Cellular immune responses induced *in vitro* by secreted proteins of *Ehrlichia ruminantium*

by

NONTOBEKO THEMA

OCTOBER 2008

Submitted in partial fulfillment of the requirements for the degree

Master of Science in the Faculty of Veterinary Science Department of Tropical Diseases

University of Pretoria

Declaration

I hereby declare that this dissertation submitted by me to the University of Pretoria for the degree Master of Science has not previously been submitted for a degree at any other University

Acknowledgements

I wish to express my gratitude to Dr Mirinda van Kleef my promoter for her guidance, patience, encouragement, valuable advice during my research and the writing of the dissertation. My thanks also go to Dr Alri Pretorius my co-promoter for her interest, valuable assistance, motivation, and her patience during the preparation of the research protocol, collection of data and the writing of the dissertation.

I would like to thank Ms Junita Liebenberg for her bioinformatics expertise and identification of vaccine candidates. I am most grateful to Ms Erika Faber for her guidance with the real-time PCR technique.

I would like to thank Ndavhe Tshikhudo for his valuable assistance with the ELISPOT assay. I would also like to express my gratitude to Ms Helena Steyn and Ms Ivy Sebatjane for their support during my studies. I am also grateful to Dr Erich Zweygarth and Antoinette Josemans for providing culture material for the purification of *Cowdria* elementary bodies and extraction of DNA.

I would like to thank the Cellular Immunology Section for their valuable assistance, for providing a positive, stimulating and happy environment during my studies. My gratefulness goes to DST students for their encouragement during my studies.

A special word of thanks to Agricultural Research Council-Onderstepoort Veterinary Institute for giving me the opportunity to be part of this excellent research institute.

I am grateful to the Department of Science and Technology and National Research Foundation for the grant that made this work possible.

Finally a special word of thanks to my husband Robert Thema and my kids for their patience, understanding, love, support and encouragement.

Table of contents

List of Tables

List of Figures

Abbreviations

APC	antigen presenting cells
A_{260}	absorbance at 260 nm
BA	bovine aorta endothelial cells
bp	base pair
BRV	bovine rotavirus
BSA	bovine serum albumin
CF-10	culture filtrate protein, 10 kDa
CMI	cell mediated immune response
cpm	counts per minute
CTL	cytotoxic T lymphocytes
CTLpred	cytotoxic T lymphocytes prediction
ºC	degrees Celsius
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
EBs	elementary bodies
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
ELISPOT	enzyme linked immunospot
ESAT6	early secreted antigen target 6 kDa
FCS	fetal calf serum
g	grams
GAPDH	glyceraldehyde-3-phosphate Dehydrogenase
GFP	green fluorescent protein
h	hour
HBSS	hank's balanced salt solution
HRV	human rotavirus
IAA	Indole 3 acetic acid
IFA	indirect immunofluorescence assay
IFN-γ	interferon gamma
IL-4	interleukin-4

IPTG	isopropyl-β-D-thiogalactopyranoside
kDa	kilodalton
kg	kilogram
LB	Luria Bertani
LPA	lymphocyte proliferation assay
LTR	long tandem repeats
MAP	major antigenic protein
MAPPP	MHC-I antigenic peptide processing prediction
MHC	major histocompatibility complex
MIC2	microneme protein
min	minutes
ml	milliliters
mM	millimolar
mRNA	messenger RNA
µg	micro gram
NO	nitric oxide
ORF	open reading frame
Ovar-DRB1	ovine major histocompatibility complex class II DRB1
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline supplemented with 0.05% (v/v) Tween-20
PCR	polymerase chain reaction
PCR-RFLP	polymerase chain reaction-restriction fragment length polymorphism
r	recombinant
RNA	ribonucleic acid
s	sheep
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SI	stimulation index
TB	tuberculosis

TE	tris ethylenediaminetetraacetic acid
Th	T helper
TNF	tumor necrosis factor
TRX	Thioredoxin
U2	urine protein 2

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Cellular immune responses induced *in vitro* by secreted proteins of *Ehrlichia ruminantium*

by

NONTOBEKO THEMA

PROMOTER: Dr Mirinda van Kleef

COPROMOTER: Dr Alri Pretorius

DEPARTMENT: Department of Veterinary Tropical Diseases, University of Pretoria

DEGREE: MSc

ABSTRACT

Ehrlichia ruminantium is an obligate intracellular pathogen and, as such, cell mediated immunity plays a key role in the control of bacterial replication and subsequent protection against heartwater in ruminants. IFN-γ has been shown to be a potent inhibitor of *E. ruminantium* growth in endothelial cells *in vitro*. Thus, identification of antigens that preferentially activate T cells to proliferate and secrete IFN-γ needs to be done so that they can be evaluated as vaccine candidates. Previously, a cocktail of four open reading frames (ORFs) that induce 100% protection after needle challenge have been identified. However, only 20% protection was obtained after tick challenge in the field, when administered as a DNA vaccine in sheep. Because only limited protection was obtained during a field vaccine trial, our research focused on the identification of additional ORFs as a mean to improve the efficacy of this vaccine. Because secreted proteins are reported to be major targets in a specific immune response we hypothesize that they may be potential heartwater vaccine candidates. Five ORFs (Erum5000, Erum5010, Erum7760, Erum8060 and Erum8610) encoding secreted *E. ruminantium* proteins were selected from the Welgevonden stock genome sequence using

bioinformatics tools. The ORFs were cloned into a pET 102/D-TOPO® vector. The corresponding recombinant (r) proteins were expressed in a bacterial expression system and the expression was confirmed by immunoblots using anti-His antibodies and sheep sera. Proteins were purified by immobilized metal ion affinity chromatography and resulted in a yield of 200 µg-2000 µg per 100 ml and 1L cultures respectively. Four of the five recombinant proteins (rErum5000, rErum7760, rErum8060 and rErum8610) could be expressed. Recombinant proteins were assayed to determine whether they induce recall cellular immune responses *in vitro.* The peripheral blood mononuclear cells used in the assays were obtained from a naïve and four heartwater immune sheep. Significant proliferative responses ($p \leq 0.01$) were evident for 3/4 recombinant proteins (rErum5000, rErum8060 and rErum8610). IFN-γ production was determined using an ELISPOT assay and 3/4 recombinant proteins (rErum5000, rErum8060 and rErum8610) induced IFN-γ production. Each recombinant protein had its own optimum concentration for inducing immune responses and the responses differed between animals. In addition, real-time PCR was used to measure IFN-γ and IL-4 gene expression by antigen stimulated immune PBMC. The real-time PCR results correlated with the ELISPOT assay results. Based on the results obtained, it can be concluded that a Th1 type immune response was elicited. Thus these proteins that induced proliferation and IFN-γ production may be important in protection against heartwater and will be tested in future vaccine studies.

CHAPTER 1

LITERATURE REVIEW

1.1. INTRODUCTION

Heartwater is a disease of cattle, sheep, goats and wild ruminants and is caused by an intracellular rickettsia previously known as *Cowdria ruminantium* (Moshkovski, 1947) and recently reclassified as *Ehrlichia ruminantium* (Dumler et al., 2001). It is a non-contagious disease characterized by high fever, hydrothorax, hydropericardium and severe nervous disorders (reviewed by Van de Pypekamp and Prozesky, 1987). Heartwater affects sub-Saharan Africa and the Caribbean where it is transmitted by ticks of the genus *Amblyomma*.

Heartwater was considered the most important endemic disease of livestock in South Africa in the 1830's (reviewed by Provost and Bezuidenhout, 1987) and has been believed to cause a serious threat to livestock development in the African continent (reviewed by Yunker, 1996). The exact economic impact of heartwater is difficult to determine because the disease is under-reported (Camus and Barré, 1982). The Deputy Director of Veterinary Services of the Eastern Cape Province of South Africa estimated that 10 percent of all stock losses, costing up to US$30 million annually were due to heartwater. This was despite an annual expenditure of between US$ 1 and 5 million for prophylaxis and vaccination procedures (Axsell, 1998). Losses from and control of heartwater is estimated to be U.S. $5.6 million per year in Zimbabwe (Mukhebi et al., 1999).

The commercial vaccine that is available against heartwater is a live blood vaccine requiring antibiotic treatment to prevent a serious course of the disease (Uilenburg, 1983; Van der Merwe, 1987). This vaccine must be kept frozen, administered intravenously, causes loss of vaccinated animals due to its virulence and does not protect against all isolates (reviewed by Oberem and Bezuidenhout, 1987). Furthermore, animals need to be under careful supervision for temperature monitoring because once a febrile reaction develops, the animals need to be treated with tetracycline. The implementation of such intensive monitoring is time consuming and

labour intensive. Therefore, the development of a recombinant vaccine against heartwater will offer an inexpensive and effective solution leading to economic and social benefits to rural and peri-urban communities and commercial farming. Heartwater is thus a major problem and it will be of major importance until an effective and safe vaccine becomes available.

1.2. ORGANISM

Previously *C. ruminantium* was classified as the only species in the genus *Cowdria*, tribe *Ehrlichiae*, family *Rickettsiaceae* and order *Rickettsiales* (Ristic and Huxoll, 1984). It was recently moved to the family Anaplasmataceae, genogroup III, order Rickettsiales and renamed *E. ruminantium* (Dumler et al., 2001). Ehrlichiae are small, gram-negative cocci that stain dark blue to purple with the Romanowsky stain (Aguero-Rosenfeld, 2003) and purplish-blue with Giemsa (Cowdry, 1926). The organism is considered an intracellular parasite that cannot survive outside a living host for more than 38 h at room temperature (Logan, 1987). These parasites are found in host membrane-bound vacuoles within the cytoplasm of vascular endothelial cells (Cowdry, 1926).

1.3. DEVELOPMENTAL CYCLE

It has been demonstrated that the developmental cycle of *E. ruminantium* resembles the life cycle of Chlamydia species (Jongejan et al., 1991c; Marcellino et al., 2005). Briefly, three developmental stages have been identified in the mammalian host, namely, recticulate bodies (RBs), intermediate bodies (IBs) and elementary bodies (EBs) (Pienaar, 1970; Jongejan et al., 1991c). Recticulate bodies are defined as the non-infectious but metabolically active form whereas elementary bodies are the infectious and metabolically inactive form of *E. ruminantium*.

The infection of ruminant host cells starts when EBs enter the intracytoplasmic vacuole of an endothelial cell. EBs divide by binary fission and matures into RBs of the same size and shape. After 3 to 4 days, RBs transform into IBs and further mature into EBs. After several rounds of division, the endothelial cell disrupts and EBs are released from the host cell and the new round of infection can start (Jongejan et al., 1991c; Marcellino et al., 2005). In addition, the developmental cycle of

E. ruminantium in the tick is believed to occur after the tick has fed on heartwater infected blood. The organism's replication takes place in the tick gut and the parasite will invade the salivary gland acini cells (Hart et al., 1991). The transmission of the parasite to the ruminant host occurs while the ticks are feeding on a new host. The organisms are horizontally transmitted from infected cells in vectors to the lymph node (Du Plessis, 1975) and from there transmitted to the blood. When elementary bodies are released they invade other endothelial cells and a new infection cycle starts as described above.

1.4. TRANSMISSION

Heartwater only occurs where the vector tick of the causative organism, *E. ruminantium* is found. There are 12 *Amblyomma* species shown to transmit *E. ruminantium,* with *Amblyomma variegatum* being the most important species because it is widely distributed in sub-Saharan Africa and in the Caribbean region (reviewed by Walker and Olwage, 1987). In South Africa heartwater is transmitted by the *A. hebraeum* tick that occurs in heartwater endemic areas (Eastern Cape, KwaZulu Natal, Limpopo, Gauteng and North West) (Uilenberg, 1983; reviewed by Bezuidenhout, 1987). Ticks of genus *Amblyomma* are three-host ticks. Ticks may obtain the infection while feeding on reacting or subclinically infected hosts. Larvae will feed on a host, drop off and molt into a nymph. The nymphae will feed again on a host, drop off as in larvae to molt and become an adult. This indicates that all three stages can transmit heartwater.

1.5. HEARTWATER SYMPTOMS

Heartwater is a disease of ruminants characterized by temperature often exceeding 41ºC with an incubation period of 14-21 days before fever. Hydrothorax and hydropericardium develops and results in excessive straw colored fluid and foam due to hemorrhaging of the trachea and heart. Animals infected with heartwater exhibit severe respiratory stress and nervous disorders. A variety of abnormal nervous behaviors, such as, twitching of facial muscles, excessive chewing movements, incoordination, walking with a high-stepping gait and head tilting may occur. These symptoms are usually followed by sudden death (reviewed by Uilenberg, 1983; reviewed by van de Pypekamp and Prozesky, 1987).

1.6. DIAGNOSIS

Clinical signs in animals infected with *E. ruminantium* are not sufficient to diagnose the disease heartwater because especially nervous disorders could be related to other diseases including rabies. Furthermore, diagnosis of the disease heartwater is best done by post mortem brain smear examinations. Therefore, a convenient, reliable, safe, sensitive and specific method for diagnosis of *E. ruminantium* is vital.

Serological diagnostic assays such as IFA (Du Plessis and Malan, 1987) and ELISA (Jongejan et al., 1991b; van Vliet et al., 1995; Mboloi et al., 1999; De Waal et al., 2000) were developed in an effort to diagnose the disease at an early stage. However they only serve to determine whether animals have been exposed to heartwater and false positive results often occurs due to cross-reaction with other Rickettsiales (reviewed by Barbet et al., 1993, Du Plessis et al., 1993). Furthermore, a real-time PCR was developed in order to quantify *E. ruminantium* (Postigo et al., 2002). Amplification was done with the use of primers targeting a fragment of the major antigenic protein (*map1*-1) gene of *E. ruminantium*. The *map1*-1 gene is one of the paralog genes of *map*1 multiple gene family (Sulsona et al., 1999). The outcome of the study indicated that the real-time PCR was found to be accurate at quantifying *E. ruminantium* in blood from clinically reacting sheep. In addition, the quantification of *E. ruminantium* using real-time PCR was also performed with the use of dsDNA SYBR Green I dye and a specific amplified PCR product, *map*1 amplicon (Peixoto et al., 2005). In this experiment, only *map*1 gene was amplified and the method was validated for four *E. ruminantium* strains (Gardel, Welgevonden, Bekuy and Sara). However, the *map*1 gene is highly polymorphic and therefore may not be suitable for diagnostics and epidemiological studies (Allsopp et al., 2001b). In contrast, the pCS20 region is conserved and therefore could be effective for detecting *E. ruminantium* infection.

The pCS20 region was used to develop a PCR/DNA probe assay specifically to detect *E. ruminantium* organisms in cell cultures, ticks and ruminants (Waghela et al., 1991). The DNA probe successfully detected *E. ruminantium* in *A. variegatum* and *A. hebraeum* ticks fed on infected and clinically ill ruminants and positively identified

eight stocks from endemic areas in Zimbabwe, South Africa, Nigeria and Carribean Island of Gaudelope (Waghela et al., 1991; Mahan et al., 1992; Yunker et al., 1993). However this assay requires radioactive labeling of the probe, it is labour-intensive and time consuming (5 days). Hence a quantitative real-time PCR (TaqMan probe) assay for the detection of *E. ruminantium* in both ticks and ruminants was developed (Steyn et al., 2008). This assay is more sensitive, does not require radioactivity and can be performed within 2 h. Presently, the pCS20 real-time PCR is used as a specific assay for the detection of *E. ruminantium* in both ruminants and ticks.

1.7. E. RUMINANTIUM *GENOTYPING*

Characterization of the *E. ruminantium* genotypes in different regions is essential in order to obtain a distribution map of different isolates. The availability of genetic variation information among isolates could assist in the development of a recombinant vaccine that protects against all genotypes circulating in endemic areas of South Africa and the surrounding countries.

More than fifty cultured stocks of *E. ruminantium* have been isolated throughout sub-Saharan Africa and Caribbean islands. One of the genes which are widely used for bacterial taxonomy and classification is the 16S ribosomal RNA gene (Allsopp, et al., 1997). Phylogenetic analysis, based on the 16S rRNA gene sequences, has shown that species classified as *Ehrlichia* are separated into three paraphyletic clades (genogroups) and *E. ruminantium* is located to genogroup III (Allsopp et al., 1996; Allsopp et al., 1997; reviewed by Allsopp et al., 2004) (Figure 1.1). There are many different 16S ribosomal RNA genotypes of *E. ruminantium* that are of interest in current research, namely, Ball3, Blaauwkrans, Crystal Springs, Gardel, Kümm1, and Kümm2, Kwanyanga, Mara 87/7, Senegal and Welgevonden. Based on 16S rDNA sequences, an isolate from South Africa (Welgevonden) was found to be similar to one from Zimbabwe (Crystal Springs) while Ball 3 and Omatjenne differed both from each other and from Crystal Springs and Senegal stocks (Allsopp et al., 1997).

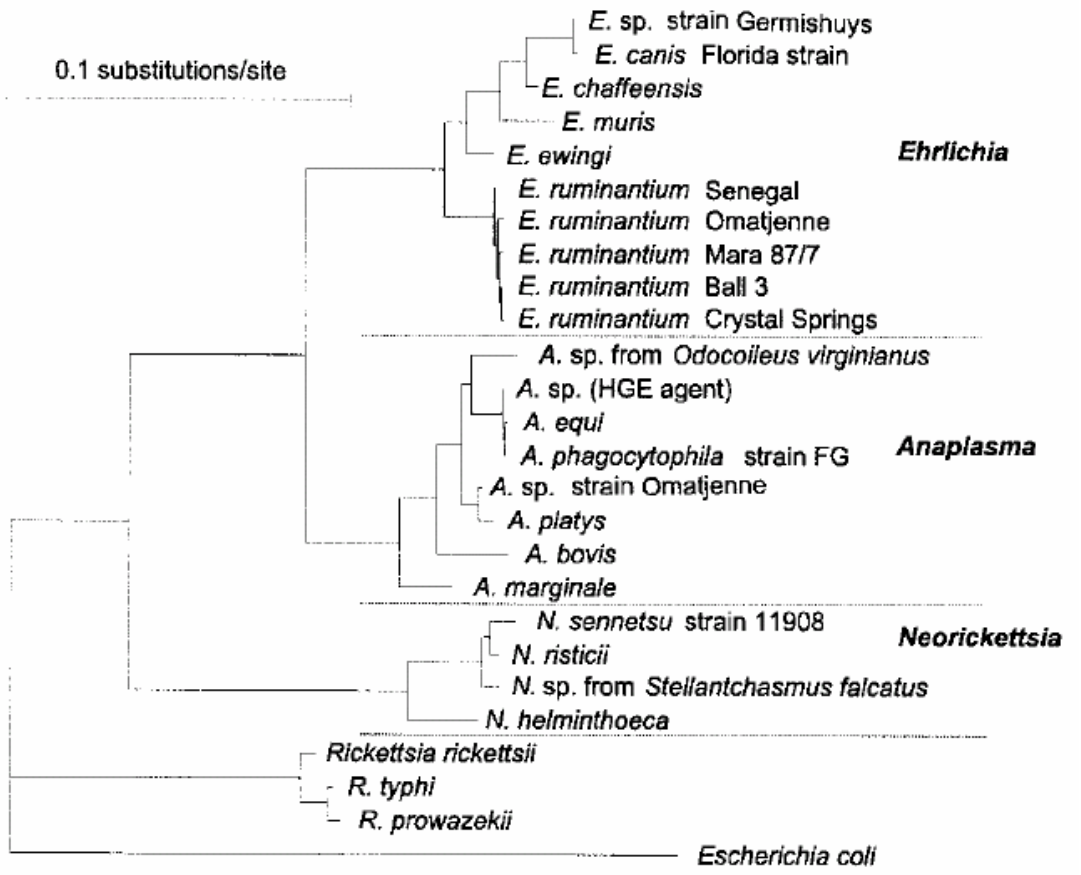


Figure 1.1. Maximum likelihood phylogenetic tree of some ehrlichial species based on comparison of 16S ribosomal RNA genes. (Allsopp and Allsopp, 2001a; reviewed by Allsopp et al., 2004).

1.8. IMMUNE MECHANISMS TO INTRACELLULAR PATHOGENS

Understanding the mechanisms behind immunology of intracellular pathogens is of increasing importance to develop vaccination strategies against a variety of established and emerging infectious diseases. There are two types of antigen specific immune responses that may be induced in response to an antigen: these are humoral and cellular immunity. Humoral immunity involves B lymphocytes and the production of antibodies, (Casadevall, 2003) and cell-mediated immunity involves $CD4^+$ T helper and $CD8^+$ T cytotoxic lymphocytes (reviewed by Johnson et al., 1994; reviewed by Guidotti et al., 1996; reviewed by Ojcius et al., 1996; reviewed by London et al., 1998; reviewed by Skapenko and Schulze-Koops, 2007). Cytotoxic $CD8^+$ T cells limit virus and bacteria replication by either killing infected cells or by releasing cytokines that mediate killing (Preston and Jongejan, 1999; Shoukry et al.,

2004; Yewdell and Haeryfar, 2005; Huster et al., 2006; reviewed by Williams and Bevan, 2007). $CD4^+$ T cells plays a critical role by providing help for priming and sustaining cytotoxic $CD8^+$ T cell responses by producing cytokines such as IFN-γ (Hoft et al., 2000; Chang et al., 2001; Bitsaktsis et al., 2004; Shoukry et al., 2004; Castellino and Germain, 2006; reviewed by Williams and Bevan, 2007).

IFN-γ is a pleotropic cytokine that plays an important role in both the innate and adaptive phases of an immune response to intracellular pathogens (reviewed by Szabo et al., 2003; reviewed by Schroder et al., 2004; reviewed by Ma et al., 2007). Different cells that secrete IFN-γ include $CD4^+$ T helper cells, cytotoxic T lymphocytes (CTL), natural killer cells and macrophages. Elevated levels of IFN-γ will activate macrophages, resulting in increased phagocytosis, increased MHC class I and II expression, and the induction of IL-12, nitric oxide and superoxide production (reviewed by Boehm, et al., 1997; Mutunga et al., 1998).

Th1 cells secrete cytokines including IFN-γ, IL-2, TNF-β and TNF-α. These cytokines are involved in the activation of macrophages, stimulate proliferation of antigen-activated lymphocytes, induce production of nitric oxide (NO) and play a role in phagocytosis. In contrast, Th2 cells produce cytokines IL-4, IL-5, IL-6 and IL-13, which are associated with the production of antibodies by stimulating proliferation and differentiation of B cells (Flynn et al., 1993; reviewed by Mosmann and Sad, 1996; reviewed by Corwin, 2000).

Cell-mediated immunity (CMI) occurs when T cells are activated after recognizing short segments of antigen (peptides) that are presented by major histocompatibility complex (MHC) class I or II molecules on the surface of antigen presenting cells (APCs) such as macrophages and dendritic cells (Guidotti et al., 1996; reviewed by Mosmann and Sad, 1996; Morrison et al., 1999; reviewed by Trombetta and Mellman, 2005). There are two pathways involved in the processing and presentation of antigens to the cell surface, the endogenous or exogenous pathway. Endogenous antigen-derived peptides from intracellular pathogens bind to MHC class I and exogenous antigen-derived peptides from extracellular pathogens bind to MHC class II. The peptide-MHC complexes are then transported to the cell surface where they can interact with T lymphocytes. Lymphocytes recognize antigens only if they are

complexed with class I or II MHC molecules and this is termed MHC restriction. They then become activated, proliferate and produce cytokines that stimulate and regulate the immune responses. $CD4^+$ and $CD8^+$ T cells interact with peptide presented by MHC class II and I respectively (Shoukry et al., 2004; reviewed by Williams and Bevan, 2007).

Several authors have presented evidence that T lymphocytes and IFN-γ play a crucial role in protection against intracellular pathogens. These include *Rickettsia conorii* (Li et al., 1987, 2003; Walker et al., 2001; de Sousa et al., 2007); *Mycobacterium tuberculosis* (Coler et al., 2001; Vesosky et al., 2006; Chang-Hong et al., 2008); *Mycobacterium avium* subspecies *paratuberculosis* (Sechi et al., 2006) and *Brucella abortus* (Luo et al., 2006).

1.9. IMMUNE RESPONSE TO E. RUMINANTIUM

Since *E. ruminantium* is an obligate intracellular rickettsial agent, CMI responses are expected to play an important role in protection (Du Plessis et al., 1982; Du Plessis et al., 1991; Du Plessis et al., 1992; Totté et al., 1997; reviewed by Totté et al., 1999). Studies in the natural hosts (cattle, sheep, and goats) and in the mouse model have confirmed that the production of IFN-γ plays an important role in protection against *E. ruminantium*. For example cattle immunized with virulent *E. ruminantium*, and treated, generated T lymphocyte responses, and heightened expression of IFN-γ (Mwangi et al., 1998). In addition, immunization of cattle with killed elementary bodies of *E. ruminantium* was characterized by Ehrlichia-specific, MHC class II-restricted, IFN-γ –producing, $CD4^+$ T lymphocytes (Totté et al., 1997). Similarly, vaccination of ruminants with killed *E. ruminantium* vaccine elicited IFN-γ responses induced by $CD8^+$ cytotoxic and $CD4^+$ helper T cells (Esteves et al., 2002; 2004a, b and c). IFN-γ has been shown to inhibit *E. ruminantium* growth in endothelial cells *in vitro* (Mahan et al., 1994; reviewed by Totté et al., 1999).

In addition to IFN-γ, there are other cytokines that are considered to be critical in attempts to understand immune responses elicited during infection. Interleukin IL-1β, IL-6 and IL-8 mRNA production was evident when bovine brain microvessel endothelial cells were infected with *E. ruminantium* (Boudourlous et al., 1995).

Moreover, IL-1 and IL-6 act as co-stimulatory signals for T-and B-cell activation (Weaver and Unanue, 1990). In addition, Totté and coworkers (1994) showed that cattle infected with *E. ruminantium* induced alpha interferon (IFN-α) that was able to slow down the infection. Moreover, nitric oxide (NO) which is produced enzymatically by activated macrophages has been shown to exert antimicrobial effects on intracellular and extracellular pathogens. These include *Listeria monocytogenes* (Boockvar et al., 1994), *Trypanosoma congolense* (Kaushik et al., 1999). It is possible that production of these cytokines could contribute to the immune response and protection against heartwater.

Several studies have demonstrated that animals immunized with killed, inactivated and/or attenuated *E. ruminantium* vaccines are protected. This suggests that the development of a recombinant vaccine against *E. ruminantium* is feasible. Studies on vaccine development for intracellular pathogens have targeted antigens that induce IFN-γ producing T lymphocytes. Both $CD4^+$ and $CD8^+$ T cells are IFN-γ-producing T lymphocytes that are required in the development of protective immunity against heartwater. Since cellular immune responses and IFN-γ have been implicated in the protection against *E. ruminantium*, it is important to identify antigens that can stimulate these responses when developing a recombinant vaccine for heartwater. For example, low molecular weight proteins of *E. ruminantium* have been identified and the evidence demonstrating their ability to induce immune responses has been established. It has been shown that these proteins induce $CD4^+$-enriched T cells from animals immunized with Welgevonden strain to proliferate and produce IFN-γ (van Kleef et al., 2000; 2002). Esteves and coworkers (2004c) similarly identified *E. ruminantium* (Gardel stock) proteins falling within the region of 14-35 kDa and these proteins may be included in vaccine development against heartwater. A subset of *E. ruminantium* genes that may play an important role in the development of a recombinant vaccine against heartwater were identified (Barbet et al., 2001). The corresponding proteins were recognized by the sheep immune sera and PBMC stimulated with the antigens induced proliferation. In addition, mice immunized with recombinant bacterial lysates were protected.

Several studies have been published on the isolation and characterization of major immunodominant proteins. An immunodominant antigenically conserved 32-

kilodalton (kDa) protein from *E. ruminantium* was identified and it was indicated that anti-sera against nine stocks of *E. ruminantium* from different regions recognized the protein (Jongejan and Thielemans, 1989). Similarly, two major immunodominant proteins of *E. ruminantium* (27 kDa and 31 kDa MAP1 proteins) which were antigenically conserved were identified (van Kleef et al., 1993). These proteins were recognized by sheep antiserum to several isolates of *E. ruminantium* (Rossouw, et al., 1990). It has been discovered that this 31kDa protein has a molecular weight that varies between isolates (Jongejan and Thielemans, 1989; Rossouw et al., 1990). From these observations, Barbet and coworkers (1994) renamed the protein as a major antigenic protein (MAP1). This MAP1 has been used for diagnosis of heartwater in an indirect MAP1-B ELISA (van Vliet et al., 1995; Bowie et al., 1999; Simbi et al., 2003; Faburay et al., 2005, 2007). Mwangi and coworkers (2002) demonstrated that immunization of cattle by infection and treatment induced MAP1- and MAP2-specific T cell responses. Therefore, these characterized proteins give a positive sign that vaccine development for heartwater that induce protective immunity is feasible.

Although cell-mediated immunity has been generally accepted to play an important role in host defence against intracellular pathogens, antibody-mediated immunity has been demonstrated to confer immunity against pathogens. These include *E. muris* (Feng and Walker, 2004); *E. chaffeensis* (Winslow et al., 2000) and *Brucella abortus* (Luo et al., 2006). Therefore, antigens of *E. ruminantium* that are able to induce an antibody response should not be excluded as potential vaccine antigens (Casadevall, 2003).

1.10. HEARTWATER VACCINES

Vaccination is one of the most important and cost-effective methods of preventing infectious diseases of animals. The goal of vaccination is to induce immune responses against pathogens, so that disease or infection can be prevented. Vaccines have significantly reduced the impact of a number of invading diseases in both companion animals and livestock (sheep, goats and cattle). Currently the majority of vaccines that are being used in the veterinary field are either live attenuated or killed. Several attempts have been made to develop an effective heartwater vaccine. The infection and treatment method is based on the infection of animals with virulent *E. ruminantium* Ball 3 stock (van der Merwe, 1987) followed by treatment with

tetracycline. The Ball 3 stock does not cross protect against all virulent field isolates as mentioned earlier.

Live attenuated vaccines are able to induce a long-lasting immunity through cell-mediated and humoral immune responses, and have low cost, making them ideally suited for applications specific to regions where the disease is endemic (Ranallo et al., 2007). However, they pose a potential risk if the vaccine agents become virulent, and they are not user-friendly because they generally require a cold chain for delivery. An attenuated vaccine was tested using the *E. ruminantium* Senegal stock which was capable of inducing protection against homologous challenge but not to heterologous challenge (Jongejan, 1991a). The Gardel stock from Guadeloupe was also attenuated *in vitro* after more that 200 passages (Martinez, 1997). *E. ruminantium* Welgevonden stock was attenuated by continuous culture in a canine macrophage-monocyte cell line (DH82) and completely protected sheep against a lethal needle challenge with the homologous and several heterologous isolates (Collins et al., 2003a; Zweygarth et al., 2005). In order to obtain protective immunity to all the isolates it would be necessary to attenuate several stocks for inclusion into an attenuated vaccine. However, not all isolates can be attenuated which limits the use of this method of vaccination.

Inactivated vaccines were developed that consisted of bovine endothelial cell culture-derived *E. ruminantium* organisms that were chemically inactivated (Mahan et al., 1995). Inactivated vaccines cannot replicate and are not infectious and can therefore be used in non endemic areas. The advantage of an inactivated vaccine is that it can be modified to include any isolate of *E. ruminantium* to overcome the lack of cross-protection against some heterologous isolates. A further advantage of this vaccine is that it can easily be administered sub-cutaneously or intra-muscularly and this is an important factor for farmers because the method is simple and effective. Several studies using inactivated *E. ruminantium* organisms indicated that they induced incomplete and variable protective immunity against homologous and heterologous challenges in goats, sheep and cattle. This vaccine needs to be mixed with either Freund's complete or Montanide ISA50 adjuvant (Mahan et al., 1995; 1998; 2001; Martinez, 1994; 1996; Totté et al., 1997). In goats, sheep and cattle, protection of vaccinated animals obtained was 65%, 79% and 100% respectively. These inactivated vaccines are not being used presently because they do not protect against a variety of

field isolates. They do not prevent infection, but decrease mortality (Mahan et al., 1998, 2001).

Recombinant vaccines could overcome the difficulties encountered with live, attenuated and inactivated vaccines. There are many advantages associated with recombinant vaccines. Firstly, they can be easily produced on a commercial scale, are reproducible, cheap and are extremely thermostable, thus, removing the need for maintaining a cold chain (Wright et al., 1992; reviewed by Liljeqvist and Ståhl, 1999). The first attempt to develop an effective DNA vaccine against *E. ruminantium* was done by Nyika and coworkers (1998). It was shown that a DNA vaccine containing the *map1* gene encoding the immunodominant MAP1 protein of the Crystals Springs stock of *E. ruminantium* induced partially protective Th1 type immune responses in DBA/2 mice. Similarly, it has been reported that boosting *map1* (*E. ruminantium*) DNA-vaccinated mice with recombinant MAP1 protein induced 67% protection against lethal homologous challenge while DNA vaccination without boosting only induced 13-27% protection (Nyika et al., 2002). In further efforts to develop a recombinant vaccine against heartwater, Collins and coworkers (2003a, b) identified four open reading frames in the sequence of a cosmid clone 1H12 that induced variable protection in a mouse model when delivered as a DNA vaccine. When a cocktail of the four 1H12 *E. ruminantium* ORFs was tested in sheep as a DNA vaccine it elicited 100% protection (Collin et al., 2003 a, b; Pretorius et al., 2007). However, only 20% protection was induced against a field challenge (Collins et al., 2003a, b). Simbi and coworkers (2006) also evaluated nine ORFs of *E. ruminantium* for protection against challenge in mice. Five out of nine ORFs offered partial protection against lethal challenge. The induction of cell-mediated or T helper 1 (Th1) type of immune responses characterized by the production of IFN-γ and IL-2 was observed. Because only limited protection was obtained during a field vaccine trial it is therefore important to identify more genes for development of an effective vaccine.

Since cellular immunity, and in particular the cytokine IFN-γ, plays an important role in protection against heartwater disease it is important for recombinant vaccine development to determine the potential of vaccine candidate genes to elicit these appropriate protective cellular immune responses. Completion of the *E. ruminantium* genome sequence now offers valuable information for identification of possible

vaccine candidates using bioinformatics tools. This study will focus on identifying *E. ruminantium* genes that encode for secreted proteins and evaluating them as vaccine candidates.

1.11. REFERENCES

AGUERO-ROSENFELD, M.E., 2003. Laboratory aspects of tick-borne diseases: lyme, human granulocytic Ehrlichiosis and Babesiosis. *The Mount Sinai Journal of Medicine*, 70:197-206.

ALLSOPP, B.A., ALLSOPP, M.T., DU PLESSIS, J.H, & VISSER, E.S., 1996. Uncharacterized *Ehrlichia* spp. may contribute to clinical heartwater. *Annals of the New York Academy of Sciences*, 791:17-23.

ALLSOPP, B.A., BEZUIDENHOUT, J.D., & PROZESKY, L., 2004. Heartwater. In: *Infectious Diseases of Livestock*. 2nd ed. Volume 1. Edited by COETZER, J.A.W., THOMPSON, G.R., & TUSTIN, R.C. Oxford University Press, pp. 507-535.

ALLSOPP, M.T.E.P., VISSER, E.S., DU PLESSIS, J.L., VOGEL, S.W., & ALLSOPP, B.A., 1997. Different organisms associated with heartwater as shown by analysis of 16S ribosomal RNA gene sequences. *Veterinary Parasitology*, 71:283-300.

ALLSOPP, M.T.E.P., & ALLSOPP, B.A., 2001a. Novel *Ehrlichia* genotype detected in dogs in South Africa. *Journal of Clinical Microbiology*, 39:4204-4207.

ALLSOPP, M.T.E.P., DORFLING, C.M., MAILLARD, J.C., BENSAID, A., HAYDON, D.T., VAN HEERDEN, H., & ALLSOPP, B.A., 2001b *Ehrlichia ruminantium* major antigenic protein gene (*map1*) variants are not geographically constrained and show no evidence of having evolved under positive selection pressure. *Journal of Clinical Microbiology*, 39:4200-4203.

AXSELL, G., 1998. Deputy Director of Veterinary Services, Private Bag X 12999, Centrahil 6006, South Africa.

BARBET, A., TEBELE, N., SEMU, S., PETER, T., WASSIK, L., & MAHAN, S., 1993. Serological diagnosis of heartwater in Zimbabwe — problems and perspectives. *Revue d'Élevage et de Médecine Vétérinaire des Pays Tropicaux*, 46:121.

BARBET, A.F., SEMU, S.M., CHIGAGURE, N., KELLY, P.J., JONGEJAN, F., & MAHAN, S.M., 1994. Size variation of the major immunodominant protein of *Cowdria ruminantium*. *Clinical and Diagnostic Laboratory immunology*, 1:744-746.

BARBET, A.F., WHITMIRE, W.M., KAMPER, S.M. SIMBI, B.H., GANTA, R.R., MORELAND, A.L., MWANGI, D.M., MCGUIRE, T.C., & MAHAN, S.M., 2001. A subset of *Cowdria ruminantium* genes important for immune recognition and protection. *Gene*, 275:287-298.

BEZUIDENHOUT, J.D., 1987. Natural transmission of heartwater. *Onderstepoort Journal of Veterinary Research*, 54:349-351.

BITSAKTSIS, C., HUNTINTON, J., & WINSLOW, G., 2004. Production of IFN-γ by CD4 T cells is essential for resolving Ehrlichia infection. *The Journal of Immunology*, 172:6894-6901.

