In vitro and *in vivo* evaluation of five low molecular weight proteins of *Ehrlichia ruminantium* as potential vaccine components

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Abstract

Low molecular weight (LMW) proteins of *E. ruminantium* can induce proliferation of immune peripheral blood mononuclear cells (PBMCs) and the production of interferon-gamma (IFN-γ) by CD4+-enriched T-cells. In this study, a reverse vaccinology approach was applied to identify additional vaccine candidates focusing on genes that encode LMW proteins smaller than 20 kDa. Five open reading frames (ORFs) were selected from the *E. ruminantium* genome and their corresponding recombinant (r) proteins were produced in a bacterial expression system. Their ability to induce proliferative responses and IFN-γ production was evaluated *in vitro* using lymphocyte proliferation and ELISPOT assays. All five recombinant proteins induced proliferation of immune PBMCs and IFN-γ production by these cells. The corresponding five genes were each individually incorporated into pCMViUBs, a mammalian expression vector and tested as a potential vaccine in sheep using a DNA prime - protein boost immunisation regimen. A cocktail of these DNA constructs protected one out of five sheep against a virulent *E. ruminantium* (Welgevonden) needle challenge. Three of the five vaccinated sheep showed an increase in their proliferative responses and production of IFN-γ before challenge. This response decreased after challenge in the sheep that succumbed to the challenge and increased in the sheep that survived. This finding indicates that sustained IFN-γ production is likely to be involved in conferring protective immunity against heartwater.

Keywords: E. ruminantium, recombinant proteins, DNA vaccine, ELISPOT

Abbreviations: LMW, low molecular weight; MAP, major antigenic protein; ORFs, open reading frames; RI, reaction index; SI, stimulation index; SFC, spot forming cells

1. Introduction

Ehrlichia ruminantium is an obligate intracellular bacterium that is transmitted by ticks of the *Amblyomma* species. It causes heartwater in domestic and wild ruminants. In South Africa, the disease is controlled either by acaricides or by an immunisation regimen in which animals are injected with infected blood from a donor animal and subsequently treated with antibiotics (Van der Merwe, 1987). This method is cumbersome and requires an uninterrupted cold storage chain. There is a risk of transmitting live organisms and the method does not protect against all isolates (Du Plessis et al., 1989; Jongejan et al., 1988). These limitations highlight the need for an improved heartwater vaccine. A recombinant subunit vaccine may be a practical and affordable alternative, but developing such a vaccine requires an understanding of the protective immune responses and the identification of antigens that elicit such responses.

Protection against heartwater is mediated by cellular immune responses characterised by the proliferation of CD4+, CD8+ and γδT-cells as well as the expression of IFN-γ, a

cytokine associated with Th1 response. Due to its inhibitory effects on the growth of *E. ruminantium in vitro*, IFN-γ is considered a key mediator of these protective immune responses (Totté et al., 1996). The search for protective antigens has therefore focused on antigens that can induce the production of IFN-γ. Several *E. ruminantium* proteins have already been investigated for their ability to induce cellular immune responses *in vitro*. For instance, electrophoreticaly separated proteins from the *E. ruminantium* Welgevonden isolate with sizes ranging from 11-21 kDa and 31 kDa induced cellular immune responses that were characterised by the proliferation of immune PBMCs and CD4+-enriched T-cells which produced enhanced levels of IFN-γ (Van Kleef et al., 2000; 2002). Similarly, antigenic proteins of 15-19 kDa and 23-29 kDa from the Gardel isolate induced IFN-γ production in immune PBMCs (Esteves et al., 2004a). These findings suggest that proteins in the low molecular weight (LMW) region of 11-19 kDa and 23-29 kDa are likely to include potential vaccine antigens. Often, however, fractionation by preparative electrophoresis results in the presence of multiple proteins within a single fraction which makes it difficult to identify the actual immunologically active protein. Reverse vaccinology uses bioinformatics to select potentially protective proteins *in silico* (Rappuoli, 2001). Several algorithms that identify genes that encode exported-, membrane- or surface-associated proteins are applied (Muzzi et al., 2007). Once selected, these antigens can then be evaluated for their ability to induce appropriate immune

responses *in vitro* before being tested *in vivo* for their efficacy. The availability of the complete genome sequence of the Welgevonden isolate of *E. ruminantium* (Collins et al., 2005) now makes it possible to apply this approach to heartwater.

Using bioinformatics, a reductive selection approach was applied to *E. ruminantium* genome to identify genes that code for potential vaccine candidates. This approach identified 102 ORFs from the whole genome. From this, five genes encoding LMW proteins of less than 20 kDa were selected and expressed in *E. coli.* The resulting recombinant proteins were tested *in vitro* for their ability to induce proliferative responses as well as IFN-y production by primed lymphocytes. These ORFs were also tested in the form of a DNA vaccine for their ability to induce protective immunity in sheep against *E. ruminantium* infection following a needle challenge.

2. Materials and Methods

2.1. Selection of ORFs

Several algorithms were used to select ORFs. The subcellular localisation was predicted using CELLO (http://cello.life.nctu.edu.tw/) and PSORTb (http://www.psort.org/psortb/). The prediction of signal peptides and transmembrane helices was performed using TMHMM and SignalP respectively, while verification of these features was performed

using Phobius (http://phobius.cgb.ki.se/ & http://phobius.binf.ku.dk/). Molecular weight and pI of the proteins were predicted using the Compute pI/MW tool (http://us.expasy.org/tools/pi_tool.html).

