Chapter 3

White rhinoceros (*Ceratotherium simum*) and Asian elephant (*Elephas maximus*) interferon-gamma specific recombinant chicken antibodies

Abstract

To isolate phage-displayed and soluble single chain variable fragment (scFv) antibodies against recombinant white rhinoceros (*Ceratotherium simum*) interferon-gamma (rRhIFN-γ) and Asian elephant (*Elephas maximus*) IFN-γ (rAsEpIFN-γ), a chicken phage-displayed antibody library was constructed. Total RNA was isolated from the spleens of two silver-hyline chickens immunized with rRhIFN-γ and reverse transcribed into cDNA. The genes of the variable parts of the heavy (V_H) and light (V_L) chain were amplified individually by polymerase chain reaction (PCR) with primers that resulted in overlapping sequences to be joined by splice overlap extension PCR. The joined V_H and V_L product was ligated into a phagemid vector, pHEN I, and transformed into *Escherichia coli* TG1 cells. The library expressing the single V_HV_L construct was panned on individual proteins. After four rounds of panning, eight clones were selected of which four were targeted against rRhIFN-γ and four against rAsEpIFN-γ. The sequencing results indicated that the four clones against rRhIFN-γ shared the same sequence. Similarly the four clones specific for rAsEpIFN-γ also shared the same sequence that, however, differed from the former. Soluble scFv antibodies, 1F5 and 6D7, were used as capture antibodies and polyclonal IgY^{uu} and monoclonal antibody 1D11 were used as detecting antibodies in different IFN-γ capture enzyme-linked immunosorbent assay (ELISA) formats to establish the detection of rRhIFN-γ and rAsEpIFN-γ. The combination of the capture scFv, 1F5, and antibody 1D11 performs best for detection of rRhIFN-γ. For the detection of rAsEpIFN-γ the 1F5 / IgY^{uu} combination is required. When the combination of 6D7 / IgY^{uu} is used there is an increase in background signals and this combination does not provide suitable detection of either rRhIFN-γ or rAsEpIFN-γ. These first steps will provide information on determining the specificity for native IFN-γ molecules and eventually may prove to be reliable tools for the diagnosis of *Mycobacterium bovis* and *M. tuberculosis* infections in rhinoceroses and elephants.

3.1 Introduction

Antibodies are antigen binding proteins present on B-cell membranes and are secreted by plasma cells. After secretion they circulate in the blood where they serve as effectors of humoral immunity. Antibodies produced in response to particular antigens are heterogeneous. Many of them are complex and contain different antigenic determinants. The immune system responds by producing antibodies to several epitopes on the antigen (Kindt, Goldsby, Babara & Kuby 2002). Since the discovery of the hybridoma technique in 1975 (Köhler & Milstein 1975), monoclonal antibodies, produced by immortalized single B-cell clones, are essential tools for research biology.

Selection of monoclonal antibodies of a desired specificity can also be achieved by bacteriophage-display of combinatorial antibody libraries (Andris-Widhopf, Rader, Steinberger *et al.* 2000). This technology allows for the display of proteins on the surface of filamentous phage such as M13, fd and f1 (McCafferty, Griffith, Winter & Chiswell 1990). In the phage-display screening format, antibodies fused to the capsid or coat proteins of filamentous bacteriophage are displayed for targeted selection on the phage particles that also encapsulate the related genes (Smith 1985; Parmley & Smith 1988). In the single chain variable fragment (scFv) format, the recombinant antibody is comprised of the variable heavy (V_H) and light (V_L) regions connected by a short peptide linker (Bird, Hardman, Jacobson *et al.* 1988; Bird & Walker 1991). Despite removal of the constant regions and the incorporation of a linker, the scFv retains the specificity of the original immunoglobulin (IgG) (Bird & Walker 1991).

In recent years, as reviewed by Bradbury & Marks (2004a), the phage-display system (Smith 1985) has been used to generate novel single chain antibodies that have high affinity with many antigens. Many researchers have isolated antibodies from naïve antibody libraries using phage-display (McCafferty *et al.* 1990; Pini & Bracci 2000). Immune libraries can also be used to generate antibodies by phage-display. Unlike naïve libraries, immune libraries are created from, amongst others, immunized humans (Vaughan, Williams, Pritchard *et al.* 1996), mice (Ames, Tornetta, Jones & Tsui 1994) or chickens (Andris-Widhopf *et al.* 2000).
In this study a phage-displayed scFv library was constructed from a chicken immunized with recombinant white rhinoceros (*Ceratotherium simum*) interferon-gamma (rRhIFN-γ). This library was used to select scFvs specific for both rRhIFN-γ and recombinant Asian elephant

(*Elephas maximus*) IFN-γ (rAsEpIFN-γ). This chapter describes the construction of an immune phage-displayed antibody library, generation of recombinant antibodies and the potential application of these reagents in an IFN-γ capture enzyme-linked immunosorbent assay (ELISA) that is able to detect recombinant antigens.

3.2 Materials and Methods

3.2.1 Immunization of chickens

Two adult silver-hyline chickens were immunized intramuscularly with 50 µg rRhIFN-γ (see 2.2.2) emulsified with an equal volume of Montanide ISA 70 adjuvant. In Week 4 the first boost immunization of 50 µg antigen was administered. The second boost immunization was administered in Week 10, this time with 100 µg of antigen. The final boost immunization was administered in Week 16 and consisted of 100 µg of antigen. The chickens were euthanased one week later and their spleens were removed for the isolation of B-cells. The immunization protocols were approved by the Animal Use and Care Committee (AUCC) of the Faculty of Veterinary Science, University of Pretoria.

3.2.2 Isolation of yolk immunoglobulin (IgY) from chickens immunized with rRhIFN-γ

Eggs from the chickens were collected on a daily basis prior to and after immunization. Yolk immunoglobulin, produced at the University of Pretoria (UP, IgY^{up}), was isolated from the eggs using a polyethylene glycol (PEG) precipitation method as described by Polson, Coetzer, Kruger *et al.* (1985). The IgY^{up} was resuspended in phosphate buffer saline solution (PBS) and stored at -20°C, to be used later in an ELISA to determine anti-IFN-γ IgY^{up} titres of the immunized chickens. The concentration of the IgY^{up} was determined with a UV/V spectrophotometer (Beckman Coulter™ DU®530) at an absorbance of 280 nm. For protein quantification an optical density (OD) reading of 1 is equal to 1.4 mg/m*l* protein.

3.2.3 ELISA to determine antibody titres of chicken immunized with rRhIFN-γ

Antibody titres in the yolk samples induced by the immunization protocol were determined in a direct ELISA. Maxisorp ELISA (Nunc) plates were coated overnight with 1 µg/m*l* of rRhIFN-γ in PBS. The next day the coating buffer was discarded and 100 µ*l* of blocking buffer comprising 2% fat-free milk powder (MP) PBS, were added to each well. This was

incubated for 1 h at 37°C. Wells were washed five times with tap water. The IgY^{up} isolated in 3.2.2, was diluted in blocking buffer to a final concentration of 5 µg/m*l* before being added to the respective wells and incubated for 1 h at 37°C. The wash step was repeated using tap water + 0.1% Tween 20. For detection, rabbit anti-chicken IgY conjugated to horse radish peroxidase (HRP) (Abcam), diluted 1:3000 in 2% blocking buffer + 0.1% Tween 20, was used. After incubation of 1 h at 37°C the wash step was repeated. Substrate *ortho*-phenylenediamine (OPD) (Sigma) was prepared with 5 mg OPD tablet, 10 m*l* 0.1 M citrate buffer pH 4.5 and 0.5 µ*l*/m*l* of 30% ($^{v}/_{v}$) H_2O_2. Prepared substrate was added to each well and after 30 min the reaction was stopped using 2 N H_2SO_4. The absorbance was read at 492 nm.

3.2.4 Isolation of total RNA from chicken spleen

After removing the spleens from the two chickens, they were placed in RNAlater solution (Ambion). The RNA was isolated from each spleen with Qiazol™ Lysis reagent (QIAgen). The spleens were sliced into small pieces and placed in 1.5 m*l* of Qiazol reagent. With the use of a mortar and pestle, the cells were homogenized. The homogenate was incubated at room temperature for 5 min to promote dissociation of nucleoprotein complexes. After the incubation, 300 µ*l* of chloroform were added to the homogenized cells and this was mixed vigorously for 15 s and subsequently left at room temperature for 2-3 min. The homogenate was centrifuged at 12 000xg for 5 min at 4°C. As a result the sample separated into three phases; an upper, colourless, aqueous phase containing the RNA, a white interphase, and a lower red organic phase. The aqueous phase was transferred to a new collection tube and to this 750 µ*l* of isopropanol were added and mixed by vortexing. The tube was then placed at room temperature for 10 min before being centrifuged at 12 000xg for 10 min at 4°C. The supernatant was discarded and the RNA pellet was visible as a white, almost gel-like, pellet at the bottom of the tube. The RNA was washed with 75% ethanol and centrifuged at 7 500xg for 5 min at 4°C. The ethanol was discarded and the RNA pellet was briefly air-dried after which it was resuspended in 300 µ*l* RNase-free water. Half of this volume of RNA was further purified using a Rneasy Minelute kit (QIAgen). For determination of RNA concentration a reading of 1 at an absorbance of 260 nm is equal to 40 µg/m*l*. The final RNA concentration, which was determined using the Nanodrop, was 1858 ng/µ*l*. The RNA was stored in aliquots of 5 µ*l* at -70°C.

3.2.5 cDNA synthesis

First strand cDNA was synthesized using the TaKaRa RNA PCR kit (AMV) ver 3.0. The procedure was performed according to the manufacturer's instructions. For this, 400 µg of total RNA was mixed with 1 µl of 10 X RT buffer, 2 µl of 5 mM $MgCl_2$, 1 µl of 1 mM deoxynucleotide mix, 2 µl 0.125 µM oligo-dT-adapter primer, 0.25 µl of RNase inhibitor, 2 µl of 2.5 units (U) of AMV Reverse Transcriptase XL (TaKaRa) and RNase free water to a final volume of 10 µl. The reaction mixture was incubated for 10 min at 30°C, followed by 1 h incubation at 42°C. The reaction was terminated by heating at 95°C for 5 min, followed by 5 min at 5°C. The first strand cDNA was used directly for amplification by the polymerase chain reaction (PCR).

3.2.6 Amplification of V_H and V_L chain genes

The libraries were generated as described by van Wyngaardt, Malatji, Mashau *et al.* (2004). Briefly, the cDNA that was synthesized in 3.2.5 was used to amplify the antibody variable domains, V_H and V_L respectively. The primary PCR described below amplified these two regions separately with overlapping linker regions added at the 3'end of the H chain and the 5'end of the L chain. The second PCR, called splicing by overlap extension (SOE) PCR, combined the V_H and V_L via the added overlapping region consisting of a glycine and serine flexible linker $(Gly_4Ser)_3$ to form a full length scFv fragment. Amplification reactions were set up in a total volume of 50 µl containing 10 µl of cDNA, 10 µl of 5 X PCR buffer, 10 pmol of each primer and 0.25 µl of 2.5 U of TaKaRa Ex Taq polymerase. The V_H gene was amplified in the primary PCR using the Sfi1L sense primer and NEWLVarH antisense primer (sequences shown in Table 3.1). The Sfi1L primer adds a Sfi1 site to the 5' side of the coding strand of the V_H chain. The V_L gene was amplified in the primary PCR using the LCNOT1 and NEWLVarL primers. The NEWLVarH primer and the NEWLVarL primers add parts of the $(Gly_4Ser)_3$ linker sequence to the 3' side of the coding strand of the V_H chain and the 5' side of the coding strand of the V_L strand respectively. The reactions were performed in an Eppendorf Mastercycler under the following conditions: 30 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min followed by a final extension at 72°C for 5 min. The amplified products were analysed on a 1% agarose gel. Amplification was repeated for increased amounts of V_H and V_L products. The final V_H and V_L products of approximately 420 and 340

basepairs (bps) respectively were gel purified from a 1.5% crystal violet agarose gel (Rand 1996) using the QIAquick gel extraction kit (QIAgen).

