ASPECTS OF THE IMMUNE RESPONSE IN RUMINANTS TO FOUR PROTECTIVE *EHRLICHIA RUMINANTIUM* GENE PRODUCTS

By
Alri Pretorius

Submitted for a Ph. D. degree in Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria
April 2007

ACKNOWLEDGEMENTS:

I would like to express my appreciation to my promoters Dr Nicola Collins and Prof Basil Allsopp. I thank them both for their mentorship, their valuable scientific contributions and the time spent in correcting my writing. Special thanks to Dr Nicola Collins whom provided me with the opportunity to obtain a Ph. D. at the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria.

I would also like to thank my project manager at the ARC-OVI, Dr Mirinda van Kleef for her immunology input and time spent in correcting my scientific writing. I am also grateful for her expert help with the lymphocyte proliferation assays (LPA) and the optimisation of the real-time PCR assays.

I would like to express my gratitude to my colleagues and ex-colleagues at the OVI for their contributions: Dr Nicola Collins who initiated the experimental work, assisted in the immunisation of animals and in the LPA; Dr Erich Zweygarth and Antoinette Josemans for providing *E. ruminantium* tissue culture material for DNA extraction; Elmarie Louw for assistance with the LPA, the optimisation of the real-time PCR assays and the immunisation of animals; Helena Steyn for screening animals with the pCS20 test, pCS20 sequence analysis, her help with the LPA and immunisation of animals; Fransie van Strijp for her assistance in immunisation and care of the experimental animals; Junita Liebenberg and Erika Faber for their support in sequencing of DNA samples, immunisation of animals and Erika for her contribution to the real-time PCR assays; and finally I would like to thank Ndavhe Tshikhudo for his help with the LPA.

A special thanks to my family and friends for their love and support during my studies.

This work was supported by the South African Department of Science and Technology (DST) LEAD biotechnology grant, the EU INCO-ICA4-CT-2000-30026 grant, the National research foundation (NRF), South Africa for the NRF Sustainable Livelihoods Focus Area" grant, entitled "Heartwater Recombinant Vaccine Development" FA2004042200063 for research support and the Belgium bilateral

institute strengthening grant, the Prince Leopold Institute of Tropical Medicine, Antwerp, Belgium and the ARC, South Africa for registration fees.

TABLE OF CONTENTS:

LIST OF ABBREVIATIONS

°C	degrees Celsius
µl	microliters
APC	antigen presenting cell
ATP	adenosine triphosphate
BCG	Bacillus Calmette Guerin
bp	base pair
CD	cluster of differentiation
CD40L	cluster of differentiation 40 ligand
CMV	cytomegalovirus
CMV-TE	cytomegalovirus promoter with a translational enhancer
cpm	counts per minute
Ct	threshold cycle
CTL	cytotoxic T cell(s)
CTLA-4	cytotoxic T lymphocyte antigen 4
CyaA	adenylate cyclase
DC	dendritic cell
DMEM/F12	Dulbecco's modified Eagle's medium/ Ham's F-12
DNA	deoxyribonucleic acid
EB	elementary body
ECOGPT	*E. coli* xanthine-guanine phosphoribosyl transferase
EF-1α	elongation factor 1α-subunit
ELISA	enzyme-linked immunosorbent assay
FCS	foetal calf serum
ffu	focus forming units
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
G/Gly	glycine
gg	gene gun
GM-CSF	granulocyte-macrophage colony stimulating factor
h	hour
HIV	human immunodeficiency virus
HRP	horseradish peroxidase

HSP70	heat shock protein 70
i.d.	intra dermal
IFA	immuno fluorescent assay
i.m.	intra muscular
IAA	isoamyl alcohol
IFN-γ	interferon gamma
IgG	immunoglobulin G
IgM	immunoglobulin M
IL	interleukin
kbp	kilo base pairs
kDa	kilo dalton
kg	kilogram
KO	knock out
LB	Luria Bertani
LD_{50}	lethal dose, 50 %
LPS	lipopolysacharide
LSDV	lumpy skin disease virus
MAP	major antigenic protein
mCTL	memory cytotoxic T cell
MDBK	Madin and Darby bovine kidney
mg	milligram
MHC	major histocompatibility complex
min	minute
ml	millilitre
mM	milli molar
mRNA	messenger ribonucleic acid
MV	myxoma virus
MyD88	myeloid differentiation factor 88
ng	nanogram
NK	natural killer
OBP	Onderstepoort Biological Products
OD_{600}	optical density of liquid medium at 600 nm
ODN	oligonucleotides

OMP	outer membrane protein
ORF	open reading frame
PAMPs	pathogen-associated microbial patterns
PBMC	peripheral blood monocytic cell
PCR	polymerase chain reaction
PVDF	hydrophilic polyvinylidene fluoride
r	recombinant
RB	reticulate body
RI	reaction index
RR	ribonuclease reductase
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
SA	South Africa
SBF	Springbokfontein
SBP	solute binding protein
SCID	severe combined immunodeficiency
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SI	stimulation index
SPG	sucrose potassium glutamate
TCR	T cell receptor
T_{H1}	T helper 1
T_{H2}	T helper 2
TLR	Toll-like receptor
TNF	tumour necrosis factor
U	enzyme units
UbC	ubiquitin C
UCT	University of Cape Town
wt	wild type

SUMMARY

ASPECTS OF THE IMMUNE RESPONSE IN RUMINANTS TO FOUR PROTECTIVE *EHRLICHIA RUMINANTIUM* GENE PRODUCTS

By

ALRI PRETORIUS

PROMOTER: Dr N.E. Collins
CO-PROMOTER: Prof B.A. Allsopp
DEPARTMENT: Veterinary Tropical Diseases
DEGREE: PhD

In the search for a better vaccine against *Ehrlichia ruminantium* infection in ruminants, four *E. ruminantium* open reading frames (ORFs) derived from the Welgevonden isolate were tested using either DNA vaccination or DNA prime-modified viral or DNA prime-recombinant protein boost strategies. Both the DNA vaccination and the DNA prime–recombinant protein boost strategy provided complete protection against *E. ruminantium* Welgevonden needle challenge, while the DNA prime–modified viral boost strategy only provided 90 % protection. The DNA prime–recombinant protein boost strategy also coincided with elevated cellular immunology as was evident from increased IFN-γ production. Furthermore, we could show that the 1H12 DNA vaccine could induce protection against heterologous needle challenge when animals were immunised with the Welgevonden-derived 1H12 ORFs and challenged with selected *E. ruminantium* stocks. Unfortunately the DNA only and the DNA prime–recombinant protein boost strategy were not protective in the field. Therefore, our results suggest that there is a vast difference between needle challenge and natural tick infestation and that *E. ruminantium* organisms transmitted by ticks have the ability to evade the protective immunity induced by immunisation with the four 1H12 ORFs.

LIST OF FIGURES

LIST OF TABLES

1. CHAPTER 1: LITERATURE REVIEW

1.1. INTRODUCTION

Ehrlichia ruminantium, a rickettsial agent transmitted to domestic and wild ruminants by *Amblyomma* tick species, is the causative agent for the disease known as heartwater or cowdriosis (Cowdry, 1925a; 1925b; 1926; Peter *et al.,* 2002). The disease is prevalent in sub-Saharan Africa, including the African islands of Sao Tome, Zanzibar, Comores, Madagascar and Mauritius. Heartwater has also been introduced into the Caribbean and was first observed on the island of Guadeloupe in 1980, and outbreaks of the disease were subsequently detected on two other islands: Marie Galante and Antigua (Muller Kobold *et al.,* 1992; Camus and Barre, 1995 and reviewed by Yunker and Allsopp, 1994). The presence of the disease on the islands of the Caribbean could also be regarded as a threat to the livestock industries of North, Central and South America.

1.1.1. History

The first record of what may have been heartwater disease can be found in the 1838 diary of Louis Trichardt, who described a fatal disease of sheep in South Africa that manifested three weeks after tick infestation was noticed on the animals. Research on heartwater started in the late 19th century, with the first major breakthrough being made by Dixon and Edington in 1898 who showed that blood inoculation could transfer disease from a sick animal to a susceptible animal. Hutcheon concluded in 1900 that heartwater was probably caused by a virus, and in the same year Lounsbury could confirm that the tick *Amblyomma hebraeum* was the vector for heartwater in South Africa (reviewed by Provost and Bezuidenhout, 1987 and by Allsopp *et al.,* 2004).

Cowdry was the first to discover that heartwater was caused by an intracellular rickettsial bacterium, which he observed microscopically in tissues from infected animals and ticks (Cowdry, 1925a; 1925b; 1926). The organism was initially named *Rickettsia ruminantium,* later renamed to *Cowdria ruminantium* (Moshkovski, 1947), and recently reclassified as *Ehrlichia ruminantium* (Dumler *et al.,* 2001).

Relatively few new discoveries were made between 1945 and 1970, other than the development of a blood vaccine at the Onderstepoort Veterinary Research Institute in the 1950s (reviewed by Provost and Bezuidenhout, 1987) and the introduction of treatments for the disease that included sulphonamide drugs and tetracycline antibiotics (Weiss *et al.,* 1952; Haig *et al.,* 1954). The discovery of an *E. ruminantium* isolate that was pathogenic for mice renewed interest in heartwater research and a mouse model for the disease was established in the early 1970s (Du Plessis and Kümm, 1971). The ability to cultivate the organism *in vitro*, regarded as perhaps the most important milestone in heartwater research (Yunker, 1995), was first achieved in 1985 (Bezuidenhout *et al.,* 1985). This technique was further improved by the development of a chemically defined medium for the long term growth of *E. ruminantium* (Zweygarth and Josemans, 2001). The cultivation of the pathogen was recently further expanded to include the growth of *E. ruminantium* in tick cell lines (Bell-Sakyi *et al.,* 2000; Bell-Sakyi, 2004). The availability of large quantities of purified *E. ruminantium* organisms has led to numerous new discoveries that included a better understanding of the intricate immunological interaction between the parasite and the host (reviewed by Totté *et al.,* 1999) and the completion of the *E. ruminantium* Welgevonden genome sequence and annotation (Collins *et al.,* 2005).

1.1.2. Tick vector

Heartwater is transmitted by *Amblyomma* ticks. In sub-Saharan Africa, *E. ruminantium* is transmitted by *Amblyomma hebraeum* and *A. variegatum* also known as the "bont" ticks. *E. ruminantium* has been detected in both tick species collected from several heartwater endemic areas (Waghela *et al.,* 1991; Peter *et al.,* 2000). It is believed that *A. variegatum* was introduced to the French Antilles during the 18th or 19th centuries (Maillard and Maillard, 1998), thus leading to the establishment of cowdriosis on Guadeloupe and Antigua (Camus and Barre, 1995). A native American species of *Amblyomma*, *A. maculatum*, has been experimentally shown to be prone to *E. ruminantium* infection and has the ability to transmit *E. ruminantium* pathogens to livestock (Mahan *et al.,* 2000). So far the American continents have remained free of the *E. ruminantium* organism but recent studies have shown *A. sparsum* ticks, isolated from Zambian reptiles illegally imported into the USA, were infected with *E. ruminantium* and could pose a great danger to the spread

of the disease to the Americas. Ruminant transmission and infectivity of the *E. ruminantium* genotype isolated from *A. sparsum* ticks still have to be investigated (Burridge *et al.,* 2000*;* 2002).

A. hebraeum and *A. variegatum* adult ticks preferably infest cattle but can also be attracted to sheep, goats and horses. Adults and nymphae usually attach on the belly or udder, while the nymphae and larvae can be located on the head and ears. *A. hebraeum* and *A. variegatum* are three-host ticks: both larvae and nymphae need to engorge on a separate host before dropping off to moult. All three life cycle stages, larvae, nymphae and adult ticks could therefore be infected with the *E. ruminantium* parasite, but only the nymphae and adult ticks are capable of transmitting heartwater from one ruminant to another (Cowdry, 1925a; 1925b; Daubney, 1930 and reviewed by Walker, 1962; 1984; 1990).

1.1.3. The lifecycle of E. ruminantium in mammalian and tick hosts

Ultra-structurally the *E. ruminantium* parasite has different morphological forms in the mammalian host epithelial cells and in the *Amblyomma* tick midgut epithelial and salivary gland acini cells (Yunker *et al.,* 1987 and reviewed by Kocan and Bezuidenhout, 1987; Prozesky, 1987b and by Prozesky and Du Plessis, 1987). *E. ruminantium* infection of the tick vector occurs after the tick (larva, nymph or adult) has fed on a heartwater-infected mammalian host. The parasite remains in the tick gut from day 1-3 of tick feeding, and will start to invade the salivary gland acini cells from day 2 (Hart *et al.,* 1991). From here the *E. ruminantium* could be transferred to the ruminant host once the tick starts to feed.

Information about the life cycle of *E. ruminantium* in the ruminant host cells was derived from studying bovine endothelial cell cultures where it was shown that the *E. ruminantium* growth pattern closely resembles that of the biphasic *Chlamydia* cycle (Jongejan *et al.,* 1991). Infection of a new host cell can only be mediated by elementary bodies (EBs), the electron dense, metabolically inactive form of the organism. Once inside the host cell the EBs transform into the more active, non-infective reticulate body (RB) stage that facilitates reproduction. The RBs multiply by binary fission or endosporulation and return to the more electron dense EB stage

before the pathogen is released from the host cell (Jongejan *et al.,* 1991; Marcelino *et al.,* 2005; and reviewed by Prozesky and Du Plessis, 1987 and by Allsopp *et al.,* 2004). Once released, the EBs are able to infect new endothelial cells and feeding ticks.

1.1.4. Symptoms

The name heartwater suggests that the disease always causes the presence of fluids in the heart sac. In fact this symptom is seen more often in sheep and goats than in cattle, where fluids are present in the whole chest cavity. Normally, heartwater symptoms start to manifest 11-18 days after tick infestation. Clinical signs include elevated temperature, an increase in respiration tempo, loss of appetite, diarrhoea, nervous symptoms (constant movement of the lower jaw and tongue, muscular twitching and squinting) and finally death. Frothy fluid can also be detected in the nostrils and in the lungs. Internal haemorrhages are visible on lung and heart tissues, and the spleen is enlarged with a softer and darker pulp than normal (reviewed by Yunker and Allsopp, 1994; Yunker, 1996 and by Allsopp *et al.,* 2004).

1.1.5. Diagnosis and detection of heartwater

The diagnosis and detection of heartwater is difficult and microscopic examination of traditional Giemsa/Diff-Quick stained brain smears (Prozesky, 1987a) is still the most reliable method used to confirm heartwater at *post mortem*. Methods of *in vivo* diagnosis include immunological tests, such as immuno-fluorescence assays (IFAs), enzyme-linked immunosorbent assays (ELISAs) and molecular genetic tests (Muller Kobold *et al.,* 1992; Perez *et al.,* 1998; Mboloi *et al.,* 1999; Sumption *et al.,* 2003). None of the immunological tests are completely reliable. False positives often occur in serological tests based on the major antigenic protein 1 (MAP1) owing to immunological cross-reactions with homologous antigens from other *Ehrlichia* species (Du Plessis *et al.,* 1993; Savadye *et al.,* 1998). DNA-based PCR and probe tests are most often used to confirm heartwater infection in animals, the targets being the pCS20 genomic region and the 16S ribosomal RNA (rRNA) gene of the organism (Waghela *et al.,* 1991; Allsopp *et al.,* 1997; 1999). A reverse line blot hybridisation assay, based on the 16S rRNA gene, is also in use (Bekker *et al.,* 2002).

1.1.6. Host immune response to E. ruminantium

Early transfer experiments using purified immunoglobulin G (IgG), isolated from *E. ruminantium* immune sheep sera, did not provide protection against a virulent *E. ruminantium* challenge in sheep (Du Plessis, 1970). This observation led to the general assumption that antibodies do not play a role in immunological protection against *E. ruminantium.* While it may be true that antibody responses will have no effect on the pathogen once it has internalised into host cells, this does not imply that an antibody response is absent or has no function in protection against *E. ruminantium*. Recent studies have shown that the role of antibodies in protection against intracellular organisms should not be disregarded (reviewed by Casadevall, 2003). Studies of numerous intracellular pathogens have shown that specific antibody responses, previously believed to be non-existent, do indeed confer protection. It is known that immune sera contain polyclonal antibodies and that the different antibodies have differences in concentration, isotype and specificity. Therefore, the preventive effect of a specific protective monoclonal antibody can easily go unnoticed (reviewed by Casadevall, 2003). We will consider some examples:

Increased protection against the Gram-positive intracellular pathogen *Corynebacterium pseudotuberculosis* was observed in sheep after immunisation with a DNA construct that promoted a humoral immune response (Chaplin *et al.*, 1999). Passive transfer of purified IgG obtained from mice immune to *Ehrlichia risticii* conferred protection on mice when antibodies were transferred immediately after infection with a lethal dose of *E. risticii* (Kaylor *et al.,* 1991). Furthermore, it became clear that these protective antibodies did not only play a role in phagocytosis by macrophages but could block cellular entry (Messick and Rikihisa, 1994) and could promote phagosomal killing of pathogens by inhibiting intracellular bacteria such as *Rickettsia conorii* from escaping from endothelial and macrophage phagosomes (Feng *et al.,* 2004). Furthermore, *E. chaffeensis* antibody complexes have been shown to induce the expression of pro-inflammatory cytokines that can contribute to suppression of pathogen growth (Lee and Rikihisa, 1997). Nevertheless, antibody responses can only be effective while the organism is outside host cells, such as after the initial infection, during intercellular spreading, or during an extracellular life stage. In fact, an extracellular life stage was confirmed in another *E. chaffeensis* study in

which it was demonstrated that the organism can survive and replicate extracellularly, thus exposing the pathogen to humoral mediation (Li and Winslow, 2003).

The role of antibodies and B cells in immuno-compromised (severe combined immunodeficiency (SCID)) mice infected with *E. chaffeensis* was also studied. Two separate studies confirmed that mice that do not have functional mature B cells, and therefore cannot express IgG or IgM, succumbed to infection, while mice that can produce normal antibodies survived (Li *et al.,* 2001; Yager *et al.,* 2005). Furthermore, passive transfer of immune serum prolonged the survival of infected SCID mice (Winslow *et al.,* 2000). Further analysis of the immune serum revealed that the protection is provided by antibodies directed to outer membrane proteins (OMPs) and it could be shown that inoculation with monoclonal antibodies directed to *E. chaffeensis* OMPs provided complete protection (Li *et al.,* 2001; 2002). These results suggest that antibodies play an important role in elimination of *E. chaffeensis* infection. It has also been shown that antibodies are probably involved in eliminating extracellular *E. chaffeensis* via complement and phagocytosis (Yager *et al*., 2005).

It is known that *E. ruminantium* is released from one host cell before infection of new host cells. This was confirmed by the induction of tissue cultures using infected plasma as starting material (Byrom *et al.,* 1991). Furthermore, immune sera obtained from sheep infected with the *E. ruminantium* Kümm stock could confer protection in mice that were challenged with low doses of the Kümm stock (Du Plessis, 1993). This finding indicates that antibodies could have a protective effect once the pathogen is released into the blood.

On the other hand, several *in vitro* and *in vivo* experiments have demonstrated the importance of a cellular immune response to *E. ruminantium* infection (Byrom *et al.,* 2000b and reviewed by Preston and Jongejan, 1999 and by Totté *et al.,* 1999). Adoptive transfer of splenocytes, cluster of differentiation 4 ($CD4^+$) T cells and cytotoxic T cells (CTL) of immune or infected animals to susceptible animals conferred protection to lethal challenge, emphasising the important role that these lymphocytes play in the protection against heartwater (reviewed by Preston and Jongejan, 1999). Furthermore, flow cytometrical analysis revealed that the $CD8^+$ T

cell population increases in mice experimentally infected with *E. ruminantium* (Du Plessis *et al.,* 1992). Experiments using $CD4^+$ and $CD8^+$ knock out (KO) mice showed that the $CD4^+$ mediated response was a prerequisite for protection against heartwater infection. It was shown that mice with no functional $CD4^+$ cells were susceptible to infection, while 43 % of the mice without $CD8^+$ T cells (only functional $CD4^+$ T cells) could survive challenge (3 LD_{50}s) (Byrom *et al.,* 2000a). Investigations into the role of $CD4^+$ and $CD8^+$ lymphocytes in other Rickettsiales revealed similar results: major histocompatibility complex (MHC) class I KO mice were highly susceptible to *Rickettsia australis* infection, thus confirming the important role of CTL in the protection against Rickettsiales (Walker *et al.,* 2001). These studies indicated that $CD4^+$ and $CD8^+$ T cells both play a crucial role in the protection against *E. ruminantium* infection. Activation of another subset of T cells, γδ T cells, was also demonstrated in cattle infected with heartwater, immune cattle and in cattle immunised with major antigenic protein 2 (MAP2) (Totté *et al.,* 1997; Mwangi *et al.,* 1998; 2002 and reviewed by Totté *et al.,* 1999). Although the function of γδ T cells in the host immune response to *E. ruminantium* infection remains unclear, these cells could be involved in interferon gamma (IFN-γ) production.

Important indicators for the activation of a cellular immune response are the production of interferons (IFN-α, IFN-β and IFN-γ). Cells that secrete IFN-γ include $CD4^+$ T helper 1 (T_{H1}) cells, CTL, macrophages and natural killer (NK) cells. Elevated levels of IFN-γ will recruit granulocytes to the infection site, and induce the secretion of nitric oxide radicals in macrophage phagosomes that will have a detrimental effect on internalised bacteria (Mutunga *et al.,* 1998). Increased IFN-α and IFN-γ expression were detected in animals that survived *E. ruminantium* infection, whereas animals that died of the infection did not show IFN upregulation (Totté *et al.,* 1994; 1999). Similarly goats that were immunised with an *E. ruminantium* killed vaccine induced CTL IFN-γ production that was significantly higher than that expressed by $CD4^+$ T cells (Esteves *et al.,* 2004b; 2004c). Up-regulation of IFN-γ has been detected in several studies using *E. ruminantium* fractionated protein samples and the *map*1 and *map*2 genes (Mwangi *et al.,* 2002; Van Kleef *et al.,* 2002; Esteves *et al.,* 2004a). Therefore, IFN-γ production can be

regarded as an important marker to evaluate if a specific protein can be considered as a potential vaccine candidate.

Endothelial cells also have some immune defence mechanisms that could counteract intracellular bacterial infection. These cells are also known as semi-professional antigen presenting cells (APCs) and have the ability to present antigen to memory lymphocytes on both MHC class II and I upon stimulation with IFN-γ (reviewed by Pober, 1999 and by Marelli-Berg and Jarmin, 2004). The presentation of antigen on MHC class II and the subsequent activation of $CD4^+$ T cells are significant characteristics that are normally reserved for specialised lymphoid cells. Once activated by cytokines such as IFN-γ, endothelial cells also have the ability to secrete nitric oxide. Killing of intracellular bacteria such as *Staphylococcus aureus* (Zhang *et al.,* 1997) and *Pseudomonas aeruginosa* (De Assis *et al.,* 2000) by activated endothelial cells has been demonstrated.

If IFN-γ stimulated endothelial cells have the ability to kill intracellular parasites, why does *E. ruminantium* infection persist in these cells? Brain endothelial cells that were infected with *E. ruminantium* did not show increased MHC class I and II protein expression (measured with flow cytometry) (Totté *et al.,* 1996; Vachiéry *et al.,* 1998), suggesting that MHC class II protein expression is inhibited by the pathogen. On the other hand it was demonstrated that addition of IFN-γ to infected cells inhibited bacterial growth (Totté *et al.,* 1996). This suggests that IFN-γ effector functions other that MHC class II upregulation must play a role in inhibition of growth. The *E. ruminantium* pathogen therefore has mechanisms in place to evade detection by the host immune response.

1.1.7. Current vaccines

1.1.7.1. The infection and treatment method of immunisation

In South Africa, heartwater is controlled either by acaricides which prevent tick transmission of the disease or ruminants are immunised with a "blood vaccine" using a method known as infection and treatment (Van der Merwe, 1987; Uilenberg, 1990). The blood of donor animals infected with the Ball3 isolate is used to infect susceptible

livestock. Once a temperature response is detected these animals are treated with tetracycline. This vaccination procedure is fairly successful in the field, but is laborious, as each animal's temperature must be closely monitored every day after the infection was initiated. The isolate used (Ball3) is not ideal because it does not completely cross-protect against all the various field strains of *E. ruminantium* (Jongejan *et al.,* 1988; Collins *et al.,* 2003). The use of more virulent isolates which may provide better cross-protection cannot be considered, as the symptom onset is faster than with Ball3 and in most cases, once fever is detected, it is too late to treat. The distribution of the vaccine is also a problem in rural areas because the delivery relies on an uninterrupted cold chain.

1.1.7.2. Attenuated *E. ruminantium* vaccines

The first attenuated vaccine tested in ruminants was derived from the virulent Senegalese stock of *E. ruminantium.* The Senegal stock was attenuated by continuous passage. Sheep and goats inoculated with pathogen obtained from the 11th passage did not show any clinical symptoms and were protected against lethal challenge with the virulent Senegal stock (Jongejan, 1991; Jongejan *et al.,* 1993). In contrast to the rapid attenuation of the Senegal stock, *E. ruminantium* Welgevonden did not attenuate spontaneously in endothelial cell culture after 231 passages (Zweygarth *et al.,* 2005). However, an attenuated *E. ruminantium* Welgevonden vaccine has recently been developed by repeated passages in a canine macrophage cell line. This vaccine conferred protection in sheep after virulent needle challenges with the Welgevonden, Gardel, Mara87/7 and Blaauwkrans stocks (Zweygarth *et al.,* 2005). These vaccines show a lot of promise as little or no fever reaction develops and the necessity for treatment with antibiotics is therefore circumvented. However, these attenuated vaccines have yet to be tested using natural tick challenge with virulent *E. ruminantium* genotypes in the field.

1.1.7.3. Inactivated *E. ruminantium* vaccines

Inactivated vaccines have also been developed and tested: cell cultures, infected tissue or tick-derived *E. ruminantium* were inactivated with β-propiolactone and then used to immunise livestock in laboratory and field trials. The use of this inactivated vaccine

showed limited protection in sheep, goats and cattle. Normally not more than 60-80 % of the test animals survived laboratory challenge. Various adjuvants have been tested in order to improve this vaccine, with Freund's adjuvant and Montanide ISA50 giving the best results. This vaccine is, however, expensive to prepare and must be purified to be free from host components to avoid autoimmune reactions (Martinez *et al*., 1994; Mahan *et al*., 1995; Martinez *et al*., 1996; Totté *et al*., 1997; Mahan *et al.,* 1998; Mahan *et al.,* 2001; Bell-Sakyi *et al.,* 2002). Inactivated heartwater vaccines do not seem to be a commercially viable possibility at this time.

1.1.7.4. Recombinant *E. ruminantium* vaccines

In most of the experiments done with the inactivated vaccine a dominant antibody response to MAP1 was detected (Perez *et al.,* 1998). This observation led to numerous attempts to develop a DNA vaccine containing the *map*1 and *map*2 genes. Some protection was observed in mice immunised with 75 µg of recombinant plasmid containing a human cytomegalovirus (CMV) promoter, the *map*1 gene sequence and growth hormone polyadenylation sequence. By using this construct, an antibody response will be favoured since the growth hormone sequence enhances secretion of the protein. The protective results were however not reproducible (88 % protection in the first trial vs. 23 % in the second) and numerous attempts were made to improve the vaccine (Nyika *et al.,* 1998). These included longer intervals between each inoculation that did not seem to make any difference in the outcome, and boosting with recombinant MAP1 protein. Priming with the DNA vaccine and boosting with the recombinant protein seemed to give the most repeatable results, but still only 50 % of the mice survived challenge (Nyika *et al.,* 2002).

Other *E. ruminantium* proteins have also been investigated to determine which proteins besides MAP1 and MAP2 would be immunogenic. Selected *E. ruminantium* proteins were expressed in *E. coli* and mice were immunised with the resulting *E. coli* protein lysate. Immunisation of single *E. ruminantium* proteins did not confer significant protection but combinations of four to five proteins were more successful. Most of these proteins were membrane proteins and were recognised by peripheral blood monocytes (PBMC) or immune sera (Barbet *et al*., 2001).

1.1.8. DNA vaccination

Immunisation using DNA is based on the principle that an open reading frame (ORF) of interest is cloned into a vector designed to express the ORF-specific protein in a mammalian host. Inoculation of the plasmid into the host cells may result in the synthesis of a protein that could, once exposed to the host immune system, activate a humoral and/or cellular immune response in the recipient animal. Therefore these plasmids must contain a promoter that will facilitate expression of the gene of interest in a mammalian host. These plasmids also contain a bacterial origin of replication and antibiotic resistance genes necessary for amplification of the plasmid in bacteria. Mammalian expression vectors also contain a poly A region (normally absent from bacterial mRNA) and terminating signals (reviewed by Hasan *et al.,* 1999 and by Gurunathan *et al.,* 2000a; 2000b). Diverse expression systems are currently available ranging from vectors designed to induce antibody (humoral) responses (mediated by group two T helper (T_{H2}) cells), to vectors responsible for activation of cellular immune responses (mediated by T_{H1} T cells) and CTLs.

1.1.8.1. The activation of humoral immunity by DNA vaccination

Activation of an antibody response requires two steps: firstly, binding of a specific antigen to B cell membrane-bound antibody that will activate clonal expansion of the B cells, and secondly, B cells require T cell "help" in order to induce IgM to IgG class switching. These helper T cells that interact specifically with B cells are known as humoral or T helper 2 (T_{H2}) cells. The T cell help is mediated by an interaction of the T cell with antigen presented on MHC class II molecules. MHC class II molecules are only expressed by a select few cells including B cells, stimulated epithelial cells and antigen presenting cells (APCs), for example macrophages and dendritic cells (DCs). Antigens presented by these cells are normally exogenous and are taken up by the APC via phagocytosis of proteins, pathogens or infected cells. These antigens are degraded and fragmented in endocytic compartments of the APCs. Suitable fragments of 12-20 amino acids in length associate and bind to MHC class II molecules resulting in the final presentation of antigen on the APCs' cell surface (reviewed by Whitton *et al.,* 1999 and by Thalhamer *et al.,* 2001).

To activate a humoral (T_{H2}) immune response with plasmid DNA, the plasmid-derived antigen should be exported out of the host cell to associate with the B cell membrane-bound antibody and for APC uptake (Drew *et al.,* 2000 and reviewed by Whitton *et al.,* 1999). Vectors that mediate a humoral immune response must therefore have a secretory signal that is covalently linked to the antigen of interest. Examples of secretory signals are: the human CD5 leader sequence (Chaplin *et al.,* 1999), glutathione S-transferase (Kang *et al.,* 1998), the human growth hormone leader sequence (reviewed by Barry and Johnston, 1997) and the cytotoxic T lymphocyte antigen 4 (CTLA-4) (Drew *et al.,* 2001).

One of the advantages of using DNA vaccination to induce antibodies as opposed to the traditional protein immunisation strategies is the fact that although initial antibody titres are lower, they are stable for longer periods if compared to the titres induced by protein immunisation. Anthrax antigen inoculated into sheep by DNA immunisation was stable for five months while protein induced antibody titres started to decrease over the same period of time (Hahn *et al.,* 2006).

1.1.8.2. The activation of cellular immunity by DNA vaccination

Cellular immunity refers to the activation of both $CD4^+$ T helper cells upon recognition of antigen presented on MHC class II molecules and $CD8^+$ T cells (or CTL) that are activated by MHC class I presentation. All cell types present samples of self antigen (endogenous antigen) on MHC class I. Cells that present antigen that is not foreign to the CTL will be tolerated, but if antigen of unknown origin is presented to the CTL, it will destroy the infected host cell by apoptosis (reviewed by Van den Eynde and Morel, 2001). The unknown antigen normally originates from oncogenic genes, viral infection or intracellular bacteria (reviewed by Lehner and Cresswell, 1996; Whitton *et al.,* 1999 and by Melief, 2003).

The expression of proteins from plasmid DNA in host cells is regarded to be equivalent to protein expression mediated by viral DNA or RNA. Therefore DNA vaccination could activate CTL mediated immunity through internal MHC class I antigen presentation (Corr *et al.,* 1996). In order to activate these cells, antigen derived from the plasmid DNA must first be introduced into the MHC class I antigen

presentation pathway via the ubiquitination of proteins. Ubiquitination directs proteins to a proteosome complex where peptide fragments are generated (reviewed by Rechsteiner *et al.,* 2000; Van den Eynde and Morel, 2001; Weissman, 2001 and by Yewdell and Bennink, 2001). These fragments are then transported to the endoplasmic reticulum where they associate with MHC class I molecules (reviewed by Hassett and Whitton, 1996 and by Yewdell and Bennink, 2001). Using an expression vector that contains the ubiquitin sequence will result in fusion proteins that are covalently linked to ubiquitin, consequently favouring a cytotoxic T cell response (Sykes and Johnston, 1999). Similarly heat shock protein 70 (HSP70) can also be used to direct recombinant protein towards the proteosome (Qazi *et al.,* 2005; Li *et al.,* 2006).

The activation of a memory CTL response by repeated boosting with plasmid DNA is not always successful. Therefore alternative vaccination strategies have been developed to improve the CTL response. So far the best results have been obtained by using a prime-boost immunisation strategy. This is achieved by priming the immune response using DNA vaccination, followed by either a modified viral or recombinant protein boost. Protection against several diseases that are controlled primarily by a cellular immune response has been demonstrated using this strategy (Hammond *et al.,* 2001; Gilbert *et al.,* 2002; Gonzalo *et al.,* 2002; Moorthy *et al.,* 2003; Stambas *et al.,* 2005).

The role of $CD4^+$ cells in the development of CTL responses still has to be elucidated. On the one hand, some scientists believe that $CD4^+$ T cell help is required for the activation of both effector and memory CTL (mCTL) (Bennett *et al.,* 1997; López-Días de Cerio *et al.,* 1999; Behrens *et al.,* 2004 and reviewed by Keene and Forman, 1982 and by Heath and Carbone, 1999). $CD4^+$ and $CD8^+$ specific epitopes derived from the one antigen must be presented on the same APC. This will facilitate interaction between $CD4^+$ T cells and $CD8^+$ T cells, thus the $CD4^+$ T cells provide direct help. There exist three models to describe this interaction between helper cells and CTL: 1) the passive three cell interaction model suggests the simultaneous recognition and interaction of $CD4^+$ and $CD8^+$ cells with antigen presented on the same APC (Bennett *et al.,* 1997), 2) the sequential two cell interaction model proposes

that the helper T cell activates the APC and this activated APC in turn activates a CTL response (Ridge *et al.,* 1998) and 3) the direct activation of CTL by $CD4^+$ helper-antigen-presenting cell model (Xiang *et al.,* 2005). It has recently been shown that $CD4^+$ cells can acquire antigen presented on APC via the transfer of MHC class I and II/antigen complexes from the APC to the $CD4^+$ T helper cell. These acquired MHC/antigen complexes can be presented on the $CD4^+$ T helper cell; thus the helper cell can act as an APC that can activate CTL via direct interaction (Xiang *et al.,* 2005). All three of these models require that epitopes derived from the same antigen are presented on both MHC class I and II molecules.

In contrast, other researchers have shown that effector CTL can be non-specifically activated by any $CD4^+$ T_{H1} cells (Elliott *et al.,* 1999; López-Días de Cerio *et al.,* 1999). A strong CTL response was observed when mice were immunised with a synthetic hepatitis C peptide containing a CTL and a $CD4^+$ specific epitope. Modification of the $CD4^+$ epitope in the peptide resulted in the absence of a CTL response in immunised mice. The CTL response was however restored when mice were co-immunised with the modified hepatitis C peptide (that rendered the $CD4^+$ epitope non-effective) and an epitope derived from a non-hepatitis specific protein: ovalbumin (López-Días de Cerio *et al.,* 1999). This indicates that $CD4^+$-derived epitopes do not have to originate from the same antigen in order to induce an effector CTL response. There is also evidence that effector CTL activation and proliferation can occur in the complete absence of $CD4^+$ T cells and can be directly activated by NK cells and DCs in a T_{H1}-independent manner (Shedlock *et al.,* 2003; Combe *et al.,* 2005 and reviewed by Sparwasser *et al.,* 2000).

All these researchers do however agree that T_{H1} $CD4^+$ specific help (i.e. $CD4^+$ T cells recognising epitopes derived from the same antigen) is essential for the development and activation of mCTLs (Gao *et al.,* 2002 and reviewed by Gao and Jakobsen, 2000 and by Wherry and Ahmed, 2004). If a strong mCTL response is required, antigen specific $CD4^+$ T cells have to be activated as well.

1.1.9. Activation of immune responses using DNA immunisation

The stimulation of a T_{H1} or T_{H2} immune response by DNA vaccination can be dependant on several factors including: the route of immunisation, the cell-types transfected, the type and origin of the expression vector promoter, the amounts of plasmid used for inoculation, the co-expression of cytokines, the expression level of the antigen, and post translational modifications in the host of the antigen itself. (Feltquate *et al.,* 1997 and reviewed by Barry and Johnston, 1997 and by Thalhamer *et al.,* 2001). We will summarise what is known about each of these factors.

1.1.9.1. The route of inoculation and the cell types that play a role in immune response activation

Strong immune responses are obtained by injection of a DNA-saline solution (i.d. or i.m.) or by direct transfection of cells using a biolistic device that will propel precipitated DNA on gold beads into the skin epidermis and dermis. Other techniques of intra dermal immunisation can also be used, including trans-epidermal immunisation, electroporation and microseeding (Tollefsen *et al.,* 2003; Scheerlinck *et al.,* 2004; Tjelle *et al.,* 2006 and reviewed by Peachman *et al.,* 2003). These procedures will induce antigen expression and will activate an immune response similar to that of gene gun immunisation. It is generally believed that intra muscular (i.m.) needle injection of an expression vector that contains secretory signals favours a cellular or T_{H1} immune response, while inoculation of the same plasmid into the skin using a biolistic device such as a gene gun (gg) promotes a humoral (antibody) immune response (Feltquate *et al.,* 1997 and reviewed by Barry and Johnston, 1997 and by Thalhamer *et al.,* 2001). However, activation of different immune responses is much more complex and can be linked to other factors such as the activation of specific cell types located at the inoculation sites:

1.1.9.1.1. Cell types that play a role in the activation of immune responses after intradermal gene gun inoculation

DNA vaccination depends on the successful delivery of the antigen encoded on the plasmid to APCs and the subsequent activation of these cells. The APCs most likely to be affected by intra dermal gene gun DNA vaccination are skin Langerhans cells (a type of DC) and keratinocytes. DCs are highly mobile cells present in most peripheral

tissues in low numbers where they specialise in antigen uptake (reviewed by Viney, 2001 and by Hartgers *et al.,* 2000). Resting or immature DCs reside in peripheral body-surface tissues, where they constantly sample and present antigen. Immature DCs process antigens through both the MHC class I and class II pathways that are required for activation of $CD8^+$ and $CD4^+$ T cells respectively.

Immune responses directed to recombinant antigen can be activated by keratinocytes (somatic cells) directly transfected with DNA (DNA attached to gold beads is propelled into the cytoplasm of the cell), which results in the expression of the protein. If secretory signals were included on the expression vector, the protein will be exported out of the host cell. The keratinocytes will continuously express antigen and an immune response will only be activated once the exported antigen is taken up by DCs. The DCs are then activated to migrate to lymph nodes where naive T cells can be activated (Corr *et al.,* 1999; Yoshida *et al.,* 2000 and reviewed by Whitton *et al.,* 1999). A small number of Langerhans cells can also be directly transfected by gene gun immunisation and can express protein for up to 2 weeks (Akbari *et al.,* 1999). There is also evidence that the DCs can phagocytose the gold beads at the inoculation site (Klinman *et al.,* 1998; reviewed by Lane and Brocker, 1999).

Uptake of unmethylated bacterial DNA acts as a maturation signal for DCs and induces chemokine production by the DCs that will in turn recruit more DCs to the inoculation site (reviewed by Lane and Brocker, 1999). Upon exposure to stimuli including: bacterial-derived lipopolysaccharide (LPS), the phagocytosis of gold beads, pathogen antigens, inflammatory cytokines and triggering of the CD40 receptor, DCs are mobilised from the site of infection to the lymph nodes where they activate antigen specific T-, B- and NK cells (Klinman *et al.,* 1998; and reviewed by Lane and Brocker, 1999; Reis e Sousa *et al.,* 1999 and by Jenkins and Mountford, 2005). During this migratory process DCs mature into APCs that specialise in the expression of high copy numbers of MHC-peptide complexes.

Once the DCs have migrated to the lymph nodes these cells will interact with $CD4^+$ T cells. It has been demonstrated that clonal expansion of helper T cells in draining lymph nodes is detectable 3-4 days post gene gun inoculation (Creusot *et al.,* 2001).

This indicates that the processes of protein synthesis, product export, phagocytosis by the DC, migration to lymphnodes and the activation of specific T cells to proliferate require a minimum of 3 days from inoculation.

Different subsets of DCs exist which will bias T_H subset development: activated $DC8\alpha^+$ DCs are known to produce IL-12 and the subsequent production of IFN-γ and IL-2 favours a T_{H1} or cellular immune response, while activated $DC8\alpha^-$ DCs induce T_{H2}-specific IL-4 production favouring a humoral response (reviewed by Hartgers *et al.,* 2000; Rengarajan *et al.,* 2000; Rea *et al.,* 2001; Théry and Amigorena, 2001; and by Melief, 2003). DCs also have the unique capacity to present exogenous internalised antigen on MHC class I through a process known as cross-presentation. It has been shown that peptide antigen-MHC class I complexes can be formed either in endocytic compartments, or in the endoplasmic reticulum where exogenous internalised proteins access the cytosol for processing by the proteosome (reviewed by Carbone *et al.,* 1998; Hartgers *et al.,* 2000 and by Théry and Amigorena, 2001). A consequence of all these observations appears to be that we can expect that both humoral and cellular immunity can be activated using gene gun inoculation.

1.1.9.1.2. Cell types that play a role in the activation of immune responses after needle challenge (i.m. or i.d.)

The reproducibility of an immune response by i.m. inoculation is dependant on the speed of injection, the type of solute, the condition of the muscle, the level of protein expression and the DNA concentration (Yoshida *et al.,* 2000). Needle challenge of DNA in solution will activate immunity in a different manner from that of directly transfected cells using gene gun inoculation. It has been shown that needle immunisation with plasmid DNA in saline recruits macrophages, $CD4^+$ and $CD8^+$ T cells to the inoculation site (reviewed by McMahon *et al.,* 1998). This recruitment of inflammatory cells can be activated by cytokine responses. DNA at the inoculation site first has to be taken up by myocytes (i.m.), keratinocytes (i.d.) or APC (both i.m. and i.d.), before expression can commence (reviewed by Barry and Johnston, 1997 and by Whitton *et al.,* 1999). Uptake of DNA will activate the innate immune response via binding and recognition of CpG motifs by host cell receptors known as Toll-like receptors (TLR). Binding of CpG motifs to TLR9 will activate secretion of

T_{H1} type cytokines including IL-12, IFN-α and IFN-γ (reviewed by Gurunathan *et al.,* 2000a; Thalhamer *et al.,* 2001 and by Abel *et al.,* 2005). Muscle cells (myocytes) can also phagocytose the plasmid DNA, and have been shown to present antigen on MHC class I. These cells do not however express the necessary co-stimulatory signals required for CTL activation. Thus CTL activation is completely dependant on MHC class I presentation on professional APC via cross-priming (reviewed by Whitton *et al.,* 1999 and by Gurunathan *et al.,* 2000a). It is more likely that the myocytes assist in expression and secretion of protein that can be taken up and processed by the APC via MHC class I and II pathways, although the mechanism is still unclear. It has been shown after i.m. inoculation that the plasmid and mRNA encoding *Plasmodium yoelii*-derived antigen only persist at the site of inoculation and could not be detected in the spleen, lymph nodes or the bone marrow of immunised mice. Since only the myocytes are stationary at the inoculation site it is believed that these cells express antigen derived from plasmid DNA (Mor *et al.,* 1995).

1.1.9.2. Other factors that play a role in immune response activation

The type of promoter used can influence the type of immune response that is activated. Promoters of cytomegaloviral (CMV) origin, combined with the *Clostridium botulinum* type F neurotoxin Hc fragment as antigen, induced predominantly IgG2a antibodies indicative of a T_{H1} response, while enhanced IgG1 responses were activated when the Hc fragment was combined with a CMV promoter that contained a translational enhancer (CMV-TE) (Jathoul *et al.,* 2004). Similarly, an increased T_{H2} response was observed when human elongation factor 1α-subunit (EF-1α) and human ubiquitin C (UbC) promoters were used (Jathoul *et al.,* 2004). In another study, comparison of six different promoters for expression of *Yersinia pestis* V antigen revealed that a CMV-TE promoter induced the highest antibody levels against the V protein derived from *Y. pestis* (Garmory *et al.,* 2004), suggesting that the choice of promoter may be gene-specific.

It is also possible to improve the immunogenicity of DNA vaccines by optimising the codon usage of the pathogen gene for mammalian expression systems (André *et al.,* 1998; Uchijima *et al.*, 1998; Ko *et al.,* 2005). Changing the codons of the *Mycobacterium tuberculosis* gene Ag85B towards human consensus codons enhanced

T_{H1} immunity in mice as was evident from enhanced IFN-γ levels in immunised mouse splenocytes (Ko *et al.,* 2005).

Furthermore, the addition into the expression vector of leader sequences or sequences coding for complete proteins will result in fusion proteins that can also influence the type of immune response activated. Such vectors may be engineered to contain a leader sequence or protein required to guide the immune response. Examples include: 1) inclusion of the human growth hormone leader sequence that assists in the extracellular secretion of protein and ultimately leads to a humoral immune response or 2) inclusion of protein-coding sequences such as ubiquitin or HSP70 that will result in fusion proteins that will be directed towards the proteosome, ultimately inducing cytotoxic T cell activation (Rodriguez *et al.,* 1997; Sykes and Johnston, 1999; Qazi *et al.,* 2005; Li *et al.,* 2006).

1.1.10. DNA vaccine safety

One important aspect to consider when developing new vaccines is their safety. While DNA immunisation has multiple advantages and is generally considered as a safe method for immunisation, several limitations and potential dangers do exist. One of the most obvious dangers is the possibility of plasmid DNA integration into the host genomic DNA. This can result in the activation of oncogenes and may lead to malignant transformation of host cells. There is evidence from malarial DNA studies that integration of 3-30 copies of vaccine DNA into the genomic DNA of the host persists for at least two months at the inoculation site. The question remains: does this pose any risk to the host? The frequency of integration is much lower than naturally occurring point mutations in a genome sequence, but the effects of these long DNA stretches inserted into host genome can be more detrimental than single point mutations (reviewed by Hassett and Whitton, 1996 and by Smith and Klinman, 2001).

The continuous low level of antigen expression by genetic vaccines could contribute to the induction of tolerance. Evidence for this occurrence is scarce. It has been shown that tolerance to a specific malarial antigen introduced by DNA vaccination developed in calves, but this was not detected if adult cattle were immunised with similar malarial antigen. The induction of tolerance therefore appears to be dependant

on the antigen itself and the age of the host animal (reviewed by Hassett and Whitton, 1996 and by Smith and Klinman, 2001). Tolerance to a specific antigen has not been shown in sheep, the ruminant model used in our study.

Experiments done in mice to determine if genetic immunisation could have an effect on autoimmunity, indicated that only a four fold increase in auto-antibody could be measured (reviewed by Smith and Klinman, 2001). Even though auto-antibodies could be measured in these studies and other human preclinical studies, the levels of auto-antibodies after DNA vaccination did not exceed levels found naturally in human serum. This has helped to ascertain that DNA vaccination is not likely to induce systemic autoimmunity. In contrast, autoimmunity could be induced when mice were immunised with DNA that contained a high level of CpG motifs. These animals developed allergic encephalomyelitis, a disease similar to multiple sclerosis (reviewed by Smith and Klinman, 2001). Because the heartwater vaccines are derived from bacterial DNA with a low GC content (30 %), the induction of autoimmunity in the sheep by CpG motifs is unlikely.

1.2. OBJECTIVES OF THIS STUDY

The aim of this study is to develop a recombinant vaccine for heartwater. Sheep will be immunised with four plasmid constructs containing an ubiquitin-ORF fusion, which will direct proteins to the MHC class I pathway for cytotoxic T cell activation (Chapter 2). The ability of this vaccine to protect against different *E. ruminantium* isolates will also be tested (Chapter 3). Enhanced cytotoxic T cell activation will be obtained by a prime-boost strategy where the animals will be primed with DNA and boosted with either recombinant live lumpy skin disease viruses (rLSDV) expressing the four ORFs, or with recombinant 1H12 (r1H12) proteins (Chapter 4). If the vaccine is successful in the laboratory, it will be evaluated in the field (tick transmission).

1.3. REFERENCES

ABEL, K., WANG, Y., FRITTS, L., SANCHEZ, E., CHUNG, E., FITZGERALD-BOCARSLY, P., KRIEG, A.M. and MILLER, C.J. 2005. Deoxycytidyl-deoxyguanosine oligonucleotide classes A, B and C induce distinct cytokine gene expression patterns in rhesus monkey peripheral blood mononuclear cells and distinct alpha interferon responses in TLR9-expressing rhesus monkey plasmacytoid dendritic cells. *Clinical and Diagnostic Laboratory Immunology* **12:** 606-621.

AKBARI, O., PANJWANI, N., GARCIA, S., TASCON, R., LOWRIE, D. and STOCKINGER, B. 1999. DNA vaccination: transfection and activation of dendritic cells as key events for immunity. *Journal of Experimental Medicine* **189:** 169-177.

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

ALLSOPP, M.T.E.P., VISSER, E.S., DU PLESSIS, J.L., VOGEL, S.W. and 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., HATTINGH, C.M., VOGEL, S.W. and ALLSOPP, B.A. 1999. Evaluation of 16S, *map*1 and pCS20 probes for detection of *Cowdria* and *Ehrlichia* species. *Epidemiology and Infection* **122:** 323-328.

ANDRÉ, S., SEED, B., EBERLE, J., SCHRAUT, W., BÜLTMANN, A. and HAAS, J. 1998. Increased immune response elicited by DNA vaccination with a synthetic gp120 sequence with optimized codon usage. *Journal of Virology* **72:** 1497-1503.

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

BARRY, M.A. and JOHNSTON, S.A. 1997. Biological features of genetic immunisation. *Vaccine* **15:** 788-791.

BEHRENS, G.M.N., LI, M., DAVEY, G.M., ALLISON, J., FLAVELL, R.A., CARBONE, F.R. and HEATH, W.R. 2004. Helper requirements for generation of effector CTL to islet B cell antigens. *The Journal of Immunology* **172:** 5420-5426.

BEKKER, C.P.J., DE VOS, S., TAOUFIK, A., SPARAGANO, O.A.E. and JONGEJAN, F. 2002. Simultaneous detection of *Anaplasma* and *Ehrlichia* species in ruminants and detection of *Ehrlichia ruminantium* in *Amblyomma variegatum* ticks by reverse line blot hybridization. *Veterinary Microbiology* **89:** 223-238.

BELL-SAKYI, L., PAXTON, E.A., MUNDERLOH, U.G. and SUMPTION, K.J. 2000. Growth of *Cowdria ruminantium*, the causative agent of heartwater, in a tick cell line. *Journal of Clinical Microbiology* **38:** 1238-1240.

BELL-SAKYI, L., PAXTON, E., WRIGHT, P. and SUMPTION, K. 2002. Immunogenicity of *Ehrlichia ruminantium* grown in tick cell lines. *Experimental and Applied Acarology* **28:** 177-185.

BELL-SAKYI, L. 2004. *Ehrlichia ruminantium* grows in cell lines from four *Ixodid* tick genera. *Journal of Comparative Pathology* **130:** 285-293.

BENNETT, S.R.M., CARBONE, F.R., KARAMALIS, F., MILLER, J.F.A.P. and HEATH, W.R. 1997. Induction of a $CD8^+$ cytotoxic T lymphocyte response by cross-priming requires cognate $CD4^+$ T cell help. *Journal of Experimental Medicine* **186:** 65-70.

BEZUIDENHOUT, J.D., PATERSON, C. and BARNARD, B.J.H. 1985. *In vitro* cultivation of *Cowdria ruminantium*. *Onderstepoort Journal of Veterinary Research* **52:** 113-120.

BURRIDGE, M.J., SIMMONS, L.A., SIMBI, B.H., PETER, T.F. and MAHAN, S.M. 2000. Evidence of *Cowdria ruminantium* infection (heartwater) in *Amblyomma sparsum* ticks found on tortoises imported into Florida. *Journal of Parasitology* **86:** 1135-1136.

BURRIDGE, M.J., SIMMONS, L.A., PETER, T.F. and MAHAN, S.M. 2002. Increasing risks of introduction of heartwater onto the American mainland associated with animal movements. *Annals of the New York Academy of Science* **969:** 269-274.

BYROM, B., YUNKER, P.L., DONOVAN, P.L. and SMITH, G.E. 1991. *In vitro* isolation of *Cowdria ruminantium* from plasma of infected ruminants. *Veterinary Microbiology* **26:** 263-268.

BYROM, B., BARBET, A.F., OBWOLO, M. and MAHAN, S.M. 2000a. $CD8^+$ T cell knockout mice are less susceptible to *Cowdria ruminantium* infection than athymic $CD4^+$ T cell knockout, and normal C57BL/6 mice. *Veterinary Parasitology* **93:** 159-172.

BYROM, B., OBWOLO, M., BARBET, A.F. and MAHAN, S.M. 2000b. A polarized T_{H1} type immune response to *Cowdria ruminantium* infection is detected in immune DBA/2 mice. *Journal of Parasitology* **86:** 983-992.

CAMUS, E. and BARRE, N. 1995. Vector situation of tick-borne diseases in the Caribbean Islands. *Veterinary Parasitology* **57:** 167-176.

CARBONE, F.R., KURTS, C., BENNETT, S.R.M., MILLER, J.F.A.P., and HEATH, W. 1998. Cross-presentation: a general mechanism for CTL immunity and tolerance. *Immunology Today* **19:** 368-373.

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

CHAPLIN, P.J., DE ROSE, R., BOYLE, J.S., MCWATERS, P., KELLY, J., TENNENT, J.M., LEW, A.M. and SCHEERLINCK, J.Y. 1999. Targeting improves the efficacy of a DNA vaccine against *Corynebacterium pseudotuberculosis* in sheep. *Infection and Immunity* **67:** 6434-6438.

COLLINS, N.E., PRETORIUS, A., VAN KLEEF, M., BRAYTON, K.A., ALLSOPP, M.T., ZWEYGARTH, E. and ALLSOPP, B.A. 2003. Development of improved attenuated and nucleic acid vaccines for heartwater. In: Vaccines for OIE list A and Emerging Animal Diseases. Edited by BROWN, F. and ROTH, J. *Developments in Biologicals Basel Karger* **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., BERTHIER, D., BOTHA, M., JOUBERT, F., CORTON, C.H., THOMSON, N.R., ALLSOPP, M.T. and 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.

COMBE, C.L., CURIEL, T.J., MORETTO, M.M. and KHAN, I.A. 2005. NK cells help to induce $CD8^+$ T-cell immunity against *Toxoplasma gondii* in the absence of $CD4^+$ T cells. *Infection and Immunity* **73:** 4913-4921.

CORR, M., LEE, D.J., CARSON, D.A. and TIGHE, H. 1996. Gene vaccination with naked plasmid DNA: mechanism of CTL priming. *Journal of Experimental Medicine* **184:** 1555-1560.

CORR, M., VON DAMM, A., LEE, D.J. and TIGHE, H. 1999. *In vivo* priming by DNA injection occurs predominantly by antigen transfer. *The Journal of Immunology* **163:** 4721-4727.

COWDRY, E.V. 1925a. Studies on the etiology of heartwater. I. Observation of a rickettsia, *Rickettsia ruminantium* in the tissues of infected animals. *Journal of Experimental Medicine* **42:** 231-252.

COWDRY, E.V. 1925b. Studies on the etiology of heartwater. II. *Rickettsia ruminantium* in the tissues of ticks transmitting the disease. *Journal of Experimental Medicine* **42:** 253-274.

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

CREUSOT, R.J., THOMSEN, L.L., VAN WELY, C.A., TOPLEY, P., TITE, J.P. and CHAIN, B.M. 2001. Early commitment of adoptively transferred $CD4^+$ T cells following particle-mediated DNA vaccination: implications for the study of immunomodulation. *Vaccine* **19:** 1678-1687.

DAUBNEY, R. 1930. Natural transmission of heartwater in sheep by *Amblyomma variegatum* (Fabricius 1974). *Parasitology* **22:** 260-274.

DE ASSIS, M.C., DA COSTA, A.O., BARJA-FIDALGO, T.C. and PLOTKOWSKI, M.C. 2000. Human endothelial cells are activated by interferon-γ plus tumour necrosis factor-α to kill intracellular *Pseudomonas aeruginosa*. *Immunology* **101:** 271-278.

DREW, D.R., LIGHTOWLERS, M. and 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:** 2522-2532.

DREW, D.R., BOYLE, J.S., LEW, A.M., LIGHTOWLERS, M.W., CHAPLIN, P.J. and STRUGNELL, R.A. 2001. The comparative efficacy of CTLA-4 and L-selectin targeted DNA vaccines in mice and sheep. *Vaccine* **19:** 4417-4428.

DU PLESSIS, J.L. 1970. Immunity in heartwater. I. A preliminary note on the role of serum antibodies. *Onderstepoort Journal of Veterinary Research* **37:** 147-150.

DU PLESSIS, J.L. and KÜMM, N.A.L. 1971. The passage of *Cowdria ruminantium* in mice. *Journal of the South African Veterinary Association* **42:** 217-221.

DU PLESSIS, J.L., GRAY, C. and 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. 1993. An *in vitro* test to demonstrate the inhibitory effect of homologous immune serum on the infectivity of *Cowdria ruminantium*. *Onderstepoort Journal of Veterinary Research* **60:** 69-73.

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

DUMLER, J.S., BARBET, A.F., BEKKER, C.P.J., DASCH, G.A., PALMER, G.H., RAY, S.C., RIKIHISA, Y. and RURANGIRWA, F.R. 2001. Reorganisation of genera in the families *Rickettsiaceae* and *Anaplasmataceae* in the order *Rickettsiales*: unification of some species of *Ehrlichia* and *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 Systemic and Evolutionary Microbiology* **51:** 2145-2165.

ELLIOTT, S.L., PYE, S., LE, T., MATEO, L., COX, J., MACDONALD, L., SCALZO, A.A., FORBES, C.A. and SUHRBIER, A. 1999. Peptide based cytotoxic T-cell vaccines; delivery of multiple epitopes, help, memory and problems. *Vaccine* **17:** 2009-2019.

ESTEVES, I., MARTINEZ, D. and 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. and 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., WALRAVENS, K., VACHIÉRY, N., MARTINEZ, D., LETESSON, J.J. and TOTTÉ, 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.

FELTQUATE, D.M., HEANEY, S., WEBSTER, R.G. and ROBINSON, H.L. 1997. Different T helper cell types and antibody isotypes generated by saline and gene gun DNA immunisation. *The Journal of Immunology* **158:** 2278-2284.

FENG, H., WHITWORTH, T., POPOV, V., and WALKER, D.H. 2004. Effect of antibody on the Rickettsia-host cell interaction. *Infection and Immunity* **72:** 3524-3530.

GAO, G.F. and JAKOBSEN B.K. 2000. Molecular interactions of coreceptor $CD8^+$ and MHC class I: the molecular basis for functional coordination with the T-cell receptor. *Immunology Today* **21:** 630-636.

GAO, F.G., KHAMMANIVONG, V., LIU, W.J., LEGGATT, G.R., FRAZER, I.H. and FERNANDO, G.J.P. 2002. Antigen-specific $CD4^+$ T-cell help is required to activate a memory $CD8^+$ T cell to a fully functional tumour killer cell. *Cancer Research* **62:** 6438-6441.

GARMORY, H.S., FREEMAN, D., BROWN, K.A. and TITBALL, R.W. 2004. Protection against plague afforded by immunisation with DNA vaccines optimised for expression of the *Yersinia pestis* V antigen. *Vaccine* **22**: 947–957.

GILBERT, S.C., SCHNEIDER, J., HANNAN, C.M., HU, J.T., PLEBANSKI, M., SINDEN, R. and HILL, A.V.S. 2002. Enhanced $CD8^+$ T cell immunogenicity and protective efficacy in a mouse malaria model using a recombinant adenoviral vaccine in heterologous prime-boost immunisation regimes. *Vaccine* **20:** 1039-1045.

GONZALO, R.M., DEL REAL, G., RODRIGUEZ, J.R., RODRIGUEZ, D., HELJASVAARA, R., LUCAS, P., LARRAGA, V. and ESTEBAN 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.

GURUNATHAN, S., KLINMAN, D.M. and SEDER, R.A. 2000a. DNA vaccines: immunology, application, and optimisation. *Annual Reviews in Immunology* **18:** 927-974.

GURUNATHAN, S., WU, C., FREIDAG, B.L. and SEDER, R.A. 2000b. DNA vaccines: a key for inducing long-term cellular immunity. *Current Opinion in Immunology* **12:** 442-447.

HAHN, U.K., AICHLER, M., BOEHM, R. and BEYER, W. 2006. Comparison of the immunological memory after DNA vaccination and protein vaccination against anthrax in sheep. *Vaccine* **24:** 4595-4597.

HAIG, D.A., ALEXANDER, R.A. and WEISS, K.E. 1954. Treatment of heartwater with tetramycin. *Journal of the South African Veterinary Association* **25:** 45-48.

HAMMOND, J.M., JANSEN, E.S., MORRISSY, C.J., GOFF, W.V., MEEHAN, G.C., WILLIAMSON, M.M., LENGHAUS, C., SPROAT, K.W., ANDREW, M.E., COUPAR, B.E.H. and JOHNSON, M.A. 2001. A prime-boost vaccination strategy using naked DNA followed by recombinant porcine adenovirus protects pigs from classical swine fever. *Veterinary Microbiology* **80:** 101-119.

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

HARTGERS, F.C., FIDGOR, C.G. and ADEMA, G.J. 2000. Towards a molecular understanding of dendritic cell immunobiology. *Immunology Today* **21:** 542-545.

HASAN, U.A., ABAI, A.M., HARPER, D.R., WREN, B.W. and MORROW, W.J.W. 1999. Nucleic acid immunisation: concepts and techniques associated with third generation vaccines. *Journal of Immunological Methods* **229:** 1-22.

HASSETT, D.E. and WHITTON, J.L. 1996. DNA immunisation. *Trends in Microbiology* **4:** 307-312.

HEATH, W.R. and CARBONE, F.R. 1999. Cytotoxic T lymphocyte activation by cross-priming. *Current Opinion in Immunology* **11:** 314-318.

JATHOUL, A.P., HOLLEY, J.L. and GARMORY, H.S. 2004. Efficacy of DNA vaccines expressing the type F botulinum toxin Hc fragment using different promoters. *Vaccine* **22:** 3942-3946.

JENKINS S.J. and MOUNTFORD, A.P. 2005. Dendritic cells activated with products released by schistosome larvae drive th2-type immune responses, which can be inhibited by manipulation of CD40 costimulation. *Infection and Immunity* **73:** 395-402.

JONGEJAN, F., UILENBERG, G., FRANSSEN, F.F., GUEYE, A. and NIEUWENHUIJS, J. 1988. Antigenic differences between stocks of *Cowdria ruminantium*. *Research in Veterinary Sciences* **44:** 186-189.

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

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

JONGEJAN, F., VOGEL, S.W., GUEYE, A. and UILENBERG, G. 1993. Vaccination against heartwater using in vitro attenuated *Cowdria ruminantium* organisms. *Revue d'Elevage et de Médecine Vétérinaire des Pays Tropicaux* **46:** 223-227.

KANG, Y., CALVO, P.A., DALY, T.M. and LONG, C.A. 1998. Comparison of humoral immune responses elicited by DNA and protein vaccines based on merozoite surface protein-1 from *Plasmodium yoelii*, a rodent malaria parasite. *The Journal of Immunology* **161**: 4211-4219.

KAYLOR, P.S., CRAWFORD, T.B., MCELWAIN, T.F. and PALMER, G.H. 1991. Passive transfer of antibody to *Ehrlichia risticii* protects mice from ehrlichiosis. *Infection and Immunity* **59:** 2058-2062.

KEENE, J. and FORMAN, J. 1982. Helper activity is required for the *in vivo* generation of cytotoxic T lymphocytes. *Journal of Experimental Medicine* **155:** 768-782.

KLINMAN D.M., SECHLER, J.M.G., CONOVER, J., GU, M. and ROSENBERG, A.S. 1998. Contribution of cells at the site of DNA vaccination to the generation of antigen-specific immunity and memory. *The Journal of Immunology* **160:** 2388-2392.

KO, H.J., KO, S.Y., KIM, Y.J., LEE, E.G., CHO, S.N and KANG, C.Y. 2005. Optimisation of codon usage enhances the immunogenisity of a DNA vaccine encoding mycobacterial antigen Ag85B. *Infection and Immunity* **73:** 5666-5674.

KOCAN, K.M. and BEZUIDENHOUT J.D. 1987. Morphology and development of *Cowdria ruminantium* in *Amblyomma* ticks. *Onderstepoort Journal of Veterinary Research* **54:** 177-182.

LANE, P.J.L and BROCKER, T. 1999. Developmental regulation of dendritic cell function. *Current Opinion in Immunology* **11:** 308-313.

LEE, E.H. and RIKIHISA, Y. 1997. Anti-*Ehrlichia chaffeensis* antibody complexed with *E. chaffeensis* induces potent proinflammatory cytokine mRNA expression in human monocytes through sustained reduction of IκB-α and activation of NF-κB. *Infection and Immunity* **65:** 2890-2897.

LEHNER, P.J. and CRESSWELL, P. 1996. Processing and delivery of peptides presented by MHC class I molecules. *Current Opinion in Immunology* **8:** 59-67.

LI, J.S., YAGER, E., REILLY, M., FREEMAN, C., REDDY, G.R., REILLY, A.A., CHU, F.K. and WINSLOW, G.M. 2001. Outer membrane protein-specific monoclonal antibodies protect SCID mice from fatal infection by the obligate intracellular bacterial pathogen *Ehrlichia chaffeensis. The Journal of Immunology* **166:** 1855-1862.

LI, J.S., CHU, F., REILLY, A. and WINSLOW, G.M. 2002. Antibodies highly effective in SCID mice during infection by the intracellular bacterium *E. chaffeensis* are of picomolar affinity and exhibit preferential epitope and isotype utilization. *The Journal of Immunology* **169:**1419-1425.

LI, J.S. and WINSLOW, G.M. 2003. Survival, replication, and antibody susceptibility of *Ehrlichia chaffeensis* outside of host cells. *Infection and Immunity* **71:** 4229-4237.

LI, X., YANG, X., LI, L., LIU, H. and LIU, J. 2006. A truncated C-terminal fragment of *Mycobacterium tuberculosis* HSP70 gene enhanced potency of HBV DNA vaccine. *Vaccine* **24**: 3321-3331.

LÓPEZ-DÍAS DE CERIO, A., CASARES, N., LASARTE, J.J., SAROBE, P., PEREZ-MEDIAVILLA, L.A., RUIZ, M., PRIETO, J. and BORRAS-CUESTA, F. 1999. T_{H1} but not T_{H0} help is efficient to induce cytotoxic T lymphocytes by immunisation with short synthetic peptides. *International Immunology* **12:** 2025-2033.

MAHAN, S.M., ANDREW, H.R., TEBELE, N., BURRIDGE, M.J. and BARBET, A.F. 1995. Immunisation of sheep against heartwater with inactivated *Cowdria ruminantium.* *Research in Veterinary Science* **58:** 46-49.

MAHAN, S.M., KUMBULA, D., BURRIDGE, M.J. and 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., PETER, T.F., SIMBI, B.H., KOCAN, K., CAMUS, E., BARBET, A.F. and BURRIDGE, M.J. 2000. Comparison of efficacy of American and African *Amblyomma* ticks as vectors of heartwater (*Cowdria ruminantium*) infection by molecular analyses and transmission trials. *Journal of Parasitology* **86:** 44-49.

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

MAILLARD, J.C. and MAILLARD, N. 1998. Historique du peuplement bovin et de l'introduction de la tique *Amblyomma variegatum* dans les îles françaises des Antilles: Synthèse bibliographique. *Ethnozootechnie* **1:** 19-36.

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

MARELLI-BERG, F.M. and JARMIN, S.J. 2004. Antigen presentation by the endothelium: a green light for antigen-specific T cell trafficking? *Immunology Letters* **93:** 109-113.

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

MBOLOI, M.M., BEKKER, C.P., KRUITWAGEN, C., GREINER, M. and 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.

MCMAHON, J.M., WELLS, K.E., BAMFO, J.E., CARTWRIGHT, M.A. and WELLS, D.J. 1998. Inflammatory responses following direct injection of plasmid DNA into skeletal muscle. *Gene Therapy* **5:** 1283-1290.

MELIEF, C.J.M. 2003. Mini review: Regulation of cytotoxic T lymphocyte responses by dendritic cells: peaceful coexistence of cross-priming and direct priming? *European Journal of Immunology* **33:** 2645-2654.

MESSICK, J.B. and RIKIHISA, Y. 1994. Inhibition of binding, entry, or intracellular proliferation of *Ehrlichia risticii* in P388D_1 cells by anti-*E. risticii* serum, immunoglobulin G, or Fab fragment. *Infection and Immunity* **62:** 3156-3161.

MOORTHY, V.S., MCCONKEY, S., ROBERTS, M., GOTHARD, P., ARULANANTHAM, N., DEGANO, P., SCHNEIDER, J., HANNAN, C., ROY, M., GILBERT, S.C., PETO, T.E.A. and HILL, A.V.S. 2003. Safety of DNA and modified vaccinia virus Ankara vaccines against liver-stage *P. falciparum* malaria in non-immune volunteers. *Vaccine* **21:** 1995-2002.

MOR, G., KLINMAN, D.M., SHAPIRO, S., HAGIWARA, E., SEDEGAH, M., NORMAN, J.A., HOFFMAN, S.L. and STEINBERG, A.D. 1995. Complexity of the cytokine and antibody response elicited by immunising mice with *Plasmodium yoelii* circumsporozoite protein plasmid DNA. *The Journal of Immunology* **155:** 2039-2046.

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

MULLER KOBOLD, A., MARTINEZ, D., CAMUS, E. and JONGEJAN, F. 1992. Distribution of heartwater in the Caribbean determined on the basis of detection of antibodies to the conserved 32-kilodalton protein of *Cowdria ruminantium*. *Journal of Clinical Microbiology* **30:** 1870-1873.

MUTUNGA, M., PRESTON, P.M. and 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. and MCKEEVER, D.J. 1998. Immunisation of cattle by infection with *Cowdria ruminantium* elicits T lymphocytes that recognize autologous, infected endothelial cells and monocytes. *Infection and Immunity* **66:** 1855-1860.

MWANGI, D.M., MCKEEVER, D.J., NYANJUI, J.K., BARBET, A.F. and 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. and 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. and MAHAN, S.M. 2002. DNA vaccination with *map*1 gene followed by protein boost augments protection against challenge with *Cowdria ruminantium*, the agent of heartwater. *Vaccine* **20:** 1215-1225.

PEACHMAN, K.K., RAO, M. and ALVING, C.R. 2003. Immunisation with DNA through the skin. *Methods* **31:** 232-242.

PEREZ, J.M., MARTINEZ, D., SHEIKBOUDOU, C., JONGEJAN, F. and BENSAID, A. 1998. Characterisation of variable immunodominant antigens of *Cowdria ruminantium* by ELISA and immunoblots. *Parasite Immunology* **20:** 613-622.

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

PETER, T.F., BURRIDGE, M.J. and MAHAN, S.M. 2002. *Ehrlichia ruminantium* infection (heartwater) in wild animals. *TRENDS in Parasitology* **18:** 214-218.

POBER, J.S. 1999. Immunobiology of human vascular endothelium. *Immunological Research* **19:** 225-232.

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

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

PROZESKY, L. 1987a. Diagnosis of heartwater at post-mortem in ruminants and the confirmation of *Cowdria ruminantium* in mice. *Onderstepoort Journal of Veterinary Research* **54:** 301-303.

PROZESKY, L. 1987b. Heartwater: The morphology of *Cowdria ruminantium* and its staining characteristics in the vertebrate host and in vitro. *Onderstepoort Journal of Veterinary Research* **54:** 173-176.

PROZESKY, L. and DU PLESSIS, J.L. 1987. Heartwater. The development and life cycle *of Cowdria ruminantium* in the vertebrate host, ticks and cultured endothelial cells. O*nderstepoort Journal of Veterinary Research* **54:** 193-196.

QAZI, K.R., WIKMAN, M., VASCONCELOS, N.M., BERZINS, K., STÅHL, S. and FERNÁNDEZ, C. 2005. Enhancement of DNA vaccine potency by linkage of *Plasmodium falciparum* malarial antigen gene fused with a fragment of HSP70 gene. *Vaccine* **23:** 1114-1125.

REA, D., JOHNSON, M.E., HAVENGA, M.J.E., MELIEF, C.J.M. and OFFRINGA, R. 2001. Strategies for improved antigen delivery into dendritic cells. *TRENDS in Molecular Medicine* **7:** 91-94.

RECHSTEINER, M., REALINI, C. and USTRELL, V. 2000. The proteosome activator 11 S REG (PA28) and class I antigen presentation. *Journal of Biochemistry* **345:** 1-15.

REIS E SOUSA, C., SHER, A. and KAYE, P. 1999. The role of dendritic cells in the induction and regulation of immunity to microbial infection. *Current Opinion in Immunology* **11:** 392-399.

RENGARAJAN, J., SZABO, S.J. and GLIMCHER, L.H. 2000. Transcriptional regulation of T_{H1}/ T_{H2} polarization. *Immunology Today* **21:** 479-483.

RIDGE, J.P., DI ROSA, F. and MATZINGER, P. 1998. A conditioned dendritic cell can be a temporal bridge between a $CD4^+$ T-helper and a T-killer cell. *Nature* **393:** 474-478.

RODRIGUEZ, F., ZHANG, J. and WHITTON, J.L. 1997. DNA immunisation: Ubiquitination of a viral protein enhances cytotoxic T-lymphocyte induction and antiviral protection but abrogates antibody induction. *Journal of Virology* **71:** 8497-8503.

SAVADYE, D.T., KELLY, P.J. and MAHAN, S.M. 1998. Evidence to show that an agent that cross-reacts serologically with *Cowdria ruminantium* in Zimbabwe is transmitted by ticks. *Experimental and Applied Acarology* **22:** 111-122.

SCHEERLINCK, J.P.Y., KARLIS, J., TJELLE, T.E., PRESIDENTE, P.J.A., MATHIESEN, I. and NEWTON, S.E. 2004. In vivo electroporation improves immune responses to DNA vaccination in sheep. *Vaccine* **22:** 1820-1825.

SHEDLOCK, D.J., WHITMIRE, J.K., TAN, J., MACDONALD, A.S., AHMED, R. and SHEN, H. 2003. Role of $CD4^+$ T cell help and costimulation in $CD8^+$ T cell responses during *Listeria monocytogenes* infection. *The Journal of Immunology* **170:** 2053-2063.

SMITH, H.A. and KLINMAN, D.M. 2001. The regulation of DNA vaccines. C*urrent opinion in Biotechnology* **12:** 299-303.

SPARWASSER, T., VABULAS, R.M., VILLMOW, B., LIPFORD, G.B. and WAGNER, H. 2000. Bacterial CpG-DNA activates dendritic cells in vivo: T helper cell-independent cytotoxic T cell responses to soluble proteins. *European Journal of Immunology* **30:** 3591-3597.

STAMBAS, J., BROWN, S.A., GUTIERREZ, A., SEALY, R., YUE, W., JONES, B., LOCKEY, T.D., ZIRKEL, A., FREIDEN, P., BROWN, B., SURMAN, S., COLECLOUGH, C., SLOBOD, K.S., DOHERTY, P.C. and HURWITZ, J.L. 2005. Long lived multi-isotype anti-HIV antibody responses following a prime-double boost immunisation strategy. *Vaccine* **23:** 2454-2464.

SUMPTION, K.J., PAXTON, E.A. and BELL-SAKYI, L. 2003. Development of a polyclonal competitive enzyme-linked immunosorbent assay for detection of antibodies to *Ehrlichia ruminantium. Clinical and Diagnostic Laboratory Immunology* **10:** 910-916.

SYKES, K.F. and JOHNSTON, S.A. 1999. Genetic and live vaccines mimic the antigenicity but not pathogenicity of live viruses. *DNA and Cell Biology* **18:** 521-531.

THALHAMER, J., LEITNER, W., HAMMERL, P. and BRTKO, J. 2001. Designing immune responses with genetic immunisation and immunostimulatory DNA sequences. *Endocrine Regulations* **35:** 143-166.

THÉRY, C. and AMIGORENA, S. 2001. The cell biology of antigen presentation in dendritic cells. *Current Opinion in Immunology* **13:** 45-51.

TJELLE, T.E., SALTE, R., MATHIESEN, I. and KJEKEN, R. 2006. A novel electroporation device for gene delivery in large animals and humans. *Vaccine* **24:** 4667-4670.

TOLLEFSEN, S., VORDERMEIER, I., OLSEN, I., STORSET, L., REITAN, L.J., CLIFFORD, D., LOWRIE, D.B., WIKER, H.G., HUYGEN, G., HEWINSON, G., MATHIESEN, I. and TJELLE, T.E. 2003. DNA injection in combination with electroporation: a novel method for vaccination of farmed ruminants. *Scandinavian Journal of Immunology* **57:** 229-238.

TOTTÉ, P., JONGEJAN, F., DE GEE, A.L.W. and 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., VACHIÉRY, N., MARTINEZ, D., TRAP, I., BALLINGALL, K.T., MACHUGH, N.D., BENSAID, A. and WÉRENNE, J. 1996. Recombinant bovine interferon gamma inhibits the growth of *Cowdria ruminantium* but fails to induce major histocompatibility complex class II following infection of endothelial cells. *Veterinary Immunology and Immunopathology* **53:** 61-71.

TOTTÉ, P., MCKEEVER, D., MARTINEZ, D. and BENSAID, A. 1997. Analysis of T-cell responses in cattle immunised 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. and MCKEEVER, D.J. 1999. Immune responses to *Cowdria ruminantium* infections. *Parasitology Today* **15:** 286-290.

UCHIJIMA, M., YOSHIDA, A., NAGATA, T. and KOIDE, Y. 1998. Optimisation of codon usage of plasmid DNA vaccine is required for the effective MHC class I-restricted T cell responses against an intracellular bacterium. *The Journal of Immunology* **161:** 5594-5599.

UILENBERG, G. 1990. Present and future possibilities for the control of cowdriosis and anaplasmosis. *Veterinary Quarterly* **12:** 39-45.