2.2. Expression of recombinant proteins

Protein expression was done using the pET102/TOPO® expression system (Invitrogen) following the manufacturer's instructions. Sequence specific primers (Table 1) were designed for each ORF to facilitate cloning into the pET vector. The recombinant plasmids pET-ORFs were sequenced to verify that nucleotide sequence was translated into the correct peptide sequence and to ensure that the His-tag was transcribed. BL21Star™ (DE3) cells (Invitrogen) were transformed with pET-ORFs and expression was induced with 1 mM IPTG. The recombinant proteins were purified using the Protino® Ni 1000 prepacked columns (Macherey-Nagel) following the manufacturer's instructions and analysed with SDS-PAGE using Criterion™XT Precast Gels, 4-12 % gradient (Bio-Rad) followed by Western Blot analysis using Anti-His_6 antibodies (Roche). The concentration of the recombinant proteins was determined using the RC DC Protein Assay (Biorad).

2.3. Immunological assay

PBMCs for immunological assays were obtained from five immune sheep (S147, S151, S849, S5400, and S5401) immunised by infection and treatment method and one naïve sheep (S5408). PBMCs isolation was carried out using the Ficoll purification method as previously described (Van Kleef et al., 2000).

2.3.1. Lymphocyte proliferation assays

Lymphocyte proliferation assays were performed as described previously (Van Kleef et al., 2000). The assay was carried out in a 96 well half-area plate in triplicates in a total volume of 100 µl complete RPMI-1640 medium. PBMCs (2 x 10^5 cells/well) were stimulated with the five test recombinant proteins individually at final concentrations of 0.1 ng/ml, 10 ng/ml and 10.0 µg/ml respectively, crude *E. ruminantium* isolated from infected bovine endothelial cells (1 µg/ml, positive antigen), negative antigen (uninfected bovine endothelial cell extract, 1 µg/ml) and ConA. For the negative control, PBMCs were stimulated with a negative recombinant protein (Neg. rprotein) which was an *E. ruminantium* recombinant protein (rErum4930) that tested negative previously. Additional control wells consisted of medium or unstimulated PBMCs. The cells were

incubated at 37 oC in a humidified atmosphere with 5 % CO_2 for 3 days and pulsed with 1 μCi/well of [^{3}H] thymidine and incubated for an additional 18 h. Cells were harvested and the radioactivity was counted using a Trilux 1450 Microbeta scintillation counter (Wallace). The results were expressed as the stimulation index (SI) calculated by the mean cpm of the test recombinant protein or negative recombinant protein (averaged from triplicate wells) divided by the mean cpm of the unstimulated PBMCs. A response was considered positive if it was two times higher than the SI of the Neg. rprotein with a significant *p*-value. Additionally the response induced by test recombinant proteins in immune sheep was compared to the response induced in naïve sheep. An SI value obtained in immune sheep was regarded positive if it was two times higher that the SI value obtained in naive sheep.

2.3.2. IFN-γ ELISPOT assay

The IFN-γ ELISPOT assay was adopted from a method published by Mwangi et al., 2002 with modifications. Briefly, the assay was carried out in triplicates in a 96 well MAIPS 4510 Multiscreen™-IP filtration plates (Millipore). The plates were coated overnight with mouse anti-bovine IFN-γ mAb CC302 (1μg/ml) at 4 °C, and washed three times with

unsupplemented RPMI-1640 followed by blocking with RPMI-1640 supplemented with 10 % FCS for 2 h at 37°C. PBMCs (2 x10^5/well) were stimulated with the same antigens as in the proliferation assay and incubated for 20 hours at 37°C in a humidified atmosphere with 5% CO_2. The plates were washed three times with 0.05% distilled water-Tween(T), three times with 0.05% PBS-T and incubated with rabbit anti-bovine IFN-γ anti-serum (Immonodiagnostik) diluted 1/1500 in PBS-T/1%BSA for 1 h at room temperature. The plates were washed four times with 0.05% PBS-T followed by incubation with anti-rabbit IgG alkaline phosphatase conjugate (Sigma) diluted 1/2000 in PBS-T/1%BSA for 1 h at room temperature. The plates were washed six times with 0.05% PBS-T followed by addition of 50 µl of substrate solution (Sigma Fast BCIP/NBT substrate tablets) and incubation in the dark for 10 -15 minutes. The plates were washed for two minutes under running water and dried overnight. Spot forming cells (SFC) were enumerated using an automated ELISPOT reader (Zeiss KS ELISPOT Compact 4.5). The number of SFC produced after stimulation of immune PBMCs with the recombinant proteins was compared to the number of SFC produced after stimulation with recombinant negative protein as well as the number of SFC produced after stimulation of naïve PBMCs with the recombinant proteins.

2.4. Cloning of the five ORFs into pCMViUBs vector

The five ORFs were PCR amplified from *E. ruminantium* genomic DNA using specific primers (Table 1) containing restriction enzyme sites for *Bam HI* and *Xba I* to facilitate directional cloning into a pCMViUBs vector. The PCR products were first cloned into pGEM-T Easy vector using 1 U T4 ligase (Roche) and clones with correct-sized inserts were sequenced with the ABI 3100 automated sequencer (Applied Biosystems). The inserts were cut out of the recombinant plasmid (pGEM T-Easy_ORF) and individually ligated into the pCMViUBs vector and sequenced as above. Large scale DNA preparation was done in *E. coli* and the resulting DNA was purified using NucleoBond'® Xtra Maxi purification Kit (Macherey-Nagel) following the manufacturer's instructions. The plasmid DNA was quantified and diluted to a final concentration of 1 mg/ml in endotoxin-free PBS (Sigma) and stored at -20 ºC until use. An aliquot of each DNA construct was sequenced before immunisation.

