

Protection against heartwater by DNA immunisation with four *Ehrlichia ruminantium* open reading frames

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Abstract

We have reported previously that a recombinant DNA vaccine consisting of four *Ehrlichia ruminantium* (Welgevonden) open reading frames (ORFs) known as the 1H12 cocktail provided protection against a virulent *E. ruminantium* (Welgevonden) needle challenge in sheep. In this study, we have investigated the vaccine effectiveness of two other cocktails of *E. ruminantium* (Welgevonden) ORFs, as well as single ORFs from the 1H12 cocktail, to protect sheep against a virulent needle challenge with the homologous strain. Each individual 1H12 ORF provided protection, but all the animals vaccinated with the other cocktails succumbed to the challenge.

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1. Introduction

Ehrlichia ruminantium, a rickettsial agent transmitted to wild and domestic ruminants by *Amblyomma* tick species, is the causative agent of the disease known as heartwater which is prevalent in sub-Saharan Africa and the Caribbean [1,2]. In South Africa, heartwater is controlled either by acaricides to prevent tick transmission or by using a method known as infection and treatment [3]. The blood of donor animals infected with the Ball3 isolate is used to infect susceptible livestock and when a temperature response is detected the 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. The isolate used (Ball3) is also not ideal because it does not completely cross-protect against all the various field genotypes of *E. ruminantium* [4]. The use of more virulent isolates which may provide better cross-protection is not feasible, as the

onset of symptoms is faster than with Ball3 and in many cases once fever is detected it is too late to treat. The distribution of the vaccine also presents a problem in rural areas because the delivery relies on an uninterrupted cold chain. There is therefore a need for an improved vaccine against heartwater. Several attempts which have been made to improve on the current vaccine include the development of inactivated vaccines [5–9], attenuated vaccines [10,11] and major antigenic protein 1 (MAP1) DNA vaccines [12,13].

We have previously reported the identification of 11 open reading frames (ORFs) in a cosmid clone containing an *E. ruminantium* DNA fragment [14] known as 1H12. Four of these ORFs (Erum2540, Erum2550, Erum2580 and Erum2590, Table 1) were shown to induce partial protection in mice and 100% protection in sheep against homologous needle challenge when inoculated as a cocktail using a DNA vaccination strategy [4,15]. In this study, we have further investigated the immunity stimulated in sheep by *E. ruminantium* ORFs, presented as a DNA vaccine, and their ability to induce protective immunity against a lethal heartwater needle challenge. We tested the 1H12 cocktail as before, and compared the results with those obtained when using each ORF individually. As an immunological control, we also tested two

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Table 1
ORFs used for the three vaccine cocktails (1H12, WL2AP1 and WL2HCS1) selected from the *E. ruminantium* (Welgevonden) fragments sequenced previously

Cocktail	Number ^a	Closest homologies	(%) GC content ^b	Number of putative CpG islands predicted by EMBOSS: cpgplot
1H12	Erum2540	Exported protein	30.11	1
	Erum2550	ATP-binding protein part of ABC transporters	31.28	2
	Erum2580	Periplasmic solute binding protein (SBP)	26.04	1
	Erum2590	ATP-binding protein part of ABC transporters	26.88	1
WL2AP1	Erum7450	Probable integral membrane protein	30.89	3
	Erum7490	Probable inorganic polyphosphate/ATP-NAD kinase	31.06	1
	Erum7510	Hypothetical protein	28.13	1
	Erum7530	Putative conjugal transfer protein	31.78	2
WL2HCS1	Erum6480	Probable peptidase	30.08	None
	Erum6490	Hypothetical protein	31.14	None
	Erum6530	Hypothetical protein	30.64	None

^a Erum numbers are taken from Collins et al. [39].

^b Determined from the annotated genome sequence.

other cocktails composed of ORFs randomly selected from large inserts that were sequenced previously [16].