Table 3.1 Primers used for V_H and V_L region amplification (van Wyngaardt *et al.* 2004)

NEWLVarH 5'CCGCCAGAGCCACCTCCACCTGAACCGCCTCCACCGGAGGAGACGATGACTTCGG 3'
SfiIL 5'GTCCTCGCAACTGCGGCCCAGCCGGCCCTGATGGCGGCCGTGACG 3'
NEWLVarL 5'TCAGGTGGAGGTGGCTCTGGCGGAGGCGGATCGGGCGCTGACTCAGCCGTCCTCGG 3'
LCNOT I 5'TGATGGTGGCGGCCGCATTGGGCTG 3'

Using the SOE PCR the DNA sequences coding for the V_H and V_L chains were joined by means of a DNA sequence coding for a 15 amino acid (aa) $(Gly_4Ser)_3$ linker. The SOE PCR was performed in two steps. The first PCR consisted of 10 µl 10 X TaKaRa buffer, 8 µl TaKaRa dNTPs, 100 ng V_H and V_L chain DNA, 0.3 µl of 2.5 U TaKaRa Ex Taq DNA polymerase, 0.5 µl of 3 U Promega *Pfu* DNA polymerase (with proof reading activity) and water to a final volume of 50 µl. The reactions were carried out in an Eppendorf Mastercycler: 15 cycles of amplification (94°C for 1 min, 60°C for 1 min, 72°C for 2 min) and a final extension at 72°C for 5 min. In the second step a pullthrough PCR, was performed, which consisted of 10 µl of 10 X TaKaRa PCR buffer, 8 µl TaKaRa dNTPs, 10 pmol Sfi1 primer, 10 pmol of LCNOT1 primer, 4 µl spliced product, 0.3 µl of 2.5 U TaKaRa Ex Taq DNA polymerase and water to a final volume of 50 µl. The pullthrough reactions were run for a further 25 cycles under the same conditions as mentioned above. The PCR products were analysed on a 0.8% agarose gel.

Before purifying the PCR products from a 1.5% crystal violet agarose gel the DNA was precipitated by adding an equal volume of 70% isopropanol to the PCR products and mixed. This solution was centrifuged for 5 min at 13 000 rpm at 4°C. The supernatant was discarded and the pellet was washed with 150 µl of 70% ethanol. The centrifugation step was repeated and the ethanol was removed. The pellet was air-dried for 5-10 min at room temperature. The DNA pellet was resuspended in 30 µl of TE (Tris, EDTA) buffer and purified from a 1.5% crystal violet agarose gel using the QIAquick protocol as mentioned above. Approximately 500 ng of the purified product (joined V_H and V_L genes) was digested overnight at 50°C with 4 µl of 10 U/µl Sfi1 (Roche) in 10 µl Buffer M (Roche) and 1 µl of 10 mg/ml of acetylated

bovine serum albumin (BSA). For the overnight digestion at 37°C with Not1, 6 µl of 2.5 M NaCl, 12 µl of 1 M Tris pH 8.0, 1 µl of 10 mg/ml of acetylated BSA and 2 µl of 40 U/µl Not1 (Roche) was used. The digested DNA was gel purified as mentioned above. The phagemid vector, pHEN I, was digested in the same manner as the joined V_H V_L fragment.

The next step was to ligate the joined DNA fragment into the phagemid vector (pHEN I, Fig. 3.2). In the first approach a rapid DNA ligation kit (Roche) was used. The vector:insert ratio was 2:1. The rapid ligation reaction was performed for 5 min at room temperature. The ligated products were transformed into *Escherichia coli* TG1 competent cells. The TG1 cells were prepared according to Inoue, Nojima & Okayama 1990, using heat shock treatment at 42°C for 30 s and then snapped cooled on ice to transfer the DNA into the cells. To increase the efficiency of the transformation a second approach was used. In this approach the ligation step was performed with T4 DNA ligase (Roche) overnight at 15°C. After the overnight incubation the ligation reaction was purified using the QIAquick PCR purification kit to remove contaminating salts. This was followed by a desalting step in which the ligated product was incubated in agarose columns for 90 min on ice (Atrazhev & Elliott 1996). These steps would prevent arcing during the electroporation of the ligated product. The desalted ligated product was electroporated into Epicuran Coli Electroporation-Competent TG1 cells (Stratagene, USA). The electroporator (Biorad Gene Pulser IT) was set at 1700 V, 200 Ω and 25 µF. In both transformation experiments the transformed TG1 cells were incubated at 37°C in a shaking incubator for 1 h. They were plated onto TYE agar (15 g agar, 10 g tryptone, 5 g yeast extract and 8 g NaCl in 1 *l* of double distilled deionised water) plates supplemented with 100 µg/ml ampicillin (A) and 2% glucose (G), for the selection of transformed cells.

To determine the library size serial dilutions (ten-fold) of the transformed cells were plated and incubated at 30°C overnight. The remainder of the transformation was also plated and incubated under the same conditions. The next day the library size was determined by counting the number off colonies that grew at a specific dilution. The bacteria were scraped of all the plates in 2xTY medium (16 g tryptone, 10 g yeast extract, 5 g NaCl dissolved in 1 *l* of double distilled deionised water) supplemented with 100 µg/ml A and 2% G. Glycerol (60%) was added to the bacterial suspensions. The samples were stored at -70°C. An aliquot, taken before the addition of glycerol, was used to inoculate 500 ml of 2xTY medium supplemented with 100 µg/ml A and 2% G and was incubated at 37°C in a shaking incubator

for 2 h or until the OD_{600} was between 0.45 and 0.55. When the required OD_{600} was reached 100 m*l* of the medium was infected with 80 µ*l* of 4 X 10^{10} phages/m*l* of [1]M13K07 helper phage. After a 30 min incubation at 37°C the cells were collected by centrifugation and resuspended in 200 m*l* pre-warmed 2xTY medium containing 100 µg/m*l* of A and 25 µg/m*l* of kanamycin (K). The culture was incubated overnight at 30°C with vigorous shaking. The next day the phages were recovered from the supernatant by precipitation at 4°C with $^1/_5$ volume 20% PEG in 2.5 M NaCl and collected by centrifugation at 1 700 rpm for 10 min. Precipitated phages were resuspended in PBS and stored at -70°C in 15 % glycerol.

3.2.7 Selection of phage antibodies (Biopanning)

Although the chickens were only immunized with rRhIFN-γ, the library was panned against rAsEpIFN-γ (see 4.2.5) as well, to see if cross-reactive clones could be obtained due to these genes sharing a homology of 89% on the nucleotide (nt) level and a 75% identity on the aa level (Fig 3.1). Maxisorp immunotubes (Nunc) were coated overnight at 4°C with 10 µg/m*l* of rRhIFN-γ or rAsEpIFN-γ diluted in PBS (Sigma). Tubes were blocked for 1 h with 2% MP in PBS. After blocking, the tubes were washed three times with PBS + 0.1% Tween 20. Panning was performed according to the protocol as described by van Wyngaardt *et al.* (2004). Briefly, 1 m*l* of PEG precipitated phages that were mixed with 3 m*l* 4% MP + PBS + 0.2% Tween 20, was added to each tube and incubated on a rotator for 30 min and left to stand at room temperature for 90 min. Subsequently the tubes were washed 20 times with PBS + 0.1% Tween 20, followed by another 20 washes with PBS. Phages were eluted using 1 m*l* 100 mM triethylamine (TEA) and neutralized with 500 µ*l* 1 M Tris pH7. The phages (1.5 m*l*) and 4 m*l* of 2xTY+AG were added to 5 m*l* of exponentially growing TG1 cells and incubated at 37° C to allow the phage to infect the cells for 30 min. Aliquots were plated onto TYE agar plates to determine phage titres and the rest were plated onto 13 cm panning plates. After an overnight incubation at 30°C the bacteria were scraped off the plates and an aliquot was used to inoculate fresh 2xTY+AG. The rest of the bacterial supernatant was made into a glycerol stock and stored at -80°C. Phages were rescued by the addition of M13K07 helper

[1] Phagemids cannot produce infective particles on their own. They require helper phages to provide the genes which are essential for phage replication and assembly, including a wild-type copy of the coat protein used for display (Carmen & Jermutus 2002).

phage (1:20 bacteria:helper phage). Four rounds of panning were performed as described for the library rescue.

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RhIFN-γ    MNYTSFILAFQLCVILGSPSYYCQAVFFKEIENLKEYFNASNPDVADGGSLFLDILKNWK  60
           MN+TS+ILAFQLC+ILGS S YCQA F KEI+NLKEY NA++ DVADGG LF+DILKNWK
AsEpIFN-γ MNFTSYILAFQLCIILGSSSCYCQATFLKEIQNLKEYLNATDSDVADGGPLFIDILKNWK  60

RhIFN-γ    EESDKKIIQSQIVSFYFKLFENLKDNQVIQKSMDIIKEDLFVKFFNSSTSKLDDFKKLIQ  120
           E+SDKKIIQSQIVSFY K+F+NLKDNQVIQ+S+  ++EDLFVKFFNSS+SK DDF K++Q
AsEpIFN-γ EDSDKKIIQSQIVSFYLKIFDNLKDNQVIQESVKTLEEDLFVKFFNSSSSKRDDFLKVMQ  120