BOEHM, U., KLAMP, T., GROOT, M., & HOWARD, J.C. 1997. Cellular responses to interferon-gamma. *Annual Reviews of Immunology*, 15:749-95.

BOOCKVAR, K.S., GRANGER, D.L., POSTON, R.M., MAYBODI, M., WASHINGTON, M.K., JR, J.B.H., & KURLANDER, R.L., 1994. Nitric oxide produced during murine listeriosis is protective. *Infection and Immunity*, 62:1089-1100.

BOURDOULOUS, S., BENSAID A., MARTINEZ, D., SHEIKBOUDOU, C., TRAP, I., STROSBERG, A.D., & COURAUD, P.O., 1995. Infection of bovine brain microvessel endothelial cells with *Cowdria ruminantium* elicits IL-1β, -6, and -8 mRNA production and expression of an unusual MHC class II DQα transcript. *The Journal of Immunology*, 154:4032-4038.

BOWIE, M.V., REDDY, G.R., SEMU, S.M., MAHAN, S.M., & BARBET, A.F., 1999. Potential value of major antigenic protein 2 for serological diagnosis of hearwater and related Ehrlichial infections. *Clinical and Diagnostic Laboratory Immunology*, 6:209-215.

CAMUS, E., & BARRÉ, N., 1982. La cowdriose (heartwater). Revue générale des connaissances. IEMV, Maisons-Alfort, France, pp. 147.

CASADEVALL, A., 2003. Antibody-mediated immunity against intracellular pathogens: Two-dimensional thinking comes full circle. *Infection and Immunity*, 71:4225-4228.

CASTELLINO, F. & GERMAIN, R.N., 2006. Cooperation between CD4+ and CD8+ T cells: when, where, and how. *Annual Reviews of Immunology*, 24:519-540.

CHANG, K.M., THIMME, R., MELPOLDER, J.J., OLDACH, D., PEMBERTON, J., MOORHEAD-LOUDIS, J., MCHUTCHISON, J.G., ALTER, H.J., & CHISARI, F., 2001. Differential $CD4^+$ and $CD8^+$ T-cell responsiveness in Hepatitis C virus infection. *Hepatology*, 33:267-276.

CHANG-HONG, S., XIAO-WU, W., HAI, Z., TING-FEN, Z., LI-MEI, W., & ZHI-KAI, X., 2008. Immune responses and protective efficacy of the gene vaccine expressing Ag85B and ESAT6 fusion protein form *Mycobacterium tuberculosis*. *DNA and Cell Biology*, 27:199-207.

COLER, R.N., CAMPOS-NETO, A., OVENDALE, P., DAY, F.H., FLING, S.P., ZHU, L., SERBINA, N., FLYNN, J.L., REED, S.G., & ALDERSON, M.R., 2001. Vaccination with the T cell antigen Mtb 8.4 protects against challenge with *Mycobacterium tuberculosis*. *The Journal of Immunology*, 166:6227-6235.

COLLINS, N.E., PRETORIUS, A., VAN KLEEF, M., BRAYTON, K.A., ALLSOPP, M.T., ZWEYGARTH, E. & ALLSOPP, B.A., 2003a. Development of improved attenuated and nucleic acid vaccines for heartwater. *Developments in biologicals*, 114:121-136.

COLLINS, N.E., PRETORIUS, A., VAN KLEEF, M., BRAYTON, K.A., ZWEYGARTH, E. & ALLSOPP, B.A., 2003b. Development of improved vaccines for heartwater. *Annals of the New York Academy of Science*, 990:474-484.

CORWIN, E. J., 2000. Understanding cytokines Part I: physiology and mechanism of action. *Biological Research for Nursing*, 2:30-40.

COWDRY, E.V., 1926. Studies on the etiology of heartwater. III. The multiplication of *Rickettsia ruminantium* within endothelial cells of infected animals and their discharge into the circulation. *Journal of Experimental Medicine*, 44:803-814.

DE SOUSA, R., ISMAIL, N., NOBREGA, S.D., FRANÇA, A., AMARO, M., ANES, M., POÇAS, J., COELHO, R., TORGAL, J., BACELLAR, F., & WALKER, D.H., 2007. Intralesional expression of mRNA of interferon-gamma, tumor necrosis factor-alpha, interleukin-10, nitric oxide synthase, indoleamine-2, 3-dioxygenase, and RANTES is a major immune effector in Mediterranean spotted fever rickettsiosis. *Journal of Infectious Diseases*, 196:770-781.

DE WAAL, D.T., MATTHEE, O., & JONGEJAN, F., 2000. Evaluation of the MAP1b ELISA for the diagnosis of heartwater in South Africa. *Annals of the New York Academy of Science*s, 916:622-627.

DUMLER, J.S., BARBET, A.F., BEKKER, C.P., DASCH, G.A., PALMER, G.H., RIKIHISA, S.C., & RURANGIRWA, F.R., 2001. Reorganisation of genera in the families Rickettsiaceae and Anaplasmataceae in the order Rickettsiales: unification of some species of *Ehrlichia* with *Anaplasma, Cowdria* with *Ehrlichia,* and *Ehrlichia* with *Neorickettsia*, descriptions of six new

species combinations and designation of *Ehrlichia*, equi and 'HGE agent' as subjective synonyms of *Ehrlichia*, *phagocytophila*. *International Journal of Systematic and Evolutionary Microbiology*, 51:2145-2165.

DU PLESSIS, J.L., 1975. Electron microscopy of *Cowdria ruminantium* infected reticulo-endothelial cells of the mammalian host. *Onderstepoort Journal of Veterinary Research*, 42:1-14.

DU PLESSIS, J.L., 1982. Mice infected with a *Cowdria ruminantium*-like agent as a model in the study of heartwater. D.V.Sc. thesis. University of Pretoria, South Africa.

DU PLESSIS, J.L., & MALAN, L., 1987. The application of indirect fluorescent antibody test in research on heartwater. *Onderstepoort Journal of Veterinary Research*, 54:319-325.

DU PLESSIS, J.L., BERCHE, P., & VAN GAS, L., 1991. T cell-mediated immunity to *Cowdria ruminantium* in mice: the protective role of Lyt-2^+ T cells. *Onderstepoort Journal of Veterinary Research*, 58:171-179.

DU PLESSIS, J.L., GRAY, C., & VAN STRIJP, M.F., 1992. Flow cytometric analysis of T cell response in mice infected with *Cowdria ruminantium*. *Onderstepoort Journal of Veterinary Research*, 59:337-338.

DU PLESSIS, J.L., BEZUIDENHOUT, J.D., BRETT, M.S., CAMUS, E., JONGEJAN, F., MAHAN, S.M., & MARTINEZ, D., 1993. The sero-diagnosis of heartwater: a comparison of five tests. *Revue d'Élevage et de Médecine Vétérinaire des Pays Tropicaux*, 46:123-129.

ESTEVES, I., BENSAID, A., MARTINEZ, D., & TOTTÉ, P., 2002. IFN-gamma as an indicator of successful immunization of goats vaccinated with a killed *Cowdria ruminantium* vaccine. *Annals of New York Academy of Science*. 969:126-130.

ESTEVES, I., MARTINEZ, D., & TOTTÉ, P., 2004a. Identification of *Ehrlichia ruminantium* (Gardel strain) IFN-γ inducing proteins after vaccination with a killed vaccine. *Veterinary Microbiology*, 100:233-240.

ESTEVES, I., VACHIÉRY, N., MARTINEZ, D., & TOTTÉ, P., 2004b. Analysis of *Ehrlichia ruminantium*-specific T1/T2 responses during vaccination with a protective killed vaccine and challenge of goats. *Parasite Immunology*, 26:95-103.

ESTEVES, I., WALREVENS, K., VACHIÉRY, N., MARTINEZ, D., LETESSON, J.J., & TTOTÉ, P., 2004c. Protective killed *Ehrlichia ruminantium* vaccine elicits IFN-γ responses by CD4+ and CD8+ T lymphocytes in goats. *Veterinary Immunology and Immunopathology,* 98:49-57.

FABURAY, B., MUNSTERMANN, S., GEYSEN, D., BELL-SAKYI, L., CEESAY, A., BODAAN, C., & JONGEJAN, F., 2005. Point seroprevalence survey of *Ehrlichia ruminantium* infection in small ruminants in the Gambia. *Clinical and Diagnostic Laboratory Immunology*, 12:508-512.

FABURAY, B., MUNSTERMANN, S., BELL-SAKYI, L., & JONGEJAN, F., 2007. Longitudinal monitoring of *Ehrlichia ruminantium* infection in Gambian lambs and kids by pCS20 PCR and MAP1-B ELISA. *BMC Infectious Diseases*, 7:85.

FENG, H.M., & WALKER, D.H., 2004. Mechanism of immunity to *Ehrlichia muris*: a model of monocytotropic Ehrlichiosis. *Infection and Immunity*, 72:966-971.

FLYNN, G.C., BECKERS, C.J.M., BAASE, W.A., & DAHLQUIST, F.W., 1993. Individual subunits of bacterial luciferase are molten globules and interact with molecules chaperones. *Proceedings of the National Academy of Sciences of United States of America*, 90:10826-10830.

GUIDOTTI, L.G., ISHIKAWA, T. & HOBBS, M.V., 1996. Intracellular inactivation of the hepatitis B virus by cytotoxic T lymphocytes. *Immunity*, 4:25-36.

HART, A., K., KATHERINE, M., BEZUIDENHOUT, J.D., & PROZESKY, L., 1991. Ultrastructural morphology of *Cowdria ruminantium* in midgut epithelial cells of adult *Amblyomma habraeum* female ticks. *Onderstepoort Journal of Veterinary Research*, 58:187-193.

HOFT, D.F., SCHNAPP, A.R., EICKHOFF, C.S., & ROODMAN, S.T., 2000. Involvement of CD4+ Th1 cells in systemic immunity protective against primary and secondary challenges with *Trypanosoma cruzi*. *Infection and Immunity*, 68:197-204.

HUSTER, K.M., STEMBERGER, C., & BUSCH, D.H., 2006. Protective immunity towards intracellular pathogens. *Current Opinion in Immunology*, 18:458-464.

JOHNSON, H.M., BAZER, F.W., SZENTE, B.E., & JARPE, M.A., 1994. How interferons fight disease. *Scientific American*, 270:40-47.

JONGEJAN, F., & THIELEMANS, M.J.C., 1989. Identification of an immunodominant antigenically conserved 32-kilodalton protein from *Cowdria ruminantium*. *Infection and Immunity*, 57:3243-3246.

JONGEJAN, F., 1991a. Protective immunity to heartwater (*Cowdria ruminantium* infection) is acquired after vaccination with in vitro- attenuated rickettsiae. *Infection and Immunity*, 59:729-731.

JONGEJAN, F., THIELEMANS, M.J.C., DE GROOT, M., VAN KOOTEN, P.J.S., & VAN DER ZEIJST, B.A.M., 1991b. Competitive enzyme-linked immunosorbent assay for heartwater disease using monoclonal antibodies against *Cowdria ruminantium*-specific 32-kilodalton protein. *Veterinary Microbiology*, 28:199-211.

JONGEJAN, F., ZANDBERGEN, T.A., VAN DE WIEL, P.A., DE GROOT, M., & UILENBERG, G. 1991c. The tick-borne rickettsia *Cowdria ruminantium* has a Chlamydia-like developmental cycle. *Onderstepoort Journal of Veterinary Research*, 58:227-237.

KAUSHIK, R.S., UZONNA, J.E., GORDON, J.R., & TABEL, H., 1999. Innate resistance to *Trypanosoma congolense* infections: differential production of nitric oxide by macrophages from susceptible BALB/c and resistant C57BI/6 mice. *Experimental Parasitology*, 92:131-143.

LI, H., JERRELLS, T.R., SPITALNY, G.L., & WALKER, D.H., 1987. Gamma interferon as a crucial host defense against *Rickettsia conorri* in vivo. *Infection and Immunity*, 55:1252-1255.

LI, Z., DIAZ-MONTERO, C.M., VALBUENA, G., YU, X.J., OLANO, J.P., FENG, H.M., WALKER, D.H. 2003. Identification of CD8 T-lymphocyte epitopes in OmpB of *Rickettsia conorii*. *Infection and Immunity*, 71:3920-3926.

LILJEQVIST S., & STÅHL, S., 1999. Production of recombinant subunit vaccines: protein immunogens, live delivery systems and nucleic acid vaccines. *Journal of Biotechnology*, 73:1-33.

LOGAN, L.L., 1987. *Cowdria ruminantium*: stability and preservation of the organism. *Onderstepoort Journal of Veterinary Research*, 54:187-191.

LONDON, C.A., ABBAS, A.K., & KELSO, A., 1998. Helper T cell subsets: Heterogeneity, functions and development. *Veterinary Immunology and Immunopathology*, 63:73-44.

LUO, D., NI, B., LI, P., SHI, W., ZHANG, S., HAN, Y., MAO, L., HE, Y., WU, Y., & WANG, X., 2006. Protective immunity elicited by a divalent DNA vaccine encoding both the L7/L12 and Omp16 genes of *Brucella abortus* in BALB/c mice. *Infection and Immunity*, 74:2734-2741.

MA, C.S., NICHOLS, K.E., & TANGYE, S.G., 2007. Regulation of cellular and humoral immune responses by the SLAM and SAP families of molecules. *Annual Reviews of Immunology*, 25:337-379.

MAHAN, S.M., WAGHELA, S.D., McGUIRE, T.C., RURANGIRWA, F.R., WASSINK, L.A., & BARBET, A.F., 1992. A cloned DNA probe for *Cowdria ruminantium* hybridizes with eight heartwater strains and detects infected sheep. *Journal of Clinical Microbiology*, 30:981-986.

MAHAN, S.M., SMITH, G.E & BYROM, B., 1994. Concanavalin A-stimulated bovine T cell supernatants inhibit growth of *Cowdria ruminantium* in bovine endothelial cells in vitro. *Infection and Immunity*, 62:747-750.

MAHAN, S.M., ANDREW, H.R., & TEBELE, N., 1995. Immunization of sheep with inactivated *Cowdria ruminantium*. *Research in Veterinary Science*, 58:46-49.

MAHAN, S.M., KUMBULA, D., BURRIDGE, M.J. & BARBET, A.F., 1998. The inactivated *Cowdria ruminantium* vaccine for heartwater protects against heterologous strains and against laboratory and field tick challenge. *Vaccine*, 16:1203-1211.

MAHAN, S.M., SMITH, G.E., KUMBULA, D., BURRIDGE, M.J., & BARBET, A.F., 2001. Reduction in mortality from heartwater in cattle, sheep and goats exposed to field challenge using an inactivated vaccine. *Veterinary Parasitology*, 97:295-308.

MARCELLINO, I., VERÍSSIMO, C., SOUSA, M.F.Q., CARRONDO, M., & ALVES, P.M., 2005. Characterization of *Ehrlichia ruminantium* replication and release kinetics in endothelial cell cultures. *Veterinary Microbiology*, 110:87-96.

MARTINEZ, D., MAILLARD, J.C., COISUE, S., SHEIKBOUDOU, C., & BENSAID, A., 1994. Protection of goats against heartwater acquired by immunisation with inactivated elementary bodies of *Cowdria ruminantium*. *Veterinary Immunology and Immunopathology*, 41:153-163.

MARTINEZ, D., PEREZ, J.M., SHEIKBOUDOU, C., DEBUS, A. & BENSAID, A., 1996. Comparative efficacy of Freund's and Montanide ISA50 adjuvants for the immunisation of goats against heartwater with inactivated *Cowdria ruminantium*. *Veterinary Parasitology*, 67:175-184.

MARTINEZ, D., 1997. Analysis of the immune response of ruminants to *Cowdria ruminantium* infection. Ph.D. thesis. University of Utrecht, Utrecht, The Netherlands.

MBOLOI, M.M., BEKKER, C.P., KRUITWAGEN, C., GREINER, M., & JONGEJAN, F., 1999. Validation of the indirect MAP1-B enzyme-linked immunosorbent assay for diagnosis of experimental *Cowdria ruminantium* infection in small ruminants. *Clinical and Diagnostic Laboratory Immunology*, 6:66-72.

MORRISON, W., TAYLOR, G., GADDIUM, R.M., & ELLIS, S.A., 1999. Contribution to advances in immunology to vaccine development. *Advances in Veterinary Medicine*, 4:181-195.

MOSHKOVSKI, S.D., 1947. Comments by readers. Science, 106:62

MOSMANN, T.R., & SAD, S., 1996. The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunology Today*, 17:138-146.

MUKHEBI, A.W., CHAMBOKO, T., O'CALLAGHAN, C.J., PETER, T.F., KRUSKA, R.L., MEDLEY, G.F., MAHAN, S.M., & PERRY, B.D., 1999. An assessment of the economic impact of heartwater (*Cowdria ruminantium* infection) and its control in Zimbabwe. *Preventive Veterinary Medicine*, 39:173-189.

MUTUNGA, M., PRESTON P.M., & SUMPTION K., 1998. Nitric oxide is produced by *Cowdria ruminantium*-infected bovine pulmonary endothelial cells in vitro and is stimulated by gamma interferon. *Infection and Immunity*, 66:2115-2121.

MWANGI, D.M., MAHAN, S.M., NYANJUI, J.K., TARACHA, E.L.N., & MCKEEVER, D.J., 1998. Immunization of cattle with *Cowdria ruminantium* elicits T lymphocytes that recognize autologous endothelial cells and monocytes. *Infection and Immunity*, 66:1855.

MWANGI, D.M., MCKEEVER, D.J., NYANJUI, J.K., BARBET, A.F., & MAHAN, S.M., 2002. Immunisation of cattle against heartwater by infection with *Cowdria ruminantium* elicits T lymphocytes that recognise major antigenic proteins 1 and 2 of the agent. *Veterinary Immunology and Immunopathology*, 85: 23-32.

NYIKA, A., MAHAN, S.M., BURRIDGE, M.J., MCGUIRE, T.C., RURANGIRWA, F., & BARBET, A.F., 1998. A DNA vaccine protects mice against the rickettsial agent *Cowdria ruminantium. Parasite Immunology*, 20:111-119.

NYIKA, A., BARBET, A.F., BURRIDGE, M.J., & MAHAN, S.M., 2002. DNA vaccination with map1 gene followed by protein boost augments protection against challenge with *Cowdria ruminantium*, the agent of heartwater. *Vaccine*, 20:1215-1225.

OBEREM, P.T., & BEZUIDENHOUT, J.D., 1987. The production of heartwater vaccine. *Onderstepoort Journal of Veterinary Research*, 54:485-488.

OJCIUS, D.M., GACHELIN, G., & DANTRY-VARSAT, A., 1996. Presentation of antigens derived from microorganisms residing in host-cell vacuoles. *TRENDS in Microbiology*, 4:53-59.

PEIXOTO, C.C., MARCELINO, I., VACHIÉRY, N., BENSAID, A., MARTINEZ, D., CARRONDO, M.J.T., & ALVES, P.M., 2005. Quantification of *Ehrlichia ruminantium* by real time PCR. *Veterinary Microbiology*, 107:273-278.

PETER, T.F., DEEM, S.L., BARBET, A.F., NORVAL, R.A.I., SIMBI, B.H., KELLY, P.J., & MAHAN, S.M., 1995. Development and evaluation of PCR assay fro detection of low levels of *Cowdria ruminantium* infection in *Amblyomma* ticks not detected by DNA probe. *Journal of Clinical Microbiology*, 33:166-172.

PETER, T.F., BARBET, A.F., ALLEMAN, A.R., SIMBI, B.H., BURRIDGE, M.J., & MAHAN, S.M., 2000. Detection of the agent of hearwater, *Cowdria ruminantium*, in *Amblyomma* ticks by PCR: validation and application of the assay to field ticks. *Journal of Clinical Microbiology*, 38:1539-1544.

PIENAAR, J.G., 1970. Electron microscopy of *Cowdria* (*Rickettsia*) *ruminantium* (Cowdry, 1926) in the endothelial cells of the vertebrate host. *Onderstepoort Journal of Veterinary Research*, 37:67-78.

POSTIGO, M., BELL-SAKYI, L., PAXTON, E., & SUMPTION, K., 2002. Kinetics of experimental infection of sheep with *Ehrlichia ruminantium* cultivated in tick and mammalian cell lines. *Experimental and Applied Acarology*, 28:187-193.

PRESTON, P.M., & JONGEJAN, F., 1999. Protective immune mechanisms to ticks and tick-borne diseases of ruminants. *Parasitology Today*, 15:255-258.

PRETORIUS, A., COLLINS, N.E., STEYN, H.C., VAN STRIJP, F., VAN KLEEF, M., & ALLSOPP, B.A., 2007. Protection against heartwater by DNA immunization with four *Ehrlichia ruminantium* open reading frames. *Vaccine*, 25:2316-2324.

PROVOST, A., & BEZUIDENHOUT, J.D., 1987. The historical background and global importance of heartwater. *Onderstepoort Journal of Veterinary Research*, 54:165-169.

RANALLO, R.T., THAKKAR, S., CHEN, Q., & VENKATESAN, M.M., 2007. Immunogenicity and characterization of WRSF2G11: a second generation live attenuated *Shigella flexneri* 2a vaccine strain. *Vaccine*, 25:2269-2278.

REDDY, G.R., SULSONA, C.R., HARRISON, R.H., MAHAN, S.M., BURRIDGE, M.J., & BARBET, A.F., 1996. Sequence heterogeneity of the major antigenic protein 1 genes from *Cowdria ruminatium* isolates from different geographical areas. *Clinical and Diagnostic Laboratory Immunology*, 3:417-422.

RISTIC, M., & HUXOLL, D.L., 1984. Tribe II. Ehrlichiae Philip 1957. *In*: KRIEG, N.R. & HOLT, J.G., (eds.) *Bergey's Manual of Systemic Bacteriology*, Baltimore: Williams & Wilkins.

ROSSOUW, M., NEITZ, A.W.H., DE WAAL, D.T., DU PLESSIS, J.L., VAN GAS, L., & BRETT, S., 1990. Identification of the antigenic proteins of *Cowdria ruminantium*. *Onderstepoort Journal of Veterinary Research*, 57:215-221.

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

SECHI, L.A., MARA, L., CAPPAI, P., FROTHINGAM, R., ORTU, S., LEONI, A., AHMED, N., & ZANETTI, S., 2006. Immunization with DNA vaccines encoding different mycobacterial antigens elicits a Th1 type immune response in lambs and protects against *Mycobacterium avium* subspecies *paratuberculosis* infection. *Vaccine*, 24:229-235.

SHOUKRY, N.H., CAWTHON, A.G., & WALKER, C.M., 2004. Cell-mediated immunity and the outcome of Hepatitis C virus infection. *Annual Reviews of Microbiology*, 58:391-424.

SIMBI, B.H., PETER, T.F., BURRIDGE, M.J., & MAHAN, S.M., 2003. Comparing the detection of exposure to *Ehrlichia ruminantium* infection on a heartwater-endemic farm by the pCS20

polymerase chain reaction assay and an indirect MAP1-B enzyme linked immunosorbent assay. *Onderstepoort Journal of Veterinary Research*, 70:231-235.

SIMBI, B.H., BOWIE, M.V., MCGUIRE, T.C., BARBET, A.F., & MAHAN, S.M., 2006. Evaluation of *E. ruminantium* genes in DBA/2 mice as potential DNA vaccine candidates for control of heartwater. *Annals of New York Academy of Science*, 1078:424-437.

SKAPENKO, A., & SCHULZE-KOOPS, H., 2007. Analysis of Th1/Th2 T-cell subsets. *Methods in Molecular Medicine*, 136:87-96.

STEYN, H.C., VAN HEERDEN, H., ALLSOPP, M.T.E.P., ALLSOPP, B.A., 2003. Variability of pCS20 gene sequences among different *Ehrlichia ruminantium* isolates. *Annals of New York Academy of Science*, 990:1-3.

STEYN, H.C., PRETORIUS, A., MCCRINDLE, C.M.E., STEINMANN, C.M.L., VAN KLEEF, M., 2008. A quantitative real-time PCR assay for *Ehrlichia ruminantium* using pCS20. *Veterinary Microbiology*, 131:258-265.

SULSONA, C.R., MAHAN, S.M., & BARBET, A.F., 1999. The *map1* gene of *Cowdria ruminantium* is a member of a multigene family containing both conserved and variable genes. *Biochemical and Biophysical Research Communications*, 257:300-305.

SZABO, S.J., SULLIVAN, B.M., PENG, S.L., & GLIMCHER, L.H., 2003. Molecular mechanisms regulating Th1 immune responses. *Annual Reviews of Immunology*, 21:713-58.

TOTTÉ, P., JONGEJAN, F., DE GEE, A.L.W., & WÉRENNE, J., 1994. Production of alpha interferon in *Cowdria ruminantium*-infected cattle and its effect on infected endothelial cell cultures. *Infection and Immunity*, 62:2600-2604.

TOTTÉ, P., MACKEEVER, D., MARTINEZ, D., & BENSAID, A., 1997. Analysis of T-cell responses in cattle immunized against heartwater by vaccination with killed elementary bodies of *Cowdria ruminantium*. *Infection and Immunity*, 65:236-241.

TOTTÉ, P., BENSAID, A., MAHAN, S.M., MARTINEZ, D., & MACKEEVER, D., 1999. Immune responses to *Cowdria ruminantium* infections. *Parasitology Today*, 15:286-290.

TROMBETTA, E.S., & MELLMAN, I., 2005. Cell biology of antigen processing in vitro and in vivo. *Annual Reviews of Immunology*, 23:975-1028.

UILENBERG, G., 1983. Heartwater (*Cowdria ruminantium* infection). Current status. *Advances in Veterinary Science and Comparative Medicine,* 27:427-480.

VAN HEERDEN, H., STEYN, H.C., ALLSOPP, M.T., ZWEYGARTH, E., JOSEMANS, A.I., & ALLSOPP, B.A., 2004. Characterization of the pCS20 region of different *Ehrlichia ruminantium* isolates. *Veterinary Microbiology*, 101:279-291.

VAN DE PYPEKAMP, H.F., & PROZESKY, L., 1987. Heartwater. An overview of the clinical signs, susceptibility and differential diagnoses of the disease in domestic ruminants. *Onderstepoort Journal of Veterinary Research*, 54:263-266.

VAN DER MERWE, L., 1987. The infection and treatment method of vaccination against heartwater. *Onderstepoort Journal of Veterinary Research*, 54:489-491.

VAN KLEEF, M., NEITZ, A.W.H., & DE WAAL, D.T., 1993. Isolation and characterization of antigenic proteins of *Cowdria ruminantium*. *Revue d'Élevage et de Médecine Vétérinaire des Pays Trop*icaux, 46**:**157-164.

VAN KLEEF, M., GUNTER, N.J. MACMILLAN, H., ALLSOPP, B.A., SHKAP, V., & BROWN, W.C., 2000. Identification of *Cowdria ruminantium* antigens that stimulate proliferation of lymphocytes from cattle immunized by infection and treatment or with inactivated organisms. *Infection and Immunity*, 68:603-614.

VAN KLEEF, M., MACMILLAN, H., GUNTER, N.J., ZWEYGARTH, E., ALLSOPP, B.A., SHKAP, V., DU PLESSIS, D.H., & BROWN, W.C., 2002. Low molecular weight proteins of *Cowdria ruminantium* (Welgevonden isolate) induce bovine CD4+-enriched T-cells to proliferate and produce interferon-gamma. *Veterinary Microbiology*, 85:259-273.

VAN VLIET, A.H., JONGEJAN, F., VAN KLEEF, M., & VAN DER ZEIST, B.A., 1994. Molecular cloning, sequence analysis and expression of the gene encoding the immunodominant 32-kilodalton protein of *Cowdria ruminantium*. *Infection and Immunity*, 62:1451-1456.

VAN VLIET, A.H., VAN DER ZEIJST, B.A., CAMUS, E., MAHAN, S.M., MARTINEZ, D., & JONGEJAN, F., 1995. Use of a specific immunogenic region on *Cowdria ruminantium* MAP1 protein in a serological assay. *Journal of Clinical Microbiology*, 33:2405-2410.

VESOSKY, B., FLAHERTY, D.K., & TURNER, J., 2006. Th1 cytokines facilitate CD8 –T-cell-mediated early resistance to infection with *Mycobacterium tuberculosis* in old mice. *Infection and Immunity*, 74:3314-3324.

WAGHELA, S.D., RURANGIRWA, F.R., MAHAN, S.M., YUNKER, C.E., CRAWFORD, T.B., BARBET, A.F., BURRIDGE, M.J., & MCGUIRE, T.C., 1991. A cloned DNA probe identifies *Cowdria ruminantium* in *Amblyomma variegatum* ticks. *Journal of Clinical Microbiology*, 29:2571-2577.

WALKER, J.B., & OLWAGE, A., 1987. The tick vectors of *Cowdria ruminantium* (Ixodoidea, genus Amblyomma) and their distribution. *Onderstepoort Journal of Veterinary Research,* 54:353-379.

WALKER, D.H., OLANO, J.P., & FENG, H.M., 2001. Critical role of cytotoxic T lymphocytes in immune clearance of rickettsial infection. *Infection and Immunity*, 69:1841-1846.

WEAVER, C.T., & UNANUE, E.R., 1990. The costimulatory function of antigen presenting cells. *Immunology Today*, 11:49-55.

WINSLOW, G.M., YAGER, E., SHILO, K., VOLK, E., REILLY, A., & CHU, F.K., 2000. Antibody-mediated elimination of the obligate intracellular bacterial pathogen *Ehrlichia chaffeensis* during active infection. *Infection and Immunity*, 68: 2187-2195.

WILLIAMS, M.A., & BEVAN, M.J., 2007. Effector and Memory CTL differentiation. *Annual Reviews of Immunology*, 25:171-192.

WRIGHT, I.G., CASU, R., COMMINS, M.A., DALRYMPLE, B.P., GALE, K.R., GOODGER, B.V., RIDDLES, P.W., WALTISBUHL, D.J., ABETZ, I., BERRIE, D.A., BOWLES, Y., DIMMOCK, C., HAYES, T., KALNINS, H., LEATCH, G., McCRAE, R., MONTAGUE, P.E., NISBET, I.T., PARRODI, F., PETERS, J.M., SCHEIWE, P.C., SMITH, W., RODE-BRAMANIS, K., & WHITE, M.A., 1992. The development of a recombinant *Babesia* vaccine. *Veterinary Parasitology*, 44:3-13.

YEWDELL, J.W., & HAERYFAR, S.M.M., 2005. Understanding presentation of viral antigens to CD8+ T cells in vivo: the key to rational vaccine design. *Annual Reviews in Immunology*, 23:51-682.

YUNKER, C.E., MAHAN, S.M., WAGHELA, S.D., McGUIRE, T.C., RURANGIRWA, F.R., BARBET, A.F., & WASSINK, L.A., 1993. Detection of *Cowdria ruminantium* by means of a DNA probe, pCS20, in infected bont ticks, *Amblyomma hebraeum*, the major vector of heartwater in Southern Africa. *Epidemiology and Infection*, 110:95-104.

YUNKER, C.E., 1996. Heartwater in sheep and goats: review. *Onderstepoort Journal of Veterinary Research*, 63:159-170.

ZWEYGARTH, E., JOSEMANS, A.I. VAN STRIJP, M.F., LOPEZ-REBOLLAR, L., VAN KLEEF, M., & ALLSOPP, B., 2005. An attenuated *Ehrlichia ruminantium* (Welgevonden stock) vaccine protects small ruminants against virulent heartwater challenge. *Vaccine*, 23:1695-1702.

CHAPTER 2

Selection, molecular cloning and expression of five *Ehrlichia ruminantium* genes encoding secreted proteins

2.1. INTRODUCTION

Pathogen secreted proteins are the most likely to interact with the host. They are involved in signaling pathways, blood coagulation, immune defense, cell envelope biogenesis and virulence (reviewed by Bonin-Debs et al., 2004). A number of secreted proteins have been successfully tested as vaccine candidates against pathogens including: *M. tuberculosis* (Pal and Horwitz, 1992; Horwitz et al., 1995; Hasløv et al., 1995; Vekemans et al., 2004; Mukherjee et al., 2005; Cho et al., 2007), *Taenia ovis* (Drew et al., 2000), *Neospora caninum* (Cho et al., 2005) and *Streptococcus equi* (Flock et al., 2006). For example, early secreted antigen target 6 kDa (ESAT-6) and culture filtrate protein 10 kDa (CFP-10) are two well studied secreted proteins of *Mycobacterium tuberculosis* important for virulence and immunogenicity (Langermans et al., 2005). They are involved in the anti-mycobacterial Th1-host immune response. Moreover, the Ag85 complex of *M. tuberculosis* was implicated in the infection pathogenesis through fibronectin-binding capacity and cell wall biogenesis (Belisle, et al., 1997). Hence, there is evidence that secreted proteins play an important role in the generation of immune responses and are considered as most promising vaccine candidates (reviewed by Bonin-Debs et al., 2004).

There have been several recent attempts to identify secreted proteins in rickettsias for vaccine development. For example, soluble and secreted antigens that may play an important role in anaplasmosis had been identified and characterized from a short term culture of a Venezuelan isolate of *A. marginale* (Leal et al., 2000). These antigens were identified by metabolic labelling with (^{35}S) methionine, followed by fractionation and

immunoprecipitation with homologous and heterologous bovine sera. Furthermore, two secreted major immunoreactive proteins of *Ehrlichia canis* and *E. chaffeensis* were identified and molecularly characterized (Doyle et al., 2006). Both proteins were shown to be glycoproteins and recognized by antibodies and these results demonstrated that these proteins might be very useful in developing immunodiagnostic assays. Moreover, a large number of secreted proteins which might be useful in vaccine development against other intracellular bacteria such as *Mycobacterium* infection were identified (Ganguly et al., 2007; Gibbons et al., 2007; Målen et al., 2007; Martinez et al., 2007).

Many Gram negative pathogens are able to transport proteins to extra-cytoplasmic locations such as the inner membrane, outer membrane, periplasmic space cell surface (external part of outer membrane), extracellular medium, or into other recipient cells (reviewed by Ding et al., 2003; Lammertyn and Anne, 2004). These proteins are secreted by bacteria through different transport mechanisms such as the general export pathway (GEP) (Pugsley, 1993; Kostakioti et al., 2005; reviewed by Saier, 2006, reviewed by DiGiuseppe and Cox, 2007). Secretory and transmembrane proteins are distinguished from other proteins synthesized in the cytoplasm by hydrophobic stretch of amino acids known as a signal peptide. The signal peptide directs proteins into or across cell membranes. In prokaryotic cells proteins are directly transported across plasma (cytoplasmic) membranes to the cell membrane (reviewed by Rapoport et al., 1991; High, 1995; reviewed by Ito, 1996; reviewed by Pohlschröder, et al., 1997; reviewed by Brodsky, 1998; Römisch et al., 2003; reviewed by Swanton and Bulleid, 2003; Ackerman et al., 2005; Marti, et al., 2005; Wickner and Schekman, 2005; Pimpl et al., 2006; reviewed by Rapoport et al., 2007; Rusch and Kendall, 2007). The signal sequence of prokaryotes contains three domains including a positively charged N-terminus, hydrophobic region and an uncharged polar region. It is approximately 20 amino acids long and is similar to the signal peptide of eukaryotes. In addition to GEP, there are several secretion systems that translocate proteins across biological membranes. For example bacteria including *Agrobacterium tumerfaciens* (Christie, 2004), *Brucella* species (Carle et al., 2006) and *Helicobacter pylori* (Bourzac and Guillemin, 2005) utilizes a Type IV system, while *Salmonella enterica* serovar Typhimurium (Remaut and

Waksman, 2004; Chen et al., 2006) utilizes a Type III system. Type IV system is capable of transporting both DNA and proteins and translocates numerous effector proteins into its eukaryotic host. Type III system is like a molecular syringe through which a bacterium (eg *Salmonella* and *Shigella*) can inject proteins into eukaryotic cells. Furthermore, *Legionella pneumophila* uses type II and IV secretion systems (Lammertyn and Anne, 2004; reviewed by Segal et al., 2005). Proteins secreted through Type II depend on the Sec system for initial transport into the periplasm. Once there, they pass through the outer membrane via a multimeric complex of secretin proteins. Prokaryotes also use a twin-arginine transporter (Tat) pathway to translocate folded protein substrates across the plasma membrane. The signal sequence has the same structure as the Sec signal sequence the only difference is the presence of a double arginine motif followed by two uncharged residues near the N-terminus (Palmer and Berks, 2003; reviewed by DiGiuseppe and Cox, 2007). There are several recombinant expressed proteins that were shown to be exported via the Tat system of *E. coli* that included the folded jellyfish green fluorescent protein (GFP) (Santini et al., 2001; Thomas et al., 2001). Once these proteins are secreted they can interact with the host.

Pathogens have developed survival strategies by expressing products on the surface and /or secreting products into the surrounding environment. Thus, secreted proteins have been implicated to play a role in immune evasion. When bacterial pathogens invade a mammalian host, they encounter defense mechanisms exerted by the host cells which include innate or adaptive immune responses (Bitsaktsis et al., 2004, 2007; Winslow and Bitsaktsis, 2005; Turcotte et al., 2007; Moore and Hutchings, 2007). The secretory protein phospholipase A_2 mediates induced phagocytosis of *Rickettsia conorii* into non-phagocytic cells where the pathogen can divide and grow without immune detection (Teysseire et al., 1995). Similarly, one of the secreted proteins (MIC2) of *Toxoplasma gondii* plays a role in attachment and invasion of host cells (Huynh et al., 2003). Clearly, selection, expression and immunological studies of the genes encoding secreted proteins of *E. ruminantium* might contribute in vaccine development against heartwater and provide an insight into how the organism manipulates the host immune response during infection.