2. Materials and methods

2.1. Cloning of ORFs into pCMViUBs vector

ORFs were selected from cosmid clone 1H12 [14] and from two LambdaGEM-11 clones designated WL2AP1 and WL2HCS1 (Table 1), sequenced previously [16]. The ORFs were amplified from *E. ruminantium* genomic DNA and the PCR products were cloned into the DNA vaccine vector pCMViUBs [17] as described previously [4,15]. Briefly, the PCR, using ORF-specific primers that contained restriction enzyme sites to facilitate directional cloning (Table 2), was performed using TaKaRa Ex TaqTM (TaKaRa Bio Inc.) in a GeneAmp PCR 9700 thermocycler (Applied Biosystems). The PCR products were first cloned into pMOSBlue (Amersham Pharmacia Biotech) according to the manufacturer's instructions, and their sequences were confirmed. The

inserts were then cut out of the pMOSBlue_ORF plasmids using the restriction enzyme sites incorporated into the primer sequences and were cloned into the pCMViUBs vector [17] using T4 DNA ligase. Clones with correctly sized inserts were sequenced to confirm that the ORFs were in-frame.

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

2.2. Immunisation of sheep with the DNA vaccines

Six to eight month old Merino sheep were obtained from heartwater-free areas of South Africa. All sheep used in these experiments tested negative for *E. ruminantium* using the pCS20 probe [18]. Sheep were divided into groups of 5 and each animal received DNA via intramuscular (i.m.) inoculations into the left and right quadriceps and tibialis anterior

Table 2
Primer sequences of ORFs used for cloning into the pCMViUBs vector

Clone	Primer	Primer sequence (5' → 3') ^a	T_a (°C) ^b
WL2AP1	Erum7450 F	CGCGGATCCATGGAGGTTATTTTGAGTAGG	
	Erum7450 R	GATC <u>CTAG</u> ATTAGTTTACATACTTTTTATTTTATATTTTATTTAG	58
	Erum7490 F	CGCGGATCCATGAGCAATTATCAAAACATTGGG	
	Erum7490 R	GATC <u>CTAG</u> ATTAATGAAAAAATTGCTCATTATAATAATTC	52
	Erum7510 F	CGCGGATCCATGCTATTTTCATACATTAGATATAAATC	
	Erum7510 R	GATC <u>CTAG</u> ATTAGTTAGTAACCAGTTTTGTATATC	52
	Erum7530 F	CGCGGATCCATGAAAAATTTAGTAGTAG	
	Erum7530 R	GATC <u>CTAG</u> ACTAGCGTTTCTTTTTATTG	45
WL2HCS1	Erum6480 F	CGCGGATCCATGTTCTTATGTTTTGGTTG	
	Erum6480 R	CTAG <u>CTAG</u> ATTATTCTAATGATTTAG	43
	Erum6490 F	CGCGGATCCATGCCAGAAATATGTATCAG	
	Erum6490 R	CTAG <u>CTAG</u> ATTAATAGTATATTTCTTCCAC	48
	Erum6530 F	CGCGGATCCATGATTGATGCACAAAACCTCG	
	Erum6530 R	CTAG <u>CTAG</u> ATTATTTTATCACAAAATGC	50

^a Restriction enzyme sites incorporated into the primers to facilitate directional cloning are underlined.

^b Annealing temperature (T_a) used for primer pair.

Table 3
Reaction index for sheep challenged with *E. ruminantium* (Modified from Collins et al. [4])

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

Body temperatures and symptoms were monitored for the duration of the experiment. After challenge, animals were scored daily according to their temperature reaction, symptoms displayed and treatment received. Scores were totalled throughout the duration of the experiment.

