bothrops* [Assakura *et al.*, 1992; Jiménez-Porras, 1964; Tan and Ponnudurai, 1992b]. The results are conflicting, as some authors support the possibility of characterising venoms by this method whilst others disagree. Our results indicate the possibility of using PAGE for the interspecies differentiation of *Bothrops* venoms, although it must be emphasised that we only used pools of venoms and so individuals and regional characteristics would have been diluted and not observed in the results. Nonetheless the results show that the venoms can be specifically identified by their pattern.

Studies on the cross-reactivities of the *Bothrops* venoms have been carried out before, and extensive interspecific cross reactions have been observed [Silles-Villarroel *et al.*, 1974]. The work of Silles-Villarroel focused on the cross-reaction of *B. jararaca* venom and its specific antivenom against other *Bothrops* venoms using the immunoelectrophoresis technique. His results showed that the anti-*B. jararaca* serum had more specificity towards the specific venom as judged by the number of precipitin lines visible after protein staining with Coomassie Blue. In our studies all the venoms and antivenoms of the *Bothrops* species in our study panel were reacted against each other. In accordance with the previous study we did find an extensive interspecies cross-reaction but could not reproduce results in relation to the number of precipitin lines of *B. jararaca* antivenom against the specific and heterologous venoms. The above cited authors used as a starting concentration of antivenom a dilution that would neutralise 4 mg/ml of the specific venom. This is a relative measure since each antivenom can present different specificity and avidity towards its specific venom, hence their initial IgG concentration for each antivenom might have been different. We used weight (a basic standard) per volume as the concentration of IgG for each antisera. In this manner all antisera had the same amount of IgG to react against their specific and heterologous venom. In contrast with the previous study the anti-*B. jararaca* showed more bands against the venoms of *B. atrox* and *B. alternatus* than against its specific venom. Our results also showed that some venoms reacted more strongly, as judged by the number of precipitin lines, with some heterologous antivenoms than with their homologous antivenom, as exemplified by the anti-*B. neuwiedi* against the *B. jararaca* venom. Unfortunately the lines were not clear enough and it was not possible to count them for a more detailed comparison.

Quantitative cross-reactivity studies of *Bothrops* venoms has been previously carried out [Dias da Silva, *et al.*, 1989]. Using end-point titration for ELISA and gel double diffusion the authors reported that some antivenoms were strong, medium or weak reactors towards their specific and heterologous venoms. End-point titration is not a reliable method, as it is

difficult to determine the end-point. The authors also did not specify the concentrations of IgG in each antiserum used. Their results show that *B. atrox* is a weak reactor even against its own venom in contrast with our results. The inhibition studies performed by us showed that *B. atrox* is the most reactive venom and by this evaluation is a strong reactor, as judged by the amount (less than 1 μ g) of the venom needed to inhibit the specific and some heterologous antivenoms. Unfortunately the amount of some of the venoms necessary to inhibit by 50% the specific and heterologous reactions fell off the curve on either side thus not allowing a more precise determination of their 50% inhibition points. Even so a classification based on the results was possible and it did correlate well with our other cross-reactivity results.

The 2D-IEP studies performed by us on snake venoms have not been reported in any other published work on *Bothrops* species. The results were interesting in showing that the patterns for each venom greatly differ from each other, indicating antigenic differences. The results where the monovalent antivenoms were used in the reference gel allowed the differentiation of the specific venoms. The use of the intermediate gel allowed the visualisation of specific components for *B. atrox* and *B. moojeni* venoms. The controls did not work properly due possibly to differences in the specificity and antigenicity of the different batches of antisera used. The failure in not showing the specific components for other venoms can be attributed to non-ideal conditions for each case. In another published study [Matthiesen, *et al.*, 1989] it was shown that some peaks were only seen by radioactivity probing, which was not used here.

The first part of the study, despite the shortcomings of some of the assays, was crucial for it demonstrated the existence of species-specific components for some of the *Bothrops* venoms. In addition it provided interesting information on the crossreactivities and antigenic composition of Bothropic venoms.

The second part of this study focused primarily on producing species-specific antivenoms, identifying species-specific components for the other Bothropic venoms in the study and on characterising and isolating these components.

The production of species-specific antivenoms was first attempted by immunising rabbits with the immune complexes of the species-specific components excised from the 2D-IEP agarose gels as shown before [Kroll, 1981]. Rabbits received the immune complexes in FCA for the first immunisation and in FIA for the subsequent injections. Immune complexes from the venoms of *B. moojeni*, *B. neuwiedi* and *B. atrox* were used. The rabbits sera were assayed by ELISA for the presence of antivenom activity towards the specific venom. Unfortunately no immune response was detected in any of the assays, probably due to insufficient amount of the immunising antigens injected and also probably due to the time gap between the immunisations.

In a second attempt to produce species-specific antivenoms the cross-reacting antibodies from the monovalent antivenoms were absorbed by affinity adsorption onto a venom adsorbent. This approach yielded excellent results and it was possible to produce species-specific antivenoms for anti-*B. jararaca*, anti-*B. jararacussu*, anti-*B. alternatus*, anti-*B. atrox*, anti-*B. moojeni*, anti-*B. neuwiedi* and anti-*L. muta*. The venom adsorbents were prepared using glutaraldehyde or polyacrylamide gel beads as the support matrix. The first yielded the best results and variations of it were tried, with species-specificity being achieved in all cases. The adsorption experiments were important also in confirming the existence of antigenic similarities between the venoms, as the adsorption onto a venom adsorbent containing a single venom would decrease the absorbed antivenom's cross-reactivity against venoms not present in the adsorbent. The antivenoms were tested for species-specificity by antigen capture or indirect antibody ELISA and species-specificity was observed in all cases throughout a range of different antivenom dilutions and venom concentrations. The cross-reactivity studies of the absorbed antivenoms indicate that the species-specificity is independent of antivenom dilution or venom concentration. In studies

carried out by other workers, antivenom or sample dilution was often used to reduce the cross-reactivity [Coulter, *et al.*, 1980]. Even so such assays proved to be unreliable, giving poor specificity [Cox, *et al.*, 1992; Theakston, 1989b]. The sensitivity of either the indirect antibody or antigen-capture assay in our studies is about 10 ng/ml, which compares well with previous workers, [Coulter, *et al.*, 1980; Cox, *et al.*, 1992; Labrousse, *et al.*, 1988] where the whole venom was detected in concentration ranges of 5 ng/ml to 50 ng/ml. Another important point to consider is that the assay developed in our study is detecting only some of the venom components and not the whole venom and yet an expected loss of sensitivity is not observed. The sensitivity of the assay was improved in an antigen-capture assay, using polyvalent antivenom as the coating layer and the absorbed antivenom as the second antibody, followed by an anti-rabbit conjugate, concentrations of 1 ng/ml could then be detected.

Monoclonal antibodies have been employed in snake venom research for the study of venom toxins and other venom components. They have been used for mapping epitopes, for neutralisation studies and cross-reactivity studies [Ménez, 1991]. We tried to produce monoclonal antibodies by immunising mice with venom immune complexes prepared by mixing together the absorbed antivenoms and their homologous venoms. The sera of the mice immunised with the *B. moojeni* and *B. neuwiedi* immune complexes, when tested for cross-reactivity against the other venoms of the study, showed an increased species-specificity, but a high degree of cross-reactivity was observed against the venoms of *B. jararaca*, *B. jararacussu* and *B. neuwiedi* for the mice immunised with the *B. moojeni* immune complex, and against *B. jararaca*, *B. jararacussu* and *B. moojeni*, for the mice immunised with the *B. neuwiedi* immune complex. These results do nevertheless indicate the possibility of raising species-specific antivenoms in animals using venom immune complexes of absorbed antivenoms. Evidently additional steps should be taken to abolish the remaining cross-reactivities and these should include: further absorption of the antivenom on the venom adsorbents, additional washing steps of the immune complexes,

avoiding the addition of anti-rabbit IgG used to increase the immune complex yield. This last step also increases the precipitation of non-specific immune complexes.

Despite the cross-reactivity observed hybridomas were established using the spleen from the mice immunised with the *B. moojeni* immune complexes. Clones showing anti-*B. moojeni* activity were further cultured but when tested for species-specificity none of the previously responding clones exhibited any antivenom activity. The failure of the clones in maintaining the antivenom acitivity could possibly be explained by most commom causes, lost of chromosomes or clones being overgrown by irrelevant cells. Infection, another cause of loss of activity did not occur in the clones tested.

The identification of species-specific venom components is an important factor for the detection, and kinetic studies, of envenoming [Ménez, 1991]. The absorbed antivenoms were used as probes in immunoblot assays to detect in their homologous venoms the existence of specific components. Species-specific components were identified for the venoms of *B. jararaca*, *B. jararacussu*, *B. alternatus*, *B. moojeni*, *B. neuwiedi*, *L. muta* and possibly for *B. atrox* and *B. cotiara*. It is interesting to note that their molecular weights vary between each species, which suggests that they could belong to different classes of proteins. Another point of interest is that the molecular weights of these components indicate that their cleareance from the victim's body by glomerular filtration should be minimal. It implies, therefore, that these components will have to be either degraded by the body's enzymatic system or depleted by antivenom which, in either case, suggests their importance in the study of the kinetics of envenoming. In fact as predicted by Ménez, [Ménez, 1991], "It should be stressed in particular that venoms are mixtures of toxic and non-toxic components which vary in proportion with the age, the sex, the environment, etc. of the animals. Such a variability makes the quantification of an injected venom in a patient difficult. Indeed, antibodies used for detection could be raised against a venom (or a mixture of venoms) which may be somewhat different from the particular injected venom. Only the

search for a defined antigen using specific antibodies can give an unambiguous answer." The components here identified appear to fulfil such requirements.

Based on preliminary venom immunoblot assays showing bands with species-specificity characteristics, polyacrylamide gel bands were cut out and their contents eluted and tested for cross-reactivity by ELISA. Venoms from *B. jararacussu*, *B. alternatus*, and *B. moojeni* were used. The protein contents eluted from the gels cross-reacted with the unabsorbed heterologous antivenoms. In all venoms, some of the bands tested were later confirmed as carriers of the specific components. These results indicate that other components are present in the bands stained by the absorbed antivenoms. The material eluted from the *B. jararacussu* gel bands were tested by immunoblot assay. The absorbed antivenom failed to react with the contents of either of the two excised bands but the unabsorbed specific antivenom and the un-absorbed heterologous antivenoms reacted with the eluted material.

In another attempt to isolate the specific components, an affinity column was prepared using absorbed anti-*B. jararaca* IgG coupled to Sepharose-CL-4B. *B. jararaca* venom was applied to the column and the bound venom antigens were eluted by 0.1 M Glycine-HCl, pH 2.5. The eluate was tested for the presence of the specific components by immunoblot. The absorbed antivenom stained 4 venom components. One of them stained very lightly is a band with approximate molecular weight of 22 kDa which also stained specifically stained by the same absorbed antivenom on the whole venom Fig. 3.39 (B). Therefore this venom component could probably be an specific component of the *B. jararaca* venom. The homologous non-absorbed antivenom stained also this band and with more intensity than the absorbed antivenom. Only the non-absorbed anti-*B. atrox* stained lightly a band with the same molecular weight. The other heterologous non-absorbed antivenoms showed reactivity with other *B. jararaca* venom components affinity isolated. The non-absorbed anti-*B. neuwiedi* shows very little reaction with only one of the venom components. The homologous absorbed and non-absorbed antivenom and the heterologous non-absorbed

antivenoms stained also a venom band of approximate molecular weight of 50 kDa. This venom band was also stained by the absorbed antivenom on the *B. jararaca* venom Fig. 3.39 (B). The results suggest that specific venom component(s) might be present in minute amounts in the *B. jararaca* venom. Also that they can share the same molecular weight of non-specific components. The use of absorbed antivenom with higher degrees of species-specificity (this could possibly be achieved by increasing the number of absorption steps) for the preparation of the affinity column, could probably ensure the isolation of only the species-specific components from the venom. However the results obtained in this study seem to indicate that an specific venom marker (approx. 22 kDa) for the *B. jararaca* venom could have been identified and partially purified.

4.1.1. General Discussion

Snake venom research can be loosely divided into three main areas. The first is a basic research area where snake venoms are studied for their composition, properties, and how the components are characterised and sometimes used as tools for further research. The second area is that of applied research where snake venom components are studied in order to be used for the prevention or treatment of diseases. The third area could be named as clinical research, which deals with the treatment of snake bites and related areas such as epidemiology of snakebites. This is not a proposition for a classification for snake venom research but it is being used here to put into perspective the work carried out, that was mainly concerned in solving problems found in the area of clinical research as described above.

The problems facing a physician having to treat a snake bite patient have not changed much since the early 1900's. As shown in the Introduction, it is necessary first to confirm the existence of a snakebite, then of envenoming, and the most important, to identify the venom causing the symptoms. Finally he must take the necessary measures for an efficient

treatment. The most important of the above problems, is the specific and safe identification of the venom, followed by the treatment to be adopted. If this is the use of antivenom, then at which dose? Present serumtherapy is empirically determined, and so little guidance can be given [Cardoso, 1990; Warrell, 1992].

Many methods have been tried to achieve this goal but none had yet succeeded. The advantages to be gained are obvious and some of the most clearly defined are as follows. It would allow the development of specific treatment measures for each species, even accounting for the geographical variations. It would make it possible to construct a clinical picture of the envenoming caused by the different species of snakes; allow the production of species-specific antivenoms or regional specific antivenoms. Epidemiological studies would be more accurate and allow for the development of preventive and treatment measures concerning a country or a specific geographical region. Antivenom therapy could be monitored and the right doses defined for each snake species.

This work is concerned primarily in generating species-specific antivenoms and identifying species-specific "marker" components for some of the Brazilian Bothropic venoms. The study creates the possibility of using these reagents for the specific detection of the snake venoms.

One of the problems for the production of antivenoms, especially where there is a great number of different species of snakes sharing a large geographical area, is venom variability. These are due to the number of species, to geographical and individual variability as well as seasonal variability of the venoms (WHO, 1981). Although cross-specific neutralisation has been shown for the Bothropic venoms [Silles-Villarroel, *et al.*, 1978/79], venom variability raises questions as to the composition of the venoms to be mixed or to be individually used to immunise the animals and in the case of polyvalent antivenom it also raises the question of appropriate proportions [Dias da Silva, *et al.*, 1989; Moura da Silva, *et al.*, 1990]. Studies [Mandelbaum and Assakura, 1988] demonstrated a relationship between

immunological characteristics and biological activity when they reported on the cross-neutralisation of two haemorrhagic factors isolated from *B. jararaca*, and one from the venom of *B. neuwiedi* that had been shown to cross-react in the immunodiffusion method. Dias da Silva *et al.*, [1989], using immunological, immunochemical and biological assays, reported an extensive cross-reaction between the Bothropic venoms studied by ELISA and immunodiffusion methods. They also confirmed the cross-specific neutralisation of the monovalent anti-Bothropic anti-venoms. Based on their results the authors suggested that the venoms of *B. jararaca*, *B. jararacussu*, *B. alternatus*, *B. moojeni* and *B. neuwiedi*, should be in the immunising "venom pool" used for the production of the polyvalent anticoagulant antivenom produced in Brazil. Moura da Silva *et al.*, [1990], studying the cross-reactivities of Bothropic venoms, reported that when the venoms of *B. alternatus*, *B. atrox* and of *B. cotiara* were used as antigens in immunoblot studies and were stained by specific and non-specific monovalent anticoagulant sera they behaved similarly. The same similarity of behaviour was found between the venoms of *B. jararacussu*, *B. neuwiedi* and *B. pradoi* to the venom of *B. moojeni*. Further the authors demonstrated that the *B. moojeni* venom shared epitopes with venom components of *B. jararaca*. They also demonstrated that the anti-*B. moojeni* could neutralise the lethal activity of the *B. jararaca* as efficiently as the specific antivenom. Leite, *et al.*, [1992] reported on the characterisation of seven Bothropic venoms by their biological activities, (caseinolytic, myotoxic, coagulant and haemorrhagic). They observed that only a few venoms would represent the activity present in venoms from the different species of snake: *B. jararaca*, *B. jararacussu* and *B. atrox* could cover for caseinolytic activity in any of the species; *B. jararacussu*, *B. moojeni* or *B. neuwiedi* would cover for myotoxic activity; *B. moojeni* and *B. jararaca* may cover for coagulant activity and *B. jararaca*, *B. jararacussu* and *B. neuwiedi* would cover for haemorrhagic activity. They observed that in considering the active protein distribution, the venoms of *B. jararaca*, *B. jararacussu*, *B. moojeni*, *B. neuwiedi* and *B. atrox* contain all the active protein determined in any of the species studied by them and that this group of venoms is very similar to the one suggested by Dias da Silva *et al.*, [1989] which consisted

of *B. jararaca*, *B. jararacussu*, *B. alternatus*, *B. moojeni* and *B. neuwiedi*. Our crossreactivity index, derived from the ELISA-inhibition studies suggests that the venoms of *B. atrox*, *B. jararaca*, *B. neuwiedi*, *B. moojeni* and *B. cotiara*, are likely candidates for the composition of the "venom pool" due to their reactivity. In the three studies the suggested composition is very similar indicating that there could be a relationship between the immunological characteristics of snake venom and some of their biological activities.

