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Novel immunoassay for human erythropoietin using camelid antibodies

Novel Immunoassay for Human Erythropoietin Using Camelid Antibodies

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Start Date: September 2017

Submission: September 2020

A dissertation submitted to the University of Bristol in accordance with the requirements for award of the degree of Cellular and Molecular Medicine (MSc) (R) in the Faculty of Biomedical Sciences

Word Count: 22077

Declaration

I declare that the work presented in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programs and that it has not been submitted for any other academic award. This project is my own work except where indicated. All text, figures, tables, data or results, which are not my own work, are indicated and the sources acknowledged. In addition, I confirm that the hardcopy and the e-submission are identical.

SIGNED: Stephanie Saghbazarian

DATE: 16/05/2020

Abstract

Background. Erythropoietin (EPO) is a glycoprotein, secreted mainly by the kidneys that regulates erythrocyte production. Clinically it is used to treat certain anemias, but elite athletes misuse it, despite prohibition of the practice by the World Anti-Doping Agency (WADA). As endogenous and recombinant forms of EPO only vary in the extent of their glycosylation, current assays used to detect this growth factor fail to completely discriminate between the two forms. Thus, there is a need for the development of assays that specifically measure only the recombinant forms. The humoral immune repertoire of camels is unique, in that they produce heavy-chain-only antibodies (HCAbs) which exhibit a broad antigen-binding repertoire. Exploiting this natural immune capability of camels may produce antibodies with unique specificities and affinities for utilization in novel assays.

Aims & objectives. This study aimed to raise camelid HCAbs and heavy chain variable fragments (V_HH fragments) directed against recombinant human EPO for future use in specific immunoassay development.

Methods. Two, male dromedary camels were immunized with recombinant human EPOs (rhEPOs), Epoietin α and β respectively, until a humoral immune response was detectable. 1. Polyclonal HCAbs were affinity (Protein G) purified, conjugated to horse radish peroxidase and used in an immunoassay to detect human urinary EPO in a volunteer injected with Epoietin β. 2. RNA was isolated from the peripheral blood lymphocytes of these animals and used to produce V_HH fragments, or single domain antibodies, by phage display technology, and selected for antigen binding.

Results. 1. The assay developed, using the HCAbs, was able to measure Epoietin β, without any signal being detected prior to the injection of recombinant. 2. The V_HH fragments produced by phage display successfully recognized antigen.

Conclusions. The camel-derived polyclonal HCAb against rhEPO distinguishes between endogenous and exogenous EPO, but, this needs to be confirmed in a larger cohort and after further assay development. This suggests that camelid polyclonal HCAbs, and V_HH fragments generated from a phage display library, have the potential to form the basis for an assay specific for recombinant EPO.

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Dedication and Acknowledgements

In the accomplishment of this project successfully, I am indebted to many for their heart pledged support. Primarily, I cannot express enough thanks to my supervisor Dr. Vidya Mohamed-Ali for her ongoing support and encouragement throughout my whole journey in this project. I offer my sincere appreciation for the learning opportunities, progress, and unlimited guidance. Her passion for research and science has been a significant driving force for me. She taught me how to ask questions and express my ideas and showed me different ways to approach a research problem and the need to be persistent to accomplish any goal. I thank Dr. David Morgan for advice and guidance in the experimental design, formulating a proper hypothesis, and how to break down my results. Thanks to Dr. Pasquale Vito for his help in facilitating the construction of the VHH library and many recommendations behind the troubleshooting processes. I also, would like to thank Dr. Christoph Wuelfing, for his continuous support and assistance throughout my study. My gratitude to Professor. Mohammed Al-Maadheed who gave me the opportunity to be part of his leadership and vision behind this program in Qatar by leading junior Scientists forward.

I also would like to thank all my colleagues at Anti-Doping Lab Qatar (ADLQ) for the continuous help and training as it has tremendously improved my skills in the lab. Thank you to my colleagues at the University of Bristol (UoB) for all their help and support during the project. Finally, I would like to dedicate my work to my parents. My dad taught me so much by example. He taught me the importance of work ethics, honesty and integrity. One of my favorite quotes that have always resonated with me is:" There is always going to be someone out there who is better looking than you or more intelligent than you. The one thing you have complete control over is how hard you work. Work the hardest."

To my family, my husband, and my sons, thank you for sharing the good and challenging moments during my studies, being a new mom has not been an easy journey but rather it has been a discovery of strengths that I had no idea I had.

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Abbreviations

Ang	angiotensin
BRP	Biological Reference Preparation
CFU-Es	Colony-Forming Units-Erythroid
CHO	Chinese Hamster Ovary
CBP	CREB-binding protein
CFU-E	Colony-forming unit-erythroid
CKD	Chronic kidney disease
CREB	cAMP response element-binding protein
CERA	Mircera
EPO	Erythropoietin
EPO-R	EPO receptor
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-linked Immunosorbent Assay
ESA	Erythropoiesis-Stimulating Agent
Hb	Haemoglobin
Hct	Haematocrit
HIF	Hypoxia-inducible factor
HNF	Hepatocyte nuclear factor
HRE	Hypoxia-response element
HCAbs	Heavy-Chain-Only Antibodies
HRPO	Horseradish Peroxidase
PHD	Prolyl hydroxylase
RBC	Red blood cell
RIN	RNA Integrity Number
RT-PCR	Real-time Polymerase Chain Reaction
rhEPO	Recombinant human Erythropoietin
VHL	Von Hippel-Lindau
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-linked Immunosorbent Assay
ESA	Erythropoiesis-Stimulating Agent
Ig	Immunoglobulins
IPTG	Ispropyl-beta-D-Thiogalactopyranoside
LB	Lysogeny Broth
OD	Optical Density
PBS	Phosphate-buffered Saline
PCR	Polymerase Chain Reaction

PVDF	Polyvinylidene Difluoride Membrane
sdAbs	Single Domain Antibodies
SDS-PAGE	Sodium Dodecyl Sulfate-Poly Acrylamide Gel Electrophoresis
WADA	World Anti-Doping Authority

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Conference proceedings

1. Saghbazarian S, Morgan D, Mohamed-Ali V. Novel immunoassay for human erythropoietin using camelid antibodies. Oral presentation at the 5th Annual Junior Symposium at ADLQ (JS ADLQ) 19th Dec 2017.
2. Saghbazarian S, Morgan D, Mohamed-Ali V. Novel immunoassay for human erythropoietin using camelid antibodies. Oral presentation at the 6th Annual Junior Symposium at ADLQ (JS ADLQ) 11th Dec 2018.

Original Articles

1. Voss SC, Orie NN, Elsaftawy W, Saghbazarian S, Al-Kaabi A, Georgakopoulos C, Athanasidou I, Mohamed-Ali V, Al Maadheed M. HRP-conjugated Anti-EPO antibodies for direct rhEPO detection – proof of concept. Drug testing analysis 2020; Accepted for publication, under revision.

CHAPTER 1
INTRODUCTION

1.1 Erythropoietin, historical perspective

A direct relationship between hypoxia and red blood cell (RBC) count was first proposed by Paul Bert as far back as 1882 (1); and Carnot and Deflandre also postulated that there was a humoral factor, 'haemopoietin', that controlled RBC production (2). In 1948 the name 'Erythropoietin' (EPO) was given to a putative humoral factor that could be transferred via the blood (3); closely followed by evidence that EPO was able to increase levels of RBC in anemia (4). Many other studies rapidly followed this, including the purification of EPO from urine samples of patients with aplastic anemia in 1977 (5), and the demonstration of its ability to treat anemia. The significant role of the hormone/growth factor EPO in the regulation of erythropoiesis and the functioning process of mature red blood cells (RBCs) and its therapeutic potential was thus established. In 1985, the EPO gene was cloned paving the way for the commercial production of recombinant human Epo (rhEPO) (6,7); which was later approved and licensed for use as a therapeutic agent in 1988 for patients with chronic renal failure caused by anemia.

EPO is a sialo glycoprotein that is synthesized primarily by the kidneys. And, to a lesser extent, erythropoietin production can be found in the liver. The kidney endothelial and epithelial cells of the tubule that form the lining of the collecting duct are reported as being the main source of EPO. One of the main essential effects of EPO is to promote haemoglobin synthesis and proliferation of differentiating erythroid cells, primarily the colony-forming units-erythroid (CFU-Es) (8). EPO production is triggered by hypoxia; and systemic and urine levels are high when tissue oxygenation is low. Therefore, when the oxygen-carrying capacity of blood is low, accompanied by decreased tissue oxygenation, the kidneys increase secretion of EPO which, in turn, stimulates production of RBCs. The normal, physiological, circulating concentration of EPO is approximately 15 U/L (≈5pmol/L), with a range between 10-25 U/L (9). Low levels of EPO can be seen in various clinical conditions which are associated with anemia; especially chronic renal failure, and during certain cancer chemotherapies, and is clinically managed with administration of rhEPO (10).

Several studies now point to roles for EPO beyond its effect in hematopoiesis. There is emerging evidence for the activity of EPO, especially in skeletal muscle, endothelial, neural, cardiovascular and renal tissues, in response to exercise or changes in metabolic demand. In these tissues EPO appears to mediate the protection against oxidative stress and angiogenesis (11).

1.2 Structure of EPO

The human EPO encoding gene is made up of five exons and four introns and post-transcriptionally generates a single polypeptide coding for a prohormone of 193 amino acids. It is located at chromosome 7q11-22 (12). The primary structure of the mature glycoprotein hormone is composed of 165 amino acids, and prior to its release into the blood circulation, the Arg-166 at the carboxyl terminal is cleaved. The molecular mass of the erythropoietin peptide backbone is approximately 18 kDa. Due to being a highly glycosylated protein, post-translational changes yield about a 40% carbohydrate moiety deriving a total mass

of 30 kDa. However, EPO is detected on a SDS-polyacrylamide gel with apparent sizes between 34-38kDa (13). Furthermore, EPO contains four cystine residues at positions 7, 29, 33, and 161. EPO contains two disulfide bonds, one which can be found between Cys 7and Cys 161, while the other is reported to be between Cys 29 and Cys 33, sandwiched between two glycosylation sites, Asn 24 and Asn 38 (14).The export of EPO is dependent on the correct processing that involves the addition of three *N*-Linked tetra antennary at Asn-24, Asn-38 and Asn-83, and one *O*-linked, at Ser-126, acidic oligosaccharide side chain, and intracellular removal of the 27 amino acid hydrophobic secretory sequence from the NH2 terminus and arginyl residue from the carboxy-terminus (12). Some alterations to the molecular mass, biological activity, electrical charge or immunoreactivity of the EPO protein can be altered due to some structural modifications or permutations of the carbohydrate chains (14). Two of the many varieties of functions that the extensive glycosylation of EPO serves are the protection of the glycoprotein from proteases and the modulation of its receptor binding affinity. The three complex-type N-linked oligosaccharides contribute significantly to specific properties of EPO, which include maintaining the conformation of the polypeptide chains, conferring stability to the systemic hormone, enhancing its *in vivo* biological activity; as well as receptor binding and interaction with other molecules. The function of the O-linked oligosaccharide at Ser-126 is still unclear, although it constitutes less than 4% of the glycosylation, it lacks significant functional importance (15). Some parts of the carbohydrates in EPO which contributes to its relatively low iso-electric pH of ~4.4 contain a minimum of 10 molecules of sialic acid. EPO occurs in several isoforms that differ from each other in their carbohydrate structure. The tertiary structure of EPO is that of other members of the class I cytokines which folds into a compact globular structure made of four α-helical bundles.

1.3 Mode of action of EPO and Epo-R

EPO controls the erythroid progenitor cells located in the bone marrow and exerts its effect by binding to a cytokine class I receptor superfamily member, the EPO-R, to stimulate erythropoiesis (16, 17). Epo-R is a transmembrane receptor that includes an extracellular domain containing a WSXWS motif, a single transmembrane hydrophobic domain and a variable cytoplasmic domain. Also, its cDNA encodes for a protein of 508 amino acids(18). When rhEPO is administered intravenously it is rapidly distributed and then significantly eliminated at first pass through the liver by zero order kinetic rate. The elimination half-life of circulating rhEPO is 6 – 8 hours. Once EPO's actions are mediated by the activation of the high affinity receptor (Epo-R), which act as homodimers of two 57 kDa transmembrane glycoproteins in human erythropoietic tissues, the primary mechanism by which EPO maintains erythropoiesis is by suppressing apoptosis. During RBCs differentiation, the number of erythropoietin receptors fluctuates. Peak presentations occur when the most primitive erythrocyte progenitors give rise to the colony forming unit-erythroid/pro-erythroblastic cells and it is undetectable amongst reticulocytes. Signal transduction pathways through the Epo-R is initiated by ligand binding and the homodimerization of the receptor. Pre-dominant pathways activated by Epo-R are: JAK2/STAT5 system, G-protein (RAS), calcium channel, and kinases. The Epo-R

mRNA is also expressed in non-erythrocytic tissues, including the cardiovascular system. However, there is some contention as to whether or not this is translated into a functional receptor protein, as some studies using specific anti-Epo-R antibody were unable to detect the Epo-R protein in normal non-erythroid human tissues (19,20). Thus, it is possible that EPO is a unique survival and growth factor for erythropoietic tissues. One of the major actions of EPO is to prevent apoptosis in EPO-dependent colony-forming units, RBCs and erythroblasts that have not undergone hemoglobin synthesis. The sensitivity of EPO production to the local oxygen environment is regulated via the production of RBCs and is enabled by induction of EPO mRNA expression by hypoxia which is mediated by the transcription factor HIF-1 (21). Loss of RBCs in an individual causes a decrease in oxygen distribution to the tissues, which stimulates the kidney tubule cells to secrete EPO into the blood to be carried to the bone marrow. This situation triggers more erythrocytes to be produced resulting in higher oxygen delivery to the tissues (22; Figure 1).

1.4 Recombinant EPOs

Endogenous EPO and recombinant (rh)EPO have the same amino acid sequence, but are somewhat different in their glycosylation status. rhEPO is routinely generated in Chinese Hamster Ovary (CHO) cell lines that are genetically modified express human erythropoietin (23). Four different rhEPOs are currently available: alpha, beta, delta, and omega. However, the only two commercially readily available are EPO-alpha and EPO-beta. Despite these rhEPOs acting on the same erythropoietin receptor, differences in some degree of glycosylation causes some variations which give rise to differences in the pharmacokinetics and pharmacodynamics. A rise in the number of glycosylation sites may improve its activity as the *N*-glycosylation confers the biological activity of rhEPO. A hyperglycosylated rhEPO, known as novel erythropoiesis stimulating protein (NESP) or Darbepoetin-alpha is also available on the market. This has been produced using site mutagenesis to modify the polypeptide backbone of the rhEPO, introducing five *N*-glycosylation sites, as opposed the three in the other rhEPOs. Compared with other rhEPOs, NESP is found to have a threefold extended half-life and holds a higher negative charge. Despite the fact that it requires a less frequent dosing schedule, the clinical outcome and safety profile in treating anemia associated with chronic renal disease and of malignancy is similar to rhEPOs (24,25). Currently, the applicability of NESP is being evaluated in supplemental clinical conditions. Another approach to enrich the biological activity of rhEPO is to protect it from being metabolized; so that its rate of elimination is decreased and its half-life prolonged. As such, microencapsulation and pegylation of rhEPO are presently being investigated in an attempt to achieve this.

1.5 Clinical applications of rhEPO

The commercial availability of large quantities of therapeutic grade rhEPO has greatly improved the treatment of anemic patients with chronic renal disease. In addition, clinical studies of administration of rhEPO in various nonuremic conditions: such as HIV infection; hematological and oncological disorders, prematurity; and perioperative therapies has shown great clinical benefit (26). Since the initial clinical introduction of

rhEPO and cloning, its therapeutic potential and usage has expanded enormously. Approximately, a third of patients with haemoglobin (Hb) levels of <10.0 g/dl, which are therefore diagnosed as clinically anemic as a result of receiving cancer chemotherapy, are treated with epoetin (27).

Other EPO analogues, that lack erythropoietic potential, have also been developed for further investigation, such as asialo-EPO, which is rapidly cleared from circulation, carbamylated EPO (CEPO; 28), which is devoid of erythropoietic activity due the neutral or negative charges of its lysine's; and ARA290, which is a truncated helix-B peptide of EPO (Helix B surface peptide, HBSP (29,30). In a rodent model of myocardial infarction (MI), daily injection of CEPO improved the recovery (31). A novel CEPO (Lu AA24493) was also tested in neurological diseases, however, in a multicenter, double-blind, placebo-controlled, phase II clinical trial this was found to be ineffective (32, 33). Dampening, or even eliminating, the erythropoietic activity is clinically attractive as elevated Hct, that accompanies EPO therapy may be adversely associated with pro-thrombotic events. ARA290, a linear peptide of 11 amino acids with a short plasma half-life of ~2 min, also appears cardioprotective in rat models of MI. It was found to reduce mortality and ameliorate MI expansion and CHF progression (34). ARA290 also appears to decrease coronary atherosclerosis by its inhibitory effects on endothelial cell apoptosis and its ability to inhibit the release of TNF-α production. Effects of ARA290 on patients with non-cardiologic indications, such as those neuropathic type 2 diabetes, have also been reported (35).

1.6 Effects of EPO on exercise performance, blood doping and skeletal muscle

In aerobic sport disciplines the total mass of Hb (Hb mass), correlates with the rate of maximal O_2 uptake (VO_2max) (36). Regrettably, EPO ESAs are also used by some non-anemic athletes to increase the mass of circulating RBCs and Hb. As a result, maximal physical exercise may improve due to: (i) an enlarged peripheral O_2 concentration, and, (ii) an increase in blood volume and, thus, cardiac output. The World Anti-Doping Agency (WADA) prohibited the misuse of EPO and ESAs in sports in 1988 (37). Maintaining an appropriate Hb levels and adequate O_2 supply to the tissues is EPO´s primary function. In sports deemed to demand a high level of aerobic fitness, such as cycling, cross-country skiing, or long-distance running the key determiner for performance are an elevated delivery of O_2 to the exercising skeletal muscles (38).
The easiest way to improve physical performance is to increase the arterial O_2 content by enhancing Hb mass. Hence, rhEPO and other ESAs are misused by sport professionals and amateurs to accelerate erythropoiesis above normal. VO_2max is also dependent on the cardiac output and the rate of peripheral O_2 extraction, but these parameters are difficult to manipulate during competitions (39).

The use of EPO to enhance exercise performance has led to research interest in elucidating its role in skeletal muscle(14). There is some evidence for the molecular mechanisms through which EPO may act on human muscle. A single EPO injection was sufficient to acutely increase the mRNA expression of the myogenic regulatory factor 4 (MRF-4), like that seen after resistance exercise, perhaps facilitating the growth of muscle

fiber. Elevate levels of Epo-R mRNA, compared to pre-exercise levels, have been reported in muscle biopsies taken pre and post training. EPO also appears to increase resting energy expenditure, muscle fiber diameter, especially in type I fibers, and glycogen content, though without an effect on insulin sensitivity. Both in rodents and in humans EPO treatment elevated skeletal muscle mitochondrial oxidative phosphorylation and maximal electron transport capacity. However, there is still some controversy regarding the direct effects of EPO on human skeletal muscle growth, repair, or metabolism. It is suggested that these may not be via EPO/Epo-R interaction, but due to better oxygen supply to the tissues. (40)

rhEPO, at doses like to those used for the treatment of anemia, does not have direct, acute effects on cardiac function. However, chronic doping with rhEPO could result in indirect cardiovascular effects, due to greater training loads because of the increased arterial O_2 content (41). In addition, increased Hct, blood viscosity and peripheral flow resistance could also cause hypertension and microvascular dysfunction. It is for these reasons that rhEPO derivatives such as asialo-EPO, CEPO, and ARA290, that lack EPO activity, have generated some interest (42). However, none of these compounds appear to have performance-enhancing potential in humans.

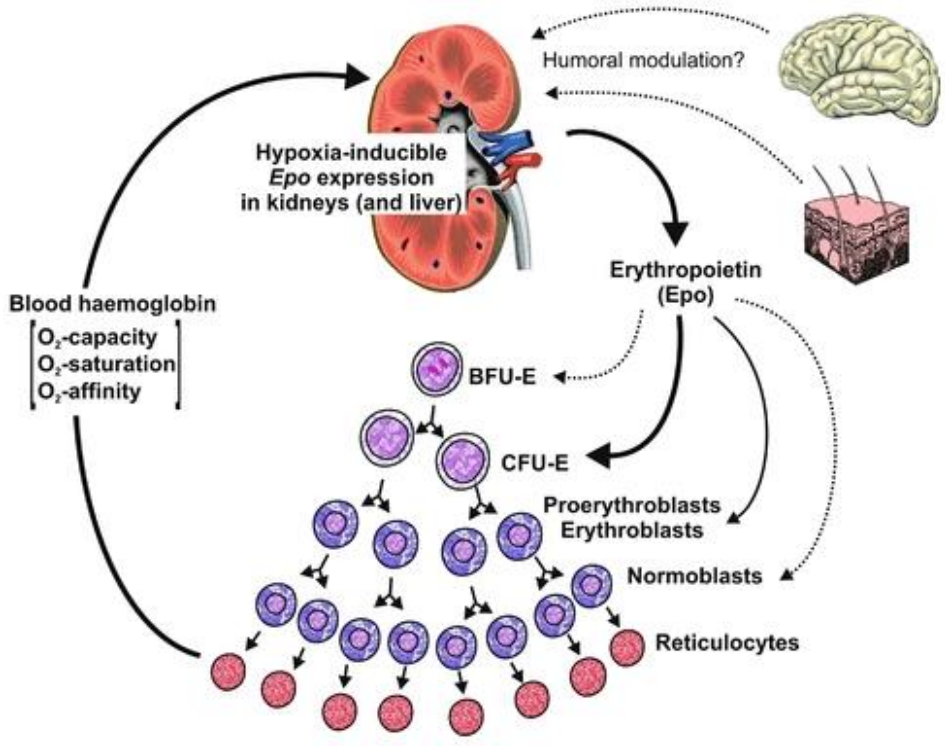


Figure 1 - Diagram of a feedback regulation of erythropoiesis

The main site for the synthesis of EPO is the kidneys and is triggered by a lack of oxygen or hypoxia. The erythrocytic progenitors that give rise to the colony-forming units-erythroid (CFU-Es) is dependent on the survival of EPO proliferation and differentiation. Once there is a response to EPO, the CFU-Es proliferate and differentiate into cohorts of proerythroblasts and normoblasts. Human haematopoietic EPO receptor (Epo-R) belongs to the cytokine class I receptor family and is composed of 484 amino acid glycoprotein that forms homodimers. Upon the binding of EPO to the Epo-R dimer, the cytoplasmic jaus kinases 2 (JAK2) causes the phosphorylation of tyrosine residues of the Epo-R and various other intracellular proteins. As erythropoiesis is a gradual acting process, it takes three to four days for a reticulocytosis to become apparent following a rise in plasma EPO. Once there is a release of reticulocytes the oxygen capacity of the blood increases (Adapted from 8).