muscles and via intradermal i.d. gene gun delivery of the same DNA. Gene gun inoculation was delivered to the lateral aspect of the pinna using the OPgunTM [19]. Before inoculation, the ear surface was depilated with ImmacTM to remove hair and dead skin, and the DNA was freshly precipitated onto gold beads as described previously [19]. The animals were immunised three times at 3-week intervals and challenged 5 weeks after the final boost with the virulent *E. ruminantium* (Welgevonden) stock (10LD₅₀) originating in South Africa [20]. Blood stabilate was prepared from an *E. ruminantium* (Welgevonden) infected animal as described previously [21]. The infected sheep blood was diluted in sucrose potassium glutamate buffer (SPG) (0.22 M sucrose, 0.01 M potassium phosphate pH 7.0, 5 mM potassium glutamate) and stored in liquid nitrogen. The stabilate was titred to determine a dose equivalent to 10LD₅₀ prior to challenge. 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) [4] with some modifications: animals that were treated were regarded as non-survivors and were therefore given a score identical to that of animals that were euthanased (Table 3). It was also decided to increase the previous score of 20 [4] for animals that were euthanased to 50 in order to differentiate more clearly between animals that succumbed to the infection and animals that survived but had a fever reaction for up to 6 days. In trial 1 sheep were euthanased in extremis using 200 mg sodium pentobarbitone (Eutha-Nase, Centaur) per kg body mass. In all subsequent animal trials it was decided to treat sheep with 200 mg oxytetracycline (Liquamycin/LA, Pfizer) per 10 kg body mass once elevated temperatures were detected combined with severe heartwa-

ter symptoms that included hyperesthesia, lacrimation and convulsions.

2.2.1. DNA vaccine trial 1: comparison of the 1H12 DNA vaccine with two other *E. ruminantium* (Welgevonden)-derived vaccine cocktails

The survival of sheep inoculated with the 1H12 cocktail was compared to that of two other cocktails composed of *E. ruminantium* ORFs selected from previously sequenced *E. ruminantium* (Welgevonden) genome fragments WL2AP1 and WL2HCS1 [16]. A total of 7 ORFs were selected: 3 from WL2HCS1 and 4 from WL2AP1 (Table 1). The groups inoculated with the 1H12 cocktail and WL2AP1 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 (five shots of each ORF containing 1 µg DNA per shot). The third group of animals was inoculated with the WL2HCS1 cocktail and received 200 µg DNA via i.m. inoculation (66.6 µg of each ORF) and 21 µg of DNA (7 µg per ORF) via gene gun inoculation. The survival of these three groups was compared to a negative challenge control group (naive, non-immunised animals), a positive challenge control group immunised using the conventional infection and treatment method using the Welgevonden isolate, and a negative DNA control group (inoculated with empty pCMViUBs vector 200 µg i.m. and 20 µg i.d.).

2.2.2. DNA vaccine trial 2: 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 Section 2.2.1. 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 these groups was compared to a negative challenge control, positive challenge control, and negative DNA control groups, as described above.

2.3. Lymphocyte proliferation assays

Peripheral blood mononuclear cell (PBMC) lymphocyte proliferation assays were performed before immunisation, and 4 weeks after the third inoculation, as described previously [22]. Briefly, a single cell suspension was prepared in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine (Sigma), 5 × 10⁻⁵ M 2-mercaptoethanol (Sigma), 0.1 mg/ml sodium benzyl penicillin (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% CO₂. In a total volume of 100 µl, 2 × 10⁵ PBMCs/well were incubated with partially purified *E. ruminantium* antigen isolated from infected bovine

endothelial cells (1 $\mu\text{g}/\text{well}$, positive antigen) or with uninfected bovine endothelial cell extract (1 $\mu\text{g}/\text{well}$, negative antigen). The *E. ruminantium* antigen was prepared by differential centrifugation as described previously [22]. Negative control wells contained PBMC without antigen and positive control wells contained PBMCs stimulated with Concanavalin A (5 $\mu\text{g}/\text{ml}$, Sigma). The cultures were incubated for 72 h and pulsed with 1 $\mu\text{Ci}/\text{well}$ of [^3H] thymidine (Amersham) for the last 6 h of the incubation period. Cells were harvested onto a 96-well glass fibre filter (Wallac) and the [^3H] 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 \pm the standard deviation. A SI greater than 2 was considered positive.