RhIFN-γ    IPVDDLQVQRKAISELIKVMNDLSPRSNLRKRKRSQGQFRGRRA  164
            PV+D  VQRKAISEL KVMNDLS RSN  KRKR Q  FRGRRA
AsEpIFN-γ TPVNDRNVQRKAISELSKVMNDLSHRSNGAKRKRRQYSFRGRRA  164
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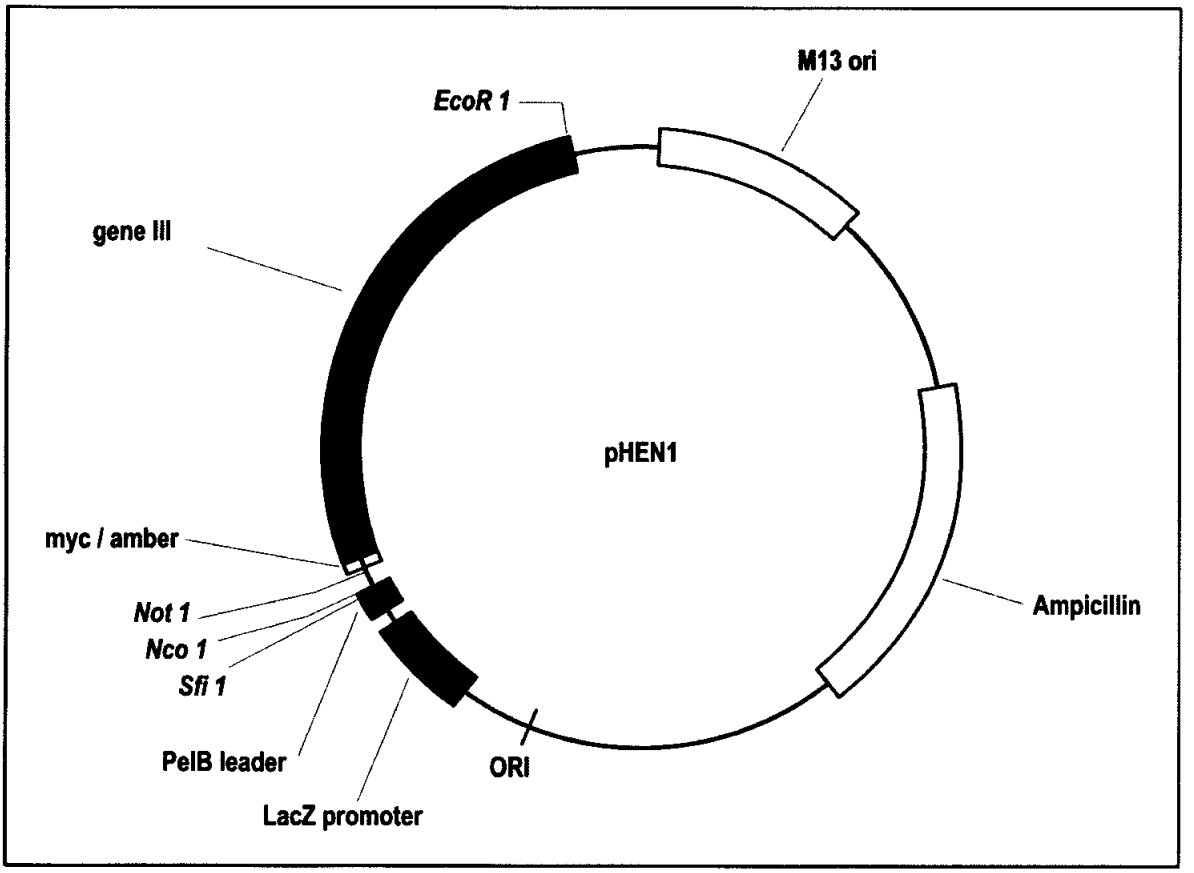
Figure 3.1 Alignment of predicted sequences for white RhIFN-γ and AsEpIFN-γ. Amino acid identities are shown in blue and a plus sign denotes a conserved substitution. The predicted signal sequence of RhIFN-γ is underlined. A blast search of the white RhIFN-γ sequence with the AsEpIFN-γ (see 4.3.2) sequence demonstrated an identity of 89% on the nt level and 75% identity on the aa level

3.2.8 Polyclonal phage ELISA

Maxisorp ELISA (Nunc) plates were coated with rRhIFN-γ or rAsEpIFN-γ at 10 µg/m*l* in PBS in a final volume of 50 µ*l*/well and incubated overnight at 4°C. The next day the overnight solution was discarded. Blocking buffer (2% MP in PBS) was added to each well (300 µ*l*) and incubated for 1 h at 37°C. After incubation the wells were washed three times with PBS + 0.1% Tween 20. The PEG precipitated phages obtained after each round of panning was used for the polyclonal ELISA. Phage was concentrated 25 times by PEG precipitation and diluted 1:100 in PBS prior to mixing with an equal volume of 4% MP + PBS + 0.2% Tween 20. From this 50 µ*l* aliquots were tested in ELISA. The plate was incubated at 37°C for 1 h. The wash step was repeated. Mouse monoclonal antibody B62-FE2 (Progen Biotechnik, GmbH), specific for the phage, was diluted to 1:1000 in blocking buffer + 0.1% Tween 20. This was added to the wells (50 µ*l*/well) and incubated for 1 h at 37°C. The wash step was followed by the addition of swine anti-mouse IgG HRP conjugate (Dako, P0260) diluted 1:1000 in 2% MP + 0.1% Tween 20. After a 1 h incubation at 37°C the final wash step was performed. Substrate (OPD) solution was added. The plates were incubated at room temperature for 1 h to allow colour development. Absorbance was measured at 492 nm after stopping the reaction with 2 N H_2SO_4.

3.2.9 Monoclonal phage ELISA

For the monoclonal phage ELISA individual colonies from the different rounds of panning were inoculated into 150 µl of 2xTY+AG medium, in a 96-well culture plate, grown overnight at 200 rpm. Aliquots of 2 µl were transferred using a replica transfer device (Sigma) to a new 96-well culture plate containing 150 µl 2xTY+AG and allowed to grow for 2.5 h in a shaking incubator at 37°C. After the incubation aliquots of 50 µl of 2xTY+AG containing 2 X 10^{12} phages/ml M13K07 helper phage were added to the wells. Cultures were incubated at 37°C for 30 min and then centrifuged at 1 700 rpm for 10 min. The glucose medium was removed with a pipette and the bacterial pellets were resuspended in 2xTY+A and 25 µg/ml K. The plates were incubated at 30°C overnight with shaking at 200 rpm. The next day the cells were pelleted and the phage-containing supernatant was used for analysis in an ELISA. The protocol was the same as that used for the polyclonal phage ELISA except that the supernatant was diluted 1:1 with 4% MP + PBS + 0.2% Tween 20. A direct ELISA was performed, using the same steps as performed in the monoclonal phage ELISA, to evaluate the specificity and or cross-reactivity of the phage-displayed antibodies to the recombinant antigens. Sixteen clones were selected for screening by direct ELISA.

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----------pelB leader-----|
 L  L  A  A  Q  P  A  M  A  Q  V  Q  L  Q  V  D  L  E  I  K  R
TTACTCGCGGCCCAGCCGGCCATGGCCCAGGTGCAGCTGCAGGTCGACCTCGAGATCAAACGG
            SfiI   NcoI          PstI   SalI  XhoI
          |---------cmyc tag-------------|
 A  A  A  E  Q  K  L  I  S  E  E  D  L  N  G  A  A (E) T  V  E
GCGGCCGCAGAACAAAAACTCATCTCAGAAGAGGATCTGAATGGGGCCGCATAGACTGTTGAA
  NotI                                          amber stop codon
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Figure 3.2 Schematic representation of the phagemid vector pHEN1. The pelB leader directs the secretion of scFv-pIII fusions to the periplasm. ScFv are cloned into the vector as Sfi1-NotI fragments. The c-myc epitope tag is used for detection of expressed scFv and the amber stop codon allows simple switching between displayed scFv and secreted scFv. The vector polylinker sequence is shown below the vector map (Bradbury & Marks 2004b)

3.2.10 Screening of soluble scFv antibody fragments by ELISA

Selection of single colonies was based on the results of the monoclonal phage ELISA (see 3.2.9). Sixteen colonies were selected for the screening. Five millilitres of 2xTY+AG medium were inoculated with single colonies. Inoculated samples were incubated overnight at 30°C with vigorous shaking. Aliquots of the overnight cultures were diluted 1:100 into fresh 2xTY+AG and incubated at 37°C in a shaking incubator for 2-3 h until the OD_{600} reached 0.9. The samples were centrifuged to pellet the bacteria and the glucose medium was discarded. The pellet was resuspended in medium containing 100 µg/m*l* A and 1 mM isopropylthio-β-D-galactosidase (IPTG) and shaken at 30°C for 16 h to induce expression of soluble scFv protein. Cells were pelleted and the supernatant contained the secreted antibody fragment. A higher concentration of soluble scFv is present in the periplasmic protein, therefore the cell pellet was resuspended in one tenth of the volume of ice cold PBS containing 1 M NaCl and 1 mM ethylenediaminetetraacetic acid (EDTA) and left on ice for 30 min. This was followed by a centrifugation step at 4°C for 10 min at 6 000 rpm to remove the cells. The resulting supernatant was transferred to a clean tube and further centrifuged at 14 000 rpm for 10 min to remove the debris. The supernatant was transferred to a clean tube and contained the periplasmic protein fraction. Screening of individual soluble scFvs was performed in a similar manner to the procedure of the monoclonal phage ELISA but was adapted as follows: Tween 20 was not included in the diluents, the wash buffer was PBS + 0.05% Tween 20, and detection of soluble scFvs was done using anti-c-myc monoclonal antibody 9E10-HRP (Roche).

3.2.11 DNA sequencing

Once the phage libraries were constructed, random single colonies were selected from titre plates and grown in 2xTY+AG medium overnight at 30°C, with vigorous shaking. The next day the plasmid DNA was isolated using the QIAprep spin miniprep kit (QIAgen). The DNA was sequenced at the Onderstepoort Veterinary Institute (OVI) sequencing facility. The sequences were analysed using the BioEdit software package. Sequencing primers OP52 (5' CCCTCATAGTTAGCGTAACG 3') and M13rev (5' CAGGAAACAGCTATGAC 3') were used (van Wyngaardt *et al.* 2004). The DNA of phagemid clones from selected positive scFvs were also prepared and sequenced using the same method.

3.2.12 Purification of soluble scFvs

Two scFvs, 1F5 and 6D7, were selected to be purified using the Aminolink Plus Immobilization Kit (Pierce Biotechnology). The anti-cmyc monoclonal antibody (Roche) was immobilized to the column, followed by scFv purification. The purification was carried out according to the manufacturer's instructions. The different elution fractions that were collected were tested in a direct ELISA to determine which fractions contained the highest amount of protein. Fractions with high amounts of protein were pooled and added to a pre-hydrated dialysis cassette (Side-A-Lyzer® dialysis cassette, Pierce Biotechnology). The cassette was incubated in a beaker containing 1 *l* of PBS with a magnet and placed on a magnetic stirrer at 4°C. The buffer was changed after 1 h and this was repeated twice. The last incubation was overnight. The next day the dialysed protein samples were removed from the cassette and stored at -20°C in 200 µ*l* aliquots. The concentration of the purified proteins was quantitated at an absorbance of 280 nm.

3.2.13 Different formats of the IFN-γcapture ELISA

Various forms of capture ELISAs were performed to determine which would give the ideal results. In the first format, the purified scFvs were used as a capture antibodies and the detecting antibody was a mouse monoclonal antibody, 1D11. Antibody 1D11 is a subclone of 1H11. Monoclonal antibody 1H11 was produced against rRhIFN-γ (see 2.3.3). In the second format, the detecting antibody was replaced with polyclonal chicken IgY produced at the Utrecht University (UU, IgY^{uu}) to IFN-γ (see 2.2.3). The methods of the different ELISAs that were used are described below.

a) *scFv Capture ELISA using mouse monoclonal antibody 1D11 as detecting antibody*

Maxisorp ELISA (Nunc) plates were coated with purified scFv 1F5 or 6D7 (see 3.2.12) at a concentration of 5 µg/m*l* and incubated at 4°C overnight. The next day the coating buffer was discarded and 300 µ*l* of 2% BSA in PBS were added to each well. The plates were incubated for 1 h at 37°C. After the incubation the blocking buffer was discarded and the plates were washed three times with PBS + 0.05% Tween 20. Next, the recombinant antigens (rRhIFN-γ or rAsEpIFN-γ) were added, diluted two-fold in 2% BSA in PBS with a starting concentration of 5 µg/m*l*. The incubation step was repeated as previously and it was followed by the same wash steps. Antibody 1D11, diluted in 2% BSA in PBS, was added to the

respective wells and also diluted from a starting concentration of 5 µg/m*l*. Incubation and wash steps were repeated. Polyclonal rabbit anti-mouse IgG HRP (Dako, Cytomation) was diluted 1:1000 in 2% BSA in PBS and added to the wells. This was followed by the incubation and wash steps. The substrate (OPD) was prepared as mentioned in 3.2.3 and added to the wells. The plates were left at room temperature for 1 h to allow colour development. The reaction was stopped with 2 N H_2SO_4 and the absorbance was measured at 492 nm. A signal (OD) of 0.8 and / or higher was considered a strong signal in these ELISAs.

b) *scFv Capture ELISA using polyclonal chicken IgY^{uu} as detecting antibody*

The same ELISA as above (a) was performed but antibody 1D11 was replaced with the chicken polyclonal IgY^{uu}. The latter was diluted two-fold with a starting concentration of 5 µg/m*l* and ending with a concentration of 0.075 µg/m*l*. For detection, polyclonal rabbit anti-chicken to IgY (Abcam) (1:3000) was used. This ELISA was repeated with scFv 6D7 as a capture antibody.