Since the complete genome sequence of *E. ruminantium* is available (Collins et al., 2005), bioinformatics could aid in the screening and identification of secreted proteins as potential vaccine candidates against heartwater. Bioinformatics refers to the search for and use of patterns and inherent structures in biological data such as genome sequences (NCBI Bioinformatics Definition URL) (reviewed by Grandi, 2001; reviewed by McDevitt and Rosenberg, 2001; reviewed by Rappuoli, 2001; Monteoliva et al., 2002; Adu-Bobie et al., 2003; reviewed by Mora et al., 2003, 2006; Meinke et al., 2004; reviewed by Yu et al., 2004; reviewed by Bansal, 2005; Scarselli et al., 2005; Muzzi et al., 2007). A variety of bioinformatics software packages exists with which to assign functions to genes and predict key features such as molecular weight, solubility and cellular location on bacterial surfaces. Putative open reading frames (ORFs) are analyzed using computer programs such as Signal P, TMHMM, PSORT, TMPRED and MOTIFS (Nakai and Kanehisa, 1991; Kanehisa and Bork, 2003; Käll et al., 2004; Gardy et al., 2005; Rey et al., 2005). These programs are used to predict signal peptide sequences, transmembrane helices, sub-cellular localization of proteins, putative hydrophobic membrane regions and lipoproteins respectively.

Bioinformatics has been successfully used to predict cell-surface and secreted proteins and to identify genes with sequence or structural homology to known virulence factors that may play a vital role in the generation of immune response and protection against pathogens. For example, ORFs from *Neisseria meningitidis* were selected and classified according to their localization on the bacterial surface (Comanducci et al., 2002). Some were shown to be outer membrane proteins, exported proteins, inner membrane proteins, lipoproteins and proteins with similarities to known virulence factors. The NadA, a surface antigen of *N. meningitides* was tested as a promising vaccine candidate: it elicited a strong antibody response and was protective in the infant rat model. In a recent study of *N. meningitidis* serogroup B, all open reading frames (ORFs) from the genome that contained a signal peptide sequence were selected for protein expression (Pizza et al., 2000; Tettelin et al 2000, reviewed by Danzig, 2004; Girard et al., 2005, 2006). The expressed proteins were then tested for their relevance to vaccine efficacy. The outcome of the experiment was that, proteins were secreted, conserved, expressed in all strains and

are therefore likely to confer protection against homologous and heterologous strains (Klein et al., 1996). In addition, five candidate genes were selected from the *Brucella melitensis* 16M genome. The selection was based on the prediction that these genes had sequence or structural homology to known virulence factors and protective antigens from other pathogens. A significant protective effect against the organism was observed from constructs encoding *B. melitensis omp*25 and *ial*B (Commander et al., 2007). Furthermore, in *Bacillus anthracis,* surface-associated or secreted proteins and virulence-associated proteins, namely toxins, adhesions, repeat proteins and enzymes were selected to determine whether they might induce immune response and protection against pathogens. Surface-bound or secreted proteins from these pathogens were able to induce immunity and protection against virulent challenges (Pizza et al., 2000; Adu-Bobie, 2003; Allan and Wren, 2003; Ariel et al., 2003; Serruto et al., 2004). Further evidence comes from the experiments done by Rollagen and his coworkers (2004) where they showed that some of the *in vivo* abundantly expressed Salmonella antigens SifA, SifB, IicA, SsaJ, SseB and Mig-14 (surface-bound/ secreted proteins) were able to induce strong and protective immune responses. Moreover, surface exposed proteins of *Porphyromonas gingivalis* (PG32 and PG33) demonstrated significant protection in the animal model (Ross et al., 2001).

The genome-based bioinformatics approach significantly reduces the time required to identify vaccine candidates. This approach also provides new solutions for those vaccines which have been difficult or impossible to develop (Rappuoli, 2001; Suerbaum et al., 2002; Raskin et al., 2006). However the vaccine candidates must still be evaluated *in vitro* and *in vivo* for their ability to induce protective immune responses and this will be the main focus in Chapter 3. In achieving recombinant protein expression, there are several factors that need to be considered. For example, for molecular cloning and expression of proteins *in vitro*, the choice of bacterial strain, medium formulation, and the promoter and expression system are critical in determining protein yields. There are two main expression systems that are commonly used for the expression of recombinant proteins. These are bacterial or eukaryotic; the latter include yeast, mammalian cells and baculovirus insect cells. The *Escherichia coli* bacterial system has many advantages

including high-level of protein production, ease to handle, low cost, rapid cell growth, ease of purification and there are lot of commercial kits available for recombinant protein expression (Sørensen and Mortensen, 2005; Dyson et al., 2004). On the other hand, the formation of inclusion bodies is a major problem if the protein should be expressed in a correct functional form. This phenomenon affects the enzymatic and functional studies of the proteins. In contrast, eukaryotic expression systems offer high levels of expression, easy purification, no inclusion body formation and proteins have intact post-translational modification. However, there are also limitations involved in this system which include the fact that eukaryotic cells grow very slow and low yields are obtained (http://www.molecularstation.com/molecular-biology-techniques/recombinant protein-expression.) Hence, the bacterial expression system was chosen for *E.ruminantium* recombinant protein expression.

The pET 102/D-TOPO® vector was used in this study because it is designed for efficient, rapid directional TOPO® cloning of blunt-end PCR products and for regulated expression in *E. coli.* This vector has a T7 promoter which is widely used for the over expression of both prokaryotic and eukaryotic proteins. It has a high specificity and processivity of T7 RNA polymerase. T7 RNA polymerase is produced from lysogenic λ prophage DE and its expression is under the control of the IPTG-inducible lacV5 promoters. This enzyme recognizes the promoter, binds to it and transcribes the gene of interest (Studier et al., 1990; reviewed by Gräslund et al., 2008).

In this chapter, we describe the molecular cloning and expression of five secreted proteins selected from the complete genome sequence of *E. ruminantium* (Welgevonden isolate) using bioinformatics tools.

2.2. MATERIALS AND METHODS

2.2.1. Materials

Materials used in this chapter are listed in Appendix A

2.2.2. Methods

2.2.2.1. *In silico* identification of secreted proteins

E. ruminantium secreted protein genes were identified by Ms Junita Liebenberg *in silico* using bioinformatics algorithms that can predict which proteins contain signal peptides, and the sub cellular localization of proteins as described below. Molecular weight, solubility and pI values of proteins were also predicted with the use of the website: http://us.expasy.org/tools/pi_tool.html.

Phobius

Phobius is a combined transmembrane topology and signal peptide predictor. Compared to TMHMM and SignalP, errors coming from cross-prediction between transmembrane segments and signal peptides are supposedly reduced substantially by Phobius. **http://phobius.cgb.ki.se/** as well as at **http://phobius.binf.ku.dk/** (Käll et al., 2004).

CELLO

CELLO is a subCELlular LOcalization predictive system for gram-negative bacteria. It utilizes a learning machine, Support Vector Machine, which is based on the multiple n-peptide composition. **http://cello.life.nctu.edu.tw/** (reviewed by Yu et al., 2004; reviewed by Yu et al., 2006).

PSORTb

PSORTb is an updated version of the PSORT algorithm designed for Gram-negative bacterial proteins. PSORTb displays the highest precision of any bacterial protein localization prediction tool available, with an overall precision of 96%. PSORT-b examines a given protein sequence for amino acid composition, similarity to proteins of

known localization, presence of a signal peptide, transmembrane alpha-helices and motifs corresponding to specific localizations. **http://www.psort.org/psortb/** (Gardy et al., 2005; Rey et al., 2005).

MAPPP

MAPPP (MHC-I Antigenic Peptide Processing Prediction) will predict possible T-cell epitope peptides to be processed and finally presented on the cell surfaces. It aids the prediction of immunodominant T-cell epitopes. **http://www.mpiib-berlin.mpg.de/MAPPP/** (Hakenberg et al., 2003)

CTLPred

CTLPred is a direct method for prediction of CTL (Cytotoxic T Lymphocyte) epitopes that play a vital role in subunit vaccine design (Bhasin and Raghava, 2004) **http://www.imtech.res.in/raghava/ctlpred/**

2.2.2.2. Purification of genomic DNA from E. ruminantium Welgevonden

E. ruminantium infected bovine aorta endothelial (BA) cells (Zweygarth et al., 1998) were harvested when they have achieved 80-100% lysis. The cell suspension was passed through a 0.4 mm needle. The cells were pelleted by centrifugation at 3000 g for 15 min at 4 °C to remove cell debris and BA cells. The elementary bodies (EBs) were pelleted by centrifugation of the resulting supernatant at 20000 g for 30 min at 4 °C. The EB pellet was resuspended in 1 ml phosphate buffered saline (PBS) by pipette aspiration and passed through a 26-28 gauge needle several times to break up the particles. A discontinuous Percoll (Sigma) gradient was prepared as previously described by Mahan et al., 1995. Briefly, a 100% Percoll (3 ml per gradient) was prepared by adding 1 ml 10x PBS (pH 7.2-7.5) to 9 ml pure Percoll. Using the buffered 100% Percoll, a 10% and 20% Percoll (10 ml of each per gradient) solutions were prepared and the gradient was prepared by starting with 9 ml PBS, followed by underlying 9 ml of 10% Percoll followed by 9 ml of 20% Percoll. One milliliter of elementary body suspension was layered on top of the gradient. The EBs were separated by centrifugation at 400 g in a Beckman JS-7.5 swing out rotor for 30 min. The top 15 ml containing the EBs was

removed and 20 ml PBS was added and centrifuged at 20000 g for 30 min at 4 °C. The pellet was carefully removed using a 1 ml syringe and passed several times through a 26-28 gauge needle to fully resuspend it. The EBs were washed once and after centrifugation at 20000 g for 30 min at 4 °C the pellet was harvested and resuspended in 350 µl of ice cold PBS. The EB suspension was treated with RNAse (0.1 mg/ml) and DNAse I (0.15 mg/ml) to remove RNA and eukaryotic DNA respectively, and incubated for 90 min at 37 °C. The reaction was stopped by adding 25 µl 0.5 M EDTA pH 8.0. The EBs were harvested by centrifugation at 20000 g for 15 min at 12 °C and the supernatant was discarded. The pellet was washed twice with 900 µl sterile Millipore H_2O and the EBs were harvested by centrifugation at 20000 g for 15 min at 12 °C. The supernatant was poured off and the pellet was resuspended in 50 µl sterile Millipore H_2O. Lysis buffer 0.1 M Tris-HCl pH 8.0, 0.2 M NaCl, 0.0075% SDS, 0.02 mg/µl Proteinase K (20 mg/ml) was added to the EBs and mixed gently. The suspension was incubated overnight at 55 °C. The EBs DNA was extracted twice with an equal volume of phenol and extracted once with an equal volume of phenol/chloroform/IAA (Indole 3 Acetic Acid) followed by an extraction with an equal volume of chloroform/IAA and precipitated by adding absolute ethanol (EtOH). The pellet was carefully washed with 70% EtOH. The pellet was dried and resuspended in 25-100 µl TE buffer pH 8.0. The quality and concentration of the genomic DNA was determined by running a 2 µl aliquot on a 0.5% agarose gel with appropriate size markers and measuring the DNA at A_{260} and A_{280} using the NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Inc.).

2.2.2.3. Recombinant protein expression in Escherichia coli

Preparation of Electrocompetent cells: TOP10, BL21 (DE3) and BL21 pLys (DE3)

A single colony of *E. coli* cells was inoculated into 15 ml LB medium and grown overnight at 37 °C with moderate shaking. The overnight culture (5 ml) was used to inoculate prewarmed LB broth (2x 500 ml). The cells were grown at 37 °C with vigorous shaking (300 rpm) until an A_{600} of 0.5-0.6 was measured.

The culture was poured into four prechilled 250 ml centrifuge bottles and cooled on ice for 20 min. The cells were harvested by centrifugation at 4000 g for 10 min at 4°C. The

supernatant was discarded and each pellet was resuspended in about 10 ml ice cold Millipore H_2O. The pellets were washed twice by adding 240 ml ice cold H_2O and mixed gently. The cells were harvested by centrifugation at 4000 g for 10 min at 4 °C. The supernatants were discarded immediately and the pellets were resuspended in 10 ml 10% glycerol incubated on ice for 30 min–1 h followed by centrifugation at 4000 g for 10 min at 4 °C. The supernatants were discarded and the pellets were resuspended in 800 µl 10% glycerol. Cells were snap-frozen until further use.

pET directional cloning: Cloning and transfection was done using the Champion™ pET Directional TOPO® Expression Kit (Invitrogen) according to the instructions of the manufacturer. Briefly, oligonucleotide polymerase chain reaction (PCR) primers were designed specific for each ORF (Table 2.1) and all signal peptides were excluded from resulting amplicon (see Table 2.2 for signal peptide lengths). The gene of interest was amplified using a proofreading polymerase that produces a blunt ended PCR product (*Pfu*, Promega). The PCR was done by adding 0.5 µl genomic DNA (~197.3 ng) in a 25 µl PCR reaction mix consisting of: *Pfu* DNA Polymerase (0.75 U), 0.2 µM of each primer (Table 2.1), 1x dNTP buffer (containing 2 µM dNTPs) and 1x reaction buffer (containing 0.2 mM Mg^{2+}). The DNA was PCR amplified using 30 cycles of amplification: hot start, (2 min at 95 °C), followed by 30 cycles of denaturation (95 °C for 30 s), annealing (50 °C for 30 s), and extension (72 °C for 1 min), this was followed by a final extension of 7 min at 72 °C) using the GeneAmp® PCR 9700 amplifier (Applied biosystems). Amplified products were analyzed by electrophoresis on 1% agarose gels stained with ethidium bromide (0.02 mg/ml). PCR products were purified using Roche kit according to manufacturer's instructions.

The amplified gene products and the amplified *LacZ* gene supplied in the kit were cloned into the pET102/D-TOPO® vector and the new plasmid constructs were transformed into One Shot®TOP10 competent *E. coli* cells using either the heat-shock or electroporation method. The cells were plated on LB-ampicillin (50 µg/ml) plates, incubated overnight at 37 °C and positive clones were selected and grown overnight in LB-ampicillin broth at 37 °C. The bacterial cells were pelleted, the plasmid DNA was purified (ROCHE

miniprep kit) and inserts were detected by PCR. The PCR was done by adding 0.5 µl of plasmid DNA in 25 µl PCR reaction mix consisting of: *TaKaRa Ex Taq*™ (0.75 U) (TaKaRa), 0.2 µM of each vector specific primers (TrxFus forward and T7 reverse, Table 2.1), 1x dNTP buffer (containing 2 µM dNTPs) and 1x reaction buffer (containing 0.2 mM Mg^{2+}). The amplification consisted of an initial denaturation step at 95 ºC for 2 min, followed by 30 cycles, including a denaturation step at 95 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 1 min. The final extension was performed for 7 min at 72 °C using the GeneAmp® PCR 9700 amplifier (Applied biosystems). Clones with the correct inserts were sequenced.

The template used for sequencing was either a PCR product (obtained from amplification with vector specific primers (Trx Fus forward and T7 reverse, Table 2.1) as described above) or plasmid DNA. The recommended DNA quantities for plasmid DNA was between 200-500 ng and for PCR product was 90 ng. DNA sequencing was performed (plasmid/PCR product) using the Big Dye® Terminator v 3.1 Cycle Sequencing kit (Applied Biosystems) and sequencing reaction mixtures were analyzed with 3100 Genetic Analyzer (Applied Biosystems). The resulting sequences were assembled using staden gap4 (Staden, et al., 1998; Martins et al., 2006) and the cloned sequences were compared with that of the *E. ruminantium* genome sequence. After confirming that the vector sequences were identical to that of the genome sequence, sequences were translated using the staden spin (Staden, et al., 1998) to make sure that the His_6-tag was intact.

Table 2.1. Primer sequences used for the amplification and cloning of *E. ruminantium* ORFs into the pET102/D-TOPO® vector.

Primer	Sequence 5'-3'	Predicted melting temperature (°c)
Erum7760F	AAGGAACGTGTTGTAAAATCTGTTG	63
Erum7760R	GTTATGGGACAATACATACCACTCAC	59
Erum5000F	GCTAAAGCTAATACAAATGATAACAAGATAAC	65
Erum5000R	AAATTCATACTTCATACCCAACAATAGAAC	61
Erum5010F	GTTGATGTATTAAGTCAGGGGAATCAG	67
Erum5010R	AAACTCCAGTTTTACACCAACTAAAAATATATTAC	61
Erum8060F	ATTAATACAAAAACAGATAACACAGTTAAAAC	62
Erum8060R	TTCTGAATAATAATGGGATATATCATGTTTAAATTC	60
Erum8610F	TATCCAAATATCATATCTAATGTAGCTTC	63
Erum8610R	TAATATAACCTTGAAATAATTGAACGTAAAAAAG	59
TrxFus F	TTCCTCGACGCTAACCTG	59
T7 R	TAGTTATTGCTCAGCGGTGG	55

Pilot expression of recombinant proteins: Plasmids containing the correct sequence were used for pilot expression. Electro-competent BL21Star™ (DE3) cells (Promega) were transformed by addition of 1 - 5 ng plasmid DNA/40 µl electrocompetent cells. The cell-plasmid mix was electroporated at 1.5 kV using the gene pulser® II (BIORAD). The transformation was incubated for 45 min at 37 °C in SOC medium and was added to 10 ml LB- ampicillin and grown overnight at 37 °C. Pilot expression was done by adding 500 µl of the overnight culture to fresh 10 ml LB-ampicillin. The cells were grown at 37 °C with shaking until an OD_{600} of 0.5-0.8 was measured (~2 h). The culture was split in two (5 ml each) and one of the two cultures was induced to express the PCR product by the addition of IPTG (Promega) to a final concentration of 1 M. Samples of both were taken at different time points starting from 1h after induction to a maximum of 5 h. Bacterial cells were pelleted by centrifugation at 16000 g for 30 s at 4 °C and were stored at -20 °C. Expression was analyzed by separating the proteins by SDS-PAGE and optimum conditions and time points were chosen for large scale expression.

Large scale expression and purification of recombinant proteins: Large scale expression was done according to instruction manual (Invitrogen). Briefly, LB-ampicillin

(10 ml) was inoculated with a fresh BL21Star™ (DE3) transformation and grown overnight at 37 °C. *E. coli* BL21 (DE3) pLysS (Promega) was also used for high-level expression of toxic proteins. A 100 ml LB-ampicillin culture was inoculated with 2 ml overnight culture and grown at 37 °C with shaking until an OD_{600} of 0.5-0.8 was measured. The protein expression was induced by the addition of IPTG (final concentration of 1M) and the culture was grown until the optimum time point as predetermined by pilot expression was reached. The cells were harvested by centrifugation at 3000 g for 10 min at 4°C.

Recombinant (His_6-tagged) proteins were purified from soluble supernatant or the inclusion bodies using the Protino® Ni 150 and 1000 prepacked columns kit (Macherey-Nagel) according to the instructions of the manufacturer. The purified proteins were assayed using SDS-PAGE analysis and anti-His_6 Western blot analyses.

SDS-PAGE analysis

All samples were separated on a 12% PAGE (See Appendix A for preparation of stacking and separating gels). The expressed proteins were thawed and resuspended in 1x SDS-PAGE sample buffer, boiled for 10 min and a 20 µl aliquot was loaded on a 12% SDS-PAGE gel. The proteins were separated by running a 12% gel at 100 V for ± 2 h. The gel was stained with BIOsafe Coomassie Blue (Bio-Rad) according to the instructions of the manufacturer. Protein bands were visualised using the Auto Chemi™ system from UVP BioImaging systems.

Western blot analyses using anti His_6 antibodies:

The proteins were again separated on a 12% SDS-PAGE as described above (http://www3.interscience.wiley.com/emrw/resolve/oid?OID=104554809). The proteins were transferred to a PVDF membrane after equilibrating the gel and membrane in transfer buffer (38 mM Tris base, 288 mM glycine, 300 ml methanol, pH ~8.3-8.4) with a semi-dry blotter (Semi-phor TE70, Hoefer scientific instruments) at 110 mA for 90 min. The membrane was incubated in 1% block buffer (1% bovine serum albumin in PBS) followed by incubation with anti-His_6 antibodies (Roche) (75 ng/100 ml) overnight at

room temperature. The membrane was washed 3x with wash buffer (1x PBS, 0.05% tween) and incubated with secondary antibody, Goat anti-mouse IgG (Zymed) for 90 min at room temperature. The blot was again washed 3x and the protein bands visualised using the SuperSignal®West Pico Chemiluminescent substrate from Pierce and exposed to a film (Roche) which was processed by X-ray film procedures.

Recombinant protein precipitation: The concentration of the purified recombinant proteins was determined using the Bio-Rad RC/DC protein assay reagents package, Bio-Rad according to the instructions of the manufacturer. The four expressed recombinant proteins were individually precipitated by adding 8x volume acetone and incubated over night at -20 °C. The proteins were collected by centrifugation at 10000 g for 10 min, where after excess salts were removed by washing with 70% ethanol. The protein pellet was air dried and stored at -20°C till for further use. The precipitated proteins were assayed using SDS-PAGE analysis and anti-His_6 Western blot analyses.

2.3. RESULTS

2.3.1. Bioinformatic selection of secreted proteins

Bioinformatics was utilized to predict secreted proteins from *E. ruminantium* Welgevonden genome sequence. Five open reading frames (ORFs) were chosen randomly out of a total of 24 predicted exported proteins. They contained signal peptides ranging from 1-27 amino acids in size and no helices, hence predicted to be exported proteins. Erum7760 and Erum8060 were predicted to be exported lipoprotein and Erum5010 to be an exported protein containing long tandem repeats (LTR). Erum7760 had the highest solubility percentage of 50.9% compared to the other ORFs. The predicted solubility percentage for Erum5000 and Erum5010 was less than 10%. The predicted molecular weight and pI values are tabulated in Table 2.2.

Table 2.2. Summary of results obtained from the selection of vaccine candidates using bioinformatics tools

Name	Closest Homologies	Base pair size (bp)	Length (aa)	pI	MW (kDa)	Signal peptides	Solubility %	Function
Erum5000	Unknown	1470	490	8.36	53.57	1-27	8.5	exported protein
Erum5010	Unknown	1690	564	8.50	62.98	1-23	8.5	exported protein (LTR)
Erum7760	Lipoprotein	750	250	6.15	29.34	1-26	50.9	exported lipoprotein
Erum8060	Lipoprotein	600	204	5.92	23.50	1-26	29.1	exported lipoprotein
Erum8610	Unknown	700	236	9.70	27.82	1-25	23.7	exported protein

2.3.2. PCR amplification of ORFs

The five ORFs of *E. ruminantium* encoding exported proteins were successfully amplified by PCR (Figure 2.1) with 5´- and 3´-specific primers (Table 2.1) and cloned into the pET102/D-TOPO® expression vector. The *LacZ* amplicon that was supplied in the Champion™ pET Directional TOPO® cloning kit was also cloned into the vector. This protein product, recombinant ß-galactosidase (rß-galactosidase) will be used as a negative control in the immunological assays described in chapter 3.

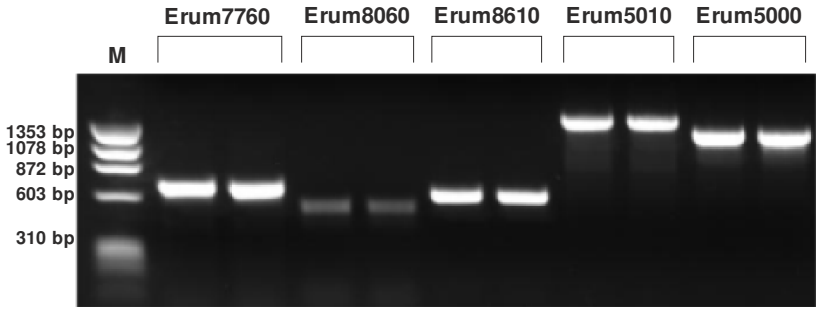


Figure 2.1. Agarose gel analysis of the PCR amplification of five ORFs: M- markers; followed by the amplified PCR products of Erum7760, Erum8060, Erum8610, Erum5010, and Erum5000 as indicated.

Repeated attempts to clone the PCR products of Erum5010 into the pET102/D-TOPO® vector failed. The bases were missing before the His tag in the sequence and a new reverse primer was used to reduce non-specific deletion and the attempt was unsuccessful.

The resulting plasmids were purified and the DNA sequences of the ORFs (Erum7760, Erum8060, Erum8610 and Erum5000) were compared to the corresponding *E. ruminantium* Welgevonden genome sequence (see Appendix B for the alignments). The DNA sequences of the selected ORFs were shown to be correct with no discrepancies and the inserts (Erum7760, Erum8060, Erum8610 and Erum5000) were cloned successfully, in-frame with the polyhistidine tail.

2.3.3. Expression and purification of recombinant proteins

The protein expression was induced in an *E. coli* BL21Star™ (DE3) host strain in conjunction with the pET102/D-TOPO® vector, where T7 RNA is produced from the λ-lysogen DE3 in the host bacterium, and its expression is under the control of the IPTG-inducible lac UV5 promoter. The pilot expression studies were investigated with four recombinant proteins (rErum5000, rErum7760, rErum8060 and rErum8610). For each time point (1-5 h), the non-induced and induced cultures were analysed on a SDS-PAGE. The optimum time of expression for rErum7760 and other recombinant proteins was shown to be between 3-5 h (Figure 2.2). This time point was subsequently used for harvesting cells in large scale production of each recombinant protein. All plasmid constructs were designed to contain six N-terminal histidine residues (His_6) that assisted in the purification of the recombinant proteins from 100 ml cultures with the Protino® Ni prepacked column kits (Macherey-Nagel). The recombinant proteins were purified from the soluble supernatant and from the inclusion bodies with yields ranging between 200 µg- 2000 µg of purified protein per 100 ml and 1 l respectively of IPTG induced culture. This system was employed successfully for the production of three recombinant proteins: rErum5000, rErum7760 and rErum8060.

Initial attempts to express the rErum8610 protein in BL21Star™ (DE3) *E. coli* cells at 37º C failed. Protein expression at 18º C rather than at 37º C was also unsuccessful. Large scale expression in 1 l instead of 100 ml culture in an attempt to express more recombinant protein also failed. Similarly, addition of 1% glucose to the bacterial medium failed. However, successful expression was obtained by using BL21 (DE3) pLysS *E. coli* cells at 37º C.

Figure 2.3 shows a Coomassie blue stained SDS-PAGE of the purified recombinant proteins. The results revealed the presence of expressed recombinant proteins migrating at the specific predicted molecular mass positions. The predicted molecular masses are shown in Table 2.4. There was a slight increase in molecular masses as expected. This increase is due to the addition of Thioredoxin (TRX) and His_6-tag sequence of

approximately 13 kDa. The TRX tags help with refolding the proteins correctly and thus enhance the solubility while the His_6-tag permits purification of recombinant proteins.

Table 2.3. Predicted molecular weights of four recombinant proteins

Name	Predicted molecular weight (kDa)	Predicted molecular weight + His-tag (kDa)
rErum7760	29.3	42.3
rErum8060	23.5	36.5
rErum5000	53.5	66.5
rErum8610	27.8	40.8

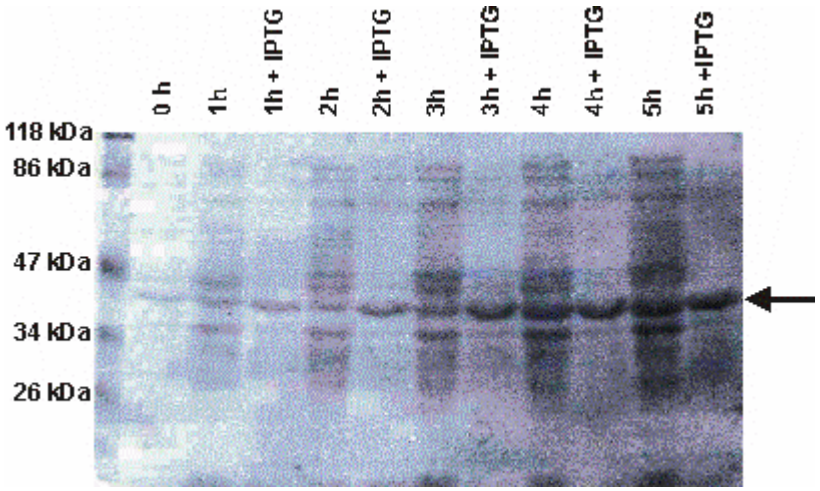


Figure 2.2. Pilot expression of recombinant protein rErum7760. Samples were analyzed on 12% SDS-PAGE and stained with Coomassie blue. Samples were collected at the indicated time points from uninduced and induced with 1 M IPTG cultures. Arrow indicates position of expressed protein.

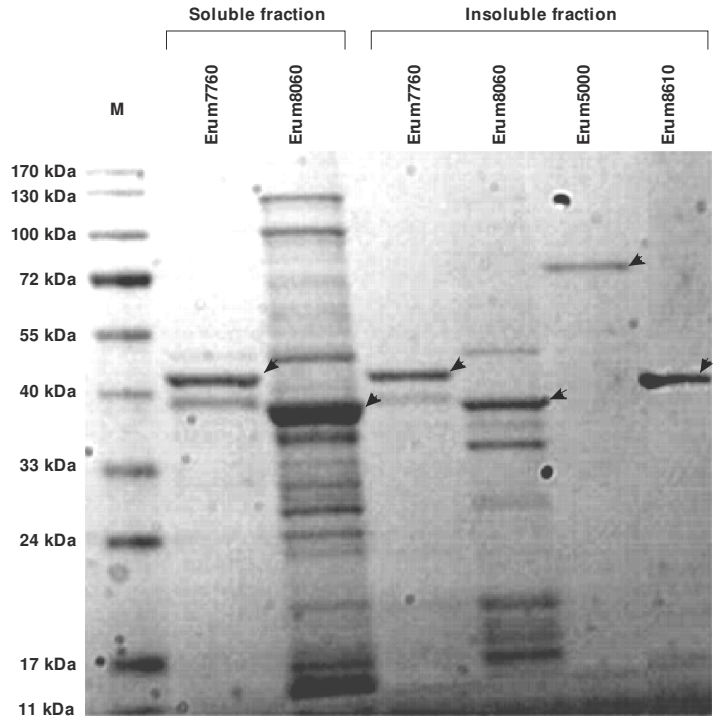


Figure 2.3. Coomassie blue stained gel of purified recombinant proteins electrophoresed on 12% SDS-PAGE gel. These proteins were expressed using Champion™ pET Directional TOPO® expression system and purified by Protino® Ni columns kit. Protein bands with the correct size are indicated with arrows.

Immunoblot blot analyses with anti-His_6 antibodies confirmed that the proteins of correct MW were expressed (Figure 2.4). Expression of the rErum7760, rErum8060, rErum8610 & rErum5000 in *E. coli*, showed distinct bands at ~42 kDa, ~36 kDa, ~41 kDa and ~70 kDa respectively in SDS-PAGE and Immunoblot blot assays thus corresponding to the predicted weights (Table 2.3). Recombinant Erum7760 and rErum8060 were purified from both the soluble and insoluble fractions while rErum5000 and rErum8610 were 100% insoluble (Figure 2.4).

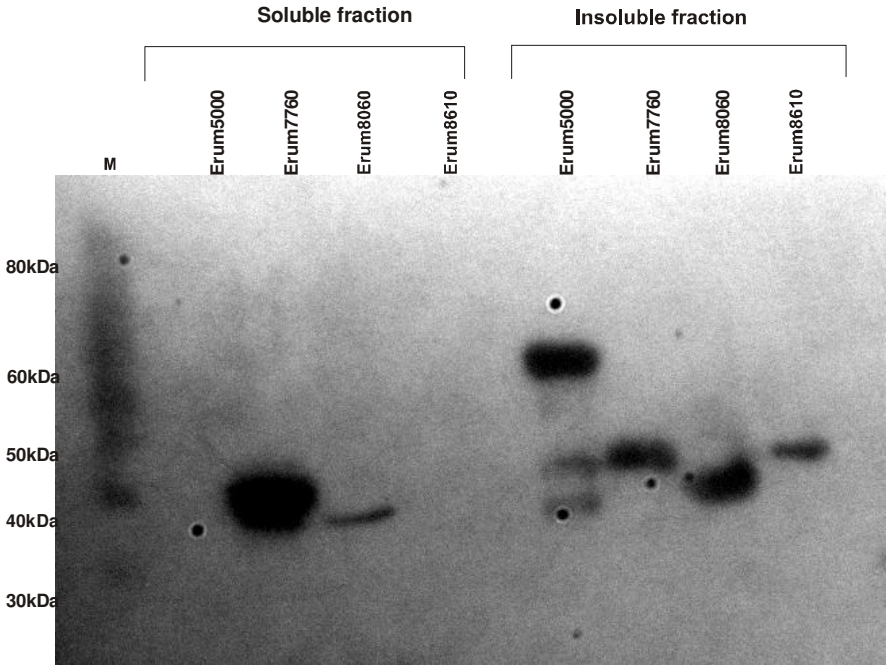


Figure 2.4. Western blot analysis of purified recombinant proteins expressed using Champion™ pET Directional TOPO® expression system. Recombinant proteins were detected by using anti-His antibodies and Chemiluminescence. M- molecular weight markers.

2.4. DISCUSSION

In this chapter, the *E. ruminantium* ORFs encoding putative proteins that were predicted to be exported were selected from the complete genome sequence of *E. ruminantium* (Welgevonden) using bioinformatics tools (annotation from Collins et al., 2005). Three of the proteins encoded by Erum5000; Erum5010 and Erum8610 did not show any similarity to known proteins in the database while those encoded by Erum7760 and Erum8060 demonstrated close homology to exported lipoproteins. Exported proteins with a lipoprotein signal peptide are retained at the extracytoplasmic side of the membrane while other proteins that have a Sec-type signal are translocated across the membrane (Fu et al., 2007). Lipoproteins have been shown to play an important role in the activation of humoral and cellular immune responses as were identified in *M. tuberculosis* (Young and Garbe, 1991), *Treponema pallidum* (Radolf et al., 1995) and *Mycoplasma hyorhinis* (Bricker et al., 1988). Hovav et al., (2003) demonstrated that a recombinant 27 kDa lipoprotein of *M. tuberculosis* is a strong elicitor of Th1 type responses. Furthermore, in a study by Neyrolles et al., 2001, a 19 kDa lipoprotein was also shown to play a vital role in the induction of a protective Th1 type immune response against *M. tuberculosis*. An immunogenic 22 kDa (P22) exported lipoprotein of *M. avium* subspecies *paratuberculosis* was found to stimulate cellular mediated immunity characterized by the production of IFN-γ (Rigden et al., 2006). These observations indicate that exported lipoproteins are inducers of Th1 cellular immune responses and therefore merit further investigation as vaccine candidates.

Four out of five ORFs were successfully cloned except Erum5010 into the pET102/D-TOPO® vector using TOP10 *E. coli* cells. It was shown in the annotation (Collins et al., 2005) that Erum5010 showed 29% amino acid sequence identity to Erum5000. The inability to clone Erum5010 into the vector could not be due to size because these ORFs were also similar in size (Erum5000 – 1470bp and Erum5010 – 1690bp). One factor that could attribute to instability of the gene in the expression vector could be the fact that Erum5010 was predicted to have long tandem repeats. Previously, it has been shown that long tandem repeats are unstable in *E. coli* (Raman et al., 1997). However, with the use

of host strain PMC107, relative stability of plasmids containing long tandem repeats was increased. Therefore, experiments designed to measure plasmid instabilities and use of different host strains to overcome these limitations need to be investigated for Erum5010.

Recombinant Erum5000, rErum7760 and rErum8060 were successfully expressed in BL21 (DE3) *E. coli* strain at 37ºC. Two recombinant proteins (rErum7760 and rErum8060) were present in both the soluble and insoluble fraction whereas rErum5000 was approximately 100% insoluble. Several attempts to initially express rErum8610 in the BL21 (DE3) *E. coli* strain were not successful. The lack of expression in BL21 (DE3) cells could be due to the over activity of T7 RNA polymerase that may result in plasmid instability and low expression. Therefore, it was necessary to optimize culture conditions in order to reduce the activity of the enzyme and thus increase plasmid stability and protein yield. Zhang and coworkers (2003) proved that by inducing expression at low temperature and adding glucose to the medium enhanced T7 RNA polymerase-based plasmid stability and improved protein yield in *E. coli*. Therefore, expression of rErum8610 was induced at 18 ºC but no expression was obtained. The addition of 1% glucose added to culture medium in an attempt to improve plasmid stability was also not successful. This led to the hypothesis that the lack of expression could be due to toxicity of the target protein to the host. Toxicity of proteins to the expression host is not uncommon. Significant expression of many eukaryotic proteins could not be obtained in different bacterial host strains due to toxicity of target proteins which lead to cell death (Ferreira and Pedersen, 1992). In BL21 (DE3) cells, background expression of the proteins was observed in the non-induced fraction of the pilot study in all four *E. ruminantium* proteins expressed. Expression of even slightly toxic gene products in BL21 (DE3) cells could lead to cell death.