2.4. Identification of CpG islands

CpG islands were identified in the *E. ruminantium* derived ORFs by using EMBOS: cpplot (<http://www.ebi.ac.uk/emboss/cpplot>). 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 spanned a minimum of 50 bases.

2.5. Statistical analysis

RI scores and lymphocyte proliferation results were compared by means of the Student's *t*-test. Significance was assessed at *p*-values of ≤ 0.01 throughout the study.

3. Results

3.1. Identification of CpG islands

All 11 ORFs had a low GC content ranging from 26.04 to 31.78% (Table 1). Putative CpG islands with a minimum of 30% GC content were identified in the 1H12 and the WL2AP1 ORFs; the putative CpG islands in these ORFs were similar in number and size. No CpG islands were found in the WL2HCS1 ORFs (Table 1).

3.2. DNA immunisation of sheep with the 1H12 cocktail compared to immunisation with WL2AP1 and WL2HCS1 cocktails

Sheep immunised with the 1H12 cocktail survived needle challenge with a lethal dose of *E. ruminantium* (Welgevonden) (Fig. 1; Table 4). The sheep in this group showed increased RI values (the average RI value for this group was 35.8; Fig. 1) compared to the RI of the infected and treated group (an average RI score of 1; Fig. 1). This was

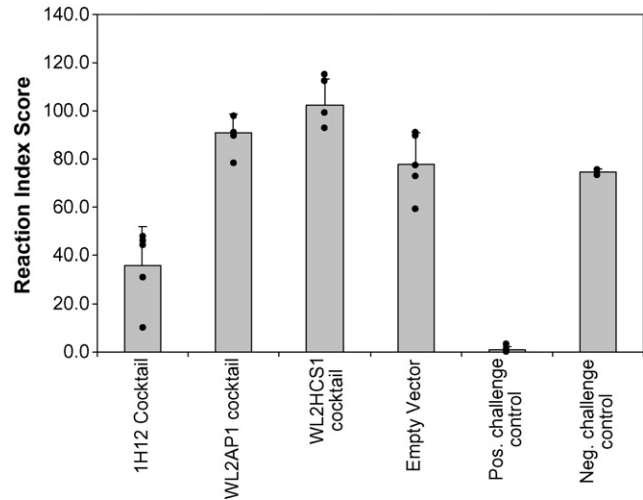


Fig. 1. The average RI of animals immunised with the 1H12 cocktail compared to animals immunised with two other *E. ruminantium* (Welgevonden) cocktails: WL2AP1 and WL2HCS1 (Table 2). The black dots represent the RI of each animal in the group.

due to temperature reactions and mild heartwater symptoms including heavy breathing and a hanging head (as observed in our previous study [4,15]), but all the animals continued to act and eat normally. In contrast, the challenge control sheep, the animals inoculated with empty vector (negative control group) and the animals immunised with the WL2AP1 and WL2HCS1 ORF cocktails, all developed severe symptoms including hyperesthesia, lacrimation and convulsions and were euthanased to prevent prolonged suffering. The RI scores of the sheep immunised with the 1H12 cocktail were significantly lower than those of the empty vector group ($p \leq 0.0006$) and those of the sheep immunised with the WL2AP1 cocktail ($p \leq 0.00006$) and the WL2HCS1 cocktail ($p \leq 0.00003$) (Fig. 1). Furthermore the RI of animals immunised with the WL2AP1 and WL2HCS1 ORF cocktails showed no significant difference ($p \leq 0.0474$ for WL2AP1 and $p \leq 0.0220$ for WL2HCS1) to the reaction indices of the negative control group immunised with empty vector (Fig. 1). However some of the animals did survive 1–4 days longer than the negative control group (Table 4) and therefore had average RI scores that were higher than that of the negative control group (Fig. 1). Sheep that were infected and treated had very low RI scores because they did not show any symptoms and only developed slight temperature reactions.

3.3. Immunisation of animals with individual 1H12 ORFs

Animals were immunised with each 1H12 ORF to determine if one or all of the individual ORFs were responsible for the protection induced by the cocktail. All the sheep immunised with the single 1H12 ORFs survived a virulent *E. ruminantium* (Welgevonden) needle challenge (Table 5).