3.3 Results

3.3.1 Antibody titres

Chickens were immunized with rRhIFN-γ. After the first immunization there was an increase in the antibody titre on Day 7. The first boost immunization was performed in Week 4, the second in Week 10 and the last one in Week 16. The boost immunization in Week 10 was successful in increasing the titre as the ELISA results (Fig. 3.3) show this increase from Day 42 to Day 98, just before the animals were euthanased. Between Day 23 and Day 42 eggs contained a constant low level of anti-RhIFN-γ IgY^{up}.

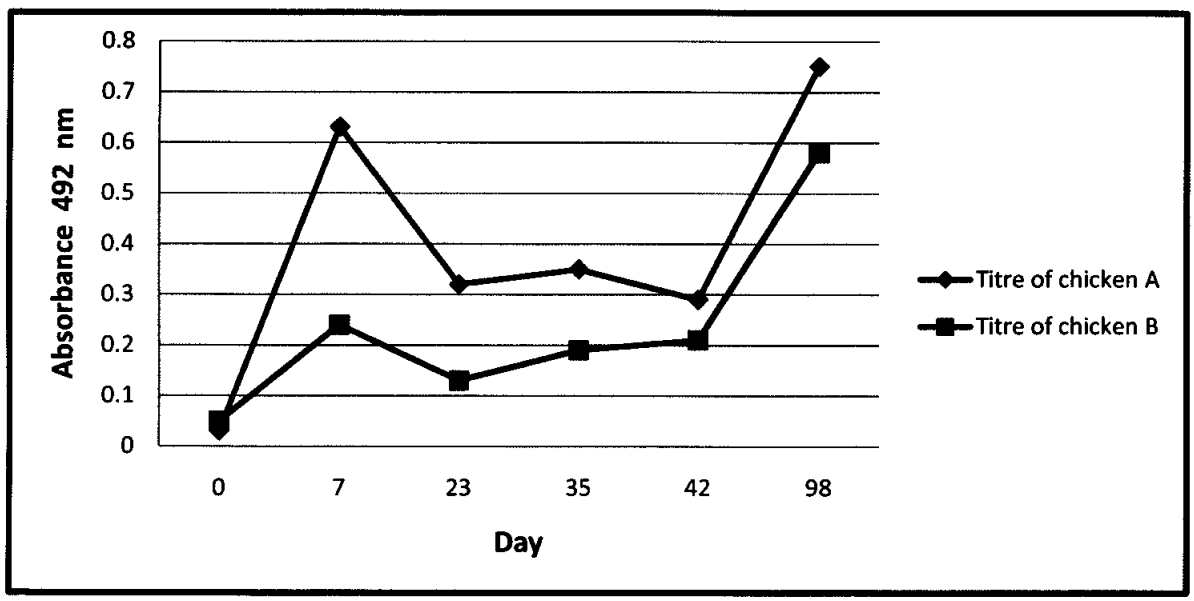

Figure 3.3 ELISA showing the antibody titres of immunized chickens from Day 0 to Day 98

3.3.2 Library construction

After confirmation that the chickens produced antibodies against rRhIFN-γ, they were euthanased and their spleens removed. RNA was extracted from their combined spleens and used to make cDNA from which the antibody variable regions were amplified by PCR. The size of the V_H fragment was 410 bps and that of the V_L fragment was 340 bps (Fig. 3.4).

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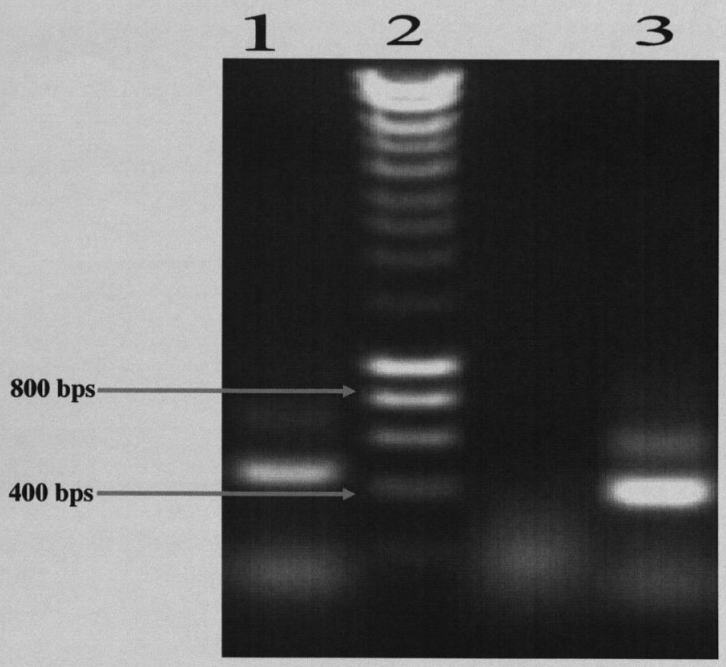

Figure 3.4 Agarose gel of the primary PCR for the amplification of V_H and V_L chains. **Lane 1:** V_H (410 bps) PCR product; **Lane 2:** Molecular weight marker (Hyperladder I, Bioline); and **Lane 3:** V_L (340 bps) PCR product

These fragments were joined via a linker sequence using a SOE PCR. After the SOE reaction the product size was approximately 800 bps (Fig. 3.5). The joined V_HV_L fragment was cloned in the pHEN1 phagemid vector. Two different ligation reactions were performed before the transformation of the resulting plasmids into *E. coli*. With the rapid ligation technique the size of the the library after transformation yielded 7 X 10^5 clones/m*l*. Switching to an overnight ligation reaction increased the number of clones generated after transformation. The size of this library was 2.4 X 10^7 clones/m*l*.

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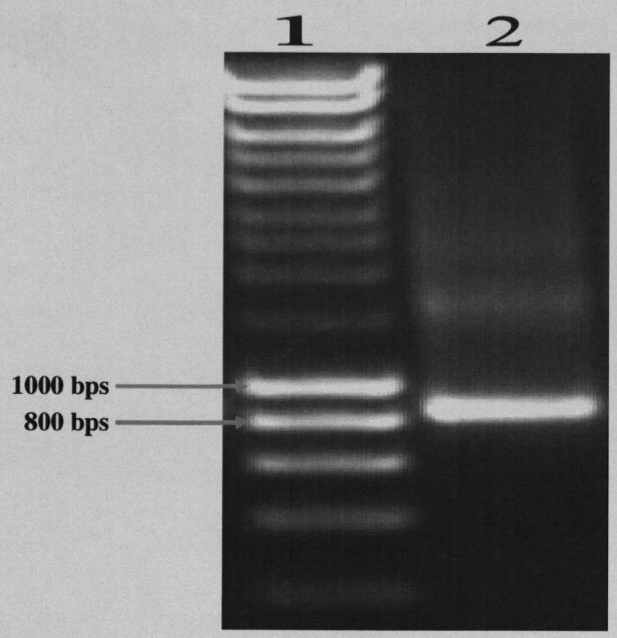

Figure 3.5 Agarose gel of the SOE PCR. **Lane 1:** Molecular weight marker (Hyperladder I, Bioline) and **Lane 2:** Joined V_H and V_L product (approximately 800 bps)

3.3.3 Selection of binders

For each recombinant antigen four consecutive rounds of panning were performed to select for specific binders against each antigen. In order to monitor the progress of the bio-panning procedure the percentage yield of phage per panning was calculated. These results showed enrichment of phage after four rounds of panning. The percentage output from each round of panning is presented in Table 3.2. Percentage output refers to the number of clones that were obtained after each round of panning.

Table 3.2 The percentage output of phages recovered after each selection round

Recombinant Elephant IFN-γ		**Recombinant Rhinoceros IFN-γ**	
Round of panning	**% Output**	**Round of panning**	**% Output**
1st	1.1×10^{-4}	1st	0.001
2nd	1.7×10^{-4}	2nd	0.23
3rd	0.082	3rd	0.29
4th	0.23	4th	0.70

The specificity of the enrichment was confirmed by increasing reactivity of the phages in the polyclonal ELISA (Fig. 3.6 and 3.7) to IFN-γ. In the polyclonal phage ELISA high signals were also obtained with the original unpanned library. However, when individual clones from the unpanned library were selected for the monoclonal phage ELISA none of the clones showed reactivity to IFN-γ (results not shown).

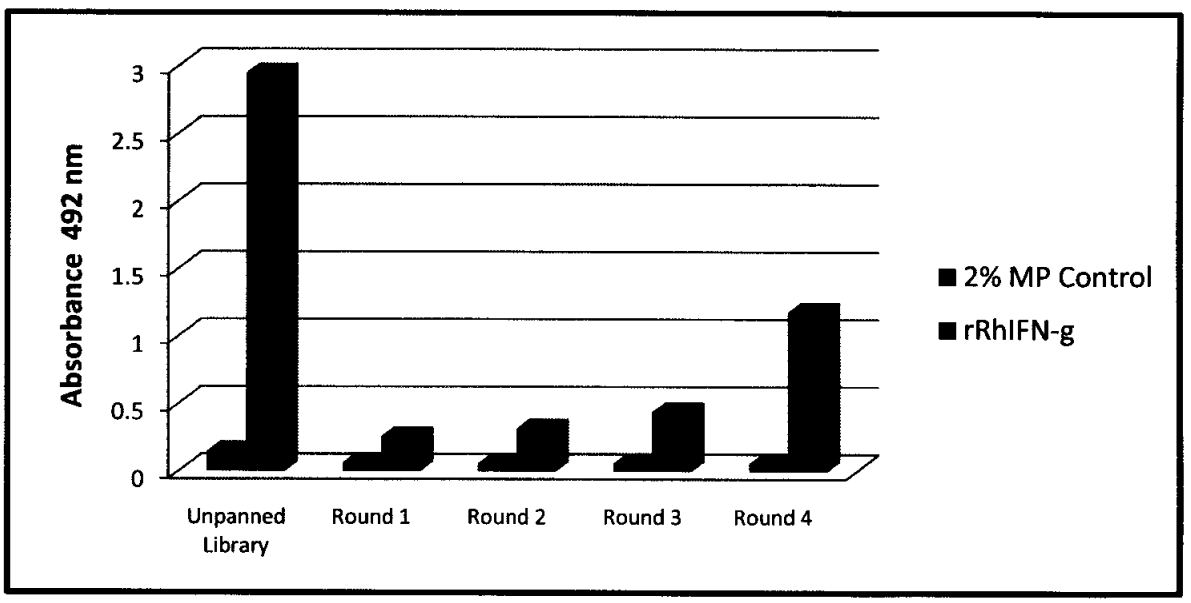

Figure 3.6 Polyclonal phage ELISA of phage pools obtained from different rounds of panning reacting to rRhIFN-γ