VACHIÉRY, N., TRAP, I., TOTTÉ, P., MARTINEZ, D. and BENSAID, A. 1998. Inhibition of MHC class I and class II cell surface expression on bovine endothelial cells upon infection with *Cowdria ruminantium*. *Veterinary Immunology and Immunopathology* **61:** 37-48.

VAN DEN EYNDE, B.J. and MOREL, S. 2001. Differential processing of class-I-restricted epitopes by the standard proteosome and the immunoproteosome. *Current Opinion in Immunology*. **13:** 147-153.

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., MACMILLAN, H., GUNTER, N.J., ZWEYGARTH, E., ALLSOPP, B.A., SHKAP, V., DU PLESSIS, D.H. and 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.

VINEY, J.L. 2001. Immune fate decided by dendritic cell provocateurs. *TRENDS in Immunology* **22:** 8-10.

WAGHELA, S.D., RURANGIRWA, F.R., MAHAN, S.M., YUNKER, C.E., CRAWFORD, T.B., BARBET, A.F., BURRIDGE, M.J. and 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. 1962. Notes on the common tick species of East Africa. *Cooper, McDougall and Robertson (E.A.) Ltd. Nairobi.* Part 1 and 3 pp 1-3, 13-18.

WALKER, A.R. 1984. The continuing problem of tick control. *Span* **27:** 102-104.

WALKER, A.R. 1990. Diseases caused by Arthropods – *Amblyomma*. In: Handbook on animal diseases in the tropics. Edited by Sewell M.M.H. and Brocklesby D.W. *Baillere Tindall, London* 5, 31-32.

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

WEISS, K.E., HAIG, D.A. and ALEXANDER, R.A. 1952. Aureomycin in the treatment of heartwater. *Onderstepoort Journal of Veterinary Research* **25:** 41-50.

WEISSMAN, A.M. 2001. Themes and variations on ubiquitylation. *Nature Reviews Molecular Cell Biology* **2:** 169-178.

WHERRY, E.J. and AHMED, R. 2004. Minireview: Memory CD8 T-cell differentiation during viral infection. *Journal of Virology* **78:** 5535-5545.

WHITTON, J.L., RODRIGUEZ, F., ZHANG, J. and HASSETT, D.E. 1999. DNA immunisation: mechanistic studies. *Vaccine* **17:** 1612-1619.

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

XIANG, J., HUANG, H. and LIU, Y. 2005. A new dynamic model of $CD8^+$ T effector cell response via $CD4^+$ T helper-antigen-presenting cells. *The Journal of Immunology* **174:** 7497-7505.

YAGER, E., BITSAKTSIS, C., NANDI, B., MCBRIDE, J.W. and WINSLOW, G. 2005. Essential role for humoral immunity during *Ehrlichia* infection in immunocompetent mice. *Infection and Immunity* **73:** 8009-8016.

YEWDELL, J.W. and BENNINK, J.R. 2001. Cut and trim: generating MHC class I peptide ligands. *Current Opinion in Immunology* **13:** 13-18.

YOSHIDA, A., NAGATA, T., UCHIJIMA, M., HIGASHI, T. and KOIDE, Y. 2000. Advantage of gene gun-mediated over intramuscular inoculation of plasmid DNA vaccine in reproducible induction of specific immune responses. *Vaccine* **18:** 1725 –1729.

YUNKER, C.E., KOCAN, K.M., NORVAL, R.A.I. and BURRIDGE, M.J. 1987. Distinctive staining of colonies of *Cowdria ruminantium* in midguts of *Amblyomma hebraeum*. *Onderstepoort Journal of Veterinary Research* **54:** 183-185.

YUNKER, C.E. and ALLSOPP, B.A. 1994. Modern biotechnological methods for diagnosis of cowdriosis – present and future trends. In: Proc. FAO expert consultation on use and applicable biotechnology. Methods for diagnosis of haemoparasites, 4-6 October 1993, Merida, Mexico. *FAO Rome.* pp. 143-155.

YUNKER, C.E. 1995. Current status of in vitro cultivation of *Cowdria ruminantium.* *Veterinary Parasitology* **57:** 205-211.

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

ZHANG, B., CENTRA, M., CAO, G.L., RATYCH, R.E., DOMACHOWSKE, J.B., MALECH, H.L. and ROSEN, G.M. 1997. Are free radicals responsible for endothelial cell killing of *Staphylococcus aureus*? *Immunology Letters* **58:** 113-120.

ZWEYGARTH, E. and JOSEMANS, A.I. 2001. A chemically defined medium for the growth of *Cowdria ruminantium*. *Onderstepoort Journal of Veterinary Research* **68:** 37-40.

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

2. CHAPTER 2: DNA VACCINATION

2.1. INTRODUCTION

Vaccination is one of the most cost-effective methods used to prevent the spread of infectious diseases. The trend in vaccine development has shifted from traditional live attenuated or killed pathogens to safer and better-defined subunit vaccines. These subunit vaccines can be administered to animals in diverse formats including DNA, RNA (Xue *et al.,* 2004), fusion- and recombinant proteins (Qazi *et al.,* 2005), as part of virosomes (viral capsid proteins) (Bungener *et al.,* 2005; Mercer and Traktman, 2005) or ghost bacteria (bacterial cell membrane) (Mayrhofer *et al.,* 2005).

Genetic immunisation, the inoculation of plasmid DNA that encodes pathogen proteins, requires the use of specifically engineered mammalian expression vectors that contain appropriate eukaryotic transcription and translation signals. The direct inoculation of plasmid DNA should result in the *in vivo* synthesis of a protein that will, after appropriate posttranslational modifications, resemble the original pathogen protein in conformation. Nucleic acid vaccination can effectively induce MHC class I-restricted cell-mediated immunity, because routine antigen synthesis and presentation sampling will include the *in vivo* expressed antigen, but it can also elicit humoral (T_{H2}) or cellular (T_{H1}) immune responses dependant on MHC class II-restricted activation of T helper cells (reviewed by Whitton *et al.,* 1999 and by Thalhamer *et al.,* 2001). DNA immunisation has a further advantage in that the type of immune response can be modulated towards cellular or humoral immunity by combining specific promoters, expression tags and the route of inoculation (i.d., mucosal and i.m.) (Fynan *et al.*, 1993; Aguiar *et al.,* 2002; De Rose *et al.,* 2002 ; Sudowe *et al.,* 2003 and reviewed by Gurunathan *et al.,* 2000a; 2000b). Furthermore, cellular immunity can be induced by the addition of CpG motifs and co-immunisation with plasmids that express cytokines such as granulocyte-macrophage colony stimulating factor (GM-CSF) or interleukin (IL)-12 and/or IL-18 (Zhu *et al.,* 2004; Coban *et al.,* 2005; O'Donovan *et al.,* 2005).

DNA vaccination has been used to activate a protective immune response to various bacterial, viral and protozoan targets, for example: *Corynebacterium pseudotuberculosis* (Chaplin *et al.,* 1999); *Mycobacterium tuberculosis* (Delogu *et al.,* 2000); *Plasmodium chabaudi* (Smooker *et al.,* 2000); *Leishmania donovani* (Ghosh *et al.,* 2001); *Leishmania major* (Méndez *et al.,* 2001; Iborra *et al.,* 2004); *Plasmodium falciparum* (Qazi *et al.,* 2005); *Pseudomonas aeruginosa* (Saha *et al.,* 2006), rabies (Cupillard *et al.,* 2005); feline leukaemia virus (O' Donovan *et al.,* 2005) and human immuno-deficiency virus (HIV) (Jiang *et al.,* 2006). Several *E. ruminantium* DNA vaccination experiments using genes including *map*1 and *gro*EL/ES have been carried out using a murine model (Brayton *et al.,* 1998; Nyika *et al.,* 1998; Pretorius *et al.,* 2002). In a previous study done in our laboratory, eleven open reading frames (ORFs) were identified in a cosmid clone containing an *E. ruminantium* DNA fragment (Collins *et al.,* 1998) designated 1H12 (Figure 2.1). Four of these ORFs (ORF 2, 3 5 and 6) were shown to induce partial protection (50-87 %) in mice against homologous challenge when used as part of a DNA vaccine (Collins *et al.,* 2003a; 2003b).

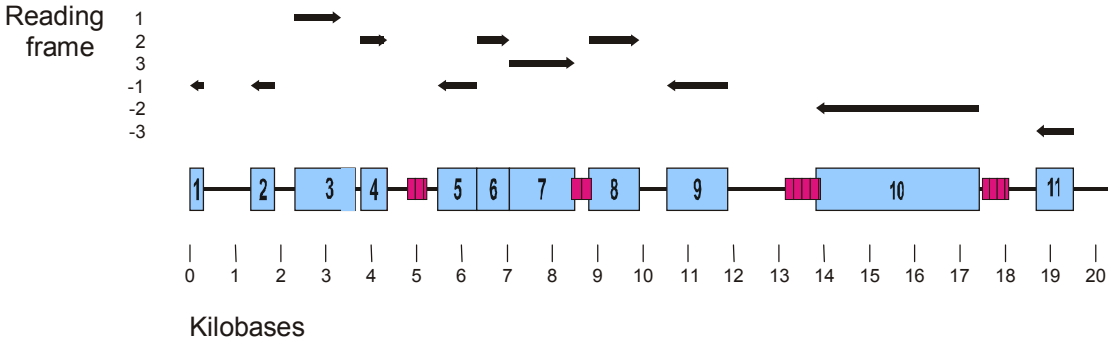


Figure 2.1: *Schematic representation of the ORFs coding for more than 90 amino acids identified in the sequence of the cosmid clone, 1H12. Blue boxes represent ORFs and the arrow above each ORF indicates its orientation. Long tandem repeats are represented by pink boxes. ORFs 2, 3, 5, and 6 were used as the 1H12 vaccine cocktail*

The four 1H12 ORFs are predicted to encode an exported protein (Erum2540 (ORF2)), two proteins that code for the adenosine triphosphate (ATP) -binding protein part of ABC transporters (Erum2590 (ORF6) and Erum2550 (ORF3)) possibly involved with import of ferric cations or zinc uptake, and a protein associated with ABC transporters (Erum2580 (ORF5)) known as a periplasmic solute binding protein (SBP) (ORF numbers are taken from Collins *et al*., 2005). Exported proteins are

considered to be good vaccine candidates for intracellular bacteria even if the function of the protein is unknown. These proteins could be exported into the endothelial cell cytoplasm where they can be presented via MHC II on the infected host cell, therefore making them useful targets for host immune responses. ABC transporters consist of two domains: an ATPase domain that binds and hydrolyses ATP for energy and a transmembrane domain consisting of up to six alpha helices that form a channel through which allocrytes are transported. Allocrytes include sugars, amino acids, peptides, polyamines, metal ions, sulfonate, iron, zinc and molybdate (Lewis *et al*., 1999; Danese *et al*., 2004 and reviewed by Garmory and Titball, 2004). Bacterial ABC transporters in the inner membrane require help from proteins known as periplasmic SBPs that bind to and direct the allocrytes towards the transporter. Transport through the outer membrane also requires additional functional proteins such as porins (reviewed by Garmory and Titball, 2004). The two *E. ruminantium* ATP-binding protein parts of ABC transporters and the SBP could be good vaccine candidates because transporters and co-proteins associated with metal uptake (also known as MET family) perform a vital function in the uptake of metal ions required for bacterial growth. Removing or blocking the function of the ABC MET transporters has been shown to have detrimental effects on bacterial growth and virulence (reviewed by Garmory and Titball, 2004) and ABC transporters have been shown to be effective targets for vaccination against *Staphylococcus aureus* and *Enterococcus faecium* infection (Burnie *et al.,* 2000; 2002).

It is known that vaccine trials using the mouse model for heartwater are not reproducible (Collins *et al.,* 2003a). The purpose of the work reported in this chapter is therefore to determine whether the increased protection observed in mice after DNA immunisation using a cocktail of the four 1H12 ORFs as a DNA vaccine, will also be induced in a ruminant host. In this study sheep will be used as the ruminant host. The results obtained with the inoculation of sheep with a cocktail consisting of the four 1H12 ORFs will be compared with those obtained from a cocktail of randomly selected *E. ruminantium* ORFs, to ensure that protection is not due to non-specific activation of a T_{H1} immune response by bacterial DNA. Lymphocyte proliferation assays will be performed to confirm expression of the 1H12 protein products and activation of a recall cellular immune response in the host.

2.2. MATERIALS AND METHODS

2.2.1. Materials

See appendix A for materials and buffer components.

2.2.2. Methods

2.2.2.1. Sequence analysis of an *E. ruminantium* Welgevonden LambdaGEM-11 phage clone

A LambdaGEM-11 phage clone (WL2AP1) was previously isolated from a large insert *E. ruminantium* Welgevonden phage library by screening the library with a ~100 kbp *Ksp* I *E. ruminantium* Welgevonden fragment. Briefly, *E. ruminantium* genomic DNA was digested with *Ksp* I yielding five fragments (De Villiers *et al.,* 2000). The largest of these fragments was used to make a probe, using a random prime labelling kit (Amersham), to detect *Ksp* I specific clones in a LambdaGEM-11 library. Several positive clones were identified and one, designated WL2AP1, was chosen to continue with sequence analysis.

2.2.2.1.1. Increasing phage titre

The titre of the isolated phage was increased by adsorbing 200 µl of phage lysate to 300 µl LE392 cells (grown to OD_{600} of 0.6–0.8). The phage/bacteria mix was incubated at 37 °C for 20 min, melted top agarose was added, and the mixture was poured onto pre-warmed LB agar plates which were incubated overnight at 37 °C. New phage lysate was prepared by soaking the top agarose with 5 ml SM buffer for at least 4 h at 4 °C. The SM buffer was removed and stored in the presence of 100 µl chloroform. This process was repeated using the newly prepared lysate until confluent phage lysis was obtained.

2.2.2.1.2. Determination of the titre of the final phage lysate

A ten-fold dilution range of the phage lysate was prepared in LB broth. Ten microliters of each dilution was added to 300 µl LE 392 cells (grown to an OD_{600} of 0.6–0.8) and plated in the presence of top agarose onto LB agar plates. Phage titre was determined by plaque counting after overnight incubation.

2.2.2.1.3. Isolation of phage genomic DNA

The pellet of an overnight culture of *E. coli* LE392 cells, grown in 50 ml LB broth supplemented with 0.2 % maltose and 10 mM $MgSO_4$, was resuspended in 10 ml 10 mM $MgSO_4$ and adjusted to an OD_{600} of 0.5 to give a concentration of approximately 10^9 *E. coli* cells/ml. Phage particles were added to obtain a multiplicity of infection (number of phage particles over number of bacterial cells) of 0.002. The phage was allowed to adsorb to the bacterial cells for 20 min at 37 °C. 100 ml LB broth supplemented with 10 mM $MgSO_4$ was inoculated with the phage/bacteria mix and the culture was allowed to grow at 37 °C. The phage growth was monitored every hour at OD_{600} until a reduction in optical density of 0.2 was observed. The cells were lysed by the addition of 100 µl of chloroform and cell debris was removed by centrifugation (~4500 *g*, 10 min, 4 °C). Phage DNA was isolated from the lysate using the Nucleobond® AX kit according to the instructions of the manufacturer.

2.2.2.1.4. Amplification of the E. ruminantium *insert*

The *E. ruminantium* insert from WL2AP1 was amplified using a long template *Taq* polymerase, TaKaRa La Taq™ (TaKaRa Bio Inc.), and SP6 and T7 primers (Table 2.1). A total of 200 ng phage DNA was added to a 100 µl reaction mixture containing final concentrations of 5 U TaKaRa LA Taq™, 250 nM of each primer, 500 µM of each dNTP and 1x reaction buffer (including 2 mM Mg^{2+}). The samples were denatured for 2 min at 94 °C, followed by 10 cycles of denaturation (94 °C for 10 s), annealing (46 °C for 30 s), and extension (68 °C for 15 min), this was followed by a further 15 cycles of denaturation (94 °C, 10 s), annealing (46 °C, 30 s), and extension (68 °C, 15 min increasing with 20 s every cycle) using a GeneAmp® PCR System 9700 amplifier (Applied Biosystems).

2.2.2.1.5. Nebulising and cloning of the amplified fragment

The amplified DNA was fragmented using a Medel jet nebuliser reservoir (Medel, Italy). A total of 25 µg amplified DNA dissolved in 2 ml TE buffer was nebulised using nitrogen at 100 kPa for 5 min. The nebulised DNA was run on a 2 % agarose gel and fragments in the 600-1500 bp size range were selected using a TaKaRa recochip (TaKaRa Bio Inc) according to the manufacturer's instructions. The ends of

the fragments were repaired using a Klenow fill-in kit from Stratagene (Stratagene cloning systems), the fragments were purified using a Qiagen mini elute kit (Qiagen Ltd), and cloned into the pMOS*Blue* plasmid vector using a blunt ended cloning kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Colonies containing insert were picked and grown overnight in LB broth containing ampicillin (50 µg/ml). Plasmid DNA was isolated using a plasmid isolation kit (Roche) and inserts were sequenced using an ABI 377 automated sequencer. The sequences were assembled and analysed using gap4 (Staden *et al.,* 1998). ORFs were identified using ORF-finder (Wheeler *et al.,* 2003) and putative functions were assigned by searching against known genes in the public nucleotide databases using BLAST (Altschul *et al*., 1997).

2.2.2.2. Identification of CpG islands

CpG islands were identified in the *E. ruminantium*-derived ORFs by using EMBOSS: cpgplot (http://www.ebi.ac.uk/emboss/cpgplot). A putative CpG island was defined as a region where the GC content was over 30 %, the calculated observed/expected ratio was over 0.6 and the conditions held for a minimum of 50 bases.

2.2.2.3. Cloning of ORFs into pCMViUBs vector

Selected ORFs identified from the WL2AP1 sequence were amplified from *E. ruminantium* genomic DNA and PCR products were cloned into the DNA vaccine vector, pCMViUBs (Sykes and Johnston, 1999).

2.2.2.3.1. Purification of E. ruminantium *genomic DNA*

E. ruminantium Welgevonden was grown in bovine endothelial cells until 80-100 % lysis was achieved. Cells were scraped off the walls of the tissue culture flasks and cell debris and nuclei were removed by centrifugation of the scraped cell material at 300 *g* for 15 min at 4 °C. The *E. ruminantium* elementary bodies (EBs) were then isolated by a second centrifugation step, where the supernatant was centrifuged for 30 min at 30 000 *g* at 4 °C. The pellet was then resuspended in 1 ml 1x PBS by pipette aspiration and passed through a 26-28 gauge needle several times to break up aggregates. The EBs were further purified by discontinuous Percoll density gradient centrifugation (Mahan *et al*., 1995). EBs in the upper 15 ml of the Percoll gradient

were isolated by another 30 min centrifugation step at 30 000 *g*. The final pellet was again resuspended in 1x PBS by pipette aspiration and by passing the pellet several times through a 26-28 gauge needle. The pellet was washed once with 30 ml 1x PBS and pelleted again by centrifugation at 30 000 *g*. The final pellet was resuspended in 1 ml 1x PBS.

Contaminating bovine DNA and RNA were removed by incubating the EBs in a mixture containing 10 µl RNAse (10 mg/ml in water) and 150 µl DNAse I (1 mg/ml in TM buffer) for 1.5 h at 37 °C. The reaction was stopped by adding 25 µl 0.5 M EDTA, pH 8. The EBs were pelleted by centrifugation (15 min, 30 000 *g* at 4 °C) and the pellet was washed twice with 1 ml dH_2O. The final EB pellet was resuspended in 50 µl dH_2O and the EBs were lysed by adding lysis buffer (1/10 of the final reaction volume) and incubating the solution overnight at 56 °C.

The genomic DNA was isolated by two equal volume phenol extractions, followed by a phenol: chloroform: isoamyl alcohol (IAA) extraction (25:24:1) and a chloroform: IAA extraction (24:1). The DNA was precipitated by addition of 100 % ethanol and 3M NaOAc pH 4, washed with 70 % ethanol and resuspended in TE buffer. The concentration and purity was determined using a spectrophotometer (Beckman DU® 640). Possible bovine DNA contamination was determined as described by Brayton *et al*. (1997b). Briefly, serial dilutions of genomic bovine DNA and the *E. ruminantium* genomic DNA were probed with bovine genomic DNA labelled with [α-^{32}P]-dATP and band intensities were compared. A bovine content of less than 10 % was accepted.

2.2.2.3.2. Cloning of ORFs into pCMViUBs vector

Selected ORFs were amplified from genomic DNA using ORF specific primers that contained restriction enzyme sites to facilitate directional cloning (Table 2.1). The PCR was performed using TaKaRa Ex Taq™ (TaKaRa Bio Inc.); a 100 µl reaction mixture contained purified genomic DNA (~100 ng), 200 µM dNTPs, 250 nM of each primer and 0.5 U *Taq* polymerase in 1x reaction buffer. Each ORF was amplified at the specific annealing temperature shown in Table 2.1. The samples were denatured for 2 min at 94 °C, followed by 30 cycles of denaturation (94 °C for 10 s), annealing

(as indicated in Table 2.1 for 30 s), and extension (68 °C for 15 min) using a GeneAmp® PCR System 9700 thermocycler (Applied Biosystems). Initial attempts to digest the PCR products and clone them directly into pCMViUBs failed. The PCR products were therefore cloned into pMOS*Blue* using a blunt ended cloning kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions, and their sequences were confirmed. The inserts were then cut out of the pMOS*Blue_ORF* plasmids using *Bam*HI and *Xba*I (Roche). The pCMViUBs vector (see Figure 2.2 for vector map) was digested with the same enzymes and dephosphorylated using shrimp alkaline phosphatase (Promega).

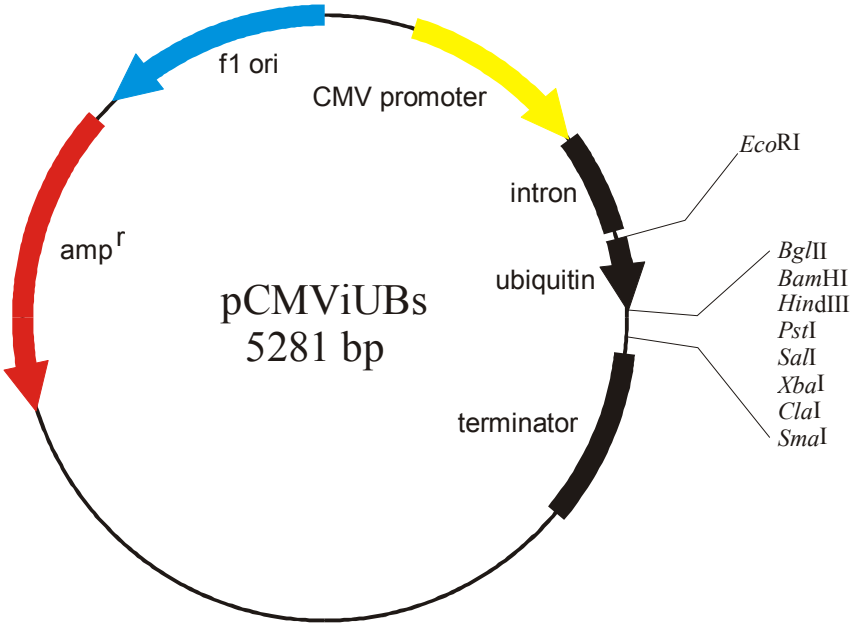


Figure 2.2: *Schematic representation of the pCMViUBs vector used for DNA immunisation.* E. ruminantium *ORFs were amplified and cloned into the cloning site in the correct orientation by incorporating specific restriction enzymes in the amplification primers.*

The ORF inserts and prepared pCMViUBs vector were purified by agarose gel electrophoresis and isolated from the gel using TaKaRa recochips (TaKaRa Bio Inc.). The inserts were ligated into linearised, dephosphorylated vector using 1 U T4 ligase. The ligation reaction was electroporated into pMOS *E. coli* cells using the Gene pulser® II (BIORAD), plated onto LB agar plates containing 50 µg/ml ampicillin and incubated overnight at 37 °C. Positive clones were grown overnight in LB broth containing 50 µg/ml ampicillin at 37 °C, and plasmid was isolated using the Roche

mini-prep kit. An aliquot of each plasmid was digested with *Bam*HI and *Xba*I to verify presence of insert. Clones with correctly-sized inserts were sequenced to confirm that their sequences were correct and that the ORFs were in-frame.

Table 2.1: *Primer sequences used for the amplification and cloning of WL2AP1 ORFs into the pCMViUBs vector.*

Primer name	Primer sequence (5' → 3')	T(a)[1]	RE[2]
T7	CTAATACGACTCACTATAGG		-
SP6	CCATTTAGGTGACACTATAG	45 °C	-
Erum7450 F	CGCGGATCCATGGAGGTTATTTTGAGTAGG		*Bam*HI
Erum7450 R	GATCTCTAGATTAGTTTACATACTTTTTATTTTTATATTTTTAG	58 °C	*Xba*I
Erum7490 F	CGCGGATCCATGAGCAATTATCAAAACATTGGG		*Bam*HI
Erum7490 R	GATCTCTAGATTAATGAAAAAATTGCTCATTAATAATTC	52 °C	*Xba*I
Erum7510 F	CGCGGATCCATGCTATTTCATACATTAGATATAAATC		*Bam*HI
Erum7510 R	GATCTCTAGATTATGTTAGTAACCAGTTTTGTATATC	52 °C	*Xba*I
Erum7530 F	CGCGGATCCATGAAAAATTTAGTAGTAG		*Bam*HI
Erum7530 R	GATCTCTAGACTAGCGTTTCTTTTTATTG	45 °C	*Xba*I

[1] *Annealing temperature used for each primer set.*

[2] *Restriction enzyme sites incorporated into the primers.*

2.2.2.4. Immunisation of animals

2.2.2.4.1. E. ruminantium *stock used for challenge material*

All animals were challenged with the virulent *E. ruminantium* Welgevonden stock originating in South Africa (Du Plessis, 1985).

2.2.2.4.2. Challenge material

Blood stabilate was prepared from an *E. ruminantium* Welgevonden infected animal as described previously (Brayton *et al.,* 2003). The infected sheep blood was diluted (1:1, v/v) in sucrose potassium glutamate buffer (SPG) (Bovarnick *et al.,* 1950) and stored in liquid nitrogen. The stabilate was titred to determine the optimum dose (10 LD_{50}s) prior to challenge.

2.2.2.4.3. Large-scale plasmid DNA preparation

Large-scale plasmid isolation was performed using the Nucleobond PC 2000 kit, according to the instructions of the manufacturer. The plasmid DNA was diluted to a final concentration of 1 µg/ml in 1x PBS and stored at –20 °C. A sample of each plasmid containing an *E. ruminantium* ORF was sequenced prior to immunisation.

2.2.2.4.4. Immunisation of sheep

Six to eight month old Merino sheep were obtained from heartwater-free areas of South Africa (SA). All sheep used in these experiments tested negative for *E. ruminantium* using a modified pCS20 probe test (Van Heerden *et al.,* 2004). Sheep were divided in groups of 5 animals per group. Each animal received DNA via i.m. inoculations into the left and right quadriceps and tibialis anterior muscles and via i.d. gene gun delivery of the same DNA. Gene gun inoculation was delivered to the lateral aspect of the pinna using the OPgun™ (Brayton *et al.,* 1997a). Before inoculation, the ear surface was depilated with Immac™ to remove hair and dead skin. DNA was precipitated onto gold beads as described previously (Brayton *et al.,* 1997a; Collins *et al.,* 2003a; 2003b). The animals were immunised three times at three-week intervals. All animals were challenged five weeks after the final boost with the virulent *E. ruminantium* Welgevonden stock (10 LD_{50}s). The animals were monitored for onset of clinical symptoms after challenge and rectal temperatures were taken daily from the start of the experiment. The severity of infection was determined by scoring the clinical signs according to a reaction index (RI) as described by Collins *et al*. (2003a) (Table 2.2) with one modification: animals that were treated were also regarded as non-survivors and were therefore given a score of 50 identical to that of animals that were euthanased. Sheep were treated with 200 mg oxytetracycline (Liquamycin/LA, Pfizer) per 10 kg body mass once elevated temperatures were combined with severe heartwater symptoms that included hyperesthesia, lacrimation and convulsions, except for trial 2 (2.2.2.4.6) where the sheep were euthanased *in extremis* using 200 mg sodium pentobarbitone (Eutha-Nase, Centaur) per kg body mass.

***Table 2.2**: Reaction index for sheep challenged with* E. ruminantium *(Modified from Collins et al., 2003a).*

	Indicator		Score (points)
	Before challenge:		
1	Record temp for each sheep for 10 days prior to challenge Determine average temperature for each sheep		
2	**After challenge:**		
	Temperature:	For every 1 °C above the average temp	+1 per day
	Early Symptoms:	Loss of appetite, heavy breathing, hanging head, stiff gait, depression, exaggerated blinking and chewing movements with anorexia	+5 per day
	Severe Symptoms:	Hyperesthesia, lacrimation, convulsions, nervous symptoms and animal cannot stand on its own.	+10 per day
	Treatment:	i.m. or i.v. treatment	+50
	Death / euthanasia:		+50

2.2.2.4.5. DNA vaccine trial 1: test the 1H12 cocktail in a ruminant host

The group of sheep inoculated with the 1H12 cocktail (Table 2.3) received a total of 200 µg of DNA via i.m. inoculation comprising a mixture of the four ORFs (50 µg each) and also received 20 µg of DNA via gene gun inoculation (5 shots of each ORF containing 1 µg DNA per shot). The survival of this group was compared to a positive challenge control group immunised with the Welgevonden isolate using the conventional infection and treatment method and a negative DNA control group inoculated with empty pCMViUBs vector (200 µg i.m. and 20 µg i.d.).

2.2.2.4.6. DNA vaccine trial 2: comparison of the 1H12 DNA vaccine with WL2AP1 DNA cocktail

The survival of sheep inoculated with the 1H12 cocktail was compared to that of sheep inoculated with the WL2AP1 cocktail. The groups inoculated with the 1H12 and WL2AP1 cocktails again received a total of 200 µg of DNA via i.m. inoculation comprising a mixture of the four ORFs (50 µg each) and also received 20 µg of DNA via gene gun inoculation (5 shots of each ORF containing 1 µg DNA per shot). The survival of sheep in these two groups was compared to a positive challenge control

group immunised with the Welgevonden isolate using the conventional infection and treatment method and a negative DNA control group inoculated with empty pCMViUBs vector (200 µg i.m. and 20 µg i.d.) (Table 2.3). Animals were not treated in this trial, but were euthanased *in extremis*.

2.2.2.4.7. DNA vaccine trial 3: Immunisation with the 1H12 cocktail compared to inoculation with the individual 1H12 ORFs

Five experimental groups were inoculated as follows: Group 1 was immunised with the 1H12 cocktail as described in 2.2.2.4.6 (Table 2.3). The remaining four groups were each inoculated with one of the four 1H12 ORFs. Each animal inoculated with a single ORF received a total of 200 µg of the ORF-plasmid DNA i.m. and 20 µg via gene gun inoculation. The survival of sheep in these groups was compared to negative challenge control, positive challenge control and negative DNA control groups as described above.

Table 2.3: *Immunisation strategy for the three animal trials described in this chapter.*

Experiment	Group	Number of animals	Inoculated with	Challenge
DNA vaccine trial 1 (2.2.2.4.5.)	Positive control	5	Infected and treated (Welgevonden)	Welgevonden
	Negative control	5	Empty pCMViUBs vector (pCMViUBs)	Welgevonden
	Experimental 1	5	1H12 DNA vaccine cocktail (pCMViUBs_1H12 ORFs)	Welgevonden
DNA vaccine trial 2 (2.2.2.4.6.)	Positive control	5	Infected and treated (Welgevonden)	Welgevonden
	Negative control	5	Empty pCMViUBs vector (pCMViUBs)	Welgevonden
	Experimental 1	5	1H12 DNA vaccine cocktail	Welgevonden
	Experimental 2	5	WL2AP1 DNA vaccine cocktail	Welgevonden
DNA vaccine trial 3 (2.2.2.4.7.)	Positive control	3	Infected and treated (Welgevonden)	Welgevonden
	Negative control	5	Empty pCMViUBs vector (pCMViUBs)	Welgevonden
	Experimental 1	5	1H12 DNA vaccine cocktail	Welgevonden
	Experimental 2	5	pCMViUBs_Erum2540	Welgevonden
	Experimental 3	5	pCMViUBs_Erum2550	Welgevonden
	Experimental 4	5	pCMViUBs_Erum2580	Welgevonden
	Experimental 5	5	pCMViUBs_Erum2590	Welgevonden

2.2.2.4.8. Sterilisation of gold beads

Gold beads were obtained from Degussa Corporation, USA. Beads (100 mg) were rinsed in 100 % ethanol at room temperature for 15 min and then washed three times with dH_2O (vortexed to mix, and pelleted by centrifugation for 15 min at 16 000 *g*). The final pellet was suspended in 1 ml sterile 50 % glycerol.

2.2.2.4.9. Precipitation of DNA onto gold beads

The plasmids were precipitated onto gold beads before each inoculation. DNA solution (1 mg/ml) was added to gold bead suspension (100 mg/ml) plus 2.5 M $CaCl_2$ and 0.1 M spermidine (Sigma) in a ratio of 2.5: 1: 2.5: 1 (Gold: DNA: $CaCl_2$: spermidine) while vortexing. The mixture was incubated on ice for 5 min, the gold beads were then pelleted by brief centrifugation and enough of the supernatant was removed to yield a final DNA concentration of 500 ng/µl. Each animal received 2 µl of bead suspension per shot (1 µg DNA in total per shot). Leftover beads (2 µl of the bead suspension i.e. 1 µg of DNA in total) were loaded on a 1 % agarose gel together with a sample of the 1 mg/ml DNA stock solution (1 µl i.e. also a total of 1 µg DNA) to ensure that the precipitation was successful. The precipitation was considered successful if the band densities of the stock DNA solution correlated with DNA precipitated on the beads. Band density was measured using Lumi-Imager (Roche) software (results not shown).

2.2.2.4.10. Lymphocyte proliferation assays

Peripheral blood mononuclear cell (PBMC) lymphocyte proliferation assays were performed before immunisation, and once a week after the third inoculation, until challenge, as described previously (Van Kleef *et al.,* 2000). Briefly, after ficoll purification of PBMC, a single cell suspension was prepared in RPMI 1640 medium supplemented with 10 % heat inactivated FCS, 2 mM L-glutamine (Sigma), 50 µM 2-mercaptoethanol (Sigma), 0.1 mg/ml sodium benzylpenicillin (Novopen, Nova Nordisk) and 0.2 mg/ml streptomycin sulphate (Novostrep, Nova Nordisk). Proliferation assays were carried out in triplicate in half-area 96-well plates (Costar) at 37 °C in a humidified atmosphere supplemented with 5 % CO_2. In a total volume of 100 µl, 2 x 10^5 PBMCs/well were incubated with partially purified *E. ruminantium* antigen isolated from infected bovine endothelial cells (1 µg/well, positive antigen) or with uninfected bovine endothelial cell extract (1 µg/well, negative antigen). The *E. ruminantium* antigen was prepared by differential centrifugation as described previously (Van Kleef *et al.,* 2000). Negative control wells contained PBMC without antigen and positive control wells contained PBMCs stimulated with Concanavalin A (ConA) (5 µg/ml, Sigma). The cultures were incubated for 72 h and pulsed with 1 µCi/well of [^{3}H] thymidine (Amersham) for the last 6 h of the incubation period.

Cells were harvested onto a 96 well glass fibre filter (Wallac) and the [^{3}H] thymidine uptake was counted after addition of 5 ml scintillation solution (Ultima gold F, Packard BioScience) using a Trilux 1450 Microbeta liquid scintillation counter (Wallac).

Results were expressed as Stimulation Index (SI) (counts per minute (cpm) of positive antigen divided by cpm of negative antigen) averaged from triplicate wells ± the standard deviation. A SI greater than 2 was considered positive.

2.2.2.5. Statistical analysis

Reaction index scores and lymphocyte proliferation results were compared by means of the one tailed distribution Student's *t*-test. Significance was assessed at P values of < 0.01 throughout the study.

2.3. RESULTS

2.3.1. Identification of CpG islands

All eight ORFs derived from 1H12 and WL2AP1 had a low GC content ranging from 26.04 - 31.78 % (Table 2.4). Putative CpG islands with a minimum of 30 % GC content were identified in the 1H12 and the WL2AP1 ORFs and the putative CpG islands in these ORFs were similar in number and size (Table 2.4).

Table 2.4: *Determination of the GC content and the prediction of putative CpG islands of the ORFs used in the 1H12 and WL2AP1 cocktails.*

Cocktail	ORF number [1]	% GC content[2]	Number of putative CpG islands predicted by EMBOSS: cpgplot
1H12	Erum2540	30.11	1
	Erum2550	31.28	2
	Erum2580	26.04	1
	Erum2590	26.88	1
WL2AP1	Erum7450	30.89	3
	Erum7490	31.06	1
	Erum7510	28.13	1
	Erum7530	31.78	2

[1] *ORF numbers are taken from Collins et al. (2005).*

[2] *% GC content determined from the annotated genome sequence.*

2.3.2. DNA immunisation of sheep using a 1H12 cocktail

Four *E. ruminantium* ORFs were previously cloned into the pCMViUBs DNA vaccine vector, and tested for their ability to induce protection against a virulent *E. ruminantium* Welgevonden needle challenge in mice. Although the experiments were not reproducible, these four ORFs did provide some protection resulting in 50 % to 87 % survival (Collins *et al.,* 2003a; 2003b). The protein products of these four ORFs showed homology to two ABC transporters (Erum2590 and Erum2550) possibly involved with import of ferric cations and zinc uptake, an exported protein (Erum2540) and a protein associated with ABC transporters (Erum2580) known as a periplasmic solute binding protein.

In order to investigate if these ORFs would confer protection in a ruminant host, sheep were immunised with a cocktail of the four 1H12 ORFs (Table 2.3). Survival of immunised sheep was compared to infected and treated sheep (positive controls) as well as to sheep inoculated with empty pCMViUBs vector (negative controls).

All the sheep immunised with the 1H12 cocktail survived needle challenge with virulent *E. ruminantium* Welgevonden (Figure 2.3). These sheep showed elevated temperatures (Figures 2.4-2.6) and some of the early heartwater symptoms including heavy breathing (Figure 2.4), but continued to eat normally. Progression of the disease to the more severe symptoms was not observed and these animals recovered without treatment. The reaction of the sheep immunised with the 1H12 cocktail differed significantly ($P < 2 \times 10^{-6}$, Table 2.5) from that of the negative control group that showed severe symptoms and elevated temperatures above 41.8 °C (Figures 2.4-2.6). Sheep in the group that received empty pCMViUBs vector had to be treated up to three times in succession before they recovered from the infection. One of the animals did not respond to treatment and was euthanased to avoid prolonged suffering (sheep number 282). Animals in the positive control group survived and showed little or no reaction after challenge, except for a mild temperature reaction (Figures 2.4-2.6). The average RI of the 1H12 cocktail group was also significantly different from the positive control group ($P < 9 \times 10^{-5}$) (Table 2.5). Although the sheep were protected, the immunity appeared to be different from that engendered by infection and treatment, since they did show early heartwater symptoms.

This experiment was repeated as a control for all the following experiments and the animals immunised with the 1H12 cocktail were consistently protected against needle challenge.

***Table2.5**: Statistical analysis of RI values as determined for each group.*

Group	Sheep number	RI_{total}	RI_{AVE}	RI_{STDEV}	P^1 value compared to	
					Vector group	1H12 cocktail group
Infected and treated	67	3.8				
	119	1.6				
	161	3.8				
	239	11.3				
	251	8.6	5.82	3.99	2×10^{-5}	9×10^{-5}
Empty pCMViUBs vector	203	84.5				
	216	87.3				
	242	99.6				
	255	85.9				
	282	82.1	87.88	6.83		2×10^{-6}
1H12 cocktail	131	52.3				
	185	39.5				
	194	39.4				
	217	45.7				
	263	41.2	41.45	5.48	2×10^{-6}	

[1] *P value was determined by one tailed distribution Student's t-test.*

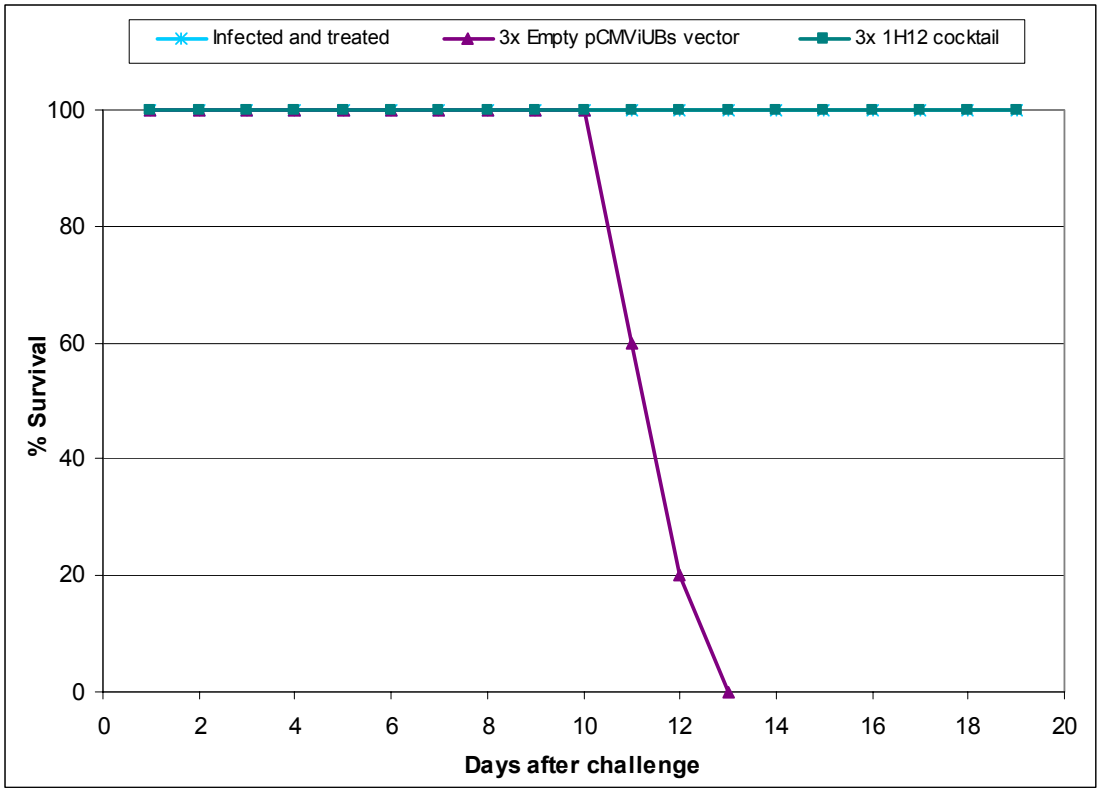


Figure 2.3: *Survival chart of animals immunised with 1H12 cocktail compared to positive and negative control groups. Animals that were treated were considered to be non-survivors.*

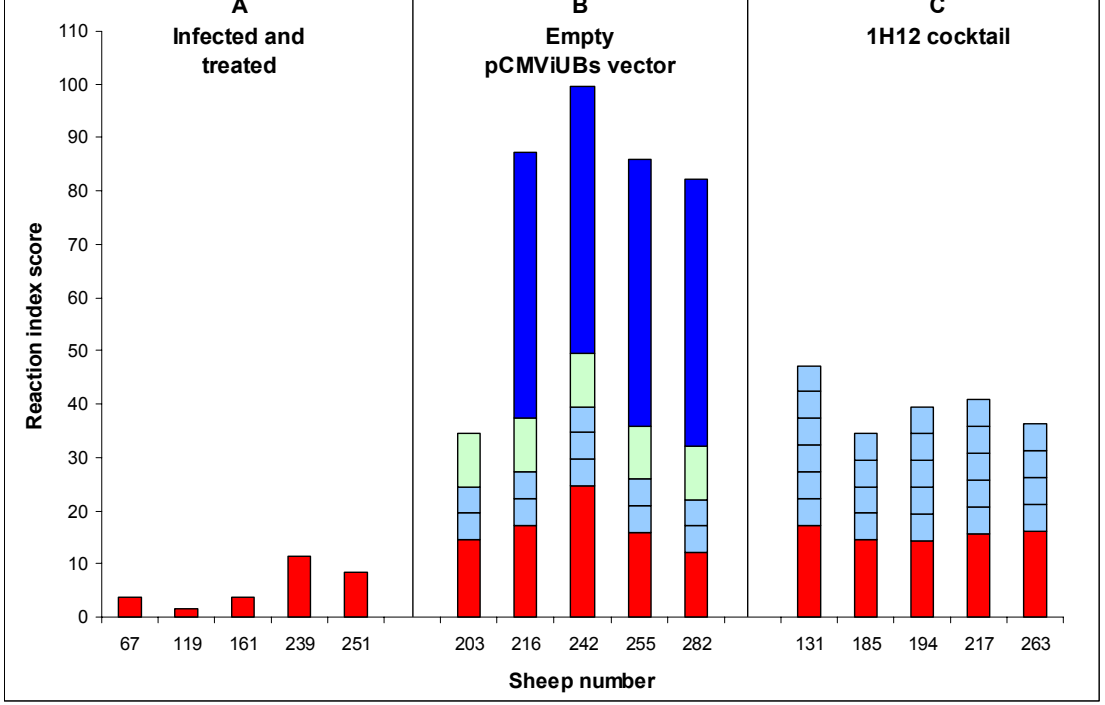


Figure 2.4: *Reaction index (RI) of the sheep immunised by infection and treatment (A), with the empty pCMViUBs vector (B) and with 1H12 cocktail (C) after challenge with virulent* E. ruminantium *Welgevonden. The red blocks indicate the total temperature reaction score 21 days after challenge, while the light blue blocks represent a RI of 5 (early heartwater symptoms measured per day), the light green a RI of 10 (severe heartwater symptoms measured for each day), and the dark blue a RI of 50 (animal was treated/ euthanased).*

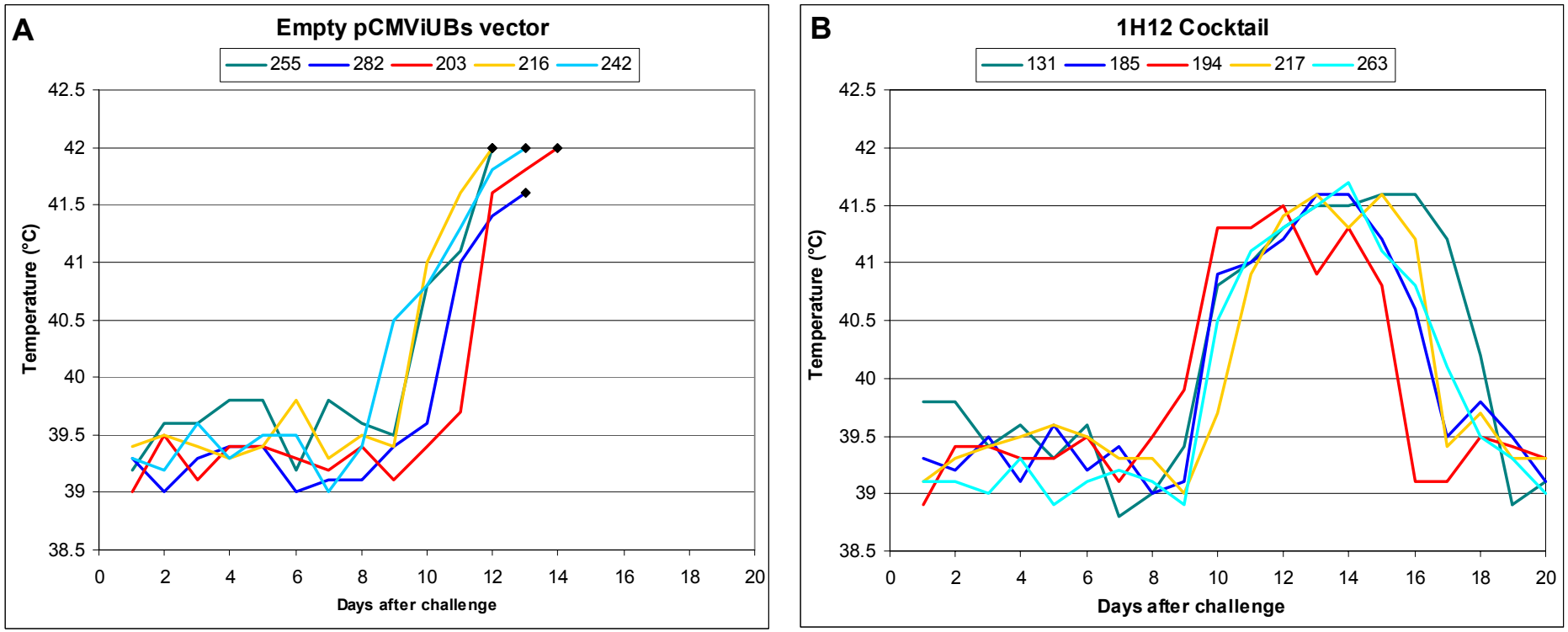


Figure 2.5: *Daily post-challenge temperatures of sheep immunised with empty pCMViUBs vector (A) and 1H12 cocktail (B). Sheep were challenged with 10 LD_{50}s of the virulent* E. ruminantium *Welgevonden isolate. Temperature measurements were terminated 20 days after challenge or as soon as the animal was treated or euthanased (represented by the black dots).*

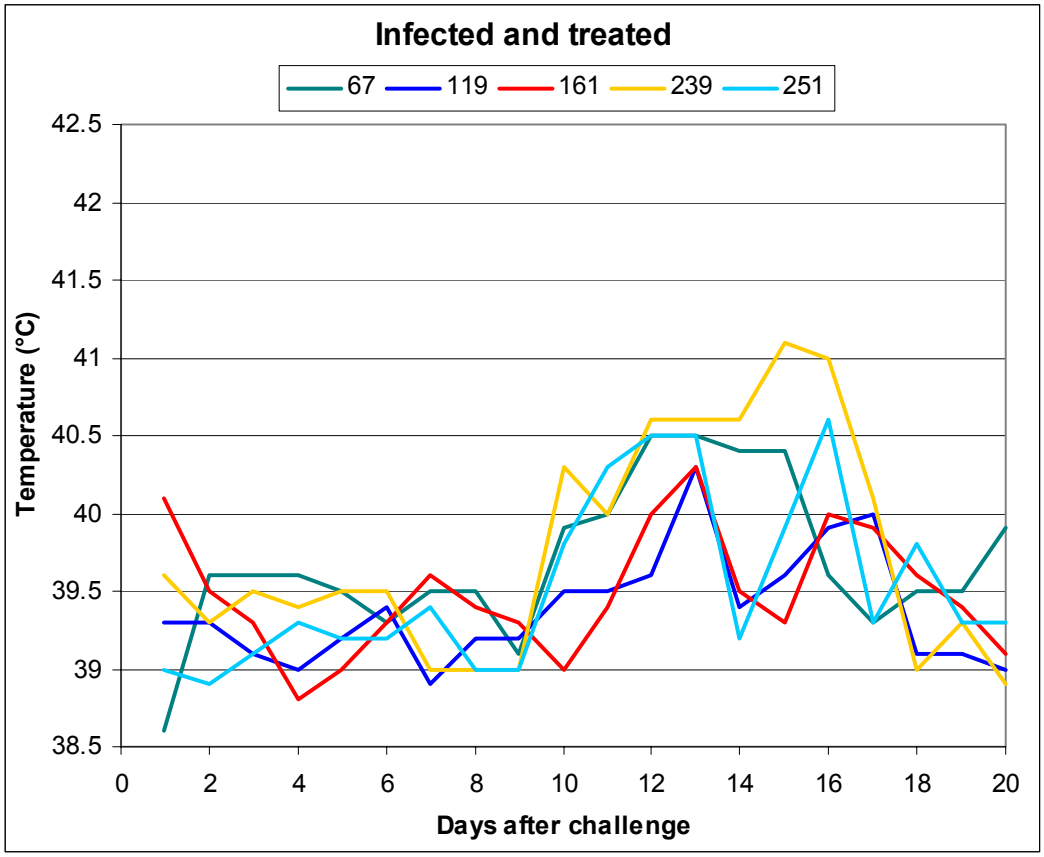


Figure 2.6: *Daily post-challenge temperatures of sheep immunised by infection and treatment. Sheep were challenged with 10 LD_{50}s of the virulent* E. ruminantium *Welgevonden isolate. Temperature measurements were terminated 20 days after challenge or as soon as the animal was treated or euthanased (represented by the black dots).*

These results indicate that a cocktail of the four *E. ruminantium* ORFs that was partially protective in mice was able to induce 100 % protection in sheep. In addition, the results were reproducible, in contrast to the variable results obtained using the mouse model.

2.3.3. DNA immunisation of sheep with 1H12 ORFs compared to immunisation with four randomly selected ORFs

Immunisation using bacterial DNA is known to activate non-specific immune responses. This occurs when unmethylated CpG motifs in bacterial DNA bind to Toll-like receptor 9 (TLR9) on host immune cells. This binding normally activates a transcriptional cascade that ultimately leads to the activation of IL-12 expression (reviewed by Takeda and Akira, 2004). Elevated levels of IL-12 can stimulate the

innate immune response to produce IFN-γ that could lead to pseudo non-specific T_{H1} protection. To ascertain that the protection against lethal *E. ruminantium* infection observed in the previous experiment was indeed due to the immune response elicited by the gene products of the ORFs, and not due to non-specific T_{H1} immune response activation, sheep were immunised with a cocktail of four randomly selected *E. ruminantium* ORFs.

These four ORFs were obtained from the sequence of a ~17 kb clone (designated WL2AP1) isolated from a LambdaGEM–11 phage library. At the time this work was done, the *E. ruminantium* genome sequence was not yet available and very few genes had been sequenced. Lambda phage clone WL2AP1 was therefore identified for sequence analysis. The clone was amplified in *E. coli* culture and phage DNA was isolated. The *E. ruminantium* insert was amplified using long template PCR, the PCR product was purified and fragmented by nebulisation. The fragments were subcloned into the pMOS*Blue* vector and sequenced.

The WL2AP1 LambdaGEM-11 clone was 16 769 bp in length and contained nine ORFs. Regions of long repeats were identified ranging from 212-237 bp (Figure 2.7 and Table 2.6).

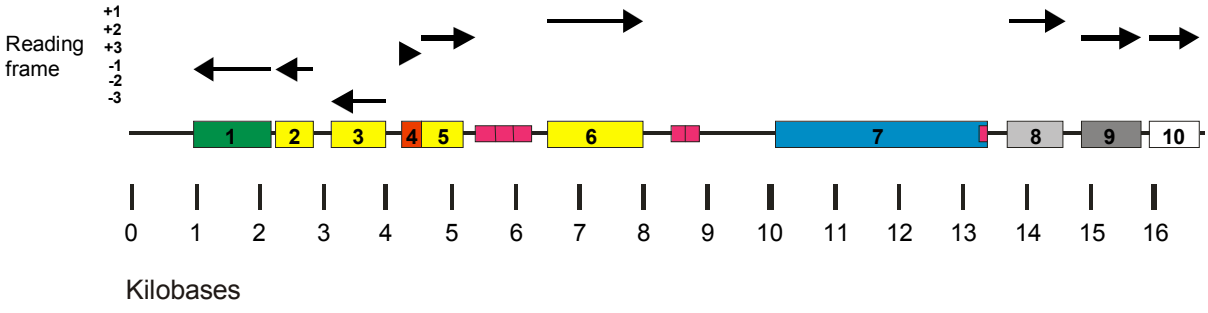


Figure 2.7: *Schematic representation of the features identified in the sequence of the WL2AP1 LambdaGEM-11 clone. Box 7 represents a ribosomal RNA gene. The other coloured boxes represent each ORF and the smaller pink boxes represent tandem repeats. The different colours are an indication of the major metabolic function in which each ORF plays a role (see Table 2.6 for detailed description of each ORF). The arrows represent the orientation and reading frame of each ORF.*

Table 2.6: *Analysis of the ORFs identified in WL2AP1. Open reading frames longer than 300 bp in length were translated and used in a PSI-BLAST search for homologies with existing proteins.*

Number (Fig. 2.7)	*Erum number*	*Size (bp)*	*Closest Homologies*	*Colour*	*Protein classification*
1	Erum7450	1227	Probable integral membrane protein	Green	Surface
2	Erum7460	606	Probable thymidylate kinase	Yellow	Central metabolism
3	Erum7470	960	Probable malonyl CoA-acyl carrier protein transacylase	Yellow	Central metabolism
4	Erum7480	225	50S ribosomal protein L31	Red	Information transfer
5	Erum7490	789	Probable inorganic polyphosphate/ ATP-NAD kinase	Yellow	Central metabolism
6	Erum7500	1455	Inosine-5'-monophosphate dehydrogenase	Yellow	Central metabolism
8	Erum7510	843	Hypothetical protein	Light grey	
9	Erum7520	987	Pyruvate dehydrogenase E1 component	Dark grey	Energy metabolism
10	Erum7530	774	Probable conjugal transfer protein	White	Pathogenicity

☐ *ORFs used for DNA immunisation.*

Four randomly selected WL2AP1 ORFs (Table 2.6; marked in green) were cloned into the pCMViUBs vector and were used to immunise sheep as described previously (Table 2.3). The survival of sheep immunised with the WL2AP1 cocktail was compared to that of sheep immunised with the 1H 12 cocktail, negative and positive controls.

Since animals immunised with the 1H12 cocktail also showed elevated temperatures after challenge in the previous experiment (Figures 2.5B and 2.6), animals were not treated with tetracycline in this experiment to rule out any bias in the decision to treat the vector group and not the 1H12 immunised animals. Prolonged suffering was prevented by euthanasia when animals had high temperature (> 41.8 °C) combined with severe symptoms including: hyperesthesia, lacrimation, convulsions, nervous symptoms and the animals were lying down.

The sheep immunised with the 1H12 cocktail again survived challenge with a lethal dose of *E. ruminantium* Welgevonden (Figure 2.8). The animals showed a temperature reaction and mild heartwater symptoms as before, but continued to act and eat normally (Figures 2.9-2.11). The animals immunised with the WL2AP1 ORF

cocktail developed severe symptoms, and the reaction indices of this group showed no significant difference ($P < 4.7 \times 10^{-2}$, Table 2.7) to the reaction indices of the negative control group immunised with empty pCMViUBs vector (Figure 2.9), although the sheep immunised with the WL2AP1 cocktail seemed to survive a little bit longer (Figure 2.8). All animals in these two groups were euthanased to prevent prolonged suffering. Sheep that were infected and treated did not show any symptoms and developed slight temperature reactions. The RI of the sheep immunised with the 1H12 cocktail differed significantly from the vector group ($P < 6.1 \times 10^{-4}$, Table 2.7) and from the sheep immunised with WL2AP1 cocktail ($P < 6 \times 10^{-5}$, Table 2.7), ruling out the possibility that the observed protective immunity was due to non-specific activation of the T_{H1} immune response by inoculation with bacterial DNA.

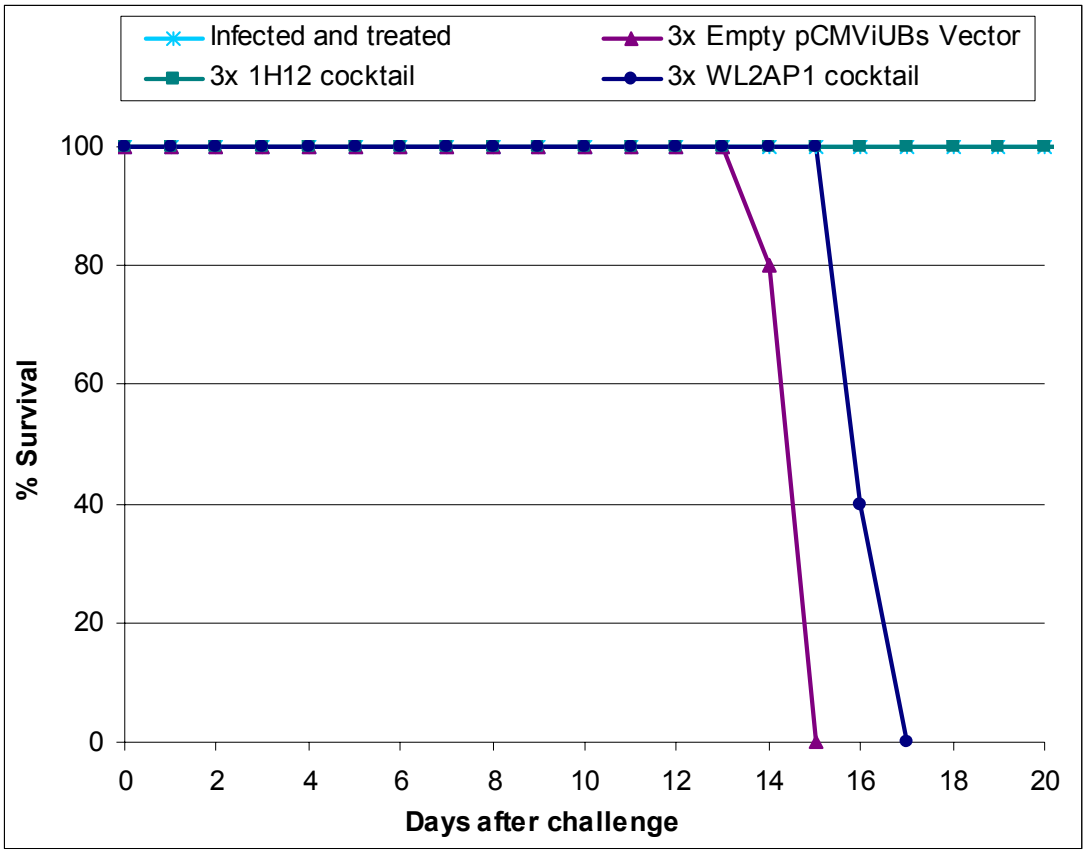


Figure 2.8: *Survival chart of sheep immunised with 1H12 cocktail compared to sheep immunised with WL2AP1 ORF cocktail, positive and negative control groups. Animals that were treated were also considered as non-survivors.*

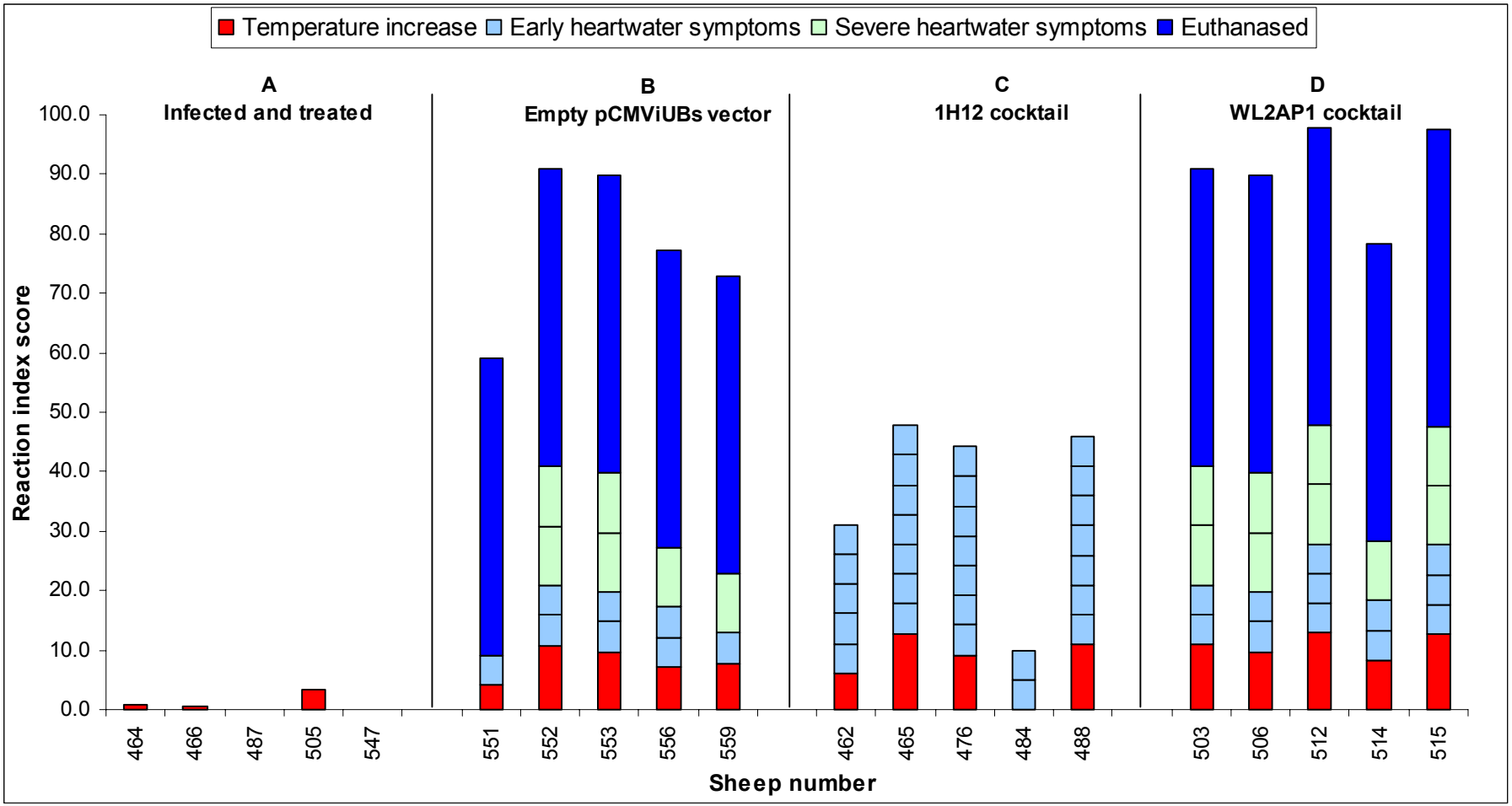


Figure 2.9: *Reaction index (RI) of the sheep immunised by infection and treatment (A), with the empty pCMViUBs vector (B), with the 1H12 cocktail (C) and the WL2AP1 cocktail (D) after needle challenge with* E. ruminantium *Welgevonden. The red blocks indicate the total temperature RI score 21 days after challenge, while the light blue blocks represent daily RI of 5 (early heartwater symptoms), the light green a RI of 10 (severe heartwater symptoms) and the dark blue a RI of 50 (animal was treated/euthanased).*

Table 2.7: *Statistical analysis of RI values as determined for each group.*

Group	Sheep number	RI_{Total}	RI_{AVE}	RI_{STDEV}	P^1 value compared to	
					Vector group	1H12 cocktail group
Infected and treated	464	0.9				
	466	0.6				
	487	0.0				
	505	3.4				
	547	0.0	0.975	1.40	9×10^{-8}	6×10^{-4}
Empty pCMViUBs vector	551	59.2				
	552	90.8				
	553	89.7				
	556	77.2				
	559	72.8	77.94	13.06		6×10^{-4}
1H12 cocktail	462	31.1				
	465	47.8				
	476	44.2				
	484	10.0				
	488	46.0	35.8	15.84	6.1×10^{-4}	
WL2AP1 cocktail	503	91.0				
	506	89.7				
	512	97.8				
	514	78.3				
	515	97.6	90.9	7.96	4.7×10^{-2}	6×10^{-5}

[1] *P value was determined by one tailed distribution Student's t-test.*

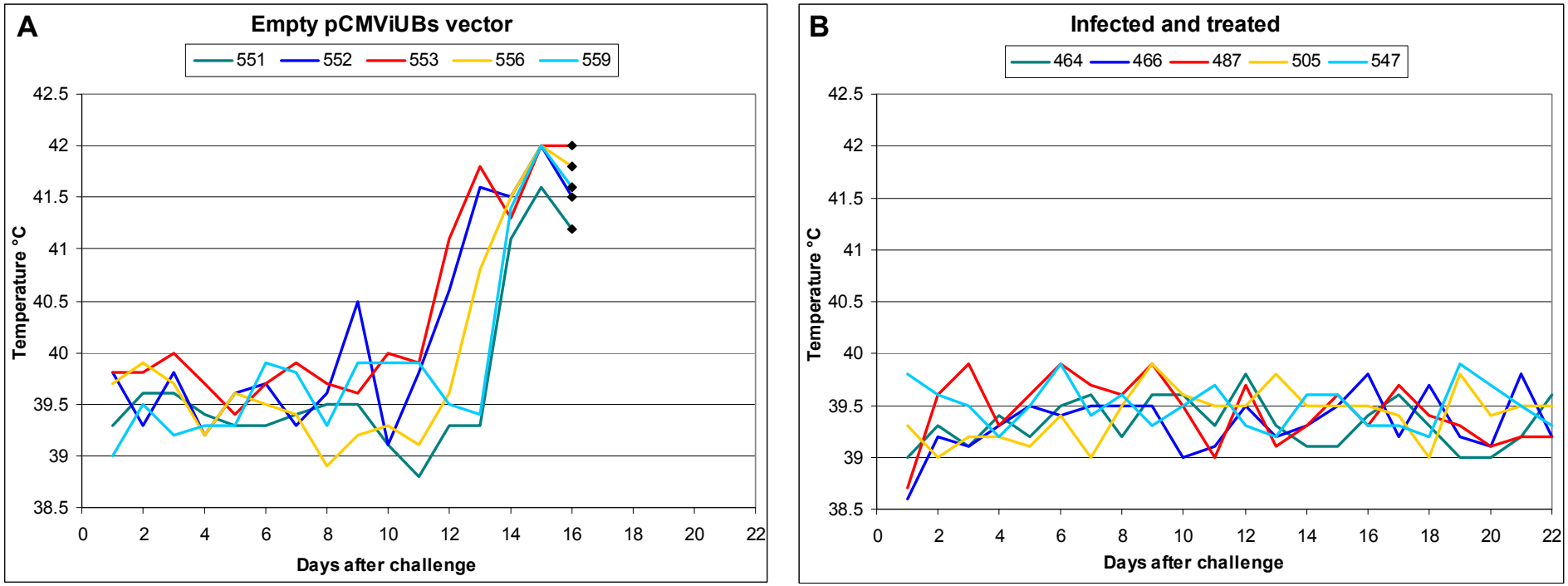


Figure 2.10: *Daily post-challenge temperatures of the negative control sheep inoculated with 3x empty pCMViUBs vector DNA (A) and the positive control animals that were infected and treated (B). Sheep were challenged with 10 LD_{50}s of the virulent* E. ruminantium *Welgevonden isolate. Temperature measurements were terminated 22 days after challenge or as soon as the animal was treated or euthanased (represented by the black dots).*

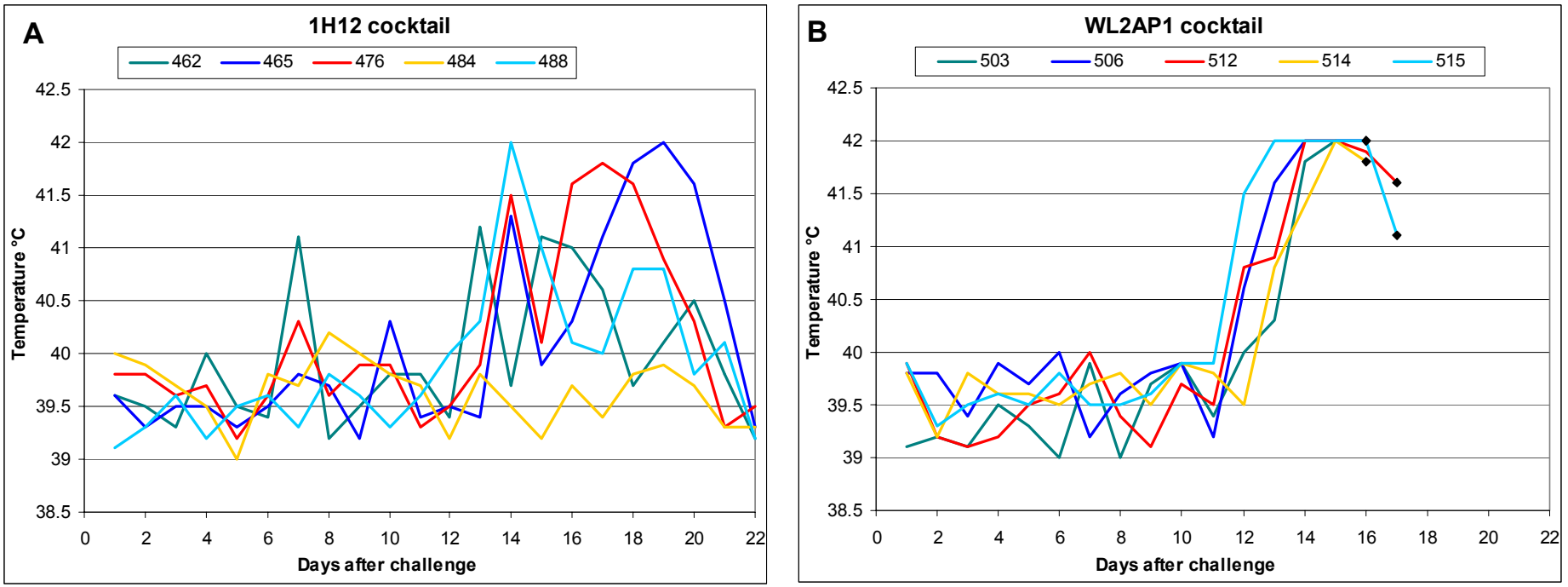


Figure 2.11: *Daily post-challenge temperatures of animals that were inoculated with the 1H12 cocktail (A) and the sheep inoculated with the WL2AP1 cocktail (B). Sheep were challenged with 10 LD_{50}s of the virulent* E. ruminantium *Welgevonden isolate. Temperature measurements were terminated 22 days after challenge as soon as the animal was treated or euthanased (represented by the black dots).*

2.3.4. Immunological assays of sheep immunised with 1H12 cocktail compared to the negative and positive controls

To further investigate the underlying immune mechanism of the protection conferred by the 1H12 ORF cocktail we performed lymphocyte proliferation assays. The assays were carried out once before immunisation and twice subsequently, at two and three weeks after the final boost. Positive proliferation results indicate that the lymphocytes were primed *in vivo* with *E. ruminantium* antigen. Thus the host cells had expressed the plasmid-derived 1H12 proteins and the sheep immune system was able to launch an immune response directed against these proteins. PBMC were isolated from the animals and stimulated in one of three ways: with Concanavalin A (as positive controls), with bovine antigen (negative controls), or with crude EB antigen containing both bovine and *E. ruminantium* antigens. The final stimulation index (SI) was determined for the PBMC of each sheep by dividing the SI obtained after immunisation (cpm of crude EB antigen divided by cpm of bovine antigen) by the SI obtained before immunisation started (cpm of crude EB antigen divided by cpm of bovine antigen).

Animals immunised three times with the empty pCMViUBs vector did not show specific proliferation to crude *E. ruminantium* antigen while 4/5 sheep PBMC showed significant proliferation two weeks after the final immunisation with the pCMViUBs_1H12 ORFs and 3/5 sheep PBMC showed proliferation after three weeks (Table 2.8). The PBMC of sheep 217 did not show proliferation at these two time points, due to high background readings determined for the naive PBMC. Although we did not see proliferation of PBMC in all of the sheep in the 1H12 vaccinated group at one single time point, this positive result was indicative of 1H12 gene product expression in the sheep host and suggests that these protein products were stimulating an immune response in the host.

E. ruminantium Welgevonden antigen. Significant proliferation is highlighted in blue (two weeks after final boost) and red (three weeks after final boost).

Group	Sheep number	SI $_{AVE}$ (SI after boost/ SI naive) Two weeks after final boost	SI $_{AVE}$ (SI after boost/ SI naive) Three weeks after final boost	P Values [1] (SI after boost compared to SI vector) Two weeks after final boost	P Values [1] (SI after boost compared to SI vector) Three weeks after final boost	Number of positive SI per group [2] Two weeks after final boost	Number of positive SI per group [2] Three weeks after final boost
Empty pCMViUBs vector (3x DNA)	203	1.5 ± 0.25	0.7 ± 0.18				
	216	1.2 ± 0.03	1.6 ± 0.63				
	242	1.4 ± 0.42	1.3 ± 0.27				
	255	1.8 ± 0.06	1.5 ± 0.21				
	282	0.8 ± 0.09	0.9 ± 0.15				
1H12 cocktail (3xDNA)	131	**6.7 ± 0.32**	**5.8 ± 0.36**	**2.0 x 10^{-6}**	**1.0 x 10^{-5}**		
	185	**2.5 ± 0.14**	1.2 ± 0.62	**6.0 x 10^{-4}**	5.0 x 10^{-1}	**4**/5	**3**/5
	194	**3.6 ± 0.31**	**4.5 ± 0.19**	**1.2 x 10^{-4}**	**2.0 x 10^{-6}**		
	217	0.5 ± 0.03	0.5 ± 0.46	3.0 x 10^{-2}	7.0 x 10^{-2}		
	263	**2.5 ± 0.25**	**2.8 ± 0.16**	**1.2 x 10^{-3}**	**1.4 x 10^{-4}**		

[1] *P value was determined by one tailed distribution Student's t-test.*
[2] *Only samples that were more than two times higher than vector and have significant p values were considered as positive.*

2.3.5. Immunisation of animals with individual ORFs

In the previous experiments, we showed that sheep survived a virulent needle challenge when immunised with the 1H12 cocktail of four *E. ruminantium* ORFs. In an attempt to determine if the individual ORFs can also confer protection, animals were immunised with each ORF individually. The survival of sheep immunised with individual 1H12 ORFs was compared to the 1H12 cocktail, negative and positive controls (Figure 2.12). Animals immunised with the single ORFs each received a total of 220 µg plasmid DNA at each inoculation (200 µg i.m. and 20 µg i.d.), while sheep immunised with the cocktail received the same dose as before (Table 2.3).

All the sheep immunised with the single 1H12 ORFs survived a virulent *E. ruminantium* Welgevonden needle challenge (Figure 2.12). Only animals immunised with the empty pCMViUBs vector developed severe heartwater symptoms and were treated (Figure 2.13). Animals immunised with the cocktail and single ORFs all developed temperatures (Figures 2.14-2.17) and early heartwater symptoms, but again continued to eat and act normally. Overall the RI of the groups immunised with the individual ORFs differed significantly ($P < 2 \times 10^{-6}$ for sheep inoculated with the pCMViUBs_Erum2540 plasmid; $P < 2 \times 10^{-5}$ for sheep inoculated with the pCMViUBs_Erum2550 plasmid; $P < 8 \times 10^{-6}$ for sheep inoculated with the pCMViUBs_Erum2580 plasmid and $P < 3 \times 10^{-7}$ for sheep inoculated with the pCMViUBs_Erum2590 plasmid; Table 2.9) from that of the negative control group. No significant difference was observed when the RI of sheep immunised with the single ORFs was compared to the 1H12 cocktail group ($P < 0.111$ for pCMViUBs_Erum2540; $P < 0.279$ for pCMViUBs_Erum2550; $P < 0.464$ for pCMViUBs_Erum2580; $P < 0.284$ for pCMViUBs_Erum2590; Table 2.9).

