Chapter 4

Cloning and sequencing of Asian (*Elephas maximus*) and African (*Loxodonta africanum*) elephant interferon-gamma and the production of monoclonal antibodies against recombinant Asian elephant interferon-gamma

Abstract

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* has been reported in captive and domesticated elephants in Asia, Europe and North America. Apart from the consequences to animal health there is also the risk to humans since they may be infected in the case of close contact with these animals. Diagnostic tests for *M. tuberculosis* infections in elephants are targeted at antibody detection which is useful for identifying infected and possibly infectious (shedders) animals. However, diagnosis by serological methods has its limitations, especially during the early stages of infection since antibodies are only produced in the later stages of the disease. The interferon-gamma (IFN-γ) assay is used for the routine diagnosis and detection of *M. bovis* infections in cattle and *M. tuberculosis* infections in humans. In this study Asian (*Elephas maximas*) and African (*Loxodonta africanum*) elephant IFN-γ (EpIFN-γ) genes were cloned and sequenced. At the predicted amino acid level, the IFN-γ of the Asian elephant showed an identity of 98% with that of the African EpIFN-γ sequence. The high similarity between the Asian and African sequences described in this study makes it likely that an IFN-γ assay for Asian elephants will also detect IFN-γ of the African species. The Asian EpIFN-γ gene (AsEpIFN-γ) was expressed as a protein and used in mice immunizations for the production of monoclonal antibodies. In a capture ELISA the monoclonal antibodies were used as capture antibodies, and biotinylated AsEpIFN-γ specific IgY^{uu} and biotinylated monoclonal antibodies were used for detection. Of the several combinations tested, the use of capture antibodies 19 and 21, in combination with polyclonal IgY^{uu} showed to detect rAsEpIFN-γ at a concentration of 15 ng. This is an important first step towards diagnosing *M. bovis* or *M. tuberculosis* infections in Asian and African elephants, while still in its early stages.

4.1 Introduction

Tuberculosis has been identified in elephants since the 1920s (Narayana 1925; Bopayya 1928; Baldrey 1930; Urbain & Dechambre 1937). From the 1970s onwards a worldwide surge in infection of both Asian (*Elephas maximus*) and African elephants (*Loxodonta africanum*) with *Mycobacterium bovis* and *M. tuberculosis* was reported (Pinto, Jainudeen & Panabokke 1973; Johnston 1981; Saunders 1983; Mikota, Larsen & Montali 2000; Harr, Isaza, Raskin *et al.* 2001; Payeur, Jarnagin, Marquardt & Whipple 2002; Lewerin, Olsson, Eld *et al.* 2005). Apart from the incidence of bovine TB (BTB) and human TB, a case of atypical mycobacteriosis was reported at Lincoln Park Zoo in Chicago, USA, when two African elephants died due to infection with *Mycobacterium szulgai* (Lacasse, Terio, Kinsel *et al.* 2007). Although *M. bovis* and / or *M. tuberculosis* infections have not yet been reported in African elephants in Africa they are of major concern in elephants throughout Asia. As in other species, infections with *M. tuberculosis* and *M. bovis* in elephants manifests as a slowly progressing infectious disease. Infected elephants (Harr *et al.* 2001; Payeur *et al.* 2002) are a risk for other elephants and wildlife as well as to humans especially those in close contact with elephants (Michalak, Austin, Diesel *et al.* 1998; Sarma, Bhawal, Yadav *et al.* 2006), and are therefore a concern in breeding and relocation programmes. Specific and sensitive diagnostic tests, as addressed by various authors (Isaza & Ketz 1999; Larsen, Salman, Mikota *et al.* 2000; Lyashchenko, Greenwald, Esfandiari *et al.* 2006), need to be in place since an *in vitro* assay will be valuable in identifying the infection, and thus helping in the management of the disease in elephants. Conventional diagnosis of TB, i.e. mycobacterial culture requires repeated trunk washes (Isaza & Ketz 1999; Isaza 2001; Janssen, Oosterhuis, Fuller & Williams 2004). This procedure, however, has its limitations since only after 4 to 8 weeks are culture results available, and its sensitivity is poor (Isaza & Ketz 1999; Mikota, Miller, Dumonceaux *et al.* 2003). Furthermore, it constitutes a health risk for the animal handlers.

Polymerase chain reaction (PCR) techniques (Collins, Radford, de Lisle & Billman-Jacobe 1994) and lymphocyte proliferation assays (Neill, Skuce & Pollock 2005), have not been validated for use in elephants. In conclusion, the diagnosis of *M. tuberculosis / M. bovis* infections in susceptible animals such as elephants is proving to be a challenge. Lyashchenko *et al.* (2006) have developed tools to target the humoral immune response in several species. A Rapid Test (RT) to diagnose TB in elephants (Elephant TB-STATPAK Kit) employing four different *M. tuberculosis* antigens uses lateral flow technology (Moller, Röken,

Petersson *et al.* 2005). It may be complemented by an assay referred to as the multi-antigen print immunoassay (MAPIA) that identifies the spectrum of IgG antibody responses to a panel of 12 different recombinant antigens of *M. tuberculosis*. Serology does not provide the ultimate solution for the diagnosis of TB since antibodies to mycobacteria may only be produced after prolonged periods of infection.

The period of detectable antibody depends on the dose of the infectious organism or pathogen. Unlike in cattle, these periods have not been defined in elephants. In experimentally infected cattle, with acute infections, antibody responses were recorded 14 days after post infection (Neill, Pollock, Bryson & Hanna 1994), and in cattle naturally infected with *M. bovis* no antibody responses were detected. Proof of an inverse relationship existing between CMI and humoral reposnses from naturally infected *M. bovis* infections in cattle have been confirmed by Ritacco, Lopez, deKantor *et al.* (1991). In experimentally infected cervids the presence of IgG antibodies was shown to start at variable time points after post infection, with the first antibodies being detectable six weeks after infection (Harrington, Surujballi, Prescott *et al.* 2008).

As an alternative for identification of infected elephants, assessment of cell mediated immunity (CMI) is considered as an important diagnostic tool. For CMI, diagnosis of *M. bovis* and / or *M. tuberculosis* infections in a majority of species, tuberculin skin tests (TST) (Monaghan, Doherty, Collins *et al.* 1994) are employed. Tuberculin skin tests and IFN-γ tests have been shown to detect experimentally infected cattle soon after an *M. bovis* infection, regardless of the infectious dose (Dean, Rhodes, Coad *et al.* 2005) and in the absence of antibody (Neill *et al.* 1994). The TST is not practical for use in elephants due to their skin anatomy and the fact that the reactions have to be read after 72 hours. Interferon-gamma (IFN-γ) assays have been used in cattle during the last decade to determine *M. bovis* specific CMI that indicates initial specific immunity against mycobacterial infections associated with Th1 responsiveness in which the production of IFN-γ is crucial (Pollock, McNair, Welsh *et al.* 2001; Richeldi, Ewer, Losi *et al.* 2004; Pollock, Welsh & McNair 2005; Lin, Yee, Klein & Lerche 2008). In diagnosing TB in humans the IFN-γ assay has been accepted as a good alternative for the TST that suffers low specificity (Andersen, Doherty, Pai & Weldingh 2007).

This study describes the cloning and sequencing of Asian (AsEpIFN-γ) and African (AfEpIFN-γ) elephant IFN-γ genes, and the comparison of their nucleotide (nt) and amino acid (aa) sequences. Furthermore, expression of the AsEpIFN-γ protein in *Escherichia coli,* its purification and the production of AsEpIFN-γ specific monoclonal antibodies are performed. These tools will allow for the development of an *in vitro* test, i.e. an IFN-γ capture enzyme-linked immunosorbent assay (ELISA) that is capable of detecting *M. bovis* or *M. tuberculosis* specific CMI responses in elephants in the early stages of infection. In Asian elephants, besides the detection of infections, the challenge will be to detect infectious animals, i.e. shedders. The search for a test correlate of shedding may not be reached by serology but may be best achieved by testing different antigens or combinations of them in the IFN-γ test, as it has been shown in cattle (Vordermeier, Chambers, Cockle *et al.* 2002) and in humans (Steingart, Henry, Laal *et al.* 2007).

4.2 Materials and Methods

4.2.1 Culture and mitogen stimulation of peripheral blood mononuclear cells (PBMCs)

Blood was collected from one adult Asian elephant (Jasmin) and from one adult African elephant (Linda) in ethylenediaminetetraacetic acid (EDTA) Vacutainer™ tubes. Peripheral blood mononuclear cells were isolated by density gradient centrifugation using Ficoll-Paque PLUS (Amersham, 17-1440-02). After 25 min of centrifugation at 2 800 rpm, mononuclear cells were taken from the interphase and washed twice with RPMI-1640 medium supplemented with L-glutamine (Sigma, R8758) and 10% heat inactivated foetal calf serum (FCS). To induce IFN-γ production, 1 m*l* purified mononuclear cells per well (1×10^6 cells/m*l* in wells of a 24-well tissue culture plate) were stimulated with 5 µg/m*l* concanavalin A (Con A) or medium as a control (Sigma, C2010-100 mg) for 18-24 h at 37°C in 5% CO_2. After the incubation period the cells were harvested, washed once with PBS, resuspended in RNAlater (RNAlater RNA Stabilization Reagent, Lady Davis Institute) and stored at -20°C for RNA isolation at a later stage.