The polyvalent anticoagulant antivenoms in Brazil are produced by the immunisation of horses with a "venom pool" with fixed proportions based on the incidence of snakebites. In the Fundação Ezequiel Dias (FUNED), this pool is composed of 50% of *B. jararaca* venom and 12.5% each of the following venoms; *B. jararacussu*, *B. alternatus*, *B. atrox* or *B. moojeni* and *B. neuwiedi*. [Domingues, *et al.*, 1990a], studying the inhibition of the haemorrhagic activity of *B. jararaca*, *B. jararacussu*, *B. alternatus*, *B. moojeni* and *B. neuwiedi*, by the anti-Bothropic antivenom from FUNED, report the lowest inhibiting potency against the venom of *B. jararaca*. The authors conclude that the criteria used to determine the composition of the "venom pool" should be revised. The same authors [Domingues *et al.*, 1990b] also demonstrated that the antivenom anti-Bothropic-Lachetic from FUNED produced, from a "venom pool" consisting of 50% *B. atrox* and 50% *L. muta*, was two times more potent in inhibiting up to 100% the haemorrhagic activity of the reference venom which consists of *B. jararaca* only. These two results taken together confirm the need for a proportional composition of the "venom pool". Our reactivity index derived from the inhibition assays provided an additional information; that is the possibility of determining a proportion for each venom to be included in the "venom pool" by their relative reactivities.

The value of venom as taxonomic criteria have been considered by many authors, [Gonçalves and Vieira, 1950; Jiménez-Porras 1964]. Some authors disputed this, arguing that venom properties are not reliable for the differentiation and classification of snake

species because of the extensive variation in venom composition due to geographical, individual, seasonal and other factors [Minton 1967; Minton and Weinstein, 1986; Schenberg, 1963]. Others studies, however, have shown that biochemical differences in venom properties could be used for the differentiation and classification of snake species [Glenn, and Straight., 1977; Jiménez-Porras 1967; Mendoza, *et al.*, 1992; Tan, and Ponnudurai, 1992b; Tu, *et al.*, 1965]. Recently Tan, and Ponnudurai, [1992a], in a large study involving more than 300 snakes from different species, genera and families, report that the biochemical characterisation of snake venoms can be used to differentiate and classify snake species. In our study differences between the species studied was observed in the PAGE assays, the IEP assays, 2D-IEP and immunoblot assays. The 2D-IEP assay venom patterns was strikingly different for all the species studied, making venom identification possible. The results of the 2D-IEP assay for the individual venoms of *B. jararacussu* indicate a constant pattern even though some individual variations are observed. Nevertheless, the identification of the venom is possible by comparison with the venom pool used as a control. When analysing the immunoblot results of the individual venoms assayed by their specific unabsorbed antivenom, again it is possible to identify the species of snake, even though individual variation can be visualised.

The specific detection of closely related species has been pursued by many authors, with little or no success [Ménez, 1991; Minton, 1987; Theakston, 1989b]. The high cross-reactivity of the venoms and poor specificity of the antivenoms were the main identified causes of failure. The attempts to develop an ELISA for the specific detection of Bothropic venoms was never successful. Barral-Neto, *et al.*, [1990], studying the development of an ELISA for the *Bothrops jararaca* venom reported a complete non-specificity of the assay with the venom of *B. atrox* being recognised more strongly by the specific anti-*B. jararaca* than the *B. jararaca* venom itself. The results of our study clearly shows that the specific identification of Bothropic venoms has been achieved in a reliable manner as well as that of *L. muta*. The specific detection of Bothropic venoms in human serum indicates that it is

possible to apply the methods here used in the clinical diagnosis of snakebite. The results show that the method here developed is specific and sensitive and also reliable as it can be repeated with ease. The absorption of monovalent antivenoms has produced a reliable method of generating species-specific antibodies.

The identification of species-specific venom "markers" is seen as the answer to the problems of non-specificity for the specific assaying of snake venoms, "Overlaps in venom components occur with regularity among species, making the consistent isolation of a species-specific "marker" component difficult. Thus, until more precise definitions of venom "fingerprints" are developed, immunodiagnostic techniques will maintain degrees of non-specificity" [Kunkel, 1987].

Intraspecific venom variability for Bothropic venoms was shown [Schenberg, 1963] for the venoms of *B. neuwiedi*. Five venom variants were shown to exist by the gel immunodiffusion method, and some subspecies would have venoms from more than one variant. No subspecies studied contained venom from all variants. Moura da Silva *et al.*, [1990], using immunoblot assays, failed to detect any venom components specifically stained by their homologous antivenom. Our results show the identification of species-specific venom components for *B. jararaca*, *B. jararacussu*, *B. alternatus*, *B. moojeni*, *B. neuwiedi* and *L. muta* and probably those of *B. atrox* and *B. cotiara* venom. In the studies with the individual venoms all presented the specific components, although differences in their amounts can be observed. The result for the *B. alternatus* venom is interesting because of the number of species-specific components visualised. It differs from the other venoms where only one or two species-specific components were observed. Some of the *B. alternatus* species-specific components could also carry regional specificity characteristics and it should be investigated further with individual venoms from different geographical regions. As the *B. alternatus* species has no record of subspecies, these proposed studies could bring additional information on the classification of the species. The results for the *B. neuwiedi* venom obtained here, and those of Schenberg, [1963], are not readily comparable.

He used a polyclonal antisera which was produced against the venoms of *B. jararaca*, *B. jararacussu*, *B. alternatus* and *B. atrox*. Thus, the antivenom could not have detected species-specific components since it had not been raised against the *B. neuwiedi* venom, but it was capable of detecting the existence of differences in the venom content of the different subspecies which, cross-reacted differently with the polyclonal antivenom. In our studies we absorbed the specific monovalent antivenoms with their heterologous venoms leaving only the antibodies reacting against venom components specific for the species. All the *B. neuwiedi* venoms from different geographical regions tested contained the specific components. This was the case also with the venoms of *B. jararacussu* and *B. moojeni*.

The results achieved here have for the first time shown the existence of species-specific snake venom components. Also it was the first time that a species-specific ELISA was developed for the Bothropic venoms. The immunoblot assays with the individual venoms demonstrate that they are a conserved component of these species.

4.1.2. Conclusion and Future work

We believe that the results obtained in this study are of fundamental importance to the area of snake venom research. They should assist in the fulfilment of most of the unachieved goals in this field of research, concerning the better understanding of snakebite envenomation past and present and the production of more efficient specific antivenoms and the characterisation of the venom's biological and immunological activities.

The future work should consist in the isolation of these components for their further physicochemical, biological and immunological characterisation.

We need to continue with the identification of specific components of other snake species and also study the venoms of specimens present in islands like the *B. insulare*s as well as

other snakes that would have evolved in parallel with the mainland snakes. Also from species on the mainland separated by natural barriers.

We need to proceed with work to develop a diagnostic kit for snakebites of Brazilian Bothropic snakes suitable for field use.

We need to develop an assay for the epidemiological study of past envenomation and use their detection in human serum for the study of antivenom therapy.

Above all at this stage we need to produce monoclonal antibodies to the specific components for use in physicochemical characterisation studies, biological activities of venom components and for use in the diagnostic test.

The amino acid sequence of purified components can be determined and used for the study of the systematic evolution of snake species. The study may answer more fundamental questions like why these components are responsible for carrying the species-specificity.

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6. APPENDICES

6.1. Appendix I

List of venomous snakes found in the different States and Territories of Brazil, from Hogue and Romano Hoge, 1978/79. In the opposite page there is a map of Brazil with the indication of the States and Territories.

ACRE

Bothrops atrox (Linnaeus)
Bothrops bilineatus smaragdinus Hoge
Bothrops brazili Hoge
Bothrops castelnaudi castelnaudi Duméril, Bibron et Duméril
Bothrops hyoprorus Amaral
Lachesis muta muta (Linnaeus)
Micrurus annelatus bolivianus Roze (provável)

ALAGOAS

Bothrops erythromelas Amaral
Bothrops leucurus Wagler
Crotalus durissus cascavella Wagler
Lachesis muta rhombeata (Wied)
Micrurus ibiboboca (Merrem)

TERRITÓRIO DO AMAPÁ

Bothrops atrox (Linnaeus)
Bothrops bilineatus bilineatus (Wied)
Bothrops brazili Hoge
Bothrops castelnaudi castelnaudi Duméril, Bibron et Duméril

Crotalus durissus (subespécie)
Lachesis muta muta (Linnaeus)
Micrurus lemniscatus lemniscatus (Linnaeus)
Micrurus lemniscatus helleri Schmidt e Schmidt
Micrurus surinamensis surinamensis (Cuvier)

AMAZONAS

Bothrops atrox (Linnaeus)
Bothrops bilineatus smaragdinus Hoge
Bothrops brazili Hoge
Bothrops castelnaudi castelnaudi Duméril, Bibron et Duméril
Bothrops hyoprorus Amaral
Bothrops neuwiedi neuwiedi Wagler
Crotalus durissus terrificus (Laurenti)
Lachesis muta muta (Linnaeus)
Micrurus albicinctus Amaral
Micrurus annelatus bolivianus Roze
Micrurus averyi (Schmidt)
Micrurus filiformis filiformis (Günther)
Micrurus filiformis subtilis Roze
Micrurus hemprichii hemprichii (Jan)
Micrurus hemprichii ortoni Schmidt
Micrurus karlchmidtii Romano
Micrurus langsdorffi langsdorffi Wagler
Micrurus lemniscatus helleri Schmidt et Schmidt
Micrurus spixii spixii Wagler
Micrurus spixii obscurus (Jan)
Micrurus surinamensis nattereri Schmidt
Micrurus surinamensis surinamensis (Cuvier)

BAHIA

Bothrops bilineatus bilineatus (Wied)
Bothrops erythromelas Amaral
Bothrops jararaca (Wied)
Bothrops jararacussu Lacerda
Bothrops leucurus Wagler

Bothrops neuwiedi neuwiedi Wagler
Bothrops neuwiedi lutzi (Miranda-Ribeiro)
Bothrops pirajai Amaral
Bothrops pradoi (Hoge)
Crotalus durissus cascavella Wagler
Lachesis muta rhombeata (Wied)
Micrurus frontalis brasiliensis Roze
Micrurus lemniscatus carvalhoi Roze

CEARÁ

Bothrops erythromelas Amaral
Bothrops neuwiedi piauhyensis Amaral
Crotalus durissus cascavella Wagler
Lachesis muta rhombeata (Wied)
Micrurus ibiboboca (Merrem)
Micrurus lemniscatus carvalhoi Roze

ESPIRITO SANTO

Bothrops bilineatus bilineatus (Wied)
Bothrops jararaca (Wied)
Bothrops jararacussu Lacerda
Bothrops neuwiedi meridionalis Müller
Bothrops pradoi (Hoge)
Lachesis muta rhombeata (Wied)
Micrurus corallinus (Merrem)

GOIAS

Bothrops castelnaudi castelnaudi Duméril, Bibron et Duméril
Bothrops itapetiningae (Boulenger)
Bothrops moojeni Hoge
Bothrops neuwiedi goyazensis Amaral
Crotalus durissus collilineatus Amaral
Lachesis muta muta (Linnaeus)
Micrurus frontalis brasiliensis Roze
Micrurus lemniscatus carvalhoi Roze

MARANHÃO

- Bothrops atrox* (Linnaeus)
Bothrops bilineatus bilineatus (Wied)
Bothrops castelnaudi castelnaudi Duméril, Bibron et Duméril
Bothrops erythromelas Amaral
Bothrops moojeni Hoge
Bothrops neuwiedi piauhyensis Amaral
Crotalus durissus cascavella Wagler
Lachesis muta muta (Linnaeus)
Micrurus ibiboboca (Merrem)
Micrurus spixii martiusi Schmidt

MATO GROSSO (incluindo Mato Grosso do Sul)

- Bothrops alternatus* Duméril et Duméril
Bothrops bilineatus smaragdinus Hoge
Bothrops brazili Hoge
Bothrops castelnaudi castelnaudi Duméril, Bibron et Duméril
Bothrops hyoprorus Amaral
Bothrops itapetiningae (Boulenger)
Bothrops jararaca (Wied)
Bothrops jararacussu Lacerda
Bothrops moojeni Hoge
Bothrops neuwiedi boliviensis Amaral
Bothrops neuwiedi diporus Cope
Bothrops neuwiedi matogrossensis Amaral
Crotalus durissus collilineatus Amaral
Crotalus durissus terrificus (Laurenti)
Lachesis muta muta (Linnaeus)
Micrurus albicinctus Amaral
Micrurus corallinus (Merrem)
Micrurus donosi Hoge, Cordeiro e Romano
Micrurus frontalis frontalis (Duméril, Bibron et Duméril)
Micrurus frontalis pyrrhocryptus (Cope)
Micrurus lemniscatus carvalhoi Roze
Micrurus spixii martiusi Schmidt

MINAS GERAIS

- Bothrops alternatus* Duméril et Duméril
Bothrops erythromelas Amaral
Bothrops fonsecai Hoge et Belluomini
Bothrops itapetiningae (Boulenger)
Bothrops jararaca (Wied)
Bothrops jararacussu Lacerda
Bothrops neuwiedi urutu Lacerda
Crotalus durissus cascavella Wagler
Crotalus durissus collilineatus Amaral
Crotalus durissus terrificus (Laurenti)
Lachesis muta rhombeata (Wied)
Micrurus frontalis brasiliensis Roze
Micrurus lemniscatus carvalhoi Roze

PARÁ

- Bothrops atrox* (Linnaeus)
Bothrops brazili Hoge
Bothrops castelnaudi Duméril, Bibron et Duméril
Bothrops marajoensis Hoge
Bothrops bilineatus bilineatus (Wied)
Crotalus durissus marajoensis (Ilha de Marajó) Hoge
Crotalus durissus (subspécie)
Lachesis muta muta (Linnaeus)
Micrurus collaris (Schlegel)
Micrurus filiformis filiformis (Günther)
Micrurus hemprichii hemprichii (Jan)
Micrurus lemniscatus helleri Schmidt et Schmidt
Micrurus lemniscatus lemniscatus (Linnaeus)
Micrurus paraensis Cunha et Nascimento
Micrurus spixii martiusi Schmidt
Micrurus surinamensis surinamensis (Cuvier)

PARAÍBA

- Bothrops erythromelas* Amaral
Crotalus durissus cascavella Wagler
Lachesis muta rhombeata (Wied)
Micrurus ibiboboca (Merrem)

PARANA

Bothrops alternatus Duméril et Duméril
Bothrops cotiara (Gomes)
Bothrops itapetiningae (Boulenger)
Bothrops jararaca (Wied)
Bothrops jararacussu Lacerda
Bothrops moojeni Hoje
Bothrops neuwiedi diporus Cope
Bothrops neuwiedi paranaensis Amaral
Crotalus durissus terrificus (Laurenti)
Micrurus corallinus (Merrem)
Micrurus decoratus (Jan)
Micrurus frontalis altirostris (Cope)
Micrurus frontalis frontalis (Duméril, Bibron et Duméril)
Micrurus lemniscatus carvalhoi Roze

RIO DE JANEIRO

Bothrops bilineatus bilineatus (Wied)
Bothrops fonsecai Hoge et Belluomini
Bothrops jararaca (Wied)
Bothrops jararacussu Lacerda
Bothrops neuwiedi meridionalis Müller
Lachesis muta rhombeata (Wied)
Micrurus corallinus (Merrem)
Micrurus decoratus (Jan)

RIO GRANDE DO NORTE

Bothrops erythromelas Amaral
Crotalus durissus cascavella Wagler
Lachesis muta rhombeata (Wied)
Micrurus ibiboboca (Merrem)
Micrurus lemniscatus carvalhoi Roze

PERNAMBUCO

Bothrops erythromelas Amaral
Bothrops neuwiedi piauhyensis Amaral
Crotalus durissus cascavella Wagler
Lachesis muta rhombeata (Wied)
Micrurus ibiboboca (Merrem)
Micrurus lemniscatus carvalhoi Roze

RIO GRANDE DO SUL

Bothrops alternatus Duméril et Bibron
Bothrops cotiara (Gomes)
Bothrops jararaca (Wied)
Bothrops neuwiedi pubescens (Cope)
Crotalus durissus terrificus (Laurenti)
Micrurus decoratus? (Jan)
Micrurus frontalis altirostris (Cope)
Micrurus frontalis pyrrhocryptus (Cope)

PIAUÍ

Bothrops erythromelas Amaral
Bothrops iglesiasi Amaral
Bothrops neuwiedii piauhyensis Amaral
Crotalus durissus cascavella Wagler
Lachesis muta rhombeata (Wied)
Micrurus ibiboboca (Merrem)

