Alpaca Immunoglobulins

Phase 2 Report

by Andrew Padula

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Foreword

Alpaca numbers in Australia exceed 130,000 head and the abundance of alpaca has driven the industry to explore alternative uses for the animal. While fibre has been the traditional saleable product from alpaca, along with live animals and to a lesser extent alpaca meat, this research explores the use of alpaca as a resource for the production of therapeutic immunoglobulins.

Alpaca blood contains molecules called immunoglobulins that have a multitude of medical uses. There is a worldwide demand for animal immunoglobulin products such as snake antivenom and the research examines the potential use of alpaca as a serum producing animal for snake antivenom manufacturing. A research herd of alpaca was hyperimmunised with various snake venoms, the immune response monitored and serum harvested for various purposes at different time periods.

The beneficiaries of this research are two-fold. Firstly, the Australian alpaca industry stands to benefit from having an alternative use for the alpaca as a producer of high value niche market products. Secondly, there are potential benefits to the global community from safer and more effective therapeutic immunoglobulin products.

This research is a continuation of work from a previous RIRDC project focused on therapeutic antibodies from alpaca. This report is an addition to RIRDC’s diverse range of over 2000 research publications and it forms part of our Animal Industries R&D program, which aims to conduct RD&E for new and developing animal industries that contribute to the profitability, sustainability and productivity of regional Australia.

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The author of this document is Andrew Padula. Andrew is a veterinary graduate of the University of Melbourne and has over 20 years of experience in veterinary practice, academia, and research.

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Abbreviations

µg: Microgram
µL: Microlitre
µm: Micrometre
AAA: Australian Alpaca Association
AGID: Agar gel immunodiffusion (assay)
BSA: Bovine serum albumin
CFT: Complement fixation test(ing)
DPI: Department of Primary Industries
ELISA: Enzyme-linked immunosorbent assay
g: Gram
h: Hour
HClgG: Heavy chain only immunoglobulin
IgA: Immunoglobulin A
IgG: Immunoglobulin G
IP: Intellectual property
Kg: Kilogram
M: Molar
mg/mL: Milligram per millilitre
mg: Milligram
mL: Millilitre
MW: Molecular weight
NaCl: Sodium chloride
nm: Nanometre
PBS: Phosphate buffered saline
PCV: Packed cell volume
PCR: Polymerase chain reaction
PLA2: Phospholipase A2
RBC: Red blood cells(s)
SCAHLs: Sub-Committee on Animal Health Laboratory Standards
SVDK: Snake Venom Detection Kit (CSL)
TGA: Therapeutic Goods Administration
U/mL: Units per millilitre
VNT: Virus neutralisation test
U: Units
°C: Degrees Celsius
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Executive summary

What the report is about

This report has explored an alternative use of alpaca other than fibre and meat; that is, for the production of therapeutic alpaca immunoglobulin products.

Who is the report targeted at?

This report is targeted at those interested in alternative markets for alpaca products.

Where are the relevant industries located in Australia?

The alpaca industry is widespread across Australia with the bulk of animal numbers located in south eastern Australia. Alpaca numbers in Australia exceed 130,000 head. Benefactors of this research are the alpaca industry and investors in biotechnology.

Background

The abundance of alpaca in Australia has driven the industry to explore alternative uses for the animal. There is a worldwide demand for animal immunoglobulin products such as snake antivenom. Currently these are made by immunising an animal against the venom and then harvesting blood and concentrating the immunoglobulins. This work has explored the use of alpaca as a serum producing animal for snake antivenom manufacturing.

Aims/objectives

The alpaca industry stands to benefit from a new market for alpaca products.

Methods used

This work is a continuation of work performed in a previous RIRDC project. A research herd of alpaca was hyperimmunised with various snake venoms, the immune response monitored and serum harvested for various purposes at different time periods.

Results/key findings

Alpaca antivenom and a range of other commercial antivenom products were examined for their anti-complementary activity which, when increased, is thought to be a measure of the likelihood of triggering adverse reactions in humans. Alpaca antivenom had significant anti-complementary activity, as measured in the assay system used in this project. The results obtained showed the more purified the immunoglobulin fraction, the greater the anti-complement activity. These results are difficult to interpret and cannot be assumed to be a reliable predictor of adverse reactions in humans. The in vitro assay system used may not be suitable for alpaca serum, and similar issues have been reported with llama and pig serum. There appears to be non-specific activation of complement, as revealed by the assay system, but this may not reflect an increased risk of adverse reactions when used therapeutically. More reliable data needs to be obtained to better interpret these results using a bioassay to measure anaphylaxis in guinea pigs or mice.

Work compared the effects of heating commercial (CSL) antivenom and experimental alpaca antivenom to 60°C for 20 hours and 72°C for 5 minutes, to simulate different pasteurisation conditions. Prior to heating, alpaca antivenom displayed excellent neutralisation of procoagulant effects of Brown Snake venom, being approximately ten times more effective than the CSL product. However, alpaca antivenom performed poorly after heating, retaining only 28% of initial activity.
compared to 52% for the CSL product. Horse-derived antivenom tolerated heat better but 60°C for 20h resulted in a gelatinous product for both antivenoms, although there was a much slower formation time for horse than alpaca product. The conclusion is that alpaca antivenom contains no inherent thermal stability properties in the form it was tested.

A quantitative enzyme-linked immunosorbent assay (ELISA) for venom antibodies was developed and correlated to a mouse bioassay. Comprehensive time course serum potency values were measured for all alpaca over 500 days. A high correlation was shown between ELISA for purified toxin antibodies and for whole venom. Alpaca were rested from immunisation for approximately six months and then boosted with two doses of venom. Immunity to venom had waned to minimal levels but was rapidly restored following the booster doses. The dataset also revealed that animals which demonstrated a high antibody response at 4 and 8 weeks were the best responders at 40 weeks. This correlation would allow for early selection of high responders. In conclusion, the ELISA is a very efficient and precise method of monitoring immune responses in alpaca to hyperimmunisation.

A number of different restraint systems for handling alpaca and sheep were examined. Each system has advantages and disadvantages. The number of animals to be bled, number of staff and the time available influence the choice in selecting the best system. An effective comprise was tested and found suitable, in which alpaca were lightly sedated with a non-cardivascular depressant sedative (Zoletil™ (Virbac); tiletamine hypochloride plus zolazepam hypochloride) and restrained in a horizontal position on a table.

A specialist patent attorney law firm was contracted to conduct an intellectual property (IP) landscape search and analysis. The goal of the work was to obtain a comprehensive understanding and professional opinion on the IP issues associated with commercialisation of alpaca antibody technology. No significant issues were found that would hinder commercial product development within Australia. The most limiting patent has now expired in August 2013 from its original filing in 1993. The pathway is thus clear for commercial product development.

A series of broad recommendations have been made for management of alpaca to minimise infectious disease risk to humans from serum products, rather than specific disease testing protocols. A detailed discussion of 17 infectious diseases of camelids is presented. Because alpaca are neither sheep nor horse, which have clearly identified zoonotic risks identified to regulatory agencies, there is scope for to establish a sound but not overly proscriptive risk-based approach to biosecurity.

Experiments were performed to see if a method of processing alpaca serum could be developed based on caprylic acid precipitation of non-immunoglobulin proteins. Alpaca serum was diluted in water to aid the precipitation process. The reaction was monitored by microscopy and compared to horse plasma processed in an identical manner. Alpaca serum failed to precipitate out cleanly using any of the combinations of dilutions. A fine white granular protein precipitate remained in solution. Alpaca serum failed to process to a high degree of purity using a standard chemical method based on caprylic acid. Heat treatment, although effective in removing the contaminating protein appears to slightly denature the immunoglobulin which may make the product more likely to trigger allergic reactions.

A new venom detection test was developed using affinity purified alpaca anti-Tiger Snake antibodies. The test had remarkable sensitivity with a limit of detection of 0.010 ng/mL making it 1,000 times more sensitive than the CSL snakevenom detection kit, and 15 times more sensitive than the currently most sensitive ELISA, which is based on rabbit antibodies.

Alpaca antivenom was extremely effective in comparison to CSL antivenom in neutralising the procoagulant effects of Tiger, Brown and Taipan venoms. The alpaca antivenom was at least 10 times more effective and showed excellent cross neutralisation of Taipan with Brown snake antivenom.

A confirmed clinical case of Tiger Snake bite was successfully treated in a dog with alpaca polyvalent antivenom. Samples collected from the dog were retrospectively assayed for Tiger Snake venom using
a new highly sensitive assay developed as part of this project. At four hours post-antivenom treatment the dog’s condition had improved markedly and serum venom concentrations were undetectable indicating complete binding by the alpaca antivenom.

**Implications for relevant stakeholders**

These results show that alpaca can be used for antivenom production as well as making antibodies for diagnostic tests. However, some barriers exist to full scale commercial production of antivenom using alpaca, including development of more effective antibody extraction methods. The field is open for commercialisation as the patent landscape search revealed no obstructions in Australia.

**Recommendations**

There are four main areas in which more basic research is required to maximise the commercial potential of alpaca immunoglobulins:

(i) to overcome the difficulties with processing of alpaca serum to obtain a higher purity product.

(ii) to further develop snake venom detection methods using alpaca antibodies to complement snake antivenom.

(iii) to better understand the likelihood of allergic reactions to alpaca serum, studies performed in animal models of allergy are needed.

(iv) to further examine the remarkable properties of alpaca antivenom for neutralising blood clotting disturbances.
Introduction

This project follows on from the previous RIRDC supported project on Alpaca Immunoglobulins (PRJ-007770) completed in 2012. The work completed in that project up to the end of 2012 proved the concept that alpaca can make therapeutic antibodies. The logical progression of that work is presented in this report titled Alpaca Immunoglobulins: Phase 2 Report.

Much of the technology used in snake antivenom production and other therapeutic immunoglobulins is almost 100 years old. Newer additions to the knowledge base have been adopted sporadically and with economic constraints by various serum producers worldwide. No commercial producer has tackled camelids. Despite this, there is a vast array of intellectual property patents that have attempted to lock up this field and take ownership. A key part of this work was to undertake an intellectual property landscape search to ascertain if there is freedom to operate.

I hope that readers of this report not merely read the facts presented but digest them and then devise their own stimulating new ideas to make forward progress in this fascinating field of biological research.
Objectives

1. *In vitro* studies on the allergenic properties of alpaca serum compared to horse serum.

2. Heat stability of alpaca antivenom to short term high temperature exposure.

Heat stability is a key property of camel serum immunoglobulins. Does alpaca serum also possess this property? Alpaca serum will be exposed to various temperatures in an accelerated stability study. The neutralising power of the antiserum will then be examined in mice. A control product will be used consisting of equine-derived antivenom (CSL).

3. Improving the ELISA for monitoring and screening alpaca for good response to venom.*

The antibody test (ELISA) will be refined so that specific toxin fractions can be assayed for. This will improve the usefulness of the ELISA. This may also become a screening test for determining which animals will be the best responders. The specific toxin fractions will be created with expert biochemistry advice from Venom Science Pty Ltd.

4. Determine correlation between ELISA values using an improved assay system and mouse testing results.*

This project is conducted with #3 (above). The predictive value of the ELISA must be correlated with the effects seen in mice. If this correlation is shown to be sufficiently strong (>80%) then the ELISA becomes a very powerful tool for selecting and monitoring animals.

The process will involve performing several mouse venom/antivenom challenge tests and correlating the results of those with ELISA values.

5. Design and construction of restraint box for bulk blood collection from alpaca.

A suitable restraint system for collecting large volumes of blood from alpaca must be developed. Suitable systems exist for sheep and it may be possible to modify the design to work for alpaca. A consultant will provide advice on this and facilitate the construction of a prototype unit.


A key piece of information required is information on patents in this field. Since 1993 there have been a number of patents applied to antibodies derived from camelids. This work will explore the patent landscape in this field. A specialist patent attorney firm will be hired to perform a search and analysis of the camelid antibody patent field.

7. Summary document outlining major biosecurity issues and strategies requiring management to achieve commercialisation of alpaca serum products.

8. Issues with procedures for concentrating immunoglobulins in alpaca serum.**

9. Neutralisation of snake venom procoagulant action by alpaca antivenom.**

10. Clinical usage of antivenom.**

*Subproject #3 and #4 are presented together in this report. **Subproject #8 and #9 and #10 are additional to the agreed objectives and included for completeness.
General methods

Animal ethics approval

Approval for experimental work involving animals was obtained from the Wildlife and Small Institutions Animal Ethics Committee, Bureau of Animal Welfare, Victorian Department of Primary Industries, Mickleham Road, Attwood. The Wildlife and Small Institutions Animal Ethics Committee approved the project in two stages. The first approval obtained (16.11) from DPI Victoria was for the hyper-immunisation and blood collection. A second application was approved (06.12) for mouse bioassay testing to determine the potency of the antivenom. A third approval, obtained via DPI Victoria from the Victorian Minister for Agriculture, was obtained to conduct the mouse protection studies as death-as-endpoint experiments. The mouse bioassay testing protocol was modified from WHO (Standardization 2010) recommendations to improve the animal welfare outcomes but still obtain meaningful results.

Alpaca sourcing and general management

The alpaca used in the previous project (Alpaca Immunoglobulins) were also used in this project.

Alpaca were sourced through the Australian Alpaca Association. The initial group of animals consisted of 20 alpaca. Within this group there was an equal mix of Suri and Huacaya alpaca types. The majority of alpaca were male castrates. Females that were used were generally infertile or had significant conformational abnormalities reducing their potential commercial value. Alpaca ranged in age from two years to eight years old. Alpaca originated from herds located in NSW or Victoria.

Alpaca were located in a five acre paddock on the outskirts of Bairnsdale, Victoria. Alpaca grazed pasture and were supplemented with lucerne hay, oats, lupins and alpaca pellets. Alpaca defaecate in communal dung piles. These piles were collected from time to time to minimise build up in the paddock.

Alpaca were monitored by faecal egg counts and treated regularly for internal parasites using injectable ivermectin (Bomectin™).

Alpaca were supplemented in early winter with a single injection of Vitamin A, D & E (Vitamec ADE Injection, AgVantage Pty Ltd, Australia).

Handling of alpaca

Alpaca are gentle animals by nature and only minimal facilities were used to handle them. All treatments were performed on the property. Cattle handling facilities were located on the property and these were used for all animal handlings. Alpaca became accustomed to being fed and were easily enticed into the yards. Alpaca do not ‘flow’ readily like sheep down a long race or chute. They have a tendency to stop and sit down, requiring human physical intervention to move them along.

To administer venom immunisation injections and collect 10mL blood samples alpaca were manually restrained individually each time. One person held the alpaca around the neck whilst a second person collected the blood sample and administered the subcutaneous injections. Physical restraint was generally minimal. Alpaca were lightly gripped around the upper neck. In most cases, there was no resistance applied by the animal. Occasionally an alpaca would spit or vocalise. Three people were generally required for each animal treatment. The third person prepared the injections and recorded all data.
Harvesting of serum was performed in the same facilities using the same personnel. Whole blood was collected into sterile 800 mL fluid collection bags.

**Health monitoring post-venom immunisation**

To ensure that the animal health implications of immunising alpaca with snake venom were understood a comprehensive health monitoring program was used for the first three immunisations. It was considered that the first three immunisations would be most likely to elicit adverse signs in the alpaca. To detect abnormalities blood samples were collected immediately prior to immunisation and again 24 hours after each of the first three immunisations. Animals were monitored clinically and had rectal temperatures recorded. Blood samples were submitted to a commercial veterinary pathology laboratory for complete biochemistry and haematology profiles including fibrinogen. The blood profiles were completed for each animal for the first three immunisations. Data was stratified by treatment group.

**Snake venom preparation for immunisation**

Venoms were diluted to stock solutions in sterile 0.9 % sodium chloride. Venoms were sterile filtered using a 0.22 µm syringe filter. Once prepared to the appropriate concentration, venoms were stored frozen at -20°C until use. Tubes containing venom were labelled with species name, volume and concentration.

**Treatment groups**

Alpaca were randomly allocated to one of five treatment groups. Treatment groups comprised a combination of monovalent and polyvalent groups. The groups were chosen based on current commercial antivenoms used in Australia and Papua New Guinea. All venoms were obtained from a commercial venom supplier, Venom Supplies Pty Ltd, Tanunda, South Australia. Venoms were stored frozen until used. The venoms of the Tiger Snake and Brown Snake were prepared as a geographically representative pool as regional differences in venom have been observed (Flight, Mirtschin et al. 2006).

Papuan Taipan venom originated from a supplier in Merauke, Bali and was supplied through Venom Supplies Pty Ltd. Papuan Taipan was chosen over the Australian Coastal Taipan because of its higher toxicity. In general it is preferable to use the most toxic of the species for immunisation. *Pseudechis australis* was chosen as the Black Snake venom. This is commonly called the King Brown Snake but is rightfully a member of the Black Snake family.
Table 1. Treatment groups used for alpaca immunisation.

<table>
<thead>
<tr>
<th>Antivenom Type</th>
<th>N</th>
<th>Common Name</th>
<th>Species Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monovalent Papuan Taipan</td>
<td>4</td>
<td>Papuan Taipan</td>
<td><em>Oxyuranus scutellatus canni</em></td>
</tr>
<tr>
<td>Monovalent Brown Snake</td>
<td>4</td>
<td>Eastern Brown Snake</td>
<td><em>Pseudonaja textilis</em></td>
</tr>
<tr>
<td>Monovalent Tiger Snake</td>
<td>4</td>
<td>Tiger Snake</td>
<td><em>Notechis scutatus</em></td>
</tr>
<tr>
<td>Polyvalent</td>
<td>4</td>
<td>Papuan Taipan</td>
<td><em>Oxyuranus scutellatus canni</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Eastern Brown Snake</td>
<td><em>Pseudonaja textilis</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tiger Snake</td>
<td><em>Notechis scutatus</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Death Adder</td>
<td><em>Acanthophis antarcticus</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>King Brown</td>
<td><em>Pseudochis australis</em></td>
</tr>
<tr>
<td>Bivalent (Tiger/Brown)</td>
<td>4</td>
<td>Tiger Snake</td>
<td><em>Notechis scutatus</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Brown Snake</td>
<td><em>Pseudonaja textilis</em></td>
</tr>
</tbody>
</table>

**Immunisation program**

Alpaca were immunised using a low-dose multi-site protocol (Chotwiwatthanakun, Pratanaphon et al. 2001). A combination of Complete Freund’s Adjuvant and Incomplete Freund’s Adjuvant was used. Emulsions were formed with the adjuvant and venom solution in a 1:1 ratio. The stability of the oily emulsion was tested by ensuring that it did not disperse when a droplet was placed onto the surface of distilled water. Injections were given at monthly intervals based on protocols used in sheep (Landon and Smith 2003). All injections were given subcutaneously on each side of the neck, lateral thorax and lumbar region. The injections were sited to be anatomically close to local draining lymph nodes to maximise exposure to the immune system (Agba, Gouro et al. 1996, Agba, Gouro et al. 1996). The purpose of the low-dose multi-site program was to expose as many lymph nodes as possible to the venom and hence create the highest immune response.
1. **In vitro** studies on the allergenic properties of alpaca antivenom

**Summary**

Alpaca antivenom and a range of other commercial antivenom products were examined for their anti-complementary activity, which is thought to be a measure of the likelihood of triggering adverse reactions in humans. Alpaca antivenom had significant anti-complementary activity as measured in the assay system used. The more purified the immunoglobulin fraction, the greater the activity. These results are difficult to interpret and cannot be assumed to be a reliable predictor of adverse reactions. The results obtained here are consistent with the general behaviour of pig and llama serum in terms of non-specific binding to complement. Further data needs to be obtained to interpret these in vitro test results using a bioassay to measure anaphylaxis in guinea pig or mice.

**Introduction**

Animal serum-derived products are associated with a relatively high incidence of adverse events following administration to humans, compared to most other injectable medications. Treatment of snakebite with antivenom can often result in allergic reactions that range from mild to life-threatening to fatal. These adverse events can be categorised into those that occur within minutes of administration (early) and those that occur 7-21 days subsequent (late). This simple categorisation system provides the basis for understanding how and why these reactions arise. Understanding the cause of and reducing the incidence of these adverse reactions is part of the constant improvement process for antivenom products.

The causes of early reactions to antivenom administration are not entirely understood. Three broad causes are postulated: firstly, contamination with viruses and endotoxins during manufacturing; secondly, factors dependent upon the physiochemical characteristics of the antivenom including purity and content of protein aggregates; and thirdly, factors dependent upon the immunocchemical profile of the antivenom including immunogenicity and anti-complementary activity.