4.2.2 African Elephant IFN-γ

4.2.2.1 RNA isolation and RT-PCR

Total RNA was isolated from frozen Con A stimulated PBMCs using the RNeasy Protocol (QIAgen). A final concentration of 100 ng of RNA was reverse transcribed in a 20 μl reaction using iScript™ cDNA synthesis kit (BIORAD). Two microlitres of the first strand reaction was used as PCR template. African EpIFN-γ primer sequences (reverse primer R10 5'-AAAATTCAAATATTGTAGGCGG-3' & forward primer F10 5'-ACTTTGGCCTAACTCTCTCTCAA-3') were designed based on the equine IFN-γ (EqIFN-γ) gene sequences (EqIFN-γ Genbank Accession Number: D28520) (Grünig, Himmler & Antczak 1994) and used to amplify the complete coding part of the AfEpIFN-γ cDNA. Each 20 μl PCR reaction mix contained 4 μl of 5 X Go *Taq* Flex colourless buffer (Promega), 1 unit (U) of Go *Taq* DNA polymerase (Promega), 10 μM of each primer (R10 and F10) and the relevant cDNA as a template. The cycling conditions were denaturation at 95°C for 30 s, annealing at 62°C, 60°C, 55.7°C and 52.8°C for 45 s and the extension for 60 s at 72°C for 35 cycles and a final extension at 72°C for 5 min. The PCR products were analysed for the correct size by 1% agarose gel electrophoresis.

4.2.2.2 Cloning and sequencing

Amplified products from independent PCRs were cloned into the pCR-TOPO®TA vector of Invitrogen (Fig. 4.1) according to the manufacturer's protocol. After transformation in *E. coli* DH5α strain, plasmid DNA was purified from colonies and sequenced to check the cloned fragment. Colonies obtained from several independent PCRs were used to exclude PCR derived inaccuracies in the sequences.

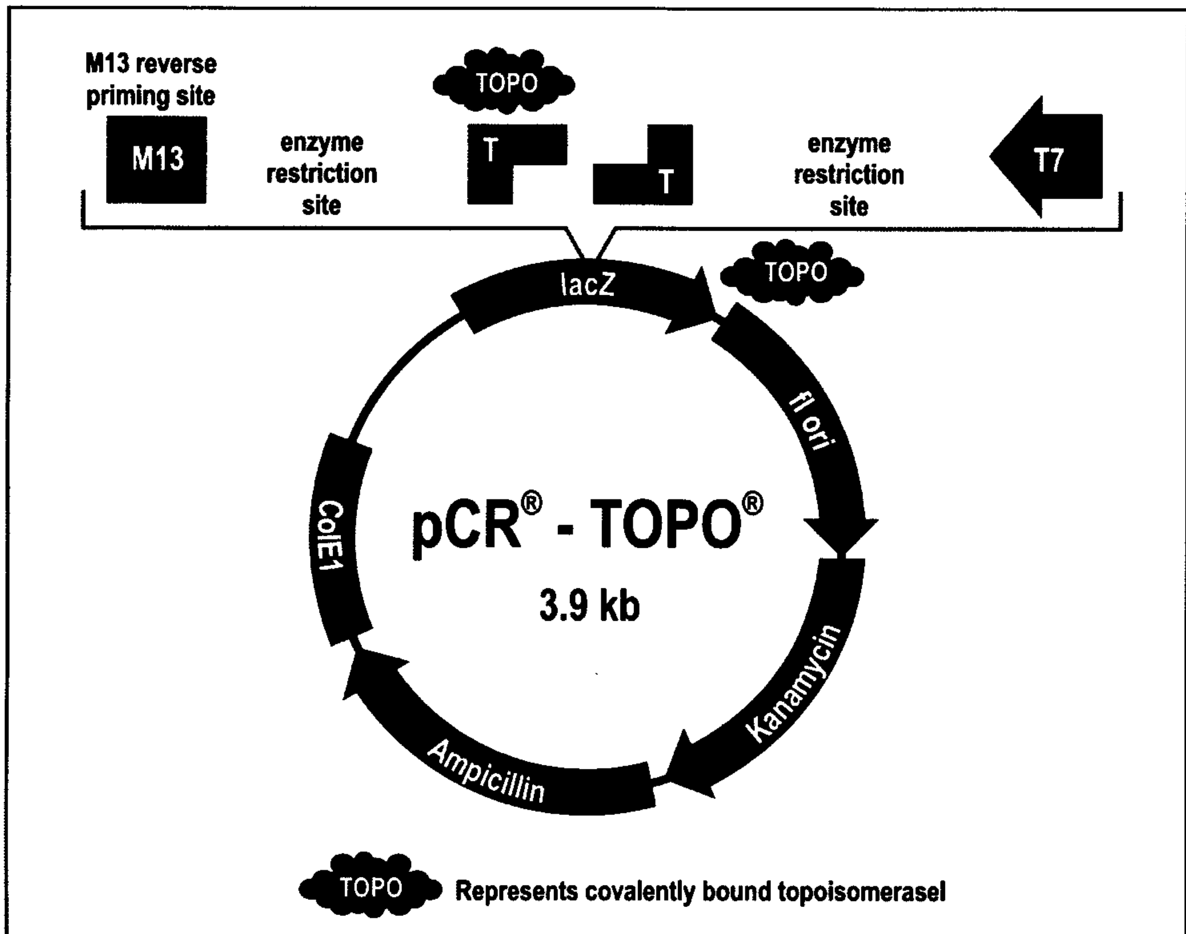

Figure 4.1 Diagrammatic representation of the TOPO Vector (Invitrogen) used for cloning of AfEpIFN-γ. Antibiotic resistance markers (ampicillin and kanamycin) are also present in the vector and the presence of these genes allowed for the selection of only those clones that contained the plasmid

4.2.3 Asian Elephant IFN-γ

4.2.3.1 RNA isolation, RT-PCR, Gateway PCR and Cloning

Total RNA was purified from Con A stimulated lymphocytes using Trizol reagent (Gibco). A touchdown PCR was performed for 35 cycles using a Biorad Thermal Cycler. Primers used for this PCR are included in Table 4.1 and the design of the primers was based on the African elephant genomic sequence (Genbank Accession Number: AC155904). Each 50 μ*l* PCR reaction mix contained 5 μ*l* of 10 X PCR buffer, 1 U KOD Hot Start DNA polymerase (Novagen), and 10 μM of each primer (D12 and E01 shown in Table 4.1). The touchdown part of the PCR reaction consisted of 18 cycles, each starting with DNA denaturation at 95°C for 30 s, followed by primer annealing at 60°C for 45 s with a decrease in temperature of

0.5°C every cycle and, finally, an extension for 1 min at 72°C. The PCR was continued for 35 cycles with denaturation at 95°C for 30 s, annealing at 55°C for 45 s and extension at 72°C for 1 min. The final step of the total PCR reaction was an extension for 10 min at 72°C. The products of amplification were analysed by 1% agarose gel electrophoresis. PCR products of the right size were cloned and sequenced as described above for the African elephant. In order to express rAsEpIFN-γ, the corresponding gene was cloned into the expression vector pET15bGW, a derivative of pET15b (Fig. 4.2) that was adapted for Gateway (GW) cloning (Invitrogen) into the corresponding sites of pET15b as described in Chapter 2 (see 2.2.1).

To enable GW cloning, one of the sequenced clones (Jasmin, 9), that represented the consensus sequence of an Asian elephant, was used as a template for a two step GW PCR. In the first step the IFN-γ sequence encoding the mature protein was amplified using GW primers, H12 and H05, shown in Table 4.1. The underlined sequences were not encoded by the IFN-γ gene, but were included as annealing sites for a second GW PCR performed with primers GW2-F2 and GW2-R2 (Table 4.1). This second PCR introduced the *att B1* and *att B2* sites, which enabled subsequent GW cloning. Both GW PCRs consisted of 18 cycles at an annealing temperature of 55°C, using the KOD Hot start proofreading polymerase (Novagen). Cloning into vector pDONR201 (Invitrogen), transformation of *E. coli* strain DH5α, and subcloning into the expression vector pET15bGW was performed as described in Chapter 2 (see 2.2.1). The resulting expression vector designated pET15-EpIFN-γ was sequenced to check that no mutations had been introduced by the GW PCRs.