The ergogenic effect of rhEPO in aerobic sports was first described 30 years ago (43). When rhEPO is administered subcutaneously (SC) at the following doses, 60-350 U/kg b.w and week (wk) for a period of 4-6 wks, the VO_2max is shown to increase and to prolong the time to exhaustion (44-46). ESAs are particularly effective in stimulating erythropoiesis when their application is combined with IV iron supplementation (47). Recent studies have shown that when rhEPO was administered in healthy volunteers in lower doses, the O_2max appears to be higher by 6-12%, once the Hct level is increased to nearly 0.50. Whereas the VO_2max at a given level is higher by up to 50% prolonging time to exhaustion (48). Treatment with rhEPO may increase submaximal endurance performance to a higher extent than the blood O2 capacity. Investigators have proposed the occurrence of non-erythropoietic ergogenic effects on rhEpo application (49). Furthermore, there have also been reports of EPO activating numerous non-haematologic factors related to enhancements in aerobic power. However, augmented erythropoiesis remains the principal mechanism by which EPO improves exercise performance in humans (50). Moreover, Lundby and Olsen have stated that there is no persuasive evidence that ESAs, other than by increasing Hb mass, causes a placebo effect and enhances exercise performance (51). In a recent review by Heuberger et al., it was concluded from reviewing the literature that there is a lack in scientific foundation to assume that the use of rhEPO would enhance the performance of elite cyclists (52), and that there is clear evidence that rhEPO has the capacity to enhance the performance in five out of 23 substance classes on the WADA Prohibited List (37, 53). These are found in the following categories: β2-adrenergic agonists, anabolic agents, stimulants (amphetamines, methylphenidate) glucocorticoids and β-blockers (52).

Furthermore, further scientific debate was also ignited after Heuberger et al. re-studied the properties of rhEPO by treating 48 inexpert male cyclists with subcutaneous injection of ~6,000 U per wk, SC, for 8 wks in a double-blinded, randomized, placebo-controlled clinical trial (38). An underperforming outcome was found when comparing the road race performance with the pertinent submaximal exercise. Despite rhEPO enhancing the laboratory parameter of maximal exercise (VO_2max) the test performance was unaltered (54,55). However, the weakness of the statistical study was a major factor of criticism and it was argued that the transferability of results to professional athletes was unclear, as well as that there were low numbers of participants and a short examination period (56). In certain studies in sports research, clinically common rhEPO doses were applied (i.e. doses similar to those administered to anemic CKD patients; ~100 U/kg b.w. per wk), or cancer patients on chemotherapy (~450 U/kg b.w. per wk). Salamin et al. have pointed out a systematic variation of cheating athletes in using micro doses of rhEPO (e.g., 10 – 20 U/ kg b.w. per treatment) to reduce the window for classic direct detection and evade noticeable alterations in the levels of blood markers (57).

The detection of rhEPO is therefore extremely challenging and despite having many parameters set in place by monitoring the reticulocyte haemoglobin levels, along with the serum transferrin receptor and packed cell volume, none of them are dependable or adequately sensitive. The detection of ESAs in cheating athletes is

based on two different approaches, namely, (i) the direct detection of the recombinant products in blood and urine, and, (ii) the indirect procedure, referred to as the 'Athlete Biological Passport', as a part of which various hematological parameters are monitored (58,59). Currently, the differentiation between the endogenous and the recombinant form of human EPO is done by direct measures, using electrophoretic analysis of the glycosylation pattern of EPO serum. Nevertheless, the method is time-consuming and is not universally available.

1.7 Detection and analysis of EPO

The biomonitoring of rhEPO is currently focused on indirect evidence based on the analyses of blood parameters of athletes, including Hct, reticulocytes, Hb, enlarged RBCs or macrocytes and serum markers, such as concentration of EPO and serum transferrin receptors. An emphasis on the inter-technique comparison has proven to be crucial for obtaining reliable results, especially for serum markers (60). The presently approved isoelectric focusing–double blotting–chemiluminescence detection method for doping control of EPO is dependent on the charge differences between isoforms of native urinary EPO and recombinant EPO and has both some advantages and limitations. The chemical basis of the differential finding is of relevance and future approaches for detection are under investigation (61).

Recombinant and endogenous human EPO differ only in their glycosylation. Therefore, the ability to detect the presence of the former to test for doping in sports is problematic and limited (62). There are two approaches that were developed for the detection of rhEPO misuse in sports. The first one was based on the recognition of indirect blood markers and on the direct detection of rhEPO in urine. The Indirect method of detection relies on the analysis of RBC percentage having a hemoglobin concentration of 28 pg and a volume above 128 fl.oz. This test is highly selective and limited by its relatively poor sensitivity. The Direct detection method identifies the consumption of the rhEPO by relying on having the biological samples, urine and blood, to be collected within 24 hours after the last rhEPO injection. Furthermore, once athletes had ceased treatment of EPO for more than three days, less than half of them could be declared positive. Seven days after rhEPO consumption no trace of rhEPO can be detected in their system (22,63). The ability to identify rhEPO abusers is hindered by the short half-life detection and the charge differences between isoforms of recombinant EPO and endogenous EPO. Hence, there is a need to improve the current assays for rhEPO that is used by doping analyses laboratories with a higher specificity and sensitivity.

There are many forms of rhEPO available on the market; some of the most used in clinics are Epoietin-α and Epoietin-β. These two forms of recombinant DNA-derived erythropoietin exhibit variances in their carbohydrate structure and pharmacological properties, but not in their respective clinical efficacies. Epoetin-β differs from epoetin-alpha by having a larger basic isoform proportion, higher in-vivo:in-vitro bioactivity ratios, and a superior portion of EPO binding to Erythrina cristagalli agglutinin. Despite similar clinical effectiveness, these slight differences between the two-isoform composition of Epoetin-α and Epoietin-β, are

a consequence of the differences in glycosylation. Thus, the difference between the two forms in some analytical systems suggests that separate international standards for these two types of rhEPO might be a necessity (64,65). As hemoglobin mass is an important factor in reaching best exercise capability, athletes have been applying the use of prohibited rhEPO in order to improve their physical performance in the sports scene. Its detectability following the administration of rhEPO has been difficult to prove directly. Even though WADA has implemented a biological passport operating guideline which monitors several parameters of mature RBCs and reticulocytes at different periods and it plays a significant role in evaluating each participating athlete with respect to manipulation, novel erythropoietic substances have been challenging to detect due to rhEPO being virtually identical to the naturally occurring form (66). Understanding the sensitivity, specificity, and utility of assays that measure anti-EPO antibodies in athletes is critical and there needs to be a consensus on a standard assay method that can distinguish between endogenous versus recombinant EPO in blood and urine samples without any cross reactivity (67). A novel approach is therefore needed, in order to obtain a highly specific assay that has a very low probability of measuring a false positive (68; and Figure 2).

Erythropoiesis stimulated by rhEPO attracts athletes wishing to improve their aerobic power; despite its misuse and the fact that is banned by the International Olympic Committee. The analyses of haematological or biochemical parameters only points to the fact that erythropoiesis has been stimulated, without a concrete confirmation blaming drug administration (69). To confirm that the hormone has been administered exogenously, it is critical for recombinant EPO to be differentiated from natural, endogenous EPO.

Unless there is a reliable method of detecting every administration of EPO and ESAs the prevalent practice of blood doping will be difficult to abolish. Despite current antibody-based assays being available to detect the former, there is a necessity for an antibody that has the characteristics of higher level of specificity and sensitivity that is able to detect and measure rhEPO solely, without cross-recognition of endogenous EPO. Novel approaches are needed to help develop better immunochemicals for this purpose. Camels have revolutionized the antibody engineering industry and by exploiting their natural immune capability, antibodies with higher specificity and affinity can be produced and potentially used in assay development. The discovery of smaller antibodies or V_HH fragments that are derived from heavy chain only antibody of camelids species (70), has shed a new light on antibody engineering as new properties could be accessed. Having an antibody-based targeting molecule containing just variable regions may lead to higher specificity, stability, and solubility and can also be readily produced using recombinant technology (71), which could give the advantage of developing a potential assay that targets anti-EPO antibodies administered only exogenously. The basis for this approach is discussed.

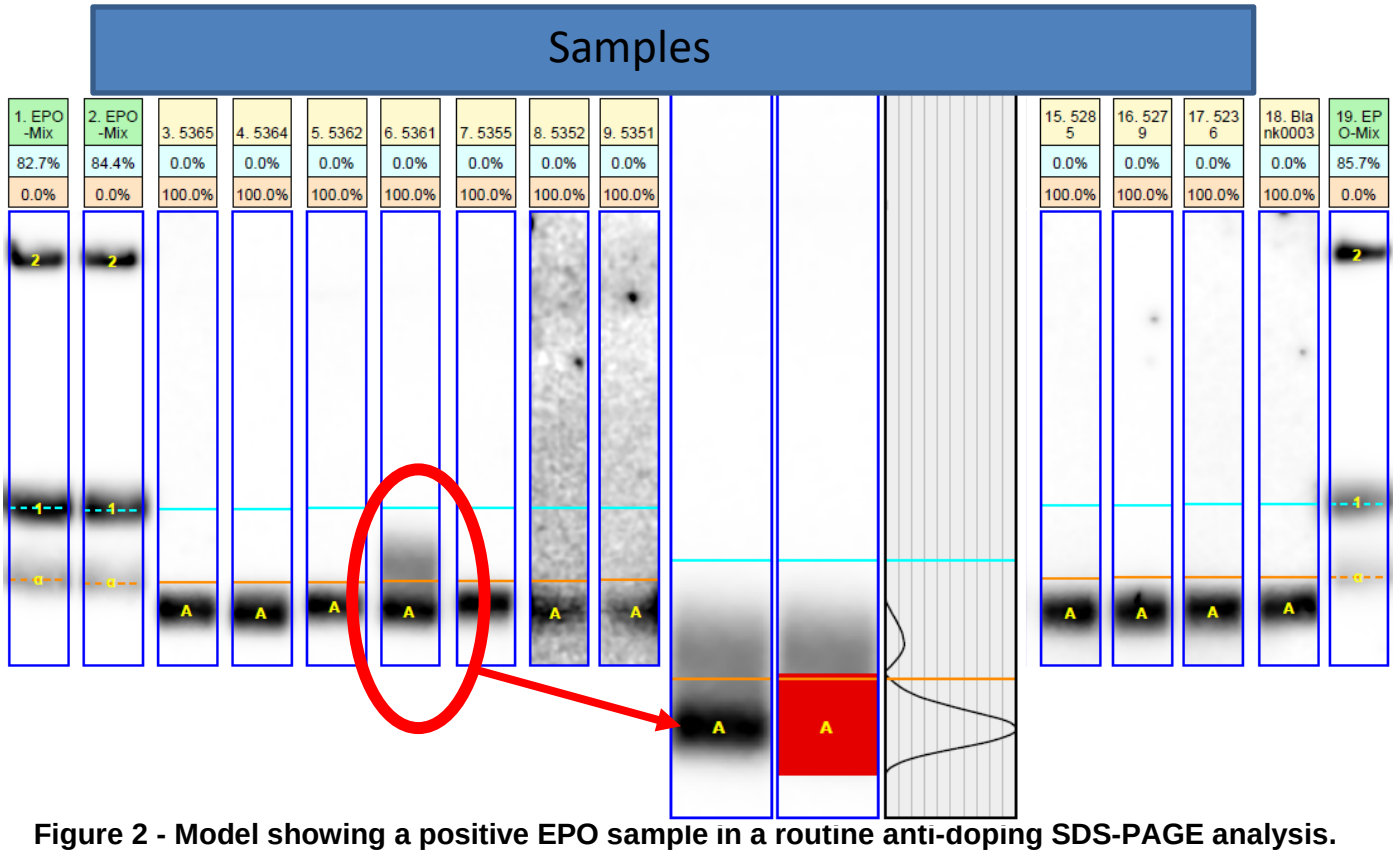


Figure 2 - Model showing a positive EPO sample in a routine anti-doping SDS-PAGE analysis.

Routine membrane, from left, lanes 1,2 and 19 represent a mixture of EPO standards (NESP (Aranesp), BRP (biological reference preparation), CERA (Mircera). All other lanes contain a selection of urine samples being tested at the anti-doping lab, including a negative urinary quality control (Blank 0003). Anti-Doping testing for rhEPO is routinely performed by gel electrophoresis followed by western blot analysis using a primary monoclonal mouse anti-human EPO antibody and a secondary anti-mouse immunoglobulin antibody conjugated to horse radish peroxidase (HRP) directed against the primary antibody to enable immunodetection by chemiluminescence. This electrophoretic analysis of glycosylated pattern of EPO in urine is a two-step procedure, that adds more than 24 hours to the testing time of a purified urine sample. Primary antibodies despite being selected for minimal cross reactivity, can potentially cross-react with Ab related fragments that increase during the immunopurification step of the urine sample preparation. Also, operator experience, with regards to interpretation of cases that are not clear, is especially worrisome when trying to achieve reproducible results when screening confirmation is solely dependent on the amount of recombinant EPO present in body fluids. In some cases, the band might be less intense or apparent, almost smear-like, and this needs confirmation, where results are subjected to a second blot (double blot) to reduce potential cross-reacting species, to help improve interpretation. Even though the method is specific and robust and has been validated for potential confounders it is a continuously evolving protocol with much room for improvement. This confirms the need for a novel approach to develop better antibodies that have the greater specificity and sensitivity, able to detect and measure rhEPO solely, without the cross recognition of endogenous EPO (Adapted from Anti-Doping Lab Qatar).

1.8 Antibodies

Antibodies are immunoglobulins (Ig) that are Y-shaped specialized molecules that are synthesized by the body's immune system in the presence of foreign substances, such as microorganisms, viruses or toxins, as well as any substances recognized as non-self or 'foreign'. Each Y-shaped protein is designed specifically for a particular epitope that is found on a particular antigen site. The part to which an Ig binds and fits into an epitope is termed as a paratope. This is referred to as a lock and key mechanism during the binding process with an antigen. Once an immune response is provoked, these glycoproteins are secreted by immune plasma B cells and bind to the designated antigen to inactivate it. Each antibody is highly specialized to recognize just one kind of immunogen (72). Immunoglobulins are generated by B cells and can be found on the cell surface, as B cell receptors, or secreted into the extracellular environment where it circulates as antibodies in the blood. Antibodies are vital elements of the adaptive immune response and are a highly specific defense mechanism. When naïve B cells come into contact with an antigen, they convert into memory B cells or antibody-secreting plasma cells. Memory B lymphocytes retain memory of specific antigens and upon future exposure can convert into plasma cells providing quicker immune responses against re-encounter of foreign molecules (73).

1.8.1 The biological properties of Immunoglobulins

Every mammalian species has several classes and subclasses of immunoglobulins (Igs). The differences between each immunoglobulin class, and among some of the subclasses, depends upon the Fc portion of the heavy chain, which has no association with the combining site (74). These heterodimeric proteins are made up of two heavy (H) and two light chains (L). Each component of the L chain contains one NH2-terminal variable domain, and one or more COOH-constant (C) domains each of which are comprised of two sandwiched β-pleated sheets that are held together via disulfide bridges between two cysteine residues (75). Each V or C domain vary in molecular mass averaging between 12,000-13,000 KDa and consists of about 110-130 amino acids. Ig H chains holds three or four constant domains, whereas both Ig L chains consist of only one C domain. A small spacer called a *hinge* region is included between the three constant domains of the H chains between the first (CH1) and the second (CH2) domains (76). The way scientists studied Ig structure was by fragmenting IgG molecules using specific enzymes. Antibody fragmentation is achieved by using reducing agents and proteases that digest or cleave selectively specific portions of the IgG protein structure. The two main antibody portions of interest are antigen binding fragments, Fab, and class defining fragments, Fc, that do not bind antigen, but anchor to the cell membrane. Digestion by the enzyme pepsin usually produces one F(ab')2 fragment and numerous small peptides of the small Fc portion, whilst digestion by papain yields a split of one or more peptide bonds in the hinge region resulting in two Fab fragment and one Fc fragment of similar size (77). Once the IgG is digested into two Fab fragments, each one can bind with an antigen and a single Fc fragment. IgG splits into an Fc fragment and a single Fab that can bind to antigens and cross-link. The Fab comprises one complete L chain in its entirety and the V and CH1 portion of one H chain (Figure 3). Fab fragments can be split into a constant fragment (Fb) composed of the CL and

CH1 domains, and a variable fragment (Fv) made up of the VH and VL domains. Furthermore, genetically enhancing the single Fv fragments leads to recapitulating the monovalent antigen binding characteristics of the original, parent antibody (75). IgGs are the most abundant immunoglobulin class that are allocated in the blood and tissue fluids. After the onset infection or artificial immunization, with time, IgG antibodies of increasingly higher avidity are produced and detected in the systemic circulation.

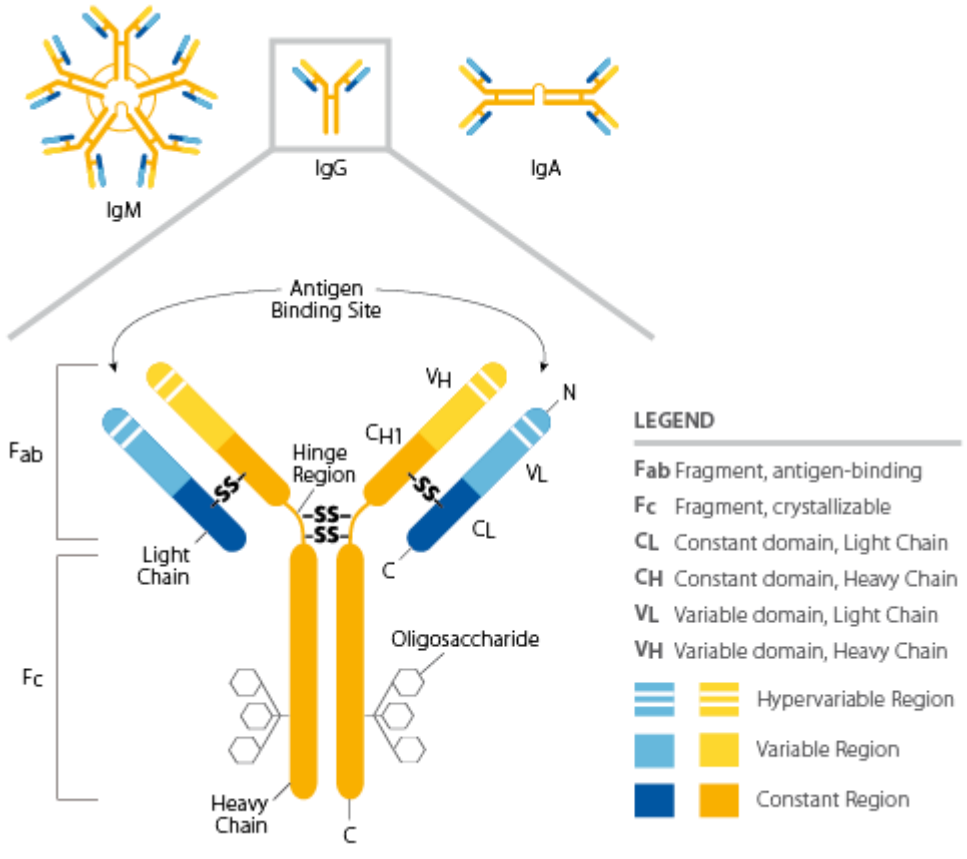


Figure 3 - Schematic design of an IgG immunoglobulin structure

Design of a full-size antibody molecule including various antibody fragment types. The IgG is comprised of a constant heavy chain, CH, a constant light chain, CL, an antigen binding fragment, Fab, a single chain variable fragment, scFv, a variable heavy chain, V_H, and a variable light chain, V_L. Useful antibody fragments can be produced by reducing the hinge disulfides region or digestion with papain, pepsin or ficin proteolytic enzymes. Some of the advantages of antibody fragmentation includes the reduction of nonspecific binding from Fc interactions, the ability to control Fc-binding in experiments, having a higher tissue penetration efficiency, lower immunogenicity for *in vivo* experiments, and higher sensitivity in antigen detection due to the reduced steric hindrance from large protein epitopes (adapted from 77).

1.8.2 Antibodies as diagnostic tools

In 1959, the very first demonstration of immunodetection was completed by Berson and Yalow; who then established the first radioimmunoassay for human insulin by using extracted guinea pigs antiserum. Their analytical method relied on recognizing a specific antibody and detecting trace amounts of the target analyte in complex matrices (78). Another milestone was set in 1975 when Köhler and Milstein came up with the hybridoma technology that enabled the production of monoclonal antibodies on a large scale and preferred specificity (79). Even though both achievements were never patented, they laid the way for the widespread use of antibodies in immunodetection assays. Different sites for antibody discovery with improved selectivity and new engineering potentials were added as molecular biology progressed along with the genetics of antibody diversity (80). Therapeutic application of antibodies has flourished and progressed, and technology has offered new ways to create new assays and biosensors since recombinant antibody fragments can be tagged and reproduced easily more readily (81). As the use of conventional antibodies has been dominated by this progress, more recent findings shed light on a salient alternative for immunosensing of the recombinant binding domain of a special type of antibody found in camels and llamas which lack light chain. Camels and llamas differ from other mammalian species by having conventional antibodies and single-domain antibodies (82). These “heavy-chain-only” antibodies (HcAbs) or camelid antibodies, both stand out as a unique tools for both diagnostic and therapeutic purposes.

1.8.3 Camelids

Conventional antibodies are made up of four polypeptide units containing two heavy chains and two light chains bound together via disulfide bonds. Each polypeptide chain has a constant region and a variable region that is specific to each antibody. Thereafter, heavy-chain-only antibodies (HCAbs) or single domain antibodies (sdAbs) consists of only two heavy chains where the binding domains constitute paired variable regions lacking the two light chains. Part of the immune response of camels is based on these HCAbs and are thought to represent 50-80% of their antibody repertoire (83). These antibodies can exhibit a broad antigen-binding repertoire by enlarging their hypervariable regions (Figure 4).

