Vaccine: Development and Therapy

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ORIGINAL RESEARCH

Recombinant *Rhipicephalus appendiculatus* gut (Ra86) and salivary gland cement (Trp64) proteins as candidate antigens for inclusion in tick vaccines: protective effects of Ra86 on infestation with adult *R. appendiculatus*

This article was published in the following Dove Press journal:
Vaccine: Development and Therapy
22 November 2011
Number of times this article has been viewed

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Abstract: *Rhipicephalus appendiculatus* gut protein Ra86 (variants Ra85A and Ra92A) and the salivary gland cement protein (Trp64) were expressed in the baculovirus-insect cell system. The recombinant gut proteins expressed as soluble proteins and the recombinant cement protein, as insoluble inclusion bodies, were used to immunize rabbits, which were then challenged with larval, nymphal, and adult stages of *R. appendiculatus* ticks. High tick mortality (23.3%) occurred on adult ticks that fed on rabbits vaccinated with the gut proteins, compared with 1.9% mortality in ticks that fed on unvaccinated naïve control rabbits. The mean weight of engorged female ticks was significantly reduced by 31.5% in rabbits vaccinated with the Ra86 recombinant protein compared with controls, as was egg production. Marked effects on these parameters were also observed in adult ticks as a result from vaccination using Trp64, but these were not statistically significant. For both antigens, there was no demonstrable effect on larval or nymphal ticks. This study demonstrates for the first time the protective efficacy of a homolog of *Boophilus microplus* Bm86 in reducing tick infestation by the adult stage of the three-host tick *R. appendiculatus*. The results demonstrate the potential of Ra86 for vaccine development against this tick and for the control of East Coast fever.

Keywords: baculovirus, Ra85A, Ra92A, *Boophilus decoloratus*, *Boophilus microplus*

Introduction

Ticks and tick-borne diseases constitute major constraints to the livestock industry, in particular for cattle in tropical and subtropical regions of the world.[1] There are over 825 described tick species in the world, which include Argasidae (soft ticks), Ixodidae (hard ticks), and the Nuttalliellidae, containing only a single species.[2,3] *Rhipicephalus appendiculatus* is a hard tick species widely distributed in eastern, central, and southern Africa and is the vector of *Theileria parva*, a protozoan that causes East Coast fever in cattle.[4] This is a devastating fatal disease, inducing high mortality in susceptible exotic breeds, and represents a major constraint to livestock development in the region.[5,6]

Currently, the main approach for tick control relies heavily on the use of chemical acaricides, a method associated with a number of significant disadvantages including chemical contamination of the food chain and environmental pollution, as well as the increasingly rapid development of acaricide resistance in the ticks.[7] The development of anti-tick vaccines represents a promising alternative/supplement to chemical

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control and has advantages such as environmental safety, low cost of production, and relative ease of product registration. Alternative approaches based on the use of vaccines containing recombinant midgut antigen from *Rhipicephalus* (*Boophilus) microplus* (Bm86) have been used for control of *R. microplus* infestations in the field in different regions of Central and South America and the Caribbean.[8–10] The effectiveness of Bm86-based vaccines includes both reduction of adult tick engorgement weights and female fecundity, as assessed in laboratory vaccination trials.[11]

Presently, Bm-gut antigen-based commercial vaccines are marketed under the trade names TickGARD® (Hoechst Australia Ltd, Melbourne, VIC, Australia) and Gavac® (Heber Biotec S.A., Havana, Cuba) and have been shown to be effective in the integrated control of *B. microplus* populations and other *Boophilus* species.[12] The potential of using TickGARD Plus to control *Amblyomma variagatum* and *R. appendiculatus* has been assessed; however, no significant protection was observed despite the description of a homolog of Bm86 in *R. appendiculatus*.[13,14] Homologs of Bm86 have been isolated, cloned, and sequenced in *R. appendiculatus* and shown to exist as two highly divergent allelic variants, namely Ra85A and Ra92A, with amino acid sequence identity of only 79% between them and an identity of 73% and 74% between the Ra86 variants and Bm86, respectively.[15–17]