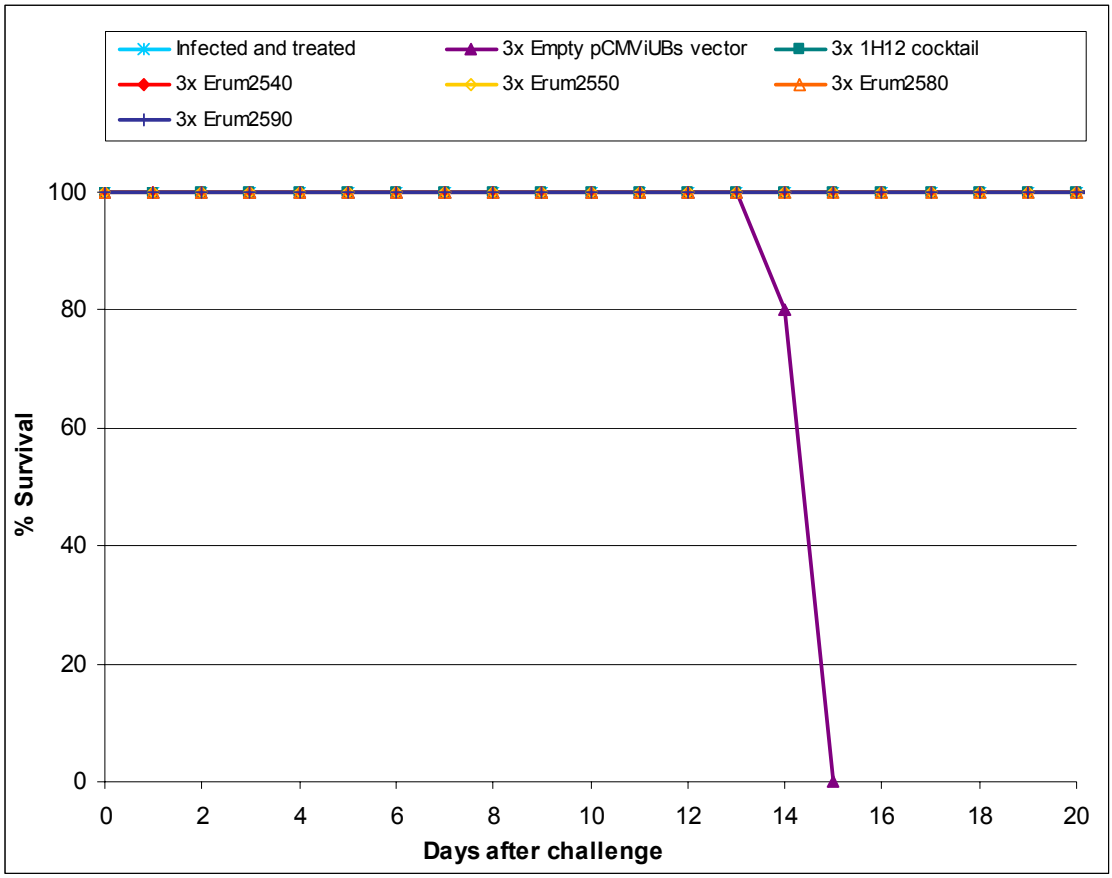


Figure 2.12: *Survival chart of sheep immunised with the 1H12 cocktail compared to sheep immunised with the individual 1H12 ORFs, positive and negative control groups. Animals that were treated were also considered as non-survivors.*

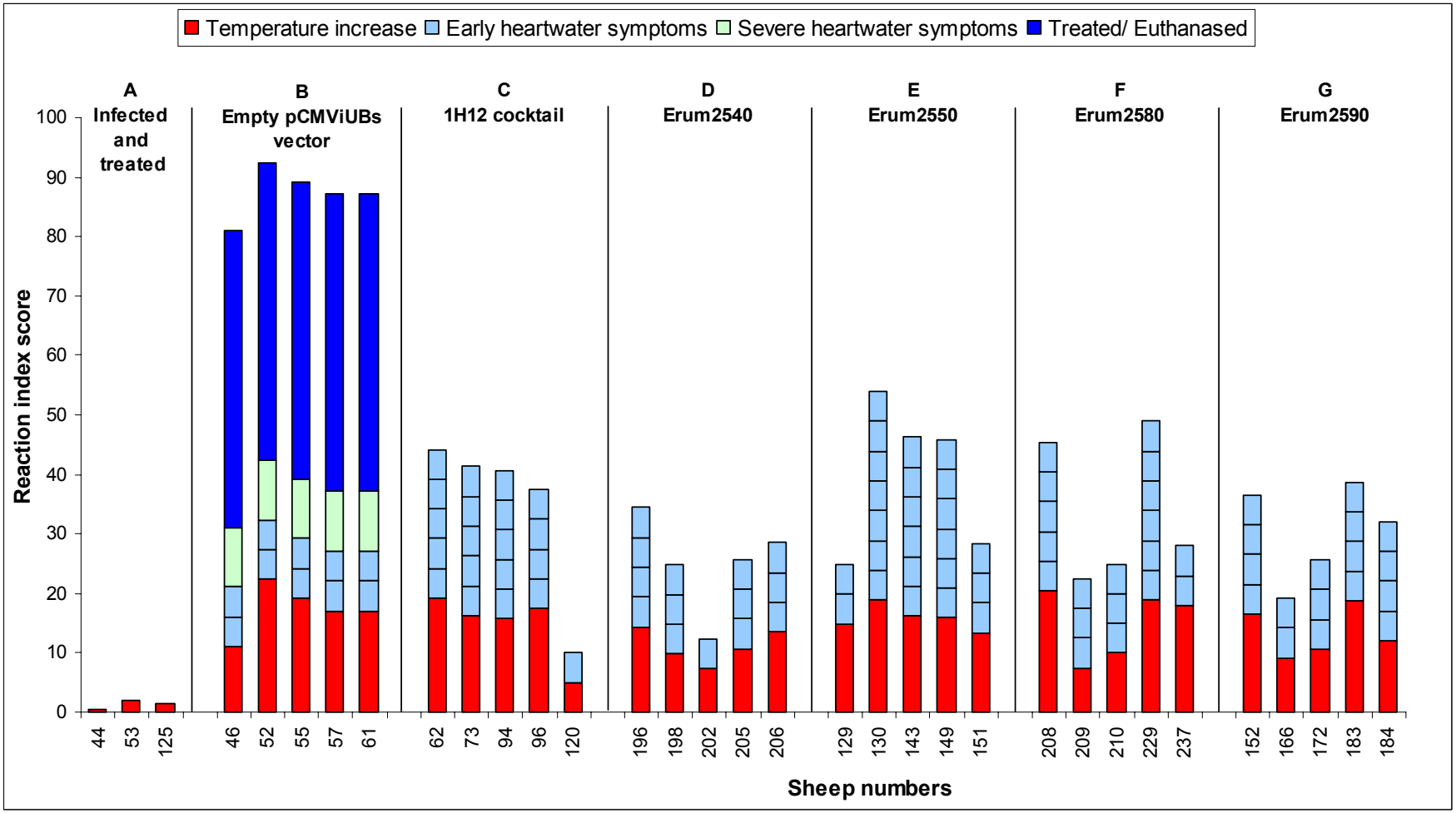


Figure 2.13: *Reaction index (RI) of sheep immunised with the infected and treated vaccine (A), the empty pCMViUBs vector (B), the 1H12 cocktail (C) and the single 1H12 ORFs (D-G) after challenge with* E. ruminantium *Welgevonden. The red blocks indicate the total temperature RI score 21 days after challenge, while the light blue blocks represent a RI of 5 (early heartwater symptoms), the light green a RI of 10 (severe heartwater symptoms) and the dark blue a RI of 50 (animal was treated/ euthanased).*

Table 2.9: *Statistical analysis of RI values as determined for each group.*

Group	Sheep number	RI_{Total}	RI_{AVE}	RI_{STDEV}	P[1] value compared to	
					Empty vector group	1H12 cocktail group
Infected and treated	44 53 125	0.5 1.9 1.5	1.30	0.72	2×10^{-7}	3.0×10^{-3}
Empty pCMViUBs vector	46 52 55 57 61	81.0 92.3 89.2 87.1 87.1	87.36	4.31		2×10^{-5}
1H12 cocktail	62 73 94 96 120	44.2 41.3 40.7 37.4 10.0	34.72	14.03	2×10^{-5}	
Erum2540	196 198 202 205 206	34.4 24.8 12.3 25.7 28.5	25.14	8.10	2×10^{-6}	1.1×10^{-1}
Erum2550	129 130 143 149 151	24.9 53.9 46.2 45.9 28.4	39.86	12.54	2×10^{-5}	2.8×10^{-1}
Erum2580	208 209 210 229 237	45.4 22.5 25.0 48.9 28.0	33.96	12.26	8×10^{-6}	4.6×10^{-1}
Erum2590	152 166 172 183 184	36.5 19.2 25.6 38.7 32.1	30.42	8.02	3×10^{-7}	2.8×10^{-1}

[1] *P value was determined by one tailed distribution Student's t-test.*

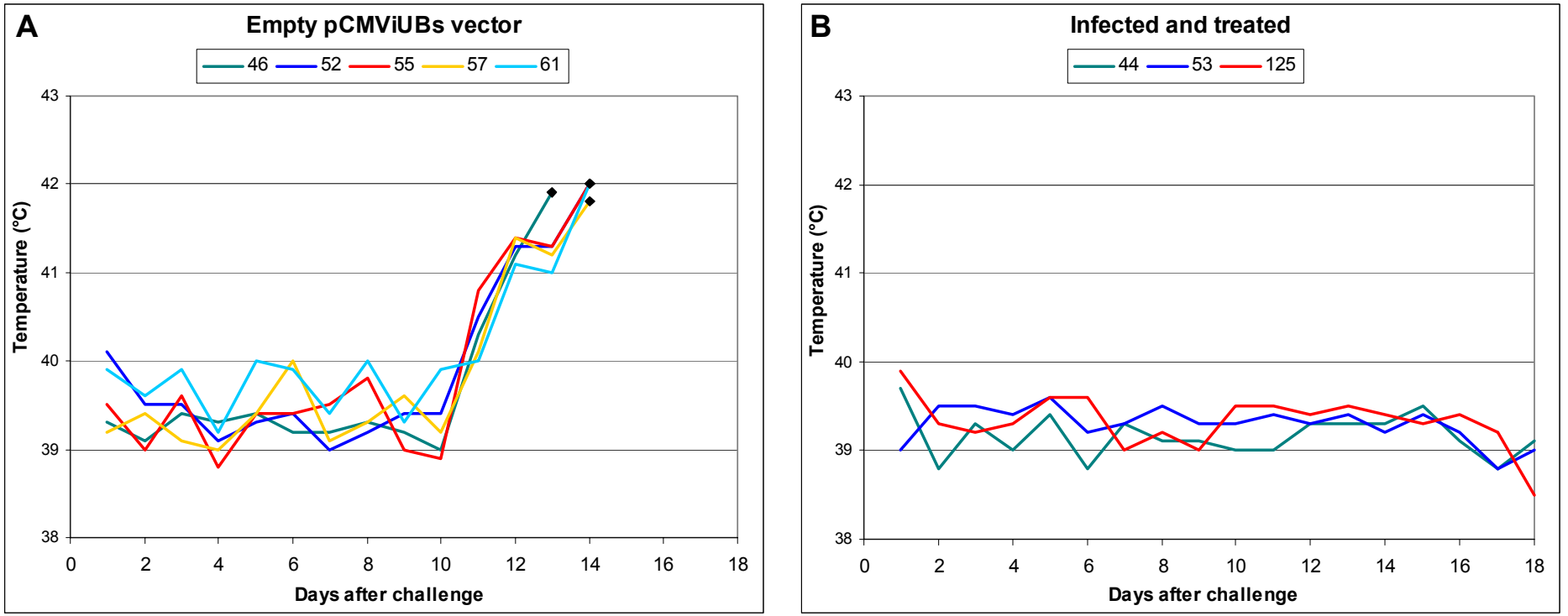


Figure 2.14: *Daily post-challenge temperatures of the negative control sheep inoculated with 3x empty pCMViUBs vector DNA (A) and the positive control animals that were infected and treated (B). Sheep were challenged with 10 LD_{50}s of the virulent* E. ruminantium *Welgevonden stock. Temperature measurements were terminated 18 days after challenge or as soon as the animal was treated or euthanased (represented by the black dots).*

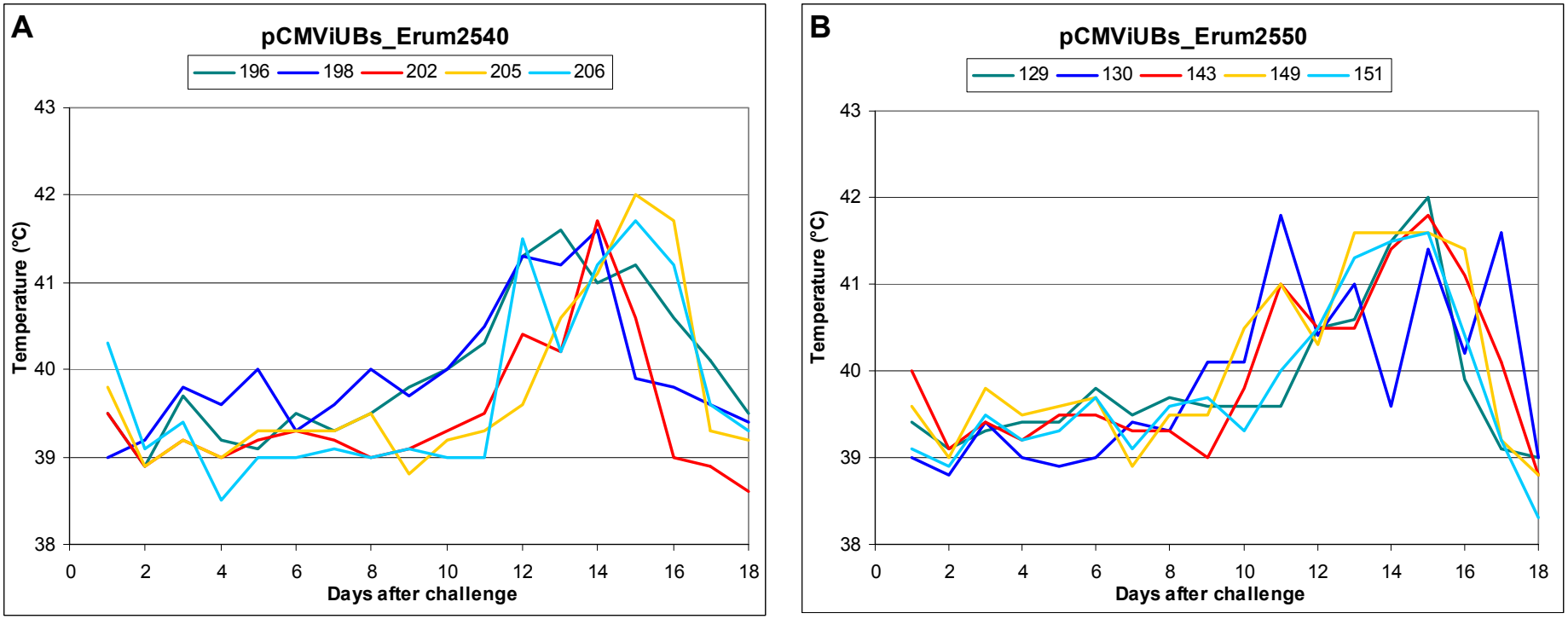


Figure 2.15: *Daily post-challenge temperatures of the sheep inoculated with pCMViUBs_Erum2540 (A) and with pCMViUBs_Erum2550 (B). Sheep were challenged with 10 LD_{50}s of the virulent* E. ruminantium *Welgevonden stock. Temperature measurements were terminated 18 days after challenge or as soon as the animal was treated or euthanased (represented by the black dots).*

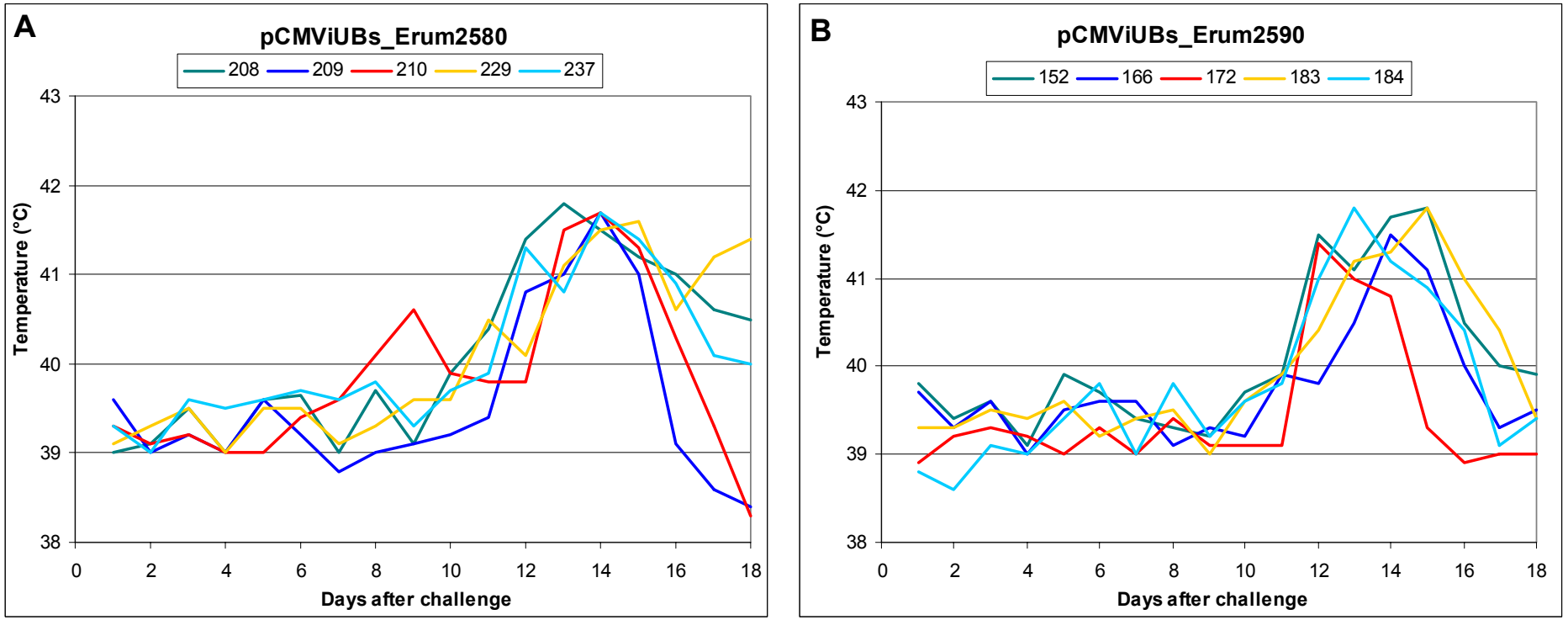


***Figure 2.16**: Daily post-challenge temperatures of the sheep inoculated with pCMViUBs_Erum2580 (A) and with pCMViUBs_Erum2590 (B). Sheep were challenged with 10 LD_{50}s of the virulent* E. ruminantium *Welgevonden stock. Temperature measurements were terminated 18 days after challenge or as soon as an animal was treated or euthanased (represented by a black dot).*

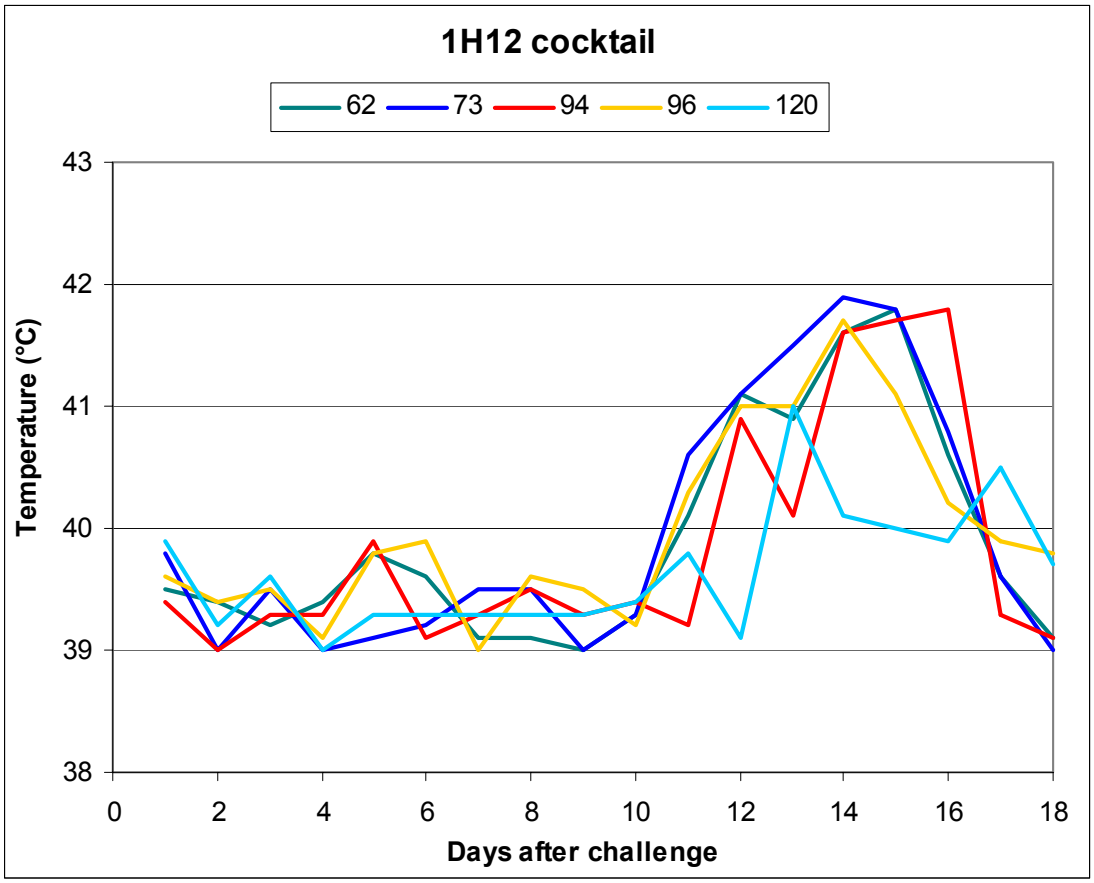


Figure 2.17: *Daily post-challenge temperatures of the sheep inoculated with the 1H12 cocktail. Sheep were challenged with 10 LD_{50}s of the virulent* E. ruminantium *Welgevonden stock. Temperature measurements were terminated 18 days after challenge or as soon as an animal was treated or euthanased (represented by a black dot).*

2.3.6. Immunological assays of sheep immunised with individual ORFs compared to the 1H12 cocktail

Lymphocyte proliferation assays were performed to confirm ORF protein product expression and priming/activation of immune responses in the host. The assay was performed as described before and PBMC were isolated at three time points: before first inoculation, three and four weeks after the final boost. The average SI of each group is represented in Table 2.10. As found in the previous experiment, the PBMC of two animals inoculated with the 1H12 cocktail showed positive proliferation both three (sheep 96 and 120) and four (sheep 62 and 120) weeks after the final boost. Four weeks after the final boost a significant increase in the SI values was observed in sheep immunised with pCMViUBs_Erum2550 (Sheep 130, 143 and 151), pCMViUBs_Erum2590 (Sheep 183 and 184), pCMViUBs_Erum2540 (Sheep 206) and pCMViUBs_Erum2580 (Sheep 208 and 229) when compared to the SI values

obtained three weeks after the final boost (see Table 2.10 for SI_{ave} of each sheep and P values).

All subsequent lymphocyte proliferation experiments were performed one week before challenge (four weeks after final boost) as most of the PBMC of immunised animals showed proliferation at this time point. The higher stimulation indices observed in animals immunised with the single 1H12 ORFs could be dose related. Although the final DNA concentration received by each animal was the same as that received by animals immunised with the 1H12 cocktail, animals immunised with single ORFs received four times higher doses of each ORF than sheep immunised with the cocktail. Nevertheless, these results indicate that all four of the 1H12 gene products are expressed in the host cells and stimulate protection.

boost) and in red (four weeks after the final boost).

Group	Sheep number	SI_{ave} (SI after boost/ SI naive) Three weeks after boost	SI_{ave} (SI after boost/ SI naive) Four weeks after boost	P Values [1] (SI after boost compared to SI vector) Three weeks after boost	P Values [1] (SI after boost compared to SI vector) Four weeks after boost	Number of positive animals per group [2] Three weeks after boost	Number of positive animals per group [2] Four weeks after boost
Vector	46	0.82 ± 0.21	1.09 ± 0.05				
	52	0.78 ± 0.03	1.22 ± 0.39				
	55	0.73 ± 0.33	0.93 ± 0.16				
	57	0.57 ±0.77	0.88 ±0.25				
	61	0.85 ± 0.13	1.16 ± 0.15				
1H12 cocktail	62	1.51 ± 0.04	**2.62 ± 0.17**	1.0×10^{-5}	**2.0×10^{-3}**	2/5	2/5
	73	1.29 ± 0.61	0.61 ± 0.16	1.4×10^{-1}	3.7×10^{-2}		
	94	1.25 ± 0.21	1.58 ± 0.12	4.0×10^{-2}	7.0×10^{-3}		
	96	**2.12 ± 0.31**	1.24 ± 0.43	**3.2×10^{-5}**	2.9×10^{-1}		
	120	**2.16 ± 0.01**	**3.89 ± 0.09**	**1.4×10^{-5}**	**6.0×10^{-3}**		
Erum2550	129	1.87 ± 0.31	1.60 ± 0.31	1.2×10^{-2}	6.8×10^{-2}	0/5	3/5
	130	1.88 ± 0.47	**2.34 ± 0.64**	4.0×10^{-2}	**1.0×10^{-3}**		
	143	1.48 ± 0.16	**2.55 ± 0.58**	2.0×10^{-2}	**2.0×10^{-3}**		
	149	1.93 ± 0.69	1.47 ± 0.02	2.0×10^{-2}	1.0×10^{-3}		
	151	1.42 ± 0.32	**2.10 ± 0.06**	1.0×10^{-3}	**3.0×10^{-5}**		
Erum2590	152	1.71 ± 0.39	1.38 ± 0.18	5.0×10^{-2}	7.7×10^{-2}	1/5	2/5
	166	0.89 ± 0.43	0.90 ± 0.15	3.3×10^{-2}	1.3×10^{-1}		
	172	1.12 ± 0.21	0.93 ± 0.08	7.0×10^{-2}	1.1×10^{-1}		
	183	1.28 ± 0.08	**2.51 ± 0.41**	1.0×10^{-3}	**1.0×10^{-2}**		
	184	**2.93 ± 0.16**	**2.69 ± 0.77**	**7.0×10^{-3}**	**3.0×10^{-5}**		
Erum2540	196	1.92 ± 0.30	0.97 ± 0.25	3.0×10^{-2}	3.9×10^{-1}	0/5	1/5
	198	1.83 ± 0.04	1.77 ± 0.09	1.0×10^{-5}	3.0×10^{-3}		
	202	1.21 ± 0.21	1.30 ± 0.10	4.0×10^{-2}	5.8×10^{-2}		
	205	1.79 ±0.35	1.55 ± 0.37	3.0×10^{-2}	1.1×10^{-2}		
	206	1.78 ±0.09	**7.34 ± 0.60**	3.0×10^{-3}	**7.0×10^{-3}**		
Erum2580	208	1.17 ± 0.39	**5.29 ± 0.83**	1.0×10^{-3}	**3.0×10^{-3}**	0/5	2/5
	209	1.03 ± 0.13	1.67 ± 0.07	1.0×10^{-3}	2.0×10^{-4}		
	229	1.09 ± 0.11	**5.16 ± 1.04**	1.0×10^{-3}	**1.0×10^{-2}**		
	237	0.93 ± 0.10	1.60 ±0.34	7.0×10^{-2}	5.8×10^{-2}		

[1] *P value was determined by one tailed distribution Student's t-test.*
[2] *Only samples that were more than two times higher than vector and have significant P values were considered as positive.*

2.4. DISCUSSION

Previous experiments done using cosmid 1H12 ORFs cloned into the mammalian expression vector pCMViUBs provided partial protection in mice against a lethal heartwater challenge. These results were not reproducible: large variations were observed in the survival rates of different groups of mice immunised with the same constructs at different times (Collins *et al.,* 2003a; 2003b). Variable results obtained in genetically immunised mice are not uncommon and have been observed previously in our laboratory (Brayton *et al.,* 1998; Pretorius *et al.,* 2002) and by other groups (Nyika *et al.,* 1998; 2002) thus indicating that the murine model is not well suited for recombinant heartwater vaccine evaluation.

In this study the potential of four of the 1H12 ORFs cloned into the mammalian expression vector pCMViUBs to protect against virulent *E. ruminantium* Welgevonden was investigated in ruminants. Here, for the first time, complete protection was observed against a virulent *E. ruminantium* needle challenge when sheep were immunised with the 1H12 ORF cocktail. We have repeated this experiment on three separate occasions with the same result every time.

Cattle immunised by infection and treatment induce $CD4^+$, $CD8^+$ and γδ lymphocyte responses that express IFN-γ, TNF-α, TNF-β and IL-2 (Mwangi *et al*., 1998). Similarly, cattle vaccinated with killed *E. ruminantium* elementary bodies (EBs) show elevated levels of IFN-γ, a cytokine that mediates cellular immunity, after challenge with virulent *E. ruminantium.* Furthermore, the treatment of *E. ruminantium* infected animals with recombinant IFN-γ also results in the inhibition of EB growth in endothelial cells (Totté *et al.,* 1996; 1997). This suggests that IFN-γ producing T cells may play an important role in protection against *E. ruminantium* infection. IFN-γ can, however, be activated via several non-specific pathways that include the activation of the innate immune response. It is known that CpG motifs in bacterial DNA can induce pseudo protective immune responses by activating DC IL-12 expression upon binding to host TLR9 (reviewed by Rothenfusser *et al.,* 2002 and by Wagner, 2002). This cytokine is known to direct the immune response towards a cellular response by activating the secretion of IFN-γ. To exclude the possibility that the protection

observed was due to the activation of a non-specific innate immune response, we included four randomly selected *E. ruminantium* ORFs in one of the immunisation experiments. Again the 1H12 ORFs conferred protection while the animals immunised with the other *E. ruminantium* ORFs developed severe heartwater symptoms and had to be euthanased. All the ORFs used to immunise the animals had similarly low GC content. Putative CpG islands with a minimum of 30 % GC content were identified in the 1H12 and the WL2AP1 ORFs. Although none of these putative CpG islands were identical to the CpG motifs previously shown to be stimulatory in sheep (Mutwiri *et al.,* 2003; Booth *et al.,* 2006), the possibility that these motifs could induce innate immunity cannot be excluded and should be further investigated. This could be done by determining if the DNA sequences of the ORFs can induce proliferation in naive sheep PBMC. However, since CpG motifs were identified in both the 1H12 and the WL2AP1 ORFs, our challenge results suggest that the protection stimulated by the 1H12 cocktail depends upon specific properties of the 1H12 protein products.

The mechanism of protection by the four 1H12 ORFs still has to be elucidated. Two of the ORFs (Erum2590 and Erum2250) demonstrated homology to ABC transporter ATP-binding/ATPase proteins. ABC transporters located in the outer membrane have been shown to be effective targets for antibody mediated immunity against *Staphylococcus aureus* (Burnie *et al.,* 2000); *Enterococcus faecium* (Burnie *et al.,* 2002); *Streptococcus pneumoniae* (Jomaa *et al.,* 2005; 2006) and *Yersinia pestis* (Tanabe *et al.,* 2006) infection not only due to their cellular location but also due to the conserved nature of these proteins. Opsonising antibodies (T_{H1}) directed against iron uptake ABC transporters of *Streptococcus pneumoniae* were shown to increase protection against a lethal dose from 0 % to 80 % survival in mice (Brown *et al.,* 2001). The authors could show in a later study that the reduction in mortality was due to the elimination of bacteria by opsonophagocytosis (Jomaa *et al.,* 2005). This theory is corroborated by the finding that *Yersinia pestis* mutants, deficient in the metal uptake ABC transporter locus, showed abridged virulence (Bearden and Perry, 1999). These results were obtained in Gram positive bacteria, where the ABC transporters are located on the outer membrane, and therefore exposure to the host immune response system is imminent. ABC transporters of *E. ruminantium* are probably located on the

inner membrane as in other Gram negative bacteria and are therefore not exposed on the cell surface. Furthermore, *E. ruminantium* is an obligate intracellular bacterium which makes exposure to the host immune system even more complicated. Opsonising antibodies might have an effect on *E. ruminantium* elementary bodies which are injected into the bloodstream by infected ticks and released when an infected host cell dies, although the antibody would still have to be able to penetrate the outer membrane of the organism to exert an effect on the transporter. The protective mechanism therefore probably does not rely on opsonising antibodies or blockage of function. The protection we observed is more likely to involve either the priming of CTL via the host MHC class I system or MHC class II presentation of *E. ruminantium* antigen by professional antigen presenting cells (APC) and activated endothelial cells.

Before CTL or $CD4^+$ T_{H1} T cells can exert any effect on infected host cells (endothelium), these cells first have to "see" the hidden *E. ruminantium*. This involves the presentation of intracellular *E. ruminantium* antigen on either MHC class I or class II molecules. It has been shown for several intracellular bacteria, including *Salmonella*, *Listeria* and *Mycobacterium,* that a CTL response is a key element of host immunity to the invading pathogens (reviewed by Soloski *et al*., 2000). Activation of CTL requires the presentation of antigen on MHC class I, a presentation molecule found on all cell types. MHC class Ia presentation has been regarded as the first line of defence against all viral infections. Viral proteins are normally synthesised in the host cytoplasm and once degraded will be presented to CTL. New evidence suggests that a similar subtype of MHC class I (known as class 1b) has the ability to present antigen that originates from intracellular bacteria (reviewed by Soloski *et al*., 2000). Under normal infective conditions bacterial proteins exposed to the host cell's cytoplasm can be degraded and entered into the MHC class 1b presentation pathway. Once these foreign peptides are presented on a host cell, CTL and γδ T cells will be activated and the infected cell will be killed (reviewed by Soloski *et al*., 2000). Although this subtype has never been tested for an ability to present *E. ruminantium* antigen, it has been shown that γδ T cells increase in bovines immunised both with killed *E. ruminantium* and by using the infection and treatment method (Tottè *et al.,* 1997). It has been shown that γδ T cells are activated by MHC class 1b molecules and

this might be regarded as an indicator of MHC class 1b presentation of *E. ruminantium* antigen.

On the other hand, MHC class II expression by professional antigen presenting cells (APC) and activated endothelial cells could also contribute to the observed protection. Host immunity to *E. ruminantium* involves complex interactions of $CD4^+$-, $CD8^+$- and γδ T lymphocytes, macrophages, natural killer cells and cytokines (Totté *et al.,* 1997; Mwangi *et al.,* 1998; Totté *et al.,* 1999; Byrom *et al.,* 2000; Mwangi *et al.,* 2002). In the early stages of infection, host immunity is alerted to infection at the tick bite site by DC uptake of *E. ruminantium* EBs or once EBs are released to infect new endothelial cells. The first line of defence relies on phagocytosis of EBs by DCs and blood monocytes resulting in the presentation of *E. ruminantium* antigens to T lymphocytes on MHC class II molecules. The resulting activation and recruitment of lymphocytes to the site of infection (artery endothelial cells) leads to increased levels of IFN-γ that in turn activate endothelial cells, which produce nitric oxide (Mutunga *et al.,* 1998). Some of the intracellular bacteria will be destroyed in the endothelial cells by nitric oxide, superoxide or peroxide and indoleamine 2,3-dioxygenase, a tryptophan-catabolising enzyme, resulting in the exposure of the host cytoplasm to bacterial antigen where the antigen can enter the MHC class II presentation pathway (De Assis *et al.,* 2000; Feng and Walker, 2000 and reviewed by Pober, 1999). Killing of intracellular bacteria such as *Staphylococcus aureus* (Zhang *et al.,* 1997), *Pseudomonas aeruginosa* (De Assis *et al.,* 2000) and *Rickettsia conorii* (Feng and Walker, 2000; Feng *et al.,* 2004) via endothelial nitric oxide expression and superoxide dismutase activity has been demonstrated.

In the immunisation strategy that we used, CTL priming was targeted by the use of an expression vector designed so that the translated protein of interest will be covalently linked to ubiquitin. In theory, ubiquitination of proteins will target these proteins to the proteosome where the protein will subsequently be cleaved into peptides and targeted for MHC class I presentation. This will ultimately lead to the activation of a CTL response against heartwater. However, there exists controversy as to whether the expression vector engineered ubiquitin-protein fusion survives in the host cell environment. Fu *et al*. (1998) reported that the addition of ubiquitin to the target

antigen did not enhance degradation or improve the CTL responses. Several papers have reported that the end sequence of the ubiquitin is very important: having a glycine (Gly) at position 76 (G_{76}) in the N terminal region of the protein will result in cleavage of the ubiquitin as soon as the fusion protein is translated (Delogu *et al.,* 2000 and reviewed by Weissman, 2001). Cleavage of the ubiquitin from the fusion protein will result in the accumulation of recombinant protein in the transfected cell cytoplasm and can result in the presentation of the antigen on MHC class II only if a professional antigen presenting cell is transfected with the plasmid DNA. Transfection of non-presenting cells can only activate immune responses if the ORF product is a protein that contains a secretory signal. These proteins can be exported from the host cell, where APC uptake and presentation will result in the activation of T_{H1} or T_{H2} type lymphocytes. The expression vector we used has a G_{76} and the ubiquitin would therefore theoretically be expected to be cleaved from the fusion protein (reviewed by Varshavsky, 1996). Nonetheless, other genetic immunisation experiments done using the pCMViUBs vector did show increased CTL responses in monkeys against HIV antigens, suggesting that the ubiquitin-protein fusion is not cleaved and that targeting of the protein for MHC class I presentation does occur (Sykes *et al.,* 2002 and reviewed by Johnston *et al.,* 2002). Transient expression experiments should be done to determine if the ubiquitin will target the protein to the proteosome for cleavage or if the ubiquitin is removed from the fusion protein resulting in the protein remaining intact in the host cells.

It is well known that a CTL immune response is greatly augmented in the presence of MHC class II-mediated $CD4^+$ T cell help. This function is believed to be especially well stimulated by DNA vaccination, because of direct transfection of DCs (by gene gun inoculation) that have the ability to present antigen on both MHC class I and II (Maecker *et al.,* 1998). Although the vector that we used is known to stimulate strong CTL responses, the possibility of MHC class II expression cannot be excluded, as mentioned before. We were not able to perform CTL assays, which would have been the preferred assays to monitor the immune response during these experiments, since target cells were not available. Expression of gene products by the recombinant pCMViUBs constructs, and activation of the immune response, was therefore confirmed by recall lymphocyte proliferation assays. Only the groups immunised

with the 1H12 cocktail and individual 1H12 ORFs showed antigen specific proliferation (confirming expression), but the SI was in most cases only marginally higher than 2 and proliferation was only detected in a few of the animals per group at a specific time point. Positive SI of one animal immunised with pCMViUBs_Erum2540 and two animals immunised with pCMViUBs_Erum2580 had higher stimulation indices than was found for the cocktail. This was surprising as one might expect that the combined effect of all four ORF protein products could increase the SI values in the cocktail group. The higher stimulation indices for sheep inoculated with pCMViUBs_Erum2540 and pCMViUBs_Erum2580 could be due to the antigenic properties of the protein products derived from these two ORFs. It is also possible that those two genes expressed better in the mammalian host cells than the Erum2550 and Erum2590 genes or it could simply be due to the inoculation of a higher concentration of plasmid DNA as compared to the cocktail. Although the final DNA concentration received by each animal was the same, animals immunised with single ORFs received four times higher doses of each ORF than sheep immunised with the cocktail. It is known that increased doses of cocktail DNA could induce stronger immunity (Nyika *et al.,* 1998). Thus stronger immune responses might be expected if we increase the dose of the cocktail plasmid DNA; this needs to be optimised and tested in future studies.

The low levels and absence of proliferation observed in this study can be attributed to numerous factors. Firstly, the crude partially purified antigens used to stimulate the PBMC were prepared from bovine endothelial cells infected with *E. ruminantium*. Although total protein isolated from the cell culture was quantified, the ratio of bovine to *E. ruminantium* antigen in the preparation was unknown, and would be very difficult to determine. In addition we have no data on the expression levels of the 1H12 proteins in *E. ruminantium* cells. The only fact of which we can be fairly certain is that the PBMCs were stimulated with a low concentration of the antigen that is encoded by the DNA with which the animals were immunised. Low concentrations of antigen would be expected to correspond to low SI. Ideally, these assays should be repeated using recombinant 1H12 (r1H12) proteins, but these were not available at the time that this work was done.

Secondly, we should consider the location of the T_H cells at the time the assay was performed. Lymphocyte proliferation assays were carried out at different time points in these experiments, and we observed differences in the SI over time. Sheep 62 did not show proliferation 3 weeks after the final inoculation, but positive proliferation was detected 4 weeks after the final inoculation (Table 2.10). In contrast sheep 96 only showed proliferation 3 weeks after the final boost and not 4 weeks after the boost. Similarly, Vachiéry *et al.* (2006) showed that enhanced immunity measured by IFN-γ production in blood plasma could not be used to predict if goats immunised with a heartwater inactivated vaccine would be protected against challenge. According to these authors the lack of IFN-γ detection in protected animals could have been due to memory T cells that were not circulating in the blood at the time the blood was collected; these memory T cells might have been detected in other immunological compartments such as the spleen and lymph nodes. Likewise, in our study the 1H12 specific memory T cells may not have been circulating when the lymphocyte proliferation assay was performed. This does not, however, imply that activated lymphocytes were not present, because proliferation was detected at a different time point. This may explain why lymphocyte proliferation was never observed in some sheep immunised with the 1H12 cocktail (e.g. sheep 73 and 94, Table 2.10), yet these sheep were protected against challenge. Enhanced proliferation by PBMC might be obtained by further optimisation of the assay, including the use of r1H12 proteins as stimulatory antigen instead of the partially purified *E. ruminantium* antigen currently used

In this study animals that were vaccinated and survived developed a fever, similar to that of the negative non-immunised control groups after needle challenge. Fever or temperature reactions can be linked to the expression and secretion of specific pyrogenic cytokines. There are four cytokines that play a major role in the induction of fever: IL-1, TNF–α and IL-6 (cytokines secreted by monocytes, endothelial cells, DCs and fibroblasts) and IFN-γ (a cytokine that is produced by T and NK cells (Mackowiak, 1998). It is difficult to distinguish between a "protective" fever and one induced by infection unless we can measure the cytokines produced at the time of infection. IFN-γ plays an important role in the protective immune response to heartwater (reviewed by Tottè *et al.,* 1999), and therefore we would expect to see a

fever in animals that are protected since elevated levels of IFN-γ will induce the secretion of TNF-α, and together these two cytokines will induce fever. On the other hand, infected endothelial cells will also induce the production of IL-1 that will induce fever by up-regulating the expression of TNF-α.

In conclusion, we have shown for the first time that immunising ruminant hosts with a DNA vaccine containing four *E. ruminantium* ORFs, either as part of a cocktail or individually, conferred protection against a lethal *E. ruminantium* Welgevonden needle challenge. Under natural conditions heartwater is transmitted by ticks. It is known that the saliva of ticks can induce immunosuppresion and that some opportunistic pathogens utilise this suppression to survive and grow within the host (reviewed by Wikel and Alarcon-Chaidez, 2001). It is therefore important that we test the 1H12 cocktail under natural field conditions in a heartwater endemic area where animals would receive a tick challenge. Another factor that has to be considered is genetic diversity of field *E. ruminantium* genotypes. We have shown that the Welgevonden-derived 1H12 ORFs protected against an *E. ruminantium* Welgevonden challenge, but will this vaccine be protective against other genotypes? These two issues will be addressed in chapter 3.

2.5. REFERENCES

AGUIAR, J.C., HEDSTORM, R.C., ROGERS, W.O., CHAROENVIT, Y., SACCI, J.B., LANAR, D.E., MAJAM, V.F., STOUT, R.R. and HOFFMAN, S.L. 2002. Enhancement of the immune response in rabbits to a malaria DNA vaccine by immunisation with a needle-free jet device. *Vaccine* **20:** 275-280.

ALTSCHUL, S.F., MADDEN, T.L., SCHÄFFER, A.A., ZHANG, J., ZHANG, Z., MILLER, W., and LIPMAN, D.J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25:** 3389-3402.

BEARDEN, S.W. and PERRY, R.D. 1999. The Yfe system of *Yersinia pestis* transports iron and manganese and is required for full virulence of plague. *Molecular Microbiology* **32:** 403-414.

BOOTH, J.S., NICHANI, A.K., BENJAMIN, P., DAR, A., KRIEG, A.M., BABIUK, L.A., and MUTWIRI, K.G. 2006. Innate immune responses induced by classes of CpG oligodeoxynucleotides in ovine lymph node and blood mononuclear cells. *Veterinary Immunology and Immunopathololology* **115:** 24-34.

BOVARNICK, M.R., MILLER, J.C. and SNYDER, J. C. 1950. The influence of certain salts, amino acids, sugars and proteins on the stability of Rickettsiae. *Journal of Bacteriology* **59:** 509–522.

BRAYTON, K.A., BOTHMA, G.C., VOGEL, S.W. and ALLSOPP, B.A. 1997a. Development of the OPgun™ for bombardment of animal tissues. *Onderstepoort Journal of Veterinary Research* **64:** 153-156.

BRAYTON, K.A., FEHRSEN, J., DE VILLIERS, E.P., VAN KLEEF, M. and ALLSOPP, B.A. 1997b. Construction and initial analysis of a representative lambda ZAPII expression library of the intracellular rickettsia *Cowdria ruminantium* cloning of map1 and three other Cowdria genes. *Veterinary Parasitology* **72:** 185-199.

BRAYTON, K.A., VOGEL, S.W. and ALLSOPP, B.A. 1998. Expression library immunisation to identify protective antigens from *Cowdria ruminantium*. *Annals of the New York Academy of Sciences* **849:** 369-371.

BRAYTON, K.A., COLLINS, N.E., VAN STRIJP, F. and ALLSOPP, B.A. 2003. Preparation of *Ehrlichia ruminantium* challenge material for quantifiable and reproducible challenge in mice and sheep. *Veterinary Parasitology* **112:** 63-73.

BROWN, J.S., OGUNNIYI, A.D., WOODROW, M.C., HOLDEN, D.W. and PATON, J.C. 2001. Immunisation with components of two iron uptake ABC transporters protects mice against systemic *Streptococcus pneumoniae* infection. *Infection and Immunity* **69:** 6702-6706.

BUNGENER, L., HUCKRIEDE, A., DE MARE, A., DE VRIES-IDEMA, J., WILSCHUT, J. and DAEMEN, T. 2005. Virosome-mediated delivery of protein antigens *in vivo*: efficient induction of class I MHC-restricted cytotoxic T lymphocyte activity. *Vaccine* **23:** 1232-1241.

BURNIE, J.P., MATTHEWS, R.C., CARTER, T., BEAULIEU, E., DONOHOE, M., CHAPMAN, C., WILLIAMSON, P. and HODGETTS, S.J. 2000. Identification of an immunodominant ABC transporter in methicillin–resistant *Staphylococcus aureus* infections. *Infection and Immunity* **68:** 3200-3209.

BURNIE, J., CARTER, T., RIGG, G., HODGETTS, S., DONOHOE, M. and MATTHEWS, R. 2002. Identification of ABC transporters in vancomycin-resistant *Enterococcus faecium* as potential targets for antibody therapy. *FEMS Immunology and Medical Microbiology* **33:** 179-189.

BYROM, B., BARBET, A.F., OBWOLO, M. and MAHAN, S.M. 2000. $CD8^+$ T cell knockout mice are less susceptible to *Cowdria ruminantium* infection than athymic, $CD4^+$ T cell knockout, and normal C57BL/6 mice. *Veterinary Parasitology* **93:** 159-172.

CHAPLIN, P.J., DE ROSE, R., BOYLE, J.S., MCWATERS, P., KELLY, J., TENNENT, J.M., LEW, A.M. and SCHEERLINCK, J.Y. 1999. Targeting improves the efficacy of a DNA vaccine against *Corynebacterium pseudotuberculosis* in sheep. *Infection and Immunity* **67:** 6434-6438.

COBAN, C., ISHII, K.J., GURSEL, M., KLINMAN, D.M. and KUMAR, N. 2005. Effect of plasmid backbone modification by different human CpG motifs on the immunogenicity of DNA vaccine vectors. *Journal of Leukocyte Biology* **78:** 647-655.

COLLINS, N.E., DE VILLIERS, E.P., BRAYTON, K.A., and ALLSOPP, B.A. 1998. DNA sequence of a cosmid clone of *Cowdria ruminantium*. *Annals of the New York Academy of Sciences* **849:** 365-368.

COLLINS, N.E., PRETORIUS, A., VAN KLEEF, M., BRAYTON, K.A., ALLSOPP, M.T., ZWEYGARTH, E. and ALLSOPP, B.A. 2003a. Development of improved attenuated and nucleic acid vaccines for heartwater. In: Vaccines for OIE list A and Emerging Animal Diseases. Edited by BROWN, F. and ROTH, J. *Developments in Biologicals Basel Karger* **114:** 121-136.

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

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., BERTHIER, D., BOTHA, M., JOUBERT, F., CORTON, C.H., THOMSON, N.R., ALLSOPP, M.T. and 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.

CUPILLARD, L., JUILLARD, V., LATOUR, S., COLOMBET, G., CACHET, N., RICHARD, S., BLANCHARD, S. and FISCHER, L. 2005. Impact of plasmid supercoiling on the efficacy of a rabies DNA vaccine to protect cats. *Vaccine* **23:** 1910-1916.

DANESE, I., HAINE, V., DELRUE, R., TIBOR, A., LESTRATE, P., STEVAUX, O., MERTENS, P., PAQUET, J., GODFROID, J., DE BOLLE, X. and LETESSON, J. 2004. The ton system, an ABC transporter, and a universally conserved GTPase are involved in iron utilization by *Brucella melitensis* 16M. *Infection and Immunity* **72:** 5783-5790.

DE ASSIS, M.C., DA COSTA, A.O., BARJA-FIDALGO, T.C. and PLOTKOWSKI, M.C. 2000. Human endothelial cells are activated by interferon-γ plus tumour necrosis factor-α to kill intracellular *Pseudomonas aeruginosa*. *Immunology* **101:** 271-278.

DE ROSE, R., TENNENT, J., MCWATERS, P., CHAPLIN, P.J., WOOD, P.R., KIMPTON, W., CAHILL, R. and SCHEERLINCK, J.Y. 2002. Efficacy of DNA vaccination by different routes of immunisation in sheep. *Veterinary Immunology and Immunopathology* **90:** 55-63.

DE VILLIERS, E.P., BRAYTON, K.A., ZWEYGARTH, E. and ALLSOPP, B.A. 2000. Genome size and genetic map of *Cowdria ruminantium*. *Microbiology* **146:** 2627-2634.

DELOGU, G., HOWARD, A., COLLINS, F.M. and MORRIS, S.L. 2000. DNA vaccination against tuberculosis: Expression of an ubiquitin-conjugated tuberculosis protein enhances antimycobacterial immunity. *Infection and Immunity* **68:** 3097-3102.

DU PLESSIS, J.L. 1985. A method for determining the *Cowdria ruminantium* infection rate of *Amblyomma hebraeum*: Effects in mice injected with tick homogenates. *Onderstepoort Journal of Veterinary Research* ***52*****:** *55-61.*

FENG, H. and WALKER, D.H. 2000. Mechanisms of intracellular killing of *Rickettsia conorii* in infected human endothelial cells, hepatocytes and macrophages. *Infection and Immunity* **68:** 6729-6736.

FENG, H., WHITWORTH, T., POPOV, V., and WALKER, D.H. 2004. Effect of antibody on the Rickettsia-host cell interaction. *Infection and Immunity* **72:** 3524-3530.

FU, T., GUAN, L., FRIEDMAN, A., ULMER, J.B., LIU, M.A. and DONNELLY, J.J. 1998. Induction of MHC class I-restricted CTL response by DNA immunisation with ubiquitin-influenza virus nucleoprotein fusion antigens. *Vaccine* **16:** 1711-1717.

FYNAN, E.F., WEBSTER, R.G., FULLER, D.H., HAYNES, J.R., SANTORO, J.C. and ROBINSON, H.L. 1993. DNA vaccines: Protective immunisations by parental, mucosal, and gene-gun inoculations. *Proceedings of the National Academy of Sciences of the United States of America* **90:** 11478-11482.

GARMORY, H.S. and TITBALL, R.W. 2004. ATP-binding cassette transporters are targets for the development of antibacterial vaccines and therapies. *Infection and Immunity* **72:** 6757-6763.

GHOSH, A., LABRECQUE, S. and MATLASHEWSKI, G. 2001. Protection against *Leishmania donovani* infection by DNA vaccination: increased DNA vaccination efficiency through inhibiting the cellular p53 response. *Vaccine* **19:** 3169-3178.

GURUNATHAN, S., KLINMAN, D.M. and SEDER, R.A. 2000a. DNA vaccines: immunology, application and optimisation. *Annual Reviews in Immunology* **18:** 927-974.

GURUNATHAN, S., WU, C., FREIDAG, B.L. and SEDER, R.A. 2000b. DNA vaccines: a key for inducing long-term cellular immunity. *Current Opinion in Immunology* **12:** 442-447.

IBORRA, S., SOTO, M., CARRIÓN, J., ALONSO, C. and REQUENA, J.M. 2004. Vaccination with a plasmid DNA cocktail encoding the nucleosomal histones of *Leishmania* confers protection against murine cutaneous leishmaniosis. *Vaccine* **22:** 3865-3876.

JIANG, W., JIN, N., CUI, S., LI, Z., ZHANG, L., WANG, H. and HAN, W. 2006. Enhancing immune responses against HIV-1 DNA vaccine by co inoculating IL-6 expression vector. *Journal of Virological Methods* **136:** 1-7.

JOHNSTON, S.A., TALAAT, A.M. and MCGUIRE, M.J. 2002. Genetic immunisation: What's in a name? *Archives of Medical Research* **33:** 325-329.

JOMAA, M., YUSTE, J., PATON, J.C., JONES, C., DOUGAN, G. and BROWN, J.S. 2005. Antibodies to the iron uptake ABC transporter lipoproteins PiaA and PiuA promote opsonophagocytosis of *Streptococcus pneumoniae*. *Infection and Immunity* **73:** 6852-6859.

JOMAA, M., TERRY, S., HALE, C., JONES, C., DOUGAN, G. and BROWN, J. 2006. Immunisation with the iron uptake ABC transporter proteins PiaA and PiuA prevents respiratory infection with *Streptococcus pneumoniae*. *Vaccine* **24:** 5133-5139.

LEWIS, D.A., KLESNEY-TAIT, J., LUMBLEY, S.R., WARD, C.K., LATIMER, J.L., ISON, C.A. and HANSEN, E.J. 1999. Identification of the *znu*A-encoded periplasmic zinc transport protein of *Haemophilus ducreyi*. *Infection and Immunity* **67:** 5060-5068.

MACKOWIAK, PA. 1998. Concepts of Fever. *Archives of Internal Medicine* **158:** 1870-1881.

MAECKER, H.T., UMETSU, D.T., DEKRUYFF, R.H. and LEVY, S. 1998. Cytotoxic T cell responses to DNA vaccination: dependence on antigen presentation via Class II MHC. *The Journal of Immunology* **161**: 6532-6536.

MAHAN, S.M., ANDREW, H.R., TEBELE, N., BURRIDGE, M.J. and BARBET, A.F. 1995. Immunisation of sheep against heartwater with inactivated *Cowdria ruminantium*. *Research in Veterinary Science* **58:** 46-49.

MAYRHOFER, P., TABRIZI, C.A., WALCHER, P., HAIDINGER, W., JECHLINGER, W. and LUBITZ, W. 2005. Immobilisation of plasmid DNA in bacterial ghosts. *Journal of Controlled Release* **102:** 725-735.

MÉNDEZ, S., GURUNATHAN, S., KAMHAWI, S., BELKAID, Y., MOGA, M.A., SKEIKY, Y.A.W., CAMPOS-NETO, A., REED, S., SEDER, R.A. and SACKS, D. 2001. The potency and durability of DNA- and protein-based vaccines against *Leishmania major* evaluated using low-dose, intradermal challenge. *The Journal of Immunology* **166:** 5122-5128.

MERCER, J. and TRAKTMAN, P. 2005. Genetic and cell biological characterization of the vaccinia virus A30 and G7 phosphoproteins. *Journal of Virology* **79:** 7146-7161.

MUTUNGA, M., PRESTON, P.M. and SUMPTION, K.J. 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.

MUTWIRI, G., PONTAROLLO, R., BABIUK, S., GRIEBEL, P., VAN DRUNEN LITTLE – VAN DEN HURK, S., MENA, A., TSANG, C., ALCON, V., NICHANI, A., IOANNOU, X., GOMIS, S., TOWNSEND, H., HECKER, R., POTTER, A. and BABIUK, L.A. 2003. Biological activity of immunostimulatory CpG DNA motifs in domestic animals. *Veterinary Immunology and Immunopathology* **91**: 89-103.

MWANGI, D.M., MAHAN, S.M., NYANJUI, J.K., TARACHA, E.L.N. and MCKEEVER, D.J. 1998. Immunisation of cattle by infection with *Cowdria ruminantium* elicits T lymphocytes that recognise autologous infected endothelial cells and monocytes. *Infection and Immunity* **66:** 1855-1860.

MWANGI, D.M., MCKEEVER, D.J., NYANJUI, J.K., BARBET, A.F. and 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. and 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. and MAHAN, S.M. 2002. DNA vaccination with the *map*1 gene followed by protein boost augments protection against challenge with *Cowdria ruminantium*, the agent of heartwater. *Vaccine* **20:** 1215-1225.

O'DONOVAN, L.H., MCMONAGLE, E.L., TAYLOR, S., BAIN, D., PACITTI, A.M., GOLDER, M.C., MCDONALD, M., HANLON, L., ONIONS, D.E., ARGYLE, D.J., JARRETT, O. and NICOLSON, L. 2005. A vector expressing feline mature IL-18 fused to IL-1β antagonist protein signal sequence is an effective adjuvant to a DNA vaccine for feline leukaemia virus. *Vaccine* **23:** 3814-3823.

POBER, J.S. 1999. Immunobiology of human vascular endothelium. *Immunologic Research* **19:** 225-232.

PRETORIUS, A., VAN STRIJP, F., BRAYTON, K.A., COLLINS, N.E., and ALLSOPP, B.A. 2002. Genetic immunisation with *Ehrlichia ruminantium* GroEL and GroES homologues. *Annals of the New York Academy of Sciences* **969:** 151-154.

QAZI, K.R., WIKMAN, M., VASCONCELOS, N., BERZINS, K., STÅHL, S. and FERNÁNDEZ, C. 2005. Enhancement of DNA vaccine potency by linkage of *Plasmodium falciparum* malarial antigen gene fused with a fragment of HSP70 gene. *Vaccine* **23:** 1114-1125.

ROTHENFUSSER, S., TUMA, E., ENDRES, S. and HARTMANN, G. 2002. Plasmacytoid dendritic cells: the key to CpG. *Human Immunology* **63:** 1111-1119.

SAHA, S., TAKESHITA, F., SASAKI, S., MATSUDA, T., TANAKA, T., TOZUKA, M., TAKASE, K., MATSUMOTO, T., OKUDA, K., ISHII, N., YAMAGUCHI, K., KLINMAN, D.M., XIN, K.Q., and OKUDA, K. 2006. Multivalent DNA vaccine protects mice against pulmonary infection caused by *Pseudomonas aeruginosa*. *Vaccine* **24:** 6240-6249.

SOLOSKI, M.J., SZPERKA, M.E., DAVIES, A. and WOODEN, S.L. 2000. Host immune response to intracellular bacteria: A role for MHC-linked class-1b antigen–presenting molecules. *Proceedings of the Society for Experimental Biology and Medicine* **224:** 231-239.

SMOOKER, P.M., SETIADY, Y.Y., RAINCZUK, A. and SPITHILL, T.W. 2000. Expression library immunisation protects mice against a challenge with virulent rodent malaria. *Vaccine* **18:** 2533-2540.

STADEN, R., BEAL, K.F. and BONFIELD, J.K. 1998. The Staden Package. In: Computer Methods in Molecular Biology. Edited by MISENER, S. and KRAWETZ, S.A. The Humana Press Inc., Totowa, NJ 07512. *Bioinformatics Methods and Protocols* **132:** 115-130.

SUDOWE, S., LUDWIG-PORTUGALL, I., MONTERMANN, E., ROSS, R. and RESKE-KUNZ, A.B. 2003. Transcriptional targeting of dendritic cells in gene gun-mediated DNA immunisation favours the induction of type 1 immune responses. *Molecular Therapy* **8:** 567-575.

SYKES, K.F. and JOHNSTON, S.A. 1999. Genetic live vaccines mimic the antigenisity but not pathogenicity of live viruses. *DNA and Cell Biology* **18:** 521-531.

SYKES, K.F., LEWIS, M.G., SQUIRES, B. and JOHNSTON, S.A. 2002. Evaluation of SIV library vaccines with genetic cytokines in a macaque challenge. *Vaccine* **20:** 2382-2395.

TAKEDA, K. and AKIRA, S. 2004. TLR signalling pathways. *Seminars in Immunology*. **16:** 3-9.

TANABE, M., ATKINS, H.S., HARLAND, D.N., ELVIN, S.J., STAGG, A.J., MIRZA, O., TITBALL, R.W., BYRNE, B. and BROWN, K.A. 2006. The ABC transporter protein OppA provides protection against experimental *Yersinia pestis* infection. *Infection and Immunity* **74:** 3687-3691.

THALHAMER, J., LEITNER, W., HAMMERL, P. and BRTKO, J. 2001. Designing immune responses with genetic immunisation and immunostimulatory DNA sequences. *Endocrine Regulations* **35:** 143-166.

TOTTÉ, P., VACHIÉRY, N., MARTINEZ, D., TRAP, I., BALLINGALL, K.T., MACHUGH, N.D., BENSAID, A. and WÉRENNE, J. 1996. Recombinant bovine interferon gamma inhibits the growth of *Cowdria ruminantium* but fails to induce major histocompatibility complex class II following infection of endothelial cells. *Veterinary Immunology and Immunopathology* **53:** 61-71.

TOTTÉ, P., MCKEEVER, D., MARTINEZ, D. and BENSAID, A. 1997. Analysis of T-cell responses in cattle immunised 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. and MCKEEVER, D.J. 1999. Immune responses to *Cowdria ruminantium* infections. *Parasitology Today* **15:** 286-290.

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

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

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

VARSHAVSKY, A. 1996. The N-end rule: Functions, mysteries, uses. *Proceedings of the National Academy of Sciences of the United States of America* **93:** 12142-12149.

WAGNER, H. 2002. Interactions between bacterial CpG-DNA and TRL9 bridge innate and adaptive immunity. *Current Opinion in Microbiology* **5:** 62-69.

WEISSMAN, A.M. 2001. Themes and variations on ubiquitylation. *Nature Reviews Molecular Cell Biology* **2:** 169-178.

WHEELER, D.L., CHURCH, D.M., FEDERHEN, S., LASH, A.E., MADDEN, T.L., PONTIUS, J.U., SCHULER, G.D., SCHRIML, L.M., SEQUEIRA, E., TATUSOVA, T.A. and WAGNER, L. 2003. Database resources of the National Center for Biotechnology. *Nucleic Acids Research* **31:** 28–33.

WHITTON, J.L., RODRIGUEZ, F., ZHANG, J. and HASSETT, D.E. 1999. DNA immunisation: mechanistic studies. *Vaccine* **17:** 1612-1619.

WIKEL, S.K. and ALARCON-CHAIDEZ, F.J. 2001. Progress toward molecular characterization of ectoparasites modulation of host immunity. *Veterinary Parasitology* **101:** 275-287.

XUE, T., STAVROPOULOS, E., YANG, M., RAGNO, S., VORDERMEIER, M., CHAMBERS, M., HEWINSON, G., LOWRIE, D.B., COLSTON, M.J. and TASCON, R.E. 2004. RNA encoding the MPT83 antigen induces protective immune responses against *Mycobacterium tuberculosis* infection. *Infection and Immun*ity **72:** 6324-6329.

ZHANG, B., CENTRA, M., CAO, G.L., RATYCH, R.E., DOMACHOWSKE, J.B., MALECH, H.L. and ROSEN, G.M. 1997. Are free radicals responsible for endothelial cell killing of *Staphylococcus aureus*? *Immunology Letters* **58:** 113-120.

ZHU, Y., REN, J., DA'DARA, A., HARN, D., XU, M., SI. J., YU, C., LIANG, Y., YE, P., YIN, X., HE, W., XU, W., CAO, G. and HUA, W. 2004. The protective effect of *Schistosoma japonicum* Chinese strain 23 kDA plasmid DNA vaccine in pigs is enhanced with IL-12. *Vaccine* **23:** 78-83.

3. CHAPTER 3: HETEROLOGOUS NEEDLE AND NATURAL TICK CHALLENGE

3.1. INTRODUCTION

An ideal recombinant vaccine should protect against all the different pathogen genotypes present in the field and should be able to induce recall immunity effective enough to overcome natural tick challenge. In the previous chapter we were able to identify four possible vaccine candidates and we demonstrated that these four 1H12 open reading frames (ORFs) protected sheep against a virulent *E. ruminantium* Welgevonden needle challenge. Several issues still need to be addressed: firstly, the ability of this vaccine to protect against different *E. ruminantium* genotypes, and secondly, whether the vaccine will be effective under natural field conditions.

Initial experiments carried out in the early 20th century showed that almost all *E. ruminantium* stocks available at the time cross-protected against each other (Neitz, 1939). This suggested that few, or no, differences existed at the serological and molecular level between the various field isolates. These studies were however done before the tools for genetic and immunological characterisation were available. There was no way of knowing if the animals used in these studies were previously exposed to *Ehrlichia* or if the stocks used to challenge and immunise the animals consisted of only one genotype.

Today there is strong evidence, both on the serological and molecular levels that several *E. ruminantium* genotypes exist in the field (Du Plessis *et al*., 1989; van Heerden *et al.,* 2004a; 2004b). Cross-protection studies have shown that the Ball3 stock, used for the "blood vaccine", does not protect against more virulent stocks such as Welgevonden and Kümm (Du Plessis *et al.,* 1989). It has been discovered recently, however, that the original Kümm stock consisted of two distinct genotypes, one of which, designated Kümm 1, is closely related to the Senegal stock as determined by the pCS20 sequence (Zweygarth *et al.,* 2002; van Heerden *et al.,* 2004b). Cross-protection studies were also done using the West African *E. ruminantium* stocks

and immunisation using the Ball3 stock did not protect against the Senegal stock (Jongejan *et al.,* 1988). A more recent cross-protection study was done using six genetically different *E. ruminantium* stocks: Ball3, Blaauwkrans, Gardel, Kwanyanga, Mara87/7 and Welgevonden. Animals used in this experiment were not previously exposed to *E. ruminantium*, as determined by the pCS20 test (van Heerden *et al.,* 2004b). Only the Welgevonden stock provided complete cross-protection in sheep against challenge using the six stocks mentioned above. Ball3, the "vaccine" stock, only provided protection against Kwanyanga (Collins *et al.,* 2003). This observed failure to cross-protect confirms the existence of genetic and antigenic differences between the various *E. ruminantium* genotypes.

These antigenic differences between *E. ruminantium* genotypes will have an influence on the immunological outcome when vaccines are designed. DNA immunisation with one or more genes will prime the host immune response to recognise the encoded protein or, more specifically, epitopes derived from that protein. After challenge with live bacteria, the host immune response will respond by presenting *E. ruminantium* antigen on MHC class I and II molecules by APC. This requires that the bacterial proteins are degraded in a sequence dependant manner into epitopes by the host cells. Recall immunity will only be activated once epitopes are identified in the bacteria that are identical to the epitopes derived from the antigen that was used to prime the host immune response. The more conserved a protein is, the more likely it will be that identical epitopes will exist in the same proteins found in other *E. ruminantium* genotypes.

In contrast a polymorphic protein will have a lower percentage of epitopes shared between two genotypes thus reducing the chance that a protein derived from one genotype can induce protection against other genotypes. A study which illustrates this point is one in which an experimental vaccine consisting of the polymorphic *E. ruminantium* Crystal Springs major antigenic protein 1 (*map*1) gene was used to immunise mice. Animals immunised with *map*1 DNA were challenged with two different *E. ruminantium* stocks: Crystal Springs and Mbizi. While 50 % of the mice survived after challenge with Crystal Springs, when the immunised animals were challenged with the Mbizi stock only 2/30 mice survived (Nyika *et al.,* 2002). This

implies that the Crystal Springs *map*1 gene has different epitopes than those which could be derived from the *map*1 gene in the Mbizi stock. Therefore, we postulate that conserved epitopes derived from one genotype will protect against other genotypes. In this chapter the genetic diversity of the four 1H12 ORFs originating from the *E. ruminantium* Welgevonden stock will be assessed and their ability to protect against six other virulent *E. ruminantium* stocks will be investigated.

The potential contribution of vector-derived factors in pathogen transmission and establishment is often neglected and it is therefore important to include studies on the effects of arthropod transmission of *E. ruminantium* when a vaccine is being developed. The nature of the tick–host relationship involves intricate countermeasures mounted by the tick against host acquired immune elements in order to ensure tick survival (Ribeiro *et al.,* 1985 and reviewed by Wikel, 1999 and Wikel and Alarcon-Chaidez, 2001). These include the down regulation of cellular immunity associated cytokines such as IFN-γ, IL-6, TNF-α and IL-8 (Leboulle *et al.,* 2002a), and superoxide and nitric oxide production (Kuthejlová *et al.,* 2001). All of these functions are pivotal for the killing of intracellular pathogens.

There is increasing evidence that tick-borne pathogens take advantage of this tick-induced modulation of the host immune response. Tick transmitted pathogens such as *Borrelia* utilise this immune suppression to avoid macrophage anti-microbial killing (Kuthejlová *et al.,* 2001). Another tick transmitted bacterium, *Francisella tularensis,* showed increased proliferation in the host after stimulation with a tick salivary gland extract **(**Krocova *et al*., 2003). This suggests that *E. ruminantium* infection via tick bite could be qualitatively different from infection by needle injection and therefore that different immune responses might be expected. The survival of animals immunised with *E. ruminantium* Welgevonden 1H12 cocktail will therefore be investigated in the field where disease transmission is by natural tick infestation.

3.2. MATERIALS AND METHODS

3.2.1. Materials

See appendix A for materials and buffer components.

3.2.2. Methods

3.2.2.1. Sequence analysis of selected 1H12 ORFs from different *E. ruminantium* isolates

3.2.2.1.1. E. ruminantium *stocks and genomic DNA preparation*

Genomic DNA isolated from *E. ruminantium* stocks originating in South Africa, West Africa and the Caribbean were used for amplification of the four 1H12 ORFs. Stocks isolated in South Africa were Ball3 (Haig, 1952), Kwanyanga (Mackenzie and Van Rooyen, 1981), Welgevonden (Du Plessis, 1985), Mara87/7 (Du Plessis *et al.,* 1989), Vosloo (Du Plessis, 1993), Kümm1 (Zweygarth *et al.,* 2002) and Blaauwkrans (an isolate from an *A. hebraeum* tick taken from an eland in the Eastern Cape province of South Africa). Gardel was isolated in Guadeloupe (Uilenberg *et al.,* 1985). West African stocks included Pokoase417 and Sankat430 from Ghana (Bell-Sakyi *et al.,* 1997), Mali (Logan *et al.,* 1985) and Senegal (Jongejan *et al.,* 1988). Genomic DNA was prepared from blood stabilates or cell cultures of each isolate using the QIAamp DNA mini kit according to the instructions of the manufacturer.

3.2.2.1.2. Amplification of the 1H12 ORFs

Erum2540, -2550, -2580 and -2590 were amplified from genomic DNA preparations of the eleven *E. ruminantium* isolates using primers located in the regions flanking the genes (Table 3.1). The PCR was carried out using TaKaRa Ex Taq™ (TaKaRa Bio Inc.) polymerase, and a 100 µl reaction mixture contained purified genomic DNA (~100 ng) and 0.5 U *Taq* polymerase in 1x reaction buffer, 200 µM dNTPs and 250 nM of each primer. The samples were denatured for 2 min at 94 °C, followed by 30 cycles of denaturation (94 °C, 10 s), annealing (55 °C, 30 s), and extension (72 °C, 15 min) using a GeneAmp® PCR System 9700 thermocycler (Applied Biosystems). For each isolate, PCR products from five separate reactions were pooled and purified, and the same primers were used to sequence the amplicons directly using a BigDye terminator cycle sequencing kit (Perkin Elmer, Applied Biosystems). The sequences

were assembled using gap4 (Staden *et al.,* 1998). Sequence alignment and phylogenetic analysis were done using CLUSTAL_X (Thompson *et al.,* 1997). Epitope mapping was done using SYFPEITHI (Rammensee *et al.,* 1999).

***Table 3.1**: Primers used to amplify the four 1H12 ORFs.*

Primer name	Primer sequence (5' → 3')	T(a)[1] °C
Erum2540 F	TAT TTT TTT TTC CAA AAA TTT ATT A	
Erum2540 R	TGT TGA GAC TTA GTT TTA T	55
Erum2550 F	ATG ATA GGT TTA CAG TTG	
Erum2550 R	TAT ATC TTA AAT AAT AAA ATT TTA	55
Erum2580 F	ATG AAA CAT ATA ATC TTT CTT T	
Erum2580 R	CTT ATG TAG TTG ATA GAC AA	55
Erum2590 F	ATG TTT CAT AAT TTA CTC G	
Erum2590 R	TAA GCA TAA GAT GAG AA	55

[1] *Annealing temperature for each primer set.*

3.2.2.2. Animal experiments

3.2.2.2.1. Animal trial 1: Heterologous needle challenge

Animals were immunised using the immunisation strategy described in chapter 2 (section 2.2.2.4). Briefly, six to eight month old Merino sheep were each given plasmid DNA cocktail containing all four 1H12 ORFs, 200 µg by i.m. inoculation and 20 µg by gene gun delivery. All animals received three immunisations given at three week intervals. Groups of four animals per group were challenged with the six *E. ruminantium* stocks (Table 3.2) five weeks after the final boost as described previously (section 2.2.2.4). Sheep were monitored daily for onset of clinical symptoms after challenge. Severity of infection was determined by scoring the animals' symptoms according to a RI (Table 2.2). Sheep were either treated with oxytetracycline or euthanased *in extremis*.

3.2.2.2.2. Animal immunisation trial 2: Natural tick challenge

All the sheep that survived the needle challenge, a 1H12 cocktail immunised group and an empty pCMViUBs vector control group were challenged in the field (Table 3.2). For field challenge, sheep were translocated to Springbokfontein farm, in the

Limpopo province, a heartwater-endemic area of South Africa. Here the sheep were subjected to natural challenge by infestation with *Amblyomma hebraeum* ticks.

3.2.2.2.3. Preparation of the challenge material for animal trial 1

Blood stabilate was prepared from the six *E. ruminantium* stocks as described previously (Brayton *et al*., 2003). The following virulent *E. ruminantium* stocks were used: Welgevonden, Ball3, Blaauwkrans, Kwanyanga, Gardel and Mara87/7. The infected sheep blood samples were diluted in SPG (1:1) and stored in liquid nitrogen. The stabilates were titred so that a dose of 10 LD_{50}s could be reproducibly given.

3.2.1.7. Statistical analysis

Reaction index scores were compared by means of the one tailed distribution Student's *t*-test. Significance was assessed at P values of < 0.01 throughout the study.

Table 3.2: *Immunisation strategy followed in this chapter. Groups of sheep were immunised with the 1H12 ORF cocktail and challenged, along with positive and negative control groups, either with various* E. ruminantium *stocks in the laboratory or exposed to a natural tick challenge in the field.*

Group	Number of sheep	Immunisation	Challenge with	Challenge method
Negative control	5	Empty pCMViUBs vector	Welgevonden	Needle (Laboratory)
Positive control	5	Infect and treat	Welgevonden	Needle (Laboratory)
Experimental	5	DNA vaccine cocktail	Welgevonden	Needle (Laboratory)
Negative control	4	Empty pCMViUBs vector	Ball3	Needle (Laboratory)
Experimental	4	DNA vaccine cocktail	Ball3	Needle (Laboratory)
Negative control	4	Empty pCMViUBs vector	Gardel	Needle (Laboratory)
Experimental	4	DNA vaccine cocktail	Gardel	Needle (Laboratory)
Negative control	4	Empty pCMViUBs vector	Kwanyanga	Needle (Laboratory)
Experimental	4	DNA vaccine cocktail	Kwanyanga	Needle (Laboratory)
Negative control	4	Empty pCMViUBs vector	Mara87/7	Needle (Laboratory)
Experimental	4	DNA vaccine cocktail	Mara87/7	Needle (Laboratory)
Negative control	4	Empty pCMViUBs vector	Blaauwkrans	Needle (Laboratory)
Experimental	4	DNA vaccine cocktail	Blaauwkrans	Needle (Laboratory)
Negative control	5	Empty pCMViUBs vector	n/a	Ticks (field)
Experimental	5	DNA vaccine cocktail	n/a	Ticks (field)

3.3. RESULTS

3.3.1. Sequence analysis of selected 1H12 ORFs from different *E. ruminantium* isolates

In the previous chapter we showed that Welgevonden-derived 1H12 proteins protect against an *E. ruminantium* Welgevonden challenge, thus indicating that the host immune response recognised and responded to the 1H12 proteins in the bacterial organism. Here we wanted to test whether the Welgevonden-derived 1H12 proteins were conserved at the amino acid level. This should give an indication if these four genes could protect against heterologous challenge.

The level of molecular conservation of each of the four 1H12 ORFs between different *E. ruminantium* stocks available in the laboratory was determined by PCR amplification and sequence analysis. The DNA sequence of each ORF was translated into its corresponding amino acid sequence, and aligned using CLUSTAL_X (Thompson *et al.,* 1997). All four of the ORFs were highly conserved and only single amino acid polymorphisms could be detected as indicated for each ORF in figures 3.1-3.4. The amino acid sequences of all four ORFs were more conserved in the West African isolates than in isolates from southern Africa. The Erum2540, Erum2580 and Erum2590 amino acid sequences of the West African isolates Pokoase417, Sankat430, Mali and Senegal were identical. The Erum2550 amino acid sequence of Pokoase417 and Sankat430 stocks were identical and grouped with Senegal, (Figure 3.5) while the Mali stock grouped with the southern African stocks. The southern African isolates showed more sequence diversity, including amino acid sequences of ORFs from the Kümm1 isolate grouping with the West African stocks. The amino acid sequences of Welgevonden and Vosloo were identical for all four ORFs tested, and Ball3, Kwanyanga and Blaauwkrans usually grouped together (Figure 3.5).