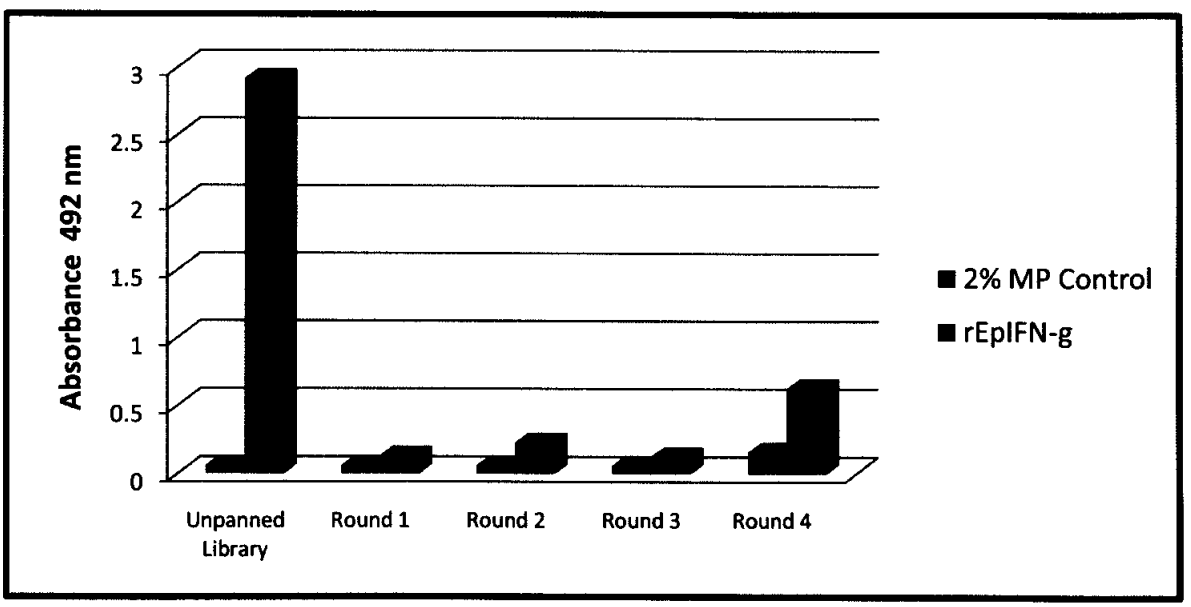

Figure 3.7 Polyclonal phage ELISA of phage pools obtained from different rounds of panning reacting to rAsEpIFN-γ

Table 3.3 summarizes the results obtained in the monoclonal phage ELISA which was used for the screening of individual binders to respective recombinant antigens. The number of clones that were tested from each round of panning for the respective antigens are also indicated in Table 3.3. Ten clones reacted with rAsEpIFN-γ, producing good signals in the monoclonal phage ELISA; but one clone, 6D9, proved to be an MP binder and was not characterized further. The remainder of the clones were sequenced. Seven clones, which reacted with rRhIFN-γ, were identified in the monoclonal phage ELISA and were selected to be sequenced.

Table 3.3 Clones that were identified as binders from four rounds of panning against the recombinant proteins

Selection rounds against rAsEpIFN-γ	**Number of clones tested**	**Binders**	**Clones**
Round 0	94	0	
Round 1	47	0	
Round 2	96	5	3B1, 3B2, 3B3, 3B4, 3C2
Round 4	47	5	6C8, 6D7, *6D9, E610, 6F10
Selection rounds against rRhIFN-γ	**Number of clones tested**	**Binders**	**Clones**
Round 0	96	0	
Round 1	47	0	
Round 3	96	4	1D10, 1E10, 1F5, 1H2
Round 4	47	3	4B10, 4C9, 4D12

*6D9 was a milk powder binder

3.3.4 Antigen specificity and cross-reactivity of phage-displayed antibodies and scFvs

Individual clones were induced to produce phage-displayed antibodies and soluble scFvs. Supernatants from 16 clones containing the phage-displayed antibodies and the soluble scFvs were screened in a direct ELISA to select antigen specific and cross-reactive antibodies. Results for the phage-displayed antibodies show that clones 4B10, 4C9 and 4D12, which were obtained from Round 4, show specificity to rRhIFN-γ. The anti-rAsEpIFN-γ antibodies, (1F5, 6C8, 6D7, E610 and 6F10), cross-react with rRhIFN-γ. These phage-displayed antibodies, with the exception of 1F5, show stronger reactivity to rAsEpIFN-γ than rRhIFN-γ in the direct ELISA (Fig. 3.8). Results of the direct ELISA against rRhIFN-γ and rAsEpIFN-γ using scFv antibodies indicate strong signals with most scFvs except for 4B10 and 4D12 (Fig. 3.9). There is no scFv that shows specificity to either recombinant antigen but stronger signals are obtained with 1F5, 6C8, 6D7, E610 and 6F10 against rRhIFN-γ compared to rAsEpIFN-γ. The scFv format of the antibodies shows a greater degree of cross-reactivity between the two recombinant antigens in the direct ELISA.

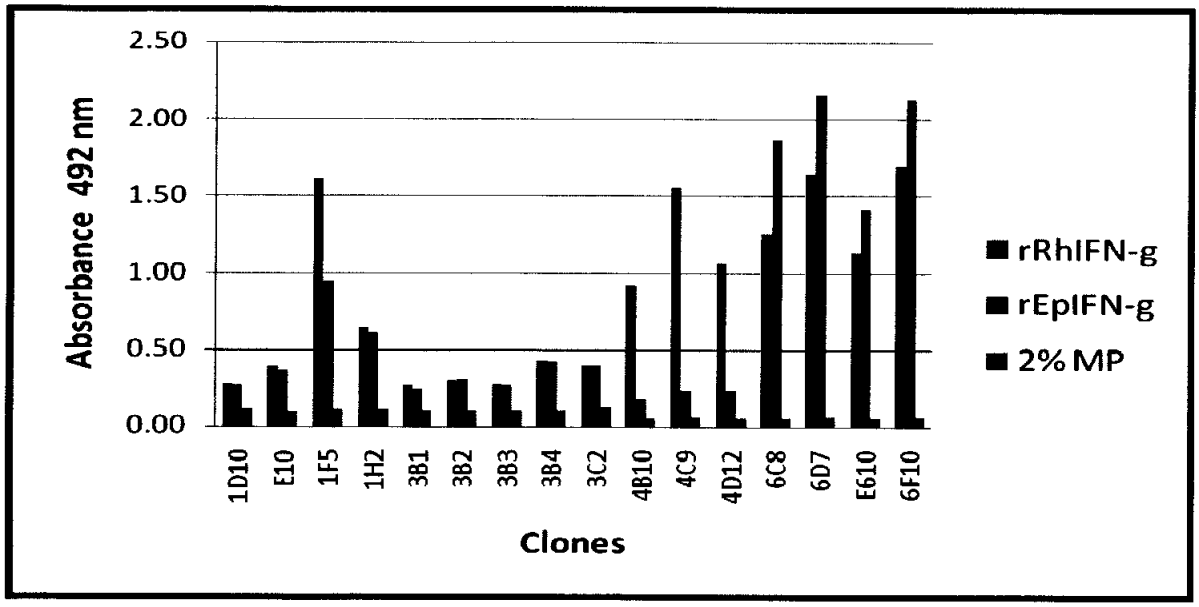

Figure 3.8 Direct ELISA to determine the antigen specificity and cross-reactivity of phage-displayed antibodies to rRhIFN-γ and to rAsEpIFN-γ

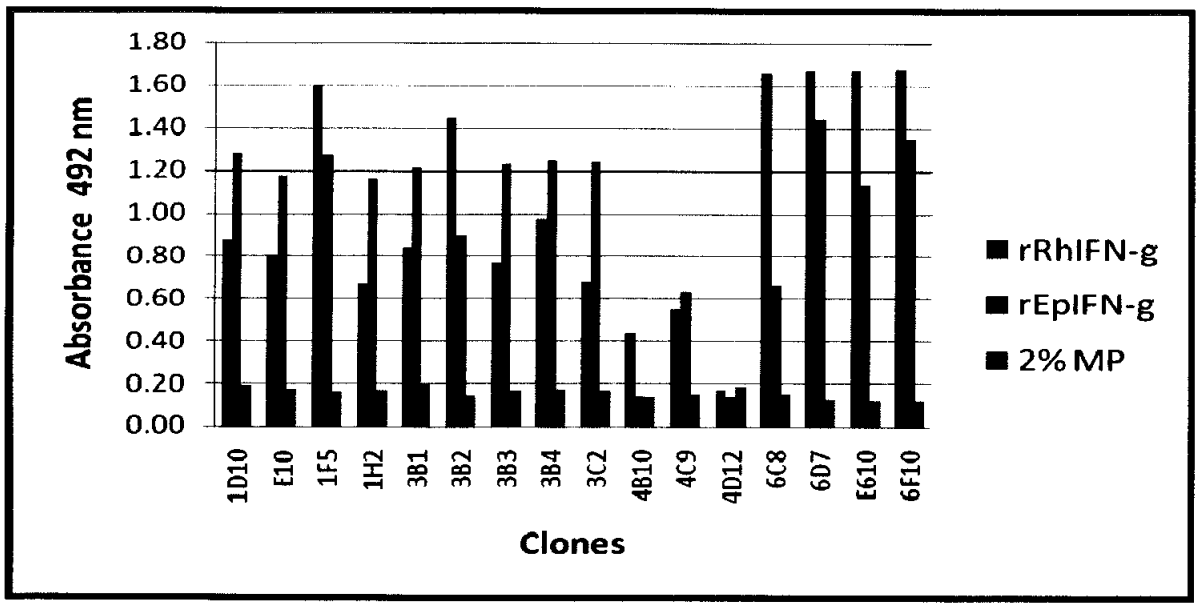

Figure 3.9 Direct ELISA to determine the antigen specificity and cross-reactivity of scFv antibodies to rRhIFN-γ and to rAsEpIFN-γ

3.3.5 Sequence analysis of selected clones

The DNA inserts of all the clones identified during the monoclonal phage ELISA were sequenced. Sequence analysis revealed that the clones (6C8, 6D7, E610 and 6F10), from the fourth round of panning, shared the same sequence. The same was true for clones 1F5, 4B10, 4C9 and 4D12 but they differed in sequence compared to the former four clones. Clones 1F5 and 6D7 were selected as representatives of the clones against rRhIFN-γ and rAsEpIFN-γ respectively. These clones were selected because they gave strong signals when used in a direct ELISA in both phage-displayed antibody (Fig. 3.8) and soluble scFv formats (Fig. 3.9). The aa sequences of the representative clones are shown in Fig. 3.10. The sequence results of the other clones were inconclusive and are not included in the results.

```
                                                    VH CDR 1
1F5   LLLLAAQPALMAAVTLDESGGGLQTPGGALSLVCKGSGFXFSNYYMFWVRQA 52
6D7   LLLLAAQPALMAAVTLDESGGGLQTPGGALSLVCKASGFTFSDYGMFWVRQA 52

                    VH CDR 2
1F5   PGKGLEYVAQISSKTGKYTYYAPAVKGRATISRDDGQSTVRLQLNNLRAENT 104
6D7   PGKGLEYVGVINDDGSWTD YGSAVKGRATISRDNGQSTVRLQLNNLRAEDT 104

                VH CDR 3                          linker
1F5   GTYYCAKDGDGSGCYGGVICAGQIDTWGHGTEVIVSSGGGGSGGGGSGGGGS 156
6D7   GTYFCAKDGGSSASWIHTGD   IDAWGHGTEVIVSSGGGGSGGGGSGGGGS 156

                          VL CDR 1                  VL CDR 2
1F5   ALTQPSSVSANPGETAKITCSGGGSDYGWYQQKSPGSAPVTVIYSNDERPSD 208
6D7   ALTQPSSVSANPGETVKITCSGGSGSYGWFQQKSPGSAPVTVIYSNDKRPSD 208

                                        VL CDR 3
1F5   IPSRFSGSKSGSTATLTITGVRADDEAVYFCGSSDSSSYSSTFGAGTTLTVL 260
6D7   IPSRFSGALSGSTGTLTITGVRAEDEAVYFCGGYDSSTDAA FGAGTTLTVL 260


1F5   GQPNAAAEQKLISEEDLNGAA* 281
6D7   GQPNAAAEQKLISEEDLNGAA* 281
```

Figure 3.10 Amino acid alignment of 1F5 (rhinoceros) and 6D7 (elephant) clones. The complementarity determining regions (CDRs) and linker regions are indicated in colour. Framework regions are in indicated in black. The "~" indicates spaces

3.3.6 scFv Capture ELISAs to detect rRhIFN-γand rAsEpIFN-γ

Two antibodies in the scFv format, 1F5 and 6D7, were isolated and affinity purified for use as capture antibodies in an IFN-γ ELISA. In the first set up, scFv 1F5 was used as the capture antibody and 1D11 was used as a detecting antibody. In these ELISAs different dilutions of the recombinant antigens and of the detecting antibody, 1D11, were used to determine optimal conditions. This ELISA format shows detection of rRhIFN-γ (Fig. 3.11). The detection limit showed 0.075 μg/m*l* rRhIFN-γ could be detected with 1.25 μg/m*l* antibody 1D11.