The coding sequence for a putative tick cement cone protein (Trp64) from the salivary gland of *R. appendiculatus* has been determined, and demonstrated that Trp64 is a glycine-proline-rich protein, with a predicted molecular mass of 15.5 kDa.[18] Trp64 shares 59% amino acid identity with a tick cement cone protein (64P) from *R. appendiculatus*, previously reported to block tick-borne viral encephalitis transmission in rodent models.[19,20] The cement cone consists of several proteins secreted by tick salivary glands during tick feeding, which are believed to aid in facilitating long-term attachment of ticks to the host.[21,22] Recombinant tick salivary gland cement proteins have been shown to protect against *R. appendiculatus* tick infestation in guinea pigs.[23] In addition, a 29 kDa extra cellular matrix-like salivary gland protein from *Haemophysalis longicornis*, identified as a cement protein with an amino acid composition superficially similar to that of collagen, was shown to protect against tick challenge and resulted in high mortalities of immature tick stages in immunized rabbits.[24]

In this study, the two Ra86 variants and the Trp64 cement protein were expressed in the baculovirus-insect cell system and these antigens were evaluated as vaccine candidates for *R. appendiculatus* tick control. The level of protection induced following vaccination of rabbits with these recombinant proteins was assessed by quantifying the effect on key tick biological parameters such as engorgement weights, egg weights, and survival.

Materials and methods

Experimental immunization and tick challenge under laboratory conditions

A total of 21 healthy, naïve, out-bred New Zealand rabbits aged 14–16 weeks raised at the International Livestock Research Institute (ILRI) in Nairobi, Kenya, were used. The experiment used a randomized block design to assign 21 rabbits into two groups of seven immunized and one group of seven nonimmunized control rabbits. They were held in individual cages and fed on rabbit pellets with cocciostats (UNGA Farm Care (EA) Ltd, Nairobi, Kenya) and water ad libitum. The immunization and tick challenge experiment were carried out at ILRI and approved by the ILRI Institutional Animal Care and Use Committee.

Group 1 rabbits were inoculated intramuscularly with each of the baculovirus-expressed gut proteins (Ra85A and Ra92A) in the left and right thigh muscle, respectively, with a priming dose followed by a booster dose 4 weeks after the initial immunization. The vaccine formulation consisted of 0.5 mL of Ra85A or 0.5 mL of Ra92A antigen containing 20 µg of the recombinant protein in a saponin-based adjuvant (Q-VAC®; NOR-VET, Hvidovre, Denmark). The two antigens were formulated separately and administered on different sides of the animal on the same day to reduce any antigenic interference. Group 2 rabbits were immunized intramuscularly with 1 mL containing 25 µg of recombinant Trp64 cement protein in two equal volumes, administered in the left and right thigh muscle, followed by a booster dose 4 weeks later. Rabbits in group 3 were not immunized and represented controls without any treatment including application of adjuvant. One rabbit from group 2 (immunized with Trp64) accidentally died before challenge; therefore only six rabbits were challenged with ticks in this group. Three weeks after the booster dose, all rabbits were simultaneously challenged with 60 adult ticks (30 female and 30 male) of *R. appendiculatus*, 500 nymphs counted, and approximately 1000 larvae (based on egg weight); each stage was placed in separate cotton bags. The adults attached to the back of the rabbits, while the nymphs and larvae were attached on the right and left ear respectively.

Ticks

The *R. appendiculatus* ticks used in this study were of the Muguga line, which was originally isolated from the central

highlands of Kenya in the 1960s and thereafter maintained at the East African Veterinary Research Organization and ILRI laboratories. Ticks were reared in a biological oxygen demand (BOD) incubator at 28°C with 80% relative humidity.

Polymerase chain reaction (PCR) amplification, cloning, and expression of *R. appendiculatus* antigens