Erum2540

```
Consensus       MLRLSFIFIV LITLLTYQNG YSAKSPDPVN IKLIVGSMDM KNRTINIGVE FRIQDGWHIY YKSPGDLGLP TVFQWQENIF KDVQIHWPQP IQHTDTTSNN  100
Welgevonden     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Vosloo          ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Blaauwkrans     ---------L ---------- ---------- ---------- ---------- ---------L ---------- ---------- ---------- ----------
Kwanyanga       ---------L ---------- ---------- ---------- ---------- ---------L ---------- ---------- ---------- ----------
Mara87/7        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Ball3           ---------L ---------- ---------- ---------- ---------- ---------L ---------- ---------- ---------- ----------
Gardel          ---------M ---------- ---------- ---------- ---------- ---------M ---------- ---------- ---------- ----------
Senegal         ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Mali            ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Sankat430       ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Kümm1           ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Pokoase417      ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

Consensus       IFHSNVYKDI VMFPISFALK HDNLNTKELS ISLRIKYAVC KDVCIPQEKV IILNRFLQDY VNQENLGLIN FWKKK             176
Welgevonden     ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----
Vosloo          ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----
Blaauwkrans     ----------- ---------- ---------- ---------- ---------- ----------- ---------- -----
Kwanyanga       ----------- ---------- ---------- ---------- ---------- ----------- ---------- -----
Mara87/7        ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----
Ball3           ----------- ---------- ---------- ---------- ---------- ----------- ---------- -----
Gardel          ----------- ---------- ---------- ---------- ---------- ----------- ---------- -----
Senegal         ---------- ---------- --T-SS---- ---------- ---------- ---------- ---------- -----
Mali            ---------- ---------- --T-SS---- ---------- ---------- ---------- ---------- -----
Sankat430       ---------- ---------- --T-SS---- ---------- ---------- ---------- ---------- -----
Kümm1           ---------- ---------- --T-SS---- ---------- ---------- ---------- ---------- -----
Pokoase417      ---------- ---------- --T-SS---- ---------- ---------- ---------- ---------- -----
```

Figure 3.1: *Comparison of the predicted amino acid sequences of Erum2540 between twelve* E. ruminantium *stocks. Differences in sequence are indicated in bold one-letter code.*

Erum2550

```
Consensus       MIGLQLENVS YKYKKGQFLL SDVNIICKKG EVICLLGPSG CGKSTVLKLI AGLENLSQGS IYINDKVIVN NDFYVPTEKR NVGLIFQHPS LFPHQTVIEN  VMFAIKEPPK MQKFQVALDI  120
Welgevonden     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Vosloo          ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Blaauwkrans     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Kwanyanga       ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Mara87/7        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Ball3           ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Gardel          ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Senegal         ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Mali            ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Sankat430       ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Pokoase417      ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Kümm1           ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

Consensus       LQSVNMGAYK DMYPDMLSGG QQQLVTIARA IAQKPAIILL DEPFSNLDTV LRLNIRRNVL SLLKSKGITV LLVTHDPEEA LEVSDVIYVM RDGHVVQHGT PHEIYYNPKD HMLARFFGEV   220
Welgevonden     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Vosloo          ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Blaauwkrans     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Kwanyanga       ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Mara87/7        ------V--- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Ball3           ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Gardel          ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Senegal         ------V--- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Mali            ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Sankat430       ------V--- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Pokoase417      ------V--- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Kümm1           ------V--- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

Consensus       PHEIYYNPKD HMLARFFGEV NHFVGIVRNS YITLPIGKIP AQSFNDGEEV VVCIRPEAII SDPNRGIKGI VEHIKFFNNM ISVCVEGHSY WMRFNNVMLP QIGDTIFILL DLNKILLFKI  341
Welgevonden     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Vosloo          ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Blaauwkrans     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- V--------- ---------- ---------- ----------
Kwanyanga       ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- V--------- ---------- ---------- ----------
Mara87/7        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Ball3           ---------- ---------- -------Q-- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Gardel          ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Senegal         ---------- ---------- -------Q-- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Mali            ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Sankat430       ---------- ---------- -------Q-- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Pokoase417      ---------- ---------- -------Q-- ---------- ---------- ---------- ---------- ---------- -----K---- ---------- ---------- ----------
Kümm1           ---------- ---------- -------Q-- ---------- ---------- ---------- ---------- ---------- -----K---- ---------- ---------- ----------
```

Figure 3.2: *Comparison of the predicted amino acid sequences of Erum2550 between twelve* E. ruminantium *stocks. Differences in sequence are indicated in bold one-letter code.*

Erum2580

```
Consensus       MKHIIFLLFL YISLYPITGY SVPKIIATIN PIYSLVDAVT EGVTKPVLLI NQQISIHDYM LKPSDKRKIR SSNVIFYVDD HLETFINKIK DKTLIKLSDV         50
Welgevonden     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Vosloo          ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Blaauwkrans     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Kwanyanga       ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Mara87/7        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Ball3           ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Gardel          ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Senegal         ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Mali            ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Sankat430       ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Kümm1           ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Pokoase417      ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

Consensus       VALLPSRYDS HFSYKVHTTS NDLHIWLSPD NAKKIVEHIK LVLCKIDPEN AEVYDKNARL TIIRITELAE KIKQLLNTVK TKPYVVTHDA YQYFEKYFGL         150
Welgevonden     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Vosloo          ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Blaauwkrans     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Kwanyanga       ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Mara87/7        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Ball3           ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Gardel          ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Senegal         ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Mali            ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Sankat430       ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Kümm1           ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Pokoase417      ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

Consensus       NFITSLSSSH NTNISVKKLT HIQKVITKNN ISCIFSESQN DKIRNLFSQH KVTFQILDPI GNIAQKESYF DIMQNIANNF FSCLSTT        250
Welgevonden     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -------
Vosloo          ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -------
Blaauwkrans     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -N-----
Kwanyanga       ---------- ---------A ---------- ---------- ---------- ---------- ---------- ---------- -N-----
Mara87/7        ---------- ---------A ---------- ---------- ---------- ---------- ---L------ ---------- -------
Ball3           ---------- ---------A ---------- ---------- ---------- ---------- ---------- ---------- -N-----
Gardel          ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -------
Senegal         ---------- ---------A ---------- ---------- ---------- ---------- ---S------ ---------- -------
Mali            ---------- ---------A ---------- ---------- ---------- ---------- ---S------ ---------- -------
Sankat430       ---------- ---------A ---------- ---------- ---------- ---------- ---S------ ---------- -------
Kümm1           ---------- ---------A ---------- ---------- ---------- ---------- ---S------ ---------- -------
Pokoase417      ---------- ---------A ---------- ---------- ---------- ---------- ---S------ ---------- -------
```

Figure 3.3: *Comparison of the predicted amino acid sequences of Erum2580 between twelve* E. ruminantium *stocks. Differences in sequence are indicated in bold one-letter code.*

Erum2590

```
Consensus       MFHNLLGKNK IFSHHDRYVN DYIINVKDLS FAYAKKKVID NVSFQVKFGE IITILGPNGG GKTTLIRILV GIYKNYLGIV EYAKNFVIGY LPQNFSVNSL         100
Welgevonden     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Vosloo          ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Blaauwkrans     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Kwanyanga       ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Mara87/7        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Ball3           ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Gardel          ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Senegal         ---------- ----R----- -------N-- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Mali            ---------- ----R----- -------N-- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Sankat430       ---------- ----R----- -------N-- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Pokoase417      ---------- ----R----- -------N-- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Kümm1           ---------- ----R----- -------N-- ---------- ---------- ---------- ---------- ---------- ---------- ----------

Consensus       IPMTVEYFLV SSYTKQRKKL NLNSVLKDVN VVKILNRQMS EISHGELQLV LLARCLMLNP DIIILDEPVS CMDINAKDSF YKLINQLILR YNLSVIMTSH         200
Welgevonden     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Vosloo          ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Blaauwkrans     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Kwanyanga       ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Mara87/7        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Ball3           ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Gardel          ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Senegal         ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Mali            ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Sankat430       ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Pokoase417      ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Kümm1           ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

Consensus       DLHFVMANSY RVICINKSIY CEGSPSEIVK NEKFLKMFSS YA                  243
Welgevonden     ---------- ---------- ---------- ---------- --
Vosloo          ---------- ---------- ---------- ---------- --
Blaauwkrans     ---------- ---------- ---------- ---------- --
Kwanyanga       ---------- ---------- ---------- ---------- --
Mara87/7        ---------- ---------- ---------- ---------- --
Ball3           ---------- ---------- ---------- ---------- --
Gardel          ---------- ---------- ---------- ---------- --
Senegal         ---------- ---------- ---------- ---------- --
Mali            ---------- ---------- ---------- ---------- --
Sankat430       ---------- ---------- ---------- ---------- --
Pokoase417      ---------- ---------- ---------- ---------- --
Kümm1           ---------- ---------- ---------- ---------- --
```

Figure 3.4: *Comparison of the predicted amino acid sequences of Erum2590 between twelve* E. ruminantium *stocks. Differences in sequence are indicated in bold one-letter code.*

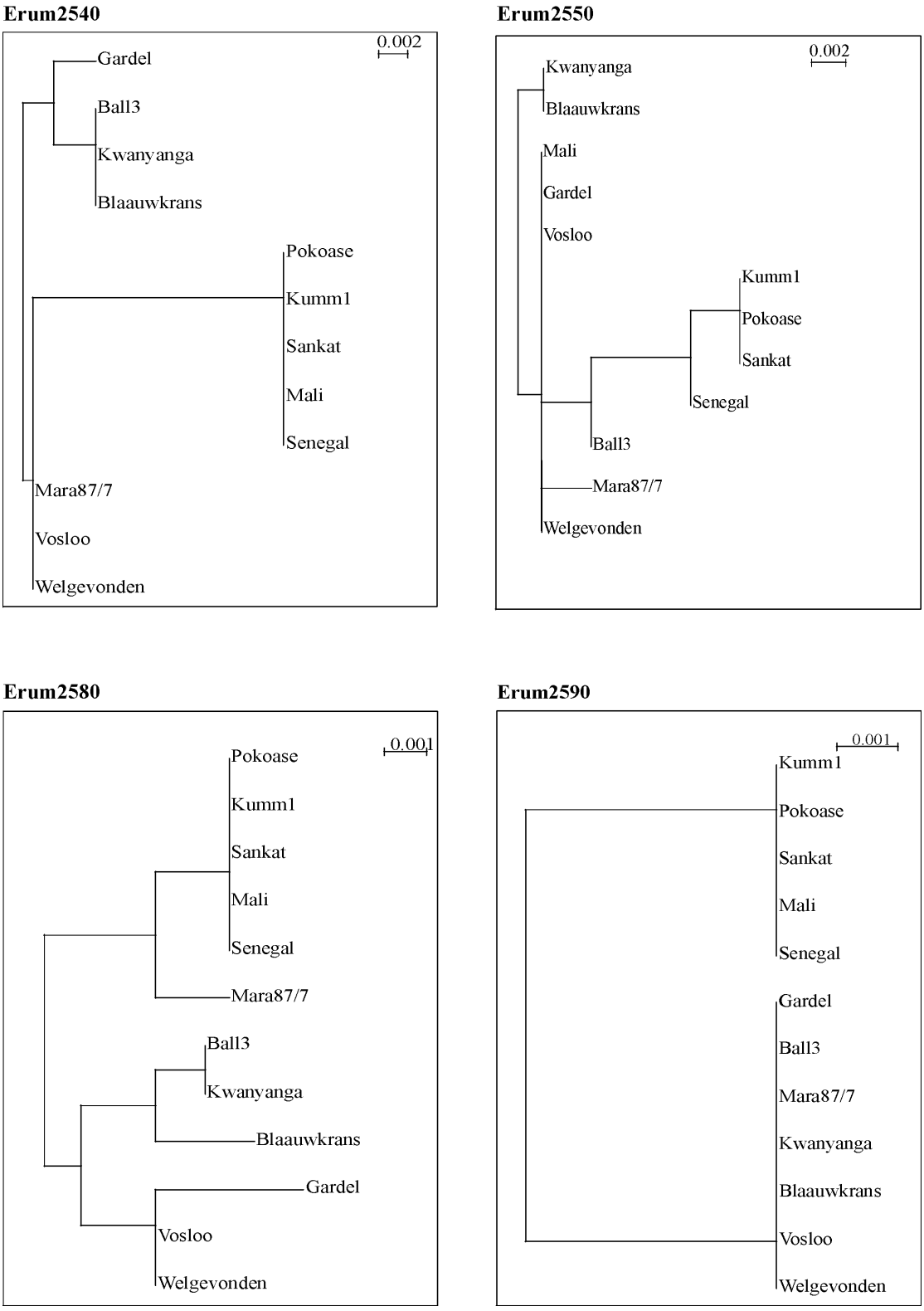


***Figure 3.5**: Phylogenetic neighbor joining trees showing relationships between the amino acid sequences of Erum2540, -2550, -2580 and -2590.*

In addition to sequence analysis, epitope prediction for each of the four 1H12 ORFs was performed using the epitope prediction program SYFPEITHI (Rammensee *et al.,* 1999). This was done to determine if the polymorphisms detected in the amino acid sequences would have an influence on predicted epitopes. The results presented here are only examples of possible epitopes that will associate with MHC class II molecules. So far epitope prediction programs can only predict epitopes that will bind to human MHC molecules, but it is likely that similar epitopes will be predicted for sheep MHC. Epitopes were predicted for all the possible sequence variations that were detected in the amino acid sequence for each ORF. Only the top scoring 15-mer epitopes (the best MHC class II binders) are presented in table 3.3.

All of the predicted top scoring epitopes were identical in the different sequence combinations obtained for Erum2550, -2580 and -2590. Epitopes predicted for Erum2540 were similar for Gardel, Senegal , Welgevonden and Blaauwkrans, but due to the slight variation in sequence the SYFPEITHI scores for each epitope were slightly different, thus making the epitopes derived from Welgevonden and closely related sequences better MHC binders. Epitope prediction was done in the same way for the more polymorphic MAP1 protein from South African (Welgevonden) and West African (Senegal) stocks. In contrast to the highly conserved 1H12 proteins, few of the MAP1 epitopes showed scores higher than 20, and only 3 of the top ten scoring epitopes were identical between the two isolates while another 2 of these epitopes had similar sequences.

This could be an indication that the Welgevonden-derived 1H12 DNA vaccine may protect against heterologous challenge since the more conserved the epitopes are between genotypes, the greater the chance that the protein products of these conserved genes will induce protective immunity against other genotypes.

Table 3.3: *Top scoring predicted epitopes from the four different amino acid sequences obtained from sequencing the ORFs from twelve* E. ruminantium *stocks and the map1 sequence obtained from the Welgevonden and Senegal stocks. Epitopes were predicted using H2-A –15mer epitopes with SYFPEITHI. (See figure 3.2 for amino acid sequences).*

Erum2540:

Sequence corresponding to Welgevonden			Sequence corresponding to Blaauwkrans			Sequence corresponding to Senegal			Sequence corresponding to Gardel		
Position	Predicted Epitopes	Score	Position	Predicted Epitope	Score	Position	Predicted Epitope	Score	Position	Predicted Epitope	Score
156	**F**LQ**D**Y**V**NQENLGLIN	26	156	**F**LQ**D**Y**V**NQENLGLIN	26	156	**F**LQ**D**Y**V**NQENLGLI	26	156	**F**LQ**D**Y**V**NQENLGLIN	26
119	**L**KH**D**N**L**NTKELSISL	24	119	**L**KH**D**N**L**NTKELSISL	24	139	**V**CK**D**V**C**IPQEKVIIL	24	119	**L**KH**D**N**L**NTKELSISL	24
139	**V**CK**D**V**C**IPQEKVIIL	24	139	**V**CK**D**V**C**IPQEKVIIL	24	63	**S**PG**D**L**G**LPTVFQWQE	20	139	**V**CK**D**V**C**IPQEKVIIL	24
63	**S**PG**D**L**G**LPTVFQWQE	20	63	**S**PG**D**L**G**LPTVFQWQE	20	36	**G**SM**D**M**K**NRTINIGVE	18	63	**S**PG**D**L**G**LPTVFQWQE	20
36	**G**SM**D**M**K**NRTINIGVE	18	36	**G**SM**D**M**K**NRTINIGVE	18	52	**R**IQ**D**G**W**HIYYKSPGD	18	36	**G**SM**D**M**K**NRTINIGVE	18
						119	**L**KH**D**T**L**SSKELSISL	18			

Erum2550:

Sequence corresponding to Welgevonden			Sequence corresponding to Blaauwkrans			Sequence corresponding to Senegal		
Position	Predicted Epitope	Score	Position	Predicted Epitope	Score	Position	Predicted Epitope	Score
209	**V**MR**D**G**H**VVQHGTPHE	26	209	**V**MR**D**G**H**VVQHGTPHE	26	209	**V**MR**D**G**H**VVQHGTPHE	26
79	**K**RN**V**G**L**IFQHPSLFP	22	79	**K**RN**V**G**L**IFQHPSLFP	22	79	**K**RN**V**G**L**IFQHPSLFP	22
145	**V**TI**A**R**A**IAQKPAIIL	22	145	**V**TI**A**R**A**IAQKPAIIL	22	145	**V**TI**A**R**A**IAQKPAIIL	22
154	**K**PA**I**I**L**LDEPFSNLD	22	154	**K**PA**I**I**L**LDEPFSNLD	22	154	**K**PA**I**I**L**LDEPFSNLD	22
313	**R**FN**N**V**M**LPQIGDTIF	20	313	**R**FN**N**V**M**LPQIGDTIF	20	313	**R**FN**N**V**M**LPQIGDTIF	20
19	**L**LS**D**V**N**IICKKGEVI	18	19	**L**LS**D**V**N**IICKKGEVI	18	19	**L**LS**D**V**N**IICKKGEVI	18
69	**V**NN**D**F**Y**VPTEKRNVG	18	69	**V**NN**D**F**Y**VPTEKRNVG	18	69	**V**NN**D**F**Y**VPTEKRNVG	18

Sequence corresponding to Mara87/7			Sequence corresponding to Pokoase417		
Position	Predicted Epitope	Score	Position	Predicted Epitope	Score
209	**V**MR**D**G**H**VVQHGTPHE	26	209	**V**MR**D**G**H**VVQHGTPHE	26
79	**K**RN**V**G**L**IFQHPSLFP	22	79	**K**RN**V**G**L**IFQHPSLFP	22
145	**V**TI**A**R**A**IAQKPAIIL	22	145	**V**TI**A**R**A**IAQKPAIIL	22
154	**K**PA**I**I**L**LDEPFSNLD	22	154	**K**PA**I**I**L**LDEPFSNLD	22
313	**R**FN**N**V**M**LPQIGDTIF	20	313	**R**FN**N**V**M**LPQIGDTIF	20
19	**L**LS**D**V**N**IICKKGEVI	18	19	**L**LS**D**V**N**IICKKGEVI	18
69	**V**NN**D**F**Y**VPTEKRNVG	18	69	**V**NN**D**F**Y**VPTEKRNVG	18

Table 3.3: *Continued.*

Erum2580*:*

Sequence corresponding to Welgevonden			Sequence corresponding to Blaauwkrans			Sequence corresponding to Kwanyanga			Sequence corresponding to Senegal*		
Pos	Predicted Epitope	Score	Pos	Predicted Epitope	Score	Pos	Predicted Epitope	Score	Pos	Predicted Epitope	Score
144	CKIDPENAEVYDKNA	24	144	CKIDPENAEVYDKNA	24	144	CKIDPENAEVYDKNA	24	144	CKIDPENAEVYDKNA	24
44	TKPVLLINQQISIHD	22	44	TKPVLLINQQISIHD	22	44	TKPVLLINQQISIHD	22	44	TKPVLLINQQISIHD	22
257	LDPIGNIAQKESYFD	22	257	LDPIGNIAQKESYFD	22	257	LDPIGNIAQKESYFD	22	257	LDPIGNIAQKESYFD	22
266	KESYFDIMQNIANNF	22	266	KESYFDIMQNIANNF	22	266	KESYFDIMQNIANNF	22	266	KESYFDIMQNIANNF	22
147	DPENAEVYDKNARLT	20	147	DPENAEVYDKNARLT	20	147	DPENAEVYDKNARLT	20	147	DPENAEVYDKNARLT	20
237	ESQNDKIRNLFSQHK	20	237	ESQNDKIRNLFSQHK	20	237	ESQNDKIRNLFSQHK	20	237	ESQNDKIRNLFSQHK	20
76	FYVDDHLETFINKIK	18	76	FYVDDHLETFINKIK	18	76	FYVDDHLETFINKIK	18	76	FYVDDHLETFINKIK	18

* *Identical epitopes were predicted for Mara87/7 sequence*

Erum2590:

Sequence corresponding to Welgevonden			Sequence corresponding to Blaauwkrans		
Position	Predicted Epitopes	Score	Position	Predicted Epitope	Score
85	NFVIGYLPQNFSVNS	22	85	NFVIGYLPQNFSVNS	22
159	NPDIIILDEPVSCMD	22	159	NPDIIILDEPVSCMD	22
213	ICINKSIYCEGSPSE	20	213	ICINKSIYCEGSPSE	20
37	KVIDNVSFQVKFGEI	18	37	KVIDNVSFQVKFGEI	18
198	TSHDLHFVMANSYRV	18	198	TSHDLHFVMANSYRV	18
13	SHHDRYVNDYIINVK	16	13	SHRDRYVNDYIINVK	16

MAP1:

Sequence corresponding to Welgevonden			Sequence corresponding to Senegal		
Position	Predicted Epitopes	Score	Position	Predicted Epitope	Score
56	IKEDSKNTQTVFGLK	24	96	YENNPFLGFAGAVGY	20
21	GVSFSDVIQEDSNPA	22	23	SFSDVIQEENNPVGS	18
99	YENNPFLGFAGAIGY	20	69	LKKDWDGVKTPSGNT	18
127	ETFDVKNPGGNYKND	18	124	ETFDVRNPGGNYKND	18
181	ACYDIMLDGMPVSPY	18	175	ACYDIMLDGMPVSPY	18
144	MYCALDTAQQSATNG	16	139	AHMYCALDTASSSTA	16
165	VMIKNENLTNISLML	16	159	VMVKNENLTDISLML	16
261	KVSTVANPGFASATL	16	165	NLTDISLMLNACYDI	16
6	IFITSTLISLVSFLP	14	6	IFITSTLISLVSFLP	14
22	VSFSDVIQEDSNPAG	14	21	GVSFSDVIQEENNPV	14

3.3.2. 1H12 vaccine tested against heterologous *E. ruminantium* challenge

Five *E. ruminantium* stocks were chosen that are known to be virulent for sheep. These were Blaauwkrans, Ball3, Gardel, Kwanyanga, Mara87/7 and Welgevonden. Sheep were immunised with the 1H12 pCMViUBs constructs and the empty pCMViUBs vector as described in Table 3.2.

All the sheep immunised with the empty pCMViUBs vector and challenged with the various *E. ruminantium* stocks succumbed to infection; sheep challenged with the Welgevonden stock died on day 15 while animals in the groups challenged with the remaining stocks died between days 15 to 19. Every animal immunised with the 1H12 cocktail survived challenge with the various *E. ruminantium* stocks used in this experiment (Figures 3.6-3.10).

Sheep immunised with the empty pCMViUBs vector and challenged with the Welgevonden stock showed similar symptoms (Figure 3.10) and temperature reactions (Figure 3.11) as had been seen in the previous experiments (Chapter 2). In this experiment however, only two of the sheep immunised with the 1H12 cocktail and challenged with Welgevonden showed elevated temperatures of 42 °C, while the remaining three showed temperatures that peaked more than once above 41 °C but never reached 42 °C (Figure 3.16). In the previous experiments all the sheep immunised with the 1H12 cocktail showed elevated temperatures above 41.5 °C.

Similarly, sheep immunised with the empty pCMViUBs vector and challenged with Ball3 (Figures 3.6, 3.10), Blaauwkrans (Figures 3.6, 3.10), Gardel (Figures 3.7, 3.10), Kwanyanga (Figures 3.7, 3.10) and Mara87/7 (Figures 3.8, 3.10) succumbed to infection while animals that were immunised with the 1H12 cocktail survived challenge with the various stocks (Figures 3.6-3.9).

All the sheep immunised with the empty pCMViUBs vector showed a temperature reaction after challenge with the Ball3 (Figure 3.11), Blaauwkrans (Figure 3.12), Gardel (Figure 3.13), Kwanyanga (Figure 3.14) and Mara87/7 (Figure 3.15) stocks. All of these sheep developed severe heartwater symptoms (Figure 3.10) and had to be treated. In contrast, a temperature reaction was detected in two sheep immunised with

the 1H12 cocktail and challenged with Ball3 (Figure 3.11; sheep 472 and 475) and Kwanyanga (Figure 3.14; sheep 513 and 518); in one 1H12-immunised animal challenged with the Blaauwkrans stock (Figure 3.12; sheep 490); and in none of the 1H12 immunised sheep challenged with Gardel (Figure 3.13) and Mara87/7 (Figure 3.15). A RI score could therefore only be assigned to animals that did show temperature reactions (Figure 3.9).

Overall the reaction indices of the sheep immunised with 1H12 cocktail differed significantly from those of sheep immunised with empty pCMViUBs vector after challenge with each of the six *E. ruminantium* stocks (See Table 3.4 for P values). Even though differences in symptom development and temperature reactions were observed in the various groups, we were able to demonstrate that the *E. ruminantium* Welgevonden-derived 1H12 ORFs protected against needle challenge with Ball3, Kwanyanga, Gardel, Mara87/7 and Blaauwkrans stocks. This confirms the hypothesis that conserved protective ORFs from one genotype could confer protection to various other genotypes.

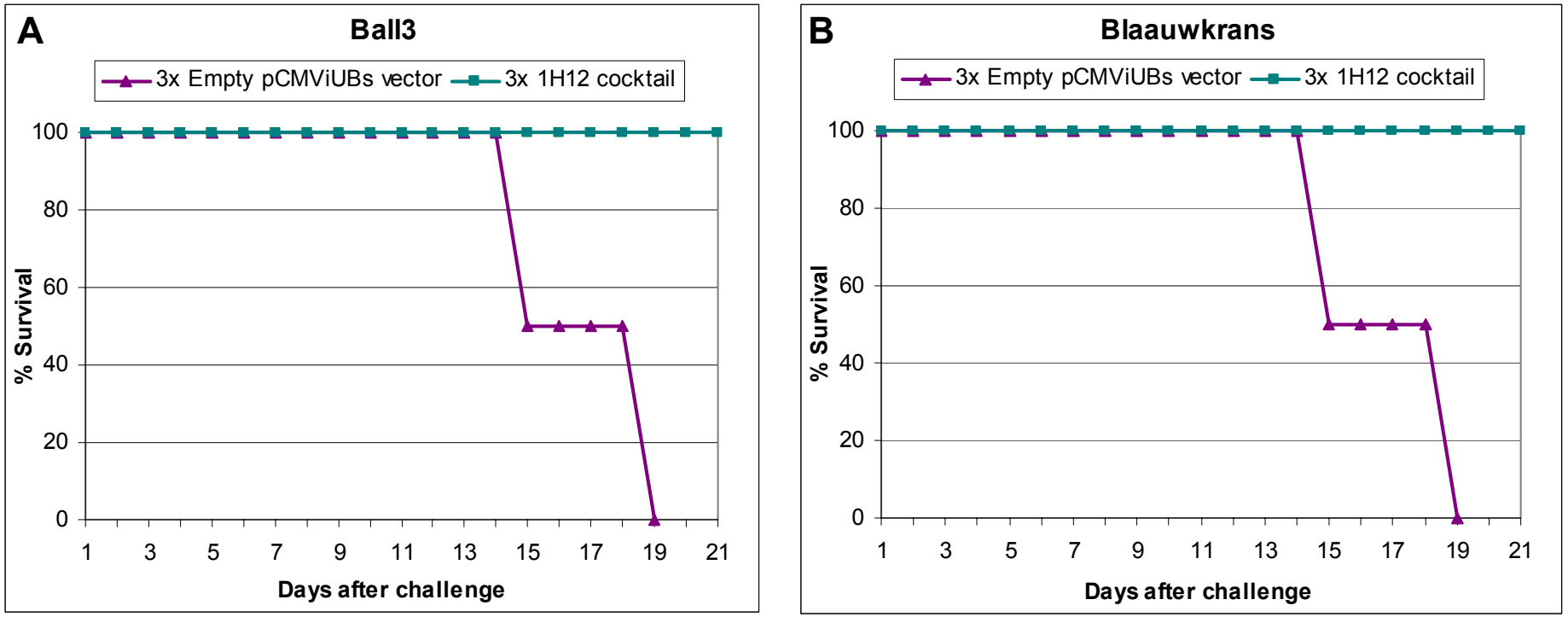


Figure 3.6: *Survival charts of animals challenged with* E. ruminantium *Ball3 (A) and Blaauwkrans (B) stocks after immunisation with 1H12 cocktail as compared to the survival of sheep immunised with empty pCMViUBs vector. Animals that were treated were also considered as non-survivors.*

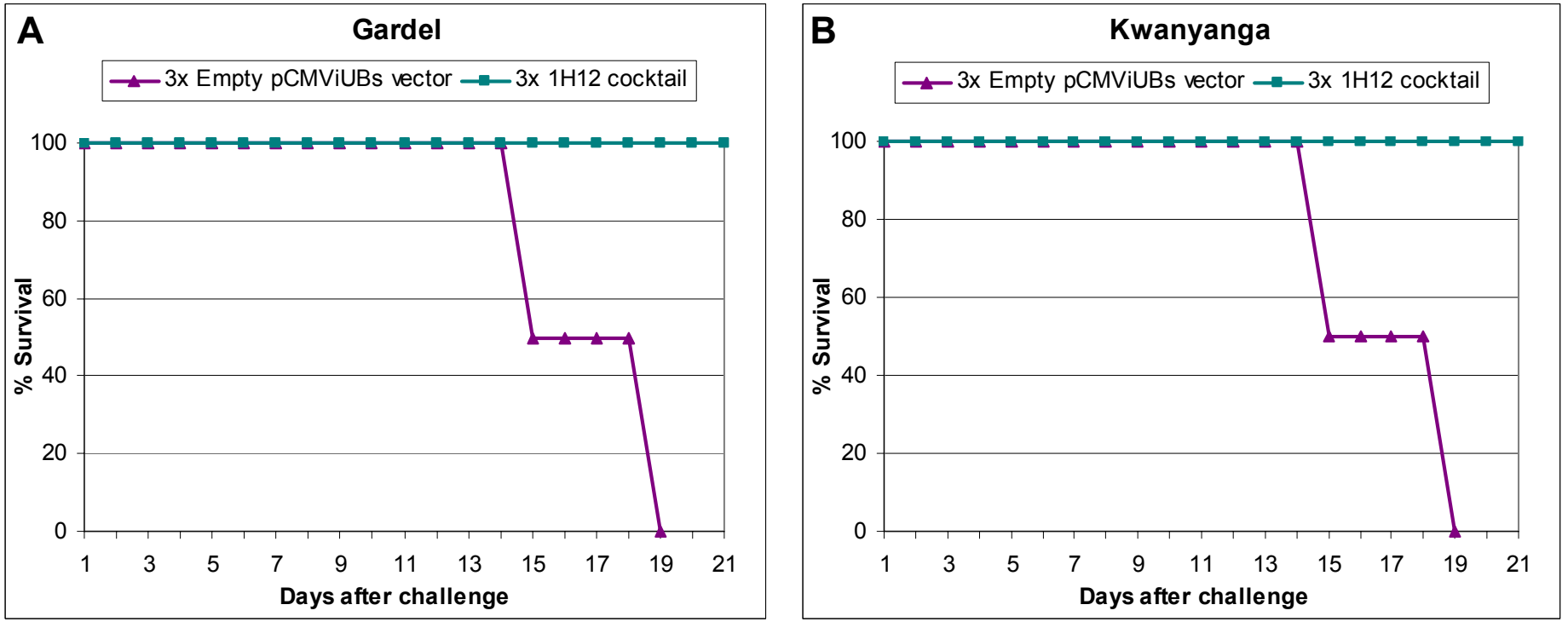


Figure 3.7: *Survival charts of animals challenged with* E. ruminantium *Gardel (A) and Kwanyanga (B) stocks after immunisation with 1H12 cocktail as compared to the survival of sheep immunised with empty pCMViUBs vector. Animals that were treated were also considered as non-survivors.*

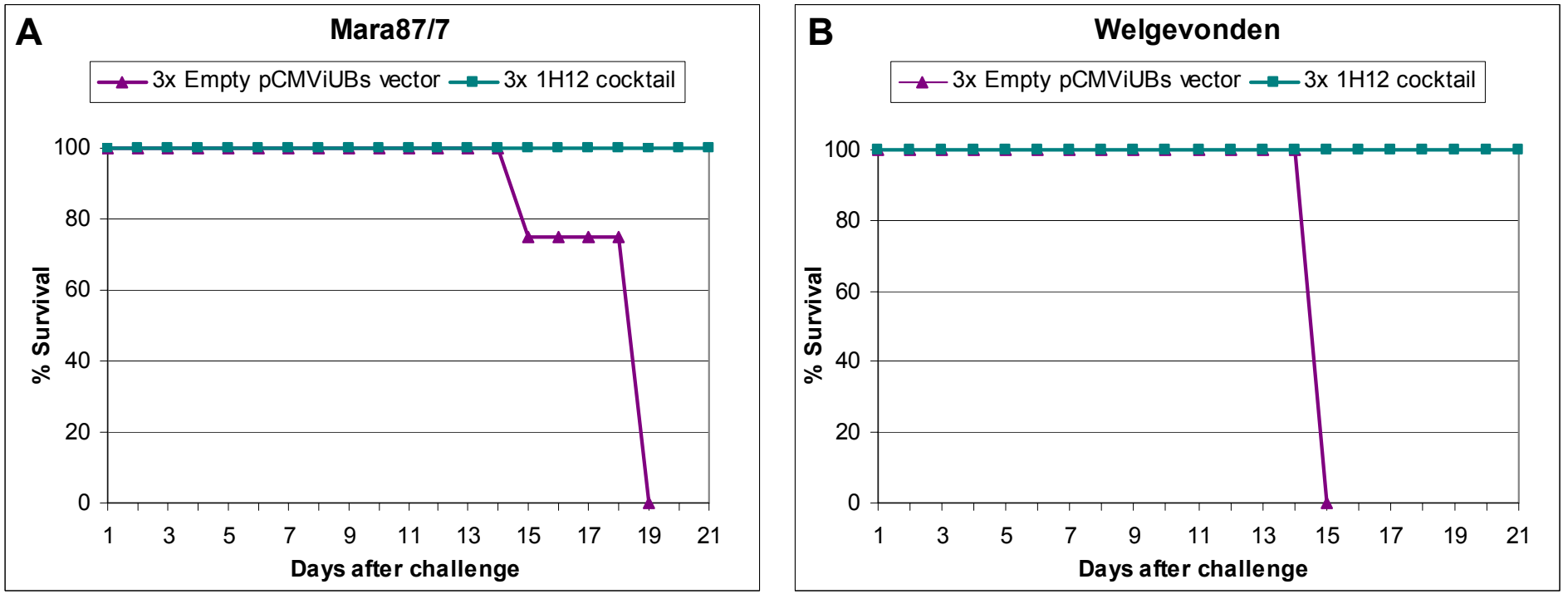


Figure 3.8: *Survival charts of animals challenged with* E. ruminantium *Mara87/7 (A) and Welgevonden (B) stocks after immunisation with 1H12 cocktail as compared to the survival of sheep immunised with empty pCMViUBs vector. Animals that were treated were also considered as non-survivors.*

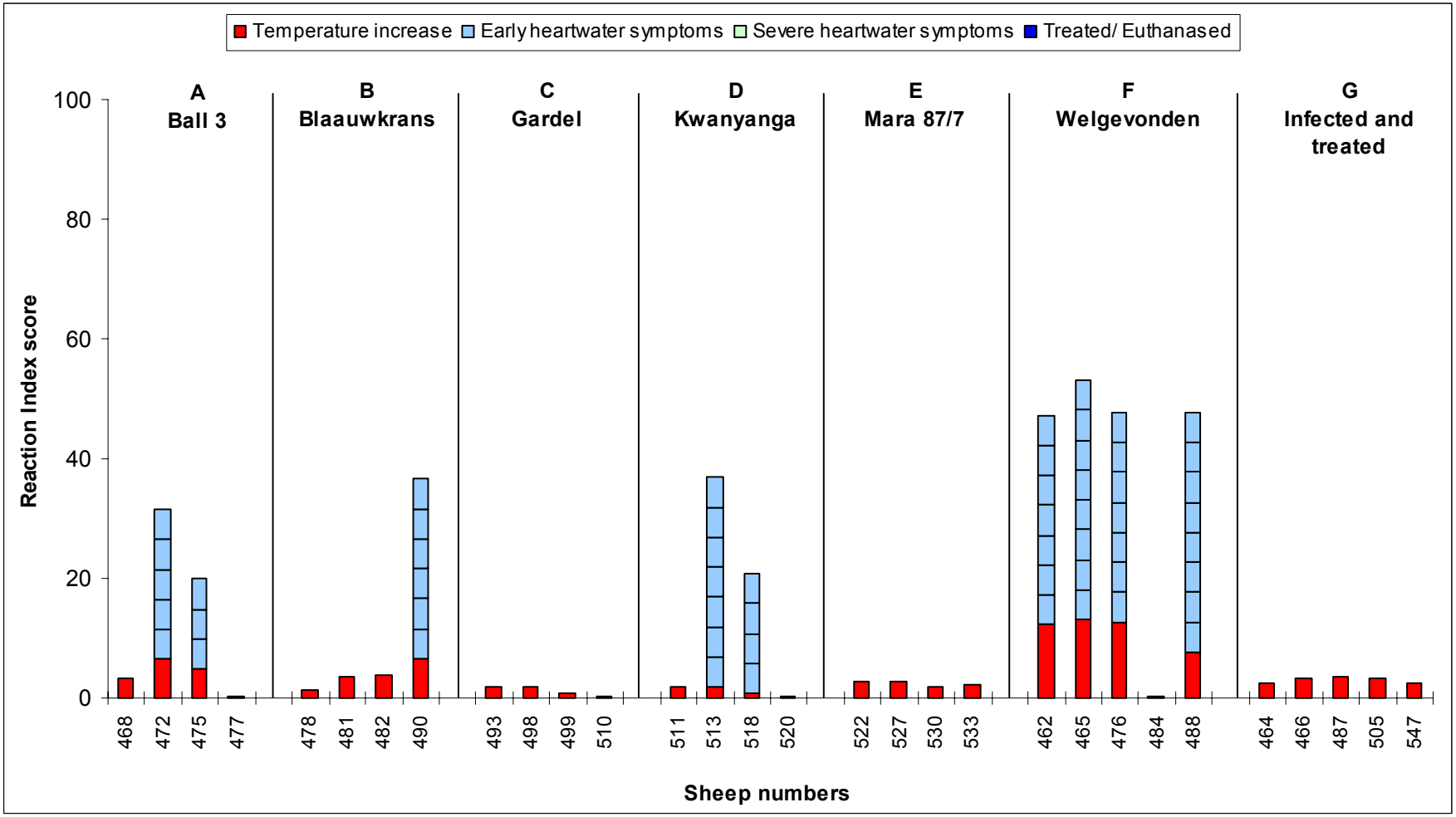


Figure 3.9: *Reaction index (RI) of the sheep immunised with 1H12 cocktail after challenge with* E. ruminantium *Ball3 (A), Blaauwkrans (B), Gardel (C), Kwanyanga (D), Mara87/7 (E) and Welgevonden (F) stocks. The positive control group was immunised by infection and treatment and was challenged with the Welgevonden stock (G). The red blocks indicate the total temperature reaction score 21 days after challenge, while the light blue blocks represent a RI of 5 (early heartwater symptoms), the light green a RI of 10 (severe heartwater symptoms), and the dark blue a RI of 50 (animal was treated/euthanased/died).*

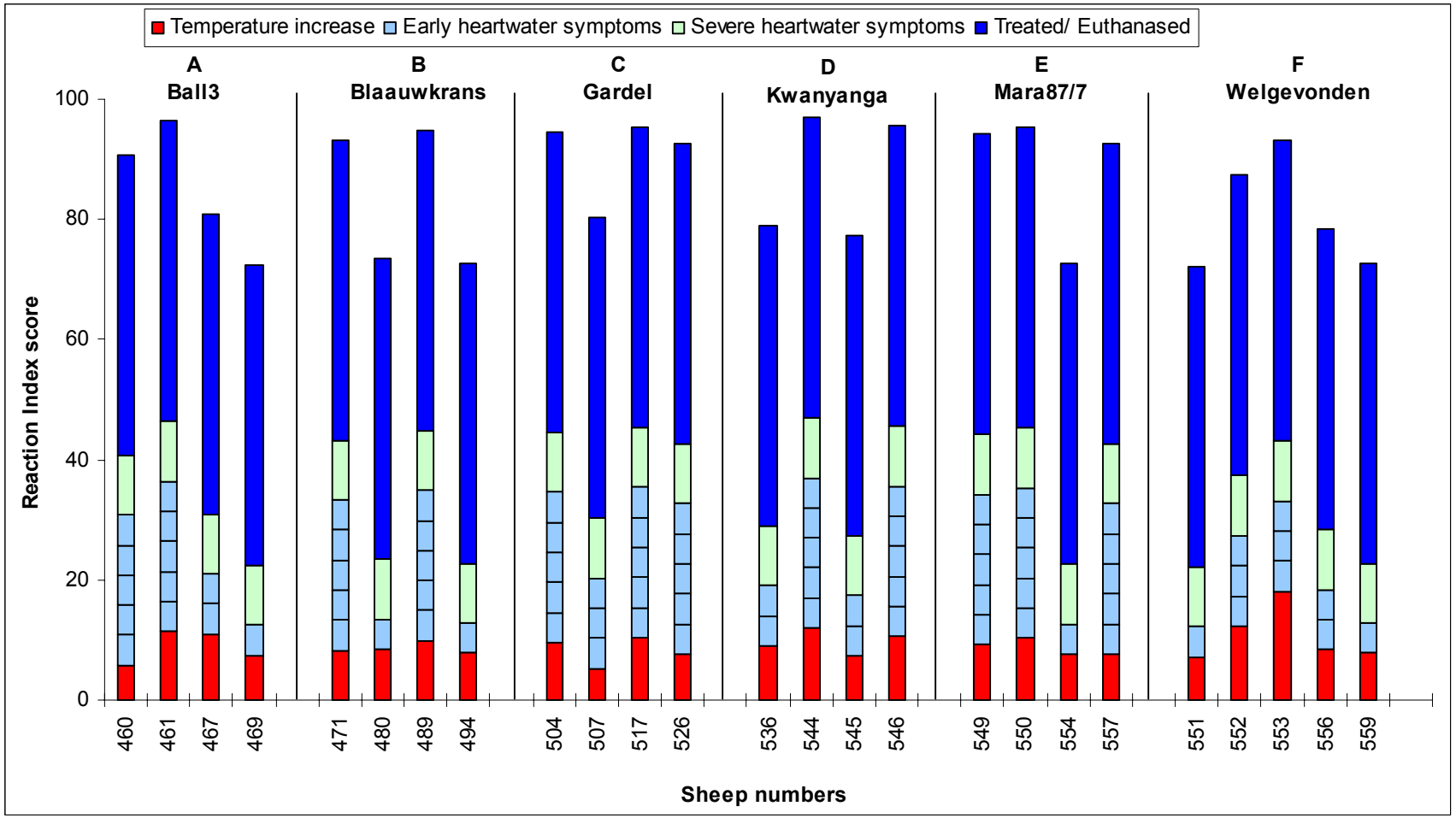


Figure 3.10: *Reaction index (RI) of the sheep immunised with empty pCMViUBs vector after challenge with* E. ruminantium *Ball3 (A), Blaauwkrans (B), Gardel (C), Kwanyanga (D), Mara87/7 (E) and Welgevonden (F) stocks. Red blocks indicate the total temperature reaction score 21 days after challenge, while the light blue blocks represent a RI of 5 (early heartwater symptoms), the light green a RI of 10 (severe heartwater symptoms), and the dark blue a RI of 50 (animal was treated/euthanased).*

Table 3.4: *Statistical analyses of RI values of sheep immunised either with 1H12 cocktail or with empty pCMViUBs vector and challenged with six* E. ruminantium *genotypes.*

Challenged with	Immunised with 1H12 cocktail		Immunised with Empty pCMViUBs vector		P value[1]
	RI_{AVE}	RI_{STDEV}	RI_{AVE}	RI_{STDEV}	
Ball3	16.22	18.98	85.17	10.57	4.0×10^{-4}
Blaauwkrans	11.32	16.88	83.63	12.12	2.0×10^{-4}
Gardel	1.17	0.81	90.75	7.06	1.0×10^{-7}
Kwanyanga	14.93	17.38	87.25	10.49	2.0×10^{-4}
Mara87/7	2.45	0.43	88.73	10.73	2.0×10^{-6}
Welgevonden	39.22	21.83	80.76	9.18	7.0×10^{-3}

[1] *P value was determined by one tailed distribution Student's t-test by comparing the 1H12 cocktail with the empty pCMViUBs vector group.*

Ball3:

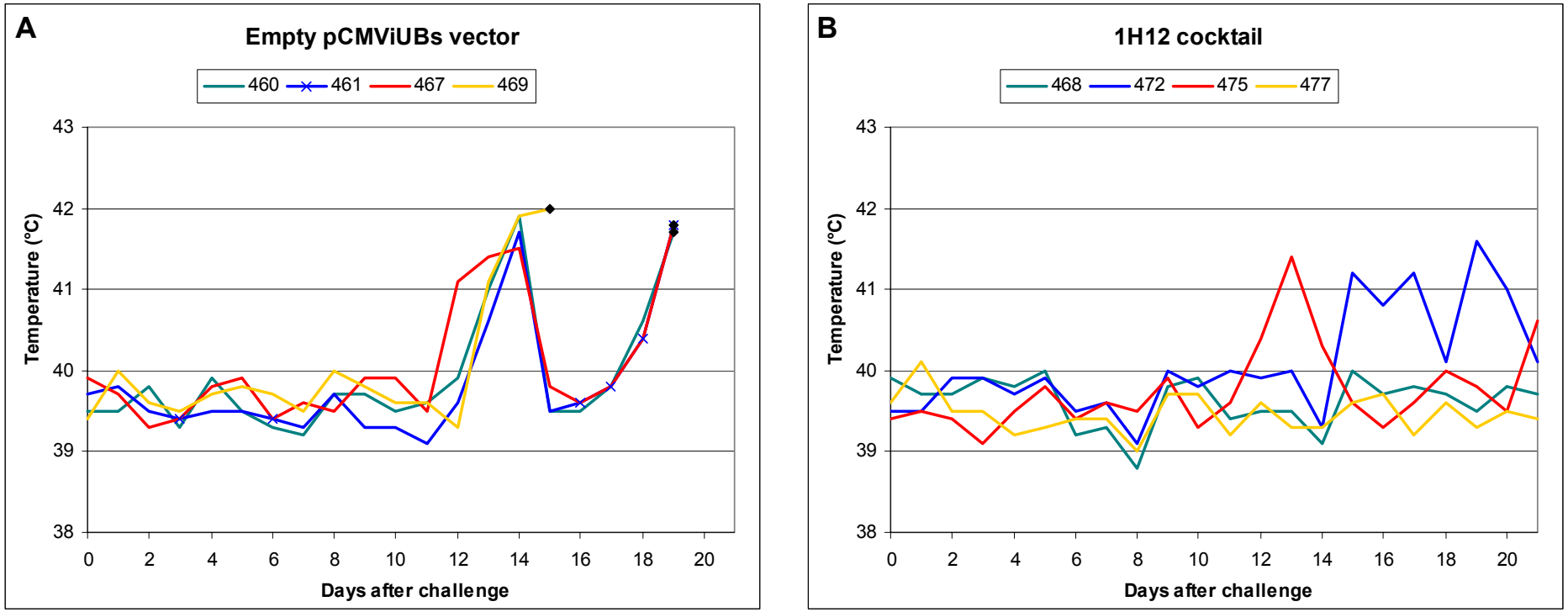


Figure 3.11: *Daily post-challenge temperatures of sheep immunised with empty pCMViUBs vector (A), and 1H12 cocktail (B). Sheep were challenged with 10 LD_{50}s of the virulent* E. ruminantium *Ball3 stock. Temperature measurements were terminated 21 days after challenge or as soon as an animal was treated or euthanased (represented by a black dot).*

Blaauwkrans:

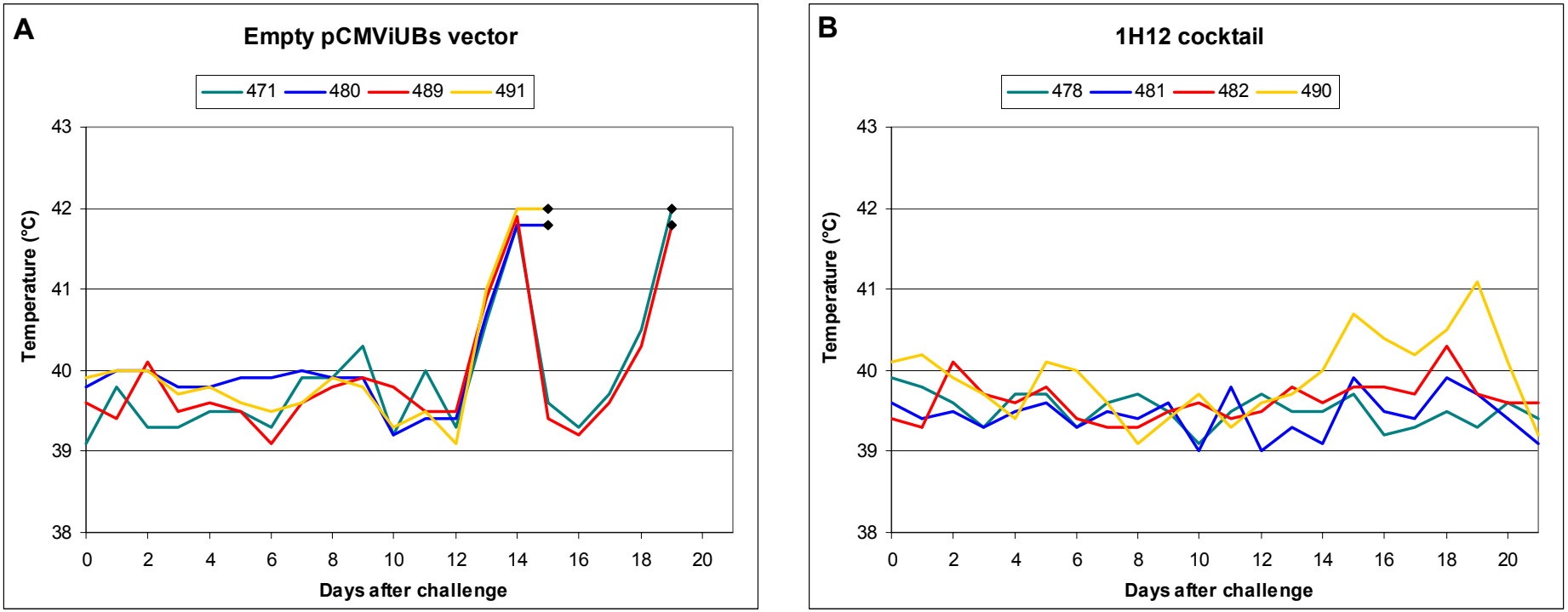


Figure 3.12: *Daily post-challenge temperatures of sheep immunised with empty pCMViUBs vector (A), and 1H12 cocktail (B). Sheep were challenged with 10 LD_{50}s of the virulent* E. ruminantium *Blaauwkrans stock. Temperature measurements were terminated 21 days after challenge or as soon as an animal was treated or euthanased (represented by a black dot).*

Gardel:

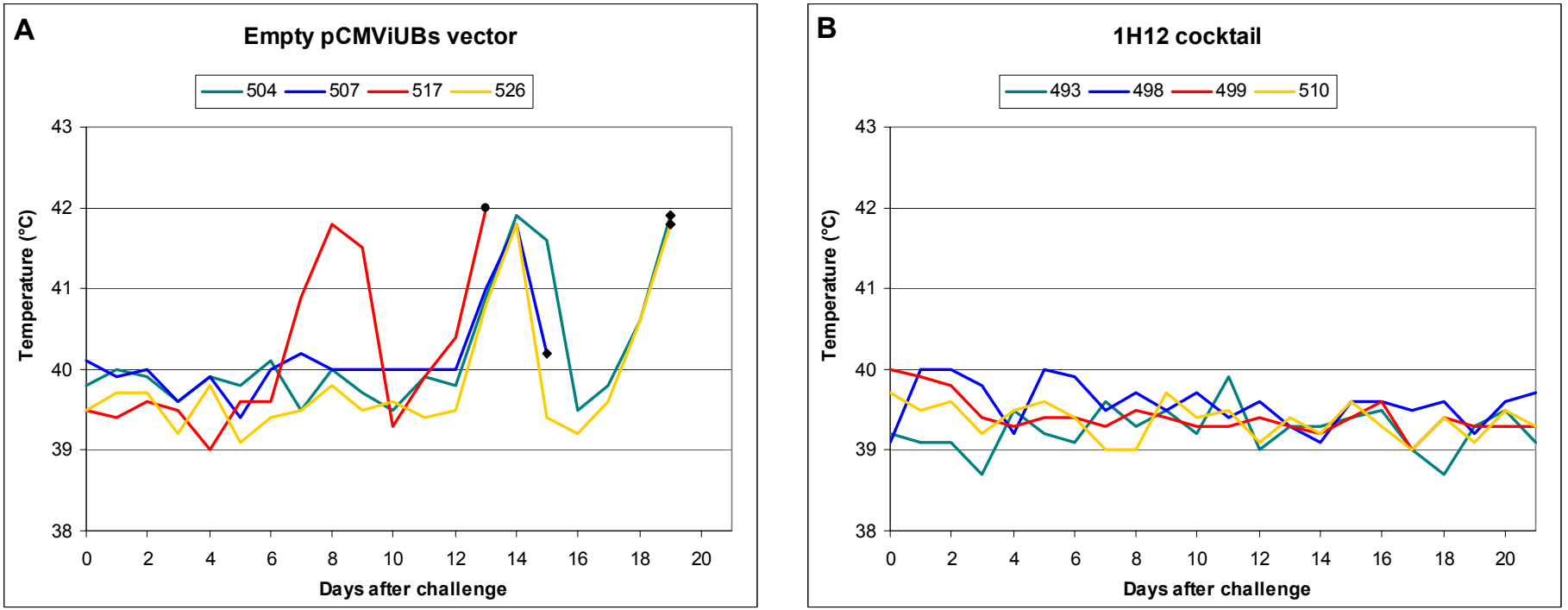


Figure 3.13: *Daily post-challenge temperatures of sheep immunised with empty pCMViUBs vector (A), and 1H12 cocktail (B). Sheep were challenged with 10 LD_{50}s of the virulent* E. ruminantium *Gardel stock. Temperature measurements were terminated 21 days after challenge or as soon as an animal was treated or euthanased (represented by a black dot).*

Kwanyanga:

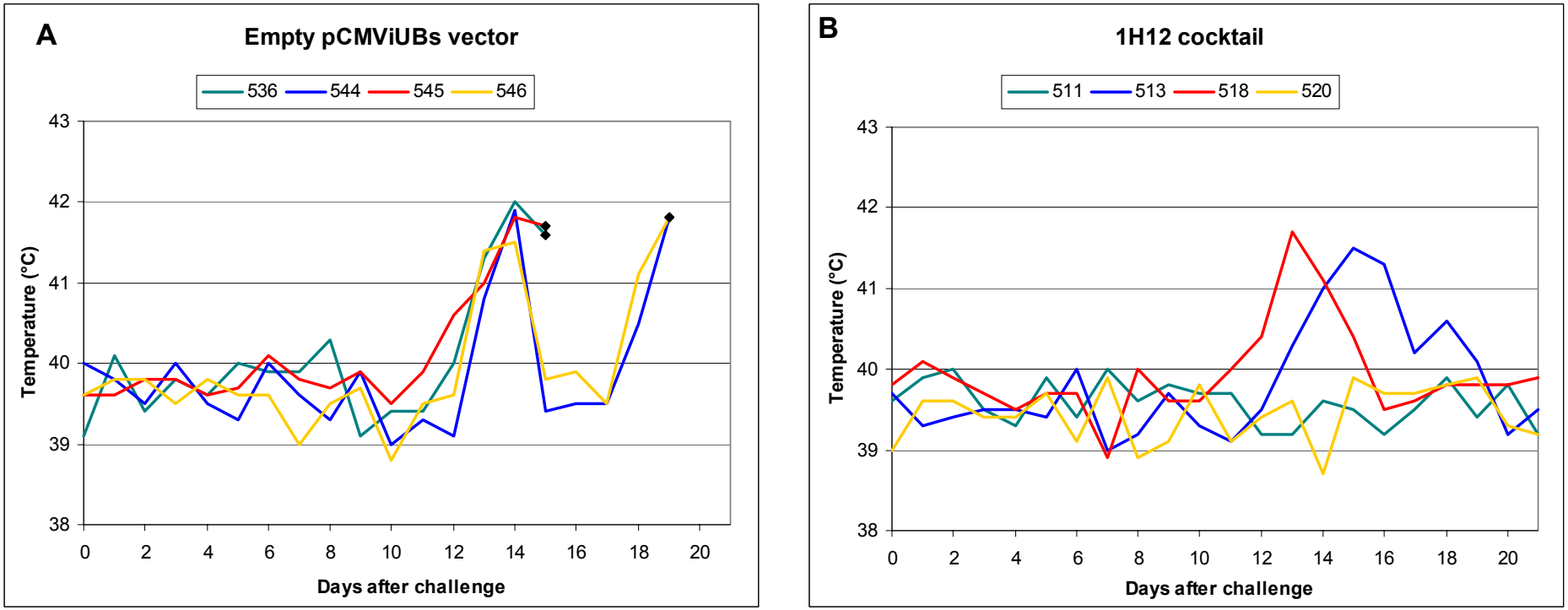


Figure 3.14: *Daily post-challenge temperatures of sheep immunised with empty pCMViUBs vector (A), and 1H12 cocktail (B). Sheep were challenged with 10 LD_{50}s of the virulent* E. ruminantium *Kwanyanga stock. Temperature measurements were terminated 21 days after challenge or as soon as an animal was treated or euthanased (represented by a black dot).*

Mara87/7:

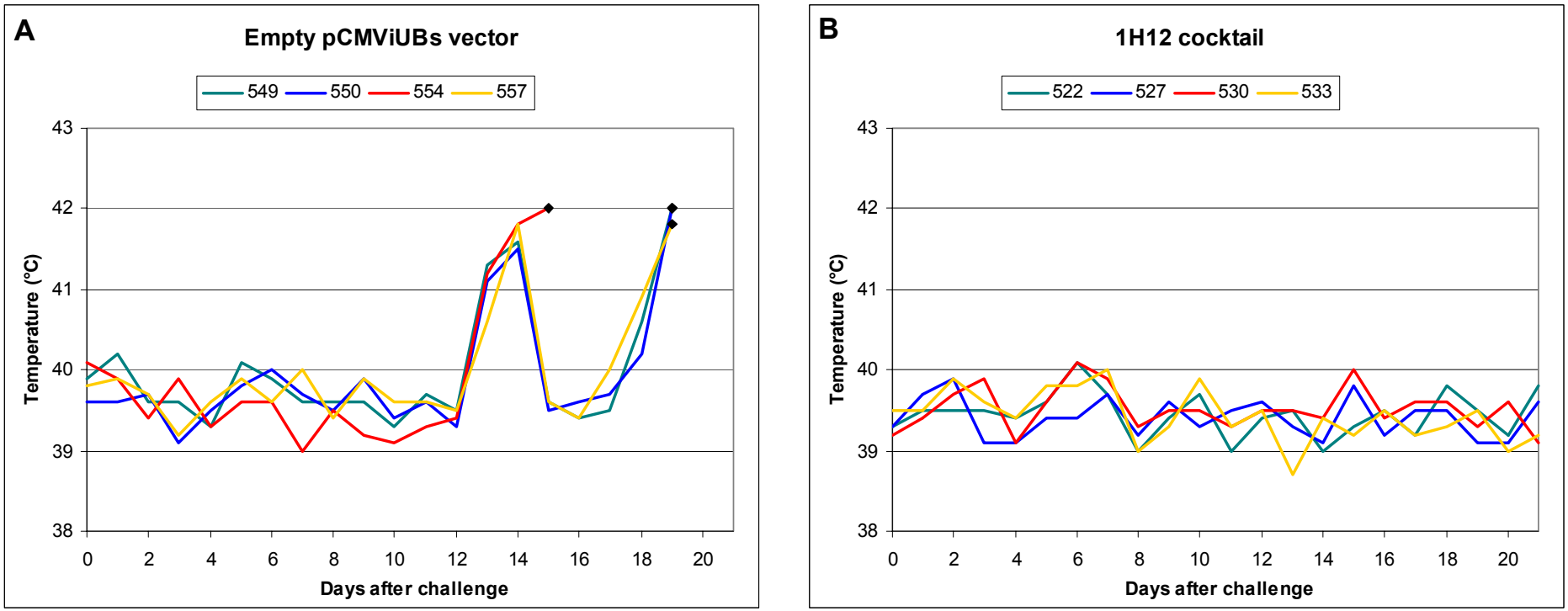


Figure 3.15: *Daily post-challenge temperatures of sheep immunised with empty pCMViUBs vector (A), and 1H12 cocktail (B). Sheep were challenged with 10 LD_{50}s of the virulent* E. ruminantium *Mara87/7 stock. Temperature measurements were terminated 21 days after challenge or as soon as an animal was treated or euthanased (represented by a black dot).*

Welgevonden:

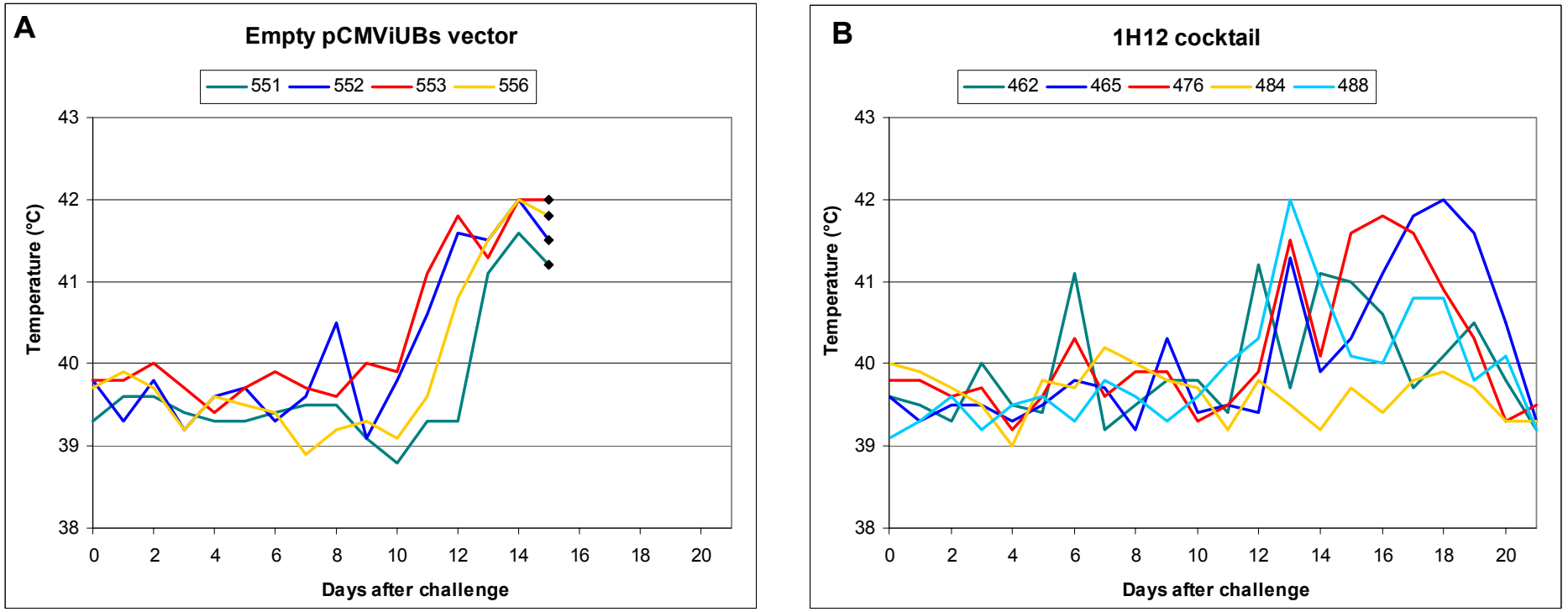


Figure 3.16: *Daily post-challenge temperatures of sheep immunised with empty pCMViUBs vector (A), and 1H12 cocktail (B). Sheep were challenged with 10 LD_{50}s of the virulent* E. ruminantium *Welgevonden stock. Temperature measurements were terminated 21 days after challenge or as soon as an animal was treated or euthanased (represented by a black dot).*

3.3.3 Field challenge on the farm Springbokfontein

3.3.3.1 Field challenge of animals immunised with 1H12 ORFs

To ascertain whether the 1H12 cocktail would provide protection to sheep in the field, animals were immunised with the 1H12 cocktail or empty pCMViUBs vector and sent to Springbokfontein farm located in the heartwater endemic area of South Africa (Figure 3.17) for natural tick challenge.

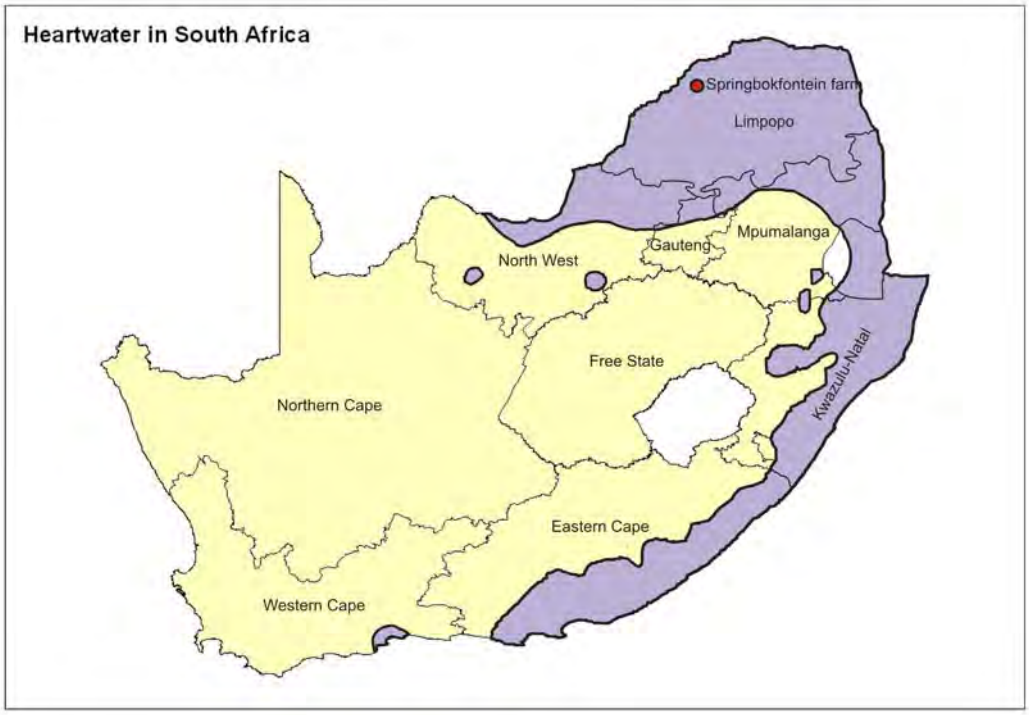


***Figure 3.17**: A map of South Africa indicating the heartwater endemic areas (highlighted in purple) and the location of Springbokfontein farm in the Limpopo province (red dot).*

All the animals immunised with the empty pCMViUBs vector died after 20 days in the field, and only 20 % (one out of five) of the animals immunised with the 1H12 cocktail survived (Figure 3.18)

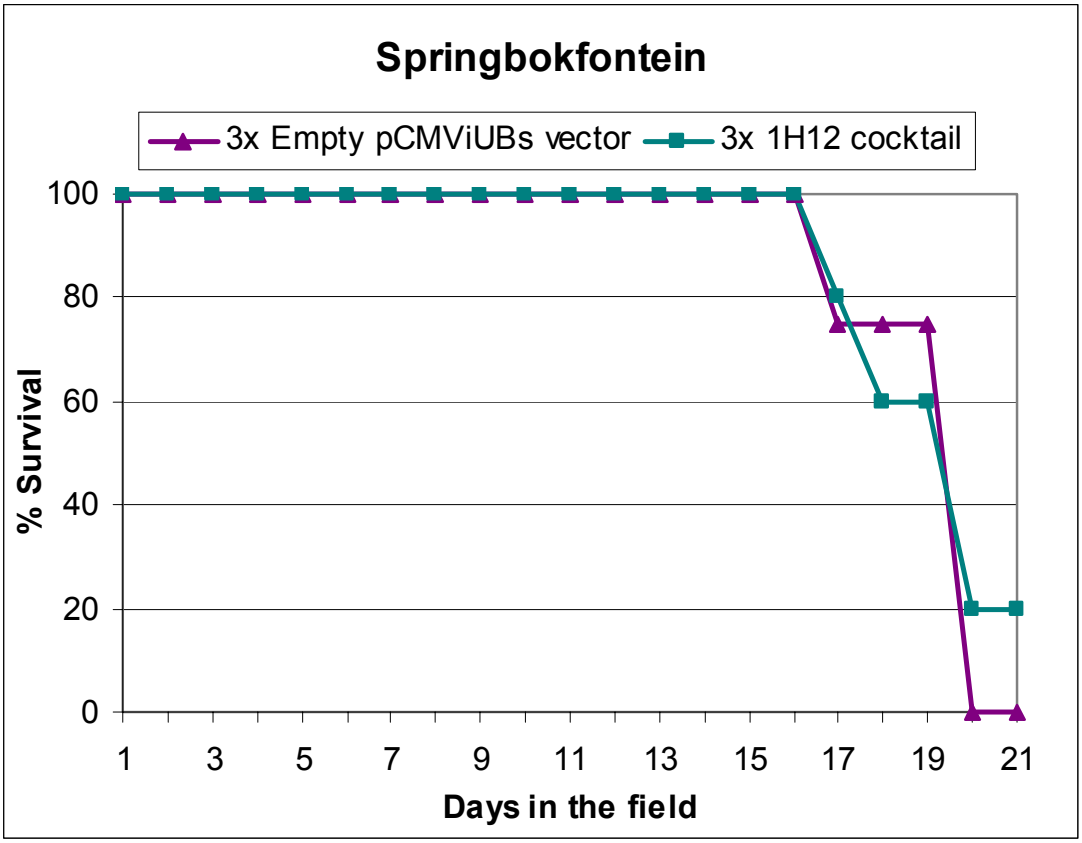


Figure 3.18: *Survival chart of sheep challenged via natural tick infestation at Springbokfontein. These animals were immunised with either the 1H12 cocktail or with empty pCMViUBs vector as indicated.*

The temperature profiles of the sheep in the empty pCMViUBs vector group and the group immunised with the 1H12 cocktail seemed to be similar. The temperatures of the animals started to increase above 41 °C after 15 days in the field (Figure 3.19).

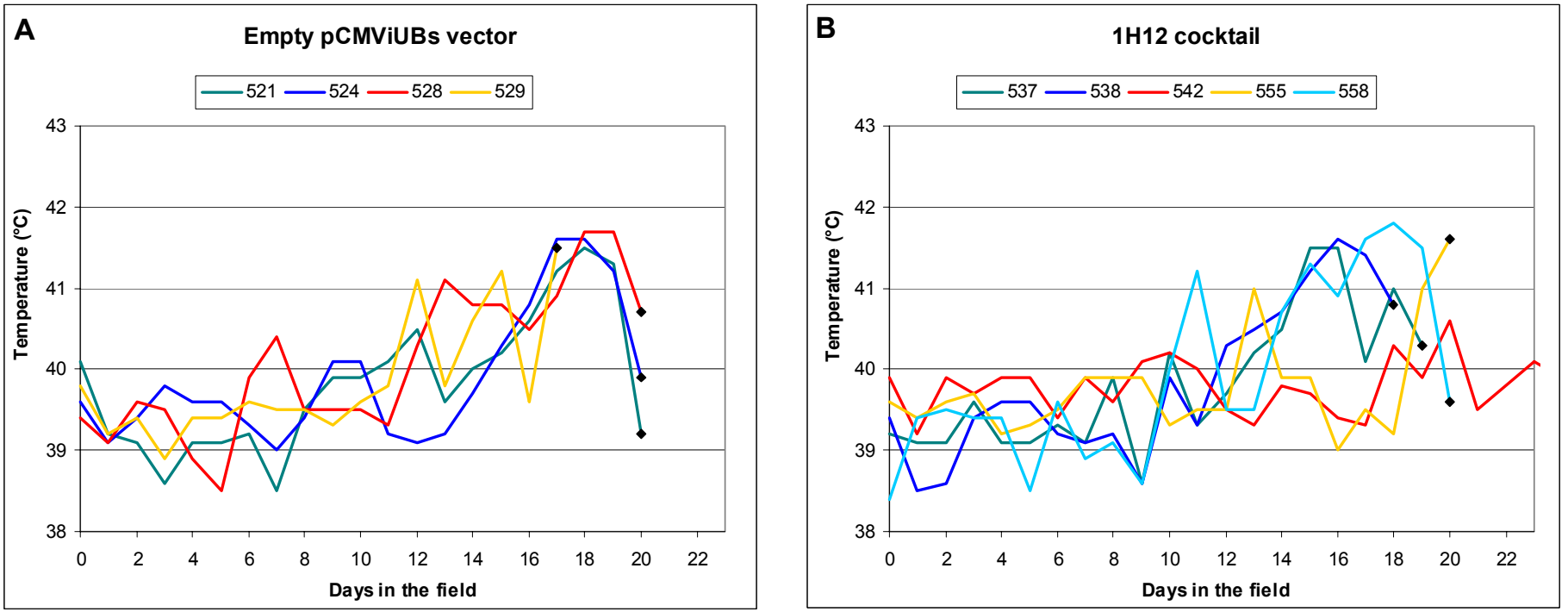


Figure 3.19: *Daily temperatures of sheep immunised with empty pCMViUBs vector (A) and with the 1H12 cocktail (B). Sheep were challenged at Springbokfontein (natural tick challenge). Temperature measurements were terminated 23 days after challenge or as soon as an animal was treated or euthanased (represented by a black dot).*

The sheep in both groups developed severe heartwater symptoms and all except one died. That one, sheep 542, was in the group that had been immunised with the 1H12 cocktail (Figures 3.19-20). No significant difference was observed between the reaction indices of the sheep in the 1H12 cocktail and the empty pCMViUBs vector group ($p \geq 0.384$), suggesting that the 1H12 cocktail does not protect against a natural tick challenge.

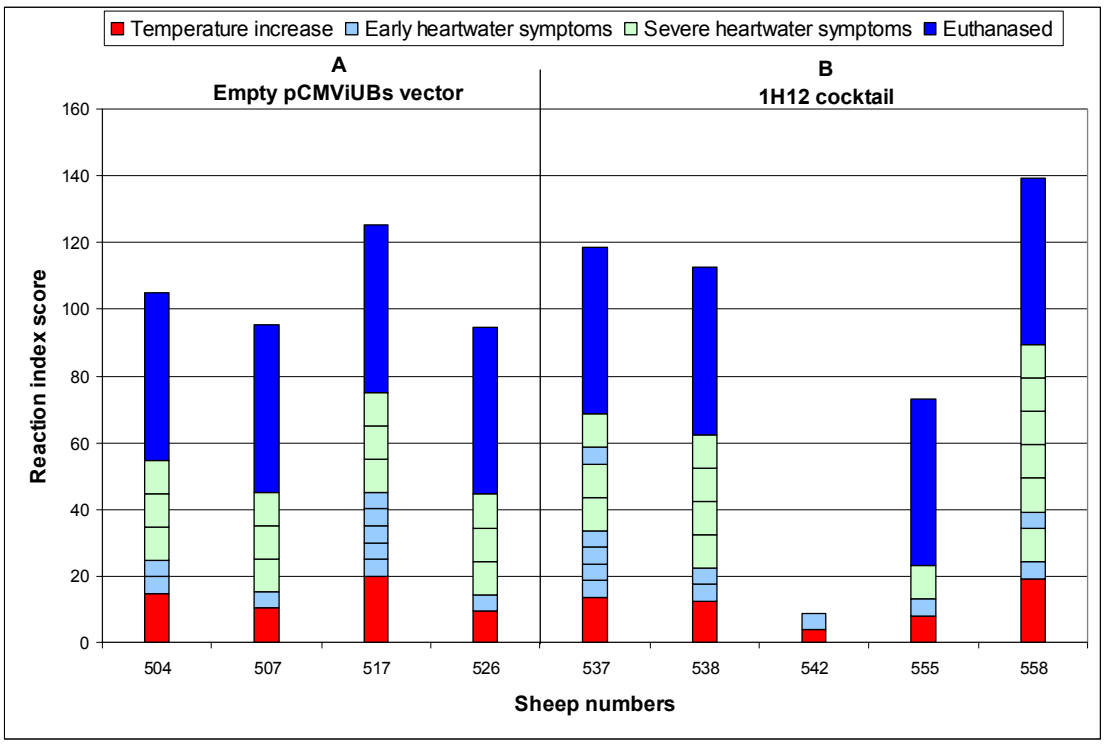


Figure 3.20: *Reaction index (RI) of the sheep exposed to tick infestation after immunisation with the empty pCMViUBs vector (A) and with the 1H12 cocktail (B). The red blocks indicate the total temperature reaction score after translocation to Springbokfontein farm, while the light blue blocks represent a RI of 5 (early heartwater symptoms), the light green a RI of 10 (severe heartwater symptoms), and the dark blue a RI of 50 (animal was euthanased).*

3.3.3.2 Field challenge of needle challenge survivors on the farm Springbokfontein

Immunisation using the blood vaccine that is prepared with the Ball3 stock is not always very effective in the field, because Ball3 does not cross-protect against more virulent *E. ruminantium* genotypes (Collins *et al.,* 2003). A cross-immunity study was done to determine if the sheep that survived needle challenge with the six *E. ruminantium* stocks would survive natural tick challenge. All the survivors of the

previous experiment were also translocated to the farm Springbokfontein, located in the heartwater endemic area in the Limpopo province of South Africa (Figure 3.17).

Animals in the groups previously challenged in the laboratory with Welgevonden, Ball3 and Kwanyanga had a 75%-80 % survival rate in the field. Fifty percent of the sheep previously challenged with Gardel and Mara87/7 survived tick challenge in the field, while only one sheep survived in the group that had been challenged with Blaauwkrans (Figure 3.21). Temperature profiles seem to be more inconsistent than for those in the laboratory challenge (Figures 3.22-3.24), but all the animals that developed heartwater and died did show critical rise in temperature of up to 42 °C.