Table 4

The survival of sheep immunised with the 1H12 cocktail compared to that of sheep immunised with WL2AP1 and WL2HCS1 cocktails and animals immunised with empty pCMViUBs vector

Group	Sheep number	Days to temperature above 40 °C	Highest temperature reached (°C)	Survival (S) or euthanased (E) on (day) shown
Empty vector	551	14	41.6	E (15)
	552	12	42	E (15)
	553	12	42	E (15)
	556	13	42	E (15)
	559	14	42	E (15)
1H12 cocktail	462	13	41.2	S
	465	14	41.8	S
	476	14	41.8	S
	484	–	39.9	S
	488	12	42	S
WL2AP1	503	12	42	E (16)
	506	12	41.9	E (16)
	512	12	42	E (17)
	514	12	42	E (16)
	515	12	42	E (17)
WL2HCS1	516	11	42	E (15)
	519	12	42	E (15)
	525	12	41.8	E (15)
	531	11	42	E (17)
	532	12	42	E (19)

Only animals immunised with the empty vector developed severe heartwater symptoms and were treated (Fig. 2). Animals immunised with the cocktail and single ORFs showed a similar increase in their RI scores (compared to the infected and treated group) as previously seen. Again this was due to the development of temperature reactions and early heartwater symptoms, but the animals continued to eat and act normally and survived without treatment (Table 5; Fig. 2). Overall the RI scores of the groups immunised with the individual ORFs were significantly lower ($p \leq 0.003$) than those

of the negative control group, while no significant difference was observed between the RI scores of sheep immunised with the single ORFs as compared to the 1H12 cocktail group (Fig. 2).

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

PBMC were isolated at two time points: before first inoculation and 4 weeks after the final boost. The SI of the PBMC of each animal per group is represented in Table 5. The PBMC of two animals (sheep 62 and 120) inoculated with the 1H12 cocktail showed positive proliferation, while none of PBMC isolated from animals inoculated with empty vector showed a significant SI increase 4 weeks after the final boost. A significant increase in the SI values was also observed in sheep immunised with Erum2540 (sheep 206), Erum2550 (sheep 130, 143 and 151), Erum2580 (sheep 208 and 229) and Erum2590 (sheep 183 and 184) (see Table 5 for SI and p -values). These results indicate that all four of the 1H12 gene products are expressed in the host cells and are able to stimulate recall immune responses.

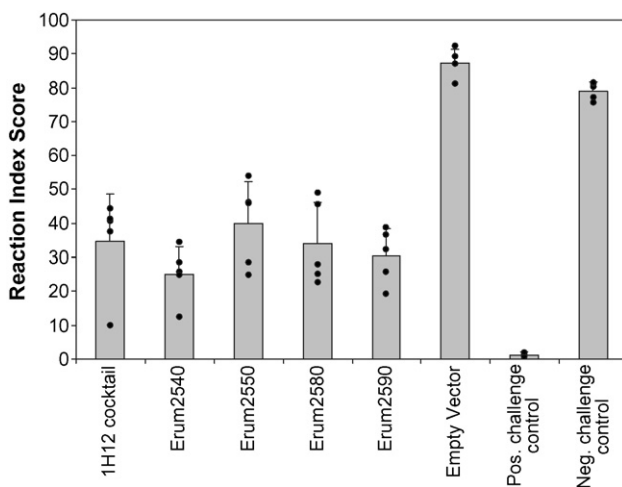


Fig. 2. The average RI of animals immunised with the 1H12 cocktail compared to animals immunised with the individual 1H12 ORFs: Erum2540, Erum2550, Erum2580 and Erum2590 (Table 2). The black dots represent the RI of each animal in the group.