2.5. Immunisation and challenge of sheep

Ten to twelve month old Merino sheep were purchased from heartwater free areas of

South Africa and tested negative for *E. ruminantium* using the pCS20 real-time PCR assay (Steyn et al., 2008). A group of five sheep were immunised at 3 week intervals with 2 inoculations of a cocktail of five DNA vaccine constructs. Each sheep received 200 μg plasmid DNA delivered by i.m. injection and 20 μg plasmid DNA delivered i.d. by gene gun as previously described by Pretorius et al., 2007. Gene gun inoculations were done using the OPgun™ (Brayton et al., 1997). This DNA prime was followed by a protein boost with a cocktail of the five corresponding recombinant proteins (150 μg of each). The negative control group *(n* = 5), was immunised with the empty pCMViUBs vector followed by 750 μg of unrelated, *E. coli* derived recombinant β-galactosidase protein. Additional controls were non immunised naïve animals *(n* = 2) and the positive control *(n* = 2) immunised by the standard infection and treatment method. Five weeks after protein boost, the animals were challenged with the virulent *E. ruminantium* Welgevonden stock ($10LD_{50}$) prepared as previously described (Brayton et al., 2003).

Body temperatures were taken daily and after challenge the animals were monitored for onset of clinical symptoms. To determine the severity of the infection, clinical signs were scored using a reaction index (RI) as described previously (Pretorius et al., 2007).

Animals with body temperatures raised above 42 ºC combined with any one of the following symptoms: loss of appetite, heavy breathing, depression, hanging head, stiff gait, exaggerated blinking, chewing movements, anorexia and signs of nervous symptoms

were euthanased using 200 mg sodium pentobarbitone (Eutha-Nase, Centaur) per kg body mass to prevent prolonged suffering. These animals were regarded as non survivors.

2.6. Cellular Immunology Assays

For cellular immunology assays all the animals were bled before immunisation (naïve), before challenge (four weeks after final boost) as well as one week after challenge. PBMCs were isolated from whole blood at the three set points and stimulated with a cocktail of the five recombinant proteins (1.0 μg/ml) for use in lymphocyte proliferation and IFN-γ ELISPOT assays as described above.

2.7. Statistical analysis

The significance of differences between lymphocyte proliferation results, ELISPOT assay results and RI scores were determined by Student's *t*-test; differences were considered significant at $p < 0.01$.

3. Results

3.1. Selection of five LMW proteins and their expression

Several standard bioinformatic methods were used to select 102 *E. ruminantium* ORFs from the complete genome sequence encoding proteins with high probability of being surface-associated or membrane proteins, secreted proteins, transporters and with similarity to proven protective antigens or known virulence factors (manuscript in preparation). From this, five ORFs that encode LMW proteins of between 13 and 18 kDa (Erum7340, Erum7350, Erum7360, Erum7380 and Erum4360; Table 2) were selected for our study because LMW proteins were previously identified to induce specific cell mediated immune responses (Van Kleef et al., 2000; 2002). Four of these proteins were predicted to be membrane associated and the remaining protein had no known function. The genes were expressed in *E. coli* as thioredoxin and His-tagged fusion proteins which added 16 kDa to their predicted molecular masses. As shown in Fig. 1A and B, all the recombinant proteins had the expected molecular masses except for rErum7380 which migrated at approximately 44 kDa on a SDS-PAGE gel instead of at 33 kDa. Although all proteins were pure, multiple bands were observed with rErum4360 (Fig. 1A) which could have been due to contamination with *E. coli* proteins or degradation due to *E. coli* proteases. The recombinant proteins were present mainly in a form of insoluble inclusion bodies although they were also present in the soluble fractions (Fig. 1A and B).

3.2. Lymphocyte proliferation assay

The ability of each recombinant protein to induce proliferative responses was evaluated using PBMCs obtained from three immune sheep designated S147, S151 and S849 as well as a naïve control animal (S5408). All the recombinant proteins specifically induced immune PBMCs to proliferate with SI values that were at least double that of naïve PBMCs. Optimum responses occurred at different concentrations of the recombinant protein with each individual animal (Table 3). Four of the proteins (rErum7340 soluble (s), rErum7350s, rErum7360s and rErum4360s) induced significant proliferation when tested with PBMCs from S147. In the case of PBMCs from S151, rErum7350 insoluble (i) and rErum4360i induced a significant proliferative response. While with PBMCs from S849, rErum7340i, rErum7350i and rErum7380i induced significant proliferation. When testing the recombinant proteins or crude *E. ruminantium* positive antigen on PBMCs from naïve S5408, no significant proliferation was induced.

3.3. Antigen-specific IFN-yproduction

Each of the five recombinant proteins was tested for its ability to induce IFN-γ production in PBMC using an ELISPOT assay (Table 4). As with the proliferative responses, they induced IFN-γ production that varied amongst the different immune animals. When tested with PBMCs from the naïve S5408, only rErum7360s and rErum7360i induced IFN-γ production. There was no significant IFN-γ production when the recombinant proteins were tested with PBMCs from S151 and S849. However, with PBMCs from S147; rErum7340, rErum7380s and rErum4360i did induce a positive IFN-

γ response. The recombinant Erum7350s, rErum7350s and rErum4360 induced a positive response with PBMCs from S5400 while rEum7340s, rErum7350 and rErum7360i gave positive responses with PBMCs from S5401. Although rErum7360 induced non-specific responses in PBMCs from the naïve sheep, the response induced by rErum7360i in PBMCs from S5401 was regarded positive because it was twice that elicited in PBMCs from the naive animal (S5408).