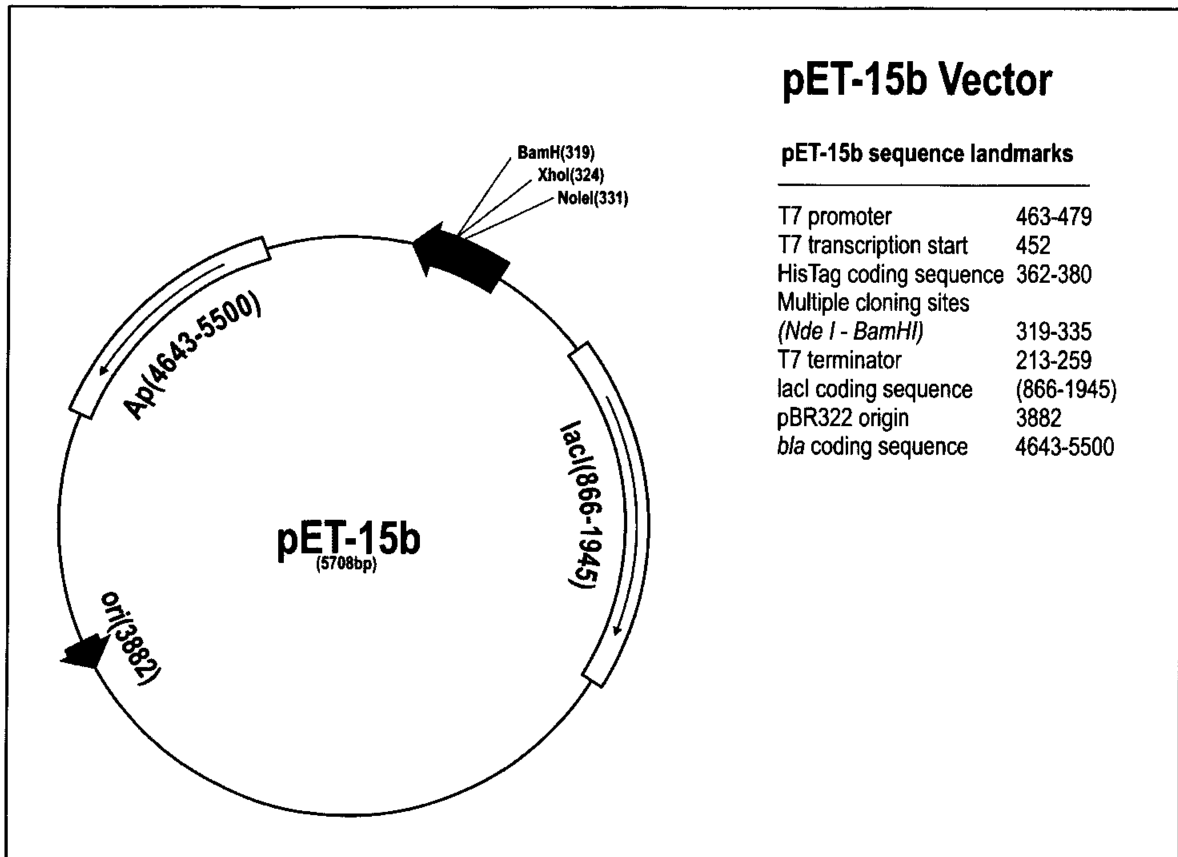

Figure 4.2 Diagrammatic representation of the pET15b vector (Novagen)

4.2.4 Sequence analysis

All sequencing reactions were performed at BaseClear sequencing services in Leiden, The Netherlands. Both the forward and reverse reactions were fully sequenced for each species (AsEpIFN-γ and AfEpIFN-γ). The sequences of both Asian and African elephant IFN-γ genes were compared using NCBI nucleotide BLAST, and were aligned and compared to protein sequence data of other AsEpIFN-γ sequences (AbdEl-Gawad, Ibrahim & Steinbach 2007; Sreekumar, Janki, Arathy *et al.* 2007), and to EqIFN-γ using the NCBI protein BLAST.

4.2.5 Expression and purification of rAsEpIFN-γ

The expression and purification of rAsEpIFN-γ was performed in a similar manner to that described in Chapter 2 (see 2.2.2) for white rhinoceros (*Ceratotherium simum*) IFN-γ. Samples obtained during the purification steps were analysed on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

Table 4.1 Primers used for isolation and cloning of AsEpIFN-γ gene

Reverse Primer AfricanIFN-γ FLRev	D12
5'-CCA TTA TTC TGA TGC TCT CCG GCC–3'	
Forward Primer AfricanIFN-γ FLFor	E01
5'-GGC CTA ACT CTC TCT GAA ACA ATG AAT TTT AC-3'	
GW Primers	
Reverse Primer elephGW.R2	H05
5'-GCG GCG GCG GGT TCG AAT TCG CCC TTC AGG-3'	
Forward Primer elephGW.F2	H12
5'-GTG CCG AGA GGG AGC ACT TTT TTG AAA GAG ATA CAG AAC CTA AAG-3'	
Forward Primer GW2-F2	
5'-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT GGT GCC GAG AGG GAG C-3'	
Reverse Primer GW2-R2	
5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GT GCG GCG GCG GG-3'	

4.2.6 The production and selection of monoclonal antibodies

4.2.6.1 Immunization protocol

A 12-week-old Balb/C mouse was immunized with the purified rAsEpIFN-γ protein. Pre-serum was collected from blood that was obtained from the mouse before immunization. The mouse was immunized subcutaneously with 80 μg rAsEpIFN-γ in 200 μ*l* in a 1:1 dilution in Stimune (Prionics). After 21 days the mouse was bled via a tail vein to collect the first serum samples after immunization. On the same day a subcutaneous boost immunization was performed with 80 μg recombinant antigen in 200 μ*l* in a 1:1 dilution of Stimune. At Day 29, blood was again obtained from its tail vein. This was the second serum sample obtained from the mouse. An ELISA against rAsEpIFN-γ, described in 4.2.6.4 was performed to determine the antibody titres in the mouse. It was given an intraperitoneal (ip) boost immunization without adjuvant three days before the isolation of the spleen. The immunization protocols were approved by the Animal Ethics Committee of the Faculty of Veterinary Medicine, Utrecht University.

4.2.6.2 Isolation of spleen cells

The mouse was euthanased by cervical dislocation 20 days after the second sampling based on antibody titres measured. Its spleen was placed in 10 m*l* of serum-free Medium A (OptiMEM containing HT + 1% PBS + 200 mM Glutamax). A cell suspension was prepared in a petri dish by gently rubbing the cut surface of the spleen several times with the base of the plunger of a 5 m*l* plastic syringe. The cell suspension was then washed in 10 m*l* serum free Medium A and centrifuged at 150xg for 10 min. The cells were now ready for fusion with the myeloma cell line (Sp2/0). Before the mouse was euthanased a sample of 0.3 m*l* of its blood was collected from its tail vein and stored at -20°C to be used as a positive control in subsequent screening ELISAs.

4.2.6.3 Fusion Technique

A hundred million cells isolated from the spleen of the mouse were fused with Sp2/0 cells to produce hybridomas. Briefly, the spleen cells from the immunized mouse were fused with the mouse myeloma Sp2/0 cells using polyethylene glycol (PEG), which allows the cell membranes to fuse. The mixed cell suspension, consisting of washed myeloma cells and a spleen cell suspension in a ratio of 1:5 (myeloma:spleen cells) was centrifuged at 150xg for 10 min. The supernatant was discarded and the pellet was suspended in 1 m*l* of 94% PEG solution (2 g PEG 4000 MERCK, 2.1 m*l* Medium A). To the cells suspended in the PEG solution 1 m*l* Medium A was added and the cell suspension was allowed to stand for 90 s. This was followed by the addition of 2 m*l* of Medium A and another addition of 5 m*l* Medium A after 2 min. After a further 2 min, 5 m*l* of Medium B (Medium A + 15% FCS) was added to the cell suspension which was centrifuged at 150xg for 10 min. The supernatant was discarded and the cell pellet was resuspended with 10 m*l* of Medium B. From this 100 μ*l* was used to count the hybridoma cells. The cells were plated in five 96-well culture plates. The hybridomas were refreshed on a regular basis during the following 14 days. On Day 14 the supernatant of the 480 hybridomas was screened for the production of antibodies against rAsEpIFN-γ.

4.2.6.4 Screening of hybridomas for antibody production

Hybridoma supernatants were screened for antibodies to rAsEpIFN-γ using the following direct ELISA protocol. Wells of an enzyme immuno-assay (EIA) 96-well co-star high

binding plate, were coated with 0.5 μg of purified rAsEpIFN-γ in 50 μ*l* phosphate buffered saline solution (PBS) and incubated at 4°C overnight. The next day the coating buffer was discarded and 200 μ*l* of blocking buffer comprising 5% bovine serum albumin (BSA) in PBS, were added to each well and incubated at room temperature for 15 min on a shaker at 100 rpm. The blocking buffer was discarded and the plates were washed three times with wash buffer (PBS + 0.1% Tween 20). A volume comprising 80 μ*l* of the hybridoma culture supernatants was added to each well of the plate at room temperature and the plate was incubated at 37°C at 7% CO_2 for 1 h. The serum of the immunized mouse was used as a positive control. The wells were then washed three times with wash buffer. Anti-AsEpIFN-γ antibody was detected using 50 μ*l* per well of goat anti-mouse IgG (1:1000) horse radish peroxidase (HRP) conjugate (Boehringer Mannheim), diluted in blocking buffer and incubated for 1 h at room temperature on a shaker at 100 rpm. After repeating the wash step, ABTS buffer (Boehringer Mannheim) was added as a substrate at 50 μ*l* per well. After 30 min the absorbance was read at 405 nm.

In a second ELISA an EIA plate was coated with 1 μg/well of recombinant EqIFN-γ (rEqIFN-γ) to test for potential cross-reactive monoclonal antibodies. The ELISA was performed as described above. Cells in wells that gave rise to a positive ELISA result were transferred to 8 m*l* cultures in 25 m*l* tissue culture flasks for further expansion. Once the cell density (2 weeks of growth) was optimal, the cultures were retested in the rAsEpIFN-γ ELISA. Clones that remained positive in the ELISA were subcloned by using FACSVantage (Becton Dickson) single cell sorting, based on forward and sideward scatter characteristics, into new 96-well tissue culture plates at a concentration of one cell per well. After growth of the subclones the supernatants were tested in a direct ELISA against rAsEpIFN-γ.

4.2.7 Western blot analysis of gene products

Purified rAsEpIFN-γ and rEqIFN-γ were loaded onto precast Criterion gels (BIORAD) at a concentration of 1 μg/lane of each protein. The samples were run at 200 V (100 amp) for 1 h. The protein on the gel was transferred to nitrocellulose paper by western blotting at 100 V for 1 h. Confirmation of transfer to nitrocellulose paper was done with Ponceau S staining before further processing. Subsequently, the blot was incubated in 50 m*l* of freshly prepared blocking reagent (0.5% BSA in PBS) for 1 h at room temperature. After discarding the

blocking reagent the blots were cut into strips and individually incubated with the supernatant produced by the following hybridoma clones: 5, 7, 11, 19, 21 and 35. After an overnight incubation at 4°C, the blots were washed four times, each wash step lasting 10 min. The next step was to incubate the blots with goat anti-mouse IgG-Peroxidase (POD) (Boehringer Mannheim), 1:1000 for 1 h. The incubation was followed by three wash steps with wash buffer. Detection was performed using 3,3'diaminobenzidine (DAB-alkaline phosphatase, SigmaFAST™) incubation at room temperature for 1 h. Finally the nitrocellulose was washed with PBS for 10 min.

