UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Development of ELISAs for the detection of interferon-gamma in rhinoceroses and elephants as diagnostic tools for *Mycobacterium bovis* and *Mycobacterium tuberculosis* infections

BY

DARSHANA MORAR

SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF PHILOSOPHIAE DOCTOR (PhD) IN THE FACULTY OF VETERINARY SCIENCE, UNIVERSITY OF PRETORIA

JULY 2009

© University of Pretoria

Acknowledgements

I wish to express my sincere appreciation and gratitude to:

Jacques Godfroid, I am fortunate to have met and to have worked with you. I am grateful to you for being my mentor and friend. As you always say "***La vie est belle***". Thank you Jacques for your support, guidance and encouragement.

A special thank you to my co-supervisors Victor Rutten and Jeanni Fehrsen. Victor, I have worked with you for a long time and in that time you have provided me with guidance, support, motivation and encouragement. I am grateful to you for this, thank you. Jeanni, thank you for your supervision, guidance and for providing me with the training and skills to work with the phage-display system.

Prof Koos Coetzer, thank you for believing in me and for those wise words "just a little bit, little bit at a time." It has helped me get through the days when I felt like giving up.

Anita L. Michel, thank you for giving me the opportunity to work on this project. Without it I would not have experienced the world of wildlife veterinary science. I have learnt much and I will always be grateful to you for this.

Jackie Picard, I am grateful to you for always giving me advice whenever I came to you for assistance.

Tshepo Matjila, thank you for all the advice and support you gave me during this project. You are a good friend and work colleague.

A special thank you to Rina Serfontein for assisting me in the formatting of this thesis.

Lilly Seshoka, Fransie Lottering and Nomsa Molekoa, thank you for assisting me with various tasks for the duration of my research.

Edwin Tijhaar, thank you for your supervision, guidance and friendship. I am very grateful that I had the opportunity to have met you and to have worked with you. It was an enjoyable experience.

A special thank you to Peter van Kooten for the production of mouse monoclonal antibodies, and for providing me with advice and guiding me throughout the process of monoclonal antibody production.

Relu Negrea, thank you for assisting in the isolation and purification of the IgY in Utrecht.

Taweepoke Angkawanish thank you for performing the capture ELISAs for the Asian elephant study.

Thank you to Ildiko van Rhijn for your supervision and guidance.

Thank you to Daphne van Haarlem and Judith Hendriks for your assistance in the lab.

Thank you to Dion du Plessis for the use of the facilities at the Immunology Division of the Onderstepoort Veterinary Institute.

A special thank you to Wouter van Wyngaardt for your guidance and advice whenever I needed it. Thank you also for providing me with the Nkuku® library and some of the ELISA reagents for the phage-display experiments.

Joy Sixholo, thank you for providing me with TG1 competent cells.

Magdeline Rakabe, thank you for providing me with the prepared affinity columns.

A special thank you to Peter Buss (Wildlife and Game Capture Unit, Kruger National Park, Skukuza, South Africa) for your advice and providing the white rhinoceros blood samples.

My appreciation goes out to Jacques Barnard, Shahn Bisschop and Jack Maphothoma of the Poultry Reference Centre for providing me with the silver-hyline chickens, the use of their facilities and their assistance during the course of the immunization study.

The group at Wildlife-Assignments, www.efafdinokeng.co.za, for providing me with the African elephant blood samples.

Willem Schafternaar from the Blijdorp Zoo, Rotterdam, The Netherlands for providing me with Asian elephant blood samples, and Jacques Kaandorp from the Beekse Bergen Safari Park in Tilburg, The Netherlands for the African elephant blood samples.

A special thank you to Prof Roy Tustin, for editing my thesis and providing me with valuable advice.

My parents, to whom I dedicate this study, thank you for your support, love and guidance.

My siblings, Kamlesh, Kalpana and Sunil, and my sister-in-law, Jayshree, thank you for your understanding and support.

Sean Leather, thank you for your love, support, patience and understanding, and most importantly thank you for always believing in me.

Jack and Vicky Leather, thank you for taking an interest in my project. I am grateful for all the support and encouragement that you gave me.

My friends, Raksha Bhoora, Anna-Mari Bosman, Thamsanqa Chiliza, Minishca Dhoogra, Akin Jenkins, Nirusha Kassen, Omer Kibeida, Miriam Maas, Cordelia Mashau, Justin Masumu, Tshepo Matjila, Yugashnee Naidoo, Marinda Oosthuizen, Visva Pillay, Kgomotso Sibeko, Joy Sixholo, Ndavhe Tshikhudo and Yusufu Woma: thank you for your support, advice and encouragement.

Utrecht Delta Scholarship – Utrecht University, The Netherlands; National Research Foundation – Thuthuka Grant, South Africa; Belgium Grant - Institutional Collaboration between Institute of Tropical Medicine in Antwerp, Belgium and Department of Veterinary Tropical Diseases, University of Pretoria (95401) Framework Agreement DGIC-ITM 2003-2007, University of Pretoria Postgraduate Abroad Programme – University of Pretoria, South Africa.

List of Contents

Acknowledgements ---------- **ii**

List of Abbreviations ---------- **x**

List of Figures ---------- **xiv**

List of Tables ---------- **xvii**

Thesis Summary ---------- **xviii**

Chapter 1 Literature review ---------- **1**

1.1 History ---------- 1

1.2 Mycobacteria of the *Mycobacterium tuberculosis* complex (MTBC) ---------- 2

1.3 Tuberculosis in humans ---------- 3

1.3.1 Diagnosis of tuberculosis in humans ---------- 5

1.4 Tuberculosis in animals ---------- 7

1.4.1 Cattle ---------- 8

1.4.2 Free-ranging wildlife ---------- 9

1.4.3 Captive wildlife with special emphasis on rhinoceroses and elephants ---------- 10

1.4.3.1 Rhinoceroses ---------- 10

1.4.3.2 Elephants ---------- 11

1.5 Diagnosis of *M. bovis* and *M. tuberculosis* infections in animals ---------- 14

1.5.1 Post-mortem ---------- 14

1.5.2 Identification of the bacteria and / or their genetic material ---------- 14

1.5.3 Genotyping of mycobacteria ---------- 15

1.5.3.1 Restriction enzyme analysis ---------- 15

1.5.3.2 Polymorphic (GC)-rich sequences ---------- 16

1.5.3.3 Restriction fragment length polymorphism ---------- 16

1.5.3.4 IS*6110*- Restriction fragment length polymorphism ---------- 16

1.5.3.5 Spoligotyping ---------- 17

1.5.3.6 Variable number of tandem repeats ---------- 17

1.5.4 *Ante-mortem* diagnosis ---------- 18

1.5.5 Immune reactions to mycobacteria ---------- 18

1.5.5.1 Cytokines ---------- 18

1.5.5.2 Immunopathogenesis ---------- 19

1.6 Exploiting immune responses against *M. bovis* and *M. tuberculosis* for diagnosis ---------- 22

1.6.1 Intradermal tuberculin test----------22
1.6.2 Mycobacterial antigens ----------23
1.6.3 Blood-based tests ----------24
1.6.3.1 Serological tests ----------24
1.6.3.2 Lymphocyte proliferation assay----------26
1.6.3.3 Interferon-gamma assay----------26
1.7 Antibodies as tools to develop IGRAs ----------27
1.8 Problem statement----------30
1.9 Research objectives ----------33
1.10 Impact ----------33
1.11 References ----------34
Chapter 2 Cloning, sequencing and expression of white rhinoceros (*Ceratotherium simum*) interferon-gamma and the production of white rhinoceros interferon-gamma specific antibodies----------63
Abstract ----------63
2.1 Introduction ----------64
2.2 Materials and Methods----------65
2.2.1 Cloning and sequencing of white RhIFN-γ ----------65
2.2.2 Expression and purification of recombinant white RhIFN-γ ----------68
2.2.3 White RhIFN-γ specific poly- and monoclonal antibodies ----------69
2.2.4 Screening of hybridomas for antibody production by indirect ELISA----------70
2.2.5 Native IFN-γ----------70
2.2.6 Prototype capture ELISA for detection of native white RhIFN-γ ----------70
2.3 Results----------71
2.3.1 Cloning and sequencing of the white RhIFN-γ gene ----------71
2.3.2 Expression and purification of white rRhIFN-γ----------73
2.3.3 Prototype capture ELISA for the detection of white RhIFN-γ----------74
2.4 Discussion ----------75
2.5 Publication----------77
2.6 References ----------78
Chapter 3 White rhinoceros (*Ceratotherium simum*) and Asian elephant (*Elephas maximus*) interferon-gamma specific recombinant chicken antibodies ----------81
Abstract ----------81

3.1 Introduction ----------82
3.2 Materials and Methods----------83
3.2.1 Immunization of chickens ----------83
3.2.2 Isolation of yolk immunoglobulin (IgY) from chickens immunized with rRhIFN-γ----------83
3.2.3 ELISA to determine antibody titres of chicken immunized with rRhIFN-γ ----83
3.2.4 Isolation of total RNA from chicken spleen ----------84
3.2.5 cDNA synthesis ----------85
3.2.6 Amplification of V_H and V_L chain genes----------85
3.2.7 Selection of phage antibodies (Biopanning) ----------88
3.2.8 Polyclonal phage ELISA ----------89
3.2.9 Monoclonal phage ELISA----------90
3.2.10 Screening of soluble scFv antibody fragments by ELISA ----------92
3.2.11 DNA sequencing----------92
3.2.12 Purification of soluble scFvs ----------93
3.2.13 Different formats of the IFN-γ capture ELISA ----------93
3.3 Results----------95
3.3.1 Antibody titres ----------95
3.3.2 Library construction ----------95
3.3.3 Selection of binders ----------97
3.3.4 Antigen specificity and cross-reactivity of phage-displayed antibodies and scFvs ---------- 100
3.3.5 Sequence analysis of selected clones ---------- 102
3.3.6 scFv Capture ELISAs to detect rRhIFN-γ and rAsEpIFN-γ---------- 103
3.4 Discussion ---------- 110
3.5 References ---------- 114
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 ---------- 118
Abstract ---------- 118
4.1 Introduction ---------- 119
4.2 Materials and Methods---------- 121

4.2.1 Culture and mitogen stimulation of peripheral blood mononuclear cells (PBMCs) ---------- 121
4.2.2 African Elephant IFN-γ ---------- 122
4.2.2.1 RNA isolation and RT-PCR ---------- 122
4.2.2.2 Cloning and sequencing ---------- 122
4.2.3 Asian Elephant IFN-γ ---------- 123
4.2.3.1 RNA isolation, RT-PCR, Gateway PCR and Cloning ---------- 123
4.2.4 Sequence analysis ---------- 125
4.2.5 Expression and purification of rAsEpIFN-γ ---------- 125
4.2.6 The production and selection of monoclonal antibodies ---------- 126
4.2.6.1 Immunization protocol ---------- 126
4.2.6.2 Isolation of spleen cells ---------- 127
4.2.6.3 Fusion Technique ---------- 127
4.2.6.4 Screening of hybridomas for antibody production ---------- 127
4.2.7 Western blot analysis of gene products ---------- 128
4.2.8 Mouse hybridoma isotyping ---------- 129
4.2.9 Establishment of a capture ELISA for recombinant AsEpIFN γ ---------- 129
4.3 Results ---------- 130
4.3.1 Cloning and sequencing of African and Asian EpIFN-γ ---------- 130
4.3.2 Sequencing results ---------- 131
4.3.3 Expression and purification of rAsEpIFN-γ ---------- 134
4.3.4 Immunization of a mouse with rAsEpIFN-γ, and the production and screening of IFN-γ specific hybridomas ---------- 135
4.3.5 Western blot ---------- 137
4.3.6 Isotyping results ---------- 138
4.3.7 Establishment of capture ELISA for recombinant AsEpIFN-γ ---------- 138
4.4 Discussion ---------- 141
4.5 References ---------- 145
Chapter 5 Discussion and concluding remarks ---------- 151
5.1 Introduction ---------- 151
5.2 Diagnosis ---------- 152
5.3 Control measures ---------- 152
5.4 Development of an IFN-γ enzyme-linked immunosorbent assay (ELISA) ---------- 154

5.5 Conclusion ---------- 160
5.6 References ---------- 161

List of Abbreviations

a	alanine
aa	amino acid
A	ampicillin
ABTS	2, 2'-azino-bis(3-ehtylbenzthiazoline-6-sulphonic acid)
AfEpIFN-γ	African elephant interferon-gamma
Ag85	antigen 85
AG	ampicillin and glucose
AK	ampicillin and kanamycin
AMI	antibody-mediated immunity
AsEpIFN-γ	Asian elephant interferon-gamma
AUCC	Animal Use and Care Committee
BCG	Bacille Calmette-Guérin
bps	base pairs
BSA	bovine serum albumin
BTB	bovine tuberculosis
cDNA	complementary DNA
CDR	complementarity determining region
CFP-10	culture filtrate protein
CMI	cell mediated immunity
Con A	concanavalin A
CSF	colony stimulating factor
DAB	diaminobenzidine
DIVA	differentiating between infected and vaccinated animals
DR	direct repeat
DTH	delayed type hypersensitivity
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EIA	enzyme immuno-assay
ELISA	enzyme-linked immunosorbent assay
ESAT-6	early secreted antigenic target-6
ETR	exact tandem repeats
FCS	foetal clone serum

G	glucose
GW	Gateway
h	hour / s
HiP	Hluhluwe-iMfolozi Park
HIV	human immunodeficiency virus
HRP	horse radish peroxidase
HT	hypoxanthine thymidine
ICGA	immuno-chromatographic assay
IUCN	International Union for Conservation of Nature
IDT	intradermal test
IFN-γ	interferon-gamma
IgG	immunoglobulin G
IGRA	interferon-gamma release assay
IL	interleukin
IgY	yolk immunoglobulin
IgY^{uu}	IgY produced at Utrecht University
IgY^{up}	IgY produced at University of Pretoria
IPTG	isopropyl-β-D-1-thiogalactopyranoside
ip	intraperitoneal
IS	insertion sequence
IMAC	immobilized metal affinity chromatography
K	kanamycin
KNP	Kruger National Park
LB	Luria broth
LBAA	latex bead agglutination assay
LTBI	latent tuberculosis infection
MAPIA	multi-antigen print immuno-assay
MBCF	*Mycobacterium bovis* culture filtrate
MDR-TB	Multi-drug resistant-tuberculosis
min	minute / s
MHC	major histocompatibility complex
MPB/T	major secreted immunogenic protein
MP	fat-free milk powder

MIRU	mycobacterial interspersed repetitive units
MTBC	*Mycobacterium tuberculosis* complex
MW	moleclular weight
NCBI	National Center for Biotechnology Information
NDSB 201	non detergent sulfobetaines
NK	natural killer cell
nt	nucleotide
OD	optical density
OIE	Organisation Mondiale de la Santé Animale / World Organisation for Animal Health
OPD	*ortho*-phenylenediamine dihydrochloride
OVI	Onderstepoort Veterinary Institute
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PGRS	polymorphic (GC)-rich sequences
POD	peroxidase
PPD	purified protein derivative
QFT	QuantiFERON®
REA	restriction enzyme analysis
rEpIFN-γ	recombinant elephant interferon-gamma
rEqIFN-γ	recombinant equine interferon-gamma
RFLP	restriction fragment length polymorphism
rMoGMCSF	recombinant mouse granulocyte macrophage colony stimulating factor
RNA	ribonucleic acid
rRhIFN-γ	recombinant rhinoceros interferon-gamma
rpm	revolutions per minute
RT	Rapid Test
RT-PCR	reverse transcriptase-PCR
s	second / s
scFv	single chain variable fragment
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SICTT	single intradermal comparative tuberculin test
SIT	single intradermal test
SOE	splice overlap extension
TB	tuberculosis
TEA	triethylamine
Th1	T-helper cell that participates in CMI
Th2	T-helper cell that participates in AMI
TMB	tetramethylbenzidine
TNF	tumour necrosis factor
TST	tuberculin skin test
2xTY	tryptone yeast medium
U	units (unit of enzyme)
UP	University of Pretoria
USDA	United States Department of Agriculture
UU	Utrecht University
v	valine
V_H	variable part of the heavy chain
V_L	variable part of the light chain
VNTR	variable number of tandem repeats
WHO	World Health Organisation

List of Figures

Figure 1.1 Diagrammatic representations of the pleiotropic functions of IFN-γ (Alvarez 2006) ----------19

Figure 1.2 During the former (less advanced) stages of the disease the CMI response predominates. As the disease progresses CMI responses tend to wane and the humoral immunity takes over. Finally, in the clinical stage of the disease, reached by only a minority of infected animals, *M. bovis* specific immunity disappears completely, while bacterial shedding occurs (Pollock & Neill 2002) ----------22

Figure 1.3 Diagrammtic representation of an IgG molecule and a single chain variable fragment----------28

Figure 1.4 Construction of a phage-display antibody library from an immunized chicken ----------30

Figure 2.1 Nucleotide sequence of RhIFN-γ gene (Genbank Accession Number: DQ305037)----------72

Figure 2.2 Alignment of predicted protein sequences for white RhIFN-γ and EqIFN-γ----------73

Figure 2.3 SDS-PAGE showing the purification of white rRhIFN-γ----------74

Figure 2.4 Detection of white RhIFN-γ in PBMC stimulated with Bovine PPD, Avian PPD and Con A using the IFN-γ capture ELISA----------75

Figure 3.1 Alignment of predicted sequences for white RhIFN-γ and AsEpIFN-γ -89

Figure 3.2 Schematic representation of the phagemid vector pHEN I----------91

Figure 3.3 ELISA showing the antibody titres of immunized chickens from Day 0 to Day 98 ----------95

Figure 3.4 Agarose gel of the primary PCR for the amplification of V_H and V_L chains ----------96

Figure 3.5 Agarose gel of the SOE PCR ----------97

Figure 3.6 Polyclonal phage ELISA of phage pools obtained from different rounds of panning reacting to rRhIFN-γ----------98

Figure 3.7 Polyclonal phage ELISA of phage pools obtained from different rounds of panning reacting to rAsEpIFN-γ ----------98

Figure 3.8 Direct ELISA to determine the antigen specificity and cross-reactivity of phage-displayed antibodies to rRhIFN-γ and to rAsEpIFN-γ---------- 101

Figure 3.9 Direct ELISA to determine the antigen specificity and cross-reactivity of scFv antibodies to rRhIFN-γ and to rAsEpIFN-γ ---------- 101

Figure 3.10 Amino acid alignment of 1F5 (rhinoceros) and 6D7 (elephant) clones 102

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 ---------- 103

Figure 3.12 Capture ELISA using capture antibody scFv 1F5, at a concentration of 5 μg/m*l*, and detection of rAsEpIFN-γ with antibody 1D11---------- 104

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 ---------- 105

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---------- 106

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 ---------- 107

Figure 3.16 Capture ELISA using capture antibody scFv 6D7, at a concentration of 5 μg/m*l*, and antibody 1D11 for the detection of rAsEpIFN-γ---------- 108

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 ---------- 109

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---------- 109

Figure 4.1 Diagrammatic representation of the TOPO Vector (Invitrogen) used for cloning of AfEpIFN-γ---------- 123

Figure 4.2 Diagrammatic representation of the pET15b vector (Novagen)--------- 125

Figure 4.3 PCR products from cDNA (African elephant, Linda) amplification --- 131

Figure 4.4 Nucleotide sequences of the AfEpIFN-γ (Linda) ---------- 133

Figure 4.5 Nucleotide sequence of the AsEpIFN-γ (Jasmin)---------- 133

Figure 4.6 Alignment of predicted protein sequences for AsEpIFN-γ, AfEpIFN-γ and EqFN-γ---------- 134

Figure 4.7 SDS-PAGE gel showing the purification of recombinant AsEpIFN-γ-- 135

Figure 4.8 Direct ELISA using rAsEpIFN-γ and rEqIFN-γ for screening of 41 antibody producing clones---------- 136

Figure 4.9 Western blots against rAsEpIFN-γ and rEqIFN-γ showing the results obtained using monoclonal antibodies 11, 19 and 35 ---------- 137

Figure 4.10 Western blots against rEqIFN-γ, rAsEpIFN-γ and rMo-GMCSF showing the results obtained using monoclonal antibodies 5, 7, and 21 ---------- 138

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 ---------- 139

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 ---------- 140

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 ---------- 140

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 ---------- 141

List of Tables

Table 1.1 Classification of the *Mycobacterium tuberculosis* Complex (Bergey & Holt 1993)---------- 2

Table 1.2 Reported incidence of TB in African and Asian elephants ----------13

Table 3.1 Primers used for V_H and V_L region amplification (van Wyngaardt *et al.* 2004)----------86

Table 3.2 The percentage output of phages recovered after each selection round 97

Table 3.3 Clones that were identified as binders from four rounds of panning combinant proteins----------99

Table 4.1 Primers used for isolation and cloning of AsEpIFN-γ gene ---------- 126

Table 4.2 Percent homology on the amino acid and nucleotide levels between the different elephant IFN-γ sequences ---------- 132

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Thesis Summary

Development of ELISAs for the detection of interferon-gamma in rhinoceroses and elephants as diagnostic tools for *Mycobacterium bovis* and *Mycobacterium tuberculosis* infections

by

Darshana Morar

Promoter: Prof. J. Godfroid
Co-Promoters: Prof. V. PMG. Rutten and Dr. J. Fehrsen

Degree: PhD in Veterinary Science

Bovine tuberculosis, caused by *Mycobacterium bovis*, has been reported in many wildlife species. In addition, it has been reported that *Mycobacterium tuberculosis* causes tuberculosis mainly in Asian elephants (*Elephas maximus*). The disease cannot be diagnosed clinically in its early stages since clinical signs only appear during the later stages of the infection. For early detection diagnosis has to be performed using cell mediated immune based techniques. For cattle, validated tests include the *in vivo* intradermal skin test (IDT) and the *in vitro* interferon-gamma (IFN-γ) based test. The IDT has not been validated for use in wildlife. In addition, this test would not be suitable for use in rhinoceroses and elephants due to their skin anatomy and the fact that animals have to be captured and immobilized twice. Bovigam™, proven to be very effective in detecting *M. bovis* infections in cattle, is used as an ancillary test but this enzyme-linked immunosorbent assay (ELISA) only recognizes the IFN-γ of cattle and of a limited number of other ruminant species. Therefore, anti-IFN-γ antibodies for different wildlife species have to be produced in order to make use of an IFN-γ test for the diagnosis of (bovine) tuberculosis in wildlife.

This thesis presents the results of a series of studies aimed towards the development of an IFN-γ capture ELISA for the early detection of *M. bovis* and *M. tuberculosis* infections, and the detection of infectious animals (shedders) in wildlife species. The first set of studies led to the production of monoclonal and polyclonal antibodies against recombinant white

rhinoceros IFN-γ (rRhIFN-γ) in mice and chickens respectively. One monoclonal antibody, 1H11 (and its subclone 1D11), was identified as a suitable antibody for the capture of both rRhIFN-γ and native RhIFN-γ, using polyclonal IgY as a detecting antibody (Chapter 2). To increase the number of IFN-γ specific antibodies to RhIFN-γ, the phage-displayed technique was utilized in the second study (Chapter 3). An immune phage-display library targeted against rRhIFN-γ was constructed. The library was panned against both rRhIFN-γ and recombinant Asian elephant IFN-γ (rAsEpIFN-γ). The antibodies, single chain variable fragments (scFvs), generated in this study (Chapter 3) were used as capture antibodies and 1D11 or IgY as detecting antibodies in an ELISA for the detection of rRhIFN-γ and rAsEpIFN-γ. The capture ELISAs proved to be most effective in detecting rRhIFN-γ. Recombinant AsEpIFN-γ could only be detected with the scFv/IgY ELISA format. In the third study (Chapter 4) efforts were concentrated at producing monoclonal antibodies in mice against rAsEpIFN-γ. Six monoclonal antibodies were identified. Three were specific to rAsEpIFN-γ and three cross-reacted with recombinant equine IFN-γ (rEqIFN-γ). These antibodies along with polyclonal IgY were used in different capture ELISAs to determine which one would provide the optimal results in detecting rAsEpIFN-γ. Results indicated detection of rAsEpIFN-γ was best achieved when a cross-reactive antibody was used as a capture antibody and a specific antibody was used as a detecting antibody.

Altogether, these results document the detection of rRhIFN-γ and rAsEpIFN-γ in different capture ELISAs. Therefore, these ELISAs provide the first steps towards the development of suitable diagnostic tools for the detection of *M. bovis* and *M. tuberculosis* infections in wildlife species.

"You are never given a wish without also being given the power to make it come true. You may have to work for it, however." (Bach 1977)

Chapter 1
Literature review

1.1 History

Phthisis is what the Greeks (ca 460 BC) called tuberculosis (TB) and it means to waste away. It was very appropriately named since emaciation is one of the most prominent clinical signs of human and bovine TB (BTB) (Herzog 1998). It was not until 1865 that Villemin (1827-1892), a French military doctor, discovered that the disease in humans or cattle could be transmitted to rabbits (Herzog 1998; Daniel 2006). By performing an experiment in animals he injected blood or sputum from humans and cattle into laboratory rabbits and found that they developed TB, whereas a similar transfer of cancerous tissue displayed no effect on the recipients (Sakula 1983). At this stage the causative agent was not yet identified. Discovering that the disease was communicable led to the understanding that the disease is caused by a specific micro-organism. These findings by Villemin were published in *Comptes rendus de l'Academie des Sciences* (Sakula 1983). Robert Koch (1882) discovered the tubercle bacillus but did not differentiate between the isolates from cattle and humans (Koch 1882; Grange & Bishop 1982). It was Professor Theobald Smith who, in 1898, differentiated between the two tubercle bacilli and noted that the designation of the two types did not imply that they were restricted to the hosts after which they were named (Sakula 1983).

During the early 1900s there was much controversy about the importance of the bovine tubercle bacillus as a source of human disease. Koch proclaimed that humans were immune or only slightly susceptible and that control measures in cattle were unnecessary (Sakula 1983). Koch, however, failed to convince many people from the veterinary profession and M'Fadyean, Ravanel and Bang (Grange & Bishop 1982) along with other researchers started obtaining data regarding this issue. Ten years later results of various researchers refuted Koch's views of *Mycobacterium bovis* and established the risk of bovine tubercle bacilli to human health, and confirmed that milk is the principal source of transmission. Measures were put in place and were made effective by the policy of eradication of infected cattle and the pasteurization of milk (Sakula 1983). The discoveries of Robert Koch and other famous scientists who contributed significantly to mycobacteriology have stimulated others to search

for preventative and curative modalities, and the development of diagnostic techniques for both TB and BTB.

1.2 Mycobacteria of the *Mycobacterium tuberculosis* complex (MTBC)

Mycobacteria belong to the order Actinomycetales, family Mycobacteriaceae, and genus *Mycobacterium* (Table 1.1). Mycobacteria of the MTBC are aerobic, non-motile bacilli with a thick prominent cell wall which is rich in mycolic acids and is hydrophobic (Rastogi, Legrand & Sola 2001). Due to this structural characteristic the Gram staining technique cannot be used to assist identification. Instead they are referred to as acid-fast bacteria because once they are stained with the Ziehl-Neelsen staining method (Van Deun, Hossain, Gumusboga & Rieder 2008) they resist decolouration with acid-alcohol. These mycobacteria are slow growers with a doubling time of 18 to 24 hours. They grow optimally at 37°C (Karlson & Lessel 1970; Rastogi *et al.* 2001).

Table 1.1 Classification of the *Mycobacterium tuberculosis* Complex (Bergey & Holt 1993)

Kingdom	Bacteria
Phylum	Actinobacteria
Order	Actinomycetales
Suborder	Corynebacterineae
Family	Mycobacteriaceae
Genus	*Mycobacterium*
Species	*M. tuberculosis, M. bovis, M. caprae, M. microti, M. pinnipedii, M. africanum*

The MTBC encompasses the following species: *M. tuberculosis* (Rastogi *et al.* 2001) the human tubercle bacillus; *M. bovis* (Karlson & Lessel 1970) the bovine tubercle bacillus; *M. microti* (Reed 1957) a rare pathogen of rodents and other small mammals; *M. africanum* causing TB in humans mainly in equatorial Africa; *M. pinnipedii* (Cousins, Francis, Gow *et al.* 1990; Cousins, Bastida, Cataldi *et al.* 2003) primarily infecting seals. *Mycobacterium caprae* (Aranaz, Cousins, Mateos & Dominguez 2003), initially classified as a subspecies of *M. bovis,* was recently recognised as a species on its own. This pathogen primarily infects goats but cattle, pigs and humans are also susceptible (Cvetnic, Katalinic-Jankovic, Sostaric *et al.* 2007).

Modern genome analysis of the MTBC has revealed, contrary to previously held dogma, that human TB has not evolved from *M. bovis* (Brosch, Gordon, Marmiesse *et al.* 2002). A separate lineage that is represented by *M. africanum*, *M. microti* and *M. bovis* branched from the progenitor of *M. tuberculosis* strains. This process was accompanied by a successive loss of DNA, which may have contributed to the appearance of more successful pathogens in new host species. *Mycobacterium canettii*, a rare tubercle bacillus could represent the most ancestral lineage of the MTBC (Brosch *et al.* 2002). *Mycobacterium canettii* and other smooth tubercle bacilli actually correspond to lineages belonging to a much broader progenitor species from which the MTBC emerged. This last common ancestor could already have affected early hominids in East Africa at least 2.6 million years ago (Gutierrez, Brisse, Brosch *et al.* 2005).

1.3 Tuberculosis in humans

Tuberculosis, caused by *M. tuberculosis*, is a major disease in humans and is now the leading cause of death in adults worldwide as a result of the human immunodeficiency virus (HIV) epidemic. The World Health Organisation (WHO) estimates, 2 billion people, about one-third of the world's population, are infected with *M. tuberculosis* (WHO 2008). In 2003 about 8.8 million people were estimated to have developed TB (incidence rate of 140 per 100 000 population), of whom 1.7 million were fatalities (mortality rate 28 per 100 000 population) with 99% of these being concentrated in developing countries, particularly Asia and Africa (Figueroa-Munoz, Palmer, Poz *et al.* 2005; Gunneberg, Reid, Williams *et al.* 2008). This situation is believed to be closely associated with the spread of HIV in developing countries. It is estimated that 70% of the 24 million people that are globally co-infected with HIV and *M. tuberculosis* live in sub-Saharan Africa (Corbett, Watt, Walker *et al.* 2003).

In 2002 it was estimated that in India 4.58 million people were co-infected (HIV-TB) (Narain & Lo 2004) and at present approximately 5% of new *M. tuberculosis* infections occur in people with HIV infection (Steinbrook 2007). Furthermore, the spread of TB is intensified by poor sanitary and living conditions due to poverty and the delay in acting against the infection (WHO 2008). Tuberculosis is a contagious disease. Individuals with TB lung lesions spread the infection by aerosol when coughing, sneezing, talking or spitting (Harries & Dye 2006a). Inhalation of a small number of tubercle bacilli is sufficient to induce infection in the individual. During the initial infection when the mycobacteria enter the body,

they settle on the host tissue, taking several weeks to multiply. The immune system recognises the foreign invasion and a cell mediated immune response is elicited. The cells of the tissues or organs, which are infected, multiply to "wall off" the bacteria thus preventing further spread of the pathogen. At this stage of infection a small tuberculous nodule is formed, called a tubercle. The tubercle is encapsulated by connective tissue and the bacteria become dormant. When the individual's immune system is weakened the pathogen multiplies and spreads to infect other regions of the lungs and body. This can lead to active pulmonary TB and, if not treated, can lead to the individual's death (Glickman & Jacobs 2001). Each person with active TB will infect on average between 10 and 15 people every year. Not all people infected with TB bacilli will necessarily develop clinical symptoms of the disease (van Lerberghe, Evans, Rasanathan & Mechbal 2008). People co-infected with HIV and TB bacilli infections are much more likely to develop frank TB. In most cases, if individuals with latent TB become infected with HIV, reactivation and progression to active TB disease occurs (Gunneberg *et al.* 2008; Pai & O'Brien 2008).

Humans are also susceptible to infection with *M. bovis.* This infection may lead to zoonotic TB (Fritsche, Engel, Buhl & Zellweger 2004). Humans may be exposed to the pathogen by consumption of unpasteurized cow's milk and its products or through close physical contact with infected cattle. Transmission of *M. bovis* between humans is rare and anecdotal, and it is clearly less relevant as compared to animal-to-human transmission (Wedlock, Skinner, de Lisle & Buddle 2002). The transmission of TB between humans due to *M. bovis* is better documented in industrialized than in developing countries (de la Rua-Domenech 2006a).
Bovine TB is more common in rural dwellers as compared to urban dwellers (Ayele, Neill, Zinsstag *et al.* 2004), that inhale dust particles containing bacteria or aerosols shed by infected animals which may give rise to pulmonary TB. Urban dwellers are more likely to suffer from extra-pulmonary TB because they acquire the infection via the gastrointestinal route (Ayele *et al.* 2004).

The prevalence of zoonotic TB caused by *M. bovis* in African and Asian countries is much higher than elsewhere in the world, and the problem is further complicated by the alarming spread of HIV in these countries (Narain & Lo 2004). Complementing this setback are the constraints in the control and diagnosis of TB (Ayele *et al.* 2004; Regassa, Medhin & Ameni 2008) as control measures and diagnostic techniques are either inadequate or, in most cases, unavailable (McCrindle & Michel 2006). *Mycobacterium bovis* was confirmed in seven of 65

(10.8%) human cervical adenitis cases in Tanzania and Uganda (Cleaveland, Shaw, Mfinanga *et al.* 2007; Oloya, Opuda-Asibo, Kazwala *et al.* 2008). In Nigeria, a recent study indicated that 5% of human isolates were found to be *M. bovis* (Cadmus, Palmer, Okker *et al.* 2006). In the UK, where the incidence of BTB is likely to be under-reported (de la Rua-Domenech 2006a), *M. bovis* represents approximately 1.1 to 1.5% of bacteriologically proven TB cases (Rowe & Donaghy 2008). The fact that *M. bovis* does contribute to the current human epidemic of TB emphasizes the importance of increasing awareness of zoonotic TB in order to implement appropriate veterinary public health control measures.

1.3.1 Diagnosis of tuberculosis in humans

Diagnostic approaches are categorized into pathogen identification and assessment of the host's immunological response to infection. Conventional TB diagnosis relies on tests such as sputum smear microscopy, culture, intradermal tuberculin tests (IDT) and chest radiography (Harries & Dye 2006a; Harries, Boxshall, Phiri & Kwanjana 2006b; Pai & O'Brien 2008). Culture and microscopy techniques are routinely used to identify the pathogen but are not necessarily advantageous to making an individual diagnosis. This is because the *M. tuberculosis* organism takes 2-3 months to become visible on culture media and such cultures are usually also used for monitoring drug sensitivity patterns in patients with recurring TB (Harries & Dye 2006a; Harries *et al.* 2006b). Another drawback is the low sensitivity of the techniques since infected individuals not shedding the pathogen will not be detected. Apart from this limitation the above-mentioned tests perform poorly in populations infected with the HIV (Pai & O'Brien 2008). The vast majority of TB patients live in low and middle income countries (Dye, Watt, Bleed *et al.* 2005). The diagnosis of TB disease, in such countries, relies primarily on the identification of acid-fast bacilli in unprocessed sputum smears using a conventional light microscope and chest X-rays, which are used to diagnose pulmonary TB.

Microscopy is used mostly for *M. tuberculosis* detection in TB-endemic countries (Toman 2004; Steingart, Henry, Laal *et al.* 2007). Microscopy has been reported to have variable sensitivity: low in some reports and high in others (range 20% to 80%) (Urbanczik 1985; Steingart, Henry, Hopewell *et al.* 2006). More importantly, sensitivity is poor for paucibacillary disease (e.g. pediatric and HIV-associated TB) (Shingadia & Novelli 2003). This lack of sensitivity of the sole diagnostic test in many parts of the world results in delays

in diagnosis, enabling the disease to progress and increasing the potential for transmission of *M. tuberculosis* (Behr, Warren, Salamon *et al.* 1999). To ensure appropriate measures to improve the control of the global TB epidemic, simple, accurate, inexpensive and, ideally, point-of-care diagnostic tools for TB are urgently needed. The choice of a diagnostic test depends upon the setting in which the test is to be performed and the intended use of the results (fit for purpose principle). If test results are to be used to exclude TB in patients with respiratory symptoms in TB-endemic countries, then tests with a high sensitivity (and thus providing high negative predictive value) are required even if the test is only moderately specific. Once a smaller "at risk" group is identified, a rigorous diagnostic work-up will be performed. In this case patients with respiratory symptoms for anti-TB treatment should be identified; therefore a test with a high specificity (and thus providing high positive predictive value) is required. A recent systematic review on commercial serological antibody detection tests for the diagnosis of pulmonary TB highlighted that none of the commercial tests evaluated performed well enough to replace sputum smear microscopy (Steingart *et al.* 2007). Thus nowadays, these commercial serological tests have little or no role in the diagnosis of pulmonary TB.

The IDT relies on the response of the individual to the injection of tuberculin and is described as a delayed type hypersensitivity (DTH) response. The test has several drawbacks, some of which include the return visit of the patient to allow reading of the test, problems in interpreting the results, variability in its application and readings, immunosuppression, and cross-reactivity in Bacille Calmette-Guérin (BCG) vaccinated people (Pottumarthy, Morris, Harrison & Wells 1999). Furthermore the IDT is not 100% specific or sensitive, and an average of 20-25% of patients with active TB do not react to the purified protein derivative (PPD) used in the IDT (Fietta, Meloni, Cascina *et al.* 2003). Recently *in vitro* tests measuring cell mediated immunity (CMI) have been developed. These include interferon-gamma release assays (IGRAs), such as the Quanti-FERON®-TB (QFT) Gold (Cellestis Inc., Victoria, Australia) and T_SPOT.TB (T_SPOT, OXFORD Immunotec). The QFT Gold and the immunospot-formatted T_SPOT measure the IFN-γ produced by T-cells in whole blood upon stimulation by *M. tuberculosis* antigens. Diel, Loddenkemper, Meywald-Walter *et al.* (2008) compared the performance of IDT, QFT test and T_SPOT test in contact investigations for TB. The authors concluded that IGRAs are more accurate indicators of the presence of latent TB infections (LTBI) than IDT. They also noted that IGRAs show excellent agreement with

each other. Other researchers (Ozdemir, Annakkaya, Tarhan *et al.* 2007; Manuel & Kumar 2008; Nienhaus, Schablon & Diel 2008) also showed that the QFT test is an ideal test for the diagnosis of LTBI. Quanti-FERON®-TB Gold shows a higher sensitivity for detecting active TB (i.e. sputum positive patients) than the IDT (Kobashi, Obase, Fukuda *et al.* 2006) and is considered as a useful diagnostic aid for the diagnosis of active TB in humans when compared to IDT (Bartu, Havelkova & Kopecka 2008). A recent report suggests that active TB can be clearly identified provided that T-cell IGRAs are performed on mononuclear cells derived from the site of disease (Jafari & Lange 2008).

1.4 Tuberculosis in animals

Mycobacterium bovis is the principal cause of BTB and the success of this pathogen is reflected by its global distribution in a diversity of mammalian host species, including domestic, captive and free-ranging animals, and humans. The impact of this disease is not restricted to the health of the species but also has economic implications because of the production and trade of these animals, and for ecotourism. Reservoir wildlife hosts have become a source of infection for domestic animals (Corner 2006) and thus may affect entire ecosystems (Renwick, White & Bengis 2007). Maintenance hosts of *M. bovis* include the African buffalo (*Syncerus caffer*) in South Africa (Bengis, Kriek, Keet *et al.* 1996); the European badger (*Meles meles*) in the UK (Nolan & Wilesmith 1994); the brushtail possum (*Trichosurus vulpecula*) in New Zealand (Corner, Stevenson, Collins & Morris 2003); bison (*Bison bison)* in Canada (Lutze-Wallace, Turcotte, Stevenson *et al.* 2006) and white-tailed deer (*Odocoileus virginianus*) in the USA (Schmitt, Fitzgerald, Cooley *et al.* 1997).

Animal-to-animal transmission of *M. bovis* can occur via contact, aerosol exposure and ingestion of contaminated material. Contact between animals can occur at water points and places where animals are likely to gather (milking parlours, transportation, market places, dipping tanks, auction stations and grazing areas) (Renwick *et al.* 2007). Aerosol exposure to *M. bovis* is considered to be the most frequent route of infection of cattle and results in the entry of the pathogen into the host.

Tuberculosis, caused by *M. tuberculosis* is not restricted to humans, its main host. According to present knowledge, *M. tuberculosis* does not appear to have an animal reservoir and the animals that become infected most probably represent accidental hosts (Thoen, Steele &

Gilsdorf 2006). For transmission of *M. tuberculosis* infection to susceptible animals there has to be prolonged and close contact with humans (Michel, Bengis, Keet *et al.* 2006; Parsons, Gous, Warren & van Helden 2008). Disease associated with *M. tuberculosis* has occurred mostly within captive settings. In countries, such as India (Srivastava, Chauhan, Gupta *et al.* 2008), Ghana (Bonsu, Laing & Akanmori 2000), Nigeria (Mawak, Gomwalk, Bello & Kandakai-Olukemi 2006), Slovenia (Pavlik, Ayele & Parmova 2003; Ocepek, Pate, Zolnir-Dovc & Poljak 2005) and the UK (de la Rua-Domenech 2006a), infection with *M. tuberculosis* has been most frequently identified in cattle. Published data also indicates that the prevalence of *M. tuberculosis* in cattle herds did not exceed 1% in the majority of studies (Ocepek *et al.* 2005). A prevalence of 6.2% and 7.4% was, however, recorded in Algeria (Boulahbal, Benelmouffok & Brahimi 1978) and Sudan (Sulieman & Hamid 2002) respectively. Other cases of *M. tuberculosis* in domestic animals includes cats (*Felis catus*) (Gunn-Moore 1994; Fernandez & Morici 1999), domestic pigs (*Sus scrofa f. domestica*) (Pavlik *et al.* 2003), and canines (*Canis lupus*) (Aranaz, Liébana, Pickering *et al.* 1996; Turinelli, Ledieu, Guilbaud *et al.* 2004; Parsons *et al.* 2008). *Mycobacterium tuberculosis* has been reported in both Asian (*Elephas maximus*) (Mikota, Larsen & Montali 2000) and African (*Loxodonta africanum*) (Gorovitz 1962) elephants. In free-living mammals it was thought to be absent (Montali, Mikota & Cheng 2001) until it was reported in free-ranging banded mongooses (*Mungos mungo*) in Botswana and suricates (*Suricata suricatta*) in South Africa (Alexander, Pleydell, Williams *et al.* 2002), its occurrence being believed to be associated with ecotourism.