3.4. DNA prime - protein boost immunisation

Inoculating sheep with a cocktail of five ORFs (Erum7340, Erum7350, Erum7360, Erum7380 and Erum4360) given as a DNA prime - recombinant protein boost, resulted in one of the five sheep surviving challenge with virulent *E. ruminantium* (Table 5). The four sheep that died showed increased body temperatures as well as severe heartwater symptoms before death as shown by their RI scores of between 76.5 and 86.3 (Fig. 2). In contrast, the single sheep that survived showed no temperature increase or heartwater symptoms and had an RI score of 1.8 (Table 5; Fig. 2). Additionally, it was observed that some of the sheep in the experimental group showed elevated body temperatures earlier than the naïve and negative control animals (Table 5). The immunised positive control animals survived challenge and did not show any reaction in terms of temperature increases and/or heartwater symptoms as shown by the low RI scores of between 1.3 and 1.6 (Table 5; Fig. 2). None of the naïve and negative control sheep survived and these animals showed temperature increases as well as severe heartwater symptoms (Table 5; Fig. 2).

3.5. Cellular immune responses in sheep immunised by DNA prime - protein boost

In an attempt to understand the immune responses induced by the vaccination and those associated with protection against *E. ruminantium* needle challenge, lymphocyte proliferation and IFN-γ ELISPOT assays were carried out. Accordingly, PBMCs were isolated before immunisation, four weeks after immunisation and one week after challenge. PBMCs of three sheep (S888, S894, and S895) inoculated with the cocktail DNA and cocktail protein had significant positive SI values compared to the sheep inoculated with the empty vector both before and after challenge. There was, however, a decrease in the SI values of two of these sheep (S888 and S894) after challenge. These animals both succumbed to infection. In contrast, the single sheep that survived challenge (S895), showed an increase in the SI value after challenge. Three sheep (S889, S894, and S895) showed a significant number of IFN-γ producing cells before challenge, while after challenge four sheep (S888, S889, S894, and S895) showed significant IFN-y production. Of the animals that were positive before challenge, S889 and S894 showed a decrease in the number of SFC after challenge and these sheep succumbed to the disease. Both S888 and S895 showed a significant increase in the number of SFC after challenge. However S888 died while S895 survived.

4. Discussion

Five ORFs that coded for proteins with low molecular weights ranging from 13.32 – 17.51 kDa and were predicted to be membrane or surface-associated except for

rErum4360 (which is unknown) were selected for this study. Membrane proteins are regarded as good vaccine candidates because they are likely to have epitopes that are exposed on the surface of the pathogen (Pizza et al., 2000). The corresponding recombinant proteins were produced in *E. coli*. One of them, rErum7380, had a molecular weight of~44 kDa which was larger than the expected 34 kDa. SDS-PAGE can yield anomalous mobility for glycoproteins or proteins with low pI (Marciani and Papamatheakis, 1978; Alves *et al.*, 2004). It is not known whether rErum7380 is a glycoprotein but it had a predicted pI value of 4.16 which was the lowest of all the five proteins and this could have affected its mobility. Both soluble and insoluble fractions were tested in the cellular immunological assays. Protein denaturation or insolubility usually does not affect the outcome of the cellular immunological assays because T-cell epitopes recognise proteins in the form of small peptide fragments (Hickling, 1998). Leung and co-workers (2004) showed that recombinant proteins in the form of inclusion bodies still induced cellular immune responses even after denaturation.

Since Th1 cellular immune responses mediated by IFN-γ are essential for protection against *E. ruminantium* (Totté et al., 1999a), the ability of the five recombinant proteins to induce recall lymphocyte proliferative responses and IFN-γ production was first investigated *in vitro*. Although specific CD4+ T-cell lines have been recommended for identifying potential vaccine candidates (Totte et al., 1999b; Van Kleef et al., 2002) immune PBMCs have also been used successfully to test *E. ruminantium* proteins (Barbet et al., 2001; Totté et al., 1998; Van Kleef et al., 2000). Every one of the five rErum proteins tested induced proliferation of immune PBMCs and the production of IFN-γ as measured in the ELISPOT assay. These results are in agreement with earlier findings

showing that LMW proteins induce IFN-γ production in CD4+ T-cell lines as well as immune PBMCs (Esteves et al., 2004a; Van Kleef et al., 2002). Although all five proteins induced proliferation of immune PBMCs and IFN-γ responses, the results varied between cells from the different immune animals. Such variable responses have been reported previously (Barbet et al., 2001). Similarly, Ag-specific IFN-γ production can also vary between different immune animals due to the genetic diversity of outbred animals (Esteves et al., 2004b). The sheep used in this study were not inbred, a factor which may explain the variable responses obtained.

A recent study showed that a DNA prime - boost immunisation with four 1H12 ORFs and their corresponding recombinant proteins protected five out of five sheep against a needle challenge with *E. ruminantium* Welgevonden (Pretorius et al., 2008). This was, however, not the case with the five ORFs tested in our study since only one of the five sheep was protected. It is not clear whether one of the five ORFs contributed to the protection obtained, or whether the effect was co-operative. Further testing of these ORFs as single gene constructs is therefore necessary. The use of multiple immunogens in DNA vaccine formulations can affect the immune response either positively (Jiang et al., 2007) or negatively (Sedegah et al., 2004). There are several factors that could lead to suppression of responses induced by cocktail DNA vaccines. These include the amount of each DNA vaccine construct used for priming, gene expression after plasmid uptake, competition for transcription/translation factors, and antigenic interference among encoded proteins. Antigenic interference results in inhibition of expression of other antigens and Ag-specific immune responses to the individual antigens (Hooper et al., 2003; Kjerrstörm et al., 2001). When testing Ag-specific immune responses in our study, a cocktail of the five

proteins was used as the stimulatory agent. It will therefore be necessary to test individual recombinant proteins to determine whether there was antigenic interference amongst the antigens.