A recent review of the topic of adverse reactions caused by antivenom cited an early adverse incidence rate of between 3% and 88% for 24 published clinical trials (Leon, Herrera et al. 2013). As a broad average, most antivenom products are reported to induce early reactions in around 20% of human patients. A number of clinical factors also influence this. Of particular importance are the rate and concentration of administration of antivenom. Rapid infusion rates are likely to lead to a higher rate of reactions; slow intravenous infusion is generally considered safer. Dilution of the antivenom in physiological saline also reduces the risk of adverse early reactions.

Of importance in triggering early reactions is the activation or binding to complement proteins in the body. The complement system helps or “complements” the ability of antibodies and phagocytic cells to clear pathogens from an organism. It is part of the immune system (called the “innate” immune system) that is not adaptable and does not change over the course of an individual's lifetime. However, it can be recruited and brought into action by the adaptive immune system. The complement system consists of a number of small proteins found in the blood, in general synthesised by the liver, and normally circulating as inactive precursors (pro-proteins). When stimulated by one of several triggers, proteases in the system cleave specific proteins to release cytokines and initiate an amplifying cascade of further cleavages. The end-result of this activation cascade is massive amplification of the response and activation of the cell-killing membrane attack complex. Over 25 proteins and protein fragments make up the complement system, including serum proteins, serosal proteins, and cell membrane...
receptors. They account for about 5% of the globulin fraction of blood serum. Complement proteins are inactivated by heating to 56°C.

The binding of antivenom to complement was first demonstrated in the 1970s (Sutherland 1977). Since then numerous studies have examined the role of complement in early adverse reactions to antivenom (Romer, Gardi et al. 1979, Malasit, Warrell et al. 1986, Garcia, Monge et al. 2002, 2005, Herrera, Leon et al. 2005, Leon, Lomonte et al. 2005).

No data is available on the anti-complementary activity of alpaca serum or immunoglobulin concentrates derived from alpaca. This work is the first study to examine the activation of the innate immune system by alpaca immunoglobulins through assessing complement activation.

Materials and methods

Samples

A range of different antivenom samples as described in the table below were analysed in the complement fixation assay.

Total protein assay

The total protein concentration was measured using the BCA method (Pierce, USA). Briefly, standards were prepared (0, 0.25, 0.5, 1 and 2 mg/mL) from a commercial bovine serum albumin (BSA) standard (Sigma-Aldrich, Australia). Unknown samples were diluted 1:100 in sterile saline. The BSA working reagent was prepared at 1:50 dilution and added to the samples at 1:20 (100 µL of sample to 2.0 mL working reagent). Samples were incubated in a constant temperature water bath for 30 minutes. The liquid was transferred to a cuvette and the absorbance read at 562 nm. A quadratic curve was fitted to the data and the unknown values interpolated using software (GraphPad Prism 6.02, USA).

Complement fixation assay

Complement fixation testing (CFT) was performed according to the protocol defined by the Australian Sub-Committee on Animal Health Laboratory Standards (SCAHLS). The protocol is well defined and a based on detection of haemolysis in sheep red blood cells (RBC) following treatment with guinea pig derived complement protein (Spencer 1981).

Briefly, samples of antivenom were prepared in doubling dilutions from 1:1 to 1:128 in veronal buffer. Samples were assayed in duplicate using a 96-well microplate system. Samples were heat inactivated at 60°C for 60 minutes prior to assay. A 25 µL aliquot of serum was applied to the first well and serially diluted. A 25 µL aliquot of guinea pig serum was added to each well. The plates were incubated at 37°C for 60 minutes to allow binding to occur. A 25 µL aliquot of haemolysin-sensitised sheep RBC was then added to each well. Plates were incubated for a further 30 minutes at 37°C. The plates were then left for 2 hours to allow unlysed RBC to settle to the bottom of each well. The plates were then read visually and scored for haemolysis. The titre was determined as the highest dilution at which 50% haemolysis was deemed to have occurred.

Data analysis

Complement fixation test titres were compared graphically using Microsoft Excel.
<table>
<thead>
<tr>
<th>No.</th>
<th>Sample</th>
<th>Preservative</th>
<th>Total Protein (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><strong>Raw horse serum.</strong> Serum collected from a clinically normal horse on 13.3.13, sterile filtered (0.22um), preservative added and stored at 4°C.</td>
<td>Thimerosal 0.01%</td>
<td>48.1</td>
</tr>
<tr>
<td>2</td>
<td><strong>Raw alpaca serum.</strong> Pooled serum from six normal alpaca that had not been immunised with any snake venom. As used in ELISA for negative control. Stored frozen at -20°C and freshly thawed immediately prior to assay.</td>
<td>None</td>
<td>44.2</td>
</tr>
<tr>
<td>3</td>
<td><strong>CSL Polyvalent Antivenom.</strong> Commercial product, sample extracted directly from vial. Phenol is contained within the antivenom product. Expiry Date on vial 06/14.</td>
<td>Phenol 0.22%</td>
<td>186</td>
</tr>
<tr>
<td>4</td>
<td><strong>CSL Brown Snake Antivenom.</strong> Commercial antivenom product, sample extracted direct from vial. Expiry 03/11. Label states 3.31 mL vial fill volume containing 1,000 Units. Batch 055910701</td>
<td>Phenol 0.22%</td>
<td>158</td>
</tr>
<tr>
<td>5</td>
<td><strong>Alpaca Polyvalent Antivenom (without phenol).</strong> No preservative. Sodium sulphate precipitation. 27.11.12. ALP-POLY-231012-SS</td>
<td>None</td>
<td>44.0</td>
</tr>
<tr>
<td>6</td>
<td><strong>Alpaca Polyvalent Antivenom (with 0.22% phenol).</strong> ALP-POLY-231012. As used for ELISA standards.</td>
<td>Phenol 0.22%</td>
<td>56.7</td>
</tr>
<tr>
<td>7</td>
<td><strong>Summerland Serums Tiger-Brown Antivenom.</strong> Exp. May 15. TB 3 12 7.</td>
<td>Phenol 0.22%</td>
<td>112.6</td>
</tr>
<tr>
<td>8</td>
<td><strong>AVSL Multi-Brown Snake Antivenom.</strong> Batch BZ. Exp. Sep 12. 10mL vial.</td>
<td>Phenol 0.22%</td>
<td>56.5</td>
</tr>
<tr>
<td>9</td>
<td><strong>Alpaca Mono Tiger Antivenom.</strong> TP=2.6g/L. Sterile filtered.</td>
<td>Phenol 0.22%</td>
<td>29.5</td>
</tr>
<tr>
<td>10</td>
<td><strong>Alpaca Mono Brown Antivenom.</strong> TP=3.9g/L. ALP-BRO-231012</td>
<td>Phenol 0.22%</td>
<td>37.4</td>
</tr>
<tr>
<td>11</td>
<td><strong>Normal horse IgG concentrate.</strong> Prepared from caprylic acid fractionation.</td>
<td>Thimerosal 0.01%</td>
<td>140</td>
</tr>
<tr>
<td>12</td>
<td><strong>Alpaca Mono Taipan antivenom.</strong> 23-10-12. Total protein = 2.6g/L. 0.22% phenol.</td>
<td>Phenol 0.22%</td>
<td>27.6</td>
</tr>
</tbody>
</table>
Results

The results are presented as the titre or highest sample dilution that resulted in a positive reaction. As can be seen from the table below high levels of anti-complementary activity were detected in alpaca antivenom samples, particularly those processed using sodium sulphate. Sodium sulphate precipitation of serum results in a high degree of purity of the immunoglobulin fraction, greater than that obtained with ammonium sulphate.

Results of a second CFT assay using alpaca affinity purified anti-Tiger Snake antivenom showed very high levels of anti-complementary activity.

Table 3. Results of CFT assay in round 1 (top) and round 2 (bottom).

<table>
<thead>
<tr>
<th>No.</th>
<th>Product</th>
<th>Titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Raw Horse Serum</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Raw Alpaca Serum</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>CSL Polyvalent Exp.06/14</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>CSL Brown Snake Exp. 03/11</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>Alpaca Poly SS</td>
<td>128</td>
</tr>
<tr>
<td>6</td>
<td>Alpaca Poly AS + phenol</td>
<td>8</td>
</tr>
<tr>
<td>7</td>
<td>Summerland TB Exp May 15</td>
<td>4</td>
</tr>
<tr>
<td>8</td>
<td>AVSL Multi Brown</td>
<td>1</td>
</tr>
<tr>
<td>9</td>
<td>Alpaca Mono Tiger SS 2.6g/L</td>
<td>8</td>
</tr>
<tr>
<td>10</td>
<td>Alpaca Mono Brown SS 3.9g/L</td>
<td>128</td>
</tr>
<tr>
<td>11</td>
<td>Normal Horse IgG Concentrate</td>
<td>4</td>
</tr>
<tr>
<td>12</td>
<td>Alpaca Mono Taipan 2.6g/L</td>
<td>128</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>No.</th>
<th>Product</th>
<th>Titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Alpaca Mono Brown Antivenom</td>
<td>32</td>
</tr>
<tr>
<td>2</td>
<td>Affinity Alpaca TSV IgG</td>
<td>256</td>
</tr>
<tr>
<td>3</td>
<td>CSL Polyvalent Exp. 06/14</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>Raw Horse Serum</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>Raw Alpaca Serum</td>
<td>16</td>
</tr>
<tr>
<td>Product</td>
<td>Titre (g/L)</td>
<td></td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>Alpaca Mono Taipan</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td>Normal Horse IgG Concentrate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alpaca Mono Brown SS</td>
<td>3.9</td>
<td></td>
</tr>
<tr>
<td>Alpaca Mono Tiger SS</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td>AVSL Multi Brown</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Summerland TB Exp May 15</td>
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<td></td>
</tr>
<tr>
<td>Alpaca Poly AS + phenol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alpaca Poly SS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CSL Brown Snake Exp. 03/11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CSL Polyclonal Exp. 06/14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Raw alpaca serum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Raw Horse Serum</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 1. Chart showing complement fixation test titre results for all antivenom products tested in round 1.
Figure 2. Results of a round 2 CFT assay using affinity purified alpaca anti-Tiger Snake venom antibodies, raw serum (horse/alpaca) and alpaca monovalent Brown Snake antivenom (x-axis = titre).
Discussion

It must be recognised that the in vitro tests for assessing allergenic potential of a substance when administered in vivo are not fully validated. Whilst it is comforting to think that these in vitro laboratory tests can predict the likelihood of an allergic reaction, the reality appears to be different. The only true test of the allergenic potential of a substance is to inject it into the target species (i.e. humans) en masse and monitor the population for allergic reactions. This is rarely done prior to releasing an antivenom product. However, this data does eventually become available through good pharmacovigilance and sound post-marketing surveillance of the product.

The complement fixation testing outcomes obtained here were unexpected. It was assumed that alpaca antivenom would not bind complement and should be less reactive than horse antivenom. It is possible that the heat treatment applied to the alpaca antivenom during processing led to increased and non-specific binding of complement, and thus apparent higher test results. As this work proceeded it became obvious that the results were not in line with the expectations of reduced complement binding.

The results of this complement fixation assay demonstrate that processed alpaca serum has complement activating activity in this assay system. This does not necessarily mean that it would activate complement in a human or lead to adverse reaction. A previous published report on this issue also showed that despite in vitro complement activation, when the antivenom was administered to rabbits intravenously there was no activation of complement in vivo (Leon, Lomonte et al. 2005). The authors went on to conclude that “...These observations raise doubts on the clinical significance of the in vitro Anti Complementary Activity (ACA) test as a predictor of ACA in vivo.”

Serum from some animal species is known to innately activate complement and for this reason CFTs cannot be used as diagnostic tests. A recently published study in the USA attempted to use CFT in llama for the diagnosis of Brucella infection status. The report concluded that the CFT could not be used as 31% of samples displayed inherent anti-complementary activity (Nielsen, Smith et al. 2007). Due to the close relationship between alpaca and llama it is likely that alpaca serum also activates complement in a high percentage of cases meaning CFT is also a meaningless test in alpaca.

Affinity purified alpaca anti-Tiger Snake venom antibodies showed the highest anti-complementary activity. This is difficult to explain. Perhaps the only conclusion that can be reached is that this test system using guinea pig complement is not appropriate for testing alpaca products.

The author (A. Padula) concludes that although the CFT assay conducted here provides interesting results it is not possible to interpret the results with a view to predicting adverse reactions.

A more useful test would be to conduct a guinea pig anaphylaxis assay (Jeter, Russell et al. 1983). This assay involves sensitising guinea pigs with a low dose of antivenom and then one month later challenging the guinea pig with a large dose of antivenom administered intravenously. The guinea pig is exquisitely sensitive to developing anaphylactic reactions which occur within minutes of administration. A similar result can be obtained with mice although with reduced sensitivity. This type of assay is best used to predict likely reactions to protein based products. However to conduct this type of bioassay requires a specific animal ethics approval to be obtained. The type of bioassay required needs a special ethics permit to perform as the laboratory animals are observed until the point of death. Obtaining the necessary permits and laboratory animals was considered beyond the scope of this project.
2. Heat stability of alpaca antivenom to short term high temperature exposure

Summary

This work compared the effects of heating commercial (CSL) antivenom and experimental alpaca antivenom to 60°C for 20 hours and 72°C for 5 minutes to simulate different pasteurisation conditions. Prior to heating, alpaca antivenom displayed excellent neutralisation of procoagulant effects of Brown Snake venom, being approximately ten time more effective than the CSL product. However alpaca antivenom performed poorly after heating retaining only 28% of initial activity compared to 52% for the CSL product. Horse-derived antivenom tolerated heat better but 60°C for 20h resulted in a gelatinous product for both antivenoms, although there was a much slower formation time for horse than alpaca product. The conclusion is that alpaca antivenom contains no inherent thermal stability properties in the form it was tested.

Introduction

Immunoglobulins are inherently heat stable molecules. Short term exposure to 56°C does not significantly affect their ability to bind to their target. Horse-derived snake antivenoms have been shown to maintain excellent neutralising ability when maintained at 37°C for 12 months (Rojas, Espinoza et al. 1990). However there is a gradual change in colour and turbidity of the solution during higher temperature storage than when 4°C is used.

During manufacturing of antivenom or other immunoglobulin products, the product must be produced in a sterile manner. Many liquid products are terminally sterilised, that is they are sterilised using heat or radiation in the vial, rather than using a pre-filling method of sterilisation. Terminal sterilisation would reduce some costs with manufacturing. Milk factories use pasteurisation to heat milk to either 72°C for 15 seconds or for ultra-high temperature (UHT) milk heating to 135°C for up to 2 seconds. Lower temperatures require longer heating times such that heating to 60°C requires at least 10 hours to achieve the same level of microbial reduction (Grandgeorge, Veron et al. 1996). Numerous human serum products have historically been heat treated to reduce their potential viral burden (Mannucci 1993) with 60°C and 144 hours used in some cases.

The advantages of having antivenom stable at tropical temperatures are obvious – no refrigeration is required. However the workaround for this is to prepare antivenom as freeze dried powder. Although more costly, freeze dried pharmaceuticals have widespread use. In fact the BTG Pty Ltd rattlesnake antivenom CroFab™ is a freeze dried formulation.

This work has examined the effect of a 10-hour 60°C treatment of alpaca antivenom on its neutralising ability for coagulation disturbances. Coagulation defects predominate with many elapid snakebites and this can be measured more precisely and without harming animals using standard blood clotting assays.

Materials and methods

Antivenom

Alpaca Brown Snake Antivenom containing a final formulation of 50g/L total protein was prepared from a single high responding alpaca using ammonium sulphate fractionation. This product had been previously heat treated to 56°C for 60 minutes during manufacture.
For comparison, a vial of CSL Brown Snake Antivenom was used as a control (Batch No. 0559-10701; 1000 Units in 3.31mL).

**Heating**

A 2mL aliquot of each antivenom was placed into a 10 mL clear polycarbonate tube (Thermo Fisher, Australia) and incubated in a digital temperature-controlled water bath (Thermo Fisher). The temperature of the water bath unit was calibrated against a digital thermocouple unit (Fluke, Australia). The water bath temperature was programmed to either 60°C or 72°C. A second sample was heated to 72°C for 5 minutes. At the completion of the heating phase the test samples were transferred to a refrigerator at 4°C until assayed for neutralisation of coagulation.

**Neutralisation of coagulation**

Whole Brown Snake venom was used to determine the ability of the antivenom to neutralise the procoagulant effects of the venom. The assayed used was as described in the section in this report on neutralisation of coagulation. Briefly, 50µL of 0.01M calcium chloride solution was added to a coagulation cuvette and incubated at 37°C for 60 seconds with 50µL of citrated canine plasma. Canine plasma was used because it has been shown to be a reliable substitute for human plasma in clotting assays (Sprivulis, Jelinek et al. 1996). The cuvette was placed into the timing chamber of a semi-automatic electronic fibrintimer. The clotting reaction was initiated by adding 100µL of venom/antivenom mixture. The fibrintimer electronically monitors clot formation and stops the clock when a clot has formed. The challenge dose of venom was chosen such that a clot formed in approximately 60 seconds. A fixed dose of venom was incubated with various dilutions of antivenom in a quantitative manner.

**Turbidity**

Turbidity of the liquid antivenom solutions before and after heating was assessed by measuring the absorbance of each solution in a spectrophotometer at 590nm and 750nm (Rojas, Jimenez et al. 1994, Otero, Leon et al. 2006). The baseline absorbance was determined using distilled water as a blank.

**Data analysis**

Coagulation tests were performed in duplicate and the mean of each neutralisation level was used. The coefficient of variation between replicates averaged less than 5%. Coagulation data was plotted in Microsoft Excel™ as log (Level) on the x-axis and clotting time on y-axis. The effective dose 50% (ED50) was defined as the dose at which the clotting time was three times the minimum coagulant dose (MCD). The Level was then visually interpolated from the x-axis.

**Results**

**Turbidity and physical appearance**

Heating of alpaca antivenom to 72°C for 5 minutes changed the liquid into an milky coloured, semi-opaque gelatinous substance with higher viscosity than prior to heating. The CSL antivenom was much less affected but appeared to be thicker in consistency than prior to heating. The absorbance measurements made on the products were consistent with the physical appearance, in that the absorbance at both wavelengths was markedly increased for alpaca antivenom but much less for horse antivenom.
The changes in antivenom appearance were time dependent. Although not assessed in detail it was obvious that 72°C caused more change after 5 minutes than 1 minute. A more detailed time dependent analysis may reveal a different result to that achieved after 5 minutes. Continuing the heating to 30 minutes at 72°C resulted in a solid white gelatinous plug of material at the bottom of the tube.

Heating of both alpaca and CSL antivenom to 60°C for 20 hours resulted in formation of a solid gelatinous mass at the bottom of each tube. This was time dependent. CSL antivenom remained liquid for approximately 10 hours whilst at this same stage the alpaca antivenom had turned into a white semi-solid gel. Further heating to 20 hours resulted in both products having the same physical appearance. These could not be used for testing. No further antivenom from the same batches was available for repeat testing at different temperatures and times (see Table 4).

Neutralisation of procoagulant

Prior to heat treatment, Alpaca Brown Snake antivenom was at least ten times more effective at neutralising the procoagulant effects of whole Brown Snake venom (*P. textilis*) compared to CSL Brown Snake Antivenom (Figure 3; Table 5. The alpaca antivenom was formulated to 50g/L total protein while the CSL antivenom was 170g/L total protein.

Heat treatment at 60°C for 20 hours rendered both antivenom products untestable for coagulation studies as they had both formed a rigid gel in the tube (Figure 4).

Heat treatment at 72°C for 5 minutes resulted in a decline to 28% of initial potency for alpaca antivenom and a decline to 52% of initial potency for CSL. The alpaca antivenom appeared to be more affected by the higher temperature than CSL antivenom.

Table 4. Effect of heat treatment on antivenom turbidity. Data presented as optical density measurements using the absorbance of distilled water as the baseline.