1.4.1 Cattle

In countries which have instituted comprehensive eradication programmes, the clinical disease in cattle is rarely seen because the IDT enables a presumptive diagnosis to be made and elimination of infected animals to be effected before the disease is advanced and clinical signs are recorded. Prior to the national TB eradication campaigns, however, the clinical signs associated with TB were commonly observed (de Lisle, Mackintosh & Bengis 2001).

1.4.2 Free-ranging wildlife

In South Africa, *M. bovis* infections were first reported in 1929 in greater kudus (*Tragelaphus strepsiceros*) and in a common duiker (*Sylvicapra grimmi*) (Cousins 2008). In Uganda, a 10% prevalence of BTB in African buffaloes and 9% prevalence in warthogs (*Phacochoerus aethiopicus*) were reported in 1982 (Woodford 1982). *Mycobacterium bovis* infections in wildlife in Zambia, Kenya, Tanzania (Cleaveland, Mlengeya, Kazwala *et al.* 2005; Cousins 2008) and later in the Kruger National Park (KNP) of South Africa were reported. In South Africa, the disease has already spilled over into chacma baboons (*Papio ursinus*), lions (*Panthera leo*), cheetahs (*Acinonyx jubatus*), leopards (*Panthera pardus*), honey badgers (*Mellivora capensis*), hyenas (*Crocuta crocuta*) (Keet, Kriek, Penrith *et al.* 1996; Keet, Kriek, Bengis *et al.* 2000; De Vos, Bengis, Kriek *et al.* 2001), warthogs (*Phacochoerus aethiopicus*), kudus (*Tragelaphus strepsiceros*) (Keet, Kriek, Bengis & Michel 2001), and a bush pig (*Potamochoerus larvatus*) (Michel, Coetzee, Keet *et al.* 2009).

In North America, *M. bovis* has been detected in white-tailed deer (*Odocoileus virginianus*) in USA, in the northeastern part of Michigan state (O'Brien, Schmitt, Fierke *et al.* 2002), in Canada in elk (*Cervus elaphus*) in Manitoba province (Fuller 1959; Nishi, Shury & Elkin 2006), and wood bison (*Bison bison athabascae*) in Alberta province (Lutze-Wallace *et al.* 2006). *Mycobacterium bovis* has spread to wildlife populations in many European countries. In Spain, it has been reported in wild boars (*Sus scrofa*), Iberian lynx (*Lynx pardus*) and deer species (Gortazar, Torres, Vicente *et al.* 2008). In Great Britain and Ireland, the extent of *M. bovis* infections in the badger population and its role in the increase of TB caused by *M. bovis* in cattle is still a matter of vigorous debates (Courtenay, Reilly, Sweeney *et al.* 2006; White, Bohm, Marion & Hutching 2008). Australia has been successful in eradicating *M. bovis* from cattle herds but there are populations of animals such as feral pigs and feral Asian water buffaloes (*Bubalus bubalis*) that have been reported to be infected with *M. bovis* in the northern parts of the country (Corner, Barrett, Lepper *et al.* 1981; McInerney, Small & Caley 1995). As in South Africa, New Zealand has a diversity of wild animals infected with *M. bovis.* Apart from the reservoir host, which is the brushtail possum, *M. bovis* infections have been reported in feral populations of cats, ferrets (*Mustela putorius*) and stoats (*Mustela erminca*) in Otago and Southland provinces (Ragg, Moller & Waldrup 1995). The continuous transmission of *M. bovis* from free-ranging wildlife reservoirs to domestic livestock results in

economic losses and is a significant barrier to the success of national eradication and control programmes worldwide.

1.4.3 Captive wildlife with special emphasis on rhinoceroses and elephants

Disease associated with *M. tuberculosis* infection has been diagnosed most frequently in animals in captive situations. It has been reported in snow leopards (*Uncia uncia*) (Helman, Russell, Jenny *et al.* 1998), Asian (Furley 1997; Mikota *et al.* 2000; Payeur, Jarnagin, Marquardt & Whipple 2002) and African elephants, terrestial tapirs (*Tapirus terrestris*), agoutis (*Dasyprocta aguti*) (Pavlik *et al.* 2003), rhinoceroses (Valandikar & Raju 1996; Oh, Granich, Scott *et al.* 2002) and ungulates such as oryx (*Oryx gazelle beisa*), black buck (*Antilope cervicapra*), bongo antelope (*Tragelaphus eurycerus*), mountain goats (*Oreamnos americanus*) and giraffes (*Giraffa camelopardlis*) (Montali *et al.* 2001). Cases of *M. tuberculosis* infections have been diagnosed in Asian elephants in zoo collections in the USA and Europe, and in circuses in the USA (Mikota *et al.* 2000; Pavlik *et al.* 2003; Lewerin, Olsson, Eld *et al.* 2005).

Animals in captivity or in domesticated situations are also susceptible to infection by *M. bovis*. *Mycobacterium bovis* infections have been reported in rhinoceroses (Mann, Bush, Janssen *et al.* 1981), baboons (*Papio harmadryas*), leopards (*Panthera uncia* and *Panthera pardus*) (Thorel, Karoui, Varnerot *et al.* 1998), European wild boars (*Sus scrofa*) and Iberian red deer (*Cervus elephas hispanicus*) (Gortazar *et al.* 2008). There have been no reports of *M. bovis* infection in captive or free-ranging African elephants. Tuberculous captive wildlife maintained on farms or animal parks (e.g. zoos or game farms) may also serve as foci of infection for domestic animals, free-ranging wildlife, and humans (Bengis *et al.* 1996; de Lisle *et al.* 2001; Moller, Röken, Petersson *et al.* 2005).

1.4.3.1 Rhinoceroses

Tuberculosis caused by either *M. bovis* (BTB) or *M. tuberculosis* (TB) has not been reported in free-living rhinoceroses. All cases of the disease in rhinoceroses have been reported in captive situations. In 1981, *M. bovis* infections were reported in captive black rhinoceroses (*Diceros bicornis*) in a zoo in the USA (Mann, Bush, Janssen *et al.* 1981). At necropsy the major lesions (nodular-like) were found in the lungs. On a histopathological level the lungs

contained multiple granulomas with giant cells and areas of alveolar wall fibrosis (Mann *et al.* 1981). In another zoo situation in the USA, Dalovisio, Stetter & Mikota-Wells (1992) reported the exposure of seven zookeepers to a southern white rhinoceros (*Ceratotherium simum simum*) infected with *M. bovis*. This animal was presumably infected via aerosols generated during cleaning of the barn in which the rhinoceros was kept. All the zookeepers who were exposed to infection demonstrated conversion by the skin test, but, apart from rhinorrhoea, none had clinical illness. In South Africa, the first case of mycobacteriosis in a black rhinoceros was diagnosed in 1970 in the Hluhluwe-iMfolozi Park (HiP) (Keep & Basson 1973).

During 1989 and 1991 at a zoo in Louisiana, seven animal handlers who were previously negative for TB tested (Mantoux) positive after an *M. bovis* outbreak in a southern white rhinoceros and monkeys (Stetter, Mikota, Gutter *et al.* 1995). Two black rhinoceroses died in 1992 and 1994 respectively at Mysore zoo in India from pulmonary TB. Post-mortem findings from the first rhinoceros were enlarged, oedematous lungs. The intralobular septa were thickened, and multiple nodules and abscesses were found on the entire surface of the lungs. In the second rhinoceros the lungs had granulomas and the liver was shrunken and necrosed with multiple nodules. The cause of the death, which was confirmed by bacteriological examination, indicated that *M. tuberculosis* was the infectious organism (Valandikar & Raju 1996). In September 1998 *M. tuberculosis* was cultured from a captive black rhinoceros but humans were not found to be responsible for the infection as genotyping evidence suggested that transmission was from another animal species in the zoo (Oh *et al.* 2002).

1.4.3.2 Elephants

Tuberculosis in Asian elephants has been reported since the 1930s (Baldrey 1930). Between August 1996 and May 2000 all isolates of elephant TB in North America were identified as *M. tuberculosis* (Mikota *et al.* 2000; Montali *et al.* 2001). Tuberculosis in captive and domestic Asian elephants has been reported in the USA, Europe and Asia (Pavlik *et al.* 2003; Lewerin *et al.* 2005), (Table 1.2). In 1996, 12 circus Asian elephant handlers were infected with *M. tuberculosis* on an exotic animal farm in Illinois, one of them showing signs consistent with the active disease after three elephants had died of TB. Medical history and testing of the handlers indicated that the elephants had been the probable source of exposure

for most of the human infections (Michalak, Austin, Diesel *et al.* 1998). In addition an outbreak of *M. tuberculosis* in Asian elephants occurred in a Swedish zoo between 2001 and 2003 (Lewerin *et al.* 2005). Five elephants were found to have been infected by four different strains of *M. tuberculosis*. In 1997, the United States Department of Agriculture (USDA) developed guidelines (http://www.elephantcare.org/protodoc) for the taking of samples from elephants for bacterial culture by using a trunk-wash method, and for the removal of TB-infected elephants from public contact (Mikota, Miller, Dumonceaux *et al.* 2008). These guidelines were revised in 2003 and again in 2008.

Clinical signs in captive elephants infected with *M. tuberculosis* include loss of appetite and weight, reluctance to do strenuous work and, in some, subcutaneous ventral oedema (Mikota *et al.* 2000; de Lisle, Bengis, Schmitt & O'Brien 2002). In some cases infected elephants show no clinical signs of disease (Lysahchenko, Greenwald, Esfandiari *et al.* 2006). Pathological examinations have indicated that the lungs, bronchi, trachea and the thoracic lymph nodes are primarily involved when the infection involves *M. tuberculosis* (Mikota *et al.* 2000; Montali *et al.* 2001; Lewerin *et al.* 2005). Characteristic histological findings include epithelioid granulomas with significant giant cell formation in the earlier stages. Lymph node and pulmonary lesions exhibiting extensive caseous and pyogranulomatous reactions occur in the advanced form of the disease. The presence of mycobacteria in the areas of caseation in the lungs is common, but is typically rare in the lymph nodes (Montali *et al.* 2001). Tuberculous elephants with extensive involvement of both lungs usually die and show severe caseo-calcareous and cavitating lesions as well as abscesses in the lungs on post-mortem examination. In the extensive cases, firm granulomatous nodules occur in the bronchial lymph nodes which are extensively enlarged and usually show a proliferative response with less caseation than the pulmonary lesions (Mikota *et al.* 2000; Lewerin *et al.* 2005).

Table 1.2 Reported incidence of TB in African and Asian elephants

Cases of Tuberculosis	**Reference**
M. tuberculosis in an Asian elephant	(Narayana 1925)
M. tuberculosis in an Asian elephant	(Bopayya 1928)
M. tuberculosis in an Asian elephant	(Baldrey 1930)
M. tuberculosis in an African elephant in a zoo in Paris	(Urbain & Dechambre 1937)
M. tuberculosis in an African elephant in a zoo	(Gorovitz 1962)
Fatal tuberculosis pneumonia in an elephant	(Seneviratna, Wettimuny & Seneviratna 1966)
M. tuberculosis in a domestic Asian elephant	(Pinto, Jainudeen, Panabokke *et al.* 1973)
M. tuberculosis in an Asian elephant	(Johnston 1981; Gutter 1981)
Pulmonary *M. tuberculosis* in an Asian elephant (circus elephant)	(Saunders 1983)
M. tuberculosis in Asian elephants (captive and circus elephants)	(Binkley 1997; Furley 1997; Whipple, Meyer, Berry *et al.* 1997)
M. tuberculosis in Asian elephants reported during 1994-1996 at an exotic animal farm Illinois, USA	(Michalak *et al.* 1998)
M. tuberculosis in 18 Asian elephants during August 1996-May 2000 in North America	(Mikota *et al.* 2000)
M. tuberculosis culture-positive elephants	(Harr, Raskin, Blue & Harvey 2001)
Mycobacterial isolations from captive Asian elephants and *M. tuberculosis* infection in an Asian elephant	(Payeur *et al.* 2002)
M. tuberculosis in an African elephant in Gdansk Zoo, Poland	(Pavlik *et al.* 2003)
M. tuberculosis in 2 Asian elephants in Assam, India	(Sarma, Bhawal, Yadav *et al.* 2006)
Outbreak of *M. tuberculosis* infection in captive Asian elephants in a Swedish Zoo	(Lewerin *et al.* 2005)
M. szulgai (atypical mycobacterium) in an African elephant at Lincoln Park Zoo, USA	(Lacasse, Terio, Kinsel *et al.* 2007)

1.5 Diagnosis of *M. bovis* and *M. tuberculosis* infections in animals

1.5.1 Post-mortem

Post-mortem diagnosis of *M. bovis* infection in cattle and bacteriological examination of samples are important steps in the diagnosis of BTB. Lesions that are found during necropsy are collected and a presumptive diagnosis using histopathology is performed. However, for a definitive diagnosis, the isolation of *M. bovis* is required (Corner 1994). For post-mortem diagnosis the method that is employed and the anatomical sites that are examined are considered important as they can affect the effectiveness of the examination (Corner, Melville, McCubbin *et al.* 1990; Corner 1994). In cattle, which have reacted positively to the IDT, essential tissues such as the mediastinal, medial retropharyngeal and bronchial lymph nodes, palatine and pharyngeal tonsils and lungs as well as the parotid, caudal cervical and superficial inguinal and mesenteric lymph nodes should be examined in order to identify all the tuberculous cattle (Corner *et al.* 1990; Corner 1994; Cassidy, Bryson & Neill 1999). In wildlife species, such as deer, tuberculous lesions are not pathognomonic of *M. bovis* infection due to gross and microscopic similarities with lesions caused by *Mycobacterium avium subsp. paratuberculosis* or *M. avium subsp. avium* (Godfroid, Delcorps, Irenge *et al.* 2005). In elephants and rhinoceroses, major lesions are found in the lungs. Hence the "gold standard" for the diagnosis of TB / BTB remains the isolation by culture and identification of *M. bovis* or *M. tuberculosis*.

1.5.2 Identification of the bacteria and / or their genetic material

A presumptive diagnosis of *M. bovis* / *M. tuberculosis* infections can be made on microscopic detection of acid-fast bacilli in smears of suspect tuberculous lesions stained by the Ziehl-Neelsen method (Van Deun *et al.* 2008). However, the absence of acid-fast organisms in a smear does not necessarily rule out a diagnosis of TB. The definitive diagnosis requires that the *M. bovis* organism be isolated from the infected tissue. A disadvantage of the latter is that *M. bovis* grows slowly in culture, taking from 4 to 6 weeks to form visible colonies. A further disadvantage of using this means of diagnosis is the resulting delay in action in managing the problem or infection by causing delays in locating potentially exposed animals. In addition, isolates have to be confirmed by biochemical evaluation, polymerase chain reaction (PCR) and other molecular typing techniques. The PCR technique, although not superior to routine culture in terms of sensitivity, specificity or reliability, does allow the rapid screening of

samples from live animals, monitoring of the environment for the presence of mycobacteria (de la Rua-Domenech, Goodchild, Vordermeier *et al.* 2006b) and classification of mycobacteria into the MTBC group or the *Mycobacterium avium* complex (Miller, Jenny & Payeur 2002). An added advantage of PCR over culturing techniques is that it does not require the presence of viable organisms. Insertion sequences, IS*6110* and IS*1081*, are specific to the mycobacteria belonging to the MTBC, and these insertion sequences are used in PCR techniques to determine the presence of *M. tuberculosis* and *M. bovis* in cattle with suspected TB (Skuce, McCorry, McCarroll *et al.* 2002). Recently, a two-step, multiplex PCR method based on genomic regions of difference was developed for the differentiation of members of the MTBC. The size of the respective PCR amplification products correspond to the presence of the different *M. tuberculosis* complex members (Warren, Gey van Pittius, Barnard *et al.* 2006).

1.5.3 Genotyping of mycobacteria

Before the development of molecular typing techniques, the available techniques of strain typing did not allow the differentiation of *M. bovis* isolates (Haddad, Monique & Durand 2004). Molecular techniques have proven to be very useful as tools for differentiating not only strains that belong to the MTBC but also between strains within the species (Gutierrez, Samper, Gavigan *et al.* 1995), thus making it possible to determine the origin of outbreaks, understand the link between different outbreaks, show relationships between domestic and wildlife TB, and also identify sources of infection (Haddad *et al.* 2004). Typing techniques used for *M. bovis* strain typing include restriction enzyme analysis (REA) (Collins & de Lisle 1985), polymorphic (GC)-rich sequences (PGRS) (Ross, Raios, Jackson & Dywer 1992), restriction fragment length polymorphism (RFLP) analysis (Skuce, Brittain, Hughes & Neill 1994), spoligotyping (Gutierrez *et al.* 1995; Kamerbeek, Schouls, Kolk *et al.* 1997; Roring, Brittain, Bunschoten *et al.* 1998) and variable number of tandem reapeats (VNTRs) (Le Fleche, Fabre, Denoeud *et al.* 2002)

1.5.3.1 Restriction enzyme analysis

In this technique high molecular weight DNA is digested with a restriction enzyme and the fragments produced are separated by gel electrophoresis. Strains are characterized on the

basis of the fragment patterns (Collins & de Lisle 1985). This technique differentiates between strains and isolates.

1.5.3.2 Polymorphic (GC)-rich sequences

Polymorphic (GC)-rich sequences are sequences with an 80% GC content and belong to the "PE multigene family". Multiple copies of these highly homogenous genes are present in mycobacteria. For typing, polymorphism is based on the number and location of the PGRS domains and is determined using PGRS-RFLP analysis (Haddad *et al.* 2004; Michel *et al.* 2009). Upon analyses, a distinction can be made between different isolates of the same species.

1.5.3.3 Restriction fragment length polymorphism

Restriction fragment length polymorphism takes place in two steps. In the first, a part of a gene common to all species of the MTBC is amplified using specific primers. These primers do not generate PCR products from other species of mycobacteria. This is followed by the second step in which the amplicons produced are digested with restriction enzymes. Upon analyses of the products, a distinction can be made between the different species of the MTBC (Skuce *et al.* 1994; Aranaz, Liebana, Mateos *et al.* 1998; Michel *et al.* 2009).

1.5.3.4 IS6110- Restriction fragment length polymorphism

The insertion sequence, IS*6110*, is regarded as a useful marker for typing *M. tuberculosis* strains (Gutierrez *et al.* 1995) as it is present in multiple copies (ranging from 0 to 25) in different locations of the genome (van Soolingen & Arbeit 2001). The number of copies of the IS*6110* element in *M. bovis* strains is usually lower (only one copy of the IS*6110* element) than in *M. tuberculosis* strains (Allix, Walravens, Saegerman *et al.* 2006) and it is always present in the same location. Thus the discriminatory power of this technique is also lower when typing *M. bovis* strains. Therefore other techniques based on direct repeats (DR) (Groenen, Bunschoten, van Soolingen & van Embden 1993) are preferred for the typing of *M. bovis* isolates.

1.5.3.5 Spoligotyping

Spoligotyping (spacer oligotyping) is a PCR-based hybridization blotting typing method. In this typing practice the genetic polymorphisms are restricted to a single genomic cluster locus, the DR cluster (Roring, Scott, Brittain *et al.* 2002; Brudey, Driscoll, Rigouts *et al.* 2006). In this locus the DRs are interspersed by unique DNA spacer sequences of 35 to 41 base pairs (bp) in length (Kremer, van Soolingen, Frothingham *et al.* 1999). The presence or absence of spacers preferably at the DR locus, between different isolates is determined by spoligotyping (Kremer *et al.* 1999) and thus allows the differentiation of strains within each species belonging to the MTBC. Spoligotyping has been widely used for typing *M. bovis* isolates. It is highly reproducible, rapid, simple and produces reliable phylogenetic data (Kamerbeek *et al.* 1997).

1.5.3.6 Variable number of tandem repeats

Variable number of tandem repeats is polymorphic loci within regions of tandemly repeated DNA and were identified during the genome sequencing projects for members of the MTBC (Cole, Brosch, Parkhill *et al.* 1998). Variable number of tandem repeats typing is based upon repeat number polymorphisms within these tandemly arranged repetitive DNA sequences (Roring *et al.* 2002). These tandem repeat loci display allelic hypervariability and include exact tandem repeats A to F (Frothingham & Meeker-O'Connell 1998) but in mycobacteria the majority of the VNTRs correspond to mycobacterial interspersed repetitive units (MIRUs) (Haddad *et al.* 2004). These are mini-satellite structures composed of 51-77 bp sequences that are scattered in 41 locations throughout the bacterial chromosome (Supply, Mazara, Lesjean *et al.* 2000; Sola, Filliol, Legrand *et al.* 2003).

The VNTRs are first amplified using different pairs of primers and the products are analysed by electrophoresis or by an automated technique using primers tagged with flourescence dye (Haddad *et al.* 2004). The use of VNTR enables exploitation for strain typing in numerous bacterial species. The availability of these different techniques has made it possible to type mycobacteria belonging to the MTBC. This is particularly useful for assisting in tracing the spread of *M. bovis* between herds and for evaluating the role of wildlife reservoirs (Haddad *et al.* 2004) in the spread and maintenance of the pathogen. These typing tools form a part of the many aspects of tackling the challenges of TB diagnostics, epidemiology and disease management. When spoligotyping is employed along with VNTRs its use is extended from

the clinical laboratory to molecular epidemiology including evolutionary and population genetics.

1.5.4 Ante-mortem diagnosis

Infection of mammals with mycobacteria of the MTBC results in a chronic, devastating disease (O'Reilly & Daborn 1995). Infected animals usually show no clinical signs during the early stages of the disease, which makes its diagnosis based on clinical signs challenging (Cousins 2008). For example, in cattle the clinical signs of *M. bovis* infection are not pathognomonic and infected cattle can become infectious long before they exhibit obvious clinical signs. As a result effective *ante-mortem* surveillance for the disease must primarily rely on the detection of infected cattle at an early stage by the use of sensitive immunodiagnostic tests (Cousins 2008). *Ante-mortem* diagnostic tests allow for the detection of disease during the preclinical stages of either *M. tuberculosis* or *M. bovis* infection. Tests targeting CMI (IDT, interferon-gamma (IFN-γ) assay and the lymphocyte proliferation test) and those targeting the humoral immunity (serology) have been adapted for use in domestic and wildlife species (de la Rua-Domenech *et al.* 2006b). New developments towards the progression of reliable and cost effective *ante-mortem* diagnostic tests, in both the human (Bartalesi, Vicidomini, Goletti *et al.* 2008) and wildlife veterinary fields, are also under investigation (Lesellier, Corner, Costello *et al.* 2008; Lyashchenko, Greenwald, Esfandiari *et al.* 2008, Greenwald, Lyashchenko, Esfandiari *et al.* 2009).

1.5.5 Immune reactions to mycobacteria

1.5.5.1 Cytokines

Cytokines are a group of proteins that have the ability to regulate functions of cells and tissues, particularly immune functions. Included in this family of cytokines are, amongst many others, interferons (IFN), interleukins (IL), chemokines, colony stimulating factor (CSF) and tumour necrosis factor (TNF) (Scheerlinck & Yen 2005). According to their roles they may be subdivided into pro- and anti-inflammatory cytokines or regulatory cytokines. In the pathogenesis of TB they may be active in different phases of the disease. Of particular interest is IFN-γ. Interferon is involved in the regulation of nearly all phases of the immune and inflammatory responses (Fig. 1.1) (Schroder, Hertzog, Ravasi & Hume 2004; Alvarez 2006) and is produced by Th1 cells. Interferon-γ activates macrophages and increases

expression of the major histocompatibility complex (MHC) class I and class II molecules thus increasing antigen presentation. In its absence both humans and experimental animal hosts become susceptible to microbial infection (Rhodes, Palmer, Graham *et al.* 2000a; Pearl, Saunders, Ehlers *et al.* 2001; Widdison, Watson, Percy *et al.* 2008). Interferon-γ is considered as highly relevant for protection in the early phases of a mycobacterial infection and is the target in diagnostic assays like the IDT and IFN-γ assays.

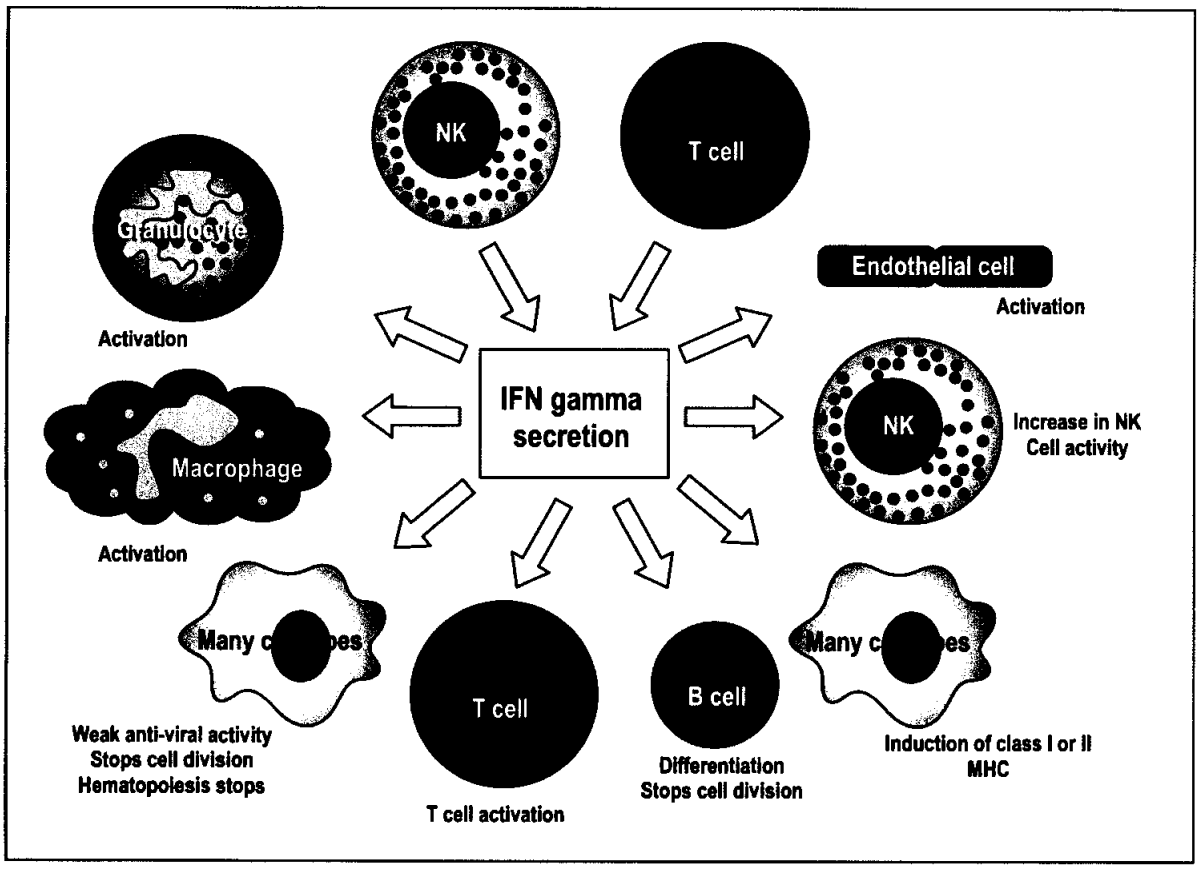


Figure 1.1 Diagrammatic representations of the pleiotropic functions of IFN-γ (Alvarez 2006)

1.5.5.2 Immunopathogenesis

When cattle are exposed to *M. bovis* various factors are produced and activated which subsequently influence the eventual outcome of infection. These factors may be inherent to the tubercle bacillus, the features of the host, and / or other factors relating to the environment (Pollock & Neill 2002). Bovine TB is primarily a pulmonary disease in cattle. The primary site of natural infection and the principal manifestation site in cattle is the respiratory tract (van Rhijn, Godfroid, Michel & Rutten 2008). Once the tubercle bacillus has entered the host

via inhalation it lodges itself within the respiratory tract in the terminal air spaces of the lungs (Pollock & Neill 2002). The host's immune response to the pathogen is influenced by the initial exposure. A high dose of infectious material will result in CMI that develops within a few weeks and a rapid production of circulating anti-*M. bovis* antibodies occurs. With lower doses there is a gradual development of CMI and little or no antibody response (Pollock, McNair, Welsh *et al.* 2001; Pollock & Neill 2002).

The preferred host cells for these pathogens are the alveolar macrophages (Glickman & Jacobs 2001). Ironically, these are key effector cells for controlling and destroying such pathogens. The pathogen is phagocytosed by macrophages and migrates in them in the lymphatic system initially to the regional lymph nodes where it may be destroyed as a result of immune responsiveness to mechanisms such as lysosomal pH, lysosomal hydrolysis, anti-bacterial peptides and superoxide (Pollock & Neill 2002; Teixeira, Abramo & Munk 2007). Mycobacteria may remain "hidden" in the phagosomes of macrophages and, in this way, are able to resist the immune systems defence mechanism. Macrophages produce IL-18, IL-12 and IL-23, and so promote Th1 cell activity. Lymphocytes release IFN-γ and TNF-α which in turn activate macrophages. This positive feedback interaction contains the infection within the T-cell-activated macrophages. It is thought that mycobacteria infect and replicate within non-activated macrophages using a variety of strategies including the avoidance of generating reactive oxygen and nitrogen intermediates, and inhibit the phagosome maturation process, thus avoiding fusion of lysosomes with the macrophage (Fairbairn, Stober, Kumararatne & Lammans 2001). This survival mechanism of mycobacteria within the macrophage can be prevented by autophagy, an intracellular pathway for the lysosomal degradation of long-lived cytoplasmic macromolecules (Harris, Master, De Haro *et al.* 2009), induced by IFN-γ. Autophagy enables infected macrophages to overcome the phagosome's maturation block, initiated by mycobacteria, and thus inhibits the intracellular survival of mycobacteria. However, on the other hand, infected macrophages release Th2 cytokines, IL-4 and IL-13, which act in inhibiting this autophagic process. Thus autophagy has the potential of producing anti-mycobacterial responses in macrophages which may be mediated by Th1 and Th2 cytokines, and shows its link with innate and adaptive immune responses against intracellular pathogens like mycobacteria (Harris *et al.* 2009).

Recognition of mycobacteria and the secretion of IL-12 by macrophages are processes initiated before the pathogen antigens are presented to T-lymphocytes. These initial stages and the production of IFN-γ in natural killer (NK) cells are induced by IL-12 (Teixeira *et al.* 2007). The primary (CMI) response helps to contain the spread of infection by induction of granuloma formation around the infective foci. Gamma-delta cells (WC1+γδ) and CD2+ T-cells are present within the granulomas during the early stages (7 days after infection) of the disease (Cassidy, Bryson, Gutierrez *et al.* 2001). Granulomas progress further becoming necrotic in their centres which undergo mineralization within 10 weeks of infection, and are partially encapsulated by fibrous connective tissue. Further, a large number of lymphoid cells are present, the majority representing CD2+ T-cells and the minority WC1+γδ T-cells (Cassidy *et al.* 2001).

Gamma-delta T-cells play an important role in lesion development and might be involved in containing the bacilli through cytokine release (IL-12 and IFN-γ) and stimulating macrophage activation (McNair, Welsh & Pollock 2007). The responding T-cells also produce a range of cytokines which aid in the protective immunity against mycobacterial infections. The cytokines that are produced include IFN-γ, TNF-α, IL-4 and IL-6. T-cells (CD4+ and CD8+) also have the ability to lyse infected macrophages (Liébana, Aranaz, Aldwell *et al.* 2000). Macrophages are also involved during the early immune response (McNair *et al.* 2007). The lysis of mycobacteria by CD8+ cytotoxic T-cells is an important immune process with many of the mycobacteria killed by lytic enzymes and mycobacteria released from the macrophages are phagocytosed by more activated macrophages. If the mycobacteria causing TB are contained effectively within a bovine host, infection may become latent. It may remain in this state for years without progression but, in some, may progress to cause clinical disease. In a mouse model, results indicated that Th1-biased immune responses were predominant in ensuring bacteria remain in the latent condition and reactivation was associated with a shift towards a Th2-biased response (Pollock & Neill 2002). Th2 produces the cytokines IL-4, IL-5 and IL-10 which are involved in B-cell activation and the production of antibodies. If CMI responses fail to contain the spread of infection, i.e. mycobacteria are not killed or are not fully contained within granulomas, the animal's humoral immune system will be activated (Fig. 1.2). Since antibodies produced by activated B-cells are ineffective against mycobacteria inside macrophages, the disease will start to disseminate. Pollock, McNair,

b17548858
i19554722

Welsh *et al.* (2001) reported that antibodies produced during the advanced stages of BTB are associated with the progression of the disease (Fig. 1.2).

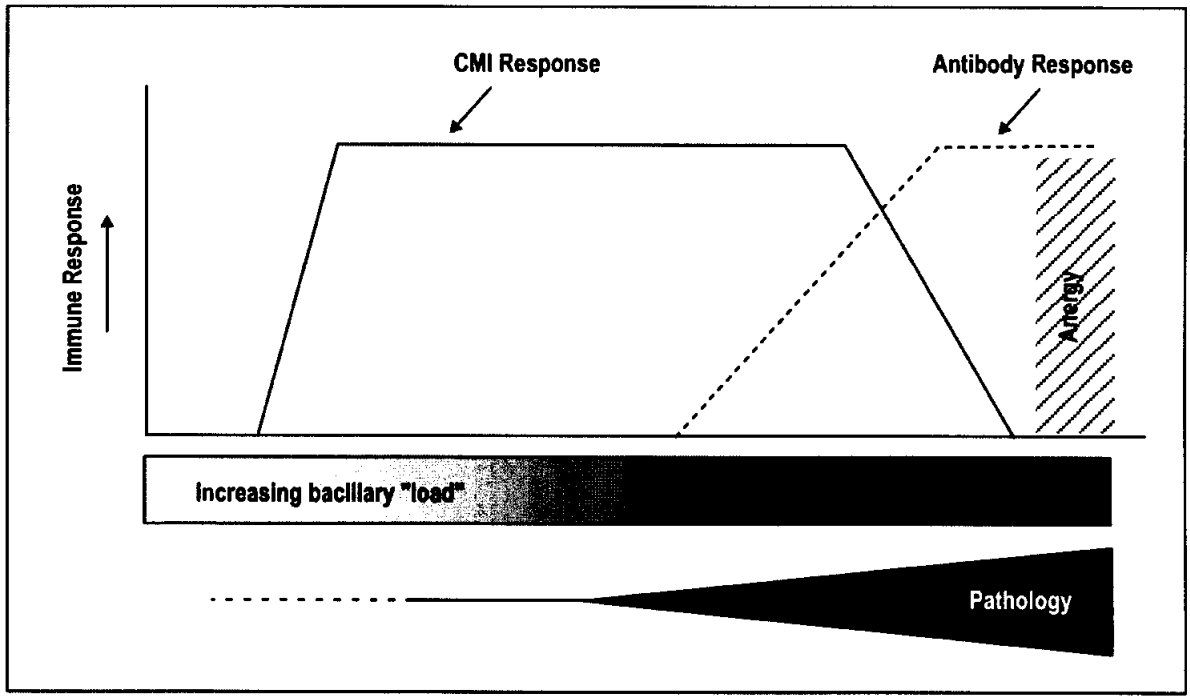


Figure 1.2 During the former (less advanced) stages of the disease the CMI response predominates. As the disease progresses CMI responses tend to wane and the humoral immunity takes over. Finally, in the clinical stage of the disease, reached by only a minority of infected animals, *M. bovis* specific immunity disappears completely, while bacterial shedding occurs (Pollock & Neill 2002)

Antibody IgG1 has been associated with lesion development and may be a useful indicator of disease status (McNair *et al.* 2007). During the progressive stage of the mycobacterial disease, which is typified by high numbers of infecting bacteria, infected animals display a lack of DTH and therefore give a negative result when tested with an IDT but have high levels of circulating antibodies. This condition is referred to as anergy (Plackett, Ripper, Corner *et al.* 1989; Neill, Cassidy, Hanna *et al.* 1994; Pollock & Neill 2002) which can be detected serologically.

1.6 Exploiting immune responses against *M. bovis* and *M. tuberculosis* for diagnosis

1.6.1 Intradermal tuberculin test

The IDT, first described by Koch (Koch 1891), is the primary method of diagnosing *M. bovis* infections in cattle and certain species of wildlife. It is based on induction of a DTH reaction,

mediated by mycobacterial antigen specific memory T-cells, upon injection of a PPD of mycobacteria into the skin. Local swelling, measured 48/72 hours later, indicates prior contact with mycobacteria (de la Rua-Domenech *et al.* 2006b). Apart from the relevant mycobacteria, environmental mycobacteria may also induce reactivity and one has to take into account false positive results. Usually two types of tuberculin, avian PPD produced from *M. avium* (D4ER and TB56 strains) and bovine PPD produced from *M. bovis* (AN5 or Vallee strains) (Monaghan, Doherty, Collins *et al.* 1994) are used in BTB testing, but other strains may be of relevance. The skin test may be applied in one of two ways i.e. the single intradermal test (SIT), and the single intradermal comparative tuberculin test (SICTT) (Monaghan *et al.* 1994). In the former test only bovine PPD is used, whereas in the SICTT both avian and bovine PPD are injected at different sites of the animal. Using a set of interpretation criteria, a comparison is made between the extent of the reaction at injection sites to differentiate between infected animals and / or those that have been exposed to environmental mycobacteria (de la Rua-Domenech *et al.* 2006b).

A disadvantage of BTB diagnosis using the IDT is that it requires capture of animals by immobilizing them in order to perform the test. Three days after release they have to be recaptured in order to read the results of the test. In addition, the IDT is associated with high costs and high levels of handling stress in animals due to the double immobilization (Grobler, Michel, De Klerk & Bengis 2002). Research on the production and evaluation of the use of recombinant antigens of *M. bovis* and *M. tuberculosis* is necessary to improve the sensitivity and specificity of the tuberculin test (Doherty, Monaghan, Bassett & Quinn 1995).

1.6.2 Mycobacterial antigens

Mycobacterium bovis and *M. tuberculosis* express proteins that have the ability to induce cellular immune responses in their hosts (Rhodes, Gavier-Widen, Buddle *et al.* 2000b). Among others, these include the major secreted immunogenic proteins (e.g. MPB70, MPB80, MPB83 and MPT63), early secreted antigen target-6 (ESAT-6) and culture filtrate protein-10 (CFP-10). The soluble antigens, MPB70 and MPB80 are closely related proteins, which are expressed within the *M. bovis* "body", whereas, MPB83 is an external lipoprotein associated with the bacterial surface (Wiker, Lyashchenko, Aksoy *et al.* 1998). The antigen, MPT63, is secreted by *M. tuberculosis* (Manca, Lyashchenko, Wiker *et al.* 1997). Early secreted antigenic target-6 is a protein secreted during the early or active stage of mycobacterial

infections (Brodin, Rosenkrands, Andersen *et al.* 2004). The ESAT-6 gene is present in *M. tuberculosis* and virulent *M. bovis* strains but absent from *M. bovis* BCG and environmental acid-fast bacteria (Harboe, Oettinger, Wiker *et al.* 1996). Both ESAT-6 and CFP-10 are potent T-cell antigens. These two antigens are used in diagnosing BTB in cattle by differentiating between non-vaccinated and BCG-vaccinated cattle (Buddle, Parlane, Keen *et al.* 1999; Vordermeier, Chambers, Cockle *et al.* 2002).

1.6.3 Blood-based tests

1.6.3.1 Serological tests

In serology, antigen-antibody complexes are measured and the results are used to aid in diagnosis of diseases of animals (Jacobson 2007). Interpretation of the results will depend largely on whether an antigen or antibody is detected. Various serological tests, such as western blots and enzyme-linked immunosorbent assays (ELISA), are available to assess the exposure of animals to pathogens, i.e. to measure the presence of antibodies. A western blot analysis is used to demonstrate the presence of antibodies against specific proteins of pathogens after their separation by electrophoresis. The ELISA is the most widely used semi-quantitive serologic assay applied for diagnosis. It is also versatile because it can be formatted in many different ways (direct, indirect and competitive) (Jacobson 2007).

For the diagnosis, in domestic animals and wildlife, serological tests have been investigated as alternatives to the IDT and other CMI-based tests. The IDT, "a *good herd test but poor animal test*", is poor at identifying individual infected animals (de la Rua-Domenech *et al.* 2006b). Serological tests for BTB diagnosis have to be sensitive, easy to use and should be able to distinguish between different mycobacteria of the MTBC (Koo, Park, Ahn *et al.* 2005). During the initial stages of *M. bovis* infection in cattle, the cellular immunity comes into play but, as the disease progresses, humoral immune responsiveness become prominent. Therefore antibody formation is considered to be related to the advanced stages of the disease (Pollock & Neill 2002), but it should also be noted that antibodies are also present in tuberculous animals with subclinical and / or latent infections. Various antigens (MPB83, MPB70, Ag 85, ESAT-6 & CFP10) have been used in sero-diagnostic tests and it has been stated that the best option for a sero-diagnostic test for TB is to have a cocktail of antigens (Lyashchenko, Singh, Colangeli & Gennaro 2000; Amadori, Lyashchenko, Gennaro *et al.* 2002; Aagaard, Govaerts, Meikle *et al.* 2006; Liu, Guo, Wang *et al.* 2007). Koo, Park, Anh *et*

al. (2005) demonstrated that ELISA, latex bead agglutination assays (LBAA) and immuno-chromatographic assays (ICGA) using ESAT-6p, a peptide containing an immuno-dominant portion of ESAT-6, and recombinant MPB70, one of the immuno-dominant antigens of *M. bovis* and also a stable and active component of bovine PPD (Fifis, Plackett, Corner & Wood 1989), have the potential for detecting both early and advanced stages of BTB.

Recently, the Rapid Test (RT) has been developed for the diagnosis of TB in various species of animals (Drewe, Dean, Michel *et al.* 2009). Different "cocktails" of selected *M. bovis* antigens are used. This simple and rapid antibody test developed by Chembio Diagnostic Systems, Inc. employs colour latex-based lateral flow technology along with relevant TB antigens. The RT was used to quickly identify positive sera for further analysis in a multi-antigen print immunoassay (MAPIA) that utilizes a panel of 12 mycobacterial antigens which includes eight recombinant proteins (ESAT-6, CFP-10, MPB64, MPB59, MPB70, MPB83, alpha-crystallin [Acr1], a 38kDa protein), two protein fusions ([CFP-10/ESAT-6], [Acr1/MPB83BCF]), and two native antigens (bovine PPD and *M. bovis* culture filtrate [MBCF]) (Lyashchenko *et al.* 2000).