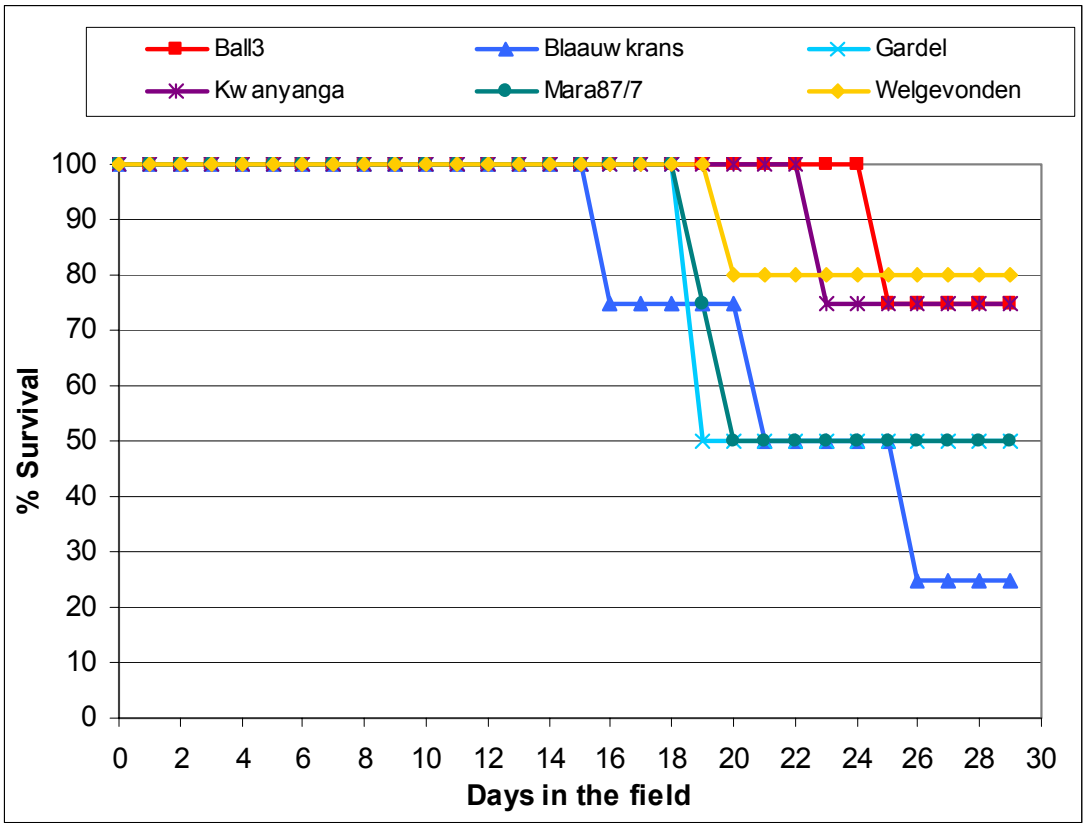


Figure 3.21: *Survival chart of sheep challenged via natural tick infestation at Springbokfontein. These animals were all immunised with 1H12 cocktail and survived challenge in the laboratory with various* E. ruminantium *stocks (as indicated).*

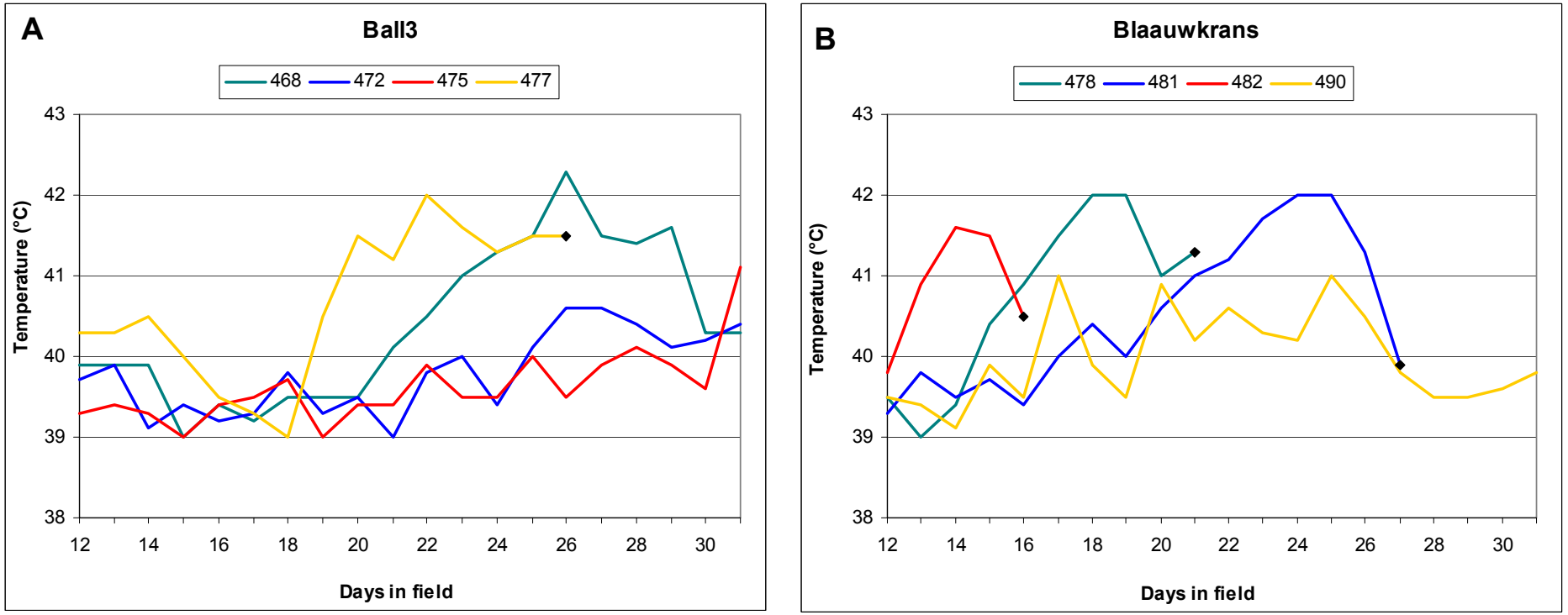


Figure 3.22: *Daily post-challenge temperatures of sheep challenged at Springbokfontein (natural tick challenge). Sheep were immunised with 1H12 cocktail and needle challenged with Ball3 (A) and Blaauwkrans (B) in the laboratory before exposure to natural tick infestation. Temperature measurements were terminated 31 days after challenge or as soon as an animal was treated or euthanased (represented by a black dot).*

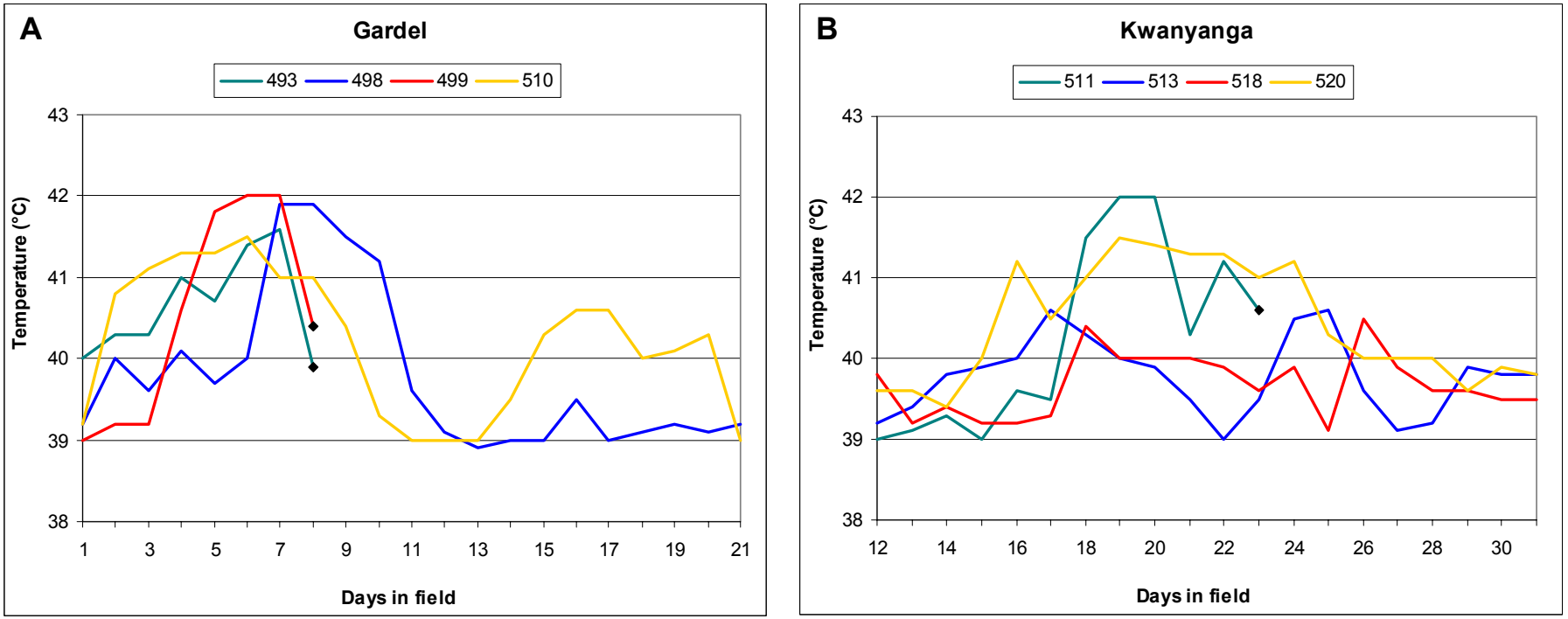


Figure 3.23: *Daily post-challenge temperatures of sheep challenged at Springbokfontein (natural tick challenge). Sheep were immunised with 1H12 cocktail and needle challenged with Gardel (A) and Kwanyanga (B) in the laboratory before exposure to natural tick infestation. Temperature measurements were terminated 31 days after challenge or as soon as an animal was treated or euthanased (represented by a black dot).*

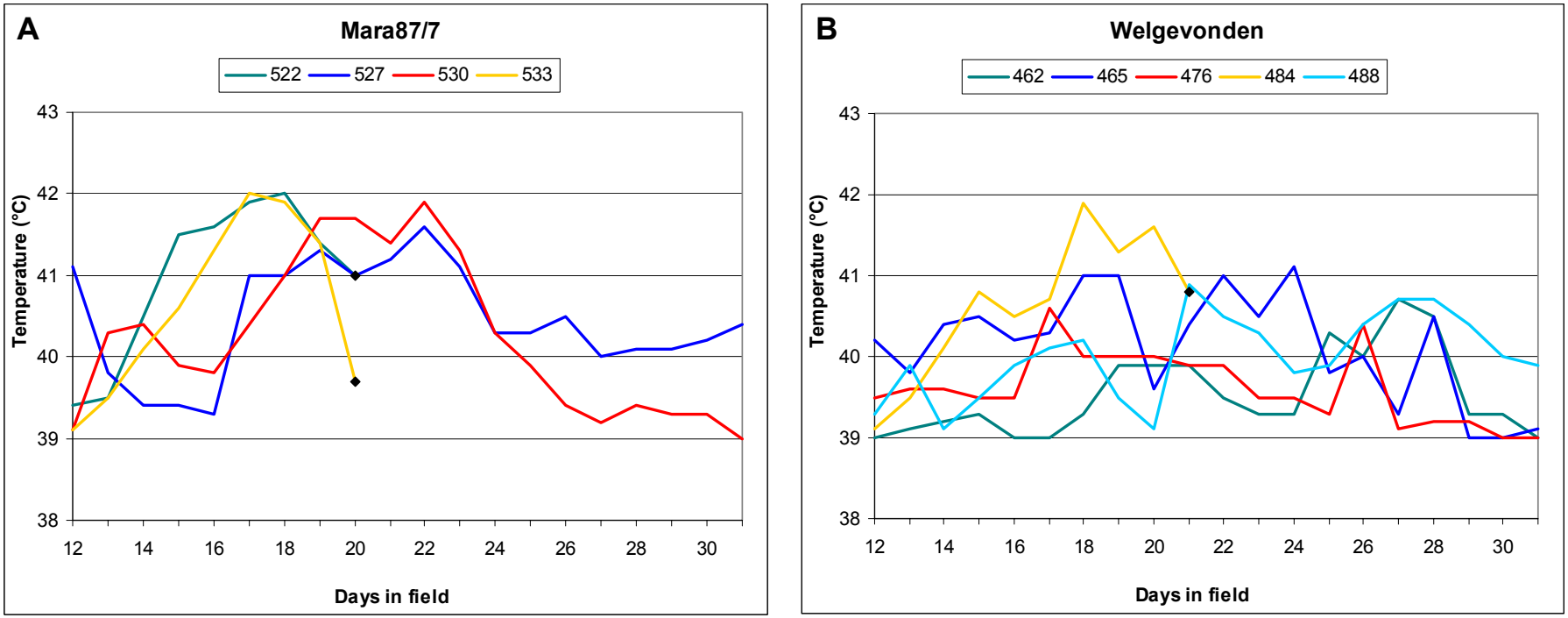


***Figure 3.24**: Daily post-challenge temperatures of sheep challenged at Springbokfontein (natural tick challenge). Sheep were immunised with 1H12 cocktail and needle challenged with Mara87/7 (A) and Welgevonden (B) in the laboratory before exposure to natural tick infestation. Temperature measurements were terminated 31 days after challenge or as soon as an animal was treated or euthanased (represented by a black dot).*

According to the RI criteria, sheep previously challenged with Welgevonden showed some heartwater symptoms when exposed to a field challenge: these included a rise in temperature and early heartwater symptoms. One animal (sheep 484) did develop severe heartwater symptoms and died (Figure 3.25). Interestingly, this was the only sheep that had not reacted after the previous needle challenge (Figure 3.9). Two sheep in each of the groups previously challenged with Ball3 and Kwanyanga developed severe heartwater symptoms upon tick challenge; of these sheep 511 (Kwanyanga) and sheep 477 (Ball3) died (Figure 3. 25). The remaining two sheep only developed slight temperature increases and early heartwater symptoms; again these sheep had shown heartwater symptoms after the earlier needle challenge (Figure 3.9). All the sheep in the Mara87/7 and Gardel needle challenged groups developed severe heartwater symptoms and two sheep in each group died. None of these sheep had shown reaction after needle challenge. One sheep (sheep 490) previously needle challenged with the Blaauwkrans stock was the only animal in its group that survived field challenge (Figure 3.25); again this animal had reacted with early heartwater symptoms after needle challenge (Figure 3.9). The rest of the sheep in the Blaauwkrans group had not previously reacted and died after field challenge.

There appears to be a correlation between animals that showed temperature and early heartwater symptoms after needle challenge, and survival in the field. Animals that were immunised with the 1H12 ORFs and survived needle challenge without showing symptoms succumbed to field challenge. It is therefore important that future immunisation experiments with the 1H12 ORFs should be tested with natural tick challenge. The fact that all the animals were not immune to field challenge suggests that there must be a difference in the immune response that was induced by needle challenge as opposed to immunity induced by tick infestation.

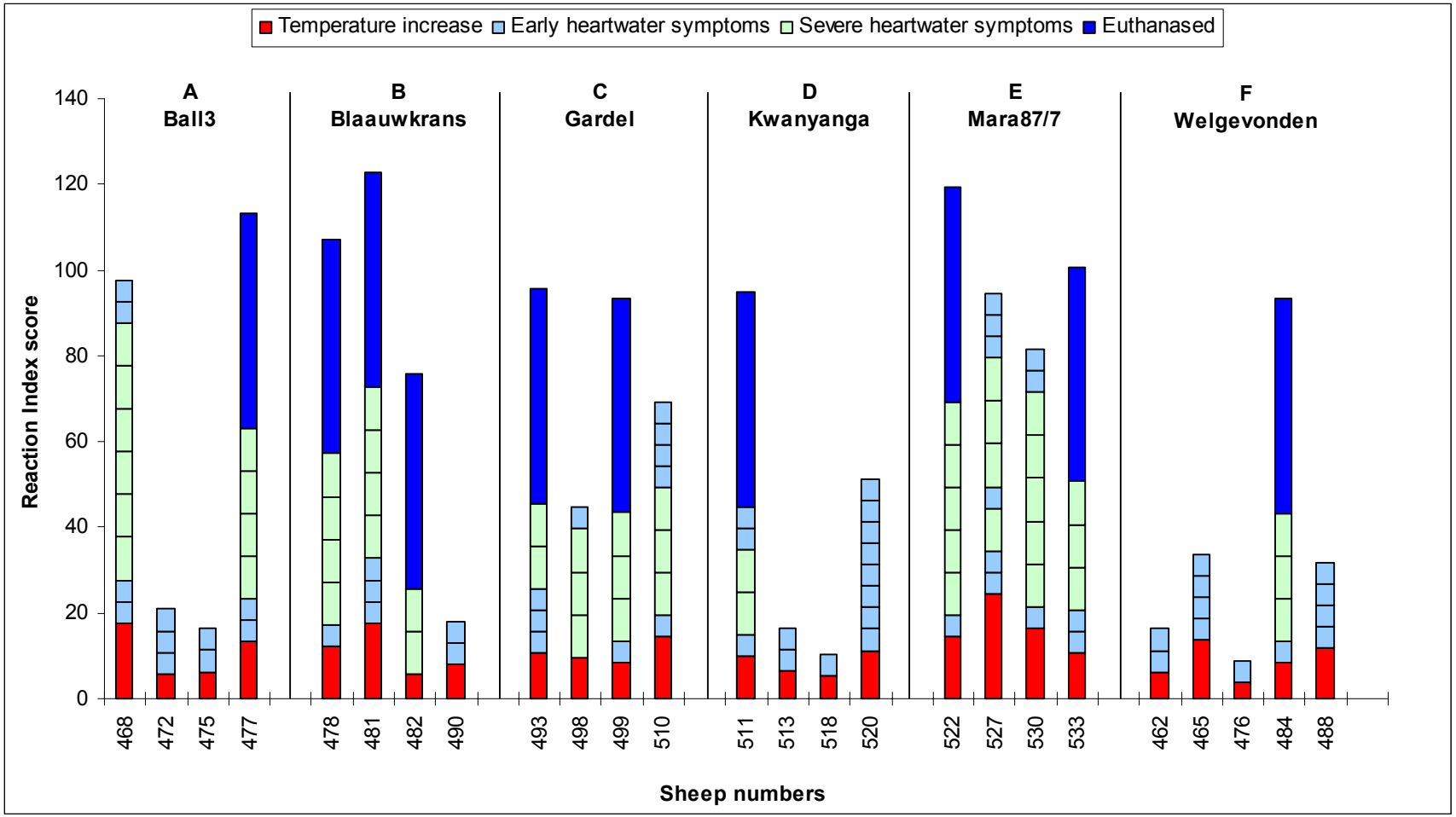


Figure 3.25: *Reaction index (RI) of the sheep exposed to tick infestation after they had been previously immunised with 1H12 cocktail followed by a needle challenge with* E. ruminantium *Ball3 (A), Blaauwkrans (B), Gardel (C), Kwanyanga (D), Mara87/7 (E) or Welgevonden (F) stocks. Red blocks indicate the total temperature reaction score during the field challenge, while the light blue blocks represent a RI of 5 (early heartwater symptoms), the light green a RI of 10 (severe heartwater symptoms), and the dark blue a RI of 50 (animal died or was euthanased).*

3.4. DISCUSSION

It is known that various *E. ruminantium* genotypes exist in the field and that these genotypes show different degrees of cross-protection (Jongejan *et al.,* 1988; Collins *et al.,* 2003). This suggests that sequence variation at amino acid level could play an important role in the host immune response to specific gene products. This variation in sequence could have an influence on the choice of antigen when vaccines are developed for *E. ruminantium.* Sequence variation among genes or open reading frames can have negative implications for recombinant vaccine development, because a protective polymorphic gene would require a multivalent vaccine containing all possible nucleotide sequences for that gene to ensure multiple epitope recognition and protection against all genotypes. In contrast, a vaccine consisting of genes that are conserved among genotypes will require a far less complex vaccine formulation. This was illustrated in a study involving DNA vaccination using *E. ruminantium* Crystal Springs *map*1. Protection in mice was observed against challenge with the Crystal Springs stock (53-67 % survival), but not against the Mbizi stock (6 % survival), suggesting that a vaccine based on the *map*1 gene will require copies of many *map*1 genotypes (Nyika *et al.,* 2002).

Determining the sequence identity of the four 1H12 ORFs in 12 *E. ruminantium* isolates revealed that the 1H12 ORFs were present in all stocks tested and that the ORFs were highly conserved. For all four of the 1H12 ORFs the South African Kümm1 amino acid sequence was identical to that of the West African isolates Sankat430 and Pokoase417, as was found previously with the pCS20 sequence (van Heerden *et al.,* 2004b). Furthermore, and again similar to pCS20, little or no variation in amino acid sequence was observed among the West African isolates (except for Erum2550) while the South African isolates have greater sequence diversity for all four of the 1H12 ORFs tested. In addition, the differences in amino acid sequence that were determined for these four ORFs did not affect the predicted epitopes between the various genotypes tested. This suggests that the four ORFs derived from Welgevonden will protect against other *E. ruminantium* genotypes.

In this study, a heterologous needle challenge was done to test the hypothesis that conserved ORFs will protect against other *E. ruminantium* genotypes. We showed that the 1H12 DNA vaccine induced protective immunity against a virulent needle challenge with six *E. ruminantium* stocks: Welgevonden, Ball3, Gardel, Kwanyanga, Mara87/7 and Blaauwkrans. Although we did not test a West African stock in this study, the epitope mapping results suggest that identical epitopes in three of the four ORFs exist. Furthermore, the 1H12 proteins are highly conserved at amino acid level with only one or two amino acid changes observed between the southern and western African isolates. This may suggest that the Welgevonden-derived 1H12 ORFs could also protect against challenge with West African stocks.

The concept of using conserved proteins as vaccine candidates has been tested in other organisms using conserved surface proteins and ABC transporter components. Good cross-protective immunity results were obtained using a conserved Streptococcal surface protein (Sip) which was used to immunise mice. The recombinant Sip protein derived from one streptococcal serotype induced protection to six other serotypes tested (Brodeur *et al.,* 2000). In a separate study *Streptococcus pneumoniae* ABC transporter components (PiuA and PiaA) were found to be highly conserved among typical pneumococci and induced protection when used as a vaccine (Brown *et al.,* 2001; Jomaa *et al.,* 2006; Whalan *et al.,* 2006). These conserved proteins also showed cross-protective immunity at the antibody level where antibodies directed to proteins derived from one *S. pneumoniae* serotype cross-reacted against the PiuA and PiaA proteins isolated from nine other serotypes, suggesting that immunising with PiuA and PiaA will induce protective immunity to these other serotypes as well (Brown *et al.,* 2001; Jomaa *et al.,* 2006). Protective immunity has also been induced by the nucleoside hydrolase component of the *Leishmania donovani* fucose mannose ligand to challenge with *L. chagasi* and *L. mexicana* (Aguilar-Be *et al.,* 2005), and mice immunised with the conserved outer membrane protein NspA derived from *Neisseria flavescens* were also protected against *N. meningitides* 608B (O'Dwyer *et al.,* 2004).

Although the 1H12 DNA vaccine provided 100 % protection in sheep against homologous and heterologous needle challenge, it did not protect animals under natural field conditions. This could be explained if the corresponding

Springbokfontein isolate 1H12 ORFs were more polymorphic than the Welgevonden-derived vaccine. However this was tested in a separate study using two Springbokfotein isolates (SBF1 and SBF2) that were cultured. The SBF1 stock showed pCS20, 16S and *map*1 sequence identity with the Welgevonden stock while the SBF2 stock showed pCS20 and *map*1 sequence identity with the Mara87/7 stock and 16S sequence identity with the Welgevonden stock. The SBF1 and SBF2 sequences of the 1H12 ORFs also corresponded to those of Welgevonden and Mara87/7 (Helena Steyn, personal communication)

We have shown that the conserved Welgevonden-derived 1H12 ORFs conferred protection against other isolates. Since the 1H12 sequences of the Springbokfontein stocks correlate with those of Welgevonden and Mara87/7, the difference in survival and protection is more likely to be a reflection of the difference between needle challenge and tick challenge. It is well known that blood feeding ticks can suppress the acquired and innate host immune responses in order to survive the host immune onslaught. Immunomodulation strategies employed by ticks include: the down regulation of the lymphocyte proliferative response to saliva mitogens that reduces the ability of the host to generate an antibody response; the inhibition of macrophage activity including the down regulation of cytokines such as IL-1, IL–2 and IL–12, tumour necrosis factor alpha (TNF–α) and IFN-γ (Ferreira and Silva, 1998; Krocova *et al*., 2003 and reviewed by Wikel and Bergman, 1997 and by Wikel and Alarcon-Chaidez, 2001); and the salivary gland extracts of some tick species, including *Dermacentor*, *Amblyomma* and *Haemaphysalis*, can induce the reduction of natural killer (NK) cell killing abilities by suppressing nitric oxide production (Kubeš *et al.,* 2002). Host immune responses to tick-borne bacteria such as *Borrelia burgdorferi* seem to be different when mice are challenged by needle (Keane-Myers and Nickell, 1995a; 1995b; Keane-Myers *et al.,* 1996) as opposed to tick infestation (Zeidner *et al.,* 1997). Furthermore, it has been shown that the addition of tick salivary extract originating from the *Ixodes ricinus* tick can inhibit macrophage induced killing of *Borrelia afzelli* by reducing the production of superoxide and nitric oxide (Kuthejlová *et al.,* 2001). These immunomodulatory effects of salivary gland extracts have been demonstrated for many tick species including *Ixodes* (Leboulle *et al.,* 2002a; 2002b), *Rhipicephalus* (reviewed by Wikel, 1999) and *Amblyomma*

variegatum (Koney *et al.,* 1994). Although not demonstrated for *Amblyomma hebraeum* ticks, the South African hosts of *E. ruminantium*, immunosuppresion almost certainly occurs in the ruminant host.

Phenotypic differences between *E. ruminantium* organisms grown in tissue cultures (used for needle challenge) and the organism in the tick host can also not be excluded. Several groups have demonstrated that *Borrelia burgdorferi* expresses certain proteins only at specific life stages (Revel *et al.,* 2002 and reviewed by de Silva and Fikrig, 1997 and by Porcella and Schwan, 2001). Outer surface proteins (OSPs) A and B are expressed in the tick host but as soon as the pathogen is translocated to the mammalian host these proteins are no longer expressed and a different OSP (OSP C) is found on the bacterial surface. Several other genes only expressed in the mammalian host and not in the tick gut have subsequently been identified (Revel *et al.,* 2002 and reviewed by de Silva and Fikrig, 1997). It is believed that temperature differences and a shift in pH could play a major role in the up and down regulation of expressed proteins in the different hosts. This temperature variation will occur when the pathogen is transferred from the warm blooded host (37 °C) to the much colder temperature in the tick gut (~23 °C) (Revel *et al.,* 2002; Caimano *et al.,* 2004 and reviewed by Porcella and Schwan, 2001). Similar results were obtained with transcriptional studies of the *E. ruminantium* surface protein gene, *map*1-1. In two separate studies, transcription of the gene was investigated, and according to the earlier study done by Bekker *et al*. (2002) *map*1-1 was not transcribed in virulent Welgevonden-, Sankat- and Senegal-infected bovine endothelial cells but was transcribed in *A. variegatum* tick cell lines infected with the Senegal and Gardel stocks. Contrary to these results, van Heerden *et al*. (2004a) reported that the *map*1-1 gene was transcribed in bovine cells grown at 30 °C and a later study done by Bekker *et al.* (2005) showed that the *map*1-1 gene was transcribed in both bovine and tick cell lines grown at 30 °C. These results do not exclude the possibility that the MAP1-1 proteins are expressed at a lower level in the bovine cells than in the tick cell line and further investigation using quantitative assays is required. Although not demonstrated, the absence or down regulation of the proteins expressed by the 1H12 ORFs in the tick could lead to failure of detection of these proteins by primed memory $CD8^+$ (induced via cross-presentation of protein epitopes on MHC class I) and/or

memory $CD4^+$ T cells (induced via MHC class II presentation) at the tick bite site, thus allowing the *E. ruminantium* pathogens to multiply in animals immunised with the 1H12 vaccine.

While sheep immunised with the 1H12 cocktail alone did not survive field challenge, sheep immunised with the 1H12 cocktail that had a temperature reaction upon needle challenge did survive field challenge. This observed protection may be as a result of the needle challenge alone. Alternatively, these results could suggest that a DNA prime followed by a boost with protein in some form may be an effective vaccination strategy to improve immunity against heartwater challenge in the field. It is known that DNA vaccination can stimulate antibody and cellular responses successfully by repeated boosting. However, repeated boosting with plasmids that target the cytotoxic T cell response does not always induce enhancement of the cytotoxic immune response or memory $CD8^+$ T cells and prime-boost immunisation strategies have been shown to be far more effective in inducing strong CTL responses (reviewed by Schneider *et al.,* 1999 and by Woodland, 2004). This prime-boost immunisation strategy will be investigated in the following chapter to see if the immunity and survival of animals challenged in the field can be improved.

In conclusion, we were able to show that the 1H12 DNA vaccines can induce protection against heterologous *E. ruminantium* needle challenge but not against natural tick challenge in the field. Further investigation is required to optimise the vaccination strategy for induction of protection under natural challenge conditions.

3.5. REFERENCES

AGUILAR-BE, I., DA SILVA ZARDO, R., DE SOUZA, E.P., BORJA-CABRERA, G.P., ROSADO-VALLADO, M., MUT-MARTIN, M., GARCÍA-MISS, M.D.R., DE SOUZA, C.B.P. and DUMONTEIL, E. 2005. Cross-protective efficacy of a prophylactic *Leishmania donovani* DNA vaccine against visceral and cutaneous murine Leishmaniasis. *Infection and Immunity* **73:** 812-819.

BEKKER, C.P.J., BELL-SAKYI, L., PAXTON, E.A., MARTINEZ, D., BENSAID, A. and JONGEJAN, F. 2002. Transcriptional analysis of the major antigenic protein 1 multigene family of *Cowdria ruminantium. Gene* **285:** 193-201.

BEKKER, C.P.J., POSTIGO, M., TAOUFIK, A., BELL-SAKYI, L., FERRAZ, C., MARTINEZ, D. and JONGEJAN, F. 2005. Transcription analysis of the major antigenic protein 1 multigene family of three in vitro-cultured *Ehrlichia ruminantium* isolates. *Journal of Bacteriology* **187:** 4782-4791.

BELL-SAKYI, L., KONEY, E.B.M., DOGBEY, O., ABBAM, J.A. and ANING, K.G. 1997. Isolation and *in vitro* cultivation in Ghana of *Cowdria ruminantium*, the causative agent of heartwater. In: Proceedings of the 22th annual congress of the Ghana veterinary medical association. Edited by Koney, E.B.M. and Aning, K.G. The information support unit, Ministry of food and agriculture Accra, Ghana.

BRAYTON, K.A., COLLINS, N.E., VAN STRIJP, F. and ALLSOPP, B.A. 2003. Preparation of *Ehrlichia ruminantium* challenge material for quantifiable and reproducible challenge in mice and sheep. *Veterinary Parasitology* **112:** 63-73.

BRODEUR, B.R., BOYER, M., CHARLEBOIS, I., HAMEL, J., COUTURE, F., RIOUX, C.R. and MARTIN D. 2000. Identification of group B Streptococcal Sip protein, which elicits cross-protective immunity. *Infection and Immunity* **68:** 5610-5618.

BROWN, J.S., OGUNNIYI, A.D., WOODROW, M.C., HOLDEN, D.W. and PATON, J.C. 2001. Immunisation with components of two iron uptake ABC transporters protects mice against systemic *Streptococcus pneumoniae* infection. *Infection and Immunity* **69:** 6702-6706.

CAIMANO, M.J., EGGERS, C.H., HAZLETT, K.R.O. and RADOLF, J.D. 2004. RpoS is not central to the general stress response in *Borrelia burgdorferi* but does control expression of one or more essential virulence determinants. *Infection and Immunity* **72:** 6433-6445.

COLLINS, N.E., PRETORIUS, A., VAN KLEEF, M., BRAYTON, K.A., ALLSOPP, M.T., ZWEYGARTH, E. and ALLSOPP, B.A. 2003. Development of improved attenuated and nucleic acid vaccines for heartwater. In: Vaccines for OIE list A and Emerging Animal Diseases. Edited by BROWN, F. and ROTH, J. *Developments in Biologicals Basel Karger* **114:** 121-136.

DE SILVA, A.M. and FIKRIG, E. 1997. Perspective series: host/pathogen interactions. Arthropod- and host-specific gene expression by *Borrelia burgdorferi*. *Journal of Clinical Investigation* **99:** 377-379.

DU PLESSIS, J.L. 1985. A method for determining the *Cowdria ruminantium* infection rate of *Amblyomma hebraeum*: effect in mice injected with tick homogenates. *Onderstepoort Journal of Veterinary Research* **52:** 55-61.

DU PLESSIS, J.L., VAN GAS, L., OLIVIER, J.A. and BEZUIDENHOUT, J.D. 1989. The heterogenicity of *Cowdria ruminantium* stocks: cross-immunity and serology in sheep and pathogenisity to mice. *Onderstepoort Journal of Veterinary Research* **56:** 195-201.

DU PLESSIS, J.L. 1993. The relationship between Cowdria and Ehrlichia: change in the behaviour of ehrlichial agents passaged through *Amblyomma hebraeum*. *Revue d Elevage et de Médecine Vétérinaire des Pays Tropicaux* **46:** 131-143.

FERREIRA, B.R. and SILVA, J.S. 1998. Saliva of *Rhipicephalus sanguineus* tick impairs T cell proliferation and IFN-γ-induced macrophage microbicidal activity. *Veterinary Immunology and Immunopathology* **64:** 279-293.

HAIG, D.A. 1952. Note on the use of the white mouse for the transport of strains of heartwater. *Journal of the South African Veterinary Medical Association* **23:** 17-70.

JOMAA, M., TERRY, S., HALE, C., JONES, C., DOUGAN, G. and BROWN, J. 2006. Immunisation with the iron uptake ABC transporter proteins PiaA and PiuA prevents respiratory infection with *Streptococcus pneumonia*. *Vaccine* **24:** 5133-5139.

JONGEJAN, F., UILENBERG, G., FRANSSEN, F.F., GUEYE, A., and NIEUWENHUIJS, J. 1988. Antigenic differences between stocks of *Cowdria ruminantium*. *Research in Veterinary Science* **44:** 186-9.

KEANE-MYERS, A. and NICKELL, S.P. 1995a. Role of IL-4 and IFN-γ in modulation of immunity to *Borrelia burgdorferi* in mice. *The Journal of Immunology* **155:** 2020-2028.

KEANE-MYERS, A. and NICKELL, S.P. 1995b. T cell subset-dependent modulation of immunity to *Borrelia burgdorferi* in mice. *The Journal of Immunology* **154:** 1770-1776.

KEANE-MYERS, A., MALISZEWSKI, C.R., FINKELMAN, F.D. and NICKELL, S.P. 1996. Recombinant IL-4 treatment augments resistance to *Borrelia burgdorferi* infections in both normal susceptible and antibody-deficient susceptible mice. *The Journal of Immunology* **156:** 2488-2494.

KONEY, E.B.M., MORROW, A.N., HERON, I., AMBROSE, N.C. and SCOTT, G.R. 1994. Lymphocyte proliferative responses and the occurrence of dermatophilosis in cattle naturally infested with *Amblyomma variegatum*. *Veterinary Parasitology* **55:** 245-256.

KROCOVA, Z., MACELA, A., HERNYCHOVA, L., KROCA, M., PECHOVA, J. and KOPECKY, J. 2003. Tick salivary gland extract accelerates proliferation of *Francisella tularensis* in the host. *Journal of Parasitology* **89:** 14-20.

KUBEŠ, M., KOCÁKOVÁ, P., SLOVÁK, M., SLÁVIKOVÁ, M., FUCHSBERGER, N. and NUTTALL, P.A. 2002. Heterogeneity in the effect of different ixodid tick species on human natural killer cell activity. *Parasite Immunology* **24:** 23-28.

KUTHEJLOVÁ, M., KOPECKÝ, J., ŠTĚPÁNOVÁ, G. and MACELA, A. 2001. Tick salivary gland extract inhibits killing of *Borrelia afzelii* spirochetes by mouse macrophages. *Infection and Immunity* **69:** 575-578.

LEBOULLE, G., CRIPPA, M., DECREM, Y., MEJRI, N., BROSSARD, M., BOLLEN, A. and GODFROID, E. 2002a. Characterisation of a novel salivary immunosuppressive protein from *Ixodes ricinus* ticks. *The Journal of Biological Chemistry* **277:** 10083-10089.

LEBOULLE, G., ROCHEZ, C., LOUAHED, J., RUTTI, B., BROSSARD, M., BOLLEN, A. and GODFROID, E. 2002b. Isolation of *Ixodes ricinus* salivary gland mRNA encoding factors induced during blood feeding. *American Journal of Tropical Medicine and Hygiene* **66:** 225-233.

LOGAN, L.L., ENDRIS R.G., BIRNIE, E.F. and MEBUS C.A. 1985. Research objectives to improve US diagnostic capabilities for heartwater disease. In: Proceedings of the 89th Annual Meeting of the United States Animal Health Association, Milwaukee, Wisconsin.

MACKENZIE, P.K.I. and VAN ROOYEN, R.E. 1981. The isolation and culture of *Cowdria ruminantium* in albino mice. In: Proceedings of the International Congress on Tick Biology and Control. *Rhodes University, Grahamstown*, pp. 33-39.

NEITZ, W.O. 1939. The immunity in heartwater. *Onderstepoort Journal of Veterinary Science and Animal Industry* **13:** 245-283.

NYIKA, A., BARBET, A.F., BURRIDGE, M.J. and MAHAN, S.M. 2002. DNA vaccination with the *map*1 gene followed by protein boost augments protection against challenge with *Cowdria ruminantium*, the agent of heartwater. *Vaccine* **20:** 1215-1225.

O'DWYER, C.A., REDDIN, K., MARTIN, D., TAYLOR, S.C., GORRINGE, A.R., HUDSON, M.J., BRODEUR, B.R., LANGFORD, P.R. and KROLL, J.S. 2004. Expression of heterologous antigens in commensal *Neisseria* spp.: Preservation of conformational epitopes with vaccine potential. *Infection and Immunity* **72:** 6511-6518.

PORCELLA, S.F. and SCHWAN, T.G. 2001. *Borrelia burgdorferi* and *Treponema pallidum*: a comparison of functional genomics, environmental adaptors, and pathogenic mechanisms. *The Journal of Clinical Investigation* **107:** 651-656.

RAMMENSEE, H., BACHMANN, J., EMMERICH, N.P., BACHOR, O.A. and STEVANOVIC S. 1999. SYFPEITHI: database for MHC ligands and peptide motifs. *Immunogenetics* **50:** 213-219 (access via: www.syfpeithi.de).

REVEL, A.T., TALAAT, A.M. and NORGARD, M.V. 2002. DNA microarray analysis of differential gene expression in *Borrelia burgdorferi*, the Lyme disease spirochete. *Proceedings of the National Academy of Sciences of the United States of America* **99:** 1562-1567.

RIBEIRO, J.M.C., MAKOUL, G.T., LEVINE, J., ROBINSON, D.R. and SPIELMAN, A. 1985. Antihemostatic, anti-inflammatory and immunosuppressive properties of the saliva of a tick, *Ixodes dammini. Journal of Experimental Medicine* **161:** 332-344.

SCHNEIDER, J., GILBERT, S.C., HANNAN, C.M., DÉGANO, P., PRIEUR, E., SHEU, E.G., PLEBANSKI, M. and HILL, A.V.S. 1999. Induction of $CD8^+$ T cells using heterologous prime-boost immunisation strategies. *Immunological Reviews* **170:** 29-38.

STADEN, R., BEAL, K.F. and BONFIELD, J.K. 1998. The Staden Package. In: Computer Methods in Molecular Biology. Edited by MISENER, S. and KRAWETZ, S.A. The Humana Press Inc., Totowa, NJ 07512. *Bioinformatics Methods and Protocols* **132:** 115-130.

THOMPSON, J.D., GIBSON, T.J., PLEWNIAK, F., JEANMOUGIN, F. and HIGGINS, D.G. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **25:** 4876-4882.

UILENBERG, G., CAMUS, E. and BARRE, N. 1985. Quelques observations sur une souche de *Cowdria ruminantium* isolée en Guadeloupe (Antilles Françaises). *Revue d'Élevage et de Médecine Vétérinaire des Pays Tropicaux* **38:** 34-42.

VAN HEERDEN, H., COLLINS, N.E., BRAYTON, K.A., RADEMEYER, C. and ALLSOPP, B.A. 2004a. Characterisation of a major outer membrane protein multigene family in *Ehrlichia ruminantium*. *Gene* **330:** 159-168.

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

WHALAN, R.H., FUNNELL, S.G.P., BOWLER, L.D., HUDSON, M.J., ROBINSON, A. and DOWSON, C.G. 2006. Distribution and genetic diversity of the ABC transporter lipoproteins PiuA and PiaA within *Streptococcus pneumoniae* and related streptococci. *Journal of Bacteriology* **188:** 1031-1038.

WIKEL, S.K. and BERGMAN, D. 1997. Tick-host immunology: Significant Advances and Challenging Opportunities. *Parasitology Today* **10:** 383-389.

WIKEL, S.K. 1999. Tick modulation of host immunity: an important factor in pathogen transmission. *International Journal of Parasitology* **29:** 851-859.

WIKEL, S.K. and ALARCON-CHAIDEZ, F.J. 2001. Progress toward molecular characterization of ectoparasites modulation of host immunity. *Veterinary Parasitology* **101:** 275-287.

WOODLAND, D.L. 2004. Jump-starting the immune system: prime-boosting comes of age. *TRENDS in Immunology* **25:** 98-104.

ZEIDNER, N., MBOW, M.L., DOLAN, M., MASSUNG, R., BACA, E. and PIESMAN, J. 1997. Effects of *Ixodes scapularis* and *Borrelia burgendorferi* on modulation of the host immune response: Induction of a T_{H2} cytokine response in Lyme disease-susceptible (C3H/HeJ) mice but not in disease-resistant (BALB/c) mice. *Infection and Immunity* **65:** 3100-3106.

ZWEYGARTH, E., JOSEMANS, A.I., VAN STRIJP M.F., VAN HEERDEN, H., ALLSOPP, M.T. and ALLSOPP, B.A. 2002. The Kümm isolate of *Ehrlichia ruminantium: In vitro* isolation, propagation and characterisation. *Onderstepoort Journal of Veterinary Science* **69:** 147-153.

4. CHAPTER 4: HETEROLOGOUS PRIME-BOOST IMMUNISATION

4.1. INTRODUCTION

We have reported in the previous chapters that, even though the 1H12 DNA vaccine protected against needle challenge with homologous and heterologous strains of *E. ruminantium*, this vaccine was not protective when used in the field. Therefore, new immunisation strategies are needed to improve the immunogenicity and protective efficacy of our recombinant heartwater vaccine. Protection against needle challenge was most likely conferred by CTL and $CD4^+$ T helper one (T_{H1}) lymphocytes. It has previously been shown that repeated boosting with the same recombinant plasmid (so-called homologous boosting) targeting a cytotoxic T cell (CTL) response does not lead to enhancement of the cytotoxic immune response (Wherry *et al.,* 2005). This could be attributed to impairment of antigen presentation and inefficient generation of appropriate inflammatory signals by repeated DNA boosting (reviewed by Gurunathan *et al.,* 2000a; 2000b and by Woodland, 2004). One strategy that has been used to circumvent this problem is the so-called prime-boost immunisation strategy (reviewed by Woodland, 2004). This involves an initial inoculation (the prime) followed by successive boosting using different antigen delivery systems, this procedure is also known as heterologous prime-boosting. In the case of a DNA vaccine, the DNA prime is followed by a recombinant protein or recombinant modified viral boost.

Several studies have revealed that a far better augmentation of the cytotoxic immune response was observed when plasmid and recombinant virus immunisation regimens were combined than was observed for DNA vaccination only. Recombinant pox- and adenoviruses are the most frequently used viral vector antigen delivery systems. These viruses have been attenuated by extensive *in vitro* passage that has resulted in the deletion of host range and virulence genes. Protection against several pathogens, which are controlled primarily by a cellular immune response, has been demonstrated using the DNA prime-modified viral boost strategy. These pathogens include: *Mycobacterium tuberculosis* (McShane *et al.,* 2002), *Plasmodium falciparum, P.*

berghei, P. yoelii and *P. knowlesi* (Sedegah *et al.,* 1998; Dégano *et al.,* 2000; Rogers *et al.,* 2001; Schneider *et al.,* 2001; Rogers *et al.,* 2002; Sedegah *et al.,* 2002), *Leishmania infantum* (Gonzalo *et al.,* 2002), *Schistosoma mansoni* (Shalaby *et al.,* 2003), classical swine fever (Hammond *et al.,* 2001) and HIV (Kent *et al.,* 1998; Gómez *et al.,* 2004). All these studies showed increased levels of protection that coincided with increased cytotoxic T cell responses and elevated levels of IFN-γ when compared to homologous DNA immunisation experiments.

DNA priming followed by recombinant protein boosting has also been used in several studies in an attempt to improve immunity to viral and bacterial diseases. One of the advantages of using a recombinant protein boost is that the outcome of immunity can be directed towards cellular or humoral pathways depending on the type of vector and adjuvant used. Induction of a humoral response was achieved in an experiment where mice were immunised with an expression vector containing the ORF for the malarial antigen Pf155/RESA (M3), followed by boosting with protein in alum (Haddad *et al.,* 1999). Strong antibody production was also induced against HIV antigen when DNA immunisation was followed by a double boost consisting of one recombinant vaccinia boost followed by a recombinant protein boost in complete Freund's adjuvant (Stambas *et al.,* 2005). On the other hand, improved cytotoxic T cell responses were induced during studies in mice and dogs when priming with DNA was followed by recombinant protein boosting using incomplete Freund's adjuvant, Quil-A or Montanide 720 as adjuvant (Nyika *et al.,* 2002; Wang *et al.,* 2004a; Rafati *et al.,* 2005).

If the 1H12 DNA vaccine does confer protection via CTL or cellular immunity, as predicted in the previous chapters, these immune responses could be improved by a prime-boost immunisation strategy. In this chapter, we have investigated if a DNA prime followed by either a viral boost or a recombinant 1H12 (r1H12) protein boost induced improved protection against heartwater as compared to DNA vaccination alone. The four 1H12 ORFs were cloned into a vaccine strain of lumpy skin disease virus (LSDV), and in addition, r1H12 proteins were expressed in *E. coli.* Three prime-boost strategies were evaluated in the laboratory and repeated in a field trial.

4.2. MATERIALS AND METHODS

4.2.1. Materials

See appendix A for materials and buffer components.

4.2.2. Methods

4.2.2.1. Preparation of recombinant lumpy skin disease virus (rLSDV)

rLSDV was prepared according to methods described by Aspden *et al.* (2002) with some modifications:

4.2.2.1.1. Cloning of 1H12 ORFs into the pAFMCR shuttle vector

The pAFMCR shuttle vector (Figure 4.1) was kindly donated by the University of Cape Town (UCT) Medical School, South Africa (Aspden *et al.,* 2002). As indicated in figure 4.1, this shuttle vector contains the *Escherichia coli* xanthine guanine phosphoribosyl transferase (*ECOGPT*) gene, the *β-galactosidase* gene and a restriction enzyme cassette that will facilitate cloning of the ORF of interest into the vector. These genes are flanked by parts of the LSDV ribonuclease reductase (RR) gene sequences. Both these genes and the ORF of interest are introduced into the RR gene of the LSDV genome by homologous recombination. The *ECOGPT* gene will introduce mycophenolic acid resistance to the resulting rLSDV while the *β-galactosidase* gene will facilitate blue/white selection of rLSDV via X-gal metabolism.

Primer sets containing either the *Bam* HI or the *Sal* I restriction sites, were designed (Table 4.1) to facilitate directional cloning of the 1H12 ORFs into the pAFMCR shuttle vector. The ORFs were amplified in 100 µl PCR reactions using 1.25 U TaKaRa Ex Taq™ (TaKaRa Bio Inc.) in buffer containing a final concentration of 1x reaction buffer, 200 µM dNTPs, 2 mM Mg^{2+} and 400 nM of each primer. The samples were denatured (94 °C for 30 s) followed by 35 cycles of denaturation (94 °C for 30 s), annealing (50 °C for 30 s) and extension (72 °C for 90 s) using an Applied Biosystems Gene Amp® 9700 PCR system. The amplicon was visualized and its size determined by separating one tenth of the amplified PCR product by electrophoresis

on a 1 % agarose gel (in 1x TBE) containing 0.5 µg/ml ethidium bromide. The products were visualized using a Lumi-Imager™ (Roche).

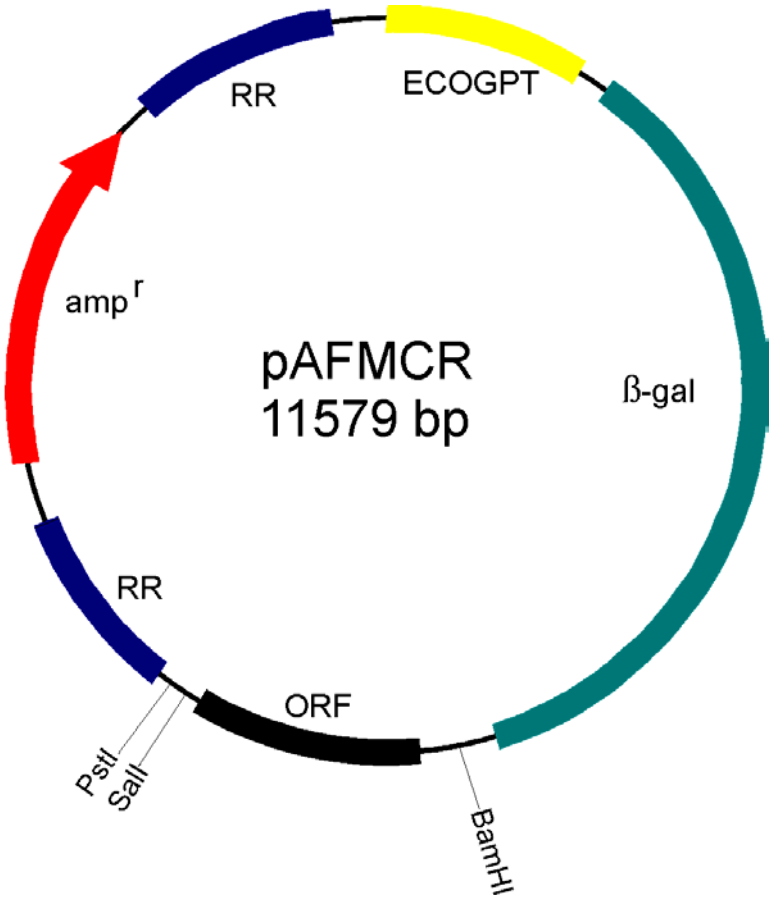


Figure 4.1: *Schematic representation of the pAFMCR shuttle vector (copied from Aspden et al., 2002) used to facilitate homologous recombination of ORFs of interest into wtLSDV.* E. ruminantium *ORFs were amplified and cloned into the cloning site in the correct orientation by incorporating specific restriction enzyme sites into the amplification primers. The flanking ribonuclease reductase (RR) regions facilitate homologous recombination.*

The amplified ORFs were purified (Concert™ rapid PCR purification system), and cloned into the pGEM®-T Easy vector (Promega) according to the manufacturer's instructions. Clones containing the correct ORF sequence ([0]verified by PCR) were digested with *Bam* HI (Roche), purified (Qiagen mini elute kit), and digested with *Sal* I (Roche). The shuttle vector (1 pMol protruding ends) was digested as described above and was dephosphorylated using 1 U shrimp alkaline phosphatase as described by the manufacturer (Promega). The inserts and linearised shuttle vector were gel

purified using TaKaRa recochips (TaKaRa Bio Inc.) according to the manufacturer's instructions. Each ORF was ligated into the linearised shuttle vector in a 50 µl reaction mixture containing 1x reaction buffer, 1 U T4 DNA ligase (Promega) and a vector: insert ratio that varied between 1:2 and 1:10. The ligated DNA was electroporated (BIORAD gene pulser® II) into MOS*Blue* cells, and grown overnight at 37 °C on LB agar plates containing ampicillin (50 µg/ml).

Clones were screened for the presence of inserts by PCR [0]using pAFMCR-specific primers, LSDVF and LSDVR (Table 4.1), and recombinants were sequenced to determine if the correct ORF sequences were present. The clones were sequenced with the same primers, LSDVF and LSDVR (Table 4.1) using the ABI Prism® BigDye™ Terminator cycle sequencing ready reaction kit (Applied Biosystems), on an ABI™ 3100 automated sequencer (Applied Biosystems). The sequences were assembled and edited using the Staden package (Staden *et al.,* 1998) and aligned with the corresponding Welgevonden genome sequences using CLUSTAL_X (Thompson *et al.,* 1997).

4.2.2.1.2. MDBK cell cultures

Madin and Darby bovine kidney (MDBK) cells (ATCC no CRL 6071) were also obtained from UCT. The cells were found to be contaminated with mycoplasma and, to eliminate the mycoplasma contamination before use, the cells were treated with 30 µg/ml cyprofloxicin for 3 days in DMEM/F12 medium substituted with 10 % foetal calf serum (FCS), 1:100 Penstrep, 1:200 Fungizone. The cells were washed with 1x PBS, and tested for mycoplasma contamination by means of a PCR test using mycoplasma specific primers, GPO3 and MGSO (Table 4.1). The test was performed by adding 2 µl of cell suspension in a 50 µl PCR reaction mix consisting of: 1 U TaKaRa Ex Taq™ (TaKaRa Bio Inc.), 400 nM of each primer, 1x dNTP buffer (final concentration of 200 µM dNTPs) and 1x reaction buffer (final concentration of 2 mM Mg^{2+}). The samples were denatured for 1 min at 94 °C, followed by 35 cycles of denaturation (94 °C for 30 s), annealing (55 °C for 1 min), and extension (72 °C for 1 min), this was followed by a final extension step of 10 min at 72 °C using the GeneAmp® PCR System 9700 thermocycler (Applied Biosystems). Amplified gene products were run on a 2 % agarose gel. Cyprofloxicin treatment was repeated until

the mycoplasma PCR test was negative. The cells were maintained in complete DMEM/F12 medium (10 % FCS (heat inactivated), 0.1 mg/ml sodium benzylpenicillin (Novopen, Nova Nordisk) and 0.2 mg/ml streptomycin sulphate (Novostrep, Nova Nordisk)) and passaged once cells had grown to form a confluent monolayer. The cells were split using a 1 in 10 ratio, after treatment with 1 ml activated trypsin (Onderstepoort Biological Products (OBP)) for 10 min at 37 °C to loosen them. Once the cells had been detached, DMEM/F12 medium containing FCS was added to inhibit trypsin action and the cells were split into new flasks (see appendix A for buffers and media).

4.2.2.1.3. Preparation of wild type LSDV (wtLSDV) stocks used for homologous recombination

MDBK cells were infected with LSDV Neethling strain in 75 cm^2 flasks in the presence of DMEM/F12 medium (supplemented with FCS and antibiotics as described before). The wtLSDV was passaged up to 6 times through MDBK cells, after which cells and medium were harvested, freeze-thawed three times, and wtLSDV was collected by 36 % sucrose density centrifugation for 2 h at 19 000 *g*. The resulting pellet was resuspended in serum-free medium, sonicated at 40 % duty cycle, output control 3 for 20 s on ice (Branson Cuphorn Sonifier 250), and frozen at -70 °C in aliquots.

The virus titre was determined by infecting MDBK cells grown to 60 % confluency in 24 well plates (in triplicate) using serial dilutions of the wtLSDV stock. Cells were infected with wtLSDV for 90 min at 37 °C, washed and grown for 72 h followed by immobilisation in agarose-virus selection medium. Foci of MDBK cells infected with wtLSDV were counted after 16 h incubation at 37 °C and the titre determined.

Table 4.1: *Primer sequences used for cloning of 1H12 ORFs into the LSDV shuttle vector and into the pET vector for protein expression. Restriction enzyme sites and TOPO cloning sites (5' CACC) are underlined where applicable.*

Primer name	Primer sequence 5' - 3'	Linker	T(a) °C[1]	PCR product (bp)
Erum2550 Bam F	CGC GGA TCC ATG ATA GGT TTA CAG TTG	*BamHI*	59	
Erum2550 Sal R	ACGC GTC GAC TAT ATC TTA AAT AAT AAA ATT TTA	*Sal I*	54	1040
Erum2590 Bam F	CGC GGA TCC ATG TTT CAT AAT TTA CTC G	*BamHI*	58	
Erum2590 Sal R	ACGC GTC GAC TTA AGC ATA AGA TGA GAA	*Sal I*	60	746
Erum2540 Bam F	CGC GGA TCC ATG TTG AGA CTT AGT TTT AT	*BamHI*	59	
Erum2540 Xba R	GCT CTA GAT TAT TTT TTT TTC CAA AAA TTT ATT A	*Xba I*	52	544
Erum2580 Bam F	CGC GGA TCC ATG AAA CAT ATA ATC TTT CTT T	*BamHI*	58	
Erum2580 Sal R	ACGC GTC GAC CTT ATG TAG TTG ATA GAC AA	*Sal I*	61	877
LSDV F	CGT TGT AAA AAC GAC GGG ATC TAG C	None	58	
LSDV R	ACA AAA AAG CAT CGT TTG GAG AAC G	None	57	Depend on insert size
LSDV ribonuclease F	GAA ACA TCT TCA AGA TTT GTA G	None	48	wtLSDV 918
LSDV ribonuclease R	ATC GTC TTT GTT AGA AAT AAC GC	None	51	
pET Erum2550 F	C ACC ATG ATA GGT TTA CAG	Topo	51	
pET Erum2550 R	TAT CTT TAA ATA ATAAAA TTT TAT TTA AGT CAA GC	No stop	50	1040
pET Erum2590 F	C ACC ATG TTT CAT AAT TTA CTC G	Topo	50	
pET Erum2590 R	AGA ATA AGA TGA GAA CAT TTT TA	No stop	46	746
pET Erum2540 F	C ACC ATG TTG AGA CTT AGT TTT AT	Topo	51	
pET Erum2540 R	TTT TTT TTT CCA AAA ATT TAT TAA TCC	No Stop	48	544
pET Erum2580 F	CACC ATG AAA CAT ATC TTT CTT T	Topo	49	
pET Erum2580 R	TGT AGT TGA TAG ACA ACT GAA AA	No Stop	50	877
TrxFus forward	TTC CTC GAC GCT AAC CTG	None	54	
T7 reverse	TGT AGT TGA TAG ACA ACT GAA AA	None	50	
GPO3	GGG AGC AAA CAG GAT TAG ATA CCC T	None	59.8	259
MGSO	TGC ACC ATC TGT CAC TCT GTT AAC CTC	None	62.1	

4.2.2.1.4. Generation of rLSDV

rLSDV were generated as described by Aspden *et al.* (2002). Briefly, MDBK cells were grown to a monolayer of ~60 % confluency in six well (35 mm) plates. Cells were infected with wtLSDV at a multiplicity of infection of 0.1 focus forming units (ffu) per well for 90 min at 37 °C. Infected cells were transfected by adding 5-10 µg shuttle vector DNA in 50 µl DOTAP liposomal transfection reagent (Roche) to each well (DNA and DOTAP were premixed at room temperature for 35 min) followed by overnight incubation at 37 °C. The DOTAP mixture was then removed and transfected cells were incubated with virus selection medium (see appendix A) for 3 days at 37 °C after which the transfection efficacy was assessed using the X-gal stain (Kitching *et al.,* 1987). After homologous recombination, only the rLSDV which contained the *ECOGPT* gene was resistant to mycophenolic acid, while the growth of the wtLSDV that does not contain the *ECOGPT* gene was inhibited. The rLSDV was passaged up to 6 times through MDBK cells. Cells containing rLSDV were isolated by immobilising a 72 h MBDK-rLSDV infected cell culture in virus selection medium containing 1 % agarose, 1 mM mycophenolic acid and 1 mM X-gal. Cells were incubated for 3 h and blue colonies were picked into 0.5 ml growth medium. Recombinant virus was purified by repeated plating of blue foci containing the rLSDV in agarose-virus selection medium until a negative wtLSDV PCR result (see section 4.2.2.1.7) was obtained. This method was repeated for each of the four 1H12 ORFS.

4.2.2.1.5. Large-scale preparation of rLSDV

A representative blue focus containing the rLSDV was selected from plated, pure rLSDV (on 6 well plates) and passaged in MDBK cells grown in 75 cm^2 flasks using virus selection medium that contained mycophenolic acid (1 mM). Cells and medium were harvested, freeze-thawed three times and rLSDV was collected by 36 % sucrose density centrifugation for 2 h at 19 000 *g*. The resulting pellet was resuspended in serum-free medium, sonicated at 40 % duty cycle, output control 3 for 20 s on ice (Branson Cuphorn Sonifier 250), aliquotted and frozen at -70 °C.

The titre of each rLSDV was determined by infecting MDBK cells grown to 60 % confluency in 24 well plates (in triplicate) using serial dilutions of the rLSDV stock.

Cells were infected with rLSDV for 90 min at 37 °C, washed and grown in virus selection medium for 72 h followed by immobilisation in agarose-virus selection medium. Blue foci were counted after 16 h incubation at 37 °C and the virus titre determined.

4.2.2.1.6. Isolation of rLSDV genomic DNA

Purified recombinant virus stock was incubated for 2 h at 37 °C after the addition of 1/10 volume of rLSDV DNA extraction buffer. The solution was extracted with phenol/chloroform (1:1 volume) followed by chloroform. The genomic DNA was precipitated from the aqueous phase by the addition of 1/10 volume of 3 M sodium acetate and 2.5 volumes of 100 % ethanol. The mixture was incubated overnight at -20 °C, the DNA was pelleted by centrifugation (>10 000 *g* for 20 min), and excess salt removed by washing the pellet with 1 ml 70 % ethanol (centrifugation >10 000 *g* for 20 min). The pellet was resuspended in 50 µl deionised water.

4.2.2.1.7. rLSDV-specific PCR to confirm transfection and purity of sample

Primers were designed (Table 4.1) to amplify across the ribonucleotide reductase (*RR*) gene (the gene used for homologous recombination). The PCR was performed by adding 2 µl of resuspended genomic DNA to a 25 µl PCR reaction mix consisting of: 0.5 U TaKaRa Ex Taq^{TM} (TaKaRa Bio Inc.), 250 nM of each primer, 1x dNTP buffer (containing 400 µM dNTPs) and 1x reaction buffer (containing 2 mM Mg^{2+}). The samples were denatured for 2 min at 94 °C, followed by 15 cycles of denaturation (94 °C for 30 s), annealing (50 °C for 30 s), and extension (72 °C for 1 min), this was followed by a further 20 cycles of denaturation (94 °C for 30 s), annealing (50 °C for 30 s), and extension (72 °C for 90 s plus 30 s with each new cycle) using a GeneAmp® PCR System 9700 thermocycler (Applied Biosystems). Amplified gene products were separated by electrophoresis on a 0.8 % agarose gel.

4.2.2.1.8. Isolation of mRNA to determine expression

Total RNA was isolated from rLSDV infected cells using the Qiagen RNA isolation kit according to the instructions of the manufacturer. First strand cDNA was prepared with Superscript I (Invitrogen) using random hexamer primers (Promega), and PCR

was performed using the same ORF-specific primers (Table 4.1) and conditions as were used for directional cloning in the pAFMCR vector (see section 4.2.2.1.1.).

4.2.2.2. Recombinant 1H12 proteins expressed in *E. coli*

4.2.2.2.1. Directional cloning into the pET vector

Cloning and transformation into the pET vector were executed according to the instructions of the manufacturer. Specific primers were designed for each ORF and the gene of interest was amplified using a proofreading DNA polymerase that produces a blunt ended PCR product (*Pfu*, Promega). The PCR was performed by adding 2 µl of *E. ruminantium* Welgevonden genomic DNA in a 50 µl PCR reaction mix containing: 1.25 U *Pfu* polymerase, 250 nM of each primer, 1x dNTP buffer (containing 200 µM dNTPs) and 1x reaction buffer (containing 2 mM Mg^{2+}). The samples were denatured for 4 min at 94 °C, followed by 40 cycles of denaturation (94 °C for 20 s), annealing (54 °C for 30 s), and extension (72 °C for 3 min), this was followed by a final extension step of 10 min at 72 °C using a GeneAmp® PCR System 9700 thermocycler (Applied Biosystems). The amplified gene products were cloned into the TOPO pET vector and the new plasmid constructs were transformed into TOP10 competent *E. coli* cells using the heat-shock method. The cells were plated on LB agar plates containing ampicillin (50 µg/ml) and incubated overnight at 37 °C. Representative recombinant clones were picked and grown overnight at 37 °C in LB broth containing ampicillin (50 µg/ml). The plasmid DNA was purified (Roche mini plasmid preparation kit) and inserts were detected by PCR as follows. The cloned ORFs were amplified by adding 2 µl of a 1/100 dilution of plasmid DNA into a PCR reaction mixture consisting of: 0.13 U TaKaRa Ex Taq™ (TaKaRa Bio Inc.), 250 nM of the pET vector specific primers, TrxFus and T7 reverse primer (Table 4.1), 1x dNTP buffer (containing 200 µM dNTPs) and 1x reaction buffer (containing a final concentration of 2 mM Mg^{2+}). The samples were denatured for 2 min at 94 °C, followed by 30 cycles of denaturation (94 °C for 30 s), annealing (58 °C for 30 s), and extension (72 °C for 1 min), this was followed by a final extension of 10 min at 72 °C using a GeneAmp® PCR System 9700 thermocycler (Applied Biosystems). Clones containing fragments of the correct size were sequenced using the primers supplied in the kit, i.e. TrxFus and T7 reverse primer (Table 4.1). Sequences were assembled and translated to check that the His_6-tag was intact (See Appendix B for alignments).

4.2.2.2.2. Pilot expression of recombinant proteins

Plasmids containing inserts with the correct sequence were used for pilot expression. Electrocompetent BL21Star™ (DE3) cells were transformed by addition of 5 ng plasmid DNA/40 µl electrocompetent cells. The cell-plasmid mix was electroporated at 1.4 kV using the BIORAD gene pulser® II. The transformation reaction mixture was incubated for 30 min at 37 °C in SOC medium and then added to 10 ml LB broth containing ampicillin (50 µg/ml) and grown overnight at 37 °C.

The next day 500 µl of the overnight culture was added to fresh LB broth (10 ml) supplemented with ampicillin (50 µg/ml). The cells were grown at 37 °C with shaking until an OD_{600} of 0.5-0.8 was reached (~2 h). The culture was split into two aliquots of 5 ml each and one aliquot was treated to induce the expression of the cloned PCR product by the addition of IPTG to a final concentration of 1 mM. Samples were taken from each aliquot at different time points, starting from 4 h after induction to a maximum of 6 h. Bacterial cells were pelleted by centrifugation (5 min, 16 000 *g*) and were stored at -20 °C. Expression was determined by separating the proteins by SDS-PAGE, performing Western blot analyses if protein concentration was too low, and optimal conditions and time points were chosen for large-scale expression.

4.2.2.2.3. Large-scale expression and purification of recombinant proteins

LB broth (20 ml) containing ampicillin (50 µg/ml) was inoculated with freshly transformed BL21Star™ (DE3) and incubated overnight at 37 °C. 500 ml LB broth containing ampicillin (50 µg/ml) was inoculated with 10 ml overnight culture and grown at 37 °C with shaking until an OD_{600} of 0.5-0.8 was reached. Protein expression was induced by the addition of IPTG (to a final concentration of 1 mM) and the culture was grown until the optimum time point was reached as predetermined by the pilot expression study. The cells were harvested by centrifugation at 3000 *g* for 10 min at 4 °C. Recombinant protein was purified using the Protino® Ni 1000 prepacked columns kit (Macherey-Nagel) according to the instructions of the manufacturer. The purified proteins were assayed using anti-His_6 Western blot analyses.

4.2.2.2.4. Western blot analyses using anti-His_6 antibodies

For analysis of expressed proteins, bacterial cell pellets were thawed and resuspended in SDS-PAGE gel loading buffer, boiled for 10 min and a 10 µl aliquot was loaded on a 12 % PAGE gel. The proteins were separated by running the gel at 100 V for approximately 2 h. The separated proteins were transferred to a PVDF membrane (Millipore Corporation) after equilibrating the gel and membrane in transfer buffer with a semi-dry blotter (Semi-phor TE70, Hoefer scientific instruments) at 110 mA for 90 min. The blot was incubated in blocking buffer (1x PBS, 1 % BSA) for 1 h and incubated in the presence of anti-His_6 antibodies (75 ng/100ml, Roche) overnight at room temperature. The membrane was washed three times with wash buffer (1x PBS, 0.05 % Tween-20) and incubated with conjugate (1/20 000 dilution, horseradish peroxidase (HRP)-goat-anti-mouse IgG (H + L); Zymed) for 1 h at room temperature. The blot was again washed three times and the recombinant His-tagged protein bands were visualised using the SuperSignal®West Pico Chemiluminescent substrate from Pierce. Fluorescence was captured using the Lumi-Imager or Lumi-film, both from Roche.

4.2.2.3. Immunisation of animals

4.2.2.3.1. E. ruminantium stock used for challenge material

All animals were challenged with the virulent *E. ruminantium* Welgevonden stock originating in South Africa (Du Plessis, 1985).

4.2.2.3.2. Challenge material

E. ruminantium Welgevonden challenge material was prepared as described in chapter 2.

4.2.2.3.3. DNA preparation

Large-scale plasmid isolation was carried out as explained in chapter 2.

4.2.2.3.4. rLSDV vaccine preparation

Each animal was inoculated with 1x 10^7 ffu/ORF. The appropriate stock was diluted in 1x PBS to the correct concentration and animals were inoculated intramuscularly with the virus/PBS mix. Negative control animals were immunised with the same concentration of live attenuated wtLSDV vaccine obtained from OBP.

4.2.2.3.5. Recombinant protein preparation

The concentration of the purified recombinant proteins was determined using the Biorad protein assay (BIORAD) Each animal received 150 µg recombinant protein per ORF (a total of 600 µg protein was needed per sheep). The four expressed recombinant proteins were individually precipitated by adding eight volumes of acetone and incubating overnight at -20 °C. The proteins were collected by centrifugation at 10 000 *g* for 10 min, whereafter excess salts were removed by washing with 70 % ethanol and centrifugation at 10 000 *g* for 30 min. The pellet was dissolved in endotoxin free PBS (Sigma), and mixed (1:1 v/v) with adjuvant (Montanide ISA50). The PBS-protein: adjuvant mixture was emulsified by syringe aspiration until a stable emulsion was obtained which was then used for immunisation by subcutaneous injection. The control insert supplied with the TOPO pET kit (*LacZ* gene) was used as the negative control recombinant protein and was prepared using identical methods as for the recombinant 1H12 proteins. Each animal in the negative control group received 600 µg of recombinant β-galactosidase (rβ-galactosidase) protein.

4.2.2.3.6. Immunisation of sheep - laboratory trial 1: DNA prime- with either a rLSDV or recombinant protein boost compared with DNA only immunisation

Sheep (10 animals per group) were immunised with 1H12 pCMViUBs_ORF constructs, and boosted a third time with either DNA, 1H12 rLSDV or r1H12 proteins (see Table 4.2 for the different groups), using the same doses of plasmid DNA as described before (Chapter 2). For the prime-boost immunisation strategy, sheep were immunised twice with the 1H12 cocktail vector followed by either 1x 10^7 rLSDV or 150 µg/ORF r1H12 protein (each animal received a total of 600 µg protein). The negative control group received empty pCMViUBs vector, followed by either wtLSDV or 600 µg of rβ-galactosidase protein prepared in the same way as the r1H12 proteins. Animals were challenged in the laboratory five weeks after the final boost. Sheep were monitored for onset of clinical symptoms after challenge and temperatures were monitored as described in chapter 2.

4.2.2.3.7. Immunisation of sheep - laboratory trial 2: DNA prime–recombinant protein boost immunisation of sheep using only i.m. DNA inoculation

In a separate experiment three groups of five sheep were immunised using a variant of the prime-boost immunisation strategy described above. The DNA prime was given by i.m. inoculation only, instead of by both i.m. inoculation and gene gun, and the boost consisted of recombinant protein only (Table 4.2). The survival of sheep primed with DNA (i.m. inoculation) followed by a r1H12 protein boost (identical to that in 4.2.2.3.6) was compared to a negative control (empty pCMViUBs vector prime-rβ-galactosidase protein boost), naive animals (negative challenge control) and infected and treated sheep (positive challenge control). Sheep were monitored for the onset of clinical symptoms after challenge and temperatures were monitored as described in chapter 2.

4.2.2.3.8. Immunisation of sheep - field trial 1: Comparison of the DNA prime-recombinant protein boost with the DNA only immunisation

A field trial was also carried out, comparing the survival of sheep immunised with the 1H12 DNA vaccine to sheep immunised using the DNA prime-recombinant protein boost strategy (Table 4.2). Five weeks after the final boost, sheep were translocated to the Springbokfontein farm (Limpopo province, South Africa) in the heartwater endemic area. Again, sheep were monitored for the onset of clinical symptoms after challenge and temperatures were monitored as described in chapter 2.

4.2.2.3.9. Lymphocyte proliferation assays

Assays were performed as described in chapter 2 with minor modifications. Briefly, the isolated peripheral blood monocytic cells (PBMC) were stimulated with a r1H12 protein cocktail (1 µg/ml final concentration) instead of crude antigen. For the negative controls, PBMC were stimulated with rβ-galactosidase (derived from the *LacZ* gene) (1 µg/ml final concentration) prepared and isolated similarly to the r1H12 antigens.

Table 4.2: *The immunisation strategy for the three animals trials described in this chapter.*

Experiment	Group	Number of sheep	Immunised with	Challenged with
Prime-boost (4.3.3)	Negative challenge control	6	None, naive	Welgevonden; needle
	Positive challenge control	10	Infected and treated	Welgevonden; needle
	Negative 1	10	3x empty pCMViUBs vector plasmid DNA(i.m. and gg[1])	Welgevonden; needle
	Negative 2	10	2x empty pCMViUBs vector plasmid DNA (i.m. and gg), 1x wtLSDV	Welgevonden; needle
	Negative 3	10	2x empty pCMViUBs vector plasmid DNA (i.m. and gg), 1x rβ-galactosidase protein	Welgevonden; needle
	Experimental 1	10	3x 1H12 cocktail (i.m. and gg)	Welgevonden; needle
	Experimental 2	10	2x 1H12 cocktail (i.m. and gg)	Welgevonden; needle
	Experimental 3	10	2x 1H12 cocktail (i.m. and gg), 1x r1H12 LSDV	Welgevonden; needle
	Experimental 4	10	2x 1H12 cocktail (i.m. and gg), 1x r1H12 protein	Welgevonden; needle
I.m. only immunisation (4.3.4)	Negative challenge control	2	None, naive	Welgevonden; needle
	Positive challenge control	2	Infected and treated	Welgevonden; needle
	Negative	5	2x empty pCMViUBs vector plasmid DNA(i.m.), 1x rβ-galactosidase protein	Welgevonden; needle
	Experimental	5	2x 1H12 cocktail (i.m.), 1x r1H12 protein	Welgevonden; needle
Field trial (4.3.5)	Negative challenge control	5	None, naive	Natural tick challenge on Springbokfontein
	Positive challenge control	5	Infected and treated	Natural tick challenge on Springbokfontein
	Negative	5	3x empty pCMViUBs vector DNA (i.m. and gg)	Natural tick challenge on Springbokfontein
	Experimental	5	3x 1H12 cocktail (i.m. and gg)	Natural tick challenge on Springbokfontein
	Negative	5	2x empty pCMViUBs vector DNA(i.m. and gg), 1x rβ-galactosidase protein	Natural tick challenge on Springbokfontein
	Experimental	5	2x 1H12 cocktail (i.m. and gg), 1x r1H12 protein	Natural tick challenge on Springbokfontein

[1]***gg*** *= intra dermal immunisation using a gene gun.*

4.2.2.3.10. Western blot analysis of immunised sheep sera

Recombinant 1H12 protein was run on a 12 % SDS-PAGE gel, and transferred to PVDF membrane as described for the anti-His_6 Western blot. Two strips of membrane, one containing 1H12 antigen and the other rβ-galactosidase, were incubated overnight with a 1/20 dilution of sheep serum. Anti-1H12 antibodies were detected by incubating with HRP-rabbit-anti-sheep-IgG (H + L) conjugate (Zymed) for 2 h followed by incubating washed strips with SuperSignal®West Pico Chemiluminescent substrate (Pierce).

4.2.2.4. Cytokine profiling using real-time PCR

4.2.2.4.1. Stimulation of cell cultures with recombinant 1H12 proteins

Cell cultures for real-time PCR analysis were performed in 1 ml volumes in 5 ml polypropylene round bottomed tubes. PBMC (4 x 10^6 cells/ml) were resuspended in complete RPMI-1640 medium and stimulated with r1H12 antigen (final concentration of 1 µg/ml). Positive controls were treated with ConA (using a previously optimised final concentration of 5.2 µg/ml) and negative controls with an unrelated recombinant protein (rβ-galactosidase). The cultures were incubated at 37 °C in a humidified atmosphere containing 5 % CO_2 for approximately 18 h. The cells were pelleted by centrifugation at 300 *g* for 5 min. The pellet was washed once with 1 ml 1x PBS to remove all medium and centrifuged at 300 *g* for 5 min. The cells were resuspended in 100 µl 1x PBS whereafter 1 ml RNA*later* (Qiagen) was added. The cells were transferred to a cryotube and stored at -70 °C until used.

4.2.2.4.2. RNA extraction from stimulated cultures

After thawing, the cells were pelleted by centrifuging at 3000 *g* for 5 min and the cell pellet was resuspended in 100 µl PBS. RNA was isolated on a MagNA Pure LC instrument using the MagNA Pure RNA Isolation-High Performance kit according to the instruction manual (Roche).

4.2.2.4.2. Reverse transcriptase reaction (RT) of total RNA

A mixture containing 25 µg total RNA and 1 µl random hexamer primers (0.5 µg/µl) was incubated for 10 min at 65 °C and cooled on ice to 4 °C to denature the RNA and anneal the primers. This was followed by the addition of 40 U/µl RNaseOUT (Invitrogen) and 50 U/µl Expand (Roche) reverse transcriptase in 1x Expand reverse

transcriptase buffer. The RT reaction was performed in a final volume of 40 µl, incubated for 10 min at 30 °C, for 45 min at 42 °C, cooled to 4 °C and stored at -20 °C until further use.

4.2.2.4.3. Primers designed for real-time PCR

mRNA sequences for ovine glyceraldehyde-3-phosphate dehydrogenase (GAPDH), IFN-γ and IL-4 were obtained from GenBank (accession numbers, U94889, Z73273 and AF17268 respectively). Amplification primers (Table 4.3) were designed using the Light Cycler Probe Design 2 software (Roche).