A BL21 (DE3) pLysS *E. coli* strain was used to provide tighter control of background expression for toxic rErum8610 and to overcome toxic effects which may result in plasmid or expression instability or even cell death (Studier et al., 1990; Dubendorff and Studier, 1991; Baneyx, 1999). The pLysS plasmid, which expresses T7 lysozyme in the bacterial cytoplasm, strongly inhibits protein expression from pET102/D-TOPO® vector

in the absence of IPTG induction, thus enabling expression of toxic proteins (Dąbrowski and Kur, 1999). Two recombinant chimaeric fusion proteins that consist of human interleukin-13 and *Pseudomonas* exotoxin were expressed in a genetically modified *E. coli* strain BL21 (DE3) pLysS and a normal strain BL21 (DE3) (Joshi and Puri, 2005). The BL21 (DE3) pLysS bacterial system produced the highest yield expression of recombinant proteins (21-28 mg/L) as compared to BL21 (DE3) (14-22mg/L). Similarly, two mutants strains of *E. coli* BL21 (DE3), called C41 (DE3) and C43 (DE3) have been used successfully (Miroux and Walker, 1996; Dumon-Seignovert et al., 2004). They also modified the expression vector in order to reduce the plasmid instability when cultures were expanded for large-scale production. This was achieved by adding the *par* locus from the plasmid pSC101 to the vector backbone (pRSETA). The pSC101 par locus stabilizes plasmid inheritance in populations of dividing cells (Meacock & Cohen, 1980; Miller & Cohen, 1993; Conley, & Cohen, 1995). Recombinant Erum8610 was successfully expressed in BL21 (DE3) pLysS *E. coli* strain at 37ºC and was shown to be insoluble. Thus even though rErum8610 was toxic to the *E. coli* host cells, enough protein was expressed using the BL21 (DE3) pLysS strain for use in the immunological assays (Lymphocyte proliferation and ELISPOT) described in Chapter 3. However, the protein could not be repeatedly expressed at the same conditions stated above. On the basis of these results, it is clear that this protein is toxic and other methods could to be investigated that include epitope mapping and modification of vectors.

Secretory proteins are known to be soluble (Georgiou and Segatori, 2005; Berrow et al., 2006; Esposito and Chatterjee, 2006). In this study most of the proteins were however purified from the insoluble fraction (rErum7760, rErum8060, rErum5000 and rErum8610). This could be a result of over-expression of proteins that leads to the formation of inclusion bodies. Inclusion bodies are proteins that are not refolded correctly. Many recombinant proteins accumulate as insoluble, biologically inactive forms when expressed in microbes (Kane and Hartley, 1991; Misawa and Kumagai, 1999; Georgiou and Segatori, 2005; Kunji et al., 2005). To overcome these folding problems, alternative host cells such as insect, yeast and mammalian cells could be used for recombinant protein production and codon-optimise the gene for expression (Rudolph

and Lillie, 1996; Baneyx, 1999). Moreover, changing the pH of the culture, media components, carbon source or growth temperature and use of modified strains could also improve the refolding of the protein (Quick and Wright, 2002). Proteins expressed in insect cells using the baculovirus system are mostly in a correctly folded state and the production yield of proteins can be high. However, there may be toxicity problems and it is cost ineffective (Pizarro et al., 2003). In addition, it has been reported that the high content of A/T repeats in the *P. falciparum* genome might influence the formation of insoluble inclusion bodies (Flick et al., 2004). Since, *E. ruminantium* genome is an A/T-rich genome it might be expected to obtain insoluble proteins as seen in the *P. falciparum* genome (Pandey et al., 2002; Singh et al., 2003; Flick et al., 2004). However, insoluble proteins have been used for immunisation especial for obtaining cellular immune responses (Bascoul et al., 1978; Kawasaki, et al., 2007) but purification requires denaturing conditions (8 M Urea). Urea can inhibit lymphocyte proliferation and therefore needs to be removed from the final product (Orendi et al., 2000). Nevertheless, a recombinant protein VP8* of human rotavirus (HRV) still in its aggregated form or solubilized in 8 M urea was found to be a good immunogen for the production of neutralizing antibodies in immunized animals, and to prime the immune system of mice (Lizano et al., 1991; Lee et al., 1995). However, since protection against intracellular pathogens is mediated by cellular immune responses, vaccine candidates should contain epitopes recognized by T cells. It is known that cellular immunity requires linear epitopes therefore conformation (solubility) of proteins will not affect the lymphocyte proliferation assays and ELISPOT assays that will be discussed in the next chapter (Mustafa et al., 2000).

The purity of proteins was also one of the problems observed. Western blot results indicated that rErum5000 was not 100% pure. Two or more bands were observed. These bands could be contaminants or due to proteolysis. In addition, there are other proteins that bind to nickel columns. For example lysozyme is one of the proteins that we are adding to the BugBuster protein extraction reagent. Because it has the poly histidine in its structure, it also binds to the nickel column. However, the size of the lysozyme is only 14 kDa and does not correlate with the sizes seen on the gels. It is not surprising to obtain

two or more bands when expressing recombinant proteins in *E. coli*. Previously, a His_6-tagged Pwo dUTPase from *Pyrococcus woesei* was successfully expressed and purified in *E. coli* where two bands corresponding to a 25kDa and 53kDa were observed (Dąbrowski and Ahring, 2003). Furthermore, the expression of human rotavirus Wa strain VP8* produced a duplex band on the SDS-PAGE gels (Kovacs-Nolan et al., 2001; Perez Filgueira et al., 2004). However, obtaining duplex or triple bands is a common feature due to digestion by bacterial proteases or contaminants (Favacho et al., 2006).

In conclusion, four recombinant proteins were successfully produced using the bacterial expression system. The production yield for three recombinant proteins was high except for Erum8610. Therefore, the availability of expressed *E. ruminantium* recombinant proteins should now allow the testing of these antigens in different immunological assays for their ability to induce immune response which will be discussed in Chapter 3.

2.5. REFERENCES

ACKERMAN, A.L., KYRITSIS, C., TAMPÉ, R., & CRESSWELL, P., 2005. Access of soluble antigens to the endoplasmic reticulum can explain cross-presentation by dendritic cells. *Nature Immunology*, 6:107-113.

ADU-BOBIE, J., CAPECCHI, B., & SERRUTO, D., 2003. Two years into reverse vaccinology. *Vaccine*, 21:605-610.

ALLAN, E. & WREN, B.W., 2003. Genes to genetic immunization: identification of bacterial vaccine candidates. *Methods*, 31:193-198.

ARIEL, N., ZVI, A., MAKAROVA, K.S., CHITLARU, T., ELHANANY, E., VELAN, B., COHEN, S., FRIEDLANDER, A.M. & SHAFFERMAN, A., 2003. Genome-based bioinformatics selection of chromosomal *Bacillus anthracis* putative vaccine candidates coupled with proteomic identification of surface-associated antigens. *Infection and Immunity*, 71:4563-4579.

BANEYX, F., 1999. Recombinant protein expression in *Escherichia coli*. *Current Opinion in Biotechnology*, 10:411-421.

BANSAL, A.K., 2005. Bioinformatics in microbial biotechnology- a mini review. *Microbial Cell Factories*, 4:1-11.

BASCOUL, S., CANNAT, A., HUGUET, M.F., & SERRE, A., 1978. Studies on the immune protection to murine experimental brucellosis conferred by Brucella fractions. *Immunology*, 35:213-221.

BELISLE, J.T., VISSA, V.D., SIEVERT, T., TAKAYAMA, K., BRENNAN, P.J., & BESRA, G.S., 1997. Role of the major antigen of *Mycobacterium tuberculosis* in cell wall biogenesis. *Science*, 276:1420-1422.

BERROW, N.S., BÜSSOW, K., COUTARD, B., DIPROSE, J., EKBERG, M., FOLKERS, G.E., LEVY, N., LIEU, V., OWENS, R.J., PELEG, Y., PINAGLIA, C., QUEVILLON-CHERUEL, S., SALIM, L., SCHEICH, C., VINCENTELLI, R., & BUSSO, D., 2006. Recombinant protein expression and solubility screening in *Escherichia coli*: a comparative study. *Acta Crystallographica*, D62:1218-1226.

BHASIN, M., & RAGHAVA, G.P.S., 2004. Prediction of CTL epitopes using QM, SVM and ANN techniques. *Vaccine*, 22:3195-3201.

BITSAKTSIS, C., HUNTINGTON, J., & WINSLOW, G., 2004. Production of IFN-gamma by CD4 T cells is essential for resolving ehrlichia infection. *Journal of Immunology*, 172:6894-6901.

BITSAKTSIS, C., NANDI, B., RACINE, R., MACNAMARA, K.C., & WINSLOW, G., 2007. T-cell-independent humoral immunity is sufficient for protection against fatal intracellular ehrlichia infection. *Infection and Immunity*, 75:4933-4941.

BONIN-DEBS, A., BOCHE, I., GILLE, H., & BRINKMANN, U., 2004. Development of secreted proteins as biotherapeutic agents. *Expert Opinion on Biological Therapy*, 4:551-558.

BOURZAC K.M., & GUILLEMIN, K., 2005. *Helicobacter pylori*-host interactions mediated by type IV secretion. *Cell Microbiology*, 7:911-19.

BRICKER, T.M., BOYER, M.J. KEITH, J., WATSON-MCKOWN, R., & WISE, K.S., 1988. Association of lipids with integral membrane surface proteins of *Mycoplasma hyorhinis*. *Infection and Immun*ity, 56:295-301.

BRODSKY, J.L., 1998. Translocation of proteins across the endoplasmic reticulum membrane. *International Review of Cytology*, 178:277-328.

CARLE, N., HÖPPER, C., ALY, K.A., YUAN, Q., DEN DULK-RAS, A., VERGUNST, A., O'CALLAGHAN, D., & BARON, C., 2006. The *Brucella suis* type IV secretion system assembles in the cell envelope of the heterologous host *Agrobacteruim tumefaciens* and increases IncQ plasmid pLS1 recipient competence. *Infection and Immunity*, 74:108-117.

CHEN, L.M., BRIONES, G., DONIS, R.O., & GALÁN, J.E., 2006. Optimization of the delivery of heterologous proteins by the *Salmonella enterica* Serovar Typhimurium Type III secretion sytem for vaccine development. *Infection and Immunity*, 74:5826-5833.

CHO, J.H., CHUNG, W.S., SONG, K.J., NA, B.K., KANG, S.W., SONG, C.Y., & KIM, T.S., 2005. Protective efficacy of vaccination with *Neospora caninum* multiple recombinant antigens against experimental *Neospora caninum* infection. *Korean Journal of Parasiotol*ogy, 43:19-25.

CHO, D., SHIN, S.J. TALAAT, A.M., & COLLINS, M.T., 2007. Cloning, expression, purification and serodiagnostic evaluation of fourteen *Mycobaterium paratuberculosis* proteins. *Protein Expression and Purification*, 53:411-420.

CHRISTIE, P.J., 2004. Type IV secretion: the *Agrobacterium* VirB/D4 and related conjugation systems. *Biochimica et Biophysica acta*. 1694:219-234.

COLLINS, N.E., LIEBENBERG, J., DE VILLIERS, E.P., BRAYTON, K.A., LOUW, E., PRETORIUS, A., FABER, F.E., VAN HEERDEN, H., JOSEMANS, A., VAN KLEEF, M., STEYN, H.C., VAN STRIJP, M.F., ZWEYGARTH, E., JONGEJAN, F., MAILLARD, J.C., BERTHEIR, D., BOTHA, M., JOUBERT, F., CORTON, C.H., THOMSON, N.R., ALLSOPP, M.T., & ALLSOP, B.A., 2005. The genome of the heartwater agent *Ehrlichia ruminantium* contains multiple tandem repeats of actively variable copy number. *Proceedings of the National Academy of Sciences of the United States of America*, 102:838-843.

COMANDUCCI, M., BAMBINI, S., BRUNELLI, B., ADU-BOBIE, J., ARICÒ, B., CAPECCHI, B., GIULIANI, M.M., MASIGNANI, V., SANTINI, L., SAVINO, S., GRANOFF, D.M., CAUGANT, D.A., PZZA, M., RAPPUOLI, R., & MORA, M., 2002. NadA, a novel vaccine candidate of *Neisseria meningitidis*. *Journal of Experimental Medicine*, 195:1445-1454.

COMMANDER, N.J., SPENCER, S.A., WREN, B.W., & MACMILLAN, A.P., 2007. Identification of two protective DNA vaccines from a panel of five plasmid constructs encoding *Brucella melitensis* 16M genes. *Vaccine*, 25:43-54.

CONLEY, D.L., & COHEN, S.N., 1995. Effects of the pSC101 partition (*par*) locus on *in vivo* DNA supercoiling near the plasmid replication origin. *Nucleic Acids Research*, 23:701-707.

DĄBROWSKI, S., & KUR, J., 1999. Cloning, overexpression, and purification of the recombinant His-tagged SSB protein of *Escherichia coli* and use in Polymerase Chain Reaction amplification. *Protein Expression and Purification*, 16:96-102.

DĄBROWSKI, S., & AHRING, B.K., 2003. Cloning, expression, and purification of the His_6-tagged hyper-thermostable dUTPase from *Pyrococcus woesei* in *Escherichia coli*: application in PCR. *Protein Expression and Purification*, 31:72-78.

DANZIG, L., 2004. Meningococcal vaccines. *The Pediatric Infectious Disease Journal*, 23:S285-S292.

DIGIUSEPPE, P.A., & COX, J.S., 2007. Protein secretion systems in Mycobacteria. *Cellular Microbiology*, 9:1876-1384.

DING, Z., ATMAKURI, K., & CHRISTIE, P.J., 2003. The outs and ins of bacterial type IV secretion substrates. *TRENDS in Microbiology*, 11:527-535.

DOYLE, C.K., NETHERY, K.A., POPOV, V.L., & MCBRIDE, J.W., 2006. Differentially expressed and secreted major immunoreactive protein orthologs of *Ehrlichia canis* and *E. chaffeensis* elicit early antibody responses to epitopes on glycosylated tandem repeats. *Infection and Immunity*, 74:711-720.

DREW, D.R., LIGHTOWLERS, M., & STRUGNELL, R.A., 2000. Humoral immune responses to DNA vaccines expressing secreted, membrane bound and non-secreted forms of the *Taenia ovis* 45W antigen. *Vaccine*, 18:2522-25-32.

DUBENDORFF, J.W., & STUDIER, F.W., 1991. Controlling basal expression in an inducible T7 expression system by blocking the target T7 promoter with lac repressor. *Journal of Molecular Biology*, 219:45-59.

DUMON-SEIGNOVERT, L., CARIOT, G., & VUILLARD, L., 2004. The toxicity of recombinant proteins in *Escherichia coli*: a comparison of overexpression in BL21 (DE3), C41 (DE3), and C43 (DE3). *Protein Expression and Purification*, 37: 203-206.

DYSON, M.R., SHADBOLT, S.P., VINCENT, K.J., PERERA, R.L., & MCCAFFERTY, J., 2004. Production of soluble mammalian proteins in *Escherichia coli*: identification of protein features that correlate with successful expression. *BMC Biotechnology*, 4:32.

ESPOSITO, D., & CHATTERJEE, D.K., 2006. Enhancement of soluble protein expression through the use of fusion tags. *Current Opinion in Biotechnology*, 17:353-358.

FAVACHO, A.R.M., KURTENBACH, E., SARDI, S.I., & GOUVEA, V.S., 2006. Cloning, expression, and purification of recombinant bovine rotavirus hemagglutinin, VP8*, in *Escherichia coli*. *Protein Expression and Purification*, 46:196-203.

FERREIRA, G.C., & PEDERSEN, P.L., 1992. Overexpression of higher eukaryotic membrane proteins in bacteria. *The Journal of Biological Chemistry*, 267:5460-5466.

FLICK, K., AHUJA, S., CHENE, A., BEJARANO, M.T., & CHEN, Q., 2004. Optimized expression of *Plasmodium falciparum* erythrocyte membrane protein 1 domains in *Escherichia coli. Malaria Journal*, 3:50.

FLOCK, M., KARLSTRÖM, Å., LANNERGÅRD, J., GUSS, B., & FLOCK, J.I., 2006. Protective effect of vaccination with recombinant proteins from *Streptococcus equi* subspecies *equi* in a strangles model in the mouse. *Vaccine*, 24:4144-4151.

FU, L.L., XU, Z.R., LI, W.F., SHUAI, J.B., LU, P., & HU, C.X., 2007. Protein secretion pathways in *Bacillus subtilis*: implication for optimization of heterologous protein secretion. *Biotechnology Advances*, 25:1-12.

GANGULY, N., GIANG, P.H., BASU, S.K., MIR, F.A., SIDDIQUI, I., & SHARMA, P., 2007. *Mycobacterium tuberculosis* 6-kDa Early Secreted Antigenic Target (ESAT-6) protein downregulates liposaccharide induced c-myc expression by modulating the extracellular signal regulated kinases 1/2. *BMC Immunology*, 8:24.

GARDY, J.L., LAIRD, M.R., CHEN, F., REY, S., WALSH, C.J., ESTER, M., & BRINKMAN, F.S.L., 2005. PSORTb v.2.0: expanded prediction of bacterial protein subcellular localization and insights gained from comparative proteome analysis. *Bioinformatics*, 21:617-623.

GEORGIOU, G., & SEGAROTI, L., 2005. Preparative expression of secreted proteins in bacteria: status report and future prospects. *Current Opinion in Biotechnology*, 16: 538-545.

GIBBONS, H.S., WOLSCHENDORF, F., ABSHIRE, M., NIEDERWEIS, M., & BRAUNSTEIN, M., 2007. Identification of two *Mycobacterium smegmatis* lipoproteins exported by a SecA2-dependent pathway. *Journal of Bacteriology*, 189:5090-5100.

GIRARD, M.P., CHERIAN, T., PERVIKOV, Y., & KIENY, M.P., 2005. A review of vaccine research and development: human acute respiratory infections. *Vaccine*, 23:5708-5724.

GIRARD, M.P., PREZIOSI, M.P., AGUADO, M.T., & KIENY, M.P., 2006. A review of vaccine research and development: meningococcal disease. *Vaccine*, 23:4692-4700.

GRANDI, G., 2001. Antibacterial vaccine design using genomics and proteomics. *TRENDS Biotechnology*, 19:181-188.

GRÄSLUND, S., NORDLUND, P., WEIGELT, J., BRAY, J., GILEADI, O., KNAPP, S., OPPERMANN, U., ARROWSMITH, C., HUI, R., MING, J., et al., 2008. Protein production and purification. *Nature Methods*, 5:135-146.

HAKENBERG, J., NUSSBAUM, A.K., SCHILD, H., RAMMENSEE, H.G., KUTTLER, C., HOLZHÜTTER, H.G., KLOETZEL, P.M., KAUFMANN, S.H., MOLLENKOPF, H.J., 2003. MAPPP: MHC class I antigenic peptide processing prediction. *Applied Bioinformatics*, 2:155-8.

HASLØV, K., ANDERSEN, Å., NAGAI, S., GOTTSCHAU, A., SØRENSEN, T., & ANDERSEN, P., 1995. Guinea pig cellular immune responses to proteins secreted by *Mycobacterium tuberculosis*. *Infection and Immunity*, 63:804.

HIGH, S., 1995. Protein translocation at the membrane of the endoplasmic reticulum. *Progress in Biophysics and Molecular Biology*, 63:233-250.

HORWITZ, M.A., LEE, B.E., DILLON, B.J. & HARTH, G., 1995. Protective immunity against tuberculosis induced by vaccination with major extracellular proteins of *Mycobacterium tuberculosis*. *Proceedings of the National Academy of Sciences of the United States of America*, 92:1530-1534.

HOVAV, A.H., MULLERAD, J., DAVIDOVITCH, L., FISHMAN Y., BIGI, F., CATALDI, A., & BERCOVIER, H., 2003. The *Mycobacterium tuberculosis* recombinant 27-Kilodalton lipoprotein induces a strong Th1-type immune response deleterious to protection. *Infection and Immunity*, 71:3146-3154.

HUYNH, M.H., RABENAU, K.E., HARPER, J.M., BEATTY, W.L., SIBLEY, L.D., & CARRUTHERS, V.B., 2003. Rapid invasion of host cells by *Toxoplasma* requires secretion of the MIC2-M2AP adhesive protein complex. *The EMBO Journal,* 22:2082-2090.

ITO, K., 1996. The major pathways of protein translocation across membranes. *Genes to Cells*, 1:337-346.

JOSHI, B.H., & PURI, R.K., 2005. Optimization of expression and purification of two biologically active chimeric fusion proteins that consist of human interleukin-1`3 and *Pseudomonas* exotoxin in *Escherichia coli*. *Protein Expression and Purification*, 39:189-198.

KÄLL, L., KROGH A. AND SONNHAMMER, E.L.L. 2004. A combined transmembrane topology and signal peptide prediction method. *Journal of Molecular Biology*, 338:027-1036.

KANE, J.F. & HARTLEY D.L., 1991. Properties of recombinant protein-containing inclusion bodies in *Escherichia coli*. *Bioprocess Technology*, 12:121-145.

KANEHISA, M., & BORK, P., 2003. Bioinformatics in the post-sequence era. *Nature Genetics Supplement,* 33:305-310.

KAWASAKI, P.M., KANO, F.S., VIDOTTO, O., & VIDOTTO, M.C., 2007. Cloning, sequencing, expression, and antigenic characterization of rMSP4 from *Anaplasma marginale* isolated from Paraná State, Brazil. *Genetics and Molecular Research*, 6:15-22.

KLEIN, R.D., GU, Q., GODDARD, A., & ROSENTHAL, A., 1996. Selection for genes encoding secreted proteins and receptors. *Proceedings of the National Academy of Sciences of the United States of America*, 93:7108-7113.

KOSTAKIOTI, M., NEWMAN, C.L., THANASSI, D.G., STATHOPOULOS, C., 2005. Mechanisms of protein export across the bacterial outer membrane. *Journal of Bacteriology*, 187:4306-4314.

KOVACS-NOLAN, J., SASAKI, E., YOO, D., & MINE, Y., 2001. Cloning and expression of human rotavirus spike protein, VP8*, in *Escherichia coli*. *Biochemical and Biophysical Research Communications*, 282:1183-1188.

KUNJI, E.R.S., CHAN, K.W., SLOTBOOM, D.J., FLOYD, S., O'CONNOR, R., & MONNÉ, M., 2005. Eukaryotic membrane protein overproduction in *Lactococcus lactis*. *Current Opinion in Biotechnology*, 16:546-551.

LAMMERTYN, E., & ANNE, J., 2004. Protein secretion in *Legionella pneumophila* and its relation to virulence. *FEMS Microbiology Letters*, 238:273-279.

LANGERMANS, J.A., DOHERTY, T.M., VERVENNE, R.A., VAN DER LAAN, T., LYASHCHENKO, K., GREENWALD, R., AGGER, E.M., AAGAARD, C., WEILER, H., VAN SOOLINGEN, D., DALEMANS, W., THOMAS, A.W., & ANDERSEN, P., 2005. Protection of macaques against *Mycobacterium tuberculosis* infection by a subunit vaccine based on a fusion protein of antigen 85B and ESAT-6. *Vaccine*, 23:2740-2750.

LEAL, M., NODA, A., REYNA-BELLO, A., CASAS, B., PRÉCIGOUT, E., ASO, P.M., GORENFLOT, A., GONZATTI, M.I., 2000. Identification and characterization of corpuscular, soluble and secreted antigens of a Venezuelan isolate of *Anaplasma marginale*. *Veterinary Parasitology*, 94:1-15.

LEE, J., BABIUK, L.A., HARLAND, R., GIBBONS, E., ELAZHARY, Y., & YOO, D., 1995. Immunological response to recombinant VP8* subunit protein of bovine rotavirus in pregnant cattle. *Journal of General Virology*, 76:2477-2483.

LIZANO, M., LÓPEZ, S., & ARIAS, C.F., 1991. The amino-terminal half of rotavirus SA114fM VP4 protein contains a hemagglutinin domain and primes for neutralizing antibodies to the virus. *Journal of Virology,* 65:1383-1391.

MAHAN, S.M., ANDREW, H.R., & TEBELE, N., 1995. Immunisation of sheep against heartwater with inactivated *Cowdria ruminantium*. *Research Veterinary Science*, 58:46-49.

MÅLEN, H., BERVEN, F.S., FLADMARK, K.E., & WIKER, H.G., 2007. Comprehensive analysis of exported proteins from *Mycobacterium tuberculosis* H37Rv. *Proteomics*, 7:1702-1718.

MARTI, M., BAUM, J., RUG, M., TILLEY, L., & COWMAN, A.F., 2005. Signal-mediated export of proteins from the malaria parasite to the host erythrocyte. *The Journal of Cell Biology*, 171:587-592.

MARTINEZ, V., CARCELAIN, G., BADELL, E., JOUAN, M., MAUGER, I., SELLIER, P., TRUFFOT, C., BRICAIRE, F., AREND, S.M., OTTENHOFF, T., AUTRAN, B., & GICQUEL, B., 2007. T-cell and serological responses to Erp, an exported *Mycobacterium tuberculosis* protein, in tuberculosis patients and healthy individuals. *BMC Infectious Diseases*, 7:38.

MARTINS, W., DE SOUSA, D., PROITE, K., GUMARÃES, P., MORETZSOHN, M., & BERTIOLI, D., 2006. New softwares for automated microsatellite marker development. *Nucleic Acids Research*, 34:1-4.

MCDEVITT, D. & ROSENBERG, M., 2001. Exploiting genomics to discover new antibiotics. *TRENDS Microbiology*, 9:611-617.

MEACOCK, P.A., & COHEN, S.N., 1980. Partitioning of bacterial plasmids during cell division: a cis-acting locus that accomplishes stable plasmid inheritance. *Cell*, 20:529-542.

MEINKE, A., HENICS, T. & NAGY, E., 2004. Bacterial genomes pave the way to novel vaccines. *Current Opinion in Microbiology*, 7:314-320.

MILLER, C.A., & COHEN, S.N., 1993. The partition (par) locus of pSC101 is an enhancer of plasmid incompatibility. *Molecular Microbiology*, 9:695-702.

MIROUX, B. & WALKER, J.E., 1996. Overproduction of proteins in *Escherichia coli*: mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. *Journal of Molecular Biology*, 260:289-298.

MISAWA, S. & KUMAGAI, I., 1999. Refolding of therapeutic proteins produced in *Escherichia coli* as inclusion bodies. *Bioplymers* (*Peptide Science*), 51:297-307.

MONTEOLIVA, L., MATAS, M.L., GIL, C., NOMBELA, C., & PLA, J., 2002. Large-scale identification of putative exported proteins in *Candida albicans* by genetic selection. *Eukaryotic Cell*, 1:514-525.

MOORE, A.C., & HUTCHINGS, C.L., 2007. Combination vaccines: synergistic simultaneous induction of antibody and T-cell immunity. *Expert Review of Vaccines*, 6:111-121.

MORA, M., VEGGI, D., SANTINI, L., PIZZA, M., & RAPPUOLI, R., 2003. Reverse vaccinology. *Drug Discovery Today*, 8:459-462.

MORA, M., DONATI, C., MEDINI, D., COVACCI, A., & RAPPUOLI, R., 2006. Microbial genomes and vaccine design: refinements to the classical reverse vaccinology approach. *Current Opinion in Microbiology*, 9:532-536.

MUKHERJEE, S., KASHINO, S.S., ZHANG, Y., DAIFALLA, N., RODRIGUES, J.V., REED, S.G., & CAMPOS-NETO, A., 2005. Cloning of the gene encoding a protective *Mycobacterium tuberculosis* secreted protein detected in vivo during the initial phases of the infectious process. *Journal of Immunology*, 175:5298-5305.

MUSTAFA, A.S., SHABAN, F.A., ABAL, A.T., AL-ATTIYAH, R., WIKER, H.G., LUNDIN, K.E.A., OFTUNG, F., & HUYGEN, K., 2000. Identification and HLA restriction of naturally derived Th1-cell epitopes from the secreted *Mycobacterium tuberculosis* antigen 85B recognized by antigen-specific human $CD4^+$ T-cell lines. *Infection and Immunity*, 68:3933-3940.

MUZZI, A., MASIGNANI, V., & RAPPUOLI, R., 2007. The pan-genome: towards a knowledge-based discovery of novel targets for vaccines and antibacterials. *Drug Discovery Today,* 12:429-439.

NAKAI, K., & KANEHISA, M., 1991. Expert system for predicting protein localization sites in gram-negative bacteria. *Proteins,* 11:95-110.

NEYROLLES, O., GOULD, K., GARES, M.P., BRETT, S., JANSSEN, R., O'GAORA, P., ERRMANN, J.L., PREVOST, M.C., PERRET, E., THOLE, J.E., & YOUNG, D., 2001. Lipoprotein access to MHC class I presentation during infection of murine macrophages with live mycobacteria. *Journal of Immunology*, 166:447-457.

ORENDI, J.M., NOTTET, H.S., DE VOS, N.M., VISSER, M.R., SNIPPE, H., BOUCHER, C.A., & VERHOEF, J., 2000. Hydroxyurea interferes with antigen-dependent T-cell activation *in vitro*. *European Journal of Clinical Investigation*, 30:162-6.

PAL, P.G., & HORWITZ, M.A., 1992. Immunization with extracellular proteins *Mycobacterium tuberculosis* induces cell-mediated responses and substantial protective immunity in a guinea pig model of pulmonary tuberculosis. *Infection and Immunity*, 60:4781-4792.

PALMER, T., & BERKS, B.C., 2003. Moving folded proteins across the bacterial cell membrane. *Microbiology*, 149:547-556.

PANDEY, K.C., SINGH, S., PATTNAIK, P., PILLAI, U., LYNN, A., JAIN, S.K., & CHITNIS, C.E., 2002. Bacterially expressed and refolded receptor binding domain of *Plasmodium falciparum* EBA-175 elicits invasion inhibitory antibodies. *Molecular and Biochemical Parasitology*, 123:23-33.

PÉREZ FILGUEIRA, D.M., MOZGONOJ, M., WIGDOROVITZ, A., DUS SANTOS, M.J., PARREÑO, V., TRONO, K., FERNANDEZ, F.M., CARRILLO, C., BABIUK, L.A., MORRIS, T.J., & BORCA, M.V., 2004. Passive protection to bovine rotavirus (BRV) infection induced by a BRV VP8* produced in plants using a TMV-based vector. *Archives of Virology*, 149:2337-2348.

PIMPL, P., TAYLOR, J.P., SNOWDEN, C., HILLMER, S., ROBINSON, D.G., & DENECKE, J., 2005. Golgi-mediated vacuolar sorting of the endoplasmic reticulum chaperone BiP may play an active role in quality control within the secretory pathway. *The plant Cell*, 18:198-211.

PIZARRO, J.C., CHITARRA, V., VERGER, D., HOLM, I., PETRES, S., DARTEVELLE, S., NATO, F., LONGACRE, S., & BENTLEY, G.A., 2003. Crystal structure of a Fab complex formed with PfMSP1-19, the C-terminal fragment of merozoite surface protein 1 from *Plasmodium falciparum*: a malaria vaccine candidate. *Journal of Molecular Biology*, 328:1091-1103.

PIZZA, M., SCARLATO, V., MASIGNANI, V., GIULIANI, M.M., ARICO, B., COMANDUCCI, M., JENNINGS, G.T., BALDI, L., BARTOLINI, E., CAPECCHI, B., GALEOTTI, C.L., LUZZI, E., MANETTI, R., MARCHETTI, E., MORA, M., NUTI, S., RATTI, G., SANTINI, L., SAVINO, S.,

SCARSELLI, M., STORNI, E., ZUO, P., BROEKER, M., HUNDT, E., KNAPP, B., BLAIR, E., MASON, T., TETTELIN, H., HOOD, D.W., JEFFRIES, A.C., SAUNDERS, N.J., GRANOFF, D.M., VENTER, J.C., MOXON, E.R., GRANDI, G. & RAPPUOLI, R., 2000. Identification of vaccine candidates against serogroup B meningococcus by whole-genome sequencing. *Science*, 287:1816-1820.

POHLSCHRÖDER, M., PRINZ, W.A., HARTMANN, E., & BECKWITH, J., 1997. Protein translocation in the three domains of life: variations on a theme. *Cell*, 91:563-566.

PUGSLEY, A.P., 1993. The complete general secretory pathway in gram-negative bacteria. *Microbiological Reviews*, 57:50-108.

QUICK, M. & WRIGHT, E.M., 2002. Employing *Escherichia coli* to functionally express, purify, and characterize a human transporter. *Proceedings of National Academy of Science of the United States of America*, 99:8597-8601.

RADOLF, J.D., ROBINSON, E.J., BOURELL, K.W., AKINS, D.R., PORCELLA, S.F., WEIGEL, L.M., JONES, J.D., & NORGARD, M.V., 1995. Characterization of outer membranes isolated from *Treponema pallidum*, the syphilis spirochete. *Infection and Immunity*, 63:4244-52.

RAMAN, V., WOODCOOK, D., & HILL, R.J., 1997. Typically unstable long tandem repeats in *Escherichia coli* show increased stability in strain PMC107 and are stable when incorporated into the *Drosophila melanogaster* genome. *Analytical Biochemistry*, 245:242-245.

RAPOPORT, T.A., 1991. Protein transport across the endoplasmic reticulum membrane: facts, models, mysteries. *FASEB Journal*, 5:2792-2798.

RAPOPORT, T.A., 2007. Protein translocation across the eukaryotic endoplasmic reticulum and bacterial plasma membranes. *Nature*, 450:663-669.

RAPPUOLI, R., 2001. Reverse vaccinology, a genome-based approach to vaccine development. *Vaccine*, 19:2688-2691.

RASKIN, D.M., SESHADRI, R., PUKATZKI, S.U., & MEKALANOS, J.J., 2006. Bacterial genomics and pathogen evolution. *Cell*, 124:703-714.

REMAUT, H. & WAKSMAN, G., 2004. Structural biology of bacterial pathogenesis. *Current Opinion in Structural Biology*, 14:161-170.

REY, S., ACAB, M., GARDY, J.L., LAIRD, M.R., DEFAYS, K., LAMBERT, C., & BRINKMAN, F.S.L., 2005. PSORTdb: protein subcellular localization database for bacteria. *Nucleic Acids Research*, 33:164-168.

RIGDEN, R.C., JANDHYALA, D.M., DUPONT, C., CROSBIE-CAIRD, D., LOPEZ-VILLALOBOS, N., MAEDA, N., GICQUEL, B., & MURRAY, A., 2006. Humoral and cellular immune responses in sheep immunized with a 22 kilodalton exported protein of *Mycobacterium avium* subspecies *paratuberculosis*. *Journal of Medical Microbiology*, 55:1735-1740.

RÖMISCH, K., COLLIE, N., SOTO, N., LOGUE, J., LINDSAY, M., SCHEPER, W., & CHENG, C.H.C., 2003. Protein translocation across the endoplasmic reticulum membrane in cold-adapted organisms. *Journal of Cell Science*, 116:2875-2883.

ROLLAGEN, C., SÖRENSEN, M., RIZOS, K., HURVITZ, R. & BUMANN, D., 2004. Antigen selection based on expression levels during infection facilitates vaccine development for an intracellular pathogen. *Proceedings of the National Academy of Sciences of the United States of America*, 101:8739-8744.

ROSS, B.C., CZAJKOWSKI, L., HOCKING, D., MARGETTS, M., WEBB, E., ROTHEL, L., PATTERSON, M., AGIUS, C., CAMUGLIA, S., REYNOLDS, E., LITTLEJOHN, T., GAETA, B., NG, A., KUCZEK, E.S., MATTICK, J.S., GEARING, D. & BARR, I.G., 2001. Identification of vaccine candidate antigens from a genomic analysis of *Porphyromonas gingivalis*. *Vaccine,* 19:4135-4124.

RUDOLPH, R., & LILIE, H., 1996. In vitro folding of inclusion body proteins. *FASEB Journal*, 10:49-56.

RUSCH, S.L., & KENDALL, D.A., 2007. Interactions that drive Sec-dependent bacterial protein transport. *Biochemistry*, 46:9665-9673.

SAIER, M.H., 2006. Protein secretion and membrane insertion systems in gram-negative bacteria. *Journal of Membrane Biology*, 214:75-90.

SANTINI, C.L., BERNADAC, A., ZHANG, M., CHANAL, A., IZE, B., BLANCO, C., & WU, L.F., 2001. Translocation of jellyfish green fluorescent protein via the Tat system of *Escherichia coli* and change

of its periplasmic localization in response to Osmotic Up-Shock. *The Journal of Biological Chemistry*, 276:8159-8164.

SCARSELLI, M., GIULIANI, M.M., ADU-BOBIE, J., PIZZA, M., & RAPPUOLI, R., 2005. The impact of genomics on vaccine design. *TRENDS in Biotechnology*, 23:84-91.

SEGAL, G., FELDMAN, M., ZUSMAN, T., 2005. The Icm/Dot type-IV secretion systems of *Legionella pneumophila* and *Coxiella burnetii*. *FEMS Microbiology Reviews*, 29: 65-81.

SERRUTO, D., ADU-BOBIE, J., CAPECCHI, B., RAPPUOLI, R., PIZZA, M. & MASIGNANI, V., 2004. Biotechnology and vaccines: application of functional genomics to *Neisseria meningitidis* and other bacterial pathogens. *Journal of Biotechnology*, 113:15-32.

SINGH, S., KENNEDY, M.C., LONG, C.A., SAUL, A.J., MILLER, L.H., & STOWERS, A.W., 2003. Biochemical and immunological characterization of bacterially expressed and refolded *Plasmodium falciparum* 42-kilodalton C-terminal merozoite surface protein 1. *Infection and Immunity*, 71:6766-6774.

SØRENSEN, H.P., & MORTENSEN, K.K., 2005. Advanced genetic strategies for recombinant protein expression in *Escherichia coli*. *Journal of Biotechnology*, 115:113-128.