<table>
<thead>
<tr>
<th></th>
<th>Initial</th>
<th>60°C x 20 hours</th>
<th>72°C x 5 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A590nm</td>
<td>A750nm</td>
<td>A590nm</td>
</tr>
<tr>
<td>Alpaca</td>
<td>0.108</td>
<td>0.128</td>
<td>ND</td>
</tr>
<tr>
<td>CSL</td>
<td>0.058</td>
<td>0.016</td>
<td>ND</td>
</tr>
<tr>
<td>dH₂O blank</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
</tbody>
</table>

ND=not determined due to gelling of antivenom
Table 5. Summary of CSL Brown Snake antivenom and Alpaca Brown Snake antivenom in neutralising the procoagulant effects of Brown Snake venom. Results are shown as mg of venom neutralised per mL of antivenom.

<table>
<thead>
<tr>
<th></th>
<th>Neutralising Capacity / ED50 (mg/mL)</th>
<th>Percentage of Initial Activity Remaining after Heating</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial 72°C for 5 minutes</td>
<td></td>
</tr>
<tr>
<td>Alpaca</td>
<td>2.90  0.80</td>
<td>28%</td>
</tr>
<tr>
<td>CSL</td>
<td>0.27  0.14</td>
<td>52%</td>
</tr>
</tbody>
</table>

Figure 3. Comparison of pre-heat treatment (initial) and post-heat treatment at 72°C for 5 minutes on capacity of either CSL Brown Snake antivenom or Alpaca Brown Snake antivenom to neutralise the procoagulant effect of Brown Snake (P. textilis) venom.
Figure 4. Appearance of CSL Brown Snake antivenom (LEFT) and alpaca Brown Snake antivenom (RIGHT) after heating to 60°C for 1 hour or 20 hours.

Discussion

The results obtained here do not provide evidence for greater heat stability of alpaca antivenom compared with commercial horse antivenom. This is an unexpected result considering previous work undertaken with camel serum-derived antivenom that did show heat stability (Cook 2010). The close genetic relationship between camels and alpaca was assumed to have resulted in similar IgG properties. However the heat stability studies that Cook performed were using highly purified immunoglobulin fractions. The aim of that work was to compare the IgG subclasses to see if heat stability differed between them. Cook reported that sheep serum turned gelatinous when heated to 80°C which is similar to what we have observed here for alpaca serum.

However, these results further demonstrate the remarkably potency of alpaca antivenom in neutralising the procoagulant effects of Brown Snake venom. Alpaca antivenom was at least ten times as effective on a comparative per mL basis as the CSL product. This result is even more remarkable because the alpaca antivenom product was only formulated at 50 g/L total protein compared to 170 g/L for the CSL product. Thus the CSL product is 3.4 (170/50) times more concentrated in terms of antibodies. Thus the true potency of the alpaca product could well exceed 30 times that of the CSL product giving alpaca antivenom a significant product marketing advantage.

Heat stability could be improved by adding chemical stabilisers such as sorbitol. However this adds to manufacturing and analytical costs and safety must also be demonstrated.

In conclusion, alpaca immunoglobulins do not appear to have any significant heat stability.
3. Improving the venom antibody ELISA

Summary

A quantitative enzyme-linked immunosorbent assay (ELISA) was developed and correlated to a mouse bioassay. Comprehensive time course serum potency values were measured for all alpaca over 500 days. A high correlation was shown between ELISA for purified toxin antibodies and for whole venom. Alpaca were rested from immunisation for approximately six months and then boosted with two doses of venom. Immunity to venom had waned to minimal levels but was rapidly restored following the booster doses. The dataset also revealed that animals which demonstrated a high antibody response at 4 and 8 weeks were the best responders at 40 weeks. This correlation would allow for early selection of high responders. In conclusion, the ELISA is a very efficient and precise method of monitoring immune responses in alpaca to hyperimmunisation.

Introduction

During Phase 1 of the Alpaca Immunoglobulin project an ELISA test was developed to measure the immune response to venom immunisation. This ELISA data was reported only as raw optical density measurements. To make the ELISA results more meaningful it is necessary to calibrate the ELISA to a known potency reference standard. This more quantitative approach increases the utility of the ELISA and the results are more directly related to potency.

Numerous studies have reported high correlation between ELISA results and mouse protection test results. Of most relevance to Australian elapid snakes was the work conducted in Thailand during the 1990s. This work correlated a newly developed venom antibody ELISA for Cobra (Naja) species with the traditional mouse protection assay (Rungsiwongse and Ratanabanangkoon 1991, Pratanaphon, Akesowan et al. 1997). The similarity between the Thai Cobra venom and Australian elapid species means that these results are highly relevant. The Thai researchers found a very strong Pearson correlation coefficient (r) of 0.95 when purified neurotoxin from Cobra venom was used as the antigen in their ELISA assay (Rungsiwongse and Ratanabanangkoon 1991). A Pearson correlation (r) of 0.82 was found when whole venom was used as the antigen.

This subproject evaluated the performance of the alpaca antibody ELISA for measuring responses to venom over time.

Materials and methods

Animals

Alpaca were immunised at approximately monthly intervals using whole venom. The details of the immunisation program are described in the general materials and methods and in the project report from Phase 1.

Alpaca serum samples

Blood samples were collected from the jugular vein using vacuum tubes from each alpaca at approximately monthly intervals for 20 months. Tubes were centrifuged following clotting and the serum separated at stored frozen at -20°C until assayed.
Venom antibody ELISA

The ELISA used was similar to that described in Phase 1 of the alpaca immunoglobulin project but differed as described below.

Reference serum

A reference standard of known potency against Brown Snake venom, Tiger Snake venom and Papuan Taipan venom was prepared. A mouse bioassay was used to determine the potency of the reference serum. The potency of the standard was reported as mg of venom neutralised per mL of antivenom. The reference standard was stored in 10 mL tubes containing 0.01% Thimerosal as a preservative. The standard was used at an initial dilution of 1:5,000. A standard curve of ELISA units was used on each 96-well microplate. Doubling dilutions of the reference standard were applied in triplicate to each plate. A standard curve was fitted using a commercial software package (Tecan Magellan 7.1 Software). Unknown values were interpolated in duplicate from this standard curve. Arbitrary ELISA units were used based on the potency obtained from the reference serum in each mouse bioassay.

Secondary antibody

Two secondary antibody products were compared.

Firstly a specific rabbit anti-alpaca IgG conjugated to horse radish peroxidase (HRP) was made. Two rabbits were immunised over 3 months with purified alpaca immunoglobulins. Once the rabbits had reach high titres they were bled out and the rabbit IgG extracted and conjugated to HRP. This preparation was used at a dilution of 1:3,000 as determined from a checkerboard titration procedure.

Secondly, a commercial secondary antibody was purchased from AbCam, USA. This consisted of a goat anti-llama IgG HRP conjugate. It was not known how well this would detect alpaca IgG so a trial was conducted to compare the above rabbit anti-alpaca IgG with the goat anti-llama IgG conjugate. This conjugate was used at a dilution of 1:10,000.

Venom antigen

High binding 96-well polystyrene microplates were coated with either whole venom or purified fractions of venom. Through a checkerboard titration it was found the optimal concentration of either whole venom or purified toxin to coat the plates was 1 µg/mL.

A purified toxin fraction of Tiger Snake (Notechis scutatus) venom was prepared by centrifugal filtration of whole venom using a 10kD and 30kD spin-up column tube (VivaSpin, USA). The supernatant containing high molecular compounds (procoagulant) was discarded. The purity of this fraction was examined by SDS-PAGE and found to contain predominantly a single band around 13kD which corresponds to the neurotoxin Notexin.

Purified textilitoxin from Brown Snake (P. textilis) venom was purchased from a commercial supplier and used for determining specific antibody levels to Brown Snake venom. The textilitoxin fraction was used at a concentration of 1µg/mL for coating microplates.

Total serum protein concentration

The total serum protein concentration was determined at every time point using a refractometer. Values were measured in grams of protein per Litre of serum.
Total serum immunoglobulin

A direct ELISA was developed in-house to measure total serum immunoglobulin concentration. High binding 96-well microplates were coated with alpaca serum at 1:100,000 dilution in carbonate coating buffer pH 9.6. Plates were incubated for 2 hours at room temperature and overnight in the fridge. Unbound sites were blocked with 0.5% casein (Sigma-Aldrich, Australia). Rabbit anti-alpaca IgG conjugated to HRP was used to detect bound alpaca IgG. Plates were washed 6 times in PBS-T20 and the substrate tetramethylbenzidine (TMB) was added. Plates were incubated until sufficient colour had developed. The reaction was then stopped with dilute sulphuric acid and immediately read at 450nm in a microplate reader.

A reference standard prepared from Protein A/G purified alpaca IgG was used. This was the same material used for making the rabbit anti-alpaca IgG. This standard was applied to each plate in doubling dilutions. A standard curve was constructed on each plate and unknown values interpolated using the plate reader software. Results are reported in mg/mL.

Results

Quantitative ELISA performance

The general performance of the venom antibody ELISA was excellent with a wide dynamic range, high sensitivity and the ability to use low concentrations of venom and purified toxins for detection of antibodies.

Textilotoxin coating concentration

A titration was initially performed to optimise the concentration of textilotoxin for coating the microplates (Figure 5). Serial dilution of the textilotoxin across the plate showed a decreasing linear response in absorbance. A concentration of 1ug/mL was deemed optimal, resulting in an A450 of around 1.0 (Figure 6).
**Textilotoxin quantitative standard curve**

![Textilotoxin Antibody Levels](image)

Figure 6. Typical standard curve for textilotoxin antibody measurement in alpaca. The curve fit was generally better than $R^2=0.998$ using a five-point Marquadt equation (Tecan Magellan 7.01 software).

Each data point corresponds to a doubling in the concentration of venom antibody.
Secondary antibody performance

There appeared to be no difference between quantitative results if the secondary antibody was either rabbit anti-alpaca or the goat anti-llama. Both conjugated antibodies performed equally well at binding to alpaca venom antibodies. The goat anti-llama however was used at a much lower concentration than the rabbit anti-llama (Figure 7).
Total serum protein concentration

There were minimal changes in total serum protein (TP) concentration over time (Figure 8). A small increase in TP occurred immediately following the beginning of immunisation. A small drop in TP resulted after the bulk bleedings but was quickly restored to normal levels.
Polyvalent alpaca – Tiger Snake venom 10-30kD fraction

There were significant differences between individual alpaca in the polyvalent (five venom types) treatment group. The chart below (Figure 9) shows responses to the purified toxic fraction of Tiger Snake (*Notechis scutatus*) venom. Alpaca No. 9 was a superior responder to all venom types. Almost no response was observed in Alpaca No.6 and No.7. Note the maximum concentration that this ELISA assay can measure was 100 Units/mL. The plateauing at 100 Units/mL of Alpaca No. 9 is a result of that animal’s antibody levels reaching the maximum recordable. It is likely that this animal reached significantly higher levels than were recordable by the assay.

Alpaca No. 9 is a super responder and quickly reached very high levels of specific antibody compared to all other animals studied. This animal responded well to all venom types, not just Tiger Snake. The conclusion was that a good responder to one venom type will also be a good responder to other venom types. The response is not venom specific.

![Graph showing antibody responses to the 10-30kD purified toxic fraction of Tiger Snake venom in four alpaca immunised with five venom types (polyvalent). The maximum measurable value is 100 Units/mL. Values higher than this are shown as 100 Units/mL.](image-url)
Polyvalent alpaca – Whole Brown Snake venom

The response of alpaca immunised only with Brown Snake Venom (*P. textilis*) showed a similar overall pattern to the Tiger Snake venom 10-30kD toxic fraction (Figure 10). Again, Alpaca No.9 showed extremely high levels of specific antibody. Alpaca No.6 and No.7 were poor responders. Note the difference in the Y-axis scale is due to the reference standard, determined in a mouse study, to have a potency of 164.9 Units per mL. This is the highest concentration measurable in this assay with samples at this dilution (1:5,000).

Figure 10. Specific antibody responses to whole Brown Snake venom (*P. textilis*) in four alpaca immunised with five venom types (polyvalent). Note the maximum measurable concentration is 164.9 units/mL.
Polyvalent alpaca – Whole Papuan Taipan venom

The response of the polyvalently immunised alpaca to whole Papuan Taipan venom (*O. s. canni*) showed a very similar pattern to their response to Tiger and Brown snake venom (Figure 11). Alpaca No. 9 was again a superior responding animal, quickly reaching very high levels of specific antibody. Taipan venom proved more difficult for the alpaca to make antibodies towards than either Brown or Tiger with a lower result for the reference standard.

![Polyvalent alpaca – Whole Papuan Taipan venom](image-url)
Mono Tiger – Whole Tiger Snake venom v 10-30KD fraction

Four alpacas that were immunised only with Tiger Snake venom were used for this comparison (Figure 12). A direct comparison was made between the antibody response in these animals to whole Tiger Snake (*P. textilis*) venom and the response to purified toxin of this venom (10-30kd fraction). Exactly the same samples were used and all other conditions were identical except that plates were either coated with Whole venom or the 10-30kD fraction. Overall there was little difference between the results. The response to whole venom was slightly greater than that of the specific toxin.

![Mean Potency Data Comparison of TSV 10-30kD v Whole TSV](image)

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The four alpaca that were immunised with only whole Tiger Snake venom showed individual variation in antibody levels to the 10-30kD toxic fraction of Tiger Snake venom (Figure 13). Alpaca No.11 was a poor responder reaching levels that were only 10-15% of that obtained by the better responding alpaca. Levels fluctuated up and down in response to bulk blood collection and booster doses of venom.
Alpaca mono Brown – Whole Brown Snake venom antibody

Three out of four alpaca that were monovalently immunised with whole Brown Snake venom produced good antibody levels (Figure 14). One alpaca (No.13) had failed to produce almost any response by 300 days and was deemed a non-responder.

![Graph showing antibody levels](image)
Alpaca mono Taipan – Whole Papuan Taipan venom

Four alpaca were immunised monovalently with Papuan Taipan venom (*Oxyuranus scutellatus canni*) (Figure 15). The immunised alpaca did not reach the same levels of potency as for the other venom types. It would appear that Taipan venom is less immunogenic in alpaca than either Brown or Tiger snake venom.

![Diagram showing responses of four alpaca to immunisation with Papuan Taipan venom.](image-url)
Alpaca mono Taipan – Total IgG v serum venom antibody level

Four alpaca that were immunised with Papuan Taipan venom were monitored for their total immunoglobulin G concentration (Figure 16). The ELISA for total alpaca IgG demonstrated a generally close relationship between total IgG in serum and the specific immune response. This data indicates that the bulk of the antibodies produced in the total IgG were specific for Taipan venom.
**Alpaca mono Brown – Textilotoxin antibody**

Purified textilotoxin, the active neurotoxin in Brown Snake venom, was used to coat microplates for detection of specific antibody (Figure 17). A coating concentration of 1ug/mL was used (see previous data on checkerboard titration).

Antibody directed against textilotoxin showed a high degree of correlation with antibody directed against whole Brown Snake venom.
Alpaca No.18 and No.19 datasets were examined for the relationship between textilotoxin antibodies and whole Brown Snake venom antibody levels (Figure 18). There was very close agreement between results obtained for both antigens. Alpaca No.18 and No.19 were immunised only with Brown Snake venom.

![Graph showing ELISA results for Alpaca No.18 and No.19](image-url)

(a) Comparison of Mono Brown Immunised Alpaca #19 and #18 ELISA plates coated with Whole BSV and Textilotoxin 1μg/mL
Alpaca No.10 and No.13 datasets were examined for the relationship between textilotoxin antibodies and whole Brown Snake venom antibody levels (Figure 19). There was very close agreement between results obtained for both antigens. Alpaca No.10 and No.13 were immunised only with Brown Snake venom.

(b) Comparison of Mono Brown Immunised Alpaca #13 and #10 ELISA plates coated with Whole BSV and Textilotoxin 1μg/mL
Alpaca Tiger + Brown – Average response to Brown and Tiger

Four alpaca immunised bivalently with both Tiger and Brown snake venom showed similar responses to both venoms (Figure 20). Response towards Brown Snake venom was slightly better than the response to Tiger. Overall antibody levels were lower than for animals immunised with only a single venom type.
Alpaca Tiger and Brown – Comparison of textilotoxin v Whole Brown Snake venom

Alpaca No. 17 and No. 21 were immunised with both Tiger and Brown Snake venom and their responses to the purified textilotoxin were examined (Figure 21). There was very close agreement between antibody levels directed towards the whole venom and those specific to textilotoxin.
**Responses following rest for six months**

Alpaca were selected into either high-responding or low-responding groups based on their responses to venom as measured by the ELISA. Only the high-responding animals were used for further work. Ten high responders were selected from the larger group and the remainder of the animals culled from the project.

Alpaca were not given any venom immunisations for a period of approximately six months during 2013. Two venom booster injections at intervals of 2 weeks were given as shown in the following charts as Booster 1 and Booster 2. A bulk bleed (blood bag collection) was performed on each animal two weeks after the second booster dose.

There was a consistent pattern to all animals following the 6-month rest period. Antibody levels in all animals had declined significantly to baseline levels. Following the first booster dose of venom there was a small rise in antibody levels when measured at two weeks and a much larger rise when measured again at 4 weeks.

Following the second booster dose of venom antibody levels had increased 20 to 50 times the levels measured after resting for 6 months.

**Mono-Brown immunised alpaca**

Two alpaca immunised only with whole Brown Snake venom show similar responses with both animals reaching the same potency after the second booster (Figure 22).
**Mono Tiger immunised alpaca**

Three alpaca that had been monovalent immunised with whole Tiger Snake venom showed different responses (Figure 23). Alpaca No. 12 obtained much higher levels of specific antibody (175 units/mL) compared to Alpaca No. 15 & 20 (~55 Units/mL) following the second booster.
**Mono-Taipan immunised alpaca**

Two alpaca deemed high responders to Taipan venom were rested for 6 months and then given two booster doses of venom (Figure 24). The response to booster doses was significantly less for Taipan venom that for either Tiger or Brown venom.

![Graph showing antibody response following a 6-month rest period in two alpaca monovalently immunised with Papuan Taipan venom.](image-url)
Polyvalent immunised alpaca

Two alpaca that had been immunised with all five venom types simultaneously (polyvalent) were selected as high responders (Figure 25). Alpaca No.9 demonstrated the most remarkable responses to both Tiger and Taipan venom.
**Tiger & Brown immunised alpaca**

One alpaca (Alpaca No. 17) was selected as a high responder to both Brown and Tiger Snake venom (Figure 26). Antibody levels rose to extremely high levels following the second booster dose of venom in this animal.
Alpaca No. 9 – Supreme responder

The case of Alpaca No. 9 deserves special mention as this animal was the best responding alpaca of all (Figure 27). This alpaca was immunised polyvalently with five venom types. The antibody levels obtained to Tiger, Brown and Taipan were the highest of any animal. Surprisingly, this alpaca only developed modest injection site swellings to the venom in oil. We had previously noted a broad relationship between the size of the lumps and antibody responses, with the larger lumps found in better responding alpacas.

[The flat lines shown in the ELISA potency data shown in Figure 28 represent the maximum values measurable in the assay as it was set up at that stage of the project. Alpaca No. 9 exceeded the upper limit and subsequent samples (>450d) were analysed with the serum diluted 1:10,000 rather than the preceding 1:5,000 dilution. This doubled the dynamic range of the assay.]

Interestingly, it again proved difficult to obtain high levels of antibody to Taipan venom, even after a prolonged rest period.

Figure 27. Alpaca No. 9 – a supreme venom antibody producer.
Figure 28: Antibody response to Tiger, Brown and Taipan venom over the duration of the project. A rest phase of approximately 6 months was implemented from days 450-620.
Correlation between antibody levels at week 8 and at week 40

Four alpaca that received monovalent immunisation with only Tiger Snake venom were examined for the correlation between antibody levels at week 8 and week 40 (Figure 29).

The Pearson correlation coefficient was extremely strong between the early response and later levels reached (r=0.98; P<0.05).

These results demonstrate that high-responding animals can be identified very early in the course of an immunisation program.
Discussion

The data generated by the ELISA testing of sequentially collected samples has provided a fascinating insight into antibody production against different venoms.

The utility of the ELISA for monitoring antibody response was excellent. The use of purified toxins for monitoring antibody levels produced results that were very closely correlated with those that were obtained when whole venom was used. Purified toxins are extremely expensive to purchase, costing approximately $3000 to $5000 per milligram. The data generated here shows small differences between ELISA results of whole venom and purified toxins. In Thailand it was shown that the correlation between ELISA results and mouse testing was slightly improved when purified toxins were used rather than whole venom (Rungsiwongse and Ratanabanangkoon 1991). However the improvement was only of the order of 10 to 15%. Nevertheless the ELISA for purified toxins clearly demonstrates that the alpaca make an all-round response to all components within the venom, and presumably with relatively equal potency.