A drawback of these assays for the diagnosis of TB in wildlife species (captive as well as free-ranging) is that the numbers of positive animals available are usually low which hinders validation of these tests according to the World Organisation for Animal Health (OIE) standards. Furthermore, their use has been questioned as they show little or no reactivity that can be used for the diagnosis of pulmonary TB (Steingart *et al.* 2007). Extensive efforts to identify and characterize antigens unique to *M. bovis* for use in a diagnostic assays have shown that the antibody response to *M. bovis* is not uniform, with no evidence of a dominant persistent response to a single antigen (Cousins & Florisson 2005; Aranaz, De Juan, Bezos *et al.* 2006; de la Rua-Domenech *et al.* 2006b) at any stage of infection (Amadori *et al.* 2002; Lyashchenko, Pollock, Colangeli & Gennaro 1998). These findings suggest that some type of multiplex assay is needed to detect animals at different stages of infection (Amadori *et al.* 2002; Aagaard *et al.* 2006). To address this Whelan, Shuralev, O'Keeffe *et al.* (2008) have developed a multiplex assay that can simultaneously detect and analyse the response to multiple antigens spotted in a single well in a 96-well plate array format. The authors demonstrated the enhanced diagnostic power of a multiplex antigen approach over that of the standard-industry methods (de la Rua-Domenech *et al.* 2006b).

1.6.3.2 Lymphocyte proliferation assay

The lymphocyte proliferation assay is an *in vitro* test that measures antigen specific reactivity of memory T-cells. When using whole blood or purified peripheral blood mononuclear lymphocytes from *M. bovis*-infected cattle, proliferation induced by bovine PPD and avian PPD and can be compared for *M. bovis*-specific responsiveness and *M. avium*-related background proliferation. Griffin & Buchan (1994) made use of the lymphocyte assay to detect BTB in deer herds. The results of the initial pilot studies have shown that the proliferation assay has a sensitivity of 95% and a specificity of 92% for BTB diagnosis in infected herds. A lymphocyte proliferation assay is a time-consuming technique and restricts the number of animals that can be tested. The cells have to be incubated in complex tissue culture media for 3-5 days, and the detection of the level of cell proliferation requires the use of radioactive nucleotides. Additional disadvantages are the complicated logistics involved in the field work and needs of the laboratory. For these reasons this form of diagnosis is considered far too complex, slow and costly (Wood, Rothel, McWaters & Jones 1990; Griffin & Buchan 1994).

1.6.3.3 Interferon-gamma assay

Interferon-gamma of several veterinary relevant species of animals has been cloned for the purposes of developing effective diagnostics especially for BTB. During the last decade IFN-γ assays have especially been used in cattle to determine *M. bovis*-specific immune reactivity (Wood, Corner, Rothel *et al.* 1991; Wood & Jones 2001; Dalley, Hogarth, Hughes *et al.* 2004; Gormley, Doyle, Fitzsimons *et al.* 2006; Rhodes, Gruffydd-Jones, Gunn-Moore & Jahans 2008a; Rhodes, Gruffydd-Jones, Gunn-Moore & Jahans 2008b). The measurement of IFN-γ produced by memory T-lymphocytes after stimulation with a specific tuberculin (bovine or avian PPD as a control) assesses prior infection of an animal with *M. bovis*. The test is performed in two stages. In stage one; whole blood samples are incubated with tuberculins (bovine and avian PPD) to stimulate the lymphocytes to produce IFN-γ. After 24 hours the plasma supernatant is harvested. In stage two, the supernatants of each blood aliquot are tested for the presence of IFN-γ using a capture ELISA with a pair of bovine IFN-γ specific monoclonal antibodies (Wood & Rothel 1994). Infected animals are identified by their relative IFN-γ responses: bovine PPD versus avian PPD. Bovigam ™ (Wood & Jones 2001) is the commercially available bovine IFN-γ test. In South Africa, Bovigam™ has

shown promising results for use in the diagnosis of *M. bovis* infections in African buffaloes (Grobler *et al.* 2002). As compared to the skin test, the IFN-γ test ensures minimal invasion and only a single manipulation of the animal.

1.7 Antibodies as tools to develop IGRAs

Antibodies are a class of proteins synthesized by B-cells and consist of four polypeptide chains, two heavy chains and two light chains that are connected by disulphide bonds to form a Y-shaped structure (Fig. 1.3). The variable domains V_H and V_L of heavy and light chains are unique to each antibody and together determine the antigen specificity of the molecule (Litman, Rast, Shamblott *et al.* 1993). In a regular immune response antibodies will be produced by all cells of the individual's B-cell repertoire that recognise epitopes in the antigen, i.e. polyclonal antibodies are produced. This is reflected in the immune serum. Köhler & Milstein (1975) generated populations of antibody-producing cells possessing the same specificity and affinity by fusing B-cells from immunized mice with myeloma (tumour) cells. This generated a population of immortal hybridomas, and clones producing monoclonal antibodies with the required specificity and affinity. Antibodies have been recognised as primary tools to distinguish and track target molecules, and are very important research tools (Leenaars & Hendriksen 2005). However, using hybridoma technology has proved to be very expensive and labour intensive, and maintenance of the hybridoma cultures is sometimes cumbersome. In addition, there is the welfare of experimental animals to consider.

An alternative to the polyclonal and monoclonal antibodies are recombinant antibodies produced by using phage-display technology (Smith 1985), where a single chain construct of V_H and V_L (single chain variable fragment [scFv], Fig. 1.3) is displayed on filamentous phage or released in soluble form by bacteria infected with this phage. Phage-display enables researchers to capitalize on target recognition qualities of antibodies and eventually produce large quantities of the selected clones. The recombinant antibodies have all the target recognition qualities of natural antibodies, and are produced using the same genes that code for the target recognition or variable region in natural antibodies from mammalian systems (Clackson, Hoogenboom, Griffiths & Winter 1991). Some advantages of recombinant antibodies over conventional poly- and monoclonal antibodies include libraries of a large variety of antibody gene fragments that can be cloned into expression vectors. These can, in turn, be displayed as scFvs on the surface of filamentous bacteriophage (Winter, Griffiths,

Hawkins & Hoogenboom 1994). Linking the protein function with its corresponding gene (phenotype-genotype link) is significant for the panning procedure and for accessing the gene, thus allowing for manipulations to be performed (Terpe 2003; ShiHua, JiBin, ZhiPing *et al.* 2006).

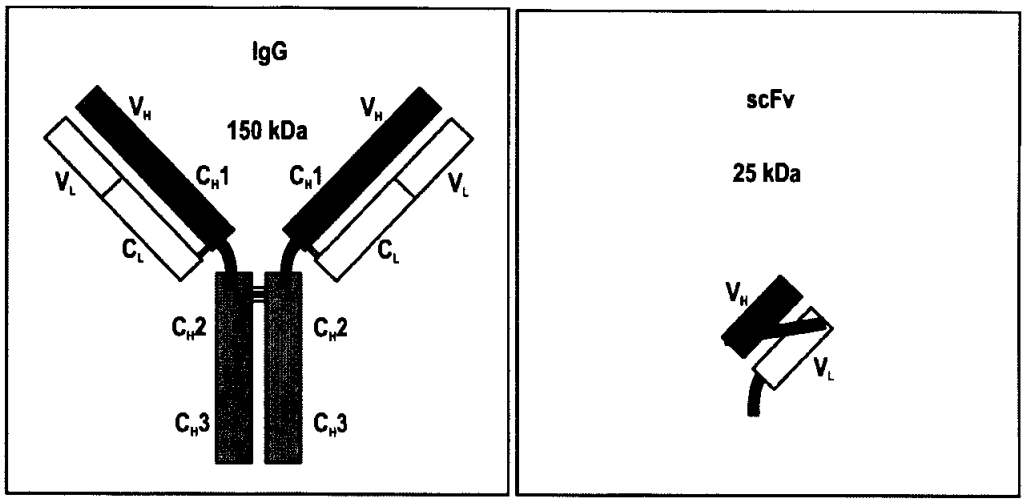


Figure 1.3 Diagrammtic representation of an IgG molecule and a single chain variable fragment

Phage libraries can be constructed from either naïve or immune libraries. The difference between the two is that the former are derived from unimmunized, rearranged, synthetic or shuffled V-genes, the last named being created from V-genes from immunized animals or humans. The immune library can have a strong bias towards antibodies of certain specificity but can also be used to select antibodies against antigens which have not been used in the immunization (Bradbury & Marks 2004). Selection of the desired clones is performed by a technique called bio-panning (Fig. 1.4), which involves the selection of specific binders from phage libraries using the protein of choice (Russel, Lowman & Clackson 1989; Clackson & Wells 1994). Briefly, the target antigen is immobilized in an immunotube. The phages which display the scFvs, bind to the antigen and unbound phages are washed away. Bound phages are eluted, amplified in *Escherichia coli* and then subjected to further rounds of panning. This should result in the enrichment of the number of phages binding to the antigen. Clones from the final round (after Round 3-4 of selection) are characterized individually. Characterization is done by ELISA, which is then followed by sequencing the DNA inserts of the reacting

clones. Once the sequences of the selected clones are known, they can be tailored according to test demands.

Chickens have been used to generate recombinant antibodies (Yamanaka, Inoue & Ikeda-Tanaka 1996; Sapats, Trinidad, Gould *et al.* 2006; Hof, Hoeke & Raats 2008). These animals diversify their immune responses by using only one recombination event in combination with a process called 'gene conversion' (Reynaud, Anquez, Grimal & Weill 1987). During this process, pseudogenes are translocated into the heavy and light chain variable regions; as a result only two primer sets are needed to construct chicken libraries (Davies, Smith, Birkett *et al.* 1995). Therefore chicken antibody libraries are easier and faster to construct compared to human and murine libraries (Hof *et al.* 2008). Chickens are more likely to evoke an immune response to epitopes that are highly conserved between mammals. Furthermore, chicken antibodies have low cross-reactivity to some proteins and display high specificity compared to other IgG antibodies (Gassmann, Thommes, Weiser & Hubscher 1990).

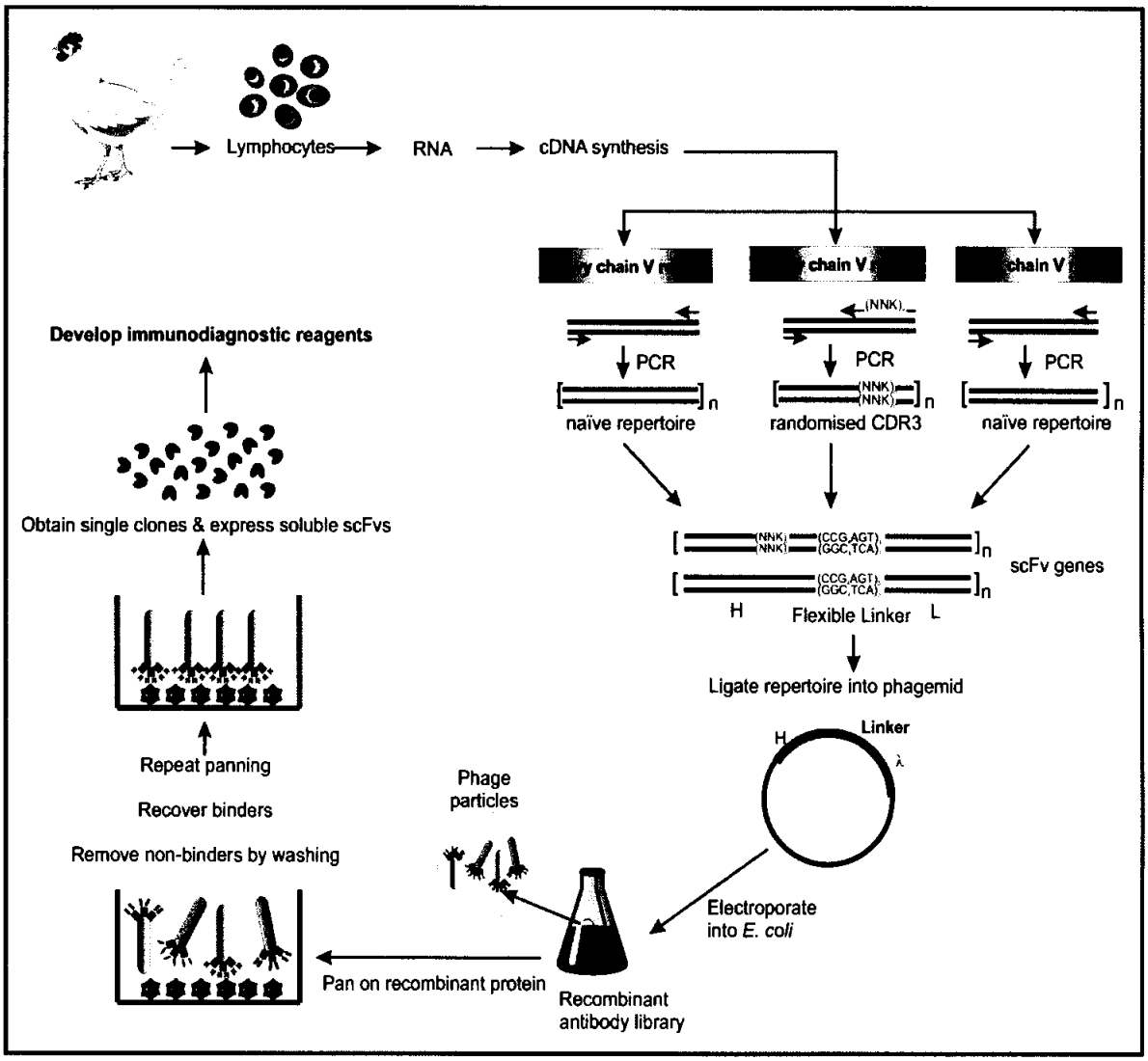


Figure 1.4 Construction of a phage-display antibody library from an immunized chicken. The diagram shows the assembly of the scFv using a three fragment assembly reaction. The construct is cloned into a vector and electroporated into electro-competent *E. coli* cells. Phage particles are then selected against an immobilized antigen by bio-panning. After repeated panning rounds single clones expressing scFv are obtained (Fehrsen, van Wyngaardt, Mashau *et al.* 2005)

1.8 Problem statement

In recent decades TB caused by *M. bovis* has spread from cattle, the primary hosts, to captive and free-ranging wildlife and may affect humans. Similarly, *M. tuberculosis* has been shown to infect domestic and wildlife species, predominantly wildlife in captivity but recently also in some that are free-ranging. At the wildlife-livestock-human interface transmission between species may occur continuously. In South African wildlife the African buffalo and,

potentially, the kudu, are the maintenance hosts of BTB. Spill-over of infection has been reported in lions, cheetahs, leopards, baboons, warthogs and honey badgers. In addition, the white rhinoceros (near threatened species) and black rhinoceros (critically endangered species) (www.iucnredlist.org) have been identified to be susceptible to both *M. bovis* (Dalovisio *et al.* 1992) and *M. tuberculosis* (Oh *et al.* 2002) infections based on culture results. The emergence of *M. tuberculosis* infection in domesticated and captive Asian elephants in the USA, Europe and Asian countries also causes major concern regarding the sensitivity of free-ranging animals.

Wildlife is one of South Africa's biggest assets from an economic standpoint, and a chronic debilitating disease which directly affects a wide spectrum of wildlife species may result in the collapse of conservation programmes. An outbreak of *M. bovis / M. tuberculosis* infections in domesticated, captive and free-ranging wildlife populations poses a significant health threat to wildlife populations in conservation areas, livestock and people in neighbouring communities and livestock farming communities that border game reserves (Michel 2002). The problem that wildlife conservationists, researchers and veterinarians are currently faced with is that there is no single test available for use in wildlife that can diagnose the infection with high specificity and sensitivity prior to the onset of clinical signs and before the animal becomes contagious (i.e. a shedder) for its congeners. Diagnostic tools are not validated or are not available for most wildlife species. There is a registered vacine for humans (BCG) but there is no registered vaccine for animals. These limitations make diagnosis, control, management and eradication of the disease, especially in free-ranging ecosystems, very challenging.

At present there is no proof that *M. bovis* infections have spread to species such as black and white rhinoceroses, and African elephants in wildlife sanctuaries in South Africa. The possibility of such an occurrence in the future cannot be ruled out, and the full effect of the present epidemic, occurring in some game reserves, may only be seen in the long term. These negative effects threaten the biodiversity of wildlife and the survival of threatened or endangered species as well as programmes for their relocation and trade.

The diagnosis of BTB is a complex, multifaceted problem in the context of the wildlife-livestock interface. Thus attempting to solve the BTB epidemic in wildlife requires an accurate understanding of its magnitude and extent of spread within a geographical area. As

for human TB, the choice of a diagnostic test depends upon the setting in which the test is to be performed and the intended use of the results (fit for purpose principle). In species in which *M. bovis* infections are enzootic, such as the buffalo populations in the KNP and HiP, surveys will provide data on the spatio-temporal distribution as well as prevalence rate estimates of the disease. However, in order to provide the parks authorities with management tools, the ideal diagnostic tool should identify the shedders so that the disease can be managed by culling such animals. In this case, a diagnostic test identifying the active disease is required and it should, in addition, be highly specific.

In species, such as domesticated and captive Asian elephants, known to be infected by *M. tuberculosis*, the identification of shedders is even more important, given the risk for humans contracting TB from these animals. In species currently free from *M. bovis / M. tuberculosis* infections (e.g. the African elephant, and black and white rhinoceroses), the ideal diagnostic tool should identify infected animals as early as possible, before an animal becomes contagious or shows clinical signs. Such animals should be quarantined and treated or euthanased to maintain a BTB-free population. In this case, the first test to be used should be highly sensitive. Once the risk animal or animals are identified a rigorous diagnostic work-up can be performed. A further challenge lies in the fact that diagnosis has to be performed in a wide diversity of species susceptible to the disease. Many tests exist for BTB diagnostics but they all suffer from one or another shortcoming when applied in the wildlife context. For example, the IDT which is the standard method of detecting *M. bovis* infections in domestic cattle is not validated in many wildlife species. Other options that have been pursued to overcome limitations associated with skin tests include serology-based assays. However, to date, no serology-based assay has shown the sensitivity and / or specificity that are required for a routine diagnostic tool. Since immune responses (including protective immunity) against mycobacteria are primarily CMI-based, diagnostic tests measuring CMI are considered to be the most important diagnostic tools for the identification of animals infected with *M. bovis* or *M. tuberculosis* (Pollock *et al.* 2001; Pollock, Welsh & McNair 2005) prior to the onset of clinical signs. Especially the IFN-γ tests, applied in some instances for the diagnosis of BTB in cattle and which, most likely, will be in the near future, the method of choice in diagnosis of TB in humans, have to be developed for relevant species. Moreover, IFN-γ tests may be performed by using different antigens. Some of these antigens are considered to be "early" antigens, for example MPB70 in cattle, while others are considered

to be correlated to active TB in humans or BTB in cattle (e.g. ESAT6 and CFP10) (Vordermeier *et al.* 2002; Jafari & Lange 2008).

1.9 Research objectives

The aim of the study was to develop IFN-γ capture ELISAs for the detection of *M. bovis* and *M. tuberculosis* infections in rhinoceroses and elephants. To achieve this goal, the reagents of the ELISA system had to be developed. The first objective was to clone, sequence and express the IFN-γ gene of the white rhinoceros and Asian elephant. This was followed by the sequencing of the AfEpIFN-γ gene and the investigation of the similarities between this gene and the AsEpIFN-γ gene. The second objective was to produce anti-rRhIFN-γ monoclonal and polyclonal antibodies in mice and chickens respectively. The third objective was to use the phage-display technique to generate recombinant antibodies against recombinant white rhinoceros and AsEpIFN-γ derived from chickens and the fourth objective was to produce anti-rAsEpIFN-γ monoclonal antibodies in mice. Antibodies that were produced and characterized during these studies were utilized in different combinations for the development of rhinoceros and elephant (recombinant) IFN-γ ELISAs.

1.10 Impact

The availability of IFN-γ detection systems for the diagnosis of *M. bovis* or *M. tuberculosis* infections in valuable wildlife species would contribute towards the efforts of controlling the infection, and will prove to be a valuable tool for relocation and breeding strategies. Furthermore, they would enable refinement of testing by the use of different recombinant antigens instead of rather crude PPD preparations according to the goal to be pursued i.e. early detection as compared to identification of shedders.

1.11 References

AAGAARD, C., GOVAERTS, M., MEIKLE, V., VALLECILLO, A.J., GUTIERREZ-PABELLO, J.A., SUAREZ-GUEMES, F., MCNAIR, J., CATALDI, A., ESPITIA, C., ANDERSEN, P. & POLLOCK, J.M. 2006. Optimizing antigen cocktails for detection of *Mycobacterium bovis* in herds with different prevalences of BTB: ESAT6-CFP10 mixture shows optimal sensitivity and specificity. *Journal of Clinical Microbiology,* 44:4326-4335.

ALEXANDER, K.A., PLEYDELL, E., WILLIAMS, M.C., LANE, E.P., NYANGE, J.F. & MICHEL, A.L. 2002. *Mycobacterium tuberculosis*: an emerging disease of free-ranging wildlife. *Emerging Infectious Diseases,* 8:598-601.

ALLIX, C., WALRAVENS, K., SAEGERMAN, C., GODFROID, J., SUPPLY, P. & FAUVILLE-DUFAUX, M. 2006. Evaluation of the epidemiological relevance of variable-number tandem-repeat genotyping of *Mycobacterium bovis* and comparison of the method with IS*6110* restriction fragment length polymorphism analysis and spoligotyping. *Journal of Clinical Microbiology,* 44:1951-1962.

ALVAREZ, D.V. 2006. Interferon-gamma (IFN-γ). http://www.biodavidson.edu.

AMADORI, M., LYASHCHENKO, K.P., GENNARO, M.L., POLLOCK, J.M. & ZERBINI, I. 2002. Use of recombinant proteins in antibody tests for bovine tuberculosis. *Veterinary Microbiology,* 85:379-389.

ARANAZ, A., LIÉBANA, E., PICKERING, X., NOVOA, C., MATEOS, A. & DOMINGUEZ, L. 1996. Use of polymerase chain reaction in the diagnosis of tuberculosis in cats and dogs. *Veterinary Record,* 138:276-280.

ARANAZ, A., LIEBANA, E., MATEOS, A., DOMINGUEZ, L. & COUSINS, D. 1998. Restriction fragment length polymorphism and spacer oligonucleotide typing: a comparative analysis of fingerprinting strategies for *Mycobacterium bovis*. *Veterinary Microbiology,* 62:311-324.

ARANAZ, A., COUSINS, D., MATEOS, A. & DOMINGUEZ, L. 2003. Elevation of *Mycobacterium tuberculosis subsp. caprae* to species rank as *Mycobacterium caprae comb. nov., sp. nov. International Journal of Systematic and Evolutionary Microbiology,* 53:1785-1789.

ARANAZ, A., DE JUAN, L., BEZOS, J., ÁLVAREZ, J., ROMERO, B., LOZANO, F., PARAMIO, J.L., LÓPEZ-SÁNCHEZ, J., MATEOS, A. & DOMÍNGUEZ, L. 2006. Assessment of diagnostic tools for eradication of bovine tuberculosis in cattle co-infected with *Mycobacterium bovis* and *M. avium subsp. paratuberculosis. Veterinary Research,* 37:593-606.

AURTENETXE, O., BARRAL, M., VICENTE, J., DE LA FUENTE, J., GORTAZAR, C. & JUSTE, R.A. 2008. Development and validation of an enzyme-linked immunosorbent assay for antibodies against *Mycobacterium bovis* in European wild boar. *BMC Veterinary Research,* 4:43-51.

AYELE, W.Y., NEILL, S.D., ZINSSTAG, J., WEISS, M.G. & PAVLIK, I. 2004. Bovine tuberculosis: an old disease but a new threat to Africa. *International Journal of Tuberculosis and Lung Disease,* 8:924-937.

BACH, R. 1977. *Illusions:* The adventures of a reluctant Messiah. United States of America: Dell Publishing Co., Inc.

BALDREY, F.S.H. 1930. Tuberculosis in an Elephant. *Journal of the Royal Army Veterinary Corps,* 1:252.

BARTALESI, F., VICIDOMINI, S., GOLETTI, D., FIORELLI, C., FIORI, G., MELCHIORRE, D., TORTOLI, E., MANTELLA, A., BENUCCI, M., GIRARDI, E., CERINIC, M.M. & BARTOLONI, A. 2008. QuantiFERON-TB Gold and TST are both useful for latent TB screening in autoimmune diseases. *European Respiratory Journal,* 33:586-593.

BARTU, V., HAVELKOVA, M. & KOPECKA, E. 2008. QuantiFERON-TB Gold in the diagnosis of active tuberculosis. *Journal of International Medical Research,* 36:434-437.

BEHR, M.A., WARREN, S.A., SALAMON, H., HOPEWELL, P.C., PONCE DE LEON, A., DALEY, C.L. & SMALL, P.M. 1999. Transmission of *Mycobacterium tuberculosis* from patients smear-negative for acid-fast bacilli. *Lancet,* 353:444-449.

BENGIS, R.G., KRIEK, N.P., KEET, D.F., RAATH, J.P., DE VOS, V. & HUCHZERMEYER, H.F. 1996. An outbreak of bovine tuberculosis in a free-living African buffalo (*Syncerus caffer*-sparrman) population in the Kruger National Park: a preliminary report. *Onderstepoort Journal of Veterinary Research,* 63:15-18.

BERGEY, D.H. & Holt, J.G. 1993. *Bergey's manual of determinative bacteriology.* Philadelphia: Lippincott Williams and Wilkins.

BINKLEY, M. 1997. Tuberculosis in captive elephants. *Proceedings of the American Association of Zoo Veterinarians*: 116-119.

BONSU, O.A., LAING, E. & AKANMORI, B.D. 2000. Prevalence of tuberculosis in cattle in the Dangme-West district of Ghana, public health implications. *Acta Tropica,* 76:9-14.

BOPAYYA, A.B. 1928. Tuberculosis in an elephant. *Indian Veterinary Journal:*142-145.

BOULAHBAL, F., BENELMOUFFOK, A. & BRAHIMI, K. 1978. Role of *Mycobacterium tuberculosis* in bovine tuberculosis. *Archives de l'Institut Pasteur d'Algerie,* 53:155-164.

BRADBURY, A.R. & MARKS, J.D. 2004. Antibodies from phage antibody libraries. *Journal of Immunological Methods,* 290:29-49.

BRODIN, P., ROSENKRANDS, I., ANDERSEN, P., COLE, S.T. & BROSCH, R. 2004. ESAT-6 proteins: protective antigens and virulence factors? *Trends in Microbiology,* 12:500-508.

BROSCH, R., GORDON, S.V., MARMIESSE, M., BRODIN, P., BUCHRIESER, C., EIGLMEIER, K., GARNIER, T., GUTIERREZ, C., HEWINSON, G., KREMER, K., PARSONS, L.M., PYM, A.S., SAMPER, S., VAN SOOLINGEN, D. & COLE, S.T. 2002. A

new evolutionary scenario for the *Mycobacterium tuberculosis* complex. *Proceedings of the National Academy of Sciences of the United States of America,* 99:3684-3689.

BRUDEY, K., DRISCOLL, J.R., RIGOUTS, L., PRODINGER, W.M., GORI, A., AL-HAJOJ, S.A., ALLIX, C., ARISTIMUNO, L., ARORA, J., BAUMANIS, V., BINDER, L., CAFRUNE, P., CATALDI, A., CHEONG, S., DIEL, R., ELLERMEIER, C., EVANS, J.T., FAUVILLE-DUFAUX, M., FERDINAND, S., GARCIA DE VIEDMA, D., GARZELLI, C., GAZZOLA, L., GOMES, H.M., GUTTIEREZ, M.C., HAWKEY, P.M., VAN HELDEN, P.D., KADIVAL, G.V., KREISWIRTH, B.N., KREMER, K., KUBIN, M., KULKARNI, S.P., LIENS, B., LILLEBAEK, T., HO, M.L., MARTIN, C., MARTIN, C., MOKROUSOV, I., NARVSKAIA, O., NGEOW, Y.F., NAUMANN, L., NIEMANN, S., PARWATI, I., RAHIM, Z., RASOLOFO-RAZANAMPARANY, V., RASOLONAVALONA, T., ROSSETTI, M.L., RUSCH-GERDES, S., SAJDUDA, A., SAMPER, S., SHEMYAKIN, I.G., SINGH, U.B., SOMOSKOVI, A., SKUCE, R.A., VAN SOOLINGEN, D., STREICHER, E.M., SUFFYS, P.N., TORTOLI, E., TRACEVSKA, T., VINCENT, V., VICTOR, T.C., WARREN, R.M., YAP, S.F., ZAMAN, K., PORTAELS, F., RASTOGI, N. & SOLA, C. 2006. *Mycobacterium tuberculosis* complex genetic diversity: mining the fourth international spoligotyping database (SpolDB4) for classification, population genetics and epidemiology. *BMC Microbiology,* 6:23-40.

BUDDLE, B.M., PARLANE, N.A., KEEN, D.L., ALDWELL, F.E., POLLOCK, J.M., LIGHTBODY, K. & ANDERSEN, P. 1999. Differentiation between *Mycobacterium bovis* BCG-vaccinated and *M. bovis*-infected cattle by using recombinant mycobacterial antigens. *Clinical and Diagnostic Laboratory Immunology,* 6:1-5.

CADMUS, S., PALMER, S., OKKER, M., DALE, J., GOVER, K., SMITH, N., JAHANS, K., HEWINSON, R.G. & GORDON, S.V. 2006. Molecular analysis of human and bovine tubercle bacilli from a local setting in Nigeria. *Journal of Clinical Microbiology,* 44:29-34.

CASSIDY, J.P., BRYSON, D.G., GUTIERREZ CANCELA, M.M., FORSTER, F., POLLOCK, J.M. & NEILL, S.D. 2001. Lymphocyte subtypes in experimentally induced early-stage bovine tuberculous lesions. *Journal of Comparative Pathology,* 124:46-51.

CLACKSON, T., HOOGENBOOM, H.R., GRIFFITHS, A.D. & WINTER, G. 1991. Making antibody fragments using phage display libraries. *Nature,* 352:624-628.

CLACKSON, T. & WELLS, J.A. 1994. *In vitro* selection from protein and peptide libraries. *Trends in Biotechnology,* 12:173-184.

CLEAVELAND, S., MLENGEYA, T., KAZWALA, R.R., MICHEL, A., KAARE, M.T., JONES, S.L., EBLATE, E., SHIRIMA, G.M. & PACKER, C. 2005. Tuberculosis in Tanzanian wildlife. *Journal of Wildlife Diseases,* 41:446-453.

CLEAVELAND, S., SHAW, D.J., MFINANGA, S.G., SHIRIMA, G., KAZWALA, R.R., EBLATE, E. & SHARP, M. 2007. *Mycobacterium bovis* in rural Tanzania: risk factors for infection in human and cattle populations. *Tuberculosis,* 87:30-43.

COLE, S.T., BROSCH, R., PARKHILL, J., GARNIER, T., CHURCHER, C., HARRIS, D., GORDON, S.V., EIGLMEIER, K., GAS, S., BARRY, E., TEKAIA, F., BADCOCK, K., BASHAM, D., BROWN, D., CHILLINGWORTH, T., CONNOR, R., DAVIES, R., DEVLIN, K., FELTWELL, T., GENTLES, S., HAMLIN, N., HOLROYD, S. HORNSBY, T., JAGELS K., KROGH, A., MCCLEAN, J., MOULE, S., MURPHY, L., OLIVER, K., OSBORNE, J., QUAIL, M.A., RAJANDREAM, M.A., RODGERS, J., RUTTER, S., SEEGER, K., SKLETON, J., SQUARES, R., SQUARES, S., SULSTONS, J.E., TAYLOR, K., WHITEHEAD, S. & BARRELL, B.G. 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature*, 393:537-544.

COLLINS, D.M. & DE LISLE, G.W. 1985. DNA restriction endonuclease anaylsis of *Mycobacterium bovis* and other members of the tuberculosis complex. *Journal of Clinical Microbiology*, 21:562-564.

CORBETT, E.L., WATT, C.J., WALKER, N., MAHER, D., WILLIAMS, B.G., RAVIGLIONE, M.C. & DYE, C. 2003. The growing burden of tuberculosis: global trends and interactions with the HIV epidemic. *Archives of Internal Medicine,* 163:1009-1021.

CORNER, L.A., BARRETT, R.H., LEPPER, A.W.D., LEWIS, V. & PEARSON, C.W. 1981. A survey of mycobacteriosis of feral pigs in the Northern Territory. *Australian Veterinary Journal,* 57:537-542.

CORNER, L.A., MELVILLE, L., MCCUBBIN, K., SMALL, K.J., MCCORMICK, B.S., WOOD, P.R. & ROTHEL, J.S. 1990. Efficiency of inspection procedures for the detection of tuberculous lesions in cattle. *Australian Veterianary Journal,* 67:389-392.

CORNER, L.A. 1994. Post-mortem diagnosis of *Mycobacterium bovis* infection in cattle. *Veterinary Microbiology,* 40:53-63.

CORNER, L.A., STEVENSON, M.A., COLLINS, D.M. & MORRIS, R.S. 2003. The re-emergence of *Mycobacterium bovis* infection in brushtail possums (*Trichosurus vulpecula*) after localised possum eradication. *New Zealand Veterinary Journal,* 51:73-80.

CORNER, L.A.L. 2006. The role of wild animal populations in the epidemiology of tuberculosis in domestic animals: How to assess the risk. *Veterinary Microbiology,* 112:303-312.

COURTENAY, O., REILLY, L.A., SWEENEY, F.P., HIBBERED, V., BRYAN, S., UL-HASSAN, A., NEWMAN, C., MACDONALD, D.W., DELAHAY, R.J., WILSON, G.J. & WELLINGTON, E.M. 2006. Is *Mycobacterium bovis* in the environment important for the persistence of bovine tuberculosis? *Biological Letters,* 1:460-462.

COUSINS, D.V., FRANCIS, B.R., GOW, B.L., COLLINS, D.M., MCGLASHAN, C.H., GREGORY, A. & MACKENZIE, R.M. 1990. Tuberculosis in captive seals: bacteriological studies on an isolate belonging to the *Mycobacterium tuberculosis* complex. *Research in Veterinary Science,* 48:196-200.

COUSINS, D.V., BASTIDA, R., CATALDI, A., QUSE, V., REDROBE, S., DOW, S., DUIGNAN, P., MURRAY, A., DUPONT, C., AHMED, N., COLLINS, D.M., BUTLER, W.R., DAWSON, D., RODRÃ GUEZ, D., LOUREIRO, J., ROMANO, M.I., ALITO, A., ZUMARRAGA, M. & BERNARDELLI, A. 2003. Tuberculosis in seals caused by a novel

member of the *Mycobacterium tuberculosis* complex: *Mycobacterium pinnipedii* sp. nov. *International Journal of Systematic and Evolutionary Microbiology,* 53:1305-1314.

COUSINS, D.V. & FLORISSON, N. 2005.A review of tests available for use in the diagnosis of tuberculosis in non-bovine species. *Revue Scientifique et Technique,* 24:1039-1059.

COUSINS, D.V. 2008. Bovidae - Bovine Tuberculosis, in *Manual of diagnostic tests and vaccines for terrestrial animals* 2008. Summary, edited by Office International des Epizooties: 683-697

CVETNIC, Z., KATALINIC-JANKOVIC, V., SOSTARIC, B., OBROVAC, M., MARJANOVIC, S., BENIC, M., KIRIN, B.K. & VICKOVIC, I. 2007. *Mycobaterium caprae* in cattle and humans in Croatia. *International Journal of Tuberculosis and Lung disease,* 11:652-658.

DALLEY, D.J., HOGARTH, P.J., HUGHES, S., HEWINSON, R.G. & CHAMBERS, M.A. 2004. Cloning and sequencing of badger (*Meles meles*) interferon and its detection in badger lymphocytes. *Veterinary Immunology and Immunopathology,* 101:19-30.

DALOVISIO, J.R., STETTER, M. & MIKOTA-WELLS, S. 1992. Rhinoceros' rhinorrhea: cause of an outbreak of infection due to airborne *Mycobacterium bovis* in zookeepers. *Clinical Infectious Diseases,* 15:598-600.

DANIEL, T.M. 2006. The history of tuberculosis. *Respiratory Medicine,* 100:1862-1870.

DAVIES, E.L, SMITH, J.S, BIRKETT, C.R, MANSER, J.M, ANDERSON-DEAR, D.V & YOUNG, J.R. 1995. Selection of specific phage-display antibodies using libraries derived from chicken immunoglobulin genes. *Journal of Immunological Methods,* 186:125–35.

DE LA RUA-DOMENECH, R. 2006a. Human *Mycobacterium bovis* infection in the United Kingdom: Incidence, risks, control measures and review of the zoonotic aspects of bovine tuberculosis. *Tuberculosis,* 86:77-109.

DE LA RUA-DOMENECH, R., GOODCHILD, A.T., VORDERMEIER, H.M., HEWINSON, R.G., CHRISTIANSEN, K.H. & CLIFTON-HADLEY, R.S. 2006b. *Ante-mortem* diagnosis of tuberculosis in cattle: a review of the tuberculin tests, gamma-interferon assay and other ancillary diagnostic techniques. *Research in Veterinary Science,* 81:190-210.

DE LISLE, G.W., MACKINTOSH, C.G. & BENGIS, R.G. 2001. *Mycobacterium bovis* in free-living and captive wildlife, including farmed deer. *Revue Scientifique et Technique,* 20:86-111.

DE LISLE, G.W., BENGIS, R.G., SCHMITT, S.M. & O'BRIEN, D.J. 2002. Tuberculosis in free-ranging wildlife: detection, diagnosis and management. *Revue Scientifique et Technique,* 21:317-334.

DE VOS, V., BENGIS, R.G., KRIEK, N.P., MICHEL, A., KEET, D.F., RAATH, J.P. & HUCHZERMEYER, H.F. 2001. The epidemiology of tuberculosis in free-ranging African buffalo (*Syncerus caffer*) in the Kruger National Park, South Africa. *Onderstepoort Journal of Veterinary Research,* 68:119-130.

DIEL, R., LODDENKEMPER, R., MEYWALD-WALTER, K., GOTTSCHALK, R. & NIENHAUS, A. 2008. Comparative performance of tuberculin skin test, QuantiFERON-TB-gold in tube assay, and T-Spot.TB test in contact investigations for tuberculosis. *Chest,* Abstract.

DOHERTY, M.L., MONAGHAN, M.L., BASSETT, H.F. & QUINN, P.J. 1995. Effect of a recent injection of purified protein derivative on diagnostic tests for tuberculosis in cattle infected with *Mycobacterium bovis. Research in Veterinary Science,* 58:217-221.

DREWE, J.A., DEAN, G.S., MICHEL, A.L., LYASHCHENKO, K.P., GREENWALD, R. & PEARCE, G.P. 2009. Accuracy of three diagnostic tests for determining *Mycobacterium bovis* infection status in live-sampled wild meerkats (*Suricata suricatta*). *Journal of Veterinary Diagnostic Investigation,* 21:31-39.

DYE, C., WATT, C.J., BLEED, D.M., HOSSEINI, S.M. & RAVIGLIONE, M.C. 2005. Evolution of tuberculosis control and prospects for reducing tuberculosis incidence,

prevalence, and deaths globally. *Journal of the American Medical Association,* 293:2767-2775.

FAIRBAIRN, I.P., STOBER, C.B., KUMARARATNE, D.S. & LAMMANS, D.A. 2001. ATP-mediated killing of intracellular mycobacteria is a $P2X_7$-dependent process inducing bacterial death by phagosome-lysosome fusion. *Journal of Immunology*, 167:3300-3307.

FEHRSEN, J., VAN WYNGAARDT, W., MASHAU, C., POTGIETER, C., GUPTA, A., CHAUDHARY, V.K., JORDAAN, F. & AND DU PLESSIS, D.H. 2005. Chicken scFvs in bluetongue virus serogroup-reactive and type-specific inhibition ELISAs. *South African Society for Biochemistry and Molecular Biology XIXth Conference, Stellenbosch University.*

FERNANDEZ, F. & MORICI, E. 1999. Feline tuberculosis due to *Mycobacterium bovis*: report of 2 cases. *Revista Argentina de Microbiologa,* 31:19-20.

FIETTA, A., MELONI, F., CASCINA, A., MOROSINI, M., MARENA, C., TROUPIOTI, P., MANGIAROTTI, P. & CASALI, L. 2003. Comparison of a whole-blood interferon-gamma assay and tuberculin skin testing in patients with active tuberculosis and individuals at high or low risk of *Mycobacterium tuberculosis* infection. *American Journal of Infection Control,* 31:347-353.

FIFIS, T., PLACKETT, P., CORNER, L.A. & WOOD, P.R. 1989. Purification of a major *Mycobacterium bovis* antigen for the diagnosis of bovine tuberculosis. *Scandinavian Journal of Immunology,* 29:91-101.

FIGUEROA-MUNOZ, J., PALMER, K., POZ, M.R., BLANC, L., BERGSTROM, K. & RAVIGLIONE, M. 2005. The health workforce crisis in TB control: a report from high-burden countries. *Human Resources for Health,* 3:2-10.

FRITSCHE, A., ENGEL, R., BUHL, D & ZELLWEGER, J-P. 2004. *Mycobacterium bovis* tuberculosis: from animal to man and back. *International Journal of Tuberculosis and Lung Disease*, 7:903-904.

FROTHINGHAM, R. & MEEKER-O'CONNELL, W.A. 1998. Genetic diversity in the *Mycobacterium tuberculosis* complex based on variable numbers of tandem DNA repeats. *Microbiology,* 144:1189-1196.

FULLER, W.A. 1959. The biology and management of bison of Wood Buffalo National Park. Ph.D. thesis, University of Wisconsin-Madison.

FURLEY, C.W. 1997. Tuberculosis in elephants. *Lancet,* 350:224.

GASSMANN, M., THOMMES, P., WEISER T. & HUBSCHER. U. 1990. Efficient production of chicken egg yolk antibodies against a conserved mammalian protein. *Federation of Amercian Societies for Experimental Biology Journal,* 4:2528–32.

GLICKMAN, M.S. & JACOBS Jr, W.R., 2001. Microbial pathogenesis of *Mycobacterium tuberculosis*: dawn of a discipline. *Cell,* 104:477-485.

GODFROID, J., DELCORPS, C., IRENGE, L.M., WALRAVENS, K., MARCHE, S. & GALA, J.L. 2005. Definitive differentiation between single and mixed mycobacterial infections in red deer (*Cervus elaphus*) by a combination of duplex amplification of p34 and f57 sequences and Hpy188I enzymatic restriction of duplex amplicons. *Journal of Clinical Microbiology,* 43:4640-4648.

GORMLEY, E., DOYLE, M.B., FITZSIMONS, T., MCGILL, K. & COLLINS, J.D. 2006. Diagnosis of *Mycobacterium bovis* infection in cattle by use of the gamma-interferon (Bovigam®) assay. *Veterinary Microbiology,* 112:171-179.