STADEN, R., BEAL, K.F., & BONFIELD, J.K., 1998. The Staden package. In: *Computer Methods in Molecular Biology, Bioinformatics Methods and Protocols*. Volume 132. Edited by Misener, S., & Krawertz, S.A. The Humana Press Inc., Totowa, NJ, pp. 115-130.

STUDIER, F.W., ROSENBERG, A.H., DUNN, J.J., & DUBENDORFF, J.W., 1990. Use of T7 RNA Polymerase to direct expression of cloned genes. *Methods in Enzymology*, 185:60-89.

SUERBAUM, S., JOSENHANS, C., CLAUS, H., & FROSCH, M., 2002. Bacterial genomics: seven years on. *TRENDS in Microbiology*, 10:351-353.

SWANTON, E., & BULLEID, N.J., 2003. Protein folding and translocation across the endoplasmic reticulum membrane (Review). *Molecular Membrane Biology*, 20:99-104.

TETTELIN, H., SAUNDERS, N.J., HEIDELBERG, J., JEFFRIES, A.C., NELSON, K.E., EISEN, J.A., KETCHUM, K.A., HOOD, D.W., PEDEN, J.F., DODSON, R.J., NELSON, W.C., GWINN, M.L., DEBOY, R., PETERSON, J.D., HICKEY, E.K., HAFT, D.H., SALZBERG, S.L., WHITE, O.,

FLEISCHMANN, R.D.,DOUGHERTY, B.A., MASON, T., CIECKO, A., PARKSEY, D.S., BLAIR, E., CITTONE, H., CLARK, E.B., COTTON, M.D., UTTERBACK, T.R., KHOURI, H., QIN, H., VAMATHEVAN, J., GILL, J., SCARLATO, V., MASIGNANI, V., PIZZA, M., GRANDI, G., SUN, L., SMITH, H.O., FRASER, C.M., MOXON, E.R., RAPPUOLI, R., & VENTER, J.C., 2000. Complete genome sequence of *Neisseria meningitides* serogroup B strain MC58. *Science*, 287:1809-1815.

TEYSSEIRE, N., BOUDIER, J.A., & RAOULT, D., 1995. *Rickettsia conorii* entry into Vero cells. *Infection and Immunity*, 63:366-374.

THOMAS, J.D., DANIEL, R.A., ERRINGTON, J., ROBINSON, C., 2001. Export of active green fluorescent protein to the periplasm by the twin-arginine translocase (Tat) pathway in *Escherichia coli*. *Molecular Microbiology*, 39:47-53.

TURCOTTE, K., GAUTHIER, S., MALO, D., TAM, M., STEVENSON, M.M., & GROS, P., 2007. Icsbp1/RF-8 is required for innate and adaptive immune response against intracellular pathogens. *Journal of Immunology*, 179:2467-76.

VEKEMANS, J., OTA, M.O.C. SILLAH, J., FIELDING, K., ALDERSON, M.R., SKEIKY, Y.A.W., DALEMANS, W., MCADAM, K.P.W.J., LIENHARDT, C.,& MARCHANT, A., 2004. Immune responses to mycobacterial antigens in the Gambian population: implications for vaccines and immunodiagnostic test design. *Infection and Immunity*, 72:381-388.

WICKNER, W., & SCHEKMAN, R., 2005. Protein translocation across biological membranes. *Science*, 310:1452-1456.

WINSLOW, G.M., & BITSAKTSIS, C., 2005. Immunity to the ehrlichiae: new tools and recent developments. *Current Opinion in Infectious Diseases*, 18:217-221.

YOUNG, D.B., & GARBE, T.R., 1991. Lipoprotein antigens of *Mycobacterium tuberculosis*. *Research in Microbiology*, 142:55-65.

YU, C.S., LIN, C.J., & HWANG, J.K., 2004. Predicting subcellular localization of proteins for Gram-negative bacteria by support vector machines based on n-peptide compositions. *Protein Science*, 13:1402-1406.

YU, U., LEE, S.H., KIM, Y.J., & KIM, S., 2004. Bioinformatics in the post-genome era. *Journal of Biochemistry and Molecular Biology*, 37:75-82.

YU, C.S. CHEN, Y.C., LU, C.H., & HWANG, J.K., 2006. Prediction of protein subcellular localization. *Proteins*, 64:643-51.

ZHANG, Y., TAIMING, L., & LIU, J., 2003. Low temperature and glucose enhanced T7 RNA polymerase-based plasmid stability for increasing expression of glucagons-like peptide-2 in *Escherichia coli*. *Protein Expression and Purification*, 29:132-139.

ZWEYGARTH, E., JOSEMANS, A.I., & HORN, E., 1998. Serum-free media for the *in vitro* cultivation of *Cowdria ruminantium*. *Annals of the New York Academy of Science*, 849:307-312.

CHAPTER 3

Evaluation of *E. ruminantium* secreted proteins for their ability to induce recall T cell responses *in vitro*

3.1. INTRODUCTION

The control of heartwater by effective vaccination remains an important goal. Cell mediated immunity has been shown to play a key role in protection against heartwater (Totté et al., 1997, 1999a, b). Therefore, characterization of purified antigens and determining their role in protective immunity against infection may lead to the development of an effective vaccine. The protective antigens of *E. ruminantium* are still not precisely characterized or defined, but several studies indicated that immunization of animals with *E. ruminantium* proteins induce immune responses that are characterized by the production of IFN-γ and proliferative responses. For example, proteins of *E. ruminantium* were shown *in vitro* to stimulate lymphocytes from cattle immunized by infection and treatment or inactivated organisms to proliferate and produce IFN-γ (van Kleef et al., 2000, 2002). Moreover, proteins of *E. ruminantium* (Gardel strain) were shown to be immunogenic in terms of IFN-γ production (Esteves et al., 2004a). Similarly, protection was augmented when DNA primed mice were boosted with recombinant proteins of *E. ruminantium* (Simbi et al., 2006). The protection was characterized by production of IFN-γ and IL-2. It was also shown that ruminants can be protected by *E. ruminantium* genes presented as a nucleic acid vaccine (Collins et al., 2003a, b; Pretorius et al., 2007).

Much effort has been spent on the identification of proteins that induce strong cell-mediated immune responses characterized by type 1 $CD4^+$ and $CD8^+$ T-cell responses (Mwangi et al., 1998). T cell responses against major antigenic proteins 1 and 2 of *E. ruminantium* were examined (Mwangi et al., 2002). Real-time PCR results demonstrated strong expression of IFN-γ, TNF-α, TNF-β, IL-2Rα transcripts and these findings indicate that these proteins can possibly be included in vaccine development against the agent.

Of particular relevance to this study is evidence suggesting that secreted proteins of *M. tuberculosis* are likely targets of protective immune responses that are thought to be primarily $CD4^+$ T cell mediated (Andersen, 1991; Andersen et al., 1991; 1994; Pal and Horwitz, 1992; Orme, 1994; Gomez et al., 2000; Coler et al., 2001; Bottai et al., 2003). Secreted proteins of *M. tuberculosis* have been shown to have an important role in inducing protective immunity. For example, six different secretory proteins of molecular weights (15, 26, 30, 41, 55 and 70 kDa) were examined for their ability to induce cell-mediated immunity. The results showed that a 30 kDa protein was a promising vaccine candidate as determined by its induction of the secretion of IFN-γ and IL-2 (Sinha et al., 1997; Carlisle et al., 2007). Promising results in animal models for *M. tuberculosis* infection were obtained based on secreted proteins such as Ag85A and ESAT-6. The production of IFN-γ in the presence of Ag85 and ESAT6 demonstrated the importance of these antigens in subunit vaccines against TB (Lozes et al., 1997; Tanghe et al., 2000; Al-Attiyah et al., 2004; Langermans et al., 2005; Ganguly et al., 2007; 2008). Furthermore, Vekemans et al. (2004) identified secretory mycobacterial antigens (Mtb8.4, Mtb12, Mtb32-C and Mtb32-N) that were able to stimulate significant IFN-γ production. In a similar study it was demonstrated that recombinant Mtb12 secreted protein was able to induce strong cellular immune responses (Webb et al., 1998). Horwitz et al., (1995) demonstrated that immunization of guinea pigs or mice with a 30 kDa major secretory protein, alone or in a cocktail with other abundant extracellular proteins of *M. tuberculosis* induced cell mediated immune responses and protection against *M. tuberculosis*.

Secreted proteins of *M. bovis* BCG within the range of 34-30 kDa played a vital role in cell-mediated immune responses and these peptides may be promising vaccine candidates in the design of subunit-based vaccine against tuberculosis (Rani et al., 2005). Mukherjee and his co-workers (2005) have managed to identify a secreted recombinant urine protein 2 (U2) that was able to induce immunity and protection against *M. tuberculosis*. Their results suggested that U2 protein might be exported to the extracellular compartment of *Mycobacterium* associated with lipid molecules. This protein was found to have epitopes recognized by T cells from *M. tuberculosis* infected mice and therefore suggests that this protein be included in subunit vaccine development against *M. tuberculosis*. Similarly, Biondo et al. (2005) have identified

12 major secreted proteins that might be of relevance in attempt to control cryptococcosis. Molecular characterization of other secreted proteins may provide vital information needed in the development of subunit-based vaccines against intracellular pathogens. Hence, development of a subunit vaccine for the intracellular pathogen *E. ruminantium* is possible and identification of secreted proteins that induce strong proliferation and IFN-γ production could play an important role.

This study used lymphocyte proliferation, the establishment of T cell lines, cytokine profiling using real-time PCR, ELISPOT, MHC typing and immunoblotting to study immune responses in sheep to *E. ruminantium* recombinant proteins. T cell lines were established in order to improve antigen specific proliferative responses *in vitro*. This was achieved by producing short-term lymphocyte cultures where immune PBMC were stimulated with *E. ruminantium* crude antigen. Several studies have reported that antigen specific proliferation can be detected in PBMC from immune animals. For example, *E. ruminantium* antigens that stimulate proliferation of lymphocytes from cattle immunized by infection and treatment or inactivated organisms were identified (van Kleef et al., 2000). T lymphocytes have been cultured for long periods in order to analyze the function and phenotype of responding cells and cytokine production profile (Kodama et al., 1987; Meij et al., 2000; van Kleef et al., 2002).

Since real-time polymerase chain reaction is now becoming universally accepted and recognized tool for measuring gene expression at transcriptional level (Giulietti et al., 2001; Mena et al., 2002; Stodeur et al., 2002a, b; Konnai et al., 2003b; Nguyen et al., 2006), this assay has been performed to test type 1 and type 2 cytokine gene expression (IFN-γ and IL-4) respectively in PBMC stimulated with recombinant proteins. Furthermore, the enzyme-linked immunospot (ELISPOT) assay which is a sensitive immunoplaque assay that has been adapted for the identification and enumeration of cytokine-producing cells at the single cell level (Kalyuzhny and Stark 2001; Britten et al., 2002; Mäkitalo et al., 2002; Shafer-Weaver et al., 2003; Malyguine et al., 2004; Díaz and Mateu, 2005) has been used to investigate if immune PBMC produce IFN-γ after stimulation with secreted proteins. Individual cytokine secreting cells are counted under the microscope. This assay is rapid and reproducible and also permits detection of low frequency antigen-specific T-cells (Guerkov et al., 2003; Pahar et al., 2003).

Animals vary in disease susceptibility, therefore it is essential to characterize MHC polymorphism that plays an important role in the initiation of the immune response to pathogen-derived peptide antigens (Konnai et al., 2003a). Thus MHC typing was used in this study. The variability in the *Ovar*-DRB1 gene might hamper attempts to understand the mechanism behind the host's response to an infection. Therefore, the different alleles need to be identified which may correlate to the immune responses obtained. Considerable progress has been made in the characterization of the highly polymorphic Ovine MHC DRB1. It has been proven that polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) might be a very powerful tool for *Ovar* typing with nine restriction enzymes. Many alleles were found in different animal models using this method (Gelhaus et al., 1995, Gilliespie et al., 1999; Konnai et al., 2003a). In addition, immunoblotting was performed in order to determine that the expressed *E. ruminantium* recombinant proteins were recognized by the immune sheep.

Based on the knowledge that secreted proteins stimulate lymphocytes to proliferate and secrete IFN-γ, four recombinant proteins which were successfully expressed as discussed in Chapter 2 will be tested for their ability to elicit cell mediated immune responses *in vitro*.

3.2. MATERIALS AND METHODS

3.2.1. Materials

Materials used are listed in Appendix A

3.2.2. Methods

3.2.2.1. Experimental animals

Six to eight month old Merino sheep (s) were obtained from heartwater-free areas of South Africa. They tested negative for *E. ruminantium* using the pCS20 real-time PCR (Steyn et al., 2008). Five sheep (s147, s151, s5400, s5401, s849) were injected with 10 LD_{50}s Welgevonden challenge material. The animals were monitored for heartwater symptoms and rectal temperatures were measured daily. The animals were treated with liquamycin (Pfizer) (1 ml / 10 kg body weight) when their temperature was raised above 41 ^{o}C for 2 days or 42 ^{o}C for 1 day combined with loss of appetite, heavy breathing, depression, hanging head, stiff gait, exaggerated blinking, chewing movements, anorexia and signs of nervous symptoms. Animals were euthanased when they did not respond to treatment to prevent prolonged suffering (when its temperature has been raised above 41.5^{o}C for several days). One sheep (s5408) was not immunized and kept as negative non-immune negative control. Peripheral mononuclear cells were collected to monitor cellular immune response to *E. ruminantium* antigen (proliferation assay, ELISPOT, cytokine detection by real time PCR etc).

3.2.2.2. MHC typing

Genomic DNA isolation: Samples of genomic DNA of each animal used in this study were obtained from whole blood collected in BD Vacutainer® K2E tubes containing EDTA. Genomic DNA purification was done using the Generation®Capture Column Kit (Gentra systems) according to the instructions of the manufacturer.

MHC typing: Typing for Ovine MHC *Ovar*-DRB1 was performed using PCR-RFLP as described by Konnai et al. (2003a). Briefly, the second exon of *Ovar*-DBR1 was amplified by nested PCR. The first round of PCR was performed with the OLA-ERBI and HLO31 primers (Table 3.2). A total of 100 ng of genomic DNA was subjected to amplification by PCR in a

total volume of 100 µl, to which 50 µl Go-taq® master mix (2x) (Promega) and 0.02 µM of each primer was added. The PCR cycling conditions were: incubation for 2 min at 94 °C, followed by 15 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min, with a final extension of 10 min at 72 °C using the GeneAmp® PCR 9700 amplifier (Applied biosystems). For the second round (nested PCR), 24 µl DNA (resulting PCR product from first round), 100 µl Go-taq® master mix (Promega), plus 0.04 µM primers (OLA-ERBI and OLA-XRBI) (Table 3.2) were added together. The conditions for the second round of amplification were one cycle of incubation for 2 min at 94 °C, followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min, with final extension of 10 min at 72 °C. Ten microliters of DNA (nested PCR product) was digested overnight at 37 °C with 5 U of either *Rsa*I, *Hae*III, *Sac*I, *Sac*II, *Dde*I, *Nci*I, *Hin1*I or *Eco*RI restriction enzymes (Roche), or at 60 °C with 5 U of *Bst*NI, in a total volume of 20 µl. The digests were resolved on a 5% agarose gel. The gels were photographed under UV light with an Auto Chemi™ system from UVP BioImaging systems. The relative migration of the DNA bands was estimated in comparison with the DNA molecular Marker VIII (Roche). The restriction patterns obtained were compared with published restriction maps (Konnai et al., 2003a).

3.2.2.3. Ficoll purification of PBMC.

PBMC were purified from whole blood under sterile conditions. A volume of 60 ml blood was collected into a syringe containing 2 ml 0.5 M EDTA. The blood was diluted 1:3 with Hanks' Balanced salt solution (HBSS, Sigma) containing 0.2% EDTA. This mixture was underlayed with 15 ml lymphoseparation medium (MP Biomedicals) and centrifuged at room temperature for 40 min at 900 g. The PBMC/Ficoll interface was collected and diluted with three volumes of Alsever's solution, followed by centrifugation at 600 g for 10 min. Red blood cells were lysed by adding 1 ml SABAX water and incubating for 30 s. Lysis was stopped by addition of Alsevers and centrifuged at 300 g for 10 min. The resulting pellet was resuspended in Alsever's solution and centrifuged at 300 g for 10 min. This wash step was repeated until the supernatant was clear. After the last wash, the pellet was resuspended in 1 ml complete RPMI-1640 medium (see Appendix A). Cells were stained with 0.4% Trypan Blue (Sigma) and counted using a haemocytometer (Sigma).

3.2.2.4. Lymphocyte proliferation assay (LPA)

LPA were carried out in triplicate wells of half-area flat bottom 96 well plates (Costar) at 37 °C in a humidified atmosphere containing 5% CO_2 for four days as described by Van Kleef et al. (2000). Each well (total volume 100 µl) contained complete RPMI-1640 medium and responder cells added at a final concentration of 4 x 10^6 PBMC per ml. The following were added to respective test wells: positive control of *E. ruminantium* crude antigen (1 µg/ml), ConA and *E. ruminantium* recombinant protein at determined optimum concentration and unrelated recombinant protein (negative control). The negative control used in this assay was unrelated recombinant protein and crude *E. ruminantiumn* antigen was used for positive control. Proliferation was determined by measuring the incorporation of 0.5 µCi of [methyl-^{3}H] thymidine added during the final 18 h of the assay. The cells were harvested, and the radioactivity was determined using a Trilux 1450 Microbeta liquid scintillation & luminescence counter (Wallac). Results were presented as a stimulation index (SI) ± standard deviation (SD), where SI is the mean counts per minute (cpm) of cells stimulated with recombinant proteins divided by cpm of cells stimulated with unrelated negative control. Unless otherwise stated a SI $\geq$ 2 was considered to be an indication of antigen-specific proliferation.

3.2.2.5. T cell line generation

T cell lines specific for *E. ruminantium* were established from PBMC of s151 and s5401 as described previously (Brown and Logan, 1992) with the following modifications: Six million Ficoll-purified PBMC were cultured per well in a volume of 1.5 ml complete RPMI in a 24-well plate (Costar) together with 1 µg/ml *E. ruminantium* antigen. After 7 days the cells were subcultured to a density of 1 x 10^6 lymphocytes/ml and restimulated with 4 x 10^6 mitomycin treated autologous PBMC/ml added as a source of antigen presenting cells (APC) (rest cycle). After 7 days the cells were subcultured to a density of 1 x 10^6 lymphocytes/ml and restimulated with 1 µg/ml *E. ruminantium* antigen together with 4 x 10^6 autologous mitomycin treated PBMC/ml (stimulation cycle). The T cell lines were maintained by alternating the rest and restimulation cycles. Lymphocyte proliferation assays were performed after a rest cycle. The

amount of cells recovered after each cycle (day 7 and day 14) were counted using a haemocytometer (Sigma). Different treatment methods for T-cell line generation are summarized in Table 3.1.

Table 3.1. Different methods for T-cell line generation

Day		Treatment methods					
		A	**B**	**C**	**D**	**E**	**F**
0	stimulate	Stimulate PBMC with crude *E. ruminantium*					
4		x	x	x	IL-2 10U/ml	IL-2 10U/ml	IL-2 10U/ml
7	rest	subculture PMBC and restimulate with auotologous APC	Subculture PMBC and restimulate with auotologous APC & IL-2	Subculture PMBC and restimulate with auotologous APC, IL-2 & ConA	Subculture PMBC and restimulate with auotologous APC, & IL-2	No subculturing, just replace previous culture medium with IL-2	No subculturing, just replace previous culture medium with IL-2 & ConA
14	Assays	Lymphocyte Proliferation using a 3 day assay (ConA assay)					

3.2.2.6. Cytokine profiling using real-time PCR.

Cell culture conditions: Cell culture for real-time PCR analysis was performed in 2 ml volumes in 15 ml centrifuge tubes. PBMC (4 x 10^6cells/ml) were resuspended in complete RPMI-1640 medium. Antigens were added at a final concentration of 1 µg/ml. The cultures were incubated at 37 ºC in a humidified atmosphere containing 5% CO_2 for 18 h. The cells were pelleted by centrifugation at 300 g for 10 min. Cells were lysed with 1 ml TRI REAGENT™ (Sigma) per 5-10 x 10^6 animal cells. The samples were allowed to stand for 5 min at room temperature to ensure complete dissociation of nucleoprotein complexes. A volume of 0.2 ml of chloroform per ml TRI REAGENT™ used was added and allowed to stand for 15 min at room temperature. The resulting mixture was centrifuged at 12000 g for 15 min at 4 ºC. Centrifugation separates the mixture into 3 phases: a red organic phase (protein), an interphase (DNA) and a colorless upper aqueous phase (RNA).

RNA isolation: The aqueous phase was transferred to a fresh tube and a volume of 0.5 ml of isopropanol per ml of TRI REAGENT™ used was added. The samples were allowed to stand at room temperature for 10 min and centrifuged at 12000 g for 10 min at 4 ºC. The supernatant was removed and the RNA pellet washed by adding 1 ml of 75% ethanol per 1 ml of TRI REAGENT™ used in the sample preparation. The samples were mixed by vortexing and centrifuged at 7.500 g for 5 min at 4 ºC. The supernatant was removed and the RNA pellet air-dried for 10 min. An appropriate volume of diethyl pyrocarbonate (DEPC) treated water was added and the RNA dissolved by mixing with a micropipette at 60 ºC for 15 min. The RNA concentration was measured using a NanoDrop® ND-10000 Spectrophotometer (NanoDrop® Technologies, Inc.).

Genomic DNA elimination: Contaminating genomic DNA after the total RNA isolation was removed using DNA-Free™ kit (Ambion).A 3 µl 10x DNase I buffer and 1.2 U rDNase was added to the 10 µl of RNA and incubated at 37 ºC for 30 min. The tube was mixed thoroughly and 3 µl of DNase Inactivation Reagent added and incubated for 2 min at room temperature. The tubes were centrifuged at 10000 g for 1.5 min. The supernatant was carefully removed and placed in to a new tube. The RNA was stored at -70 ºC until use.

Reverse transcriptase of mRNA: A mixture containing 24 µl RNA and 0.5 µg random primers (1 µl) (Promega) was incubated for 10 min at 65 ºC and cooled on a PCR block, GeneAmp® PCR 9700 amplifier (Applied biosystems) to 4 ºC. The following was added to this mixture: 8 µl of 5x Expand reverse transcriptase buffer (250 mM Tris-HCl, 200 mM KCl, 25 mM $MgCl_2$, 2.5% Tween 20 (v/v), pH 8,3), 4 µl 10 mM DTT, 2 µl 0.5 µM dNTP, 0.5 µl 40 U/µl RNaseOUT, 1 µl 50 U/µl Expand reverse transcriptase. The reaction was performed in 40 µl final volume by incubating for 10 min at 30 ºC and thereafter for 45 min at 42 ºC and cooled to 4 ºC and stored at -20 ºC until further use.

Primers for real-time PCR: mRNA sequences for ovine glyceraldehyde-phosphate-dehydrogenase (GAPDH), IFN-γ and IL-4 were obtained from GenBank at accession numbers, U94889, Z73273 and AF17268 respectively. Primers for real-time PCR were designed using

the Light Cycler Probe Design 2 software. All oligonucleotide sequences are listed in Table 3.2.

Table 3.2. Primers used for real-time PCR reaction and MHC typing.

Target	Sequence 5'-3'
GAPDH F	TCA CTG CCA CCC AGA AGA
GAPDH R	CTC AGG GAT GAC CTT GC
IFN-γ F	CTT GGT GTT ATT GTG ACT GTT G
IFN-γ R	ATG AAT CCC TCC TAA ATC TCT GTA
IL-4 F	TGA CAG GAA TCT CAG CAG
IL-4 R	TTC TCC CTC ATA ATA GTC TTT AGC
OLA-ERBI F	(GC) CCG GAA TTC CCG TCT CTG CAG CAC ATT TCT T
HLO31 R	TTT AAA TTC GCG CTC ACC TCG CCG CT
OLA-ERB1 F	(GC) CCG GAA TTC CCG TCT CTG CAG CAC ATT TCT T
OLA-XRBI R	AGC TCG AGC GCT GCA CAG TGA AAC TC

Real-time PCR analysis of IFN-γ and IL-4 mRNA expression: Real-time PCR for GAPDH, IFN-γ and IL-4 was performed as described by Pretorius, (2007). LightCycler® FastStart DNA Master^{PLUS} SYBR Green 1 kit was used according to the instruction manual (Roche) and the LightCycler instrument. Thermal cycling conditions were 10 min at 95 ºC and 50 cycles of 10 sec at 95 ºC, 10 sec at 57 ºC and 5 sec at 72 ºC followed by cooling for 30 sec at 40 ºC.

Gene expression was measured by relative quantitation which compares the delta threshold cycle (ΔCt) of the sample of interest (PBMC stimulated with antigen) to the ΔCt generated by a reference sample referred to as the calibrator (non-stimulated PBMC or stimulated with unrelated recombinant protein (β galactosidase), incubated for the same time period as stimulated PBMC). GAPDH, a common housekeeping gene, was used to normalize cytokine gene expression by subtraction of Ct to provide the ΔCt values. The ΔΔCt was calculated as the difference between ΔCt values for antigen stimulated and non stimulated PBMC. The relative difference in cytokine expression between naïve and immune sheep was determined using the equation $2^{-\Delta\Delta Ct}$ (Livak et al., 1995; Mena et al., 2002).

eg.

ΔCt = Ct (target gene, IL-4) – Ct (GAPDH)

ΔΔCt = ΔCt (sample) - ΔCt (calibrator)

The amount of target relative to calibrator = $2^{-\Delta\Delta Ct}$

3.2.2.7. IFN-γ ELISPOT Assay

ELISPOT 96 well filtration plates (Millipore MAIPS 4510) were coated with 50 µl/well mouse anti-bovine IFN-γ mAb CC302 (Serotec 0.5 mg/vial) coating antibody at 1 µg/ml in sterile coating buffer and incubated at 4 ºC for 16 h. Plates were washed twice with washing medium (RPMI-1640 unsupplemented) at 200 µl/well then incubated for 2 h at room temperature with 200 µl/well blocking buffer (RPMI-1640 with 10% FCS). The blocking media was flicked off and replaced with 5 x 10^6 PBMC/ml and filtered antigen solution (at indicated concentration) at 50 µl/well and incubated for 20 h at 37 ºC and humidified 5% CO_2 incubator. Cells were removed by washing the plates three times with distilled water-0.05% Tween 20 (dH_2O-T), and three times with phosphate buffer saline-0.05% Tween 20 (PBS-T). Rabbit anti-bovine IFN-γ antiserum was diluted 1:1500 in PBS-0.05% Tween 20, 0.1% bovine serum albumin (BSA) buffer, was filtered, added to each well at 50 µl/well and incubated for 1 h at room temperature. Thereafter, the plates were washed four times with PBS-T and 50 µl of filtered anti-rabbit IgG alkaline phosphate conjugate (1:2000 in PBS-T/BSA) was added to each well and incubated for 1 h at room temperature. Plates were washed 6 times with PBS-T. Excess PBS-T was removed by tapping the plates on paper towels and 50 µl of filtered substrate solution consisting of 5-bromo-4-chloro-3-indolyphosphate and nitroblue tetrazolium (Sigma) was added in each well and incubated in the dark for 15 min until spots emerged. The plates were washed for 2 min with tap water and air-dried before spots were counted using an automated Zeiss KS ELISPOT Reader. Samples with 2x the number of spots/million cells of the negative control were considered positive.

3.2.2.8. Western blot analyses of sheep sera

Preparing serum from whole blood: Blood samples were obtained from the six sheep (s147, s151, s5400, s5401, s5408 and s849) by bleeding into BD Vacutainer® SST™ II Advance tubes. The blood was incubated at 37 ºC for 30-60 min to clot. A Pasteur pipette was used to separate the clot from the sides of the glass tube. The tubes were incubated at 4 ºC overnight to allow

the clot to contract. The serum was decanted from the clot into a new centrifuge tube and was centrifuged at 300 g for 10 min to remove the remaining clots/red blood cells and other insoluble material. The serum was aliquoted and stored at -70 ºC until further use.

Immunoblot assay: The recombinant proteins (rErum7760, rErum8060 and rErum5000) were separated on a 12% SDS-PAGE gel as described in Chapter 2. Proteins were transferred to a PVDF membrane after equilibrating the gel and membrane in transfer buffer (see appendix A). The electrophoretic transfer was carried out with a semi-dry blotter (Semi-phor TE70, Hoefer scientific instruments) at 110 mA for 90 min. The membrane was cut into strips and air dried. The strips were incubated in 1% block buffer (1% BSA in PBS) followed by incubation with diluted sheep sera (1:5) overnight at room temperature. Preliminary testing was done to optimize the sheep sera working dilutions. The membrane was washed 3x with wash buffer (PBS-T) and incubated with Rabbit anti-Sheep IgG (Zymed) for 90 min at room temperature. The blot was again washed 3x and the protein bands visualized using the SuperSignal®West Pico Chemiluminescent substrate from Pierce and exposed to a film (Roche) which was processed by usual photographic procedures.

3.2.2.9. Statistical analysis

The significance of SI between test samples and negative control was determined using the Student's *t*-test. Only samples with SI's two times higher than the negative control and that have significant p values ≤ 0.01 were considered as positive.

3.3. RESULTS

3.3.1. PCR-RFLP typing of the *Ovar*-DRB1 gene from six sheep

The polymorphism of the ovine major histocompatibility complex class II DRB1 second exon (*Ovar*-DRB1) was studied in six sheep, (s147, s151, s849, s5400, s5401 and s5408) by PCR-RFLP. When each amplified DNA product was cleaved by the restriction enzymes, different patterns were observed. Gel images in Figures 3.1-3.3 show results obtained when PCR amplified products were digested with nine restriction enzymes. Patterns and *Ovar*-DRB1 alleles based on restriction endonuclease enzyme digestion are listed in Table 3.3. Three different alleles (*0332 and *0323, *0333) which were similar to alleles reported by Konnai et al., 2003a were obtained for sheep 147 and 5401. The alleles detected for sheep (s849, s5400, s5408 and s151) were unknown and not reported previously in the scientific literature. Furthermore, some patterns (151, 849 and 5408) were not identical to those published but closely related while some allele patterns (5400) were unique (Table 3.3). In summary, using PCR-RFLP, published, new and unknown alleles were found in this study.

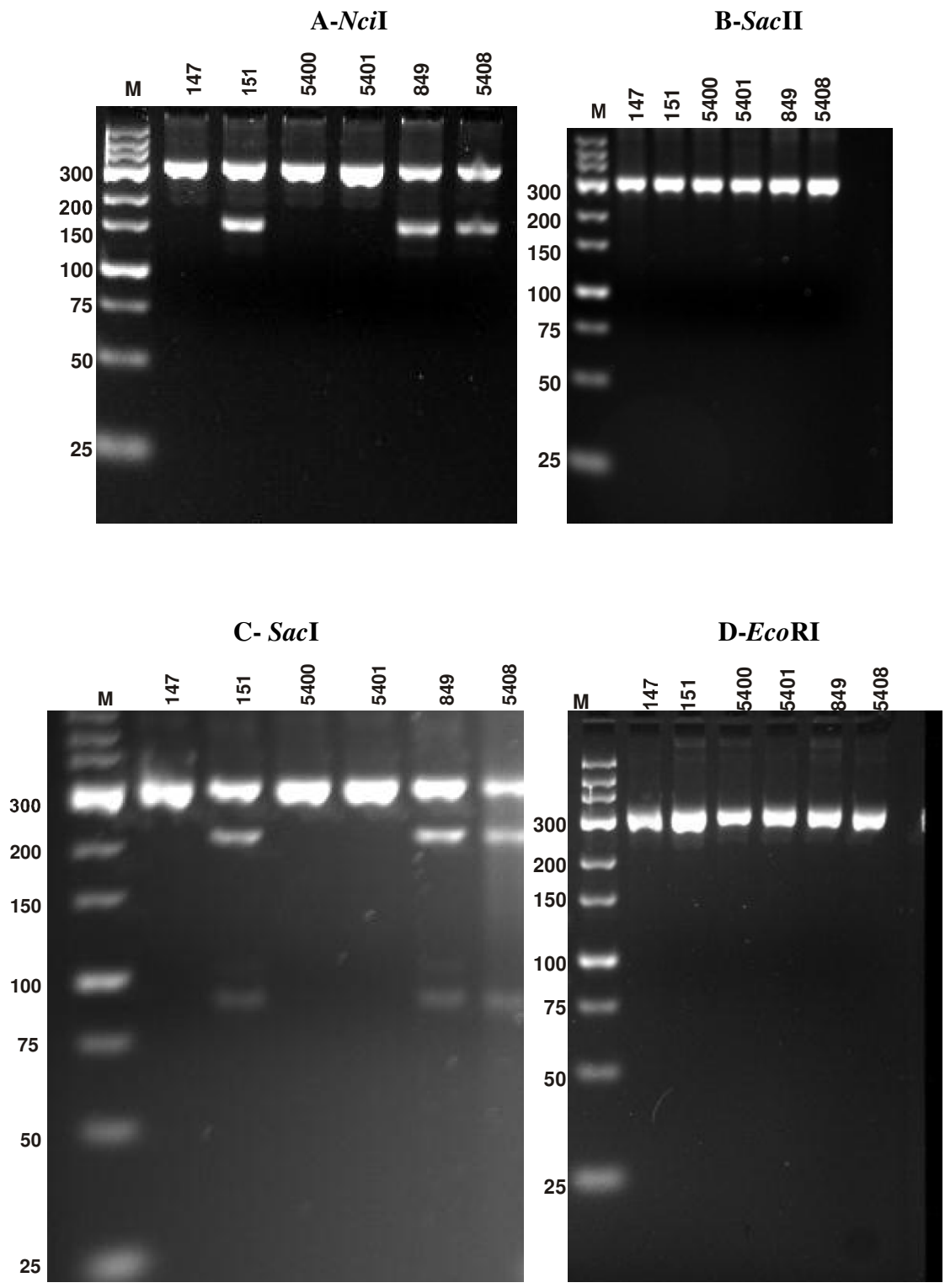


Figure 3.1. The PCR-RFLP analysis of exon 2 of the *Ovar-DRB1* gene from six sheep, s147, s151, s5400, s5401, 5408 and s849. The PCR- amplified products of DNA from the nested PCR were digested with A-*Nci*I, B-*Sac*II, C- *Sac*I, D-*Eco*RI. M-DNA fragment size marker.

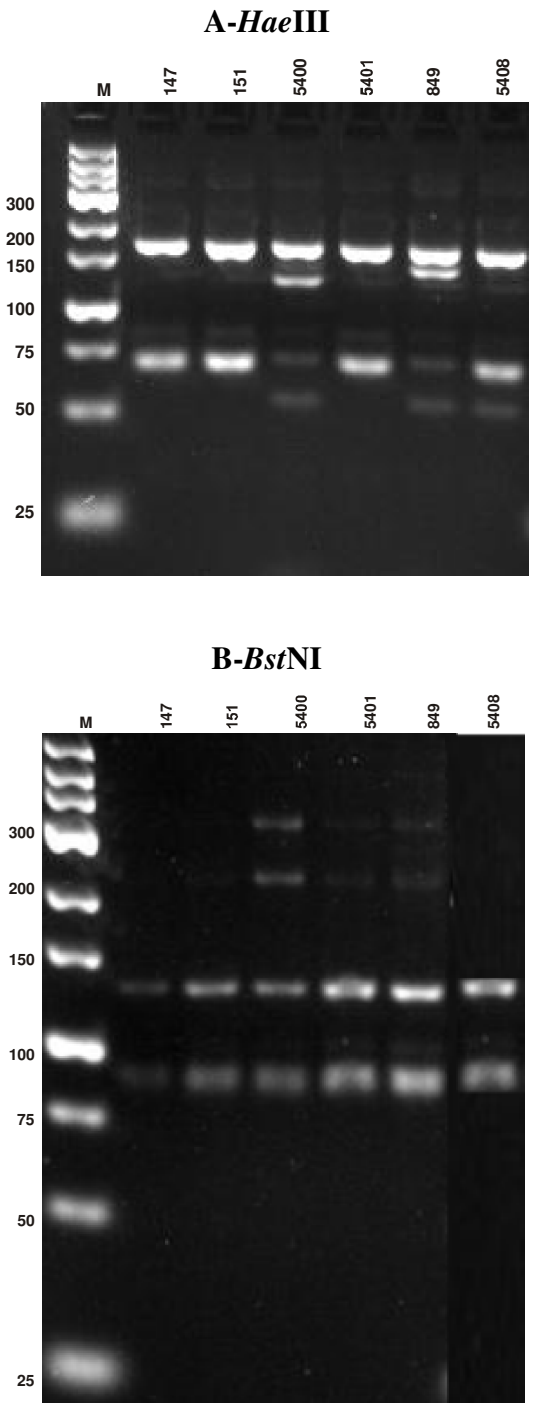


Figure 3.2. The PCR-RFLP analysis of exon 2 of the *Ovar-DRB1* gene from six sheep, s147, s151, s5400, s5401, 5408 and s849. The PCR- amplified products of DNA from the nested PCR were digested with A-*Hae*III, B-*Bst*NI. M-DNA fragment size marker.