In a second western blot experiment the gel was loaded with rAsEpIFN-γ, rEqIFN-γ and recombinant mouse granulocyte-macrophage colony stimulating factor (rMo-GMCSF). These three recombinant antigens were all expressed using the pET15bGW vector which has the histidine tag for purification purposes. To confirm that the antibodies which were screened against rAsEpIFN-γ were indeed against the protein and not the histidine tag, rMo-GMCSF was included as a proper negative control in the experiment. The protocol was repeated as mentioned above.

4.2.8 Mouse hybridoma isotyping

In order to determine the immunoglobulin class of the mouse monoclonal antibodies, Beadlyte® mouse immunoglobulin isotyping (IgM, IgA, IgE, IgG1, IgG2a, IgG2b and IgG3 heavy chains, kappa and lamba light chains) was performed with Luminex 100. The procedure was performed according to the manufacturer's instructions.

4.2.9 Establishment of a capture ELISA for recombinant AsEpIFN γ

The monoclonal antibodies 5, 11, 19, 21 and 35, and irrelevant antibodies serving as negative isotype controls 1F4F4, JJ319 and Ox62, were employed as capture antibodies. Antibody 7 was not included in capture ELISAs. The antibodies 5, 19, 35, and affinity-purified chicken yolk immunoglobulin, produced at the Utrecht University (UU), IgY^{uu} (Chapter 2, see 2.2.3), were biotin-labelled using a biotin protein labelling kit (Roche Diagnostics) according to the manufacturer's instructions, and used as detecting antibodies. ELISA high binding (Co-star) plates were coated with the different capture antibodies at a final concentration of 0.5 μg/well in a final volume of 50 μ*l*. Each step after coating was performed at 37°C for 1 h except for

the last step, which took place at room temperature. Blocking buffer (0.5% BSA in PBS) was used at a final volume of 100 μl/well after the coating buffer was discarded. Wash steps, using PBS + 0.1% Tween 20, were performed after each incubation step. Recombinant AsEpIFN-γ was used at a starting concentration of 250 ng/well and two-fold dilutions were performed in the ELISA wells. For detection biotin-labelled antibodies were used at a final concentration of 0.1 μg/well. Horse radish peroxidase labelled streptavidin was used to bind to the biotin-labelled detecting antibodies. The addition of ABTS substrate resulted in a colour reaction which was measured at 405 nm after 15 min.

4.3 Results

4.3.1 Cloning and sequencing of African and Asian EpIFN-γ

For AfEpIFN-γ, at an annealing temperature of 60°C, a high yield of the expected product was obtained (approximate size 530 bps) (Fig. 4.3). At the higher annealing temperature, of 62°C, only a weak band of the correct size was seen while at the lower tested temperatures non-specific PCR products became more abundant. Products of several independent PCRs were sequenced to come to a consensus sequence. This was essential to exclude PCR derived inaccuracies in the sequences. For AsEpIFN-γ, a PCR product was obtained at an annealing temperature of 55°C. The products from several independent PCR reactions were cloned and sequenced. The obtained consensus nt sequences of both African and Asian EpIFN-γ are shown in Fig. 4.4 and 4.5 respectively.

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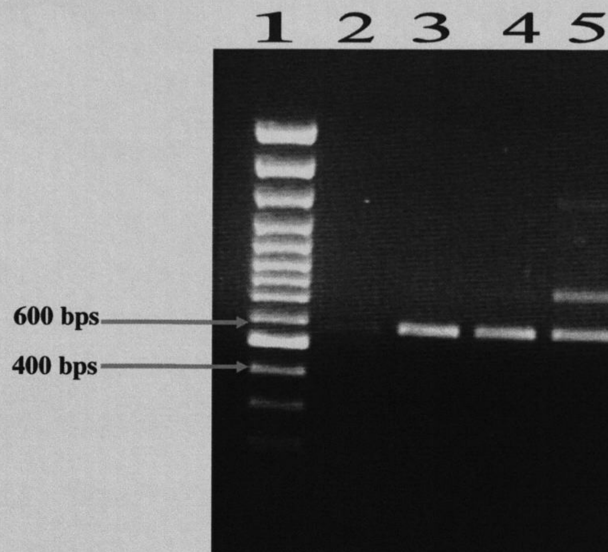

Figure 4.3 PCR products from cDNA (African elephant, Linda) amplification. **Lane 1:** 100 bp O' gene ruler (Fermentes); **Lane 2:** Weak PCR product obtained at annealing temperature of 62°C; **Lane 3:** PCR product obtained at annealing temperature of 60°C; **Lane 4:** PCR product obtained at annealing temperature of 55.7°C; and **Lane 5:** PCR product obtained at annealing temperature of 52.8°C

The predicted aa sequences of the Asian and African EpIFN-γ genes are shown in Fig. 4.6 aligned with the published sequences of AbdEl-Gawad *et al.* (2007) and Sreekumar *et al.* (2007), and the genomic AfEpIFN-γ sequence, and EqIFN-γ (Grunig *et al.* 1994).

4.3.2 Sequencing results

The sequence of the AfEpIFN-γ (Linda) is completely identical at the predicted aa level to that of the genomic AfEpIFN-γ sequence (GenBank Accession Number: AC155904). The AsEpIFN-γ (Jasmin) and the AfEpIFN-γ (Linda) sequences were compared at the nt (Fig. 4.4 & Fig. 4.5) and the aa levels (Fig. 4.6) with each other and with AsEpIFN-γ sequences obtained by Sreekumar *et al.* (2007) (Genbank Accession Number: EF203241), AbdEl-Gawad *et al.* (2007) (Genbank Accession Number: EU000432) and with the EqIFN-γ sequence (Genbank Accession number D28520) (Grunig *et al.* 1994). Results of the sequence homology on the nt and aa levels between the different EpIFN-γ sequences are summarized in Table 4.2.

Table 4.2 Percent homology on the amino acid and nucleotide levels between the different elephant IFN-γ sequences

Gene	% Homology (aa level)	% Homology (nt level)	Sequence
AfEpIFN-γ (Linda)	98%	98%	AsEpIFN-γ (Jasmin)
AfEpIFN-γ (Linda)	95%	97%	AsEpIFN-γ (Sreekumar *et al.* 2007)
AfEpIFN-γ (Linda)	90%	95%	AsEpIFN-γ (AbdEl-Gawad *et al.* 2007)
AfEpIFN-γ (Jasmin)	75%	82%	Equine IFN-γ (Grunig *et al.* 1994)
AsEpIFN-γ (Jasmin)	97%	99%	AsEpIFN-γ (Sreekumar *et al.* 2007)
AsEpIFN-γ (Jasmin)	90%	96%	AsEpIFN-γ (AbdEl-Gawad *et al.* 2007)
AsEpIFN-γ (Jasmin)	75%	82%	Equine IFN-γ (Grunig *et al.* 1994)
AsEpIFN-γ (Sreekumar *et al.* 2007)	95%	96%	AsEpIFN-γ (AbdEl-Gawad *et al.* 2007)

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Linda
ACTTTGGCCTAACTCTCTCTGAAACA   AATTTTACAAGTTATATCTTAGCTTTTCAGCTT
TGCATCATTATGGGTTCTTCTAGCTGCTACTGCCAGGCTACTTTTTTGAAAGAGATACAGAA
CCTAAAGGAATATCTTAATGCAACTGACTCAGATGTAGCGGATGGTGGGCCTCTTTTCATAG
ATATTTTGAAGAACTGGAAAGAGGACAGTGACAAAAAAATAATTCAGAGCCAGATCGTTTCC
TTTTACCTCAAAATCTTTGACAACCTGAAAGACAACCAGGTCATCCAAGAGAGCGTGAAGAC
CCTTGAGGAAGACCTCTTTGTTAAGTTCTTCAATAGCAGCTCCAGCAAACGGGATGACTTCC
TAAAAGTGATGCAAACTCCGGTAAATGACCAGAACATCCAGCGCAAAGCCATAAGTGAGCTC
GCCAAGGTGATGAATGACCTGTCACACAGATCTACCGGGTCAAAACGAAAAAGGAGACCGTA
TTCGTTTCGAGGCCGGAGAGCATCAGAA   TGGTCACCCTGCCTACAATATTTGAATTTT
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Figure 4.4 Nucleotide sequences of the AfEpIFN-γ (Linda). Selected primers used for the cloning of the African IFN-γ gene is marked with arrows. The reverse primer is shown in red font and the forward primer is shown in blue font