GOROVITZ, C. 1962. Tuberculosis in an African elephant. *Nordisk Veterinaer Medicin,* 14:351-352.

GORTAZAR, C., TORRES, M.J., VICENTE, J., ACEVEDO, P., REGLERO, M., DE LA FUENTE, J., NEGRO, J.J. & AZNAR-MARTIN, J. 2008. Bovine tuberculosis in Donana Biosphere Reserve: the role of wild ungulates as disease reservoirs in the last Iberian lynx strongholds. *PLoS ONE,* 3:e2776.

GRANGE, J.M. & BISHOP, P.J. 1982. 'Uber tuberkulose'. A tribute to Robert Koch's discovery of the tubercle bacillus, 1882. *Tubercle,* 63:3-17.

GREENWALD, R., LYASHCHENKO, O., ESFANDIARI, J., MILLER, M., MIKOTA, S., OLSEN, J.H., BALL, R., DUMONCEAUX, G., SCHMITT, D., MOLLER, T., PAYEUR, J.B., HARRIS, B., SOFRANKO, D., WATERS, W.R. & LYASCHENKO, K.P. 2009. Highly accurate antibody assays for early and rapid detection of TB in African and Asian elephants. *Clinical and Vaccine Immunology*, 16:605-612.

GRIFFIN, J.F.T. & BUCHAN, G.S. 1994. Aetiology, pathogenesis and diagnosis of *Mycobacterium bovis* in deer. *Veterinary Microbiology,* 40:193-205.

GROBLER, D.G., MICHEL, A.L., DE KLERK, L.M. & BENGIS, R.G. 2002. The gamma-interferon test: its usefulness in a bovine tuberculosis survey in African buffaloes (*Syncerus caffer*) in the Kruger National Park. *Onderstepoort Journal of Veterinary Research,* 69:221-227.

GROENEN, P.M.A., BUNSCHOTEN, A.E., VAN SOOLINGEN, D. & VAN EMBDEN, J.D.A. 1993. Nature of DNA polymorphisms in the direct repeat cluster of *Mycobacterium tuberculosis*; application for strain differentiation by a novel typing method. *Molecular Microbiology*, 10:1057-1065.

GUNNEBERG, C., REID, A., WILLIAMS, B.G., FLOYD, K. & NUNN, P. 2008. Global monitoring of collaborative TB-HIV activities. *International Journal of Tuberculosis and Lung Disease,* 12:2-7.

GUNN-MOORE, D.A. 1994. Tuberculosis in cats. *Veterinary Record,* 134:395.

GUTIERREZ, M., SAMPER, S., GAVIGAN, J.-A., MARIN, J.F.G. & MARTIN, C. 1995. Differentiation by molecular typing of *Mycobacterium bovis* strains causing tuberculosis in cattle and goats. *Journal of Clinical Microbiology*, 33:2953-2956.

GUTIERREZ, M.C., BRISSE, S., BROSCH, R., FABRE, M., OMAIS, B., MARMIESSE, M., SUPPLY, P. & VINCENT, V. 2005. Ancient origin and gene mosaicism of the progenitor of *Mycobacterium tuberculosis*. *PLoS Pathogens,* 1:e5.

GUTTER, A. 1981. *Mycobacterium tuberculosis* in an Asian elephant. *Proceedings of the American Association of Zoo Veterinarians*: 105-106.

HADDAD, N., MASSELOT, M. & DURAND, B. 2004. Molecular differentiation of *Mycobacterium bovis* isolates. Review of main techniques and applications. *Research in Veterinary Science*. 76:1-18.

HARBOE, M., OETTINGER, T., WIKER, H.G., ROSENKRANDS, I. & ANDERSEN, P. 1996. Evidence for occurrence of the ESAT-6 protein in *Mycobacterium tuberculosis* and virulent *Mycobacterium bovis* and for its absence in *Mycobacterium bovis* BCG. *Infection and Immunity,* 64:16-22.

HARR, K., ISAZA, R., RASKIN, R., BLUE, J. & HARVEY, J. 2001. Laboratory findings in *Mycobacterium tuberculosis* culture-positive Asian elephants compared to clinically normal elephants. *Veterinary Clinical Pathology,* 30:150-151.

HARRIES, A.D. & DYE, C. 2006a. Tuberculosis. *Annals of Tropical Medicine and Parasitology,* 100:415-431.

HARRIES, A.D., BOXSHALL, M., PHIRI, S. & KWANJANA, J. 2006b. Managing HIV and tuberculosis in sub-Saharan Africa. *Lancet,* 367:1817-1818.

HARRIS, J., MASTER, S.S, DE HARO, S.A., DELGADO, M., ROBERTS, E.A., HOPE, J.C., KEANE, J. & DERETIC, V. 2009. Th1-Th2 polarisation and authophagy in the control of intracellular mycobacateria by macrophages. *Veterinary Immunology and Immunpathology*, 128:37-43.

HELMAN, R.G., RUSSELL, W.C., JENNY, A., MILLER, J. & PAYEUR, J. 1998. Diagnosis of tuberculosis in two snow leopards using polymerase chain reaction. *Journal of Veterinary Diagnostic Investigation,* 10:89-92.

HERZOG, H. 1998. History of tuberculosis. *Respiration,* 65:5-15.

HOF, D., HOEKE, M.O. & RAATS, J.M. 2008. Multiple-antigen immunization of chickens facilitates the generation of recombinant antibodies to autoantigens. *Clinical and Experimental Immunology,* 151:367-377.

JACOBSON, E.R. 2007. *Infectious diseases and pathology of reptiles.* Boca Raton: CRC Press.

JAFARI, C. & LANGE, C. 2008. Suttons's Law: Local imunodiagnosis of tuberculosis. *Infection,* 36:510-514.

JOHNSTON, J.M. 1981. Tuberculosis in a circus elephant. *CDC Veterinary Public Health Notes,* 16:18.

KAMERBEEK, J., SCHOULS, L., KOLK, A., VAN AGTERVELD, M., VAN SOOLINGEN, D., KUIJPER, S., BUNSCHOTEN, A., MOLHUIZEN, H., SHAW, R., GOYAL, M. & VAN EMBDEN, J. 1997. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. *Journal of Clinical Microbiology,* 35:907-914.

KARLSON, A.G. & LESSEL, E.F. 1970. *Mycobacterium bovis* norm. nov. *International Journal of Systematic Bacteriology,* 20:273-282.

KEEP, M.E. & BASSON, P.A. 1973. Mycobacteriosis in a black rhinoceros (*Diceros bicornis* Linnaeus 1758). *Journal of the South African Veterinary Association,* 44:285-286.

KEET, D.F., KRIEK, N.P.J., PENRITH, M.L., MICHEL, A. & HUCHZERMEYER, H.F. 1996. Tuberculosis in buffaloes (*Syncerus caffer*) in the Kruger National Park: Spread of the disease to other species. *Onderstepoort Journal of Veterinary Research,* 63:239-244.

KEET, D.F., KRIEK, N.P., BENGIS, R.G., GROBLER, D.G. & MICHEL, A. 2000. The rise and fall of tuberculosis in a free-ranging chacma baboon troop in the Kruger National Park. *Onderstepoort Journal of Veterinary Research,* 67:115-122.

KEET, D.F., KRIEK, N.P., BENGIS, R.G. & MICHEL, A.L. 2001. Tuberculosis in kudus (*Tragelaphus strepsiceros*) in the Kruger National Park. *Onderstepoort Journal of Veterinary Research,* 68:225-230.

KOBASHI, Y., OBASE, Y., FUKUDA, M., YOSHIDA, K., MIYASHITA, N. & OKA, M. 2006. Clinical reevaluation of the QuantiFERON TB-2G test as a diagnostic method for differentiating active tuberculosis from nontuberculous mycobacteriosis. *Clinical Infectious Disease,* 43:1540-1546.

KOCH, R. 1882. Die aetiologie der tuberculose. *Berlin Klinische Wochenschrift,* 19:221-230.

KOCH, R. 1891. Weitere mittheilungen uber das tuberkulin. *Deutsche Medizinische Wochenschrrift*, 17:1189-1192.

KÖHLER, G. & MILSTEIN, C. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature,* 256:495-497.

KOO, H.C., PARK, Y.H., AHN, J., WATERS, W.R., PALMER, M.V., HAMILTON, M.J., BARRINGTON, G., MOSAAD, A.A., PARK, K.T., JUNG, W.K., HWANG, I.Y., CHO, S.N., SHIN, S.J. & DAVIS, W.C. 2005. Use of rMPB70 protein and ESAT-6 peptide as antigens for comparison of the enzyme-linked immunosorbent, immunochromatographic, and latex bead agglutination assays for serodiagnosis of bovine tuberculosis. *Journal of Clinical Microbiology,* 43:4498-4506.

KREMER, K., VAN SOOLINGEN, D., FROTHINGHAM, R., HAAS, W.H., HERMANS, P.W.M., MARTIN, C., PALITTAPONARNPIM, P., PLIKAYTIS, B.B., RILEY, L.W., YAKRUS, M.A., MUSSER, J.M. & VAN EMBDEN, J.D.A. 1999. Comparison of methods based on different molecular epidemiological markers for typing of *Mycobacterium tuberculosis* complex starins: interlaboratory study of dsicriminator power and reproducibility. *Journal of Clinical Microbiology*, 37:2607-2618.

LACASSE, C., TERIO, K., KINSEL, M.J., FARINA, L.L., TRAVIS, D.A., GREENWALD, R., LYASHCHENKO, K.P., MILLER, M. & GAMBLE, K.C. 2007. Two cases of atypical

mycobacteriosis caused by *Mycobacterium szulgai* associated with mortality in captive African elephants (*Loxodonta africanum*). *Journal of Zoo and Wildlife Medicine*, 38:101-107.

LE FLECHE, P., FABRE, M., DENOEUD, F., KOECK, J.L. & VERGNAUD, G. 2002. High resolution, on-line identification of strains from the *Mycobacterium tuberculosis* complex based on tandem repeat typing. *BMC Microbiology*, 2:37-49.

LEENAARS, M. & HENDRIKSEN, C.F. 2005. Critical steps in the production of polyclonal and monoclonal antibodies: evaluation and recommendations. *Institute of Laboratory Animal Resources Journal,* 46:269-279.

LESELLIER, S., CORNER, L., COSTELLO, E., SLEEMAN, P., LYASHCHENKO, K., GREENWALD, R., ESFANDIARI, J., SINGH, M., HEWINSON, R.G., CHAMBERS, M. & GORMLEY, E. 2008. Antigen specific immunological responses of badgers (*Meles meles*) experimentally infected with *Mycobacterium bovis*. *Veterinary Immunology and Immunopathology,* 122:35-45.

LEWERIN, S.S., OLSSON, S.L., ELD, K., ROKEN, B., GHEBREMICHAEL, S., KOIVULA, T., KALLENIUS, G. & BOLSKE, G. 2005. Outbreak of *Mycobacterium tuberculosis* infection among captive Asian elephants in a Swedish zoo. *Veterinary Record,* 156:171-175.

LIÉBANA, E., ARANAZ, A., ALDWELL, F.E., MCNAIR, J., NEILL, S.D., SMYTH, A.J. & POLLOCK, J.M. 2000. Cellular interactions in bovine tuberculosis: release of active mycobacteria from infected macrophages by antigen-stimulated T cells. *Immunology*, 99:23-29.

LITMAN, G.W., RAST, J.P., SHAMBLOTT, M.J., HAIRE, R.N., HULST, M., ROESS, W., LITMAN, R.T., HINDS-FREY, K.R., ZILCH, A. & AMEMIYA, C.T. 1993. Phylogenetic diversification of immunoglobulin genes and the antibody repertoire. *Molecular Biology and Evolution,* 10:60-72.

LIU, S., GUO, S., WANG, C., SHAO, M., ZHANG, X., GUO, Y. & GONG, Q. 2007. A novel fusion protein-based indirect enzyme-linked immunosorbent assay for the detection of bovine tuberculosis. *Tuberculosis,* 87:212-217.

LUTZE-WALLACE, C., TURCOTTE, C., STEVENSON, D.A., ELKIN, B., KOLLER-JONES, M., NISHI, J. & WOBESER, G. 2006. Isolation of *Mycobacterium bovis* from a wood bison in a wildlife conservation project in the Northwest Territories. *Canadian Veterinary Journal,* 47:317-318.

LYASHCHENKO, K.P., POLLOCK, J.M., COLANGELI, R. & GENNARO, M.L. 1998. Diversity of antigen recognition by serum antibodies in experimental bovine tuberculosis. *Infection and Immunity*, 66:5344-53-49.

LYASHCHENKO, K.P., SINGH, M., COLANGELI, R. & GENNARO, M.L. 2000. A multi-antigen print immunoassay for the development of serological diagnosis of infectious diseases. *Journal of Immunological Methods,* 242:91-100.

LYASHCHENKO, K.P., GREENWALD, R., ESFANDIARI, J., OLSEN, J.H., BALL, R., DUMONCEAUX, G., DUNKER, F., BUCKLEY, C., RICHARD, M., MURRAY, S., PAYEUR, J.B., ANDERSEN, P., POLLOCK, J.M., MIKOTA, S., MILLER, M., SOFRANKO, D. & WATERS, W.R. 2006. Tuberculosis in elephants: antibody responses to defined antigens of *Mycobacterium tuberculosis*, potential for early diagnosis, and monitoring of treatment. *Clinical and Vaccine Immunology,* 13:722-732.

LYASHCHENKO, K.P., GREENWALD, R., ESFANDIARI, J., CHAMBERS, M.A., VICENTE, J., GORTAZAR, C., SANTOS, N., CORREIA-NEVES, M., BUDDLE, B.M., JACKSON, R., O'BRIEN, D.J., SCHMITT, S., PALMER, M.V., DELAHAY, R.J. & WATERS, W.R. 2008. Animal-side serologic assay for rapid detection of *Mycobacterium bovis* infection in multiple species of free-ranging wildlife. *Veterinary Microbiology*, 132:283-292.

MANCA, C., LYASHCHENKO, K., WIKER, H.G., USAI, D., COLANGELI, R. & GENNARO, M.L. 1997. Molecular cloning, purification, and serological characterization of

MPT63, a novel antigen secreted by *Mycobacterium tuberculosis. Infection and Immunity,* 65:16-23.

MANN, P.C., BUSH, M., JANSSEN, D.L., FRANK, E.S. & MONTALI, R.J. 1981. Clinicopathologic correlations of tuberculosis in large zoo mammals. *Journal of the American Veterinary Medical Association,* 179:1123-1129.

MANUEL, O. & KUMAR, D. 2008. QuantiFERON-TB Gold assay for the diagnosis of latent tuberculosis infection. *Expert Review of Molecular Diagnostics,* 8:247-256.

MAWAK, J., GOMWALK, N., BELLO, C. & KANDAKAI-OLUKEMI, Y. 2006. Human pulmonary infections with bovine and environment (atypical) mycobacteria in Jos, Nigeria. *Ghana Medical Journal,* 40:132-136.

MCCRINDLE, C.M.E. & MICHEL, A.L. 2006. Pathobiologics International: Status for controlling bovine tuberculosis in Africa. http://www.pathobiologics.org/ivphc/ref/MCCRINDLE_SHANGAI_2006.pdf

MCINERNEY, J., SMALL, K.J. & CALEY, P. 1995. Prevalence of *Mycobacterium bovis* infection in feral pigs in the Northern Territory. *Australian Veterinary Journal,* 72:448-451.

MCNAIR, J., WELSH, M.D. & POLLOCK, J.M. 2007. The immunology of bovine tuberculosis and progression toward improved disease control strategies. *Vaccine,* 25:5504-5511.

MICHALAK, K., AUSTIN, C., DIESEL, S., BACON, M.J., ZIMMERMAN, P. & MASLOW, J.N. 1998. *Mycobacterium tuberculosis* infection as a zoonotic disease: transmission between humans and elephants. *Emerging Infectious Diseases,* 4:283-287.

MICHEL, A.L. 2002. Implications of tuberculosis in African wildlife and livestock. *Annals of the New York Academy of Sciences,* 969:251-255.

MICHEL, A.L., BENGIS, R.G., KEET, D.F., HOFMEYR, M., KLERK, L.M.D., CROSS, P.C., JOLLES, A.E., COOPER, D., WHYTE, I.J., BUSS, P. & GODFROID, J. 2006.

Wildlife tuberculosis in South African conservation areas: Implications and challenges. *Veterinary Microbiology,* 112:91-100

MICHEL, A.L., COETZEE, M.L., KEET, D.F., MARE, L., WARREN, R., COOPER, D., BENGIS, R.G., KREMER, K. & VAN HELDEN, P. 2009. Molecular epidemiology of *Mycobacterium bovis* isolates from free-ranging wildlife in South African game reserves. *Veterinary Microbiology,* 133:335-343.

MIKOTA, S.K., LARSEN, R.S. & MONTALI, R.J. 2000. Tuberculosis in elephants in North America. *Zoo Biology,* 19:393-403.

MIKOTA, S.K., MILLER, M., DUMONCEAUX, G., MASLOW, J.N., TERREL, S.P., SOFRANKO, D. & LYASHCHENKO, K.P. 2008. National Tuberculosis Working Group for Zoo & Wildlife Species. *Guidelines for the control of tuberculosis in elephants.* Washington, D.C. United States Department of Agriculture, Animal and Plant Health InspectionService, Washington, D.C. [Online] http://www.elephantcare.org/protodoc

MILLER, J.M., JENNY, A.L. & PAYEUR, J.B. 2002. Polymerase chain reaction detection of *Mycobacterium tuberculosis* complex and *Mycobacterium avium* organisms in formalin-fixed tissues from culture-negative ruminants. *Veterinary Microbiology,* 87:15-23.

MOLLER, T., RÖKEN, B.O., PETERSSON, L., VITAUD, C. & LYASCHSHENKO, K. 2005. Preliminary results of a new serological test for detection of TB-infection (*Mycobacterium tuberculosis*) in elephants (*Elephas maximus* and *Loxodonta africanum*) - Swedish case studies. *Proceedings of the Institute for Zoo and Wildlife Research,* Berlin: 173-181.

MONAGHAN, M.L., DOHERTY, M.L., COLLINS, J.D., KAZDA, J.F. & QUINN, P.J. 1994. The tuberculin test. *Veterinary Microbiology,* 40:111-124.

MONTALI, R.J., MIKOTA, S.K. & CHENG, L.I. 2001. *Mycobacterium tuberculosis* in zoo and wildlife species. *Revue Scientifique et Technique,* 20:291-303.

NARAIN, J.P. & LO, Y.R. 2004. Epidemiology of HIV-TB in Asia. *Indian Journal of Medical Research,* 120:277-289.

NARAYANA, R.S.N. 1925. A case of tuberculosis in an elephant. *Journal of Comparative Pathology and Therapeutics,* 38:96-97.

NEILL, S.D., CASSIDY, J., HANNA, J., MACKIE, D.P., POLLOCK, J.M., CLEMENTS, A., WALTON, E. & BRYSON, D.G. 1994. Detection of *Mycobacterium bovis* infection in skin test-negative cattle with an assay for bovine interferon-gamma. *Veterinary Record,* 135:134-135.

NIENHAUS, A., SCHABLON, A. & DIEL, R. 2008. Interferon-gamma release assay for the diagnosis of latent TB infection-analysis of discordant results, when compared to the tuberculin skin test. *PLoS One,* 3:e2665.

NISHI, J.S., SHURY, T. & ELKIN, B.T. 2006. Wildlife reservoirs for bovine tuberculosis (*Mycobacterium bovis*) in Canada: strategies for management and research. *Veterinary Microbiology,* 112:325-338.

NOLAN, A. & WILESMITH, J.W. 1994. Tuberculosis in badgers (*Meles meles*). *Veterinary Microbiology*, 40:179-191.

O'BRIEN, D.J., SCHMITT, S.M., FIERKE, J.S., HOGLE, S.A., WINTERSTEIN, S.R., COOLEY, T.M., MORITZ, W.E., DIEGEL, K.L., FITZGERALD, S.D., BERRY, D.E. & KANEENE, J.B. 2002. Epidemiology of *Mycobacterium bovis* in free-ranging white-tailed deer, Michigan, USA, 1995-2000. *Preventive Veterinary Medicine*, 54:47-63.

OCEPEK, M., PATE, M., ZOLNIR-DOVC, M. & POLJAK, M. 2005. Transmission of *Mycobacterium tuberculosis* from human to cattle. *Journal of Clinical Microbiology,* 43:3555-3557.

OH, P., GRANICH, R., SCOTT, J., SUN, B., JOSEPH, M., STRINGFIELD, C., THISDELL, S., STALEY, J., WORKMAN-MALCOLM, D., BORENSTEIN, L., LEHNKERING, E., RYAN, P., SOUKUP, J., NITTA, A. & FLOOD, J. 2002. Human exposure following

Mycobacterium tuberculosis infection of multiple animal species in a metropolitan zoo. *Emerging Infectious Diseases*, 8:1290-1293.

OLOYA, J., OPUDA-ASIBO, J., KAZWALA, R., DEMELASH, A.B., SKJERVE, E., LUND, A., JOHANSEN, T.B. & DJONNE, B. 2008. Mycobacteria causing human cervical lymphadenitis in pastoral communities in the Karamoja region of Uganda. *Epidemiology and Infection,* 136:636-643.

O'REILLY, L.M. & DABORN, C.J. 1995. The epidemiology of *Mycobacterium bovis* infections in animals and man: A review. *Tubercle and Lung Disease,* 76:1-46.

OZDEMIR, D., ANNAKKAYA, A.N., TARHAN, G., SENCAN, I., CESUR, S., BALBAY, O. & GUCLU, E. 2007. Comparison of the tuberculin skin test and the quantiferon test for latent *Mycobacterium tuberculosis* infections in health care workers in Turkey. *Japanese Journal of Infectious Diseases,* 60:102-105.

PAI, M. & O'BRIEN, R. 2008. New diagnostics for latent and active tuberculosis: state of the art and future prospects. *Seminars in Respiratory and Critical Care Medicine*, 29:560-568.

PARSONS, S.D., GOUS, T.A., WARREN, R.M. & VAN HELDEN, P.D. 2008. Pulmonary *Mycobacterium tuberculosis* (Beijing strain) infection in a stray dog. *Journal of the South African Veterinary Association*, 79:95-98.

PAVLIK, I., AYELE, W.Y. & PARMOVA, I. 2003. *Mycobacterium tuberculosis* in animal populations in animal and human popultions in six Central European countries during 1990-1999. *Veterinární Medicina*, 48:83-89.

PAYEUR, J.B., JARNAGIN, J.L., MARQUARDT, J.G. & WHIPPLE, D.L. 2002. Mycobacterial isolations in captive elephants in the United States. *Annals of the New York Academy of Sciences*, 969:256-258.

PEARL, J.E., SAUNDERS, B., EHLERS, S., ORME, I.M. & COOPER, A.M. 2001. Inflammation and lymphocyte activation during mycobacterial infection in the interferon-gamma-deficient mouse. *Cellular Immunology*, 211:43-50.

PINTO, M.R.M., JAINUDEEN, M.R. & PANABOKKE, R.G. 1973. Tuberculosis in a domesticated Asiatic elephant, *Elephas maximus*. *Veterinary Record,* 93:662-664.

PLACKETT, P., RIPPER, J., CORNER, L.A., SMALL, K., DE WITTE, K., MELVILLE, L., HIDES, S. & WOOD, P.R. 1989. An ELISA for the detection of anergic tuberculous cattle. *Australian Veterinary Journal*, 66:15-19.

POLLOCK, J.M., MCNAIR, J., WELSH, M.D., GIRVIN, R.M., KENNEDY, H.E., MACKIE, D.P. & NEILL, S.D. 2001. Immune responses in bovine tuberculosis. *Tuberculosis*, 81:103-107.

POLLOCK, J.M. & NEILL, S.D. 2002. *Mycobacterium bovis* infection and tuberculosis in cattle. *Veterinary Journal*, 163:115-127.

POLLOCK, J.M., WELSH, M.D. & MCNAIR, J. 2005. Immune responses in bovine tuberculosis: towards new strategies for the diagnosis and control of disease. *Veterinary Immunology and Immunopathology*, 108:37-43.

POTTUMARTHY, S., MORRIS, A.J., HARRISON, A.C. & WELLS, V.C. 1999. Evaluation of the tuberculin gamma interferon assay: potential to replace the Mantoux skin test. *Journal of Clinical Microbiology*, 37:3229-3232.

RAGG, J.R., MOLLER, H. & WALDRUP, K.A. 1995. The prevalence of bovine tuberculosis (*Mycobacterium bovis*) infections in feral populations of cats (*Felis catus*), ferrets (*Mustela furo*) and stoats (*Mustela erminea*) in Otago and Southland, New Zealand. *New Zealand Veterinary Journal*, 43:333-337.

RASTOGI, N., LEGRAND, E. & SOLA, C. 2001. The mycobacteria: an introduction to nomenclature and pathogenesis. *Revue Scientifique et Technique*, 20:21-54.

REED, G. 1957. Genus *Mycobacterium* (species affecting warm-blooded animals except causing leprosy), in *Bergey's manual of determinants, bacteriology*, edited by R.S. Breed, E.G.D. Murray & N.R. Smith. Baltimore: The William and Wilkins. Co.

REGASSA, A., MEDHIN, G. & AMENI, G. 2008. Bovine tuberculosis is more prevalent in cattle owned by farmers with active tuberculosis in central Ethiopia. *Veterinary Journal*, 178:119-125.

RENWICK, A.R., WHITE, P.C. & BENGIS, R.G. 2007. Bovine tuberculosis in southern African wildlife: a multi-species host-pathogen system. *Epidemiology and Infection*, 135:529-540.

REYNAUD, C.A, ANQUEZ, V., GRIMAL, H. & WEILL, J.C. 1987. A hyperconversion mechanism generates the chicken light chain preimmune repertoire. *Cell*, 48:379–88.

RHODES, S.G., PALMER, N., GRAHAM, S.P., BIANCO, A.E., HEWINSON, R.G. & VORDERMEIER, H.M. 2000a. Distinct response kinetics of gamma interferon and interleukin-4 in bovine tuberculosis. *Infection and Immunity*, 68:5393-5400.

RHODES, S.G., GAVIER-WIDEN, D., BUDDLE, B.M., WHELAN, A.O., SINGH, M., HEWINSON, R.G. & VORDERMEIER, H.M. 2000b. Antigen specificity in experimental bovine tuberculosis. *Infection and Immunity*, 68:2573-2578.

RHODES, S.G., GRUFFYDD-JONES, T., GUNN-MOORE, D. & JAHANS, K. 2008a. Interferon-gamma test for feline tuberculosis. *Veterinary Record*, 162:453-455.

RHODES, S.G., GRUFFYDD-JONES, T., GUNN-MOORE, D. & JAHANS, K. 2008b. Adaptation of IFN-gamma ELISA and ELISPOT tests for feline tuberculosis. *Veterinary Immunology and Immunopathology*, 124:379-384.

RORING, S., BRITTAIN, A., BUNSCHOTEN, A.E., HUGHES, M.S., SKUCE, R.A., VAN EMBDEN, J.D.A. & NEILL, S.D. 1998. Spacer oligotyping of *Mycobacterium bovis* isolates compared to typing by restriction fragment length polymorphism using PGRS, DR and IS*6110* probes. *Veterinary Microbiology*, 61:111-120.

RORING, S., SCOTT, A., BRITTAIN, D., WALKER, I., HEWINSON, G., NEILL, S. & SKUCE, R. 2002. Development of variable-number tandem repeat typing of *Mycobacterium*

bovis: comparison of results with those obtained by using existing exact tandem repeats and spoligotyping. *Journal of Clinical Microbiology*, 40:2126-2133.

ROSS, B.C., RAIOS, K., JACKSON, K. & DWYER, B. 1992. Molecular cloning of a highly repeated DNA element from *Mycobacterium tuberculosis* and its use as an epidemiological tool. *Journal of Clinical Microbiology*, 30:942-946.

ROWE, M.T. & DONAGHY, J. 2008. *Mycobacterium bovis*: the importance of milk and dairy products as a cause of human tuberculosis in the UK. A review of taxonomy and culture methods, with particular reference to artisanal cheeses. *International Journal of Dairy Technology*, 61:317.

RUSSEL, M., LOWMAN, H.B. & CLACKSON, T. 1989. Introduction to phage biology and phage display, in *Phage Display: A Pratical Approach*, edited by T. Clackson & H.B. Lowman. Oxford: Oxford University Press: 1-26

SAKULA, A. 1983. Robert Koch: Centenary of the discovery of the tubercle bacillus, 1882. *Canadian Veterinary Journal*, 24:127-131.

SAPATS, S.I., TRINIDAD, L., GOULD, G., HEINE, H.G., VAN DEN BERG, T.P., ETERRADOSSI, N., JACKWOOD, D., PAREDE, L., TOQUIN, D. & IGNJATOVIC, J. 2006. Chicken recombinant antibodies specific for very virulent infectious bursal disease virus. *Archives of Virology*, 151:1551-1566.

SARMA, K.K., BHAWAL, A., YADAV, V.K., SAIKIA, G. & JOGIRAJ, D. 2006. Investigation of tuberculosis in captive Asian elephants of Assam vis-a-vis its cross infections with the handlers. *Intas Polivet,* 7:269-274.

SAUNDERS, G. 1983. Pulmonary *Mycobacterium tuberculosis* infection in a circus elephant. *Journal of the American Veterinary Medical Association,* 183:1311-1312.

SCHEERLINCK, J.Y. & YEN, H. 2005. Veterinary applications of cytokines. *Veterinary Immunology and Immunopathology*, 108:17-22.

SCHMITT, S.M., FITZGERALD, S.D., COOLEY, T.M., BRUNING-FANN, C.S., SULLIVAN, L., BERRY, D., CARLSON, T., MINNIS, R.B., PAYEUR, J.B. & SIKARSKIE, J. 1997. Bovine tuberculosis in free-ranging white-tailed deer from Michigan. *Journal of Wildlife Diseases*, 33:749-758.

SCHRODER, K., HERTZOG, P.J., RAVASI, T. & HUME, D. 2004. Interferon-γ: An overview of signals, mechanisms and functions. *Journal of Leukocyte Biology*, 75:163-189.

SENEVIRATNA, P., WETTIMUNY, S.G. & SENEVIRATNA, D. 1966. Fatal tuberculosis pneumonia in an elephant. *Veterinary Medicine Small Animal Clinician,* 60:129-132.

SHIHUA, W., JIBIN, Z., ZHIPING, Z., YAFENG, Z., RUIFU, Y., JIA, C., YONGCHAO, G., FAN, Y. & XIANEN, Z. 2006. Construction of single chain variable fragment (scFv) and biscFv-alkaline phosphatase fusion protein for detection of *Bacillus anthracis*. *Analytical Chemistry*, 78:997-1004.

SHINGADIA, D. & NOVELLI, V. 2003. Diagnosis and treatment of tuberculosis in children. *Lancet Infectious Diseases*, 3:624-632.

SKUCE, R.A., BRITTAIN, D., HUGHES, M.S., BECK, L-A. & NEILL, S.D. 1994. Genomic fingerprinting of *Mycobacterium bovis* from cattle by restriction fragment length polymorphism analysis. *Journal of Clinical Microbiology*, 32:2387-2392.

SKUCE, R.A., MCCORRY, T.P., MCCARROLL, J.F., RORING, S.M., SCOTT, A.N., BRITTAIN, D., HUGHES, S.L., HEWINSON, R.G. & NEILL, S.D. 2002. Discrimination of *Mycobacterium tuberculosis* complex bacteria using novel VNTR-PCR targets. *Microbiology*, 148:519-528.

SMITH, G.P. 1985. Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science*, 228:1315-1317.

SOLA, C., FILLIOL, I., LEGRAND, E., LESJEAN, S., LOCHT, C., SUPPLY, P. & RASTOGI, N. 2003. Genotyping of the *Mycobacterium tuberculosis* complex using MIRUs:

association with VNTR and spoligotyping for molecular epidemiology and evolutionary genetics. *Journal of Molecular Epidemiology and Evolutionary Genetics Infectious Diseases*, 3:125-133.

SRIVASTAVA, K., CHAUHAN, D.S., GUPTA, P., SINGH, H.B., SHARMA, V.D., YADAV, V.S., SREEKUMARAN, THAKRAL, S.S., DHARAMDHEERAN, J.S., NIGAM, P., PRASAD, H.K. & KATOCH, V.M. 2008. Isolation of *Mycobacterium bovis* & *M. tuberculosis* from cattle of some farms in north India-possible relevance in human health. *Indian Journal of Medical Research*, 128:26-31.

STEINBROOK, R. 2007. Tuberculosis and HIV in India. *New England Journal of Medicine*, 356:1198-1199.

STEINGART, K.R., NG, V., HENRY, M., HOPEWELL, P.C., RAMSAY, A., CUNNINGHAM, J., URBANCZIK, R., PERKINS, M.D., AZIZ, M.A. & PAI, M. 2006. Sputum processing methods to improve the sensitivity of smear microscopy for tuberculosis: a systematic review. *Lancet Infectious Diseases*, 6:664-674.

STEINGART, K.R., HENRY, M., LAAL, S., HOPEWELL, P.C., RAMSAY, A., MENZIES, D., CUNNINGHAM, J., WELDINGH, K. & PAI, M. 2007. A systematic review of commercial serological antibody detection tests for the diagnosis of extrapulmonary tuberculosis. *Postgraduate Medical Journal*, 83:705-712.

STETTER, M.D., MIKOTA, S.K., GUTTER, A.F., MONTERROSO, E.R., DALOVISIO, J.R., DEGRAW, C. & FARLEY, T. 1995. Epizootic of *Mycobacterium bovis* in a zoologic park. *Journal of the American Veterinary Medical Association*, 207:1618-1621.

SULIEMAN, M.S. & HAMID, M.E. 2002. Identification of acid fast bacteria from caseous lesions in cattle in Sudan. *Journal of Veterinary Medicine, Infectious Diseases and Veterinary Public Health*, 49:415-418.

SUPPLY, P., MAZARA, E., LESJEAN, S., VINCENT, V., GICQUEL, B. & LOCHT, C. 2000. Variable human minisatellite-like regions in the *Mycobacterium tuberculosis* genome. *Molecular Microbiology*, 36:762-771.

TEIXEIRA, H.C., ABRAMO, C. & MUNK, M.E. 2007. Immunological diagnosis of tuberculosis: problems and strategies for success. *Jornal Brasileiro de Pneumologia*, 33:323-334.

TERPE, K. 2003. Overview of tag protein fusions: from molecular and biochemical fundamentals to commercial systems. *Applied Microbiology and Biotechnology*, 60:523-533.

THOEN, C.O., STEELE, J.H. & GILSDORF, M.J. 2006. *Mycobacterium bovis* infection in animals and humans, 2nd ed. Massachusetts: Willey-Blackwell Publishing.

THOREL, M.F., KAROUI, C., VARNEROT, A., FLEURY, C. & VINCENT, V. 1998. Isolation of *Mycobacterium bovis* from baboons, leopards and a sea-lion. *Veterinary Research*, 29:207-212.

TOMAN, K. 2004. What are the advantages and disadvantages of fluorescence microscopy? in *Toman's tuberculosis: Case detection, treatment, and monitoring-questions and answers.* edited by T. Frieden. Geneva: World Health Organisation: 31-34

TURINELLI, V., LEDIEU, D., GUILBAUD, L., MARCHAL, T., MAGNOL, J.P. & FOURNEL-FLEURY, C. 2004. *Mycobacterium tuberculosis* infection in a dog from Africa. *Veterinary Journal of Clinical Pathology*, 33:177-181.

URBAIN, A. & DECHAMBRE, E. 1937. TB in an African Elephant. *Bulletin de la Societe de Pathologie Exotique,* 30:834-886.

URBANCZIK, R. 1985. Present position of microscopy and of culture in diagnostic mycobacteriology. *Zentralblatt für Bakteriologie, Mikrobiologie, und Hygiene*, 260:81-87.

VALANDIKAR, S.C. & RAJU, R. 1996. Pulmonary tuberculosis in a black rhinoceros (*Diceros bicornis*) in Mysore Zoo. *Zoos Print*, 3:16-17, www.rhinoresourcecenter.com.

VAN DEUN, A., HOSSAIN, M.A., GUMUSBOGA, M. & RIEDER, H.L. 2008. Ziehl-Neelsen staining: theory and practice. *International Journal of Tuberculosis and Lung Disease*, 12:108-110.

VAN LERBERGHE, W., EVANS, T., RASANATHAN, K. & MECHBAL, A. 2008. *The World Health Report 2008: Primary health care now more than ever*, 1:1-148.

VAN RHIJN, I., GODFROID, J., MICHEL, A. & RUTTEN, V. 2008. Bovine tuberculosis as a model for human tuberculosis: advantages over small animal models. *Microbes and Infection*, 10:711-715.

VAN SOOLINGEN, D. & ARBEIT, R.D. 2001. Dealing with variation in molecular typing of *Mycobacterium tuberculosis*: low-intensity bands and other challenges. *Journal of Medical Microbiology*, 50:749-751.

VORDERMEIER, H.M., CHAMBERS, M.A., COCKLE, P.J., WHELAN, A.O., SIMMONS, J. & HEWINSON, R.G. 2002. Correlation of ESAT-6-specific gamma interferon production with pathology in cattle following *Mycobacterium bovis* BCG vaccination against experimental bovine tuberculosis. *Infection and Immunity*, 70:3026-3032.

WARREN, R.M., GEY VAN PITTIUS, N.C., BARNARD, M., HESSELING, A., ENGELKE, E., DE KOCK, M., GUTIERREZ, M.C., CHEGE, G.K., VICTOR, T.C., HOAL, E.G. & VAN HELDEN, P.D. 2006. Differentiation of *Mycobacterium tuberculosis* complex by PCR amplification of genomic regions of difference. *International Journal of Tuberculosis and Lung Disease*, 10:818-822.

WATERS, W.R., PALMER, M.V., BANNANTINE, J.P., WHIPPLE, D.L., GREENWALD, R., ESFANDIARI, J., ANDERSEN, P., MCNAIR, J., POLLOCK, J.M. & LYASHCHENKO, K.P. 2004. Antigen recognition by serum antibodies in white-tailed deer (*Odocoileus virginianus*) experimentally infected with *Mycobacterium bovis*. *Clinical and Diagnostic Laboratory Immunology*, 11:849-855.

WEDLOCK, D.N., SKINNER, M.A., LISLE, G.W.D. & BUDDLE, B.M. 2002. Control of *Mycobacterium bovis* infections and the risk to human populations. *Microbes and Infection*, 4:471-480.

WHELAN, C., SHURALEV, E., O'KEEFFE, G., HYLAND, P., KWOK, H.F., SNODDY, P., O'BRIEN, A., CONNOLLY, M., QUINN, P., GROLL, M., WATTERSON, T., CALL,

S., KENNY, K., DUIGNAN, A., HAMILTON, M.J., BUDDLE, B.M., JOHNSTON, J.A., DAVIS, W.C., OLWILL, S.A. & CLARKE J. 2008. Multiplex immunoassay for serological diagnosis of *Mycobacterium bovis* infection in cattle. *Clinical and Vaccine Immunology*, 15:1834-1838.

WHIPPLE, D.L., MEYER, R.M., BERRY, D.F., JARNAGIN, J.L. & PAYEUR, J.B. 1997. Molecular epidemiology of tuberculosis in wild white-tailed deer in Michigan and elephants. *Proceedings of the 101st and 1st Annual Meeting of the United States Animal Health Association,* USA: 543-546.

WHITE, P.C., BOHM, M., MARION, G. & Hutching, M.R. 2008. Control of bovine tuberculosis n British livestock: there is no "silver bullet". *Trends in Microbiology*, 9:420-427.

WIDDISON, S., WATSON, M., PIERCY, J., HOWARD, C. & COFFEY, T.J. 2008. Granulocyte chemotactic properties of *Mycobacterium tuberculosis* versus *M. bovis*-infected bovine alveolar macrophages. *Molecular Immunology*, 45:740-749.

WIKER, H.G., LYASHCHENKO, K.P., AKSOY, A.M., LIGHTBODY, K.A., POLLOCK, J.M., KOMISSARENKO, S.V., BOBROVNIK, S.O., KOLESNIKOVA, I.N., MYKHALSKY, L.O., GENNARO, M.L. & HARBOE, M. 1998. Immunochemical characterization of the MPB70/80 and MPB83 proteins of *Mycobacterium bovis. Infection and Immunity*, 66:1445-1452.

WINTER, G., GRIFFITHS, A.D., HAWKINS, R.E. & HOOGENBOOM, H.R. 1994. Making antibodies by phage display technology. *Annual Review of Immunology*, 12:433-455.

WOOD, P.R., ROTHEL, J.S., MCWATERS, P.G.D. & JONES, S.L. 1990. Production and characterization of monoclonal antibodies specific for bovine gamma-interferon. *Veterinary Immunology and Immunopathology*, 25:37-46.

WOOD, P.R., CORNER, L.A., ROTHEL, J.S., BALDOCK, C., JONES, S.L., COUSINS, D.B., MCCORMICK, B.S., FRANCIS, B.R., CREEPER, J. & TWEDDLE, N.E. 1991. Field

comparison of the interferon-gamma assay and the intradermal tuberculin test for the diagnosis of bovine tuberculosis. *Australian Veterinary Journal*, 68:286-290.

WOOD, P.R. & ROTHEL, J.S. 1994. *In vitro* immunodiagnostic assays for bovine tuberculosis. *Veterinary Microbiology*, 40:125-135.

WOOD, P.R. & JONES, S.L. 2001. Bovigam™: an *in vitro* cellular diagnostic test for bovine tuberculosis. *Tuberculosis*, 81:147-155.

WOODFORD, M.H. 1982. Tuberculosis in wildlife in the Ruwenzori National Park, Uganda (Part II). *Tropical Animal Health and Production*, 14:155-160.

WORLD HEALTH ORGANIZATION. 2008. *Tuberculosis Fact Sheet*, 2.

YAMANAKA, H.I., INOUE, T. & IKEDA-TANAKA, O. 1996. Chicken monoclonal antibody isolated by a phage display system. *Journal of Immunology*, 157:1156-1162.