After immunisation, recall lymphocyte proliferation assays showed that Ag-specific proliferation was induced. This finding confirmed that the recombinant pCMViUBs constructs expressed gene products and that an immune response was stimulated. Additionally, the ELISPOT assay showed a significant increase in the number of IFN-γ producing cells. These responses decreased after challenge in the sheep that succumbed to the disease and increased in the animal that survived challenge. Differences in proliferative and IFN-γ responses upon challenge of vaccinated animals were reported previously in cattle infected with *Babesia bovis* and *Anaplasma mariginale* (East et al., 1997; Abbott et al., 2005). A decrease in antigen-specific immune responses upon immunisation or challenge may be due to development of a regulatory response which could be confirmed by analysing additional cytokines like IL-10 and TGF-β1. Another possible explanation for the decrease in IFN-γ production could be that pathogen-specific lymphocytes were not circulating in the peripheral blood compartment at the time of isolation. Instead they may have been in other immunological compartments such as lymph nodes where *E. ruminantium* is initially detected after challenge before entering the blood stream (Du Plessis, 1970). As observed in other studies, analysis of cellular immune responses in other immunological compartments like lymph nodes and spleen is essential in challenge studies (Esteves et al., 2004c; Vachiery et al., 2006). IFN-y is regarded as a key mediator of protective immunity against *E. ruminantium* infection (Totté et al., 1996) and the production of this cytokine could be used as an indicator for

successful immunisation (Esteves et al., 2002). However, a lack of correlation between IFN-y production and protective immunity against *E. ruminantium* has been reported (Vachiery et al., 2006). In accordance with this finding, some of the sheep in our study showed significant IFN-y production after vaccination but succumbed to the disease with evidence of a decreased number of IFN-y producing cells after challenge. Reduction of both Ag-specific proliferation and IFN-γ producing cells in mice
was shown to be associated with susceptibility to fatal *Ehrlichia* infection with the highly virulent *Ixodes ovatis* ehrlichia strain. Additionally IFN-γ production by CD8+ T-cells did not protect mice suggesting that production of IFN-γ by CD4+ T-cells might be essential for protection (Ismail et al., 2004). In our study, it is possible that CD8+ T-cells were activated since the pCMViUBs vector used is known to activate CD8+ cytotoxic T-cell responses (Andersson and Barry 2004). CD8+ T-cells could therefore, have been the dominating sub-population and source of IFN-γ in the unprotected sheep.

In conclusion, we have shown that five recombinant LMW proteins of *E. ruminantium* induced re-call lymphocyte proliferative responses and IFN-γ production *in vitro*. Additionally, when the corresponding ORFs were tested in sheep as a cocktail DNA vaccine using the prime-boost strategy, significant cellular immune responses characterised by Ag-specific proliferative responses and IFN-γ production were induced. Our data also showed that these Ag-specific responses were reduced upon challenge in

sheep that succumbed to the disease and increased in the sheep that survived challenge. This highlights the need to analyse additional cytokines and the phenotype of the cells producing them for use as indicators of protection. Future research should include analysis of memory T-cell responses in other immunological compartments as well as testing of the ORFs as single gene constructs.

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Table 1

Primer sequences used for the amplification of the five ORFs for cloning into pET vector as well as for cloning into the pCMViUBs vector with restriction enzyme sites underlined.

Table 2

Characteristics of five ORFs encoding LMW proteins selected from *E. ruminantium* genome.

Table 3

Proliferative responses of PMBC from immune S147, S151, S849 and naïve S5408 to five recombinant proteins of **£** *ruminantium.*

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Proliferative responses and IFN-y production induced in sheep immunised with cocktail DNA vaccine in comparison to sheep immunised with the empty vector before and after challenge.

Fig. 1.

Western blot analysis of rErum proteins expressed in *E. coli.* Proteins were purified from both the soluble cellular fraction ("s") and inclusion bodies ("i"). Lane M represents molecular weight markers. The rproteins were detected using anti His antibodies. After purification, an equivalent amount of the four precipitated rErum proteins was loaded on to the gel (A). An aliquot of the purified rErum7340 was loaded on the gel before precipitation (B).

Fig. 2.

Average RI scores of sheep immunised with cocktail DNA vaccine using prime - boost strategy compared to sheep immunised with the empty vector. Sheep were needle challenged with the lethal dose of *E. ruminantium* Welgevonden stock and only sheep 895 (indicated with an arrow) and sheep in the infected and treated group survived challenge. The black dots represent the RI score of each sheep in the group.

Table 1. Primer sequences used for the amplification of the five ORFs for cloning into pET vector as well as for cloning into the pCMViUBs vector with restriction enzyme sites underlined.