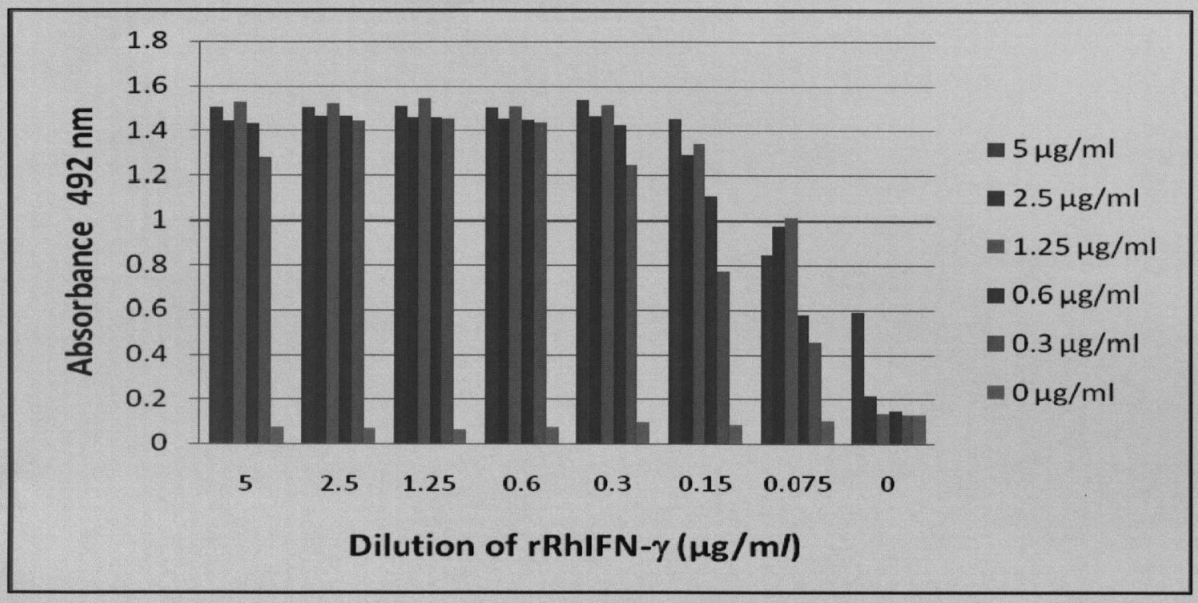

Figure 3.11 Capture ELISA using capture antibody scFv 1F5, at a concentration of 5 μg/m*l*, and detection of rRhIFN-γ with antibody 1D11. The legend on the right indicates the dilution of the detecting antibody 1D11

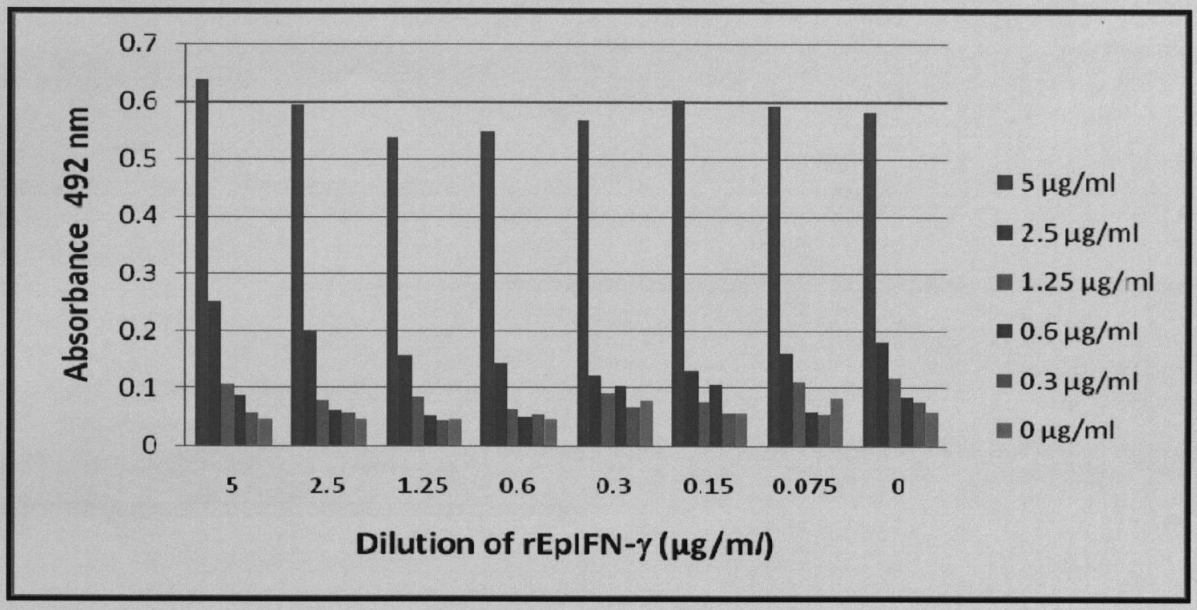

Figure 3.12 Capture ELISA using capture antibody scFv 1F5, at a concentration of 5 µg/ml, and detection of rAsEpIFN-γ with antibody 1D11. The legend on the right shows the dilution of detecting antibody 1D11

This combination of a capture ELISA (1F5/1D11) shows no detection of rAsEpIFN-γ. The signals (OD_{492}) obtained for all dilution combinations ranged from 0.6 and lower. These background signals observed could be a result of non-specific binding of the capture antibody and conjugate (Fig. 3.12).

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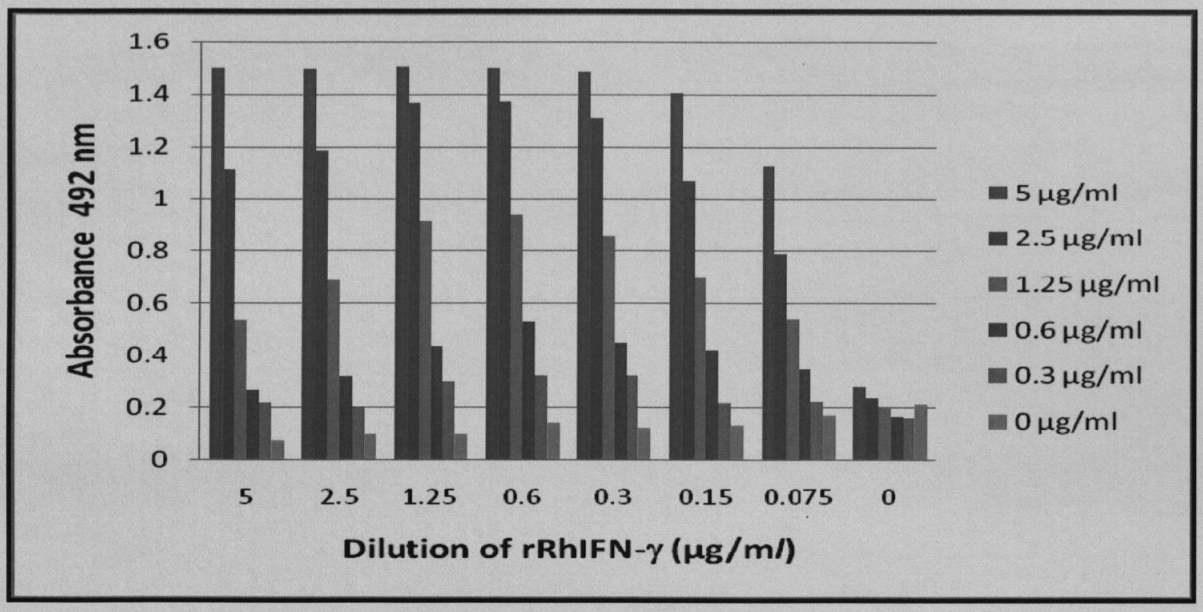

Figure 3.13 Capture ELISA using capture antibody scFv 1F5, at a concentration of 5 µg/m*l*, and IgY^{uu} for the detection of rRhIFN-γ. The legend on the right represents the dilution series of IgY^{uu}

For the scFv/IgY^{uu} ELISA combination, rRhIFN-γ can be detected and the signal strength drops in a stepwise manner (Fig. 3.13). The detection limit of this ELISA indicates that 0.15 µg/m*l* antigen can be detected with 2.5 µg/m*l* IgY^{uu}. At 0.07 µg/m*l* rRhIFN-γ higher concentrations of detecting antibody (IgY^{uu}) are required to produce a signal. Background signals remain at 0.2 or lower at an absorbance of 492 nm.