Chapter 2

Cloning, sequencing and expression of white rhinoceros (*Ceratotherium simum*) interferon-gamma and the production of white rhinoceros interferon-gamma specific antibodies

Abstract

Bovine tuberculosis (BTB) is endemic in African buffaloes (*Syncerus caffer*) in the Kruger National Park (KNP). In addition to buffaloes, *Mycobacterium bovis* has been found in at least 14 other mammalian species in South Africa, including kudus (*Tragelaphus strepsiceros*), chacma baboons (*Papio ursinus*) and lions (*Panthera leo*). This has raised concern about the spillover into other potentially susceptible species such rhinoceroses, thus jeopardising breeding and relocation projects aiming at the conservation of biodiversity. Hence procedures to screen for and diagnose BTB in the black rhinoceros (*Diceros bicornis*) and white rhinoceros (*Ceratotherium simum*) need to be in place. The interferon-gamma (IFN-γ) assay is used as a routine diagnostic tool to determine infection of cattle and, recently, African buffaloes, with *M. bovis* and other mycobacteria. The aim of this study was to develop reagents to set up a rhinoceros IFN-γ (RhIFN-γ) assay. The white RhIFN-γ gene was cloned, sequenced and expressed as a mature protein. Amino acid sequence analysis revealed that RhIFN-γ shares a homology of 90% with equine IFN-γ. Monoclonal antibodies, as well as polyclonal chicken antibodies (Yolk Immunoglobulin-IgY) with specificity for recombinant RhIFN-γ, were produced. Using monoclonal 1H11 as a capture antibody and polyclonal IgY for detection, it was shown that recombinant as well as native white RhIFN-γ was recognised. This preliminary IFN-γ enzyme-linked immunosorbent assay has the potential to be developed into a diagnostic assay for *M. bovis* infection in rhinoceroses.

2.1 Introduction

Mycobacterium bovis is the causative agent of bovine tuberculosis (BTB) and has mostly presented itself as a problem in cattle (O'Reilly & Daborn 1995; Pollock, Rodgers, Welsh & McNair 2006) and related species including goats (Cousins, Francis, Casey & Mayberry 1993; Liébana, Aranaz, Urquia *et al.* 1998) worldwide. More recently *M. bovis* has also been found to infect wildlife species such as the lion (*Panthera leo*), kudu (*Tragelaphus strepsiceros*), African buffalo (*Syncerus caffer*) and chacma baboon (*Papio ursinus*) in South Africa (Michel, Bengis, Keet *et al.* 2006), European badger (*Meles meles*) (Cheeseman, Wilesmith & Stuart 1989) in the United Kingdom and the brushtail possum (*Trichosurus vulpecula*) in New Zealand (Buddle, Skinner & Chambers 2000). In the Kruger National Park (KNP) and elsewhere, potential spillover into other species, such as the rhinoceros, for which currently no validated *ante-mortem* (indirect) diagnostic tools exist, may jeopardise breeding and relocation projects aimed towards the conservation of biodiversity. The development of practical and reliable procedures to diagnose BTB in black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceroses have therefore been identified as a priority area for research by conservation bodies.

Although BTB has to date not been diagnosed in pachyderms in South Africa, it is of utmost importance to be able to provide an additional guarantee on the TB-free status of these animals and to provide conservation bodies with an early warning system should BTB enter the rhinoceros population. While tests traditionally available for diagnosing BTB include microscopic and bacterial culture techniques, as well as tuberculin skin tests (TST) (Wood, Rothel, McWaters & Jones 1990b; Monaghan, Doherty, Collins *et al.* 1994), these are of little value for screening purposes in pachyderms. Culture techniques are the most reliable and specific, but have the drawback that they generally require post-mortem specimens, and results are obtained only after 6 to 8 weeks. Specificity and sensitivity of TST have been determined mostly for domestic animals, especially cattle (Monaghan *et al.* 1994), but diagnosing *M. bovis* infection in wildlife is proving to be a challenge.

In pachyderms, such as the rhinoceros and elephant, TST are not practical both due to difficulties in defining suitable injection sites and the fact that these reactions have to be read after approximately 72 hours, which necessitates the recapture of the animals. As an alternative to TST, interferon-gamma (IFN-γ) assays, for example the Bovigam™ test

(Wood, Corner & Plackett 1990a; Wood *et al.* 1990b; Wood & Jones 2001), have been used during the last decade to determine *M. bovis*-specific immune responses in ruminants. These tests consist of antibody-based capture enzyme-linked immunosorbent assays (ELISA) that will detect IFN-γ produced by specific T-cells after incubation of heparinized blood with *M. bovis* antigens. Infected animals are identified by their high IFN-γ responses, due to *M. bovis* antigen specific T-cells induced by the mycobacterial infection.

In this chapter, the first steps in developing an IFN-γ-based capture ELISA for the detection of *M. bovis* infection in the white rhinoceros are described. Furthermore, the first evaluation of the specificity of the test in known TB-free rhinoceroses is reported. The TB-free status of these white rhinoceroses was determined based mainly on the epidemiological evidence of the absence of an *M. bovis* infection in rhinoceroses in South Africa. Indeed to date, after more than 10 years of translocation programmes, no single case of *M. bovis*- or *M. tuberculosis*-infected rhinoceros has been documented (Michel *et al.* 2006).

The set up of the test includes the cloning, sequencing and the expression of the white rhinoceros IFN-γ (RhIFN-γ) gene and the production of RhIFN-γ specific monoclonal and polyclonal antibodies. Thus, the IFN-γ produced *in vitro* by antigen stimulation of sensitized T-lymphocytes can be measured to serve as a sensitive and specific indicator of *M. bovis* exposure. Development of this assay could ultimately yield a vital tool for detecting *M. bovis* infection in rhinoceroses prior to the development of clinical signs.

2.2 Materials and Methods

2.2.1 Cloning and sequencing of white RhIFN-γ

Blood from an adult white rhinoceros was collected in ethylenediaminetetraacetic acid (EDTA) Vacutainer™ tubes. Peripheral blood mononuclear cells (PBMC) 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 in RPMI-1640 medium supplemented with L-glutamine (Sigma, R8758) and 10% heat inactivated foetal calf serum (FCS). To induce IFN-γ production, purified mononuclear cells (1 X 10^6 cells/m*l* in wells of a 24-well plate) were stimulated with 5 μg/m*l*

concanavalin A (Con A) (Sigma, C2010-100mg) for 18 to 24 h at 37°C in a 5% CO_2 incubator.

Total RNA was purified from stimulated lymphocytes using Trizol reagent (Gibco BRL, Life Technologies, 15596-018) and 1 µg of total RNA was subjected to first strand cDNA synthesis using reverse transcriptase Rnase H SuperscriptII™ (Gibco BRL, Life Technologies, 18064-014) and oligo-$(dT)_{12-18}$ as a primer for RT-PCR. The cDNA produced in this way was used as a template for a polymerase chain reaction (PCR) using *Pwo* DNA polymerase (Roche, 1644 947) according to the manufacturer's instructions. PCR primers (5'-endprimer sequence: 5'-GCCGCGCGGGAGCCAGGCCGCGTTTTTTAAAGAAATAG -3' and 3'-end primer sequence: 5'-GCGGCGGCGGGAATTCAAATATTGCAGGCAGG-3') used were designed to amplify the part of the white RhIFN-γ gene that encodes the mature protein (without signal sequence). The sequence of the primers was based on the equine IFN-γ (EqIFN-γ) gene (Genbank Accession Number - D28520), because this species has a close phylogenetic relationship with rhinoceroses.

A gradient PCR was performed for 35 cycles using a BioRad Thermal iCycler. One cycle consisted of: DNA denaturation at 95°C for 30 s, primer annealing at 56°C, 58°C, 61°C or 64.7°C (depending on the position of the PCR tube in the gradient) for 45 s and primer extension at 72°C for 60 s. The underlined sequences in the primers above are not part of the IFN-γ sequence, but were included as annealing sites for a second PCR performed with the forward primer GW2- F (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTGGTGCCG CGCGGGAGC-3') and the reverse primer GW-R (5'-GGGGACCACTTTGTACAAGAA AGCTGGGTGCGGCGGCGGG-3'). This second PCR, introduced the *attB1* and *attB2* sites, which enabled subsequent Gateway® (GW) cloning (Invitrogen). The conditions for this second PCR were similar to those described for the first PCR, apart from the annealing temperature that was set at 56 °C. Following DNA electrophoresis, the PCR product was harvested from the low melting point agarose gel and inserted into the vector pDONR201 (Invitrogen) by the BP GW reaction (Invitrogen – Gateway™ BP Enzyme Mix, 11789-013) performed according to the manufacturer's instructions.

After transformation of *Escherichia coli* strain DH5α, plasmid DNA was purified from selected colonies and sequenced to check the cloned fragment. Subsequently, the IFN-γ gene

was subcloned into the expression vector pET15bGW by the LR GW reaction (Invitrogen, Life Technologies - Gateway™ LR Clonase Mix, 11791-091). The resulting expression vector was designated pET15-RhIFN-γ. Vector pET15bGW is a derivative of pET15b (Novagen) that was adapted for GW cloning (Invitrogen) by ligation of the GW cassette containing *XbaI-HindIII* fragment of pDEST17 (Invitrogen) into the corresponding sites of pET15b. The resulting plasmid was purified from liquid culture from ampicillin- and chloroamphenicol-resistant colonies obtained after transformation of *E. coli* DB3.1 (Invitrogen).

To determine the 5'-end of the complete coding part of the RhIFN-γ-gene (including the sequence encoding the signal sequence), this end was cloned separately. First, it was amplified under the PCR using *KOD* hotstart polymerase (Novogen) according to the manufacturer's instructions. The white rhinoceros cDNA described above was used as a template. The forward primer F3 (3'-CCTGATCAGCTTAGTACAGAAGTGA-5') was based on the published EqIFN-γ sequence upstream of the start codon and the reverse primer R3 (5'-TCCTCTTTCCAGTTCTTCAAGATATC-3') based on the RhIFN-γ gene sequence encoding the mature protein that had been cloned as described above. A gradient PCR, with annealing temperatures between 65°C and 55°C, resulted in a weak PCR band of the expected size for the reaction performed at 57°C. To obtain enough material for cloning, additional PCR rounds were required. To avoid amplification of the smear of unspecific PCR bands present, a half-nested PCR was performed on the original PCR product to increase the specificity. For this half-nested PCR, the same forward primer (F3) was used, because the 5'-end sequence was not known, and a reverse primer R7 (5'-TCATTCATCACTTTGATGAGTTCA-3') which anneals to a sequence upstream of primer R3 (internal reverse primer) was used. For the first 20 cycles, the annealing temperature was reduced by 0.5°C for each cycle (touch down PCR), starting at 65°C. The following 20 cycles were performed at a constant annealing temperature of 55°C. The resulting PCR product was analysed on a 1.5% low melting point agarose gel and a dominant PCR band of the expected size (approximately 450 bps) was cut out. After melting the agarose at 65°C, this PCR band was used as a template for another half-nested PCR, using the same forward primer as a reverse primer R4 (5'-CCTCTTTCCAGTTCTTCAAGATATC-3') which anneals to a sequence upstream of primer R7. The PCR conditions were identical to those described for the previous half-nested PCR, except that only ten cycles were performed, once the touch

down PCR reached an annealing temperature of 55°C. The obtained PCR product was cloned into the vector pCR4, using the "Zero-blunt-TOPO-PCR-cloning-kit" (Invitrogen) according to the manufacturer's instructions. After transformation of *E. coli* strain DH5α, plasmid DNA was purified from selected colonies and sequenced to verify the cloned fragment. The successive rounds of this half-nested PCR approach were performed to obtain enough material to allow efficient cloning and to increase the specificity.

2.2.2 Expression and purification of recombinant white RhIFN-γ

Vector pET15-RhIFN-γ was used to transform *E. coli* BL21-codon$^{+®}$ (DE3)-RIL competent cells (Stratagene, 230245). A single ampicillin- and chloramphenicol-resistant colony was spread on a Luria Broth (LB) agar plate containing ampicillin, chloramphenicol and 1% ($^{w}/_{v}$) glucose. The glucose was added to repress expression of the recombinant protein. After overnight incubation at 37°C, the bacteria were harvested from the plate with an inoculation loop and resuspended in 10 m*l* LB medium. After resuspension the bacteria were transferred to 500 m*l* LB medium containing ampicillin and chloramphenicol and incubated at 37°C, with shaking, until the optical density at 600 nm (OD_{600}) reached 0.6-0.9. Gene expression was induced with 1 mM isopropylthio-β-D-galactosidase (IPTG) and incubation continued for 4 h, after which cells were harvested by centrifugation at 5 000xg for 15 min. Cell pellets were resuspended in 40 m*l* of Buffer B (20 mM Tris HCl pH8, 500 mM NaCl) containing 0.1 mg/m*l* of lysozyme. The cell suspension was transferred to a 50 m*l* tube and was incubated at room temperature under rotation for 30 min. This was followed by addition of 5 m*l* Buffer C (100 mM DTT, 50 mM EDTA, 10% Triton X 100). The contents of the tube were mixed by inverting the tubes several times and the lysate was prepared by repeating (three times) alternate freezing and thawing steps at –20°C and room temperature respectively. After the last freeze / thaw step, 1 500 units of benzonase (Novagen) and 1.5 m*l* of a 0.5 M $MgCl_2$ solution were added and the mixture incubated at room temperature for 30 min to break down the DNA and reduce the viscosity. The total protein lysate was centrifuged at 5 000xg for 15 min. The pellet, containing the IFN-γ inclusion bodies, was washed with Buffer B containing 1% Triton X 100. After a final centrifugation step (5 000xg for 15 min) the pellet was dissolved in 10 M urea prepared freshly in Buffer B containing 20 mM imidazole at room temperature. Any remaining insoluble material was removed by centrifugation at 5 000xg for 20 min at room temperature. The hexa-histidine tagged recombinant IFN-γ was purified by immobilized metal affinity chomatography (IMAC) on chelating sepharose fast flow

(Amersham-Biosciences, 17-0575-02) charged with Ni^{2+} according to the manufacturer's instructions. After equilibration of the column with Buffer B containing 20 mM imidazole and 8 M urea, the solved inclusion bodies were applied to the column. The bound protein was washed with ten column volumes of Buffer B containing 20 mM imidazole and 8 M urea. The protein was refolded on the column by fast replacement (rapid switching) of wash buffer with two column volumes of refolding buffer (50 mM Tris HCl pH8, 2 mM oxidized gluthathione, 0.22 mM reduced gluthathione, 1 M NDSB201 [Merck], 0.5 M L-Arginine) and incubated at 4°C for 40 h. Refolding buffer was discarded and the column washed twice with one column volume PBS containing 1 M NDSB201. The refolded IFN-γ was eluted with a total of five column volumes of PBS containing 50 mM EDTA. The protein was dialysed against 1 X PBS and subsequently centrifuged (5 000xg for 30 min) to remove any protein that had precipitated during the dialysis. The protein solution was mixed with an equal volume of glycerine and then sterile-filtered using a 0.2 μM filter and stored at -20°C. Samples were taken during the whole purification process for analysis by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

2.2.3 White RhIFN-γ specific poly- and monoclonal antibodies

Mice were immunized with recombinant RhIFN-γ (rRhIFN-γ) according to routine procedures using Specol, an oil-based adjuvant (Bokhout, van Gaalen & van der Heijden 1981; Hall, Molitor, Joo & Pijoan 1989; Boersma, Bogaerts, Bianchi & Claassen 1992) and boosted on three occasions. When desired serum antibody titres were achieved, as determined by direct rRhIFN-γ ELISA, mice spleen cells were fused to SP2/0 cells to obtain hybridomas and plated in 96-well tissue culture plates (Köhler & Milstein 1975). Supernatants from wells containing colonies of hybridoma cells were tested in the ELISA for the presence of rRhIFN-γ specific monoclonal antibodies. Positive colonies were subcloned by FACSVantage (Becton Dickson) single cell sorting, based on forward / sideward scatter characteristics, and tested again.

For the production of polyclonal antibodies, chickens were immunized intramuscularly using Specol as an adjuvant, and boosted on a regular basis to maintain antibody titres. Eggs were collected for up to 1 year following the first booster immunization and stored at 4°C till further processing. Finally, antibodies were purified from the egg yolk by the "water dilution method" followed by ammonium sulphate precipitation according to the procedure described

by Hansen, Scoble, Hanson & Hoogenraad (1998). After extensive dialysis against PBS, poly- and monoclonal antibodies were sterile filtered using a 0.2 μM filter, aliquoted and stored at 4°C until use. The immunization protocols were approved by the Animal Ethics Committee of the Faculty of Veterinary Medicine, Utrecht University.

2.2.4 Screening of hybridomas for antibody production by indirect ELISA

Fifty microlitres per well of the rRhIFN-γ protein diluted to 1 μg/m*l* in carbonate buffer (0.1 M, pH9.6) were used to coat 96-well Costar high binding ELISA plates overnight at 4°C. After removal of the coating buffer, the plates were blocked with 100 μ*l*/well of 2% fat-free milk powder (Protifar) in PBS for 1 h at 37°C. Plates were then washed five times using tap water with 0.1% Tween 20 using an ELISA plate washer. Fifty microlitres of hybridoma supernatants diluted 1:1 with 2% Protifar in PBS containing 0.1% Tween 20, were added to the wells. After 1 h at 37°C and five additional washings, goat anti-mouse IgG (1:2000) horse radish peroxidase (HRP) conjugate (Boehinger Mannheim, 1047523) in Protifar in PBS containing 0.1% Tween 20 was added and plates were incubated for 1 h at 37°C. After five additional washes, ABTS (5 mg/m*l*) (Roche, 1112597) was used as the substrate for the colour reaction. After 30 min at room temperature plates were read spectrophotometrically (BioRad) at 492 nm.

2.2.5 Native IFN-γ

Blood was collected in EDTA Vacutainer™ tubes from three TB-free white rhinoceroses. Purified PBMCs were isolated as described above. To induce IFN-γ production, purified PBMCs (1 X 10^6 cells/m*l* in wells of a 24-well plate) were stimulated with 10 μg/m*l* Con A (Sigma, C2010-100mg) at 37°C in a 5% CO_2 incubator. A control sample was included consisting of PBMC cultured without mitogen. After 18 to 24 h incubation, cell cultures were collected and centrifuged at 3 200 rpm for 10 min and the supernatant harvested. Production of IFN-γ was analysed in the capture ELISA described below.

2.2.6 Prototype capture ELISA for detection of native white RhIFN-γ

Microwell™ polysorb ELISA plates (Nunc, C96 446140) were coated with 50 μ*l* of monoclonal antibody 1H11 at 1 μg/m*l* and incubated overnight at 4°C. Wells were blocked

with 100 µ*l* block buffer (Protifar in PBS) and incubated at 37°C for 1 h. The plates were washed with wash buffer (H_2O/0.1% Tween 20) five times. As a positive control white rRhIFN-γ was diluted in PBS to 1 µg/m*l* and tested in duplicate. Undiluted supernatants (50 µ*l*) collected from overnight stimulated PBMCs were added to the remainder of the wells. After the incubation the wells were washed five times with wash buffer and incubated with 50 µ*l* polyclonal antibodies to white RhIFN-γ (chicken IgY 700 µg/m*l*, 1:100 dilution in block buffer) per well. After 1 h the plates were washed five times with wash buffer and rabbit polyclonal to chicken IgY H&L (HRP) (Abcam, ab6753) antibody was added (1:3000 dilution). The wash step was repeated and the addition of *ortho*-phenylenediamine (OPD) (Sigma, P3804) substrate followed. The reaction was stopped after 20 min with 50 µ*l* of 2 N H_2SO_4 and the absorbance was read 10 min later at 492 nm.

2.3 Results

2.3.1 Cloning and sequencing of the white RhIFN-γ gene

Initially the part of the IFN-γ gene encoding the mature protein was amplified by reverse transcriptase-PCR (RT-PCR) using primers that were based on the EqIFN-γ sequence. A single PCR band was obtained (results not shown) that was cloned into the GW vector pDONR201. Sequencing demonstrated strong homology of the cloned PCR fragment with the EqIFN-γ gene. The RhIFN-γ gene was subsequently cloned into an *E. coli* expression vector (pET15b-GW). To determine the total coding sequence of the RhIFN-γ gene, the missing 5'-end was cloned separately, using a forward primer that was based on the equine 5'-end IFN-γ sequence and reverse primers based on the cloned sequence encoding the mature part of white RhIFN-γ. The complete coding sequence was composed of this 5'-end sequence and the previously determined sequence encoding the mature IFN-γ. The nucleotide (nt) and predicted amino acid (aa) sequences of white RhIFN-γ are shown in Fig. 2.1 and 2.2 respectively. The coding part of the white RhIFN-γ gene is 501 nucleotides long and encodes a protein with a predicted molecular weight (MW) of 19.4 kDa. According to the SignalP 3.0 prediction server (www.cbs.dtu.dk/services/SignalP/) the most likely signal peptidase cleavage site is located between aa 25 and 26, which would yield a mature protein of 141 aa and a signal peptide of 25 aa. The predicted amino acid sequences of white RhIFN-γ and EqIFN-γ (Grunig, Himmler & Antczak 1994) are aligned in Fig. 2.2.

```
CCTGATCAGCTTAGTACAGAAGTGATCAGCTTAGCACAGAAGCTACTGATTTCAACTGCTTTGGCCTAA
                                F3
CTCTCTCCGAAACAATGAATTATACAAGTTTTATCTTAGCTTTTCAGCTTTGCGTGATTTTGGGTTCTC

CCAGCTATTACTGCCAGGCTGTGTTTTTTAAAGAAATAGAAAACCTTAAGGAATATTTTAATGCAAGCA

ATCCAGACGTAGCGGATGGTGGATCTCTTTTCCTAGATATCTTGAAGAACTGGAAAGAGGAGAGTGACA
                                                        R4
AAAAAATAATTCAGAGCCAGATCGTCTCCTTCTACTTCAAACTCTTTGAAAATTTAAAAGATAACCAGG

TCATTCAAAAGAGCATGGATATCATCAAGGAAGACCTGTTTGTTAAGTTCTTCAACAGCAGCACCAGCA

AACTGGATGACTTCAAAAAGCTGATTCAGATTCCGGTAGATGATCTGCAGGTCCAACGCAAGGCGATAA

GTGAACTCATCAAAGTGATGAATGACCTGTCCCCGAGATCTAACCTAAGAAAGCGGAAGAGGAGTCAGG
                R7                                              R3
GTCAGTTTCGAGGCCGGAGAGCACTGAACTAATGGTCATCCTGCCTGCAATATTTGAATTCCCGCCGCC

GCACCCAGCTTTCTTGTACAAAGTGGTTGATTCGAGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTG
```

Figure 2.1 Nucleotide sequence of RhIFN-γ gene (Genbank Accession Number: DQ305037). Bold arrows indicate annealing regions used by PCR primers for cloning the sequence encoding the mature protein. Primers employed for determining the 5′-end of the gene are indicated with thin arrows. The start codon (ATG) and the stop codon (TAA) are in bold and underlined

```
Rhino    MNYTSFILAFQLCVILGSPSYYCQAVFFKEIENLKEYFNASNPDVADGGSLFLDILKNWK 60
         MNYTSFILAFQLC ILGS +YYCQA FFKEIENLKEYFNASNPDV DGG LFLDILKNWK
Horse    MNYTSFILAFQLCAILGSSTYYCQAAFFKEIENLKEYFNASNPDVGDGGPLFLDILKNWK 60

Rhino    EESDKKIIQSQIVSFYFKLFENLKDNQVIQKSMDIIKEDLFVKFFNSSTSKLDDFKKLIQ 120
         E+SDKKIIQSQIVSFYFKLFENLKDNQVIQKSMD IKEDLFVKFFNSSTSKL+DF+KLIQ
Horse    EDSDKKIIQSQIVSFYFKLFENLKDNQVIQKSMDTIKEDLFVKFFNSSTSKLEDFQKLIQ 120

Rhino:   IPVDDLQVQRKAISELIKVMNDLSPRSNLRKRKRSQGQFRGRRALN 166
         IPV+DL+VQRKAISELIKVMNDLSP++NLRKRKRSQ  FRGRRAL
Horse    IPVNDLKVQRKAISELIKVMNDLSPKANLRKRKRSQNPFRGRRALQ 166
```

Figure 2.2 Alignment of predicted protein sequences for white RhIFN-γ and EqIFN-γ. Amino acid identities are shown in blue and a plus sign denotes conserved substitution. The predicted signal sequence of RhIFN-γ is underlined. A blast search of the white RhIFN-γ sequence demonstrated a 90% identity on the nt as well as on the aa level with EqIFN-γ

2.3.2 Expression and purification of white rRhIFN-γ

The rRhIFN-γ protein with a hexa-histidine tag (his-6-tag) at its N-terminal end and the additional aa's derived from the GW recombination sequence and thrombin cleavage site was expressed in *E. coli* by plasmid pET15-RhIFN-γ. Upon IPTG induction a strong protein band with the expected molecular weight for the tagged rRhIFN-γ was induced (Fig. 2.3, Lane 3). The major part of the expressed rRhIFN-γ was present in the insoluble fraction as inclusion bodies (Fig. 2.3, Lane 5). After solubilization of the inclusion bodies in 8 M urea (Fig. 2.3, Lane 6), the majority of the his-6-tag rRhIFN-γ bound to a column with immobilized Ni^{2+} (some of the column matrix was removed and loaded, Fig. 2.3, Lane 8) and a minor part failed to bind and showed up in the flow-though fraction (Fig. 2.3, Lane 7). After washing, the bound protein was refolded on the column. After elution and dialysis the refolded IFN-γ had a purity of at least 95% (Fig. 2. 3, Lane 9).

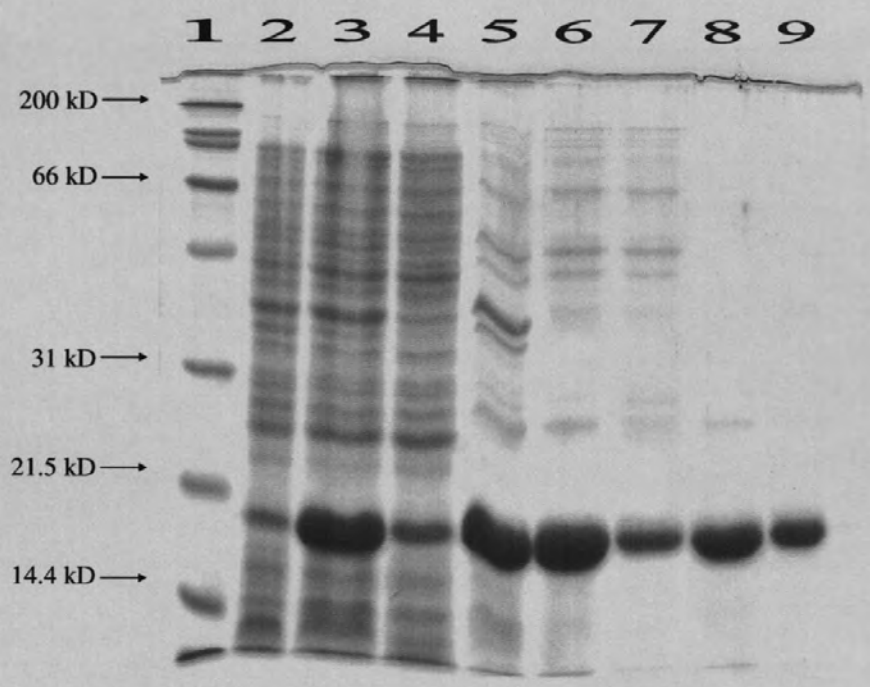


Figure 2.3 SDS-PAGE showing the purification of white rRhIFN-γ. **Lane 1:** Broad Range Mw Marker; **Lane 2:** Total bacterial lysate (uninduced); **Lane 3:** Total bacterial lysate (IPTG-induced); **Lane 4:** Soluble fraction; **Lane 5:** Insoluble fraction (inclusion bodies); **Lane 6:** Solved inclusion bodies (8 M urea); **Lane 7:** Flow-through Ni^{2+} column; **Lane 8:** Protein bound on column matrix; and **Lane 9:** Eluted protein

2.3.3 Prototype capture ELISA for the detection of white RhIFN-γ

The purified white rRhIFN-γ was used to generate specific monoclonal mouse antibodies and polyclonal chicken antibodies (IgY). The antibodies were used to develop a capture ELISA using monoclonal antibody 1H11 as a capture antibody and the anti-IFN-γ polyclonal IgY as detecting antibody. This capture ELISA was able to detect recombinant white RhIFN-γ (Fig. 2.4). The capture ELISA could also detect native IFN-γ as was demonstrated by the strong signal obtained with supernatants of PBMC of three white rhinoceroses that had been stimulated with Con A to induce the expression of this cytokine (Fig. 2.4).

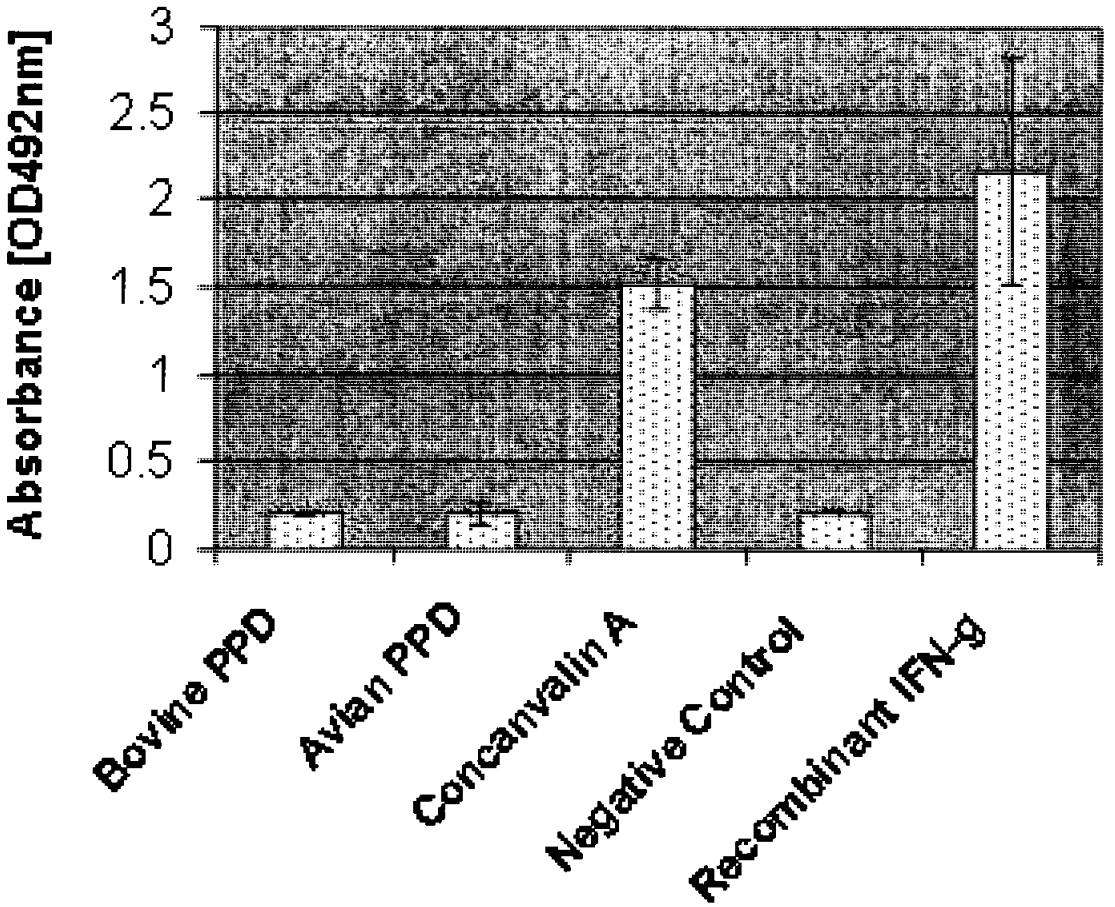


Figure 2.4 Detection of white RhIFN-γ in PBMC stimulated with Bovine PPD, Avian PPD and Con A using the IFN-γ capture ELISA. Results are expressed as the mean value from three rhinoceroses. "I" indicates the standard deviations

2.4 Discussion

Mycobacterium bovis has been found to have an exceptionally wide host range which includes domestic ruminants and captive and free-ranging wildlife (Buddle *et al.* 2000; Michel *et al.* 2006). The high BTB prevalence among buffalo herds in the southern region of the KNP has facilitated the spillover of *M. bovis* infections into a number of animal species and poses a real threat to valuable species, thus jeopardising breeding and relocation projects of, amongst others, rhinoceroses in the context of conservation and biodiversity. Although not yet diagnosed in South Africa, rhinoceroses have been reported to be susceptible to both *Mycobacterium tuberculosis* and *M. bovis* (Mann, Bush, Janssen *et al.* 1981; Dalovisio, Stetter & Mikota-Wells 1992; Stetter, Mikota, Gutter *et al.* 1995; Michel 2002), but little is

known concerning the pathogenesis of the disease in them. Diagnostic tests available for *M. bovis* infections are often limited to certain species or lack validation in others, such as the TST in pachyderms. As a consequence, the specificity and sensitivity of these tests are not known in these animal species. Control of infection in the individual animal or in groups of animals strongly depends on early diagnosis. The IFN-γ test has proven to be highly successful in demonstrating mycobacterial infections in domestic and non-domestic species, including cattle, goats, bison, African buffaloes (Bovigam™) (Grobler, Michel, De Klerk & Bengis 2002), deer (Cervigam™) (Waters, Palmer, Thacker *et al.* 2008) and primates (Primagam™) (Garcia, Yee, Bouley *et al.* 2004). In wildlife species the test is considered practical, as it requires minimal invasion and manipulation.

This chapter reports the successful cloning, sequencing and expression of IFN-γ from white rhinoceroses, and the production of monoclonal antibodies specific for rRhIFN-γ, as essential tools for development of assays to detect the IFN-γ response to *M. bovis* infection in this animal species. As expected from the close phylogenetic relationship, the highest homology was observed with the EqIFN-γ sequence on the DNA as well as the protein level (both 90%). The RhIFN-γ gene is predicted to encode a signal sequence of 25 aa's and a mature protein of 141 aa residues. For expression purposes in *E. coli*, the sequence encoding the mature protein was initially cloned. The forward primer that was used to clone the mature IFN-γ was based on the EqIFN-γ sequence, because of its relatedness to the rhinoceros. Potentially this equine forward primer might have had one or a few mismatches with the rhinoceros sequence, which would consequently result in different aa's at the N-terminal end of the mature white RhIFN-γ. The sequence of the 5' end was therefore cloned separately using forward primers corresponding to EqIFN-γ sequences upstream of the start codon and reverse primers based on the obtained white RhIFN-γ sequence. Based on the new set of primers derived from those sequences, the final nt at the 5' end was verified. Indeed, the equine forward primer used to clone the sequence encoding the mature RhIFN-γ contained two mismatches with the real rhinoceros sequence. Only the second mismatch results in a different amino acid after translation. This aa, the first of the predicted mature RhIFN-γ, turned out to be a valine (v) instead of the alanine (a) (Fig. 2.2). Apparently, the aa at this position does not form an essential part of the epitope recognized by monoclonal antibody 1H11, as a prototype capture ELISA could be developed, using this antibody in combination with polyclonal chicken antibodies, that is able to detect both recombinant and native white RhIFN-γ.

To date tests have only been performed in the white rhinoceros and have yet to be performed in black rhinoceroses (*Diceros bicornis*). A first preliminary validation of the specificity of the test was done on TB-free white rhinoceroses. No IFN-γ was detected after *M. bovis* stimulation; whereas it was shown that a signal was detected after Con A stimulation. Sensitivity of the tests is presently unknown and might be evaluated in *M. bovis*- / *M. tuberculosis*-infected rhinoceroses. In conclusion: although further development and validation of the described capture ELISA is necessary, it demonstrates a promising approach towards a diagnostic assay for *M. bovis* infection in rhinoceroses.

2.5 Publication

This chapter was published in the *Journal of Veterinary Immunology and Immunopathology* in 2007. Herewith is the reference:

Morar, D., Tijhaar, E., Negrea, A., Hendriks, J., van Haarlem, D., Godfroid, J., Michel, A.L. & Rutten, V.P.M.G. 2007. Cloning, sequencing and expression of white rhinoceros (*Ceratotherium simum*) interferon-gamma (IFN-γ) and the production of rhinoceros IFN-γ specific antibodies. *Journal of Veterinary Immunology and Immunopathology*, 115:146-154.

2.6 References

BOERSMA, W.J., BOGAERTS, W.J., BIANCHI, A.T. & CLAASSEN, E. 1992. Adjuvant properties of stable water-in-oil emulsions: evaluation of the experience with Specol. *Research in Immunology*, 143:503-512.

BOKHOUT, B.A., VAN GAALEN, C. & VAN DER HEIJDEN, P. 1981. A selected water-in-oil emulsion: Composition and usefulness as an immunological adjuvant. *Veterinary Immunology and Immunopathology*, 2:491-500.

BUDDLE, B.M., SKINNER, M.A. & CHAMBERS, M.A. 2000. Immunological approaches to the control of tuberculosis in wildlife reservoirs. *Veterinary Immunology and Immunopathology*, 74:1-16.

CHEESEMAN, C.L., WILESMITH, J.W. & STUART, F.A. 1989. Tuberculosis: the disease and its epidemiology in the badger, a review. *Epidemiology and Infection*, 103:113-125.

COUSINS, D.V., FRANCIS, B.R., CASEY, R. & MAYBERRY, C. 1993. *Mycobacterium bovis* infection in a goat. *Australian Veterinary Journal*, 70:262-263.

DALOVISIO, J.R., STETTER, M. & MIKOTA-WELLS, S. 1992. Rhinoceros' rhinorrhea: cause of an outbreak of infection due to airborne *Mycobacterium bovis* in zookeepers. *Clinical Infectious Diseases*, 15:598-600.

GARCIA, M.A., YEE, J., BOULEY, D.M., MOORHEAD, R. & LERCHE, N.W. 2004. Diagnosis of tuberculosis in macaques, using whole-blood in vitro interferon-gamma (PRIMAGAM) testing. *Comparative Medicine*, 54:86-92.

GROBLER, D.G., MICHEL, A.L., DE KLERK, L.M. & BENGIS, R.G. 2002. The gamma-interferon test: its usefulness in a bovine tuberculosis survey in African buffaloes (*Syncerus caffer*) in the Kruger National Park. *Onderstepoort Journal of Veterinary Research*, 69:221-227.

GRUNIG, G., HIMMLER, A. & ANTCZAK, D.F. 1994. Cloning and sequencing of horse interferon-gamma cDNA. *Immunogenetics*, 39:448-449.

HALL, W., MOLITOR, T.W., JOO, H.S. & PIJOAN, C. 1989. Comparison of protective immunity and inflammatory responses of pigs following immunization with different *Actinobacillus pleuropneumoniae* preparations with and without adjuvants. *Veterinary Immunology and Immunopathology*, 22:175-186.

HANSEN, P., SCOBLE, J.A., HANSON, B. & HOOGENRAAD, N.J. 1998. Isolation and purification of immunoglobulins from chicken eggs using thiophilic interaction chomatography. *Journal of Immunological Methods*, 215:1-7.

KÖHLER, G. & MILSTEIN, C. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature*, 256:495-497.

LIÉBANA, E., ARANAZ, A., URQUIA, J.J., MATEOS, A. & DOMINGUEZ, L. 1998. Evaluation of the gamma-interferon assay for eradication of tuberculosis in a goat herd. *Australian Veterinary Journal*, 76:50-53.

MANN, P.C., BUSH, M., JANSSEN, D.L., FRANK, E.S. & MONTALI, R.J. 1981. Clinicopathologic correlations of tuberculosis in large zoo mammals. *Journal of the American Veterinary Medical Association*, 179:1123-1129.

MICHEL, A.L. 2002. Implications of tuberculosis in African wildlife and livestock. *Annals of the New York Academy of Sciences*, 969:251-255.

MICHEL, A.L., BENGIS, R.G., KEET, D.F., HOFMEYR, M., KLERK, L.M.D., CROSS, P.C., JOLLES, A.E., COOPER, D., WHYTE, I.J., BUSS, P. & GODFROID, J. 2006. Wildlife tuberculosis in South African conservation areas: Implications and challenges. *Veterinary Microbiology*, 112:91-100.

MONAGHAN, M.L., DOHERTY, M.L., COLLINS, J.D., KAZDA, J.F. & QUINN, P.J. 1994. The tuberculin test. *Veterinary Microbiology*, 40:111-124.

O'REILLY, L.M. & DABORN, C.J. 1995. The epidemiology of *Mycobacterium bovis* infections in animals and man: A review. *Tubercle and Lung Disease*, 76:1-46.

POLLOCK, J.M., RODGERS, J.D., WELSH, M.D. & MCNAIR, J. 2006. Pathogenesis of bovine tuberculosis: The role of experimental models of infection. *Veterinary Microbiology*, 112:141-150.

STETTER, M.D., MIKOTA, S.K., GUTTER, A.F., MONTERROSO, E.R., DALOVISIO, J.R., DEGRAW, C. & FARLEY, T. 1995. Epizootic of *Mycobacterium bovis* in a zoologic park. *Journal of the American Veterinary Medical Association*, 207:1618-1621.

WATERS, W.R., PALMER, M.V., THACKER, T.C., ORLOSKI, K., NOL, P., HARRINGTON, N.P., OLSEN, S.C. & NONNECKE, B.J. 2008. Blood culture and stimulation conditions for the diagnosis of tuberculosis in cervids by the Cervigam assay. *Veterinary Record*, 162:203-208.

WOOD, P.R., CORNER, L.A. & PLACKETT, P. 1990a. Development of a simple, rapid *in vitro* cellular assay for bovine tuberculosis based on the production of gamma interferon. *Research in Veterinary Science*, 49:46-49.

WOOD, P.R., ROTHEL, J.S., MCWATERS, P.G.D. & JONES, S.L. 1990b. Production and characterization of monoclonal antibodies specific for bovine gamma-interferon. *Veterinary Immunology and Immunopathology*, 25:37-46.

WOOD, P.R. & JONES, S.L. 2001. BOVIGAM™: an *in vitro* cellular diagnostic test for bovine tuberculosis. *Tuberculosis*, 81:147-155.