TERRITÓRIO DE RONDÔNIA

Bothrops atrox (Linnaeus)
Bothrops bilineatus smaragdinus Hoge
Bothrops brazili Hoge
Bothrops castelnaudi castelnaudi Duméril, Bibron et Duméril
Bothrops hyvoratus Amaral

Crotalus durissus terrificus (Laurenti)
Lachesis muta muta (Linnaeus)
Micrurus spixii martiusi Schmidt
Micrurus spixii spixii Wagler

TERRITÓRIO DE RORAIMA

Bothrops atrox (Linnaeus)
Bothrops bilineatus smaragdinus Hoge
Bothrops castelnaudi castelnaudi Duméril, Bibron et Duméril
Crotalus durissus ruruima Hoge
Crotalus durissus sp.
Lachesis muta muta (Linnaeus)
Micrurus lemniscatus helleri Roze
Micrurus surinamensis nattereri Schmidt

SANTA CATARINA

Bothrops alternatus Duméril et Duméril
Bothrops jararaca (Wied)
Bothrops jararacussu Lacerda
Crotalus durissus terrificus (Laurenti)
Micrurus corallinus (Merrem)
Micrurus decoratus (Jan)
Micrurus frontalis frontalis (Duméril, Bibron et Duméril)
Micrurus frontalis altirostris (Cope)
Micrurus frontalis baliocorynphus (Cope)

SÃO PAULO

Bothrops alternatus Duméril et Duméril
Bothrops cotiara (Gomes)
Bothrops fonsecai Hoge et Belluomini
Bothrops insularis (Amaral) (Ilha de Queimada Grande)
Bothrops itapetiningae (Boulenger)
Bothrops jararaca (Wied)
Bothrops jararacussu Lacerda
Bothrops moojeni Hoge

Bothrops neuwiedi pauloensis Amaral
Bothrops neuwiedi urutu Lacerda
Crotalus durissus collilineatus Amaral
Crotalus durissus terrificus (Laurenti)
Micrurus corallinus (Merrem)
Micrurus decoratus (Jan)
Micrurus frontalis altirostris (Cope)
Micrurus lemniscatus carvalhoi Roze

SERGIPE

Bothrops erythromelas Amaral
Bothrops leucurus Wagler
Crotalus durissus cascavella Wagler
Lachesis muta rhombifera (Wied)
Micrurus ibiboboca (Merrem)

6.2. Appendix II

6.2.1. Institutions and Suppliers

6.2.1.1. Institutions

Fundação Ezequiel Dias (FUNED)
Rua Conde Pereira Carneiro, 80 - Gameleira,
30550-010 Belo Horizonte, MG.
Brazil

Instituto Butantan
Avenida Vital Brazil, 1500 - Butantan
05504-901 São Paulo, SP
Brazil

Universidade Federal de Minas Gerais (UFMG)
Av. Antônio Carlos 6627 - Pampulha
31270-901 Belo Horizonte, MG
Brazil

6.2.1.2. Suppliers.

Bench centrifuge: C.411 model. S. A. Jouan, 130 Western Road, Tring, Hertz HP23, 4BU, UK.

CO₂ incubator: Flow Laboratories, Woodcock Hill , Harefield Road, Rickmansworth, Hertz WD3 1PQ, UK

Dialysis tubing and Dialysis tubing clips: Medicell International Ltd. 239, Liverpool Road, London, N1, UK

Large electrophoresis tank: Chemical Laboratory (Chemlab) Instruments Ltd. 129 Upminster Road, Hornchurch, Essex RM11 3XJ, UK

Electrophoresis units: Paines and Byrne Ltd. Pabyrn Laboratories, 177/179 Bilton Road, Perivale, Greenford, Middlesex UB6 7HG, UK

Inverted Microscope: Olympus CK, Olympus Optical Co., 43-2 Hatagaya 2 Chome, Shibuya-Ku, Tokio, Japan.

Laminar flow tissue culture hood: Flow Laboratories, Woodcock Hill, Harefield Road, Rickmansworth, Hertz WD3 1 PQ, UK

Glass Agar plates (8 X 8 cm) and (5 X 3 cm) from the University of Birmingham UK

Liquid Nitrogen containers: Cryoservices, Blackpole Trading Estate, Blackpole Road, Worcester WR3 8SG, UK

Microtitration plates: Flow Laboratories, Woodcock Hill, Harefield Road, Rickmansworth, Hertz WD3 1PQ, UK

Gel plate storage boxes: Sterilin Ltd., Lampton House, Lampton Road, Hounslow, Middlesex, TW3 4EE, UK.

Micropipettes, (Finnpipette): Jencon Scientific Ltd, Oxford, Cherry Court Way, Industrial Estate, Leighton Buzzard, Bedford LU7 8 UA, UK

SDS-PAGE: Mini apparatus: Pharmacia/LKB Biotechnology, Pharmacia House, Midsummer boulevard, Milton Keynes MK9 3HP, UK.

Western Blot Apparatus: Biorad. Laboratories Ltd. Caxton Way Holywell Industrial Estate, Watford, Hertz WD1 8RP, UK

Millipore Filters: (0.2 and 0.45 µm pore size): Millipore (UK) Ltd. 11/15 Peterborough Road, Harrow, Middlesex HA1 2YH, UK.

Shaker Incubator: New Brunswick Scientific (UK) Ltd. 26-34 Emerald Street, London, WC1N 3QA, UK

Tissue culture 24-well plate: Linbro, Flow Laboratories, as above

Tissue culture 96 well plate: Linbro, Flow Laboratories, as above

Tissue culture flat-bottomed flasks and disposable pipettes: Sterilin Ltd. as above.

FCA and FIA: Difco Laboratories Ltd, PO Box 14 B, Central Avenue, East Moseley, Surrey, KT8 0SE, UK

DEAE-Sephadex, Protein A-Shepharose CL 4B, Pharmacia/LKB as above.

Enzyme, (HRP), Molecular weight markers, Agarose, Bromophenol Blue, Coomassie Brilliant Blue R, Enzyme substrates, Trypan Blue, Tween-20, Pristane, and PEGs: Sigma Chemical Co. Ltd.

Foetal Calf Serum, HRPMI, RPMI-1640 with L-glutamine, Penicillin, Streptomycin: Gibco Europe, Ltd., UK.

Methotrexate: Lederle Laboratories Division, American Cyanamid Company, Pearls River, New York, USA.

6.3. Appendix III

6.3.1. Recipes for solutions and buffers

Conjugate preparation: Horse-radish peroxidase (HRP).

Enzyme: Horse-radish peroxidase (HRP) Type VI-A, RZ approx. 3.0, salt-free powder

Sodium Borohydride Crystalline, approx. 98%.

Sodium *m*-periodate, Crystalline.

Antibody. For Conjugate: Sheep IgG anti-rabbit IgG. supplied by The Binding Site, UK.

Buffers:

Phosphate buffered saline (PBS) 0.1 M pH 7.2-7.4

NaCl 8.0 g

KH₂PO₄ 0.2 g

Na₂HPO₄ 2.8 g

KCL 0.2 g

Make up to 1.0 litre in dw.

Sodium acetate 1.0 mM pH 4.4

Solution A- 8.2 g anhydrous sodium acetate in 1 litre of dw

Solution B- 6.005 g of glacial acetic acid in 1 litre of dw

Add one part of solution A to two parts of solution B. Dilute to 1:100 in dw to obtain working solution.

Sodium carbonate/bicarbonate buffer (1), pH 9.5

Na₂CO₃ 1.59 g (0.015 M)

NaHCO₃ 2.93 g (0.035 M)

Dissolve together in dw to make 1 litre

Sodium carbonate/bicarbonate buffer (2), 0.2 M pH 9.5

Na₂CO₃ 21.2 g, dissolved to 1 litre in dw.

NaHCO₃ 16.8 g, dissolved to 1 litre in dw

Add sufficient volume of the Na₂CO₃ solution to the 1 litre of bicarbonate to achieve pH 9.5

Borate buffer, 0.1 M pH 7.4

Boric acid 24.732 g, made up to 4 litres of dw

Borax 19.0 g made up to 500 ml in dw.

Add 115 ml of borax solution to the 4 litres of boric acid solution and adjust to pH 7.4 by further small additions.

Sodium borohydride solution

4.0 mg/ml sodium borohydride in dw.

Enzyme Assays ELISA and Immunoblotting.

ELISA.

Reagents:

o-Phenylenediamine Free Base (OPD).
Hydrogen Peroxide, 30% (w/w) solution.
Polyoxyethylenesorbitan monolaurate (Tween-20)

Buffers:

Coating buffer - Carbonate/bicarbonate buffer 0.05 M pH 9.6

Na₂CO₃ 1.59 g(0.015 M)
NaHCO₃ 2.93 g (0.035 M)

Dissolve both salts in 1 litre of dw adjust pH by addition of monobasic or dibasic salt solution of the same molarity.

Dilution/incubation buffer (PBS - Tween-20)

PBS, 0.1 M pH 7.4 (see above for Conjugate) + 0.05% (v/v) Tween-20

Washing solution:

0.9% (w/v) saline (NaCl) with 0.05% (v/v) Tween-20

Blocking solution:

Dilution/Incubation buffer + 5% (w/v) Low fat milk (J Sainsbury.plc.)

Substrate buffer for OPD pH 5.0

Citric acid 7.3 g
Na₂HPO₄.2H₂O 11.86 g

Dissolve both in 1 litre of dw adjust pH with acid or dibasic salt at the same molar concentration

Substrate solution:

Made up fresh, before. Dissolve OPD 40 mg/100 ml of substrate buffer, mix and add 20μl of 30% H₂O₂ (Hydrogen Peroxide)

Stopping solution:

20 % (v/v) H₂SO₄ in dw.

Alkaline Phosphatase buffer.

0.1 M Diethanolamine+ HCl + 1 mM Mg Cl₂ pH 9.8

95 ml of Diethanolamine

800 ml of dw

100 mg of MgCl₂. 6H₂O + 200 g NaN₃

Adjust pH to 9.8 with 1 N HCl. Complete to 1 litre.

Soluble substrate

N-p-nitrophenil alkaline phosphatase (PNPP). Tablets of 5 mg each.

Substrate solution for Alkaline phosphatase

Dissolve 10 mg of PNPP in 10 ml of substrate buffer.

Immunoblotting:

Electroblot apparatus from Bio-Rad

Nitrocellulose membrane 0.45 µm (Schleicher and Schuell)

Enzyme Substrate:

Diaminobenzidine (DAB)

Hydrogen Peroxide (H₂O₂)

Incubation/ Dilution/Washing/Blocking buffer.

PBS + 0.1 % (v/v) Tween-20

Transfer buffer. Same as Polyacrylamide electrode buffer but without SDS and with 20% (v/v) methanol.

Substrate buffer (DAB)

Tris 50 mM pH 7.2.

Tris 1.81 g. Dissolve in 200ml of dw. Fix pH with 5 N HCl. Complete volume to 300 ml and check pH.

Substrate solution

10 mg of DAB/20 ml of substrate buffer. After dissolution filter in heavy grade filter paper, add 5 µl of 30% H₂O₂.

Agarose gel techniques:

Reagents:

Agarose suitable for immunoelectrophoresis.

Polyethylene Glycol (PEG) 6,000 mol wt.

Bromophenol Blue, sodium salt.

Buffers and solutions

Barbitone buffer: 0.05 M pH 8.6.

9.21 g of barbital (5.5 diethyl barbituric acid)

51. 54 g of sodium barbitone. Dissolve both to a final volume of 3.0 litre of dw. Mix and add 5 g of sodium azide. Make up to 5 litres.

PBS:prepare as shown above.

Protein destaining solution

Mix dw, glacial acetic acid and methanol in proportions 5:1:5 to volume required.

Protein staining solution.

Dissolve 5 g of Coomassie Brilliant Blue R to 1 litre of the destaining solution.

Agarose solution:

Dissolve 5 g of agarose powder to 500 ml of hot barbitone buffer. Heat and stir until total dissolution of agarose. When dissolved completely add 15 g of PEG and mix until dissolved.

Adsorbents Preparation:

Reagents:

Polyacrilamide beads (P 200) Bio-Rad Laboratories.

Glutaraldehyde 25% aqueous solution.

PBS 0.1 M pH 7.2-7.4

Glycine (free base) >99%.

KH₂PO₄ anhydrous.

Ethanolamine (free base) approx. 98%.

Buffers and solutions:

0.2 M acetate buffer pH 5.0:

Solution A. Acetic acid. 11.55 ml in 100 ml of dw.

Solution B. Sodium acetate. C₂H₃O₂Na 16.4 g Make up to 100 ml of dw.

Mix 14.8 ml of A + 35.2 ml of B, or multiples of these volumes.

0.2 M HCl-glycine buffer. pH 2.8 or 2.2.

To 100 ml of 2 N HCl and enough 2 M glycine until pH drops to 2.8 or 2.2. Complete volume to 1 litre with dw. Verify the pH.

1 M Ethanolamine pH 7.4.

Add 3 ml of Ethanolamine 99% to 17 ml of dw. Adjust pH with conc. HCl. Complete volume to 50 ml with dw. Check pH.

1M KH₂PO₄

Dissolve 136.1 g of KH₂PO₄ to 1 litre of dw.

Antivenom Absorptions:

PBS 0.1 M pH 7.2-7.4

0.1 M Glycine-HCl pH 2.5 prepared as above for Adsorbents preparation and diluted 1:2.

Polyacrylamide gel electrophoresis.

Reagents:

Acrylamide purity \geq 99%

N,N'-Methylene-bis-Acrylamide purity \geq 98%.

TEMED approx. 99%

Glycerol $>$ 99%

Molecular weight markers: MW-SDS-Blue and SDS-6 (both from Sigma)

2-Mercaptoethanol \geq 98% purity.

Ammonium persulfate (APS) purity \geq 98%

Bromopheno blue, sodium salt.

Solutions:

Acrylamide solution.

Acrylamide 29.1 g

N,N'-bis-methylene-acrylamide 0.8 g

Dissolve both reagents to a final volume of 100 ml with dw. Filter in filter paper and store at 4° C. in an ambar flask.

Resolving gel buffer (1.5M Tris-HCl, pH 8.8

27.23 g Trizma dissolved in approximate 80 ml of dw. Adjust to pH 8.8 with 1 N HCl.

Make up to 150 ml with dw. nd store at 4° C.

Stacking gel buffer 0.5 M Tris-HCl, pH 6.8

6 g Trizma dissolved in approximate 60 ml of dw. Adjust to pH 6.8 with 1 N HCl. Make up to 100ml with dw and store at 4° C.

10% SDS

Dissolve 10 g SDS in dw with gentle mixing and make up to 100 ml with dw.

Sample buffer

Distilled water 4.4 ml

0.5 M Tris-HCl, pH 6.8 1.0 ml

Glycerol 0.80 ml

10% (w/v) 1.6 ml

0.05% (w/v) bromopheno blue. 0.2 ml

10X Electrode buffer, pH 8.3

Glycine 144.2 g

Trizma 30.3 g

Dissolve in 800 ml of dw. Complete to 1.0 litre, pH should be around pH 8.3 without adjustment. Dilte 1:10 to use. Store at 4° C.

10%Ammonium pesulphate (APS)

Dissolve 0.5 g of APS to final volume of 5.0 ml with dw. Make up fresh every day prior to use.

Gel Recipes

Stacking gel preparation (SDS)	3%	5%
Acrylamide solution	0.5 ml	0.8 ml
Distilled water	3.9 ml	3.6 ml
0.5 M pH Tris-HCl, pH 6.8	0.5 ml	0.5 ml
10% SDS	50 µl	50 µl
TEMED	5.0 µl	5.0 µl
10% ammonium persulphate	17.0 µl	17.0 µl

In the non-SDS procedure the SDS volume was substituted with dw.

Resolving gel preparation	7.5%	10.0%
Acrylamide solution	3.8 ml	5.0 ml
Destilled water	8.0 ml	6.8 ml
1.5 M Tris-HCl, pH 8.8	3.0 ml	3.0 ml
TEMED	7.5 µl	7.5 µl
10% APS	50 µl	50 µl

In the non-SDS procedure the SDS volume was substituted with dw.

IgG purification DEAE.

Gel matrix: DEAE Sephadex. A-25 (Pharmacia-LKB Technology)

Eluting and Sample application buffer

Phosphate buffer 0.02 M pH 7.2

NaH₂PO₄ 0.778 g

Na₂HPO₄.12H₂O 5.16 g

Dilute both to a final volume of 2 litres with dw.

Column Washing buffer

Eluting buffer + 1 M KCl

KCl 74.55 g make up to 1 litre in Eluting buffer.

Preparation of affinity column for *B. jararaca* venom components

Reagents:

Protein A-Shepharose CL-4B

Gel beads were received pre swollen in PBS.

Dimethyl Pimelimidate dihydrochloride crystalline.

0.2 M ethanolamine pH 8.0 prepared as above.

Buffer:

0.2 M Borate buffer pH 9.0.

Solution A. 0.2 M boric acid

12.4 g in 1.0 litre of dw.