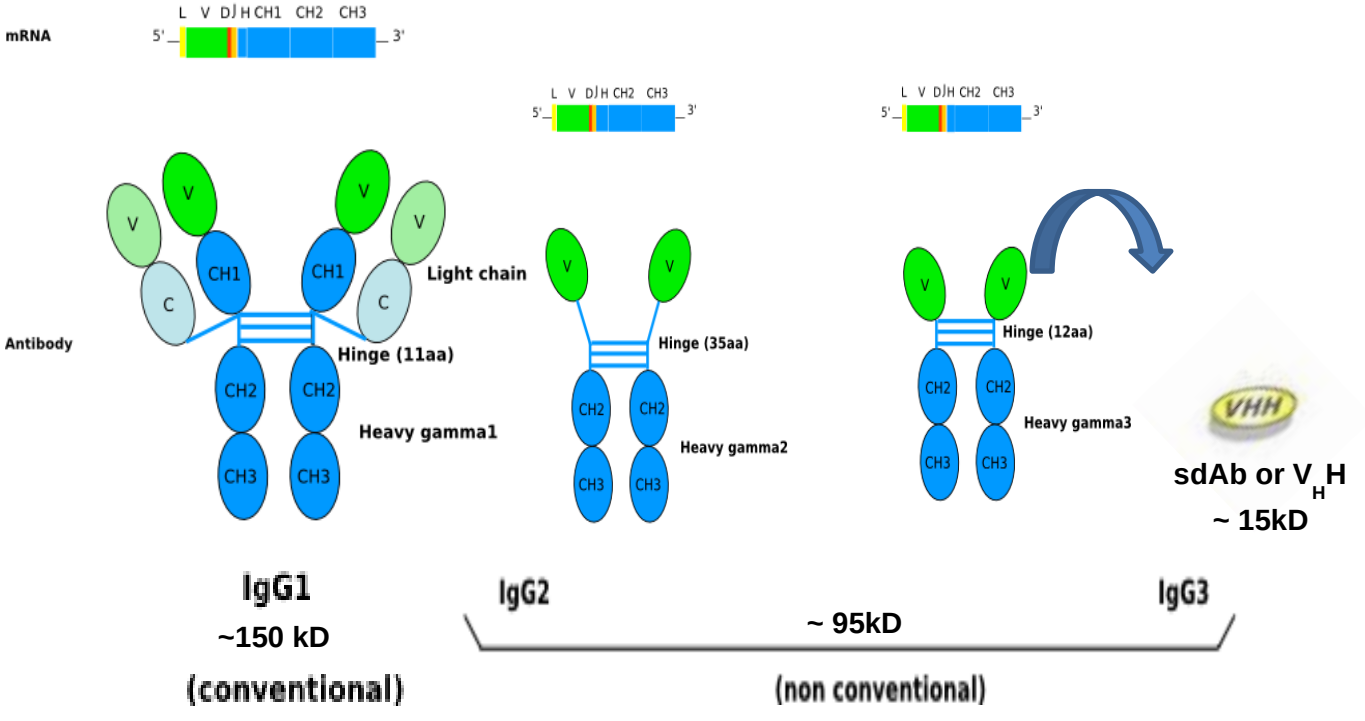


Figure 4 - Repertoire of camel antibodies

A schematic representation of the camel's antibody repertoire is shown. It consists of 2 heavy chains and 2 light chains bound together with disulphide bonds in a Y shape (IgG_1), as well as heavy-chain-only antibodies (HcAbs). These HcAbs consist of two heavy chains where the binding domains constitute paired variable regions lacking two light chains, for that reason they also lack the CH1 region but still bear an antigen binding domain at their N-terminus. Conventional Ig require the association of variable regions from both heavy and light chains to allow a high diversity of antigen-antibody interactions. The unique feature of HcIgG is the capacity of their monomeric antigen binding regions to bind antigens with specificity, affinity, and diversity, comparable to conventional antibodies. This feature is mainly due to a major variation within the amino acid sequence of the variable region of the two heavy chains, which induce deep conformational changes when compared to conventional Ig. These antibodies can exhibit a broad antigen binding repertoire by enlarging their hypervariable regions, that are called single domain Ab or V_HH, and have many advantages such good tissue penetration and highly specific, as they have high affinity for only one cognate target against a range of diverse epitopes. Their smaller antigen binding site contributes to maximized complementarity (adapted from the IMGT Biotechnology).

Camelids are able to produce functional IgG that fold autonomously and are known to be the smallest recombinant antigen-binding domain produced around ≈ 15 kDa. The sdAbs are structured in four conserved regions, referred as framework regions (FRs), and only three complementary determining regions (CDRs) are responsible for the antigen recognition site (84). Due to their intrinsic characteristics, the use of sdAbs exhibit several advantages. Their small size allows for good tissue penetration *in vivo*, and are highly specific as they have high affinity for only one cognate target against wide-ranging structurally diverse epitopes (85). Also, due to the antigen binding sites being smaller than conventional antibodies, the complementarity is maximized due to the physical-chemical properties of the V_HH as the reduced paratope and flexibility of the CDR3 makes them mainly capable of binding concave and hidden epitopes. This unique reactivity in their binding capabilities makes the recombinant form of their variable domain much easier to produce and handle in comparison to conventional antibodies (86). Moreover, it extends their analytical and diagnostic performances, and it has been considered to have great potential in various industrial applications (87). They are considered to have thermal stability and high solubility in comparison to most mice and human derived antibody fragments and can be easily expressed using bacteria or yeasts (88). Similarly, they have been utilized in several assays and protein crystallization in structural studies due to their advantageous biochemical and economic properties (89). Importantly, V_HH fragments have been developed to be great diagnostic tools and novel therapeutic agents for several infectious diseases and cancers (90).

These attributes have led to recent developments in the use of these camelid antibodies in different fields, including immunosensing applications. Several studies have shown they have a great versatility as affinity building block, and easy adaptation to screening and immune specificity in display libraries (91). Thus, this difference in unique biological properties that camelids possess with their antigenicity, size, specificity, and affinity could be the edge needed to produce a very different assay for erythropoietin, one that has not been produced previously.

Heavy chain antibodies can be formed in animals belonging to all species of the Camelidae family. Due to their smaller size, llamas are easier to raise and breed, but camels have a higher ratio of heavy chain antibodies to regular antibodies. Camel V_HHs have previously been acquired from libraries prepared from RNA of immunized animals (92), which is the protocol expected to be followed for this current project. Animals were immunized with 1 mg or less of antigen, and in previous reports several different antigens have been injected simultaneously. Anti-sera titers were monitored, mostly starting from about five weeks after immunization. Complete Freund adjuvant has been used for the first injection and incomplete Freund adjuvant for the following injections. Lymphocytes, that are the source of mRNA, were extracted from peripheral blood. Although V_HH libraries have been prepared by grafting randomized CDR regions into heavy chain scaffolds, V_HHs that are matured in vivo are found to be more stable and have a higher affinity in comparison than those from non-immune or synthetic pools. The V_HHs libraries obtained were selected for

binding to the selected antigens. Solid-phase phage display is the most frequently used method in which amplified cDNAs encoding variable regions were expressed on the surface of filamentous phages. To isolate and clone the high affinity ligands presenting variable regions that bind to the antigen a procedure called panning was used in repeated rounds.

The method or technique for selecting novel protein and producing a camel antibody library is by phage display (phage, bacteria, yeast, mammalian cell and ribosome). The three main steps included in this technique are as follows: 1) construction of the antibody library and display onto phage surface; 2) several rounds of selection by means of panning against target antigen (Ag), and 3) screening till the preferred specificity is obtained. The antibody library construction is conducted from diverse immunoglobulin-variable-region gene segments amplified from camel B cells of immune or non-immune sources. After, the library is cloned for display on the surface of the phage. Selection is performed against targeted antigen using the phage display, and antibodies that are not of interest and do not bind are washed away. Moreover, binders are eluted and amplified via infection of the bacteria *Escherichia coli*. The desired specificity is reached once several rounds of selection is completed using enzyme-linked immunosorbent assay (ELISA) or methods such as fluorescent-activated cell sorting (FACS) if the target is a cell-membrane bound protein. Furthermore, the genes of antibody variable regions can be cloned into whole human IgG expression vectors and transfected into cell lines to produce fully human mAbs once preferred specificity is attained (93). V_HHs have some distinctive advantages compared to conventional antibodies, combining the targeted features of mAbs with some other beneficial properties of small molecule drugs. Some of these advantages include: higher affinity; possibility of formatting or multimerization; higher stability and solubility; deeper fat tissue penetration and rapid blood clearance; recognition of hidden epitopes; immunogenicity and less production costs (94).

1.9 Hypothesis & Rationale

Raising, identifying and characterizing camelid HCAbs and V_HH fragments directed against rhEPO provides a potentially useful analytical tool for specific, high affinity, immunoassay, without cross reactivity to the endogenous species.

1.9.1 Aims and objectives

1. Immunization of camel(s) with commercially available, therapeutic forms of recombinant EPO to raise polyclonal antibodies directed against rhEPO.
2. Purification and characterization of the rhEPO specific, polyclonal HCAbs.
3. Utilizing the polyclonal HCAbs to develop a specific and sensitive assay for rhEPO, without cross reaction to the endogenous form.
4. Application of the developed assay to detect rhEPO in a human urine sample from a volunteer, before and after injection of rhEPO,
5. Production of rhEPO specific single domain antibody V_HH fragments using phage display technique.

CHAPTER 2
MATERIALS AND METHODS

2.1 Study Plan and Design

The following schema outlines the methodological steps undertaken during this research project.

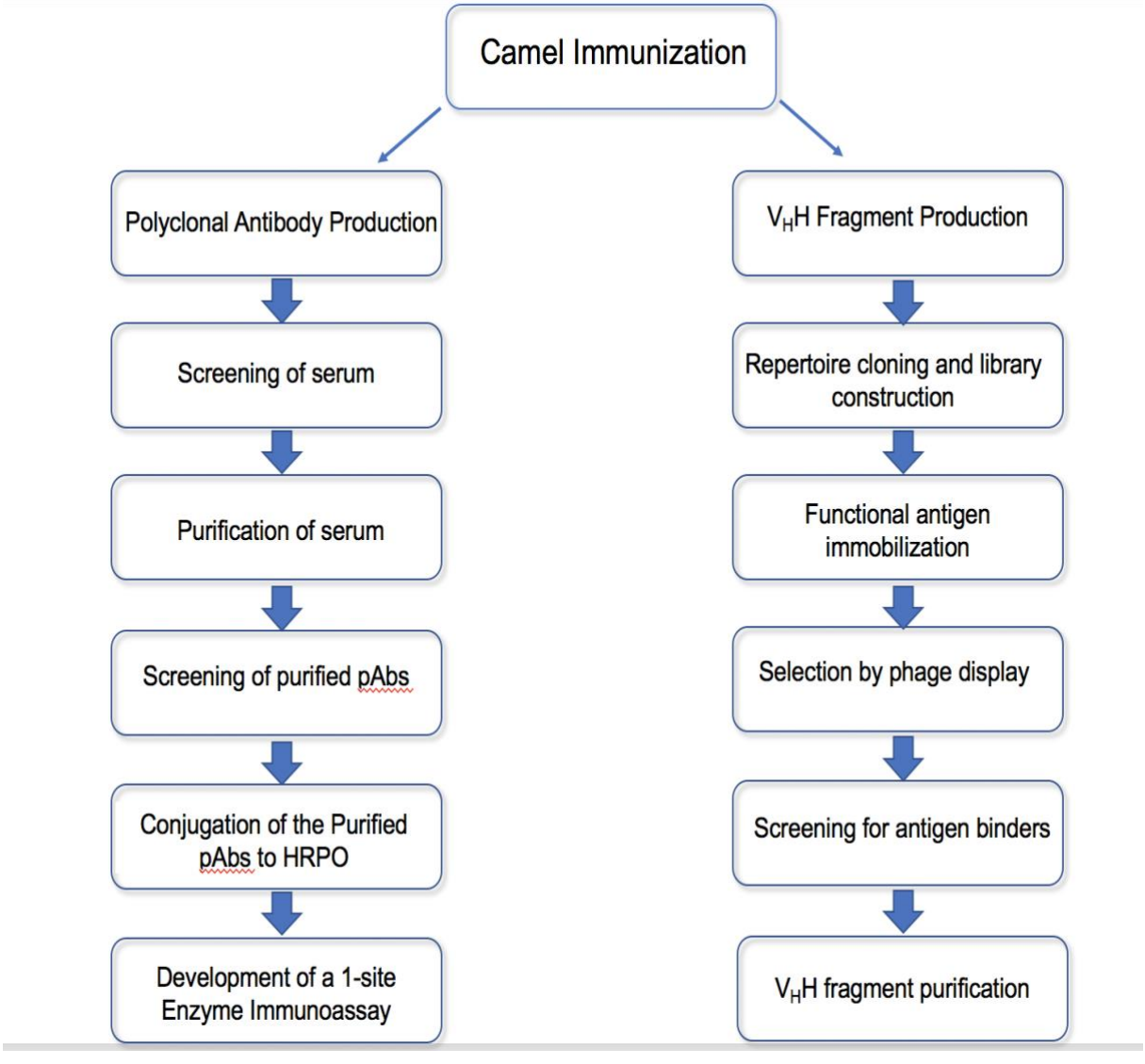


Figure 5 - Experimental schema of the methods carried out in this project for raising Camelid antibodies and the generation of V_HH fragment.

2.2 Ethical Approval and Sample Collection

The process of obtaining ethical approval for the research started a few years prior to the receipt of the offer for the MRes by the University of Bristol in September 2017. This was particularly challenging as there was no appropriate institutional review committee that could deal with questions related to research in camels. A special application was submitted to the Ministry of Public Health (MoPH), Doha, Qatar, in December 2015. All attempts were made to ensure that the rights of the camels involved in the research were protected and they were not subjected to any undue suffering. A state registered veterinarian was appointed to help with animal care. The guidelines and policies provided by the MoPH were followed during the application process, and official documentation was produced. This process took three months of preparation and submission. The first application covered the period January 2016 – May 2018, which covered the period of this project. This was then renewed until May 2020. See Appendix I and II for the letters of approval from the MoPH. All experiments were conducted in compliance with their recommendations.

For the project, the following facilities were identified, and available as soon as ethical approval was obtained. A facility to raise/immunize camels was identified in the Shahaniya area, Qatar, within an already existing herd. This would minimize the trauma to the animals of being removed to alien premises. Arrangements were negotiated with a trained camel-keeper extensively experienced in looking after the experimental animals. The camels were bought from the trained camel-keepers (male and aged between 1-2 years). A maximum of 2 animals were kept at any one time. Further, the services of a veterinarian was available for the administering the injections and also for obtaining blood samples.

The procedure was very similar to that which is carried for routine vaccinations in camels and was therefore classified as a mild risk. The animals were checked weekly, routinely during the time course of the experiment. Any indication of discomfort was reported by the camel-keeper to the vet. All necessary medical care was administered if thought appropriate, whether as a direct cause of the immunization or not. In the very unusual circumstances that anesthesia was to be used, it would have been either:

A. Xylazine Hcl 0.25 - 0.5 mg / kg IM

B. Ketamine Hcl 5 mg / kg + Xylazine Hcl 0.25 mg / kg IMAT

The end of the experimental period animals were kept for at least 3 months, testing that they were free of the immunogen in the circulation, with at least monthly observation by the vet until declared free for sale.

2.3 Materials

2.3.1. Reagents

The reagents used in this project and the companies from which they were sourced are outlined in the following table:

Table 1 - A list of reagents and purchasing information

Reagent	Purchasing
Epofit	Pharmaceutical Companies
Epoetin α	India/Greece
Epoetin β	
NeoRecormon	
4000 IU syringes	
5000 IU syringes	
Complete Freund's Adjuvant	Sigma-Aldrich (Germany)
Incomplete Freund's Adjuvant	
Paxgene Blood RNA kit	SABiosciences-Qiagen, (Germany)
BD Vacutainer K2E (EDTA)	BD (UK)
PAXgene® Blood RNA tubes	
High-Capacity RNA-cDNA reverse transcription kit	ThermoFisher Scientific (UK)
Nunc Microwell 96-Well Microplates	
Enzyme linked Plus Activated Peroxidase	
Goat Anti-Camel IgG-HRP Conjugate secondary antibody	Alpha Diagnostic
Hi Trap® Protein G HP 1 mL antibody purification columns	GE Healthcare (UK)
RNA chips for Agilent 2100 Bioanalyzer	Agilent
QIAquick gel extraction kit	Invitrogen
Quick Start Bradford Protein Assay	Biorad (U.S)

2.3.2. Solutions

The solutions required to carry out this study and their protocols are described below in Table 2.

Table 2 - Solutions and Protocols

Solutions	Protocol
Coating Buffer (CB)	1.325g of Na2CO3;1.05g of NaHCO3 pH to 9.6 in 1L of distilled water
Blocking Solution (BS)	Phosphate Buffered Saline (PBS) containing 1% (w/v) BSA
Washing Buffer (WB)	
Substrate Buffer (SB)	0.05M Phosphate-citrate buffer, pH 5.0: (2.82g of dibasic sodium phosphate (0.2M) in 100 ml distilled water) (1.92g of citric acid (anhydrous) (0.1M) in 00ml distilled water) 25.7ml of the 0.2M dibasic sodium phosphate, 24.3 ml of 0.1M citric acid and 50mL distilled water
3,3',5,5'-Tetramethylbenzidine (TMB) substrate solution	pH 5.0 (5mg of TMB in 5ml of DMSO and 45ml of 0.05M phosphate-citrate buffer). Immediately prior to use, 2µl of 30% hydrogen peroxide per 10ml of TMB substrate buffer solution was added.
Primary antibody	100 µl of the camel anti-sera diluted in wash buffer
Secondary antibody	100 µl Mouse anti-camel HRPO - in WB at 1:5000 dilution
Binding Buffer	
Start Buffer	
Elution Buffer	
Neutralizing Buffer	
De-staining Solution	100mL of 100% methanol, 100mL of glacial acetic acid, 800mL of distilled water
2x SDS gel Loading buffer	2 mL Tris (1M pH 6.8), 4.6 mL glycerol (50% v/v), 1.6mL SDS (10% w/v), 0.4 mL Bromophenol blue (0.5% w/v), 0.4 mL β-mercaptoethanol, 1.4 mL distilled water
Running Buffer	0.3g ± 10% EDTA, 1g ± 10% N-Lauroylsarcosin, 6.06 ± 1% Trizma-Base, 0.46g ± 1% MOPS in 1L of distilled water
Transfer Buffer	0.0375g ± 10% N-Lauroylsarcosin, ±1% Glycin, 5.81g ±1% Trizma-Base in 1L of distilled water
Blocking Buffer	5g of ± 2% low fat milk in 200 mL of water

Phosphate-buffered Saline	5 Sigma PBS tablets in 1L of distilled water

2.4 Methods

2.4.1 Polyclonal Antibody production

2.4.1.2 Immunization Outline and Immunogen Detail

Pre-immunization bleeding and a primary immunization with recombinant Epoetin β and α; 450µL of Epoetin β (7500 IU; 41.5 µg/0.3 mL) was mixed with 450µL of Complete Freund's Adjuvant for a total injection volume of 900µL, and 2 mL of Epoetin α (8000 IU; 33.6 µg/mL) was mixed with 2 mL of Complete Freund's Adjuvant for a total injection volume of 4mL.

Post-immunization bleeding and a 2nd immunization was completed after 4 weeks with recombinant Epoetin β and α; 450µL of Epoetin β (7500 IU; 41.5 µg/0.3 mL) was mixed with 450µL of Incomplete Freund's Adjuvant for a total injection volume of 900µL, and 2 mL of Epoetin α (8000 IU; 33.6 µg/mL) was mixed with 2 mL of Incomplete Freund's Adjuvant for a total injection volume of 4mL.

Post-immunization bleeding and a 3rd immunization was completed after 4 weeks with recombinant Epoetin β and α; 450µL of Epoetin β (7500 IU; 41.5 µg/0.3 mL) was mixed with 450µL of Incomplete Freund's Adjuvant for a total injection volume of 900µL, and 2 mL of Epoetin α (8000 IU; 33.6 µg/mL) was mixed with 2 mL of Incomplete Freund's Adjuvant for a total injection volume of 4mL. The immunization schedule is shown in Figure 6.

2.4.1.3 Lymphocyte Preparation

Post immunization bleeding were collected from the jugular vein in PAXgene blood RNA collection tubes. The blood samples were inverted several times to inhibit coagulation and transferred to the lab on ice to be processed immediately.

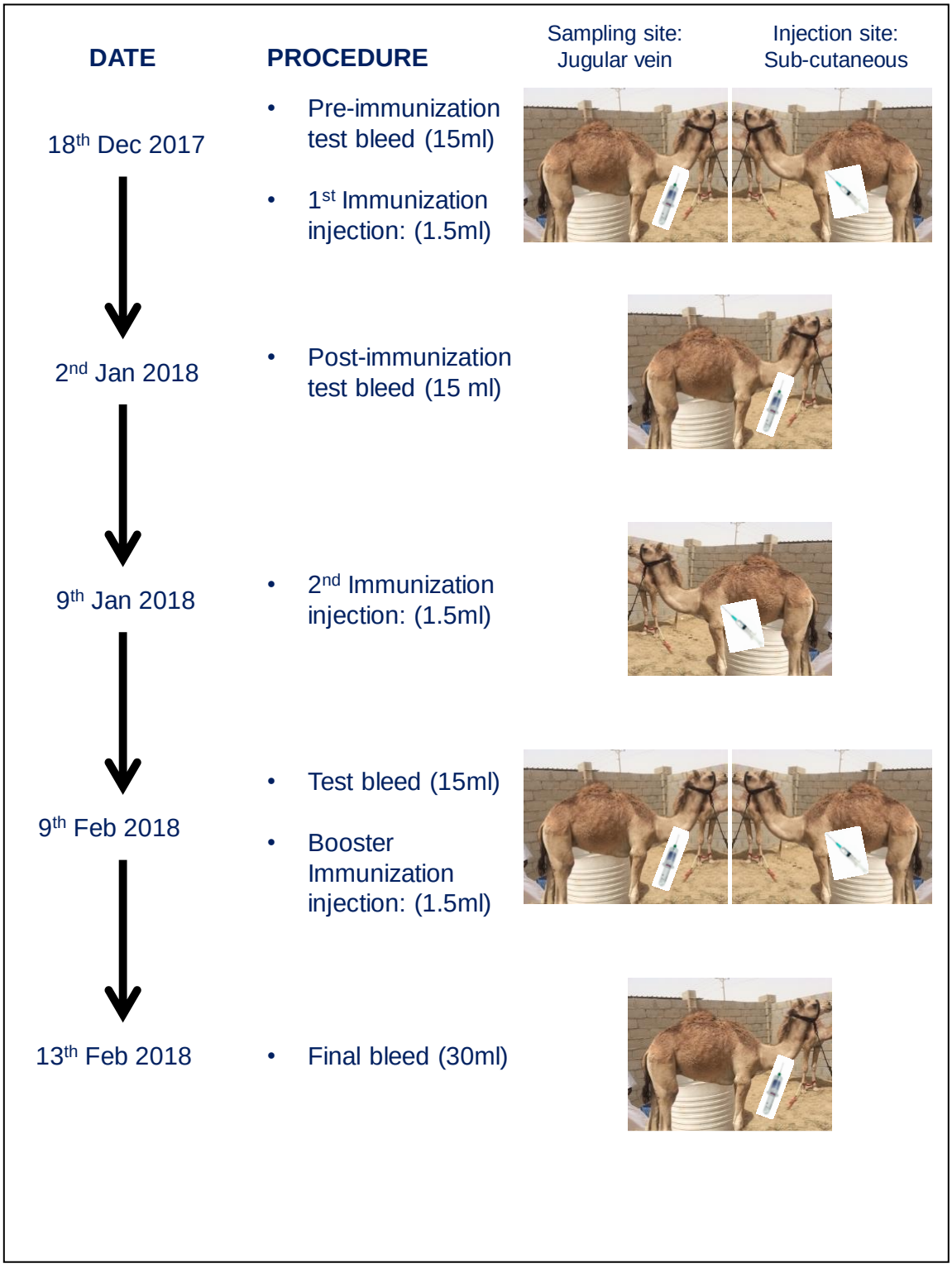


Figure 6 - Schedule of immunization of camels for antibody production.