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 µ*l* 10 X TaKaRa buffer, 8 µ*l* TaKaRa dNTPs, 100 ng V_H and V_L chain DNA, 0.3 µ*l* of 2.5 U TaKaRa Ex Taq DNA polymerase, 0.5 µ*l* of 3 U Promega *Pfu* DNA polymerase (with proof reading activity) and water to a final volume of 50 µ*l*. 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 µ*l* of 10 X TaKaRa PCR buffer, 8 µ*l* TaKaRa dNTPs, 10 pmol Sfi1 primer, 10 pmol of LCNOT1 primer, 4 µ*l* spliced product, 0.3 µ*l* of 2.5 U TaKaRa Ex Taq DNA polymerase and water to a final volume of 50 µ*l*. 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 µ*l* 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 µ*l* 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 µ*l* of 10 U/µ*l* Sfi1 (Roche) in 10 µ*l* Buffer M (Roche) and 1 µ*l* of 10 mg/m*l* 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.

```
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
```

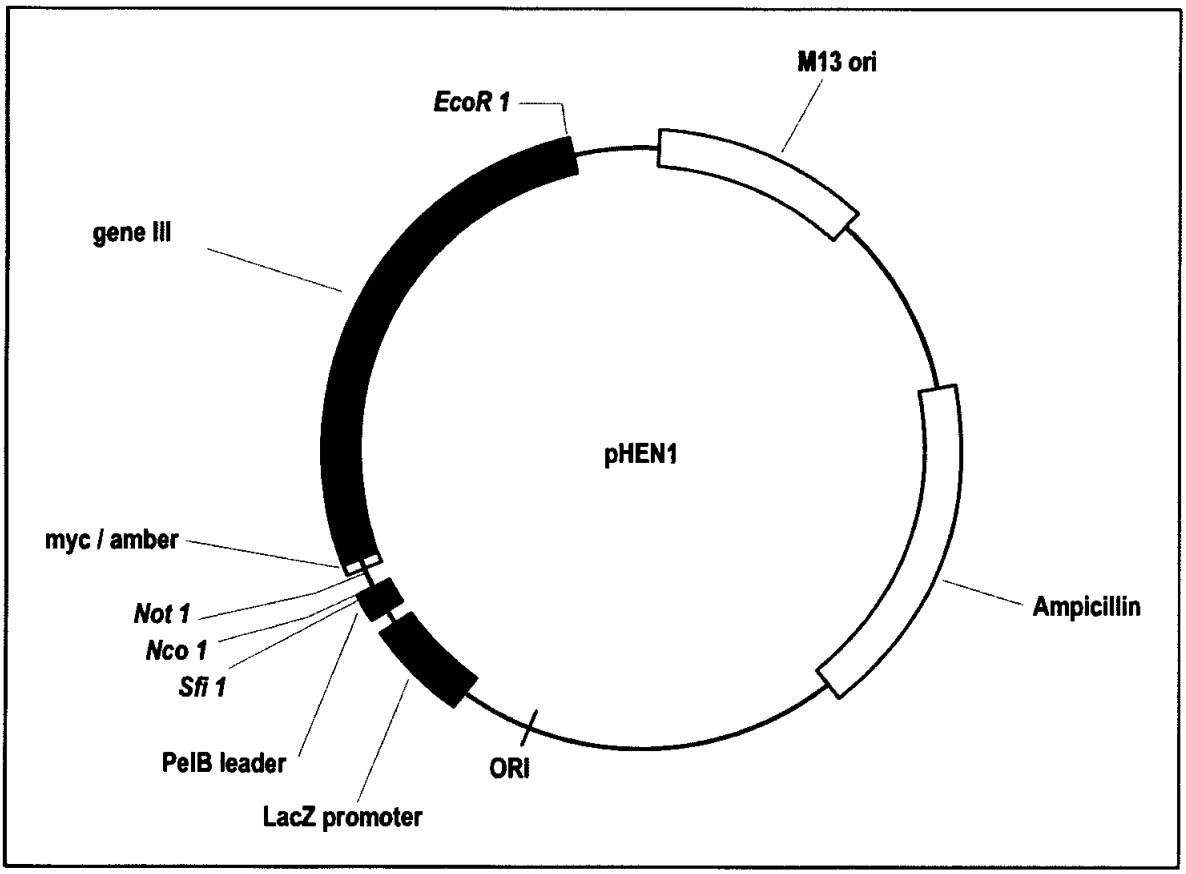
Figure 3.1 Alignment of predicted sequences for white RhIFN-γ and AsEpIFN-γ. Amino acid identities are shown in blue and a plus sign denotes a conserved substitution. The predicted signal sequence of RhIFN-γ is underlined. A blast search of the white RhIFN-γ sequence with the AsEpIFN-γ (see 4.3.2) sequence 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.




```
-----------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
```

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}.

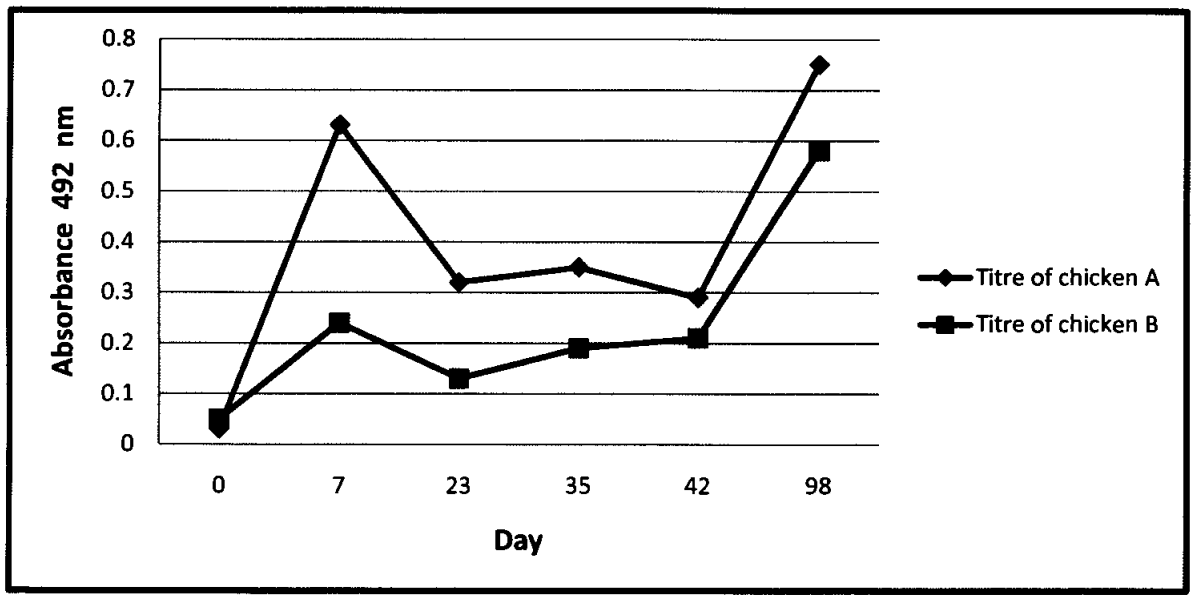


Figure 3.3 ELISA showing the antibody titres of immunized chickens from Day 0 to Day 98

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).

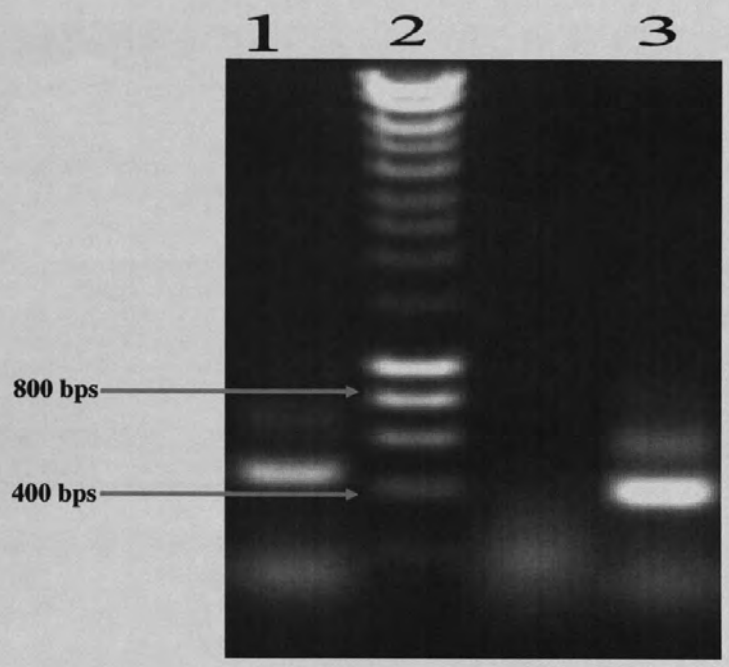


Figure 3.4 Agarose gel of the primary PCR for the amplification of V_H and V_L chains. **Lane 1:** V_H (410 bps) PCR product; **Lane 2:** Molecular weight marker (Hyperladder I, Bioline); and **Lane 3:** V_L (340 bps) PCR product

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*.

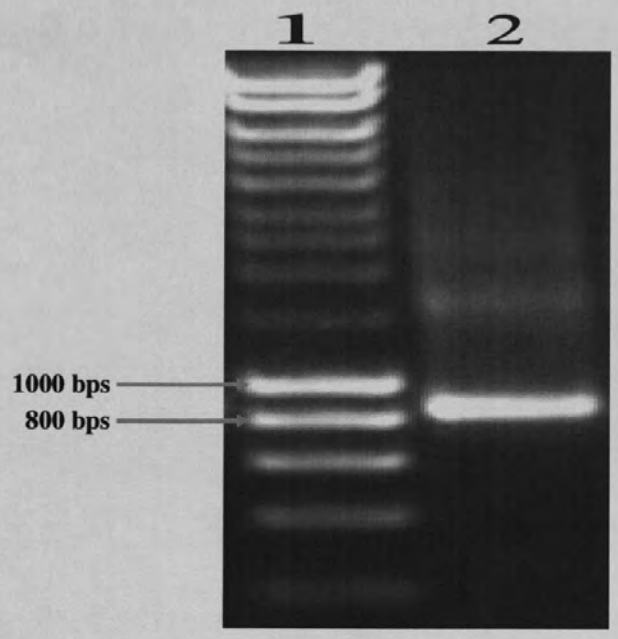


Figure 3.5 Agarose gel of the SOE PCR. **Lane 1:** Molecular weight marker (Hyperladder I, Bioline) and **Lane 2:** Joined V_H and V_L product (approximately 800 bps)

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).

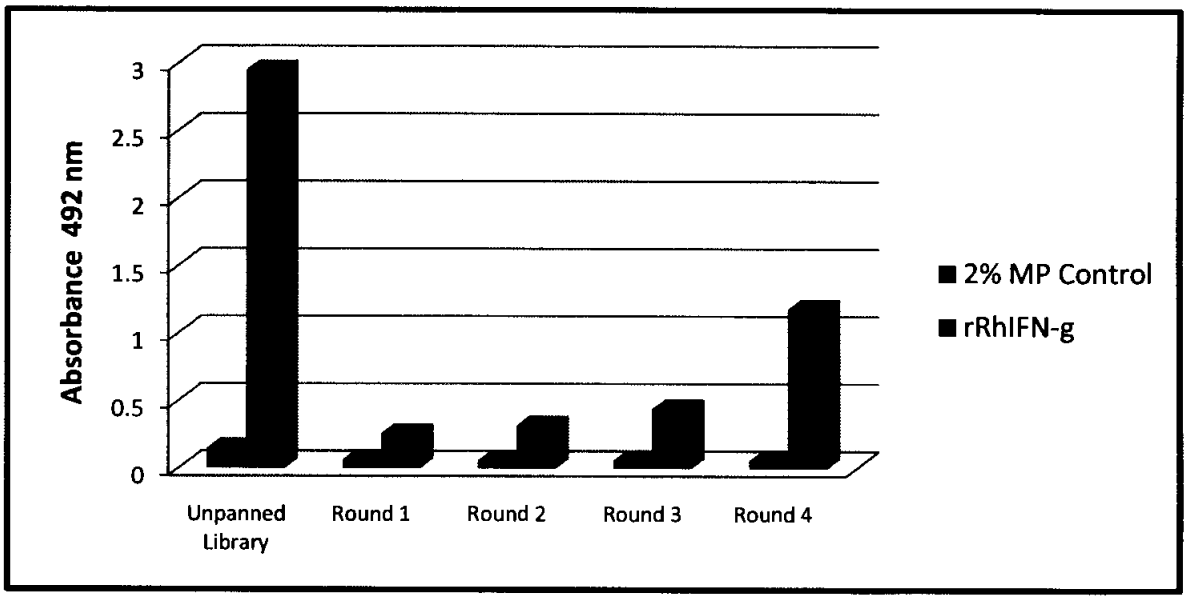


Figure 3.6 Polyclonal phage ELISA of phage pools obtained from different rounds of panning reacting to rRhIFN-γ

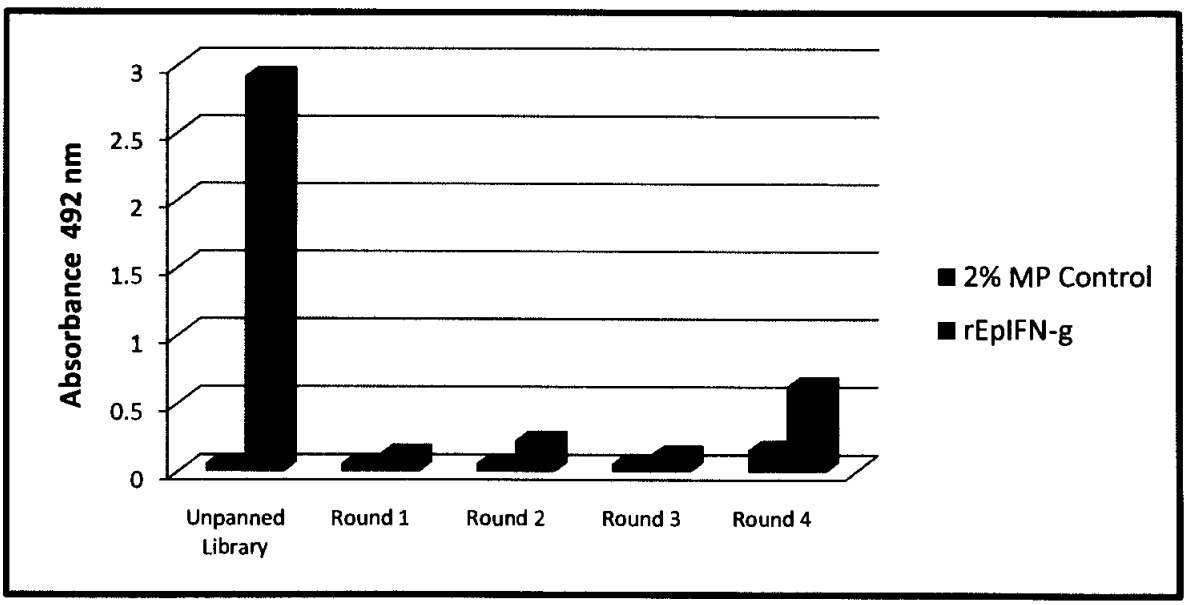


Figure 3.7 Polyclonal phage ELISA of phage pools obtained from different rounds of panning reacting to rAsEpIFN-γ

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.

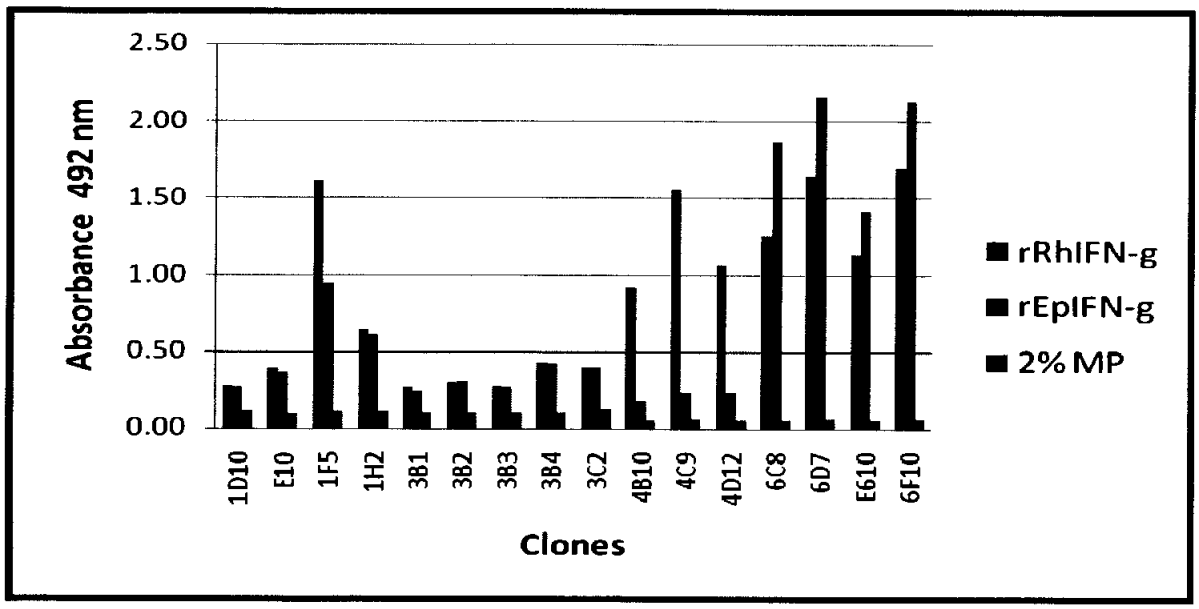


Figure 3.8 Direct ELISA to determine the antigen specificity and cross-reactivity of phage-displayed antibodies to rRhIFN-γ and to rAsEpIFN-γ

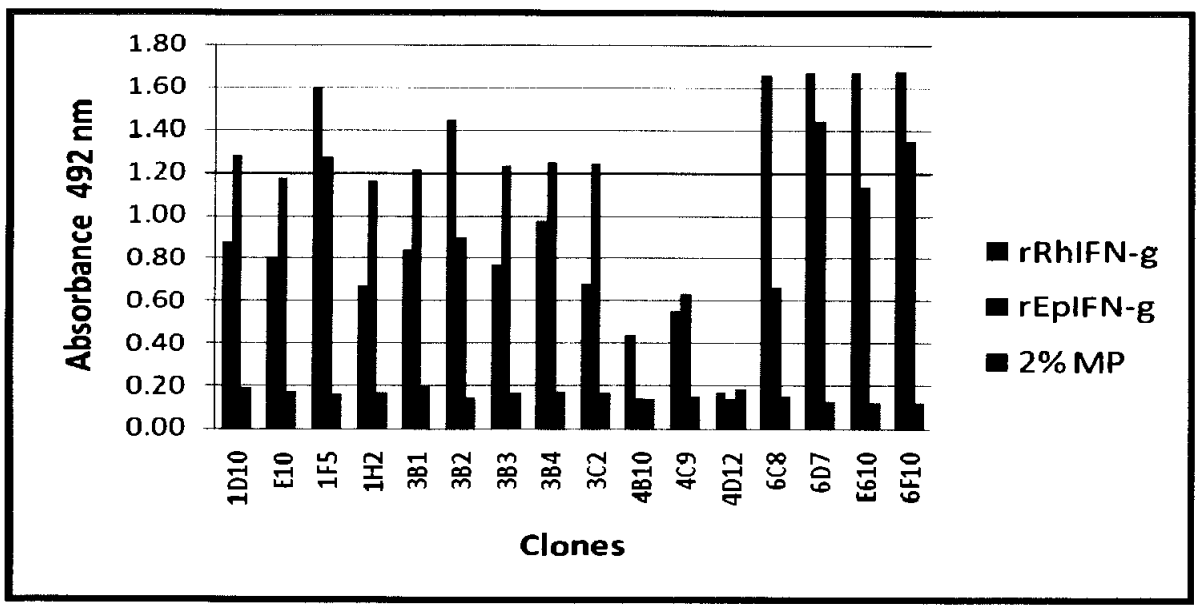


Figure 3.9 Direct ELISA to determine the antigen specificity and cross-reactivity of scFv antibodies to rRhIFN-γ and to rAsEpIFN-γ

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.

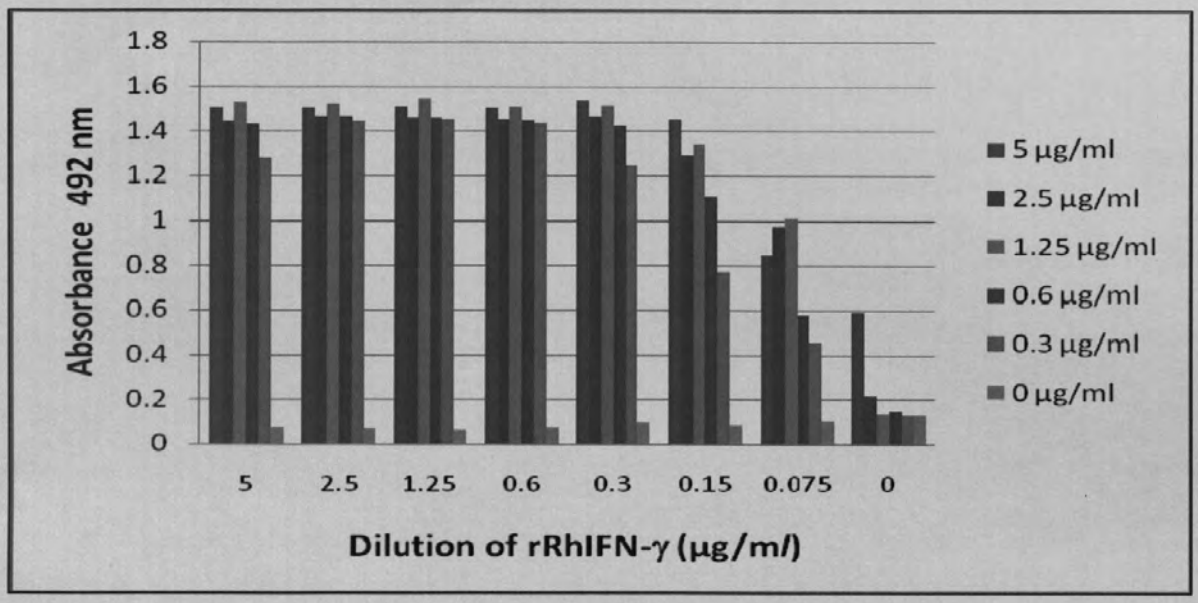


Figure 3.11 Capture ELISA using capture antibody scFv 1F5, at a concentration of 5 μg/m*l*, and detection of rRhIFN-γ with antibody 1D11. The legend on the right indicates the dilution of the detecting antibody 1D11

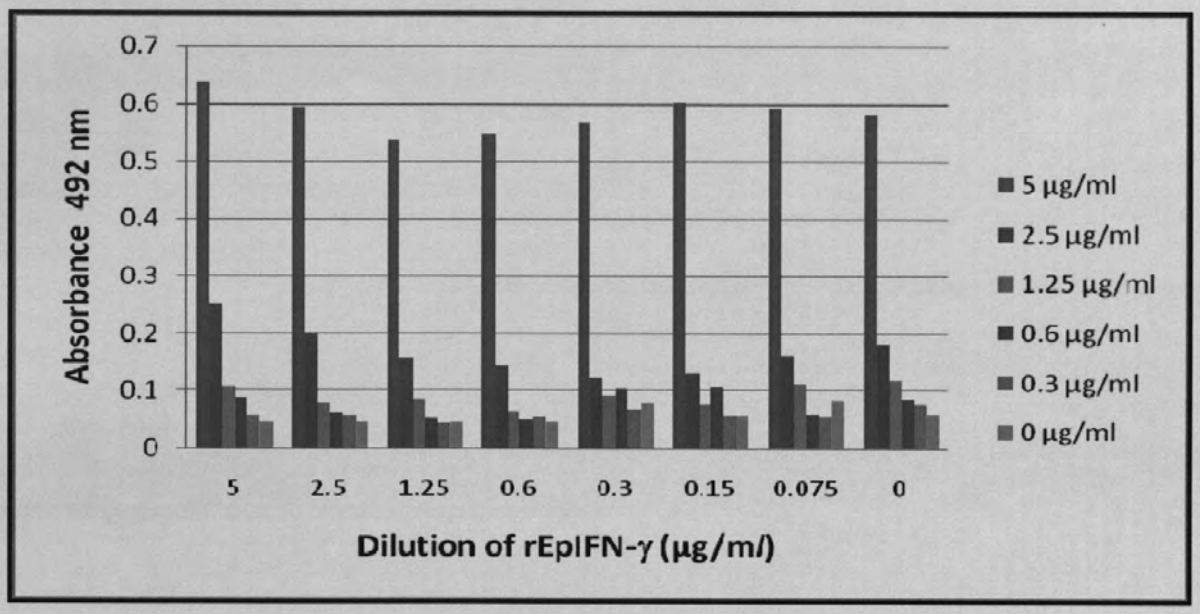


Figure 3.12 Capture ELISA using capture antibody scFv 1F5, at a concentration of 5 µg/m*l*, and detection of rAsEpIFN-γ with antibody 1D11. The legend on the right shows the dilution of detecting antibody 1D11

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).

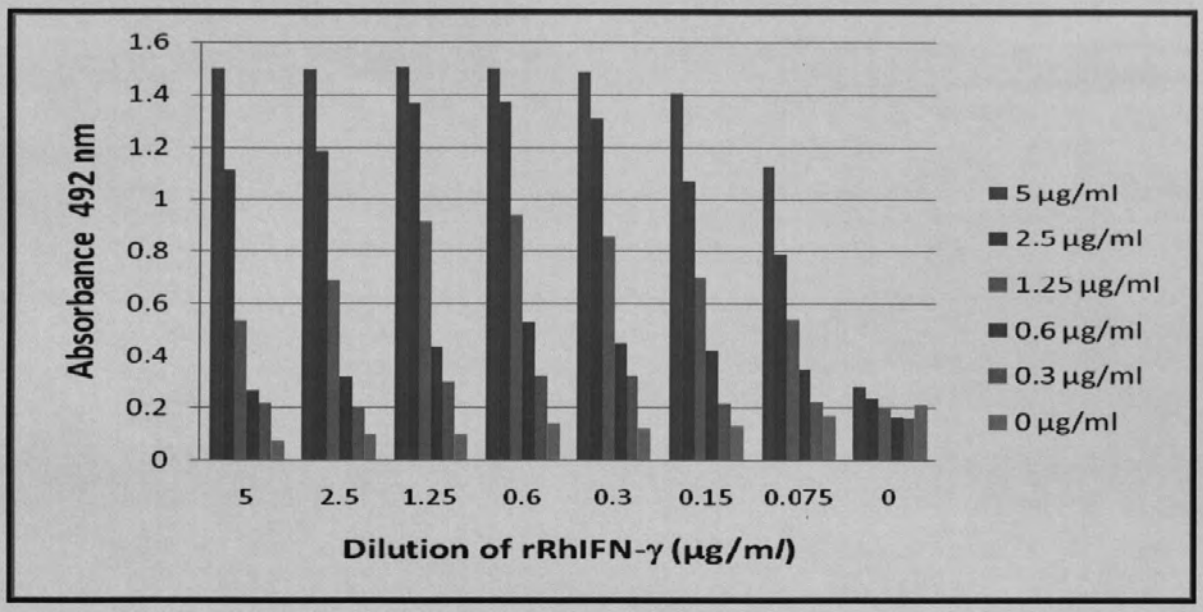


Figure 3.13 Capture ELISA using capture antibody scFv 1F5, at a concentration of 5 µg/m*l*, and IgY^{uu} for the detection of rRhIFN-γ. The legend on the right represents the dilution series of IgY^{uu}

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.

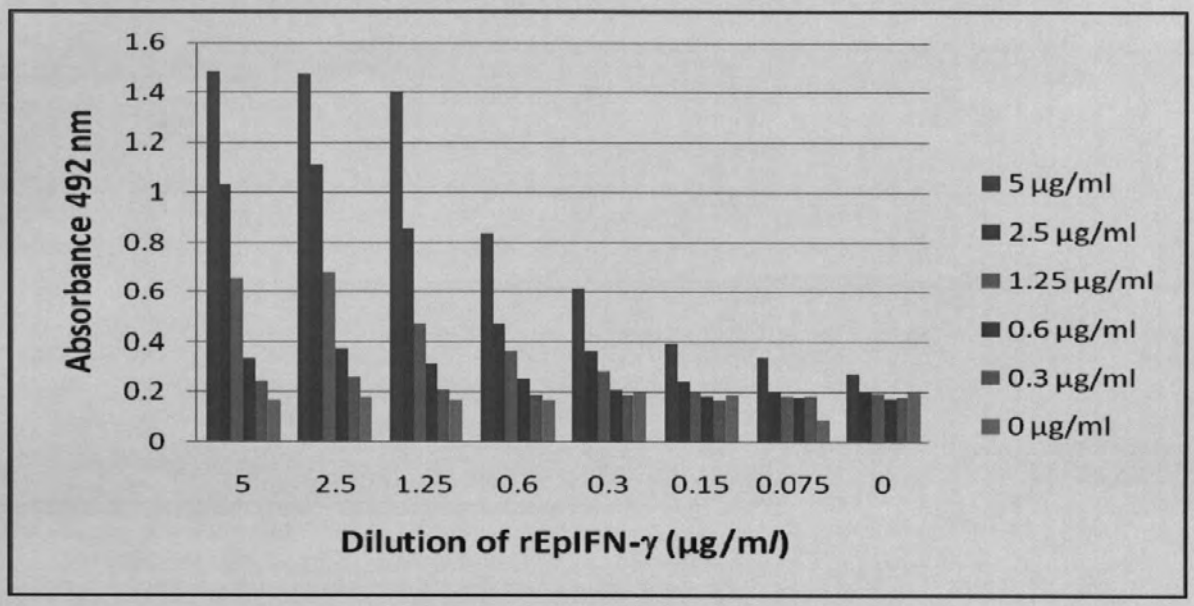


Figure 3.14 Capture ELISA using capture antibody scFv 1F5, at a concentration of 5 µg/m*l*, and IgY^{uu} for the detection of rAsEpIFN-γ. The legend on the right represents the dilution series of IgY^{uu}

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*.

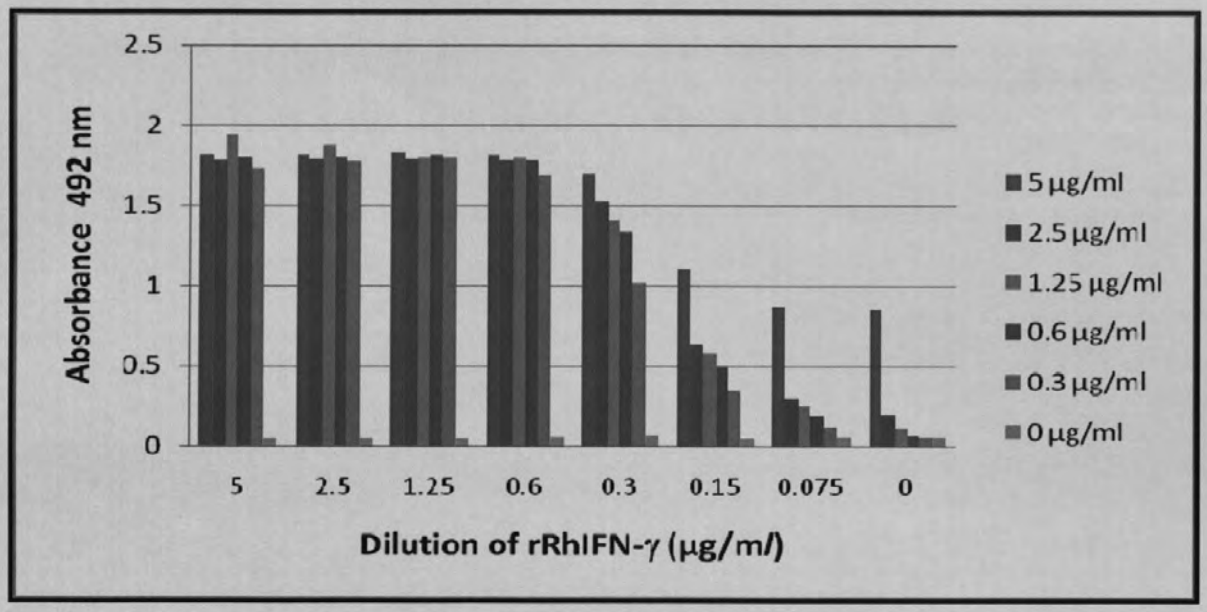


Figure 3.15 Capture ELISA using capture antibody scFv 6D7, at a concentration of 5 μg/m*l*, and antibody 1D11 for the detection of rRhIFN-γ. The legend on the right represents the dilution series of 1D11

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).

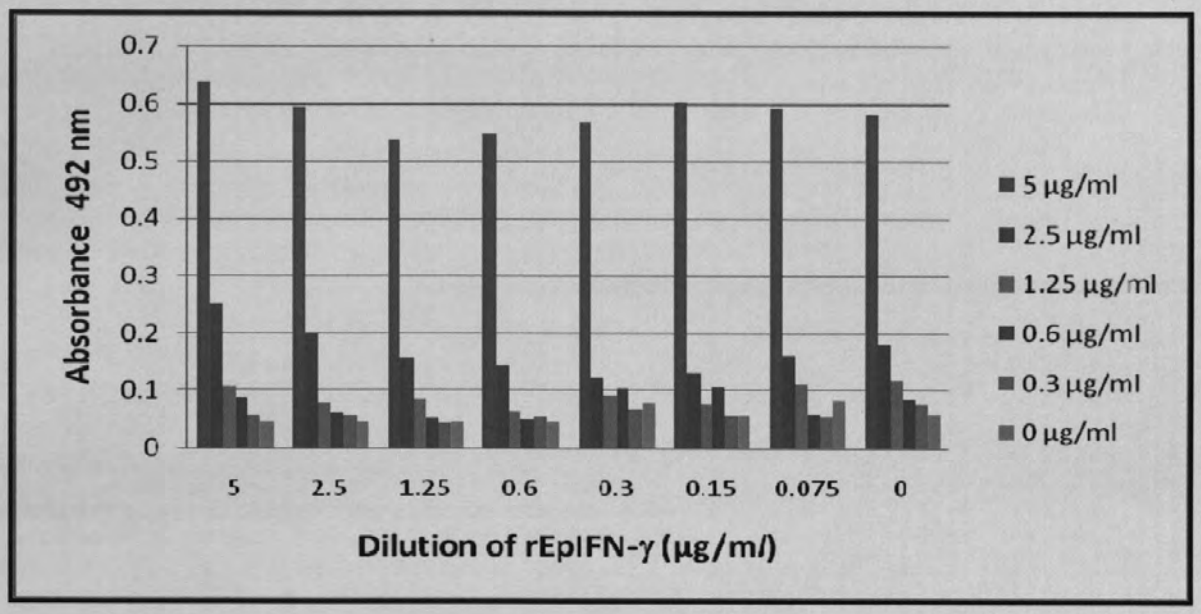


Figure 3.16 Capture ELISA using capture antibody scFv 6D7, at a concentration of 5 µg/ml, and antibody 1D11 for the detection of rAsEpIFN-γ. The legend on the right represents the dilution series of 1D11

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.

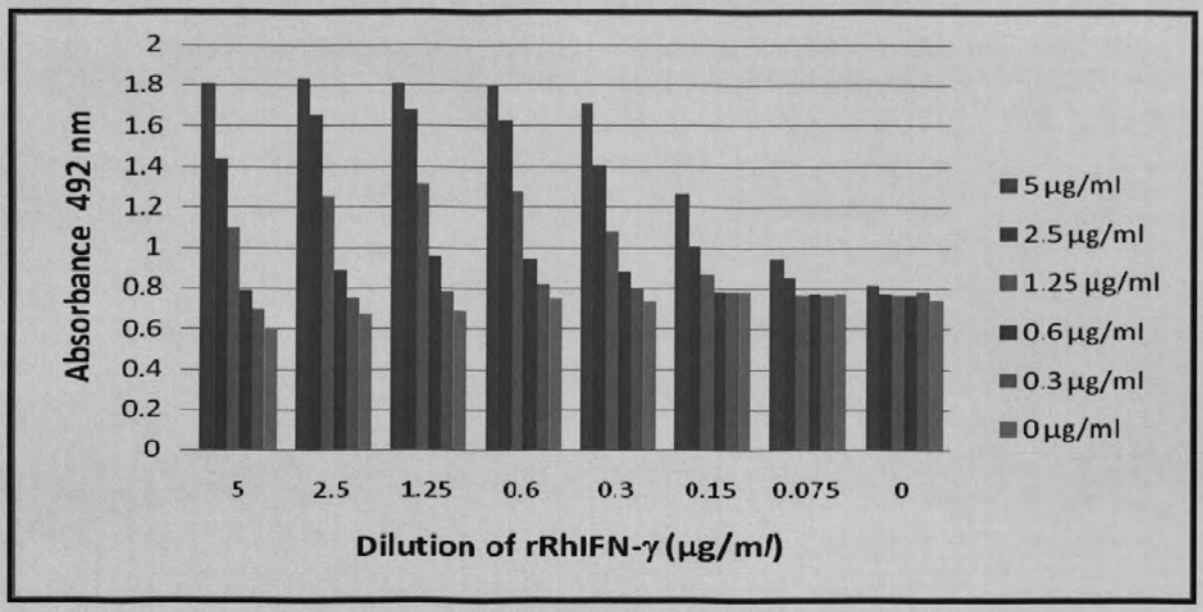


Figure 3.17 Capture ELISA using capture antibody scFv 6D7, at a concentration of 5 μg/m*l*, and IgY^{uu} for the detection of rRhIFN-γ. The legend on the right represents the dilution series of IgY^{uu}

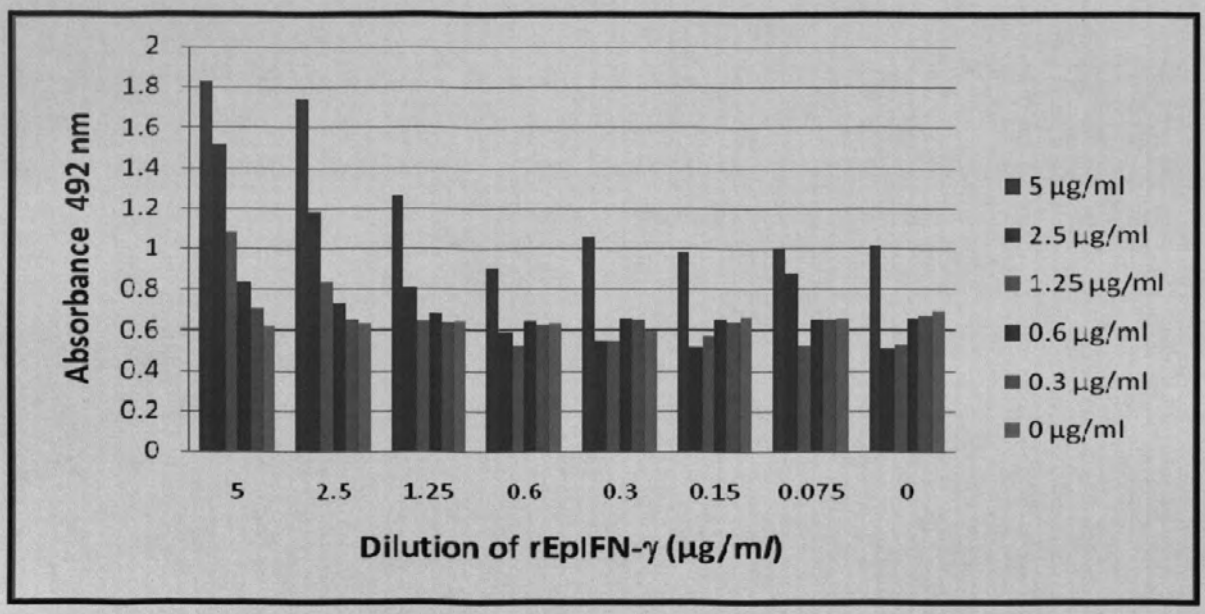


Figure 3.18 Capture ELISA using capture antibody scFv 6D7, at a concentration of 5 μg/m*l*, and IgY^{uu} for the detection of rAsEpIFN-γ. The legend on the right represents the dilution series of IgY^{uu}

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

AMES, R.S., TORNETTA, M.A., JONES, C.S. & TSUI, P. 1994. Isolation of neutralizing anti-C5a monoclonal antibodies from a filamentous phage monovalent Fab display library. *Journal of Immunology,* 153:910-920.

AMES, R.S., TORNETTA, M.A., DEEN, K., JONES, C.S., SWIFT, A.M. & GANGULY, S. 1995. Conversion of murine Fabs isolated from a combinatorial phage-display library to full length immunoglobulins. *Journal of Immunological Methods,* 184:177-186.

ANDRIS-WIDHOPF, J., RADER, C., STEINBERGER, P., FULLER, R. & BARBAS III, C.F. 2000. Methods for the generation of chicken monoclonal antibody fragments by phage-display. *Journal of Immunological Methods,* 242:159-181.

ATRAZHEV, A.M. & ELLIOTT, J.F. 1996. Simplified desalting of ligation reactions immediately prior to electroporation into *E. coli. BioTechniques,* 21:1024.

BIRD, R.E., HARDMAN, K.D., JACOBSON, J.W., JOHNSON, S., KAUFMAN, B.M., LEE, S.M., LEE, T., POPE, S.H., RIORDAN, G.S. & WHITLOW, M. 1988. Single-chain antigen-binding proteins. *Science,* 242:423-426.

BIRD, R.E. & WALKER, B.W. 1991. Single chain antibody variable regions. *Trends in Biotechnology,* 9:132-137.

BRADBURY, A. 1997. Recent advances in phage display: the report of the phage club first meeting. *Immunotechnology,* 3:227-231.

BRADBURY, A.R. & MARKS, J.D. 2004a. Antibodies from phage antibody libraries. *Journal of Immunological Methods,* 290:29-49.

BRADBURY, A.R. & MARKS, J.D. 2004b. Phage antibody libraries, in *Phage-display - A practical approach,* edited by T. Clackson & H.B. Lowman. Oxford: Oxford University Press: 243-286.

BUTLER, J.E., NAVARRO, S.J. & SUN, J. 1997. Adsorption-induced antigenic changes and their significance in ELISA and immunological disorders. *Immunological Investigations,* 26:36-54.

CARMEN, S. & JERMUTUS, L. 2002. Concepts in antibody phage display. *Briefings in Functional Genomics and Proteomics,*1:189-203.

CHILIZA, T.E., VAN WYNGAARDT, W. & DU PLESSIS, D.H. 2008. Single-chain antibody fragments from a display library derived from chickens immunized with a mixture of parasite and viral antigens. *Hybridoma,* 27:413-421.

COOMBER, D.W. 2002. Panning of antibody phage-display libraries. Standard protocols. *Methods in Molecular Biology,* 178:133-145.

DAVIES, E.L., SMITH, J.S., BIRKETT, C.R., MANSER, J.M., ANDERSON-DEAR, D.V. & YOUNG, J.R. 1995. Selection of specific phage-display antibodies using libraries derived from chicken immunoglobulin genes. *Journal of Immunological Methods,* 186:125-135.

DONG, L., CHEN, S., BARTSCH, U. & SCHACHNER, M. 2003. Generation of affinity matured scFv antibodies against mouse neural cell adhesion molecule L1 by phage display. *Biochemical and Biophysical Research Communications,* 301:60-70.

HAGA, M., SHIMURA, T., NAKAMURA, T., KATO, Y. & SUZUKI, Y. 1987. Effect of ultrasonic irradiation on the dissociation of antigen-antibody complexes. Application to homogeneous enzyme immunoassay. *Chemical & Pharmaceutical Bulletin,* 35:3822-3830.