Solution B. 0.05 M solution of Borax.

19.5 g in 1.0 litre of dw.

Mix 50 ml of A + 4.9 ml of B. dilute to a total of 200 ml.

Radiolabelled Conjugate**Reagents:**

^{125}I in the form of NaI. (Radiochemicals, Amersham)

Chloramine T, sodium salt, Hydrate Crystalline.

Sodium Metabisulfite, 2.5 mg /ml in phosphate buffer.

3% BSA. 3mg in 100 ml of 0.05 M Tris-HCl pH 8.0.

10% Trichloroacetic acid (TCA). Dissolve 10 g of TCA to a final volume of 100 ml of dw.

Buffers:

0.05 M Tris-HCl pH 8.0

Dissolve 6.057 g in 500 ml of dw. Ajust pH to by adding dropwise 1 N HCl. Complete volume to 1.0 litre with dw.

0.5 M Phosphate buffer, pH 7.6

Solution A. NaHPO_4 27.8 g. Make up to 1.0 litre with dw.

Solution B. $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ 53.65 g. Make up to 1.0 litre with dw.

Mix 13 ml of A to 87 ml of B make up to 200 ml. Dilute 1:2 before use.

Monoclonal Antibodies**Reagents**

RPMI-1640 medium with L-glutamine (Gibco Europe)

Foetal Calf Serum (FCS) (Gibco Europe)

Cell line: BALB/c plasmacytoma X63/Ag8.653 (653 for short).

Solutions**Thioguanine**

Dilute 33.4 mg of Thioguanine in 100 ml of dw. Mix thouroughly for 30 min. before fixing the pH. Warm to 80° C to help dilution. Add 1 M NaOH stepwise until pH is 9.5. Filter sterilize and aliquot in 5 ml volume into sterile universals.

Aminopterin (Methotrexate):

Stock solution at 25 mg/ml (sealed vial). Dilute 90 μl to 80 ml of dw. Ajust the pH to 7.5 with 0.01 M NaOH or 0.01 M HCl. Filter to sterilize aliquot and store at -20° C.

2-Mercaptoetanol:

Two drops of concentrated 2-Me to 10 ml of saline. Filter to sterilize, aliquot and store at -20° C.

Foetal Calf Serum (FCS)

Inactivate by heating FCS flask in a water bath at 56° C for 30 min.

HT medium**A. Hypoxantine:**

Add 408 mg of Hpxoxantine in 80 ml of dw with constant stirring. Add slowly 1M NaOH until complete dissolution of Hypoxantine. Wait a couple of minutes between additions of NaOH. Complete volume to 100 ml.

B.Thymidine:

Dissolve 114 mg of thymidine in 100 ml of dw.

Combine A and B and make up to 300 ml with dw. Mix thoroughly before adjusting the pH to 10.0 with 1 M NaOH. Filter to sterilize and aliquot in volumes of 5 ml. Store at -20°C.

HAT Medium

1. Add 1 ml of Penicillin and Streptomycin solution into 500 ml of RPMI.
2. Add 100 ml of heat inactivated Foetal Calf Serum (20 %)
3. Add 5 ml each of HT and Methotrexate.
4. Add 10 drops of 2-Me. (2 drops per 100 ml of RPMI)

Polyethylene Glycol (PEG)

Weigh out 8 g of PEG 1500 into a glass universal.

Prepare 1mM NaOH in dw add a drop of phenol red to it.

Add 8 ml of this solution to the PEG and place at 37° C to dissolve the PEG.

Sterilize by autoclaving with the universal loosely capped. Mark liquid volume for detection of any loss. If some liquid is lost return to original volume by adding 1 mM NaOH (sterile).

Allow to cool, prepare 3 ml aliquots in sterile flasks and store at 4° C.

Hepes-buffered RPMI medium (HRPMI)

Is the RPMI-1640 medium buffered with 4.6 g/l Hepes. Supplement with 2%FCS.

HYBRIDOMA TECHNIQUE

Plamacytoma cell line:

The HAT-sensitive BALB/c mouse plasmacytoma X63/Ag8.653 or (653 was used). This cell line does not produce heavy or light chains of immunoglobulin.

Production of Monoclonal antibodies using X63-Ag 8.653 cell

Pre-warm Thioguanine-RPMI (ThRPMI) at 37° C in a water bath. Leave at this temperature inside the incubator.

Prepare a bucket of ice to place the cell vial from the liquid nitrogen container. Take the cells from the container and bring to the water bath inside the ice bucket.

Place the cell vial in the water bath and thaw completely. Wipe tube and place it inside the hood.

Dilute cells with 1 ml of pre-warmed medium Thioguanine-RPMI (ThRPMI) in any sterile tube.

Centrifuge at 1500 rpm. Take off supernatant. Re-suspend in 5 ml of ThRPMI pre-warmed at 37 °C.

Take out four drops, place them in a Elisa plate well and add 1 drop of Trypan blue. Count the number of viable cells in a Hemocytometer.

If needed adjust cell concentration (spin down as above, flick cells and add necessary volume of ThRPMI medium). To fuse : 1.0×10^7 cells are needed. To grow and select good cells: 0.1×10^6 cells per well are needed in a 24 well Linbro plate. In this case fill wells with ThRPMI up to a final volume of 1.5 - 2.0 ml. Grow for two weeks to harvest good cells. Change medium whenever needed. (ThRPMI medium becomes yellow, when number of cells in well is large)

FUSION PROTOCOL

1. Count the .653 cells (1.0×10^7) and spin at 1700 rpm for 5 min. Discard supernatant and re-suspend in HRPML, approx. 20 ml. Prepare two 10 ml sterile plastic syringes with 10 ml each of HRPML and leave it aside with the 26 gauge needle capped.
2. Kill mouse by ether inhalation or neck displacement and remove spleen aseptically using a flame. Place it in a sterile Petri dish with 10 ml of HRPML and wash carefully by gentle mixing. Repeat the procedure using a second Petri dish and place spleen in a third dish with 5 ml of medium.

3. Hold spleen with one syringe and puncture it with the other throughout its surface and blow out cells by injecting slowly medium from one syringe into the near surface of the spleen. Repeat with other syringe changing from one place to another, until spleen has a whitish appearance. Place four drops of the cell suspension into a well of a non-sterile microtitre plate. Add a drop of 1% Trypan blue to the cell suspension of the microtitre plate, mix cells with a pasteur pipette and count viable cells in a Hemocytometer. Usually $0.6-1.2 \times 10^8$ are fused with 10^7 plamacytoma cells.
4. Spin separately the .653 and spleen cells at 500 g for 5 min. Discard supernatants and flick cells. To each cell pellet add 10 ml of RPMI and mix gently. Mix both cell suspensions, by pouring both into a sterile round bottomed glass tube and spin down at 400 g rpm for 7 min. Remove supernatant carefully by pipetting out.
5. Add 5 drops of alkaline RPMI (room temperature), warm pellet at 37 °C in the water bath and add PEG (0.8 ml) quickly, using the pre-prepared syringe left in the incubator. Rotate tube in the water bath for $2\frac{1}{2}$ min. Add slowly 2 ml of pre-warmed media (37 °C), over a period of 2 min.
6. Add slowly 20 ml of RPMI over a period of 5 min. with a pipette.
7. Spin at 400 g for 15 min. .
8. Take out supernatant by pouring off and add 20 ml of HAT medium, pre-warmed at 37 °C.
9. Replace cap and invert tube to resuspend cells. Dispense with the aid of a Pasteur pipette one drop of the cell suspension into each well of a 96 well tissue culture Linbro plate. Skip outer wells to avoid contamination. Then add 100 µl of HAT medium to each well. Incubate at 37° C in the presence of 5% CO₂.
10. Add 50 µl of HAT (pre-warmed) on day 5 to each well.
11. Change the HAT medium on day 9 or 11. Depends on growth of cells.
12. **CHECK FOR CONTAMINATION EVERY DAY!!**

HYBRID CELLS MAINTENANCE (FUSED CELLS)

Changing of the medium on day 9 or 11 is done by taking out 3/4 of the supernatant of each well and adding 150 µl of HAT medium at 37 °C.

After change, keep growing cells until clones are big enough to be screened.

To screen clones take out individually most of the supernatant from each well and test for MAb production by Radioimmunoassay (RIA).

Top up wells with HAT at 37 °C with approx. 150 µl of medium.

Positive wells have to be transferred to 24 well Linbro plate with only 1.0 ml of HAT at the most.

After transferring cells to Linbro plates, and adding medium, mix and transfer a drop to the original well.

Keep growing hybridomas and split when necessary to the same plate if possible, identifying each with the number of the well on the original plate, e.g. A1 or F2.

Transfer all of the cells of one well to a small flask and add 5 to 10 ml of HAT medium. Top up well on the 24 well plate again.

At this stage it is important to freeze some cells and inject others into mice pre-sensitized with Pristane. Use cells from one flask.

6.4. Appendix IV

6.4.1. Reactivity index for the preparation of the venom adsorbents with proportionally added venoms.

As the original glutaraldehyde adsorbents each contained equal amounts of each venom included in that particular adsorbent, and accepting that all venoms were equally insolubilized, it can be assumed that any remaining cross-reactivity of the absorbed antivenom would be proportional to the antigenic similarity of the venoms. If one takes the ELISA results of antivenoms absorbed in such adsorbents, and fix the homologous reaction in all as 100%, and compares these with the heterologous reactions, as a relative percentage, then a reactivity index can be determined. Then, choosing a total venom content

for the adsorbent, the relative proportional amounts of each venom can be calculated, based on their percentage values..

Crossreactivity index obtained from the ELISA of the absorbed antivenoms.

Anti-*Bothrops atrox* venom % reactivity based on OD of absorbed antivenom.

Assay No.	% Reactivity								
	<i>B. atrox</i>	<i>B. jararaca</i>	<i>B. jararacussu</i>	<i>B. alternatus</i>	<i>B. moojeni</i>	<i>B. neuwiedi</i>	<i>B. cotiara</i>	<i>L. muta</i>	C. d. c
1	100	79.8	72.0	77.0	48.5	76.6	92.3	75.0	84.0
2	100	24.0	24.0	103	77.6	27.7	85.0	18.5	16.6
3	100	20.5	32.1	91.0	85.7	35.7	80.3	25	—
Average	100	41.4	42.7	90.4	71.2	46.6	85.8	39.5	33.5
$\Sigma = 451.1 = 100\%$	9.17	9.46	20.0	15.7	10.3	19.0	8.75	7.42	$\Sigma = 451.1$

Anti-*Bothrops jararacussu* venom % reactivity based on OD of absorbed antivenom.

Assay No.	% Reactivity								
	<i>B. jararacussu</i>	<i>B. jararaca</i>	<i>B. alternatus</i>	<i>B. atrox</i>	<i>B. moojeni</i>	<i>B. neuwiedi</i>	<i>B. cotiara</i>	<i>L. muta</i>	C. d. c
1	100	—	2.6	—	12.6	33.3	4.6	—	—
2	100	—	5.9	—	15.4	46.1	—	—	—
3	100	—	—	—	19.3	36.6	8.13	—	—
4	100	6.3	3.5	—	10.46	32.5	48.2	—	—
5	100	—	30.5	39.0	29.3	27.5	21.9	—	—
6	100	—	30.5	39.0	117.0	89	21.9	45.1	2.92
Average	100	1.05	7.08	6.5	34	44.0	41.4	15.45	0.48
$\Sigma = 150 = 100\%$	0.7	4.72	4.3	22.6	29.3	27.6	10.3	0.32	$\Sigma = 150$

Anti-*Bothrops moojeni* venom % reactivity based on OD of absorbed antivenom.

Assay No.	% Reactivity								
	<i>B. moojeni</i>	<i>B. jararaca</i>	<i>B. jararacussu</i>	<i>B. alternatus</i>	<i>B. atrox</i>	<i>B. neuwiedi</i>	<i>B. cotiara</i>	<i>L. muta</i>	C. d. c
1	100	7.8	53.8	24.7	24.0	65.5	29.0	14.6	9.7
2	100	10.0	44.3	26.0	30.7	48.4	19.0	24.8	11.2
3	100	26.6	25.5	24.6	24.6	37.0	21.0	20.0	20.0
4	100	12.7	75.6	46.0	44.0	60.0	54.0	32.0	18.4
Average	100	14.3	49.8	25.0	30.8	52.85	30.7	15.3	10.3
$\Sigma = 229 = 100\%$	6.2	21.7	11.0	13.4	23.0	13.4	6.68	4.5	$\Sigma = 229$

Anti-*Bothrops alternatus* venom % reactivity based on OD of absorbed antivenom.

Assay No.	% Reactivity								
	<i>B. alternatus</i>	<i>B. jararaca</i>	<i>B. jararacussu</i>	<i>B. atrox</i>	<i>B. moojeni</i>	<i>B. neuwiedi</i>	<i>B. cotiara</i>	<i>L. muta</i>	C. d. c
1	100	—	9.0	19.4	14.0	8.0	38.4	—	—
2	100	12.7	18.0	69.6	6.0	31.3	80.0	17.6	5.88
3	100	21.6	20.8	23.7	18.2	18.6	44.8	18.6	17.3
4	100	17.3	27.2	13.0	11.8	15.4	32.0	9.45	10.6
5	100	14.0	11.0	34.4	27.0	12.0	32.0	11.0	7.0
Average	100	13.0	17.2	32.0	13.4	17.0	45.0	11.3	8.1
$\Sigma = 157 = 100\%$	8.3	11.0	20.4	8.5	11.0	29.0	7.2	5.2	$\Sigma = 157$

To find the amount of venom to add from each species, the numbers in bold in the last row of the tables were used. So from a total of 20 mg of venom, in the example of *B. alternatus* in table IV, it would be as follows:

<i>B. jararaca</i>	8.30% of 20 mg	1.7 mg
<i>venom</i>	=	
<i>B. jararacussu</i>	11.0% of 20 mg	2.2 mg
<i>venom</i>	=	
<i>B. atrox venom</i>	20.4% of 20 mg	4.1 mg
	=	
<i>B. moojeni</i>	8.50% of 20 mg	1.7 mg
<i>venom</i>	=	
<i>B. neuwiedi</i>	11.0% of 20 mg	2.2 mg
<i>venom</i>	=	
<i>B. cotiara venom</i>	29.0% of 20 mg	5.8 mg
	=	
<i>L. muta venom</i>	7.20% of 20 mg	1.4 mg
	=	
<i>C.d. collineatus</i>	5.20% of 20 mg	1.0 mg
<i>venom</i>	=	
	TOTAL=	20.0 mg

6.5. Appendix V

6.5.1. Decrease in the IgG content of the absorbed antivenoms.

IgG content decrease in the absorbed antivenoms

Purified IgG (DEAE)	Un-absorbed Conc. of IgG (mg/ml)	No. of absorptions	Absorbed Conc. of IgG (mg/ml)	Decrease ratio	No. of absorptions	Absorbed Conc. of IgG (mg/ml)	Decrease ratio
anti- <i>B. jararaca</i>	9.3	2	1	9	4	0.66	14.0
anti- <i>B. jararacussu</i>	10.1	2	1.7	6	4	1.1	9.2
anti- <i>B. alternatus</i>	7.6	2	1.25	6	4	0.77	9.9
anti- <i>B. atrox</i>	12.0	2	1.03	12	4	0.44	27.0
anti- <i>B. moojeni</i>	11.3	2	1.36	8	4	0.4	28.0
anti- <i>B. neuwiedi</i>	19.3	2	3.6	5	4	0.37	52.0

The concentration of IgG was measured by the absorption at 280nm using the extinction coefficient shown in the Material and Methods section.

6.6. Appendix VI

Here are presented the results of the RIA for the screening of clones for antivenom activity and the screening for *B. moojeni* venom specificity of clones and ascitic fluids.

Results from the screening of clones for anti-*B. mojeni* reactivity. Positive clones were further cultured by the limiting dilution method.