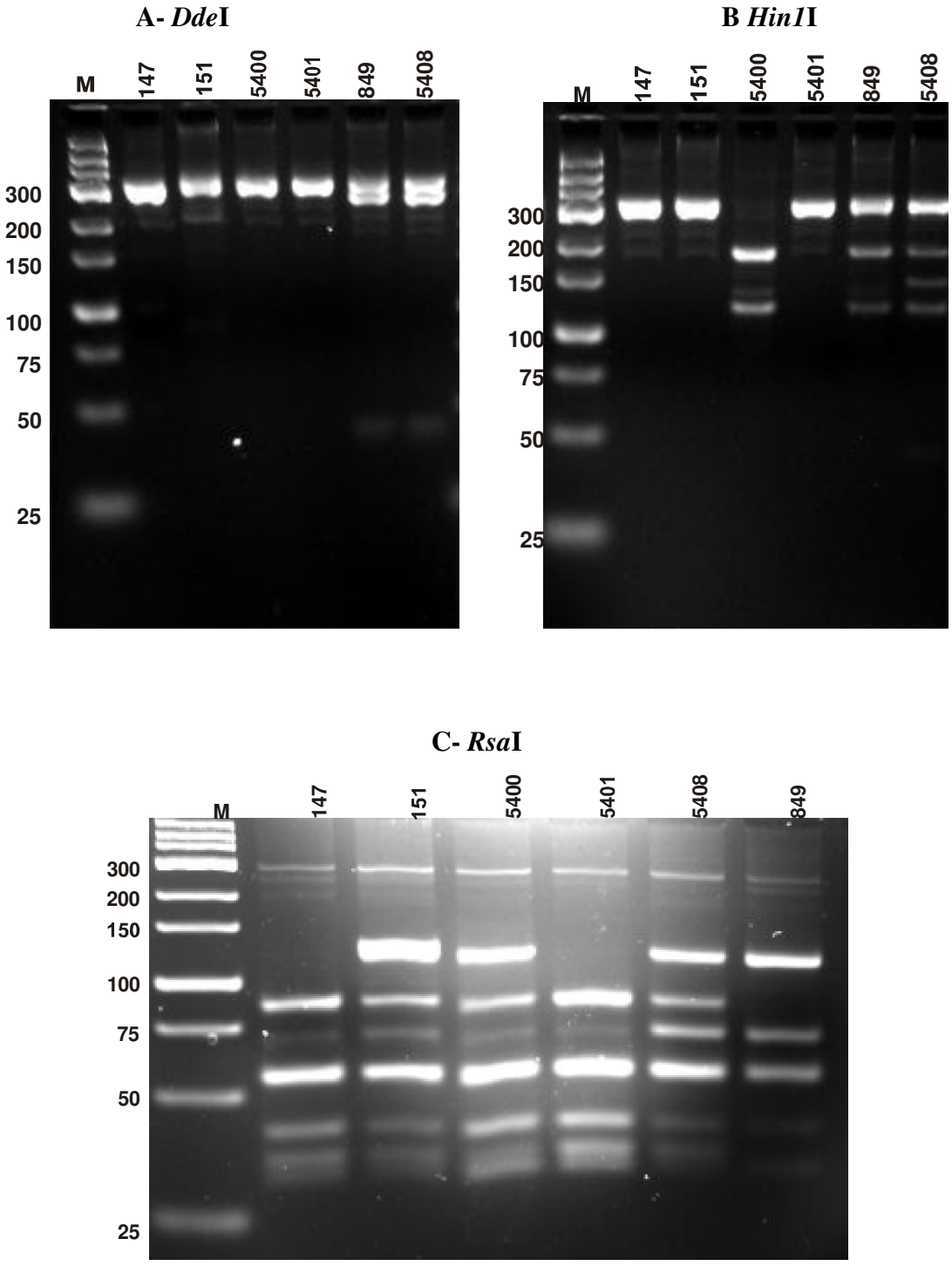


Figure 3.3. The PCR-RFLP analysis of exon 2 of the *Ovar-DRB1* gene from six sheep, s147, s151, s5400, s5401, 5408 and s849. The PCR- amplified products of DNA from the nested PCR were digested with A *DdeI, B Hin1*I and C- *Rsa*I. M-DNA fragment size marker.

Table 3.3. The PCR-RFLP patterns and *Ovar-DRB1* alleles obtained using nine restriction enzymes.

Sheep number	*Rsa*I	*Hae*II	*Sac*I	*Sac*II	*Dde*I	*Hin1*I	*Nci*I	*Eco*RI	*Bst*NI	*Ovar-DRB1* alleles
147	e	a	a	a	a	a	a	a	a	*0332 except for
	f	a	a	a	a	a	a	a	a	*Hae*II & *Nci*I
										*0323, *0333
										except for *Hae*II
151	c	a	a	a	a	a	a	a	a	Unknown 1
	e	a	b	a	a	a	b	a	a	Unknown 2
5400	c	a	a	a	a	b	a	a	a	Unknown 3
	f	?[@]	a	a	a	b	a	a	b	Unknown 4
5401	e	a	a	a	a	a	a	a	a	*0332 except for
	f	a	a	a	a	a	a	a	a	*Hae*II & *Nci*I
										*0323, *0333
										except for *Hae*II
5408	c	a	a	a	a	a	a	a	a	Unknown 1
	e	?	b	a	c	b	b	a	a	Unknown 5
849	c	a	a	a	a	a	a	a	a	Unknown 1
	c	e	b	a	c	?	b	a	b	Unknown 6

[@]Does not correlate with known restriction patterns

a,b,c,e,f- RFLP patterns as identified by Konnai et al., 2003a

3.3.2. Proliferative responses of PBMC stimulated with four recombinant proteins

LPA was carried out to test whether *E. ruminantium* recombinant proteins induce immune PBMC to proliferate. PBMC were isolated from five immune sheep (s) (s147, s849, s5400, s5401 & s151) and a naïve sheep (s5408) and stimulated with the four expressed recombinant proteins (rErum8060, rErum7760, rErum8610 and rErum5000). Results are expressed as Stimulation Index (SI) (counts per minute (cpm) of cells stimulated with recombinant proteins divided by cpm of cells stimulated by unrelated negative control). To confirm the positive proliferation, only samples with SI's two times higher than unrelated negative protein and that have significant p values ≤ 0.01 were

considered as positive. PBMC from the naïve animal (s5408) were stimulated with recombinant proteins at a concentration of 0.1 µg/ml (Table 3.5) to test if the proteins were recognized by the sheep PBMC. No significant proliferation was observed for the PBMC stimulated with the recombinant proteins as expected except for rErum8060s that did show a significant increased proliferation with a SI of 2 ($SI_{stimulated}/SI_{negative}$). Therefore, a SI $\geq$ 4 was considered antigen-specific for rErum8060 at a concentration of 0.1 µg/ml.

The results shown in Table 3.4 were obtained from s147 PBMC stimulated with the four recombinant proteins at concentrations of 10, 1 and 0.1 µg/ml. Recombinant Erum5000i induced significant lymphocyte proliferation at 1 µg/ml (SI value of 6) while rErum7760s showed a positive proliferation at 0.1 µg/ml (SI value of 2.5) but it was not significant. Recombinant Erum8060i induced proliferative responses at a concentration of 10 µg/ml with the SI value of 3.1 which was not significant. Recombinant Erum7760i and rErum8610i did not induce lymphocyte proliferation irrespective of the antigen concentration tested.

PBMC from sheep 151 stimulated with recombinant proteins at a concentration of 10 µg/ml also showed no significant proliferation responses (Table 3.5). Recombinant proteins were also tested using sheep 5400 at a concentration of 10 µg/ml and 0.1 µg/ml (Table 3.6). Proliferative responses that were not significant were obtained with rErum8060s (SI values of 2 and 5) and rErum7760i (SI values of 1.7 and 1.8) at both concentrations.

To further investigate the CMI response induced by recombinant proteins in immune animals, proliferative T-cell responses were analyzed using PBMCs from sheep 849 at concentrations of 10 µg/ml and 0.1 µg/ml (Table 3.7). Recombinant Erum7760i induced significant lymphocyte proliferation at 10 µg/ml with a SI value of 2.4. Recombinant Erum8610i showed increased proliferation at 0.1 µg/ml with a SI value of 2.2 but it was not significant. Recombinant Erum8060 induced proliferation at 10 µg/ml but it was not

significant. Proliferation of PBMC stimulated with rErum5000i and rErum7760s was not detected at the concentrations tested.

Table 3.4. Lymphocyte proliferation assays of PBMC from sheep number 147 stimulated with recombinant proteins at concentration of 10, 1 & 0.1 µg/ml. Values in red bold indicate a SI value 2x higher than the unrelated negative protein and significant.

	10 µg antigen/ml			**1 µg antigen/ml**			**0.1 µg antigen/ml**		
Group	**Average SI[a]**	**Average SI[b]**	**P values[c]**	**Average SI[a]**	**Average SI[b]**	**P values[c]**	**Average SI[a]**	**Average SI[b]**	**P values[c]**
rErum7760s	1.3±0.2	1	0.1	4.5±0.1	2.5	0.007	2.5±0.8	**2.5**	**0.02**
rErum7760i	0.7±0.1	0.5	0.4	2.5±1.8	1.4	0.250	1.7±0.9	1.7	0.1
rErum8060s	3.1±1.2	2.4	0.01	5.8±1.5	3.2	0.148	0.9±0.9	0.9	0.5
rErum8060i	4.1±2.1	**3.1**	**0.1**	1.7±0.8	0.9	0.078	0.8±0.4	0.8	0.3
rErum5000i	0.5±0.0	0.4	0.1	10.8±0.1	**6**	**0.0004**	0.8±0.4	0.8	0.3
rErum8610i	0.8±0.4	0.6	0.4	2.5±1.3	0.7	0.197	1.0±0.3	1	0.5
Negative	1.3±0.4			1.8±0.1			0.9±0.1		
Ag+	2.6±0.2			29.9±2.6			2.6±0.2		
ConA	1187±64			267±8.6			1187±64		

[a] $\mathbf{SI_{rprotein}/\ SI_{PBMC}}$

[b] $\mathbf{SI_{rprotein}/\ SI_{negative}}$

[c] p Determined by Student *t*-test method. Compared to Negative

Table 3.5. Lymphocyte proliferation assays of PBMC from sheep number 5408 (naïve) and 151 stimulated with recombinant proteins. Values in red bold indicate a SI value 2x higher than the unrelated negative protein and significant.

	Sheep 151 (10 µg antigen/ml)			Sheep 5408 (0.1 µg antigen/ml)		
Group	**Average SI[a]**	**Average SI[b]**	**P values[c]**	**Average SI[a]**	**Average SI[b]**	**P values[c]**
rErum7760s	1.5±0.78	1.25	0.241	-0.2±0.6	0	0.008
rErum7760i	0.9±0.73	0.8	0.261	-0.8±0.3	0	0.001
rErum8060s	0.5±0.28	0.4	0.02	**4.4±0.02**	**2**	**0.002**
rErum8060i	0.7±0.15	0.6	0.02	4.1±0.97	1.8	0.073
rErum5000i	0.8±0.14	0.7	0.05	1.7±1.8	0.73	0.336
rErum8610i	0.7±0.19	0.6	0.02	ND		ND
negative	1.2±0.19			2.2±0.5		
Ag+	3.5±2.13			5.7±1.2		
ConA	361.8±66.83			81.7±30.7		

[a] $SI_{rprotein}/ SI_{PBMC}$

[b] $SI_{rprotein}/ SI_{negative}$

[c] p Determined by Student *t*-test method. Compared to Negative

ND not done

Table 3.6. Lymphocyte proliferation assays of PBMC from sheep number 5400 stimulated with recombinant proteins. Values in bold indicate a SI value 2x higher than the unrelated negative protein and p value is not significant.

	10 µg antigen/ml			0.1 µg antigen/ml		
Group	**Average SI[a]**	**Average SI[b]**	**P values[c]**	**Average SI[a]**	**Average SI[b]**	**P values[c]**
rErum7760s	0.8±0.1	1.1	0.3	0.6±0.7	0.8	0.398
rErum7760i	1.2±0.3	**1.7**	**0.1**	1.4±1.2	**1.8**	**0.256**
rErum8060s	1.4±0.3	**2**	**0.1**	4.0±5.5	**5**	**0.186**
rErum8060i	0.8±0.2	1.1	0.4	1.4±1.8	1.8	0.325
rErum5000i	0.9±0.2	1.3	0.2	0.6±1.6	0.8	0.437
rErum8610i	0.7±0.1	1	0.5	ND		ND
negative	0.7±0.2			0.8±1.1		
Ag+	2.8±0.5			2.8±0.5		
ConA	139.7±33.5			139±33.5		

[a] $SI_{rprotein}/ SI_{PBMC}$

[b] $SI_{rprotein}/ SI_{negative}$

[c] p Determined by Student *t*-test method. Compared to Negative

Table 3.7. Lymphocyte proliferation assays of PBMC from sheep number 849 stimulated with recombinant proteins. Values in red indicate a SI value 2x higher than the unrelated negative protein and significant.

	10 µg antigen/ml			0.1 µg antigen/ml		
Group	**Average SI[a]**	**Average SI[b]**	**P values[c]**	**Average SI[a]**	**Average SI[b]**	**P values[c]**
rErum7760s	0.5±0.2	0.7	0.02	1.0±0.4	0.9	0.4
rErum7760i	1.7±0.2	**2.4**	**0.01**	1.5±0.5	1.4	0.1
rErum8060s	1.6±0.6	**2.3**	**0.1**	3.3±0.3	3	0.004
rErum8060i	1.2±0.2	**1.7**	**0.1**	3.1±0.2	2.8	0.001
rErum5000i	1.1±0.2	1.6	0.2	1.1±0.1	1	0.2
rErum8610i	1.2±0.2	1.7	0.1	2.5±0.5	2.2	0.020
negative	0.7±0.2			1.1±0.2		
Ag+	2.6±0.09			2.6±0.09		
ConA	684.2±88.4			684.2±88.4		

[a] $\mathbf{SI_{rprotein}/\ SI_{PBMC}}$

[b] $\mathbf{SI_{rprotein}/\ SI_{negative}}$

[c] p Determined by Student *t*-test method. Compared to Negative

3.3.3. Generation of E. ruminantium T cell lines

E. ruminantium specific T cell lines were generated by stimulating PBMCs of immune sheep 151 and 5401 with *E. ruminantium* crude antigen. This was done in an attempt to improve antigen specific proliferative responses. Several protocols were tested to determine which one resulted in the T cell line with highest SI and viability. Proliferation assay results showed that T cell lines obtained by method C (sheep 151 PBMC) gave the highest SI of 27.1 as compared to PBMC SI of 5.3 (Table 3.8). After stimulating the cells with antigen for 7 days and resting for 7 days, the number of cells recovered from sheep 151 was once again the highest when method C was used (Table 3.9). In contrast, a larger decrease in cell recovery from other methods was observed.

Table 3.8. Proliferative responses of T cells lines prepared from sheep 151 and sheep 5401.

	S151	**S5400**
Treatment method	**Average SI**	**Average SI**
A	17.9±4.5	0
B	5.6±1.7	0
C	**27.1±0.6**	8.3±3.8
D	3.1±1.3	2.1±3.8
E	0	1.3±0.6
F	6.5±0.2	14.7±9.2
PBMC	5.3±2.7	2.6±1.0

Table 3.9. Number of viable lymphocytes recovered on day 7 & 14.

Sheep 151			**Sheep 5401**		
Treatment method	**Number of PBMC/ml cells recovered**		**Treatment method**	**Number of PBMC/ml cells recovered**	
	Day 7	**Day 14**		**Day 7**	**Day 14**
A	$6x10^5$	$1.5x10^5$	A	0	0
B	$11x10^5$	$1.5x10^5$	B	$1.5x10^5$	$1.5x10^5$
C	**$14x10^5$**	**$8.5x10^5$**	C	$3.5x10^5$	0
D	$9x10^5$	$2.5x10^5$	D	$11x10^5$	$2.5x10^5$
E	0	0	E	$3x10^5$	0
F	$3.5\ x10^5$	0	F	$1x10^5$	0

3.3.4. Measurement of IFN-γ and IL-4 mRNA expression using real-time PCR

Two different cytokines were analysed using real-time PCR, IFN-γ (Th1 cytokine) and IL-4 (Th2 cytokine). The expression was assessed in PBMC from immune sheep (s147) and a naïve sheep (s5408). Isolated PBMC were stimulated with recombinant proteins

(rErum7760, rErum8060 and rErum5000) and two positive controls (*E. ruminantium* antigen and ConA) in order to determine antigen induced cytokine mRNA expression. All the samples (PBMC + Medium; PBMC + ConA, PBMC + recombinant proteins) and RNA controls came up at crossing point between 27-37 (Figure 3.4 and 3.5). Thus indicating that genomic DNA was not totally removed from the mRNA sample.

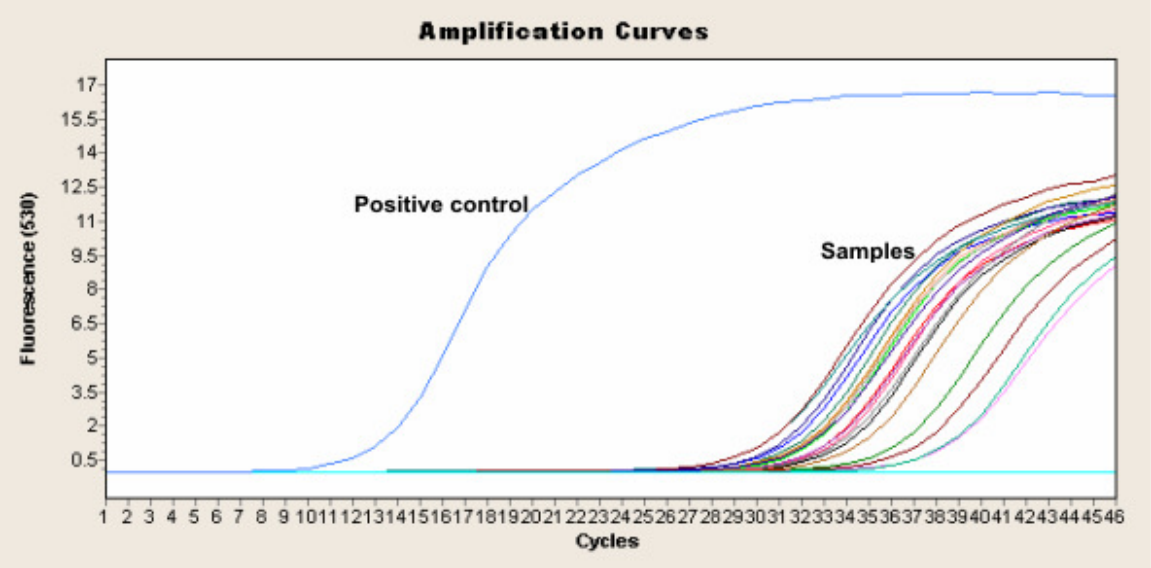


Figure 3.4. Real-time PCR analysis of IFN-γ gene expression by antigen-stimulated PBMC. PBMC from sheep 147 was stimulated with rErum7760, rErum8060, rErum5000, ConA and crude *E. ruminantium* antigen. RNA was extracted at 16 h. rDNase treated RNA was reverse transcribed and used as a template in cytokine gene expression real-time PCR assays.

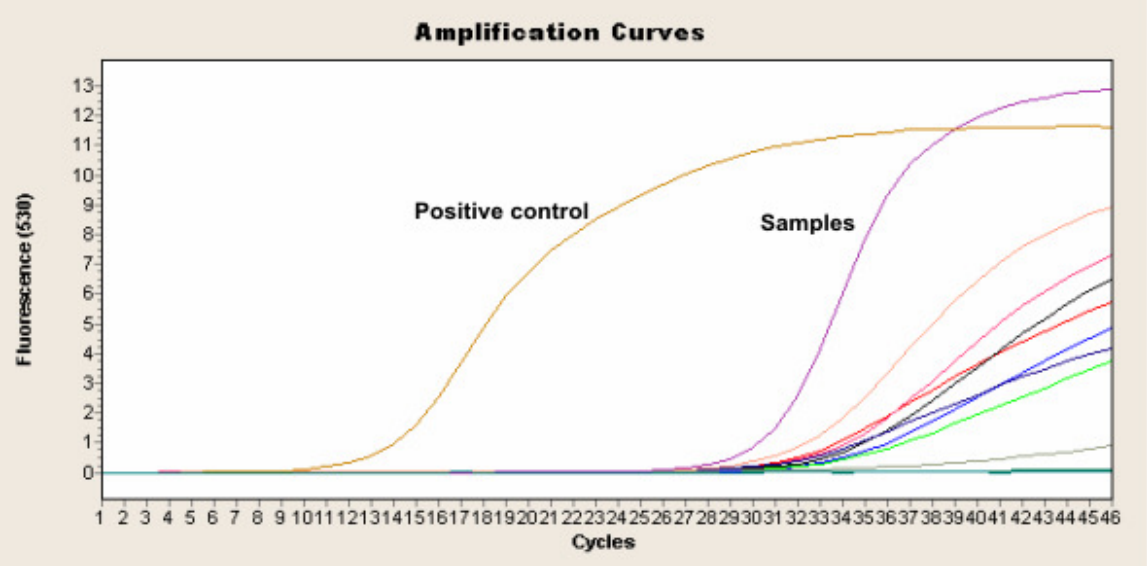


Figure 3.5. Real-time PCR analysis of IFN-γ gene expression by antigen-stimulated PBMC. PBMC from sheep 147 was stimulated with rErum7760, rErum8060, rErum5000, ConA and crude *E. ruminantium* antigen. RNA samples were used as templates in cytokine gene expression to test the presence of genomic DNA.

The contaminating genomic DNA was removed from RNA with a DNA- Free™ kit (Ambion). The resulting IFN-γ and IL-4 mRNA expression profiles after stimulation with recombinant proteins are shown in Table 3.10. The results are expressed as fold increase in cytokine production by antigen stimulated cells from immune sheep compared to similar cells from naïve sheep. Data revealed IFN-γ mRNA expression was induced in PBMC after stimulation with the rErum7760, rErum8060 and rErum5000 except rErum8610. In addition, the IFN-γ mRNA expression from immune sheep was greater than the expression from naïve sheep (Table 3.10). Relative expression varied among individual antigens (fold increase ranged from 75-2486). PBMC stimulated with rErum7760 expressed higher levels of IFN-γ mRNA than other proteins. In addition, IL-4 mRNA expression was also assessed in PBMC stimulated with recombinant proteins. No significant IL-4 expression was observed as shown in Table 3.10.

Table 3.10. Differences in the expression of mRNA for IFN-γ and IL-4 in immune and naïve sheep.

147 (immune sheep)			**5408 (naïve sheep)**	
Sample	IFN-γ[a]	IL-4[a]	IFN-γ[a]	IL-4[a]
Ag+	498	0	0.05	0
ConA	145433	0	0	0
Erum7760s	2486	0	11	0
Erum7760i	2195	0	0.01	0
Erum8060s	1418	0	0	0
Erum8060i	75	0	0.13	0
Erum5000i	129	0	4.4	0
Negative control	0.03	0	5	0

[a] fold increase of IFN-γ and IL-4 mRNA expression

3.3.5. ELISPOT IFN-γ responses of PBMC stimulated with four recombinant proteins

The ELISPOT assay was used to determine which of the four *E. ruminantium* recombinant proteins induce immune PBMC (s147, s849, s5400, s5401 & s151) and a naïve (s5408) to secrete IFN-γ. The naïve sheep displayed no IFN-γ production to the four recombinant proteins as expected. IFN-γ production by PBMC from sheep 849 (10 µg antigen/ml), 151 (2.5 µg antigen/ml), 5401 (2.5 µg antigen/ml) and 5400 (2.5 µg antigen/ml) was tested. Poor production of cytokine was detected after stimulation of PBMC from immune s151, s5400, s5401 & s849 with the four recombinant proteins. IFN-γ production by PBMC from immune s147 at an antigen concentration of 10 µg antigen/ml and 2.5 µg/ml was also tested. No significant IFN-γ production was observed at a concentration of 2.5 µg antigen/ml. However, substantial IFN-γ production by PBMC from immune sheep 147 stimulated with rErum8060s, rErum8060i, rErum8610i and rErum5000i (10 µg antigen/ml) was obtained (Figure 3.6) compared to that of negative. Interestingly, IFN-γ production with the soluble fraction of rErum8060 was higher than the insoluble fraction. In addition, no IFN-γ production was detected when cells were stimulated with rErum7760 (high mRNA levels Table 3.10). Nevertheless, PBMC from

immune sheep 147 stimulated with recombinant proteins at a concentration of 10 µg antigen/ml gave the highest production of IFN-γ. This response was specific for *E. ruminantium* recombinant proteins since IFN-γ production due to unrelated protein stimulation was not detected.

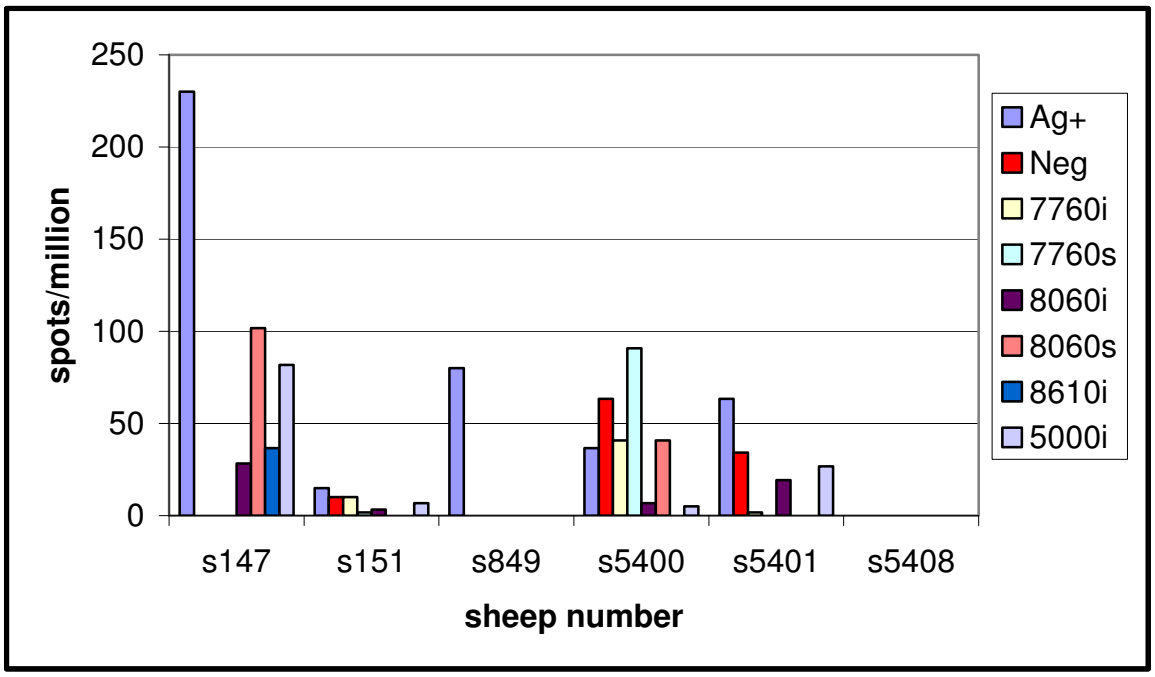


Figure 3.6. ELISPOT assay where PBMC from immune sheep were stimulated with recombinant proteins. (s) represents soluble fraction and (i) represents insoluble fraction. Only samples with 2x the number of spots /million cells of the unrelated negative protein were considered positive. Ag^+ represents *E. ruminantium* antigen and Neg represents unrelated negative protein.

3.3.6. Immunoblotting

The immunogeneicity of the recombinant proteins was analyzed by Western Blot with the sheep sera. Serum was obtained from the five immune sheep (s147, s151, s849, s5400, and s5401) and a naïve sheep (s5408). The results indicated that all recombinant proteins except rErum8610 were recognized by immune sheep sera (Figure 3.7). Recombinant

Erum8610 was not tested in the immunoblot. The naïve sheep did not react with any of the recombinant proteins (data not shown).

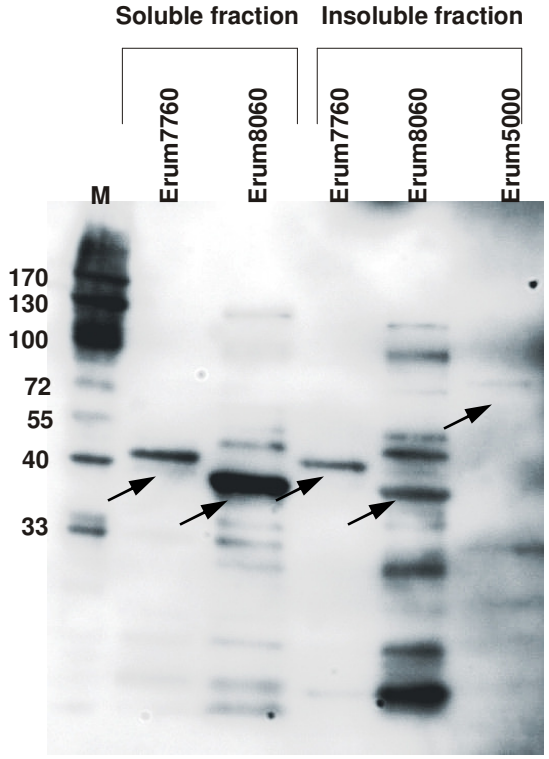


Figure 3.7. Western Blot analysis of rErum7760, rErum8060 and rErum5000 recombinant proteins with sera from immune sheep (147). After SDS-PAGE, purified recombinant proteins were transferred to the PVDF membrane. The blot was developed with the sera of immune sheep. M, prestained molecular marker.

3.4. DISCUSSION

In this chapter it was demonstrated that four recombinant proteins (rErum5000, rErum7760, rErum8060 and rErum8610) induced significant cellular immune responses, as evaluated by various immunological assays. These results confirm that the heartwater immune sheep are exposed to these proteins during infection and that recall immune responses developed in the host. Proliferation of immune PBMC stimulated with recombinant proteins was observed. Recombinant Erum7760, rErum8060, rErum5000 and rErum8610 induced cellular immune responses as evaluated by lymphoproliferation assays. It was demonstrated that both the soluble and insoluble fractions of recombinant proteins could induce recall T-cell responses. However, each recombinant protein had its own optimum concentration for inducing immune responses and differed between animals. This can be expected because out bred animals were used for these assays and each animal would react differently to the recombinant proteins. Lymphocyte proliferation assays have been shown to be very important for monitoring T cell immune responses. Lymphocytes stimulated by antigens become activated and a series of events happens (Sottong et al., 2000). These include synthesis of cytokines. In this study proliferating cells also produced IFN-γ in response to recombinant protein stimulation. Previously similar T cell responses were observed for recombinant proteins (MAP1 and MAP2) (Mwangi et al., 2002). These proteins have been implicated in protection against *E. ruminantium* (Nyika et al., 1998).

Strong IFN-γ responses were detected to three recombinant proteins tested here, rErum5000, rErum8060 and rErum8610. Only in one animal out of four the responses were obtained. The IFN-γ production was the highest for PBMC obtained from sheep 147 at antigen concentration of 10 µg/ml. The IFN-γ production could be considered to be the result of antigen-specific recall response since PBMCs from naïve sheep did not produce significant levels of IFN-γ when stimulated with *E. ruminantium* recombinant proteins. While in sheep 849, at the same concentration, no significant IFN-γ production was observed. In addition, no significant IFN-γ production was observed in s5400, s151 and s5400 at antigen concentration of 2.5 µg/ml. The antigen concentration could be

responsible for the low or absent proliferative responses and IFN-γ production observed in other sheep.

For this study, an antigen concentration range of 0.1-10 µg/ml was used. Further optimization of the protein concentration for each animal could result in significant responses for those animals where no response was detected. In previous studies using human PBMC, antigens were added such that the final concentration should be between 5 µg/ml (Lawn et al., 2007) and 10 µg/ml (Jennes et al., 2002). In addition, for human studies they use up to 100 µg/ml antigen concentration and IFN-γ production was observed only at high concentration of antigens (Farina et al., 2001). Therefore, presumably higher concentration of protein could show a positive result in our animals. However, lower concentrations gave better results in our study as seen in sheep 147. Clearly, additional studies designed to assess the concentration related effects should be performed.

Cytokine expression was measured to determine the type of immune response induced using real-time PCR. It was demonstrated by real-time PCR that PBMC isolated from immune s147 stimulated with three recombinant proteins (rErum5000, rErum7760 and rErum8060) express increased IFN-γ mRNA while no IL-4 expression was obtained. These results suggest that proteins tested in this study appear to induce Th1 responses based on the higher expression of IFN-γ mRNA. In addition to these results, there was a correlation between lymphocyte proliferation and IFN-γ production when PBMC were stimulated with rErum5000 and rErum8610. Although stimulation with rErum8060 did induce significant IFN-γ production using both ELISPOT and real-time PCR, significant proliferation was not detected when compared to the stimulation of naïve PBMC. The responses induced by PBMC isolated from naïve sheep are non-specific as the sheep was not immunized with the organism. The naïve sheep might have been previously exposed to a similar protein from another organism with similar epitopes as that of rErum8060. Immune animals should have been tested before they were vaccinated and this could have helped to interpret the results obtained from the naïve and immune sheep better.

There was a correlation between the IFN-γ mRNA production as was measured with real-time PCR and the IFN-γ protein expression determined by ELISPOT using s147 PBMC stimulated with rErum8060 and rErum5000. In contrast, rErum7760 which was not detected from ELISPOT assay expressed high levels of IFN-γ mRNA. It could be that this protein might induce IFN-γ production (ELISPOT assay) at an optimised recombinant protein concentration. Furthermore, it could be that PBMC stimulated with rErum7760 had low cytokine concentration that could not be detected or longer *in vitro* stimulation time for the production of the cytokine was necessary. Thus, the presence of mRNA does not necessarily mean that the product is expressed. It is therefore important to do ELISPOT that measures the IFN-γ product. Previously, it was demonstrated that for real-time PCR and ELISPOT assays, concentration of antigens, cytokine and antigen tested, target cells, time of incubation of cells stimulated with antigens need to be investigated in future (Esnault etal., 1996; Asai et al., 2000; Listvanova et al., 2003; Lawn et al., 2007). Therefore, thorough optimisation should be performed to be sure not to miss the cellular response induced by each protein. Nevertheless, the results obtained from real-time PCR suggest that this protein may be important and could have induced other cytokines and need to be included in vaccine development studies. There are several reasons stated in the literature that demonstrate the importance of rErum7760. For example rErum7760 has been predicted to be a lipoprotein. Lipoproteins have been shown to induce strong Th1-type immune responses and might be appropriate vaccine candidates (Brightbill et al., 1999; Yeremeev et al., 2000; Neyrolles et al., 2001; Hovav et al., 2003). In addition to these results, rErum8610 results were not obtained for real-time PCR and immunoblotting assay. The protein could not be repeatedly expressed using the same conditions as described in Chapter 2 and was finished before real-time PCR and immunoblotting were done. This explains why the results of rErum8610 do not appear in these assays.

T cell lines were generated in order to try to improve antigen specific proliferative responses. The highest increase in SI was obtained with T cell lines compared to PBMC. These results showed that the generation of T cell lines might be the best method to improve recall responses *in vitro*. Kodama and coworkers (1987) established and

characterized T-cell line specific for *Rickettsia tsutsugamushi*. It was shown that a T cell line which can be maintained in long-term culture can be a valuable tool for investigating T cell response. However, the method seemed not suitable when using sheep. The viability of cells decreased when generating T cell lines and insufficient quantity of cells were obtained to test recombinant proteins using T cell lines from sheep. This is because for bovine a lot of blood (~120 ml) can be obtained whereas for sheep only 60 ml can be obtained weekly. For example, bovine was used to prepare *E. ruminantium* specific short-term lymphocyte cultures (Van Kleef et al., 2002). Enough cells were obtained to perform LPA and flow cytometry analysis. Therefore, bovine might be a better animal for generating T cell lines for testing proteins with LPA, ELISPOT and real-time PCR assays. Interestingly, it was also demonstrated that the SI values from T cell lines and PBMC (s5401 and s151) stimulated with *E. ruminantium* crude antigen varied between animals. Hence MHC typing was done in order to identify different alleles which may correlate to the responses obtained.

To determine the contribution of MHC in the development of immune response, the highly polymorphic *Ovar*-DRB1 exon 2 of the sheep was characterized. PCR-RFLP alleles were identified by combining the restriction patterns for the *Ovar*-DRB1 axon 2 obtained with nine restriction enzymes. The restriction patterns obtained with *Rsa*I were the most polymorphic as demonstrated previously (Van Eijk et al., 1992; Gilliespie, et al., 1999; Miretti, et al., 2001; Behl et al., 2007). Three different alleles (*0332 and *0323, *0333) which were similar to alleles reported by Konnai et al., (2003a) were obtained. In addition, new unknown alleles were revealed in this study. New restriction patterns were obtained that could not be related to the known alleles found in literature. This could be due to the fact that previous studies were done in Japan using Suffolk sheep (Konnai et al., 2003a) whereas in this study South African Merino sheep were used thus genetic diversity would be expected. The results presented here indicate the Merino sheep exhibit differences in their allelic profiles. The genetic variation found among the sheep tested is important for future vaccine studies that include the determination of recombinant protein epitopes that are recognized by MHC class II loci within the Merino population. Different alleles that might be related to the variation of immune responses obtained from immune

sheep (s147, s5400, s151, s849 and s5401) were obtained. For example, s147 and s5401 alleles were both the same but they responded differently to antigens. Therefore, this lack of correlation between conserved alleles and immune response indicate that more work still need to be done. In contrast, for s5408, s151 and s849, one of the patterns were similar and the immune response among these sheep varied. This could be due to the fact that the MHC class II molecules are highly polymorphic and different alleles vary in their peptide binding specificity (Groothuis et al., 2005; Sommer, 2005). This can contribute to the variability of immune response among individuals. This technique is important in examining whether allelic diversity at the MHC class II loci influences protection against tick-borne parasite (Ballingall et al., 2004).