ORF	**Primer sequence (5′–3′)**	T_a (°C)[a]
Erum7340 F	CACCATGTTAGATGCTAGTAAAGATGTATCTATTCAAGAGC	
Erum7340 R	TGACTGCTCAACAGTTTTACCATTAACACC	53
Erum7350 F	CACCATGGTGAGTATTTGTTGTCAAGGTACC	
Erum7350 R	GAAAGAGTGACGTCTAGTATTATGTCCCG	52
Erum7360 F	CACCATGATAGGTAGGTGTAATAATAATGGTCGG	
Erum7360 R	GATAGAATTACGTCTCAAACTACAATTCAATGC	52
Erum7380 F	CACCATGGTAGTTATAAGTATTATGTCTCTCTTATTC	
Erum7380 R	TATTGATTGACAGCCTTCCACACTAC	51
Erum4360 F	CACCATGAGCGATGACTCTAAAAAAACAGGTG	
Erum4360 R	AAATTCATCTTCTATTTTCTTTACTATATCTTGTGG	54
Erum7340 F	GGATCCATGTTAGATGCTAGTAAAGATG	
Erum7340 R	TCTAGATTATTCTATTGACTGCTCAACAG	50
Erum7350 F	GGATCCGTGGTGAGTATTTTGTTG	
Erum7350 R	TCTAGATTAGAAAGAGTGACGTCTAGTATTATG	50
Erum7360 F	GGATCCATGATAGGTAGGTGTAATAATAATGG	
Erum7360 R	TCTAGATCAGATAGAATTACGTCTCAAACTAC	50
Erum7380 F	GGATCCATGGTAGTTATAAGTATTATGTCTC	
Erum7380 R	TCTAGATCATATTGATTGACAGCCTTC	50
Erum4360 F	GGATCCATGAGCGATGACTC	
Erum4360 R	TCTAGATTAAACACCAAATTCATCTTCTATTTTC	50

[a] Annealing temperature (T_a) used for primer pair.

Table 2. Characteristics of five ORFs encoding LMW proteins selected from *E. ruminantium* genome.

ORF	**Name/Function of protein**	**Mw (kDa)**	**Predicted Mw after expression**	**Actual Mw after expression**
Erum7340	Membrane protein	13.32	29	29
Erum7350	Membrane protein	16.17	32	32
Erum7360	Membrane protein	16.13	32	32
Erum7380	Membrane protein	17.23	33	~**44**
Erum4360	Unknown	17.51	33	33

Table 3. Proliferative responses of PMBCs from immune S147, S151, S849 and naïve S5408 to five recombinant proteins of *E. ruminantium*.

Test protein/Antigen	**Siam for sheep number:**				
	147[b]	**151**[b]	**5408**[b]	**849**[c]	**5408**[c]
Neg. rprotein	3.8 ± 0.1	1.2 ± 0.2	<1	1.3 ± 0.3	<1
rErum7340s	**9.0 ± 2.2**[*]	1.3 ± 0.4	<1	1.6 ± 0.5	1.3 ± 0.010
rErum7340i	5.2 ± 1.7	1.6 ± 0.6	<1	**4.0 ± 1.3**[*]	1.1 ± 0.17
rErum7350s	**14.8 ± 1**[*]	1.0 ± 0.1	1.9 ± 0.093	1.7 ± 0.3	1.0 ± 0.023
rErum7350i	5.6 ± 0.8	**2.9 ± 0.3**[*]	1.0 ± 0.0085	**3.8 ± 1.0**[*]	1.0 ± 0.504
rErum7360s	**15.8 ± 2.1**[*]	1.9 ± 0.7	<1	2.3 ± 0.7	1.0 ± 0.095
rErum7360i	5.2 ± 1.2	0.9 ± 0.05	<1	2.0 ± 0.3	<1
rErum7380s	5.7 ± 0.3	1.3 ± 0.5	<1	2.1 ± 0.2	1.2 ± 0.10
rErum7380i	3.7 ± 0.3	0.9 ± 0.06	<1	**3.0 ± 0.0**[*]	<1
rErum4360s	5.7 ± 0.5	2.0 ± 0.3	<1	1.4 ± 0.1	1.3 ± 0.129
rErum4360i	**15.4 ± 0.4**[*]	**5.8 ± 1.1**[*]	<1	1.4 ± 0.1	<1
Pos. antigen	37 ± 4.8	4.5 ± 1.6	<1	4.7 ± 1.2	1.3 ± 0.19
Con A	1024.5 ± 76.7	371.7 ± 51	64.1 ± 6.1	350 ± 18.5	64.1 ± 6.1

The SI values of the rErum proteins were compared to the SI value of the neg. rprotein. Only SI values that were two times higher than the SI of the neg. rprotein and had significant p values ($^{*}p \leq 0.01$ as determined by Student's t-test) were regarded as positive and these are indicated in bold.

[a] cpm rErum protein/cpm unstimulated PBMCs.

[b] Recombinant proteins were tested at a concentration of 1 µg/ml.

[c] Recombinant proteins were tested at a concentration of 10 µg/ml.

Table 4. IFN-γ production by PBMCs from immune S147, S151, S849, S5400, S5401 and naïve S5408 in response to five recombinant proteins. Recombinant proteins were tested at 2.5 μg/ml.

Test protein/Antigen	**Number of SFC/million PBMCs for sheep number**					
	147	**151**	**849**	**5400**	**5401**	**5408**
Neg. rprotein	7 ± 0.70	10 ± 3.46	0	10 ± 1.15	13	0
rErum7340s	**17 ± 3.21***	3 ± 0.57	0	0	**67 ± 1.41***	0
rErum7340i	**15 ± 1.00***	3 ± 0.57	0	0	20 ± 0.57	0
rErum7350s	13 ± 2.31	2 ± 0.57	0	**36 ± 4.94***	**44 ± 4.94***	0
rErum7350i	2 ± 1.53	8 ± 0.57	0	6 ± 0.70	**42 ± 5.65***	0
rErum7360s	12 ± 2.08	0	0	37 ± 7.23	0	19 ± 0.70
rErum7360i	0	3 ± 0.70	0	0	**72 ± 5.65***	10 ± 1.527
rErum7380s	**20 ± 1.41***	0	0	0	0	0
rErum7380i	0	0	0	0	0	0
rErum4360s	0	0	0	**96 ± 4.94***	0	0
rErum4360i	**23 ± 3.05***	5 ± 1.00	0	**67 ± 4.50***	0	0
Pos. antigen	427 ± 4.93	20 ± 0.00	80 ± 6.55	37 ± 2.51	63 ± 1.52	0
Con A	352 ± 5.13	352 ± 7.57	512 ± 13.57	232 ± 18.58	442 ± 1.73	

The number of SFC of the rErum proteins were compared to the number of SFC of the neg. rprotein. Only the number of SFC that were two times higher than the number of SFC of the Neg. rprotein and had significant *p* values ($^{*}p \leq 0.01$ as determined by Student's *t*-test) were regarded as positive and these are indicated in bold.