HOF, D., HOEKE, M.O. & RAATS, J.M. 2008. Multiple-antigen immunization of chickens facilitates the generation of recombinant antibodies to autoantigens. *Clinical and Experimental Immunology,* 151:367-377.

HOLLIGER, P. & WINTER, G. 1993. Engineering bispecific antibodies. *Current Opinion in Biotechnology,* 4:446-449.

INOUE, H., NOJIMA, H. & OKAYAMA, H. 1990. High efficiency transformation of *Escherichia coli* with plasmids. *Gene,* 96:23-28.

KINDT, T.J., GOLDSBY, R.A., BABARA, A. & KUBY, J. 2002. *Kuby immunology*, 6th ed. England: W.H. Freeman Press.

KÖHLER, G. & MILSTEIN, C. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature,* 256:495-497.

KORTT, A.A., LAH, M., ODDIE, G.W., GRUEN, C.L., BURNS, J.E., PEARCE, L.A., ATWELL, J.L., MCCOY, A.J., HOWLETT, G.J., METZGER, D.W., WEBSTER, R.G. & HUDSON, P.J. 1997. Single-chain Fv fragments of anti-neuraminidase antibody NC10 containing five- and ten-residue linkers form dimers and with a zero-residue linker a trimer. *Protein Engineering,* 10:423-433.

KORTT, A.A., DOLEZAL, O., POWER, B.E. & HUDSON, P.J. 2001. Dimeric and trimeric antibodies: high avidity scFvs for cancer targetting. *Biomolecular Engineering*, 18:95-108.

LI, Y., COCKBURN, W., KILPATRICK, J.B. & WHITELAM, G.C. 2000. High affinity scFvs from a single rabbit immunized with multiple haptens. *Biochemical and Biophysical Research Communications,* 268:398-404.

LUNDER, M., BRATKOVIC, T., URLEB, U., KREFT, S. & STRUKELJ, B. 2008. Ultrasound in phage-display: a new approach to nonspecific elution. *BioTechniques,* 44:893-900.

MCCAFFERTY, J., GRIFFITH, A.D., WINTER, G. & CHISWELL, D.J. 1990. Phage antibodies: filamentous phage-displaying antibody variable domains. *Nature,* 348:552-554.

PARMLEY, S.F. & SMITH, G.P. 1988. Antibody selectable filamentous fd phage vectors: affinity purification of target genes. *Gene*, 73:305-318.

POLSON, A., COETZER, T., KRUGER, J., VON MALTZAHN, E. & VAN DER MERWE, K.J. 1985. Improvements in the isolation of IgY from the yolks of eggs laid by immunized hens. *Immunological Investigations,* 14:323-327.

PINI, A. & BRACCI, L. 2000. Phage-display of antibody fragments. *Current Protein & Peptide Science*, 1:155-169.

RAND, K.N. 1996. Crystal violet can be used to visualise DNA bands during gel electrophoresis and to improve cloning efficiency. *Technical Tips Online, www.biomednet.com/db/tto.*

RATCLIFFE, M.J. 2006. Antibodies, immunoglobulin genes and the bursa of Fabricius in chicken B cell development. *Developmental and Comparative Immunology,* 30:101-118.

SMITH, G.P. 1985. Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science*, 228:1315-1317.

VAN WYNGAARDT, W., MALATJI, T., MASHAU, C., FEHRSEN, J., JORDAAN, F., MILTIADOU, D. & DU PLESSIS, D.H. 2004. A large semi-synthetic single-chain Fv phage-display library based on chicken immunoglobulin genes. *BMC Biotechnology,* 4:6-14.

VAUGHAN, T.J., WILLIAMS, A.J., PRITCHARD, K., OSBOURN, J.K., POPE, A.R., EARNSHAW, J.C., MCCAFFERTY, J., HODITS, R.A., WILTON, J. & JOHNSON, K.S. 1996. Human antibodies with sub-nanomolar affinities isolated from a large non-immunized phage-display library. *Nature Biotechnology,* 14:309-314.

YAMANAKA, H.I., INOUE, T. & IKEDA-TANAKA, O. 1996. Chicken monoclonal antibody isolated by a phage-display system. *Journal of Immunology,* 157:1156-1162.

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 X 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 μ*l* 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 μ*l* PCR reaction mix contained 4 μ*l* 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.

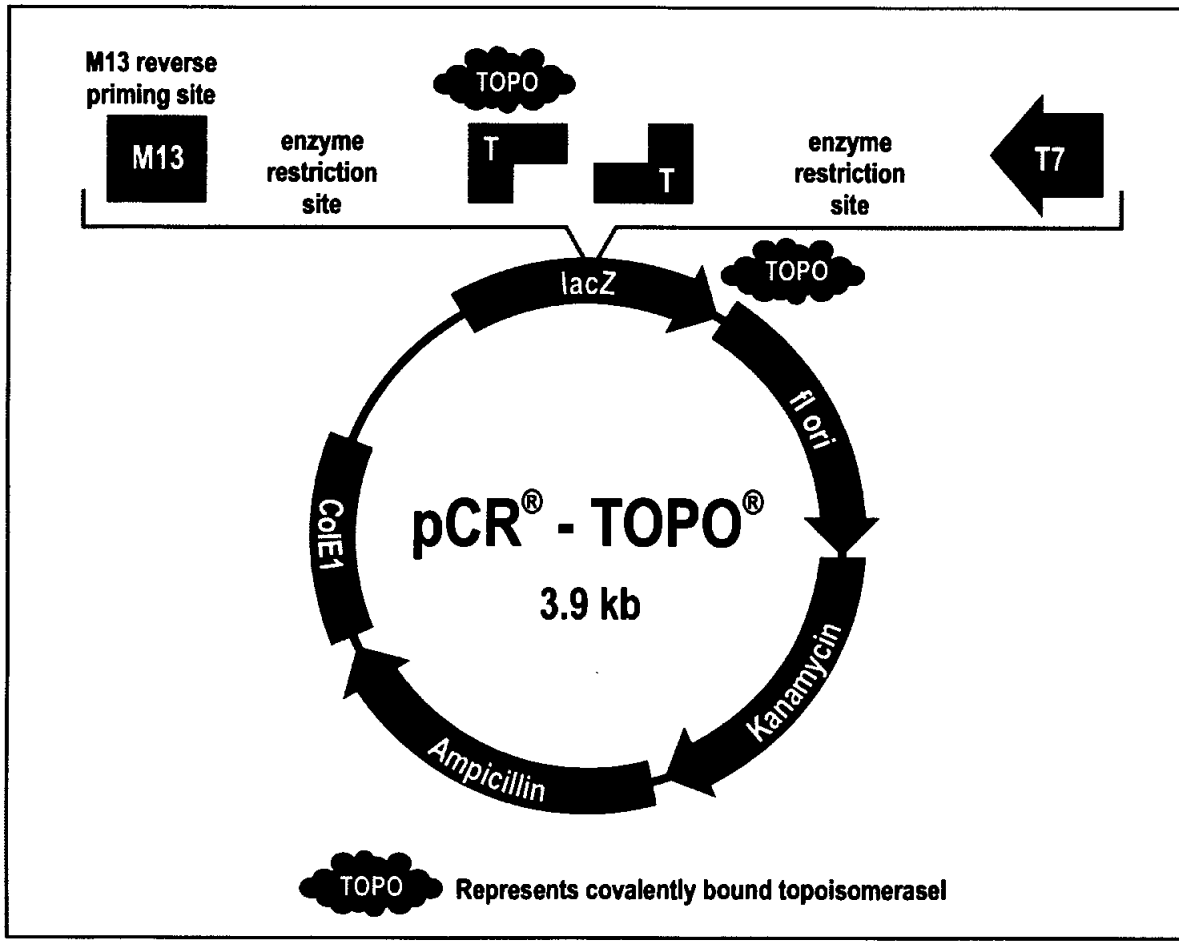


Figure 4.1 Diagrammatic representation of the TOPO Vector (Invitrogen) used for cloning of AfEpIFN-γ. Antibiotic resistance markers (ampicillin and kanamycin) are also present in the vector and the presence of these genes allowed for the selection of only those clones that contained the plasmid

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.

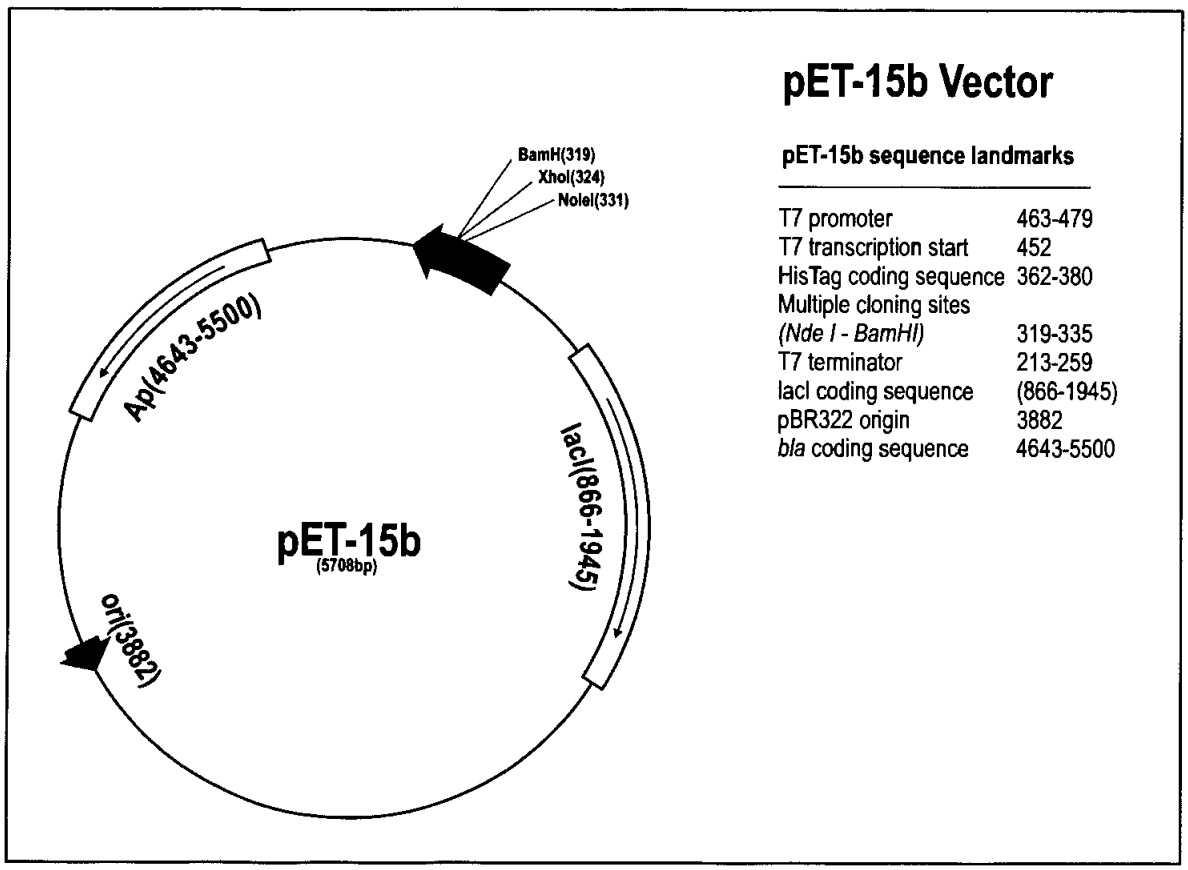


Figure 4.2 Diagrammatic representation of the pET15b vector (Novagen)

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 μl 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 μl 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.

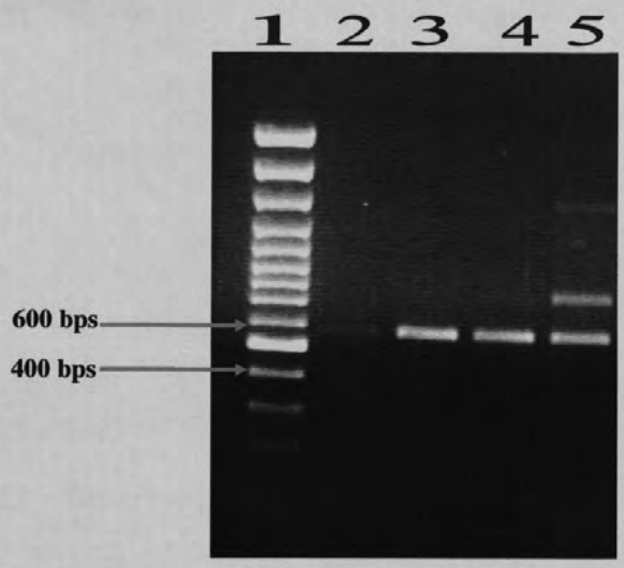


Figure 4.3 PCR products from cDNA (African elephant, Linda) amplification. **Lane 1:** 100 bp O′ gene ruler (Fermentes); **Lane 2:** Weak PCR product obtained at annealing temperature of 62°C; **Lane 3:** PCR product obtained at annealing temperature of 60°C; **Lane 4:** PCR product obtained at annealing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

```
Linda
ACTTTGGCCTAACTCTCTCTGAAACA[redacted]AATTTTACAAGTTATATCTTAGCTTTTCAGCTT
TGCATCATTATGGGTTCTTCTAGCTGCTACTGCCAGGCTACTTTTTTGAAAGAGATACAGAA
CCTAAAGGAATATCTTAATGCAACTGACTCAGATGTAGCGGATGGTGGGCCTCTTTTCATAG
ATATTTTGAAGAACTGGAAAGAGGACAGTGACAAAAAAATAATTCAGAGCCAGATCGTTTCC
TTTTACCTCAAAATCTTTGACAACCTGAAAGACAACCAGGTCATCCAAGAGAGCGTGAAGAC
CCTTGAGGAAGACCTCTTTGTTAAGTTCTTCAATAGCAGCTCCAGCAAACGGGATGACTTCC
TAAAAGTGATGCAAACTCCGGTAAATGACCAGAACATCCAGCGCAAAGCCATAAGTGAGCTC
GCCAAGGTGATGAATGACCTGTCACACAGATCTACCGGGTCAAAACGAAAAAGGAGACCGTA
TTCGTTTCGAGGCCGGAGAGCATCAGAA[redacted]TGGTCACCCTGCCTACAATATTTGAATTTT
```

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

```
Jasmin
GGCCTAACTCTCTCTGAAACA[redacted]AATTTTACAAGTTATATCTTAGCTTTTCAGCTTTGCAT
CATTTTGGGTTCTTCTAGCTGCTACTGCCAGGCTACTTTTTTGAAAGAGATACAGAACCTAA
AGGAATATCTTAATGCAACTGATTCAGATGTAGCGGATGGTGGGCCTCTTTTCATAGATATT
TTGAAGAACTGGAAAGAGGACAGTGACAAAAAAATAATTCAGAGCCAGATCGTTTCCTTTTA
CCTCAAAATCTTTGACAACTTGAAAGACAACCAGGTCATCCAAGAGAGCGTGAAGACCCTTG
AGGAAGACCTCTTTGTTAAGTTCTTCAATAGCAGCTCCAGCAAACGGGATGACTTCCTAAAA
GTGATGCAAACTCCGGTAAATGACCGGAACGTCCAGCGCAAAGCCATAAGTGAGCTCTCCAA
GGTGATGAATGACCTGTCACACAGATCTAACGGGGCAAAACGAAAAAGGAGACAGTATTCGT
TTCGAGGCCGGAGAGCATCAGAA[redacted]TGG
```