Alpaca that were immunised with only a single venom type on average performed slightly better than those immunised with multiple venoms. This has implications for commercial production. The two options generally presented are either to immunise animals with a single venom type or to post-blend the final product. A third option is to immunise animals polyvalently with all venom types and vary the volume of product administered.

The period of prolonged rest for 6 months prior to administering booster doses of venom demonstrated the immunological ‘memory’ effect. When booster doses were ceased antibody levels gradually fell back to basal levels. This phenomenon has been observed in horses (Pratanaphon, Akhesowan et al. 1997). Despite the marked decline of 20 to 50-fold antibody potency, within four weeks of receiving booster doses antibody levels had risen markedly, and in some cases higher than prior to the beginning of the rest period. This effect has important implications for managing alpaca used for hyperimmunisation. Firstly, two boosters appear to be required to restore immunity back to very high levels, as a single dose failed to generate a large response. Secondly, when not needed for production alpaca can be safely allowed to decline in immunity with the confidence that this can be restored very quickly.

One of the most significant findings from these profiles is the wide variation between individual animals in their response to venom immunisation. Some alpaca failed to respond at all to repeated doses out to 300 days of immunisation. These animals seem unable to produce high levels of specific antibody but appear healthy and normal in every other respect.

The high correlation between the early immune response to venom and the mature immune response could be predicted using the ELISA. Week 8 blood antibody levels were strongly correlated in individual animals with their mature response. This information would mean that a screening test could be applied early to cull poorly responding animals. A strategy may be to start with 50% to 100% more animals than ultimately needed, culling poor responders to leave a high responding herd.

Alpaca No.19 proved to be a superior antibody producer. This animal appeared no different to the remainder of the animals in physical appearance. It more than doubled its antibody levels between day 100 and day 175. If enough animals were available to select from then working only with a small herd of No.9-type animals is likely to provide a vast improvement over current procedures used in horses. Selection pressure for high responders is generally not applied with horses due to their high cost. An alpaca anti-venom herd might be established by starting with (say) 1,000 animals and screening these down to 50 to 100 excellent responders. The capacity to select out the highest responders early in the primary immunisation phase gives alpaca significant advantage over horses. It would be possible to select the best horses and cull the low responders but the cost of this would be significantly greater.
than for alpaca. Although we do not have enough data and can only cross reference to published literature on horses, it appears that the variance seen in alpaca responses is much greater than seen with horses. That is, horses’ antibody responses seem to be less spread out, with most horses clustered around the mean, whereas alpaca seem to be spread over a wider potency range.
4. Restraint of alpaca for bulk blood collection and handling

Summary

This project explored a number of different restraint systems for handling alpaca and sheep. Each system has advantages and disadvantages. The number of animals to be bled, number of staff and the time available influence the choice in selecting the best system. An effective comprise was tested and found suitable in which alpaca were lightly sedated with a non-cardiovascular depressant sedative (Zoletil™ (Virbac); tiletamine hypochloride plus zolazepam hypochloride) and restrained in a horizontal position on a table.

Introduction

A safe restraint system suitable for alpaca, which can be used for veterinary procedures such as hyperimmunisation and bulk blood collection, is required for upscaling to greater animal numbers. During Phase 1 of Alpaca Immunoglobulins we used manual restraint of individual alpaca for injections, blood samples and bulk bleeding. Whilst this worked reasonably well it was unsafe in some instances for both the animal and operator. The problem was especially apparent when performing the bulk bleed. Any movement by the animal during this procedure can lead to trauma to the carotid artery, trachea and tissues of the neck of the alpaca.

Many automated handling systems have been developed for sheep. The systems are commonly used by sheep farmers to reduce the manual labour required when handling large numbers of sheep. These devices also improve the occupational health and safety aspects of sheep handling. Many of these systems are portable and can be run on a 12 volt power supply.

The objective of this project was to examine various methods of restraining alpaca that may prove useful for producing and harvesting immunoglobulins from serum.

Materials and methods

The approach taken in this section was to explore and document various systems that are used for blood collection in sheep and identify existing devices that may be useful for alpaca or their adaptation. A specialist consultant, formerly with a large sheep antibody-producing company was interviewed. Animal scientists and animal handlers from a scientific research institute were also consulted for their comments.
Results

General sheep handling systems

Figure 30. Portable Peak Hill Industries sheep handling system.

A number of manufacturers produce sheep handling equipment. Shown in the above photos (Figure 30) are the Peak Hill Industries systems. Large numbers of sheep can be handled efficiently. BTG Pty Ltd in the Barossa Valley, South Australia, uses these systems for immunising sheep with rattlesnake venom. The underside of the sheep is easily accessed and injections can be given into the axilla and groin regions of each sheep with minimal effort. However these systems are not immediately applicable to alpaca with their longer necks and different group behaviour.
Bulk blood collection systems

Figure 31. Automated handling system as used at IMVS, Adelaide, for bulk blood collection in sheep.

The Institute of Medical and Veterinary Sciences (IMVS) in South Australia has a long history with antibody production in various species including sheep. The device shown above (Figure 31) is used for bulk blood collection in sheep. The sheep walks into a crush area and the device squeezes onto the sheep and then flips it over horizontally. The head is then restrained backward and the jugular vein accessed for blood collection. The squeeze crush is mounted on wheels and can be moved around as required.
Figure 32. Older style sheep bleeding crate system previously used at IMVS.

A bleeding crate system is shown above (Figure 32). This system was previously used at IMVS for bulk blood collections. The sheep walks into the V-shaped unit and is gently squeezed between the side panels. The head is restrained to the side using a padded device shown on the left.
Alpaca specific systems

Figure 33. Alpaca crush system designed by Phillip Dawees, Donnelly Park Alpacas, NSW.

The alpaca specific system shown in the above photos (Figure 33) would appear to provide excellent restraint for bulk blood collection with minor modification.
Figure 34. Automated plasmapheresis of a goat using a modified Panepinto sling for restraint.

The restraint system shown above (Figure 34) is a sling system that supports the weight of the animal whilst plasma is collected. This is a commercially available system used in laboratories to harvest plasma via plasmapheresis machines. The system takes some training of the animals but the stainless steel frame is mounted on wheels and can be wheeled around. This type of system is especially suited to processing small numbers of goats or sheep.
Figure 35. Alpaca lightly sedated being gently restrained on a portable table in stock handling yards for bulk blood collection from the jugular vein.

The system shown above (Figure 35) is a compromise between speed, equipment investment and animal comfort. The alpaca are lightly sedated with Zoletil™ (Virbac); tiletamine hypochloride plus zolazepam hypochloride) which does not depress blood pressure like some other sedatives. Alpaca are gently lifted onto the table and placed on their side. The left side jugular is readily visible when clipped and sprayed with 70% alcohol. Blood readily flows into sterile blood collection bags which hang below the table.
Discussion

There are many different methods that can be used to restrain animals for bulk blood collection. A number of factors need to be balanced as to what constitutes the best system. If speed of collection is the goal, with the aim to process hundreds of animals in a morning, then a simple flip-table system would probably be very effective. If plasmapheresis was used, with a collection time of 45 minutes per alpaca, then a system similar to what is used in sheep and goats would be required. However considerable investment would need to be made in constructing restraint boxes at an approximate cost of $5,000 each for custom manufacturing.

As a compromise, mild sedation using Zoletil™ (tiletamine, zolazepam; 100mg/mL) provides the advantage of excellent chemical restraint and alpaca can be bled on a table in a stock yard (see photo). The downside is that alpaca take some time to become ambulatory after sedation and that care must be taken that they are not trodden on by other alpaca during recovery. The use of chemical restraint for shearing of rams is now commonplace in shearing sheds, reducing operator injuries and better fleece recovery from the ram.

All manual restraint systems require training of the animals to enter and leave the unit. Stress during blood collection can lead to haemolysed serum and an inferior product.

We have noted that bleeding of alpaca in a horizontal position obtains better visualisation and filling of the jugular vein. This leads to fewer carotid artery punctures and overall a cleaner and safer blood collection for the animal.
5. **Intellectual property landscape search on camelid antibodies**

**Summary**

A specialist patent attorney law firm was contracted to conduct an intellectual property (IP) landscape search and analysis. The goal of the work was to obtain a comprehensive understanding and professional opinion on the IP issues associated with commercialisation of alpaca antibody technology. No significant issues were found that would hinder commercial product development within Australia. The most limiting patent has now expired in August 2013 from its original filing in 1993. The pathway is thus clear for commercial product development.

**Introduction**

Since the discovery in 1993 of the unique molecular structure of camel antibodies much of the intellectual property associated with these molecules has been patented. Despite the therapeutic potential of the molecules, and the 20 years that has elapsed since then, no commercial camelid polyclonal antibody products have reached the marketplace.

The researchers who published the first work on camelid antibodies took out a vast range of patents associated with making and manipulating these antibodies. What initially would appear to be a non-patentable concept – immunising an animal (camelid species) – has since had patents applied. The presence of these patents has meant that commercialisation either requires licensing of this technology from the patent holder, generally by payment of royalties or other agreed amount, or avoidance of the method documented in the patent.

It is important to understand the IP issues associated with working and developing camelid antibody products. This work was undertaken to document a pathway through the intellectual property landscape of camelid antibodies.

**Materials and methods**

The patent search and analysis work was undertaken by a specialist biotechnology patent attorney firm (Davies, Collison and Cave, 1 Nicholson Street, Melbourne, Victoria, 3000). The search identified 356 patents of relevance. Each patent was analysed in respect of its potential impact.

**Results & discussion**

A verbatim copy of the summary report from Davies, Collison and Cave is included in the following pages. A full discussion of the search and opinion findings is in the final section of patent attorney’s report.
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EXECUTIVE SUMMARY

We have formed the view that the vast majority of the patent families identified in the Patent Landscape Search are not of relevance to your freedom in Australia to commercialise the subject of the search, i.e., polyclonal antibodies directed to snake venom obtained from immunised alpaca (the "Alpaca Technology").

Many of the patent families identified do not have Australian applications and several, upon closer analysis, were deemed to be of no relevance. In relation to those patents/patent applications identified that we consider to be of some relevance to your freedom to operate, we have at this stage provided only preliminary freedom to operate advice. We would be happy to consider any of the identified patents or patent applications in detail in the context of your commercial plans in order to provide you with comprehensive freedom to operate advice, on your instructions.

Australian Patent No. 701578 in the name of Vrije Universiteit Brussel would arguably have been highly relevant but has ceased, rendering the infringement question moot.

Australian Patent No. 738133 is a divisional patent of Australian Patent No. 701578. The granted claims are directed to fragments of antibodies and accordingly it is arguable that the Alpaca Technology would not infringe the claims of this patent assuming that this technology is restricted to generating whole antibodies and not fragments of the antibodies. The patent is due to expire on 18 August 2013 which will then render the infringement question moot.

Australian Patent No. 740043 in the name of Vlaam Interuniversitair Instituut Voor Biotechnologie is currently in force and is set to expire on 27 June 2017. The specification of granted Patent No. 740043 relates to camelid heavy chain antibodies and to the CDR3 loop, or a modified form thereof which is proposed to be able to bind to the active site of a target molecule. In particular, recombinant VHH or camelised VH antibodies are proposed. We consider that Claims 1, 2, 8, 9, 10, 12, 21, 22, 24 and 25 prima facie encompass the Alpaca Technology comprising the production of polyclonal antibodies in alpaca, and their use as anti-venom.

The options for dealing with this infringement issue are to;

i) Delay commencement of commercial activities until after the patent expires on 27 June 2017;

ii) Seek revocation or amendment of the patent;

iii) Negotiate a licence from Vlaam Interuniversitair Instituut Voor Biotechnologie VZW, who owns the patent.

We understand that option i) is a feasible option. It is also by far the simplest option.

In relation to item ii) and iii) above, we note that to the extent that any of the claims of Patent No. 740043 encompass the Alpaca Technology and specifically cover the use of intact immunoglobulins, these claims may arguably lack novelty and therefore validity in light of prior art disclosures of naturally occurring camelid antibodies. We therefore recommend conducting a validity analysis of Australian Patent No. 740043 to determine...
THE ALPACA TECHNOLOGY

The technology proposed to be commercialised (the "Alpaca Technology") and the subject of the Patent Landscape Search (the "Search") is the production in alpaca and commercialisation of polyclonal antibodies (immunoglobulins) directed against snake venom.

Alpaca (llama pacos) are members of the family, Camelidae. We understand there are several important and unusual characteristics of cameld polyclonal antibodies relative to conventional antibodies from non-camelids. These characteristics include their structure, stability and immunogenicity. We further understand that the polyclonal antibodies derived from the alpaca will comprise a mixture of antibody structures including "heavy chain" antibodies and "conventional" antibodies, provoked by immunising alpaca with snake venom antigen. Heavy chain antibodies are composed of (generally) two heavy polypeptide chains and they lack a CH1 domain and any light chains. Each heavy chain has a variable domain, referred to as "VHH" to distinguish it from the distinct variable domains of the heavy chain of conventional camelid (or other species) antibodies (referred to as VH). Importantly, we understand that intact antibodies are proposed for commercial use rather than antibody fragments derived from intact antibody or via other routes such as by genetic engineering.

SEARCHING INSTRUCTIONS

In light of the fact that you are proposing to commercialise the production and use of polyclonal anti-venom antibodies raised in alpaca we were asked to investigate your freedom to perform the Alpaca Technology in Australia. To this end, the issues which we believe are relevant to freedom to operate are as follows:

(i) Patent rights in relation to intact unfragmented cameld antibodies that are not produced recombinantly.

(ii) Patent rights in relation to the use of intact cameld antibodies as anti-venom (in particular, please note that such rights may exist where rights in relation to the antibody itself may not exist).

(iii) Patent rights in relation to the processing of serum from an immunised animal.

(iv) Patent rights in relation to modifications made to the isolated antibodies or sera.
Patent rights in relation to formulations, methods of treatment or modes of administration.

FIELDS SEARCHED

As you can appreciate, the costs involved in performing a freedom to operate analysis in relation to (i) – (v) detailed above would be very high. That being the case, it was decided to focus the Patent Landscape search and analysis on the core aspects of the project, these being points (i) (but further limited to alpaca) and (ii), above, as agreed.

Accordingly, the Search was not a complete freedom to operate search as it does not aim to identify all potential infringement risks associated with your activities in bringing the polyclonal alpaca anti-venom antibodies to market. For example, the search strategy adopted did not specifically include key words relating to the processing of polyclonal antibodies or any particular modes or protocols of administration, or formulations. Further searches should be conducted to address issues (iii) – (v).

JURISDICTIONS

In terms of the jurisdictions in respect of which this search was to be focussed, we did bring your attention to the fact that there were inherent limitations in searching across a wide range of jurisdictions, due largely to the quality of access to patent literature which is made available by any given patent office.

In general, very good full text searching is made available in the context of United States patent applications, while Canadian and European applications can be extensively searched via various international databases. Unfortunately, the Australian Patent Office does not provide full text searching, this being a significant limitation of the Australian Patent Office database. Accordingly, unless an Australian patent application is a National Phase application of an International (PCT) application, it will not be detectable by keyword based searching. Published PCT applications are fully searchable and since we conduct patent family searches on any PCT applications of interest, an Australian National Phase application deriving from such a PCT application will be identified. In general, most Australian biotech patent applications are National Phase entries of PCT applications, rather than being filed directly at the Australian Patent Office. However, it nevertheless sometimes occurs that a third party files a patent application directly at the Australian Patent Office which is not related to a PCT application. Where this occurs, there is no mechanism currently available to search for those documents. The Australian Patent Office is hoping to enable key word text searching of Australian patent applications.

We also draw your attention to the fact that there are a large number of jurisdictions that do not publish patent applications in English. For example, most Asian jurisdictions, including Japan, and most European jurisdictions publish in that jurisdiction’s official language only. This can make freedom to operate analyses quite complex in that if you were proposing to pursue a commercial strategy in any of these jurisdictions, it would be necessary to instruct a local searching company to conduct a freedom to operate search in the local language.

Based on your consideration of jurisdictions of interest versus relative costs, we were
instructed to conduct a Patent Landscape Search focussing on Australia.
PATENT LANDSCAPE OPINION

GENERAL COMMENTS

A "freedom to operate" opinion is a written opinion which provides the results of a detailed search for patent documents which might be infringed if a party was to commercialise one or more technologies without an appropriate licence, together with an analysis of whether the proposed activities would infringe any of the identified patent documents. The opinion would also generally include an analysis of whether any such relevant third party patent document is valid. Such advice would closely consider the language of the patent claims and the nature of proposed commercial activities to form a view as to whether an Australian court would be likely to uphold the patent and find infringement.

A "patent landscape" opinion is a written opinion providing general patent infringement advice on the results of a search to locate patent documents which may relate to the subject matter identified in the instruction section below. As noted previously, such a search, and therefore such an opinion, can provide merely a useful indication as to whether there are competing patent rights in existence in the general field of interest.

In the absence of formally achieving revocation of a patent, or appropriate amendment to the granted claims, the exploitation of the rights claimed in that patent, in the absence of a licence, will still amount to an infringement for as long as the patent has not been revoked or suitably amended, even if the patent is arguably invalid. Patentability opinions are quite complex, and, to the extent that one is considering the impact of a foreign patent, a opinion must be sought from an attorney practising in the jurisdiction of interest.

That being the case, and in light of our understanding of the instructions provided to us, we are providing you with a patent landscape opinion in respect of your potential requirement to seek licences in order to perform the Alpaca Technology in light of relevant third party rights which may exist in relation to the Alpaca Technology. This has involved an multi-jurisdictional patent search and patent family analysis in order to identify Australian patent documents which are arguably relevant to this issue. To this end, however, we do wish to point out that no detailed analysis has been provided in relation to the issue of potential infringement of third party rights and we have not provided an opinion in respect of the validity of any of the granted patents or patent applications which have been identified as potentially impacting on the issue of your freedom to operate since. We would be pleased to do so upon request.

In relation to those patents/patent applications identified that we consider to be of some relevance to your freedom to operate, we have at this stage provided only preliminary freedom to operate advice. We would be happy to consider any of the identified patents or patent applications in detail in the context of your commercial plans in order to provide you with comprehensive freedom to operate advice, on your instructions.

INFRINGEMENT/LICENSING

For infringement, you would appreciate that where a party has secured patent rights in
respect of an invention in a particular jurisdiction, any third party who exploits that invention in the same jurisdiction without the consent of the patentee is infringing the patentee’s rights.

In order to determine the scope of the patentee’s rights and infringement, reference is made to the claims of the patent in issue. The claims define the invention in respect of which rights were granted. Infringement exists when all of the limitations specified in a claim (i.e., numbered clauses usually found towards the end of the patent specification) are found in an accused product or process in the same relationship as in the claim.

An important distinction is drawn here between what is claimed in the patent and what is disclosed in the body of the patent specification. Often, the disclosure in the body of the specification is broader than the invention which the granted claims define. This may have occurred, for example, because the patent examiner did not regard certain embodiments or aspects of the invention as novel and inventive, thereby forcing the applicant to narrow the scope of the claimed invention. Infringement will only occur if there is unauthorised use of the claimed invention and not the use of any broader or unrelated disclosures detailed in the body of the specification.

Where a third party proposes to use an invention in respect of which patent rights exist, infringement can be avoided by seeking a licence from the patentee in respect of the patented invention. It should be noted, however, that where that third party has itself obtained patent rights in respect of a more specific invention which falls within the scope of a broader patent, obtaining these more specific rights, (where there is no practising of the more specific invention) would not of itself constitute an infringement. It is only where practise of the invention occurs that a potentially infringing act occurs.

In considering the issue of infringement, it is therefore necessary to determine the precise scope of the claims of each patent in issue. In this regard, some of the patent documents detailed here correspond to patent applications. An analysis can, therefore, only be performed in respect to the claims as originally filed or currently pending (if the latter are publicly available). Since claims are often amended during the prosecution of an application, a further analysis would be required following granting of the patent application in order to accurately determine the scope of the invention in respect of which rights have ultimately been granted. Further, it should be remembered that any international patent applications which have been identified are likely to proceed as national and regional phase applications in several jurisdictions and variation may therefore occur in terms of the precise scope of the claims which are ultimately granted in each jurisdiction.