Table 5. Summary of survival of sheep immunised with a cocktail DNA vaccine and cocktail protein boost.

Group	**Sheep number**	**Days to temperature above 40 °C**	**Highest temperature reached**	**Survival (S) or ethanased (E) or Dead (D) on (day) shown**
Naïve	885	12	42.0	E (15)
	898	13	41.1	D (16)
Infected and treated	5400	–	39.2	S
	5401	–	39.0	S
Empty vector prime—unrelated protein boost	887	13	41.9	D (17)
	890	13	41.9	D (17)
	893	13	41.7	E (15)
	896	13	42.0	D (17)
	897	14	42.0	E (16)
E. ruminantium DNA Cocktail prime—boost	888	11	42.0	E (15)
	889	11	42.0	D (17)
	891	13	41.5	D (16)
	894	11	42.0	E (16)
	895	–	39.9	S

Table 6. Proliferative responses and IFN-γ production induced in sheep immunised with cocktail DNA vaccine in comparison to sheep immunised with the empty vector before and after challenge.

Group	**Sheep number**	**SI_{AVE}[a] (Before challenge)**	**SI_{AVE}[b] (After challenge)**	**SFC/10^6 PBMC[c] (Before challenge)**	**SFC/10^6 PBMC[d] (After challenge)**
Empty vector prime—unrelated protein boost	887	2.9 ± 0.02	2.7 ± 0.58	0	0
	890	0.5 ± 0.03	0.6 ± 0.15	0	10 ± 0.58
	893	1.3 ± 0.15	2.4 ± 0.21	0	3 ± 0.70
	896	1.3 ± 0.18	3.1 ± 0.15	0	13 ± 0.40
	897	3.4 ± 0.56	0.6 ± 0.03	0	0
E. ruminantium DNA Cocktail prime—boost	888	4.4 ± 0.44*	3.8 ± 0.46*	4 ± 0.70	**44 ± 0.70***
	889	2.2 ± 0.67	2.2 ± 0.34	**115 ± 6.66***	**58 ± 0.65***
	891	1.3 ± 0.23	2.2 ± 0.43	5 ± 0.70	0
	894	12.1 ± 0.59*	6.9 ± 2.43*	**53 ± 0.70***	**22 ± 0.56***
	895	5.4 ± 0.39*	10.0 ± 2.43	**19 ± 0.70***	**112 ± 2.08***

SI_{AVE} values of the sheep in the cocktail DNA prime-boost group were compared to the empty vector group. Only SI_{AVE} that were greater than two times the average SI of the empty vector group and have significant p values ($^*p \leq 0.01$ as determined by Student's t-test) were regarded as positive and are in bold. The number of IFN-γ producing cells (SFC) of sheep in the cocktail DNA prime-boost group before and after challenge was compared to the naïve number of SFC. Only SFC that were twice the naïve SFC or more and had significant $^*p$ values ($p \leq 0.01$ as determined by student-t test) were regarded as positive and these are indicated in bold.

[a] SI before challenge/SI naïve.
[b] SI after challenge/SI naïve.
[c] SFC before challenge-SFC naïve.
[d] SFC after challenge-SFC naïve.