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Jasmin
GGCCTAACTCTCTCTGAAACA   AATTTTACAAGTTATATCTTAGCTTTTCAGCTTTGCAT
CATTTTGGGTTCTTCTAGCTGCTACTGCCAGGCTACTTTTTTGAAAGAGATACAGAACCTAA
AGGAATATCTTAATGCAACTGATTCAGATGTAGCGGATGGTGGGCCTCTTTTCATAGATATT
TTGAAGAACTGGAAAGAGGACAGTGACAAAAAAATAATTCAGAGCCAGATCGTTTCCTTTTA
CCTCAAAATCTTTGACAACTTGAAAGACAACCAGGTCATCCAAGAGAGCGTGAAGACCCTTG
AGGAAGACCTCTTTGTTAAGTTCTTCAATAGCAGCTCCAGCAAACGGGATGACTTCCTAAAA
GTGATGCAAACTCCGGTAAATGACCGGAACGTCCAGCGCAAAGCCATAAGTGAGCTCTCCAA
GGTGATGAATGACCTGTCACACAGATCTAACGGGGCAAAACGAAAAAGGAGACAGTATTCGT
TTCGAGGCCGGAGAGCATCAGAA   TGG
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Figure 4.5 Nucleotide sequence of the AsEpIFN-γ (Jasmin). Selected primers used for the cloning of the AsEpIFN-γ gene are marked with arrows. The reverse primer is shown in red font and the forward primer is shown in blue font

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Linda(African)     MNFTSYILAFQLCIILGSSSCYCQATFLKEIQNLKEYLNATDSDVADGGPLFIDILKNWK 60
Genomic(African)   MNFTSYILAFQLCIILGSSSCYCQATFLKEIQNLKEYLNATDSDVADGGPLFIDILKNWK 60
Jasmin (Asian)     MNFTSYILAFQLCIILGSSSCYCQATFLKEIQNLKEYLNATDSDVADGGPLFIDILKNWK 60
Gawad (Asian)      MKYTSYFLAFQLCIILGSSSCCCQATFLKEIQNLKEYLNATDSDVADGGPLFIDILKNWK 60
Sreekumar (As.)    MNFTSYILAFQLCIILGSSSCCCQATFLKEIQNLKEYLNATDSDVADGGPLFIDILKNWK 60
Equine             MNYTSFILAFQLCAILGSSTYYCQAAFFKEIENLKEYFNASNPDVGDGGPLFLDILKNWK 60

Linda(African)     EDSDKKIIQSQIVSFYLKIFDNLKDNQVIQESVKTLEEDLFVKFFNSSSSKRDDFLKVMQ 120
Genomic(African)   EDSDKKIIQSQIVSFYLKIFDNLKDNQVIQESVKTLEEDLFVKFFNSSSSKRDDFLKVMQ 120
Jasmin (Asian)     EDSDKKIIQSQIVSFYLKIFDNLKDNQVIQESVKTLEEDLFVKFFNSSSSKRDDFLKVMQ 120
Gawad (Asian)      EESDKKIVQSQIVSFYLKIFDNLKDNQVIQESVKTLEEDLFVKFFNSSSSKRDDFPKVMQ 120
Sreekumar (As.)    EDSDKKIIQSQIVSFYLEIFDNLKDNQVIQESVKTLEEDLFVKFFNSSSSKRDDFLKVMQ 120
Equine             EDSDKKIIQSQIVSFYFKLFENLKDNQVIQKSMDTIKEDLFVKFFNSSTSKLEDFQKLIQ 120