Infringement, and therefore the requirement to seek licences, is only relevant where an invention is being practised in a jurisdiction in which a granted and in force patent exists claiming that invention. Accordingly, although a published patent application will be relevant to considerations of novelty and inventiveness irrespective of whether it is in a granted, pending, lapsed or withdrawn state, the only issue relevant to considerations of infringement and licensing is the scope of the claims as granted and whether the patent is in force. However, please note that the practise of an invention which is claimed in an application may ultimately constitute an infringement (depending upon the scope of claims ultimately granted) if the application is granted, since patent rights generally run from the publication date of the application.

We also draw your attention to the fact that some jurisdictions including Australia recognise the principle of “indirect” (or “contributory”) infringement. Such infringement may occur, for example, where a product is imported into a jurisdiction where some aspect of the manufacture of that product would have infringed a patent which exists in
the country of importation, had the manufacture been performed in that country. If it becomes necessary, we can provide further advice in relation to this issue.

Since some of the patent documents which were obtained by our search are applications (i.e., have not yet proceeded to grant), we have based the our opinion on the claims as they appear in the published applications herewith attached.

In relation to patents or patent applications which have ceased for failure to pay a renewal fee by the due date please note that there is a grace period extending by a further six months the deadline for paying renewal fees. Also, there exists in Australia generous provisions under the *Patents Act* for seeking an extension of time for undertaking a relevant act within a certain time frame (such as paying a renewal fee by the due date).

These provisions mean that a ceased patent or patent application may in special circumstances be restored. Briefly, the special circumstances are that the person responsible intended to maintain the patent or application and failed to do so by some error, omission or through circumstances beyond their control. Alternatively, if the person concerned took all due care to ensure that the action was done within the time frame available but still the action was not done. However, having decided to abandon a patent or application, the patentee/licensee cannot change their mind and seek restoration.

Finally, we note that although the term of an Australian patent is 20 years from the filing date, extensions of patent term are available where the delay in obtaining regulatory approval for a pharmaceutical substance that falls within the scope of the claims of a patent has been substantial, i.e., more than five years. We can advise further on these issues upon request.

**JURISDICTIONAL ISSUES**

Please be aware that a patent landscape opinion is, necessarily, an opinion in relation to whether or not actions which are proposed to be taken in a particular jurisdiction might infringe the claims as granted or currently pending in the patents and patent applications filed in that jurisdiction. Accordingly, such analysis is necessarily entirely dependent on the infringement laws of the jurisdiction of interest. Similarly, to the extent that one may seek to assess whether or not a given patent document is arguably invalid (thereby potentially providing a ground under which to seek revocation proceedings) this will also entirely depend on the patent validity laws of the jurisdiction of interest. As you will appreciate, although there are common themes in these laws across jurisdictions, for example the basic premise that an invention must be both novel and inventive, there do nevertheless exist significant differences in terms of the nature, scope and application of these laws. It is for this reason that the scope and type of claims which are ultimately obtained may vary from one jurisdiction to the next.

Since we practise as attorneys in Australia, we can provide you with a concluded opinion in relation to issues of infringement and validity as they relate to Australian patents. However, to the extent that you require infringement and/or validity opinions in relation to foreign patents, we are not able to provide you with a concluded opinion. Rather, we can conduct a detailed patent family and status search on an international level and, further, identify for you those applications which we believe are relevant and the major issues which we believe require consideration. However, other jurisdictions, in particular the United States, are unique in the context of infringement and validity laws and a
concluded opinion in relation to these issues could only be provided by an attorney in that jurisdiction who can consider and apply the nuances of the laws of that jurisdiction. In the context of the present opinion, this issue is moot since the opinion relates to Australian law. However, to the extent that further analysis is requested this may become an issue. In this case, if you required a concluded opinion in relation to your position in a jurisdiction such as the United States or Europe, it would be necessary to forward this opinion and all the relevant documentation to an attorney in that jurisdiction with instructions for providing an opinion.
PATENT LANDSCAPE SEARCH FOR ALPACA TECHNOLOGY

We have been instructed to conduct a patent landscape search to identify both patents and published patent applications that have been filed in the past 25 years and that may generally relate to snake anti-venom antibody production in alpaca as instructed by you on 24 January 2013. The search strategy that was originally proposed on 6 March 2012 was modified in line with a reduced scope to remove all species of camelid other than alpaca and to remove keywords relating to the processing of the alpaca antibodies. We understand you may instruct us at a later date to extend the search to such aspects.

SEARCH AND ANALYSIS STRATEGY

The search strategy used to identify patent publications that may relate to snake anti-venom antibody production in alpaca is summarised in Table 1 in Appendix A.

The patent landscape search was conducted on 27 February 2013 using the PatBase database which includes patent publications from a number of jurisdictions including Australia, United States, Europe, China and Japan.

The patent landscape search retrieved 356 patent publication records. A copy of the PatBase Search Transcript that includes the 356 PatBase records retrieved is provided herewith in Appendix B. The records appear in the transcript in descending order of earliest priority date.

A PatBase record corresponds to a patent family as opposed to an individual patent publication within a patent family. Accordingly, the search retrieved 356 PatBase patent families. As PatBase records correspond to patent families, PatBase selects the title and abstract of one of the patent family members to be the representative title and abstract for the entire family.

We have reviewed the search results, together with corresponding full Australian or International (PCT) patent documents where necessary, and we concur with the observations made by you concerning which patent families contained potentially relevant documents and were to be analysed further.

We restricted our further analyses to only those patent families with an in force Australian application, or patent, or more recent cases with an International (PCT) application yet to enter the national phase in Australia.

We identified 36 patent families which we considered required more detailed analysis. We then went on to conduct application and status searches in the Australian Patent Office Database in order to determine the Australian patent family members, if any, and whether any patent or applications were still currently pending or had lapsed. If the patent or application was still in force, we determined the precise status of that application (to the extent that this information was available), reviewed the specification and analysed the claims. As noted above, our analysis focussed on whether the Alpaca Technology is captured by the claims. Also as noted above, for any application, unlike a
patent, the scope of the claims of the application may change during prosecution as the specification undergoes examination.

We draw your attention to the fact that although the databases which were searched purport to provide current information in relation to published patent documents, there is often a delay between actual publication of an application and uploading of that data onto these databases. Accordingly, any application which has been published during the last few months should appear on these databases. However, it should be remembered that if there is any delay which has occurred in relation to the uploading of that information, we have no means available to us of identifying such an application. In this regard, we also take this opportunity to remind you that patent applications are usually not published until approximately 18 months after their earliest priority date. In the context of some of the older United States patent applications, these are not published until grant. Accordingly, any applications claiming priority within the last 18 months will not yet be published and are unlikely to be available on any of the databases which we have searched.

Whilst all due care and attention was taken in the conduct of the search, there are certain inherent limitations in conducting database searches. A summary of these limitations is attached herewith in Appendix C.
SEARCH RESULTS

The search results and initial analysis revealed documents falling into one of the six following categories:

(i) Patent documents which were entirely irrelevant.

(ii) Patent documents with no Australian counterpart or the counterpart has ceased.

(iii) Australian patent documents relating to camelid antibody but directed to unrelated (i.e., not anti-venom) antigens or to humanized antibodies.

(iv) Australian patent documents related to camelid antibody fragments.

(v) Australian patent documents related to camelid antibodies which we regarded as potentially relevant to practising the Alpaca Technology.

(vi) An Australian patent application where we have identified a conflict.
OPINION

(i) IRRELEVANT PATENT DOCUMENTS

We have formed the view that a large number of patent documents identified were irrelevant to the Alpaca Technology. These documents are derived from the 356 patent families identified in the search (Appendix B), minus the 36 patent families identified as of potential relevance described below. We have not analysed these documents other than to the extent that was required to determine their non-relevance.

(ii) PATENT DOCUMENTS WITH NO AUSTRALIAN COUNTERPART OR THE AUSTRALIAN COUNTERPART HAS CEASED

These documents are considered to be of no relevance.

Of the 36 patent families initially identified as being of potential relevance, 22 families did not appear to have an Australian counterpart and have not been considered any further. These are patent families identified by Search No. 321, 319, 293, 288, 234, 233, 222, 192, 191, 147, 134, 351, 347, 346, 341, 104, 108, 116, 87, 78, 53, 46.

In addition, some documents were identified as of potential relevance until it was determined that the Australian family members had ceased. These were:

1. Australian Patent No. 2002256866 and Australian Patent No. 2002311544 (Search No. 313)

Since these patents ceased in 2008 and 2007, respectively, through non-payment of a renewal fee, the issue of infringement is moot; and

2. Australian Patent No. 701578 (Search No. 353)

This patent in the name of Vrije Universiteit Brussel names Cecile Casterman and Raymond Hamers as inventors and has a filing date of 18 August 1993. The specification provides what may be the first disclosure of an immunoglobulin comprising two heavy polypeptide chains which lack a CH1 domain but form an antigen binding site in the absence of any light polypeptide chains. The granted claims are directed inter alia to such immunoglobulins. The Patent Office database indicates that the patent ceased before 21 March 2013, and was, in any event set to expire on 18 August 2013, the issue of infringement is moot.
(iii) AUSTRALIAN PATENT DOCUMENTS LIMITED TO UNRELATED (i.e., ANTI-VENOM) ANTIGENS OR TO HUMANIZED ANTIBODIES

1. Australian Patent No. 2003286002 (Search No. 290)
   ~ Granted claims directed to single domain antibodies against tumour necrosis factor.

2. Australian Patent No. 776824 (Search No. 333)
   ~ Granted claims directed to single variable domain antibodies capable of binding to a virus.

3. Australian Application No. 2009269698 (Search No. 102)
   ~ Application and claims directed to VHH domain antibodies binding glial fibrillary acid protein (GFAP).

4. Australian Application No. 2008281889 (Search No. 128)
   ~ Application and claims directed to camelid antibodies that bind VPS of rotavirus.

5. Australian Application No. 2011203408 (Search No. 58)
   ~ Application and claims directed to humanized antibodies.

We do not consider that the Alpaca Technology will infringe the claims of these patents or the claims of these application (if ultimately granted). A watch could be placed on the applications to monitor the claims ultimately granted on the applications.

(iv) AUSTRALIAN PATENT DOCUMENTS RELATED TO CAMELID ANTIBODY FRAGMENTS

1. Australian Patent No. 738133 (Search No. 353)

This patent is a divisional application of ceased Patent No. 701578 (see (ii) above).

Of potential relevance, granted claims 1 to 12 are directed to fragments of single heavy domain immunoglobulins wherein the fragments comprise an antigen binding site and wherein the fragment can be a heavy chain or a variable region thereof.

However, we consider that the Alpaca Technology will not infringe the claims of this patent provided that the Alpaca Technology does not involve production or use of immunoglobulin fragments. The patent is currently in force but is set to expire on 18 August 2013 after which date the issue of infringement will be moot. We note that there is no extension of patent term granted for this patent.
2. **Australian Application No. 2008272541 (Search No. 133)**

The specification and claims of this application are directed to variable fragments of camelid single chain antibodies for preparation of a peptide vector.

We do not consider that the Alpaca Technology will infringe the claims of this application because the Alpaca Technology does not involve production or use of antibody fragments.

3. **Australian Application No. 2008272541 (Search No. 133)**

The specification and claims of this application are directed to the use of VHH antibody fragments to vector substances across the blood brain barrier.

We do not consider that the Alpaca Technology will infringe the claims of this application because the Alpaca Technology does not involve production or use of antibody fragments.

4. **Australian Patent No. 2003295139 (Search No. 255)**

The granted claims are directed to single domain effector group immunoglobulins. The immunoglobulins are not isolated by animal immunisation and are not dual heavy chain or conventional camelid antibodies.

We do not consider that the Alpaca Technology will infringe the claims of this patent because the Alpaca Technology does not involve production or use of antibody fragments.

(v) **AUSTRALIAN PATENT DOCUMENTS RELATED TO CAMELID ANTIBODIES WHICH ARE REGARDED AS POTENTIALLY RELEVANT TO PRACTISING THE ALPACA TECHNOLOGY**

1. **Australian Patent No. 2003283137 (Search No. 291)**

The patent is in the name of Ablynx and has an expiry date of 7 November 2023. The specification relates to administering a polypeptide construct comprising one or more single domain antibodies by a non-invasive method to treat a disorder susceptible to modulation by an anti-target compound. The specification also relates to polypeptide constructs comprising at least one single domain antibody directed against an internalising cellular receptor and at least one therapeutic polypeptide or agent or antigen. The granted claims are directed to methods of treatment comprising administering a polypeptide construct comprising one or more single domain antibodies directed against one or more target molecules by a non-invasive route. Claims have also granted (Claim 25) to the use of a polypeptide construct comprising one or more single domain antibodies directed against a target molecule for the manufacture of a medicament wherein the medicament comprises an anti target compound and is suitable for delivery by a non-invasive route.
Privileged and Confidential

- 16 -

26 July, 2013

We believe that the skilled person would consider that a naturally derived antibody purified from alpaca as proposed in the Alpaca Technology, although likely to comprise single domain antibodies, would not be considered as a "polypeptide construct" and accordingly that the Alpaca Technology will not in our opinion fall within the scope of the granted claims. Additionally, provided the Alpaca Technology is formulated for intravenous administration, or another "non-invasive" route of administration, the Alpaca Technology would also not be covered by the granted claims for this reason.

2. Australian Patent No. 2010226938 (Search No. 289)

This patent is a divisional patent of 1. (above) and therefore its disclosure is similar.

Claim 1 of the granted patent is directed to a polypeptide construct comprising at least one single domain antibody directed against an internalising receptor, and at least one therapeutic polypeptide or agent or antigen, and wherein said polypeptide is not activating said cellular receptor.

Granted Claim 2 is directed to a polypeptide construct comprising at least one single domain antibody directed against an internalising receptor, and at least one single domain antibody directed against a therapeutic target, and wherein said polypeptide is not activating said cellular receptor.

We believe that the skilled person would consider that naturally derived antibodies purified from alpaca as proposed in the Alpaca Technology, although likely to comprise single domain antibodies, would not be considered as a "polypeptide construct" and accordingly that the Alpaca Technology will not in our opinion fall within the scope of the granted claims. Additionally, the naturally occurring alpaca antibodies are unlikely to comprise the bi-specificity required by Claim 1 and Claim 2. Granted Claims 3 to 20 are, in our opinion, of even less relevance.

3. Australian Patent No. 740043 (Search No. 344)

This granted patent is in the name of Vlaam Interuniversitair Instituut Voor Biotechnologie VZW. It has a filing date of 27 June 1997 and an expiry date of 27 June 2017. The patent claims an earliest priority date of 27 June 1996.

The specification relates to cameliid heavy chain antibodies and to the CDR3 loop, or a modified form thereof which is proposed to be able to bind to the active site of a target molecule. In particular, recombinant VHH or "camellised" VH antibodies are proposed.

Claim 1 is directed to:

A recognition molecule capable of interacting with an active site or cleft of a target molecule, wherein said recognition molecule has a basic recognition unit which comprises an exposed loop structure wherein the exposed loop structure consists of the CDR3 fragment of a cameliid species heavy chain antibody, or a derived version of such a CDR3 fragment having a binding specificity for said active site or cleft of the target molecule.

Claim 2 is directed to a recognition molecule of Claim 1 wherein the CDR3 loop structure comprises about 15 amino acids.
Both Claim 1 and Claim 2 prima facie cover the Alpaca Technology in so far as they cover naturally occurring intact camelid heavy chain antibodies.

Claims 3 to 7 relate to modified recognition molecules comprising a modified CDR3 domain or wherein the CDR3 domain is derived from a specific cAb-Lys VH H antibody. Claim 3 to 7 do not cover the Alpaca Technology.

Claims 8 to 11 require that the recognition molecule, as defined in the previous claims, is suitable for use in neutralising a biological function of a target molecule, or inter alia in therapy and vaccines, wherein the target molecule is a toxic constituent from snake venom.

Claim 8, Claim 9 and Claim 10 prima facie cover the Alpaca Technology in so far as they cover the use of naturally occurring intact camelid heavy chain antibodies.

Claims 12 to 25 are directed inter alia to compositions comprising a recognition molecule of one of the previous claims and to methods of producing or using same. Claim 21 and 24 are notable as method or uses of the subject recognition molecules “for the treatment of the deleterious effects of a toxic substance”. It is, in our opinion, surprising that these claims were not limited during prosecution to the use of recognition molecules that recognise and interact with the active site residues of a target molecule.

Claim 12, 21, 22, 24 and 25 prima facie cover the Alpaca Technology in so far as they cover the use of naturally occurring intact camelid heavy chain antibodies.

Claims 26 to 35 are omnibus claims limited to the exemplified embodiments described in the Examples of the specification. The exemplified examples do not include anti-snake venom and therefore these omnibus claims do not cover the Alpaca Technology.

To the extent that Claims 1, 2, 8, 9, 10, 12, 21, 22, 24 and 25 prima facie cover the Alpaca Technology comprising the production of polyclonal antibodies in alpaca, and their use as anti-venom, we note these claims may not be novel in light of prior art disclosures. Accordingly, the options for dealing with this freedom to commercialise issue are to;

i) Delay commencement of commercial activities until after the patent expires on 27 June 2017;
ii) Seek revocation or amendment of the patent;
iii) Negotiate a licence from Vlaam Interuniversitair Instituut Voor Biotechnologie VZW, who owns the patent.

As above, we note that to the extent that any of the claims of Patent No. 740043 cover the Alpaca Technology and specifically the use of intact immunoglobulins, these claims may arguably lack novelty and therefore they may arguably lack validity in light of prior art disclosures of naturally occurring camelid VH H antibodies. In view of the potential relevance of this patent to your proposed commercial activities you may wish to instruct us to provide you with detailed freedom to operate and patent validity advice in respect of this patent to determine whether it is prone to challenge. It might be possible to invalidate the patent or at least to restrict the scope of the claims to thereby avoid infringement. Alternatively, if upon further analysis the potentially blocking claims of the patent are clearly prone to challenge, the patentee may well be prepared to offer a licence on reasonable terms and we would be pleased to investigate this option on your behalf.

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(vi) **NO OPINION WITH RESPECT TO AUSTRALIAN APPLICATION NO. 2009265278 (SEARCH NO. 103)**

1. **Australian Application No. 2009265278 (Search No. 103)**

Please note that we have identified a conflict in relation to Australian Application No. 2009265278 as Davies Collison Cave is currently prosecuting this application and we cannot, therefore, provide you with an opinion on this patent application. We would be pleased to make a recommendation for an alternative attorney firm to provide you with an opinion, if required.
6. Biosecurity management of alpaca serum

Summary

A series of broad recommendations for management of alpaca to minimise infectious disease risk to humans from serum products have been made, rather than specific disease testing protocols. A detailed discussion of 17 infectious diseases of camelids is presented. Because alpaca are neither sheep nor horse, which have clearly identified zoonotic risks identified to regulatory agencies, there is scope for to establish a sound but not overly prescriptive risk-based approach to biosecurity.

Introduction

A theoretical risk exists for the transfer of infectious agents and toxins from alpaca to humans. This work was conducted to assess the risk and make some recommendations as to how best to manage this risk. Whilst no disease transmission has ever been documented from commercial animal serum products to humans the risks must be identified and mitigated.

Materials and methods

A list of infectious agents was compiled from various sources including:


- European Pharmacopeia Version 5.0


- Animal Health Australia.

Results

The key document for guidance on the issue of disease spread from animals to humans is published by the European Agency for the Evaluation of Medicinal Products. This document is cited by the Australian TGA for their publications.

It is important to note that these documents are guidelines only and do not mandate testing or controlling for each of these specific diseases. The list shown in Table 6 from the European Agency document is a list of infectious agents considered to be a risk from sheep and goats. Although not primarily relevant to alpaca this list is useful as a starting point and demonstrates how the European Agency considers risk. This listing is not a list of what diseases sheep and goats should be tested for. Risk is a subjective assessment and regulators naturally adopt a lower risk posture than other, commercial parties. It is the opinion of the author (AP) that the European Agency document carries
little relevance for Australian conditions but should products be exported to Europe then compliance with it becomes more important.

We must be mindful that due to limitations with testing, particularly the sensitivity of the various diagnostic tests, test results do not provide absolute assurance of freedom from the disease. Risk assessment is a subjective process and in turn will depend upon the attitude and aptitude of the risk assessor. Consequently the notes produced here on alpaca are only intended as a discussion material rather than absolute criteria. The process is more one of negotiation rather than prescription.

**Discussion**

For the majority of infectious agents that may infect alpaca, blood tests and other relatively non-invasive tests are mostly unvalidated. Until such time that these tests are tested in alpaca under commercial conditions the results are of dubious value.