4. Discussion

It is well established that IFN- γ producing T cells play an important role in protection against *E. ruminantium* infection [23–25]. However, CpG motifs in bacterial DNA can also induce pseudo-protective immune responses

Table 5
Survival and lymphocyte proliferation data of sheep inoculated with the single 1H12 ORFs compared to the 1H12 cocktail

Group	Sheep number	Days to temperature above 40 °C	Highest temperature reached (°C)	Survival (S) or Treated (T) on (day)	SI ^a (SI after boost/SI naïve)	Significance of SI ^b (<i>P</i> values)
Empty vector	46	10	41.9	T (12)	1.09 ± 0.05	
	52	10	42.0	T (13)	1.22 ± 0.39	
	55	10	42.0	T (13)	0.93 ± 0.16	
	57	10	41.8	T (13)	0.88 ± 0.25	
	61	10	42.0	T (13)	1.16 ± 0.15	
1H12 cocktail	62	10	41.8	S	2.62 ± 0.17	Yes (≤0.002)
	73	10	41.8	S	0.61 ± 0.16	No
	94	11	41.8	S	1.58 ± 0.12	No
	96	11	41.7	S	1.24 ± 0.43	No
	120	12	41.0	S	3.89 ± 0.09	Yes (≤0.006)
	129	11	42.0	S	1.60 ± 0.31	No
Erum2550	130	8	41.8	S	2.34 ± 0.64	Yes (≤0.001)
	143	10	41.8	S	2.55 ± 0.58	Yes (≤0.002)
	149	9	41.6	S	1.47 ± 0.02	No
	151	10	41.6	S	2.10 ± 0.06	Yes (≤0.00003)
Erum2590	152	11	41.8	S	1.38 ± 0.18	No
	166	12	41.5	S	0.9 ± 0.15	No
	172	11	41.4	S	0.93 ± 0.08	No
	183	11	41.8	S	2.51 ± 0.41	Yes (≤0.01)
	184	11	41.8	S	2.69 ± 0.77	Yes (≤0.00003)
Erum2540	196	9	41.6	S	0.97 ± 0.25	No
	198	10	41.6	S	1.77 ± 0.09	No
	202	11	41.7	S	1.30 ± 0.10	No
	205	12	41.7	S	1.55 ± 0.37	No
	206	11	41.7	S	7.34 ± 0.6	Yes (≤0.007)
Erum2580	208	10	41.8	S	5.29 ± 0.83	Yes (≤0.003)
	209	11	41.7	S	1.67 ± 0.07	No
	229	12	41.7	S	5.16 ± 1.04	Yes (≤0.01)
	237	11	41.7	S	1.60 ± 0.34	No

The average SI for the PBMC obtained from sheep immunised with 1H12 cocktail, and the individual ORFs were compared to that of the vector group. Only samples that were more than two times higher than vector and have significant *p* values were considered as positive and are indicated in bold.

^a SI values were averaged from triplicate wells.

^b Determined by Student's *t*-test; *p* values of *p* ≤ 0.01 were considered as significant.

by the induction of IFN- γ secretion by IL-12, expressed upon binding to host Toll-like receptor 9 [26]. Research done previously in our lab has shown that DNA immunisation using pCMViUBs plasmid constructs containing four ORFs derived from *E. ruminantium* (Welgevonden) stock protected sheep against virulent *E. ruminantium* challenge [4,15]. To exclude the possibility that the protection was due to non-specific IFN- γ secretion induced by CpG motifs in the bacterial ORF sequences, the survival of sheep immunised with the 1H12 cocktail was compared to that of animals immunised with two other cocktails derived from WL2AP1 and WL2HCS1, two *E. ruminantium* genomic clones sequenced previously [16]. As was seen in the Collins et al. [4,15] study, the 1H12 ORF cocktail conferred protection, but the animals immunised with the other *E. ruminantium* ORFs developed severe heartwater symptoms and had to be euthanased. Animals immunised with the WL2AP1 and the WL2HCS1 cocktails did survive a few days longer than the experimental control animals, which could suggest that non-specific bacterial DNA-induced

IFN- γ can delay the onset of the disease but not the final outcome.