Table 4.3: *Primers used for real-time PCR reaction.*

Target	Forward primer 5'-3'	Reverse primer 5'-3'
GAPDH	TCA CTG CCA CCC AGA AGA	CTC AGG GAT GAC CTT GC
IFN-γ	CTT GGT GTT ATT GTG ACT GTT G	ATG AAT CCC TCC TAA ATC TCT GTA
IL-4	TGA CAG GAA TCT CAG CAG	TTC TCC CTC ATA ATA GTC TTT AGC

4.2.2.4.4. Real-time PCR analysis of IFN-γ and IL-4 mRNA expression

Real-time PCR was performed using the LightCycler® FastStart DNA Master^{PLUS} SYBR Green 1 kit according to the instruction manual (Roche) and the LightCycler Instrument. Thermal cycling conditions were 10 min for 95 °C and 50 cycles of 10 s for 95 °C, 10 s for 57 °C and 5 s for 72 °C followed by cooling for 30 s at 40 °C.

IFN-γ or IL-4 gene expression was measured by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001; Mena *et al.,* 2002; Mahajan *et al.,* 2003). Relative quantitation was determined by comparing threshold cycle (Ct) of a sample (PBMC stimulated with r1H12 antigen) to the Ct generated by a reference sample known as the calibrator (PBMC stimulated with unrelated recombinant protein). Here cytokine gene expression was normalized to GAPDH (a housekeeping enzyme) expression by subtraction of Ct_{GAPDH} from the $Ct_{IFN\text{-}\gamma \text{ or IL-4}}$ to provide ΔCt values for each of the samples of interest and the calibrator. The ΔΔCt was calculated as the difference between ΔCt_{sample} and $\Delta Ct_{calibrator}$. The relative difference in cytokine expression between PBMC stimulated with r1H12 antigen and cells stimulated with unrelated recombinant protein was determined using the expression $2^{-\Delta\Delta Ct}$.

eg.

$\Delta Ct_{sample} = Ct_{sample}$ (target gene, IFN-γ or IL-4) – Ct_{sample} (GAPDH)

$\Delta Ct_{calibrator} = Ct_{calibrator}$ (target gene, IFN-γ or IL-4) – $Ct_{calibrator}$ (GAPDH)

$\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{calibrator}$

The fold increase of sample relative to calibrator = $2^{-\Delta\Delta Ct}$

4.2.2.5. Statistical analysis

Reaction index scores and lymphocyte proliferation results were compared by means of the one tailed distribution Student's *t*-test. Significance was assessed at P values of < 0.01 throughout the study.

4.3. RESULTS

4.3.1. Preparation of recombinant LSDV

The four 1H12 ORFs were cloned into the pAFMCR shuttle vector and transfected into wtLSDV infected MDBK cells after confirmation of correct sequence (see Appendix B for alignments). The rLSDV clones were selected after repeated passages and large preparations of each recombinant virus were made. To ascertain if the four 1H12 rLSDV viral stocks contained the appropriate 1H12 insert and were free from wtLSDV, rLSDV genomic DNA was used in amplification reactions containing 1H12 specific primers and primers that amplify the ribonuclease reductase gene (Table 4.1). All four of the 1H12-specific primer sets were included in the PCR to ensure that each rLSDV stock contained a single ORF. A single band of the correct size was detected in the rLSDV genomic DNA for each cloned 1H12 ORF (Figure 4.2B: lanes 2-5). The purity of the viral isolates was confirmed by amplification of the LSDV RR gene. If the transfection and homologous recombination was successful, the gene of interest, the *β-galactosidase* gene and a marker gene *ECOGPT* would have been transfected into the RR gene thus making the total size of the insert ~5 kb (Figure 4.2A). The RR gene in the wild type virus is normally 918 bp in size. If wtLSDV was still present in the sample, two bands would be visible i.e. the 918 bp and the ~5 kb band, while only the ~5 kb fragment would be amplified from a pure rLSDV sample. A 918 bp product was detected only in the wtLSDV sample while no 918 bp band was present in the 1H12 rLSDV viral genomic DNA (Figure 4.2B: lanes 9-14). Only the ~5 kb band (consisting of the 1H12 ORF, the *ECOGPT* and *β-galactosidase* genes and the RR flanking regions) was visible in the rLSDV PCR products. These results confirmed that wtLSDV was successfully transfected and the resulting rLSDV stocks were free from PCR-detectable wtLSDV. Sequence analysis using the ORF-specific primers revealed that all four of the cloned 1H12 ORFs had the correct DNA sequence except for Erum2540, where a single base pair difference was seen but this did not affect the translated amino acid sequence (see Appendix B for alignments).

Normally, protein expression by the rLSDV would be confirmed using antibodies directed against the ORF-specific protein. Unfortunately, antibodies directed to the 1H12 proteins were not available at the time this experiment was performed and

expression could only be inferred by testing if the ORFs were transcribed. Transcription of the 1H12 proteins in the four rLSDV stocks was determined by isolating total RNA from MDBK cells infected with the respective rLSDV stocks, and RT-PCR established that three of the ORFs: Erum2550, -2590 and -2580 were transcribed (Figure 4.3). We could not show that Erum2540 was transcribed, but a very poor yield of RNA was obtained from that specific culture which may explain this result. The amino acid sequence inferred from the amplified mRNA of each ORF showed no differences from the amino acid sequence obtained from genomic Welgevonden DNA. Due to low concentrations of mRNA the cDNA sequence of Erum2540 could not be obtained and Erum2580 cDNA was only partially sequenced (see Appendix B for alignments).

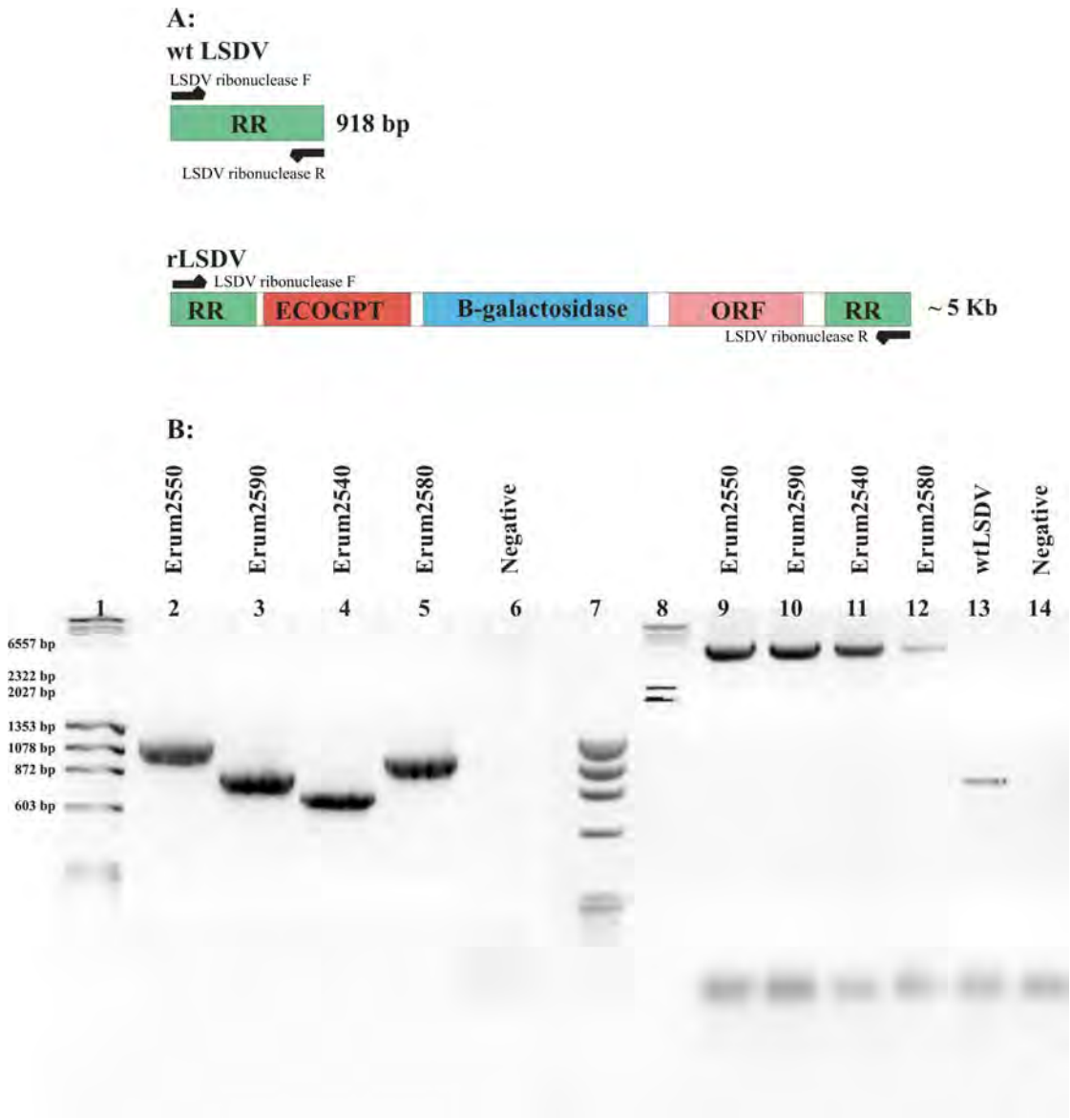


***Figure 4.2**: A: Schematic representation of the ribonucleotide reductase (RR) gene in wild type LSDV (wtLSDV) and recombinant LSDV (rLSDV) The positions of the LSDV RR primers used for PCR amplification across the RR gene are shown. B: PCR to confirm that each of the 1H12 ORFs was successfully transfected into LSDV and the resulting rLSDV stocks were free of contaminating wtLSDV. Genomic DNA was isolated from each of the four purified 1H12 rLSDV preparations. Two PCRs were done: one using 1H12 specific primers (all four primer sets were included in the master mix) (lanes 2-6) and one using the LSDV RR primer set (Table 4.1) (Lanes 9-14). Lane 1: lambda HindIII and PhiX174 HaeIII markers, Lane 7: PhiX174 HaeIII marker, Lane 8: lambda HindIII marker.*

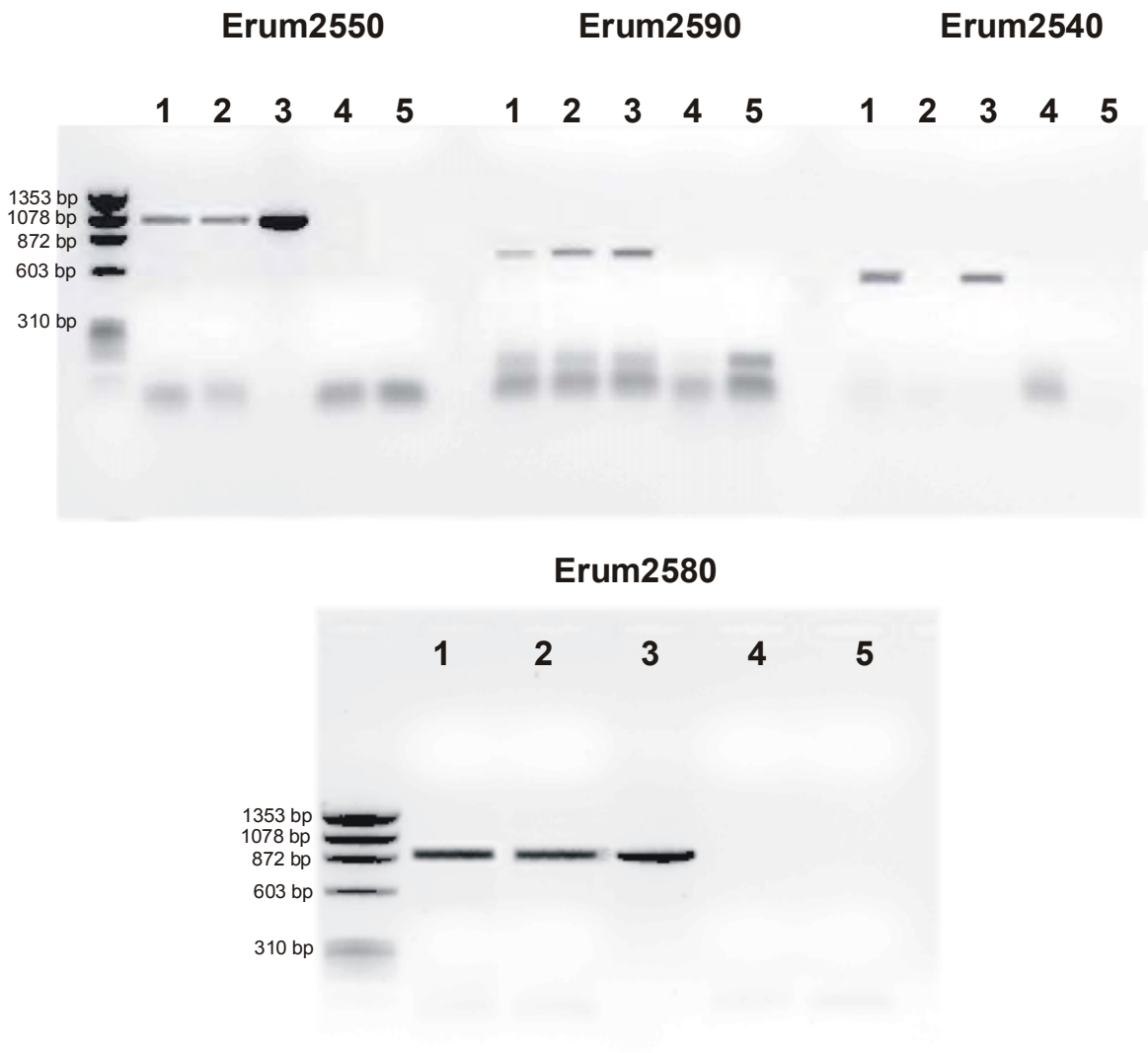


Figure 4.3: *Confirmation of transcription of three of the four 1H12 ORFs cloned in lumpy skin disease virus (LSDV). For each ORF lane 1 contains rLSDV genomic DNA; lane 2 contains cDNA prepared from bovine kidney cells; lane 3 contains a positive control (each ORF cloned in pCMViUBs plasmid); lane 4 contains total RNA; and lane 5 contains water for negative control. Each sample (genomic, cDNA, plasmid DNA, total RNA and water) was amplified with 1H12 ORF-specific primers (Table 4.1).*

4.3.2. Expression of the 1H12 recombinant proteins

For the DNA prime-recombinant protein boost immunisation strategy we expressed the 1H12 ORFs by cloning them into the TOPO TOOLS pET (Invitrogen) vector. Sequence analysis of clones revealed that the inserts were cloned in-frame with the polyhistidine tail for all four of the 1H12 ORFs (see Appendix B for sequence alignments and translations). Protein expression was confirmed by Western blot analysis using anti-His_6 antibodies. The concentrations of proteins expressed from pET_Erum2550 and pET_Erum2590 were much higher than those of pET_Erum2540 and pET_Erum2580. The sizes of three of the four recombinant proteins correlated with their predicted sizes (Figure 4.4, Table 4.4). Interestingly, the size of recombinant Erum2580 protein did not correlate with the predicted size (52.52 kDa), but was much smaller (~28 kDa). The pET_Erum2580 plasmid DNA was isolated from BL21 cells and sequenced. No modifications were observed at the DNA level suggesting either early termination of transcription and/or translation or that posttranslational modification of the expressed protein occurred in *E. coli.*

Table 4.4: *Predicted sizes of the four r1H12 protein products with or without thioredoxin and His6 tags (Protein sizes were predicted using a size prediction tool: http://bioinformatics.org/sms/prot_mw.html).*

1H12 ORF	Predicted protein size	Predicted size including the Thioredoxin and H_6 tags
Erum2550	38.29 kDa	57.60 kDa
Erum2590	27.75 kDa	47.16 kDa
Erum2540	20.48 kDa	39.89 kDa
Erum2580	33.10 kDa	52.52 kDa

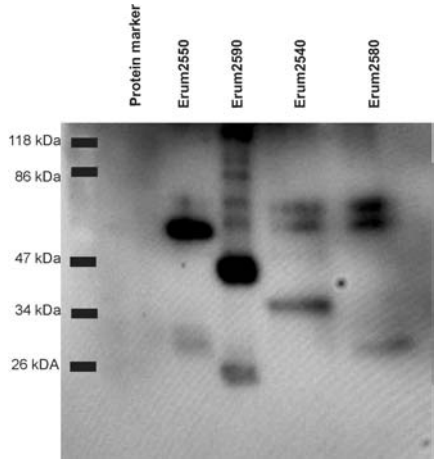


Figure 4.4*: Anti-His_6 Western blot of the four 1H12 ORFs expressed in E. coli.*

4.3.3. Immunisation of sheep using the prime-boost strategy

To determine whether a heterologous prime-boost immunisation strategy will effectively protect against heartwater, the four 1H12 ORFs were either cloned and transfected into a Capripoxvirus vaccine strain, LSDV, or the gene products were expressed in *E. coli*. Animals were immunised as before with DNA vaccine alone, or a DNA vaccine prime was followed by a heterologous protein boost using either rLSDV or recombinant protein (Table 4.2). The sheep were inoculated three times with a three week interval as done before and five weeks after the final immunisation, the sheep were needle-challenged with a virulent dose of *E. ruminantium* Welgevonden stock. Immune responses were monitored with lymphocyte proliferation, Western blot analysis and cytokine profiling using real-time PCR technology.

All of the animals in the negative control groups and in the challenge control group had to be treated after needle challenge with virulent *E. ruminantium* Welgevonden (Figure 4.5). Animals in the challenge control group and in the group immunised twice with empty pCMViUBs vector followed by wtLSDV boost were treated first (starting from day 14) while animals immunised 3x with empty pCMViUBs vector and animals immunised twice with empty pCMViUBs vector followed by recombinant protein boost (rβ-galactosidase) survived a day or two longer and were only treated starting on day 16 (see Figures 4.5 for survival and Figures 4.6-4.10 for temperature reactions). All of the negative control animals developed severe heartwater symptoms within 3 days after the first rise in temperature was detected (Figures 4.11 and 4.12).

Sheep inoculated with 2x 1H12 cocktail and sheep inoculated with 2x 1H12 cocktail followed by rLSDV boost showed similar symptoms at the same time points. One sheep in each of these groups developed a febrile reaction (Figures 4.5, 4.7B and 4.10B) and had to be treated 16 days after challenge. Five of the remaining sheep in the group boosted with rLSDV as well as two sheep in the 2x 1H12 cocktail group developed a slight temperature increase and early heartwater symptoms (Figures 4.11 and 4.12). The average RI of the group immunised with 2x 1H12 cocktail ($P < 3 \times 10^{-11}$) and the group immunised with 2x DNA prime-rLSDV boost

($P < 7 \times 10^{-06}$) did however differ significantly from their respective controls (Table 4.5). Even though one animal died (this animal showed a sudden rise in temperature on day 14 (Figure 4.10B) and unfortunately died overnight) in the group boosted with rLSDV, the average RI of this group (Table 4.5) did not differ significantly from that of the sheep immunised with 3x DNA ($P < 0.09$). Only sheep immunised with 3x 1H12 cocktail and sheep immunised with 2x 1H12 cocktail followed by r1H12 protein had a 100 % survival rate (Figure 4.5). These animals did not have a critical rise in temperature (Figures 4.7A and 4.9B) and only a few animals in each group developed early heartwater symptoms (Figures 4.11 and 4.12). As expected, significant differences were obtained between the 1H12 inoculated groups (3x DNA, 2x DNA followed by rLSDV boost and 2x DNA followed by rprotein boost) and their respective negative controls (Table 4.5).

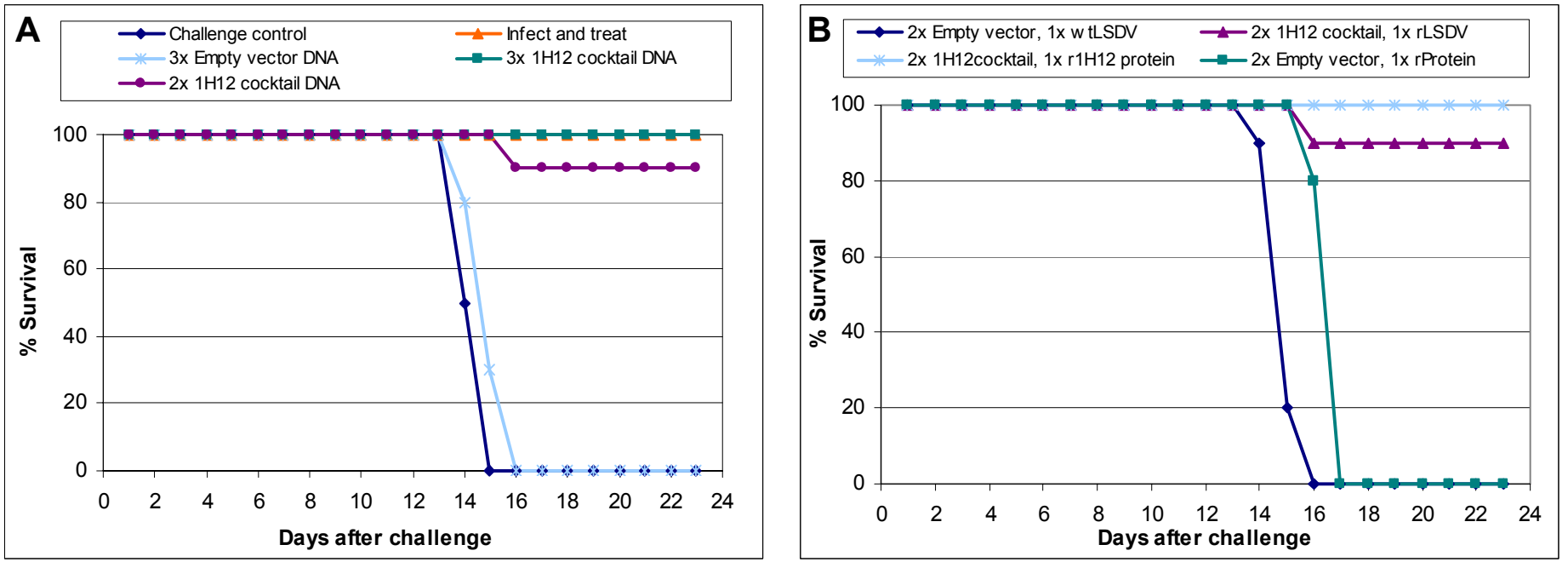


***Figure 4.5**: Survival charts of challenge control sheep compared to sheep immunised with empty pCMViUBs vector, 2x 1H12 cocktail and 3x 1H12 cocktail (A) and sheep immunised with DNA (1H12 cocktail/empty pCMViUBs vector) and boosted with rLSDV/wtLSDV or r1H12 protein/rβ-galactosidase protein (B). Animals that were treated were also considered as non-survivors.*

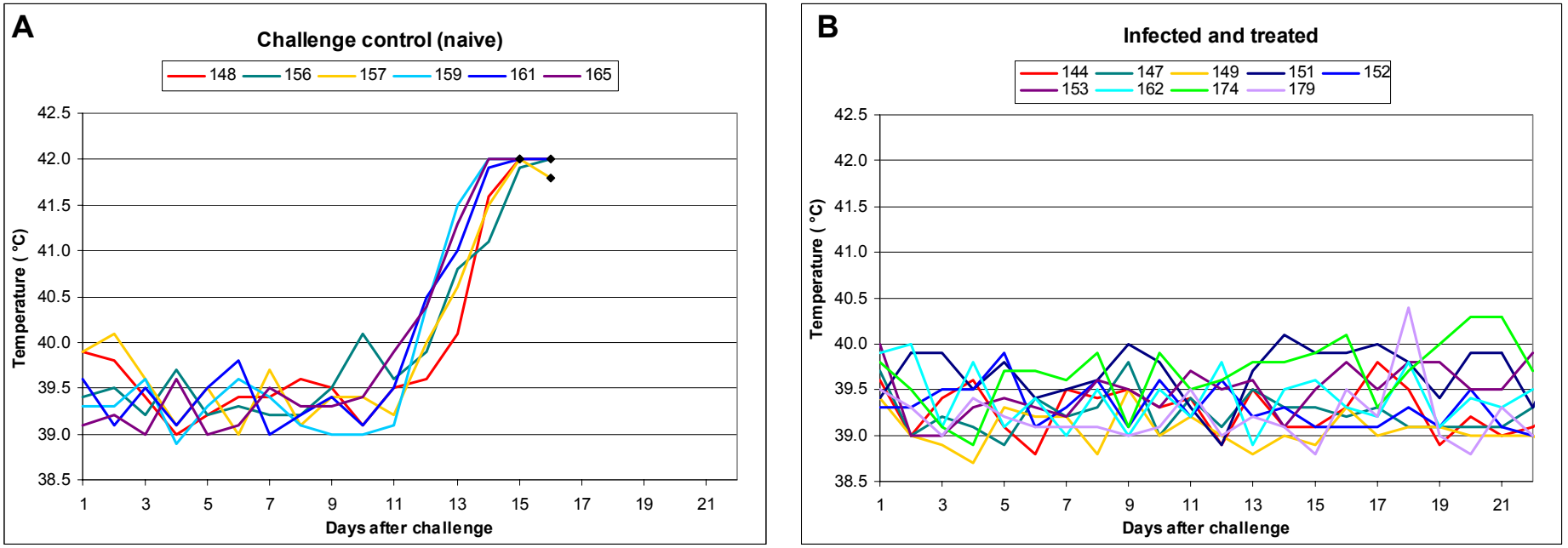


Figure 4.6: *Daily post-challenge temperatures of challenge control (naive) (A) and positive control (infected and treated) sheep (B). Sheep were challenged with 10* LD_{50}*s of the virulent* E. ruminantium *Welgevonden stock. Temperature measurements were terminated 22 days after challenge or as soon as an animal was treated or euthanased (represented by a black dot).*

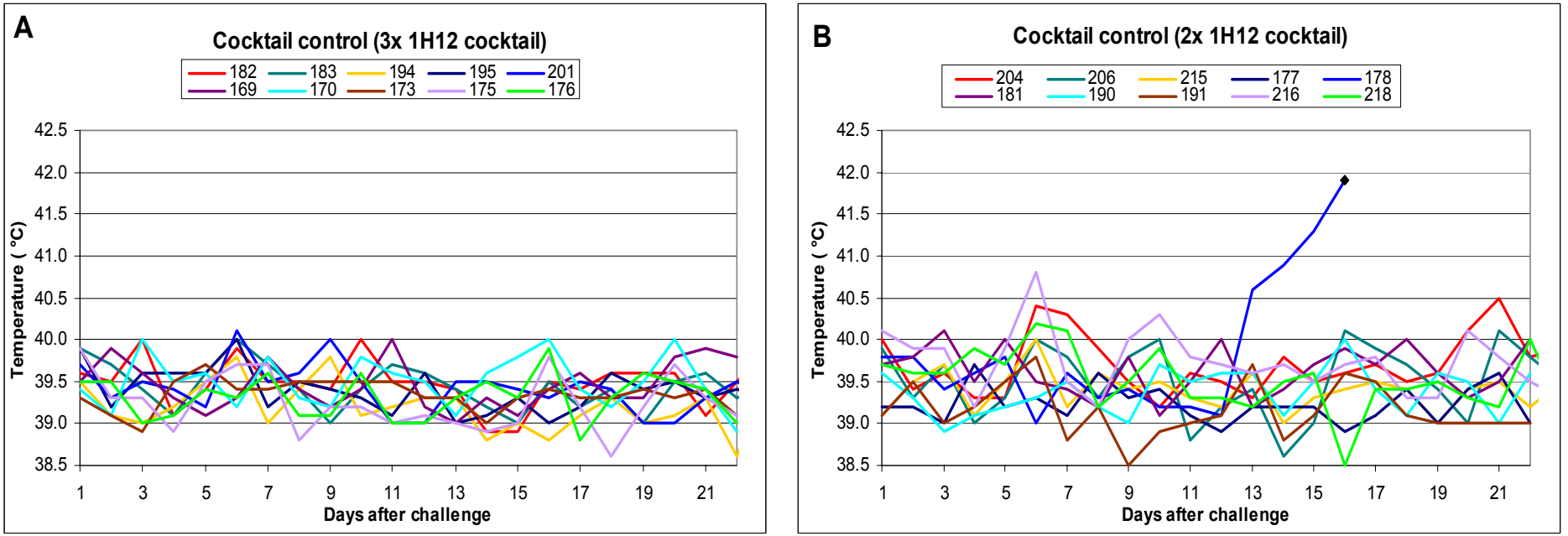


Figure 4.7: *Daily post-challenge temperatures of sheep immunised 3x with 1H12 cocktail (A)and 2x with 1H12 cocktail (B). Sheep were challenged with 10 LD_{50}s of the virulent* E. ruminantium *Welgevonden stock. Temperature measurements were terminated 22 days after challenge or as soon as an animal was treated or euthanased (represented by a black dot).*

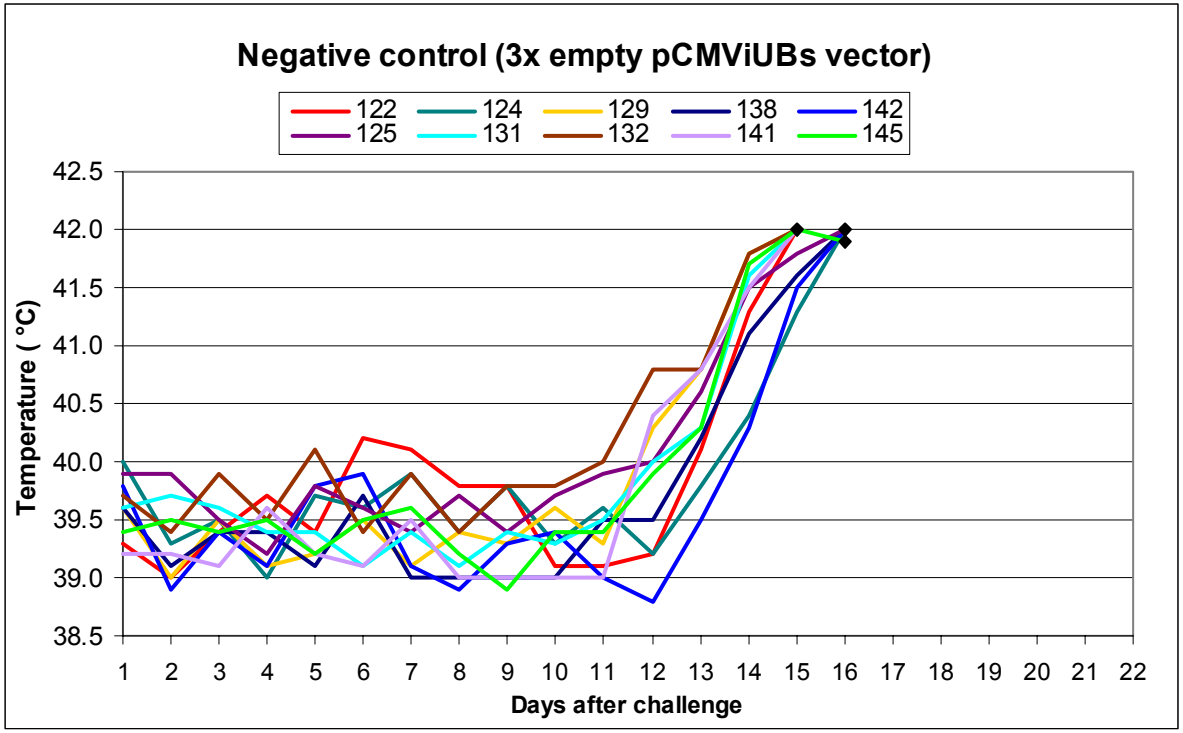


Figure 4.8: *Daily post-challenge temperatures of sheep immunised with 3x with empty pCMViUBs vector. Sheep were challenged with 10 LD_{50}s of the virulent* E. ruminantium *Welgevonden stock. Temperature measurements were terminated 22 days after challenge or as soon as an animal was treated or euthanased (represented by a black dot).*

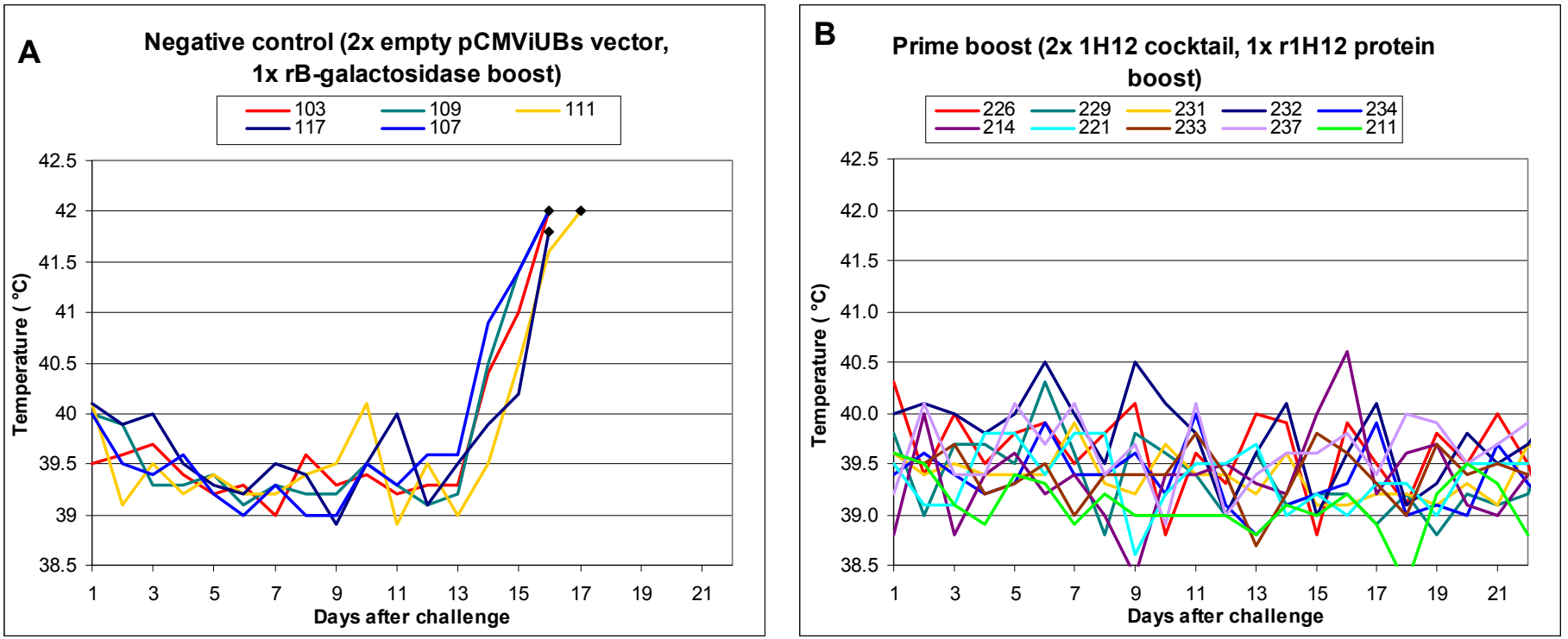


Figure 4.9: *Daily post-challenge temperatures of sheep immunised using a heterologous prime-boost immunisation strategy: 2x with empty pCMViUBs vector followed by an unrelated protein (rβ-galactosidase) boost (A) and 2x with 1H12 cocktail followed by 1x r1H12 protein boost (B). Sheep were challenged with 10 LD_{50}s of the virulent* E. ruminantium *Welgevonden stock. Temperature measurements were terminated 22 days after challenge or as soon as an animal was treated or euthanased (represented by a black dot).*

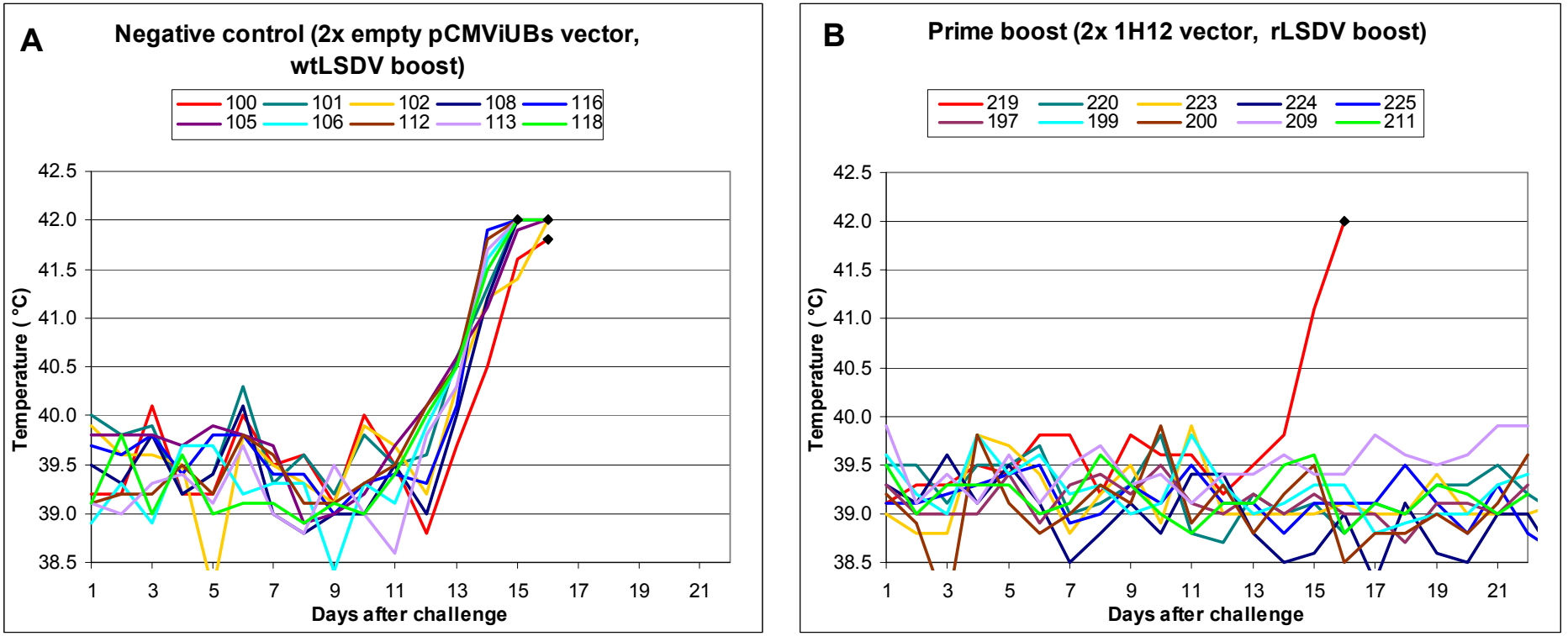


Figure 4.10: *Daily post-challenge temperatures of sheep immunised using a heterologous prime-boost immunisation strategy: 2x with empty pCMViUBs vector followed by a wtLSDV boost (A) and 2x with 1H12 cocktail followed by 1x rLSDV boost (B). Sheep were challenged with 10 LD_{50}s of the virulent* E. ruminantium *Welgevonden stock. Temperature measurements were terminated 22 days after challenge or as soon as an animal was treated or euthanased (represented by a black dot).*

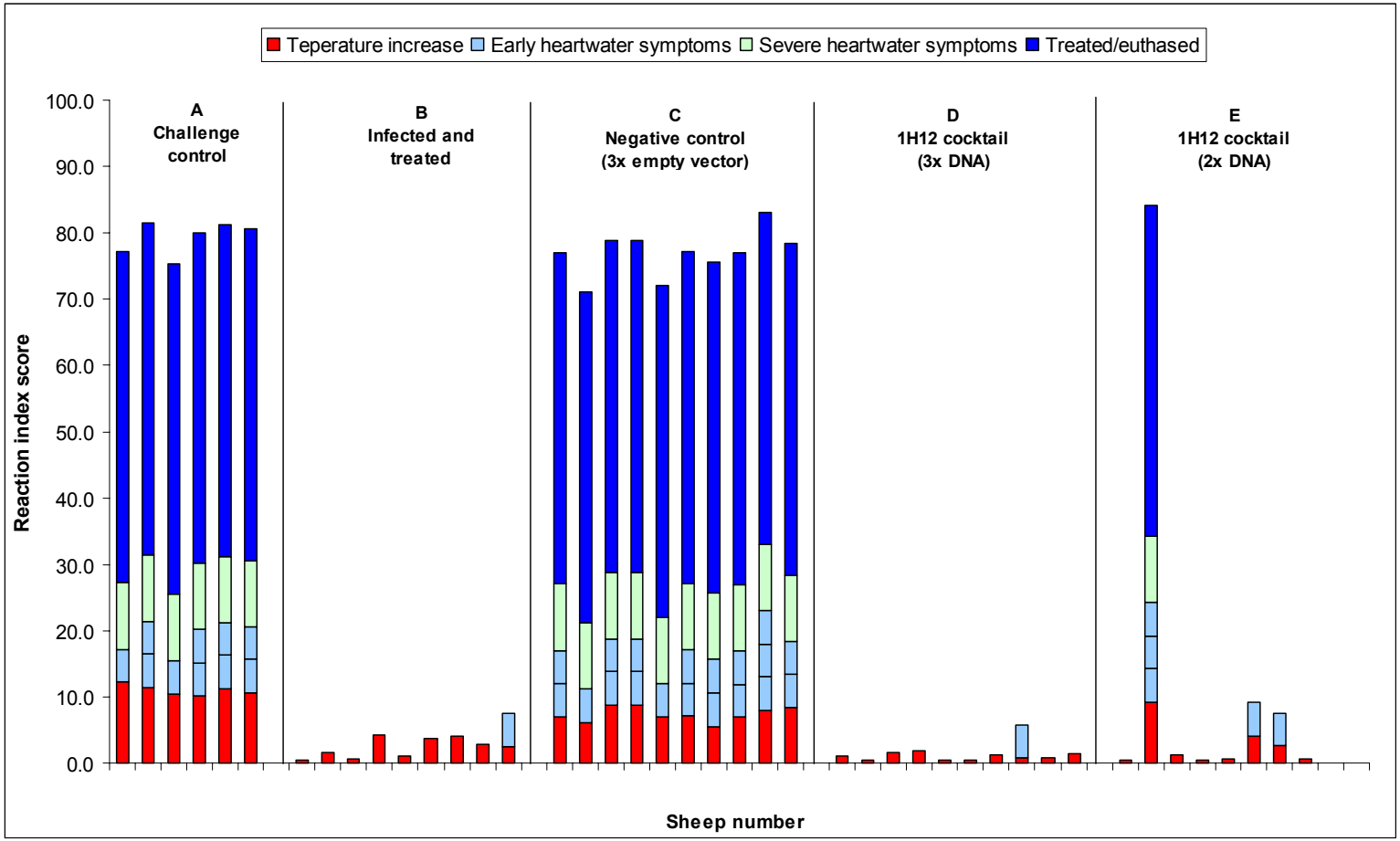


Figure 4.11: *Reaction index (RI) of the naive challenge control sheep (A) and infected and treated sheep (positive control) (B), sheep immunised with the empty pCMViUBs vector (C) and with the 1H12 cocktail with three inoculations (D) and with two inoculations (E) after challenge with* E. ruminantium *Welgevonden. Red blocks indicate the total temperature RI score 21 days after challenge, while the light blue blocks represent a RI of 5 (early heartwater symptoms), the light green a RI of 10 (severe heartwater symptoms) and the dark blue a RI of 50 (animal was treated/euthanased).*

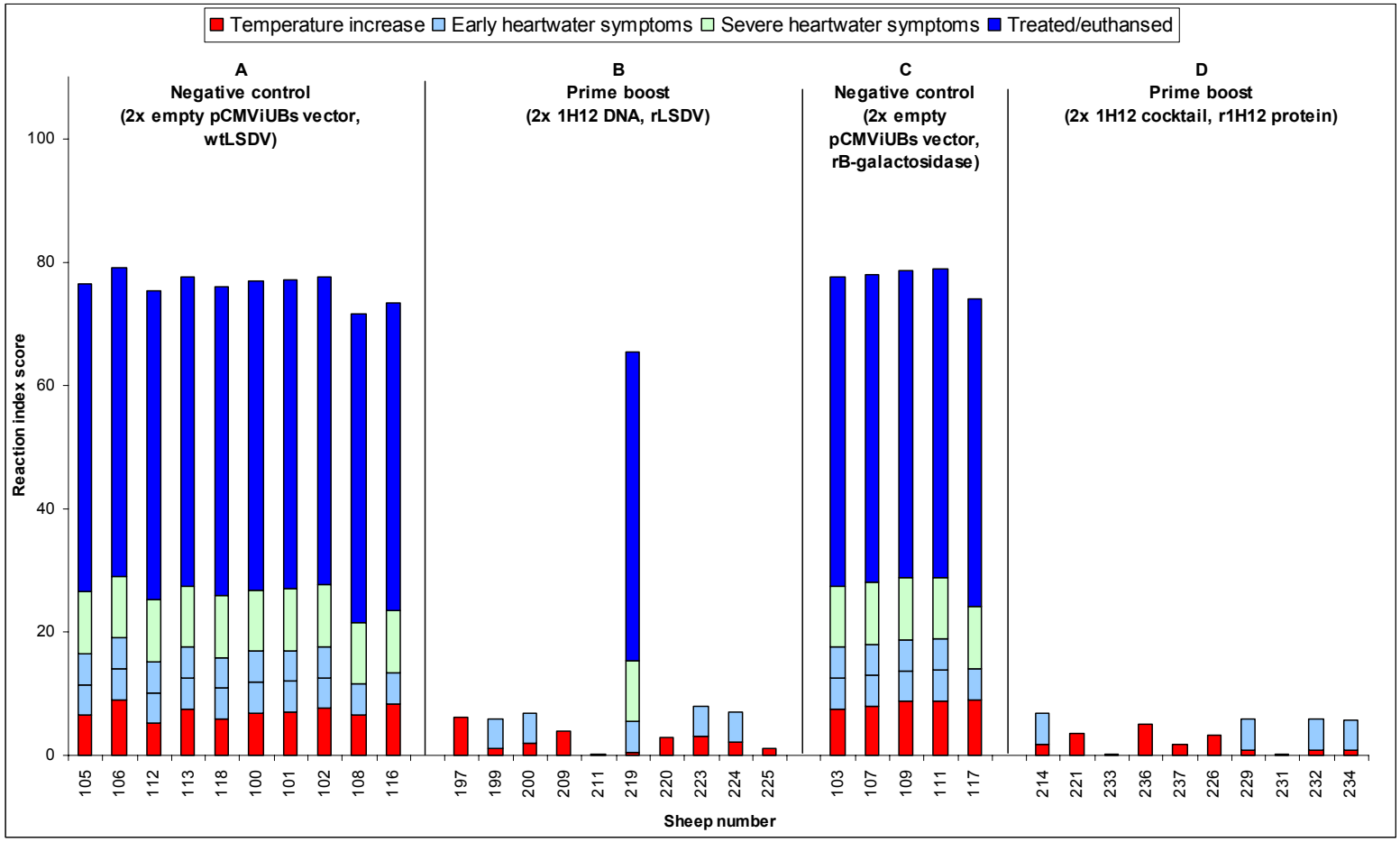


Figure 4.12: *Reaction index (RI) of sheep immunised with empty pCMViUBs vector followed by wtLSDV boost (A), 1H12 DNA prime–rLSDV boost (B), empty pCMViUBs vector DNA prime - unrelated protein (rβ-galactosidase) boost (C) and 1H12 cocktail prime–r1H12 protein boost (D) after challenge with* E. ruminantium *Welgevonden. Red blocks indicate the total temperature RI score 21 days after challenge, while the light blue blocks represent a RI of 5 (early heartwater symptoms), the light green a RI of 10 (severe heartwater symptoms) and the dark blue a RI of 50 (animal was treated/euthanased).*

Table 4.5: *Statistical analysis of RI values as determined for each group.*

	Sheep numbers	RI_{TOTAL}	RI_{AVE}	RI_{STDEV}	P value[1] compared to: Infected and treated	1H12 cocktail (3x DNA)	Empty pCMViUBs vector (3x DNA)	Empty pCMViUBs vector (2x), wtLSDV	Empty pCMViUBs vector (2x), rProtein
Cocktail control (3x 1H12 vector, DNA only)	169	1							
	170	0.5							
	173	1.6							
	175	1.8							
	176	0.5							
	182	0.4							
	183	1.2							
	194	5.8							
	195	0.8							
	201	1.5	1.51	1.6	1.2×10^{-1}		3×10^{-11}		
Cocktail control (2x 1H12 vector, DNA only)	177	0.4							
	178*	69.2							
	181	1.3							
	190	0.5							
	191	0.6							
	204	9.1							
	206	7.6							
	215	0.7							
	216	0							
	218	0	8.94	21.4	2×10^{-1}	3.0×10^{-11}	7.0×10^{-7}		

[1] *P value was determined by one tailed distribution Student's* t-test.
* *Note that the values of these animals, that developed heartwater and died, were included in the RI statistical calculations.*

Table 4.5: *Continued*

	Sheep numbers	RI TOTAL	RI_{AVE}	RI STDEV	P value[1] compared to: Infected and treated	1H12 cocktail (3x DNA)	Empty pCMViUBs vector (3x DNA)	Empty pCMViUBs vector (2x), wtLSDV	Empty pCMViUBs vector (2x), rProtein
Prime-boost (2x 1H12, 1x rLSDV)	197	6.2							
	199	6.0							
	200	6.9							
	209	4.0							
	211	0.3							
	219*	85.4							
	220	2.9							
	223	8.0							
	224	7.1							
	225	1.0	12.78	25.7	1.0×10^{-1}	9.0×10^{-2}		7×10^{-6}	
Prime-boost (2x 1H12, 1x r1H12 protein)	214	6.7							
	221	3.5							
	233	0.2							
	236	5.1							
	237	1.7							
	226	3.2							
	229	5.9							
	231	0.2							
	232	5.9							
	234	5.8	3.82	2.4	1.0×10^{-1}	3.0×10^{-2}			2×10^{-4}

[1] *P value was determined by one tailed distribution Student's* t*-test.*
** Note that the values of these animals, that developed heartwater and died, were included in the RI statistical calculations.*

Lymphocyte proliferation assays were done on the PBMC isolated from 5 sheep per group. No significant proliferation was detected in any of the groups when the PBMC were stimulated with r1H12 proteins, except for the group boosted with 2x 1H12 cocktail followed by r1H12 protein (Table 4.6). Two of the sheep in this group (sheep 229 and 232) showed significant stimulation indices (SI) of 5.5 ($P < 1.8 \times 10^{-4}$) and 3.9 ($P < 2 \times 10^{-3}$) respectively before challenge while the PBMC of three sheep had significant SI after challenge (Table 4.6).

It has been shown previously that protection against a virulent *E. ruminantium* infection requires elevated levels of IFN-γ (Tottè *et al.,* 1997; 1999). Here, real-time PCR was used to detect relative IL-4 and IFN-γ levels in PBMC stimulated with recombinant proteins. Only the infected and treated group and DNA prime-recombinant protein boost group showed increased IFN-γ before challenge (Table 4.7). This increase in IFN-γ production was maintained only in the infected and treated group after challenge. Increased IFN-γ expression was detected after challenge in the three times DNA group while the DNA prime-recombinant protein group did not show any IFN-γ after challenge except for one animal (sheep 231) showing increased IFN-γ and IL-4 expression (Table 4.7). Interestingly, this was also the only animal with detectable antibodies specific to r1H12 antigen as determined by Western blot analysis (results not shown).

Table 4.6: *Lymphocyte proliferation assays using the PBMCs from sheep immunised with empty pCMViUBs vector followed by a rβ-galactosidase protein boost and sheep immunised with 2x 1H12 cocktail DNA followed by a r1H12 protein boost. Lymphocyte proliferation was done at three time points: before immunisation, before and after challenge. PBMC were stimulated with a cocktail of the four 1H12 recombinant proteins. Significant proliferation is highlighted in blue (before challenge) and red (after challenge).*

		SI_{AVE} (SI after boost/ SI naive)		**P Values [1] (SI (2x1H12 cocktail, rprotein boost) compared to SI (2x empty pCMViUBs vector, unrelated protein boost)**		**Number of positive animals per group[2]**	
Group	**Sheep number**	**Before challenge**	**After challenge**	**Before challenge**	**After challenge**	**Before challenge**	**After challenge**
2x empty pCMViUBs vector ; 1x rβ-galactosidase protein boost	103	0.6 ± 0.9	0.5 ± 0.1				
	107	0.7 ± 0.04	1.2 ± 0.4				
	109	1.0 ± 0.1	1.1 ± 0.2				
	111	0.7 ± 0.7	0.7 ± 0.9				
	117	1.1 ± 0.6	1.1 ± 0.4				
2x 1H12 cocktail ; 1x r1H12 protein boost	226	0.8 ± 0.8	**2.5 ± 0.4**	4.9×10^{-1}	**1.8×10^{-3}**		
	229	**5.2 ± 0.6**	0.5 ± 0.4	**1.8×10^{-3}**	1.1×10^{-1}	**2**/5	**3**/5
	231	**3.9 ± 0.5**	**2.2 ± 0.5**	**2.0×10^{-3}**	**1.0×10^{-2}**		
	232	0.3 ± 0.1	0.4 ± 0.3	3.0×10^{-2}	2.8×10^{-1}		
	234	1.1 ± 0.2	**2.3 ± 0.5**	4.8×10^{-2}	**1.0×10^{-2}**		

[1] *P value was determined by one tailed distribution Student's t-test.*
[2] *Only samples with an SI more than two times higher than that of the negative control (empty pCMViUBs vector) and significant p values were considered as positive.*

Table 4.7: *Cytokine profiling results: Cytokine expression was determined by doing cyber green relative quantification using the ΔCt method. Fold increase values that are more than 2x higher than values obtained for the negative control groups are highlighted in **red** (IFN-γ) and **blue** (IL-4).*

Group	Sheep number	Fold increase ($2^{-\Delta\Delta Ct}$ value of each animal before or after challenge compared to its naive $2^{-\Delta\Delta Ct}$ value)				Number of animals positive in group (as compared to negative)			
		Pre-challenge		After challenge		Pre-challenge		After challenge	
		IFN-γ	IL-4	IFN-γ	IL-4	IFN-γ	IL-4	IFN-γ	IL-4
Infect and treat	147	2.3	0.0	2.3	0.0				
	149	0.26	0.0	0.16	0.0				
	152	0.3	0.0	**20.3**	0.0				
	153	**467.9**	0.0	**20.0**	0.0				
	174	**54.6**	0.0	**8.4**	0.0	**2**/5	0/5	**3**/5	0/5
3x Empty pCMViUBs vector	122	0.0	0.1	1.2	0.0				
	124	0.1	0.0	2.6	18.3				
	129	2.5	1.2	0.9	0.2				
	138	0.2	0.0	0.1	1.1				
	142	0.3	0.0	0.0	2.7				
3x 1H12 cocktail	182	1.4	0.0	**12.9**	5.5				
	183	2.1	**7.5**	n/d	0.7				
	194	0.1	0.0	**13.3**	0.0				
	195	0.2	0.7	2.7	2.6				
	201	0.1	0.0	3.5	0.0	0/5	**1**/5	**2**/5	0/5
2x 1H12 cocktail	204	1.8	0.2	**537.5**	0.0				
	206	0.1	1.6	n/d	n/d				
	215	2.0	**79.3**	0.3	0.0				
	216	2.1	**29.9**	**32.2**	18.3				
	218	**22.8**	**14.9**	n/d	n/d	**1**/5	**3**/5	**2**/5	0/5
2x Empty pCMViUBs vector, 1x wtLSDV virus	100	7.0	0.0	0.2	0.6				
	101	2.8	1.9	284.0	1.4				
	102	1.1	10.1	4.5	0.8				
	108	1.1	1.0	0.6	0.4				
	116	0.5	0.0	98.4	4.6				
2x 1H12 cocktail, 1x rLSDV	219	0.0	**42.5**	0.0	0.0				
	220	0.2	0.0	1.3	0.0				
	223	0.1	1.5	0.0	**32.2**				
	224	0.4	**3516.7**	0.0	0.0				
	225	n/d	n/d	n/d	n/d	0/5	**2**/5	0/5	**1**/5
2x Empty pCMViUBs vector, 1x unrelated recombinant protein	103	0.2	0.0	0.1	0.0				
	107	1.6	0.0	1.7	0.0				
	109	0.7	0.3	0.1	0.0				
	111	1.6	2.2	2.5	0.3				
	117	06	0.0	1.5	0.0				
2x 1H12 cocktail, 1x r1H12 protein	226	**4.4**	2.2	0.2	0.0				
	229	**25.1**	0.0	0.4	0.0				
	231	**3.5**	1.3	**107.6**	**382.7**				
	232	**3.3**	**13.3**	0.4	**2.4**				
	234	0.9	1.1	0.2	0.0	**4**/5	**1**/5	**1**/5	**2**/5

n/d: mRNA extraction was not successful or could not be repeated.

4.3.4. Prime-boost immunisation of sheep using the i.m. method of DNA inoculation only

In a subsequent experiment, animals were immunised using the DNA prime-recombinant protein boost strategy by i.m. inoculation only (instead of the usual i.m. and i.d. inoculations with a gg used in all the previous experiments) to determine if the cumbersome gg immunisation can be eliminated. The experimental group was inoculated two times with 1H12 cocktail DNA using the i.m. inoculation route only, this was followed by a r1H12 protein boost. Negative control animals were inoculated with empty pCMViUBs vector followed by an unrelated rβ-galactosidase protein boost. Positive control infected and treated and naive challenge control animals were included as shown in table 4.3.

In the previous experiment all the animals immunised with 1H12 constructs by both routes (i.m. and i.d. with a gg.) survived, but when sheep were immunised with the 1H12 cocktail by i.m. inoculation alone, followed by recombinant protein boost, only one of the five sheep (20 %) did not need treatment after a virulent needle challenge (Figure 4.13). Even this animal developed a temperature response (Figure 4.15) and early heartwater symptoms (Figure 4.16). Animals that were immunised with the 1H12 cocktail (i.m. inoculation only) followed by recombinant protein boost did seem to survive a little bit longer than animals immunised with empty pCMViUBs vector followed by rβ-galactosidase boost (Figure 4.13), but nevertheless four of the five animals succumbed to the challenge. All the sheep in the negative control groups developed febrile reactions (Figure 4.14) and severe heartwater symptoms (Figure 4.16).

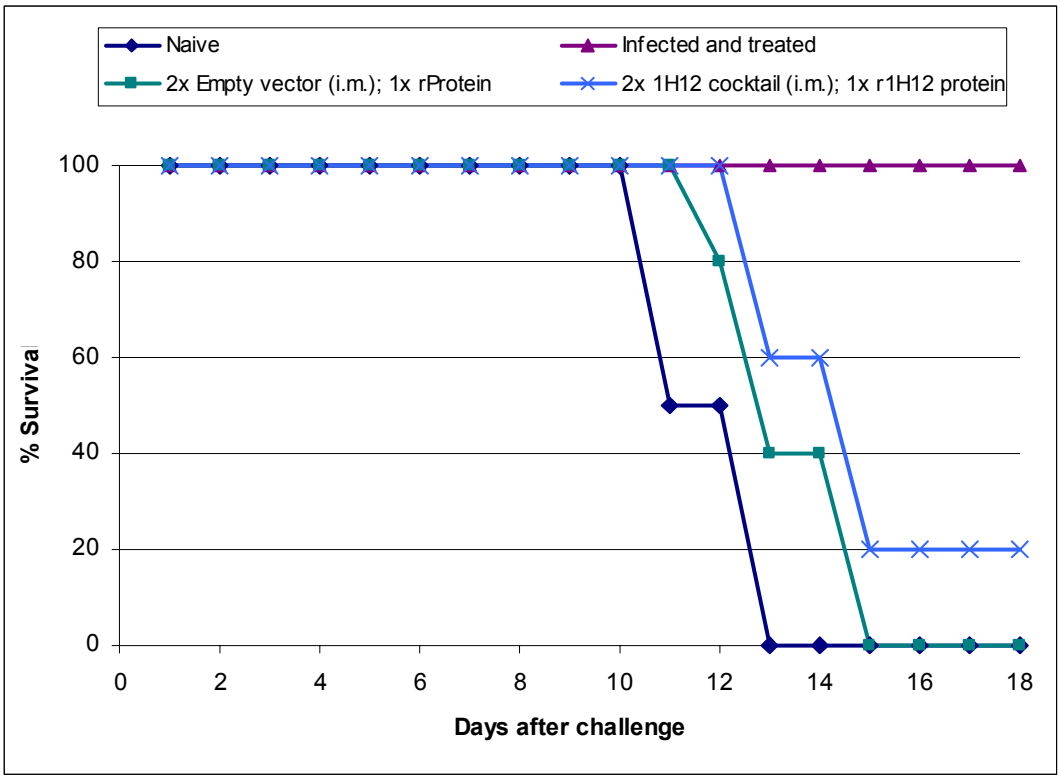


Figure 4.13: *Survival of challenge control sheep, infected and treated sheep, negative prime-boost sheep and sheep immunised with the 1H12 vaccine using a DNA prime (i.m. only) followed by a r1H12 protein boost. Sheep were needle challenged with virulent* E. ruminantium *Welgevonden. Animals that were treated were also considered as non-survivors.*

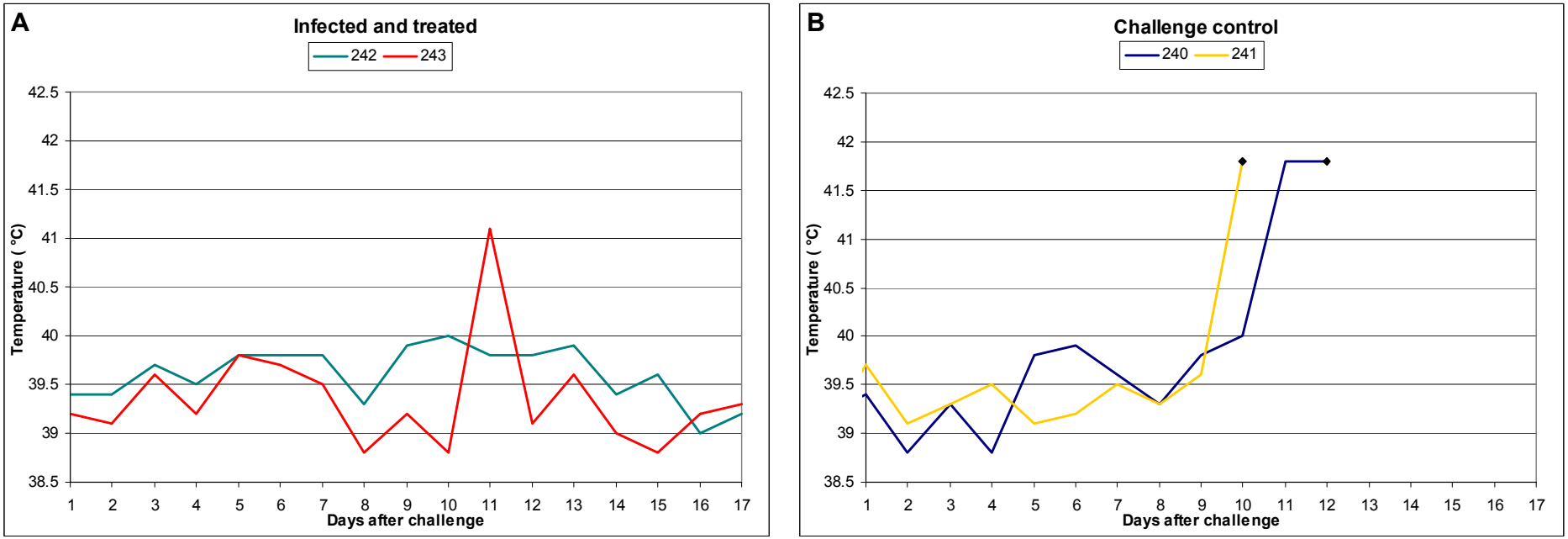


Figure 4.14: *Daily post-challenge temperatures of sheep immunised using the DNA prime (i.m. only) -recombinant protein boost immunisation strategy: Infected and treated (A), Challenge control (B). Sheep were challenged with 10 LD_{50}s of the virulent* E. ruminantium *Welgevonden stock. Temperature measurements were terminated 17 days after challenge or as soon as an animal was treated or euthanased (represented by a black dot).*

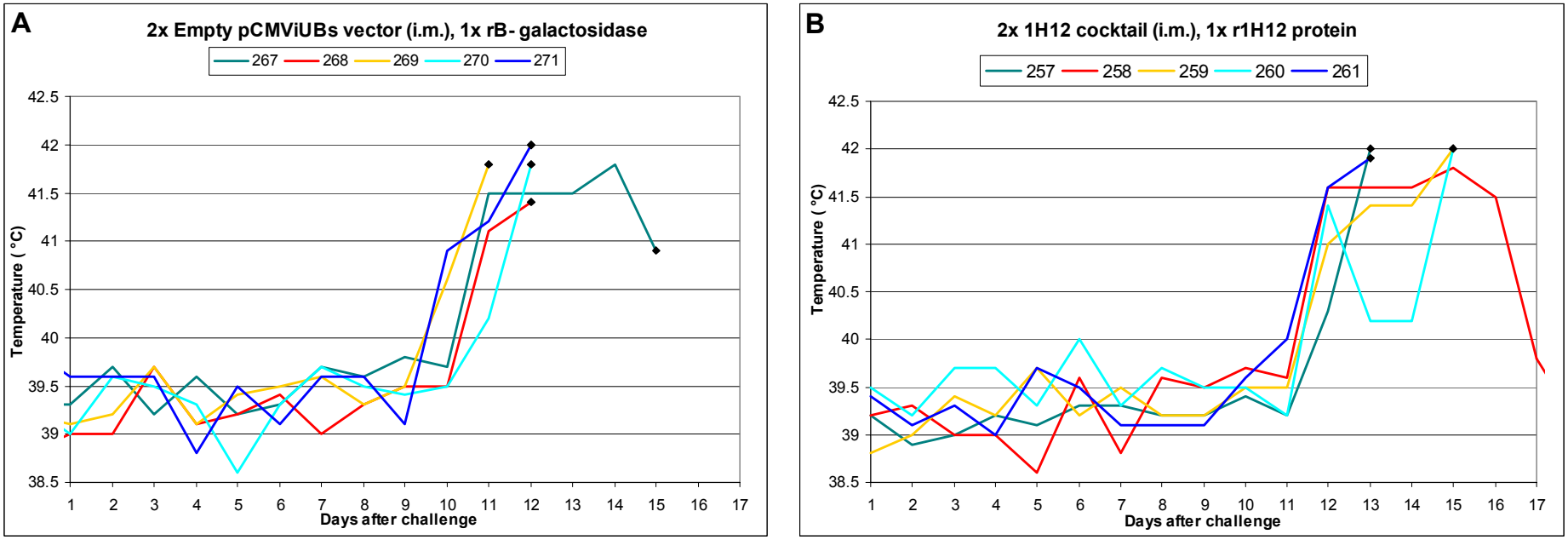


Figure 4.15: *Daily post-challenge temperatures of sheep immunised using the DNA prime (i.m. only) -recombinant protein boost immunisation strategy: prime-boost control (2x with empty pCMViUBs vector (i.m. only) followed by an unrelated protein boost (rβ-galactosidase) (A) and the 1H12 prime-boost group (2x 1H12 cocktail (i.m. only), 1x r1H12 protein) (B). Sheep were challenged with 10 LD_{50}s of the virulent* E. ruminantium *Welgevonden stock. Temperature measurements were terminated 17 days after challenge or as soon as an animal was treated or euthanased (represented by a black dot).*

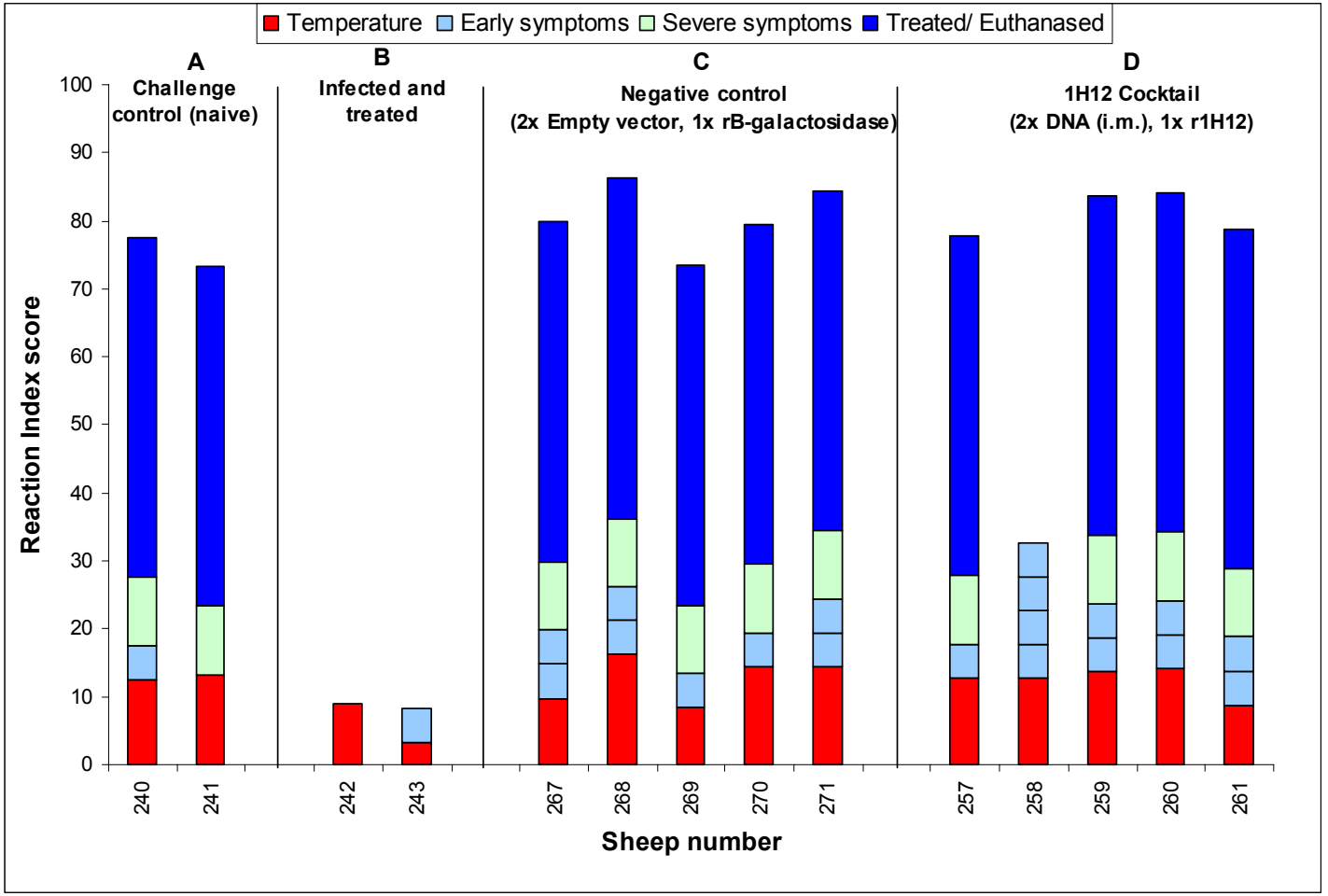


Figure 4.16: *Reaction index (RI) of challenge control sheep (A), sheep immunised by infection and treatment (B), sheep immunised 2x with empty pCMViUBs vector (i.m. only) followed by unrelated protein boost (rβ-galactosidase) (C) and 2x 1H12 DNA prime (i.m. only)– r1H12 protein boost (D) after challenge with* E. ruminantium *Welgevonden. Red blocks indicate the total temperature RI score 21 days after challenge, while the light blue blocks represent a RI of 5 (early heartwater symptoms), the light green a RI of 10 (severe heartwater symptoms) and the dark blue a RI of 50 (animal was treated/euthanased/died).*

No statistically significant difference was obtained between the negative control group and the group immunised with 1H12 using a DNA prime- (i.m. only), recombinant protein boost strategy (Table 4.8) and no lymphocyte proliferation or IFN-γ production could be detected (results not shown). It would therefore seem that either not enough DNA was administered using the i.m. route of inoculation or that immunisation using the i.d. route is very important to establish protective immunity to virulent *E. ruminantium* infection.

***Table 4.8**: Statistical analysis of the RI values obtained from sheep immunised with 1H12 using a DNA prime- (i.m. only), r1H12 protein boost immunisation strategy.*

Group	Sheep number	RI $_{TOTAL}$	RI_{AVE}	RI $_{STDEV}$	P^1 value compared to: Infected and treaded group	P^1 value compared to: Negative prime-boost
Challenge control	240	77.6				
	241	73.3	75.5	3.0	1.0×10^{-3}	1.0×10^{-1}
Infected and treated	242	9.1				
	243	8.2	8.6	0.6		1.4×10^{-6}
Negative prime-boost	267	79.8				
	268	86.2				
	269	73.5				
	270	79.5				
	271	84.4	80.7	5.0	1.4×10^{-6}	
1H12 prime-boost (i.m. only)	257	77.8				
	258	32.7				
	259	83.7				
	260	84.2				
	261	78.8	71.4	21.8	1.5×10^{-3}	2.0×10^{-1}

[1] *P value was determined by one tailed distribution Student's t-test.*

4.3.4. Field challenge of sheep immunised using the prime-boost immunisation strategy compared to DNA immunisation

In the previous chapter, we reported that only 20 % of animals immunised with the 1H12 DNA vaccine were protected from challenge under natural field conditions. To ascertain if the prime-boost immunisation strategy will improve protection under natural field conditions, two groups of sheep were either inoculated three times with the 1H12 DNA cocktail or two times with DNA (1H12 cocktail) followed by a r1H12

protein boost. Appropriate negative control, a challenge control (naive animals) and the positive infected and treated control groups were included as shown in table 4.2. Five weeks after immunisation animals were transported to Springbokfontein farm for natural tick challenge.

The first animals started to get sick after fifteen days on the farm whereafter new animals showed reactions until day 45 (end of the experiment). Three out of five animals in the challenge control group survived (Figure 4.17) suggesting that not all the animals received a lethal challenge. Similarly, two sheep from each group including the infected and treated group succumbed to infection, except for the 1H12 DNA prime-recombinant protein group where four sheep died (Figure 4.17). Post mortem results indicated that all these animals died of heartwater.

All the sheep in the remaining groups that survived showed typical heartwater symptoms, including high fever (Figures 4.18-20), but recovered from the infection. The severity of infection was also not the same in all the animals as can be seen by the RI (Figure 4.21) and statistical analysis on each group could not be done due to a high standard deviation. The diverse degrees of reaction from mild infection to severe symptoms resulting in death suggest that the challenge dose received by each animal was different. Nonetheless, the animals immunised with the prime-boost immunisation strategy all seem to have been infected with a high challenge dose and succumbed, except for one animal, sheep 846, which reacted and survived challenge. This implies that the prime-boost immunisation strategy does not provide improved protection of sheep to tick challenge.