However, tests that locate, visualise or culture the physical presence of an infectious agent are of much more meaning: for example, identification of Johne’s disease in alpaca by culturing *Mycobacterium avium paratuberculosis* (MAP) directly from faeces. Yet even for this example the result is contentious as passive shedding of MAP through ingestion of faeces from cattle will give the same result. These identification tests are often less sensitive, though, than indirect measures such as presence of antibodies.

The key point is that for almost every one of these infectious agents documented in this section there is considerable room for discussion on what constitutes a sound risk management approach. Those more risk averse than others will desire to seek assurances on as many levels as possible while for the less risk averse no disease testing but strong biosecurity measures will suffice.
Recommendations

A series of general recommendations on handling alpaca are summarised below.

General quarantine procedures for new alpaca arrivals

1. Obtain animals from low disease-risk herds such as Q-Alpaca certified.
2. Quarantine newly-introduced animals for 14 d prior to introduction to the herd and observe daily for clinical signs of disease.
3. House new arrivals in a dedicated quarantine area well away from other alpaca under conditions such that physical contact between animals cannot occur.
4. Treat all new arrivals with single injection of long acting oxytetracycline (Engemycin®, Coopers Animal Health) to eradicate Leptospira.

Procedures to reduce serum viral and bacterial load in harvested serum

1. Do not harvest serum from animals showing signs any disease. Check rectal temperature.
2. Immediately isolate any animal showing signs of illness.
3. Undertake routine annual immunisation with Clostridial disease vaccines (7in1).
4. Provide regular anthelmintic treatments.

Processing steps to reduce viral and bacterial load

1. Undertake viral filtration using 35nm viral filter of serum (validation required).
2. Apply processing conditions that reduce viral load. For example, use caprylic acid to reduce risks with lipid-enveloped viruses. Robust inactivation is achieved with pH <6.

*Tetanus: safety test of serum. Inject into guinea pigs and observe for 7 days.
Table 6. European medicines agency list of infectious disease of concern for sheep and goats.

<table>
<thead>
<tr>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foot and mouth disease virus*</td>
</tr>
<tr>
<td>Wesselbron virus*</td>
</tr>
<tr>
<td>Louping ill virus*</td>
</tr>
<tr>
<td>Rift valley fever complex*</td>
</tr>
<tr>
<td>Tick-borne encephalitis virus*</td>
</tr>
<tr>
<td>Bluetongue virus*</td>
</tr>
<tr>
<td>Vesicular stomatitis virus*</td>
</tr>
<tr>
<td>Parapoxvirus*, Capripoxvirus*, Cowpox virus*</td>
</tr>
<tr>
<td>Parainfluenze virus type 3*</td>
</tr>
<tr>
<td>Borna disease virus*</td>
</tr>
<tr>
<td>Reovirus 1-3</td>
</tr>
<tr>
<td>Respiratory syncytial virus</td>
</tr>
<tr>
<td>Rotavirus</td>
</tr>
<tr>
<td>Akabane virus</td>
</tr>
<tr>
<td>Ovine herpes virus 2</td>
</tr>
<tr>
<td>Bovine herpes virus type 1,2,4</td>
</tr>
<tr>
<td>Ovine/bovine papilloma virus</td>
</tr>
<tr>
<td>Border disease virus</td>
</tr>
<tr>
<td>Retroviruses (CAE, Maedi-Visna, Jaagsiekte, Bovine Leukaemia)</td>
</tr>
<tr>
<td>Epizootic haemorrhagic disease virus</td>
</tr>
<tr>
<td>Peste des petits ruminants</td>
</tr>
<tr>
<td>Adenoviruses</td>
</tr>
<tr>
<td>Nairobi sheep disease</td>
</tr>
<tr>
<td>Ross river virus</td>
</tr>
</tbody>
</table>

*Virus classified by European Medicines Agency as pathogenic for humans
Contagious Ecthyma (also called Scabby Mouth)

Infectious agent

- A virus, member of parapoxviridae. Specific virus is *Parapoxvirus ovis*.

Epidemiology

- Disease of sheep, goats and wild ruminants.

Zoonotic potential

- Documented transmission to humans from handling infected animals resulting in scabby and crusty skin lesions typically on the hands and forearm.

Clinical signs

- Clinical disease obvious and develops 2 to 5 days post-infection.
- Crust raised skin lesions around mouth and lips.

Reported occurrence in alpaca and camelids in Australia

- Described in camelid species, both Old and New World Camelids.

Testing of individual animals

- Clinical signs obvious (Figure 36). Not feasible to test individuals. Confirmatory diagnostic testing only.

Testing of harvested serum

- Not viable.

Availability of diagnostic tests in Australia

- Testing is only used to confirm presence of the virus and definitively establish diagnosis. Electron microscopy of scrapings from lesions to visualise virus. Not suitable for screening.

Critical control points

- Individual animal or pooled serum testing not feasible.
- Avoid contact with sheep, goats and other wild ruminants likely to be infected with Parapoxivirus. Avoid co-grazing with sheep and goats.
- Vaccination available for sheep but not evaluated in alpaca. No long lasting immunity following either natural infection or vaccination.
- In case of infection, isolate infected animals until lesions have resolved. Observe sound hygiene practices between handling infected animals and non-infected animals.
- Humans with suspect skin lesions and prior exposure to infected sheep and goats should wear gloves when handling alpaca.
• Apply a 14 d quarantine period when introducing new animals and observe for signs.

References


Figure 36. Lesions around the mouth of an alpaca consistent with scabby mouth (source A.Padula)
Johne’s Disease (also called Paratuberculosis)

Infectious agent

- *Mycobacterium avium paratuberculosis* (MAP)

Epidemiology

- Common in dairy cattle in south eastern Australia

Zoonotic potential

- Conflicting and ongoing debate about the role of MAP in Crohn’s disease in humans. Recommended to apply precautionary principle and reduce human exposure to MAP.
- Bacteraemia only encountered in animals with terminal clinical signs.

Clinical signs

- Weight loss, death.

Reported occurrence in alpaca and camelids in Australia

- No clinical cases reported in last 15 years so presumed not present in alpaca in Australia but possible risk of transmission from cattle.
- Johne's disease was diagnosed in 10 alpaca (*Lama pacos*) in Australia between February 1993 and May 1994. Eight of the animals were between 12 and 24 months of age, one was a 6-year-old female, and one was a 4-year-old male. Five, including the 6-year-old and the 4-year-old alpaca, showed weight loss and diarrhoea before death or slaughter. The other cases showed no clinical signs of Johne's disease but 4 gave a positive result on faecal culture and one gave a positive result on testing with the caprine agar gel immunodiffusion (AGID) assay and had acid-fast organisms in its faeces. At necropsy, all cases had grossly enlarged mesenteric lymph nodes. Johne's disease was diagnosed after histological examination of the lymph nodes with conventional culture and polymerase chain reaction testing of tissue samples.

Testing of individual animals

- Low sensitivity in detecting infected animals either by AGID on serum or faecal culture.
- Pooled faecal culture annually?

Testing of harvested serum

- Ineffective.

Availability of diagnostic tests in Australia

- Faecal culture readily available.
- AGID – Department of Primary Industries, Elizabeth Macarthur Agricultural Institute (EMAI).
Critical control points

- Sourcing of animals from Q-Alpaca certified herds or AlpacaMAP certified herds.
- Autopsy all animals that die with testing of lymph nodes and ileum for histopathology signs of *M. paratuberculosis*.
- Establishment of ‘closed herd’.
- Avoid co-grazing with susceptible species such as cattle and goats.

References

Australian Johne’s Disease Market Assurance Programs, Accessed 8 Sep 2012. URL


Ross River Virus

Infectious agent

- Mosquito-transmitted Alphavirus.

Epidemiology

- Over 30 mosquito species implicated in transmission.
- Warm wet conditions promote mosquito development and transmission of Ross River Virus.
- Most human cases are reported from Qld and Northern Australia. Enveloped virus.

Zoonotic potential

- Well documented clinical disease in humans.
- Various animal species (incl. horses) known to become infected
- Ross River Fever the most commonly reported Arbovirus in Australia in humans.

Clinical signs

- Clinical cases not documented in alpaca.
- Clinical signs in horses are initially high temperature, joint pain, lameness, swelling in lower limbs. Chronic signs result in poor exercise tolerance.

Reported occurrence in alpaca and camelids in Australia

- Not reported to date.

Testing of individual animals

- IgG antibodies indicate previous exposure.
- IgM antibodies indicate recent infection.

Testing of harvested serum

- Not useful due to low sensitivity

Availability of diagnostic tests in Australia

- Ross River Virus Neutralisation Test – DPI Vic, Attwood.

Critical control points

- Do not harvest serum from animals showing signs of generalised illness (weight loss, febrile).
- Insect vector control.
Barmah Forest Virus

Infectious agent
- Alphavirus.

Epidemiology
- Similar to Ross River Virus. Mosquito-spread.

Zoonotic potential
- Common in humans.

Clinical signs
- Similar to Ross River.

Reported occurrence in alpaca and camelids in Australia
- Not reported to date.

Testing of individual animals
- Virus neutralisation test (VNT) to detect presence of circulating antibody.

Testing of harvested serum
- Limited sensitivity.

Availability of diagnostic tests in Australia
- VNT – DPI Victoria, Attwood.

Critical control points
- Insect vector control.
- Do not harvest serum from febrile animals.
West Nile Virus

Infectious agent

- Flavivirus.
- Closely related to Japanese Encephalitis Complex.

Epidemiology

- Mosquito transmission.

Zoonotic potential

- Yes, documented human cases.

Clinical signs

- Not described for alpaca.
- In horses encephalitis is common.

Reported occurrence in alpaca and camelids in Australia

- Not described for alpaca.

Testing of individual animals

- VNT – EMAI Laboratory, NSW

Testing of harvested serum

- Limited sensitivity.

Availability of diagnostic tests in Australia

- Common antigen test available.

Critical control points

- Insect vector control.
Kunjin Virus

Infectious agent

- Flavivirus.
- Subtype of West Nile Virus.

Epidemiology

- Mosquito transmission.

Zoonotic potential

- Yes.

Clinical signs

- Mild disease.

Reported occurrence in alpaca and camels in Australia

- Not reported

Testing of individual animals

- Useful.

Testing of Harvested Serum

- Limited sensitivity.

Availability of diagnostic tests in Australia

- VNT – EMAI Lab, NSW DPI

Critical control points

- Insect vector control.
- Do not harvest serum from febrile animals.
Parainfluenza-3 Virus

Infectious agent
- Parainfluenza-3 virus.

Epidemiology
- Cattle-associated disease.

Zoonotic potential
- Unknown but listed by European Medicines Agency as a risk.

Clinical signs
- Respiratory signs.

Reported occurrence in alpaca and camels in Australia
- Unknown.

Testing of individual animals
- No alpaca validated tests.

Testing of harvested serum
- Unknown.

Availability of diagnostic tests in Australia

Critical control points
- Do not harvest serum from febrile animals.
Bovine Virus Diarrhoea

Infectious agent

- Pestivirus.

Epidemiology

- Cattle and sheep disease.
- Persistent infection occurs when foetus is exposed to virus.

Zoonotic potential

- No.

Clinical signs

- Illthrift.

Reported occurrence in alpaca and camelids in Australia

- Not reported to date in alpaca in Australia.
- Has been reported in USA.

Testing of individual animals

- Antigen detection.

Testing of harvested serum

- Polymerase chain reaction (PCR) on pooled serum possible but needs validation for alpaca.

Availability of diagnostic tests in Australia

- Yes.

Critical control points

- Initial antigen test to ensure alpaca negative for persistently-infected state.
- Antibody testing to monitor for signs of exposure to BVDV.
- Avoid pregnant animals as offspring may become persistently infected.

References


Equine Herpes Virus - Type 1

Infectious agent

- Herpesvirus.

Epidemiology

- Horse-associated virus.

Zoonotic potential

- Listed by European Medicines Agency as zoonotic potential.

Clinical signs

- Blindness, retinal degeneration and encephalitis.

Reported occurrence in alpaca and camelids in Australia

- Not reported.

Testing of individual animals

- Unknown.

Testing of harvested serum

- Unknown.

Availability of diagnostic tests in Australia

- VNT – DPI Victoria, Attwood.

Critical control points

- Avoid contact between alpaca and horses.
- Testing poor efficiency for monitoring.
Bovine Herpes Virus - Type 1

Infectious agent

- Herpesvirus.

Epidemiology

- BHV-1 is the cause of infectious bovine rhinotracheitis.

Zoonotic potential

- Listed by European Medicines Agency as zoonotic potential.

Clinical signs

- Blindness, retinal degeneration and encephalitis.

Reported occurrence in alpaca and camelids in Australia

- BHV-1.1 only subtype recorded in cattle in Australia.

Testing of individual animals

- Unknown for alpaca.

Testing of harvested serum

- Unknown.

Availability of diagnostic tests in Australia

- VNT – DPI Victoria, Attwood.

Critical control points

- Avoid contact between alpaca and cattle.
- Testing poor efficiency for monitoring.
Bluetongue Virus

Infectious agent

- Orbivirus (Arbovirus).

Epidemiology

- Northern Australia only. Geographical zone for Bluetongue.

Zoonotic potential

- No disease syndrome described in humans.

Clinical signs

- Clinical Bluetongue disease not observed in Australia

Reported occurrence in alpaca and camelids in Australia

- None, but no survey data available.
- Sporadic cases reported internationally.

Testing of individual animals

- Testing not necessary if animals are sourced and located outside Bluetongue zone
- PCR (DNA) testing of tissues may be diagnostic.

Testing of harvested serum

- Not necessary if animals located in Southern Australia (Figure 37).

Availability of diagnostic tests in Australia

- Unknown for alpaca but could be adapted from other species.

Critical control points

- Locate animals in Vic/Tas outside the Arbovirus zone.
- Initial test negative for Bluetongue then no further testing.
- Insect control.

References

Figure 37. Current arbovirus zone map from National Arbovirus Monitoring Program, Animal Health Australia.
Encephalomyocarditis Virus

Infectious agent

- Cardiovirus from Picornaviridae family.

Epidemiology

- Pigs are the domestic host and the most susceptible to infection.

Zoonotic potential

- No reports of human disease but antibodies have been detected.

Clinical signs

- Febrile illness, death.

Reported occurrence in alpaca and camels in Australia

- Has been recorded in an alpaca herd in Queensland, Australia. Multiple alpaca deaths described. Presence of virus confirmed.

Testing of individual animals

- Post-mortem diagnosis.

Testing of harvested serum

- Unknown

Availability of diagnostic tests in Australia


Critical control points

- Prevent rodents accessing animal feed. Rodent contamination of water supply.

- Testing of individual animals not as effective as good hygiene control measures and early detection of clinical cases.

- Avoid harvesting serum from febrile alpaca.

References

Murray Valley Encephalitis

Infectious agent

- Flavivirus. Related to Kunjin virus.

Epidemiology

- Incursion of the virus into Victoria has occurred in recent years. A number of equine clinical cases have been documented.

Zoonotic potential

- Yes.

Clinical signs

- Febrile illness.

Reported occurrence in alpaca and camelids in Australia

- Not reported to date.

Testing of individual animals

- Unknown for alpaca.

Testing of harvested serum

- Unknown.

Availability of diagnostic tests in Australia

- VNT – DPI Victoria, Attwood Laboratory.

Critical control points

- Insect vector control.

- Periodic monitoring of individual animals by VNT during peak risk times (mosquito breeding season; warm, wet weather).

- Avoid harvesting serum from febrile alpaca.
Q-Fever

Infectious agent
- *Coxiella burnetti*.

Epidemiology
- Well documented in camels.

Zoonotic potential
- Yes.

Clinical signs
- Minimal clinical signs.
- Abortion reported in sheep and goats.

Reported occurrence in alpaca and camelids in Australia
- Not described but Q-Fever occurs in other species.

Testing of individual animals
- Detection of antibodies to Coxiella via CFT – EMAI Lab, NSW DPI or Attwood.

Testing of harvested serum
- PCR for Coxiella on pooled serum.
- CFT – DPI Victoria, Attwood Laboratory.

Availability of diagnostic tests in Australia
- Yes, microbiological tests.

Critical control points
- General hygiene.
Leptospirosis

Infectious agent

- Various spirochaete organisms of the Leptospira family.

Epidemiology

- Water- and rodent-associated.

Zoonotic potential

- Yes.

Clinical signs

- Clinical disease not reported in South American camelids.

Reported occurrence in alpaca and camelids in Australia

- No.

Testing of individual animals

- Not applicable.

Testing of harvested serum

- Not applicable.

Availability of diagnostic tests in Australia

- Microbiological tests for organism identification.

Critical control points

- Treatment of new arrivals with long acting oxytetracycline injection.

- Vaccination not recommended for camelids.
Akabane Virus

Infectious agent
- Arbovirus.

Epidemiology
- Similar to Bluetongue virus.

Zoonotic potential
- Not reported in humans but listed by European Medicines Agency as risk.

Clinical signs
- Neurological, foetal effects.

Reported occurrence in alpaca and camelids in Australia
- Not reported but antibodies found in camels in USA.

Testing of individual animals
- Possible but low sensitivity.

Testing of harvested serum
- Possible but low sensitivity.

Availability of diagnostic tests in Australia
- Limited availability.

Critical control points
- National Arbovirus Monitoring Program (NAMP, Animal Health Australia).
- Insect vector control.
- Locate and source animals from outside the geographical Arbovirus zone in Australia.
- Testing not required in Victoria.

References
Animal Health Australia
Tuberculosis

Infectious agent
- *Mycobacterium bovis* (cattle TB).

Epidemiology
- Numerous cases documented in camelids in the UK.

Zoonotic potential
- Yes.

Clinical signs
- Abscess/nodule formation.

Reported occurrence in alpaca and camelids in Australia
- No.

Testing of individual animals
- Yes.

Testing of harvested serum
- No.

Availability of diagnostic tests in Australia
- Yes.

Critical control points
- International borders.

References
Animal Health Australia
7. **Procedures for concentrating immunoglobulins in alpaca serum**

**Summary**

Experiments were performed to see if a method of processing alpaca serum could be developed based on caprylic acid precipitation of non-immunoglobulin proteins. Alpaca serum was diluted in water to aid the precipitation process. The reaction was monitored by microscopy and compared to horse plasma processed in an identical manner. Alpaca serum failed to precipitate out cleanly using any of the combinations of dilutions. A fine white granular protein precipitate remained in solution. Alpaca serum failed to process to a high degree of purity using a standard chemical method based on caprylic acid. Heat treatment, although effective in removing the contaminating protein appears to slightly denature the immunoglobulin which may make the product more likely to trigger allergic reactions.

**Introduction**

The removal of non-immunoglobulin proteins from serum and plasma is recognised as an important step to improve product quality and reduce allergic reactions. The most commonly used methods of removing these non-IgG proteins are the chemical precipitation methods. Ammonium sulphate solution can be used to ‘salt-out’ immunoglobulins. When added to serum at a concentration of 50% the immunoglobulins become insoluble and by centrifuging or filtering the mixture they can be recovered. Caprylic acid is a fatty acid and when added to acidified serum at 6% concentration it causes precipitation of non-IgG proteins, leaving the IgG in solution. The caprylic acid method is very simple and can be used on undiluted horse plasma. Ammonium sulphate precipitation works better with diluted serum (1:2) meaning larger volumes must be processed.

Caprylic acid processing has become more popular with horse plasma due to its simplicity and the potentially higher degree of IgG purity obtained (Rojas, Jimenez et al. 1994). In the previous RIRDC project work on alpaca immunoglobulins a method of processing alpaca serum using ammonium sulphate was successfully developed.

The aim of this work was to develop a caprylic acid processing protocol that could be used with alpaca serum.

**Materials and methods**

**Reagents**

Chemicals were purchased from Sigma-Aldrich. Alpaca serum was harvested from clotted whole blood and filtered using a cartridge 0.22 µm filter. Horse plasma was harvested from a clinically normal horse into citrated blood collection bags. Total protein was measured using a refractometer. Microscopy was performed with an Olympus microscope and images captured using a Canon 7D camera mounted on a microscope eyepiece.
Protocol

Alpaca serum

Serum from hyperimmune alpaca previously immunised with snake venom was used. Alpaca serum was diluted with distilled water (dH₂O) at either 1:1, 1:2, 1:3 or 1:4. This solution was then acidified to pH 5.7 using dropwise addition of glacial acetic acid. To this was added caprylic acid at a ratio of 6mL per 100mL of undiluted plasma. The mixture was stirred briskly on a magnetic stirrer and then centrifuged at 4200 rpm for 15 minutes at 4°C. To test the filtration a sample was aspirated into a 20mL syringe and passed through a 0.22 µm syringe filter (Millipore, Australia).