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 but not in the WL2HCS1 ORFs. Although none of these putative CpG islands were identical to the CpG motifs previously shown to be stimulatory in sheep [27,28], 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.

Two of the 1H12 ORFs (Erum2590 and Erum2250) demonstrated extensive homology to ABC transporter ATP-binding/ATPase proteins. ABC transporters located in the

outer membrane of Gram-positive bacteria have been shown to be effective targets for antibody mediated immunity against *Staphylococcus aureus* [29]; *Enterococcus faecium* [30]; *Streptococcus pneumoniae* [31] and *Yersinia pestis* [32]. Opsonising antibodies (Th1) directed against iron uptake ABC transporters of *Streptococcus pneumoniae* were shown to increase survival of mice, after exposure to a lethal dose of bacteria, from 0 to 80% via blockage of transporter function [33]. This is corroborated by the finding that *Yersinia pestis* mutants, deficient in the metal uptake ABC transporter locus, showed abridged virulence [34]. These results were obtained in Gram-positive bacteria, where the ABC transporters are located on the outer membrane and are therefore exposed to the host immune response system. 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 protection we observed is more likely to involve either the priming of cytotoxic T cells (CTLs) 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. 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. Other genetic immunisation experiments done using the same pCMViUBs vector did show increased CTL responses against HIV antigens in monkeys, suggesting that targeting of the protein for MHC class I presentation does occur [35,36].

To determine if the protective effect was due to the combined immunological influence of all four of the ORFs or to the attributes of a single 1H12 ORF gene product, sheep were immunised with the individual 1H12 ORFs. Surprisingly, the gene products of each of the four 1H12 ORFs were able to induce protective immunity to a virulent *E. ruminantium* (Welgevonden) needle challenge. Expression of gene products by the recombinant pCMViUBs constructs, and activation of the immune response, was confirmed by recall lymphocyte proliferation assays in which only the groups immunised with the 1H12 cocktail and individual 1H12 ORFs showed antigen-specific proliferation. However, a significantly raised SI value was only detected in a few of the animals per group at a specific time point, and even then the values were in most cases only marginally higher than 2, nonetheless this does confirm that expression of the ORFs had taken place. Sheep that did not show proliferation were protected against challenge, suggesting that one can expect little correlation between protection and positive

lymphocyte proliferation results in this heartwater experimental system. Similarly, Vachierey et al. [37] 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. Enhanced proliferation by PBMC might be obtained by further optimisation of the assay, including the use of recombinant 1H12 proteins as stimulatory antigen instead of the partially purified *E. ruminantium* antigen currently used. On the other hand, CTL priming was targeted in this immunisation strategy by using the pCMViUBs vector, therefore a CTL assay might give a better indication of adaptive immune activation by the in vivo expressed 1H12 proteins. Future studies will include the optimisation of ovine specific lymphocyte proliferation and CTL assays and the measurement of IFN- γ , using real time PCR and ELISPOT techniques.

Interestingly, animals that were protected showed increased temperatures of up to 42 °C (Tables 4 and 5), but continued to act and eat normally. Fever or temperature reactions can be linked to the expression and secretion of specific pyrogenic cytokines including: IL-1, TNF- α , IL-6 and IFN- γ [38]. Protected animals producing IFN- γ would also have a fever 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 [25] that will induce fever by up-regulating the expression of TNF- α [38]. It is therefore difficult to distinguish between a “protective” fever and one induced by infection, without measuring the individual cytokine levels produced at the time of infection.

In conclusion, we have shown that immunising ruminant hosts with a DNA vaccine containing four specific *E. ruminantium* ORFs, either as part of a cocktail or individually, conferred protection against a lethal *E. ruminantium* (Welgevonden) needle challenge. Our results suggest that this protection was a direct result of the antigenic properties of the ORF protein products, not to the induction of non-specific immune pathways. These four ORFs are promising new vaccine candidates against heartwater infection and are currently being evaluated using DNA prime-recombinant protein/modified viral boost strategies.

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