2.4.1.4 Screening of Purified Polyclonal Antibodies via Enzyme-Linked Immunosorbent Assay

In order to determine if the immune response induced by the antigen was EPO-specific, titers of the pre-immune and post-immune serum were analyzed and compared quantitatively via an enzyme linked immunosorbent assay (ELISA). Microtiter plates were coated with 100 µl of a 2 µg/ml antigen solution of Epoetin β and α, mixed with coating buffer (CB). Plates were incubated overnight at 4°C. The next day, the plates were washed 3 times with 250 µl of wash buffer (WB), 100 µl of blocking solution (BS) was added and incubated for 1 hour at 37°C. The plates were washed 4 times in WB, and 100 µl of the primary antibody solution was added. This was incubated for 1 hour at 37°C. The plates were then washed 3 times in WB, and 100 µl secondary antibody was added to each well. The plates were then incubated for a further 1 hour at 37°C. After this, the plates were then washed 3 times in WB and 100 µl of substrate buffer (SB) was added to each well. This was incubated at room temperature, in the dark, for 30 minutes. After incubation, 50 µl of 2M H_2SO_4 was added to stop the reaction. Thorough mixing was ensured and the absorbance within 30 minutes was measured at an optical density (OD) of 450nm.

2.4.1.5 Purification of serum using HiTrap® Protein G column by Affinity Chromatography

Post Immune serum with good titers (OD 450nm > 0.10), were centrifuged at 5000 rpm to remove cellular debris, and supernatant was collected in a new tube. In order to adjust the serum sample to the composition of the binding buffer, serum was diluted in binding buffer at a 1:1 ratio. The column was equilibrated with 5 mL of start buffer at a flow rate of 0.5 mL/min (60 cm/hr). Serum sample was applied using a syringe attached to a Luer connector at a 500 µl volume, and fractions were collected in 1 mL total volume. The column was then washed with 3 mL of start buffer whilst collecting unbound protein or “breakthrough”. After, the protein (IgG) was eluted with the elution buffer in a total of 3 mL volume. The second sharp peak was collected in 1 mL fraction containing the IgG, neutralized with 50 µl neutralizing buffer and stored at -20˚C for further analysis. The breakthrough samples were also collected and retained at 4˚C.

2.4.1.6 Detection of Purified Antibodies by Coomassie Blue Staining and Western Blotting

Two 10 % sodium dodecyl sulphate (SDS) polyacrylamide gels were prepared. 15 µL of each purified serum and breakthrough samples were mixed with 15 µL of 2x SDS gel loading buffer. This was heat shocked at 100˚C for 10 min. 20µL of the reduced samples were loaded on the gel and run at 130 mV for a total of 1 hour. One gel was stained with Coomassie blue staining for 2-4 hours and de-stained using the de-staining solution for 2 and half hours. The second gel was transferred to a polyvinylidene difluoride membrane (PVDF) for 1 hour and 30 minutes. Prior to transfer the PVDF membrane, this was activated by soaking it in 100% methanol for 30 seconds, and then equilibrated in transfer buffer for 2 minutes. The membrane was blocked on shaker in blocking buffer (BB) for 1 hour and washed with Phosphate buffered saline (PBS) a total of 5 times on shaker. The membrane was incubated in a 1:5000 dilution of primary Antibody-Mouse anti-Camel HRPO in 2% low fat milk for two hours. This was then washed in PBS 4 times on shaker. The membrane

was developed with 1.5 mL stable peroxide buffer and 1.5 mL Luminol enhancer, provided from the Pierce Signal West Femto kit. The image was captured using the ImageQuant LAS 4000 Camera.

2.4.1.7 Statistical Analysis

The software used to analyse the data were the Statistical Package for the Social Sciences (SPSS, version 25.0) or GraphPad prism (version 6.02, GraphPad Inc. La Jolla, Ca. USA). Data are expressed as mean ± SD (or SEM) for normally distributed data and median (interquartile range, IQR) for skewed data. Antigen titre response curves were constructed on a log scale and data fitted as a histogram fitting routine in Prism. Significant effects were determined using paired and unpaired t-test or two-way ANOVA (with post-hoc correction) as appropriate. Significance was defined as $p<0.05$. and "n" = number of samples or experiments.

2.4.2 V_HH Fragment Production

2.4.2.1 Isolation of peripheral blood lymphocytes

For the collection of pre-immunized camel's blood, PAXgene blood RNA tubes were used. Separation of lymphocytes from erythrocytes was completed using the Paxgene Blood RNA Kit by QIAGEN for PreAnalytiX, following the manufacturer's guidelines. Prior to extraction and centrifugation, the blood samples needed to be at room temperature. Centrifugation conducted at 2,000 rpm, 20 ℃ without any breaks for a period of 30 minutes. Once done, the concentration and purity of the isolated RNA were assessed using a NanoDrop 2000 Spectrophotometer with measuring OD at 260 nm (OD 260) and 280 nm (OD 280).The purity of the extracted RNA was evaluated as a ratio of OD260:OD280 (A_{260}/A_{280} ratio), in which a value of 2 indicated pure RNA, and a reading between 1.7 and 2.0 is considered acceptable purity. The isolated RNA was further analyzed for sizing, quality and quantification control using the Agilent 2100 Bioanalyzer. The electro kinetic forces moving in pico-liter volumes through the

channels of a polymer chip, are the way RNA integrity number (RIN) are determined. The data was presented in high quality digital gel-like image, electropherogram. A RIN ≥ 5-6 was considered to be of acceptable purity.

2.4.2.2 Construction of phage display library

To generate the phage display library, lymphocytes from the rhEPO immunized camels were used to synthesize single-stranded cDNA from the mRNA transcripts using the High-Capacity cDNA Reverse Transcription (RT) Kit, according to the manufacturer's instructions. 10ul of RNA was added to 10ul of 2X RT Master Mix. The protocol for cDNA synthesis is illustrated in the following figure 7.

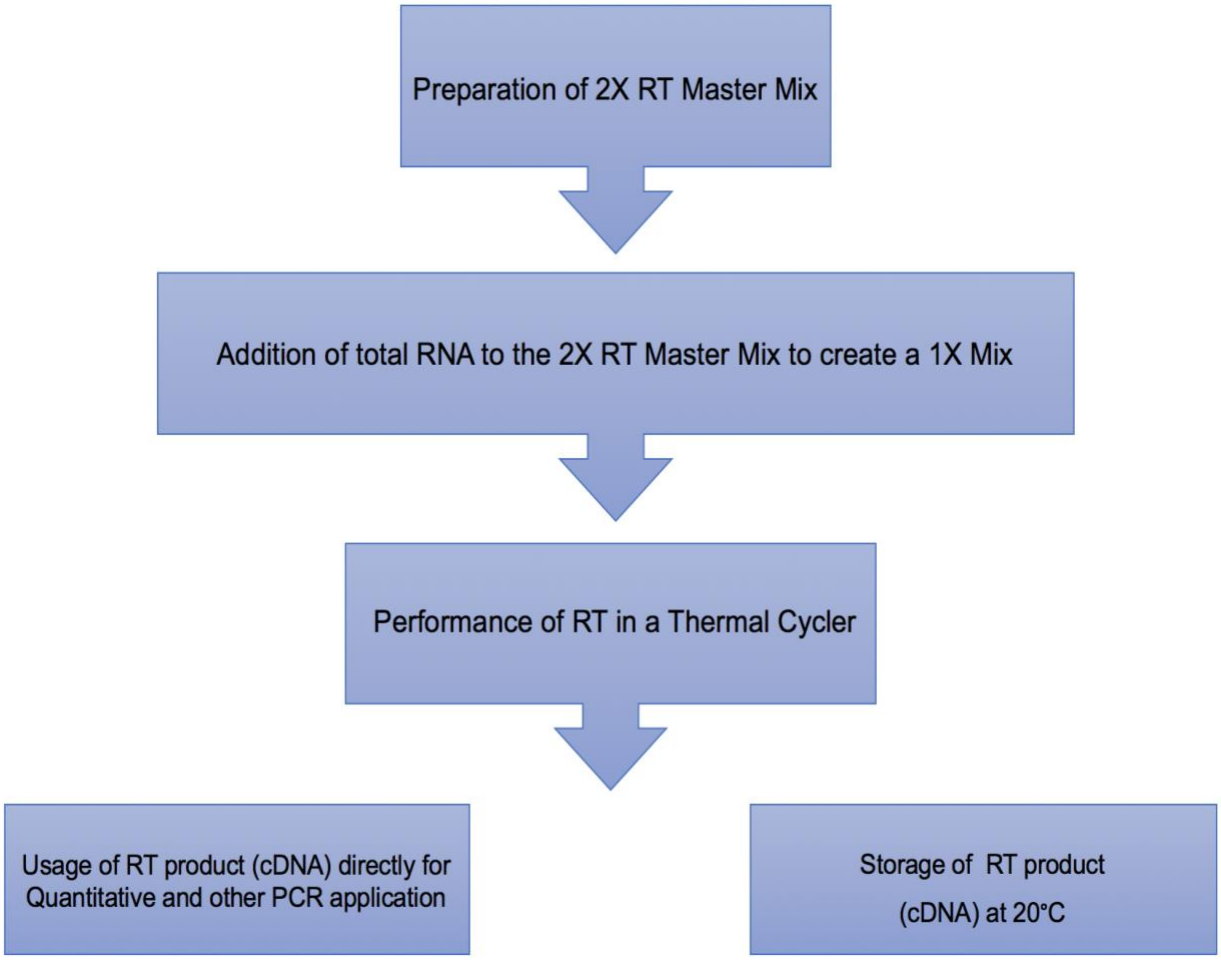


Figure 7 - Schematic design of the first strand cDNA synthesis

The PCR reaction mixture is outlined in Table 3, using the High Capacity cDNA RT Kit without RNase inhibitor.

Table 3 - Polymerase Chain Reaction (PCR) mix for cDNA conversion

Component	Volume/Reaction (µL)
10X RT Buffer	2.0
25× dNTP Mix (100 mM)	0.8
10× RT Random Primers	2.0
MultiScribe™ Reverse Transcriptase	1.0
Nuclease-free H_2O	4.2
Total per Reaction	**10.0**

The reaction mixture was then placed in a thermal cycler for amplification, using the conditions described in the table below:

Table 4 - PCR Conditions for Reverse Transcription

	Step 1	Step 2	Step 3	*Step 4*
Temperature (°C)	25	37	85	*4*
Time (min)	**10**	**120**	**5**	**∞**

2.4.2.3 Amplification of the variable domains

The camel VH and V_HH regions were amplified from the cDNA using two gene-specific nested primers. In the first PCR, the forward primer CALL001 anneals to framework 1 region of the camel VH and V_HH while the reverse primer CALLL002 anneals to the CH2 region. These primers amplify fragments between 650-900 base pairs, that corresponds to the homodimer heavy chain immunoglobulins IgG3, IgG2 respectively with short and long hinge regions as well as the heavy chain heterotetrametric immunoglobulins IgG1. The reaction mixture for this is described in Table 5.

Table 5 - PCR mix for Amplification of camel VH and V_HH Genes

PCR Mix Preparation

Master Mix	25 µL
CALL001	0.4 µL
CALL002	0.4 µL
cDNA	1.0 µL
H20	23.2 µL
Total Volume	**50 µL**

The thermo cycling parameters used to amplify the camel VH and V_HH genes are described in Table 6.

Table 6 - PCR conditions for amplification

	Step 1	Step 2			Step 3	Step 4
	1 Cycle	2-31 Cycles			32 Cycles	
Temperature (°C)	95	94	57	72	72	4
Time	**3min**	**1min**	**1min**	**45 sec**	**7**	**∞**

The PCR products corresponding to the two VH and V_HH fragments, represented by the ~700 bp bands, were analyzed on a 1 % agarose gel electrophoresis, cut out and purified using the QIAquick gel extraction kit according to the manufacturer's instructions. This was used as template for second round of PCR. The extracted gel can be stored at -20 ˚C for weeks without losing diversity. For the second PCR, the forward (sense) primers annealing to the framework 1 region of the V_HH, and the reverse (anti-sense) primers annealing at the framework 4 region, were used to generate a PCR fragment of around 400 bps long. The reaction mixture involved ADL2 sense and ADLR sense primers for the forward reaction, and ADL Anti-sense with ADL2 Anti-sense as the reverse primers. This is reaction is shown in Table 7.

Table 7 - PCR Primers Reaction Mixture for Amplification of V_HH

Reaction	Components
Sense ***(Forward Reaction)***	90 µL H_2O
	5 µL ADL2 sense
	5 µL of ADLR sense
Anti-Sense ***(Reverse Reaction)***	90 µL H_2O
	5 µL ADL Anti-sense
	5 µL of ADL2 Anti-sense

The PCR reaction system is described below (Table 8) as well as the thermocycling parameters for the amplification of V_HH genes (Table 9).

Table 8 - PCR Reaction System for Amplification of V_HH

Component	Volume/Reaction (µL)
PCR Master Mix	200
Sense Reaction	3.2
Anti-Sense Reaction	3.2
Gel Purified PCR product	20
H_2O	173.2
Total Reaction Volume	**400**

Table 9 - Thermo cycling parameters and annealing temperature

	Step 1 1 Cycle	Step 2 2-16 Cycles			Step 3 17 Cycles	Step 4
Temperature (°C)	94	94	55	72	72	4
Time	**5 min**	**30 sec**	**30 sec**	**30 sec**	**7 min**	**∞**

The vector used to carry the V_HH genes was the pADL™- 22c Phagemid Vector. The following figure illustrates the main features of the vector:

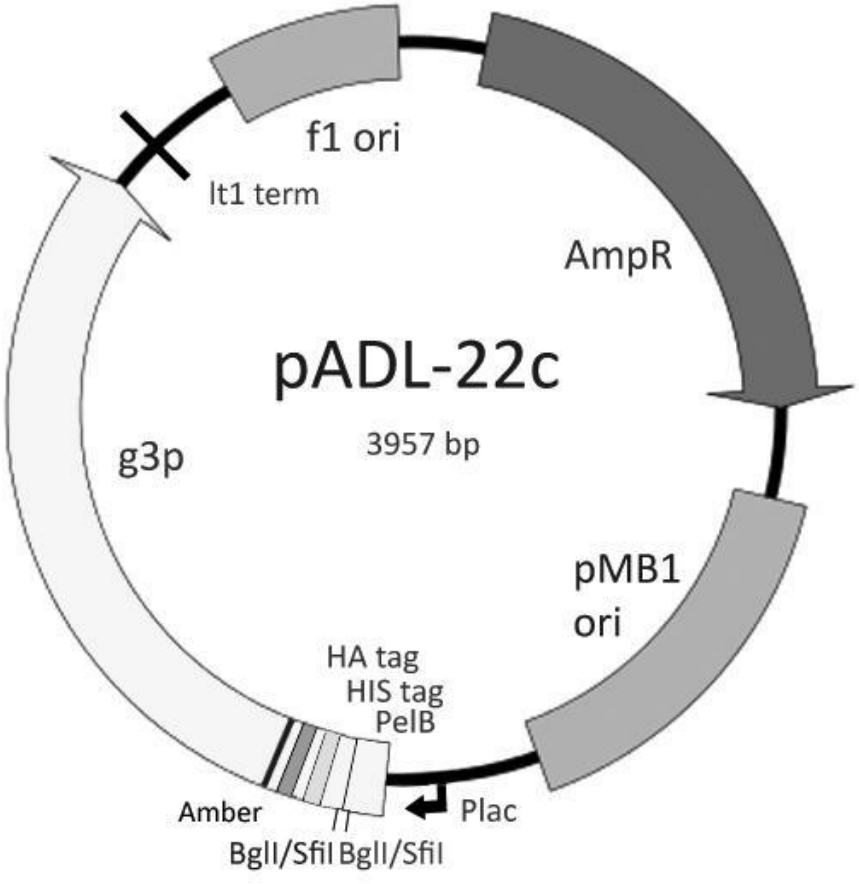


Figure 8 - Vector Map of pADL™- 22c Phagemid Vector

The figure above demonstrates the main key features of pADL^{tM} - 22c phagemid vector with a total length of 3957 bp. It is a type 3+3 phage display vector that is designed especially to be used for the display of peptides, antibodies, or proteins on the cloning site at the N-terminal side of the gene III protein of filamentous bacteriophage. A Polyhistandine (HIS) tag and a Hemagglutinin (HA) tag, followed by an amber codon are located before the gene III protein. The phage display was carried out using bacterial strains that suppress the amber codon, while growth on non-suppressive strains resulted in the expression of free single chain variable fragments (scFv) in the periplasmic space. The means of detection of this vector is its binding through the HA tag and production of free scFvs (Adapted from Antibody Design Laboratories manual)

2.4.2.4 Restriction Enzyme digestion, DNA purification and Ligation

The PCR reaction was purified with the Invitrogen PCR Purification Kit, according to the manufacturer's instructions and analyzed on a 1% agarose gel. The bands were cut out of the gel, and purified using the QIAquick gel extraction kit.

The restriction digestion of DNA and vector was done according to the recommendations of the enzyme supplier and left on a heat block overnight at 37˚C (refer to the reaction system below). CIP enzyme was added to the vector so the DNA can be catalyzed, and to remove the phosphate groups at 5' end. The PCR product and the vector were purified using the Invitrogen PCR purification kit, and could be stored for a maximum of 6 months without loss of cloning efficiency. The below tables (10 and 11) show the digestion reactions and their components:

Table 10 - Fragment Digestion Reaction

Components	Volume/Reaction (µL)
10x Buffer	20
BgLI	5
PCR Product	50
H_2O	125
Total Reaction Volume	**200**

Table 11 - Vector Digestion Reaction

Components	Volume/Reaction (µL)
10x Buffer	20
BgLI	5
PCR Product	50
H_2O	125
Total Reaction Volume	**200**

2.4.2.5 Transformation

To control the amount needed of vector and insert, a test Ligation was carried to generate a library of at least 10^7 individual transformants. 10 ng of the digested vector along with 30 ng of the digested PCR product, and 100 ng of the vector alone was made up in 10 µL reactions, using 0.5 units of ligase. These were left on the bench at room temperature for 2 hours. The ligation mixtures were purified using the Qiaquick PCR purification kit according to the manufacturer's instructions. The ligation reaction is described below in Table 12.

Table 12 - Ligation Reaction

Vector (µL)	Fragment (µL)	T4DNA Ligase Buffer (µL)	T4 Ligase (µL)	Distilled Water (µL)
1	0	1	0.5	7.5
1	1	1	0.5	6.5
1	3	1	0.5	4.5
1	0.1	1	0.5	7.4

2.4.2.6 Transformation of Cells

On ice, a tube of DH 5-alpha Competent *E. coli* cells was thawed for about 10 minutes. While working on ice, 80 µl of cells were pipetted into a transformation tube and 1µl (containing 1 pg-100 ng) of our plasmid DNA was added to the cell mixture. The tube was flicked 4-5 times to mix cells and DNA. The mixture remained on ice for 30 minutes. Afterwards, using a heat block, at exactly 42°C, the mixture of cells were heat shocked for 30 seconds, then placed on ice for 5 minutes. 950 µl of room temperature Lysogeny Broth (LB) was pipetted into the mixture, and the tubes were placed horizontally on shaker at 37°C, 250 rpm for 60 minutes. The samples were centrifuged for 20 seconds, the supernatant discarded, and the cell pellet resuspended with 50 µl of LB. Several 10-fold serial dilutions in LB were performed, and 50-100 µl of each dilution was placed onto LB agar plates with Ampicillin. This was spread using a spreader, and incubated overnight at 37°C.

2.4.2.7 Screening of the recombinant clones by Colony PCR

A colony PCR is a fast method of detection for screening the numerous colonies from the test ligation. In this method, a PCR master mix was made up, and distributed to PCR tubes whose number corresponded to the number of colonies to screen. The colonies were picked by using a toothpick and added to the PCR tubes. The presence of the proper size of amplified fragments were analyzed on a 1% agarose gel. The PCR reaction was carried out as follows in table 13.

Table 13 - Colony PCR Reaction System

Components	Volume (µL)
PCR Master Mix	12.5
PhIS4	0.3
PsIR2	3.2
H_2O	11.9
Total Reaction Volume/PCR tube	**25**

2.4.2.8 Transformation reaction system via electroporation

Once transformants have been confirmed to have the majority of insert, they were ligated by increasing the amount of the test ligation of digested vector and insert by 15-25 times, and left overnight at room temperature. The ligation mixture was purified using the Invitrogen PCR purification kit according to the manufacturer's instructions. In electro-transformation, 25 µL of the TG1 electrocompetent E.coli cells were mixed with 5 µL of the purified ligation on a pre-chilled 2mm cuvette then kept on ice for one minute before the mixture was electroporated with an automated electroporation apparatus (Gene Pulser Xcell modular system). The cells were immediately diluted with 1 mL of recovery medium and incubated at 37˚C for 1 hour with shaking (200 rpm). After 1 hour, these were plated on LB-ampicillin (100 µg ml^{-1}), 2 % glucose and grown at 37˚C overnight. The cells were collected using LB media with a sterile scraper in a 50 mL falcon tube, and mixed with glycerol. This was then divided into 10-20 aliquots and stored at -80˚C for long term preservation.