Linda(African)     TPVNDRNIQRKAISELAKVMNDLSHRSTGSKRKRRQYSFRGRRASE 166
Genomic(African)   TPVNDRNIQRKAISELAKVMNDLSHRSTGSKRKRRQYSFRGRRASE 166
Jasmin (Asian)     TPVNDRNVQRKAISELSKVMNDLSHRSNGAKRKRRQYSFRGRRASE 166
Gawad (Asian)      TPVNDRNVQRKAISELAKVMNDLSPKSSETKRKRGQYSFRGRRASK 166
Sreekumar (As.)    TPVNDRNVQRKAISELSKVMNDLSHRSNGAKRKRRQYSFRGRRAST 166
Equine             IPVNDLKVQRKAISELIKVMNDLSPKANLRKRKRSQNPFRGRRALQ 166
```

Figure 4.6 Alignment of predicted protein sequences for AsEpIFN-γ, AfEpIFN-γ and EqFN-γ. Differences in aa identities between the sequences are indicated in red font

4.3.3 Expression and purification of rAsEpIFN-γ

Recombinant AsEpIFN-γ was expressed in *E. coli* by plasmid pET15-AsEpIFN-γ. Upon isopropyl-β-D-1-thiogalactopyranoside (IPTG) induction a strong protein band was induced with a size that corresponds well to the theoretical molecular weight of 19.8 kD for the tagged rAsEpIFN-γ (Fig. 4.7, lane 3). The major part of the expressed rAsEpIFN-γ was present in the form of (insoluble) inclusion bodies (Fig. 4.7, lane 5). After solubilization of the inclusion bodies in 8 M urea (Fig. 4.7, lane 6) the majority of the protein bound to a column with immobilized Ni^{2+} (Fig. 4.7, lane 8) and a minor part showed up in the flow through fraction (Fig. 4.7, lane 7). Following the wash step the bound protein was refolded on the column and eluted. The eluted protein was dialysed. The purity of the protein was at least 90% (Fig. 4.7, lane 9).

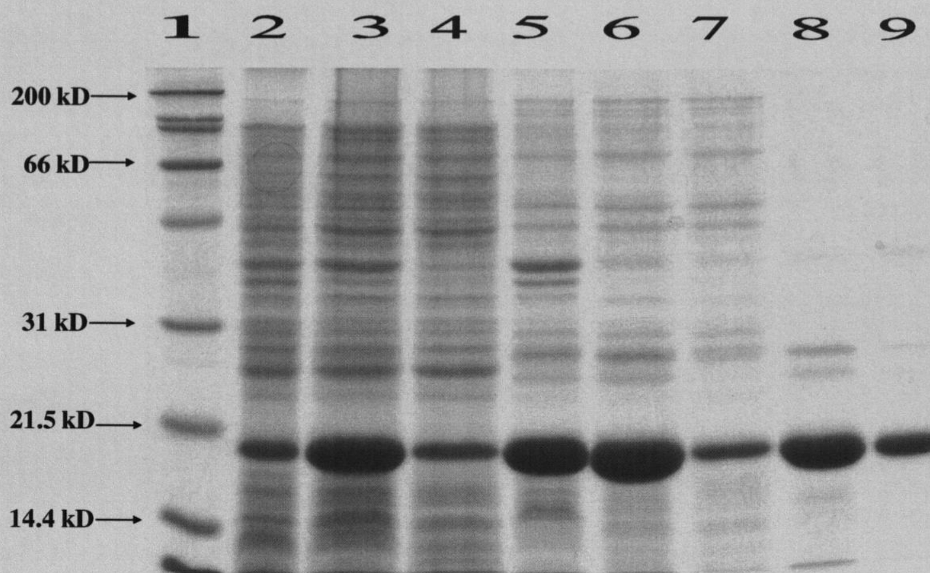

Figure 4.7 SDS-PAGE gel showing the purification of recombinant AsEpIFN-γ. **Lane 1:** Broad Range Mw Marker; **Lane 2:** Total bacterial lysate (uninduced); **Lane 3:** Total bacterial lysate (IPTG-induced); **Lane 4:** Soluble fraction; **Lane 5:** Insoluble fraction (inclusion bodies); **Lane 6:** Solved inclusion bodies (10 M urea); **Lane 7:** Flow-through Ni^{2+} column; **Lane 8:** Protein bound on column matrix; and **Lane 9:** Eluted protein

4.3.4 Immunization of a mouse with rAsEpIFN-γ and the production and screening of IFN-γ specific hybridomas

The presence of antibodies against rAsEpIFN-γ, in the immunized mouse, was determined using an indirect ELISA. The OD_{405} value obtained at Day 0 (before immunization) was 0.202 and at Day 21 and Day 29 it was 0.723 and 0.783 respectively. These results indicated that after immunization and boosting, the mouse showed an increase in antibodies produced against rAsEpIFN-γ. After fusion of spleen cells to Sp2/0 tumour cells, the supernatants of 480 hybridoma clones were screened for rAsEpIFN-γ specificity by ELISA and 41 positive clones were selected. These hybridoma clones were expanded in 25 m*l* tissue culture flasks. In a second ELISA, wells were coated with rAsEpIFN-γ and rEqIFN-γ at a concentration of 1 μg/well. Three clones (11, 19 and 35) showed cross-reactivity to EqIFN-γ and three clones (5, 7 and 21) were specific to rAsEpIFN-γ (Fig. 4.8). Two additional clones, 8 and 12, showed cross-reactivity but the signal obtained with the detection of rAsEpIFN-γ was comparable to

background noise. These two clones showed specificity to rEqIFN-γ and were, therefore, not tested further.

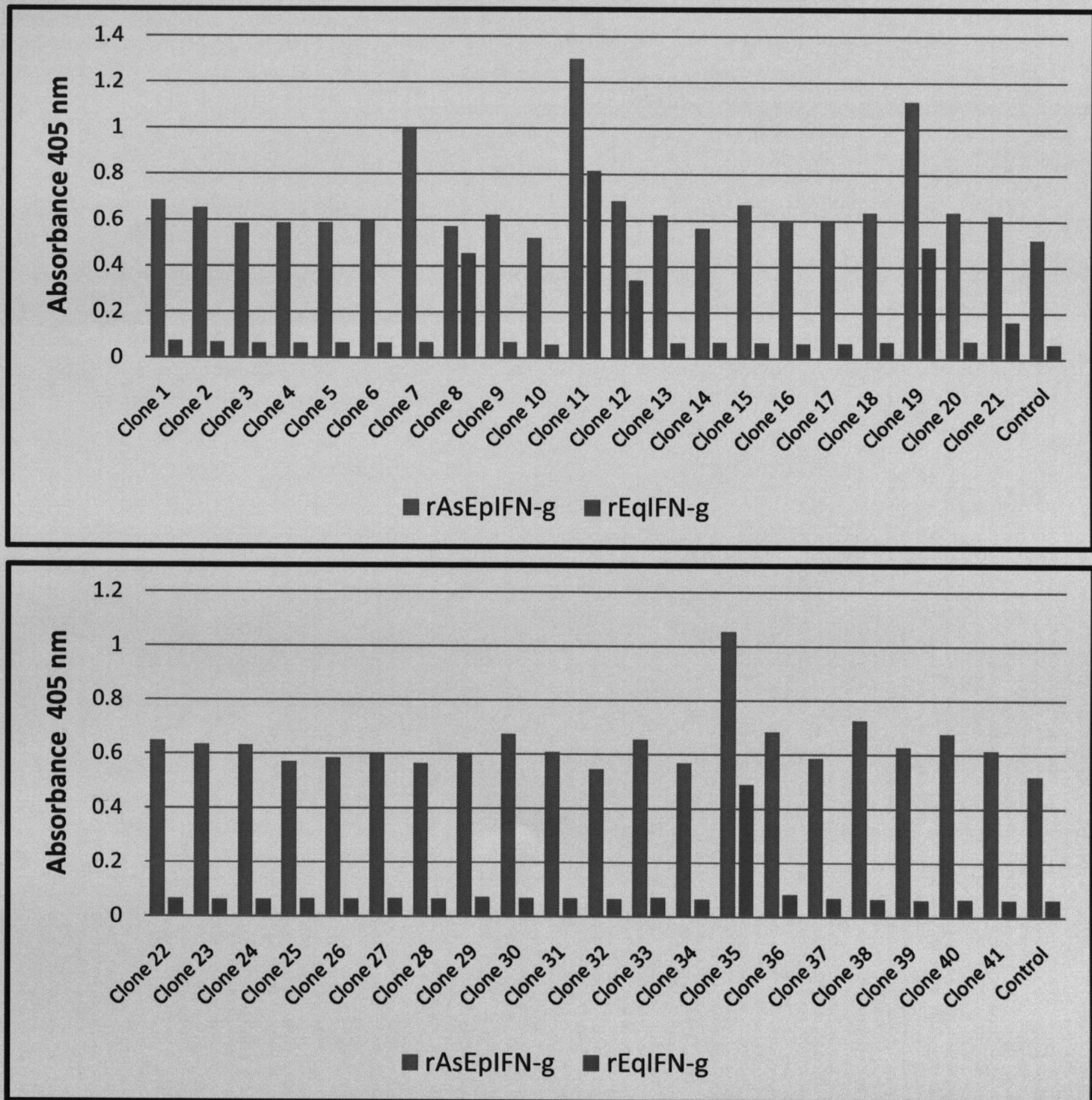

Figure 4.8 Direct ELISA using rAsEpIFN-γ and rEqIFN-γ for screening of 41 antibody producing clones

4.3.5 Western blot

Results of the first western blot that was performed using rAsEpIFN-γ and rEqIFN-γ indicated that the antibody-producing clones 11, 19 and 35 (Fig. 4.9) were cross-reactive with rEqIFN-γ and that clones 5, 7 and 21 were only reacting to rAsEpIFN-γ (results not shown). Results of the second western blot that was performed using three recombinant antigens confirmed that antibody-producing clones 5, 7 and 21 (Fig. 4.10) were specific to rAsEpIFN-γ and did not react with the histidine tag control rMo-GMCSF or with rEqIFN-γ (Fig. 4.10). This second blot also confirmed that antibody-producing clones 11, 19, and 35 (results not shown) were cross-reactive to EqIFN-γ. Results are shown on individual strips of nitrocellulose paper.

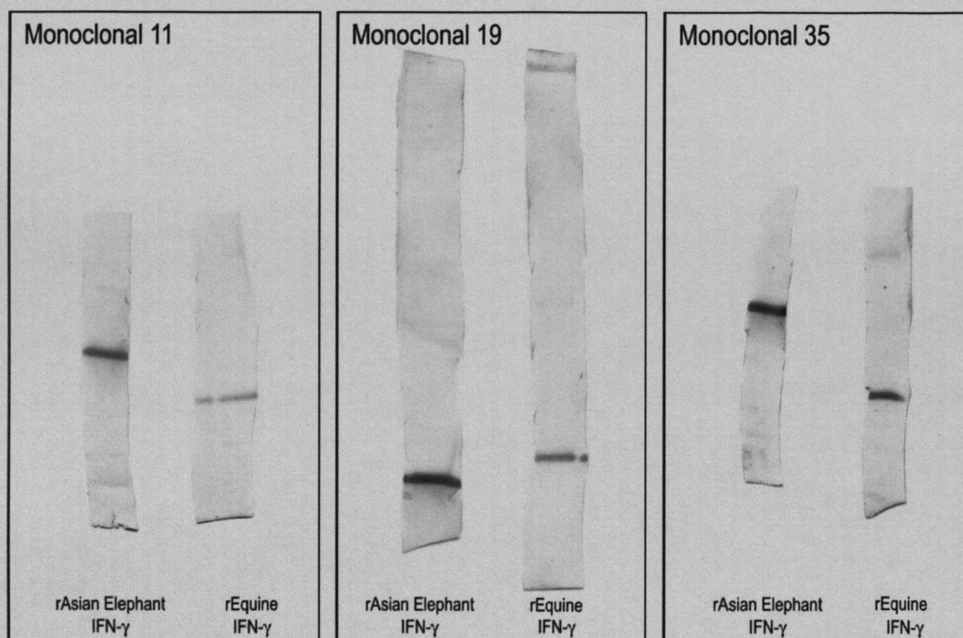

Figure 4.9 Western blots against rAsEpIFN-γ and rEqIFN-γ showing the results obtained using monoclonal antibodies 11, 19 and 35

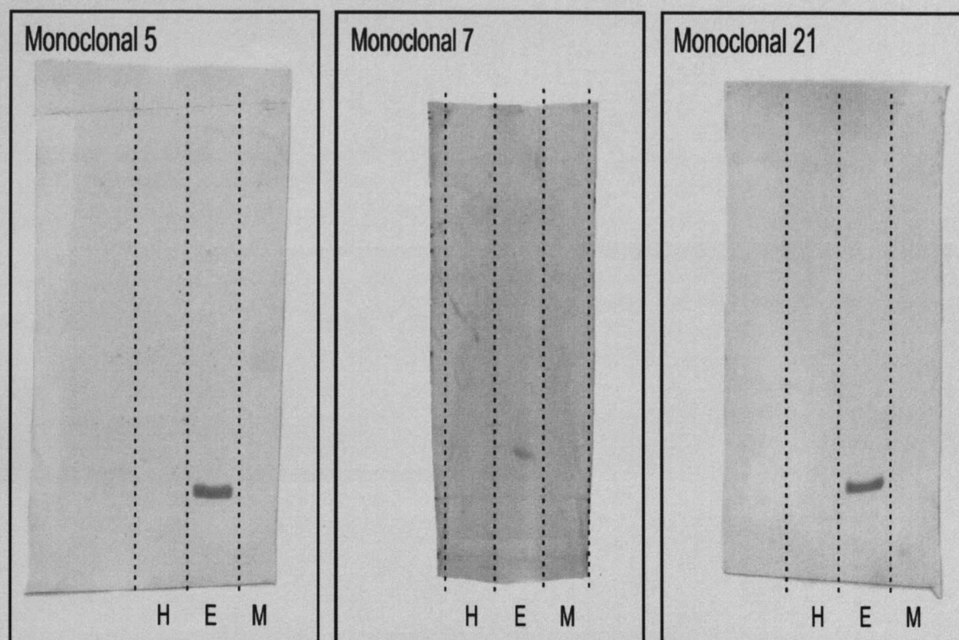

Figure 4.10 Western blots against rEqIFN-γ, rAsEpIFN-γ and rMo-GMCSF showing the results obtained using monoclonal antibodies 5, 7, and 21. H = rEqIFN-γ, E = rAsEpIFN-γ and M = rMo-GMCSF

4.3.6 Isotyping results

All clones were of the IgG1 isotype with kappa light chains.

4.3.7 Establishment of capture ELISA for recombinant AsEpIFN-γ

Antibodies 5, 11, 19, 21 and 35 along with irrelevant antibodies 1F4F4, JJ319 and Q62.1 were used as capture antibodies and three antibodies, 5, 19 and 35 along with purified IFN-γ specific IgY^{uu}, were biotin-labelled and used as antibodies for the detection of rAsEpIFN-γ in a capture ELISA. Antibody 7 did not provide an ideal result during the western blot as compared to the other hybridoma clones that were tested and therefore was not included in the capture ELISAs. Biotin-labelled IgY^{uu} when used as a detecting antibody (Fig. 4.11) gave good results with all relevant capture antibodies but strong signals were also obtained with the irrelevant antibodies (1F4F4, JJ319, Ox62.1) at high concentration of recombinant antigen. The combination of capture antibodies 19 and 21 with detecting antibody IgY^{uu} provided the best detection limits (7 ng of antigen) in this ELISA. When detection was performed with biotin-labelled antibody 5, the most favourable signals were obtained with capture antibodies 11, 19 and 21 (Fig. 4.12) with a detection limit of 125 ng of antigen. When