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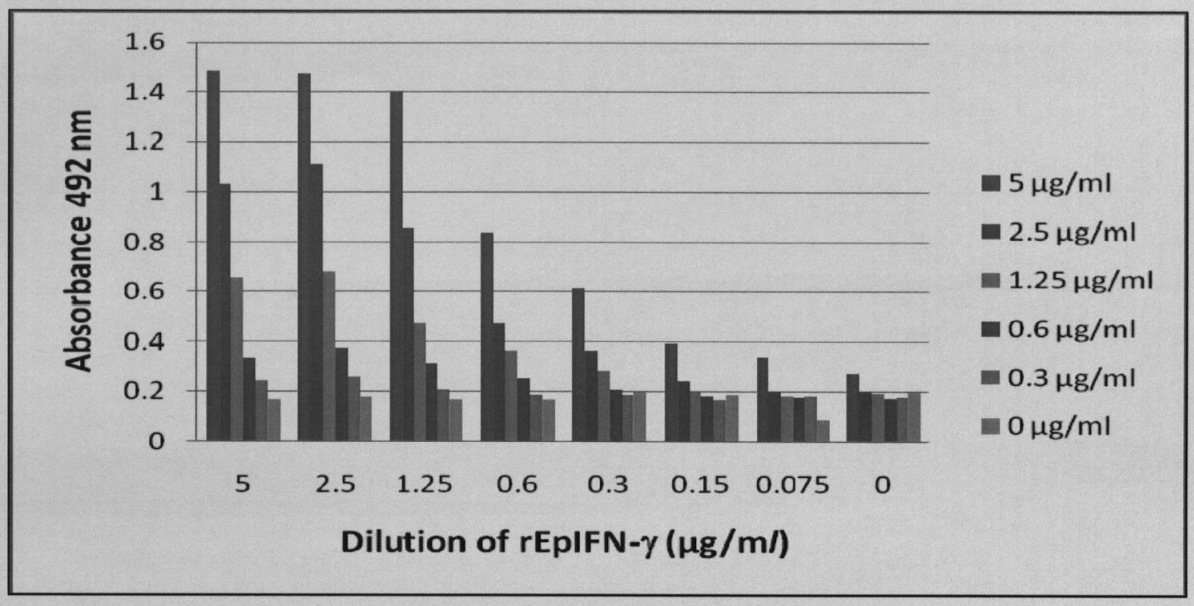

Figure 3.14 Capture ELISA using capture antibody scFv 1F5, at a concentration of 5 µg/m*l*, and IgY^{uu} for the detection of rAsEpIFN-γ. The legend on the right represents the dilution series of IgY^{uu}

Detection of rAsEpIFN-γ using this format of the capture ELISA indicates that high concentrations of antigen and detecting antibody (between 5 µg.m*l* and 1.25 µg/m*l*) is required to obtain a good signal (Fig. 3.14). The detection limit of this ELISA shows 1.25 µg/m*l* rAsEpIFN-γ can be detected with 2.5 µg/m*l* IgY^{uu}. There was no difference in signal strength if the concentration of the recombinant antigen was 5 µg/m*l* or 2.5 µg/m*l*.

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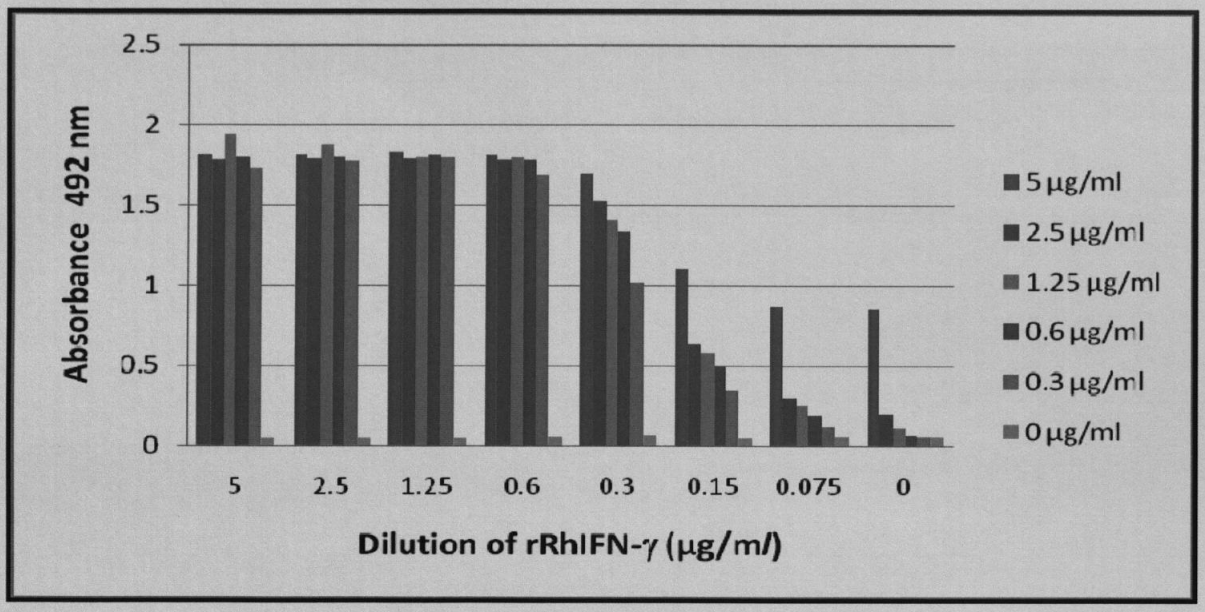

Figure 3.15 Capture ELISA using capture antibody scFv 6D7, at a concentration of 5 µg/m*l*, and antibody 1D11 for the detection of rRhIFN-γ. The legend on the right represents the dilution series of 1D11

In the next ELISA format scFv, 6D7, was used as a capture antibody and 1D11 as a detecting antibody. Recombinant RhIFN-γ could be detected with this ELISA format (Fig. 3.15). The detection limit of this ELISA is 0.3 µg/m*l* of both antigen and antibody 1D11. Strong signals are observed for different concentrations of both rRhIFN-γ and detecting antibody, 1D11. Signal strength (OD_{492}) is slightly higher in this ELISA compared to the 1F5/1D11 combination (Fig. 3.11). However, the detection limit is better with the latter ELISA combination. When the concentration of the recombinant antigen and the antibody is reduced to 0.15 µg/m*l* and 2.5 µg/m*l* respectively the signal tapers down in a stepwise manner. There is no difference in signal if 5 µg/m*l*, 2.5 µg/m*l*, 1.25 µg/m*l*, 0.6 µg/m*l* or 0.3 µg/m*l* of rRhIFN-γ is used in the ELISA (Fig 3.15).

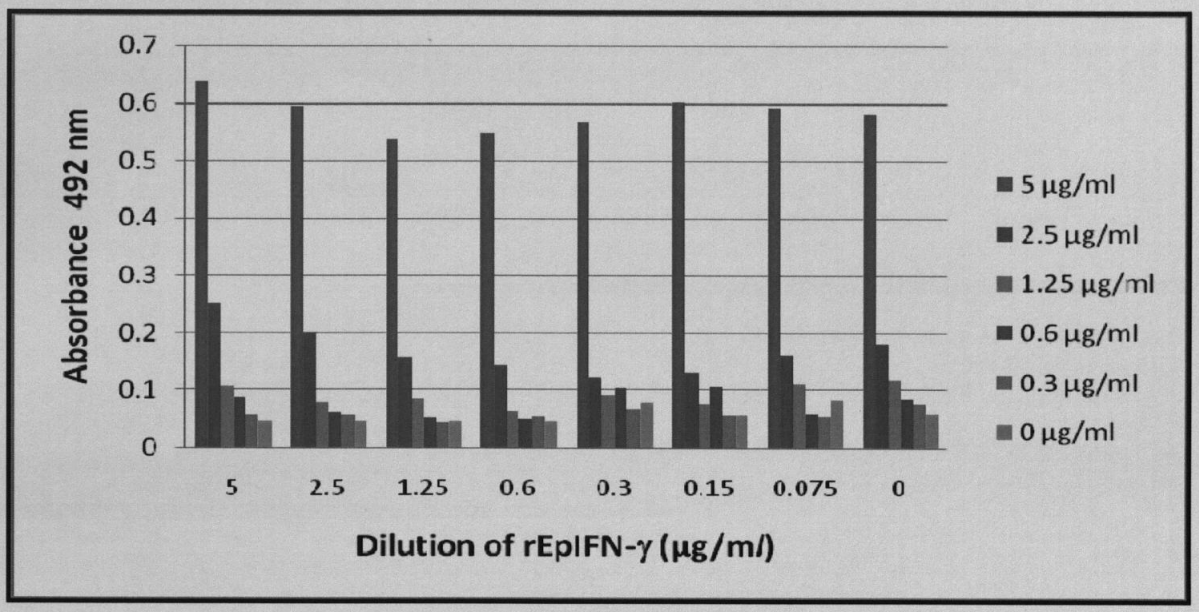

Figure 3.16 Capture ELISA using capture antibody scFv 6D7, at a concentration of 5 µg/ml, and antibody 1D11 for the detection of rAsEpIFN-γ. The legend on the right represents the dilution series of 1D11

The results of the 6D7/1D11 ELISA for the detection of rAsEpIFN-γ mirror the results of the 1F5/1D11 ELISA for detection of rAsEpIFN-γ. Recombinant AsEpIFN-γ could not be detected using this ELISA system. The signals obtained are equivalent to background noise (Fig. 3.16).

When 1D11 was substituted with IgY^{uu} as a detecting antibody, both rRhIFN-γ (Fig. 3.17) and rAsEpIFN-γ (Fig. 3.18) were detected with a signal strength between 1.2 and 1.8 respectively, at an absorbance of 492 nm. Furthermore, the background noise increased from the previous ELISA (Fig. 3.15) from 0.2 to 0.8.

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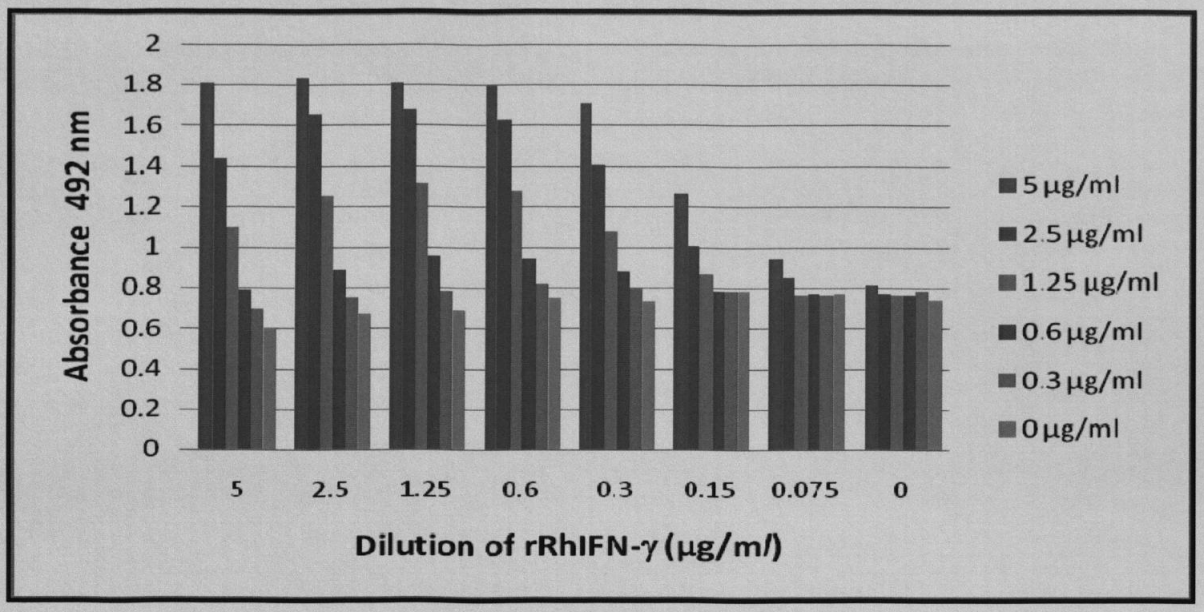

Figure 3.17 Capture ELISA using capture antibody scFv 6D7, at a concentration of 5 µg/m*l*, and IgY^{uu} for the detection of rRhIFN-γ. The legend on the right represents the dilution series of IgY^{uu}