2.4.2.9 Rescue of phagemids by M13K07 helper phage and Panning

One aliquot, ~ 10^9 cells, of the library were grown in 2xTY medium, containing 100 µg ml^{-1} ampicillin and 2% glucose until they reached mid logarithmic phase (OD_{600} 0.5-0.6). Once centrifuged and pelleted for 15 min at 4000 rpm they were resuspended in 2xTY medium with 100 µg ml^{-1} ampicillin and kanamycin (70µg/mL), and transferred into a sterile baffled flask. M13K07 bacteriophages ($1.4x10^{12}$pfu/mL) were added and kept at room temperature without any shaking for optimum phage adsorption, and the culture was incubated at 37℃ overnight with shaking at 200 rpm. The subsequent day, the bacteria were transferred to a 50 mL falcon tube and pelleted by centrifugation. The phagemid particles found in the supernatant were concentrated with precipitation by adding 20% PEG/2.5M NaCL on ice for one hour. The pellet was resuspended in PBS, centrifuged for 1 min and supernatant transferred to 2 mL tube.

For Panning, microtiter well plates (Nunc;Maxisorp) were coated with 100µL of Epoietin β at a concentration of 100 µg/mL in coating buffer (Na_2C0_3, $NaHC0_3$ pH 9.6) and incubated at 4℃ overnight. The following day; the wells were washed with PBS. Residual protein binding was blocked with blocking solution containing PBS and 1% milk at room temperature for 2 hours. The blocking solution was washed off and rinsed numerous times with PBS+0.1% tween 20 and afterwards, solely with PBS. In each well, 200 µL of phagemid

virion + 200 µL of PBS with 5% milk and a negative control (PBS+milk) were coated and incubated at room temperature for 2 hours. 250µL of grown TG1 cells (OD_{600}=0.5-0.6) were coated onto wells for 30 min at 37℃. Subsequently the infected cells were added to 10 mL of selective media (2xTY medium, 100 µg ml^{-1} ampicillin and 2% glucose) and incubated overnight at 37℃ on shaker. The adsorption of phages on immobilized antigen and elution conditions were done according to nature protocols vol.9 NO.3 (2014). Three rounds of panning were carried respectively. After the last round, the eluted phages were tittered out by plating various dilutions on LB with ampicillin plates, then several colonies were tested via colony PCR for the detection of insert and for binding activity in ELISA.

2.4.2.10 Plasmid DNA extraction and Sequence determination

In 15 mL falcon tubes, 2 mL of LB with ampicillin and 2% glucose was added, and one colony was inoculated in each tube using a toothpick. This was incubated at 37℃ overnight on shaker. The following day, the grown cells were transferred to a 1.5 mL microcentrifuge tube and centrifuged at max speed for 1 minute. The plasmids were extracted using the protocol found in the Purelink quick plasmid miniprep kit. The sequencing reaction plate was as follows in table 14.

Table 14 - Sequencing Reaction System

Components	Volume/(µL)
Master Mix 360	12.5
Primer 1 (PhiS4)	0.5
Primer 2 (PsiR2)	0.5
Plasmid DNA	3
H_2O	8.5
Total Reaction Volume/PCR tube	**20**

The sequencing plate was sent to the University College London to be processed and to determine the amino acid sequences.

2.4.2.11 Protein Preparation

The expressed recombinant V_HH proteins were produced in accordance with Neu and Heppel 1965 paper, with the main difference being the removal of the wash steps before resuspending the cells in the sucrose solution. Selected plasmids were transformed in 5 mL of LB with ampicillin, with prepared competent SSC 320 *E.coli* cells and grown at 37˚c overnight. Competent cells were prepared for the transformation in the following manner: on LB agar, not including antibiotics, SSC 320 cells were streaked and incubated at 37˚C overnight. These were then inoculated with 100 mL LB, while shaking at 37˚C, until they reached the following logarithmic phase OD_{600} 0.5. 40 mL of the grown cell culture was transferred and divided into 2 falcon tubes and 10 mL of ice cold 0.1 M $CaCL_2$ was added and kept on ice for 45 minutes. Centrifugation followed for 5 minutes at 2000 rpm, and 4˚C. The pellet was resuspended with 1 mL of ice cold 0.1M $CaCL_2$, pooled together, and another 1 mL of $CaCL_2$ was added for a total of 3 mL final volume. 10% glycerol was added and the cells aliquoted into 300 µL volume per tube and stored at -80˚C.

While working on ice, 80 µL of competent cells with 3 µL of plasmid were taken and left for 20 minutes. Using a heat block at 42˚C, the cells were heat shocked for 1 minute and 30 seconds, and transferred immediately to a 15 mL falcon tube. 900 µL of LB was added and left shaking for 1 hour horizontally. To collect the pellet, the tubes were centrifuged at 8000 rpm for 1 min, and the supernatant was discarded. 50 µL of LB was added, and the mixture plated on LB agar with ampicillin and 2% glucose. The plates were incubated at 37˚C overnight without exceeding 16 hours.

One colony of the SSC 320 cells harboring the plasmids were taken and grown in 5 mL of LB and ampicillin overnight. The following day, 1 mL of the grown cells were inoculated in 250 mL of LB medium with 100 µg ampicillin until they reached OD_{600} 0.5 approximately. Protein expression was induced by addition of 1mM ispropyl-β-D-thiogalactopyranoside (IPTG) to the culture which was incubated for 3 hours at 37˚C with shaking. The cells were harvested by centrifugation for 15 minutes 4000 rpm at 4˚C and the pellet dissolved in 20% sucrose, 1mM EDTA, and 30 mM Tris-HCL at pH 8. The cells were kept at room temperature for 10 min while shaking before centrifugation at 4˚C for 10 minutes. The pellets were drained well and given an osmotic shock by resuspending with ice cold pure water, then kept on ice for 10 minutes. After incubation on ice, the periplasmic protein extracts were recovered in the supernatant after centrifuging the mixture at 13000 rpm for 10 minutes. The periplasmic extract was used for the detection of recombinant protein in SDS-PAGE and western blotting.

2.4.2.12 SDS-PAGE and Western Blotting

15 µL of the periplasmic protein extracts were mixed with 15 µL of 2x SDS gel loading buffer and incubated at 100˚C for 10 min to denature proteins. 20µL of the reduced mixture was then run on a 17.5% SDS gel at 130 mV for 1 hour, alongside a standard protein molecular weight marker. This was then transferred to a

nitrocellulose membrane by creating a stack using sponges and filter paper. Once the stack was made, with the gel and membrane adjacent to each other, this was run in transfer buffer at a voltage of 30V for 2 hours. After 2 hours, to confirm that the proteins had transferred onto the membrane, Ponceau staining was used and washed off with PBST. Using blocking buffer, the membrane was blocked for 1 hour. Anti-HA or Anti-HIS antibodies were used as primary antibodies and left to incubate at 4°C overnight. This was then washed off with washing buffer, and an anti-mouse/anti-Rabbit IgG alkaline phosphatase conjugate was used as a secondary antibody. 2mL chemiluminescence reagent was placed on the membrane to allow for visualization. In a dark room, the luminescent membrane was exposed to an X-Ray film, which was then developed and fixed to show the expression of the recombinant V_HH proteins.

CHAPTER 3
RESULTS

In this chapter, the results are presented in the same order as shown in Figure 5, page 32, describing the schema of the project.

3.1 Anti-EPO Polyclonal Antibody Production

3.1.1 Subject of immunization

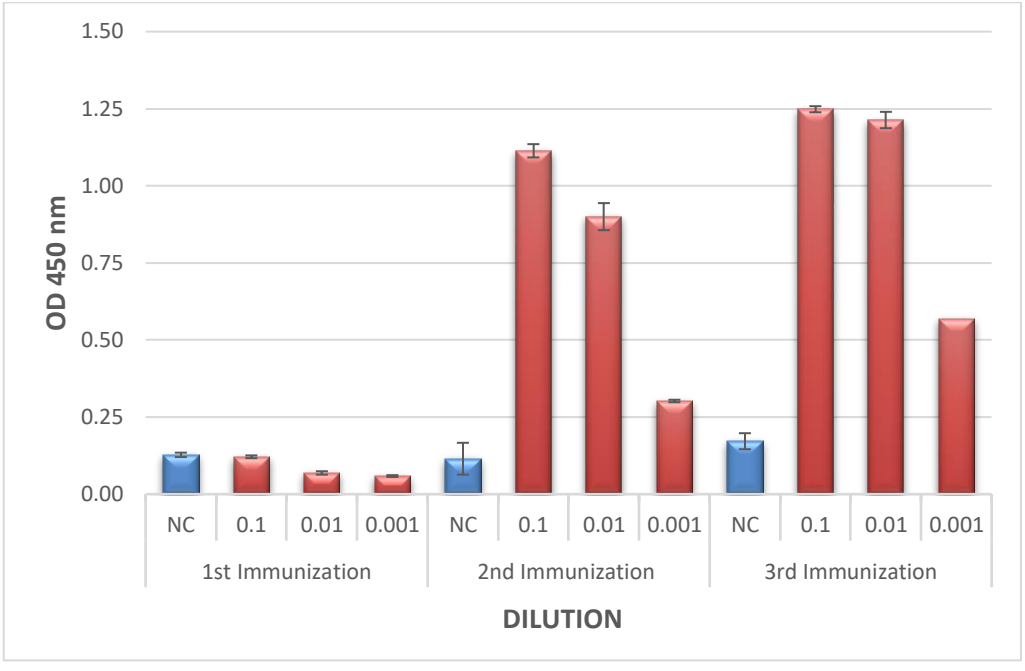
A male camel, 12 months of age, was injected in multiple sub-cutaneous sites with recombinant human erythropoietin α (Epoetin α), at a concentration of 33.6 µg/mL in Complete Freund's adjuvant. This was followed by injection with the antigen in Incomplete Freund's adjuvant. Samples were taken prior to, and at 4 weeks, 8 weeks and 12 weeks after each injection. Another male camel, 12 months of age, was injected in multiple sub-cutaneous sites, with recombinant human erythropoietin β (Epoetin β) at a concentration of 41.5 µg/0.3 mL mixed in Complete Freund's adjuvant; the next immunization was followed by injection with the antigen in Incomplete Freund's adjuvant. Samples were also taken prior to, and 4 weeks, 8 weeks and 12 weeks after each injection.

Serum from these immunizations were screened for immunogen specific antibody production using an in-house ELISA with antigen coated plates**.** Three dilutions were used for testing each anti-serum, and the absorbance at an OD of 450 nm was measured. The OD was used as a measure of titer of antibody produced by the immunization as an initial screening method because it is directly proportional to the humoral response. It is also an accepted, rapid and convenient way to screen for the immunization effectiveness. The results for both antigens are shown in Figure 9. Comparing the two antigens, the overall titer for Epoetin α following the 3rd immunization was 1:1000, as opposed to 1:100 for Epoetin β, and Epoetin α may also be marginally better at eliciting an immune response, both in magnitude and earlier, as seen even after the second immunization, and with higher ODs. The optimal response was seen after the 3rd injection for both antigens and these samples were used for further analysis. The results for this are shown in Figure 9.

3.1.2 Purification of serum

The immunised serum that showed optimal immunogen binding came from the third immunisation. These samples were then purified to extract IgGs using a HiTrap protein G column. The column contains protien G, coupled to sepharose beads, that bind the Fc portion of the IgG, which can then be washed and eluted off the column. 500µL of serum was applied on the column at pH 5.8 and eluted at pH 2.7. The eluted peak was collected as 1mL fractions. The results for this are shown in Figure 10.

A.



B.

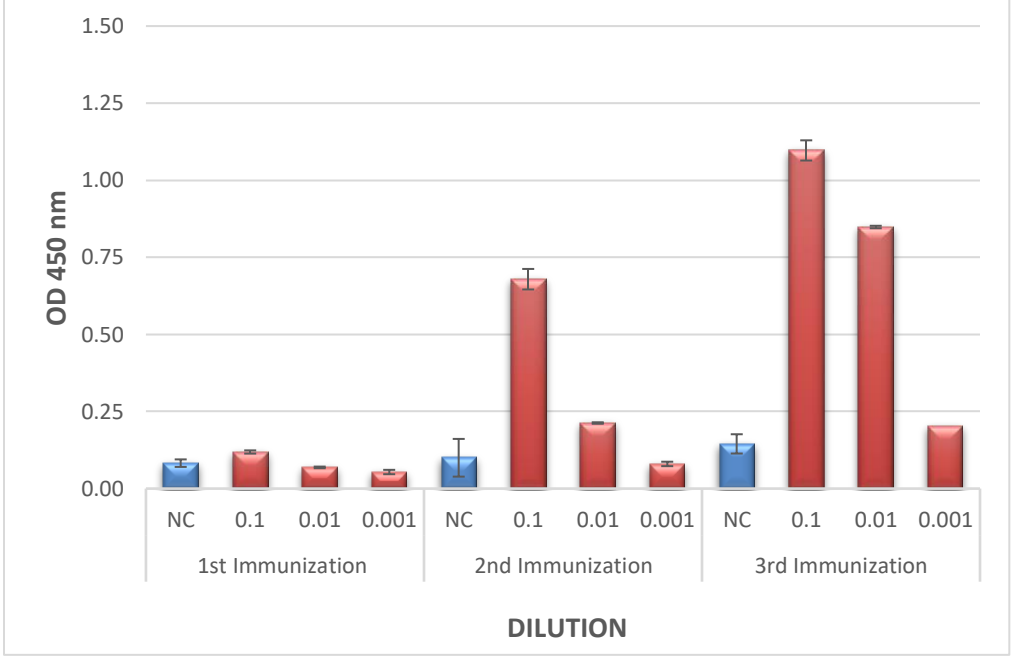
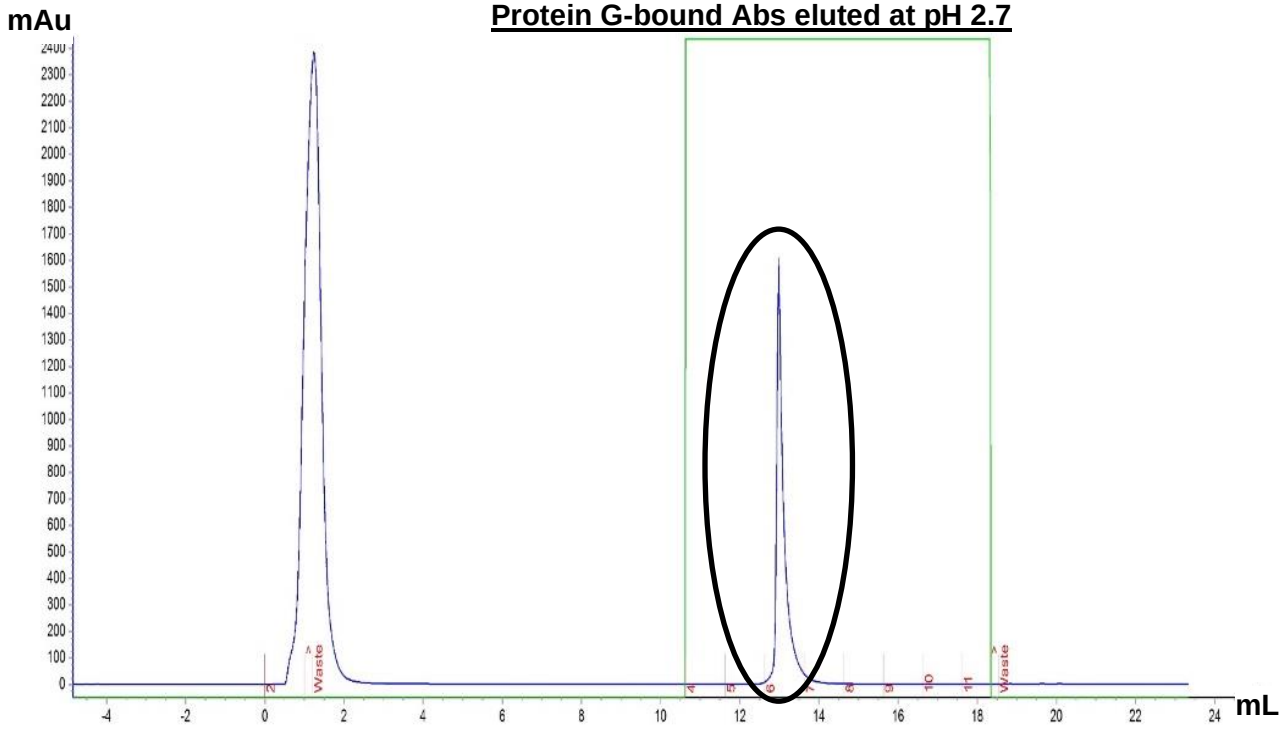


Figure 9 - Immune response following immunization with Epoetin α (A) and Epoetin β (B)

The OD is used to produce a maximal titer for the antibody following each immunization. Negative Control (blue bar) refers to the non-immunized control serum. The red bars refer to the diluted serum from the camel after immunizations (1, 2 and 3). The titer is 0.1 , 0.01 and 0.001 after 1st, 2nd and 3rd immunization respectively. Therefore, the after the 3rd immunization a 1:1000 dilution of the anti-sera was detectably higher than NC.

A.



B.

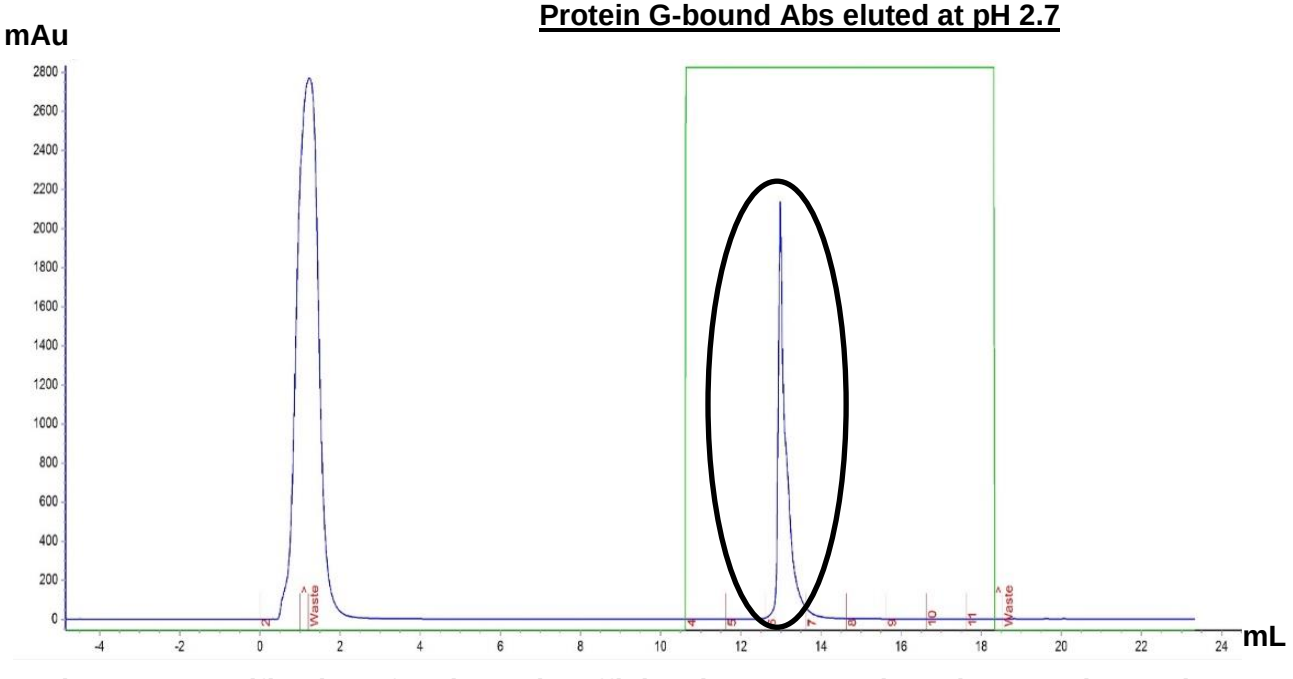


Figure 10 - Purification of anti-sera by affinity chromatography using protein G columns.

The initial peak represents unbound proteins, 'breakthrough', while the second peak represents the protein G-bound antibodies eluted at pH 2.7. The y-axis represents milli-absorbance unit (mAU) and the x axis represents milliliter (mL). A: Epoetin α, B: Epoetin β.

3.1.3 Detection of purified antibodies

The polyclonal antibodies from two serum samples were purified for each camel. The success of the purification was confirmed by running the eluted fractions (EF) and breakthrough (BT) on a 10 % SDS-PAGE gel, followed by coomassie staining for total protein. A high recovery of polyclonal antibodies can be seen in the gel, with both the conventional Ab (Hc + Lc) and the heavy chain only Ab apparent. The gel was repeated due to over-exposure, which led to an inability to see the heavy chain only antibodies, as they have a lower molecular weight of 40 kDa, as opposed to 50KDa, and the light chain at 25 KDa. The two gels showing both the BT and EF of both camels are shown in Figure 11. Following Epoetin α immunisation there seems to be equal amounts of conventional and HCAbs. But, Epoetin β seemed to favour an HCAb response, with less of the conventional Abs.

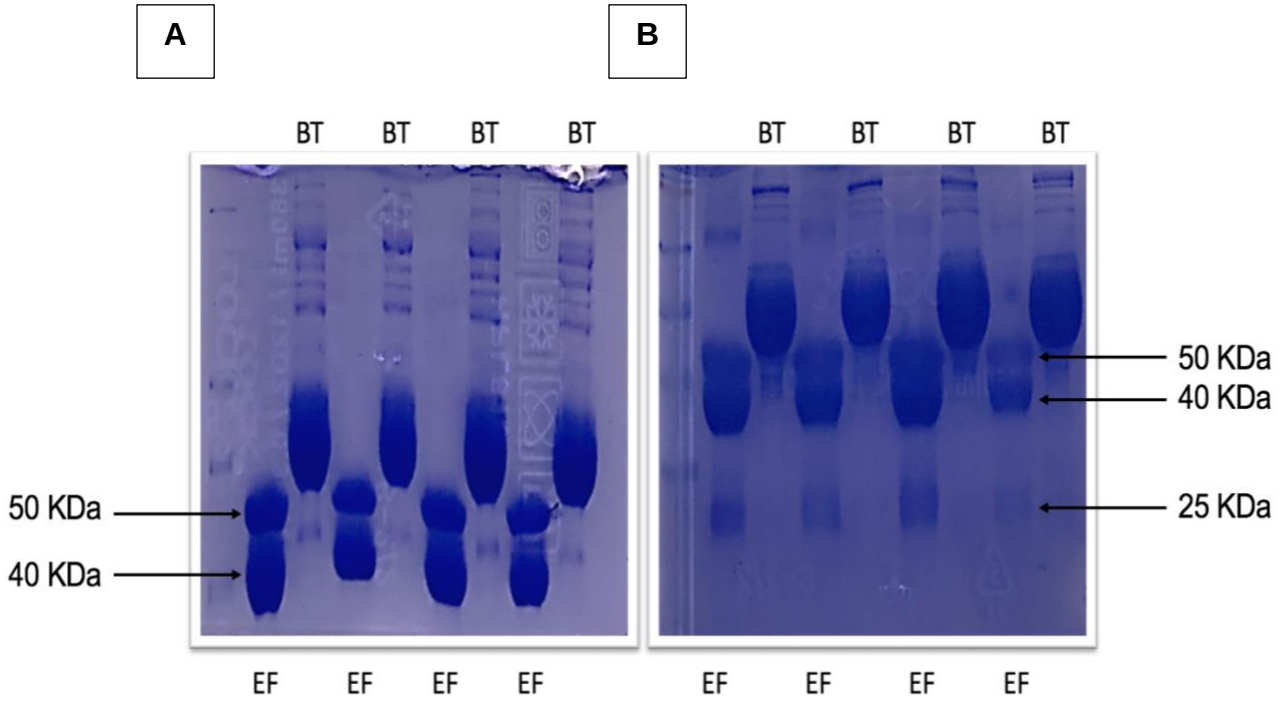


Figure 11 - Protein gels stained with Coomassie Blue

Gel electrophoresis followed by coomassie staining showing purified antibodies and unbound proteins. The purified antibodies, after Epoetin α (A) and after Epoetin β (B) immunisations, consisted of conventional antibodies and heavy chain only antibodies. The eluted fraction (EF) lanes represented the purified antibodies and the breakthrough (BT) lanes represent the unbound proteins. This membrane represents both the conventional and HCAbs. The bands indicated by the 50 KDa are of the heavy chain of the conventional abs while the 40 KDa bands are of the heavy chain of the sdAbs, and the 25 KDa bands are of the light chain of the conventional abs. This result indicated a succesful completion and optimization of the antibody purification, as no overloading of column is detected in the staining along with any additional loss of proteins in the BT.