the trapping combination of the ELISA was changed to detecting antibody 19 (biotin-labelled) positive signals were obtained with capture antibodies 5 and 11 (Fig. 4.13). In this ELISA the detection limit was 125 ng and 31 ng antigen respectively. In the final ELISA where antibody 35 (biotin-labelled) was used as a detecting antibody, capture antibodies 5 and 11 gave favourable signals (Fig. 4.14) and the detection limit ranges between 125 and 62 ng for both combinations.

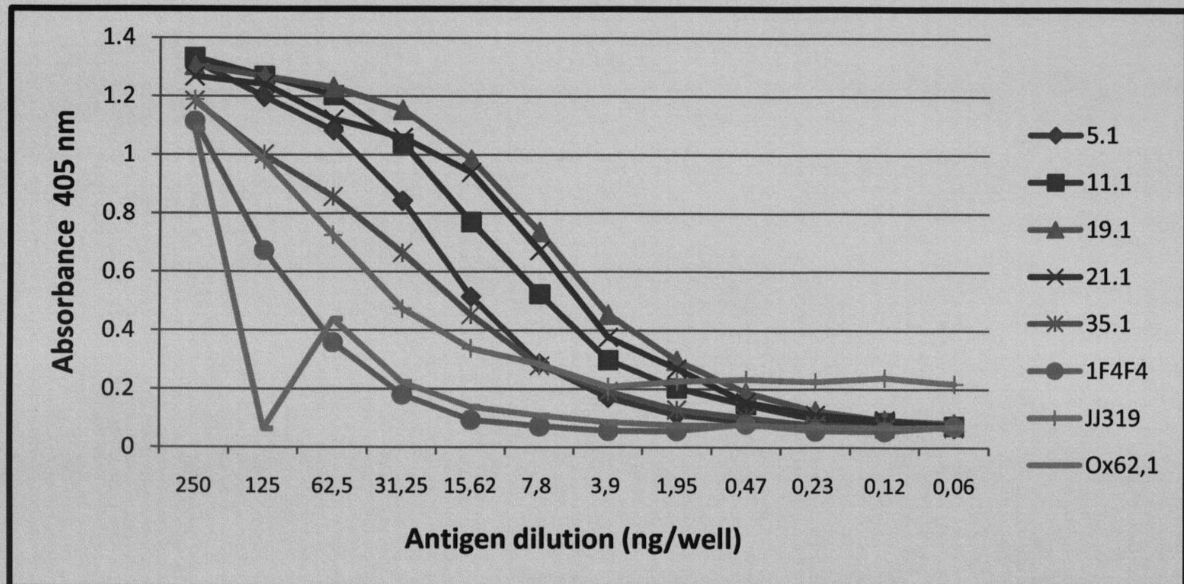

Figure 4.11 Capture ELISA using two-fold dilutions of the antigen (rAsEpIFN-γ) with a starting concentration of 250 ng/well using IgY^{uu} (0.1 μg/well) as detecting antibody. Legend on the right represents the capture antibodies (0.5 μg/well) that were used against rAsEpIFN-γ except 1F4F4, JJ319, Ox62.1 that served as negative control isotypes. The highest OD_{405} signals were obtained with capture antibodies 11, 19 and the 21

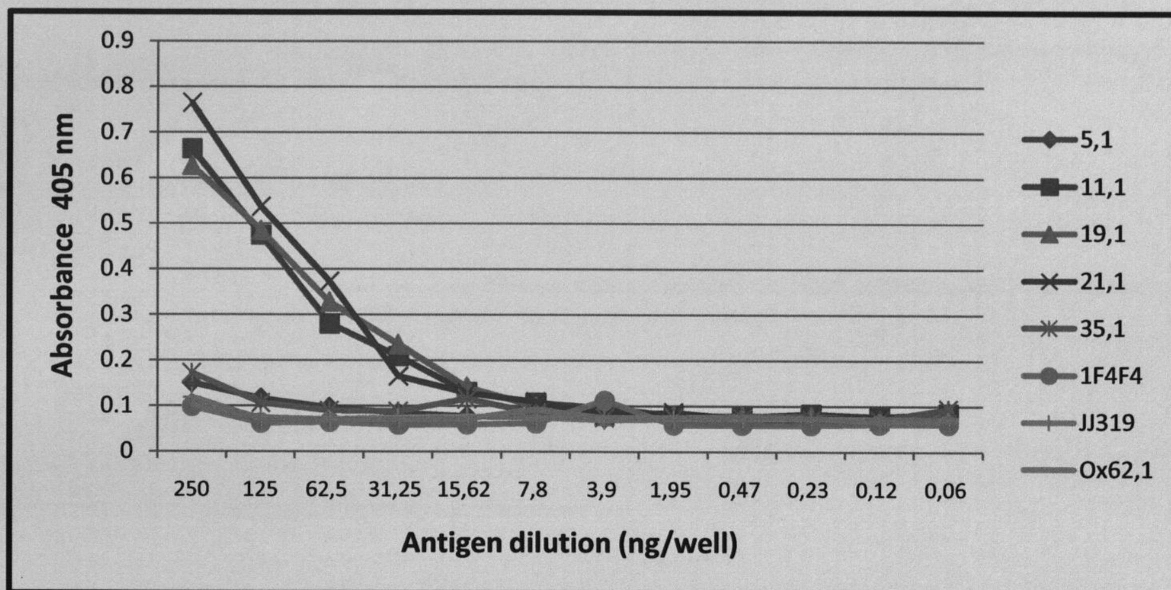

Figure 4.12 Capture ELISA using two-fold dilutions of the rAsEpIFN-γ with a starting concentration of 250 ng/well using monoclonal antibody 5 (0.1 µg/well) as detecting antibody. Legend on the right represents the capture antibodies (0.5 µg/well) that were used against rAsEpIFN-γ except 1F4F4, JJ319, Ox62.1 that served as negative control isotypes. Optimal signals were obtained with capture antibodies 11, 19 and 21

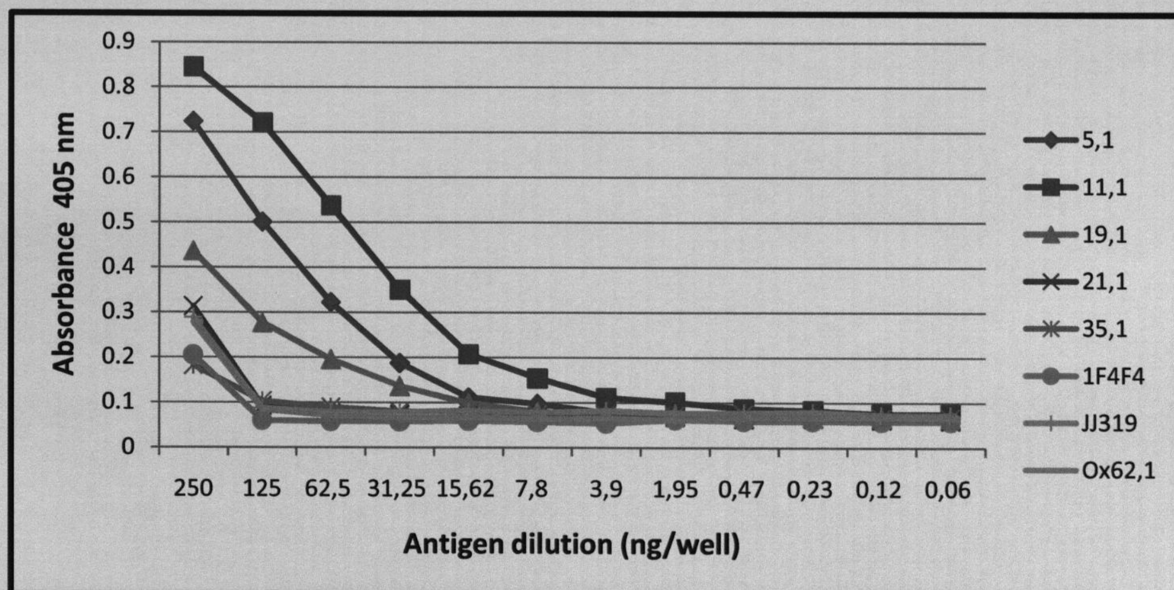

Figure 4.13 Capture ELISA using two-fold dilutions of the antigen (rAsEpIFN-γ) with a starting concentration of 250 ng/well using monoclonal antibody 19 (0.1 µg/well) as detecting antibody. Legend on the right represents the capture antibodies (0.5 µg/well) that were used against rAsEpIFN-γ except 1F4F4, JJ319, Ox62.1 that served as negative control isotypes. Strongest signals are obtained with capture antibodies 5 and 11

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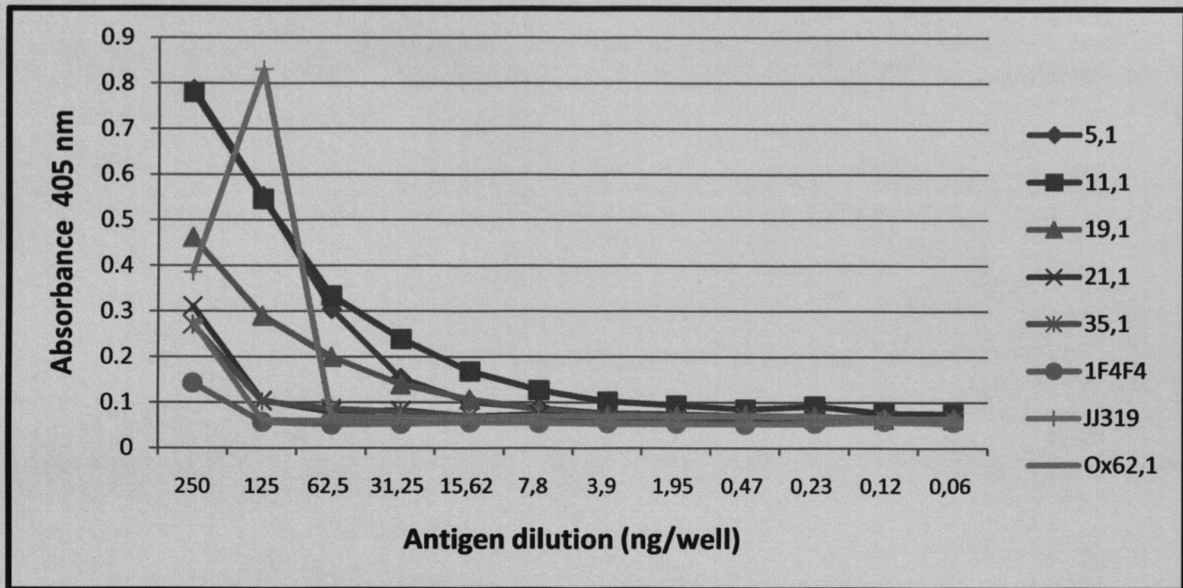

Figure 4.14 Capture ELISA using two-fold dilutions of the antigen (rAsEpIFN-γ) with a starting concentration of 250 ng/well using monoclonal antibody 35 (0.1 µg/well) as detecting antibody. Legend on the right represents the capture antibodies (0.5 µg/well) that were used against rAsEpIFN-γ except 1F4F4, JJ319, Ox62.1 that served as negative control isotypes. Optimal signals were obtained with capture antibodies 5 and 11

4.4 Discussion

Tuberculosis has occurred in zoological elephant populations in the USA and Sweden during the last decade (Mikota *et al.* 2000; Lewerin *et al.* 2005). Diagnostic tests such as MAPIA and the Chembio Elephant Vet Stat-Pak (Larsen *et al.* 2000; Lyashchenko, Singh, Colangeli & Gennaro 2000) target the humoral immune system in order to diagnose whether an elephant is infected with *M. bovis* or *M. tuberculosis*. Despite these, and many other diagnostic tools, there is still a disturbing lack of knowledge concerning the pathogenesis of the disease, its early diagnosis, and correlation of infection and shedding in elephants. Even if elephants do not display clinical signs of the disease they may shed the organism (Lyashchenko *et al.* 2006). In elephants it is not known when shedding begins after an animal is infected. It could be that different doses of the infectious organisms and different routes of infection can affect variations and patterns of shedding (McCorry, Whelan, Welsh *et al.* 2005). In cattle it has been shown that there is a clear dose-response effect between the level of inoculum and the frequency of shedding (McCorry *et al.* 2005), and it is possible that IDT negative animals show no lesions on post-mortem examination yet they may have acted as potential sources of infection. Herein lies the importance of identifying *M. tuberculosis*-

infected and -infectious elephants. The latter are more likely to spread the infection to other elephants, other species of animals and humans (Lyashchenko *et al.* 2006).

In the early phase of infection, assessment of CMI responses in cattle have been shown to be more sensitive than diagnosis based on TB-specific antibodies (Wood, Rothel, McWaters & Jones 1990). The search to find a test correlate of shedding might be best achieved by testing different antigens in the IFN-γ test in these animals. By doing so the development of the IFN-γ ELISA is not restricted to the early detection of infections in elephants especially in Asian elephants in captive situations. It has, however, been established that cellular responses of *M. bovis* infected cattle to individual antigens varies between animals and changes over the time course of infection (Rhodes, Gavier-Widen, Buddle *et al.* 2000b). A dominant antigen, ESAT-6, can be recognised by T-cells following infection in cattle (Pollock & Anderson 1997). In a study performed by Rhodes, Palmer, Graham *et al.* (2000a), IFN-γ responses to tuberculin and ESAT-6 were detected within 4 weeks of initial infection, the IFN-γ responses being observed in 20 of 21 naturally infected cattle. Interferon-γ tests have also proven to successfully identify cattle shedding the organism even if the animals tested negative on IDT and showed no gross lesions during post-mortem (McCorry *et al.* 2005).

This study was aimed at the development of a capture ELISA for the detection of IFN-γ in cell culture supernatants. Interferon-γ production can be detected in whole blood or PBMC cultures when stimulated with the relevant *M. bovis* or *M. tuberculosis* antigens; this may serve as an indication of CMI responses in animals exposed to the infectious organisms. The sequences of Asian and African EpIFN-γ genes were defined and compared with those available from Genbank and the literature. The nt sequences of AsEpIFN-γ and AfEpIFN-γ gene encodes a 166 aa protein. They share a 98% indentity at both the nt and aa level. These sequences are also highly homologous to those of Sreekumar *et al.* (2007) and AbdEl-Gawad *et al.* (2007) with more than 95% homology on the nt level. There is a small difference at the aa level with the Sreekumar sequence (97% identity) and a homology of 90% at the aa level with the AbdEl-Gawad sequence (Table 4.2). When both the Asian (Jasmin) and African (Linda) EpIFN-γ sequences were compared to the EqIFN-γ gene (Genbank Accession Number: D28250) homology at the aa level was 75 %. Despite this difference on the aa level, antibodies specific to and cross-reacting with EqIFN-γ were also identified during the