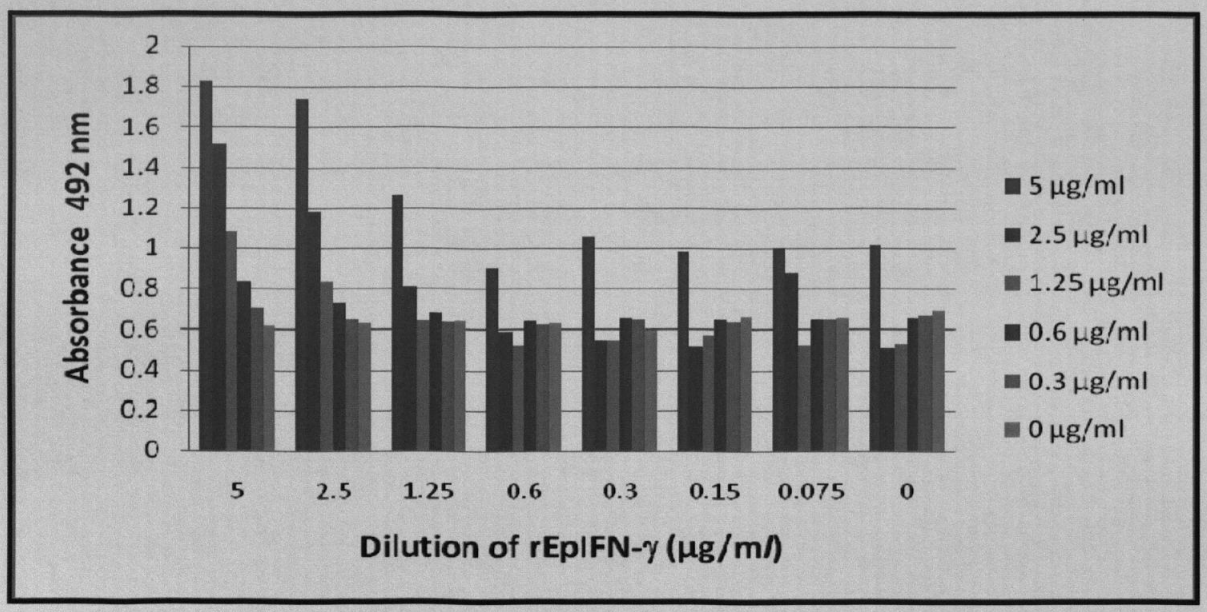

Figure 3.18 Capture ELISA using capture antibody scFv 6D7, at a concentration of 5 µg/m*l*, and IgY^{uu} for the detection of rAsEpIFN-γ. The legend on the right represents the dilution series of IgY^{uu}

3.4 Discussion

This study describes the construction of a phage-displayed antibody library against rRhIFN-γ, and the selection and isolation of scFv antibodies. Apart from mice (Ames, Tornetta, Deenvet *et al.* 1995) and rabbits (Li, Cockburn, Kilpatrick & Whitelam 2000), chickens are regarded as excellent sources for the generation of antibodies (Davies, Smith, Birkett *et al.* 1995; Ratcliffe 2006; Chiliza, Van Wyngaardt & Du Plessis 2008). Recombinant antibodies have been isolated successfully from chicken phage-display libraries (Yamanaka, Inoue & Ikeda-Tanaka 1996; Andris-Widhopf *et al.* 2000; Hof, Hoeke & Raats 2008). Immune B cells were used for the library because they would be highly biased towards the expression of V-genes specific for the immunizing protein and this approach has the potential to produce antibodies of greater specificity and affinity than those that would be isolated from a naïve library (Bradbury & Marks 2004a).

Before making an immune library against rRhIFN-γ, the Nkuku® (chicken) library, a semi-synthethic scFv phage-displayed library based on chicken immunoglobulin genes (van Wyngaardt *et al.* 2004) was used for the selection of antibodies against rRhIFN-γ and rAsEpIFN-γ. Since it is a semi-synthetic phage library and could be directed against a wide variety of antigens (van Wyngaardt *et al.* 2004), the expectation was that this library could provide binders to the antigens of interest. Unfortunately this library did not yield any binders to these antigens.

Previously (Chapter 2) 1H11 and 1D11, a subclone of monoclonal antibody 1H11 (see 2.2.3 & 2.3.6), was produced. This antibody was used in a capture ELISA with IgY^{uu} and was able to detect white RhIFN-γ. Since this was the only antibody that was isolated the prospect to isolate more antibodies and test new combinations of capture ELISAs was still required. It was also the aim to develop a capture ELISA for the detection of rAsEpIFN-γ, but at this stage steps were still in process to express and purify rAsEpIFN-γ (Chapter 4). In this study the chickens were immunized with recombinant white RhIFN-γ (see 2.3.2) but the library was panned against both rRhIFN-γ and rAsEpIFN-γ. The reason for this was that at the time of immunization no rAsEpIFN-γ was available. Therefore, by panning against rAsEpIFN-γ it was anticipated that cross-reactive clones to rAsEpIFN-γ would be obtained, since the IFN-γ genes of these two antigens share a homology of 89% on the nt level and a 75% identity on the aa level (Fig. 3.1).

The V_H and V_L fragments were assembled together with a glycine-serine linker by overlap extension PCR and cloned into vector pHEN I. A library was prepared with the use of *E. coli* TG1 cells and M13K07 helper phages. The results of four rounds of panning showed an increase in the selection of binders in Round 4 for each antigen. An unexpected result was the signal obtained for the unpanned library. This signal was stronger than those obtained for the panned rounds. When single colonies from the unpanned rounds were selected for screening, in the monoclonal phage ELISA, no binders were identified. This result could indicate two opposing scenarios. Firstly, during the panning steps the binders were very weak and were removed during the wash steps. Secondly, they could be very strong binders and the elution method was unable to break the strong virion-target interactions (Lunder, Bratkovic, Urleb *et al.* 2008). A possible solution could be the use of a new elution approach, where a low-pH elution buffer and sonication could aid in releasing the peptide-antibody interaction and also be involved in detaching the target molecule from the immobilization surface (Haga, Shimura, Nakamura *et al.* 1987; Lunder *et al.* 2008). In addition, the probability of finding binders might be higher if more clones were screened.

Single colonies from Round 4 for each antigen, were screened using the monoclonal phage ELISA and only eight clones in total were identified as possible binders. Single colonies were also selected from Round 3 for rRhIFN-γ and from Round 2 for rAsEpIFN-γ. An additional eight clones were identified as possible binders and these clones along with the previous eight clones obtained in the first screening were selected for further analysis.

Sequences of the clones, which were recognised as strong binders in the monoclonal phage ELISA, directed at rRhIFN-γ were identical and those directed at rAsEpIFN-γ were also identical. Clones 1F5 and 6D7 gave the strongest signals in the direct ELISA and were selected as representatives against the respective antigens for the remainder of the experiments. All the clones, except 1F5, that were obtained from Round 2, against rAsEpIFN-γ, and Round 3, against rRhIFN-γ, did not give complete sequences. Clone 1D10 did not have the V_HV_L construct. These clones were therefore excluded from the remainder of experiments. Sequencing results indicated that clones 1F5 and 4C9 were identical and that they differed from 6D7. Clone 1F5 and 6D7 were affinity purified and used as capture antibodies in the capture ELISA format. In the direct ELISA a larger number of clones showed reactivity in the scFv format than in the phage-display format. Clones were also

tested for cross-reactivity against the two antigens. Antibodies produced by the clones selected against rAsEpIFN-γ cross-reacted with rRhIFN-γ. However, the antibodies produced by the clones selected against rRhIFN-γ did not react with rAsEpIFN-γ. The chickens were immunized with rRhIFN-γ and not with rAsEpIFN-γ. This could be a reason as to why the antibodies have a greater affinity to binding with rRhIFN-γ.

The next objective was to determine if these antibodies could be used as capture antibodies in an IFN-γ capture ELISA. Several different capture ELISAs were performed to determine this. Both 1F5 and 6D7 were used as capture antibodies and mouse monoclonal antibody, 1D11, and polyclonal IgY^{uu} were used as detecting antibodies. In the scFv/1D11 combination rRhIFN-γ can be detected. The 6D7/1D11 ELISA shows slightly higher signals than the 1F5/1D11 ELISA but the latter ELISA presents better detection limits. These results may indicate that the capture antibodies, 1F5 and 6D7, and detecting antibody 1D11 recognise different epitopes on the same antigen (rRhIFN-γ). Results for rAsEpIFN-γ using either 1F5/1D11 or the 6D7/1D11 show no detection of rAsEpIFN-γ. Failure of 1D11 to detect rAsEpIFN-γ could mean that 1D11 recognises the same epitope as the capture scFvs or the antibody does not recognise the epitope in the format as presented by the capture scFv. When polyclonal chicken IgY^{uu} was used as the detecting antibody results with 1F5 were better than with 6D7 for both antigens, but stronger signals were obtained for the detection of rRhIFN-γ than for the rAsEpIFN-γ. Since IgY^{uu} is a polyclonal antibody it would recognise more than one epitope on the antigen and is more likely to bind to the antigen and could therefore detect the recombinant protein. With 6D7 as capture and IgY^{uu} as detection antibodies there is a high degree of background. This could be due to a reaction between the conjugate and the capture antibody, 6D7, because in the absence of both antigen and IgY^{uu} a signal of 0.8 is recorded. In these ELISAs the concentration of recombinant IFN-γ required to obtain an ideal detection signal was very high. The capture antibody concentrations were kept constant, at 5 μg/m*l*, but the recombinant proteins and the detecting antibodies were serially diluted to determine optimal amounts required for antigen detection. In conclusion the best combinations were with scFv 1F5 and 1D11 for detection of rRhIFN-γ and scFv 1F5 and IgY^{uu} for detection of rAsEpIFN-γ. The detection limits obtained for these combinations are, however, not optimal as they are almost 1000 times higher than in IFN-γ tests for domestic cattle.

Although the testing of native samples has yet to be made, the results of this study have helped in the development of an IFN-γ ELISA to be used for the diagnosis of *M. bovis* and *M. tuberculosis* infections in wildlife such as rhinoceroses and elephants. Since only 10% of the clones (Table 3.4) were found reactive to IFN-γ, a large proportion of clones were not selected. Only two clones were isolated using the panning technique, this may be attributed to the small size of IFN-γ (18 kDa) and / or strong binding of the protein to the immunotube. As this protein is so small it is likely that a conformational change may have occurred to the epitope due to its immobilization on the immunotube, thus limiting the ability of a greater number of phage-displayed antibodies to bind to the altered epitopes (Butler, Navarro & Sun 1997). Another approach during panning could make use of the histidine tag as a means of trapping the recombinant protein before panning. An alternative to solid phase panning is solution panning by making use of magnetic beads coated with a ligand specific for the tag (Coomber 2002). In addition, there are a number of ways to obtain high binding affinity antibodies: chain shuffling, error prone PCR, mutagenesis and random mutagenesis (Bradbury 1997). By introducing mutations in the CDRs the affinity of the antibody can be increased since these domains are important regions for the formation of antigen-antibody complexes (Dong, Chen, Bartsch & Schachner 2003). Single chain variable fragments can multimerise (Holliger & Winter 1993; Kortt, Lah, Oddie *et al.* 1997) to form diabodies (two scFv molecules) and tribodies (three scFv molecules) which increase the avidity of recombinant antibody fragments (Kortt, Dolezal, Power & Hudson 2001). In this form the V_H of one scFv molecule is linked to the V_L of another by reducing the length of the scFv linker, thus providing increased avidity (Holliger & Winter 1997, Kortt *et al.* 2001).

Exploring the use of the phage-display technique for the selection of antibodies against rRhIFN-γ and rAsEpIFN-γ has allowed for a generation of recombinant antibodies. These antibodies can be used as potential diagnostic tools in an IFN-γ diagnostic test for the detection of *M. bovis* and *M. tuberculosis* infections in rhinoceroses and elephants. With further improvements to the current tools and the use of different panning methods, selection for additional recombinant antibodies with high affinity to rAsEpIFN-γ should be addressed.

3.5 References

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