3.1.4 Screening for antigen binding via Western blot analysis

To test for presence of immunoglobulin, under denaturing conditions, both samples of the purified camel serum (Epoetin α and Epoetin β) were transferred from SDS gel to a PVDF membrane. After blocking and incubating the membrane with a 1:2500 dilution of anti-Camel IgG HRP, a chemiluminescent substrate was used to develop the membrane, which generated the following image (see Figure 12).

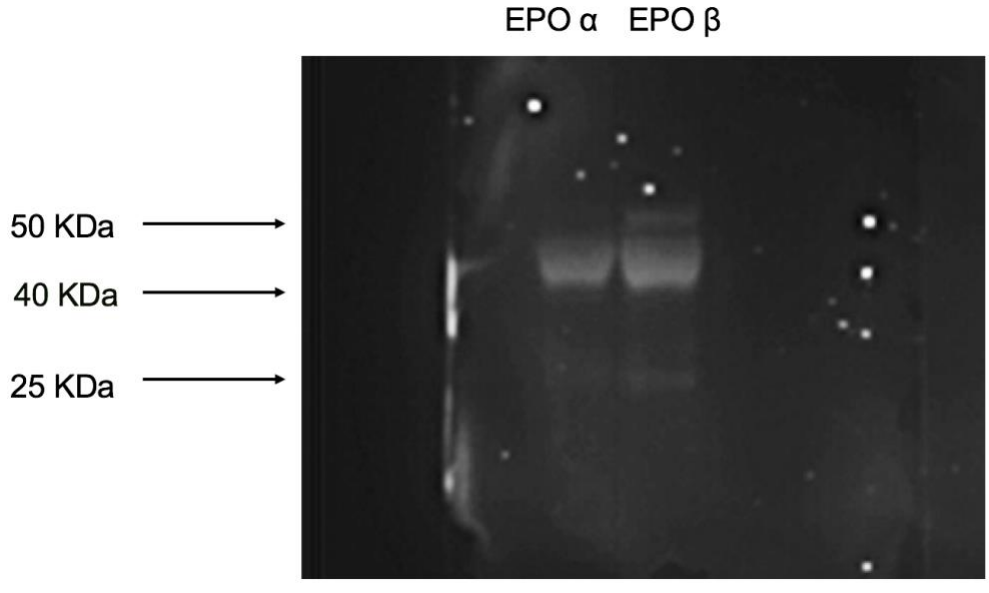


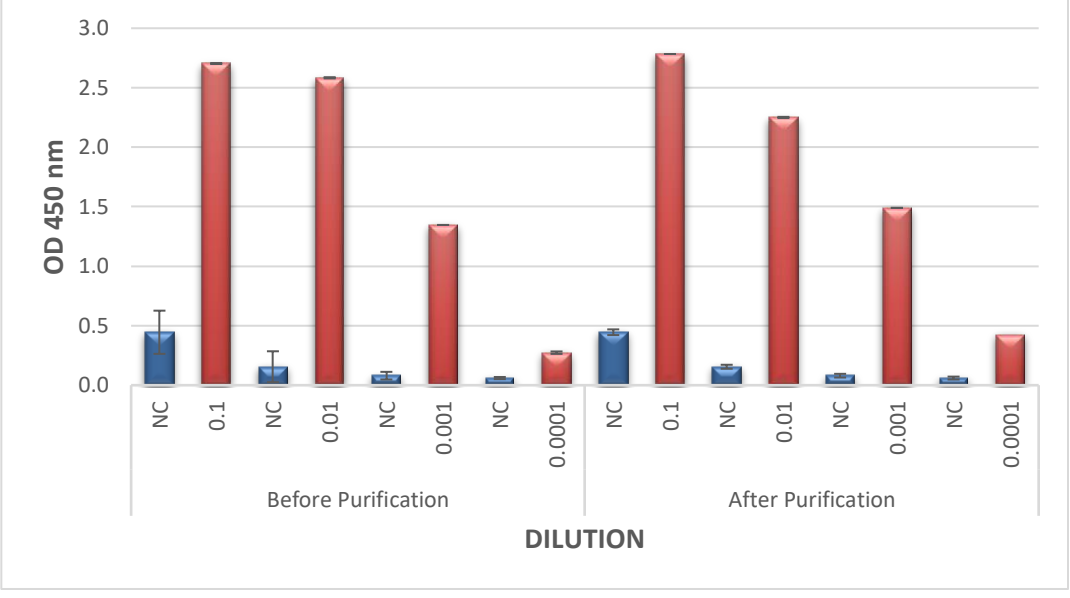
Figure 12 - Identification of purified serum by protein immunoblotting

The image shows the presence of purified forms of the conventional and heavy chain only Abs, due to the band size shown in the Western Blot and Coomassie Brilliant Blue staining appearing as expected. The clearer Band (≈ 40KDa) represent camelid heavy-chain only Abs which lack the CH1 domain and appear to be more concentrated in both the samples, than conventional Abs, but slightly more in the sample from the camel immunized with Epoetin β. Based on the results, it was estimated that a much higher propotion of the circulating polyclonal consisted of the heavy chain species, compared to the presence of conventional antibodies. The methodology for the western blot analysis was as described in Chapter 2. Purified camel serum, after being immunised with Epoetin α, showed only one band at molecular weight of 40 kDa, representing the heavy chain only antibodies. After injection with Epoetin β, the western blot shows protein bands at molecular weight of 50, 40, and 25 KDa.

3.1.5 Screening of Purified Polyclonal Antibodies via ELISA

Purified polyclonal antibodies (pAbs) from the camel injected with Epoetin α were screened by ELISA. Data are shown as optical density (OD) at 450 nm, and were compared to the standard curve of the primary antibody. Purified sera were diluted at 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} in wash buffer. In parallel, non-immunized serum that was collected prior to immunization was used to compare with serum samples post 3rd immunization, before purification. The secondary antibody was a dilution of 1:5000 of a mouse anti-camel HRPO (see Figure 13).

A.



B.

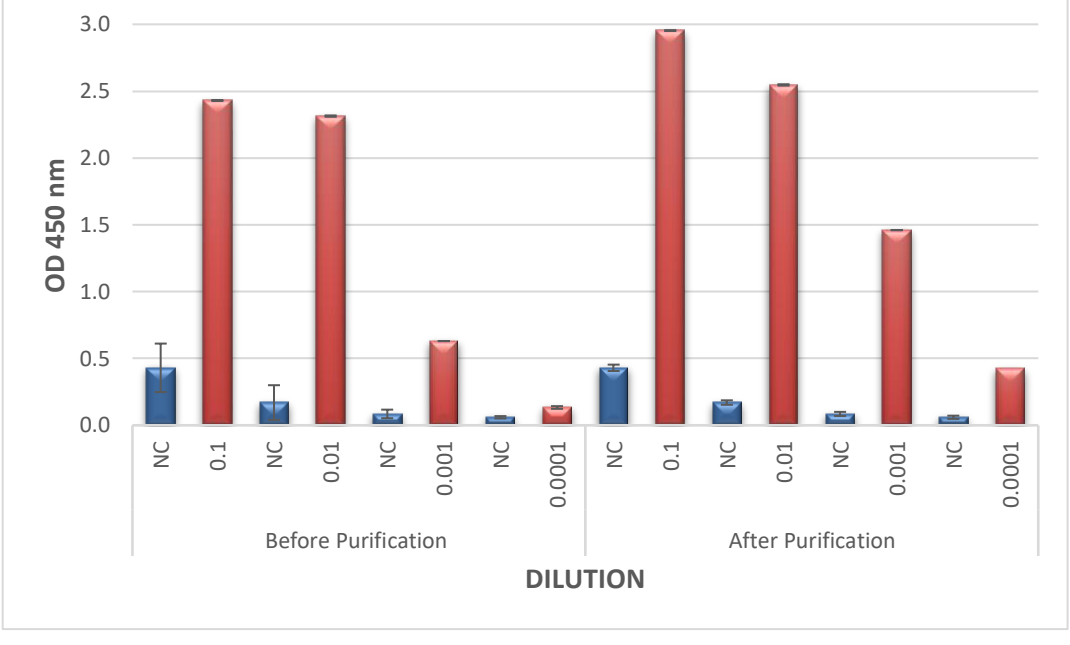


Figure 13 - Antigen specific immune response: Epoetin α (A) and Epoetin β (B)

The results of the assays conducted with the purified pAbs following immunization with both forms of Epoetin, compared to non-immunized serum, suggest that there is an antigen specific immune response (Figure 12). The purification was successful, as shown in Figures 11 and 12. The purified polyclonal antibody was conjugated to horse radish peroxidase (HRPO) and used in the development of a competitive assay, using Epoetin α and β as the standards. The ability of the assay to detect injected, recombinant hormone was then investigated in urine samples from a volunteer, before and after an injection of Epoetin β. See principles of the assay shown in Figure 14.

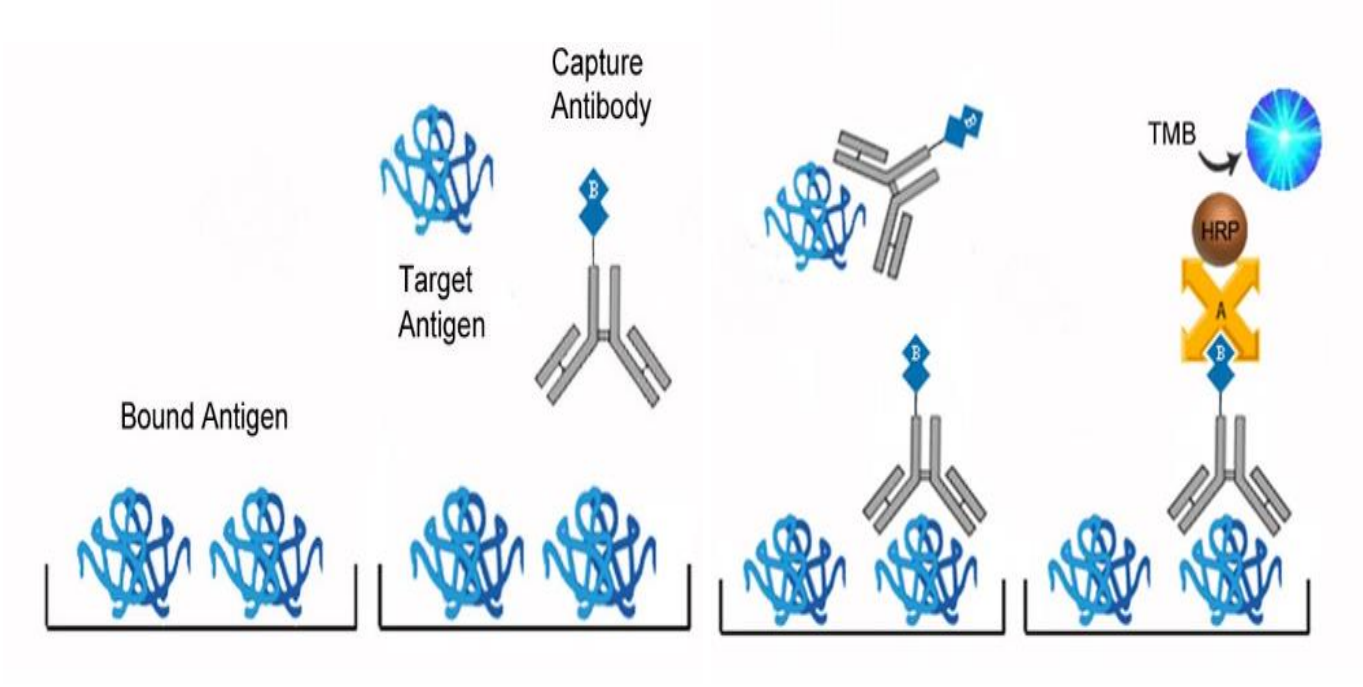


Figure 14 - Principle behind the Competitive ImmunoEnzyme Assay (EIA) protocol

A known antigen (eg. rhEPO) was used to coat a multiwell plate. Following blocking of non-specific sites with BSA, and washing steps, samples containing unknown concentrations of the antigen (eg rhEPO calibrants or endogenous EPO) were added. A labeled (eg horse radish peroxidase, HRP) antibody was then applied for detection using relevant substrates (e.g. 3,3',5,5'-Tetramethylbenzidine or TMB). If there is a high concentration of antigen in the sample, a significant reduction in signal output will be observed. In contrast, if there is very little antigen in the sample, there will be very little reduction in the expected signal output. In the example shown in this figure, there would be a reduction in signal output.

A target specific capture antibody was pre-coated onto each well of the supplied microtiter plate. Also, a fixed quantity of Horseradish Peroxidase (HRP)-conjugated detection antibody were added to the wells as a set of known concentration of standards or samples. This allows the free antigens, represented by the standards or samples, and antigens bound to the plate to compete together in order to bind to the detection antibody. During the washing phase, only the bound HRP-conjugated detection antibody would be washed away. A 3,3',5,5'-Tetramethylbenzidine (TMB) Liquid Substrate was used to react with the HRP enzyme resulting in color development.

After 30 min, H_2SO_4 was added to stop the assay by terminating the color development reaction. The components of the wells were measured by determining their optical density (OD) at a wavelength of 450 nm (± 2 nm). The ODs generated were analyzed; the unknown sample against the standard curve with known antigen concentrations. The concentration of Epoetin in the sample was determined from this. In contrast to typical 2-site, sandwich ELISA, in a competitive assay, there is an inverse relationship between concentration and the OD, the greater the amount of antigen in the sample, the lower the color development and OD reading.

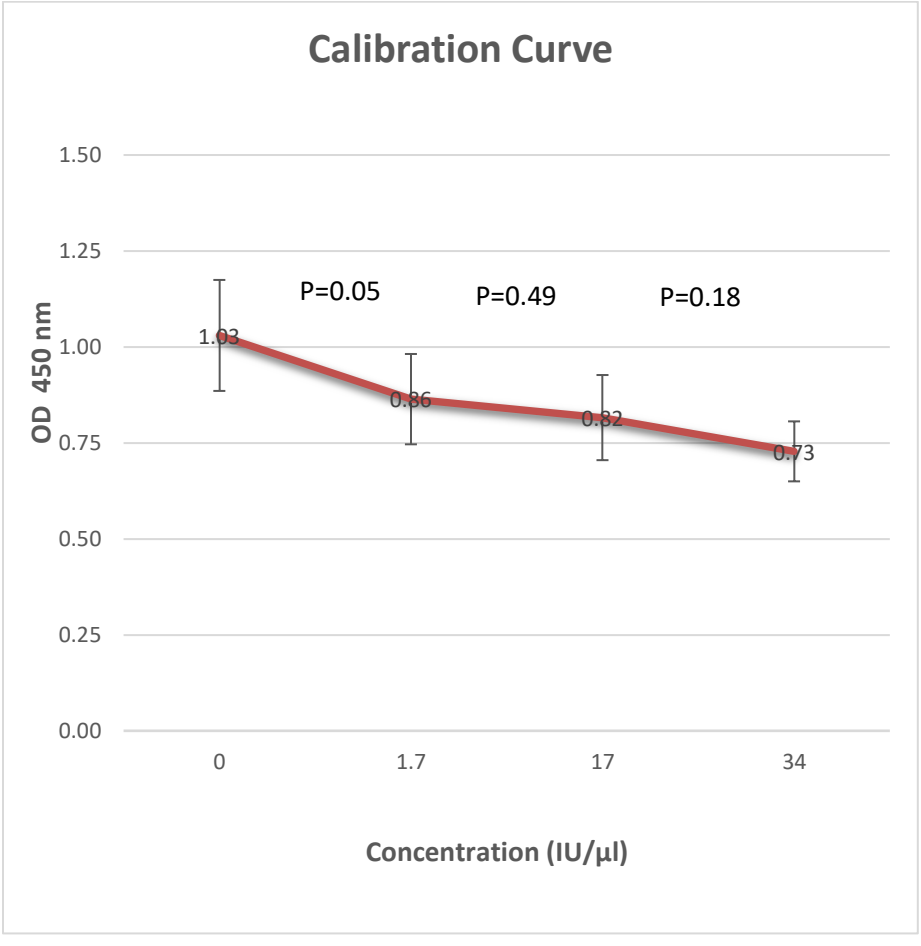


Figure 15 - Calibration curve of Competitive Enzyme Immuno Assay

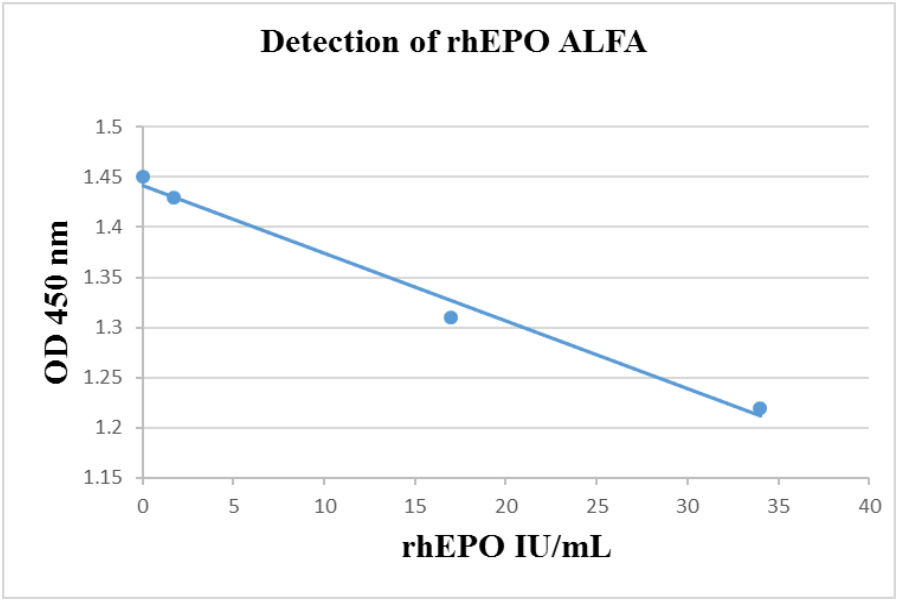
Concentration range covered by the standard curve was between 0 to 34 IU/µL (0, 1.7, 17, 34 IU/µL), with Epoetin β as the calibrant. The assay was able to detect only differences between 0 and 1.7 IU/µL significantly (p=0.05 by t-test). The curve at the higher concentrations lacked sensitivity. However, as the expected concentration was below 2 U/mL it was deemed adequate for use.

Table 15 - Competitive (EIA) using Anti-EPO Camel Ig-HRPO from the Pabs

	Concentration (IU/µL)		
	0 – 1.7	1.7 - 17	17 - 34
Spiked urine (α)	+/-	+/+	+
Spiked urine (β)	+/-	+/+	-
Before EPO		-	
After EPO		+	

The standard curve was used to determine the concentration of Epoetin β in the urine sample of a volunteer (Caucasion male, age 57 years), before and after one injection of Epoetin β, concentration of 41.5µU/0.3 mL (4940 U). The samples (before and after injection) were tested repeatedly 8 times and the mean (SD) calculated. None of the assays detected any signal in the urine before the injection. The urine, after the injection always showed detectable levels of Epoetin: mean (SD) 0.7 (0.04) IU/µl. The inter and intra-assay coefficients of variation were all below 10. There was no Epoetin detected in sample prior to injection. After the injection Epoetin was detected in the sample for up to 24 hours. Got recovery through all the phases but it had poorer detection of beta as it plateaued out from 17-34. EPO is relatively short-lived and has a half-life in serum of 8.5 ± 2.4 hours once administered intravenously, and 19.4 ± 10.7 hours when administered subcutaneously. EPO remains in the body around 48 hours. However, whether the assay truly did not detect of endogenous EPO could not be confirmed, as the sample had not been prepared as per procedure for the usual direct method, which involves concentrating by filtration. Therefore, the lack of detection of EPO prior to injection may be due to lack of sensitivity of the current assay. This part of the methodology requires further development and validation. Further development of the assay was not possible because of time constraints.

A.



B.

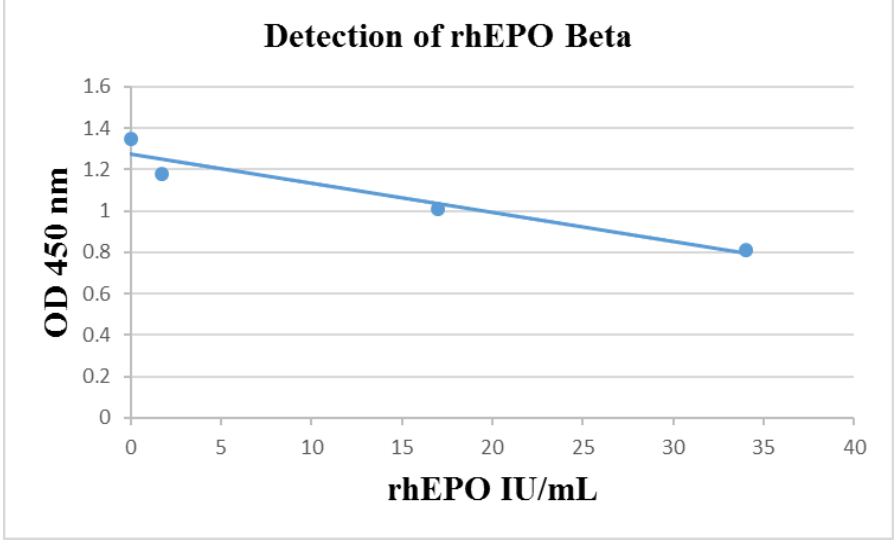


Figure 16 - Competitive assay with unpurified Pab-HRP conjugate to check reproducibility of the calibration curve using Epoetin α (A) and Epoetin β (B)

Because of the differences in the amount of HCAbs seen in the anti-serum following Epoetin α and β immunisation the competetive assay was set using both Epoetin α and β as calibrants and unpurified polyclonal abs. The concentration range covered by the standard curve was between 0 to 34 IU/µL (0, 1.7, 17, 34 IU/µL), with Epoetin α (A) and Epoetin β (B) as the calibrants. The calibration curves were found to be reproducible (see Figure 15), with the same sensitivities, between 0 and 1.7 IU/µL significantly (p=0.05 by t-test). The curves, as per the previous, lacked range at the higher concentrations. However, as the expected concentration was below 2 U/mL it was deemed adequate for use.The standard curve with the anti-serum from Epoetin α immunised camel showed slightly better discrimination between the standard.