Serum of an immune animal detected the recombinant proteins (Figure 3.8) confirming that all proteins were secreted and exposed to the humoral immune system of the host during infection. However when measuring IL-4 (associated with Th2 type responses) IL-4 production was not detected thus the antibodies detected could be opsonising antibodies (IgG2) associated with a Th1 response. Alternatively the proteins may contain cross-reacting epitopes of a protein that induces a humoral immune response. It has been indicated that antibody-mediated immunity for intracellular parasites is not unusual (Winslow et al., 2000; Casadevall and Pirofski, 2003; Feng and Walker, 2004; Doyle et al., 2006). The use of an isotyping method to investigate the involvement and importance of antibody-mediated immunity is hampered by the fact that isotyping antibodies for sheep are not commercially available.

In *E. ruminantium* and other diseases caused by intracellular pathogens, protective immunity is mainly mediated by T-lymphocytes. It is the $CD4^+$ T-cell subset (Th1) of T-lymphocytes which is believed to be responsible for protection against heartwater (Totté et al., 1997). Our results, clearly demonstrated that the recombinant antigens tested trigger a Th1 type response as judged by higher production of IFN-γ and the absence of IL-4 production (real-time PCR). These findings strongly suggest the potential of rErum5000, rErum7760, rErum8060 and rErum8610 for the development of an anti-heartwater vaccine. These results are in agreement with experimental work of other researchers who have shown that antigens of mycobacteria including secreted proteins

activate the Th1 type of T cells, which secrete IFN-γ (Lozes et al., 1997; Tanghe et al., 2000; Al-Attiyah et al., 2004; Ganguly et al., 2007; 2008). For example, DNA vaccines expressing secreted proteins of *M. tuberculosis* had a protective efficacy associated with IFN-γ secreting T cells (Kamath et al., 1999). Similarly, immunogenicity of *M. tuberculosuis* secreted proteins was assessed in baboons. It was shown that all animals tested, produced antibody and cellular proliferative responses (Pehler et al., 2000). In addition, Dupont and coworkers (2005) showed that an exported 22kDa putative lipoprotein of *Mycobacterium avium* subspecies *paratuberculosis* induced cell-mediated immune response and can be included in a subunit vaccine against Johne's disease.

Thus, rErum5000, rErum7760, rErum8060 and rErum8610 have several attributes of being good heartwater vaccine candidate antigens. This stems from observation that these proteins were predicted to be exported proteins. In addition, a strong immune response characterized by the proliferation of PBMC and the production of IFN-γ was obtained. This is a compelling argument that secreted proteins are promising vaccine candidates and might be able to improve the protection against heartwater and it may be useful to include these antigens in future vaccine studies. To our knowledge, this study is the first to evaluate genes of *E. ruminantium* encoding secreted proteins to induce cellular immune responses.

3.5. REFERENCES

AL-ATTIYAH, R., MUSTAFA, A.S., ABAL, A.T., EL-SHAMY, A.S.M., DALEMANS, W., & SKEIKY, Y.A.W., 2004. In vitro cellular immune responses to complex and newly defined recombinant antigens of *Mycobacterium tuberculosis*. *Clinical and Experimental Immunology*, 138:139-144.

ANDERSEN, P., 1991. T-cell proliferative response to antigens secreted by *Mycobacterium tuberculosis*. *Infection and Immunity*, 59:1558-1563.

ANDERSEN, P., ASKGAARD, D., LJUNGQVIST, L., BENNEDSEN, J., & HERON, I., 1991. Proteins released from *Mycobacterium tuberculosis* during growth. *Infection and Immunity*, 59:1905.

ANDERSEN, P., 1994. Effective vaccination of mice against *Mycobacterium tuberculosis* infection with a soluble mixture of secreted mycobacterial proteins. *Infection and Immunity*, 62:2536.

ASAI, T., STORKUS, W.J., & WHITESIDE, T.L., 2000. Evaluation of the modified ELISPOT assay for gamma interferon production in cancer patients receiving antitumor vaccines. *Clinical and Diagnostic Laboratory Immunology*, 7:145-154.

ATTANASIO, R., PEHLER, K., & MCCLURE, H.M., 2000. Immunogenicity and safety of *Mycobacterium tuberculosis* culture filtrate proteins in non-primates. *Clinical and Experimental Immunology*, 119:84-91.

BALLINGALL, K.T., LUYAI, A., ROWLANDS, G.J., SALES, J., MUSOKE, A.J., MORZARIA, S.P., & MCKEEVER, D.J., 2004. Bovine leukocyte antigen major histocompatibility complex class II DRB3*2703 and DRB3*1501 alleles are associated with variation in levels of protection against *Theileria parva* challenge following immunization with the sporozoite p67 antigen. *Infection and Immunity*, 72:2738-2741.

BEHL, J.D., VERMA, N.K., BEHL, R., MUKESH, M., & AHLAWAT, S.P.S., 2007. Characterization of genetic polymorphism of the bovine lymphocyte antigen DRB3.2 locus in Kankrej cattle (*Bos indicus*). *Journal of Dairy Science*, 90:2997-3001.

BIONDO, C., MANCUSO, G., MIDIRI, A., BOMBACI, M., MESSINA, L., BENINATI, C., & TETI, G., 2006. Identification of major proteins secreted by *Cryptococcus neoformans*. *FEMS Yeast Research*, 6:645-651.

BOTTAI, D., BATONI, G., ESIN, S., MAISETTA, G., PARDINI, M, FLORIO, W., RINDI, L., GARZELLI, C., & CAMPA, M., 2003. Expression of SA5K, a secretion antigen of *Mycobacterium tuberculosis*, inside human macrophages and in sputum from tuberculosis patients. *FEMS Microbiology Letters*, 226:229-235.

BRIGHTBILL, H.D., LIBRATY, D.H., KRUTZIK, S.R., YANG, R.B., BELISLE, J.T., BLEHARSKI, J.R., MAITLAND, M., NORGARD, M.V., PLEVY, S.E., SMALE, S.T., BRENNAN, P.J., BLOOM, B.R., GODOW0SKI, P.J., & MODLIN, R.L., 1999. Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors. *Science*, 285:732-736.

BRITTEN, C.M., MEYER, R.G., KREER, T., DREXLER, I., WÖLFGANG, H., 2002. The use of HLA-A* 0201-transfected K562 as standard antigen-presenting cells for $CD8^+$ T lymphocytes in IFN-γ ELISPOT assays. *Journal of Immunological Methods*, 259:95-110.

BROWN, W.C., & LOGAN, K.S., 1992. *Babesia bovis*: Bovine helper T cell lines reactive with soluble and membrane antigens of merozoites. *Experimental Parasitology*, 74:188-199.

CARLISLE, J., EVANS, W., HAJIZADEH, R., NADAF, M., SHEPHERD, B., OTT, R.D., RICHTER, K., & DRAKE, W., 2007. Multiple *Mycobacterium* antigens induce interferon-γ production from sarcidosis peripheral blood mononuclear cells. *Clinical and Experimental Immunology*, 150:460-468.

CASADEVALL, A., & PIROFSKI, L.A., 2003. Antibody-mediated regulation of cellular immunity and the inflammatory response. *TRENDS in Immunology*, 24:474-478.

COLER, R.N., CAMPOS-NETO, A., OVENDALE, P., DAY, F.H., FLING, S.P., ZHU, L., SERBINA, N., FLYNN, J.L., REED, S.G., & ALDERSON, M.R., 2001. Vaccination with the T cell antigen Mtb 8.4 protects against challenge with *Mycobacterium tuberculosis*. *The Journal of Immunology*, 166:6227-6235.

COLLINS, N.E., PRETORIUS, A., VAN KLEEF, M., BRAYTON, K.A., ZWEYGARTH, E. & ALLSOPP, B.A., 2003a. Development of improved vaccines for heartwater. *Annals of the New York Academy of Science*, 990:474-484.

COLLINS, N.E., PRETORIUS, A., VAN KLEEF, M., BRAYTON, K.A., ALLSOPP, M.T., ZWEYGARTH, E. & ALLSOPP, B.A., 2003b. Development of improved attenuated and nucleic acid vaccines for heartwater. *Developmental in biologicals*, 114:121-136.

DÍAZ, I., & MATEU, E., 2005. Use of ELISPOT and ELISA to evaluate IFN-γ, IL-10 and IL-4 responses in conventional pigs. *Veterinary Immunology and Immunopathology*, 106:107-112.

DOYLE, C.K., NETHERY, K.A., POPOV, V.L., & MCBRIDE, J.W., 2006. Differentially expressed and secreted major immunoreactive protein orthologs of *Ehrlichia canis* and *E. chaffeensis* elicit early antibody responses to epitopes on glycosylated tandem repeats. *Infection and Immunity*, 74:711-720.

DUPONT, C., THOMPSON, K., HEUER, C., GICQUEL, B., & MURRAY, A., 2005. Identification and characterization of an immunogenic 22kDa exported protein of *Mycobacterium avium* subspecies *paratuberculosis*. *Journal of Medical Microbiology*, 54:1083-1092.

ESNAULT, S., BENBERNOU, N., LAVAUD, F., SHIN, H.C., POTRON, G., & GUENOUNOU, M., 1996. Differential spontaneous expression of mRNA for IL-4, IL-10, IL-13, IL-2 and interferon-gamma (IFN-γ) in peripheral blood mononuclear cells (PBMC) from atopic patients. *Clinical and Experiment Immunology*, 103:111-118.

ESTEVES, I., MARTINEZ, D., & TOTTÉ, P., 2004. Identification of *Ehrlichia ruminantium* (Gardel strain) IFN-γ inducing proteins after vaccination with a killed vaccine. *Veterinary Microbiology*, 100:233-240.

FARINA, C., BERGH, F.T., ALBRECHT, H., MEINL, E., YASSOURIDIS, A., NEUHAUS, O., & HOHLFELD, R., 2001. Treatment of multiple sclerosis with Copaxone (COP) Elispot assay detects COP-induced interleukin-4 and interferon-γ response in blood. *Brain*, 124:705-719.

FENG, H.M., & WALKER, D.H., 2004. Mechanisms of immunity to *Ehrlichia muris*: a model of monocytotropic Ehrlichiosis. *Infection and Immunity*, 72:966-971.

GANGULY, N., GIANG, P.H., BASU, S.K., MIR, F.A., SIDDIQUI, I., & SHARMA, P., 2007. *Mycobacterium tuberculosis* 6-kDa Early Secreted Antigenic Target (ESAT-6) protein downregulates lipopolysaccharide induced c-myc expression by modulating the Extracellular Signal Regulated Kinases 1/2. *BMC Immunology*, 8:24.

GANGULY, N., GIANG, P.H., GUPTA, C., BASU, S.K., SIDDIQUI, I., SALUNKE, D.M., SHARMA, P., 2008. *Mycobacterium tuberculosis* secretory proteins CFP-10, ESAT-6 and the CFP-10:ESAT6 complex inhibit lipopolysaccharide-induced NF-kappaB transactivation by downregulation of reactive oxidative species (ROS) production. *Immunology and Cell Biology*, 86:98-106.

GELHAUS, A., SCHNITTGER, L., MEHLITZ, D., HORSTMANN, R.D., & MEYER, C.G., 1995. Sequence and PCR-RFLP analysis of 14 novel BoLA-DRB3 alleles in Jersey cows. *Animal Genetics*, 26:147-153.

GILLIESPIE, B.E., JAYARAO, B.M., DOWLEN, H.H., & OLIVER, S.P., 1999. Analysis and frequency of bovine lymphocyte antigen DRB3.2 alleles in Jersey cows. *Journal of Dairy Science*, 82:2049-2053.

GIULIETTI, A., OVERBERGH, L., VALCKX, D., DECALLONNE, B., BOUILLON, R., & MATHIEU, C., 2001. An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. *Methods*, 25:386-401.

GOMEZ, M., JOHNSON, S., & GENNARO, M.L., 2000. Identification of secreted proteins of *Mycobacterium tuberculosis* by a bioinformatic approach. *Infection and Immunity*, 68:2323-2327.

GROOTHUIS, T.A.M., GRIEKSPOOR, A.C., NEIJSSEN, J.J., HERBERTS, C.A., & NEEFJES, J.J., 2005. MHC class I alleles and their exploration of the antigen-processing machinery. *Immunological Reviews*, 207:60-76.

GUERKOV, R.E.M., TARGONI, O.S., KREHER, C.R., BOEHM, B.O., HERRERA, M.T., TARY-LEHMANN, M., LEHMANN, P.V., & SCHWANDER, S.K., 2003. Detection of low-frequency antigen-specific IL-10-producing $CD4^+$ T cells via ELISPOT in PBMC: cognate vs. Nonspecific production of the cytokine. *Journal of Immunological Methods*, 279:111-121.

HOVAV, A.H., MULLERAD, J., DAVIDOVITCH, L., FISHMAN Y., BIGI, F., CATALDI, A., & ERCOVIER, H., 2003. The *Mycobacterium tuberculosis* recombinant 27-Kilodalton lipoprotein induces a strong Th1-type immune response deleterious to protection. *Infection and Immunity*, 71:3146-3154.

HORWITZ, M.A., LEE, B.E., DILLON, B.J. & HARTH, G., 1995. Protective immunity against tuberculosis induced by vaccination with major extracellular proteins of *Mycobacterium tuberculosis*. *Proceedings of the National Academy of Sciences of the United States of America*, 92:1530-1534.

JENNES, W., KESTENS, L., NIXON, D.F., & SHACKLETT, B.L., 2002. Enhanced ELISPOT detection of antigen-specific T cell responses from cryopreserved specimens with addition of both IL-7 and IL-15- the Amplispot assay. *Journal of Immunological Methods*, 270:99-108.

KALYUZHNY, A., & STARK, S., 2001. A simple method to reduce the background and improve well-to well reproducibility of staining in ELISPOT assays. *Journal of Immunological Methods*, 257:93-97.

KAMATH, A.T., FENG, C.G., MACDONALD, M., BRISCCOE, H., BRITTON, W.J., 1999. Differential protective efficacy of DNA vaccines expressing secreted proteins of *Mycobacterium tuberculosis*. *Infection and Immunity*, 67:1702-1707.

KODAMA K., KAWAMURA, S., YASUKAWA, M.M., & KOBAYASHI, Y., 1987. Establishment and characterization of a T cell-line specific for *Rickettsia tsutsugamushi*. *Infection and Immunity*, 55:2490-2495.

KONNAI, S., NAGAOKA, Y., TAKESIMA, S., ONUMA, M., & AIDA, Y., 2003a. Technical note: DNA typing for Ovine MHC DRB1 using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP). *Journal of Dairy Science*, 86:3362-3365.

KONNAI, S., USUI, T., OHASHI, K., & ONUMA, M., 2003b. The rapid quantitative analysis of bovine cytokine genes by real-time RT-PCR. *Veterinary Microbiology*, 94:283-294.

LANGERMANS, J.A., DOHERTY, T.M., VERVENNE, R.A., VAN DER LAAN, T., LYASHCHENKO, K., GREENWALD, R., AGGER, E.M., AAGAARD, C., WEILER, H., VAN SOOLINGEN, D., DALEMANS, W., THOMAS, A.W., & ANDERSEN, P., 2005. Protection of macaques against *Mycobacterium tuberculosis* infection by a subunit vaccine based on a fusion protein of antigen 85B and ESAT-6. *Vaccine*, 23:2740-2750.

LAWN, S.D., BANGANI, N., VOGT, M., BEKKER, L.G., BADRI, M., NTOBONGWANA, M., DOCKRELL, H.M., WILKINSON, R.J., & WOOD, R., 2007. Utility of interferon-γ ELISPOT assay responses in highly tuberculosis-exposed patients with advanced HIV infection in South Africa. *BMC Infectious Diseases*, 7:99.

LISTVANOVA, S., TEMMERMAN, S., STORDEUR, P., VERSCHEURE, V., PLACE, S., ZHOU, L., LOCHT, C., MASCART, F., 2003. Optimal kinetics for quantification of antigen-induced cytokines in human peripheral blood mononuclear cells by real-time PCR and by ELISA. *Journal of Immunological Methods*, 281:27-35.

LIVAK, K.J., FLOOD, S.J., MARMARO, J., GIUSTI, W., & DEETZ, K., 1995. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. *PCR Methods Applications*, 4:357-362.

LOZES, E., HUYGEN, K., CONTENT, J., DENIS, O., MONTGOMERY, D.L., YAWMAN, A.M., VANDENBUSSCHE, P., VAN VOOREN, J.P., DROWART, A., ULMER, J.B., & LIU, M.A., 1997. Immunogenicity and efficacy of a tuberculosis DNA vaccine encoding the components of the secreted antigen 85 complex. *Vaccine*, 15:830-833.

MÄKITALO, B., ANDERSON, M., ARESTRÖM, I., KARLÉN, K., VILLINGER, F., ANSARI, A., PAULIE, S., THORSTENSSON, R., & AHLBORG, N., 2002. ELISpot and ELISA analysis of spontaneous, mitogen-induced and antigen-specific cytokine production in cynomolgus and rhesus macaques. *Journal of Immunological Methods*, 270:85-97.

MALYGUINE, A., STROBL, S.L., SHAFER-WEAVER, K., ULDERICH, T., TROKE, A., BASELER, M., KWAK, L.W., & NEELAPU, S.S., 2004. A modified human ELISPOT assay to detect specific responses to primary tumor cell targets. *Journal of Translational Medicine*, 2:9.

MEIJ, P., BLOEMENA, E., PALMEN, N., BRINK, A., VERVOORT, M.B.H.J., MEIJER, C.J.L.M., & MIDDELDORP, J.M., 2001. Functional $CD4^+$ and $CD8^+$ T-cell responses induced by autologous mitomycin C treated Epstein-Barr virus transformed lymphoblastoid cell lines. *Cellular Immunology*, 208:25-33.

MENA, A., IOANNOU, X.P., VAN KESSEL, A., VAN DRUNEN LITTLE-VAN DEN HURK, S., POPOWYCH, Y., BABIUK, L.A., & GODSON, D.L., 2002. Th1/Th2 biasing effects of vaccination in cattle as determined by real-time PCR. *Journal of Immunological Methods*, 263:11-21.

MIRETTI, M.M., FERRO, J.A., LARA, M.A., & CONTEL, E.P.B., 2001. Restriction Fragment Length Polymorphism (RFLP) in exon 2 of the BoLA-DRB3 gene in South American cattle. *Biochemical Genetics*, 39:311-324.

MWANGI, D.M., MAHAN, S.M., NYANJUI, J.K., TARACHA, E.L.N., & MCKEEVER, D.J., 1998. Immunization of cattle with *Cowdria ruminantium* elicits T lymphocytes that recognize autologous endothelial cells and monocytes. *Infection and Immunity*, 66:1855.

MWANGI, D.M., MCKEEVER, D.J., NYANJUI, J.K., BARBET, A.F., & MAHAN, S.M., 2002. Immunisation of cattle against heartwater by infection with *Cowdria ruminantium* elicits T lymphocytes that recognize major antigenic proteins 1 and 2 of the agent. *Veterinary Immunology and Immunopathology*, 85:23-32

NEYROLLES, O., GOULD, K., GARES, M.P., BRETT, S., JANSSEN, R., O'GAORA, P., HERRMANN, J.L., PREVOST, M.C., PERRET, E., THOLE, J.E., & YOUNG, D., 2001. Lipoprotein access to MHC class I presentation during infection of murine macrophages with live mycobacteria. *Journal of Immunology*, 166:447-457.

NGUYEN VAN, N., TAGLINGER, K., HELPS, C.R., TASKER, S., GRUFFYDD-JONES, T.J., DAY, M.J., 2006. Measurement of cytokine mRNA expression in intestinal biopsies of cats with inflammatory enteropathy using quantitative real-time RT-PCR. *Veterinary Immunology and Immunopathology*, 113:404-414.

NYIKA, A., MAHAN, S.M., BURRIDGE, M.J., MCGUIRE, T.C., RURANGIRWA, F., & BARBET, A.F., 1998. A DNA vaccine protects mice against the rickettsial agent *Cowdria ruminantium. Parasite Immunology*, 20:111-119.

ORME, I., 1994. Protective and memory immunity in mice infected with *Mycobacterium tuberculosis*. *Immunobiology*, 191:503.

PAHAR, B., LI, J., ROURKE, T., MILLER, C.J., & MCCHESNEY, M.B., 2003. Detection of antigen-specific T cell interferon γ expression by ELISPOT and cytokine flow cytometry assays in rhesus macaques. *Journal of Immunological Methods*, 282:103-115.

PAL, P.G., & HORWITZ, M.A., 1992. Immunization with extracellular proteins of *Mycobacterium tuberculosis* induces cell-mediated immune responses and substantial protective immunity in a guinea pig model of pulmonary tuberculosis. *Infection and Immunity*, 60:4781.

PEHLER, K., BRASKY, K.M., BUTLER, T.M., & ATTANASIO, R., 2000. *Mycobacterium tuberculosis*-secreted protein antigen: immunogenicity in baboons. *Journal of Clinical Immunology*, 20:306-316.

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

PRETORIUS, A., COLLINS, N.E., STEYN, H.C., VAN STRIJP, F., VAN KLEEF, M., & ALLSOPP, B.A., 2007. Protection against heartwater by DNA immunization with four *Ehrlichia ruminantium* open reading frames. *Vaccine*, 25:2316-2324.

PRETORIUS, A., 2007. Aspects of the immune response in ruminants to four protective *Ehrlichia ruminantium* gene products. Ph.D. thesis. University of Pretoria, South Africa.

RANI, H.S., LAKSHMI, V.V., SUMANLATHA, G., & MURTHY, K.J.R., 2005. Cell-mediated immune response in children towards secreted proteins of *Mycobacterium bovis* BCG. *Tuberculosis*, 85:89-93.

SHAFER-WEAVER, K., SAYERS, T., STROBL, S., DERBY, E., ULDERICH, T., BASELER, M., & MALYGUINE, A., 2003. The Granzyme B ELISPOT assay: an alternative to the 51Cr-release assay for monitoring cell-mediated cytotoxicity. *Journal of Translational Medicine*, 1:14.

SIMBI, B.H., BOWIE, M.V., MCGUIRE, T.C., BARBET, A.F., & MAHAN, S.M., 2006. Evaluation of *E. ruminantium* genes in DBA/2 mice as potential DNA vaccine candidates for control of heartwater. *Annals of New York Academy of Science*, 1078:424-437.

SINHA, R.K., VERMA, I., & KHULLER, G.K., 1997. Immunobiological properties of a 30 kDa secretory protein of *Mycobacterium tuberculosis* H_{37}Ra. *Vaccine*, 15:689-699.

SOMMER, S., 2006. The importance of immune gene variability (MHC) in evolutionary ecology and conservation. *Frontiers in Zoology*, 2:16

SOTTONG, P.R., ROSEBROCK, J.A., BRITZ, J.A., & KRAMER, T.R., 2000. Measurement of T-lymphocyte responses in whole-blood cultures using newly synthesized DNA and ATP. *Clinical and Diagnostic Laboratory Immunology*, 7:307-311.

STEYN, H.C., PRETORIUS, A., MCCRINDLE, C.M.E., STEINMANN, C.M.L., VAN KLEEF, M., 2008. A quantitative real-time PCR assay for *Ehrlichia ruminantium* using pCS20. *Veterinary Microbiology*, 131:258-265.

STODEUR, P., POULIN, L.F., CRACIUN, L., ZHOU, L., SCHANDENÉ, L., DE LAVAREILLE, A., GORIELY, S., GOLDMAN, M., 2002a. Cytokine mRNA quantification by real-time PCR. *Journal of Immunological Methods*, 259: 55-54.

STODEUR, P., ZHOU, L., GOLDMAN, M., 2002b. Analysis of spontaneous mRNA cytokine production in peripheral blood. *Journal of Immunological Methods*, 261:195-197.

TANGHE, A., DENIS, O., LAMBRECHT, B., MOTTE, V., VAN DEN BERG, T., & HUYGEN, K., 2000. Tuberculosis DNA vaccine encoding Ag85A is immunogenic and protective when administered by intramuscular needle injection but not by epidermal gene gun bombardment. *Infection and Immunity*, 68:3854-3860.

TOTTÉ, P., MACKEEVER, D., MARTINEZ, D., & BENSAID, A., 1997. Analysis of T-cell responses in cattle immunized against heartwater by vaccination with killed elementary bodies of *Cowdria ruminantium*. *Infection and Immunity*, 65:236-241.

TOTTÉ, P., BENSAID, A., MAHAN, S.M., MARTINEZ, D., & MACKEEVER, D., 1999a. Immune responses to *Cowdria ruminantium* infections. *Parasitology Today*, 15:286-290.

TOTTÉ, P., NYANJUI, J., BENSAID, A., & MACKEEVER, D., 1999b. Bovine $CD4^+$ T-cell lines reactive with soluble and membrane antigens of *Cowdria ruminantium*. *Veterinary Immunology and Immunopathology*, 70:269-276.

VAN EIJK, M.J.T., STEWART-HAYNES, J.A., & LEWIN, H.A., 1992. Extensive polymorphism of the BoLA-DRB3 gene distinguished by PCR-RFLP. *Animal Genetics*, 23:483-496.

VAN KLEEF, M., GUNTER, N.J. MACMILLAN, H., ALLSOPP, B.A., SHKAP, V., & BROWN, W.C., 2000. Identification of *Cowdria ruminantium* antigens that stimulate proliferation of lymphocytes from cattle immunized by infection and treatment or with inactivated organisms. *Infection and Immunity*, 68:603-614.

VAN KLEEF, M., MACMILLAN, H., GUNTER, N.J., ZWEYGARTH, E., ALLSOPP, B.A., SHKAP, V., DU PLESSIS, D.H., & BROWN, W.C., 2002. Low molecular weight proteins of *Cowdria ruminantium* (Welgevonden isolate) induce bovine $CD4^+$-enriched T-cells to proliferate and produce interferon-gamma. *Veterinary Microbiology*, 85:259-273.

VEKEMANS, J., OTA, M.O.C. SILLAH, J., FIELDING, K., ALDERSON, M.R., SKEIKY, Y.A.W., DALEMANS, W., MCADAM, K.P.W.J., LIENHARDT, C.,& MARCHANT, A., 2004. Immune responses to mycobacterial antigens in the Gambian population: implications for vaccines and immunodiagnostic test design. *Infection and Immunity*, 72:381-388.

WEBB, JR., VEDVICK, T.S., ALDERSON, M.R., GUDERIAN, J.A., JEN, S.S., OVENDALE, P.J., JOHNSON, S.M., REED, S.G., & SKEIKY, Y.A.W., 1998. Molecular cloning, expression, and immunogenicity of MTB12, a novel low- molecular weight antigen secreted by *Mycobacterium tuberculosis*. *Infection and Immunity*, 66:4208-4214

WINSLOW, G.M., YAGER, E., SHILO, K., VOLK, E., REILLY, A., & CHU, F.K., 2000. Antibody-mediated elimination of the obligate intracellular bacterial pathogen *Ehrlichia chaffeensis* during active infection. *Infection and Immunity*, 68:2187-2195.

YEREMEEV, V.V., LYADOVA, I.V., NIKONENKO, B.V., APT, A.S., ABOU-ZEID, C., INWALD, J., & YOUNG, D.B., 2000. The 19-kDa antigen and protective immunity in murine model of tuberculosis. *Clinical and Experimental Immunology*, 120:274-279.

CHAPTER 4

CONCLUDING DISCUSSION

Regardless of the numerous recombinant vaccines under investigation, vaccination against *E. ruminantium* still relies on the infection and treatment method (van der Merwe, 1987). However, this vaccine does not cross-protect against all *E. ruminantium* genotypes, requires the use of tetracycline antibiotics and is difficult to distribute because it requires a cold chain. In addition, despite the 100% success of the 1H12 DNA vaccine trial against *E. ruminantium* using both homologous and heterologous needle challenge, only 20% protection against tick challenge was obtained (Collins et al., 2003a,b; Pretorius et al., 2007). There is therefore a need to improve the 1H12 recombinant heartwater vaccine by the selection of additional antigens that will induce a protective immune response to *E. ruminantium* infection in an outbred population in the field. The selection and investigation of antigens is usually based on their capacity to interact with a component of the immune response during infection and must play an important role in protection and pathogenesis. Moreover, promising candidate antigens must be expressed during disease, accessible (surface-bound or secreted) for functional antibodies or effector immune cells, conserved among strains, immunogenic and essential for *in vivo* survival in order to avoid counter-selection (Pizza et al., 2000; Rappuoli, 2000, 2001; reviewed by Mora et al., 2003, 2006; Muzzi, et al., 2007).

In this study antigens were selected using the complete genome sequence of *E. ruminantium* Welgevonden (Collins et al., 2005). The size and complexity of the *E. ruminantium* genome makes it impossible to evaluate each gene for its ability to induce immune responses. Therefore, in this study, a reductive strategy was employed whereby genes were grouped according to their putative functions and location. Five *E. ruminantium* open reading frames predicted to code for secreted proteins were selected randomly from the Welgevonden isolate using bioinformatics tools. Three of these expressed recombinant proteins (rErum5000; -5010 and -8610) did not show any

sequence or peptide motif similarity to known proteins in the database while rErum7760 and -8060 demonstrated homology to exported lipoproteins. Four of these recombinant proteins (rErum5000, -7760, -8060 and 8610) were successfully expressed in a bacterial expression system (Chapter 2).

The approaches used to identify vaccine candidates for recombinant vaccine development are, in general, guided by the type of immune responses that are likely to mediate protection. It is thought that a coordinated response of the cellular immunity is fundamental in destruction of intracellular pathogens like *E. ruminantium*. Th1 responses have been shown to be necessary for the resolution of, and induction of immune responses against *E. ruminantium*. Consequently, IFN-γ is believed to play an important role in protection against *E. ruminantium* (Totté et al., 1997, 1999). Hence, the four expressed *E. ruminantium* Welgevonden recombinant proteins were tested in different immunological assays for their ability to induce recall T responses and IFN-γ production (Chapter 3).

Lymphocyte proliferation (recall T cell responses), real-time PCR (IFN-γ/IL-4 mRNA expression) and ELISPOT (IFN-γ protein determination) assays were used to test which type of immune responses might be induced by these antigens. Significant proliferative responses and IFN-γ production were induced by three recombinant proteins (rErum5000, rErum8060 and rErum8610) using both ELISPOT and real-time PCR while only increased IFN-γ mRNA was detected in PBMC stimulated with rErum7760. Each recombinant protein had its own optimum concentration for proliferation that differed between animals. Each animal tested proliferated in response to a different recombinant protein. This can be expected because out bred animals with variable MHC class II *ovar*-DRB1 alleles were used for these assays and therefore each animal could react differently to the recombinant proteins. PBMC from sheep 147 responded most significantly to *E. ruminantium* antigens than PBMC form other immune sheep. Even though positive responses were obtained in the LPA and ELISPOT the overall responses were low and each animal responded to a different protein. This variability problem in outbred populations of sheep may require the addition of more antigens to a recombinant vaccine

so to introduce more epitopes that would fit all the variable sites in the MHC molecules and stimulate all sheep to respond (Outteridge, 1993). Moreover, addition of cytokines like IL-15 and IL-7 to antigen-stimulated PBMC has been shown to significantly enhance specific IFN-γ production by $CD4^+$ and $CD8^+$ T cells (Jennes et al., 2002). This method may be useful for sheep with low-level antigen-specific T cell responses that have been difficult to detect. Furthermore, the strong and weak response to recombinant protein might be due to the concentration of antigen used for immunological assays. Comparing to human studies, they used up to 100 µg/ml antigen concentration and IFN-γ production was evident at higher concentrations (Farina et al., 2001). Only 2.5 µg/ml and 10 µg/ml of antigen concentrations were used in this study. From the data presented here it is clear that the concentration of the antigen can affect the efficiency of immune response induced. Therefore, use of a serial dilution of antigen might give a positive feedback in attempt to produce good immune responses *in vitro*.

It is well known that intracellular pathogens induce cell mediated immune responses characterized by the production of IFN-γ but the induction of humoral immunity can not be ruled out (reviewed by Casadevall, 2003). Therefore, the cytokine (IL-4) that mediates antibody production that may play an important role in protection against heartwater was tested using real-time PCR. None of the proteins tested in this study induced IL-4 indicating that they may not be inducers of a Th2 response. In contrast, the serum of immune animals did detect all four the recombinant proteins, suggesting that Th1-type antibodies developed during *E. ruminantium* infection. This may also indicate that the secreted proteins were exported and exposed to the host immune system during infection or they may contain cross reacting epitopes. Thus rErum5000, rErum8060 rErum8610 were able to induce a Th1 immune response. Further studies are therefore needed to determine the isotype of the antibodies that cross reacted with the recombinant proteins. Recombinant Erum7760 showed no significant proliferative responses except in s849. Moreover, real-time PCR results showed that the level of IFN-γ mRNA expression by PBMC stimulated with rErum7760 was high. Real-time PCR showed that it could detect low amounts of specific mRNA, even when the corresponding proteins can barely be

detected by ELISPOT assay. Recombinant Erum7760 can therefore not be excluded as a possible vaccine candidate.

Several methods (real-time PCR, ELISA, ELISPOT assay, flow cytometry etc) need to be compared in order to understand the complexity of immune responses induced by these antigens. These methods are usually selected for their sensitivity and reliability. They evaluate different levels of activation, transcription, secretion and membrane expression and/or secretion, and phenotype of proliferating cells, respectively. Therefore, depending on the specific aim of investigation, combination of methods have to be chosen carefully in order to achieve significant changes in cytokine production patterns following stimulation of immune PBMC with recombinant proteins.

Lymphocyte proliferation, real-time PCR and ELISPOT assays are highly sensitive and specific methods to measure recall immune responses. Furthermore, ELISPOT was shown to be a reliable method to measure antigen-specific T helper responses (Bennouna et al., 2002). None of these techniques could however be used to determine the type of cells that are activated after stimulation with the recombinant proteins. Thus, it will be very useful to determine the phenotype of the proliferating cells by flow cytometry and test if a specific T cell ($CD4^+$ or $CD8^+$) expresses more than one cytokine. This information could be used to subsequently improve vaccine development. Numerous cases have been reported where flow cytometry technique showed a substantial difference in measuring phenotype of the proliferating cells from lymphocyte subpopulation (Elson et al., 1995; Sopp and Howard, 2001; Wang et al., 2007). Unfortunately, there are no antibodies for sheep cytokines that can be used in the flow cytometry.

IFN-γ is not the only cytokine that is involved in the induction of a protective cellular immune response, therefore, investigation of other cytokines as the measure for protection and long lasting immunity could be a solution to the efficacy of heartwater vaccine. Given the tremendous impact that cytokines have from the immunological point of view, it is important to understand their role in the control of infectious diseases.

Several studies have shown the correlation between IFN-γ and the resistance against pathogens (Esteves et al., 2004; Simbi et al., 2006; Dedieu et al., 2005). However, a single cytokine may be insufficient to confer full protection. This statement is in agreement with the experiment done by Leclerq and coworkers (2002). The immunogenicity and protective efficacy of a DNA vaccine encoding *GroEL* heat shock gene from *Brucella abortus* induced Th1-type of immune response characterized by high levels of IFN-γ but no significant level of protection was obtained. These findings should stimulate further investigations of determining the role of other cytokines in prevention against intracellular pathogens. For example, the use of Th1 cytokines, IL-12, IL-18 and IL-23 to modulate the immune responses has been investigated (Tuo et al., 2000; Ha et al., 2004; Williman et al., 2006; Wozniak et al., 2006; Leng et al., 2008; Patel et al., 2008). The results suggested that these cytokines would be effective as adjuvants and enhances protective efficacy of a DNA vaccine. IL-2 is a Th1 cytokine which induces proliferation and plays an important role in the development of T cell immunologic memory. It was demonstrated that Natural Killer cells activated /incubated with IL-2 play a significant role in defence against infection caused by *Leishmania amazonensis* (Aranha et al., 2005). Activated Natural Killer (NK) cells are very important for the lysis of damaged cells including tumor cells and cells infected with intracellular pathogens and resistance of macrophages to *L. amazonensis*. In the current study, IFN-γ production was not observed in the ELISPOT assay when immune PBMC were stimulated with rEum7760. This shows that rErum7760 could have induced other cytokines. Therefore, recombinant proteins and their epitopes must now be further tested to determine what other cytokines (IL-1β, IL-6, IL-8, IL-12, IL-18 and IL-23) they induce, cytotoxic activity which includes tumor necrosis factor, macrophages, nitric oxide and superoxide production and other cellular immune responses (IFN-γ-producing T lymphocytes $CD4^+$ and $CD8^+$ T cells) that are important in prevention against the pathogen.

Our results show that four recombinant proteins tested in this study induce cellular immune responses characterized by the production of IFN-γ. It is generally accepted that induction of IFN-γ correlates with protection and appears to be necessary to inhibit the growth of intracellular pathogens. However, the overall contribution of IFN-γ to immune

protection against intracellular pathogens is still not known. In some cases even if IFN-γ was detected, no protection was obtained. For example, an increased cellular immunity in immunized animals did not elicit protection against tuberculosis (Majlessi et al., 2006). It was also found that animals were protected against *E. ruminantium* but no IFN-γ detected (Vachiery et al., 2006). Hence, testing these antigens *in vivo* could help select proteins that induce significant immune response and protection against heartwater. Therefore, the ultimate test would be to clone these genes to a mammalian expression vector and test them in animals. Prime-boost regime could be used to test these recombinant proteins for their ability to improve the protection obtained by 1H12 DNA vaccine in the field. Recent reports indicate that in order to obtain better immunity against infectious agents, a DNA priming/protein boosting strategy is better than using DNA or protein alone (Tanghe et al., 2001; Gonzalo et al., 2002; Skinner et al., 2003; Taracha et al., 2003; Penttilä et al., 2004; Wang et al., 2004; Wu, et al., 2004; Boyer et al., 2006; Liang et al., 2008).