4	1	32	.9	.9
4	2	38	3.8	805.1 AF 5
4	3	39	4.2	676.8
4	4	40	4.3	636.5 AG 9
4	5	41	4.2	625.6
4	6	42	3.8	833.1 AG 10
4	7	43	3.6	863.3
4	8	44	3.9	818.1 BB 5
4	9	45	4.3	651.5
4	10	46	12.1	125.0 - V 0
4	11	47	43.2	21.8
4	12	48	57.7	15.2

5	1	49	-99.9	.2
5	2	50	3.2	1000.4 BB 6
5	3	51	3.6	933.8
5	4	52	7.5	247.6 BB 1
5	5	53	6.7	271.6 BC 7
5	6	54	4.3	662.3
5	7	55	5.4	414.5
5	8	56	3.4	1024.3 BC 11
5	9	57	4.2	673.8
5	10	58	-99.9	8.5
5	11	59	35.6	27.3
5	12	60	51.4	17.3

6	1	61	93.2	8.8
6	2	62	3.7	837.2 BD 3
6	3	63	3.8	811.9
6	4	64	3.6	869.6 BD 5
6	5	65	3.7	802.6
6	6	66	8.3	7225.4 BD 10
6	7	67	7.2	251.1
6	8	68	6.9	291.7 BD 11
6	9	69	6.5	314.7
6	10	70	-99.9	2.1
6	11	71	47.4	19.6
6	12	72	34.1	22.9

7	1	73	70.0	12.1
7	2	74	3.3	1035.0 BE 3
7	3	75	3.7	850.3
7	4	76	16.3	82.7 BE 4
7	5	77	11.3	118.6
7	6	78	3.6	914.1 BE 8
7	7	79	4.0	712.3
7	8	80	3.7	904.9 BE 10
7	9	81	3.7	878.4
7	10	82	63.2	16.9
7	11	83	32.3	30.7
7	12	84	-99.9	5.7

8	1	85	.0	.0
8	2	86	-99.9	7.5
8	3	87	58.9	16.9
8	4	88	4.9	496.0
8	5	89	5.8	351.6
8	6	90	3.7	886.8
8	7	91	9.9	151.8
8	8	92	4.8	547.6
8	9	93	5.9	495.2
8	10	94	-99.9	7.4
8	11	95	63.8	14.0
8	12	96	-99.9	7.8
9	1	97	56.5	15.3
9	2	98	3.8	796.5
9	3	99	3.8	817.4
9	4	100	8.3	213.8
9	5	101	7.1	245.6
9	6	102	4.0	755.4
9	7	103	4.3	625.4
9	8	104	3.6	911.6
9	9	105	3.7	852.8
9	10	106	2.8	1464.4
9	11	107	45.2	20.2
9	12	108	39.2	23.7

10	1	109	45.4	19.6
10	2	110	3.4	977.2
10	3	111	9.9	161.8
10	4	112	3.6	884.9
10	5	113	3.8	741.6
10	6	114	3.9	818.9
10	7	115	3.8	782.6
10	8	116	4.3	657.9
10	9	117	11.7	119.4
10	10	118	2.7	1496.2
10	11	119	38.2	25.1
10	12	120	57.7	15.2

11	1	121	-99.9	5.6
11	2	122	3.6	886.3
11	3	123	52.7	19.1
11	4	124	4.3	628.3
11	5	125	4.8	496.6
11	6	126	3.5	957.9
11	7	127	3.5	981.6
11	8	128	3.8	829.3
11	9	129	3.8	817.2
11	10	130	2.7	1510.0
11	11	131	-99.9	7.3
11	12	132	61.6	14.2

12	1	133	47.7	18.6
12	2	134	4.5	591.3
12	3	135	4.6	573.6
12	4	136	6.4	323.9
12	5	137	5.8	349.6
12	6	138	3.9	895.7
12	7	139	3.8	790.9
12	8	140	5.6	414.6
12	9	141	5.8	381.4
12	10	142	12.8	115.4
12	11	143	55.9	16.2
12	12	144	34.1	27.9

13	1	145	43.4	20.7
13	2	146	7.6	233.1
13	3	147	9.0	182.1
13	4	148	4.2	664.9
13	5	149	4.0	692.6
13	6	150	9.8	125.0
13	7	151	9.1	124.6
13	8	152	4.9	518.3
13	9	153	5.0	541.4
13	10	154	-99.9	2.4
13	11	155	59.6	15.1
13	12	156	.0	.0

14	1	157	47.7	18.6
14	2	158	6.5	310.1
14	3	159	7.0	284.8
14	4	160	4.4	604.9
14	5	161	4.2	628.6
14	6	162	4.9	542.9
14	7	163	4.9	495.1
14	8	164	4.9	525.0
14	9	165	5.2	460.3
14	10	166	-99.9	1.1
14	11	167	68.8	12.9
14	12	168	71.3	12.1

15	1	169	33.4	20.3
15	2	170	3.7	828.6
15	3	171	3.7	869.0
15	4	172	3.6	861.4
15	5	173	3.5	852.6
15	6	174	4.7	567.9
15	7	175	4.5	579.9
15	8	176	3.6	912.8
15	9	177	4.1	216.0
15	10	178	82.6	12.7
15	11	179	31.4	31.8
15	12	180	37.8	24.7

16	1	181	.0	.0
16	2	182	7.8	226.7
16	3	183	7.6	247.5
16	4	184	5.5	408.4
16	5	185	4.8	491.6
16	6	186	5.3	472.8
16	7	187	4.9	493.4
16	8	188	17.8	68.5
16	9	189	16.9	70.0
16	10	190	-99.9	3.2
16	11	191	45.2	20.7
16	12	192	23.2	14.8

17	1	193	64.7	13.2
17	2	194	3.6	872.4
17	3	195	3.6	926.1
17	4	196	6.4	321.9
17	5	197	5.8	350.6
17	6	198	3.7	824.7
17	7	199	3.9	726.8
17	8	200	4.5	639.6
17	9	201	4.7	554.8
17	10	202	2.9	1382.9
17	11	203	29.7	34.0
17	12	204	51.4	17.3

18	1	205	.0	.0
18	2	206	4.2	651.1
18	3	207	15.8	82.8
18	4	208	4.3	643.6
18	5	209	4.7	594.6
18	6	210	3.9	797.8
18	7	211	4.4	587.2
18	8	212	6.0	370.6
18	9	213	5.7	396.9
18	10	214	2.9	1382.8
18	11	215	36.9	+ve
18	12	216	61.6	14.2
19	1	217	56.5	15.3
19	2	218	6.5	319.8
19	3	219	7.3	269.2
19	4	220	4.3	637.5
19	5	221	4.1	659.6
19	6	222	3.7	839.2
19	7	223	3.7	830.2
19	8	224	5.6	410.1
19	9	225	5.5	419.2
19	10	226	7.8	1434.8
19	11	227	59.6	+ve
19	12	228	49.8	15.1
				22.6

20	1	229	56.5	15.3
20	2	230	5.7	386.6
20	3	231	5.9	371.6
20	4	232	5.0	493.9
20	5	233	4.7	585.6
20	6	234	4.2	712.7
20	7	235	4.2	653.5
20	8	236	4.1	729.0
20	9	237	4.1	693.2
20	10	238	10.2	161.0
20	11	239	34.4	28.4
20	12	240	71.3	12.1

21	1	241	-99.9	2.4
21	2	242	4.3	641.5
21	3	243	4.5	618.6
21	4	244	4.4	642.9
21	5	245	4.2	628.6
21	6	246	4.3	629.8
21	7	247	4.8	589.4
21	8	248	4.9	528.4
21	9	249	7.0	271.3
21	10	250	67.1	15.9
21	11	251	32.3	30.7
21	12	252	54.4	16.3

22	1	253	-99.9	4.5
22	2	254	4.2	651.1
22	3	255	4.3	659.3
22	4	256	7.2	265.9
22	5	257	6.1	315.6
22	6	258	6.6	319.5
22	7	259	6.4	311.1
22	8	260	6.5	312.6
22	9	261	6.1	344.7
22	10	262	-99.9	4.2
22	11	263	49.9	18.4
22	12	264	28.9	34.3

23	1	265	93.2	8.8
23	2	266	3.8	785.8
23	3	267	4.1	731.2
23	4	268	3.7	812.6
23	5	269	3.5	858.6
23	6	270	4.5	631.6
23	7	271	4.7	535.5
23	8	272	3.9	843.4
23	9	273	3.9	746.1
23	10	274	-99.9	2.1
23	11	275	30.5	32.9
23	12	276	46.5	19.5

24	1	277	.0	.0
24	2	278	5.2	419.1 DF5
24	3	279	4.9	520.2
24	4	280	5.1	463.4 DF7
24	5	281	14.2	84.6
24	6	282	5.0	506.8 DF8
24	7	283	5.1	460.8
24	8	284	3.9	811.2 DF10
24	9	285	3.8	825.0
24	10	286	-99.9	9.5
24	11	287	39.7	24.0
24	12	288	33.1	29.0

25	1	289	-99.9	3.4
25	2	290	4.1	603.2 DF11
25	3	291	4.3	652.7
25	4	292	4.9	501.1 DG2
25	5	293	4.6	529.6
25	6	294	4.1	744.4 DG3
25	7	295	4.4	590.3
25	8	296	46.2	21.2 DG4
25	9	297	57.3	15.6
25	10	298	3.9	1296.1
25	11	299	36.9	26.2
25	12	300	48.0	18.4

26	1	301	76.2	11.0
26	2	302	4.7	540.5 DG5
26	3	303	7.8	234.3
26	4	304	5.2	460.3 DG6
26	5	305	35.1	26.6
26	6	306	3.9	799.2 DG9
26	7	307	3.9	743.3
26	8	308	32.1	32.5 DG10
26	9	309	5.6	404.7
26	10	310	2.9	1306.7 + Ve
26	11	311	41.4	22.9
26	12	312	44.4	20.5

27	1	313	-99.9	5.6
27	2	314	10.8	134.7 EB2
27	3	315	13.2	106.9
27	4	316	18.6	69.4 EB6
27	5	317	13.8	88.6
27	6	318	5.7	410.4 EB8
27	7	319	5.9	358.6
27	8	320	-99.9	7.7 EB10
27	9	321	4.6	571.5
27	10	322	2.9	1334.2
27	11	323	32.3	30.7
27	12	324	-99.9	7.8

28	1	325	" 0	" 0
28	2	326	12.8	104.8] EC3
28	3	327	14.6	92.2]
28	4	328	14.8	93.9]
28	5	329	10.9	124.6]
28	6	330	4.6	591.0] EC11
28	7	331	4.4	636.8] ED3
28	8	332	40.6	24.6
28	9	333	41.9	22.2
28	10	334	14.5	97.4] -ve
28	11	335	55.9	16.2
28	12	336	85.2	9.9

29	1	337	-99.9	2.4
29	2	338	21.0	53.5] ED4
29	3	339	19.9	63.0]
29	4	340	8.1	220.0] ED5
29	5	341	7.0	232.6]
29	6	342	17.1	79.7] ED6
29	7	343	19.0	57.2]

Test for specificity of the cultured clones that displayed anti-*B. moojeni* venom reactivity and of ascitic fluid from some of the positive clones.

EST 100% > 100%

Specificity test for *B. moegni* venom.
ASCITIC FLUIDS AND CLONES SUPERANTIGEN

AUTO

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PARAMETERS    01
01 ID          00-125   ->
02 TIME (SEC) 00 30   ->
03 IS (125-I ) 01   ->
04 REPLICATES 01   ->
05 PRINTOUTS      1,2,3,10,20,23,23,24,24,25,25,9,20,20,0
06 FIRST SEQ.NO 001   ->
07 METHOD (1-6) 00   ->
08 SPILL (0-1) 00   ->
09 SEQ-CODE     010... ->
BAL DET SEQ.      SIGMA      CORR.CPM

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1	1	1	26.2	11.0
1	2	2	.0	.0
1	3	3	31.6	34.5 B. jan
1	4	4	.0	.0 B. ju
1	5	5	-99.9	6.6 B. alt
1	6	6	41.9	22.2 B. atr
1	7	7	27.0	39.1 B. moj
1	8	8	57.1	16.7 B. red
1	9	9	95.5	8.9 B. rot
1	10	10	.0	.0
1	11	11	9.5	166.2 +ve
1	12	12	.0	.0

2	1	13	10	10
2	2	14	16.8	71.6
2	3	15	18.9	65.2
2	4	16	17.1	77.6
2	5	17	12.7	99.6
2	6	18	21.1	51.1
2	7	19	13.4	98.9
2	8	20	14.4	92.2
2	9	21	14.8	84.5
2	10	22	10.5	70.9
2	11	23	17.7	66.2
2	12	24	19.9	4.7

EE31
17-11-91

4	1	37	-99.9	4.5
4	2	38	-99.9	8.6
4	3	39	55.6	18.9 B.jan
4	4	40	"0	"0 B.jan
4	5	41	63.3	13.6 B.att
*	4	42	"0	"0 Balr
4	7	43	-99.9	1.9 B.may
4	8	44	83.2	11.1 B.nov
4	9	45	77.7	11.1 B.cet
4	10	46	-99.9	4.2
4	11	47	41.4	22.9 1+3
4	12	48	-99.9	7.8

EE31
SUPERNATANT

5	1	49	-99.9	5.6
5	2	50	"0	"0
5	3	51	"0	"0
5	4	52	"0	"0
5	5	53	"0	"0
*	5	54	"0	"0
5	7	55	57.3	16.3
5	8	56	60.8	15.6
5	9	57	40.3	18.9
5	10	58	"0	"0
5	11	59	90.5	9.6
5	12	60	-99.9	5.7

EE33
17-11-91

6	1	61	-99.9	3.4
6	2	62	-99.9	6.4
6	3	63	-99.9	8.1
6	4	64	"0	"0
6	5	65	-99.9	4.6
*	6	66	"0	"0
6	7	67	57.3	16.3
6	8	68	83.2	11.6
6	9	69	77.7	11.1
6	10	70	"0	"0
6	11	71	47.4	19.6
6	12	72	46.5	19.5

SPECIES-SPECIFIC DETECTION OF VENOM ANTIGENS FROM SNAKES OF THE *BOTHROPS* AND *LACHESIS* GENERA

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L. G. D. HENEINE and D. CATTY. Species-specific detection of venom antigens from snakes of the *Bothrops* and *Lachesis* genera. *Toxicon*, **31**, 591-603, 1993.—Venom-insoluble adsorbents were employed to absorb out the cross-reacting antibodies from monovalent polyclonal antivenoms. The absorbed antivenoms were tested by enzyme-linked immunosorbent assay against homologous and heterologous venoms and showed species-specificity throughout a range of venom concentrations. The same absorbed antisera were used in immunoblots under non-reducing conditions as probes to reveal species-specific antigens. In all cases studied this was achieved. The range of mol. wts of specific antigens was between 20,000 and 120,000, approximately. Venoms added to human serum experimentally were specifically detected by their homologous absorbed antivenom antibodies. The work here described could be important in the development of diagnostic assays for envenomings involving snakes from the *Bothrops* and *Lachesis* genera.

INTRODUCTION

SNAKE envenomings causing death or disability are a major public health problem in many countries. Specific diagnosis is made difficult by the large numbers of venomous species present within some regions and the lack of specific diagnostic reagents which could be applied to a simple and robust test system. The lack of appropriate reagents, the need for expensive equipment and/or low sensitivity and specificity of results to date, have hampered the widespread use of routine diagnostic test such as RIA and ELISA in most countries (MINTON, 1987; NELSON, 1989). The first step in providing a simple specific test is the preparation of highly specific antivenom reagents. In Brazil, specimens from the genera *Bothrops*, *Lachesis*, *Crotalus* and *Micruurus* have overlapping distributions (HOGE and ROMANO-HOGE, 1978/79). *Bothrops* species account for 90% of the snakebites in Brazil (MINISTÉRIO DA SAÚDE, 1986). Statistics based on confirmed diagnosis by the visual identification of the snake carried out by particular regional medical centres is not, however, representative of the country as a whole. CARDOSO and BRANDO (1982) showed that *Bothrops jararaca* was responsible for 93.5% of the snakebites treated in the Hospital Vital Brazil, in the state of São Paulo. KOUYOUMDJIAN and POLIZELLI (1988) showed,

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however, that *Bothrops moojeni* was the prevailing cause of snakebite (88%), with a lower prevalence of *B. alternatus* (9.5%) and *B. jararaca* (1.5%), in the northern part of the same state. The identification of species-specific epitopes of antigens is the first step towards providing the tools for the accurate diagnosis of specific venoms in the blood of patients and for epidemiological and clinical studies of evenoming in the community, and also for studies of the geographic distribution and phylogenetic relationship of snakes. We have reported previously (HENEINE *et al.*, 1990) the identification of species-specific antigens from some Brazilian bothropic species. In the present study we report on species-specific venom detection by ELISA, and the identification of species-specific antigens by immuno-blots of Brazilian snake venoms of the *Bothrops* and *Lachesis* genera.

MATERIALS AND METHODS

Venoms

The venoms of *Bothrops* species (*B. jararaca*, *B. jararacussu*, *B. alternatus*, *B. atrox*, *B. moojeni* and *B. neuwiedi*), of *Crotalus* species, (*C. durissus terrificus* and *C. durissus collineatus*) and *Lachesis muta* were provided by Fundação Ezequiel Dias, Belo Horizonte, M. G., Brazil. Venom from *B. cotiara* was a gift from Dr I. F. HENEINE (Universidade Federal de Minas Gerais, Belo Horizonte, M. G., Brazil). Venoms of each species were constituted from a pool of donor snakes and represent therefore the best approach to being representative of venom constituents of the species as a whole.

Antivenoms

Monovalent rabbit anti-*Bothrops* (*B. jararaca*, *B. jararacussu*, *B. alternatus*, *B. atrox*, *B. moojeni*, *B. neuwiedi* and *B. cotiara*), and *L. muta* antivenoms and monovalent rabbit antivenoms conjugated to alkaline phosphatase were supplied by Dr R. D. G. THEAKSTON (Liverpool School of Tropical Medicine, Liverpool, U.K.).