3.2 Generation and Selection of Recombinant in vivo-matured Camel V_HH Fragments

3.2.1 Lymphocyte Isolation

Total RNA was isolated from blood samples collected from the camels after every immunization. The quantity and quality of 1μg isolated RNA were assessed by observing the absorbance at 260 and 280 nm using the Nanodrop 2000. As nucleic acids have absorbance maxima at 260 nm, the ratio of this absorbance maximun to the absorbance at 280 nm was used to measure the purity of the extracted RNA. A_{260}/A_{280} ratio of ~ 2.0 is generally accepted as acceptable quality of RNA. The below tables show the RNA concentration and purity for samples extracted after each immunisation, for both Epoetin α (Table 16) and Epoetin β (Table 17).

Table 16 - RNA Concentration data using the NanoDrop 2000 Spectrophotometer for Epoietin α

Sample ID	RNA Concentration (mg/mL)	A_{260}/A_{280}
Post 1st Immunization	0.583	0.49
Post 2nd Immunization	0.910	0.44
Post 3rd Immunization	1.418	0.43

Table 17 - RNA Concentration data using the NanoDrop 2000 Spectrophotometer for Epoietin β

Sample ID	RNA Concentration (mg/mL)	A_{260}/A_{280}
Post 1st Immunization	1.069	0.52
Post 2nd Immunization	0.810	0.42
Post 3rd Immunization	1.734	0.57

Yields and purity of total RNA isolated from each camel after every immunization were carried out using the described method. The quality of isolated RNA based on the nanodrop results were showed lower values than expected. The instrument was found to be displaying some innaccuracies in the reading, as values were fluctuating when repeated. For this reason, further analysis was needed.

Isolated RNA was further analyzed and quantified with the Agilent 2100 Bioanalyzer. A RNA integrity number higher or equal to ≥ 5-6 is of acceptable purity. The results of the Bioanalyzer are shown in Figure17.

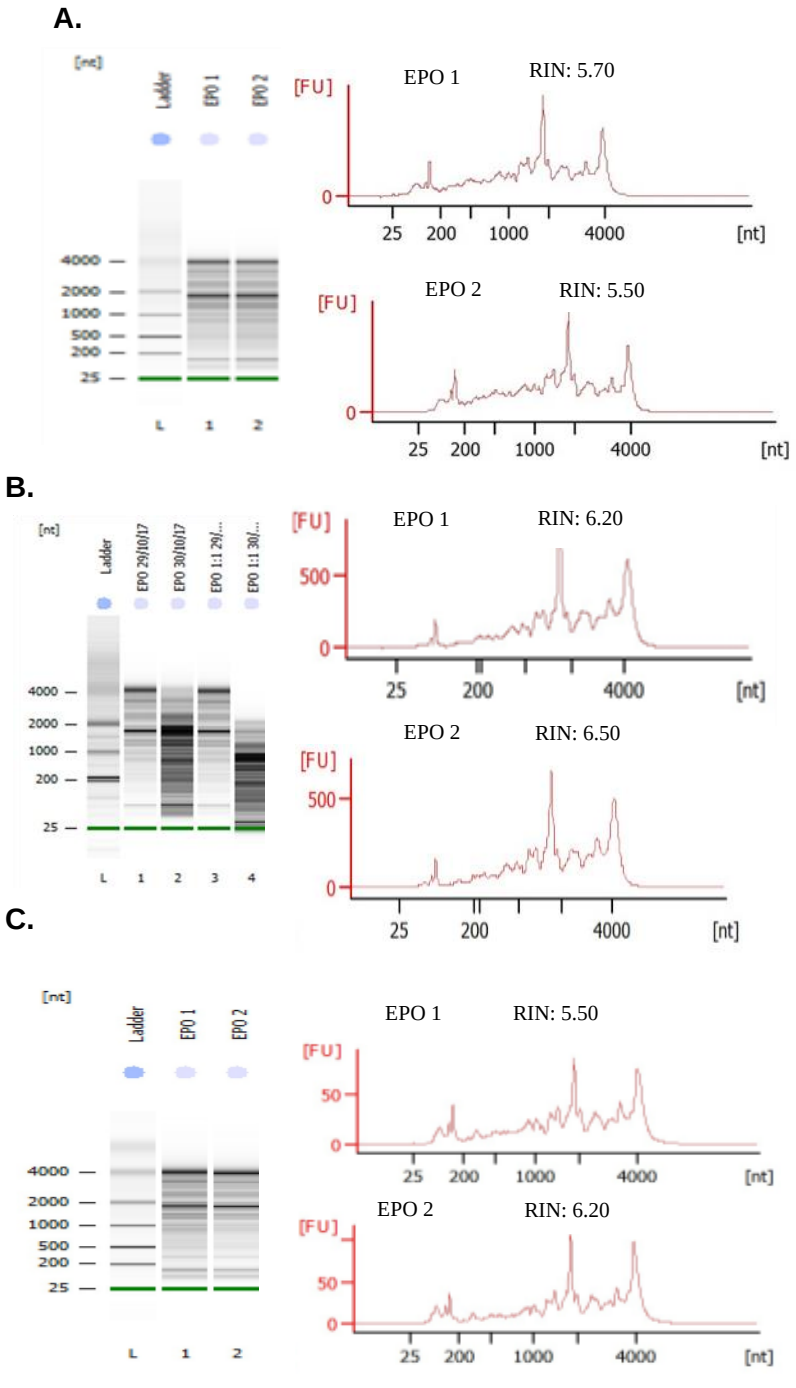


Figure 17 - Integrity of total RNA isolated using the Agilent 2100 Bioanalyzer

The above electropherogram images represent an electropherogram of total RNA, showing clear peaks of rRNAs and intact RNA of good quality. The yields of RIN extracted from camel blood post immunization with Epoetin α (EPO 1) and Epoetin β (EPO 2) ranged from 5.50 to 6.50, assigned by the 2100 expert software respectively, indicating no degradation of RNA and were suitable for RT-PCR and construction of cDNA library and expression analysis. The y- axis represents fluorescence units (FU) and the x-axis represents seconds (s).

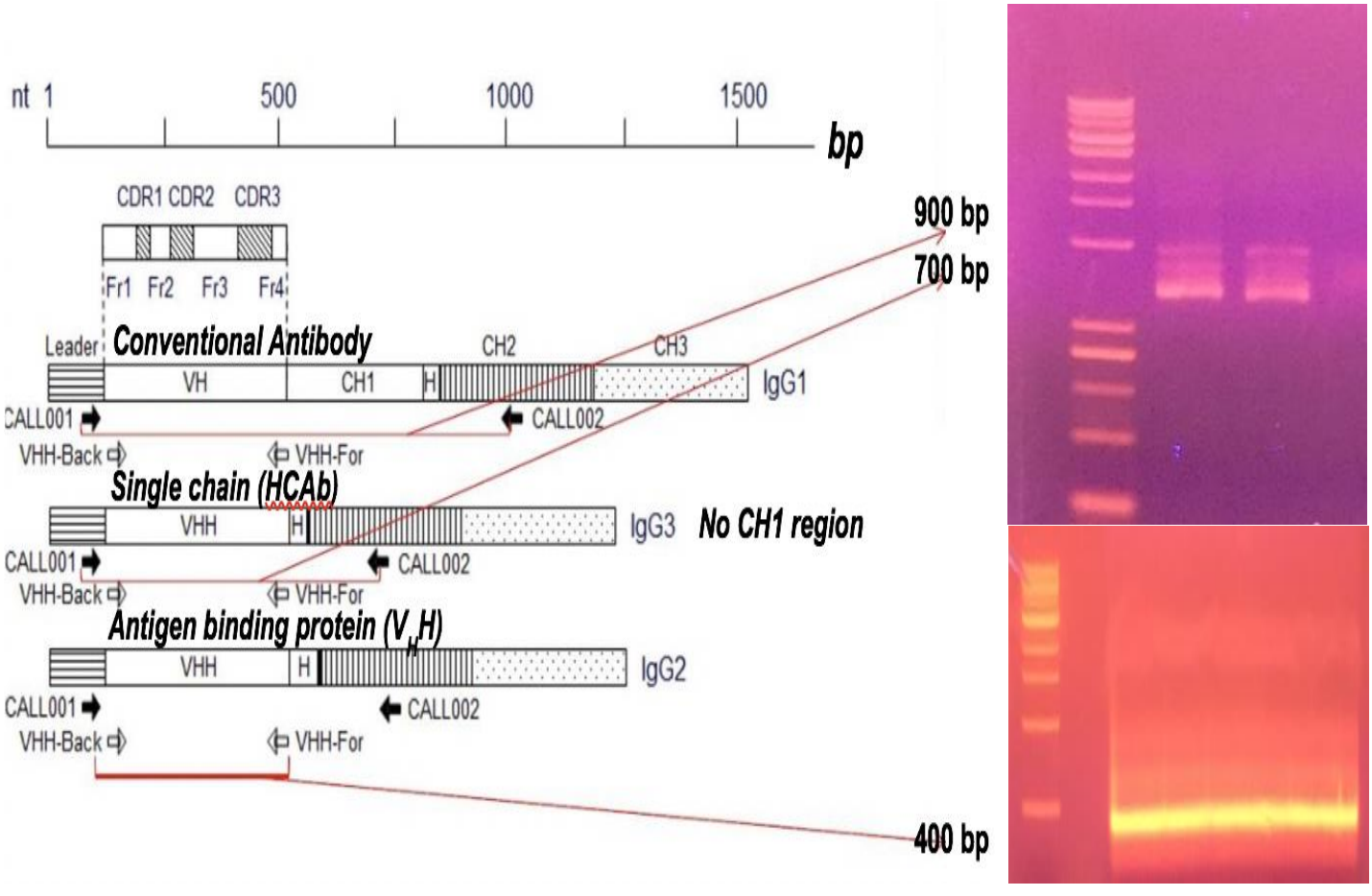


Figure 18 - Representation of the cDNA isolation without the CH1 Location (HCAb) and construction of the immune library by amplification of the VHH fragment encoding genes

Lymphocytes from rhEPO immunized camels post 3rd immunization were used to synthesize single-stranded cDNA from the mRNA transcripts using a Superscript Reverse Transcriptase. The variable domains of all immunoglobulin heavy chains from the cDNA were amplified with two gene specific primers CALL001 annealing to FR1, and CALL002 annealing to CH1. The RT-PCR products of the first 700 bp represents the heavy chain only ab repertoire, whilst the 900 bp corresponds to the heavy chain of the conventional Abs. Re-amplification of the V_HH fragment encoding genes, the antigen binding protein (V_HH), with forward primer annealing to FR1 of the V_HH and reverse primer annealing to FR4 region, yielded the 400 bp band (see Figure 19 and 20).

3.2.2 cDNA synthesis

The Superscript Reverse Transcriptase kit was used to convert total RNA transcripts into 1st strand cDNA as per manufacturers instruction from RNA extracted post 3rd immunization. Two gene specific PCR primer pairs CALL001, and CALL002 were used for the amplification of the variable domains of all the immunoglobulin heavy chains (VHs,V_HHs). PCR was analyzed on 1% TAE Agarose gel. The amplification was repeated and loaded on agarose gel at a higher volume and purified using Genelute extraction kit following the manufacturer's instructions.

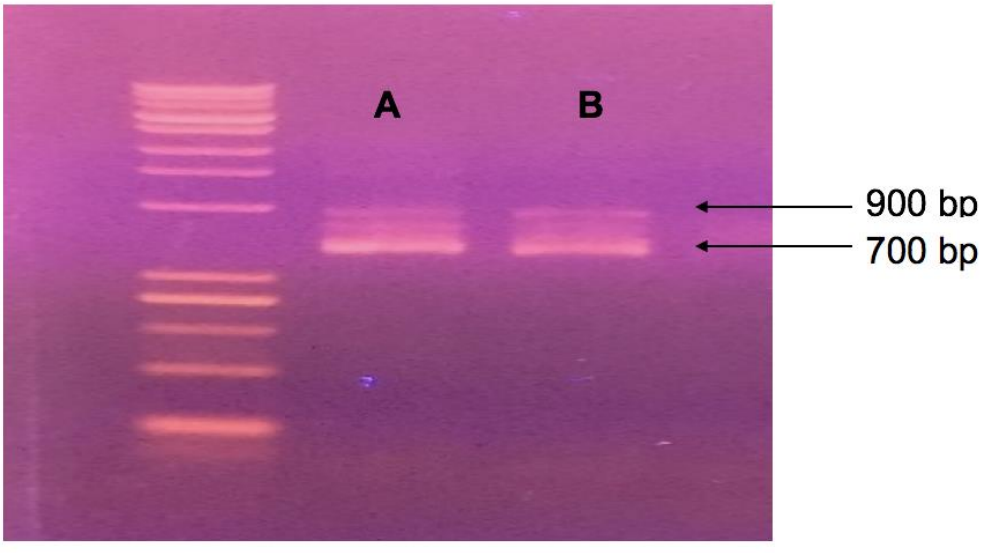


Figure 19 - 1st strand cDNA synthesis. RT of RNA after injection with Epoetin α (A) Epoetin β (B)

The above image shows 1% agarose gel electrophoresis of the RT-PCR products, illustrating the 700 bp band of the heavy chain only ab repertoire, and the 900 bp corresponding to the conventional ab, as well a DNA Ladder (100bp).

3.2.3 Re-amplification of the V_HH fragment encoding genes

The purified 700 bp PCR fragment was re-amplified using the following encoding genes with nested primers V_HH-back and V_HH-forward annealing at framework 1 and framework 4 by combining PCR components in order to separate and purify the fragment from the 900 bp fragment. To continue further, several agarose gels were completed in order perfect result to obtain a clear band.

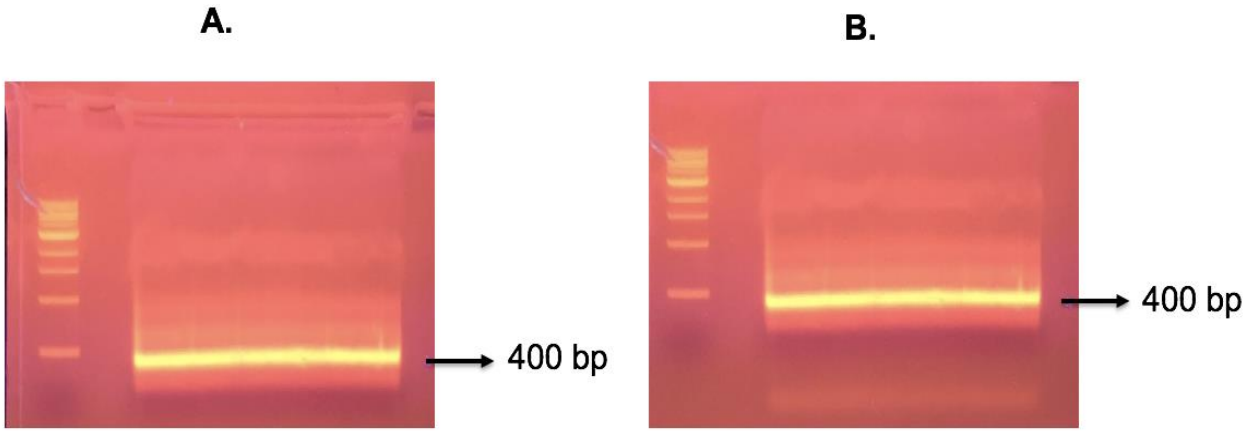


Figure 20 - Antigen binding Protein (VHH) for Epoetin α (A) and Epoetin β (B)

The above figure shows agarose gel electrophoresis of the re-amplification of the V_HH fragment encoding gene represented by the 400 bp with a DNA Ladder (1Kb).

3.2.4 Digestion

The PCR product was purified using the Genelute extraction kit following the manufacturer's instructions, digested using BgLI restriction enzyme in 200 µL reaction mix, and left overnight on heat block at 37˚C. The vector (PADL-22C) (200ng/µL) was prepared and digested as described in chapter 2. The diagram below illustrates the vector map.

3.2.5 Ligation Preparation and transformation

1 µL CIP enzyme, an alkaline phosphatase that catalyzes the dephosphorylating of 5' and 3' end of DNA, was added to the vector after overnight incubation, and left on a heat block at 37˚C for one hour. Purelink Invitrogen PCR Purification Kit was used to purify both vector and fragment before continuing with ligation.

Table 18- The ligation reactions were as follows

Reaction	Fragment	Vector	T4 DNA Ligase Buffer 10x	T4 Ligase	MilliQ water
1	0 µL	1 µL	1 µL	0.5 µL	7 µL
2	1 µL	1 µL	1 µL	0.5 µL	6.5 µL
3	3 µL	1 µL	1 µL	0.5 µL	4.5 µL
4	0.1 µL	1 µL	1 µL	0.5 µL	7 µL

Table 19- Transformation results from plates

Reaction	Result
1	Growth
2	Growth
3	Growth
4	Growth

Total Ligation volume of 10 µL was left overnight at room temperature. Transformation was done using 80 µL of competent Dh5 alpha and 3 µL of each ligase reaction, plated on LB agar + ampicillin media and incubated overnight at 37 ˚C. Results indicated that the digestion could not have been efficient as there was growth on the plates without fragments “0(F):1(V)”. Digestion efficiency was checked by running the vector and one of the fragments on a 1 % agarose and its nucleic acid concentration estimated using Nanodrop 2000. Results of this are shown in Table 20 and Figure 21.

Table 20 - Protein concentration via NanoDrop 2000 Spectrophotometer

Sample ID	Nucleic acid Concentration (ng/µL)	A_{260}/A_{280}
PADL-22C (undigested)	201.3	1.88
PADL-22C + BgLI	182	1.88
Fragment	21.8	1.80

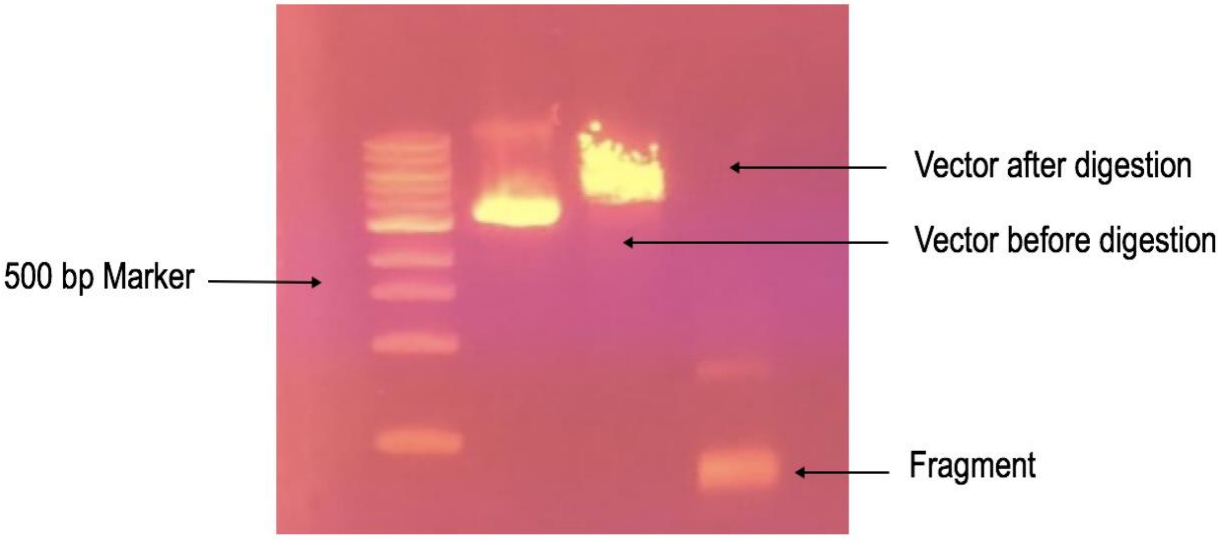


Figure 21 - Gel Electrophoresis analysis of Vector

Based on the results shown in Table 16 and Figure 21, the vector appeared to be successfully digested. Thus for troubleshooting purposes, the digestion was repeated whilst using a new BgLI enzyme and a higher dilution reaction. Ligation reaction mix was prepared using a fragment generated from RNA obtained in a preliminary immunization with newly digested vector:

Table 21- Test Ligations to determine efficiency of digested vector

Reaction	Fragment	Digested Vector	T4 DNA Ligase Buffer 10x	T4 Ligase	MilliQ water
1	0 µL	1 µL	1 µL	0.5 µL	7.5 µL
2	1 µL	0 µL	1 µL	1 µL	7 µL
3	3 µL	1 µL	1 µL	0.5 µL	4.5 µL

Table 22- Transformation results from plates:

Reaction	Result
1	No Growth
2	Growth
3	Growth

Transformation was completed using 80 µL of competent Dh5 alpha and 3 µL of each ligase reaction and plated on LB agar + ampicillin media for an overnight incubation period.
The ligation was repeated, and a transformation step was completed using the newly digested vector with fragments produced from the amplified PCR product for Epoietin α and Epoietin β.

Table 23- Repeated ligation reactions were as follows:

Reaction	Fragment	Vector	T4 DNA Ligase Buffer 10x	T4 Ligase	MilliQ water
1	0 µL	1 µL	1 µL	0.5 µL	7 µL
2	1 µL	1 µL	1 µL	0.5 µL	6.5 µL
3	3 µL	1 µL	1 µL	0.5 µL	4.5 µL
4	0.1 µL	1 µL	1 µL	0.5 µL	7 µL

Table 24- Transformation results of repeated ligation reaction from plates:

Reaction	Result
1	No Growth
2	Growth
3	Growth
4	Growth

As described in chapter 2, tenfold of serial dilutions were made using the recovered transformed cells and from colonies on each plate. The electroporation efficiency was calculated using the number of colonies and multiplying them with the corresponding dilution factor. To confirm that the majority of transformants had an insert of the proper size of the V_HH fragment, several separate colonies were picked, and a colony PCR reaction was performed. The image below shows the gel.