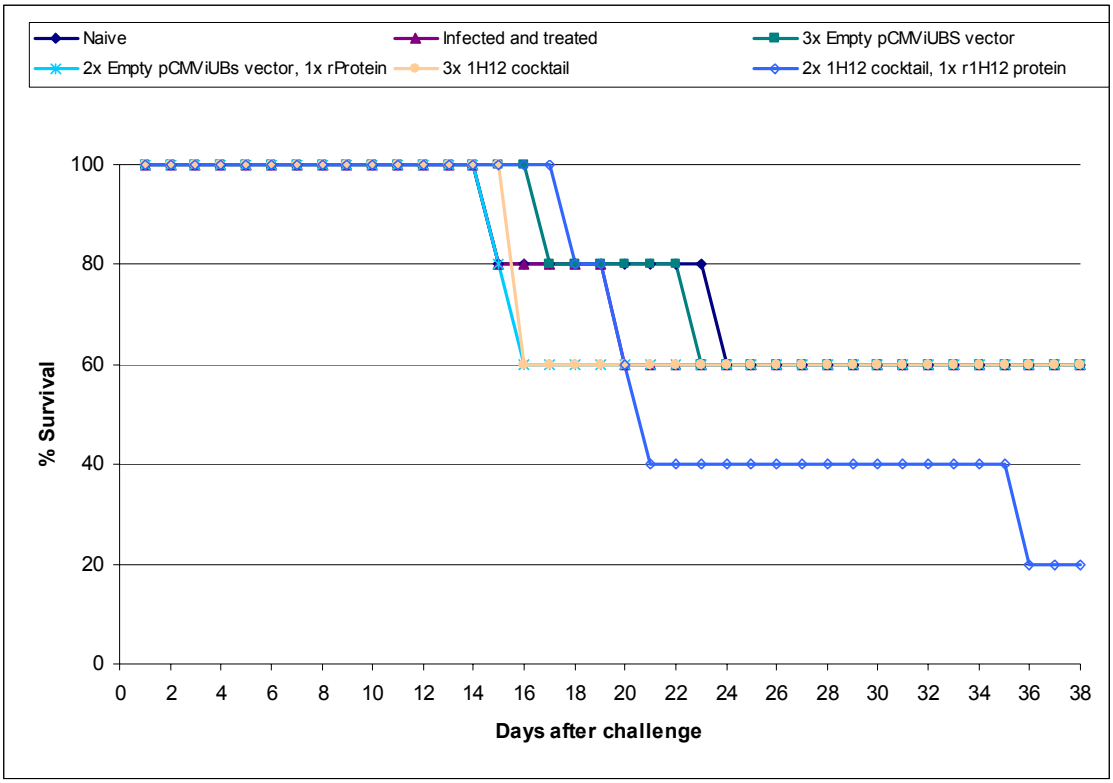


Figure 4.17: *Survival of challenge control sheep, infected and treated sheep, negative DNA immunisation and prime-boost sheep, animals immunised with the 1H12 DNA vaccine and sheep immunised using a DNA prime (i.m. and gg) followed by a r1H12 protein boost. Sheep were challenged by natural tick infestation on Springbokfontein farm.*

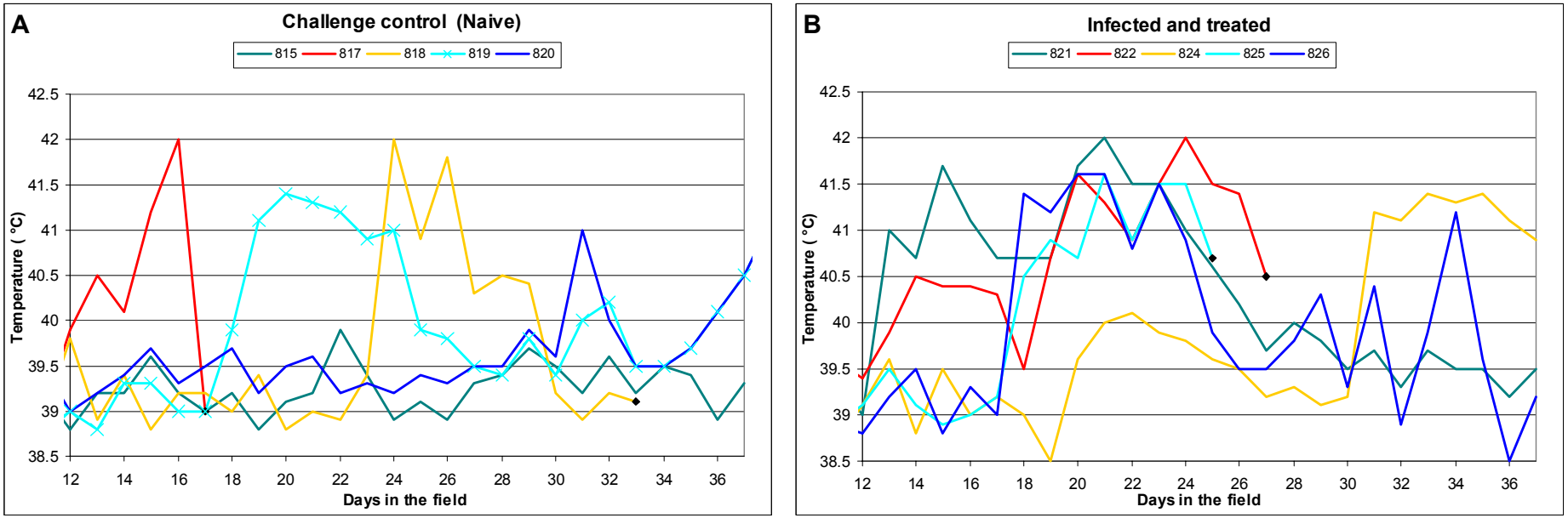


***Figure 4.18**: Daily post-challenge temperatures of the challenge control group (A) and the infected and treated group (B) challenged under natural field conditions. Temperature measurements were terminated after 37 days in the field or as soon as an animal was euthanased (represented by a black dot).*

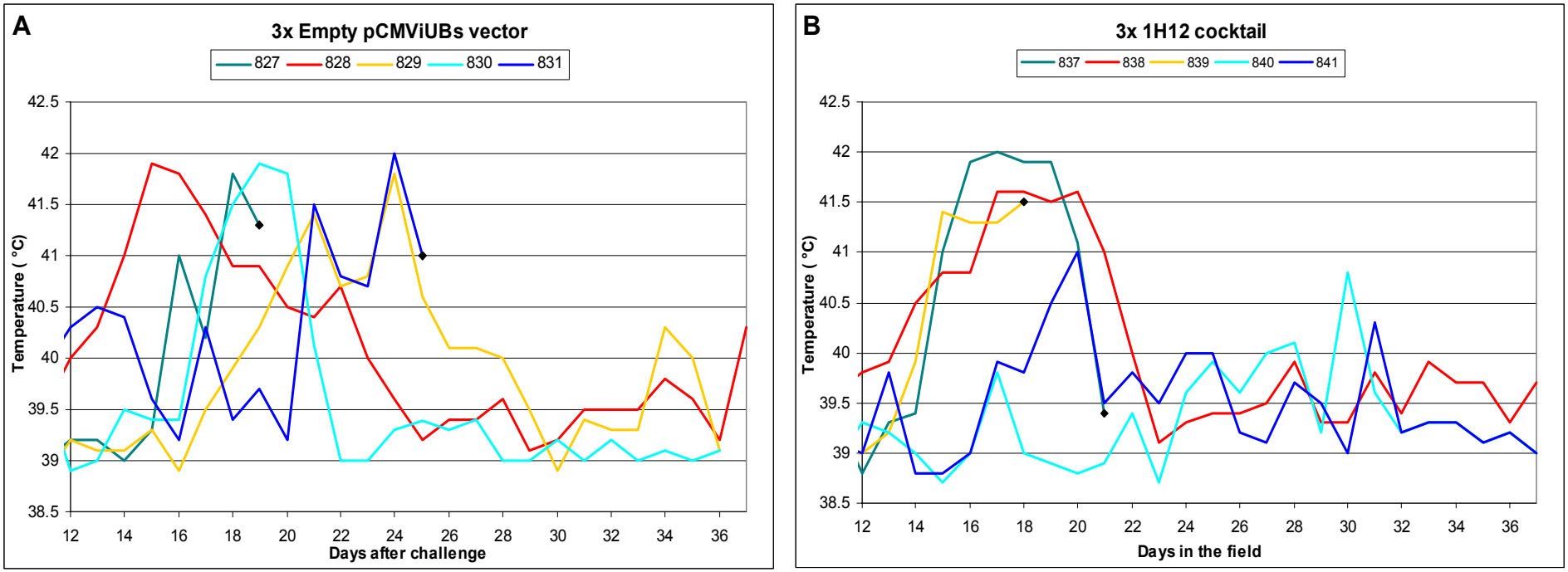


Figure 4.19: *Daily post-challenge temperatures of the negative DNA only group (3x empty pCMViUBs vector) (A) and the group immunised with 3x 1H12 DNA cocktail (B) challenged under natural field conditions. Temperature measurements were terminated after 37 days in the field or as soon as an animal was euthanased (represented by a black dot).*

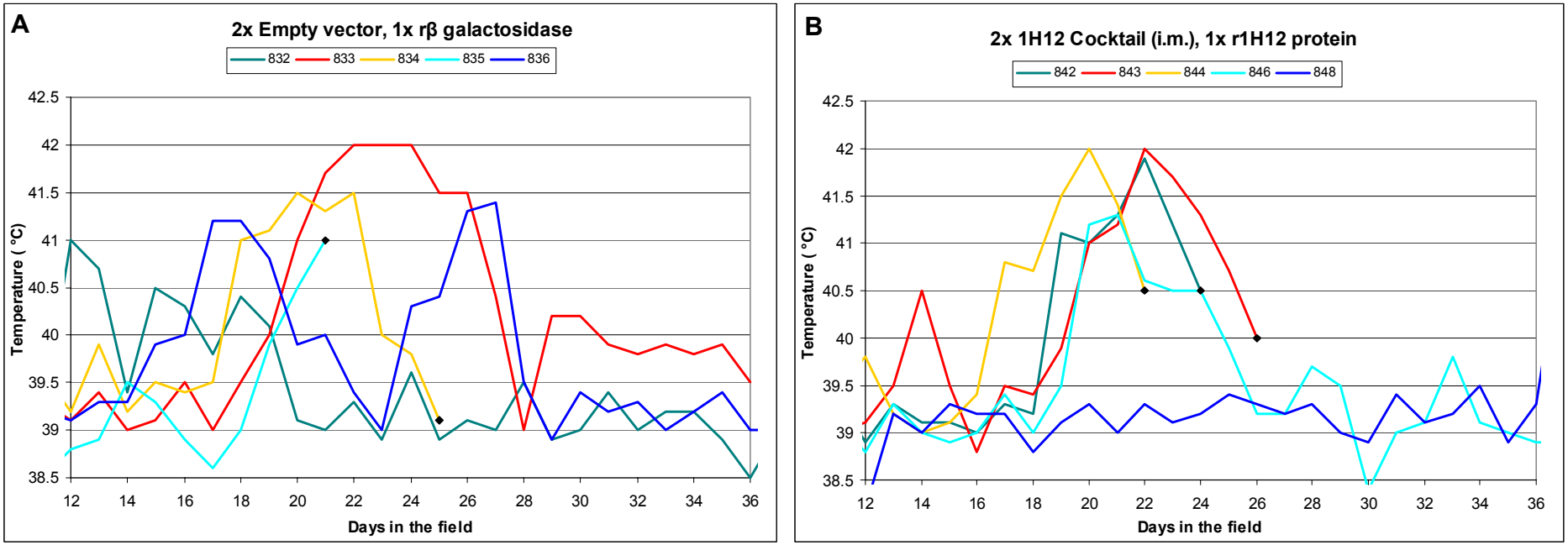


Figure 4.20: *Daily post-challenge temperatures of the negative prime-boost group (2x empty pCMViUBs vector followed by a rβ-galactosidase boost) (A) and the prime-boost vaccinated group (2x 1H12 DNA followed by a r1H12 protein boost) (B) challenged under natural field conditions. The DNA only immunisation strategy was compared with the DNA prime-r1H12 protein boost immunisation strategy. Sheep number 848 died after transport back to the laboratory. Temperature measurements were terminated after 37 days in the field or as soon as an animal was euthanased (represented by a black dot).*

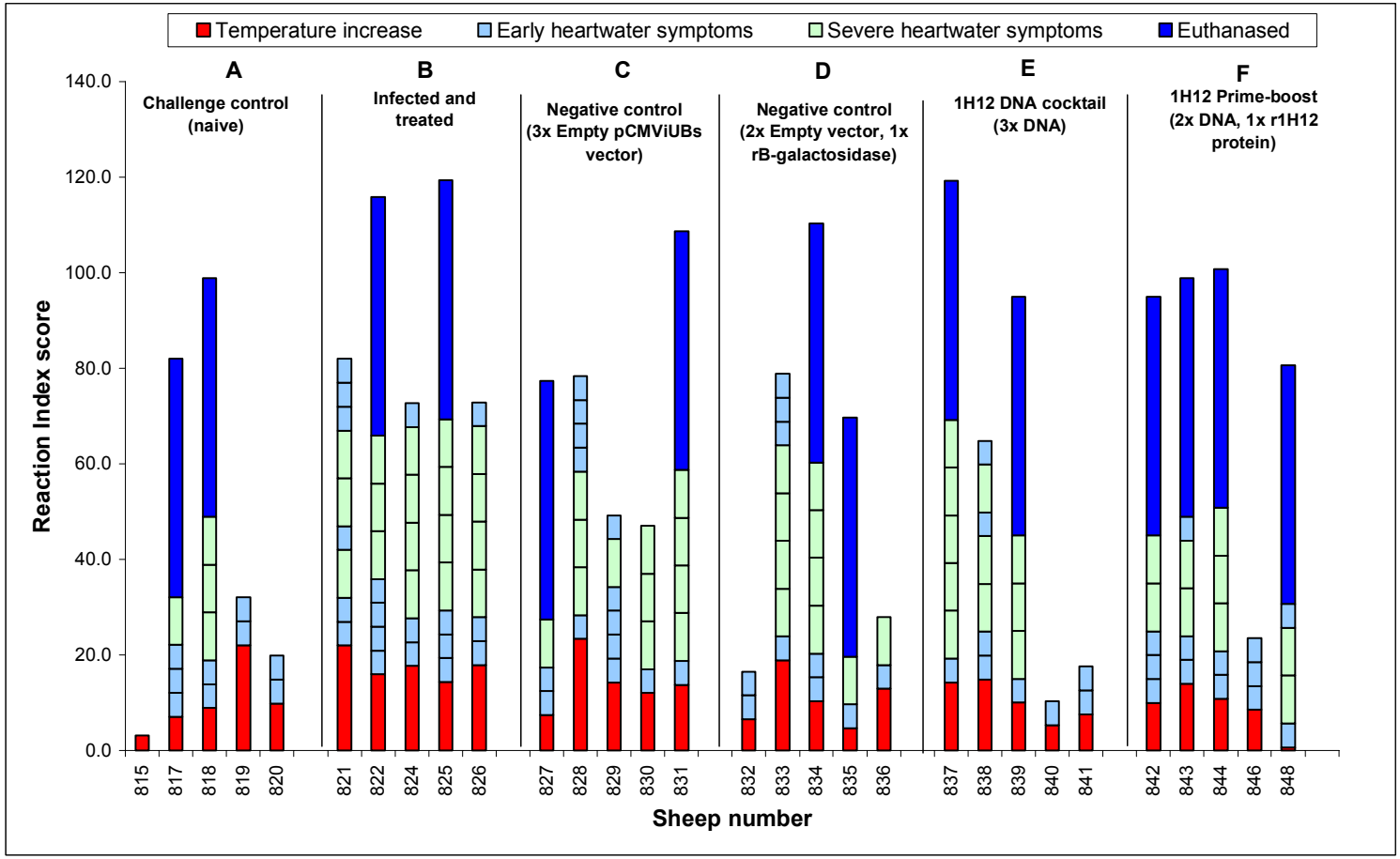


Figure 4.21: *Reaction index (RI) of challenge control sheep (A), sheep immunised by infection and treatment (B), negative control sheep immunised with empty pCMViUBs vector (C), prime-boost negative control sheep (D), sheep immunised with the 1H12 DNA vaccine (E) and sheep immunised with the 1H12 DNA prime followed by a r1H12 protein boost (F). Red blocks indicate the total temperature RI score 21 days after challenge, while the light blue blocks represent a RI of 5 (early heartwater symptoms), the light green a RI of 10 (severe heartwater symptoms) and the dark blue a RI of 50 (animal was euthanased or died).*

In an attempt to try to determine if each animal was challenged with the same genotype, the brain samples of the animals that died were tested for the presence of the parasite using the pCS20 PCR test. All the samples were positive and preliminary results revealed that five of the animals (sheep 818, 825, 836, 837 and 842) were infected with an *E. ruminantium* genotype that had a pCS20 sequence identical to that of Welgevonden and the previously obtained SBF1 isolate (Table 4.9). The pCS20 sequence from two other samples (sheep 831 and 834) was identical to the Mara87/7 stock while the remaining samples had pCS20 mixtures of the Welgevonden/Mara87/7 isolates, similar to the SBF2 culture obtained before (Chapter 3), or mixtures of Welgevonden/Blaauwkrans pCS20 sequences (Helena Steyn, personal communication).

Table 4.9*: pCS20 sequencing results obtained from brain samples of the animals that died in the field. Sequences were compared to those of known* E. ruminantium *stocks.*

Sample number	pCS20 Sequence homology	Group
817	Welgevonden/Mara87/7	Naive
818	Welgevonden	Naive
822	Blaauwkrans/Mara87/7	Infected and treated
825	Welgevonden	Infected and treated
831	Mara87/7	3x empty pCMViUBs vector
833	Welgevonden/Mara87/7	3x empty pCMViUBs vector
834	Mara87/7	2x vector 1x rβ-galactosidase
836	Welgevonden	2x vector 1x rβ-galactosidase
837	Welgevonden	3x 1H12 cocktail
839	Welgevonden/Mara87/7	3x 1H12 cocktail
841	Welgevonden/Mara87/7	2x 1H12 cocktail, 1x r1H12 protein
842	Welgevonden	2x 1H12 cocktail, 1x r1H12 protein
843	Blaauwkrans/Mara87/7	2x 1H12 cocktail, 1x r1H12 protein
844	Welgevonden	2x 1H12 cocktail, 1x r1H12 protein

The surviving sheep were subsequently challenged in the laboratory with 10 LD_{50}s of the virulent *E. ruminantium* Welgevonden stock. All the animals survived a lethal challenge except for the two challenge control naive sheep and three experimental animals: sheep numbers 815, 840 and 846. Sheep 815 had not shown a temperature reaction in the field, and thus was probably not challenged by tick infestation.

4.4. DISCUSSION

Heterologous prime-boost immunisation was investigated to see if we could improve the protection obtained by our 1H12 DNA vaccination strategy in the field. First, we assessed whether priming with DNA followed by boosting with either 1H12 rLSDV or r1H12 proteins would confer similar protection to virulent *E. ruminantium* needle challenge as was obtained using DNA vaccination alone. Sheep immunised with a DNA prime (two inoculations) followed by a r1H12 protein boost (Montanide ISA50 as adjuvant) showed 100 % protection to needle challenge, while one animal died when boosted with the 1H12 rLSDV vaccine after a DNA prime. This suggests that the recombinant protein boost strategy confers better protection than the rLSDV boost strategy.

Previously we hypothesized that the protection observed for DNA immunisation relies mainly on the induction of a CTL immune response that coincides with the induction of $CD4^+$ T_{H1} specific immunity towards the r1H12 antigens. If the protection observed in animals inoculated by DNA immunisation after needle challenge was based on a CTL response, improvement of a CTL response should correspond with elevated levels of IFN-γ. Thus an increase in IFN-γ production by PBMC would be expected before and after challenge of sheep immunised using the DNA prime-rLSDV boost immunisation strategy. Surprisingly, IFN-γ expression and antigen specific proliferation were completely absent in the group boosted with rLSDV, the strategy described in the literature to best activate and promote CTL responses (Kent *et al.,* 1998; Dégano *et al.,* 2000; Rogers *et al.,* 2001; 2002; Gonzalo *et al.,* 2002; Gómez *et al.,* 2004 to name a few). In fact, the immune response and the survival of animals boosted with the rLSDV correlated with that of sheep immunised with two times DNA alone, suggesting either that the rLSDV did not present the *E. ruminantium* proteins in the host or that the infection dose of the boost was too low. Alternatively, a T_{H2} polarized immune response may have developed instead of the expected cellular immune response.

One of the factors that can influence Th polarisation is the inoculation site of the DNA prime. It has previously been shown that a DNA prime (i.m.) followed by a recombinant fowl pox virus (rFPV) boost failed to induce T_{H1} associated IgG2a

antibodies against HIV-1 in mice, while priming with DNA (i.d.) induced high IgG2a titres after boosting with rFPV (Kent *et al.,* 1998). Moreover, i.d. immunisation of the rFPV induced higher levels of protection against HIV-1 challenge in macaques, suggesting that the epidermal route of inoculation induces stronger cellular immunity (Kent *et al.,* 1998). In our experiment, both the i.m. and gg (i.d.) routes of inoculation were used for the DNA prime but it seems as if the viral boost (given via the i.m. route) did not boost a cellular immune response. In addition, only one out of five sheep immunised by administration of a DNA prime by the i.m. route alone, followed by a recombinant protein boost, survived challenge. Cytokine profiling results indicated that the PBMC of two out of these five sheep showed increased IL-4 production before challenge (Sheep 219 with a 42 fold increase and sheep 224 with a 3516 fold increase) while none of these animals' PBMC showed IFN-γ production. This suggests that priming with DNA via the i.m. route of inoculation primed a humoral immune response.

One could argue that due to the large genome size of rLSDV and other pox viruses (and the subsequent large number of transcribed proteins), the expressed protein of interest (1H12 antigen) would be in strong competition for presentation on MHC class I and II. This could have reduced the number of 1H12 epitopes presented and ultimately led to a weak induction of 1H12-specific immunity. Nonetheless, immunisation with rLSDV, although not successful at this stage, could be improved by further optimisation, including experiments to determine the optimal rLSDV dose and the best inoculation site. For now, these experiments will not be done due to expenses and time constraints. In addition, South African government legislation requires that all animals immunised with rLSDV must be kept in isolation stables until the impact of these genetically altered viruses on the environment can be assessed, making such experiments prohibitively expensive.

The group immunised with 1H12 pCMViUBs DNA construct and boosted with r1H12 protein (with ISA50 as adjuvant) showed 100 % protection, as was obtained for the sheep immunised with the 1H12 DNA vaccine cocktail. The protection observed in the DNA prime-r1H12 protein boost group also corresponded with elevated levels of PBMC IFN-γ production before challenge. While many studies have shown that a

DNA prime-recombinant protein boost immunisation strategy will induce humoral immunity (Haddad *et al.,* 1999; Stambas *et al.,* 2005), cellular immunity was successfully induced using this strategy in several other studies (Nyika *et al.,* 2002; Wang *et al.,* 2004a; Wang *et al.,* 2004b; Rafati *et al.,* 2005). Nyika *et al.* (2002) was the first group to report a DNA prime–recombinant protein boost immunisation strategy for the development of subunit vaccines against heartwater using the major antigenic protein (MAP1) as antigen. These researchers were able to show that a DNA prime-recombinant protein boost, using Quil A as adjuvant, improved survival in mice against a virulent *E. ruminantium* challenge, while immunisation with the recombinant protein alone did not induce protection at all. The observed protection also coincided with elevated levels of IFN-γ in the mice splenocytes. Similar results were also obtained in a study done by Wang *et al*. (2004a): in an attempt to improve vaccine strategies against *Mycobacterium tuberculosis*, a DNA prime-recombinant protein boost using the ESAT6 gene not only improved the protection against lethal *M. tuberculosis* but also improved lymphocyte proliferation and increased IFN-γ production. Both Wang *et al.* and Nyika *et al.* concluded that DNA priming is essential for the enhancement of a T_{H1} immune response after protein boosting.

It is known that IFN-γ expression plays a central role in protective immunity to *E. ruminantium* infection (Totté *et al.,* 1997; 1999). These findings were corroborated in the infected and treated control group which was protected against challenge. Here, an increase in IFN-γ expression was detected in two out of five animals after the initial infection and three out of five animals showed increased IFN-γ after challenge. However, of the experimental groups that were protected against challenge, only the group boosted with recombinant protein showed an increase in PBMC IFN-γ expression before challenge (PBMC of 3/5 sheep), while the PBMC of one animal showed elevated IFN-γ and IL-4 levels that were more IL-4 polarised according to the IFN-γ/IL-4 ratio. After challenge 2/5 the animals in this group showed an increase in IL-4 expression; this could be linked to a humoral boosting process during challenge. The absence of IFN-γ after challenge could be related to the time of PBMC isolation, as it is possible that CTL and memory $CD4^+$ lymphocytes were not circulating when the animals were bled. There was no direct correlation between proliferation and IFN-γ expression except for sheep 229 and 231 in the DNA prime–recombinant

protein group. IFN-γ is a cytokine that is expressed by many cell types including CTL, NK and T_{H1} $CD4^+$ lymphocytes. The cytokine profiling assay measures IFN-γ production, after stimulation with recombinant antigen, from all of these cell types that are present in the PBMC at the time of isolation. In contrast, the lymphocyte proliferation assay measures the expansion of memory $CD4^+$ T cells in response to antigen stimulation, but other cell types can also proliferate, including B cells and $CD8^+$ T cells. It has been shown that purified $CD8^+$ T cells can induce proliferation in response to DCs pulsed with Bacillus Calmette Guerin (BCG) antigen, but 3-10 fold less than that obtained for purified $CD4^+$ T cells stimulated with the same antigen (Hope *et al.,* 2000). It is therefore important to know what cell types induced the observed IFN-γ expression, as this could clarify the lack of correlation between lymphocyte proliferation and PBMC IFN-γ expression.

In all the previous experiments reported here, animals were inoculated by both i.m. and i.d. immunisation routes. For practical immunisation purposes, i.m. immunisation would be much more suitable in rural areas, especially if large numbers of animals/herds are considered. However, we found that immunisation using the i.m. inoculation route alone did not provide protection against *E. ruminantium* needle challenge, while immunisation using both i.m. and gg (i.d.) did. Only one of the sheep immunised with a DNA prime (i.m. only) followed by an r1H12 protein boost survived challenge. In addition, no lymphocyte proliferation or cytokine induction (results not shown) was detected in these animals. These results are inconsistent with work done by De Rose *et al*. (2002), who demonstrated that only i.m. DNA immunisation resulted in an effective T_{H1}-like immune response to *Corynebacterium pseudotuberculosis* in sheep, whereas i.d. immunisation using a gg induced meagre immune responses. This phenomenon was attributed by the researchers to differences in animal dermal tissue: the skin of sheep is much thinner than that of other animals resulting in a regeneration time of less than 3 days. They concluded that immunisation in the skin of sheep will result in total exposure to the antigen for a maximum of 3 days if antigen was expressed by keratinocytes and this would not be sufficient to stimulate an immune response. However, these researchers did not take into account that professional APC are not stationary and are known to circulate to the lymph nodes as soon as they are activated by antigen. This was demonstrated in a

similar study where sheep were inoculated with plasmid DNA expressing green fluorescent protein. DCs were detected in draining lymph nodes within 24h after intradermal inoculation (Watkins *et al.,* 1999). Detectable amounts of protein were observed in the skin and in the lymph nodes persisting for up to two months (Watkins *et al.,* 2005). Even a low level of antigen has been effective in activation of T cells by DCs in lymph nodes (reviewed by Thalhamer *et al*., 2001). Removal of skin at the inoculation site within five hours of i.d. gg inoculation has also not hampered immunity to a specific antigen in mice (Klinman *et al.,* 1998). Bearing these experiments in mind, three days exposure would be more than sufficient for an immune response to develop. The lack of an immune response in the De Rose *et al*. (2002) study may rather be linked to the immunisation site. The sheep flank skin, used in the De Rose study, was covered in wool that was only clipped short. We immunised the sheep in the pinna of the ear, it is the only place where no wool is present and the hair and outer skin layer is easily removable using depilation cream.

The fact that i.m. immunisation alone did not confer protection also suggests that protection against *E. ruminantium* infection relies on activation of skin Langerhans cells, a subset of DCs, and the subsequent presentation of antigen on both MHC class I and II. This will ultimately lead to the activation of memory T cells in the lymph nodes. Immature DCs do not have MHC class II molecules on their surface until the cells are activated to mature by external signals, including CD40 triggering and cytokine stimulation. It has been reported that DCs can be activated to present MHC class II on the cell surface by recognition of CpG motifs by Toll like receptors (TLRs) (reviewed by Pasare and Medzhitov, 2004). In contrast, MHC class I turnover and antigen presentation is maintained in both the activated and non-activated state (reviewed by Rea *et al.,* 2001). This suggests that the direct transfection of DCs by i.d. inoculation using a gg would result in continuous presentation of antigen on MHC class I, and the subsequent activation of CTL. The mechanism of DC maturation and antigen presentation after subcutaneous protein boost can be directed by the co-administration of inflammatory compounds such as adjuvants (reviewed by Rea *et al.*, 2001). Thus boosting with recombinant protein in the presence of adjuvant could induce maturation of the DCs and subsequent presentation of antigen on MHC class II. Furthermore, DCs have the unique capacity to present exogenous antigen on MHC

class I, through a process known as cross-presentation, thus recombinant protein antigen could be presented on both MHC class I and II (reviewed by Heath and Carbone, 1999; Théry and Amigorena, 2001 and by Heath *et al.,* 2004). If this cell type does indeed play a critical role in the observed protection against virulent *E. ruminantium* challenge, the vaccination strategy could be improved by addition of adjuvants that stimulate and recruit DCs to the site of inoculation.

The results obtained from the field trial were inconclusive. Three animals from the naive, non-inoculated group survived field challenge, although two of these animals did show a temperature reaction and early heartwater symptoms, but were able to recover from infection. The same types of results were observed in all the other groups: animals that survived showed a mild reaction and survived. In contrast, the sheep that died developed severe heartwater symptoms including nervous symptoms and had to be euthanased. This suggests that either the animals were not challenged with the same parasite dose or the sheep were infected with different *E. ruminantium* genotypes with varying degrees of virulence. Some sheep could have been infected by a single tick resulting in low parasite numbers enabling the animals' innate immunity to overcome the infection, while other sheep could have been infested with more ticks resulting in high parasitemia and a severe infection. The number of ticks on each animal could not be monitored daily as the animals were feeding in the field. From titration experiments we know that naive animals can survive low doses of Welgevonden needle challenge but succumb to higher doses (Brayton *et al.,* 2003).

Another factor that must be considered is the virulence of the *E. ruminantium* genotype(s) found on Springbokfontein farm. Two sheep immunised with the Welgevonden stock by infection and treatment succumbed to heartwater infection on Springbokfontein farm. Again this could be dose related, but these results may also suggest that the genotype(s) on Springbokfontein farm could be more virulent than the Welgevonden stock. Preliminary results suggest that the pCS20 sequence obtained from some of the animals that were tested correlated with previous findings, i.e. that the genotypes seem to be *E. ruminantium* Welgevonden and Mara87/7 combinations, although the sequences were not identical to those identified in previously characterised SBF stocks. This also corroborates previous findings in our laboratory

that the SBF2 stock, obtained from Springbokfontein about five years before this study was performed, was more virulent than the Welgevonden stock in a preliminary cross-protection study.

Animals in the DNA prime-recombinant protein boost group seemed to have the most severe reaction (only one animal survived), indicating that this heterologous prime-boost immunisation strategy did not confer protection in the field. This was surprising because the cytokine assays suggested that priming and boosting induced elevated production of IFN-γ, and therefore increased protection was expected. Although elevated mRNA levels can only be regarded as an indication of protein expression, preliminary proliferation and ELISPOT results also indicate that the r1H12 proteins induce enhanced lymphocyte proliferation (Erum2550 and 2590) and IFN-γ protein expression (all four ORFs) by PBMC isolated from an animal immune to *E. ruminantium* Welgevonden (Ndavhe Tshikudo, personal communication). Similar results were however obtained in a heartwater inactivated vaccine study and a tuberculosis prime-boost study. Goats immunised with an inactivated vaccine did not produce significant IFN-γ in plasma but were protected against heartwater challenge (Vachiery *et al.,* 2006). In another study, mice were inoculated with BCG and boosted with *M. tuberculosis*-derived recombinant 85A or ESAT-6 protein fused to *Bordetella pertussis* adenylate cyclase (CyaA). CyaA is known to enhance MHC class II presentation of antigen. This strategy resulted in increased lymphocyte proliferative responses and the up-regulation of IFN-γ production, but not in enhanced protection against *M. tuberculosis* challenge (Majlessi *et al.,* 2006). These authors suggested that *M. tuberculosis* could somehow evade the immunological protective effects induced by the ESAT-6 and 85A specific T cells. If a similar mechanism of immune evasion is true in *E. ruminantium* infection, it was only evident in animals challenged by tick infestation and not when sheep were challenged by needle (sheep were protected against homologous and heterologous needle challenge). This implies that tick challenge introduces different factors and mechanisms of immune evasion that can render the increased cellular immunity induced by these four genes ineffective. As mentioned in chapter 3, this evasion could involve immunosuppresion induced by factors in the tick saliva itself or could be due to different expression

profiles between *E. ruminantium* organisms multiplying in the tick and those surviving in the ruminant host.

In conclusion, we could show that using a DNA prime-recombinant protein boost immunisation strategy induced improved cellular immunity, evidenced by increased IFN-γ mRNA production as compared to DNA vaccination alone, and these animals were solidly immune to *E. ruminantium* needle challenge. A DNA prime followed by rLSDV boost did not improve protection (90 % survival). However, the DNA prime-recombinant protein boost immunisation strategy did not confer protection against a field challenge. These results indicated that there is a vast difference between needle and field challenge. Future research will benefit from a controlled tick challenge in the laboratory that is not dependant on a good rainy season, where we know what genotype is transmitted (not a mixture of genotypes as is found in the field), the number of ticks to be fed on each animal can be controlled and the infection rate in the ticks can be monitored.

4.5. REFERENCES

ASPDEN, K., VAN DIJK, A.A., BINGHAM, J., COX, D., PASSMORE, J. and WILLIAMSON, A. 2002. Immunogenicity of a recombinant lumpy skin disease virus (Neethling vaccine strain) expressing the rabies virus glycoprotein in cattle. *Vaccine* **20:** 2693–2701.

BRAYTON, K.A., COLLINS, N.E., VAN STRIJP, F. and ALLSOPP, B.A. 2003. Preparation of *Ehrlichia ruminantium* challenge material for quantifiable and reproducible challenge in mice and sheep. *Veterinary Parasitology* **112:** 63-73.

DE ROSE, R., TENNENT, J., MCWATERS, P., CHAPLIN, P.J., WOOD, P.R., KIMPTON, W., CAHILL, R. and SCHEERLINCK, J.Y. 2002. Efficacy of DNA vaccination by different routes of immunisation in sheep. *Veterinary Immunology and Immunopathology* **90:** 55-63.

DÉGANO, P., SCHNEIDER, J., HANNAN, C.M., GILBERT, S.C. and HILL, A.V.S. 2000. Gene gun intradermal DNA immunisation followed by boosting with modified vaccinia virus Ankara: enhanced $CD8^+$ T cell immunogenicity and protective efficacy in the influenza and malaria models. *Vaccine* **18:** 623-632.

DU PLESSIS, J.L. 1985. A method for determining the *Cowdria ruminantium* infection rate of *Amblyomma hebraeum*: Effects in mice injected with tick homogenates. *Onderstepoort Journal of Veterinary Research* ***52****: 55-61.*

GÓMEZ, C.E., ABAITUA, F., RODRÍGUEZ, D. and ESTEBAN, M. 2004. Efficient $CD8^+$ T cell response to the HIV-env V3 loop epitope from multiple virus isolates by a DNA prime-vaccinia virus boost (rWR and rMVA strains) immunisation regime and enhancement by the cytokine IFN-γ. *Virus Research* **105:** 11-22.

GONZALO, R.M., DEL REAL, G., RODRIGUEZ, J.R., RODRIGUEZ, D., HELJASVAARA, R., LUCAS, P., LARRAGA, V. and ESTEBAN, 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.

GURUNATHAN, S., KLINMAN, D.M. and SEDER, R.A. 2000a. DNA vaccines: immunology, application and optimisation. *Annual Reviews in Immunology* **18:** 927-974.

GURUNATHAN, S., WU, C., FREIDAG, B.L. and SEDER, R.A. 2000b. DNA vaccines: a key for inducing long-term cellular immunity. *Current Opinion in Immunology* **12:** 442-447.

HADDAD, D., LILJEQVIST, S., STÅHL, S., HANSSON, M., PERLMANN, P., AHLBORG, N. and BERZINS, K. 1999. Characterisation of antibody responses to a *Plasmodium falciparum* blood-stage antigen induced by a DNA prime-protein boost immunisation protocol. *Scandinavian Journal of Immunology* **49:** 506-514.

HAMMOND, J.M., JANSEN, E.S., MORRISSY, C.J., GOFF, W.V., MEEHAN, G.C., WILLIAMSON, M.M., LENGHAUS, C., SPROAT, K.W., ANDREW, M.E., COUPAR, B.E.H. and JOHNSON, M.A. 2001. A prime-boost vaccination strategy using naked DNA followed by recombinant porcine adenovirus protects pigs from classical swine fever. *Veterinary Microbiology* **80:** 101-119.

HEATH, W.R. and CARBONE, F.R. 1999. Cytotoxic T lymphocyte activation by cross-priming. *Current Opinion in Immunology* **11:** 314-318.

HEATH, W.R., BELZ, G.T., BEHRENS, G.M.N., SMITH, C.M., FOREHAN, S.P., PARISH, I.A., DAVEY, G.M., WILSON, N.S., CARBONE, F.R. and VILLADANGOS, J.A. 2004. Cross-presentation, dendritic cell subsets, and the generation of immunity to cellular antigens. *Immunological Reviews* **199:** 9-26.

HOPE, J.C., KWONG, L.S., SOPP, P., COLLINS, R.A. and HOWARD, C.J. 2000. Dendritic cells induce $CD4^+$ and $CD8^+$ T-cell responses to *Mycobacterium bovis* and *M. avium* antigens in Bacille Calmette Guérin vaccinated and nonvaccinated cattle. *Scandinavian Journal of Immunology* **52**: 285-291.

KENT, S.J., ZHAO, A., BEST, S.J., CHANDLER, J.D., BOYLE, D.B. and RAMSHAW, I.A. 1998. Enhanced T-cell immunogenisity and protective efficacy of a human immunodeficiency virus type-1 vaccine regimen consisting of consecutive priming with DNA and boosting with recombinant Fowlpox virus. *Journal of Virology* **72:** 10180-10188.

KITCHING, R.P., HAMMOND, J.M. and TAYLOR, W.P. 1987. A single vaccine for the control of capripox infection in sheep and goats. *Research in Veterinary Sciences* **42:** 53-60.

KLINMAN, D.M., SECHLER, J.M.G., CONOVER, J., GU, M. and ROSENBERG, A.S. 1998. Contribution of cells at the site of DNA vaccination to the generation of antigen-specific immunity and memory. *The Journal of Immunology* **160:** 2388-2392.

LIVAK, K.J. and SCHMITTGEN, T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods* **25:** 402-408.

MAHAJAN, S.D., SCHWARTZ, S.A. and NAIR, M.P.N. 2003. Immunological assays for chemokine detection in *in-vitro* culture of CNS cells. *Biological Procedures Online* **5:** 90-102.

MAJLESSI, L., SIMSOVA, M., JARVIS, Z., BRODIN, P., ROJAS, M.J, BAUCHE, C., NOUZÉ, C., LADANT, D., COLE, S.T., SEBO, P. and LECLERC, C. 2006. An increase in antimycobacterial T_{H1}-cell responses by prime-boost protocols of immunisation does not enhance protection against tuberculosis. *Infection and Immunity* **74:** 2128-2137.

MCSHANE, H., BEHBOUDI, S., GOONETILLEKE, N., BROOKES, R. and HILL, A.V.S. 2002. Protective immunity against *Mycobacterium tuberculosis* induced by dendritic cells pulsed with both $CD8^+$- and $CD4^+$-T-cell epitopes from antigen 85A. *Infection and Immunity* **70:** 1623-1626.

MENA, A., IONNOU, X.P., VAN KESSEL, A., VAN DRUNEN LITTLE-VAN DEN HURK, S., POPWYCH, Y., BABIUK, L.A. and GODSON, D.L. 2002. T_{H1}/T_{H2} biasing effects of vaccination in cattle as determined by real-time PCR. *Journal of Immunological Methods* **263:** 11-21.

NYIKA, A., BARBET, A.F., BURRIDGE, M.J. and MAHAN, S.M. 2002. DNA vaccination with the *map*1 gene followed by protein boost augments protection against challenge with *Cowdria ruminantium*, the agent of heartwater. *Vaccine* **20:** 1215-1225.

PASARE, C. and MEDZHITOV, R. 2004. Toll-like receptors: linking the innate and adaptive immunity. *Microbes and Infection* **6:** 1382-1387.

RAFATI, S., NAKHAEE, A., TAHERI, T., TASLIMI, Y., DARABI, H., ERAVANI, D., SANOS, S., KAYE, P., TAGHIKHANI, M., JAMSHIDI, S. and RAD, M.A. 2005. Protective vaccination against experimental canine visceral leishmaniasis using a combination of DNA and protein immunisation with cysteine proteinases type I and II of *L. infantum*. *Vaccine* **23:** 3716-3725.

REA, D., JOHNSON, M.E., HAVENGA, M.J.E., MELIEF, C.J.M. and OFFRINGA, R. 2001. Strategies for improved antigen delivery into dendritic cells. *TRENDS in Molecular Medicine* **7:** 91-94.

ROGERS, W.O., BAIRD, J.K., KUMAR, A., TINE, J.A., WEISS, W., AGUIAR, J.C., GOWDA, K., GWADZ, R., KUMAR, S., GOLD, M. and HOFMAN, S.L. 2001. Multistage multiantigen heterologous prime-boost vaccine for *Plasmodium knowlesi* malaria provides partial protection in rhesus macaques. *Infection and Immunity* **69:** 5565-5572.

ROGERS, W.O., WEISS, W.R., KUMAR, A., AGUIAR, J.C., TINE, J.A., GWADZ, R., HARRE, J.G., GOWDA, K., RATHORE, D., KUMAR, S. and HOFFMAN, S.L. 2002. Protection of rhesus

macaques against lethal *Plasmodium knowlesi* malaria by a heterologous DNA priming and poxvirus boosting immunisation regimen. *Infection and Immunity* **70:** 4329-4335.

SCHNEIDER, J., LANGERMANS, J.A.M., GILBERT, S.C., BLANCHARD, T.J., TWIGG, S., NAITZA, S., HANNAN, C.M., AIDOO, M., CRISANTI, A., ROBSON, K.J., SMITH, G.L., HILL, A.V.S. and THOMAS, A.W. 2001. A prime-boost immunisation regimen using DNA followed by recombinant modified vaccinia virus Ankara induces strong cellular immune responses against the *Plasmodium falciparum* TRAP antigen in chimpanzees. *Vaccine* **19:** 4595-4602.

SEDEGAH, M., JONES, T.R., KAUR, M., HEDSTORM, R., HOBART, P., TINES, J.A. and HOFFMAN, S.L. 1998. Boosting with recombinant vaccinia increases immunogenicity and protective efficacy of malaria DNA vaccine. *Proceedings of the National Academy of Sciences of the United States of America* **95:** 7648-7653.

SEDEGAH, M., BRICE, G.T., ROGERS, W.O., DOOLAN, D.L., CHAROENVIT, Y., JONES, T.R., MAJAM, V.F., BELMONTE, A., LU, M., BELMONTE, M., CARUCCI, D.J. and HOFFMAN, S.L. 2002. Persistence of protective immunity to malaria induced by DNA priming and poxvirus boosting: Characterisation of effector and memory $CD8^+$-T-cell populations. *Infection and Immunity* **70:** 3493-3499.

SHALABY, K.A., YIN, L., THAKUR, A., CHRISTEN, L., NILES, E.G. and LOVERDE, P.T. 2003. Protection against *Schistosoma mansoni* utilising DNA vaccination with genes encoding Cu/Zn cytosolic superoxide dismutase, signal peptide-containing superoxide dismutase and glutathione peroxidase enzymes. *Vaccine* **22:** *130-136.*

STADEN, R., BEAL, K.F. and BONFIELD, J.K. 1998. The Staden Package. In: Computer Methods in Molecular Biology. Edited by MISENER, S. and KRAWETZ, S.A. The Humana Press Inc., Totowa, NJ 07512. *Bioinformatics Methods and Protocols* **132:** 115-130.

STAMBAS, J., BROWN, S.A., GUTIERREZ, A., SEALY, R., YUE, W., JONES, B., LOCKEY, T.D., ZIRKEL, A., FREIDEN, P., BROWN, B., SURMAN, S., COLECLOUGH, C., SLOBOD, K.S., DOHERTY, P.C. and HURWITZ, J.L. 2005. Long lived multi-isotype anti-HIV antibody responses following a prime-double boost immunisation strategy. *Vaccine* **23:** 2454-2464.

THALHAMER, J., LEITNER, W., HAMMERL, P. and BRTKO, J. 2001. Designing immune responses with genetic immunisation and immunostimulatory DNA sequences. *Endocrine Regulations* **35:** 143-166.

THÉRY, C. and AMIGORENA, S. 2001. The cell biology of antigen presentation in dendritic cells. *Current Opinion in Immunology* **13:** 45-51.

THOMPSON, J.D., GIBSON, T.J., PLEWNIAK, F., JEANMOUGIN, F. and HIGGINS, D.G. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, **25:** 4876-4882.

TOTTÉ, P., MCKEEVER, D., MARTINEZ, D. and 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. and MCKEEVER, D.J. 1999. Immune responses to *Cowdria ruminantium* infections. *Parasitology Today* **15:** 286-290.

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

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

WANG, R., EPSTEIN, J., CHAROENVIT, Y., BARACEROS, F.M., RAHARDJO, N., GAY, T., BANANIA, J., CHATTOPADHYAY, R., DE LA VEGA, P., RICHIE, T.L., TORNIEPORTH, N., DOOLAN, D.L., KESTER, K.E., HEPPNER, D.G., NORMAN J., CARUCCI, D.J., COHEN, J.D. and HOFFMAN, S.L. 2004b. Induction in humans of $CD8^+$ and $CD4^+$ T cell and antibody responses by sequential immunisation with malaria DNA and recombinant protein. *The Journal of Immunology* **172:** 5561-5569.

WATKINS, C., LAU, S., THISTLETHWAITE, R., HOPKINS, J. and HARKISS, G.D. 1999. Analysis of reporter gene expression in ovine dermis and afferent lymph dendritic cells *in vitro* and *in vivo*. *Veterinary Immunology and Immunopathology* **72:** 125-133.

WATKINS, C., HOPKINS, J. and HARKISS, G. 2005. Reporter gene expression in dendritic cells after gene gun administration of plasmid DNA. *Vaccine* **23:** 4247-4256.

WHERRY, E.J., BLATTMAN, J.N. and AHMED, R. 2005. Low $CD8^+$ T-cell proliferative potential and high viral load limit the effectiveness of therapeutic vaccination. *Journal of Virology* **79:** 8960-8968.

WOODLAND, D.L. 2004. Jump-starting the immune system: prime-boosting comes of age. *TRENDS in Immunology* **25:** 98-104.

5. CHAPTER 5: CONCLUDING DISCUSSION

In the search for a better vaccine against *Ehrlichia ruminantium* infection in ruminants, four *E. ruminantium* open reading frames (ORFs) derived from the Welgevonden isolate were tested in the form of a DNA vaccine. Two of these ORFs showed homology to the ATP-binding protein part of ABC transporters, one was a possible periplasmic SBP and the fourth ORF showed characteristics of an exported protein. These ORFs were initially tested in mice and were found to be partially protective using a DNA vaccination strategy (Collins *et al.,* 2003a; 2003b). In this study, DNA vaccination using a cocktail of the four 1H12 ORFs was found to be totally protective in sheep against *E. ruminantium* needle challenge. This indicated that the mouse model could not be used to predict the protection observed in sheep, the ruminant model used in this study. In our laboratory the mouse model has subsequently become redundant. Complete protection against virulent *E. ruminantium* Welgevonden needle challenge was also observed when sheep were immunised with each individual ORF. Furthermore, we could show that the 1H12 DNA vaccine could induce protection against heterologous needle challenge when animals were immunised with the Welgevonden-derived 1H12 ORFs and challenged with other virulent *E. ruminantium* stocks.

Even though we identified four genes that showed protection against homologous and heterologous *E. ruminantium* needle challenge when inoculated as single genes or as part of a cocktail, the main problem with our pilot DNA vaccine was that it did not protect against tick challenge in the field. Interestingly, animals that survived the vaccine trial (i.e. had been immunised with the DNA vaccine and needle challenged with *E. ruminantium* Welgevonden), were able to survive a field challenge, and this led to our next hypothesis: Immunisation of animals using a DNA prime followed by an *E. ruminantium* recombinant protein or modified live viral boost could improve the protection observed in the field. To test this hypothesis we used a prime-boost immunisation strategy followed by needle and natural field challenge. The results indicated that animals immunised with a two times DNA prime followed by a rLSDV boost showed 90 % protection against a needle challenge with *E. ruminantium* Welgevonden, while boosting with r1H12 protein conferred 100 % protection, as was

found with the 3x DNA immunisation strategy. An increase in IFN-γ expression in 90 % of the sheep PBMC tested by real-time PCR was found only in the group immunised twice with DNA followed by a recombinant protein boost. Interestingly, although this prime-boost immunisation strategy enhanced IFN-γ mRNA expression, and presumably cellular immunity in the immunised animals, the vaccine did not improve survival when the sheep were challenged in the field. This failed to support our hypothesis that enhanced cellular immunity would provide increased protection in the field. As mentioned in chapter 4, similar results were obtained in a *Mycobacterium tuberculosis* study where enhanced cellular immunity in immunised animals did not induce protection against a lethal challenge (Majlessi *et al.,* 2006). Our results suggest that there is a vast difference between needle challenge and natural tick infestation and that *E. ruminantium* organisms transmitted by ticks have the ability to evade the protective immunity induced by immunisation with the four 1H12 ORFs. As discussed in chapter 3, this difference could be related to the challenge dose or to genotypes with varying levels of virulence introduced by tick challenge, but most importantly this difference in survival could be due to immunological modifications introduced by the tick or phenotypical modifications of the organism secreted by the tick host.

It has been shown recently that mice that lack some of the elements required for the induction of innate immunity were much more susceptible to *Borrelia burgdorferi* transmitted by ticks than normal mice (Bockenstedt *et al.,* 2006). This emphasises the important role that the innate immune response has in activating a protective immune response to invading pathogens. The biggest challenge for our vaccines, then, is that they should be able to induce an immune response strong enough to overcome any immunomodulation produced by the tick itself. This might be achieved by enhancing the innate immune response, optimising the vaccination regime and the addition of appropriate genetic adjuvants.

It has been postulated that activation of microbe-specific T cells requires the initiation of the innate immune response (reviewed by Doherty and Arditi, 2004). Thus the immune response has to be convinced that the presented antigen is of microbial origin before the adaptive immune response will react. Once *E. ruminantium* infects a ruminant host the first line of host defence will be the activation of the innate immune

response via Toll-like receptor (TLR) activation. Innate immunity is activated once antigen of bacterial origin is recognised by lymphocytes via TLR interaction. Binding of bacterial antigens, also known as pathogen-associated microbial patterns (PAMPs), to TLR leads to the secretion of several cytokines, including TNF-α (a cytokine known to suppress auto immunity), IL-6, IL-12 and IFNs. PAMPs include HSP70, LPS, single and double stranded RNA (of viral origin) and bacterial DNA. Bacterial DNA is known to contain specific unmethylated cytosine-phosphate-guanosine sequences, known as CpG motifs, and these have been shown to activate IL-12 by binding to TLR9. In this way the host immune system is alerted to pathogen invasion and can act accordingly (Zhang *et al*., 2004 and reviewed by Gurunathan *et al.,* 2000a; 2000b; Kirman and Seder, 2003; Wagner, 2002; Kadowaki and Liu, 2002). Moreover, it has been shown that this activation of TLRs primarily controls the subsequent induction of Th1 immune responses. TLR induction can take place on the surface of the host cells or after phagocytosis, i.e. in the cytoplasm of the host cells (reviewed by Doherty and Arditi, 2004). This will ultimately contribute to the maturity of T_{H1} memory lymphocytes that will form part of the adaptive immune response (reviewed by Pasare and Medzhitov, 2004). Secondary infection will induce the deployment of adaptive immune defences, an immune response that will specifically target and destroy a specific pathogen. Therefore an effective immune response to *E. ruminantium* will require successful interactions between the innate and adaptive immune responses. When animals are immunised with DNA vaccines this interaction between innate and adaptive immune responses will have to be mimicked in order to activate host immunity strong enough to overcome infection.

We attempted to activate the innate immune response by induction of TLR9 via binding with CpG motifs in the case of DNA vaccination, or using ISA50 adjuvant when animals were boosted with recombinant protein, or by boosting with modified live virus. Adjuvants contain LPS and other bacterial compounds that will activate immune responses by TLR interaction. Thus combining DNA immunisation with a recombinant protein boost in adjuvant can lead to the induction of strong innate and adaptive immune responses. The non-specific activation of the innate immune response could perhaps explain why stronger IFN-γ production could be detected in animals immunised twice with DNA followed by a recombinant protein boost in adjuvant.

Several TLRs (TLR3, TLR7, TLR8 and TLR9) are known to participate in the recognition of viruses and the subsequent activation of protective immunity. Therefore boosting with a live attenuated organism such as rLSDV should induce the innate immune response. Surprisingly, enhanced cellular immunity was not detected in animals immunised with DNA followed by a rLSDV boost and this immunisation strategy only protected 90 % of the animals after needle challenge. In fact the protective result and cytokine profile were very similar to those seen in the 2x DNA only control group. This led us to the conclusion that perhaps the virus did not replicate in the host or that humoral immunity was activated instead of cellular immunity.

The lack of cytokine stimulation and the reduced protective immunity that we observed might be avoided by optimising the dose concentration and vaccination regime of the rLSDV boost, although it may be better to investigate the use of a different viral vector altogether. Investigation of poxviruses and their immunomodulation methods reveals a very complex picture that has to be considered before choosing a viral vector as a vaccine delivery system. There are many different poxviruses, with highly diverse genome sequences, all of which have evolved mechanisms to evade host immune responses: some have the ability to express multiple copies of cytokine receptors, others express proteins that can down regulate the expression of surface CD4 molecules, and the expression of MHC class I has also been shown to be reduced. All three of these mechanisms are crucial for protection against intracellular bacteria such as *E. ruminantium*. It has been shown that MHC class I presentation is extensively reduced in some poxvirus infections such as Myxoma virus (MV) while viruses such as vaccinia virus only down regulate MHC class I moderately. This down regulation is mediated by a protein, identified in the genome of the MV virus, known as MV-LAP. This protein can also down regulate CD4 presentation on lymphocytes (reviewed by Johnston and Mcfadden, 2003). Interestingly, a homologue for this protein was found in the LSDV genome (Früh *et al.,* 2002). Taking all this into account one can argue that if a functional homologue for this MV-LAP protein does exist in LSDV, the cytotoxic T cell immune pathway may not be activated using this virus as a vaccine delivery system. This could explain the lack of detection of cellular immunity in the group boosted

with rLSDV. It may be possible to circumvent this down regulatory property of LSDV, by mutating or removing the MV-LAP homologue from the attenuated LSDV vaccine strain to produce a virus that will not down regulate MHC class I presentation. Alternatively, a different pox virus strain that does not contain MV-LAP, such as vaccinia, could be used. Furthermore, some of the pox viruses express an IFN-γ receptor molecule that binds to and inactivates the function of IFN-γ (reviewed by Johnston and Mcfadden, 2003). If these mechanisms exist in LSDV, it is possible that LSDV was not the ideal viral vector to use to activate cellular immunity but is rather a viral vector that could be used to induce humoral immunity, as has been shown by research done in vaccines to rabies (Aspden *et al.,* 2002).

Future immunisation experiments may therefore be more successful if a different viral vector is used. However, the use of live modified organisms has several drawbacks, including the possibility of reversion to virulence. This would have grave implications, especially to cattle which are the natural host of LSDV. Another concern that has to be addressed is the growing public fear of using genetically modified organisms and the impact of these on the natural environment. The addition of foreign genes with unknown functions to viral vectors might have catastrophic results, virulence might be increased or unexpected viral mutations might occur resulting in more severe disease. Therefore, further studies, especially those that evaluate potential environmental impact, would have to be incorporated before viral vectors could be used commercially.

Although we could show that the four 1H12 ORFs could induce a protective immune response against *E. ruminantium* needle challenge, the final formulation of the vaccine still has to be improved in order to induce protection against real life tick challenge. Not only do we require extensive tick challenge trials to test the prime-boost immunisation strategy, but further optimisation of the vaccination regime is necessary. This will include the manner by which the DNA is to be administered, the amount of plasmid DNA and protein to be inoculated, the number of booster doses to be given, and the optimisation of the time interval between boosters. It has recently been found that the time interval between boosters is crucial for the induction of an effective CTL response, since a boost will be effective only if the host has acquired efficient memory CTLs (reviewed by Wherry and Ahmed, 2004). This can only be

optimised if we can identify the cell types that are activated at different time points after the initial prime, which we may be able to do using flow cytometry. If CTLs do play a major role in the observed protection, then we will have to optimise the booster time points in future.

Another factor that can play a role in the induction of an efficient immune response is the plasmid dose. It has been shown that human and primate immunisation requires a higher DNA dose to induce CTL immunity than that used in mice (reviewed by Johnston *et al.,* 2002). Immunising with the individual ORFs was done using four times higher concentrations of plasmid DNA (i.e. 200 µg of each pCMViUBs_ORF) to immunise the animals than was used in the cocktail (mixture of 50 µg of each pCMViUBs_ORF). The PBMC isolated from sheep inoculated with the individual constructs (pCMViUBs_Erum2540, pCMViUBs_Erum2580 and pCMViUBs_Erum2590) showed higher SI than animals inoculated with the pCMViUBs_ORF cocktail. Therefore we believe that the immunity induced by individual inoculation of each of the four 1H12 ORFs was enhanced by increased plasmid DNA dosage. Human clinical trials revealed that substantial CTL responses could only be induced when relatively high DNA doses were used (reviewed by Donnelly *et al.,* 2003). It has also been shown that there is a correlation between the level of epitope expression and the number of memory CTLs specific for that epitope (Wherry *et al.,* 2002). In general higher concentrations of an epitope or antigen will result in a bigger memory CTL pool, but if the epitope concentration is too high (or too low) the CTL immune response can be negatively affected (Wherry *et al.,* 2002). Perhaps increasing the amount of DNA used in the cocktail would augment the CTL memory immune response and might result in higher survival rates in the field, but this would have to be carefully optimised.

The effectiveness of the vaccine could be further enhanced by optimisation of the DNA prime because the more accurately specific cells are targeted in the initial prime, the stronger the immunological outcome will be after the protein boost. Several techniques can be used to achieve this, including changing the number of CpG motifs on the vector backbone (Coban *et al.,* 2005), co-immunisation with plasmids containing genetic adjuvants, or co-immunisation with plasmids that will induce both CTL and $CD4^+$ T cells. As mentioned before, unmethylated CpG motifs have been

shown to have potent adjuvant properties and are known to activate host immunity via TLR9 (reviewed by Pasare and Medzhitov *et al.,* 2004). Addition of CpG oligonucleotides (ODN) or the CpG-modification of plasmids has been shown to induce increased protection and improved T_{H1} immunity to pathogens, including *Leishmania donovani, Burkholderia pseudomallei* and *Plasmodium falciparum* (Tewary *et al.,* 2004; Coban *et al.,* 2005; Chen *et al.,* 2006). Recently it has been shown that suppressive ODN motifs exist (poly-G or GC sequences) (Yamada *et al.,* 2004). When we examined the 1H12 *E. ruminantium* ORFs, some suppressive motifs could be identified (results not shown). This could indicate that the host immune response might be down regulated after immunisation with these DNA constructs unless there are enough CpG motifs on the vector backbone to balance out the suppressive effect.

The co-delivery of genetic adjuvants that contain immunogens and cytokines could be used to enhance the protective immunity we have observed in this study. Early T_{H1} activation and expansion is mediated by several cytokines, including IL-12, -18, -23 and -27. IL-12, -23 and -27 are heterodimeric proteins derived from homologous protein monomers: IL-12 contains a p40 and a p35 subunit, IL-23 consists of the IL-12 p40 and a p35-related protein known as p19, while IL-27 consists of two subunits closely related to those of IL-12 (Brombacher *et al.,* 2003). All of these cytokines have been tested for their ability to enhance cellular immunity. A decrease in Th2 antibody production was observed when a plasmid expressing IL-18 was co–delivered with the DNA inoculations in a prime–boost immunisation strategy using the *Nef* HIV gene as antigen (Billaut-Mulot *et al.,* 2001). The co-delivery of an IL-18 expression plasmid enhanced IFN-γ production and protection by a DNA vaccine directed to mouse herpes simplex virus (HSV-1) (Zhu *et al.,* 2003). In two similar studies done by Triccas *et al*. (2002) and Wozniak *et al.* (2006), protective immunity against *Mycobacterium tuberculosis* (MTB) was increased by co-immunisation with plasmids expressing IL-12, IL-18, IL-23 or IL-27. Co-delivery of IL-12, IL-18 and IL-23 cytokines resulted in an increased lymphocyte proliferation response directed to a specific MTB antigen (Ag85B) and in the up regulation of IFN-γ production, whereas the effects of IL-27, a cytokine known to activate early T_{H1} responses, were insignificant. The efficiency of a genetic vaccine containing the *gag/pol* and *env* genes of feline leukaemia virus (FeLV) was enhanced when kittens were also

immunised with IL-12 and IL-18 genetic vaccines. In contrast, increased viremia was observed when kittens were co-immunised with only the IL-12 genetic adjuvant (O'Donovan *et al.,* 2005).

We found that 100 % protection against virulent *E. ruminantium* needle challenge could be reproducibly induced in sheep immunised using both i.m. and i.d. routes of inoculation, while sheep immunised using the i.m. route only were not protected. Therefore we believe that the activation of dendritic cells in the skin is perhaps one of the most important events in the induction of a successful immune response using the four 1H12 ORFs. The inclusion of adjuvants in the vaccination regime, to recruit dendritic cells and activate them at the site of inoculation, could enhance the immune response. It has been shown that the inoculation of animals with plasmids that express GM-CSF three days before immunisation with antigen-expressing plasmids enhanced immunity to HIV and papilloma virus (Kusakabe *et al.,* 2000; Hu *et al.,* 2004). As an alternative to recruiting dendritic cells to the site of inoculation, animals could be inoculated with such cells stimulated with *E. ruminantium* antigen *in vitro.* This procedure was shown to stimulate strong memory CTL immunity when dendritic cells primed with *Listeria monocytogenes* antigen were used to immunise mice (Badovinac *et al.,* 2005). It should be noted that the addition of cytokines as adjuvants does not enhance protection in all cases, and an increase in mortality has been reported (Johnston *et al.,* 2002). Therefore different antigen – cytokine combinations will have to be tested to optimise the procedure.

The vector that we used here, as mentioned previously, was designed to target the expression products, by means of ubiquitin fusion, towards the proteosome for MHC class I processing and activation of CTL. It is known that $CD4^+$ T cells are required to induce memory CTLs (mCTLs) as mentioned in chapter 1, but are not necessary for effector CTL responses (Bennett *et al.,* 1997; Behrens *et al.,* 2004 and reviewed by Keene and Forman, 1982 and by Heath and Carbone, 1999). Therefore, a successful vaccination strategy against *E. ruminantium* will require mCTLs directed against the 1H12 antigens and this necessitates the activation and recruitment of 1H12 specific $CD4^+$ T cells. Activation of $CD4^+$ T cells requires the availability of secreted protein that can be taken up by professional antigen presenting cells (APC). Using our vaccination strategy, MHC class II presentation would only be possible if some of

the 1H12 recombinant proteins were intact, i.e. were not degraded by the proteosome as directed by the ubiquitin fusion. These intact proteins would also need to be secreted by the transfected host cells for MHC class II presentation. Perhaps, when using the current expression vector, not enough intact proteins were available for MHC class II presentation. If this was the case, protection might be increased if we used expression vectors containing a secretory leader sequence, that would allow recombinant protein export and ultimately 1H12-specific $CD4^+$ T cell activation via MHC class II presentation. In the prime-boost experiment the inoculated recombinant protein would have been processed via APC uptake and could stimulate both MHC class I and II processing via cross-priming, thus both T helper cells and CTL could be activated. However a much stronger immune response would develop if the T_{H1} $CD4^+$ T cells were successfully primed by DNA immunisation.

In conclusion, we have identified four ORFs, derived from the Welgevonden stock of *E. ruminantium,* which showed great promise as recombinant vaccine candidates. Although these four ORFs were found not to be protective in the field, this lack of protection may rather be a reflection of the vaccine formulation than an intrinsic lack of ability of their expression products to induce a sufficiently protective immune response.

5.1 REFERENCES

ASPDEN, K., VAN DIJK, A.A., BINGHAM, J., COX, D., PASSMORE, J. and WILLIAMSON, A. 2002. Immunogenicity of a recombinant lumpy skin disease virus (Neethling vaccine strain) expressing the rabies virus glycoprotein in cattle. *Vaccine* **20:** 2693–2701.

BADOVINAC, V.P., MESSINGHAM, K.A.N., JABBARI, A., HARING, J.S. and HARTY, J.T. 2005. Accelerated $CD8^+$ T-cell memory and prime-boost response after dendritic-cell activation. *Nature Medicine* **11:** 748-756.

BEHRENS, G.M.N., LI, M., DAVEY, G.M., ALLISON, J., FLAVELL, R.A., CARBONE, F.A. and HEATH, W.R. 2004. Helper requirements for generation of effector CTL to islet B cell antigens. *The Journal of Immunology* **172:** 5420-5426.

BENNETT, S.R.M., CARBONE, F.R., KARAMALIS, F., MILLER, J.F.A.P. and HEATH, W.R. 1997. Induction of a $CD8^+$ cytotoxic T lymphocyte response by cross-priming requires cognate $CD4^+$ T cell help. *Journal of Experimental Medicine* **186:** 65-70.

BILLAUT-MULOT, O., IDZIOREK, T., BAN, E., KREMER, L., DUPRÉ, L., LOYENS, M., RIVEAU, G., LOCHT, C., CAPRON, A., and BAHR, G.M. 2001. Interleukin-18 modulates immune responses induced by HIV-1 Nef DNA prime/protein boost vaccine. *Vaccine* **19:** 95-102.

BOCKENSTEDT, L.K., LIU, N., SCHWARTZ, I. and FISH, D. 2006. MyD88 deficiency enhances acquisition and transmission of *Borrelia burgdoferi* by *Ixodes scapularis* ticks. *Infection and Immunity* **74:** 2154-2160.

BROMBACHER, F., KASTELEIN, R.A. and ALBER, G. 2003. Novel IL-12 family members shed light on the orchestration of T_{H1} responses. *Trends in Immunology* **24:** 207-212.

CHEN, Y., HSIAO, Y., LIN, H., LIU, Y. and CHEN, Y. 2006. CpG-modified plasmid DNA encoding flagellin improves immunogenicity and provides protection against *Burkholderia pseudomallei* infection in BALB/C mice. *Infection and Immunity* **74:** 1699-1705.

COBAN, C., ISHII, K.J., GURSEL, M., KLINMANN, D.M. and KUMAR, N. 2005. Effect of plasmid backbone modification by different human CpG motifs on the immunogenicity of DNA vaccine vectors. *Journal of leukocyte biology* **78:** 647-655.

COLLINS, N.E., PRETORIUS, A., VAN KLEEF, M., BRAYTON, K.A., ALLSOPP, M.T., ZWEYGARTH, E. and ALLSOPP, B.A. 2003a. Development of improved attenuated and

nucleic acid vaccines for heartwater. In: Vaccines for OIE list A and Emerging Animal Diseases. Edited by BROWN, F. and ROTH, J. *Developments in Biologicals Basel Karger* **114:** 121-136.

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

DOHERTY, T.M. and ARDITI, M. 2004. TB, or not TB: that is the question- does TLR signalling hold the answer? *The Journal of Clinical Investigation* **114:** 1699-1703.

DONNELLY, J., BERRY, K., and ULMER, J.B. 2003. Technical and regulatory hurdles for DNA vaccines. *International Journal for Parasitology* **33:** 457-467.

FRÜH, K., BARTEE, E., GOUVEIA, K. and MANSOURI, M. 2002. Immune evasion by a novel family of viral PHD/LAP-finger proteins of gamma-2 herpes viruses and poxviruses. *Virus Research* **88:** 55-69.

GURUNATHAN, S., KLINMAN, D.M. and SEDER, R.A. 2000a. DNA vaccines: immunology, application and optimisation. *Annual Reviews in Immunology* **18:** 927-974.

GURUNATHAN, S., WU, C., FREIDAG, B.L. and SEDER, R.A. 2000b. DNA vaccines: a key for inducing long-term cellular immunity. *Current Opinion in Immunology* **12:** 442-447.

HEATH, W.R. and CARBONE, F.R. 1999. Cytotoxic T lymphocyte activation by cross-priming. *Current Opinion in Immunology* **11:** 314-318.

HU, J., CLADEL, N.M., WANG, Z., HAN, R., PICKEL, M.D. and CHRISTENSEN, N.D. 2004. GM-CSF enhances protective immunity to cottontail rabbit papillomavirus E8 genetic vaccination in rabbits. *Vaccine* **22:** 1124-1130.

JOHNSTON, J.B. AND MCFADDEN, G. 2003. Minireview: Poxvirus immunomodulatory strategies: Current perspectives. *Journal of Virology*, **77:** 6093-6100.

JOHNSTON, S.A., TALAAT, A.M. and MCGUIRE, M.J. 2002. Genetic immunisation: What's in a Name? *Archives of Medical Research* **33:** 325-329.

KADOWAKI, N. and LIU, Y. 2002. Natural type I interferon-producing cells as a link between innate and adaptive immunity. *Human Immunology* **63:** 1126-1132.

KEENE, J. and FORMAN, J. 1982. Helper activity is required for the *in vivo* generation of cytotoxic T lymphocytes. *Journal of Experimental Medicine* **155:** 768-782.

KIRMAN, J.R. and SEDER, R.A. 2003. DNA vaccination: the answer to stable, protective T-cell memory? *Current Opinion in Immunology* **15:** 471-476.

KUSAKABE, K., XIN, K., KATOH, H., SUMINO, K., HAGIWARA, E., KAWAMOTO, S., OKUDA, K., MIYAGI, Y., AOKI, I., NISHIOKA, K., KLINMAN, D. and OKUDA, K. 2000. The timing of CM-CSF expression plasmid administration influences the T_{H1}/ T_{H2} response induced by an HIV-1-specific DNA vaccine. *Journal of Immunology* **164:** 3102-3111.

MAJLESSI, L., SIMSOVA, M., JARVIS, Z., BRODIN, P., ROJAS, M., BAUCHE, C., NOUZÉ, C., LADANT, D., COLE, S.T., SEBO, P. and LECLERC, C. 2006. An increase in antimycobacterial T_{H1}-cell responses by prime-boost protocols of immunisation does not enhance protection against tuberculosis. *Infection and Immunity* **74:** 2128-2137.

O'DONOVAN, L.H., MCMONAGLE, E.L., TAYLOR, S., BAIN, D., PACITTI, A.M., GOLDER, M.C., MCDONALD, M., HANLON, L., ONIONS, D.E., ARGYLE, D.J., JARRETT, O. and NICOLSON, L. 2005. A vector expressing feline mature IL-18 fused to IL-1β antagonist protein signal sequence is an effective adjuvant to a DNA vaccine for feline leukaemia virus. *Vaccine* **23:** 3814-3823.

PASARE, C. and MEDZHITOV, R. 2004. Toll-like receptors: linking the innate and adaptive immunity. *Microbes and Infection* **6:** 1382-1387.

TEWARY, P., SUKUMARAN, B., SAXENA, S. and MADHUBALA, R. 2004. Immunostimulatory oligodeoxynucleotides are potent enhancers of protective immunity in mice immunised with recombinant ORFF leishmanial antigen. *Vaccine* **22:** 3053-3060.

TRICCAS, J.A., SUN, L., PALENDIRA, U. and BRITTON, W.J. 2002. Comparative affects of plasmid-encoded interleukin 12 and interleukin 18 on the protective efficacy of DNA vaccination against *Mycobacterium tuberculosis*. *Immunology and Cell Biology* **80:** 346-350.

WAGNER H. 2002. Interactions between bacterial CpG-DNA and TRL9 bridge innate and adaptive immunity. *Current Opinion in Microbiology* **5:** 62-69.

WHERRY, E.J., MCELHAUGH, M.J. and EISENLOHR, L.C. 2002. Generation of $CD8^+$ T cell memory in response to low, high and excessive levels of epitope. *The Journal of Immunology* **168:** 4455-4461.

WHERRY, E.J. and AHMED, R. 2004. Minireview: Memory $CD8^+$ T-cell differentiation during viral infection. *Journal of Virology* **78:** 5535-5545.

WOZNIAK, T.M., RYAN, A.A., TRICCAS, J.A. and 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.

YAMADA, H., ISHII, K.J. and KLINMAN, D.M. 2004. Suppressive oligodeoxynucleotides inhibit CpG-induced inflammation of the mouse lung. *Critical Care Medicine* **32:** 2045-2049.

ZHANG, D., ZHANG, G., HAYDEN, M.S., GREENBLATT, M.B., BUSSEY, C., FLAVELL, R.A. and GHOSH, S. 2004. A toll-like receptor that prevents infection by uropathogenic bacteria. *Science* **303:** 1522-1526.

ZHU, M., XU, X., LIU, H., LIU, X., WANG, S., DONG, F., YANG, B. and SONG, G. 2003. Enhancement of DNA vaccine potency against herpes simplex virus 1 by co-administration of an interleukin-18 expression plasmid as a genetic adjuvant. *Journal of Medical Microbiology* **52:** 223-228.

6. APPENDIX A: MATERIALS, BUFFERS, MEDIA AND SOLUTIONS

6.1. MATERIALS

Table 1. *Suppliers of the materials used to complete the experimental work.*

Supplier	**Products**
Merck Biosciences	Acrylamide, calcium chloride, chloroform, citric acid, ethanol, glucose, isoamylalcohol, methanol, potassium chloride, sodium carbonate, sodium mono-phosphate, tri-sodium citrate, tryptone powder, Tween®-20.
TAKARA Shuzo Co., LTD	TAKARA LA Taq^{TM}, TAKARA Ex Taq^{TM} TAKARA recochip
Roche	DNAse I, LightCycler® FastStart DNA Master^{PLUS} SYBR Green 1 kit, PCR purification mini-kit, plasmid purification mini-kit, restriction enzymes: *Ksp* I, *Sal* I *Bam* HI, *Xba* I, *Bgl* II RNAse I,
Qiagen Ltd	Qiagen mini elute kit
	Nucleobond® AX PC 2000 plasmid isolation kit, Nucleobond® AX L 50 phage genomic DNA isolation kit, Protino® Ni 1000 protein isolation kit.
Stratagene cloning systems	Klenow fill-in kit
Amersham Pharmacia Biotech	$^{[32]}$P-dATP, pMOS*Blue* blunt ended cloning kit, [^{3}H] thymidine,
Promega	Shrimp alkaline phosphatase, Pfu DNA polymerase
Pfizer	Liquamycin/LA,
Centaur	Eutha-Nase,
Degussa Corporation	Gold beads
Sigma	Bromophenol blue, concanavalin A, DMSO, 1,4-dithio-DL-threitol (DTT), HEPES, hypoxanthine, L-glutamine, M-2-mercaptoethanol, mycophenolic acid, percoll, phenol, spermidine, trypan blue, trypsin, xanthine, Lymphocep
Costar	Half-area 96-well plates
Nova Nordisk	Sodium benzylpenicillin, streptomycin sulphate
Wallac	96 well glass fibre filters
Packard BioScience	Ultima gold F scintillation solution.
Highveldt biologicals	Foetal calf serum, DMEM, Hams F12
BDH	Glycine, glycerol, N,N-methylenebisacrylamide, magnesium chloride, maltose, potassium bi-phosphate,
Supplier	**Products**

USB	Agar, boric acid, EDTA, ethidium bromide, gelatin, HCl, magnesium sulphate, potassium bi-phosphate, proteinase K, SDS, sodium chloride, tris-base.
UniLAb	Sodium hydroxide,

6.2. BUFFERS, MEDIA AND SOLUTIONS

6.2.1 Buffers for general laboratory use

Ampicillin (LB both)

A 10 mg/ml stock solution was prepared by dissolving 50 mg ampicillin in 5 ml dH_2O. The solution was filter sterilised and stored in aliquots at -20 °C.

EB lysis buffer (isolation of E. ruminantium*)*

Buffer contained 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 dH_2O and pH adjusted to 8.

Ethidium Bromide (Agarose gels)

1 g ethidium bromide was dissolved in 100 ml dH_2O.

LB agar plates

Buffer was prepared the same as LB broth but 15 g agar was added prior to autoclaving. Appropriate antibiotics and IPTG/X-gal were added before plates were poured.

LB broth

This buffer contained 10 g Tryptone, 5 g yeast and 10 g NaCl per litre. Buffer was sterilised by autoclaving and appropriate antibiotics added prior to use.

10x Phosphate Buffered Saline (PBS)

The buffer consisted of 137 mM NaCl (8 g), 27 mM KCl (2 g), 8.8 mM KH_2PO_4 (1.2 g) and 73 mM $Na_2HPO_4.2H_2O$ (11.4 g) were dissolved in 900 ml dH_2O and the pH adjusted to 7.5. The solution was made up to a final volume of 1000 ml, autoclaved and stored at room temperature.

SM buffer

Buffer contained 0.01 % gelatin; 50 mM Tris pH7.5; 100 mM NaCl and 8 mM $MgSO_4$. The solution was autoclaved and stored at 4 °C.

SOC medium

This buffer consisted of 20 g tryptone, 5 g yeast extract, 8.5 mM NaCl (0.5 g) and 2.5 ml 1 M KCl. Buffer pH was adjusted to 7 with NaOH, made up to 970 ml and autoclaved. Prior to use 10 ml sterile 1 M $MgCl_2$ and 20 ml sterile 1 M glucose were added.

20x SSC

The solution containing 3 M NaCl (175.3 g) and 0.3 M sodium citrate (88.2 g) was made up to 1 000 ml, and stored at room temperature.

10 x TBE

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

TE

The buffer contains 10 mM Tris–Cl and 1 mM EDTA, pH 7.5

TM buffer

The buffer contained 25 mM Tris base pH 8, 10 mM $MgCl_2$

Top agarose

A mixture of 17 g Bacto-tryptone, 10 g Bacto-yeast extract, 5 g NaCl and 7 g agarose were prepared in deionised, dH_2O to a total volume of 1 l. If necessary, the pH was adjusted to 7.0 with 5 M NaOH and autoclaved to sterilise.

6.2.2. Buffers used for immunising animals (Chapters 2, -3 and 4)

Spermidine stock

Spermidine stock solution (6.9 M) was prepared by diluting 1 g of spermidine in 1 ml dH_2O. 14.4 µl of stock was diluted in 1ml dH_2O to obtain the 0.1 M working solution.

SPG

The buffer contained 0.22 M sucrose, 0.01 M potassium phosphate pH 7.0, 0.005 M potassium glutamate. Buffer was autoclaved and stored at 4 °C.

6.2.3. Buffers used for lymphocyte proliferation (Chapters 2, -3 and 4)

Alsevers medium (Lymphocyte proliferation)

Buffer contains 110 mM glucose, 30 mM sodium citrate, 3 mM citric acid and 70 mM NaCl.

Complete RPMI 1640 medium

RPMI (225 ml), HEPES (1.49 g) and 2.5 ml of a 2 mM L-glutamine buffer were mixed and the pH adjusted to 7.2. S-mercapto-ethanol (0.9 µl), 2.5 ml FCS (heat inactivated), 250 µl sodium benzylpenicillin (Novopen), 150 µl streptomycin sulphate (Novostrep) were added and the solution was filter sterilised through a dual filter system containing 0.45 µm and 0.2 µm filters and stored at 4 °C. If the solution was stored for more than two weeks, more filter sterilised L-glutamine was added to the solution.

Hanks balanced salt solution (HBSS) (Lymphocyte proliferation)

The buffer contains 5 mM KCl, 400 µM KH_2PO_4, 140 mM NaCl, 4 mM $NaHCO_3$, 300 µM $Na_2HPO_4.7H_2O$ and 5mM glucose. The buffer was supplemented with 2 ml EDTA/ 1 l HBSS before use.

Trypan blue solution

5 g Trypan blue was dissolved in sterile 1x PBS pH 7.2. The solution was filtered through Whattman number 1 paper and finally filter sterilised and stored at room temperature.

6.2.4. Buffers used for preparation of rLSDV (Chapter 4)

Activated trypsin

Activated trypsin solution was obtained from Onderstepoort Biological Products (OBP) and was supplemented with 100 µl sodium benzylpenicillin (Novopen, Nova Nordisk) and 60 µl streptomycin sulphate (Novostrep, Nova Nordisk), filter sterilised and stored at 4 °C.

Cell lysis buffer (rLSDV genomic DNA isolation)

100 mM Tris, 100 mM NaCl and 0.5 % NP-40 was mixed and pH adjusted to 8.0.

Dulbecco's Modified Eagle's Essential Medium (DMEM)/ Hams F12

DMEM powder (6.78 g), Hams F12 powder (5.36 g) and 23mM $NaHCO_3$ (2.44 g) were dissolved in 1000 ml dH_2O, the pH adjusted to 7.1 using HCl and sterilised using a 0.2 µm filter. The solution was stored at 4 °C.

Freeze medium

10 % DMSO was mixed with sterile heat inactivated foetal calf serum.

HEPES (1M)

HEPES (23.8 g) was dissolved in 80 ml 0.1 M NaOH and the pH adjusted to 7.2. The solution was made up to a final volume of 100 ml, filter sterilised and stored at 4 °C.

Hypoxanthine (stock solution)

Hypoxanthine (0.1 g) was dissolved in 10 ml 0.1 M NaOH, filter sterilised and stored at 4 °C.

Mycophenolic acid

Mycophenolic acid (0.1 g) was dissolved in 10 ml 0.1 M NaOH, filter sterilised, divided into 100 µl aliquots and stored in the dark at -20 °C.

rLSDV DNA extraction buffer

This buffer contained 20 mM EDTA, 20 mM Tris-Cl pH 7.5 and 0.5 % SDS.
Immediately before use 1.0 mg/ml proteinase K was added.