Venom adsorbents

Glutaraldehyde insolubilization was performed following the method of TERNYCK and AVRAMEAS (1976). Batches of various absorbing venoms (a total of 20 mg in each case) were mixed in glass containers prior to insolubilization. The proportion of venoms to be added was derived from a cross-reactivity index calculated from previous ELISA tests (results not shown). For the *B. jararaca*-specific adsorbent we used: *Bothrops* (*B. jararacussu*, 1.34 mg; *B. alternatus*, 3.2 mg; *B. atrox*, 3.8 mg; *B. moojeni*, 3.4 mg; *B. neuwiedi*, 3.28 mg; *B. cotiara*, 3.2 mg); *L. muta*, 1.34 mg, and *C. d. collineatus*, 0.48 mg, making a total of 20 mg. The same methodology was applied to the other adsorbents.

Antivenom absorption

Two millilitres of each antivenom was diluted with 1 ml of 0.1 M PBS, pH 7.4, and added to its respective adsorbent not containing the homologous venom. All were rotated for 2 hr at room temperature, centrifuged at 3000 g for 40 min, and the supernatant recovered. After a second absorption the supernatants were tested for specificity by ELISA. Repeat absorptions were performed depending on individual results. The adsorbents were washed between absorptions with 0.1 M glycine-HCl, pH 2.5, until the 280 nm optical density of the supernatants was below 0.05. The pH was raised with 1 M K₂HPO₄, and PBS was used to re-equilibrate the adsorbent. The supernatants were filtered through Millipore filters (0.45 µm) after each absorption step.

Immunoglobulin purification

This was carried out by anion-exchange as described by HARLOW (1988). Briefly, the antivenoms, after absorption to remove cross-reacting antibodies to other venom species, were dialysed overnight against three changes of 0.02 M phosphate buffer, pH 7.2. Antivenom (1.2 ml of each) was then added to plastic test tubes containing 2 ml of DEAE-Sepharose (Pharmacia LKB) equilibrated in the same buffer and rotated for 2 hr at room temperature. The tubes were then centrifuged for 30 min at 3000 g at 4°C. The supernatant containing the purified immunoglobulin (mainly IgG) was taken off and the protein concentration calculated by optical density at 280 nm, using

$$\sum_{1 \text{ mg/ml}}^{\text{A}280} = 1.35.$$

The purity was checked by acetate cellulose electrophoresis in 0.05 M barbital buffer, pH 8.6, on prepacked agarose gel plates (Corning Ltd). Traces of albumin were visible in all samples. The protein concentration was assumed to be only IgG-derived.

Species-specific venom detection

Antibody-capture and antigen-capture ELISA systems were used.

Antibody-capture ELISA. The assay was carried out essentially as described by THEAKSTON *et al.* (1977): 96-well ELISA plates (Nunc Maxisorb F96, A/S NUNC, Denmark), were coated with whole snake venoms at 1 µg/ml, 100 µl/well. The plates were then washed three times with 0.1 M phosphate buffered saline (PBS), pH 7.4, containing 0.05% of Tween-20. The absorbed antivenoms (100 µl/well) were added to the plates and incubated for 1 hr at 37°C. After washing again, sheep IgG anti-rabbit IgG conjugated to horseradish peroxidase, diluted 1 : 20,000 in PBS-Tween 0.05%, was added. Following incubation and a washing step, the substrate solution *ortho*-phenylenediamine (OPD), 40 mg/100 ml, with 20 µl of 30% (v/v) hydrogen peroxide, in citrate buffer pH 5, was added at 100 µl/well. The reaction was stopped with 20% (v/v) sulphuric acid, 50 µl/well, after 30 min incubation in the dark at 37°C. The optical density at 492 nm was measured in an ELISA reader (Titertek Multiscan, Flow Labs).

Antigen-capture ELISA. Affinity-absorbed antivenoms, as IgG fractions, at 40 µg/ml, were coated overnight onto microtitre plates. After blocking with 5% (w/v) low fat milk (J. Sainsbury plc) in coating buffer, different venom preparations, in concentrations ranging from 0.01–10 µg/ml, were added either in incubation buffer or in 1 : 50 diluted normal human serum. After incubation and washing steps, non-absorbed monovalent rabbit antivenoms as IgG fractions, conjugated to alkaline phosphatase, were added in two dilutions (1 : 50 or 1 : 500) to the wells coated with the homologous absorbed antivenom IgG and 'captured' venom antigen. The substrate, *p*-nitrophenyl phosphate (SIGMA Chemical Co.), in 10 mM diethanolamine (pH 9.5) containing 0.5 mM MgCl₂, in the presence of 30% (v/v) hydrogen peroxide was added, and the plate incubated at 37°C. The optical density was determined at 405 nm without stopping the reaction. All incubation and washing steps were as described above for the antibody-capture assay. All volumes were 50 µl/well.

Gel electrophoresis

This was performed in a non-reducing SDS/PAGE, discontinuous buffer system (LAEMMLI, 1970). Twenty-five micrograms of each venom in sample buffer was applied in a volume of 2.5 µl to a 10% acrylamide gel (slab). The gels were run at a constant current of 20 mA/gel for 1.5 hr on a Midget Electrophoresis Unit (Pharmacia LKB). Prestained standard solution of MW-SDS-7-B were used as mol. wt markers (Sigma Chemical Co.)

Immunoblotting

Western blotting procedures used were similar to that reported by HENEINE *et al.* (1990). Venom samples separated on 10% polyacrylamide gel were transferred to nitrocellulose paper using 20 mM Tris, 192 mM glycine and 20% methanol (TOWBIN *et al.*, 1979) at 0.45 A for 60 min using a Bio-Rad Trans-blot electrophoresis cell. Absorbed antivenom IgGs against *B. jararaca* and *B. alternatus* were used at 1 : 800 dilution; absorbed anti-*B. jararacussu* IgG was used at 1 : 600. Non-absorbed monovalent antivenoms were used at 1 : 2000 dilution.

RESULTS

Venom adsorbents

The glutaraldehyde method of protein insolubilization proved to be reliable and met the requirements. Large amounts of protein can be insolubilized, yielding a relatively small volume of adsorbent.

Antivenom absorption

The absorption of cross-reacting antibodies of monovalent antivenoms on venom adsorbents yielded absorbed reagents with species-specificity. The number of absorptions needed to produce species-specific antivenoms varied between the samples studied. Some antivenoms required only two absorption steps, and some had to be absorbed three to four times. Some batches of antivenom required further absorptions on adsorbents

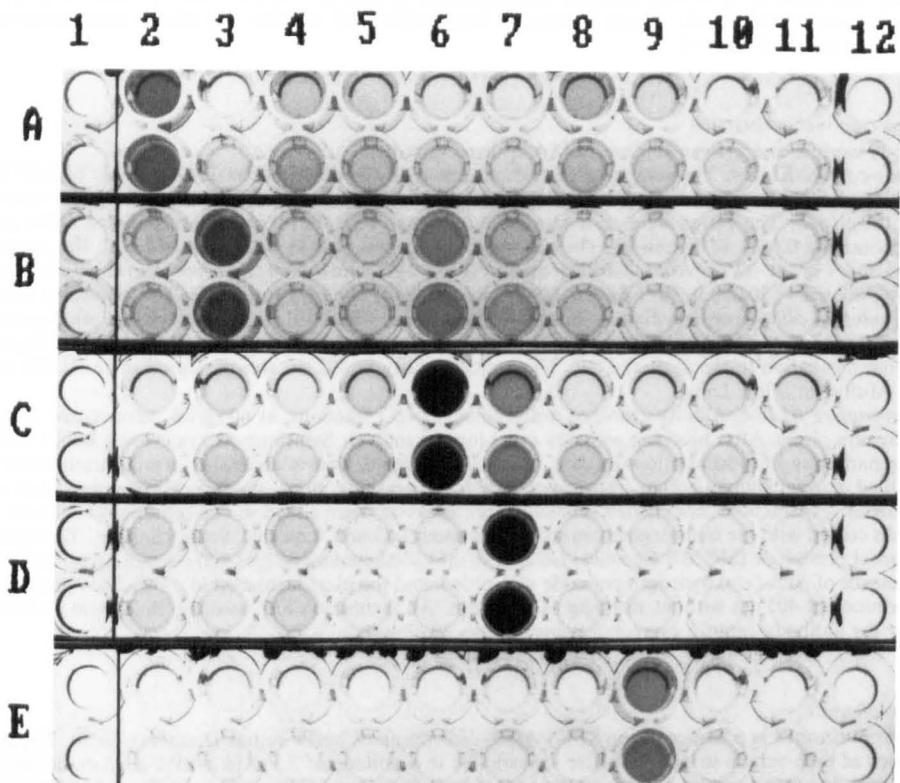


FIG. 1. REACTIVITY OF ABSORBED ANTIVENOMS AGAINST HOMOLOGOUS AND HETEROLOGOUS VENOMS. The ELISA plate was coated with venom solutions at $1 \mu\text{g}/\text{ml}$, $100 \mu\text{l}/\text{well}$. Absorbed antivenoms were added at different dilutions: A, anti-*B. jararaca*, 1 : 100; B, anti-*B. jararacussu*, 1 : 100; C, anti-*B. moojeni*, 1 : 200; D, anti-*B. neuwiedi*, 1 : 200; E, anti-*L. muta*, 1 : 200. Venoms are coated as: 2, *B. jararaca*; 3, *B. jararacussu*; 4, *B. alternatus*; 5, *B. atrox*; 6, *B. moojeni*; 7, *B. neuwiedi*; 8, *B. cotiara*; 9, *L. muta*; 10, *Crotalus durissus terrificus*; 11, *Crotalus durissus collineatus*. Column 1, buffer alone; Column 12, venom + conjugate. Conjugate, sheep anti-rabbit IgG-HRP, used at 1 : 20,000, $100 \mu\text{l}/\text{well}$.

containing a single cross-reacting venom in order to increase species-specificity. The batch procedure for the absorptions was chosen in order to avoid excessive dilution of the antivenom with the need then for subsequent concentration which might have produced a loss of antibody activity. The filtration of the supernatant was important in removing any small support matrix particles containing cross-reacting bound antibodies. These could interfere with protein quantitation and with the specificity assays.

Absorbed antivenom species-specificity

The antibody capture assay was used to assess whether the absorption process for removal of cross-reacting antibodies on venom absorbents was producing species-specific reagents, and to test the reproducibility of the absorption method. In Fig. 1, a single

TABLE 1. SUMMARY OF THE REACTIVITY (AS PERCENTAGE OF THE OD READING) BETWEEN THE SPECIES-SPECIFIC ABSORBED ANTIVENOMS AND THE HOMOLOGOUS AND HETEROLOGOUS VENOMS

Antivenoms against	Venoms							
	JAR	JSU	ALT	ATR	MOJ	NEU	COT	MUT
B. jar	100%	29 (14)	50 (5)	50 (7.5)	38 (2)	30 (2)	50 (3.5)	21 (5.8)
B. jsu	20 (5.5)	100%	12 (2)	14 (1.6)	47 (3)	30 (3)	1	18
B. atr	50 (3.5)	38 (8)	54 (2)	100%	50 (7)	50 (14)	50 (20)	24 (14)
B. moj	19 (1.7)	25 (8)	9 (2)	20 (3)	100%	41 (13)	11 (1)	8 (1.8)
B. neu	17 (4)	10 (4)	16 (3)	3.8	6.8 (1)	100%	6	8 (2)
L. mut	16 (9)	30 (5)	14	1.6	1.8	30 (15)	15 (5)	100%

Homologous reaction is 100%. Numbers in brackets are standard deviation of two experiments. Antivenoms: B. jar, anti-*B. jararaca*; B. jsu, anti-*B. jararacussu*; B. atr, anti-*B. atrox*; B. moj, anti-*B. moojeni*; B. neu, anti-*B. neuwiedi*; L. mut, anti-*L. muta*. Venoms: JAR, *B. jararaca*; JSU, *B. jararacussu*; ALT, *B. alternatus*; ATR, *B. atrox*; MOJ, *B. moojeni*; NEU, *B. neuwiedi*; COT, *B. cotiara*; MUT, *L. muta*.

dilution of absorbed antivenom was reacted with the different venoms studied. The absorbed anti-*Bothrops moojeni* (C), anti-*Bothrops neuwiedi* (D) and anti-*Lachesis muta* (E) show almost complete species-specificity. Anti-*Bothrops jararaca* (A) and anti-*Bothrops jararacussu* (B) show substantial but not complete species-specificity. To test the reproducibility of the absorption technique, two different batches of the antivenoms were then absorbed four times each on different occasions. These were added to ELISA plates coated with the different venoms in the same coating concentration, 1 µg/ml, 100 µl/well. Table 1 shows these results expressed as the mean percentage difference in OD reading between the homologous and heterologous reaction where the homologous reaction is set at 100%. All absorbed antivenoms retained high reactivity towards the homologous venom. The smallest difference between homologous and heterologous reaction of the absorbed antivenom is 46%. The standard deviation of the replicate experiments for each test is shown in brackets.

Antigen and antibody capture ELISA tests show that the species-specificity was maintained throughout a range of venom concentrations (Figs 2 and 3). In the antibody capture assay (Fig. 2), anti-*B. jararaca* (Fig. 2A), anti-*B. jararacussu* (Fig. 2B), and anti-*B. neuwiedi* (Fig. 2E) gave homologous reactions which are at least two times greater (by OD reading) than the heterologous ones, throughout the range of venom concentrations used (0.1–10 µg/ml). Anti-*B. alternatus* (Fig. 2C) showed a more significant cross-reaction against *B. cotiara* venom, as also did anti-*B. moojeni* against *B. neuwiedi* venom (Fig. 2D). Similar results were observed with the antigen-capture assay (Fig. 3), using the absorbed IgG preparations as the coating layer. The alkaline phosphatase conjugates used were from rabbit monovalent antivenom sera, which were unabsorbed. Figure 4 shows the homologous reaction of the absorbed anti-*B. atrox* antivenom, and that against *L. muta* venom (Fig. 4A), and vice versa (Fig. 4B); in both cases the species-specific reaction observed is independent of venom and antivenom concentration.

Specific venom detection in human serum

The sensitivity of the specific detection of the different venoms was tested. Venoms added to normal human serum previously diluted to 1:50 were assayed by antigen capture ELISA using the absorbed antibody IgG fractions as the coating antigen-capture

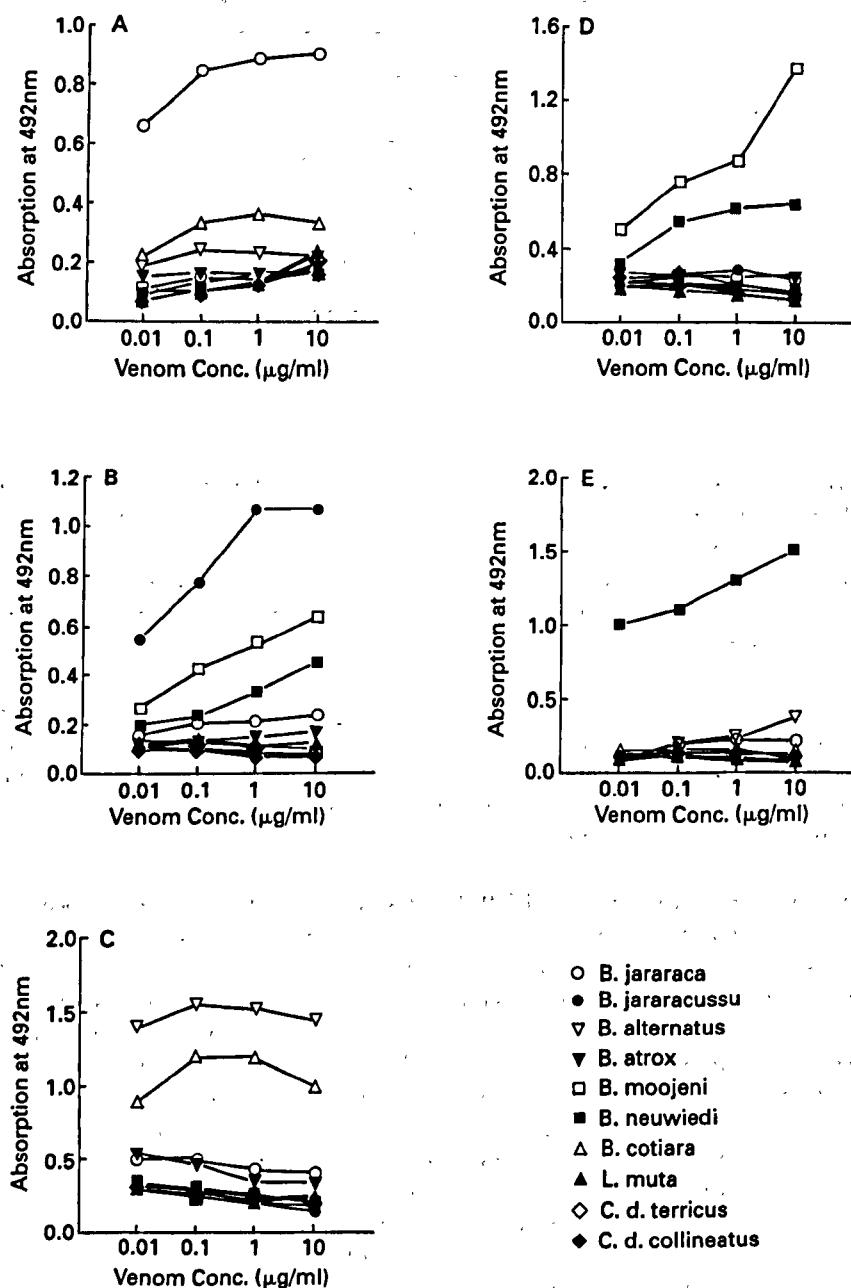


FIG. 2. ELISA REACTIVITY OF ABSORBED ANTIVENOMS AGAINST INCREASING CONCENTRATIONS OF HOMOLOGOUS AND HETEROLOGOUS VENOMS.