Furthermore, research directed at elucidating the epitope of these recombinant proteins will provide a better understanding of which fragment of the protein is immunogenic or inhibitory. Previously, in order to identify the epitope-containing region, the *E. canis* gp19 gene fragments (C-terminal, N-terminal, N_1, N_2 and N_{1c}) were expressed. It was shown that antibody reacted strongly with the N-terminal recombinant fragment but did not react with the C-terminal, suggesting that an epitope was located on the N-terminal region of the protein (McBride et al., 2007). Furthermore, peptides of a NcSRS2, *N. caninum* tachyzoite surface protein were tested to identify candidate peptides for vaccine development against bovine neosporosis abortion (Staska et al., 2005). The NcSRS2 region spanning amino acids 137 and 135 was recognized by $CD4^+$ CTL and IFN-γ-secreting T lymphocytes. Moreover, Viudes and coworkers (2004) showed that the C-terminal epitope of 58-kDa surface mannoprotein of *Candida albicans* (mp58) represents a protective epitope during candidiasis. This stems from the observation that there was an increase in antibody reactivity towards C-terminal epitope in patients who survived the infection and protection associated with this region was observed in a murine model. In this study, toxicity of rErum8610 to the expression host BL21 (DE3)

E. coli strain was encountered. To overcome problematic antigens (e.g. toxic proteins) it may be advisable to break down these genes into sub-domains or epitopes to inactivate their toxicity prior to insertion into over-expression plasmids (Barry and Johnston, 1997).

Peptide mapping may also aid in the discovery of bacterial/viral inhibitors. For example, mapping of aspartic protease (PR) an enzyme essential for human immunodeficiency virus (HIV) replication led to identification of potent inhibitors. These include two monoclonal antibodies (antibody 1696 and antibody F11.2.32) that inhibit the PR activity by binding to the enzyme epitopes (Bartonova et al., 2008). In addition, HIV-1 auxiliary protein Vif (N-terminal fragment) binds and inhibits the PR activity (Friedler et al., 1999). Furthermore, *Helicobacter pylori* urease is an enzyme essential for the survival and pathogenesis of the organism. HpU-2, HpU-18 and L2 were monoclonal antibodies that recognized urease epitopes and strongly inhibited the enzymatic activity (Fujii et al., 2004; Hirota et al., 2001). Furthermore, peptide-25 (aa240-254) of Ag85B has been designated as a Th1-inducing peptide. It is considered as a major T cell epitope. Immunization of mice with this epitope was capable of inducing significant protection against *M. tuberculosis* (reviewed by Takatsu and Kariyone, 2003). In addition, a linear peptide containing T- and B- cell epitopes of *Plasmodium falciparum* surface circumsporozoite (CS) protein was found to be highly immunogenic. Antibody and IFN-γ-secreting T cell responses were elicited (Calvo-Calle et al., 2006).

In conclusion cellular immune responses characterized by proliferative responses and IFN-γ production were obtained. The recognition of four ORFs encoding recombinant proteins (rErum5000, rErum8060, rErum7760 and rErum8610), selected from Welgevonden isolate, by immune PBMC provides evidence that they may play a important role during development of heartwater. Therefore, they need to be tested in animal trials to determine their protective efficacy.

4.1. REFERENCES

ARANHA, F.C.S., RIBEIRO JR, U., BASSE, P., CORBETT, C.E.P., & LAURENTI, M.D., 2005. Interleukin-2-activated Natural Killer cells may have a direct role in the control of *Leishmania* (*Leishmania*) *amazonensis* promastigote and macrophage infection. *Scandinavian Journal of Immunology*, 62:334-341.

BARTONOVA, V., KRÁL, V., SIEGLOVÁ, I., BRYNDA, J., FÁBRY, M., HOŘEJŠÍ, M., KOŽÍŠEK, M., ŠAŠKOVA, K.G., KONVALINKA, J., SEDLÁČEK, J., ŘEZÁČOVÁ, P., 2008. Potent inhibition of drug-resistant HIV protease variants by monoclonal antibodies. *Antiviral Research*, 78:275-277.

BENNOUNA, J., HILDESHEIM, A., CHIKAMATSU, K., GOODING, W., STORKUS, W.J., & WHITESIDE, T.L., 2002. Application of IL-5 ELISPOT assays to quantification of antigen-specific T helper responses. *Journal of Immunological Methods*, 261:145-156.

BOYER, J.D., ROBINSON, T.M., MACIAG, P.C., PENG, X., JOHNSON, R.S., PAVLAKIS, G., LEWIS, M.G., SHEN, A., SILICIANO, R., BROWN, C.R., WEINER, D.B., & PATERSON, Y., 2005. DNA prime *Listeria* boost induces a cellular immune response to SIV antigens in the rhesus macaque model that is capable of limited suppression of SIV239 viral replication. *Virology*, 333:88-101.

CALVO-CALLE, J.M., OLIVEIRA, G.A., WATTA, C.O., SOVEROW, J., PARRA-LOPEZ, C., & NARDIN, E.H., 2006. A linear peptide containing minimal T- and B-cell epitopes of *Plasmodium falciparum* circumsporozoite protein elicits protection against transgenic sporozoite challenge. *Infection and Immunity*, 74:6929-6939.

CASADEVALL, A., 2003. Antibody-mediated immunity against intracellular pathogens: Two-dimensional thinking comes full circle. *Infection and Immunity*, 71:4225-4228.

COLLINS, N.E., PRETORIUS, A., VAN KLEEF, M., BRAYTON, K.A., ZWEYGARTH, E. & ALLSOPP, B.A., 2003a. Development of improved vaccines for heartwater. *Annals of the New York Academy of Science*, 990:474-484.

COLLINS, N.E., PRETORIUS, A., VAN KLEEF, M., BRAYTON, K.A., ALLSOPP, M.T., ZWEYGARTH, E. & ALLSOPP, B.A., 2003b. Development of improved attenuated and nucleic acid vaccines for heartwater. *Developmental in biologicals*, 114:121-136.

COLLINS, N.E., LIEBENBERG, J., DE VILLIERS, E.P., BRAYTON, K.A., LOUW, E., PRETORIUS, A., FABER, F.E., VAN HEERDEN, H., JOSEMANS, A., VAN KLEEF, M., STEYN, H.C., VAN

STRIJP, M.F., ZWEYGARTH, E., JONGEJAN, F., MAILLARD, J.C., BERTHEIR, D., BOTHA, M., JOUBERT, F., CORTON, C.H., THOMSON, N.R., ALLSOPP, M.T., & ALLSOPP, B.A., 2005. The genome of the heartwater agent *Ehrlichia ruminantium* contains multiple tandem repeats of actively variable copy number. *Proceedings of the National Academy of Sciences of the United States of America*, 102:838-843.

DEDIEU, L., BALCER-RODRIGUES, V., YAYA, A., HAMADOU, B., CISSE, O., DIALLO, M., & NIANG, M., 2005. Gamma interferon-producing CD4 T-cells correlate with resistance to *Mycoplasma mycoides* subsp. *mycoides* S.C. infection in cattle. *Veterinary Immunology and Immunopathology*, 107:217-233.

ELSON, L.H., NUTMAN, T.B., METCALFE, D.D., & PRUSSIN, C., 1995. Flow cytometric analysis for cytokine production identifies T helper 1, T helper 2, and T helper 0 cells within the human $CD4^{+}CD27^{-}$ lymphocyte subpopulation. *The Journal of Immunology*, 154:4294-4301.

ESTEVES, I., MARTINEZ, D., & TOTTÉ, P., 2004. Identification of *Ehrlichia ruminantium* (Gardel strain) IFN-γ inducing proteins after vaccination with a killed vaccine. *Veterinary Microbiology*, 100:233-240.

FARINA, C., BERGH, F.T., ALBRECHT, H., MEINL, E., YASSOURIDIS, A., NEUHAUS, O., & HOHLFELD, R., 2001. Treatment of multiple sclerosis with Copaxone (COP) Elispot assay detects COP-induced interleukin-4 and interferon-γ response in blood. *Brain*, 124:705-719.

FRIEDLER, A., BLUMENZWEIG, I., BARAZ, L., STEINITZ, M., KOTLER, M., GILON, C., 1999. Peptides derived from HIV-1 Vif: a non-substrate based novel type of HIV-1 protease inhibitors. *Journal of Molecular Biology*, 287:93-101.

FUJII, R., MORIHARA, F., OKU, T., HIFUMI, E., & UDA, T., 2004. Epitope mapping and features of the epitope for monoclonal antibodies inhibiting enzymatic activity of *Helicobacter pylori* urease. *Biotechnology and Bioengineering*, 86:434-444.

GONZALO, R.M., DEL REAL, G., RODRIGUEZ, J.R., RODRIGUEZ, D., HELJASVAARA, R., LUCAS, P., LARRAGA, V., & ESTEN, M., 2002. A heterologous prime-boost regime using DNA and recombinant vaccinia virus expressing the *Leishmania infantum* P36/LACK antigen protects BALB/c mice from cutaneous leishmaniasis. *Vaccine*, 20:1226-1231.

HA, S.J., KIM, D.J., BAEK, K.H., YUN, Y.D., SUNG, Y.C., 2004. IL-23 induces stronger sustained CTL and Th1 immune responses than IL-12 in Hepatitis C virus Envelope Protein 2 DNA immunization. *The journal of Immunology*, 172:525-531.

HIROTA, K., NAGATA, K., NOROSE, Y., FUTAGAMI, S., NAKAGAWA, Y., SENPUKU, H., KOBAYASHI, M., & TAKAHASHI, H., 2001. Identification of an antigenic epitope in *Helicobacter pylori* urease that induces neutralizing antibody production. *Infection and Immunity*, 69:6597-6603.

LECLERQ, S., HARMS, J.S., ROSINHA, G.M.S., AZEVEDO, V., & OLIVEIRA, S.C., 2002. Induction of a Th1-type of immune response but not protective immunity by intramuscular DNA immunisation with *Brucella abortus* GroEL heat-shock gene. *Journal of Medical Microbiology*, 51:20-26.

LENG, J., YAO, H., SHEN, J., WANG, K., ZHUO, G., & WANG, Z., 2008. Co-expression of IL-18 binding protein and IL-4 regulates Th1/Th2 cytokine response in murine collagen-induced arthritis. *Acta Biochimica et Biophysica Sinica*, 40:116-124.

LIANG, R., VAN DEN HURK, J.V., LANDI, A., LAWMAN, Z., DEREGT, D., TOWNSEND, H., BABIUK, L.A., & VAN DRUNEN LITTLE-VAN DEN HURK, S., 2008. DNA prime protein boost strategies protect cattle from bovine viral diarrhea virus type 2 challenge. *Journal of General Virology*, 89:453-466.

MAJLESSI, L., SIMSOVA, M., JARVIS, Z., BRODIN, P., ROJAS, M., BAUCHE, C., NOUZÉ, C., LADANT, D., COLE, S.T., SEBO, P., & LECLERC, C., 2006. An increase in antimycobacterial Th1-cell responses by prime-boost protocols of immunization does not enhance protection against tuberculosis. *Infection and Immunity*, 74:2128-2137.

MCBRIDE, J.W., DOYLE, C.K., ZHANG, X., CARDENAS, A.M., POPOV, V.L., NETHERY, K.A., & WOODS, M.E., 2007. Identification of a glycosylated *Ehrlichia canis* 19-Kilodalton major immunoreactive protein with a species-specific serine-rich glycopeptide epitope. *Infection and Immunity*, 75:74-82.

MORA, M., VEGGI, D., SANTINI, L., PIZZA, M., & RAPPUOLI, R., 2003. Reverse vaccinology. *Drug Discovery Today*, 8:459-462.

MORA, M., DONATI, C., MEDINI, D., COVACCI, A., & RAPPUOLI, R., 2006. Microbial genomes and vaccine design: refinements to the classical reverse vaccinology approach. *Current Opinion in Microbiology*, 9:532-536.

MUZZI, A., MASIGNANI, V., & RAPPUOLI, R., 2007. The pan-genome: towards a knowledge-based discovery of novel targets for vaccines and antibacterials. *Drug Discovery Today,* 12:429-439.

OUTTERIDGE, P.M., 1993. High and low responsiveness to vaccines in farm animals. *Immunology and Cell Biology*, 71:355-366.

PATEL, B.A., GOMIS, S., DAR, A., WILLSON, P.J., BABIUK, L.A., POTTER, A., MUTWIRI, G., & TIKOO, S.K., 2008. Oligonucleotides containing CpG motifs (CpG-ODN) predominantly induce Th1-type immune response in neonatal chicks. *Developmental and Comparative Immunology*, 32:1041-1049.

PENTTILÄ, T., TAMMIRUUSU, A., LILJESTRÖM, P., SARVAS, M., MÄKELÄ, P.H., VUOLA, J.M., & PUOLAKKAINEN, M., 2004. DNA immunization followed by a viral vector booster in a C*hlamydia pneumoniae* mouse model. *Vaccine*, 22:3386-3394.

PIZZA, M., SCARLATO, V., MASIGNANI, V., GIULIANI, M.M., ARICO, B., COMANDUCCI, M., JENNINGS, G.T., BALDI, L., BARTOLINI, E., CAPECCHI, B., GALEOTTI, C.L., LUZZI, E., MANETTI, R., MARCHETTI, E., MORA, M., NUTI, S., RATTI, G., SANTINI, L., SAVINO, S., SCARSELLI, M., STORNI, E., ZUO, P., BROEKER, M., HUNDT, E., KNAPP, B., BLAIR, E., MASON, T., TETTELIN, H., HOOD, D.W., JEFFRIES, A.C., SAUNDERS, N.J., GRANOFF, D.M., VENTER, J.C., MOXON, E.R., GRANDI, G. & RAPPUOLI, R., 2000. Identification of vaccine candidates against serogroup B meningococcus by whole-genome sequencing. *Science*, 287:1816-1820.

PRETORIUS, A., COLLINS, N.E., STEYN, H.C., VAN STRIJP, F., VAN KLEEF, M., & ALLSOPP, B.A., 2007. Protection against heartwater by DNA immunization with four *Ehrlichia ruminantium* open reading frames. *Vaccine*, 25:2316-2324.

RAPPUOLI, R., 2000. Reverse vaccinology. *Current Opinion in Microbiology*, 3:445-450.

RAPPUOLI, R., 2001. Reverse vaccinology, a genome-based approach to vaccine development. *Vaccine*, 19:2688-2691.

SIMBI, B.H., BOWIE, M.V., MCGUIRE, T.C., BARBET, A.F., & MAHAN, S.M., 2006. Evaluation of *E. ruminantium* genes in DBA/2 mice as potential DNA vaccine candidates for control of heartwater. *Annals of New York Academy of Science*, 1078:424-437.

SKINNER, M.A., BUDDLE, B.M., WEDLOCK, D.N., KEEN, D., DE LISLE, G.W., TASCON, R.E., FERRAZ, J.C., LOWRIE, D.B., COCKLE, P.J., VORDERMEIER, H.M., & HEWINSON, R.G., 2003. A DNA prime-*Mycobacterium bovis* BCG boost vaccination strategy for cattle induces protection against bovine tuberculosis. *Infection and Immunity*, 71:4901-4907.

SOPP, P., & HOWARD, C.J., 2001. IFN-γ and IL-4 production by CD4, CD8 and WC1 γδ TCR^{+}T cells from cattle lymph nodes and blood. *Veterinary Immunology and Immunopathology*, 81:85-96.

STASKA, L.M., DAVIES, C.J., BROWN, W.C., MCGUIRE, T.C., SUAREZ, C.E., PARK, J.Y., MATHISON, B.A., ABBOTT, J.R., & BASZLER, T.V., 2005. Identification of vaccine candidate peptides in the NcSRS2 surface protein of *Neospora caninum* by using $CD4^{+}$ cytotoxic T lymphocytes and gamma interferon- secreting T lymphocytes of infected Holstein cattle. *Infection and Immunity*, 73: 1321-1329.

TAKATSU, K., & KARIYONE, A., 2003. The immunogenic peptide for Th1 development. *International Immunopharmacology*, 3:783-800.

TANGHE, A., D'SOUZA, S., ROSSEELS, V., DENIS, O., OTTENHOFF, T.H.M., DALEMANS, W., WHEELER, C., & HUYGEN, K., 2001. Improved immunogenicity and protective efficacy of a tuberculosis DNA vaccine encoding Ag85 by protein boosting. *Infection and Immunity*, 69:3041-3047.

TARACHA, E.L.N., BISHOP, R., MUSOKE, A.J., HILL, A.V.S., & GILBERT, S., 2003. Heterologous priming-boosting immunization of cattle with *Mycobacterium tuberculosis* 85A induces antigen-specific T-cell responses. *Infection and Immunity*, 71:6906-6914.

TOTTÉ, P., MACKEEVER, D., MARTINEZ, D., & BENSAID, A., 1997. Analysis of T-cell responses in cattle immunized against heartwater by vaccination with killed elementary bodies of *Cowdria ruminantium*. *Infection and Immunity*, 65:236-241.

TOTTÉ, P., BENSAID, A., MAHAN, S.M., MARTINEZ, D., & MACKEEVER, D., 1999. Immune responses to *Cowdria ruminantium* infections. *Parasitology Today*, 15:286-290.

TUO, W., PALMER, G.H., MCGUIRE, T.C., ZHU, D., & BROWN, W.C., 2000. Interleukin-12 as an adjuvant promotes Immunoglobulin G and Type 1 cytokine recall responses to Major Surface Protein 2 of the Ehrlichia pathogen *Anaplasma marginale*. *Infection and Immunity*, 68:270-280.

VACHIÉRY, N., LEFRANÇOIS, T., ESTEVES, I., MOLIA, S., SHEIKBOUDOU, C., KANDASSAMY, Y., MARTINEZ, D., 2006. Optimisation of the inactivated vaccine dose against heartwater and in vitro quantification of *Ehrlichia ruminantium* challenge material. *Vaccine*, 24:4747-4756.

VAN DER MERWE, L., 1987. The infection and treatment method of vaccination against heartwater. *Onderstepoort Journal of Veterinary Research*, 54:489-491.

VIUDES, A., LAZZELL, A., PEREAS, S., KIRKPATRICK, W.R., PEMAN, J., PATTERSON, T.F., MARTINEZ, J.P., & LÓPEZ-RIBOT, J.L., 2004. The C-terminal antibody binding domain of *Candida albicans* mp58 represents a protective epitope during candidiasis. *FEMS Microbiology Letters*, 232:133-138.

WANG, Q.M., SUN, S.H., HU, Z.L., YIN, M., XIAO, C.J., ZHANG, J.C., 2004. Improved immunogenicity of a tuberculosis DNA vaccine encoding ESAT6 by DNA priming and protein boosting. *Vaccine*, 22:3622-3627.

WANG, Y.S., CHI, K.H., LIAO, K.W., LIU, C.C., CHENG, C.L., LIN, Y.C., CHENG, C.H., CHU, R.M., 2007. Characterization of canine monocyte-derived dendritic cells with phenotypic and functional differentiation. *The Canadian Journal of Veterinary Research*, 71:165-174.

WILLIMAN, J., LOCKHART, E., SLOBBE, L., BUCHAN, G., & BAIRD, M., 2006. The use of Th1 cytokines, IL-12 and IL-23, to modulate the immune response raised to a DNA vaccine delivered by gene gun. *Vaccine*, 24:4471-4474.

WOZNIAK, T.M., RYAN, A.A., TRICCAS, J.M., & BRITTON, W.J., 2006. Plasmid interleukin-23 (IL-23), but not plasmid IL-27, enhances the protective efficacy of a DNA vaccine against *Mycobacterium tuberculosis* infection. *Infection and Immunity*, 74:557-565.

WU, H.H., CHEN, C.T., LIN, Y.L., & LEE, S.T., 2004. Sub-fragments of the envelope gene are highly protective against the *Japanese encephalitis* virus lethal infection in DNA priming-protein boosting immunization strategies. *Vaccine*, 22:793-800.

APPENDIX A

MATERIALS, BUFFERS, MEDIA AND SOLUTIONS

1. Materials

Table 1. Suppliers of the materials used in this study

Supplier	Product
Ambion	DNA-Free™ Kit
Biorad	Bio-Rad DC Protein Assay Reagents Package
Gentra systems	Generation®Capture Column Kit
Invitrogen	pET102/D-TOPO® vector
	One Shot®Top10 competent E. coli cells
Macherey-Nagel	Protino®Ni 150 & 1000 prepacked columns kit
MP Biomedicals	Lympho separation medium
	10 000 U/ml penicillin /10 mg/ml streptomycin
Promega	Isopropyl-β-D-thiogalactopyranoside (IPTG)
	Pfu DNA Polymerase, GoTaq® green master mix
	Ethidium bromide solution (10mg/ml),
Roche	Anti-His6
	High Pure PCR product purification kit
	High Pure Plasmid Isolation kit
	LightCycler® FastStart DNA MasterPLUS SYBR Green 1 kit
	Hexaprimer
	Expand reverse transcriptase buffer
	Expand reverse transcriptase
	RNaseOUT™
	Restriction endonucleases (RsaI, HaeIII, SacI, SacII, DdeI, NciI, Hin1I or EcoRI and BstNI)
	DNAse 1 (3000 units/mg protein)
Sigma	TRI REAGENT™
	Percoll®
	0.4% Trypan Blue
	RNAse (85-140 units/mg protein)
	Proteinase K (13 units/mg protein)
	Mitomycin
White Sci	Agarose (100 g)

2. Preparation of buffers, media and solutions

Ampicillin

Prepare a stock solution of 50 µg/ml by dissolving 0.05 g ampicillin in 1ml Millipore water. Aliquote and freeze (-20 ºC)

Block buffer

Contains 100 ml 1x PBS and 1 g bovine serum albumin

Blocking medium

RPMI-1640 medium supplemented with 10% heat inactivated FCS

Coating antibody

Mouse anti-bovine IFN-γ mAb CC302 (Serotec; 05 mg/vial). Dilute to 100 µg/ml in sterile PBS, aliquot and store at -20ºC

Complete RPMI 1640 medium

RPMI-1640 medium: 25 mM HEPES, 2 mM L-glutamine, 10% fetal calf serum, 5 x 10^{-5} mercapto-ethanol, 50 U/ml penicillin, 0.05 mg/ml streptomycin.

DEPC water

Incubate at 37 ºC overnight. Autoclave to remove the DEPC

dH_2O-T

distilled water with 0.05% Tween-20

DNAse 1 (1 mg/ml)

Roche 3000 units/mg protein. Make up in TM buffer at 1 mg/ml. Store in 150 µl aliquots at -20 ºC

EB lysis buffer

Buffer consists of 10 mM Tris-HCl pH 8, 142 mM NaCl, 1.43% SDS, 3 µg/µl Proteinase K. The Tris, Sodium chloride and SDS were diluted in water and was adjusted to pH 8

1 M IPTG

1.19 g isopropyl-β-D-thiogalactopyranoside was dissolved in 50 ml dH2O, sterilized by filtration, aliquoted and stored at -20 ºC.

LB Agar plates

Dissolve 10 g bacto-tryptone, 5 g yeast extract and 10 g NaCl and 15 g bacto-agar in 1000 ml dH_2O. and sterilize by autoclaving. Allow the medium to cool at 55 ºC and before pouring the plates add the appropriate amount of antibiotics.

LB broth

Dissolve 10 g bacto-tryptone, 5 g yeast extract and 10 g NaCl in 800 ml dH_2O. Adjust volume to 1000 ml with dH_2O. Aliquot into 100 ml per bottle and sterilize by autoclaving. Before use add 50 µg/ml ampicillin final concentration and store at 4 ºC for 1 month.

10x PBS

The buffer consisted of 137 mM NaCl (8 g), 27 mM KCl (2 g), 8.8 mM KH2PO4 (1.2 g) and 73 mM Na2HPO4.2H2O (11.4 g) were dissolved in 900 ml dH_2O, pH 7.5 The solution was made up to a final volume of 1 L, autoclaved and stored at room temperature.

PBS-T

Dulbecos PBS with 0.05% Tween-20

PBS-T/BSA

PBS-T with 0.1% Bovine serum albumin

Rabbit anti-bovine IFN-γ antiserum

Ammonium sulphate purified polyclonal antibody from serum after immunization with recombinant bovine IFN- γ; www.immundiagnostik. Com; AS 101 9.1/AS 1019.2. Filter through a 0.22 µm filter

Monoclonal anti-rabbit IgG alkaline phosphate conjugate

SIGMA, clone RG-96 Substrate: SIGMA Fast BCIP/NBT substrate tablets. Dissolve 1 tablet/10 ml dH_2O at room temperature for 30 min and filter through a 0.22 µm filter.

Proteinase K (20 mg/ml)

Sigma 13 units/mg protein. Make up in sterile Millipore dH_2O at 20 mg/ml and store aliquots at -20 ºC.

RNAse (10 mg/ml)

Sigma type III-A 85-140 units/mg protein. Make up in sterile Millipore d H_2O at 10 mg/ml. Heat 10 min at 90 ºC, cool on ice, centrifuge briefly. Store 100 µl aliquots at -20 ºC.

10x TBE

Buffer contains 889 mM Tris base, 889 mM boric acid and 25 mM EDTA.

Transfer buffer

38 mM Tris base (9.1 g), 288 mM (43.25 g) glycine was dissolved in 1700 ml dH2O and 300 ml methanol was added, pH ~8.3-8.4. It was stored at room temperature

Wash buffer for Western Blot (PBS- Tween, 0.05%)

Dissolve 500 µl Tween®-20 in 1000 ml 1x PBS

Washing medium: RPMI-1640 unsupplemented

Table 2. Recipes for Polyacrylamide separating and stacking gels**. (***Current Protocols in Molecular Biology).*

SEPARATING GEL

stock solution	Final acrylamide concentration in separating gel (%)									
	5	6	7	7.5	8	9	10	12	13	15
30% acrylamide/0.8 bisacrylamide	2.50	3.00	3.50	3.75	4.00	4.50	5.00	6.00	6.50	7.50
4x Tris -Cl/SDS, pH 8.8	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75
H_2O	8.75	8.25	7.75	7.50	7.25	6.75	6.25	5.25	4.75	3.75
10% (w/v) ammonium persulfate	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
TEMED	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01

Preparation of separating gel

In a 25 ml side-arm flask, mix 30% acrylamide /0.8 bisacrylamide solution, 4x Tris-HCl/SDS, pH 8.8 and H_2O. Degas under vacuum ~5 min. Add 10% ammonium persulfate and TEMED. Swirl gently to mix. Use immediately.

Preparation of stacking gel

In a 25 ml side-arm flask, mix 0.65 ml 30% acrylamide/0.8% bisacrylamide solution, 1.25 ml 4x Tris-HCl/SDS, pH 6.8, and 3.05 ml H_2O. Swirl gently to mix. Add 25 µl of 10% ammonium persulfate and 5 µl TEMED. Swirl gently to mix. Use immediately. Failure to form a firm gel usually indicates a problem with ammonium persulfate, TEMED, or both.

APPENDIX B

SEQUENCE ALIGNMENTS

CLUSTAL X (1.81) MULTIPLE SEQUENCE ALIGNMENT

File: /home/topsy/alignments/clustal/Erum8060aa.ps **Date: Wed Mar 26 08:33:00 2008**

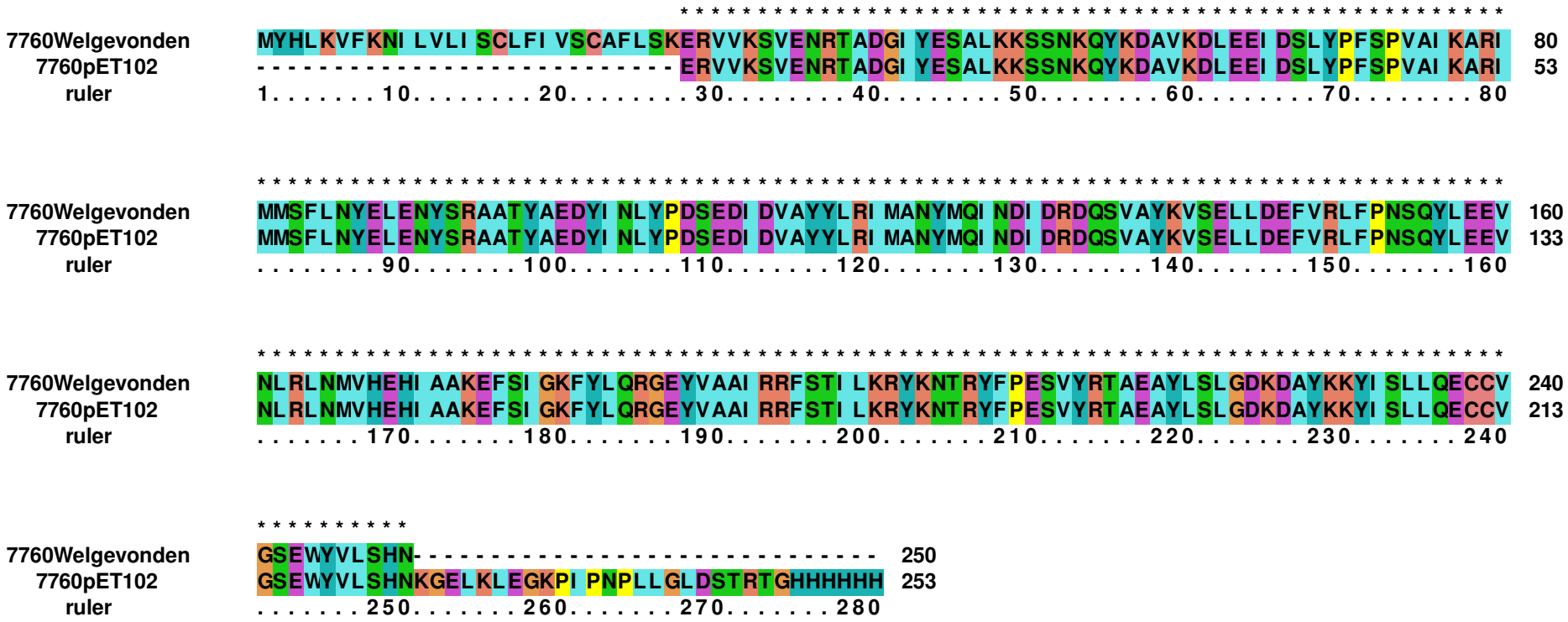
```
                                             ******************************************************
8060Welgevonden   MQLRKLLCIFCLAVLLNSYYICNAYSINTKTDNTVKTNSLTYFDAVRSFKADFMQISSSEDIPRYGYVLMKKPGLLKWDY   80
8060pET102        --------------------------INTKTDNTVKTNSLTYFDAVRSFKADFMQISSSEDIPRYGYVLMKKPGLLKWDY   54
ruler             1.......10........20........30........40........50........60........70........80

                  ********************************************************************************
8060Welgevonden   YPPTPVSIVIKGTTISYYDRELEEYSYTIVNNPIIGLLSSDIKNISTIDFINIDTVNNQKIVTLHDKKSDLYAEVIFNTD  160
8060pET102        YPPTPVSIVIKGTTISYYDRELEEYSYTIVNNPIIGLLSSDIKNISTIDFINIDTVNNQKIVTLHDKKSDLYAEVIFNTD  134
ruler             ........90.......100.......110.......120.......130.......140.......150.......160

                  ********************************************
8060Welgevonden   PITIVGLNILNPDSKTSIQFYNISSNIPIDKREFKHDISHYYSE------------------------------  204
8060pET102        PITIVGLNILNPDSKTSIQFYNISSNIPIDKREFKHDISHYYSEKGELKLEGKPIPNPLLGLDSTRTGHHHHHH  208
ruler             .......170.......180.......190.......200.......210.......220.......230....
```

CLUSTAL X (1.81) MULTIPLE SEQUENCE ALIGNMENT

File: /home/topsy/alignments/clustal/Erum7760aa.ps Date: Wed Mar 26 08:32:44 2008



CLUSTAL X (1.81) MULTIPLE SEQUENCE ALIGNMENT

File: /home/topsy/alignments/clustal/Erum5000aa.ps **Date: Fri Feb 1 10:42:32 2008**

```
                                           ************************************************
5000Welgevonden  MCKFSLTTAFVSLLFLYNVDAFAVDNLAKANTNDNKITVNTKKNHSDMKRVVLRKLAKGYHRIELGGQILSYAWC  75
5000pET102       ---------------------------AKANTNDNKITVNTKKNHSDMKRVVLRKLAKGYHRIELGGQILSYAWC  48
ruler            1.......10........20........30........40........50........60........70.....

                 ***************************************************************************
5000Welgevonden  TDNVNQSQKHGVGVSGILNVKSVSENPNLGISYGASLQIGVPAIKDKYFIPTVKAYNRGAQLFIDSSYGNISMGY  150
5000pET102       TDNVNQSQKHGVGVSGILNVKSVSENPNLGISYGASLQIGVPAIKDKYFIPTVKAYNRGAQLFIDSSYGNISMGY  123
ruler            ...80........90.......100.......110.......120.......130.......140.......150

                 ***************************************************************************
5000Welgevonden  QEGVDSIMKIDAFTIGSGDNSITWVQNLNILDKDKNLVYHVFPGLYTESLFNGSESLALLSIQGRGKDFINNLPF  225
5000pET102       QEGVDSIMKIDAFTIGSGDNSITWVQNLNILDKDKNLVYHVFPGLYTESLFNGSESLALLSIQGRGKDFINNLPF  198
ruler            .......160.......170.......180.......190.......200.......210.......220.....

                 ***************************************************************************
5000Welgevonden  RISYQSPSFAGLKVAVSYAPLGYPTSLFEHADRDDYKVDKLTIPPLLGPLQEKTADKKIEEIVAVLGDKIALQDV  300
5000pET102       RISYQSPSFAGLKVAVSYAPLGYPTSLFEHADRDDYKVDKLTIPPLLGPLQEKTADKKIEEIVAVLGDKIALQDV  273
ruler            ..230.......240.......250.......260.......270.......280.......290.......300

                 ***************************************************************************
5000Welgevonden  EMEGLADSGKVKFLGPNYKNIINAGLSYSHSFDSIDFQASVIAEYASNDVEALKKSSYSGPLVPFNGLSGIAVGT  375
5000pET102       EMEGLADSGKVKFLGPNYKNIINAGLSYSHSFDSIDFQASVIAEYASNDVEALKKSSYSGPLVPFNGLSGIAVGT  348
ruler            .......310.......320.......330.......340.......350.......360.......370.....

                 ***************************************************************************
5000Welgevonden  SVAYDSVIVAASYGYLGKSGQPINVKGNAYYWTLGAKYTYDNASISTSYFRSNNFGNEFQDFGIGIDCNLSANSK  450
5000pET102       SVAYDSVIVAASYGYLGKSGQPINVKGNAYYWTLGAKYTYDNASISTSYFRSNNFGNEFQDFGIGIDCNLSANSK  423
ruler            ..380.......390.......400.......410.......420.......430.......440.......450

                 ****************************************
5000Welgevonden  YKGQYKVFGNYHRFNVKNISSTSSGSYAGNVLLLGMKYEF------------------------------  490
5000pET102       YKGQYKVFGNYHRFNVKNISSTSSGSYAGNVLLLGMKYEFKGELKLEGKPIPNPLLGLDSTRTGHHHHHH  493
ruler            .......460.......470.......480.......490.......500.......510.......520
```

CLUSTAL X (1.81) MULTIPLE SEQUENCE ALIGNMENT

File: /home/topsy/alignments/clustal/Erum8610aa.ps

Date: Wed Mar 26 08:35:47 2008

```
                                             *******************************************************
8610Welgevonden    MNNKKIFKYSLLCGLISFLLLTSFNYPNIISNVASYSSISSFYNSIIIEHSNLRTYSSRDIIISSTPNPVSYTTLIYPNS   80
8610pET102         -------------------------YPNIISNVASYSSISSFYNSIIIEHSNLRTYSSRDIIISSTPNPVSYTTLIYPNS   55
ruler              1.......10........20........30........40........50........60........70........80

                   ********************************************************************************
8610Welgevonden    YQLYSEFKELVKISTNKLIYHIPVNHNYCLSCNCNVIKDNKVTDNSTTFIFQHPQKVLFSNFVTYYKSVLDHSVLLNSNI   160
8610pET102         YQLYSEFKELVKISTNKLIYHIPVNHNYCLSCNCNVIKDNKVTDNSTTFIFQHPQKVLFSNFVTYYKSVLDHSVLLNSNI   135
ruler              ........90.......100.......110.......120.......130.......140.......150.......160

                   ****************************************************************************
8610Welgevonden    KKFFLSFSKQLYKFFYKKKFMTSDPISFNFHIYQSKKMLVSLFKSVKNNIERTISTVRYLNLLHTFLRYNYFKVIL----   236
8610pET102         KKFFLSFSKQLYKFFYKKKFMTSDPISFNFHIYQSKKMLVSLFKSVKNNIERTISTVRYLNLLHTFLRYNYFKVILKGEL   215
ruler              .......170.......180.......190.......200.......210.......220.......230.......240


8610Welgevonden    --------------------------   236
8610pET102         KLEGKPIPNPLLGLDSTRTGHHHHHH   241
ruler              .......250.......260......
```