Virus selection medium

For 10 ml of virus selection medium, DMEM/Hams F12 medium was supplemented with 25 µl Mycophenolic acid stock solution 250 µl xanthine stock solution, 15 µl hypoxanthine stock solution, 10 % foetal calf serum (Sterilab), 10 µl sodium benzylpenicillin (Novopen, Nova Nordisk) and 6 µl streptomycin sulphate (Novostrep, Nova Nordisk). The solution was filter sterilised and stored at 4 °C.

Xanthine (stock solution)

Xanthine (0.1 g) was dissolved in 10 ml 1 M NaOH, filter sterilised through a 0.2 µm filter and stored at 4 °C.

X-gal

400 mg 5-bromo-4-chloro-3-indolyl-β-Dgalactoside (X-gal) was dissolved in 20 ml N,N'-dimethyl formamide, divided in aliquots and stored.

6.2.5 Buffers used for preparation of recombinant protein (Chapter 4)

30 % Acrylamide/ bisacrylamide (SDS-PAGE)

30 g acrylamide was mixed with 0.8 g N,N–methylenebisacrylamide in 50 ml dH_2O. After the chemicals were dissolved, the solution was made up to 100 ml, filter sterilised and stored in the dark at 4 °C.

1M IPTG (Protein expression)

1.19g isopropyl-β-D-thiogalactopyranoside was dissolved in 50 ml dH_2O, filter sterilised, aliquoted and stored at -20 °C.

PBS-Tween (0.05 %) (Western blot)

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

2x SDS-PAGE Gel loading buffer

SDS gel loading buffer contains 1 ml Tris-Cl (pH 6.8), 2 ml dithiothreitol, 4 ml 10 % SDS and 0.2 % bromophenol blue. The volume was adjusted to 10 ml and the solution was aliquoted and stored at -20 °C.

Stacking gel buffer (SDS-PAGE) (4x Tris .Cl/SDS pH 6.8)

50 mM Tris base (6.05 g) was dissolved in 40 ml dH_2O and the pH adjusted to 6.8 with 1M HCl. The total volume was adjusted to 100 ml, and 0.4 g SDS added. The solution was stored at 4 °C.

Separating gel buffer (SDS-PAGE) (4x Tris .Cl/SDS pH 8.8)

50 mM Tris base (6.05 g) was dissolved in 40 ml dH_2O and the pH adjusted to 8.8 with 1 M HCl. The total volume was adjusted to 100 ml, and 0.4 g SDS added. The solution was stored at 4 °C.

10x SDS-PAGE electrophoresis buffer

This buffer contained 250 mM Tris base (30.2 g), 2 M glycine (144 g), and 17 mM SDS (5 g). Buffer was diluted to 1000 ml with dH_2O, and stored at 4 °C.

12 % SDS-PAGE

See table 6.1 for protocol

Transfer buffer (Western blot)

25 mM Tris base (6 g) and 192 mM glycine (28.8 g) was dissolved in 1700 ml dH_2O and 300 ml methanol added. The solution was stored at room temperature.

Table 0.1: *Recipes for Polyacrylamide Separating and Stacking Gels. (Taken from Current Protocols in Molecular Biology)*

SEPARATING GEL

Stock solution[b]	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–Cl/SDS, pH 8.8 and H_2O. Degas under vacuum ~5 min. Add 10% ammonium persulfate and TEMED. Swirl gently to mix. Use immediately.

STACKING GEL ***(3.9% acrylamide)***

In a 25-ml side-arm flask, mix 0.65 ml of 30% acrylamide/0.8% bisacrylamide, 1.25 ml of 4x Tris-Cl/SDS, pH 6.8, and 3.05 ml H_2O. Degas under vacuum 10 to 15 min. 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 the persulfate, TEMED, or both.

7. APPENDIX B: SEQUENCE ALIGNMENTS

Erum2540 sequence alignments:

CLUSTAL X (1.8) MULTIPLE SEQUENCE ALIGNMENT

File: /usr/people/alri/final_results/Erum2540.ps **Date: Tue Jul 19 11:50:41 2005**

Page 1 of 3

```
                 ********************************************************************************

LSDV7_stock      ATGTTGAGACTTAGTTTTATATTTATAGTGTTAATCACATTACTTACATACCAGAATGGGTATTCTGCAAAATCC  75
pCMViUBs         ATGTTGAGACTTAGTTTTATATTTATAGTGTTAATCACATTACTTACATACCAGAATGGGTATTCTGCAAAATCC  75
Welgevonden      ATGTTGAGACTTAGTTTTATATTTATAGTGTTAATCACATTACTTACATACCAGAATGGGTATTCTGCAAAATCC  75
pET              ATGTTGAGACTTAGTTTTATATTTATAGTGTTAATCACATTACTTACATACCAGAATGGGTATTCTGCAAAATCC  75
ruler            1.......10.........20.........30.........40.........50.........60.........70.....

                 ********  ***************************************************************************

LSDV7_stock      CCTGATCCAGTGAACATAAAACTAATAGTAGGATCAATGGATATGAAAAATAGAACTATCAACATAGGAGTTGAA  150
pCMViUBs         CCTGATCCTGTGAACATAAAACTAATAGTAGGATCAATGGATATGAAAAATAGAACTATCAACATAGGAGTTGAA  150
Welgevonden      CCTGATCCTGTGAACATAAAACTAATAGTAGGATCAATGGATATGAAAAATAGAACTATCAACATAGGAGTTGAA  150
pET              CCTGATCCTGTGAACATAAAACTAATAGTAGGATCAATGGATATGAAAAATAGAACTATCAACATAGGAGTTGAA  150
ruler            ...80.........90.......100.......110.......120.......130.......140.......150

                 ********************************************************************************

LSDV7_stock      TTTAGAATTCAGGATGGATGGCATATTTATTATAAATCACCAGGAGATCTTGGATTGCCAACAGTATTTCAATGG  225
pCMViUBs         TTTAGAATTCAGGATGGATGGCATATTTATTATAAATCACCAGGAGATCTTGGATTGCCAACAGTATTTCAATGG  225
Welgevonden      TTTAGAATTCAGGATGGATGGCATATTTATTATAAATCACCAGGAGATCTTGGATTGCCAACAGTATTTCAATGG  225
pET              TTTAGAATTCAGGATGGATGGCATATTTATTATAAATCACCAGGAGATCTTGGATTGCCAACAGTATTTCAATGG  225
ruler            .......160.......170.......180.......190.......200.......210.......220.....
```

CLUSTAL X (1.8) MULTIPLE SEQUENCE ALIGNMENT

File: /usr/people/alri/final_results/Erum2540.ps **Date: Tue Jul 19 11:50:41 2005**

Page 2 of 3

```
                 * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
LSDV7_stock      CAAGAGAACATTTTTTAAAGATGTACAAATACACTGGCCACAACCTATACAACATACAGATACAACAAGTAACAAC 300
pCMViUBs         CAAGAGAACATTTTTTAAAGATGTACAAATACACTGGCCACAACCTATACAACATACAGATACAACAAGTAACAAC 300
Welgevonden      CAAGAGAACATTTTTTAAAGATGTACAAATACACTGGCCACAACCTATACAACATACAGATACAACAAGTAACAAC 300
pET              CAAGAGAACATTTTTTAAAGATGTACAAATACACTGGCCACAACCTATACAACATACAGATACAACAAGTAACAAC 300
ruler            . . 230. . . . . 240. . . . . 250. . . . . 260. . . . . 270. . . . . 280. . . . . 290. . . . . 300

                 * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
LSDV7_stock      ATCTTCCATAGCAATGTTTACAAAGACATTGTAATGTTCCCTATTTCATTTGCTCTCAAACATGACAACCTAAAC 375
pCMViUBs         ATCTTCCATAGCAATGTTTACAAAGACATTGTAATGTTCCCTATTTCATTTGCTCTCAAACATGACAACCTAAAC 375
Welgevonden      ATCTTCCATAGCAATGTTTACAAAGACATTGTAATGTTCCCTATTTCATTTGCTCTCAAACATGACAACCTAAAC 375
pET              ATCTTCCATAGCAATGTTTACAAAGACATTGTAATGTTCCCTATTTCATTTGCTCTCAAACATGACAACCTAAAC 375
ruler            . . . . . 310. . . . . 320. . . . . 330. . . . . 340. . . . . 350. . . . . 360. . . . . 370. . . . .

                 * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
LSDV7_stock      ACTAAAGAACTAAGTATTAGTTTACGTATCAAATACGCAGTATGCAAGGATGTATGTATTCCCCAAGAAAAAGTT 450
pCMViUBs         ACTAAAGAACTAAGTATTAGTTTACGTATCAAATACGCAGTATGCAAGGATGTATGTATTCCCCAAGAAAAAGTT 450
Welgevonden      ACTAAAGAACTAAGTATTAGTTTACGTATCAAATACGCAGTATGCAAGGATGTATGTATTCCCCAAGAAAAAGTT 450
pET              ACTAAAGAACTAAGTATTAGTTTACGTATCAAATACGCAGTATGCAAGGATGTATGTATTCCCCAAGAAAAAGTT 450
ruler            . . 380. . . . . 390. . . . . 400. . . . . 410. . . . . 420. . . . . 430. . . . . 440. . . . . 450
```

CLUSTAL X (1.8) MULTIPLE SEQUENCE ALIGNMENT

File: /usr/people/alri/final_results/Erum2540.ps **Date: Tue Jul 19 11:50:41 2005**

Page 3 of 3

```
            ************************************************************************
LSDV7_stock ATTATATTAAATCGTTTTTTACAGGATTACGTTAATCAAGAAAATTTAGGATTAATAAATTTTTGGAAAAAAAAA 525
pCMViUBs    ATTATATTAAATCGTTTTTTACAGGATTACGTTAATCAAGAAAATTTAGGATTAATAAATTTTTGGAAAAAAAAA 525
Welgevonden ATTATATTAAATCGTTTTTTACAGGATTACGTTAATCAAGAAAATTTAGGATTAATAAATTTTTGGAAAAAAAAA 525
pET         ATTATATTAAATCGTTTTTTACAGGATTACGTTAATCAAGAAAATTTAGGATTAATAAATTTTTGGAAAAAAAAA 525
ruler       .......460.......470.......480.......490.......500.......510.......520.....
```

```
             *
LSDV7_stock TAA-
pCMViUBs    TAA-
Welgevonden TAA-
pET         AAGG
ruler       ..53
```

Erum2540 amino acid alignments

CLUSTAL X (1.8) MULTIPLE SEQUENCE ALIGNMENT

File: /usr/people/alri/final_results/Erum2540pep.ps Date: Thu Jul 21 12:46:40 2005

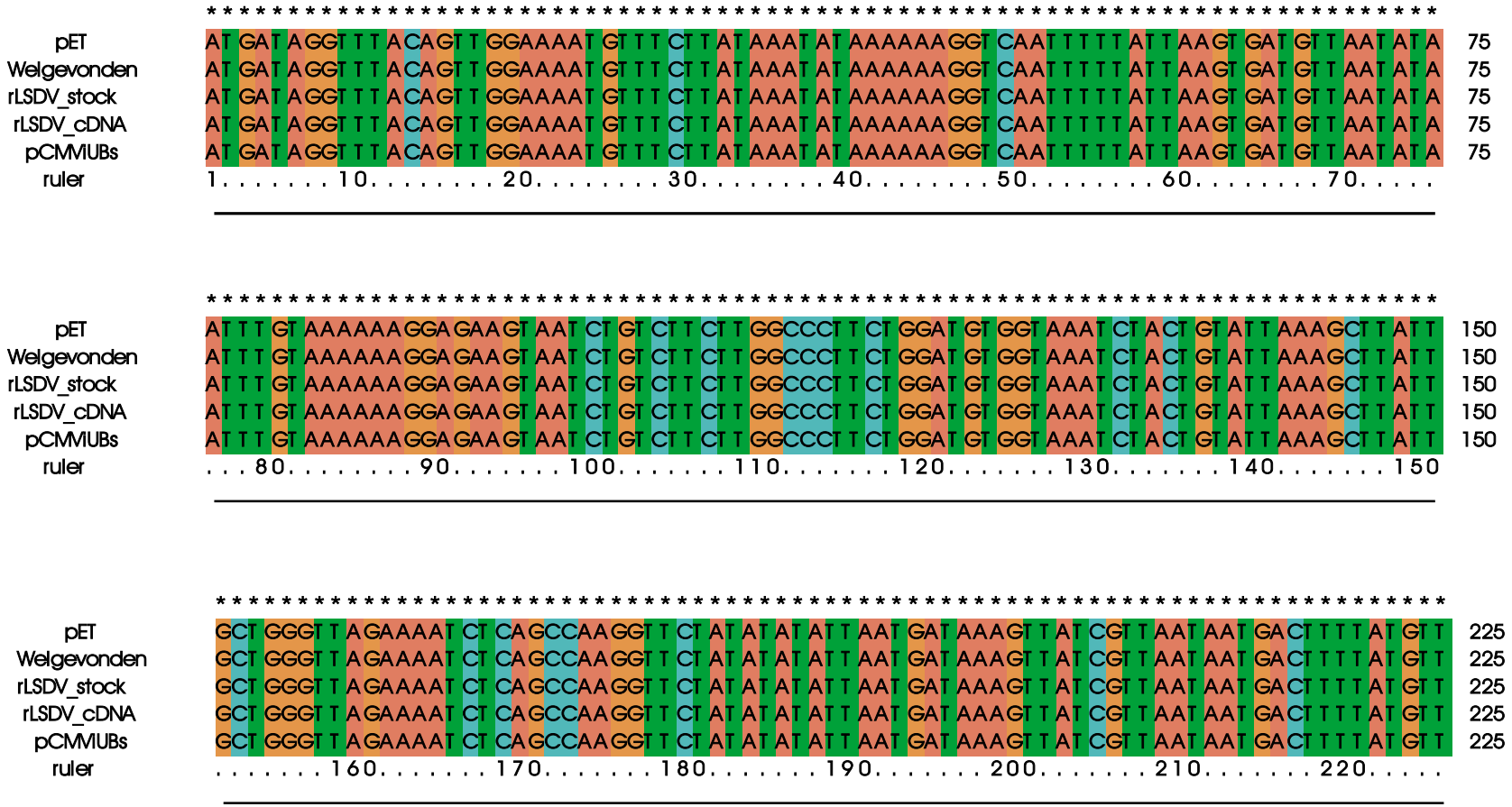
Page 1 of 1

```
                 ********************************************************************************
rLSDV_stock      MLRLSFIFIVLITLLTYQNGYSAKSPDPVNIKLIVGSMDMKNRTINIGVEFRIQDGWHIYYKSPGDLGLPTVFQW 75
pCMVIUBs         MLRLSFIFIVLITLLTYQNGYSAKSPDPVNIKLIVGSMDMKNRTINIGVEFRIQDGWHIYYKSPGDLGLPTVFQW 75
pET              MLRLSFIFIVLITLLTYQNGYSAKSPDPVNIKLIVGSMDMKNRTINIGVEFRIQDGWHIYYKSPGDLGLPTVFQW 75
Welgevonden      MLRLSFIFIVLITLLTYQNGYSAKSPDPVNIKLIVGSMDMKNRTINIGVEFRIQDGWHIYYKSPGDLGLPTVFQW 75
ruler            1.......10.........20.........30.........40.........50.........60.........70.....

                 ********************************************************************************
rLSDV_stock      QENIFKDVQIHWPQPIQHTDTTSNNIFHSNVYKDIVMFPISFALKHDNLNTKELSISLRIKYAVCKDVCIPQEKV 150
pCMVIUBs         QENIFKDVQIHWPQPIQHTDTTSNNIFHSNVYKDIVMFPISFALKHDNLNTKELSISLRIKYAVCKDVCIPQEKV 150
pET              QENIFKDVQIHWPQPIQHTDTTSNNIFHSNVYKDIVMFPISFALKHDNLNTKELSISLRIKYAVCKDVCIPQEKV 150
Welgevonden      QENIFKDVQIHWPQPIQHTDTTSNNIFHSNVYKDIVMFPISFALKHDNLNTKELSISLRIKYAVCKDVCIPQEKV 150
ruler            ...80.........90.......100.......110.......120.......130.......140.......150

                 **************************
rLSDV_stock      IILNRFLQDYVNQENLGLINFWKKK---------------------------- 175
pCMVIUBs         IILNRFLQDYVNQENLGLINFWKKK---------------------------- 175
pET              IILNRFLQDYVNQENLGLINFWKKKKGELKLEGKPIPNPLLGLDSTRTGHHHHHH 205
Welgevonden      IILNRFLQDYVNQENLGLINFWKKK---------------------------- 175
ruler            .......160.......170.......180.......190.......200.....
```

Erum2550 **sequence alignments**

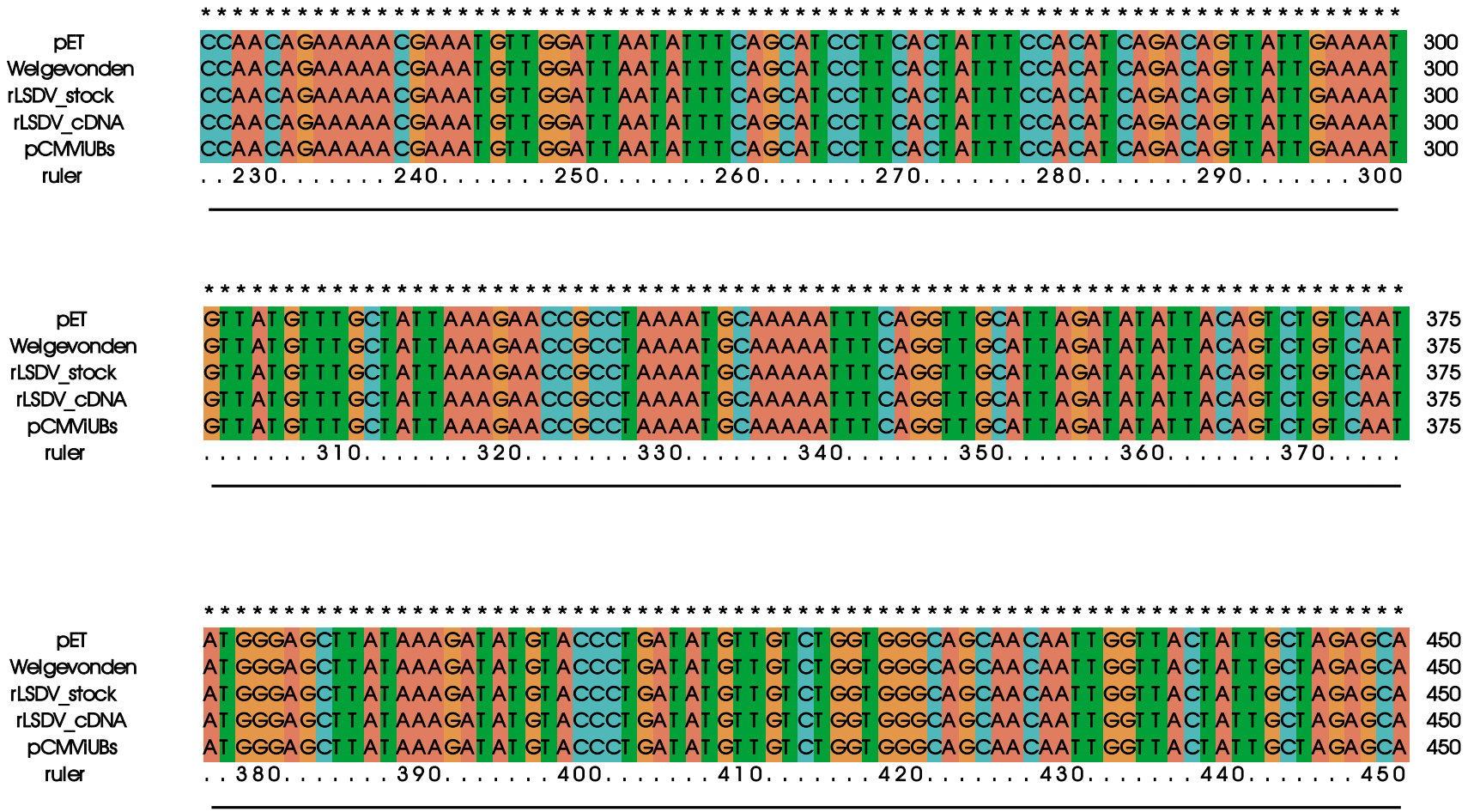
CLUSTAL X (1.8) MULTIPLE SEQUENCE ALIGNMENT

File: /usr/people/alri/final_results/Erum2550.ps Date: Tue Jul 19 12:13:42 2005
Page 1 of 5



CLUSTAL X (1.8) MULTIPLE SEQUENCE ALIGNMENT

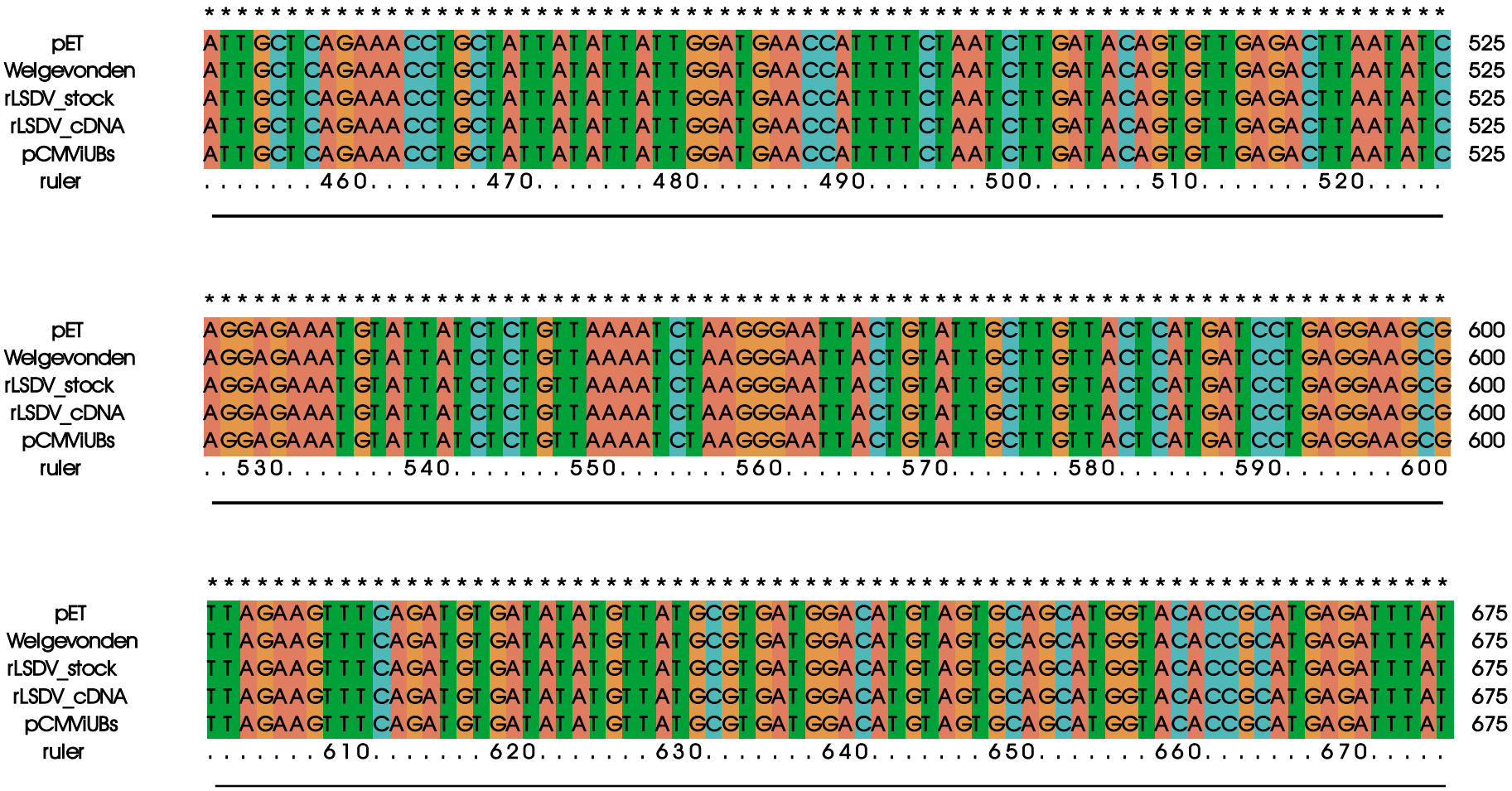
File: /usr/people/alri/final_results/Erum2550.ps Date: Tue Jul 19 12:13:42 2005
Page 2 of 5



CLUSTAL X (1.8) MULTIPLE SEQUENCE ALIGNMENT

File: /usr/people/alri/final_results/Erum2550.ps Date: Tue Jul 19 12:13:42 2005

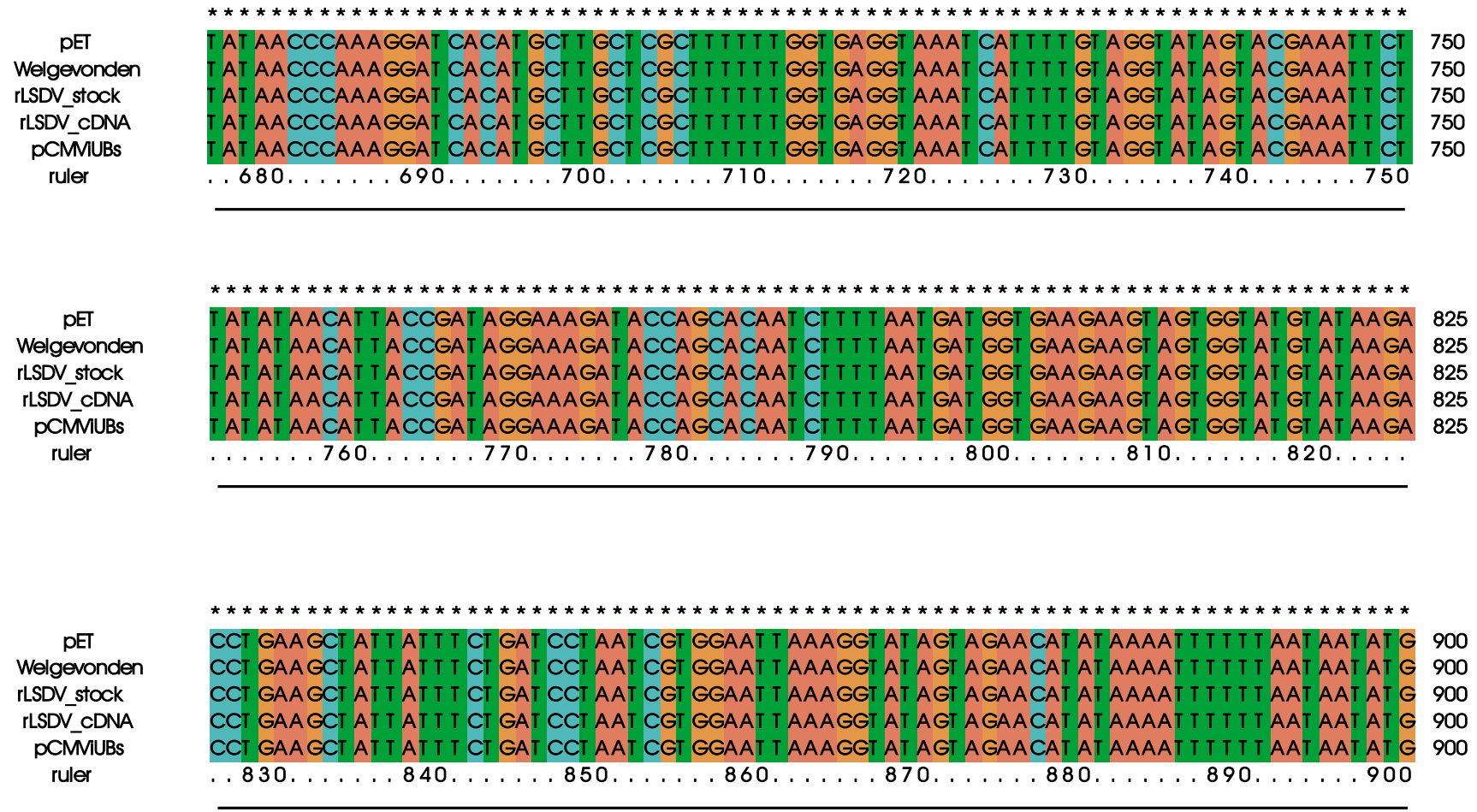
Page 3 of 5

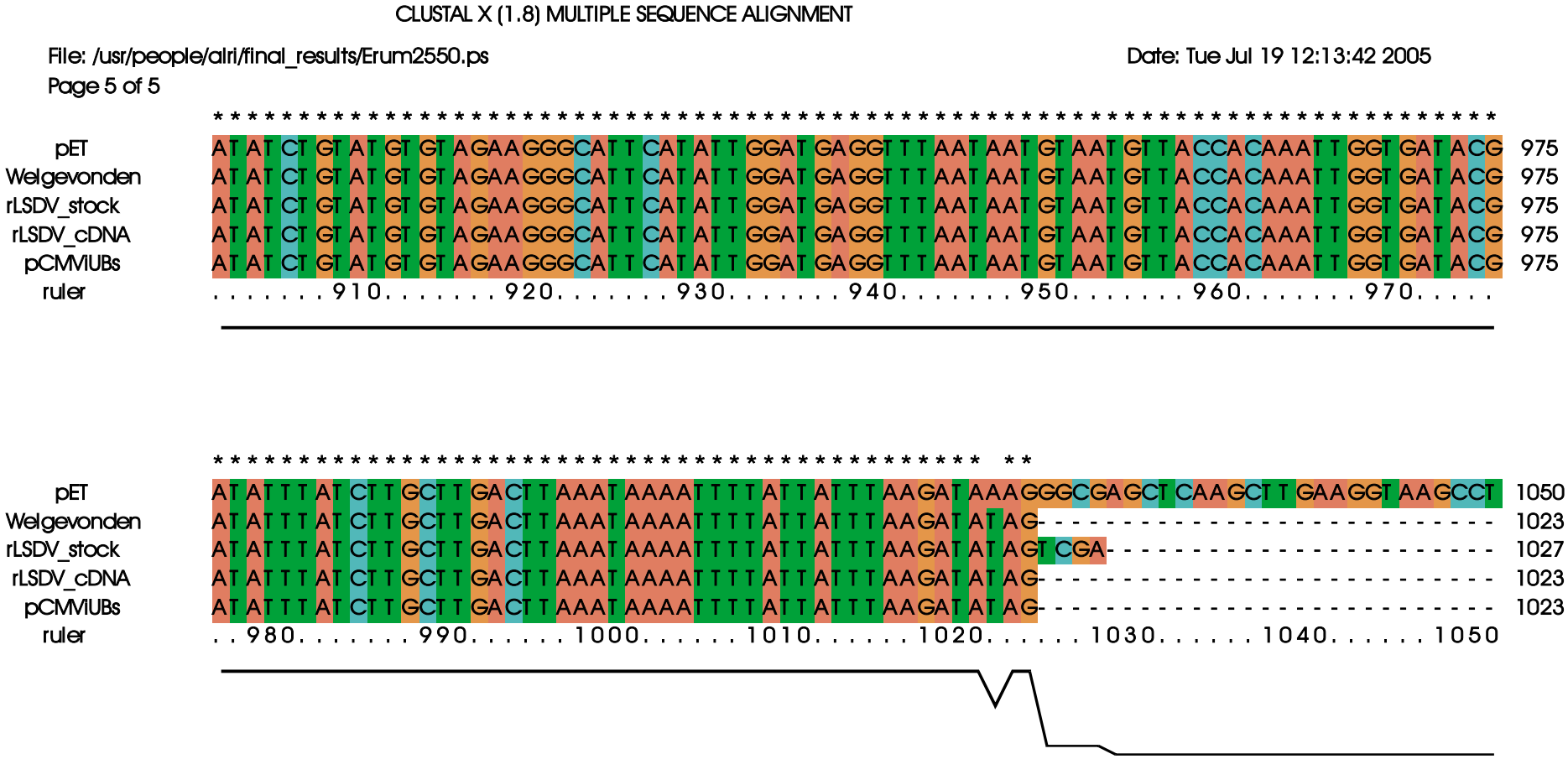


CLUSTAL X (1.8) MULTIPLE SEQUENCE ALIGNMENT

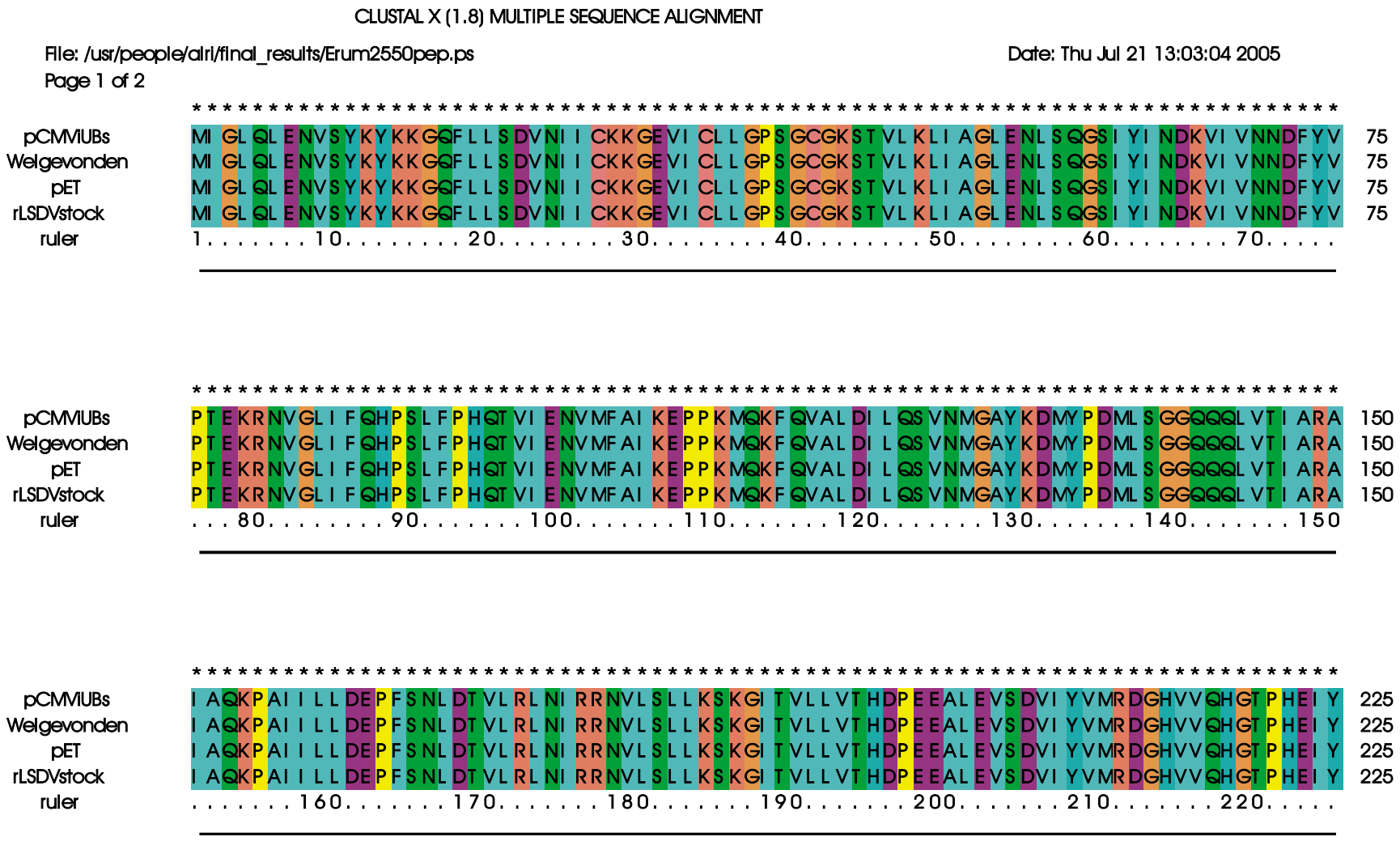
File: /usr/people/alri/final_results/Erum2550.ps Date: Tue Jul 19 12:13:42 2005

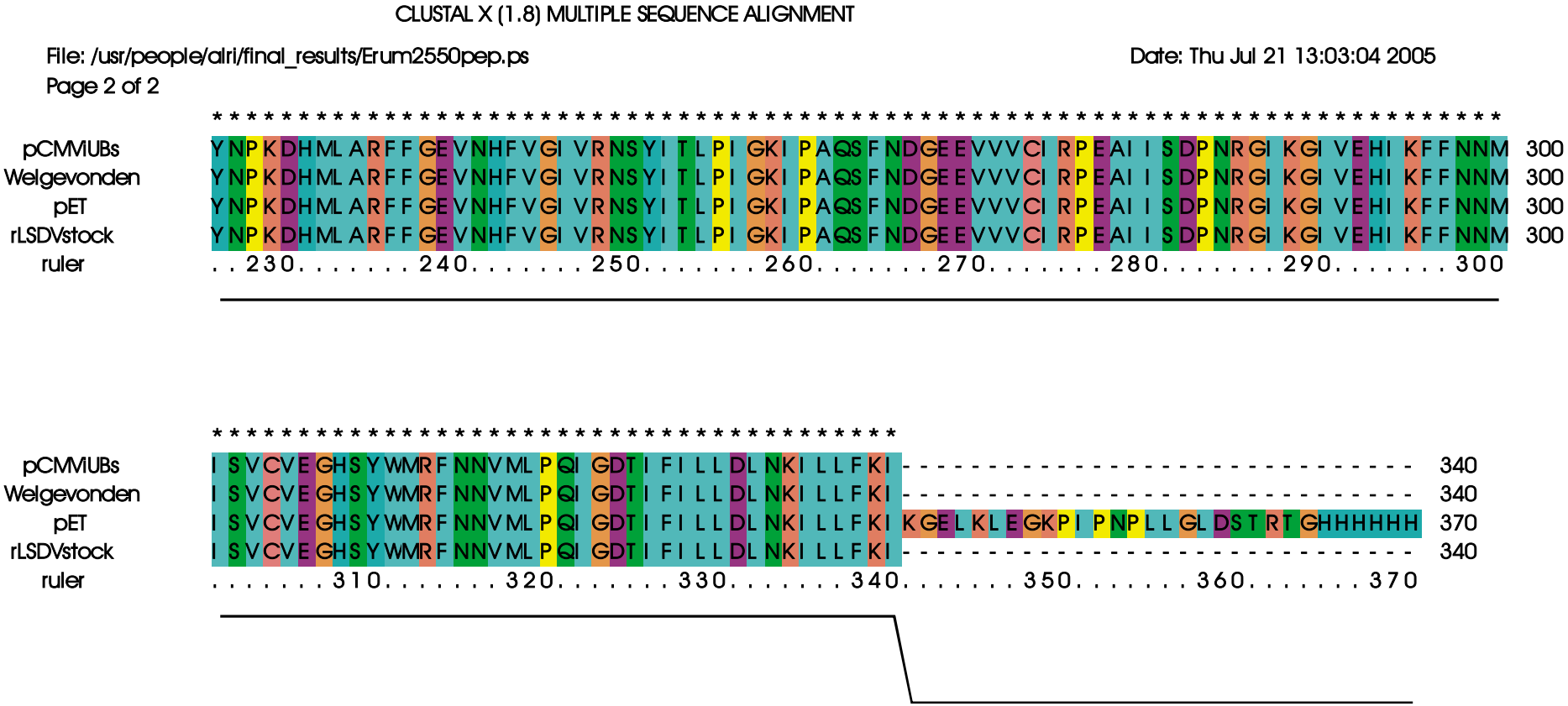
Page 4 of 5




CLUSTAL X (1.8) MULTIPLE SEQUENCE ALIGNMENT
File: /usr/people/alri/final_results/Erum2550.ps
Date: Tue Jul 19 12:13:42 2005
Page 5 of 5
pET ATATCTGTATGTGTAGAAGGGCATTCATATTGGATGAGGTTTAATAATGTAATGTTACCACAAATTGGTGATACG 975
Welgevonden ATATCTGTATGTGTAGAAGGGCATTCATATTGGATGAGGTTTAATAATGTAATGTTACCACAAATTGGTGATACG 975
rLSDV_stock ATATCTGTATGTGTAGAAGGGCATTCATATTGGATGAGGTTTAATAATGTAATGTTACCACAAATTGGTGATACG 975
rLSDV_cDNA ATATCTGTATGTGTAGAAGGGCATTCATATTGGATGAGGTTTAATAATGTAATGTTACCACAAATTGGTGATACG 975
pCMVIUBs ATATCTGTATGTGTAGAAGGGCATTCATATTGGATGAGGTTTAATAATGTAATGTTACCACAAATTGGTGATACG 975
ruler .....910.....920.....930.....940.....950.....960.....970.....
pET ATATTTATCTTGCTTGACTTAAATAAAATTTTATTATTTAAGATAAAGGGCGAGCTCAAGCTTGAAGGTAAGCCT 1050
Welgevonden ATATTTATCTTGCTTGACTTAAATAAAATTTTATTATTTAAGATATAG---------- 1023
rLSDV_stock ATATTTATCTTGCTTGACTTAAATAAAATTTTATTATTTAAGATATAGTCGA---------- 1027
rLSDV_cDNA ATATTTATCTTGCTTGACTTAAATAAAATTTTATTATTTAAGATATAG---------- 1023
pCMVIUBs ATATTTATCTTGCTTGACTTAAATAAAATTTTATTATTTAAGATATAG---------- 1023
ruler ..980.....990.....1000.....1010.....1020.....1030.....1040.....1050

Erum2550 amino acid alignments



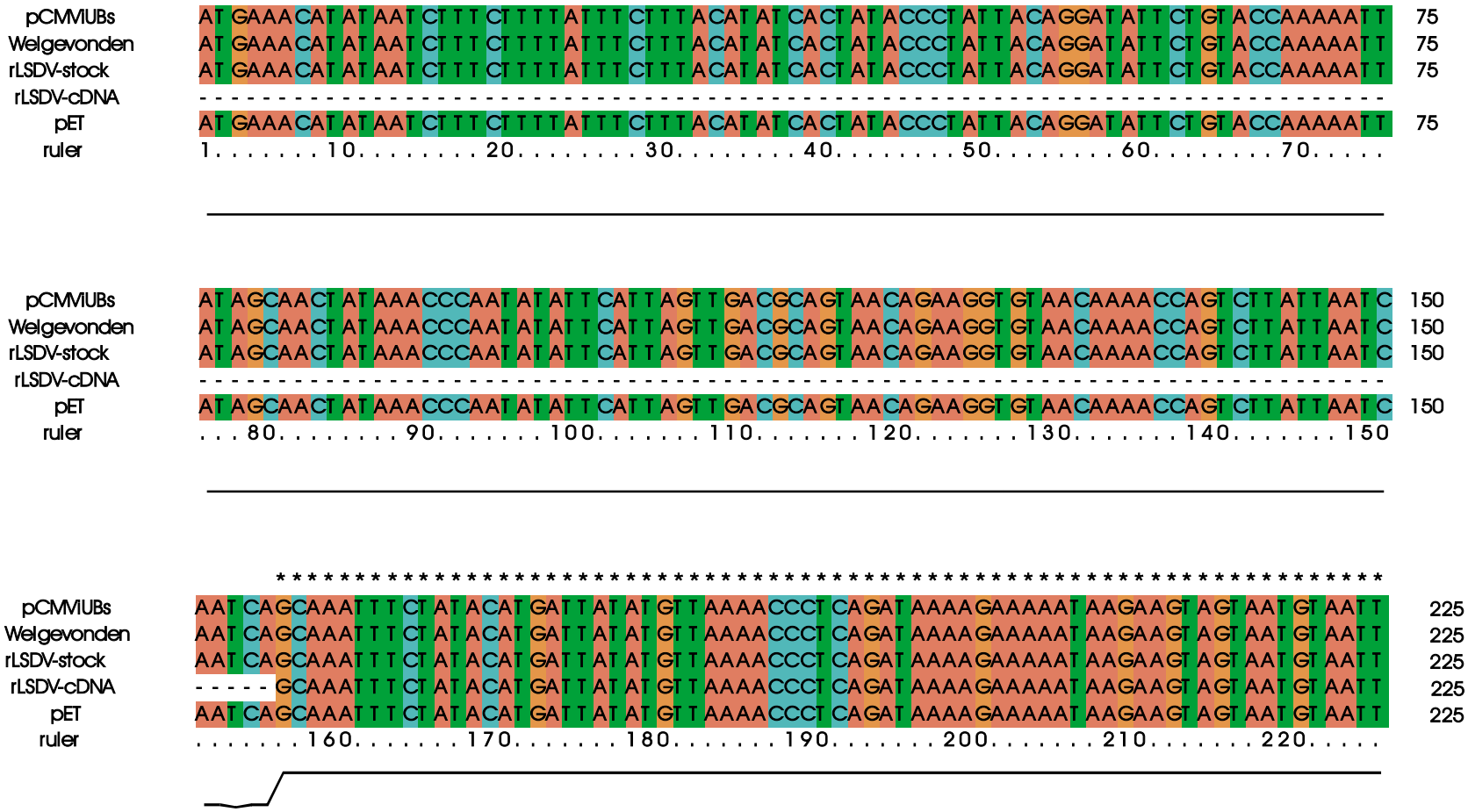

CLUSTAL X (1.8) MULTIPLE SEQUENCE ALIGNMENT
File: /usr/people/alri/final_results/Erum2550pep.ps
Date: Thu Jul 21 13:03:04 2005
Page 2 of 2
pCMVIUBs
Welgevonden
pET
rLSDVstock
ruler
. . 230. . . . . 240. . . . . 250. . . . . 260. . . . . 270. . . . . 280. . . . . 290. 300
. . . . . 310. . . . . 320. . . . . 330. . . . . 340. . . . . 350. . . . . 360. 370

Erum2580 sequence alignments

CLUSTAL X (1.8) MULTIPLE SEQUENCE ALIGNMENT

File: /usr/people/alri/final_results/Erum2580.ps Date: Tue Jul 19 12:14:03 2005

Page 1 of 4



CLUSTAL X (1.8) MULTIPLE SEQUENCE ALIGNMENT

File: /usr/people/alri/final_results/Erum2580.ps

Date: Tue Jul 19 12:14:03 2005

Page 2 of 4

```
             ***************************************************************************
pCMViUBs     TTTTATGTAGATGATCATCTAGAAACTTTTATAAACAAAATAAAAGATAAAACTCTAATTAAGTTATCTGATGTA 300
Welgevonden  TTTTATGTAGATGATCATCTAGAAACTTTTATAAACAAAATAAAAGATAAAACTCTAATTAAGTTATCTGATGTA 300
rLSDV-stock  TTTTATGTAGATGATCATCTAGAAACTTTTATAAACAAAATAAAAGATAAAACTCTAATTAAGTTATCTGATGTA 300
rLSDV-cDNA   TTTTATGTAGATGATCATCTAGAAACTTTTATAAACAAAATAAAAGATAAAACTCTAATTAAGTTATCTGATGTA 145
pET          TTTTATGTAGATGATCATCTAGAAACTTTTATAAACAAAATAAAAGATAAAACTCTAATTAAGTTATCTGATGTA 300
ruler        ..230.......240.......250.......260.......270.......280.......290.......300

             ***************************************************************************
pCMViUBs     GTAGCATTACTACCATCAAGATATGACTCTCATTTCTCATACAAAGTTCACACAACATCAAATGATTTACACATA 375
Welgevonden  GTAGCATTACTACCATCAAGATATGACTCTCATTTCTCATACAAAGTTCACACAACATCAAATGATTTACACATA 375
rLSDV-stock  GTAGCATTACTACCATCAAGATATGACTCTCATTTCTCATACAAAGTTCACACAACATCAAATGATTTACACATA 375
rLSDV-cDNA   GTAGCATTACTACCATCAAGATATGACTCTCATTTCTCATACAAAGTTCACACAACATCAAATGATTTACACATA 220
pET          GTAGCATTACTACCATCAAGATATGACTCTCATTTCTCATACAAAGTTCACACAACATCAAATGATTTACACATA 375
ruler        .......310.......320.......330.......340.......350.......360.......370.....

             ***************************************************************************
pCMViUBs     TGGTTAAGTCCAGACAATGCAAAAAAAATAGTAGAACACATAAAGTTAGTACTATGTAAAATAGATCCAGAGAAC 450
Welgevonden  TGGTTAAGTCCAGACAATGCAAAAAAAATAGTAGAACACATAAAGTTAGTACTATGTAAAATAGATCCAGAGAAC 450
rLSDV-stock  TGGTTAAGTCCAGACAATGCAAAAAAAATAGTAGAACACATAAAGTTAGTACTATGTAAAATAGATCCAGAGAAC 450
rLSDV-cDNA   TGGTTAAGTCCAGACAATGCAAAAAAAATAGTAGAACACATAAAGTTAGTACTATGTAAAATAGATCCAGAGAAC 295
pET          TGGTTAAGTCCAGACAATGCAAAAAAAATAGTAGAACACATAAAGTTAGTACTATGTAAAATAGATCCAGAGAAC 450
ruler        ..380.......390.......400.......410.......420.......430.......440.......450
```

CLUSTAL X (1.8) MULTIPLE SEQUENCE ALIGNMENT

File: /usr/people/alri/final_results/Erum2580.ps   Date: Tue Jul 19 12:14:03 2005
Page 3 of 4

```
             ***************************************************************************
pCMViUBs     GCAGAAGTATATGATAAAAATGCTCGTTTAACAATAATACGAATAACAGAACTAGCAGAGAAAATTAAGCAATTA  525
Welgevonden  GCAGAAGTATATGATAAAAATGCTCGTTTAACAATAATACGAATAACAGAACTAGCAGAGAAAATTAAGCAATTA  525
rLSDV-stock  GCAGAAGTATATGATAAAAATGCTCGTTTAACAATAATACGAATAACAGAACTAGCAGAGAAAATTAAGCAATTA  525
rLSDV-cDNA   GCAGAAGTATATGATAAAAATGCTCGTTTAACAATAATACGAATAACAGAACTAGCAGAGAAAATTAAGCAATTA  370
pET          GCAGAAGTATATGATAAAAATGCTCGTTTAACAATAATACGAATAACAGAACTAGCAGAGAAAATTAAGCAATTA  525
ruler        .......460.......470.......480.......490.......500.......510.......520.....

             ***************************************************************************
pCMViUBs     CTTAATACTGTTAAAACAAAACCTTATGTTGTTACCCACGATGCATATCAATATTTTGAAAAGTATTTTGGACTT  600
Welgevonden  CTTAATACTGTTAAAACAAAACCTTATGTTGTTACCCACGATGCATATCAATATTTTGAAAAGTATTTTGGACTT  600
rLSDV-stock  CTTAATACTGTTAAAACAAAACCTTATGTTGTTACCCACGATGCATATCAATATTTTGAAAAGTATTTTGGACTT  600
rLSDV-cDNA   CTTAATACTGTTAAAACAAAACCTTATGTTGTTACCCACGATGCATATCAATATTTTGAAAAGTATTTTGGACTT  445
pET          CTTAATACTGTTAAAACAAAACCTTATGTTGTTACCCACGATGCATATCAATATTTTGAAAAGTATTTTGGACTT  600
ruler        ..530.......540.......550.......560.......570.......580.......590.......600

             ***************************************************************************
pCMViUBs     AATTTTATAACATCACTTTCTTCAAGTCATAATACAAATATTAGTGTTAAAAAGTTAACACATATACAAAAAGTT  675
Welgevonden  AATTTTATAACATCACTTTCTTCAAGTCATAATACAAATATTAGTGTTAAAAAGTTAACACATATACAAAAAGTT  675
rLSDV-stock  AATTTTATAACATCACTTTCTTCAAGTCATAATACAAATATTAGTGTTAAAAAGTTAACACATATACAAAAAGTT  675
rLSDV-cDNA   AATTTTATAACATCACTTTCTTCAAGTCATAATACAAATATTAGTGTTAAAAAGTTAACACATATACAAAAAGTT  520
pET          AATTTTATAACATCACTTTCTTCAAGTCATAATACAAATATTAGTGTTAAAAAGTTAACACATATACAAAAAGTT  675
ruler        .......610.......620.......630.......640.......650.......660.......670.....
```

CLUSTAL X (1.8) MULTIPLE SEQUENCE ALIGNMENT

File: /usr/people/alri/final_results/Erum2580.ps Date: Tue Jul 19 12:14:03 2005

Page 4 of 4

```
             ******************************************************************
pCMViUBs     ATCACTAAAAACAATATATCATGTATTTTTTCTGAGTCTCAAAATGATAAAATTAGAAATTTATTTTCTCAACAT 750
Welgevonden  ATCACTAAAAACAATATATCATGTATTTTTTCTGAGTCTCAAAATGATAAAATTAGAAATTTATTTTCTCAACAT 750
rLSDV-stock  ATCACTAAAAACAATATATCATGTATTTTTTCTGAGTCTCAAAATGATAAAATTAGAAATTTATTTTCTCAACAT 750
rLSDV-cDNA   ATCACTAAAAACAATATATCATGTATTTTTTCTGAGTCTCAAAATGATAAAATTAGAAATTTATTT--------- 585
pET          ATCACTAAAAACAATATATCATGTATTTTTTCTGAGTCTCAAAATGATAAAATTAGAAATTTATTTTCTCAACAT 750
ruler        ..680.......690.......700.......710.......720.......730.......740.......750

pCMViUBs     AAAGTAACATTTCAAATACTGGATCCTATAGGGAATATTGCACAAAAAGAATCGTACTTTGACATTATGCAAAAC 825
Welgevonden  AAAGTAACATTTCAAATACTGGATCCTATAGGGAATATTGCACAAAAAGAATCGTACTTTGACATTATGCAAAAC 825
rLSDV-stock  AAAGTAACATTTCAAATACTGGATCCTATAGGGAATATTGCACAAAAAGAATCGTACTTTGACATTATGCAAAAC 825
rLSDV-cDNA   --------------------------------------------------------------------------- 585
pET          AAAGTAACATTTCAAATACTGGATCCTATAGGGAATATTGCACAAAAAGAATCGTACTTTGACATTATGCAAAAC 825
ruler        .......760.......770.......780.......790.......800.......810.......820.....

pCMViUBs     ATTGCTAATAATTTTTTCAGTTGTCTATCAACTACATAA------------------------------------ 864
Welgevonden  ATTGCTAATAATTTTTTCAGTTGTCTATCAACTACATAA------------------------------------ 864
rLSDV-stock  ATTGCTAATAATTTTTTCAGTTGTCTATCAACTACATAA------------------------------------ 864
rLSDV-cDNA   --------------------------------------------------------------------------- 585
pET          ATTGCTAATAATTTTTTCAGTTGTCTATCAACTACAAAGGGCGAGCTCAAGCTTGAAGGTAAGCCTATCCCTAAC 900
ruler        ..830.......840.......850.......860.......870.......880.......890.......900
```

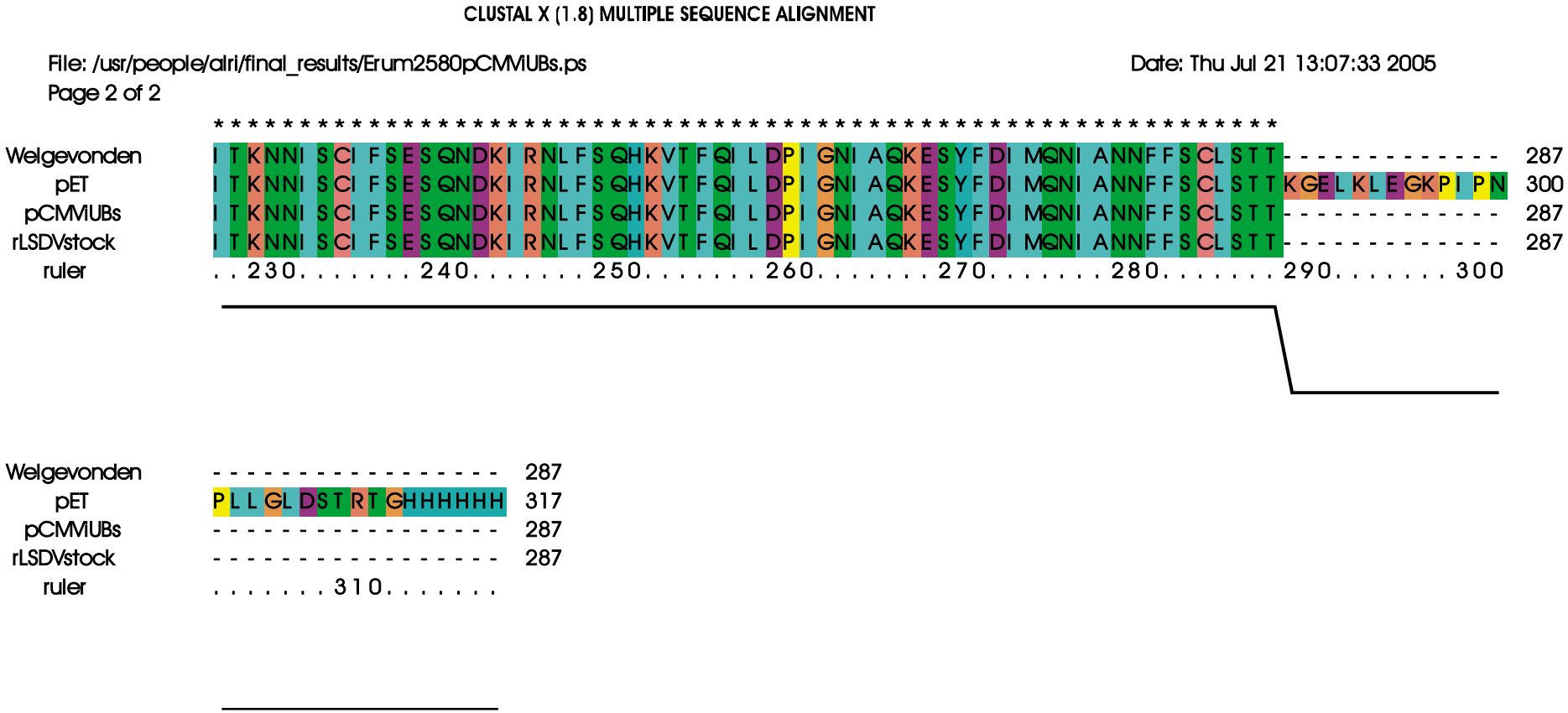
Erum2580 amino acid alignments

CLUSTAL X (1.8) MULTIPLE SEQUENCE ALIGNMENT

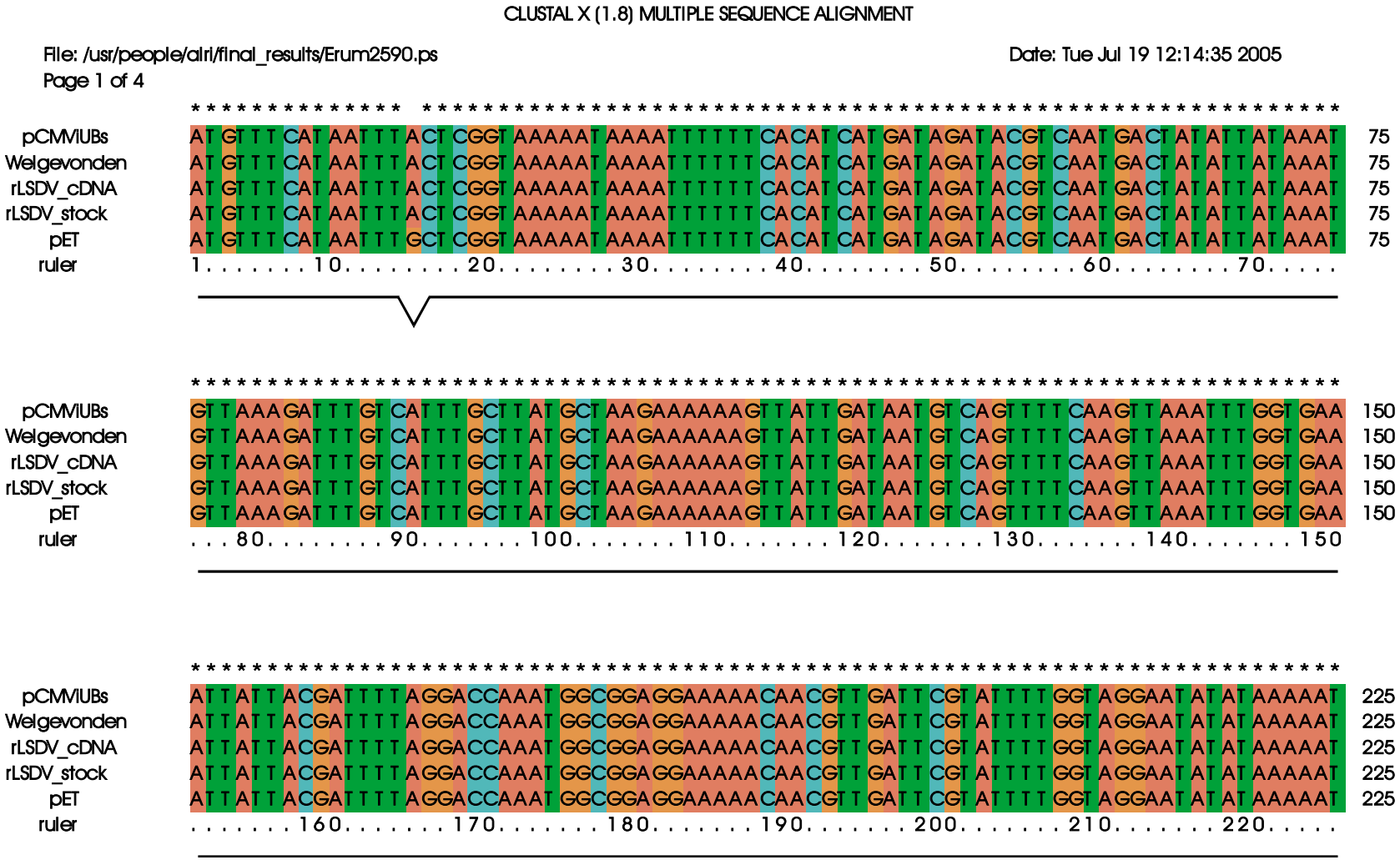
File: /usr/people/alri/final_results/Erum2580pCMVUBs.ps Date: Thu Jul 21 13:07:33 2005

Page 1 of 2

```
                 ********************************************************************************
Welgevonden      MKHIIFLLFLYISLYPITGYSVPKIIATINPIYSLVDAVTEGVTKPVLLINQQISIHDYMLKPSDKRKIRSSNVI  75
pET              MKHIIFLLFLYISLYPITGYSVPKIIATINPIYSLVDAVTEGVTKPVLLINQQISIHDYMLKPSDKRKIRSSNVI  75
pCMVUBs          MKHIIFLLFLYISLYPITGYSVPKIIATINPIYSLVDAVTEGVTKPVLLINQQISIHDYMLKPSDKRKIRSSNVI  75
rLSDVstock       MKHIIFLLFLYISLYPITGYSVPKIIATINPIYSLVDAVTEGVTKPVLLINQQISIHDYMLKPSDKRKIRSSNVI  75
ruler            1.......10.........20.........30.........40.........50.........60.........70.....

                 ********************************************************************************
Welgevonden      FYVDDHLETFINKIKDKTLIKLSDVVALLPSRYDSHFSYKVHTTSNDLHIWLSPDNAKKIVEHIKLVLCKIDPEN  150
pET              FYVDDHLETFINKIKDKTLIKLSDVVALLPSRYDSHFSYKVHTTSNDLHIWLSPDNAKKIVEHIKLVLCKIDPEN  150
pCMVUBs          FYVDDHLETFINKIKDKTLIKLSDVVALLPSRYDSHFSYKVHTTSNDLHIWLSPDNAKKIVEHIKLVLCKIDPEN  150
rLSDVstock       FYVDDHLETFINKIKDKTLIKLSDVVALLPSRYDSHFSYKVHTTSNDLHIWLSPDNAKKIVEHIKLVLCKIDPEN  150
ruler            ...80.........90.......100.......110.......120.......130.......140.......150

                 ********************************************************************************
Welgevonden      AEVYDKNARLTIIRITELAEKIKQLLNTVKTKPYVVTHDAYQYFEKYFGLNFITSLSSSHNTNISVKKLTHIGKV  225
pET              AEVYDKNARLTIIRITELAEKIKQLLNTVKTKPYVVTHDAYQYFEKYFGLNFITSLSSSHNTNISVKKLTHIGKV  225
pCMVUBs          AEVYDKNARLTIIRITELAEKIKQLLNTVKTKPYVVTHDAYQYFEKYFGLNFITSLSSSHNTNISVKKLTHIGKV  225
rLSDVstock       AEVYDKNARLTIIRITELAEKIKQLLNTVKTKPYVVTHDAYQYFEKYFGLNFITSLSSSHNTNISVKKLTHIGKV  225
ruler            .......160.......170.......180.......190.......200.......210.......220.....
```


CLUSTAL X (1.8) MULTIPLE SEQUENCE ALIGNMENT
File: /usr/people/alri/final_results/Erum2580pCMVIUBs.ps
Date: Thu Jul 21 13:07:33 2005
Page 2 of 2
Welgevonden ITKNNISCIFSESQNDKIRNLFSQHKVTFQILDPIGNIAQKESYFDIMQNIANNFFSCLSTT---------- 287
pET ITKNNISCIFSESQNDKIRNLFSQHKVTFQILDPIGNIAQKESYFDIMQNIANNFFSCLSTTKGELKLEGKPIPN 300
pCMVIUBs ITKNNISCIFSESQNDKIRNLFSQHKVTFQILDPIGNIAQKESYFDIMQNIANNFFSCLSTT---------- 287
rLSDVstock ITKNNISCIFSESQNDKIRNLFSQHKVTFQILDPIGNIAQKESYFDIMQNIANNFFSCLSTT---------- 287
ruler ..230.....240.....250.....260.....270.....280.....290.....300
Welgevonden ---------- 287
pET PLLGLDSTRTGHHHHHH 317
pCMVIUBs ---------- 287
rLSDVstock ---------- 287
ruler .....310.....

Erum2590 sequence alignments



CLUSTAL X (1.8) MULTIPLE SEQUENCE ALIGNMENT

File: /usr/people/alri/final_results/Erum2590.ps   Date: Tue Jul 19 12:14:35 2005

Page 2 of 4

```
             ***************************************************************************
pCMViUBs     TATCTAGGAATAGTGGAATATGCAAAAAATTTTGTGATAGGATATTTGCCACAGAATTTTAGTGTAAACTCTCTT 300
Welgevonden  TATCTAGGAATAGTGGAATATGCAAAAAATTTTGTGATAGGATATTTGCCACAGAATTTTAGTGTAAACTCTCTT 300
rLSDV_cDNA   TATCTAGGAATAGTGGAATATGCAAAAAATTTTGTGATAGGATATTTGCCACAGAATTTTAGTGTAAACTCTCTT 300
rLSDV_stock  TATCTAGGAATAGTGGAATATGCAAAAAATTTTGTGATAGGATATTTGCCACAGAATTTTAGTGTAAACTCTCTT 300
pET          TATCTAGGAATAGTGGAATATGCAAAAAATTTTGTGATAGGATATTTGCCACAGAATTTTAGTGTAAACTCTCTT 300
ruler        ..230.......240.......250.......260.......270.......280.......290.......300

             ***************************************************************************
pCMViUBs     ATTCCAATGACTGTTGAATATTTTTTAGTATCTAGTTATACAAAACAGAGAAAAAAATTAAATCTAAATAGTGTG 375
Welgevonden  ATTCCAATGACTGTTGAATATTTTTTAGTATCTAGTTATACAAAACAGAGAAAAAAATTAAATCTAAATAGTGTG 375
rLSDV_cDNA   ATTCCAATGACTGTTGAATATTTTTTAGTATCTAGTTATACAAAACAGAGAAAAAAATTAAATCTAAATAGTGTG 375
rLSDV_stock  ATTCCAATGACTGTTGAATATTTTTTAGTATCTAGTTATACAAAACAGAGAAAAAAATTAAATCTAAATAGTGTG 375
pET          ATTCCAATGACTGTTGAATATTTTTTAGTATCTAGTTATACAAAACAGAGAAAAAAATTAAATCTAAATAGTGTG 375
ruler        .......310.......320.......330.......340.......350.......360.......370.....

             ***************************************************************************
pCMViUBs     TTGAAAGATGTGAATGTTGTAAAAATCTTGAATAGGCAAATGTCAGAAATATCTCATGGGGAATTACAATTAGTG 450
Welgevonden  TTGAAAGATGTGAATGTTGTAAAAATCTTGAATAGGCAAATGTCAGAAATATCTCATGGGGAATTACAATTAGTG 450
rLSDV_cDNA   TTGAAAGATGTGAATGTTGTAAAAATCTTGAATAGGCAAATGTCAGAAATATCTCATGGGGAATTACAATTAGTG 450
rLSDV_stock  TTGAAAGATGTGAATGTTGTAAAAATCTTGAATAGGCAAATGTCAGAAATATCTCATGGGGAATTACAATTAGTG 450
pET          TTGAAAGATGTGAATGTTGTAAAAATCTTGAATAGGCAAATGTCAGAAATATCTCATGGGGAATTACAATTAGTG 450
ruler        ..380.......390.......400.......410.......420.......430.......440.......450
```

CLUSTAL X (1.8) MULTIPLE SEQUENCE ALIGNMENT

File: /usr/people/alri/final_results/Erum2590.ps

Date: Tue Jul 19 12:14:35 2005

Page 3 of 4

```
             ***************************************************************************
pCMViUBs     TTACTTGCTAGATGTTTAATGCTTAATCCTGATATTATAATTTTAGATGAGCCTGTGAGTTGTATGGATATTAAT  525
Welgevonden  TTACTTGCTAGATGTTTAATGCTTAATCCTGATATTATAATTTTAGATGAGCCTGTGAGTTGTATGGATATTAAT  525
rLSDV_cDNA   TTACTTGCTAGATGTTTAATGCTTAATCCTGATATTATAATTTTAGATGAGCCTGTGAGTTGTATGGATATTAAT  525
rLSDV_stock  TTACTTGCTAGATGTTTAATGCTTAATCCTGATATTATAATTTTAGATGAGCCTGTGAGTTGTATGGATATTAAT  525
pET          TTACTTGCTAGATGTTTAATGCTTAATCCTGATATTATAATTTTAGATGAGCCTGTGAGTTGTATGGATATTAAT  525
ruler        .......460.......470.......480.......490.......500.......510.......520.....

             ***************************************************************************
pCMViUBs     GCAAAAGATAGTTTTTATAAGTTAATTAATCAGCTGATATTGAGATATAATCTAAGTGTGATTATGACCTCTCAT  600
Welgevonden  GCAAAAGATAGTTTTTATAAGTTAATTAATCAGCTGATATTGAGATATAATCTAAGTGTGATTATGACCTCTCAT  600
rLSDV_cDNA   GCAAAAGATAGTTTTTATAAGTTAATTAATCAGCTGATATTGAGATATAATCTAAGTGTGATTATGACCTCTCAT  600
rLSDV_stock  GCAAAAGATAGTTTTTATAAGTTAATTAATCAGCTGATATTGAGATATAATCTAAGTGTGATTATGACCTCTCAT  600
pET          GCAAAAGATAGTTTTTATAAGTTAATTAATCAGCTGATATTGAGATATAATCTAAGTGTGATTATGACCTCTCAT  600
ruler        ..530.......540.......550.......560.......570.......580.......590.......600

             ***************************************************************************
pCMViUBs     GATTTGCATTTTGTTATGGCTAACTCTTATCGTGTGATATGTATCAATAAGAGTATCTATTGTGAAGGATCTCCT  675
Welgevonden  GATTTGCATTTTGTTATGGCTAACTCTTATCGTGTGATATGTATCAATAAGAGTATCTATTGTGAAGGATCTCCT  675
rLSDV_cDNA   GATTTGCATTTTGTTATGGCTAACTCTTATCGTGTGATATGTATCAATAAGAGTATCTATTGTGAAGGATCTCCT  675
rLSDV_stock  GATTTGCATTTTGTTATGGCTAACTCTTATCGTGTGATATGTATCAATAAGAGTATCTATTGTGAAGGATCTCCT  675
pET          GATTTGCATTTTGTTATGGCTAACTCTTATCGTGTGATATGTATCAATAAGAGTATCTATTGTGAAGGATCTCCT  675
ruler        .......610.......620.......630.......640.......650.......660.......670.....
```

CLUSTAL X (1.8) MULTIPLE SEQUENCE ALIGNMENT

File: /usr/people/alri/final_results/Erum2590.ps   Date: Tue Jul 19 12:14:35 2005

Page 4 of 4

```
             **************************************************
pCMViUBs     AGTGAAATTGTAAAAAATGAAAAATTTTTAAAAATGTTCTCATCTTATGCTTAA--------------------- 729
Welgevonden  AGTGAAATTGTAAAAAATGAAAAATTTTTAAAAATGTTCTCATCTTATGCTTAA--------------------- 729
rLSDV_cDNA   AGTGAAATTGTAAAAAATGAAAAATTTTTAAAAATGTTCTCATCTTATGCTTAA--------------------- 729
rLSDV_stock  AGTGAAATTGTAAAAAATGAAAAATTTTTAAAAATGTTCTCATCTTATGCTTAA--------------------- 726
pET          AGTGAAATTGTAAAAAATGAAAAATTTTTAAAAATGTTCTCATCTTATGCTAAGGGCGAGCTCAAGCTTGAAGGT 750
ruler        ..680.......690.......700.......710.......720.......730.......740.......750
```

Erum2590 amino acid alignments

CLUSTAL X (1.8) MULTIPLE SEQUENCE ALIGNMENT

File: /usr/people/alri/final_results/Erum2590pCMViUBs.ps   Date: Thu Jul 21 13:13:09 2005

Page 1 of 2

```
             ***************************************************************************
Welgevonden  MFHNLLGKNKIFSHHDRYVNDYIINVKDLSFAYAKKKVIDNVSFQVKFGEIITILGPNGGGKTTLIRILVGIYKN  75
pET          MFHNLLGKNKIFSHHDRYVNDYIINVKDLSFAYAKKKVIDNVSFQVKFGEIITILGPNGGGKTTLIRILVGIYKN  75
pCMViUBs     MFHNLLGKNKIFSHHDRYVNDYIINVKDLSFAYAKKKVIDNVSFQVKFGEIITILGPNGGGKTTLIRILVGIYKN  75
rLSDVstock   MFHNLLGKNKIFSHHDRYVNDYIINVKDLSFAYAKKKVIDNVSFQVKFGEIITILGPNGGGKTTLIRILVGIYKN  75
ruler        1.......10........20........30........40........50........60........70.....

             ***************************************************************************
Welgevonden  YLGIVEYAKNFVIGYLPQNFSVNSLIPMTVEYFLVSSYTKQRKKLNLNSVLKDVNVVKILNRQMSEISHGELQLV  150
pET          YLGIVEYAKNFVIGYLPQNFSVNSLIPMTVEYFLVSSYTKQRKKLNLNSVLKDVNVVKILNRQMSEISHGELQLV  150
pCMViUBs     YLGIVEYAKNFVIGYLPQNFSVNSLIPMTVEYFLVSSYTKQRKKLNLNSVLKDVNVVKILNRQMSEISHGELQLV  150
rLSDVstock   YLGIVEYAKNFVIGYLPQNFSVNSLIPMTVEYFLVSSYTKQRKKLNLNSVLKDVNVVKILNRQMSEISHGELQLV  150
ruler        ...80........90.......100.......110.......120.......130.......140.......150

             ***************************************************************************
Welgevonden  LLARCLMLNPDIIILDEPVSCMDINAKDSFYKLINQLILRYNLSVIMTSHDLHFVMANSYRVICINKSIYCEGSP  225
pET          LLARCLMLNPDIIILDEPVSCMDINAKDSFYKLINQLILRYNLSVIMTSHDLHFVMANSYRVICINKSIYCEGSP  225
pCMViUBs     LLARCLMLNPDIIILDEPVSCMDINAKDSFYKLINQLILRYNLSVIMTSHDLHFVMANSYRVICINKSIYCEGSP  225
rLSDVstock   LLARCLMLNPDIIILDEPVSCMDINAKDSFYKLINQLILRYNLSVIMTSHDLHFVMANSYRVICINKSIYCEGSP  225
ruler        .......160.......170.......180.......190.......200.......210.......220.....
```

CLUSTAL X (1.8) MULTIPLE SEQUENCE ALIGNMENT

File: /usr/people/alri/final_results/Erum2590pCMViUBs.ps **Date: Thu Jul 21 13:13:09 2005**

Page 2 of 2

```
                    * * * * * * * * *
Welgevonden         SEIVKNEKFLKMFSSYA- - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  242
pET                 SEIVKNEKFLKMFSSYAKGELKLEGKPIPNPLLGLDSTRTGHHHHHH                                  272
pCMViUBs            SEIVKNEKFLKMFSSYA- - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  242
rLSDVstock          SEIVKNEKF- - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  234
ruler               . . 230. . . . . . . 240. . . . . . . 250. . . . . . . 260. . . . . . . 270. .
```

8. APPENDIX C: PUBLICATIONS AND ETHICS

8.1 PUBLICATIONS

The research presented in this thesis has been published in the following articles:

1. Pretorius A, Collins NE, Steyn HC, van Strijp F, van Kleef M, Allsopp BA. 2007. Protection against heartwater by DNA immunisation with four *Ehrlichia ruminantium* open reading frames. *Vaccine* **25**: 2316-24.

2. Collins NE, Liebenberg J, de Villiers EP, Brayton KA, Louw E, Pretorius A, Faber FE, van Heerden H, Josemans A, van Kleef M, Steyn HC, van Strijp MF, Zweygarth E, Jongejan F, Maillard JC, Berthier D, Botha M, Joubert F, Corton CH, Thomson NR, Allsopp MT, Allsopp BA. 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 U S A.* **102:** 838-43.

3. Collins NE, Pretorius A, Van Kleef M, Brayton KA, Zweygarth E, Allsopp BA. 2003. Development of improved vaccines for heartwater. *Annals of the New York Academy of Sciences* **990:** 474-84.

4. Collins NE, Pretorius A, van Kleef M, Brayton KA, Allsopp MT, Zweygarth E, Allsopp BA. 2003. Development of improved attenuated and nucleic acid vaccines for heartwater. *Developments in Biologicals* **114:** 121-36

5. Pretorius A, Collins NE, Steyn HC, Allsopp BA. 2002. Sequence analysis of three *Ehrlichia ruminantium* LambdaGEM-11 clones. *Annals of the New York Academy of Sciences* **969:**155-8.

Article in preparation for publication (Chapter 4):

1. Pretorius A, Collins NE, van Strjip F, Louw E, Faber FE, Thikhudo, N, Allsopp BA, and Van Kleef M. A DNA prime- recombinant protein boost immunisation strategy protects against *E. ruminantium* needle challenge but not against a field challenge. *Infection and Immunity*

8.2 ETHICS

The research reported in this thesis was approved by the Animal Ethics committees at the ARC-Onderstepoort Veterinary Institute and the University of Pretoria Animal Use and Care committee (under protocol V017/04)

Letters of approval are attached