Different venom solutions were coated onto different wells. Absorbed antivenoms were separately added to wells containing homologous and heterologous venom. A, Anti-*B. jararaca*, at 1:100 dilution (dil.); B, anti-*B. jararacussu*, 1:100 dil.; C, anti-*B. alternatus*, 1:100 dil.; D, anti-*B. moojeni*, 1:200 dil.; E, anti-*B. neuwiedi*, 1:200 dil. Coating venoms are as shown in the key. Conjugate used as in Fig. 1.

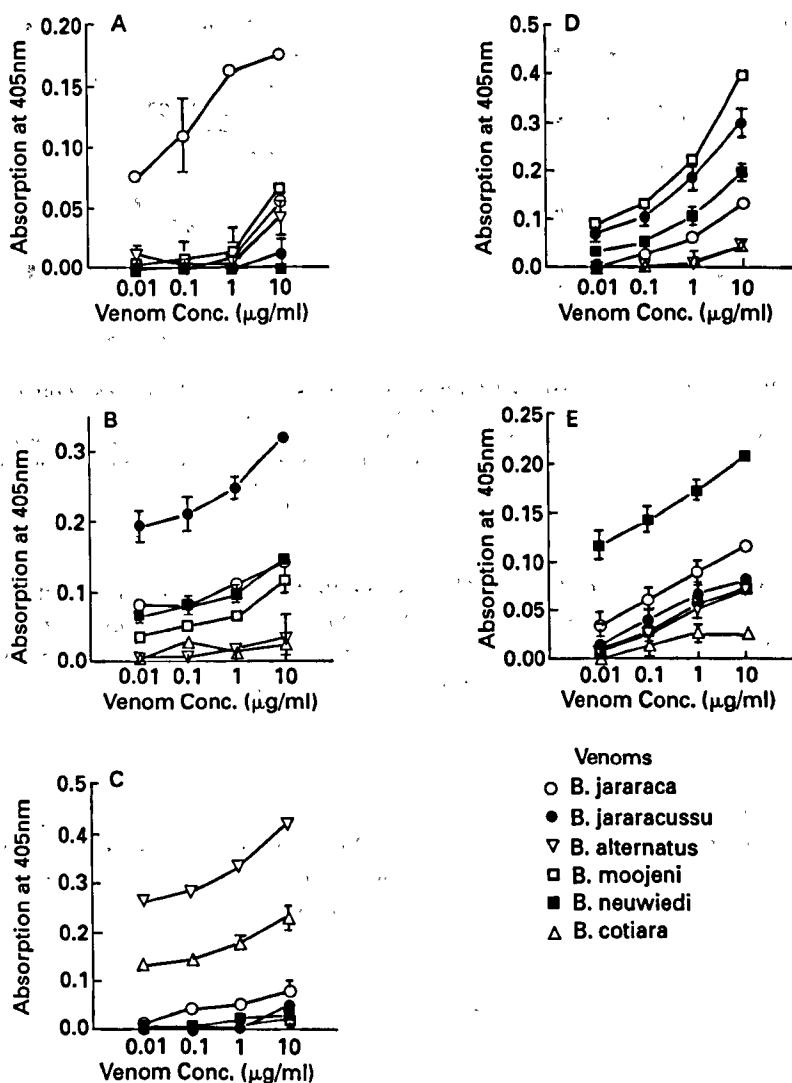


FIG. 3. ANTIGEN-CAPTURE ASSAY (ELISA) OF HOMOLOGOUS AND HETEROLOGOUS VENOM.

Absorbed antivenom IgGs, were coated onto ELISA plates at 40 µg/ml. Different venom solutions were added to different wells containing the homologous and heterologous antivenom IgG. Unabsorbed monovalent rabbit antivenom-IgG, conjugated to alkaline phosphatase, was added to wells coated with the homologous antivenom-IgG at 1 : 500. Antivenoms; A, anti-*B. jararaca*; B, anti-*B. jararacussu*; C, anti-*B. moojeni*; D, anti-*B. alternatus*; E, anti-*B. neuwiedi*. The error bars represent the standard deviation of two assays.

layer. Normal human serum was used as a negative control. Figure 5 shows the capacity of coating antibodies to detect homologous venoms diluted in serum across a range of concentrations. Sensitivity for three venoms is about 0.01 µg of venom/ml of serum or less and for *B. jararaca* venom about 0.1 µg/ml. The species-specificity of the system was also tested using a single concentration of the homologous and heterologous venoms (1 µg/ml) added to the panel of coating absorbed antivenom antibodies. Figure 6 shows the results

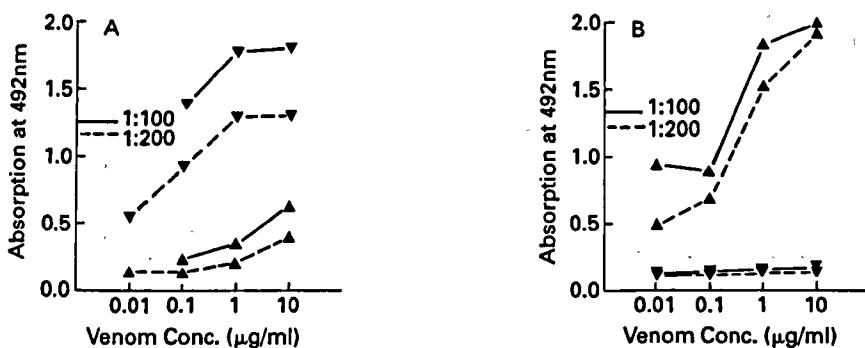


FIG. 4. REACTIVITY OF ABSORBED ANTI-*B. atrox* (A) AND ANTI-*L. muta* (B) AGAINST HOMOLOGOUS AND HETEROLOGOUS VENOMS BY ANTIBODY-CAPTURE ELISA.

Different venom solutions were coated into different wells at 100 $\mu\text{l}/\text{well}$. Absorbed antivenoms to *L. muta* and *B. atrox* venoms were separately added to different wells containing the homologous and heterologous venoms. A, Absorbed anti-*B. atrox* at 1:100 and 1:200 dil. B, Absorbed anti-*L. muta* at 1:100 and 1:200 dil. Venoms: *B. atrox* ▼; *L. muta* ▲.

for four coating antibody specificities. These show that the species-specific detection of the homologous venom was almost complete. Absorbed anti-*B. alternatus* (Fig. 6C) showed only a significant degree of residual cross-reaction to the *B. cotiara* venom and the absorbed anti-*B. neuwiedi* (Fig. 6D) to the venom of *B. jararaca*.

Detection of species-specific venom antigens

Absorbed antivenoms, which by ELISA had shown good species-specificity, were used on a cross-panel analysis of venoms, to investigate the existence of species-specific venom components. Replicate nitrocellulose membranes with the separated venom components

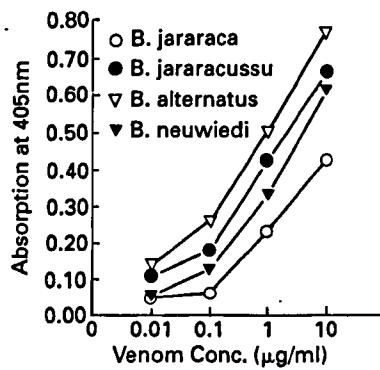


FIG. 5. ANTIGEN-CAPTURE (ELISA) TO DETECT HOMOLOGOUS VENOM ADDED TO NORMAL HUMAN SERUM DILUTED TO 1:50.

Absorbed antivenoms (IgG fractions) were coated onto microtitre plates at 40 $\mu\text{g}/\text{ml}$. Different venom concentrations (0.01 to 10.0 $\mu\text{g}/\text{ml}$) in serum were added to different wells containing the homologous absorbed antibody. Non-absorbed rabbit antivenoms (IgG fractions), conjugated to alkaline phosphatase, was used at a 1:50 dilution. Results are the mean of three experiments.

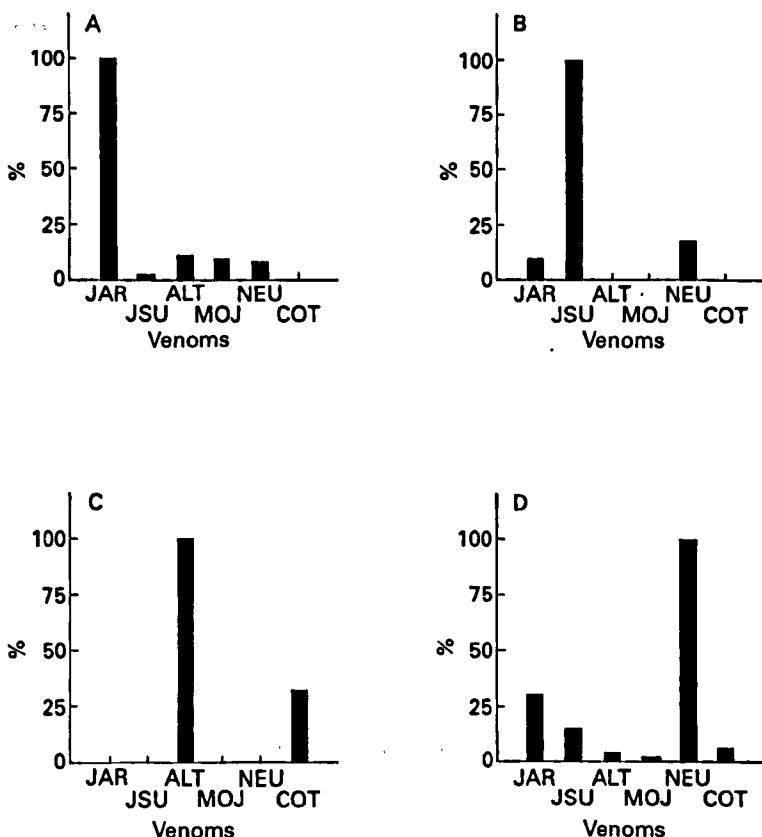


FIG. 6 SPECIES-SPECIFIC DETECTION OF SNAKE VENOMS IN HUMAN SERUM BY ANTIGEN-CAPTURE ELISA. Absorbed antivenoms (IgG fractions) were coated onto plates at 40 µg/ml and reacted with a single concentration (1 µg/ml) of homologous and heterologous venoms added to normal human serum diluted at 1:50. A, anti-*B. jararaca*; B, anti-*B. jararacussu*; C, anti-*B. alternatus*; D, anti-*B. neuwiedi*. Alkaline phosphatase conjugates were used at 1:100 dil. Volumes were of 50 µl/well. Venoms: JAR, *B. jararaca*; JSU, *B. jararacussu*; ALT, *B. alternatus*; MOJ, *B. mojeni*; NEU, *B. neuwiedi*; COT, *B. cotiara*. Results are expressed as percentage of optical density readings with the homologous reaction set at 100%.

were reacted against homologous and heterologous absorbed antivenoms, with unabsorbed antivenom as the control. Figure 7 shows the pattern of venom components revealed by the reaction of the absorbed anti-*B. jararaca* and its homologous and heterologous venoms. The absorbed anti-*B. jararaca* revealed two species-specific bands, one at the range of 14,000–20,000 mol. wt and the second at the range of 48,000–64,000. Absorbed anti-*B. jararacussu*, Fig. 8, stained two specific bands at the range of 84,000–110,000. However, a strong cross-reacting band is seen with the *B. neuwiedi* venom above 120,000. Figure 9 shows the species-specific bands revealed by the absorbed anti-*B. alternatus*. There is one band at the range of 20,000–24,000, three at 36,000–45,000, one at 64,000–90,000 and two around 119,000. In general, absorbed antivenoms stained a variable number of species-specific antigen bands of widely different mol. wt, ranging from 20,000 to 120,000.

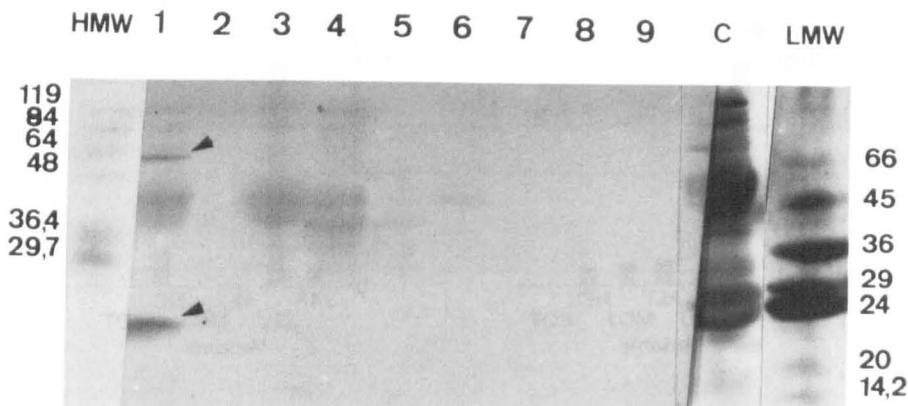


FIG. 7. VENOM ANTIGENS SEPARATED BY SDS/PAGE UNDER NON-REDUCING CONDITIONS WERE STAINED BY ABSORBED ANTI-*B. jararaca* IgG.

Venoms: (1) *B. jararaca*; (2) *B. jararacussu*; (3) *B. alternatus*; (4) *B. atrox*; (5) *B. moojeni*; (6) *B. neuwiedi*; (7) *B. cotiara*; (8) *L. muta*; (9) *C. d. terrificus*. C: control, *B. jararaca* venom stained by non-absorbed anti-*B. jararaca*. HMW and LMW, high and low mol. wt markers, respectively.

DISCUSSION

Using the venom absorption technique, monovalent antivenom sera were totally or partially depleted of cross-reacting antibodies towards other venoms in the panel studied. For some antivenoms the species-specific reactivity was rendered absolute or was greatly increased. In consequence it is possible to identify individual venom components that could be useful in the specific identification of species of snake venoms. Different batches of antivenoms of the same specificity required different numbers of absorption steps to achieve species-specificity. Also, the cross-reactivity was not abolished at the same rate for

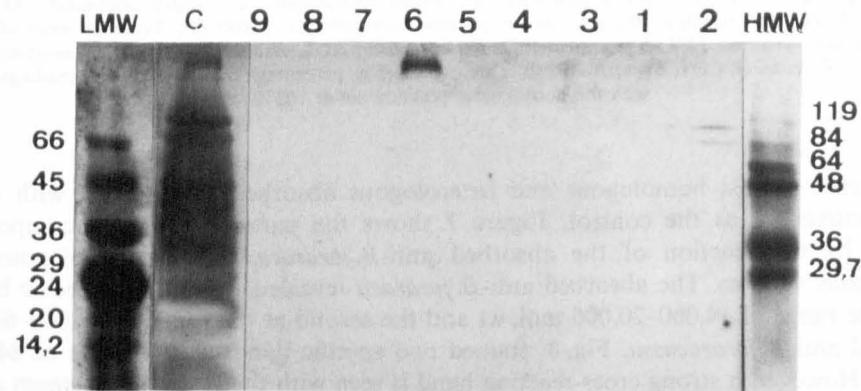


FIG. 8. VENOM ANTIGENS SEPARATED BY SDS/PAGE UNDER NON-REDUCING CONDITIONS WERE STAINED BY ABSORBED ANTI-*B. jararacussu* IgG.

Venoms: (1) *B. jararaca*; (2) *B. jararacussu*; (3) *B. alternatus*; (4) *B. atrox*; (5) *B. moojeni*; (6) *B. neuwiedi*; (7) *B. cotiara*; (8) *L. muta*; (9) *C. d. terrificus*. C: control, *B. jararacussu* venom stained by non-absorbed anti-*B. jararacussu*. HMW and LMW, high and low mol. wt markers, respectively.