screening of monoclonal antibodies to rAsEpIFN-γ. This proved useful in developing different formats of capture ELISAs for the detection of rAsEpIFN-γ.

The fact that the AsEpIFN-γ sequence (Jasmin) shares a 98% homology with the AfEpIFN-γ (Linda) at the aa level makes it highly likely that an IFN-γ assay developed for either of the species may cross-react with the IFN-γ of the other. The AsEpIFN-γ (Jasmin) sequence obtained in this study is based on multiple independent sequences and differs somewhat from the previously published Asian elephant sequences. It is also most strongly related to the African genomic sequence. This suggests that this sequence is most similar to the real consensus Asian elephant sequence than the previously published Asian sequences. The highest degree of variation between the Asian and African sequences occurs from aa 145-150.

A total of 480 hybridoma clones were screened and 41 clones were selected for further analysis. Since the elephant and equine IFN-γ genes share a 75% homology at the aa level and rEqIFN-γ was available in the laboratory, it was used to determine if any of the hybridoma clones would detect rEqIFN-γ. The results from the analysis by ELISA showed that three clones (11, 19 and 35) were cross-reacting with rEqIFN-γ and three clones (5, 7 and 21) were specific to rAsEpIFN-γ. These clones, except for hybridoma clone 7, also showed strong immunoreactivity to rAsEpIFN-γ on western blot. Results of the cross-reactivity reactions allowed the differentiation of specific and non-specific (cross-reactive) antibodies. The probability of these antibodies recognising the same epitopes were minimal. Therefore, using this information it provided the opportunity to test variations of capture and detection antibodies in the establishment of the AsEpIFN-γ capture ELISA. Cross-reactive antibodies 11, 19, and 35 and specific antibodies 5 and 21 were used as capture antibodies. The combination of capture antibody 21 and detecting antibody 5 does detect rAsEpIFN-γ and gives the highest signal in this ELISA. Since both antibodies 5 and 21 are specific antibodies, this could indicate that these two antibodies react with different epitopes on the same antigen. Strong signals are also seen with capture antibodies 11 and 19 (both cross-reactive antibodies), and therefore are recognising different epitopes. Although in this ELISA the detection signals (OD_{405}) of the irrelevant antibodies remained between 0.2 and 0.05, the lowest concentration at which this ELISA could detect antigen was 125 ng/well. In the ELISA where cross-reactive antibody 19 is used as a detecting antibody, the results correlate

with the previous ELISA in that antibodies 5 and 19 recognise different epitopes on the antigen. It is clear that antibodies 11 and 19 also recognise different epitopes. The detection limit of this ELISA indicated that 62 ng of protein could be detected with antibody 11 at an OD_{405} reading of 0.85. The other capture antibodies gave much lower signals. The OD_{405} signals of the irrelevant antibodies were below 0.1. In the last ELISA (Fig. 4.13) cross-reactive antibody 35 was used as a detecting antibody. No signal was obtained when antibody 5 was used as a detection antibody in this ELISA, but when the capture ELISA combination is reversed a signal is detected. This may be due to conformational changes of the antibody resulting in its binding to the plastic (Butler, Ni, Nessler *et al.* 1992; Butler, Navarro & Sun 1997). This ELISA also gave the highest detection limit, 250 ng of antigen. The combinations of this ELISA were found to be unsuitable for an IFN-γ capture ELISA.

Polyclonal antibody IgY^{uu}, which was obtained from the previous study (see Chapter 2, 2.2.3), along with antibodies 5, 19 and 35 were biotin-labelled and used as detecting antibodies. The detection of rAsEpIFN-γ was best achieved at a concentration of 7 ng/well with capture antibodies 19 and 21. When the concentration of the antigen was between 250 ng/well and 31 ng/well there was an increase in background values as well, since irrelevant antibodies gave high signals at these concentrations of antigen. The IgY^{uu} served as a good detecting antibody, possibly because of its polyclonal nature that would enable it to bind to different epitopes on the antigen. This capture ELISA would be ideal to use for the detection of lower concentrations of antigen, preferably between 15 to 3 ng/well, but only with capture antibodies 19 and 21.

With regards to the detection limit of the ELISAs, it is low compared to already established IFN-γ tests like Bovigam™ (Wood & Jones 2001). The detection limit of these commercial tests (Bovigam™ and Primagam™) range at picogram levels. The next step is to determine if the test detects native IFN-γ from both elephant species. This will be followed by optimizing the capture ELISA. The ELISA performed on rAsEpIFN-γ demonstrates the potential approach to diagnosing TB / BTB in both Asian and African elephants. Another step will be to test different antigens (ESAT-6 and CFP-10) in order to detect early infection in African elephants and to identify shedders (Asian elephants).

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