Horse plasma

For comparative purposes, undiluted horse plasma was processed by acidifying to pH 5.7 using glacial acetic acid. Caprylic acid was added at a rate of 6mL per 100mL of plasma. The mixture was stirred on a magnetic stirrer for 30 minutes. The suspension was then transferred to a 1 Litre centrifuge bottle and centrifuged at 4200rpm for 15 minutes at 4°C. The supernatant was then harvested and the pellet discarded. To test the filtration a sample was aspirated into a 20mL syringe and passed through a 0.22 µm syringe filter (Millipore, Australia). The supernatant was dialysed against PBS for 48 hours.

Results

Horse plasma

Following caprylic acid processing, as expected, horse plasma processed very efficiently and was easily filtered through a syringe filter. Following centrifugation the mixture separated very cleanly into two layers. The supernatant layer was mildly opaque and contained occasional granular material. A thin (1mm) floating layer of lipoprotein material was visible. Microscopically the mixture contained mostly small granular material with occasional large smooth walled structures.

Alpaca serum

Following processing with caprylic acid, alpaca serum would not filter through the 0.22µm syringe filter until it was diluted at least 1:3 with distilled water. At all dilutions there was a cloudy supernatant following centrifugation. The precipitate separated into two distinct layers. The microscopic appearance was distinctly different to that seen with horse plasma. There was a much smaller particle size than with horse plasma. A white cloudy appearance was obvious in the supernatant at all dilutions but was less visible at 1:4 dilution. Following prolonged refrigeration at 4°C there was only limited gravity sedimentation of the white granular material.

Discussion

Alpaca serum does not process satisfactorily using caprylic acid. In contrast, horse plasma processed very efficiently. The reasons for the failure of the alpaca serum to fractionate like horse plasma are not immediately clear. Caprylic acid has been adopted by many antivenom manufacturers because of the simplicity and low cost of the method. In horses the method results in a very high degree of purity of product. A clinical trial of antivenom manufactured using caprylic acid showed a low incidence of adverse reactions in patients envenomed by Bothrops asper (Otero, Leon et al. 2006).

Heat treatment of alpaca serum provides a workable method of concentrating the immunoglobulins. Heat treatment is more complicated to perform under sterile pharmaceutical manufacturing conditions. Heating has also been shown to induce aggregation of proteins (Leon, Lomonte et al. 2005) which in turn can lead to triggering of allergic reactions in patients.
More work needs to be done to develop a simple, non-heat based method of concentrating immunoglobulins from alpaca serum. Alternatively, a higher technology and higher cost method based on chromatographic methods (such as affinity chromatography) could be developed. However this is beyond the scope of the current project. A small quantity of affinity purified anti-Tiger Snake alpaca antiserum was made for use in the Tiger Snake venom ELISA.
Figure 38. Undiluted horse plasma (non-hyperimmune) processed at pH 5.7 with 5mL of caprylic acid per 100mL of plasma. Mixed briskly for 15 minutes. Photographed at 40x magnification prior to centrifugation.

Notes on Figure 38:
Mostly very small granules
Occasional very large size smooth walled protein material
Post-centrifugation supernatant very clear, floating layer of lipoprotein
Easily filters through 0.22 um syringe filter
Starting total protein 72 g/L (refractometer)
Figure 39. Alpaca hyperimmune serum diluted 1:1 with dH₂O and processed with serum caprylic acid at 5 mL per 100mL. Mixed briskly for 15 minutes. Processed at a serum pH 5.76. Photographed at 40x magnification prior to centrifugation. Note formation of very large particles, intermediate sized particles and very small particles.

Notes on Figure 39:

Suspension will not filter through 0.22μm syringe filter

Post-centrifugation very cloudy suspension. Will not separate into distinct layers. Remains cloudy.

Three types of aggregates: large smooth walled folded material (same as horse), intermediate sized granular shaped and very small spherical particles.

Starting total protein 67 g/L
Figure 40. Alpaca serum diluted 1:2 with dH$_2$O. Processed with caprylic acid at 5 mL per 100mL of original serum volume. Mixed for 15 minutes. Photographed at 40x magnification prior to centrifugation.

Notes on Figure 40:

Two sizes of aggregates: very large smooth folded objects and very small fine material.

Will not filter easily through 0.22um syringe filter. Clogs filter after 0.5 mL.

Possible presence of globules of caprylic acid that have not mixed thoroughly (see above).

Semi-clear appearance to supernatant post-centrifugation.
Figure 41. Alpaca serum diluted 1:3 with dH₂O. Processed with caprylic acid at 5 mL per 100mL of original serum volume. Photographed at 40x magnification prior to centrifugation. Mixed for 15 minutes.

Notes on Figure 41:

Filters easily through 0.22μm syringe filter.

Aggregates composed of very large smooth objects and much smaller granular material that is larger than when diluted either 1:1 or 1:2.

Post-centrifugation the suspension separates easily into two distinct layers.

Very clear supernatant (but not as clear as 1:4 dilution).
Figure 42. Alpaca serum diluted 1:4 with dH₂O. Photographed at 40x magnification prior to centrifugation.

Notes on Figure 42:

Post-centrifugation the supernatant is very clear and translucent with some flakes of lipoprotein material floating in solution and some floating on the top layer.

Easily filters via 0.22um syringe filter with no clogging.

Microscopic aggregates composed of mostly small aggregations of protein and occasion larger ‘flake’ like material.

1 part serum to 4 parts dH₂O appears to be optimal dilution.
Figure 43. Alpaca serum diluted 1:4 with dH2O and processed with caprylic acid at 5 mL per 100mL of serum. Image taken after pouring out clear supernatant after centrifugation.

Notes on Figure 43:

Note separation into two distinct layers after centrifugation at 4,200 rpm x 5 minutes.

These layers probably represent the different sized aggregates observed prior to centrifugation.

Microscopically (40x) the top white layer contains uniformly sized and very small particles whilst the brown bottom layer contains the much larger smoother particles.
Figure 44. Alpaca serum diluted 1:4 with dH20 and processed with caprylic acid at 5 mL per 100mL of serum.

Notes on Figure 44:

Note floating ‘flakes’ in the supernatant (lipoproteins?).

Floating layer on top of supernatant (lipoprotein?).
Figure 45. Undiluted horse plasma processed with caprylic acid at 5mL per 100mL plasma. Centrifuged at 4,200 rpm for 15 minutes.

Notes on Figure 45:
Supernatant has slightly cloudy colour with some floating material
Floating layer on top (lipoprotein).
Sediment appears to be uniform colour.
Figure 46. Appearance of alpaca immunoglobulin preparation diluted 1:4 with distilled water and fractionated using caprylic acid (LEFT) and same solution after gentle agitation (RIGHT). Note the cloudy and particulate suspension at the bottom layer of the bottle.
8. Development of a sensitive ELISA for the detection of Tiger Snake venom

Summary

A new venom detection test was developed using affinity purified alpaca anti-Tiger Snake antibodies. The test had remarkable sensitivity with a limit of detection of 0.010 ng/mL making it 1,000 times more sensitive than the CSL snake venom detection kit and 15 times more sensitive that the currently most sensitive ELISA which is based on rabbit antibodies. This increased sensitivity increases the utility of the test. This is especially so in the veterinary context, where there is often a significant delay between envenomation and presentation to a vet. Delayed presentation means lower serum venom levels which are currently difficult to detect with the sensitivity of current tests, leading to false negative diagnoses.

Introduction

The diagnosis of snakebite or other envenomation can be very challenging. Diagnosis is generally made through interpretation of clinical signs, various non-specific clinical pathology tests and the patient’s description of snakebite. Occasionally a victim will be found dead and a retrospective diagnosis of snakebite must be made through pathological examination. In the veterinary context snakebite can be very difficult to diagnose. Venom levels in serum decline very quickly and after 8 hours they reach non-detectable levels (Moisidis, James et al. 1996). A more sensitive diagnostic test would improve the diagnosis and treatment outcomes.

CSL released to the market in the early 1990s (Cox, Moisidis et al. 1992) a revised version of their previously released Snake Venom Detection Kit (SVDK). The test kit was patented (Cox 1988) and has not been revised since then. Although appealing in concept, the SVDK has attracted considerable criticism. Its use in human snakebite patients has shown that while it is useful it suffers from a lack of sensitivity in detecting low concentrations of venom in serum. Veterinarians rarely use the SVDK due to the high number of negative test results obtained, largely due to inadequate sensitivity. The SVDK is designed to be run in the clinic with minimal facilities, taking only around 20-30 minutes to complete.

RIRDC has previously invested in a project (Church and Forbes 2011) to evaluate the CSL SVDK in horses. Diagnosis of snakebite in horses is difficult. The conclusions of the RIRDC report were that the limit of detection of the SVDK was around 10ng/mL in horse urine, but there is no evidence to suggest that these levels are actually reached in clinically envenomed horses.

The limit of detection of the SVDK for venom in serum is around 10 ng/mL (Table 7). Envenomed patients have been reported to have median Tiger Snake venom levels prior to antivenom treatment of 3.2 ng/mL (Isbister, O'Leary et al. 2012) with a range of 0.17 ng/mL to 152ng/mL. The problem with the SVDK is evident from this published data showing that it provides diagnostic information when venom levels are relatively high but returns negative results with more average venom levels seen in human patients.

The SVDK uses antibodies created in rabbits to ‘capture’ free venom in test samples. The ELISA is novel in that all reagents are added simultaneously including a freeze dried conjugated enzyme.

In this work we created an improved version of the SVDK in the laboratory with greatly improved sensitivity using affinity purified alpaca anti-Tiger Snake Venom antibodies bound to a polystyrene microplate.
Materials and methods

Antiserum

Serum was pooled from four alpaca that had been immunised monovalently with Tiger Snake venom (Notechis scutatus).

Affinity purification

The anti-Tiger Snake Venom antibodies were extracted from the pooled raw serum using Sepharose matrix in a chromatography column (Figure 47).

ELISA

The purified alpaca anti-Tiger Snake venom antibodies were linked to biotin and a commercial streptavidin-Horse Radish Peroxidase conjugate used as a detector. The substrate used was TMB (Ultra TMB, Thermo Fisher, Australia). The assay procedure was essentially the same as that described previously (Kulawickrama, O'Leary et al. 2010). Venom was diluted in 0.5% BSA in PBS-T20. Unbound sites were also blocked with BSA.

Polystyrene 96-well microplates (Greiner, USA) were coated with alpaca anti-Tiger Snake venom antibodies at a concentration of 2 µg/mL. Plates were incubated overnight at 4°C. Unbound sites were blocked with 0.5% BSA in PBS-T20. Plates were washed and venom added pre-diluted in 0.5% BSA PBS-T20. Plates were incubated at 37°C for 15 minutes and washed 3 times using PBS-T20. Next, biotinylated alpaca IgG was added at 0.15µg/mL to each well and incubated for 15 minutes at 37°C before washing 6 times in PBS-T20. A commercial horseradish peroxidase conjugated (1:20,000) to streptavidin was added and incubated for 15 minutes at 37°C before 6 washes with PBS-T20. The chromogen (TMB) was added and colour development allowed to continue (4-10 minutes). The enzymatic reaction was stopped by addition of 100µL/well of 10% H2SO4. The intensity of the colour reaction was read in a microplate reader at 450nm. Standard curves were generated using Magellan™ software. The Limit of Detection was defined as three times the standard deviation of the mean of the blank wells.

Results

Affinity purification

Highly specific antibodies directed against whole Tiger Snake venom were successfully obtained using the affinity purification procedure.
Figure 47. Chart of chromatography fraction and protein concentration as measured by UV absorbance at 280nm.

**Gel:** CNBr-TSV-Sepharose  
**Gel size:** 12 ml (5 mg/ml TSV*)  
**Sample:** hyperimmune alpaca anti-TSV serum (50 ml)  
**Elution buffer:** 0.1 M glycine pH 2.8  
*TSV: tiger snake venom
ELISA

The ELISA showed a wide dynamic range and extraordinary sensitivity in detecting whole Tiger Snake venom (Figure 48, 49). Four standard curves were generated on a single plate to determine the Limit of Detection (LoD). The LoD for Tiger Snake venom was measured at 0.010 ng/mL.

Table 7. Comparison of the limit of detection of three assay systems.

<table>
<thead>
<tr>
<th>Assay System</th>
<th>Limit of Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSL Snake Venom Detection Kit (Cox, Moisidis et al. 1992, Church and Forbes 2011)</td>
<td>10 ng/mL</td>
</tr>
<tr>
<td>Rabbit anti-Tiger antibody (Isbister, O'Leary et al. 2012)</td>
<td>0.15 ng/mL</td>
</tr>
<tr>
<td>Alpaca anti-Tiger antibody</td>
<td>0.010 ng/mL</td>
</tr>
</tbody>
</table>
Figure 4. Standard curves for whole Tiger Snake venom diluted in blocking buffer. The dotted red line shows the limit of detection at 3 x standard deviation of the absorbance of the negative wells.
Figure 4. Standard curves for whole Tiger Snake venom diluted in blocking buffer. The dotted red line shows the limit of detection at 3 x standard deviation of the absorbance of the negative wells.
Discussion

The ELISA developed here for whole Tiger Snake venom showed extremely high sensitivity. A recently published ELISA for Tiger Snake venom reported a LoD of 0.15ng/ml (Isbister, O'Leary et al. 2012). The ELISA using alpaca antibodies LoD described here was 0.010 ng/mL. In contrast, the CSL SVDK is capable of detecting a minimum of 10ng/mL of venom. Thus the alpaca antibody-based assay was 15 times more sensitive than the best described assay to date and 1,000 times more sensitive than the CSL SVDK.

This result is extraordinary and may yield a new diagnostic test for snakebite which would have widespread implications, especially in the veterinary context. The assay we describe here is only for Tiger Snake but the same processes are applicable for the other venom types. The key to improving the assay sensitivity appears to be in using antibodies that are highly purified (e.g. affinity chromatography) and from animals with very high titres to the specific venom type.

A number of forensic investigations have concluded probable snakebite but the diagnosis has been elusive due to test limitations. The ELISA described here could have potential in these type of forensic investigations.

This exciting discovery warrants further investigation. The process would involve immunising groups of alpaca monovalently with the specific venom types, performing affinity purification and testing the ELISA system. The system described here could be adapted to a ‘rapid’ type of ELISA assay producing results in minutes. It is likely that this biotin-based ELISA system could be adapted to measure other substances using alpaca antibodies.
9. Neutralisation of snake venom procoagulant toxins by alpaca antivenom

Summary

Alpaca antivenom was extremely effective in comparison to CSL antivenom for neutralising the procoagulant effects of Tiger, Brown and Taipan venoms. The alpaca antivenom was at least 10 times more effective and showed excellent cross neutralisation of Taipan with Brown snake antivenom.

Introduction

Many venomous Australian elapid snakes contain potent toxins that prevent blood from clotting normally. Of particular importance are the Brown Snake, Taipan and Tiger Snakes. Death from blood clotting disturbances is a significant risk with clinical management of patients bitten by these snakes. Clotting disturbances begin within a few minutes of envenomation. Consequences can be severe internal bleeding and potential fatality from brain haemorrhages. These clotting disturbances are the result of the venom consuming the available clotting factors leading to a consumptive coagulopathy.

Neutralisation of the clotting disturbances induced by Brown, Taipan and Tiger by equine antivenom is poor (Tibballs, Sutherland et al. 1991). Tibballs demonstrated in both in vitro and in vivo models (anaesthetised dogs) that a dose of antivenom 25 times greater than that recommended on the label was required to prevent clotting disturbances. An experimental sheep serum and chicken egg yolk antivenom was much more effective than equine antivenom at stopping clotting disturbances (Madaras, Mirtschin et al. 2005).

The effectiveness of alpaca antivenom in neutralising the procoagulant effects of snake venom was examined in this study. For comparison two different commercial CSL Brown Snake antivenom batches were tested alongside a veterinary antivenom.

Materials and methods

Venom

Lyophilised venoms were obtained from a commercial venom supplier and stored at -20°C until ready for use. The venoms used were from Brown Snake (*Pseudonaja textilis*), Papuan Taipan (*Oxyuranus scutellatus canni*) and Tiger Snake (*Notechis scutatus*).

Antivenom

A range of commercial human and veterinary antivenoms were tested against the alpaca products (Table 8).
Table 8. Antivenom products.

<table>
<thead>
<tr>
<th>Code</th>
<th>Antivenom</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSL_B</td>
<td>CSL Brown Snake Antivenom Exp. 03/11 3.31 mL / 1,000 Units</td>
</tr>
<tr>
<td>SL_TB</td>
<td>Summerland Serums Tiger-Brown Exp. Apr-12, TB09-10</td>
</tr>
<tr>
<td>ALP_B</td>
<td>Alpaca Monovalent Brown 231012 Total Protein 39g/L</td>
</tr>
<tr>
<td>AVSL</td>
<td>AVSL Multi Brown Snake Antivenom (ovine)</td>
</tr>
<tr>
<td>CSL_P</td>
<td>CSL Polyvalent Antivenom. Expiry date 06/14</td>
</tr>
<tr>
<td>ALP_T</td>
<td>Alpaca Mono Tiger 231012 Total protein 35g/L</td>
</tr>
</tbody>
</table>

Neutralisation of Venom

Eight dilutions of antivenom was premixed with a fixed challenge dose of venom. The challenge dose of venom was determined from a preliminary assay in which venom and re-calcified citrated plasma were mixed together.

Clotting Assay

A standard clotting assay was used throughout. The protocol used was as recommended by the World Health Organisation for assessing antivenom neutralisation of procoagulants (Standardization 2010). A dual channel electronic fibrinometer was used to accurately monitor the progress of coagulation.

Data Interpretation

Data is shown with neutralisation level on the x-axis. This is the ratio of the amount of venom neutralised per mL of antivenom. The y-axis data is the actual clotting time of the plasma as determined by the machine. All assays were run with duplicate samples and the results are presented as the mean of the samples.

Results

Brown Snake Venom

The addition of Brown Snake venom to re-calcified plasma resulted in rapid clotting of the plasma. A dose of venom (2.5µg) was chosen that resulted in a clotting time of around 60 seconds.

Alpaca Brown Snake antivenom was very effective at neutralising the procoagulant contained within Brown Snake venom. Alpaca antivenom neutralised the procoagulant at a concentration of antivenom 10 times lower than that of the CSL Brown Snake antivenom.

However the most effective antivenom for neutralising the procoagulant was the AVSL commercial sheep-derived veterinary antivenom for Brown Snake. The least effective antivenom was the veterinary Summerland Serums Tiger-Brown antivenom, being approximately 20 times less effective per mL than the AVSL sheep-derived product.
Figure 50. Comparison of six antivenoms for their ability to neutralize the procoagulant effects of Brown Snake venom. The dotted line showing 3xMCD is the point at which the data is interpreted. The further to the right (i.e., higher neutralizing level), the more potent the antivenom.

Figure 51. Summary of the relative potency of each antivenom for neutralizing the procoagulant effect of Brown Snake venom (the higher the number the more potent). The bar chart above (Figure 51) shows the relative neutralizing capacity shown as mg of venom neutralized per mL of antivenom. It is difficult to compare these products as they are of different total protein concentrations. The alpaca antivenom contained only 39 g/L total protein compared to CSL which is 165 g/L. This effectively means that the alpaca product was compared at 39/165 or about a quarter of the strength of the CSL, meaning that the final result would be around 4 times better than shown if they were diluted to the same total protein concentration. In conclusion, the alpaca antivenom could be up to 40 times more potent on a ‘per gram of protein’ basis than the CSL antivenom.
Taipan Venom

Figure 52. Comparison of antivenom for the neutralisation of the procoagulant contained within Taipan venom.

The chart above shows a comparison between six different antivenom products and their ability to neutralise the procoagulant contained within Taipan venom. The most effective products for Taipan venom were the AVSL Brown Snake (ovine) antivenom, Alpaca Mono Brown antivenom, and the Alpaca Mono Taipan antivenom. Alpaca Mono Tiger antivenom was the least effective against Taipan venom.
Tiger Snake Venom

Alpaca Tiger Snake antivenom was extremely effective at neutralising the procoagulant effect of Tiger Snake venom (Figure 53, 54). The alpaca antivenom was formulated at only 35g/L total protein compared to CSL Tiger antivenom at 170g/L. This effectively means that the alpaca product was up to 4-5 times better if it was concentrated to the same total protein as the CSL product giving a final result of up to 20-30 times more potent than CSL Tiger antivenom.