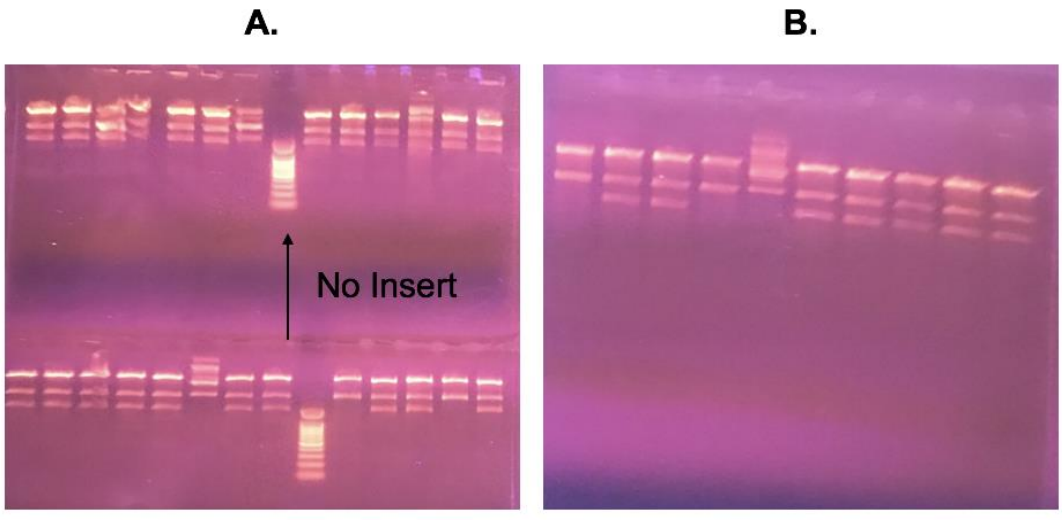
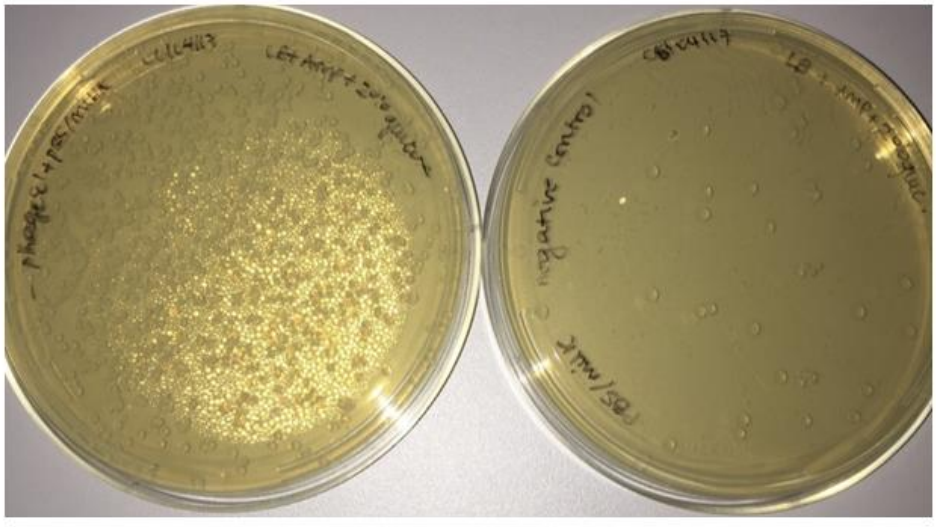


Figure 22 - Gel for electroporation efficiency: Epoietin α (A) and Epoietin β (B)

Based on the results of the colony PCR on the agarose gel, 90% of the transformants contained the V_HH fragment insert, meaning that the test ligation was sufficient, and the electroporation efficiency gave an adequate amount of transformants to reach a library size of 10^7. A new ligation reaction mix was prepared with the digested vector and insert by 15-25 times the amount of the test ligation.:

19 µL (F): 20 µL (V) +5 µL T4 DNA Ligase Buffer 10x + 2 µL T4 Ligase + 4 µLMilliQ water

After completing the transformation with TG1 electro competent cells, the library was recovered and all the cells were collected and mixed with sterile glycerol (20% (vol/vol) final concentration). The below image (figure 23) shows the agar plates containing the colonies.



Binding solution
Phage + PBS w/5% Milk

Negative Control
PBS w/5% Milk

Figure 23 - Rescue and amplification of phage from the immune library and Antigen presentation and phage selection by panning

Before each round of panning, the single domain displaying phage was rescued and amplified by inoculating 48 copies of each library clone (represented by six OD 600 units) in a selective medium (2xTY medium with antibiotic and glucose). Once cells reached logarithmic phase of 0.5 OD 600, cells were superinfected with helper Phage M13K07 to make the viral protein coat. Once the plasmid was incorporated into the viral genome and replicated, the phage was selected and recovered. After 3 rounds of selection, as described in methods, the infected cells were added directly on a selective medium alongside a negative control. Chosen plasmids were only selected against Epoetin β for sequence determination. Plasmid extraction was conducted following the protocol of the quick plasmid mini-prep kit, sequence reaction plate was prepared and sent out to the DNA Core Lab at University College London to determine the amino acid sequence of each plasmid.

3.2.6 Plasmid DNA selection and Sequence determination

Initial Plasmids expressed were the following:

Table 25- 1st set of Sequences:

Well	Sequence
D09	[redacted]HHHHHH
E09	[redacted]HHHHHH
E11	[redacted]HHHHHH
G12	[redacted]HHHHHH
F10	[redacted]HHHHHH
H08	[redacted]QHHHHHH

His-Tag

The lysate of the following were checked via ELISA the results yielded was poor (see table 28(A)) therefore, the expression was repeated with 6 different set of plasmid sequences.

Table 26- 2nd set of Sequences:

Well	Sequence
B07	[redacted]HHHHHH[redacted]
G10	[redacted]HHHHHH[redacted]
A07	[redacted]HHHHHH[redacted]
B12	[redacted]HHHHHH[redacted]
E10	[redacted]HHHHHH[redacted]
B07	[redacted]QHHHHHH[redacted]

His-Tag

The detailed sequences could not be shown here as they are part of a patent application. The expression of free scFs or Fab fragments in the periplasmic space and detection of the vectors binding through the HA tag was the classical application of the pADL^{tM} -22c vector. The following plasmids were transformed using SSC competent cells followed by induction and separation of the bacterial periplasm by osmotic shock as described in chapter 2. There was a slight improvement in the lysates from second set of plasmids.

Table 27 – ELISA results of the lysates from both sets of chosen plasmids

A.

	1	3	4	5
A	0.0683	0.1192	0.0753	0.124
B	0.3088	0.4811	0.0709	0.1019
C	0.2754	0.2887	0.1679	0.3887
D	0.213	0.267	0.1685	0.1632

B.

	1	3	4	5
A	0.1833	0.1336	0.1831	0.1741
B	0.382	0.4578	0.4339	0.4299
C	0.3218	0.2776	0.5058	0.3053
D	0.2408	0.2338	0.4678	0.428

A) Lysate from first set of plasmids B) Lysate from second set of plasmids

The plates were coated with Epoetin β overnight as described in chapter 2, then incubated with the Lysate, primary Ab Anti-HA, and secondary Ab Anti-Mouse IgG. Absorbance values were read at OD 450 nm. The highlighted wells show some immunoreactivity to Epoietin β. Those selected plasmids were antigen reactive while some of the other plasmids shown above have little or no antigen reactivity. The plasmids with the highest absorbance value were chosen for expression and selectivity.

3.2.7 Protein expression and Western Blot

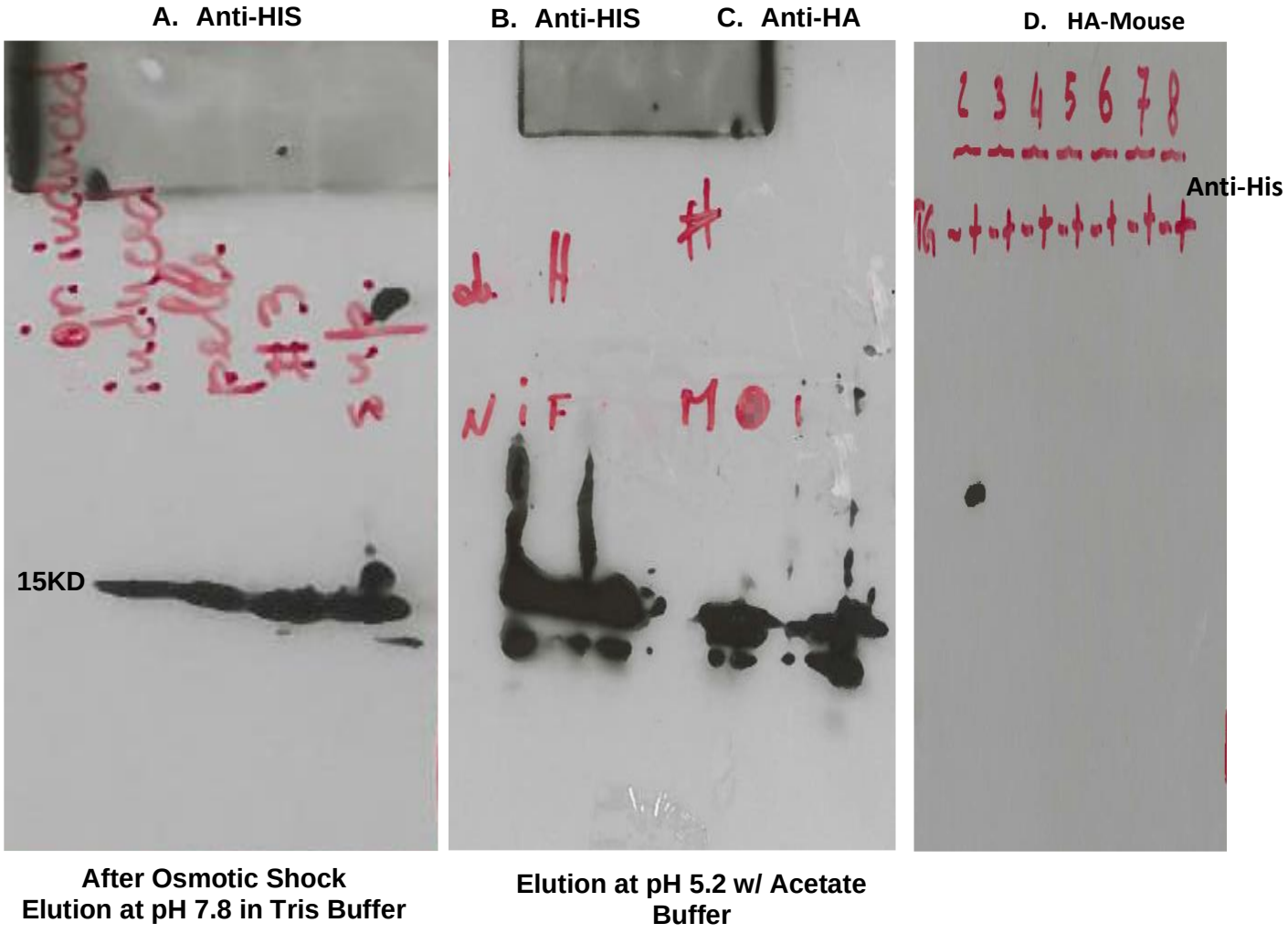


Figure 24. Expressing the V_HH Ab in extracted Plasmids

Purification of the His-Tagged V_HH fragments from the chosen plasmids were expressed with an experienced colleague only once due to very limited time constraints. Western blot was used to detect the expression of the V_HH insert in the plasmid. Loaded well lanes starting from the left included: bacterial pellet non-induced (before Osmotic shock), induced, pellet after osmotic shock, #3 (positive control), and supernatant (lysate soup). 1M IPTG (isopropyl beta-D-thiogalactoside) was used to induce the clones and the HIS-tagged proteins were purified using a nickel column. Very low levels of expression were detected and need further optimization and can be done as future work to increase selectivity and solubility of the protein.

CHAPTER 4

DISCUSSION & FUTURE EXPERIMENTS

One of the most challenging tasks of antibody engineering involves reducing the size of the antigen binding protein. Currently, therapeutic needs are being addressed based on the knowledge behind an antibody's structure relating to function. This has paved a way to create desired antibodies and antibody related biologics with the suitable functional and biophysical properties. Improving efficacy and manufacturability is applied to antibodies, antibody fragments, antibody fusion products, affinity modulations and stability enhancement (91). The analysis of IgG molecules in the serum of dromedary camels have forever changed the view of antibodies as molecules constituted of two heavy chains and two light chains. The finding of antibodies devoid of light chains, heavy chain only antibodies displaying high antigen binding affinities highly improved the prospects of developing single domain antibodies. Thus, dromedary camels, along with other members of the camelidae, such as llamas and alpacas, are rather unique hosts and are rapidly becoming highly utilized for antibody production for both diagnostic and therapeutic purposes. They differ from other mammalian species by having these conventional antibodies and single-domain antibodies. The HCAbs, or sdAbs, consisting of only two heavy chains where the binding domains constitute paired variable regions lacking the two light chains, have generated significant interest in bio engineering world. Part of the immune response of camels is based on these HCAbs, as it's thought to represent the majority of their antibody repertoire. These antibodies exhibit a broad antigen-binding repertoire by enlarging their hypervariable regions. Exploiting this natural immune capability of camels could produce antibodies with specificities and affinities that can be potentially used in assay development, especially when conventional immunoreagents have failed to produce assays with the required specificity, affinity and/or sensitivity.

Epoetin (rhEPO) specific immunoassay. Early preliminary data, prior to the start of this project, had suggested that camels may be apt as hosts to produce antibodies that are able to discriminate between the exogenous and recombinant EPO. These trial antibodies, derived from animals that had been immunized with various forms of the drug, did not bind, or, only bound the endogenous form very weakly, and therefore paved the way for further investigation described in the current report.

Antibodies were successfully raised to both Epoetin α and β, the most readily available of the rhEPOs. Interestingly, even though both of drugs do not vary in their amino acid sequence, but only differ in their glycosylation, there was a difference in the immune response they generated. The response to Epoetin α seemed to favor the production of HCAbs to a greater extent than the one to Epoetin β. Whether there was a difference in the Ig subclass was not tested but would have been interesting to investigate.

rhEPO specific immunoassay using HCAb. The calibration curve generated using this polyclonal HCAb was able to discriminate between samples spiked with the rhEPO in vitro and also after in vivo injection, compared to samples that did not contain the drug. These results suggest that these antibodies may be able to mount a response dependent on the extent, and also perhaps type of glycosylation.

The competitive assay developed with this antibody was also able to detect urinary EPO after injection of the recombinant form in vivo in a human volunteer. However, no EPO was detected in the urine sample taken prior to the injection. This suggests that the HCAb used in this assay was able to differentiate between the endogenous and recombinant species. However, the poor sensitivity of the assay needs to addressed and further development is needed to check the specificity under conditions where the endogenous EPO levels are naturally high, or are concentrated.

Epoetin specific V_HH fragments. Using molecular biology and phage display techniques V_HH fragments were generated successfully. These fragments were also found to recognize the rhEPO in a screening assay. However, expressing enough of the V_HH fragments to carry out more detailed characterization, or for use in an assay proved unsuccessful within the time allocated for this study. The sequences for several of these fragments have, however, been generated as a resource for other studies.

Future directions. The current study was able to generate some interesting antibodies and V_HH fragments with some suggestion of being different from those currently produced in the usual hosts, such as rabbits and rodents. Due to time constraints the assay development with the HCAbs could not be completed satisfactorily. This would be an important and interesting avenue to pursue further. In addition, a combination of the V_HH fragments generated and the HCAbs in a two-site ELISA may yield better results, with the ability to specifically measure rhEPO, while not detecting the endogenous hormone. While there has been much interest in the camelid antibodies as therapeutics the results obtained here suggest that camel antibodies, both HCAbs and perhaps the V_HH fragments, do have the potential discriminatory immunogenicity for application in the doping analysis field.

Furthermore, a most recent study was conducted and submitted to Drug Testing and Analysis, to test the concept of using a directly-conjugated horseradish-peroxidase (HRP)-conjugated anti-Epo primary antibody; without the need of a secondary antibody in order to reduce the analysis time and remove any nonspecific cross-reactivity with secondary antibodies. Thus, an in-house periodate and three anti-human Epo_HRP that are commercially available were assessed for sensitivity and specificity against rhEPO standards, negative human urine, and urine samples provided from an EPO excretion study. The mouse monoclonal, anti-human Epo clone was directly conjugated to HRP by using an in-house periodate labelling and the potential effect of the alterations to the assay on the antibodies' sensitivity and specificity to minimize the sample turnaround time were evaluated. The current anti-doping analysis for rhEPO is performed via gel electrophoresis and proteins are then transferred by western blotting onto a polyvinylidene difluoride membrane as a final step. Even though this method has been validated for potential confounders and is highly specific and robust, there is much room for improvements as it is a continuous evolving protocol. A primary monoclonal mouse, anti-human EPO antibody and a secondary anti-mouse immunoglobulin antibody conjugated to HRP directed against the primary antibody to enable immunodetection is used in the testing process of single blots. In the

case of a double blot analysis of EPO from serum or plasma samples the same primary anti-human EPO antibody, a secondary antibody conjugated to Biotin and a Streptavidin-HRP complex are used. Recently, a step has been reduced by the successful application and validation of the primary mouse anti-human EPO antibody clone that has been directly biotinylated, followed by combination with a Streptavidin-HRP complex. Unfortunately, selected secondary antibodies have always imposed the potential of cross-reactivity with antibody related fragments possibly from the immune-purification step in sample preparation. Therefore, the hypothesis behind the use of a direct HRP labelled antibody could prove to overcome or further reduce previous limitations. The results of the modified method that fulfilled the criteria of WADA, showed a successful reduction in analytical time using the direct labelling of the primary antibody which in turn can serve as an advantage in major Olympic competitions during a fast turnaround time.

As the current data from, the HRP-conjugated Anti-EPO antibodies for direct rhEPO detection, study provide a promising proof of concept and a good strategy, future studies can be applied in a larger scale using either camel polyclonal or V_HHs instead of monoclonal mouse antibodies directed against EPO. Without having any time constraints, raising anti-Epo camel antibodies and labelling it to HRP have the potential of not only reducing analytical time as previously investigated but also, being able to discriminate against the endogenous versus the recombinant form of EPO.

Therapeutic camelid V_HH fragments. As the main patent claims on camelid antibody fragments expired in Europe and America, major advances in the field using camelid antibodies in therapeutics received an enormous boost once. Ablynx Inc, initially established as a university spinout company from scientists at Ghent University, strived for expansion with the addition of more than 20 preclinical and clinical programs by collaborating with large biopharma players, such as Merck, Boehringer Ingelheim and Sanofi. In 2018, Sanofi acquired Ablynx for an aggregate equity value of approximately €3.9 billion. Caplacizumab, which is a drug composed of bivalent anti-vWF nanobody for the treatment of rare blood clotting disorders, was considered to be the first V_HH-based drug that reached the market in February 2019 (www.ablynx.com). Meanwhile, biotechnology companies have been more interested in the commercialization of these domain antibodies as therapeutics, diagnostics, and research reagents since IP limitations on the composition of matter of V_HHs are diminishing.

While diagnostics are not as profitable as therapeutics the need for them in clinical medicine is also of paramount importance, especially in fields such as diagnostic imaging. Camelid antibodies hold great promise as very exciting resources in both the therapeutics and diagnostics of the future.

CHAPTER 5

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APPENDICES

APPENDIX I- Ethical Approval
Ethical Approval Letter.

المجلس الأعلى للصحة
Supreme Council Of Health

دولة قطر
State Of Qatar

December 03, 2015

Exceptional Ethical Animal Approval

This exceptional ethical animal research approval is granted to the ADL by the Research Division at Supreme Council of Health (RD-SCH) on the project titled *"Development of single-domain antibodies for therapeutic and diagnostic applications"*.
Considering the nature of the animal research applied in the study and the lack of capacity and decline of existing local IACUC to review and approve this type of animal research. *The project has been reviewed by RD-SCH* with modification of protocol to include the below information:

1- The total number of camels used in the study;
2- Whether there will be collection of lymph nodes.
3- The volume of blood that will be collected from the animal at one time point;
4- Number of blood collections from the animal over the experiment timeframe;
5- The fate of the experimental animals after the experiment is completed;
6- The type of anesthesia that will be used to comfort the animal, if needed, and dose and frequency of applying drug substance; and,
7- The Name of the vet and a copy of his license as a Veterinarian.

If we can be of further assistance, please contact us at 974-4407-0363 or via email at IRB@sch.gov.qa

Sincerely,

Dr. Eman Sadoun
Manager, Research Division
Supreme Council of Health
dresadoun@sch.gov.qa

State Of Qatar دولة قطر
المجلس الأعلى للصحة
Supreme Council Of Health
إدارة الأبحاث الطبية
Dept .of Biomedical Research
004

APPENDIX II- Ethical Approval
Second Ethical Approval Letter.

May 20, 2018

Exceptional Ethical Animal Approval

This exceptional ethical animal approval is granted to the ADL by the Research Division at the ministry of public health (RD-MOPH) on the project titled "*Development of single-domain antibodies for therapeutic and diagnostic applications*".

Considering the nature of the animal research applied in the study and the lack of capacity and decline of existing local IACUC to review and approve this type of animal research. *The project has been reviewed and approved by RD-MOPH.*

This approval is effective from May 20, 2018 to May 19, 2020.

If we can be of further assistance, please contact us at 974-4407-0363 or via email at IRB@moph.gov.qa

Sincerely,

Dr. Eman Sadoun
Manager, Research Division
Ministry of Public Health
dresadoun@moph.gov.qa

APPENDIX III- Gantt Chart

GANTT CHART showing the timelines (year 1)

	1 Sept 2017	2 Nov 2017	3 Oct 2017	4 Dec 2017	5 Jan 2018	6 Feb 2018	7 Mar 2018	8 Apr 2018	9 May 2018	10 Jun 2018	11 Jul 2018	12 Aug 2018
Chapter one												
Chapter two												
Writing up	chapters 1 & 2 and the review article						Chapter 3, 4 & 5					
Chapter three & four	Recruitments and retrospective data analysis (descriptive)											
Lab work Pts and control			1. Immunization 2. Collection of Lymphocyte samples from each immunization 3. Methods of antibody purification and assay development 4. Monoclonal antibodies and assay development									
Assay Validation- Internal/External												
Final writing									Maternity leave Extension (May,2020)			