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

```
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).

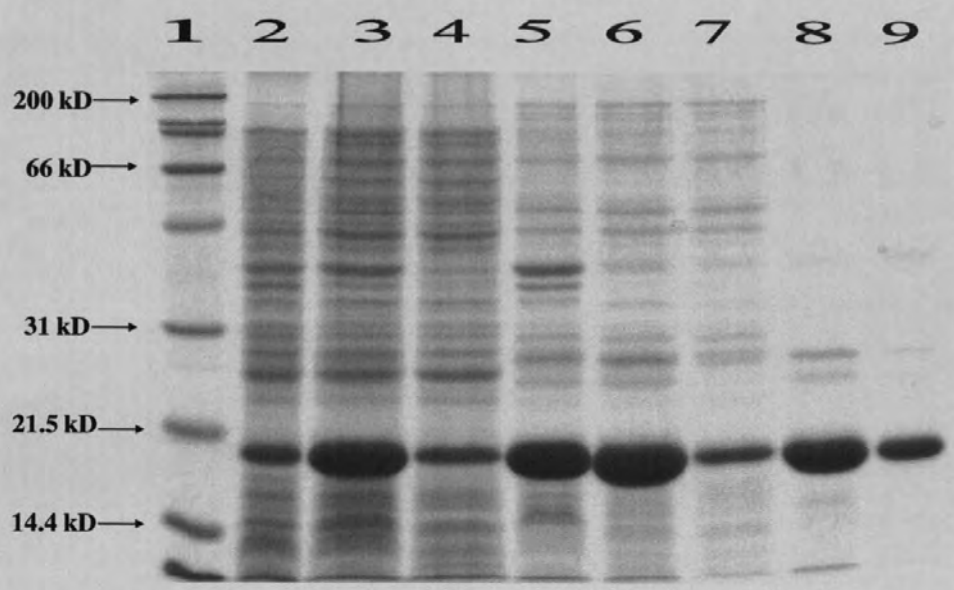


Figure 4.7 SDS-PAGE gel showing the purification of recombinant AsEpIFN-γ. **Lane 1:** Broad Range Mw Marker; **Lane 2:** Total bacterial lysate (uninduced); **Lane 3:** Total bacterial lysate (IPTG-induced); **Lane 4:** Soluble fraction; **Lane 5:** Insoluble fraction (inclusion bodies); **Lane 6:** 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-γspecific 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.

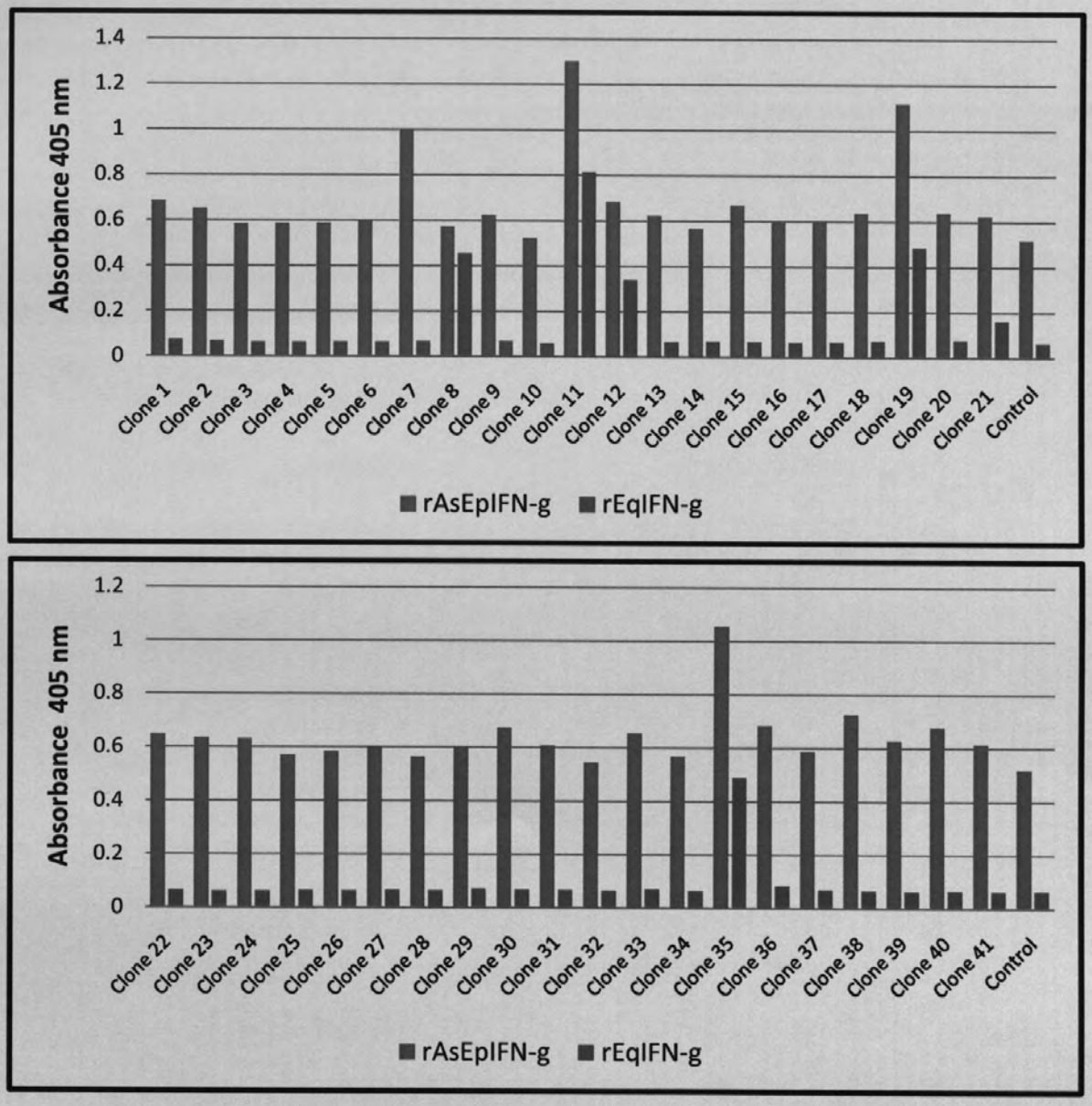


Figure 4.8 Direct ELISA using rAsEpIFN-γ and rEqIFN-γ for screening of 41 antibody producing clones

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.

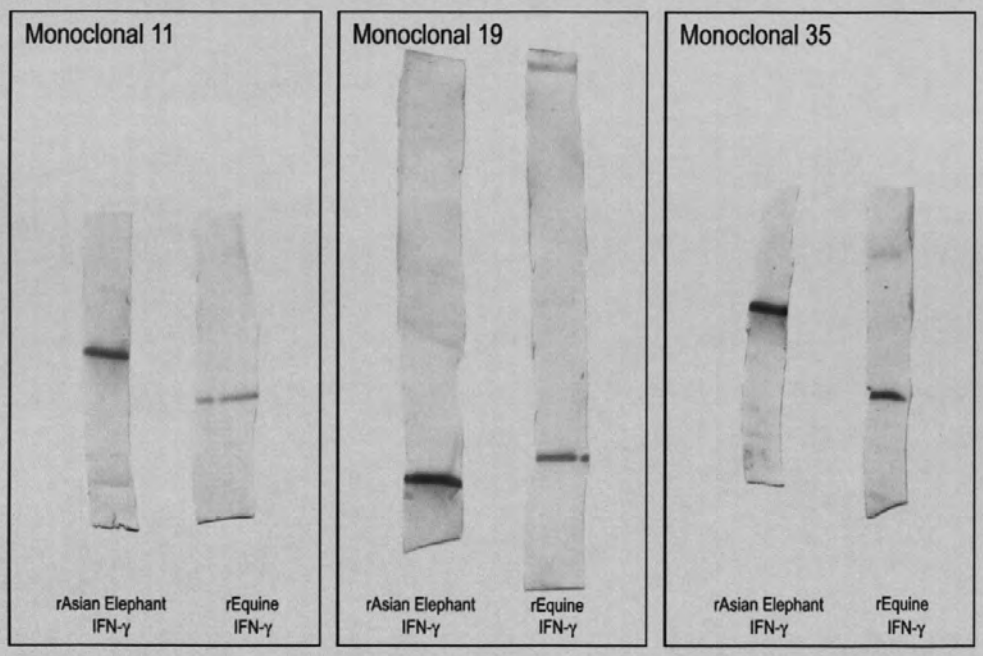


Figure 4.9 Western blots against rAsEpIFN-γ and rEqIFN-γ showing the results obtained using monoclonal antibodies 11, 19 and 35

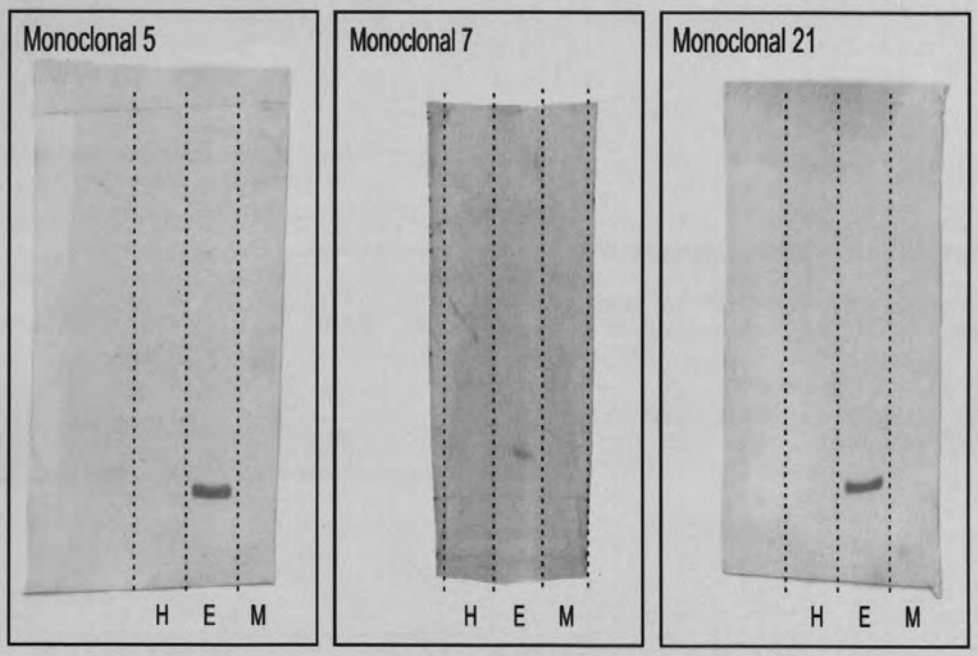


Figure 4.10 Western blots against rEqIFN-γ, rAsEpIFN-γ and rMo-GMCSF showing the results obtained using monoclonal antibodies 5, 7, and 21. H = rEqIFN-γ, E = rAsEpIFN-γ and M = rMo-GMCSF

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.

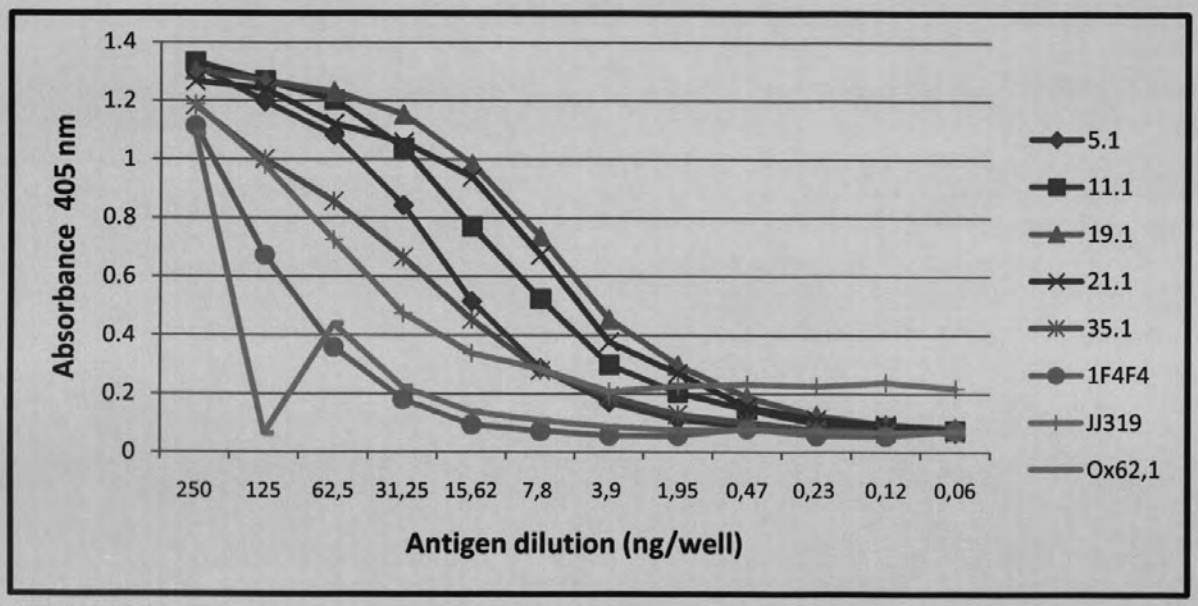


Figure 4.11 Capture ELISA using two-fold dilutions of the antigen (rAsEpIFN-γ) with a starting concentration of 250 ng/well using IgY^{uu} (0.1 µg/well) as detecting antibody. Legend on the right represents the capture antibodies (0.5 µg/well) that were used against rAsEpIFN-γ except 1F4F4, JJ319, Ox62.1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

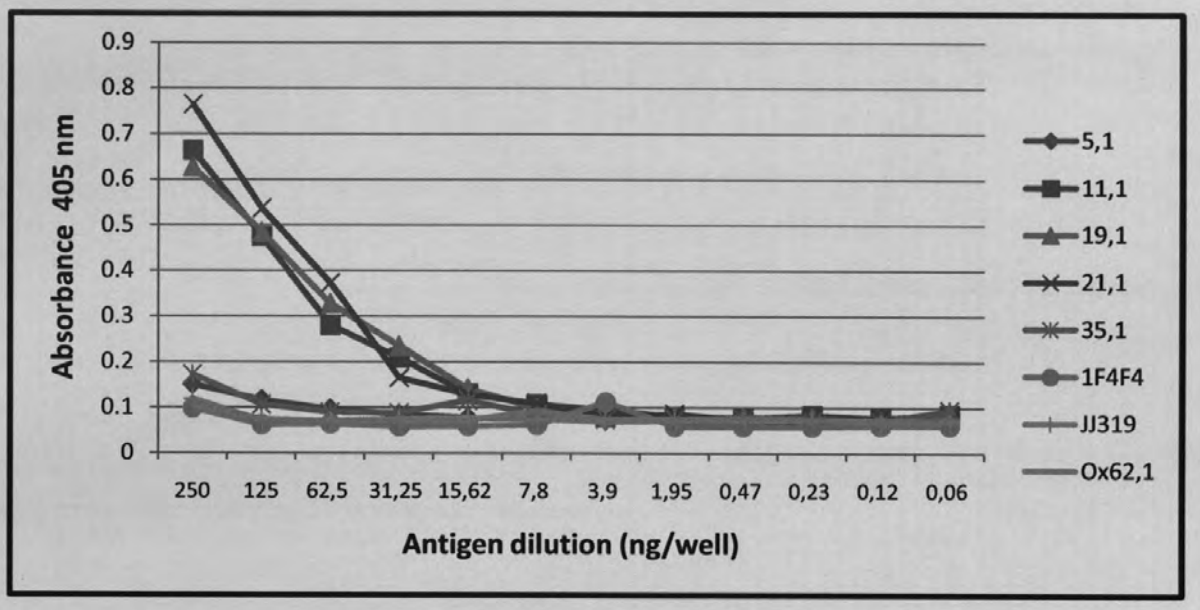


Figure 4.12 Capture ELISA using two-fold dilutions of the rAsEpIFN-γ with a starting concentration of 250 ng/well using monoclonal antibody 5 (0.1 µg/well) as detecting antibody. Legend on the right represents the capture antibodies (0.5 µg/well) that were used against rAsEpIFN-γ except 1F4F4, JJ319,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

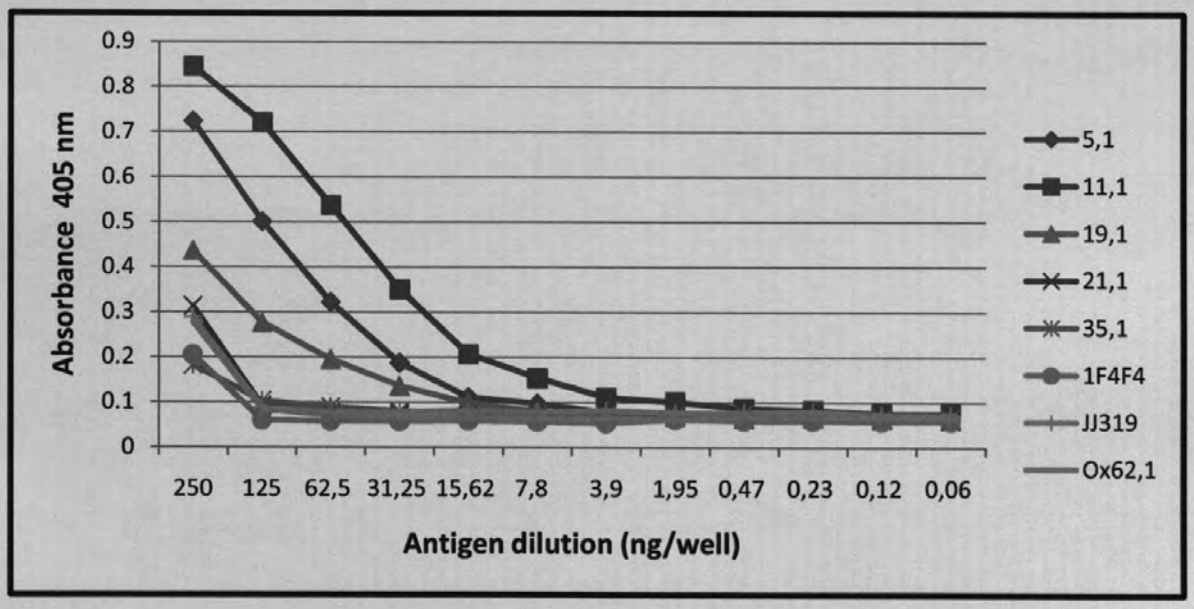


Figure 4.13 Capture ELISA using two-fold dilutions of the antigen (rAsEpIFN-γ) with a starting concentration of 250 ng/well using monoclonal antibody 19 (0.1 µg/well) as detecting antibody. Legend on the right represents the capture antibodies (0.5 µg/well) that were used against rAsEpIFN-γ except 1F4F4,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

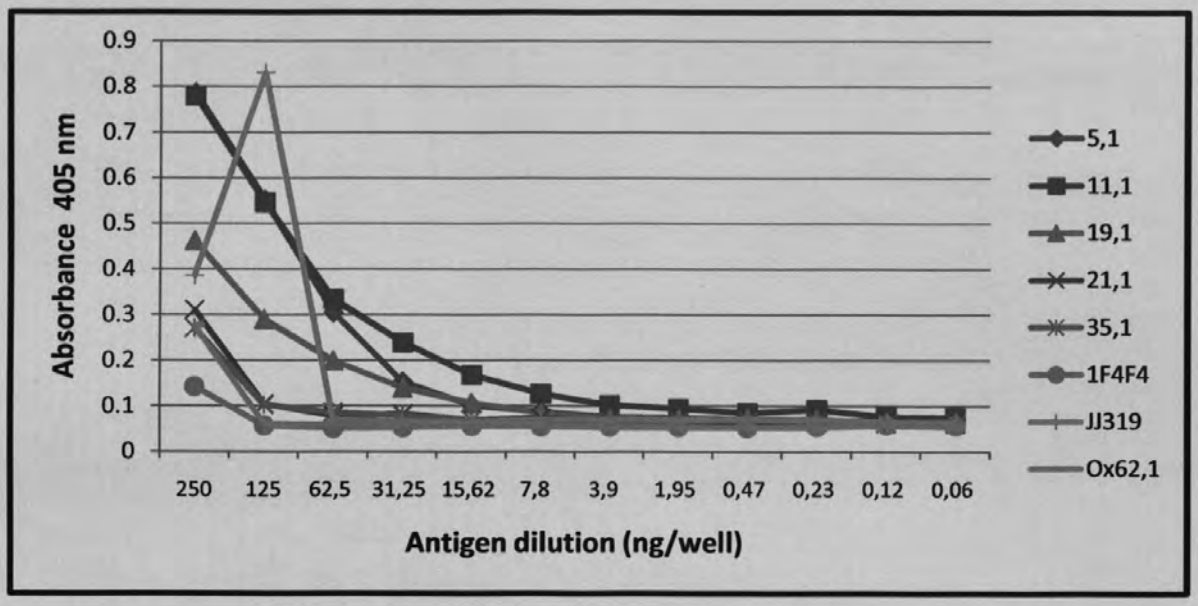


Figure 4.14 Capture ELISA using two-fold dilutions of the antigen (rAsEpIFN-γ) with a starting concentration of 250 ng/well using monoclonal antibody 35 (0.1 μg/well) as detecting antibody. Legend on the right represents the capture antibodies (0.5 μg/well) that were used against rAsEpIFN-γ except 1F4F4, 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).

4.5 References

ABDEL-GAWAD, E., IBRAHIM, S. & STEINBACH, F. 2007. Cloning of IFN-gamma, IL-4 and CD28 from equids and selected zoo animal species. *Genbank* EU000432.

ANDERSEN, P., DOHERTY, T.M., PAI, M. & WELDINGH, K. 2007. The prognosis of latent tuberculosis: can disease be predicted? *Trends in Molecular Medicine,* 13:175-182.

BALDREY, F.S.H. 1930. Tuberculosis in an Elephant. *Journal of the Royal Army Veterinary Corps,* 1:252.

BOPAYYA, A.B. 1928. Tuberculosis in an elephant. *Indian Veterinary Journal,* 142-145.

BUTLER, J.E., NI, L., NESSLER, R., JOSHI, K.S., SUTER, M., ROSENBERG, B., CHANG, J., BROWN, W.R. & CANTARERO, L.A. 1992. The physical and functional behaviour of capture antibodies adsorbed on polystyrene. *Journal of Immunological Methods,* 150:77-90.

BUTLER, J.E., NAVARRO, P. & SUN, J. 1997. Adsorption-induced antigenic changes and their significance in ELISA and immunological disorders. *Immunological Investigations,* 26:39-54.

COLLINS, D.M., RADFORD, A.J., DE LISLE, G.W. & BILLMAN-JACOBE, H. 1994. Diagnosis and epidemiology of bovine tuberculosis using molecular biological approaches. *Veterinary Microbiology,* 40:83-94.

DEAN, G.S., RHODES, S.G., COAD, M., WHELAN, A.O., COCKLE, P.J., CLIFFORD, D.J., HEWINSON, R.G. & VORDERMEIER, M. 2005. Minimum infectious dose of *Mycobacterium bovis* in cattle. *Infection and Immunity*, 73:6467-6471.

GRUNIG, G., HIMMLER, A. & ANTCZAK, D.F. 1994. Cloning and sequencing of horse interferon-gamma cDNA. *Immunogenetics,* 39:448-449.

HARRINGTON, N.P., SURUJBALLI, O.P., PRESCOTT, J.F., DUNCAN, J.R., WATERS, W.R., LYASHCJENKO, K. & GREENWALD, R. 2008. Antibody responses of cervids (*Cervus elephus*) following experimental *M. bovis* infection and the inplication for immunodiagnosis. *Clinical and Vaccine Immunology,* 1650-1658

HARR, K., ISAZA, R., RASKIN, R., BLUE, J. & HARVEY, J. 2001. Laboratory findings in *Mycobacterium tuberculosis* culture-positive Asian elephants compared to clinically normal elephants. *Veterinary Clinical Pathology,* 30:150-151.

ISAZA, R. & KETZ, C.J. 1999. A trunk wash technique for the diagnosis of tuberculosis in elephants. *Verhandlungsbericht Erkrankungen der Zootiere,* 39:121-124.

ISAZA, R. 2001. The elephant trunk wash - An update. *Elephant Managers Association Annual Conference,* Orlando, Florida.

JOHNSTON, J.M. 1981. Tuberculosis in a circus elephant. *CDC Veterinary Public Health Notes,* 16:18.

LACASSE, C., TERIO, K., KINSEL, M.J., FARINA, L.L., TRAVIS, D.A., GREENWALD, R., LYASHCHENKO, K.P., MILLER, M. & GAMBLE, K.C. 2007. Two cases of atypical mycobacteriosis caused by *Mycobacterium szulgai* associated with mortality in captive African elephants (*Loxodonta africanum*). *Journal of Zoo and Wildlife Medicine,* 38:101-107.

LARSEN, R.S., SALMAN, M.D., MIKOTA, S.K., ISAZA, R., MONTALI, R.J. & TRIANTIS, J. 2000. Evaluation of a multiple-antigen enzyme-linked immunosorbent assay for detection of *Mycobacterium tuberculosis* infection in captive elephants. *Journal of Zoo and Wildlife Medicine,* 31:291-302.

LEWERIN, S.S., OLSSON, S.L., ELD, K., RÖKEN, B., GHEBREMICHAEL, S., KOIVULA, T., KALLENIUS, G. & BOLSKE, G. 2005. Outbreak of *Mycobacterium tuberculosis* infection among captive Asian elephants in a Swedish zoo. *Veterinary Record,* 156:171-175.

LIN, P.L., YEE, J., KLEIN, E. & LERCHE, N.W. 2008. Immunological concepts in tuberculosis diagnostics for non-human primates: a review. *Journal of Medical Primatology,* 37:44-51.

LYASHCHENKO, K.P., SINGH, M., COLANGELI, R. & GENNARO, M.L. 2000. A multi-antigen print immunoassay for the development of serological diagnosis of infectious diseases. *Journal of Immunological Methods,* 242:91-100.

LYASHCHENKO, K.P., GREENWALD, R., ESFANDIARI, J., OLSEN, J.H., BALL, R., DUMONCEAUX, G., DUNKER, F., BUCKLEY, C., RICHARD, M., MURRAY, S., PAYEUR, J.B., ANDERSEN, P., POLLOCK, J.M., MIKOTA, S., MILLER, M., SOFRANKO, D. & WATERS, W.R. 2006. Tuberculosis in elephants: antibody responses to defined antigens of *Mycobacterium tuberculosis*, potential for early diagnosis, and monitoring of treatment. *Clinical and Vaccine Immunology,* 13:722-732.

MCCORRY, T., WHELAN, A.O., WELSH, J., MCNAIR, J., WALTON, E., BRYSON, D.G., HEWINSON, R.G., VORDERMEIER, H.M. & POLLOCK, J.M. 2005. Shedding of *Mycobacterium bovis* in the nasal mucus of cattle infected experimentally with tuberculosis by the intranasal and intratracheal routes. *Veterinary Record*, 157:613-618.

MICHALAK, K., AUSTIN, C., DIESEL, S., BACON, M.J., ZIMMERMAN, P. & MASLOW, J.N. 1998. *Mycobacterium tuberculosis* infection as a zoonotic disease: transmission between humans and elephants. *Emerging Infectious Diseases,* 4:283-287.

MIKOTA, S.K., LARSEN, R.S. & MONTALI, R.J. 2000. Tuberculosis in elephants in North America. *Zoo Biology,* 19:393-403.

MIKOTA, S.K., MILLER, M., DUMONCEAUX, G., MASLOW, J.N., TERREL, S.P., SOFRANKO, D. & LYASHCHENKO, K.P. 2008. National Tuberculosis Working Group for Zoo & Wildlife Species. *Guidelines for the control of tuberculosis in elephants*. Washington, D.C. United States Department of Agriculture, Animal and Plant Health InspectionService, Washington, D.C. [Online] http://www.elephantcare.org/protodoc

MOLLER, T., RÖKEN, B.O., PETERSSON, L., VITAUD, C. & LYASCHSHENKO, K. 2005. Preliminary results of a new serological test for detection of TB-infection (*Mycobacterium tuberculosis*) in elephants (*Elephas maximus* and *Loxodonta africanum*) - Swedish case studies. *Proceedings of the Institute for Zoo and Wildlife Research,* Berlin, 173-181.

MONAGHAN, M.L., DOHERTY, M.L., COLLINS, J.D., KAZDA, J.F. & QUINN, P.J. 1994. The tuberculin test. *Veterinary Microbiology,* 40:111-124.

NARAYANA, R.S.N. 1925. A case of tuberculosis in an elephant. *Journal of Comparative Pathology and Therapeutics,* 38:96-97.

NEILL, S.D., POLLOCK, J.M., BRYSON, D.B. & HANNA, J. 1994. Pathogenesis of *Mycobacterium bovis* infections in cattle. *Veterinary Microbiology*, 40:41-52.

NEILL, S.D., SKUCE, R.A. & POLLOCK, J.M. 2005. Tuberculosis - new light from an old window. *Journal of Applied Microbiology,* 98:1261-1269.

PAYEUR, J.B., JARNAGIN, J.L., MARQUARDT, J.G. & WHIPPLE, D.L. 2002. Mycobacterial isolations in captive elephants in the United States. *Annals of the New York Academy of Sciences,* 969:256-258.

PINTO, M.R.M., JAINUDEEN, M.R. & PANABOKKE, R.G. 1973. Tuberculosis in a domesticated Asiatic elephant, *Elephas maximus*. *Veterinary Record,* 93:662-664.

POLLOCK, J.M. & ANDERSEN, P. 1997. Predominant recognition of the ESAT-6 protein in the first phase of interferon with *Mycobacterium bovis* in cattle. *Infection and Immunity,* 65:2587-2592.

POLLOCK, J.M., MCNAIR, J., WELSH, M.D., GIRVIN, R.M., KENNEDY, H.E., MACKIE, D.P. & NEILL, S.D. 2001. Immune responses in bovine tuberculosis. *Tuberculosis,* 81:103-107.

POLLOCK, J.M., WELSH, M.D. & MCNAIR, J. 2005. Immune responses in bovine tuberculosis: towards new strategies for the diagnosis and control of disease. *Veterinary Immunology and Immunopathology,* 108:37-43.

RICHELDI, L., EWER, K., LOSI, M., HANSELL, D.M., ROVERSI, P., FABBRI, L.M. & LALVANI, A. 2004. Early diagnosis of subclinical multidrug-resistant tuberculosis. *Annals of Internal Medicine,* 140:709-713.

RITACCO, V., LOPEZ, B., DEKANTOR, I.N., BARRERA, L., ERRICO, F. & NADER, A. 1991. Reciprocal and cellular immune responses in BTB. *Research in Veterinary Science,* 50:365-367.

RHODES, S.G., PALMER, N., GRAHAM, S.P., BIANCO, A.E., HEWINSON, R.G. & VORDERMEIER, H.M. 2000a. Distinct response kinetics of gamma interferon and interleukin-4 in bovine tuberculosis. *Infection and Immunity*, 68:5393-5400.

RHODES, S.G., GAVIER-WIDEN, D., BUDDLE, B.M., WHELAN, A.O., SINGH, M., HEWINSON, R.G. & VORDERMEIER, H.M. 2000b. Antigen specificity in experimental bovine tuberculosis. *Infection and Immunity*, 68:2573-2578.

SARMA, K.K., BHAWAL, A., YADAV, V.K., SAIKIA, G. & JOGIRAJ, D. 2006. Investigation of tuberculosis in captive Asian elephants of Assam vis-a-vis its cross infections with the handlers. *Intas Polivet,* 7:269-274.

SAUNDERS, G. 1983. Pulmonary *Mycobacterium tuberculosis* infection in a circus elephant. *Journal of the American Veterinary Medical Association,* 183:1311-1312.

SREEKUMAR, E., JANKI, M.B., ARATHY, D.S., HARIHARAN, R., PREMRAJ, C.A. & RASOOL, T.J. 2007. Molecular characterization and expression of interferon-gamma of Asian elephants (*Elephas maximus*). *Veterinary Immunology and Immunopathology,* 118:75-83.

URBAIN, A. & DECHAMBRE, E. 1937. TB in an African Elephant. *Bulletin de la Societe de Pathologie Exotique,* 30:834-886.

WOOD, P.R., ROTHEL, J.S., MCWATERS, P.G.D. & JONES, S.L. 1990. Production and characterization of monoclonal antibodies specific for bovine gamma-interferon. *Veterinary Immunology and Immunopathology,* 25:37-46.

WOOD, P.R. & JONES, S.L. 2001. BOVIGAM™: an *in vitro* cellular diagnostic test for bovine tuberculosis. *Tuberculosis*, 81:147-155.

Chapter 5

Discussion and concluding remarks

5.1 Introduction

In many countries, despite the enormous expenditure on programmes for the control and eradication of bovine tuberculosis (BTB), it remains a serious problem (Pollock & Neill 2002), because of the persistence of infected wildlife reservoirs that have contact with cattle (Schmitt, Fitzgerald, Cooley *et al.* 1997; Corner, Stevenson, Collins & Morris 2003; Renwick, White & Bengis 2007). In reservoir hosts the infection persists through horizontal transfer in the absence of other sources of *Mycobacterium bovis*. Depending on the prevalence and the susceptibility of a species to a *M. bovis* infection, *M. bovis* may be transmitted to spill-over hosts such as chacma baboons (*Papio ursinus*), lions (*Panthera leo*), cheetahs (*Acinonyx jubatus*) and leopards (*Panthera pardus*) (Michel, Bengis, Keet *et al.* 2006; Etter, Donado, Jori *et al.* 2006). In spill-over hosts, except in primates, infections may occur sporadically or persist within these populations if a true maintenance host is present in the ecosystem (Buddle, Skinner & Chambers 2000; Etter *et al.* 2006). In the South African context the overall prevalence in cattle is low but it does occur in all provinces. However, in wildlife it is restricted to a few reserves where it occurs at high prevalence rates (Michel, Hlokwe, Coetzee *et al.* 2008).

Mycobacterium tuberculosis infections have mostly been reported in Asian elephants (*Elephas maximus*) (Mikota, Miller, Dumonceaux *et al.* 2008). It is estimated that countries in the South-East Asian Region have a 36% incidence of human TB (WHO 2008). This high prevalence makes it likely that many elephants within Asia are also infected. India is reported to have the largest burden of human TB with an estimated incidence of 1.8 million new cases annually (Steinbrook 2007; WHO 2008) and it is also home to half of all wild Asian elephants along with approximately 3 500 captive elephants (Vidya, Fernando, Melnick & Sukumar 2005; Hammattt 2007). Not only do infected humans pose a threat to these valuable animals but *M. tuberculosis*-infected elephants are potential reservoirs that could infect wildlife and humans who may come in close contact with them (Michalak, Austin, Diesel *et al.* 1998). The improper management of TB in humans is the major cause of emerging multi-drug resistant-TB (MDR-TB). Herein lies the concern for the transmission of MDR-TB from infected humans to elephants. One case has already occurred in the USA (Lyashchenko,

Greenwald, Esfandiari *et al.* 2006). No cases have been reported in Asia yet. If a new form of MDR-TB evolves in elephants it could be fatal to humans as well as elephants that may already be resistant to currently used drugs (Hammattt 2007).

5.2 Diagnosis

Ante-mortem detection of *M. bovis* and *M. tuberculosis* infections in wildlife is difficult because of the delayed appearance of clinical signs (Ashford, Whitney, Raghunathan & Cosivi 2001) and deficiencies in available diagnostic tests (de Lisle, Bengis, Schmitt & O'Brien 2002). Interferon-gamma (IFN-γ) tests are *in vitro* cell mediated assays that monitor cell responses to specific antigens, such as purified protein derivatives (PPDs) by the detection of IFN-γ produced. These *in vitro* blood tests provide a distinct advantage especially with wild animals as they are only confined once (Cousins 2008). Even in humans for whom the Mantoux reaction is well established worldwide, there is a strong tendency for its replacement by the IFN-γ assay (Pottumarthy, Morris, Harrison & Wells 1999; Herrmann, Simonney & Lagrange 2007). In children these IFN-γ tests, which make use of both the early secretory antigenic target (ESAT-6) and culture filtrate (CFP-10) proteins, are easier to perform and have a greater specificity, especially since they do not cross-react with the Bacillus Calmette-Guérin (BCG) vaccine and most other environmental bacteria (Blanc, Dubus, Garnier *et al.* 2008).

5.3 Control measures

In the African context, since no cases of *M. bovis* and / or *M. tuberculosis* infections have been reported in free-ranging or captive species of rhinoceros and African elephants (*Loxodonta africanum*), the underlying issue for diagnosis is early detection because the goal is to keep the population free of infection. In captive situations, if mycobacterial infections are not diagnosed in these animals there is the risk of infection to other rhinoceroses, elephants, and other species of animals, as well as to caretakers and visitors (Dalovisio, Stetter & Mikota-Wells 1992; Lyashchenko *et al.* 2006).

It is known that elephants can show no signs of clinical disease but may shed the organism (Lyashchenko *et al.* 2006). If these animals are diagnosed early in the course of the infection they can be isolated and specifically treated with antibiotics thus reducing the risk of

exposure to other zoo animals, handlers and visitors to the exhibits. Guidelines and protocols for the treatment of elephants are defined in the *Guidelines for the Control of Tuberculosis in Elephants* http://www.elephantcare.org/protodoc (Mikota *et al.* 2008). Starting treatment at the earliest stages of disease, before shedding, could potentially lead to improved cure rates, a decreased likelihood of the development of MDR-TB strains and reduced transmission of infection (Lyashchenko *et al.* 2006). Treatment of infected elephants with antibiotics (Maslow, Mikota, Zhu *et al.* 2005; Peloquin, Maslow, Mikota *et al.* 2006) is an expensive procedure as drugs may cost up to $50 000 to treat one elephant for one year (Hammattt 2007). The possibility of euthanasia should be considered for those animals found to have MDR-TB as they would pose a greater risk to other animals and humans with whom they come in contact if not identified and quarantined (Hammattt 2007).

Identification of antigens for use in an IFN-γ ELISA for the detection of infectious animals, which present a high risk of contamination to humans, elephants and other mammals is required (Greenwald, Lyashchenko, Esfandiari *et al.* 2009). Currently, serological tests for TB diagnostics in elephants are based on antigen cocktails, which usually detect animals in the active or advanced stages of the disease (Lyashchenko *et al.* 2006). In many of these cases the host is already exhibiting terminal disease and serology-based tests therefore do not play a significant role in defining infected hosts during the early stages of infection and have not yet proven to identify shedders. In humans serology-based tests have no value in identifying TB and it is for this reason that efforts are being put in place for IFN-γ testing by searching for appropriate antigens (Steingart, Henry, Laal *et al.* 2007). This can be achieved by testing antigens or combinations of antigens as has been done in cattle by differentiating between infected and vaccinated animals (DIVA strategy) (Rhodes, Gavier-Widen, Buddle *et al.* 2000b; Vordermeier, Whelan, Cockle *et al.* 2001; Koo, Park, Ahn *et al.* 2005). Antigens, such as, MPB70, MPB83 and PPDs (Rhodes, Buddle, Hewinson & Vordermeier 2000a) can be used for early detection. Immuno-dominant antigens ESAT-6 (Pollock & Andersen 1997) and CFP-10 can be used for the detection of active disease (Rhodes *et al.* 2000a; Koo *et al.* 2005). The IFN-γ tests established in this thesis can make use of such antigens to identify animals excreting the infectious organisms and thus posing an immediate threat to other animals.

Studies leading towards the development of real-time reverse transcription-PCR assays to quantify IFN-γ in many wildlife species (Harrington, Surujballi, Waters & Prescott 2007) and other cytokines, such as TNF-α, IL-2, IL-10 and IL-12 in Asian elephants (Landolfi, Schultz, Mikota & Terio 2009), show improvements to diagnostic techniques targeting CMI response. These tests may assist in monitoring the prevalence of disease in various wildlife populations and can be used as screening tools for animals entering disease-free zones or herds (Harinngton *et al.* 2007).

5.4 Development of an IFN-γ enzyme-linked immunosorbent assay (ELISA)

The present study demonstrates the use of different techniques towards the production of antibodies for the development of IFN-γ ELISAs for the detection of *M. bovis* and *M. tuberculosis* infections in rhinoceroses and elephants. *Mycobacterium bovis* infections are a major problem in wildlife species and the detection of pro-inflammatory cytokines (IFN-γ, TNF-α) have been used to identify animals during the early stages of infection. To date IFN-γ assays have been developed for cattle (Rothel, Jones, Corner *et al.* 1990), humans (Desem & Jones 1998) non-human primates (Vervenne, Jones, van Soolingen *et al.* 2004), cervids (Waters, Palmer, Thacker *et al.* 2008), badgers (Dalley, Davé, Lesellier *et al.* 2008) and cats (Rhodes, Gruffydd-Jones, Gunn-Moore & Jahans 2008).

For such a test to be developed and validated for rhinoceroses and elephants, much research is yet to be performed in order to define antigens which could be chosen according to the epidemiological situation and used to stimulate PBMCs and / or blood. The primary approach for rhinoceroses and African elephants in wildlife conservation areas is to provide an additional laboratory-based guarantee that documents these animals as free of *M. bovis* and *M. tuberculosis* infections. Under these conditions, it is equally important to detect infection as early as possible in these populations, in order to avoid the spread to congeners which would jeopardize any further translocation of these valuable animals, and thus putting all the conservation efforts at risk. In view of the prevalence of MTBC infection in Asian elephants the approach would lean towards detecting active disease with the use of antigens such as ESAT-6 and CFP-10. For animals in zoo collections and in circuses, the first step would be to guarantee that animals are not infected. In case of infection, it will become critical to demonstrate that animals are not contagious and to monitor this over time and / or to follow the efficacy of treatment.

A major part of this study was the production of both monoclonal and polyclonal antibodies which were used to develop different ELISA formats to detect recombinant RhIFN-γ and AsEpIFN-γ. The hybridoma technique was used to generate murine monoclonal antibodies against rRhIFN-γ (Chapter 2) and rAsEpIFN-γ (Chapter 4). Chickens were used for the production of polyclonal antibodies (Chicken IgY; Chapter 2). In Chapter 3 the phage-display technique was applied to generate chicken antibodies against recombinant RhIFN-γ and AsIFN-γ.

For production of polyclonal antibodies, chicken housing proved to be inexpensive, egg collection non-invasive, and the isolation and affinity purification of IgY antibodies showed to be fast and simple. In addition chicken IgY was used successfully in ELISA formats where it was used as a detecting antibody. Monoclonal antibody production proved to be a more expensive technique, was time consuming and required a higher level of skills to be performed. Despite these drawbacks once made they served as a constant and renewable source of antibody.

In Chapter 2 only one monoclonal antibody, 1H11, proved to be suitable for the capture of rRhIFN-γ using polyclonal IgY^{uu} as detecting antibody. The capture ELISA was used to determine whether both the recombinant and native forms of white RhIFN-γ could be detected. Results indicated that both forms of the IFN-γ can be detected in this format of the ELISA. Although the applicability of IFN-γ ELISA has been shown for native white RhIFN-γ this is not yet the case for black rhinoceroses (*Diceros bicornis*). In addition, the detection limits, sensitivity and the specificity of the test has yet to be determined in both species of rhinoceroses, and conditions such as non-specific sensitization caused by other mycobacteria potentially influencing the test outcome need to be investigated.

When the clone 1H11 was no longer secreting antibody the switch to the subclone 1D11 was made for further secretion of the required antibody. This antibody was used as a detecting antibody in ELISA formats using the single chain variable fragment (scFv) as the capture antibody (Chapter 3). Since monoclonal antibodies only recognize one epitope on any one antigen they are less likely to cross-react with other proteins and because of their specificity they are excellent as a primary antibody in an assay and will give significantly less background than polyclonal antibodies. Using the scFv and 1D11 combination (Chapter 3)

proved successful for the detection of rRhIFN-γ, but not for rAsEpIFN-γ indicating specificity of antibody 1D11 towards the rRhIFN-γ. When 1D11 was replaced with IgY as a detecting antibody in the scFv ELISA format, this polyclonal antibody detected both rRhIFN-γ and rAsIFN-γ, however background did increase.

In Chapter 4 the combination of monoclonal antibodies 21 and 5 for capture and detection of rAsEpIFN-γ worked well. Since it is preferable to include a monoclonal pair for the IFN-γ ELISA, this combination along with the 1F5/1D11 combination mentioned above, provided an ideal result as these antibodies react with different epitopes on the same antigen. Studies conducted by Dalley *et al.* (2008) indicated that IFN-γ ELISAs based on monoclonal antibody pairs were more sensitive than using polyclonal serum. In addition, they mention that it is more sustainable compared to a finite supply of polyclonal reagents. Bovigam® (Wood & Jones 2001), the human gamma-interferon assay (Desem & Jones 1998) and the gamma-interferon assay for badgers (Dalley *et al.* 2008) make use of monoclonal antibody pairs for the capture and detection of IFN-γ.

Initially the phage-display approach proved to be rather disappointing, as attempts at finding clones using the naïve semi-synthetic Nkuku® library (van Wyngaardt, Malatji, Mashau *et al.* 2004) proved unsuccessful. Various bio-panning steps were performed and each panning resulted in no enrichment. This could mean that none of the antibodies displayed in this library had an affinity towards the recombinant antigens. Consequently a phage-display immune library targeted against rRhIFN-γ was constructed which, like the hybridoma technique, necessitated the immunization of an animal. Chickens were used as a source of immunoglobulin genes to construct the antibody phage library. Previous studies (Davies, Smith, Birkett *et al.* 1995; Yamanaka, Inoue & Ikeda-Tanaka 1996; Wyngaardt *et al.* 2004) report the successful production of chicken recombinant scFvs by phage display.

Another difference between the traditional hybridoma technique and the phage-display method of generating antibodies in this study was the use of chickens instead of mice, although mice (Okamoto, Mukai, Yoshioka *et al.* 2004) have also been used to construct scFv phage-display libraries. Unlike mice, chickens have only single functional V and J segments for the heavy and light chain loci (McCormack, Tjoelker & Thompson 1991). The genes formed by these rearrangements are diversified in the bursa of Fabricus by gene conversion.

This process introduces long tracts of nucleotides from upstream V pseudogene segments into the rearranged V regions by nonreciprocal homologous recombination (Reynaud, Anquez, Grimal & Weill 1987; Thompson & Neiman 1987; Reynaud, Dahan, Anquez & Weill 1989).

Immune libraries are different from naïve semi-synthetic libraries in that they are smaller in size and therefore diversity is far less (Burton 1995) because the amplified V-region genes will already have undergone affinity maturation and clonal selection *in vivo*. However, an immune library would be biased towards the immunizing antigen and the probability of isolating binders would be much higher than using a naïve and or a semi-synthetic library in this situation. Good quality antibody phage libraries are reliable sources of useful antibodies when panned against purified antigens (Winter, Griffiths, Hawkins & Hoogenboom 1994) or even complex antigen mixtures (Marks, Ouwehand, Bye *et al.* 1993)

Since the rhinoceros and the elephant IFN-γ genes share a high nucleotide (nt) and amino acid (aa) similarity (Fig. 3.1) it provided the opportunity to pan the RhIFN-γ library against recombinant Asian elephant IFN-γ (rAsEpIFN-γ) assuming that possible binders against this antigen could also be recovered. After initial panning the polyclonal phage ELISA indicated that there was enrichment of specific binders to both the recombinant antigens (rRhIFN-γ and rAsEpIFN-γ). Single colonies were selected for screening in a monoclonal phage ELISA and binders were selected against each of the recombinant antigens. Selected clones were sequenced and showed that clones against rRhIFN-γ shared the same sequence. Similarly the clones selected against rAsEpIFN-γ showed identical sequences. The sequence of the clone against rRhIFN-γ differed when compared to the sequence of the clone identified against rAsEpIFN-γ. Direct ELISAs that were performed showed cross-reactivity between the anti-rRhIFN-γ and AsEpIFN-γ binders. The two clones (1F5 & 6D7) of different sequences, which were identified in Chapter 3, showed a higher affinity towards rRhIFN-γ. Although clone 1F5 showed an affinity to rAsEpIFN-γ, it showed a much stronger affinity to rRhIFN-γ. This point emphasizes the fact that in order to get high affinity antibodies to a specific antigen, the animal should be immunized by that antigen to elicit an immune response. By immunizing with rRhIFN-γ the response was restricted to that antigen and therefore both scFvs (1F5 & 6D7) showed a greater affinity to rRhIFN-γ than to rAsEpIFN-γ.

The end result was disappointing as only eight clones were found and only two clones did not share the same sequence. Baek, Suk, Kim & Cha (2002) mention that phagemid vector systems have very low display levels of antibody:pIII fusions, and that the possibility of useful antibodies can be missed during the selection process. Since this library was based on the above-mentioned vector system with the scFv inserted within pIII region could be a possible explanation why only two clones were isolated. Another limitation during the isolation of specific binders from the RhIFN-γ phage-display library was the concentration of the panning antigen. A concentration of 10 μg/m*l* was used during all rounds of bio-panning but only half this concentration was used during the screening. In addition, the washing stringency was maintained during all rounds of bio-panning. Selection of high affinity antibodies is favoured by increasing stringency in the washing steps after the first round and by reducing the antigen concentration during subsequent rounds of panning (Bradbury and Marks 2004).

Selection which involves two antibodies with different affinities will favour the antibody with the higher affinity. Two factors that adversely affect the selection efficiency are toxicity to the bacterial host and display levels (function of antibody folding efficiency, susceptibility to proteolysis and tendency to aggregate). It is for these reasons that if selections are continued for many rounds, diversity usually becomes reduced to a single clone (Bradbury and Marks 2004). It may be therefore better to asses diversity earlier rather (after round two or three) than later as diversity may be reduced with more bio-panning steps. Furthermore the result also raises questions regarding the quality of the immune library that was constructed. It could mean that the immune library was of a poor quality since it failed to yield a larger number of clones. It is worth noting that less than a 100 clones were screened. If more clones were screened the possibility of finding a greater number of clones could have increased.

Although only two antibodies were isolated using the phage-display approach, it did prove to be advantageous as it was cost effective and not as time consuming as the conventional hybridoma method. The antibodies produced have the potential to be manipulated to improve their specificities for use in diagnostic tests. These antibodies were rapidly derived and characterized using standard molecular techniques. With the hybridoma approach whole antibodies (IgG molecules) were derived whereas with the phage-display library approach fragments of antibodies (scFvs) were derived and thus could be expressed in *E. coli*. The

scFv format is better tolerated by bacteria, less likely to be degraded, can form dimers (diabodies), is a single protein molecule and the $(Gly_4Ser)_3$ linker can be shortened. However, they are less stable than IgG molecules, and a fraction of expressed scFv can be non-functional (Bradbury & Marks 2004). The scFvs isolated in this study have to be tested for stability since they are going to be used as potential diagnostic reagents. This will be performed as outlined by Chiliza, van Wyngaardt & du Plessis (2008).

The detection limits of the capture ELISAs developed in this study do not compare favourably with those already established for commercial cattle and primate IFN-γ capture ELISAs, and thus need to be optimised further. However, they do provide a first test to optimise the ELISA for the detection of native IFN-γ of Asian elephants. Since the AfEpIFN-γ gene has been shown to share a high homology on both the nt and aa level (Chapter 4) a single test would most likely serve for the detection of mycobacterial infections in both species. In addition, scFv antibodies will be tested in combination with those antibodies produced against rAsEpIFN-γ (Chapter 4) in order to improve the analytical and diagnostic sensitivity of these IFN-γ tests. Possible ways of increasing the sensitivity of the ELISAs include the incubation of the various steps at room temperature and comparison with that of ELISAs performed at 37°C, increasing the stringency of the wash steps, conjugation of the detecting antibodies with HRP and also substitution of the substrate, OPD, with tetramethylbenzidine (TMB) substrate. The latter has been used in IFN-γ detection ELISAs for cattle (Rothel *et al.* 1990), humans (Desem & Jones 1998) and badgers (Dalley *et al.* 2008).

The development of the IFN-γ assay may be critical in antigen discovery. It is important to re-emphasise that different antigens may be used for different purposes (the OIE "fit for purpose" strategy), and thus be utilized as a tool for differentiating infected, infectious, vaccinated and non-vaccinated animals as well as latent carriers. Furthermore by identifying target antigens and using them to determine correlates of disease severity, the assay could help in predicting the success or failure of vaccination studies (Hope & Villarreal-Ramos 2007) and of antibiotic treatment.

The field application of the IFN-γ test in wildlife settings will prove challenging as blood samples have to be processed within 8 hours of collection (Rothel *et al.* 1990; Schiller,

Waters, Vordermeier *et al.* 2009). In addition, other factors such as pre- and post-collection parameters will affect the accuracy of the IFN-γ test in field scenarios. Schiller *et al.* (2009) address such concerns regarding the whole blood IFN-γ assay in cattle. These findings, although established for cattle, will assist in improving the IFN-γ tests developed in the present study and further work linked to the validation of these ELISAs.

5.5 Conclusion

The overall endeavour of this study was to make use of molecular- and immunology-based techniques to develop a capture ELISA for the detection of IFN-γ in wildlife species, such as rhinoceroses and elephants. The diagnostic tools developed in the study have resulted in the set up of various capture ELISAs that will contribute to the establishment of *ante-mortem* diagnostic tests for the detection of *M. bovis* and *M. tuberculosis* infections in both rhinoceroses and elephants. These tools may be used in parallel and complementary to existing serological tests and thus contribute to the management and control of mycobacterial infections in these four species. It is anticipated that the IFN-γ test for TB / BTB diagnostics for rhinoceroses and elephants will prove to be advantageous in detecting not only infected animals but also infectious animals (shedders). Future research requires the detection of native IFN-γ of both rhinoceroses and elephants, using the tools generated in this study and the identification of antigens that can be used in the IFN-γ ELISA for the detection of infectious animals before the onset of clinical signs. Once this is established, steps can be taken to validate the diagnostic test with samples of known TB and BTB positive animals, and subsequently the sensitivity and specificity of the test may be determined. When this is achieved it will provide a management tool for not only screening, detection and diagnosis of *M. bovis* and *M. tuberculosis* infections in rhinoceroses and elephants, and also the identification of infectious animals.

5.6 References

ASHFORD, D.A., WHITNEY, E., RAGHUNATHAN, P. & COSIVI, O. 2001. Epidemiology of selected mycobacteria that infect humans and other animals. *Revue Scientifique et Technique,* 20:325-337.

BAEK, H., SUK, K-H., KIM, Y-H. & CHA, S. 2002. An improved helper pahge system for efficient isolation of specific antibody molecules in phage display. *Nucleic Acids Research,* 30:*e*18.

BLANC, P., DUBUS, J.C., GARNIER, J.M., BOSDURE, E. & MINODIER, P. 2008. Utility of interferon gamma assays for diagnosis of tuberculosis in children. *Archives de Pédiatrie,* 15:75-82.

BRADBURY, A.R. & MARKS, J.D. 2004. Antibodies from phage antibody libraries. *Journal of Immunological Methods,* 290:29-49.

BUDDLE, B.M., SKINNER, M.A. & CHAMBERS, M.A. 2000. Immunological approaches to the control of tuberculosis in wildlife reservoirs. *Veterinary Immunology and Immunopathology*, 74:1-16.

BURTON, D. 1995. Phage display. *Immunotechnology*, 1:87-94.

CHILIZA, T.E., VAN WYNGAARDT, W. & DU PLESSIS, D.H. 2008. Single-chain antibody fragments froma display library derived from chickens immunized with a mixture of parasite and viral antigens. *Hybridoma,* 27:413-421.

CORNER, L.A., STEVENSON, M.A., COLLINS, D.M. & MORRIS, R.S. 2003. The re-emergence of *Mycobacterium bovis* infection in brushtail possums (*Trichosurus vulpecula*) after localised possum eradication. *New Zealand Veterinary Journal,* 51:73-80.

COUSINS, D.V. 2008. Bovidae - Bovine tuberculosis, in *Manual of diagnostic tests and vaccines for terrestrial animals - Summary,* edited by *Office International des Épizooties*: 683-697.

DALLEY, D., DAVÉ, D., LESELLIER, S., PALMER, S., CRANSHAW, T., HEWINSON, R.G. & CHAMBERS, M. 2008. Development and evaluation of a gamma-interferon assay for tuberculosis in badgers (*Meles meles*). *Tuberculosis*, 88:235-243.

DALOVISIO, J.R., STETTER, M. & MIKOTA-WELLS, S. 1992. Rhinoceros' rhinorrhea: cause of an outbreak of infection due to airborne *Mycobacterium bovis* in zookeepers. *Clinical Infectious Diseases,* 15:598-600.

DAVIES, E.L., SMITH, J.S., BIRKETT, C.R., MANSER, J.M., ANDERSON-DEAR, D.V. & YOUNG, J.R. 1995. Selection of specific phage-display antibodies using libraries derived from chicken immunoglobulin genes. *Journal of Immunological Methods,* 186:125-135.

DE LISLE, G.W., BENGIS, R.G., SCHMITT, S.M. & O'BRIEN, D.J. 2002. Tuberculosis in free-ranging wildlife: detection, diagnosis and management. *Revue Scientifique et Technique,* 21:317-334.

DESEM, N. & JONES, S.L. 1998. Development of a human gamma interferon enzyme immunoassay and comparison with tuberculin skin testing for detection of *Mycobacterium tuberculosis* infection. *Clinical and Diagnostic Laboratory Immunology,* 5:531-536.

ETTER, E., DONADO, P., JORI, F., CARON, A., GOUTARD, F. & ROGER, F. 2006. Risk analysis and bovine tuberculosis, a re-emerging zoonosis. *Annals of New York Academy of Sciences*, 1081:61-73.

GREENWALD, R., LYASHCHENKO, O., ESFANDIARI, J., MILLER, M., MIKOTA, S., OLSEN, J.H., BALL, R., DUMONCEAUX, G., SCHMITT, D., MOLLER, T, PAYEUR, J.B., HARRIS, B., SOFRANKO, D., WATERS, W.R & LYASHCHENKCO, K.P. 2009. Highly accurate antibody assays for early and rapid detection of tuberculosis in African and Asian elephants. *Clinical Vaccine Immunology*, 16:605-612.

HAMMATTT, H. 2007. Elephant Care International: TB Initiative. *Meeting of Zoos and Aquariums Committed to Conservation*:1-30.

HARRINGTON, N.P., SURUJBALLI, O.P., WATERS, W.R. & PRESCOTT, J.F. 2007. Development and evaluation of a real-time reverse transcription-PCR assay for quantification of gamma interferon mRNA to diagnose tuberculosis in multiple animal species. *Clinical and Vaccine Immunology*, 14:1563-1571.

HERRMANN, J.L., SIMONNEY, N., LAGRANGE, P.H. 2007. Advantages and drawbacks of *in vitro* interferon-gamma / T cell assays compared to the Mantoux test for the diagnosis of tuberculosis. *Archives de Pediatrie,* 14:207-211.

HOPE, J.C. & VILLARREAL-RAMOS, B. 2008. Bovine TB and the development of new vaccines. *Comparative Immunology, Microbiology and Infectious Diseases*, 21:77-100.

KOO, H.C., PARK, Y.H., AHN, J., WATERS, W.R., PALMER, M.V., HAMILTON, M.J., BARRINGTON, G., MOSAAD, A.A., PARK, K.T., JUNG, W.K., HWANG, I.Y., CHO, S.N., SHIN, S.J. & DAVIS, W.C. 2005. Use of rMPB70 protein and ESAT-6 peptide as antigens for comparison of the enzyme-linked immunosorbent, immunochromatographic, and latex bead agglutination assays for serodiagnosis of bovine tuberculosis. *Journal of Clinical Microbiology,* 43:4498-4506.

LANDOLFI, J.A., SCHULTZ, S.A., MIKOTA, S.K. & TERIO, K.A. 2009. Development and validation of cytokine quantitive, real-time RT-PCR assays for characterization of Asian elephant immune responses.

LYASHCHENKO, K.P., GREENWALD, R., ESFANDIARI, J., OLSEN, J.H., BALL, R., DUMONCEAUX, G., DUNKER, F., BUCKLEY, C., RICHARD, M., MURRAY, S., PAYEUR, J.B., ANDERSEN, P., POLLOCK, J.M., MIKOTA, S., MILLER, M., SOFRANKO, D. & WATERS, W.R. 2006. Tuberculosis in elephants: antibody responses to defined antigens of *Mycobacterium tuberculosis*, potential for early diagnosis, and monitoring of treatment. *Clinical and Vaccine Immunology*, 13:722-732.

MARKS, J.D., OUWEHAND, W.H., BYE, J.M., FINNERN, R., GORICK, B.D., VOAK, D., THORPE, S.J., HUGHES-JONES, N.C. & WINTER, G. 1993. Human antibody fragments specific for human blood group antigens from a phage-display library. *Biotechnology*, 11: 1145–1149.

MASLOW, J.N., MIKOTA, S.K., ZHU, M., ISAZA, R., PEDDIE, L.R., DUNKER, F., PEDDIE, J., RIDDLE, H. & PELOQUIN, C.A. 2005. Population pharmacokinetics of isoniazid in the treatment of *Mycobacterium tuberculosis* among Asian and African elephants (*Elephas maximus* and *Loxodonta africanum*). *Journal of Veterinary Pharmacology and Therapeutics,* 28:21-27.

MCCORMACK, W.T., TJOLKER, L.W. & THOMPSON, C.B. 1991. Avian B-cell development: generation of an immunoglobulin repertoire by gene conversion. *Annual Review of Immunology*, 9:219-241.

MICHALAK, K., AUSTIN, C., DIESEL, S., BACON, M.J., ZIMMERMAN, P. & MASLOW, J.N. 1998. Mycobacterium tuberculosis infection as a zoonotic disease: transmission between humans and elephants. *Emerging Infectious Diseases*, 4:283-287.

MICHEL, A.L., BENGIS, R.G., KEET, D.F., HOFMEYR, M., DE KLERK, L.M., CROSS, P.C., JOLLES, A.E., COOPER, D., WHYTE, I.J., BUSS, P. & GODFROID, J. 2006. Wildlife tuberculosis in South African conservation areas: implications and challenges. *Veterinary Microbiology*, 112:91-100.

MICHEL, A.L., HLOKWE, T.M., COETZEE, M.L., MARÉ, L. CONNOWAY, L., RUTTEN, V.P.M.G. & KREMER, K. 2008. High *Mycobacterium bovis* genetic diversity in a low prevalence setting. *Veterinary Microbiology,* 126:151-159.

MIKOTA, S.K., MILLER, M., DUMONCEAUX, G., MASLOW, J.N., TERREL, S.P., SOFRANKO, D. & LYASHCHENKO, K.P. 2008. National Tuberculosis Working Group for Zoo & Wildlife Species. *Guidelines for the control of tuberculosis in elephants*. Washington, D.C. United States Department of Agriculture, Animal and Plant Health InspectionService, Washington, D.C. [Online] ww.aphis.usda.gov/ac/Elephant/TB2003.pdf.

OKAMOTO, T., MUKAI, Y., YOSHIOKA, Y., SHIBATA, H., KAWAMURA, M., YAMAMOTO, Y., NAKAGAWA, S., KAMADA., H., HAYAKAWA., T., MAYUMI, T. & TSUTSUMI., Y. 2004. Optimal construction of non-immune scFv phage display libraries from mouse bone marrow and spleen established to select specific scFvs efficiently binding to antigen. *Biochemical and Biophysical Research Communications*, 323:583-591.

PELOQUIN, C.A., MASLOW, J.N., MIKOTA, S.K., FORREST, A., DUNKER, F., ISAZA, R., PEDDIE, L.R., PEDDIE, J. & ZHU, M. 2006. Dose selection and pharmacokinetics of rifampicin in elephants for the treatment of tuberculosis. *Journal of Veterinary Pharmacology and Therapeutics,* 29:581-585.

POLLOCK, J.M. & ANDERSEN, P. 1997. The potential of the ESAT-6 antigen secreted by virulent mycobacteria for specific diagnosis of tuberculosis. *Journal of Infectious Diseases,* 175:1251-1254.

POLLOCK, J.M. & NEILL, S.D. 2002 *Mycobacterium bovis* infection and tuberculosis in cattle. *Veterinary Journal,* 163:115-127.

POTTUMARTHY, S., MORRIS, A.J., HARRISON, A.C. & WELLS, V.C. 1999. Evaluation of the tuberculin gamma interferon assay: potential to replace the Mantoux skin test. *Journal of Clinical Microbiology,* 37:3229-3232.

RENWICK, A.R., WHITE, P.C. & BENGIS, R.G. 2007. Bovine tuberculosis in southern African wildlife: a multi-species host-pathogen system. *Epidemiology and Infection,* 135:529-540.

REYNAUD, C.A, ANQUEZ, V., GRIMAL, H. & WEILL, J.C. 1987. A hyperconversion mechanism generates the chicken light chain preimmune repertoire. *Cell,* 48:379–88.

REYNAUD, C.A., DAHAN, A., ANQUEZ, V. & WEILL, J.C. 1989. Somatic hyperconversion diversifies the single V_H gene of the chicken with a high incidence in the D region. *Cell,* 59:171-183.

RHODES, S.G., BUDDLE, B.M., HEWINSON, R.G. & VORDERMEIER, H.M. 2000a. Bovine tuberculosis: immune responses in the peripheral blood and at the site of active disease. *Immunology,* 99:195-202.

RHODES, S.G., GAVIER-WIDEN, D., BUDDLE, B.M., WHELAN, A.O., SINGH, M., HEWINSON, R.G. & VORDERMEIER, H.M. 2000b. Antigen specificity in experimental bovine tuberculosis. *Infection and Immunity,* 68:2573-2578.

RHODES, S.G., GRUFFYDD-JONES, T., GUNN-MOORE, D. & JAHANS, K. 2008. Interferon-gamma test for feline tuberculosis. *Veterinary Record*, 162:453-455.

ROTHEL, J.S., JONES, S.L., CORNER, L.A., COX, J.C. & WOOD, P.R. 1990. A sandwich enzyme immunoassay for bovine interferon-gamma and its use for the detection of tuberculosis in cattle. *Australian Veterinary Journal*, 67:134-137

SCHILLER, I., WATERS, W.R., VORDERMEIER, H.M., NONNECKE, B., WELSH, M., KECK, N., WHELAN, A., SIGAFOOSE, T., STAMM, C., PALMER, M., THACKER, T., HARDEGGER, R., MARG-HAUFE, B., RAEBER, A. & OESCH, B. 2009. Optimization of a whole-blood gamma interferon assay for detection of *Mycobaterium bovis*-infected catlle. *Clinical and Vaccine Immunology*, 16:1196-1202.

SCHMITT, S.M., FITZGERALD, S.D., COOLEY, T.M., BRUNING-FANN, C.S., SULLIVAN, L., BERRY, D., CARLSON, T., MINNIS, R.B., PAYEUR, J.B. & SIKARSKIE, J. 1997. Bovine tuberculosis in free-ranging white-tailed deer from Michigan. *Journal of Wildlife Diseases,* 33:749-758.

STEINBROOK, R. 2007. Tuberculosis and HIV in India. *New England Journal of Medicine*, 356:1198-1199.

STEINGART, K.R., HENRY, M., LAAL, S., HOPEWELL, P.C., RAMSAY, A., MENZIES, D., CUNNINGHAM, J., WELDINGH, K. & PAI, M. 2007. A systematic review of commercial serological antibody detection tests for the diagnosis of extrapulmonary tuberculosis. *Postgraduate Medical Journal*, 83:705-712.

THOMPSON, C.B. & NEIMAN, P.E. 1987. Somatic diversification of the chicken immunoglobulin light chain gene is limited to the rearranged variable gene segment. *Cell*, 48:369-378.

VAN WYNGAARDT, W., MALATJI, T., MASHAU, C., FEHRSEN, J., JORDAAN, F., MILTIADOU, D. & DU PLESSIS, D.H. 2004. A large semi-synthetic single-chain Fv phage display library based on chicken immunoglobulin genes. *BMC Biotechnology,* 4:6.

VERVENNE, R.A.W., JONES, S.L., VAN SOOLINGEN, D., VAN DER LAAN, T., ANDERSEN, P., HEIDT, P.J, THOMAS, A.W. & LANGERMANS. 2004. TB diagnosis in non-human primates: comparison of two interferon-γ assays and the skin test for identification of *Mycobacterium tuberculosis* infection. *Veterinary Immunology and Immunopathology*, 100:61-71.

VIDYA, T.N.C.; FERNANDO, P., MELNICK, D.J. & SUKUMAR, R. 2005. Population differentiation within and among Asian elephant (*Elephas maximus*) populations in Southern India. *Heredity*, 94:71-80.

VORDERMEIER, H.M., WHELAN, A., COCKLE, P.J., FARRANT, L., PALMER, N. & HEWINSON, R.G. 2001. Use of synthetic peptides derived from the antigens ESAT-6 and CFP-10 for differential diagnosis of bovine tuberculosis in cattle. *Clinical and Diagnostic Laboratory Immunology,* 8:571-578.

WATERS, W.R., PALMER, M.V., THACKER, T.C., ORLOSKI, K., NOL, P., HARRINGTON, N.P., OLSEN, S.C. & NONNECKE, B.J. 2008. Blood culture and stimulation conditions for the diagnosis of tuberculosis in cervids by the Cervigam assay. *Veterinary Record,* 162:203-208.

WINTER, G., GRIFFITHS, A.D., HAWKINS, R.E. & HOOGENBOOM, H.R. 1994. Making antibodies by phage display technology. *Annual Review of Immunology*, 12:433–455.

WOOD, P.R. & JONES, S.L. 2001. BOVIGAM™: an *in vitro* cellular diagnostic test for bovine tuberculosis. *Tuberculosis,* 81:147-155.

WORLD HEALTH ORGANIZATION. 2008. Tuberculosis in South-East Asia Region, the regional report: 2008. *WHO Report No.: SE ICP TUB* 1-75.

YAMANAKA, H.I., INOUE, T. & IKEDA-TANAKA, O. 1996. Chicken monoclonal antibody isolated by a phage display system. *Journal of Immunology*, 157:1156-1162.