The chart below shows the comparison.
Alpaca Tiger Snake antivenom (35g/L) was extremely effective at neutralising the procoagulant effects of venom. The chart below shows this comparison. Alpaca Brown Snake antivenom was incompletely effective against Tiger Snake venom.
Discussion

The results here conclusively demonstrate that alpaca antivenom is significantly more effective at neutralising procoagulants in venom in an *in vitro* test system than equine-derived antibodies. The improvement is of the order of 20- to 50-fold which is a fascinating discovery. Also, alpaca Brown Snake antivenom was equally effective at neutralising Taipan procoagulant but significantly less effective for Tiger Snake venom. There are a number of reasons to explain these findings.

Alpaca (camelid) antibodies have been postulated to be more effective at neutralising enzymes by ‘reaching into’ enzyme molecules due to their smaller size. Our results here demonstrate that for the procoagulant enzymes found in Tiger, Brown and Taipan venom this appears to be the case, whilst horse antibodies are much less effective. Noteworthy is the observation that sheep antibodies are also extremely effective at neutralising the toxic procoagulant enzymes found in venom.

Sheep-derived antibodies appear to be extremely effective at neutralising snake venoms as demonstrated in these results. The work contained here supports previous work (Ariaratnam, Meyer et al. 1999, Jones, Lee et al. 1999, Landon and Smith 2003, Madaras, Mirtschin et al. 2005) showing good neutralising capacity for various snakes when sheep were used to make antivenom. The AVSL product appears to be nothing more than unprocessed hyperimmune sheep serum however it has excellent neutralising properties. Even though alpaca antivenom was good it was not quite as good as the sheep serum.

The alpaca antivenoms tested are likely to be inherently more active as they are formulated as whole IgG molecules. The CSL antivenoms are enzymatically digested during production to remove the non-binding Fc portion of the molecule. The theory is that this renders the molecule less allergenic but may reduce its effectiveness to bind venom.

The cross-neutralisation between Brown and Taipan is not surprising as the toxic procoagulant enzyme has molecular similarities. Tiger Snake procoagulant belongs to a different family of enzymes to Taipan and Brown so the finding that it is less effectively cross-neutralised is consistent with previous work (Isbister, O'Leary et al. 2010).

In conclusion, these results show that alpaca antivenom markedly overcomes one of the limitations of horse antivenoms in neutralising blood clotting disturbances. Alpaca immunoglobulins could be blended with horse plasma to increase the potency of procoagulant neutralisation. This would allow one to gain some of the benefits of the large blood volume collected from a horse with the improved neutralising abilities of alpaca antivenom for procoagulants.
10. Clinical case report: Successful treatment of a dog bitten by Tiger Snake with alpaca antivenom

Summary

A confirmed clinical case of Tiger Snake bite was successfully treated in a dog with alpaca polyvalent antivenom. Samples collected from the dog were retrospectively assayed for Tiger Snake venom using a new highly sensitive assay developed as part of this project. At 4 h post-antivenom treatment the dog’s condition had improved markedly and serum venom concentrations were undetectable indicating complete binding by the alpaca antivenom.

Introduction

This report is the first description of the use of the alpaca antivenom in a clinically envenomed animal presented to the author’s veterinary clinic.

Case report

The animal was treated under the framework of the Australian Pesticides and Veterinary Medicines Authority (APVMA) small scale use of immunobiologics, Category 7250, Small Trial Permit. Guidance on animal ethics was obtained from the Dr Dania Maver, Principal Veterinary Officer, Bureau of Animal Welfare, DEPI Victoria, Attwood. All procedures were performed as an Act of Veterinary Science by a registered veterinarian, Dr Andrew Padula.

History, clinical notes and treatment

A 7 year old desexed female cross bred dog called “Snoopy” was presented to the author’s veterinary clinic having been seen to have been bitten by a 75cm Tiger Snake in the owner’s back yard. The dog was presented for treatment within 20 minutes of being bitten.

On initial examination the dog was walking, appeared agitated, but was drooling saliva. The offending snake was also brought in and positively identified as a Tiger Snake (Figure 55). A blood sample was collected for preliminary tests.

A CSL SVDK test was run on serum from the dog which returned negative result (Figure 57). This result was unexpected considering the owner had observed the bite and presented the snake.

An activated clotting time test was performed using a commercial tube incubated at 37°C in a water bath. The blood did not clot. The venepuncture site oozed blood following needle removal. A blood sample was centrifuged and the serum was grossly haemolysed. A urine sample was collected which was dark red and contained +++ glucose and ++++ blood.

During the time (30 mins) that the above tests were being run the dog’s condition deteriorated and it started to vomit. A serum sample showed evidence of haemolysis (Figure 56).

Based on the history, prolonged clotting time and blood in the urine a diagnosis of Tiger Snake envenomation was made.
The dog was placed on intravenous fluids (0.9% saline) and 2 vials (50 mL total) of alpaca polyclonal antivenom was administered via slow intravenous infusion. “Snoopy” was premedicated with antihistamine and dexamethasone prior to antivenom treatment.

There was no adverse reaction to the infusion of alpaca antivenom. Within 2 hours the dog appeared clinically normal.

At 4 hours post-antivenom treatment a blood sample was collected for an activated clotting time. The blood still did not clot but the dog appeared normal.

The dog was hospitalised overnight. Next day the dog was bright and alert and had a good appetite (Figure 59). The oozing of blood from the previous venepuncture site had stopped. A blood sample was collected to assess the clotting time which had returned to normal (1 minute 20 seconds). A urine sample was collected which contained no blood and appeared grossly normal. “Snoopy” was discharged from the clinic and sent home approximately 24 h after the bite.

**Venom ELISA**

Serum and urine samples were retrospectively tested for the presence of whole Tiger Snake venom using the sensitive ELISA we have developed (see description in this RIRDC report). The serum sample collected prior to antivenom was strongly positive for venom (Table 9). Immediately after antivenom administration Tiger Snake venom could not be detected in serum. The pre-antivenom urine sample was strongly positive for Tiger Snake venom (>10,000 ng/mL).

Standards and test samples were assayed in triplicate. A standard curve showed good dynamic range of the assay (Figure 58). The coefficient of variation averaged less than 5% for all samples and standards.

**Coagulation testing**

**Activated clotting time**

The activated clotting time test was performed in accordance with the manufacturer’s instructions (Helena Laboratories). Briefly, 2mL of freshly collected whole blood was added to a pre-warmed (37°C) tube containing the clotting activator substance. The tube was placed in a water bath and gently mixed whilst observing for clot formation by rocking back and forward. The moment of clot formation was recorded using a digital timer.

**Partial Thromboplastin time**

The Partial Thromboplastin (PT) time was measured using a commercial thromboplastin reagent (Helena Laboratories) and an electronic fibrintimer. A volume of 50µL was added to the microcuvette and allowed to warm to 37°C. A volume of 100µL of commercial thromboplastin reagent was added to initiate clotting. The electronic fibrintimer automatically detected clot formation. All samples were assayed in duplicate. Variation between duplicates averaged less than 5%.
Table 9. “Snoopy”’ serum and urine venom levels and coagulation parameters pre and post-antivenom.

<table>
<thead>
<tr>
<th></th>
<th>Pre-antivenom [1 hour post-bite]</th>
<th>4 h Post-antivenom</th>
<th>16 h Post-antivenom</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine Venom Conc.</td>
<td>11,640 ng/mL</td>
<td>*&lt;0.08 ng/mL</td>
<td>*&lt;0.08 ng/mL</td>
</tr>
<tr>
<td>Serum Venom Conc.</td>
<td>365 ng/mL</td>
<td>*&lt;0.08 ng/mL</td>
<td>*&lt;0.08 ng/mL</td>
</tr>
<tr>
<td>Prothrombin Time**</td>
<td>&gt;300 seconds (no clot)</td>
<td>47.4 ± 2.6 seconds</td>
<td>12.2 ± 0.4 seconds</td>
</tr>
<tr>
<td>Activated Clotting Time</td>
<td>&gt;10 minutes (no clot)</td>
<td>&gt;10 minutes (no clot)</td>
<td>1 minute 20 seconds (normal)</td>
</tr>
</tbody>
</table>

*Limit of Detection of the ELISA for Tiger Snake venom is defined as 3 x standard deviation of the mean of the blank values (=0.08 ng/mL).

**Normal PT time for a dog is 8-20 seconds. Samples from two normal healthy dogs were run as controls in this assay and returned values of 8.4 ± 0.1 seconds and 11.7 ± 0.8 seconds.

Discussion

This is the first ever recorded case of an animal being treated with alpaca antivenom for snakebite and demonstrates the “full circle” of antivenom production, processing, testing and clinical use. This case also highlights a number of issues that the Alpaca Immunoglobulins Project has been addressing.

The negative result on the CSL Snake Venom Detection Kit (SVDK) is a common problem in veterinary clinics and human hospitals. The sensitivity of the SVDK is probably too low to be useful when applied to plasma or serum. Urine samples are more likely to be positive. However collecting urine samples from animals is not always practical for many reasons. When these same samples were assayed using the new alpaca antibody-based ELISA it was strongly positive. This is not surprising since the ELISA the author has developed using adsorbed alpaca anti-Tiger Snake venom antibodies is up to 1,000 times more sensitive the CSL rapid test kits. The CSL test kits cost approximately $450 ex GST (wholesale price) which can test 3 samples.

“Snoopy’ suffered no adverse effects during or after administration of alpaca antivenom. The dose of polyvalent antivenom was more than twice that required but it is always better to administer more than less. The results of the complement fixation test would have predicted some form of reaction but none was observed. The author routinely administers antihistamines and dexamethasone to reduce immunological reactions, both early and late.

Blood clotting returned to normal within the time frame expected. The prolonged clotting time was still obvious at 4 hours post antivenom but by 16 hours was normal. It has been reported in humans that at least 6 hours is required between antivenom administration and restoration of clotting function. This is because the consumed clotting factors must be regenerated from the patient’s own liver.

This case also highlights the usefulness of monitoring clotting function as an indicator of effective antivenom treatment. Vomiting in dogs is a characteristic clinical sign that the dog has received a lethal dose of venom. The sooner the onset of vomiting the greater the dose received (Lewis 1994).
Vomiting in this case occurred within 1 hour of envenomation. Based on the work of Lewis this roughly correlates with the dog receiving 1-2 lethal doses of venom.

Tiger Snake bite is the most common type of snakebite seen in this geographical area. Diagnosis can be difficult. The false negative SVDK result is not uncommon and limits the usefulness of these test kits aside from the confusion it creates. Secondary signs of envenomation such as clotting disturbances and urine testing can be remarkably informative.

In this case the dog presented probably having received a lethal dose of venom but had not yet developed signs of paralysis. Paralysis from Tiger Snake venom does not rapidly reverse with antivenom due to the pre-synaptic neurotoxins. A more prolonged recovery would be expected if that were the case.

To the author’s knowledge this is the first time ever that venom concentrations have been recorded in a dog following a natural snakebite. The levels seen in “Snoopy” are consistent with previous publications of Tiger Snake venom levels in cats (Moisidis, James et al. 1996) and monkeys (Sutherland, Coulter et al. 1979) injected with fixed quantities of venom. The study in cats was performed by CSL using an ELISA they had developed in-house. Cats were injected with a fixed dose of venom corresponding to 0.1mg/kg bodyweight or approximately 0.3mg per cat. Serum venom concentrations peaked at around 70ng/mL and urine levels >1,000ng/mL. This dose of venom is substantially lower than that which would be naturally injected. When Tiger Snake venom is collected from living specimens the amount collected averages around 35 mg (Mirtschin, Dunstan et al. 2006). The higher serum and urine levels seen in “Snoopy” likely reflect a much larger dose of venom having been received by the dog. In an earlier study performed by CSL using Macaque monkeys (2.3kg bodyweight), a dose of 0.3mg of venom resulted in serum levels of Tiger Snake venom peaking at over 500 ng/mL within 1 hour of injection (Sutherland, Coulter et al. 1979). Studies in humans where Tiger Snake venom levels in serum have been measured report levels up to 152ng/mL (Isbister, O'Leary et al. 2012) and a median level of 3.2 ng/mL. These levels probably reflect the higher bodyweight of humans and the application of pressure bandaging first-aid measures which dramatically lower serum venom levels.

Alpaca antivenom was very effective at binding all circulating venom and venom in urine. The absence of detectable venom at 4 hours after receiving antivenom would indicate the dose was sufficient. The absence of venom from urine at 4 hours was unexpected. This may indicate that some alpaca antivenom has passed through into the urinary tract. The larger size of IgG molecules generally limits the rate at which they leave circulation via renal excretion.
Figure 55. Tiger snake (*Notechis scutatus*) brought in by the owner of “Snoopy” contained within a plastic shopping bag.

Figure 56. Grossly haemolysed plasma collected from “Snoopy”.

Figure 57. Negative CSL Snake Venom Detection Kit result on serum obtained from “Snoopy” immediately after presentation. Only the positive control well changed colour.
Figure 58. ELISA standard curve for Tiger Snake venom detection.
Figure 59. Photograph of “Snoopy” the day after receiving alpaca antivenom for a confirmed Tiger Snake bite.
General discussion of project results

This project has generated significant new data on the production and use of alpaca immunoglobulins. The work has focused on the specific application of treatment and diagnosis of snakebite.

The major findings are that:

(i) Alpaca antivenom is extremely effective for neutralising blood clotting disturbances induced by snake venom.

(ii) Alpaca antibodies proved remarkably effective when used as a capture antibody for a new Tiger Snake venom enzyme immunoassay with a sensitivity 15 times better than the most sensitive ELISA published on Tiger Snake venom, and 1,000 times more sensitive than a commercial venom detection rapid test kit.

(iii) Alpaca serum is difficult to process using chemical purification systems commonly used for horses and sheep. Higher purity can be obtained using affinity chromatography methods but these are more expensive to apply on an industrial scale.

(iv) Antibody ELISA is a very useful tool for monitoring the immune response of alpaca to hyperimmunisation.

(v) There are few, if any, real zoonotic disease risks that cannot be managed by simple biosecurity practices rather than through use of unvalidated diagnostic tests.

(vi) The intellectual property landscape is clear and there is freedom to operate and commercialise alpaca-derived antibody technology in Australia.
Implications

In this work, we have shown that alpaca are very suitable for antivenom production. They can be used to produce high quality antivenom that shows excellent properties for neutralising both procoagulant and neurotoxins. With the strategic use of an ELISA to monitor antibody levels, a larger group of alpaca could be culled down to a smaller ‘super-producing’ group. A culling ratio of at least 50% would ensure only the best responders are used, improving the potency and thus safety of the final product. We have shown that alpaca can respond to five venom types simultaneously and some animals produce remarkably high antibody levels in response to this polyvalent immunisation.

We have also shown in this work that alpaca antibodies can be used as diagnostic test reagents. Our Tiger Snake venom ELISA using affinity purified antibodies demonstrated exquisite sensitivity and could have wide application in diagnosis of snakebite. Further work would be needed to develop monovalent affinity purified antibodies for other snakes.

The intellectual property search conducted as part of this project revealed no significant obstructions to commercialisation in this field.

Alpaca antiserum has some benefits when compared to sheep and horse serum (Table 10). A detailed economic model would be required to determine the cost efficiencies of each species, taking into account management factors, yields, potency, immunisation costs and every other factor that contributes to the cost of production. This is not a straightforward exercise as the processing costs are method dependent and pilot studies to calculate yields would be required.

The biggest limitation of alpaca for commercial antivenom, and other bulk immunoglobulin preparations, is the difficulty in processing serum. Heat treatment to 56°C for 30-60 minutes appears to be an obligate requirement to obtain a clear final product. Unfortunately, heat treatment is difficult to implement in a sterile manner in a pharmaceutical processing plant; not impossible, but challenging. The failure of caprylic acid to produce a low turbidity immunoglobulin product is also a limiting factor. When affinity chromatography is used to extract specific antibodies, the resulting product is of extremely high quality and purity. However, affinity chromatography is orders of magnitude more expensive to utilise for processing on an industrial scale.
Table 10. Comparison of antivenom product between horse, sheep and alpaca.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Horse</th>
<th>Sheep</th>
<th>Alpaca</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume of serum per blood collection</td>
<td>4,000 mL</td>
<td>350 mL</td>
<td>200 mL</td>
</tr>
<tr>
<td>Serum purifies with routine low cost methods</td>
<td>++++</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>Commercial viability proven</td>
<td>Yes &gt;100yrs</td>
<td>Yes &gt;20yrs</td>
<td>No</td>
</tr>
<tr>
<td>Tolerance of oil based adjuvants</td>
<td>Poor</td>
<td>Good</td>
<td>Moderate</td>
</tr>
<tr>
<td>Longevity as serum producer</td>
<td>&gt;20 yrs</td>
<td>5 to 8 yrs</td>
<td>?</td>
</tr>
<tr>
<td>Snake procoagulant toxin neutralising antibodies</td>
<td>Medium</td>
<td>Excellent</td>
<td>Excellent</td>
</tr>
<tr>
<td>Snake neurotoxin neutralising antibodies</td>
<td>Medium</td>
<td>Medium</td>
<td>Medium</td>
</tr>
<tr>
<td>Animal purchase price</td>
<td>$1,000*</td>
<td>$200</td>
<td>$200</td>
</tr>
<tr>
<td>Disease testing protocols defined with TGA</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Patient safety profile of commercial products</td>
<td>Moderate</td>
<td>Excellent</td>
<td>None</td>
</tr>
<tr>
<td>Freeze dried formulations effective</td>
<td>Yes</td>
<td>Yes</td>
<td>?</td>
</tr>
<tr>
<td>General procedures well defined</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Circulating antibody type in final product</td>
<td>IgG &amp; IgG\text{\textsubscript{T}}</td>
<td>IgG</td>
<td>IgG\text{\textsubscript{1,2,3}}</td>
</tr>
<tr>
<td>Heat stability of antibodies</td>
<td>Medium</td>
<td>Medium</td>
<td>Unproven\textsuperscript{1}</td>
</tr>
<tr>
<td>Economical to select only high responders</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Alternative high value animal products</td>
<td>No</td>
<td>Wool</td>
<td>Fibre</td>
</tr>
<tr>
<td>Strong market for culled poor responders</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Serum immunoglobulin concentration</td>
<td>10-15 g/L</td>
<td>15-25g/L</td>
<td>5-15g/L</td>
</tr>
<tr>
<td>Zoonotic diseases of significance in Australia</td>
<td>Emerging (Hendra)</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>Animal tolerance of tropical climates</td>
<td>Yes</td>
<td>Yes (?)</td>
<td>?</td>
</tr>
<tr>
<td>Separation of red blood cells by gravity</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Intellectual property restrictions</td>
<td>No</td>
<td>No</td>
<td>Partial</td>
</tr>
<tr>
<td>Animal rights activists interest level</td>
<td>High</td>
<td>Low</td>
<td>?</td>
</tr>
</tbody>
</table>

*Approximate price at market value, cull animals lower price.
Recommendations

Commercialisation of alpaca immunoglobulins requires more work, to better understand some of the issues exposed in this work. Alpacas do offer some benefits over other animal species for antibody production, but there are limiting factors that must be overcome.

*Further research to explore usefulness for diagnostic tests:* Alpaca antibodies appear to be particularly useful as diagnostic test kits. The binding to a solid-phase in an ELISA appears exceptional leading to high assay sensitivity.

*Further research to examine neutralisation of procoagulant effects on snake venom:* Alpacas appear to possess extraordinarily powerful antibodies for neutralising clotting disorders, at least 10 times better than horse serum.

*Further research on improving antibody extraction methods from serum:* A major limiting factor at present is the difficulty in obtaining high purity antibodies from serum. This work would also include a detailed examination of the costs of different purification methods.

*Further research to better understand the likelihood of allergic reactions to alpaca serum:* In particular, studies performed in animal models of allergy are needed. If alpacas are to be presented as producers of low allergy serum products, this must be evaluated in standard animal models such as the guinea pig anaphylaxis model, as *in vitro* tests are not conclusive.
References


Alpaca Immunoglobulins
Phase 2 Report

by Andrew Padula

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RIRDC Project No PRJ-008834