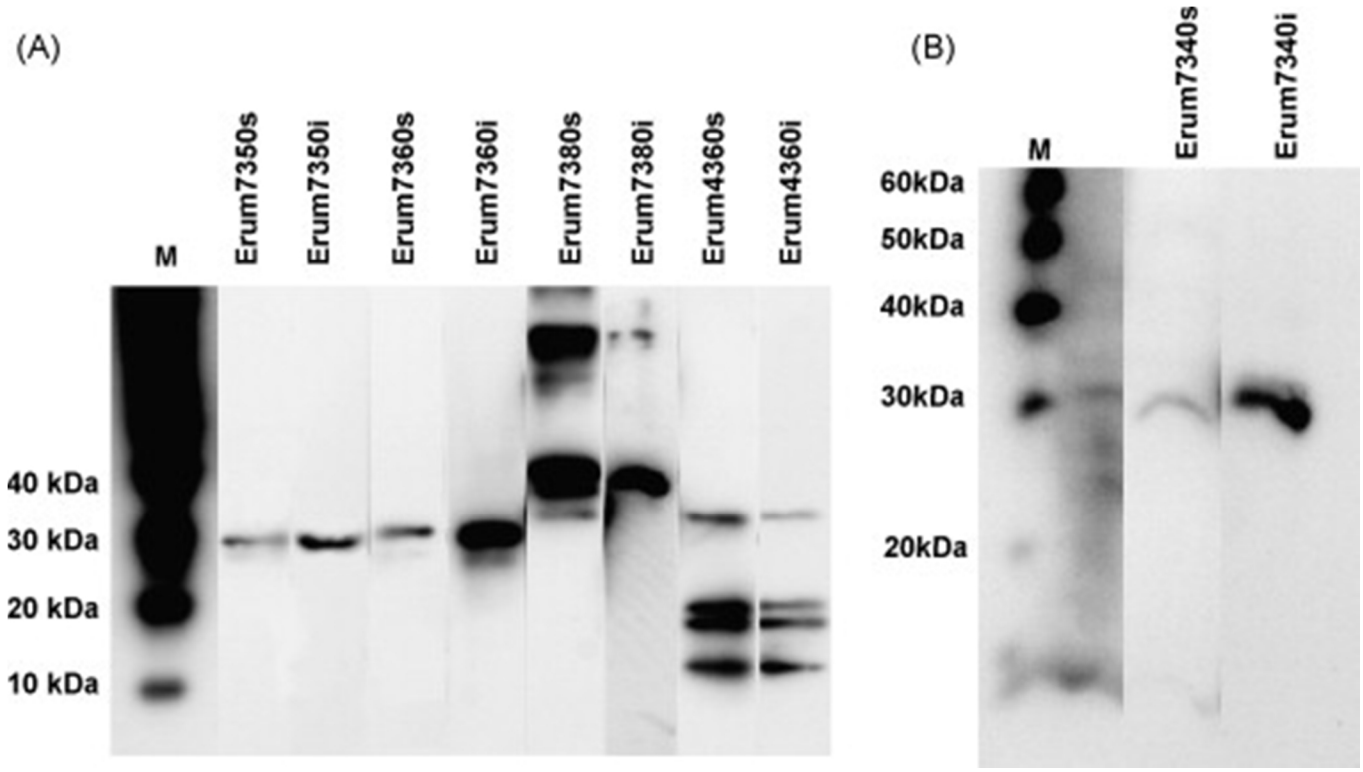

Figure 1. Western blot analysis of rErum proteins expressed in *E. coli*. Proteins were purified from both the soluble cellular fraction ("s") and inclusion bodies ("i"). Lane M represents molecular weight markers. The rproteins were detected using anti His antibodies. After purification, an equivalent amount of the four precipitated rErum proteins was loaded on to the gel (A). An aliquot of the purified rErum7340 was loaded on the gel before precipitation (B).

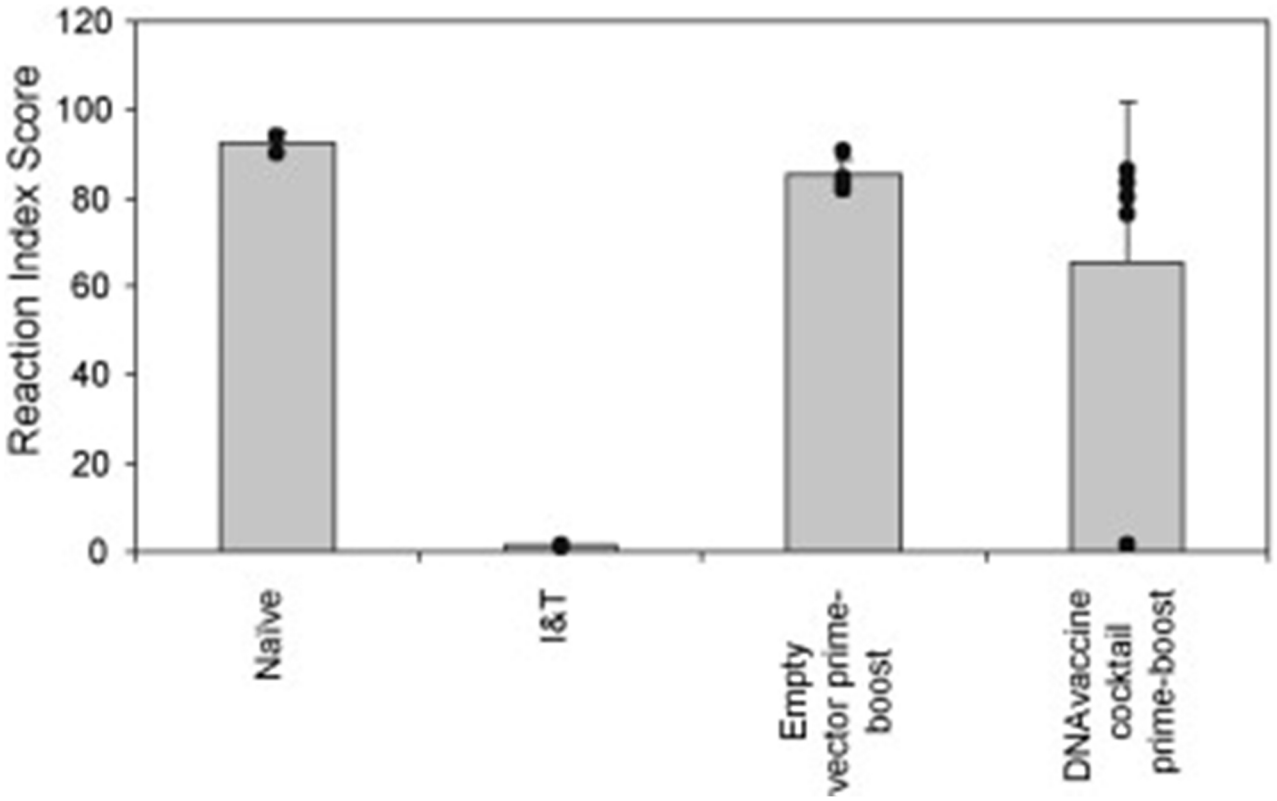

Figure 2. Average RI scores of sheep immunised with cocktail DNA vaccine using prime—boost strategy compared to sheep immunised with the empty vector. Sheep were needle challenged with the lethal dose of *E. ruminantium* Welgevonden stock and only sheep 895 (indicated with an arrow) and sheep in the infected and treated group survived challenge. The black dots represent the RI score of each sheep in the group.