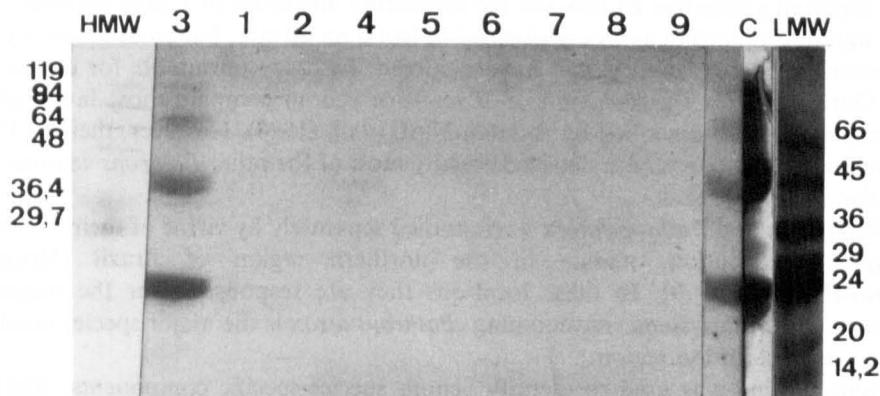


FIG. 9. VENOM ANTIGENS SEPARATED BY SDS/PAGE UNDER NON-REDUCING CONDITIONS WERE STAINED BY ABSORBED ANTI-*B. alternatus* IgG.

Venoms: (1) *B. jararaca*; (2) *B. jararacussu*; (3) *B. alternatus*; (4) *B. atrox*; (5) *B. moojeni*; (6) *B. neuwiedi*; (7) *B. cotiara*; (8) *L. muta*; (9) *C. d. terrificus*. C: control, *B. alternatus* venom stained by non-absorbed anti-*B. alternatus*. HMW and LMW, high and low mol. wt markers, respectively.

all the heterologous venoms. For example, after one or two absorption steps absorbed anti-*B. jararacussu* antivenom retained significant cross-reactivity mainly towards the *B. moojeni* and *B. neuwiedi* venoms. Anti-*B. moojeni* would behave similarly towards *B. neuwiedi*, and not against *B. jararacussu*. A possible explanation could be that some venoms contain higher concentrations of the cross-reacting component(s) than other venoms, thus eliciting an antivenom with higher concentration of cross-reacting antibodies which would make it more difficult to absorb out with the heterologous venom, carrying a lower concentration of the antigen. More absorption steps would be required to further deplete the remaining cross-reacting antibodies in such cases. Also, possibly, the binding of the antivenom onto a venom's cross-reacting component(s), present in the adsorbent, was not as efficient as in the reverse situation due to differences in affinity, or the antigen(s) may not have been bound to the adsorbent in the appropriate proportion(s).

Antigenic cross-reactivity of the *Bothrops* venoms has been previously reported. SILLE-S-VILLARROEL *et al.* (1974), using immunoelectrophoresis and agarose gel double diffusion techniques, reported extensive cross-reactivity between the venom of *B. jararaca*, and those of other *Bothrops* sp. (*B. alternatus*, *B. moojeni*, *B. insulare*, *B. cotiara*). There was less cross-reactivity with *B. jararacussu* venom. DIAS DA SILVA *et al.* (1989), using ELISA and other techniques to study cross-reactivity of ten bothropic venoms, found similar results. The absorbed antivenoms studied here were able to detect specifically the homologous venom in a concentration range of 0.01–10 µg/ml by ELISA. There are no data on the level of venom concentration derived from Brazilian pit vipers in the sera of envenomed humans. HO *et al.* (1986) reported levels of 0–600 ng/ml of venom from the Malayan pit viper (*Calloselasma rhodostoma*) in the plasma of envenomed patients. MINTON (1987) reports venom levels of 1–100 µg/ml of blood for American pit vipers in envenomed patients. Lower concentrations were not tested here but the sensitivity of the test may be improved by utilising both affinity-purified antibody preparations and enzyme amplification systems available for example with avidin/biotin conjugates. Previous attempts to develop a sensitive and rapid diagnostic test, and this includes Brazilian venoms, have not been successful (MINTON, 1987; THEAKSTON, 1989). LABROUSSE *et al.*

(1988) reported a sensitive ELISA test for measuring low levels of *Vipera ammodytes* (less than 1 ng/ml of blood) in assays performed in less than 20 min, but cross-reactivity of the monovalent antiserum used would have rendered the assay unsuitable for clinical diagnosis. Our results for the detection of *B. jararaca* venom compare most favourably for specificity with those described by BARRAL-NETO *et al.* (1990). However, their ELISA for detection of *B. jararaca* venom detected equally most of the other *Bothrops* venoms and in particular *B. atrox* venom.

Lachesis muta and *Bothrops atrox* were studied separately by virtue of their overlapping geographic distribution, mainly in the northern region of Brazil (HOGE and ROMANO-HOGE, 1978/79). In these locations they are responsible for the majority of snakebites involving systemic envenoming. *Bothrops atrox* is the major species involved in ophidic accidents in the region.

Immunoblotting was used to identify venom species-specific components. Blots were carried out in non-reducing conditions. The absorbed antivenoms revealed different patterns of specific components when reacted with their homologous venoms. Some of these results are in accordance with those presented previously by us (HENEINE *et al.*, 1990). Absorbed antivenom-IgG anti-*B. jararaca*, anti-*B. jararacussu* and anti-*B. alternatus* revealed species-specific stained bands with the homologous venom. MOURA DA SILVA *et al.* (1990), investigating the antigenic cross-reactivity of bothropic venoms, reported no species-specific antigens recognised by the homologous antivenom, and argued that the region of above 30,000 mol. wt contained the venom antigens mainly responsible for the cross-reactivities. Those results differ from ours; our absorbed antivenoms recognised species-specific venom components on the immunoblots with mol. wt greater than 30,000.

Our results show the possibility of detecting species-specific snake venom antigens from bothropic and lachetic snakes. In the cases where a more significant cross-reaction is still present after absorption, we recognise that it may not be possible to achieve complete specificity in a polyclonal system, operating at a level offering sensitive immunoassays. The adoption of monoclonal antibody methods may in these circumstances be the only way forward. In any event the ability to define species-specific venom antigens and to prepare substantially species-specific polyclonal reagents is a major aid in the steps to production of useful monoclonal antibodies.

The results here reported could be of importance in the development of snakebite diagnostic assays and in the study of the epidemiology of snake accidents and the phylogeny of closely related snake species.

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DEVELOPMENT OF A SPECIES-SPECIFIC ELISA FOR BRAZILIAN PIT-VIPER VENOMS

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Antigenic cross-reactivity between venoms of the genus *Bothrops* has been shown to be an extensive problem. However, some venom components are species-specific. In this study we have produced species-specific antivenoms against some members of the genus *Bothrops*. Monospecific rabbit antivenoms (IgG) were absorbed on venom affinity adsorbents. The species-specificity was tested by ELISA assays and immunoblots. The results of both assays showed complete species-specificity in some cases and highly increased species-specificity in others. These reagents can be used to determine the envenomating species in snake bite patients as an aid to improved serotherapy.

Key words: *Bothrops* venoms, species-specific antivenoms.

The majority of snake bite accidents in Brazil are caused by the genus *Bothrops* (1-3) which comprises a large number of species living in overlapping areas of the country (4). The pool of venoms used for the production of a polyvalent antivenom does not contain all venoms of the genus. The efficacy of these sera in treatment of patients envenomed by species not included in the pool has been questioned (2). The epidemiological data about snake bite accidents in South America are poor (5). A specific and reliable method for snake venom identification from the blood of victims would be of great value by providing a more precise knowledge of the species involved in present and past envenomations. In the present study, venoms from *B. jararaca*, *B. jararacussu*, *B. alternatus*, *B. atrox*, *B. moojeni*, *B. neuwiedi*, *B. cotiara*, *Crotalus durissus terrificus*, *C. durissus collineatus* and *Lachesis muta* were used, together with their homologous rabbit antisera, or IgG fractions of these. Glutaraldehyde adsorbents were prepared (6), each containing only nine of the ten venoms above (0.5 mg per venom). Antisera (or IgG), in volumes of 400 µl, were added to the adsorbents not containing the homologous venom. The mixtures were rotated for 2 h at room temperature and then centrifuged at 4000 g for 30 min, at 4°C. The supernatants were recovered for study as absorbed reagents. The adsorbents were washed 3 times with 0.1 M glycine-HCl buffer, pH 3.2, followed by repeated washings with PBS until the pH reached

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7.2-7.4. After a second absorption step the supernatant solutions were stored at -20°C. The decrease in the IgG content of the samples was measured by absorbance at 280 nm, $E_{13.5}^{-1}$ mg/ml. The ELISA assay was done essentially as described by Theakston et al. (7). ELISA plates (Nunc Immunosorb) were coated with all of the ten venoms in 1 μ g/ml solution using 100 μ l per well. The plates were incubated overnight at 4°C and washed. Dilutions of only one of the absorbed sera (or IgG fraction), 100 μ l/well, were then added to each plate and the material was incubated for 1 h at 37°C. Horseradish peroxidase-conjugated anti-rabbit IgG was used at 1:20,000 dilution in PBS-Tween, 100 μ l/well, and incubated for 1 h at 37°C. The substrate *o*-phenylenediamine was added in 0.1 M citric acid buffer, pH 4.5, 100 μ l/well, and incubated for 30 min at 37°C. The reaction was stopped with 50 μ l of 20% (v/v) sulphuric acid and the optical density was read at 492 nm. Figure 1 shows that the immunoabsorption of the anti-*B. moojeni*, -*B. alternatus* (IgG) and -*B. jararacussu* antisera yielded venom species-specific reagents while anti-*B. neuwiedi* IgG showed an increased degree of species-specificity against the panel of venoms. The statistical difference between reactions to homologous and heterologous venoms was calculated in all plates showing cross-reactivity. Specific reactions were found to be significant at $P < 0.05$. Polyacrylamide electrophoresis of venoms for the immunoblots was carried out under non-SDS and non-

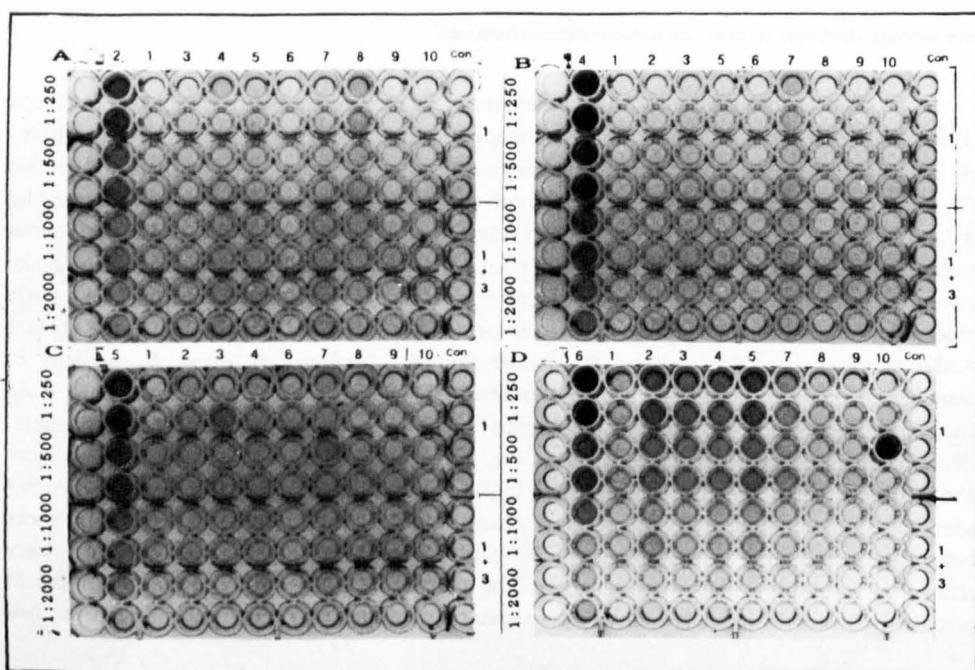


Figure 1 - ELISA plates showing the reaction of the ten venoms against the absorbed antivenoms. A, Antivenom to *B. jararacussu* venom; B, antivenom to *B. alternatus* venom; C, antivenom to *B. moojeni* venom; D, antivenom to *B. neuwiedi* venom. Venoms are from the following snakes: 1, *B. jararaca*; 2, *B. jararacussu*; 3, *B. atrox*; 4, *B. alternatus*; 5, *B. moojeni*; 6, *B. neuwiedi*; 7, *B. cotiara*; 8, *L. muta*; 9, *C.d. collineatus*; 10, *C.d. terrificus*. Antivenom dilutions are shown on the left. Controls (Con) are 1, venom alone; 1+3, venom plus conjugate.

denaturing conditions on a 7.5% gel, with a 3.0% stacking gel (8). Twenty-five µg of each venom was loaded in a volume of 2.5 µl and a current of 28 mA was applied for 50 min. The blots (9) were carried out over 50 min at 36 mA. Nitrocellulose was blocked for 1 h with 0.1% PBS-Tween. The absorbed antisera (or IgG) were added in the same buffer at a dilution of 1:1000 and the unabsorbed antisera were used as controls at a dilution of 1:2000. All incubation steps took 1 h at 37°C followed by 30-min washings with two changes of washing buffer. The substrate 3,3'-diaminobenzidine was added in 50 mM Tris buffer, pH 7.4, with 30% H₂O₂. The reactions were developed until a satisfactory clear pattern was achieved. Figure 2 shows the immunoblots for *B. jararacussu*, *B. alternatus*, *B. moojeni* and *B. atrox* venoms. Prestained molecular weight markers (Sigma-MW-SDS Blue) were used for reference. The absorbed antisera (or IgG) were shown to be reactive to only some of the components of their homologous venoms when compared with the staining pattern of the unabsorbed sera. The absorbed sera (or IgG) showed little or no cross-reactivity against the other venoms of the genus *Bothrops*.

We have demonstrated for the first time that venom affinity absorption of anti-venom sera yields species-specific antibodies against the homologous snake venom

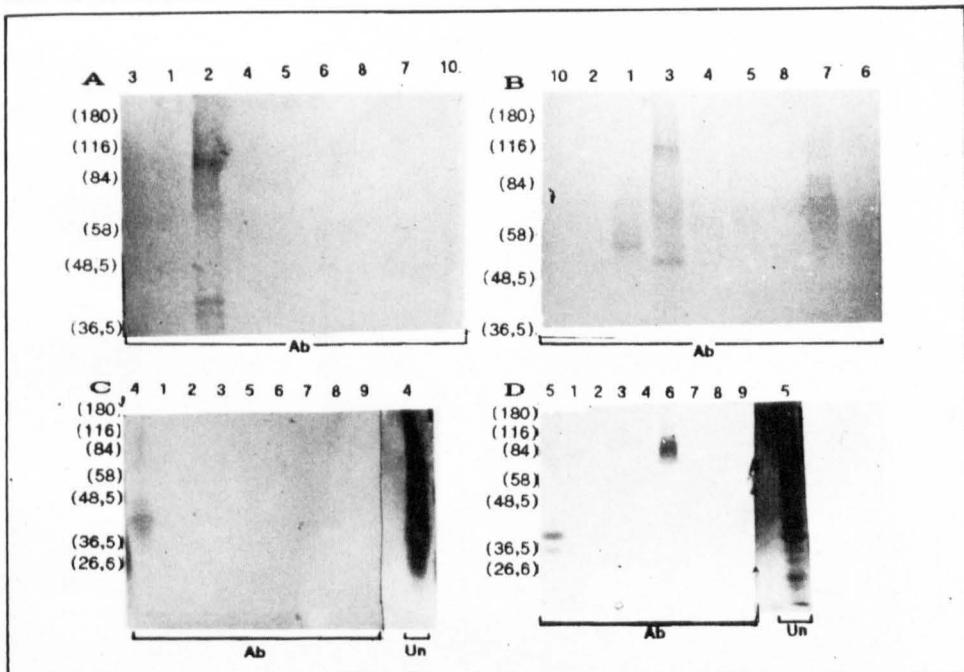


Figure 2 - Immunoblots showing the reaction of the ten venoms against the absorbed and unabsorbed antivenoms. *A*, Antivenom to *B. jararacussu* venom; *B*, antivenom to *B. atrox* venom; *C*, antivenom to *B. alternatus* venom; *D*, antivenom to *B. moojeni* venom. Antivenoms are labelled as: *Ab*, Absorbed; *Un*, unabsorbed. Numbers in parentheses indicate positions of the molecular weight markers $\times 10^3$ daltons. Venoms are labelled as in Figure 1 and are from the following snakes: 1, *B. jararaca*; 2, *B. jararacussu*; 3, *B. atrox*; 4, *B. alternatus*; 5, *B. moojeni*; 6, *B. neuwiedi*; 7, *B. cotiara*, 8, *L. muta*; 9, *C.d. collineatus*; 10, *C.d. terrificus*.

within the genus *Bothrops*. The molecules of the venoms acting as specific elements have been identified by their molecular weight. The absorbed specific sera are being used to isolate the specific antigens for characterization and for production of monoclonal antibodies.

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