The two naturally occurring *R. appendiculatus* gut protein (Ra86) variants (Ra85A and Ra92A; GenBank accession numbers FJ8500978 and FJ850976, respectively)[17] and the salivary cement (Trp64) protein (GenBank accession number AX003897) were expressed in the baculovirus/insect cell expression system.[25] The antigens were prepared in this expression system so as to obtain a near authentic post-translation of the Ra86 variant glycoproteins. The Ra86 (Ra85A) open reading frame (ORF) was amplified, including the predicted native signal sequence, but without the putative transmembrane region (aa 668–689) using primers specifically designed to introduce a StuI restriction site upstream of the ORF and a 6× His-tag, a stop codon, and a NotI site downstream of the ORF (Table 1). The PCR product was first inserted into a pGEM-T-easy vector (Promega, Fitchburg, WI) before cloning downstream of the polyhedrin (polh) promoter in pFBD-p10GFP, a derivative of pFastBac-Dual (Invitrogen, Carlsbad, CA) with a GFP (green fluorescent protein) marker gene incorporated under p10 promoter control to monitor the infection in insect cells.[26] The Ra86 (Ra92A) was amplified without the native predicted signal peptide (aa 1–19) and putative transmembrane region (aa 674–693) with a StuI site and start codon introduced by the forward primer and 6x His-tag, followed by a stop codon and XhoI and SacI sites introduced by the reverse primer (Table 1). The Ra92A amplicon was cloned downstream of the honeybee mellitin signal sequence[27] in pFastBac-Mellit, kindly provided by Intervet (Boxmeer, The Netherlands) and described previously.[28] The Trp64 cement protein homolog ORF was amplified without the predicted signal peptide (aa 1–19) as an EcoRI-HindIII fragment and cloned into pFastBac-HTa (Invitrogen), providing an N-terminal 6x His-tag using customer designed primers (Table 1).

The three recombinant vectors (pFBD-His-Ra85A, pFBD-His-Ra92A, and pFB-His-Trp64) were used to transform DH10β *Escherichia coli* competent cells, carrying an AcMNPV bacmid with a deletion of the c*hiA* (chitinase) and *v-cath* (cathepsin) genes (AcBacΔCC), as previously described.[28] The authenticity of the resulting recombinant bacmids was confirmed by sequence analysis using the universal primers M13F and M13R, and the gentamycin-specific primer GentaF (Table1). Recombinant bacmid DNA (deoxyribonucleic acid) was used to transfect *Trichoplusia ni* (High Five™; Invitrogen) insect cells using standard methods.[29] Expression was tested in High Five or Sf21 cells infected at a multiplicity of infection (MOI) of 10 $TCID_{50}$ (50% tissue culture infective dose) units/cell. Protein expression was analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blot analysis with anti-His antibody and antigen specific rabbit antiserum to recombinant Ra86 gut antigen of *R. appendiculatus*.

Table 1 Sequence of oligonucleotide primers used for PCR amplification of *Rhipicephalus appendiculatus* gut (Ra85A and Ra92A) and cement (Trp64) DNA, and expression of the recombinant protein

Primer	Sequence 5′ → 3′	Comments
85A-F	ccaggcctcgagatgcgtgacctcgctttg	Forward primer with StuI to amplify RA85A with signal peptide
85A-R	cagcggccgcttagtgatggtgatggtgatgggcagcactcgactttccag	Reverse primer with NotI, and 6x His-tag for use with 85A-F
92ASPF	aaggcctagaatcatccatttgttctg	Forward primer with StuI to amplifies Ra92A without signal peptide
92A (TMR) HTR	cgctcgagatggtgatggtgatggtggtgctcggcagcacttgactttccag	Reverse primer with XhoI and Sac1 for use with 92ASPF
HT-pBS35-F	gtgaattccgagatgaccgccgtcttgg	Forward primer with EcoRI to amplify Trp64 without signal peptide
HT-pBS35-R	gtaagctttcatgccacagaaccaccgc	Reverse primer with HindIII for use with HT-pBS35-F
M13/PUC-F	ccagtcacgacgttgtaaaacg	Forward primer for PCR analysis of bacmid DNA
M13/PUC-R	agcggataacaatttcacacagg	Reverse primer for use with M13/PUC-F
Genta-F	agccacctactcccaacatc	Forward primer for use with M13/PUC-R to verify transposition
GST-TRP-F	gaggatcccgagatgaccgccgtcttg	Forward primer with BamHI for expression of TRP in *E. coli*
GST-TRP-R	gtgaattctcatgccacagaaccaccg	Reverse primer with EcoRI for use with GST-TRP-F
Ra85-F	ctggatcctcatccatttgctccgacttc	Forward primer with BamHI for expression of RA85 in *E. coli*
Ra85-R	gtgaattcctaggcagcactcgactttccagg	Reverse primer with EcoRI for use with RA85A
GST-Ra92AF	ccggatccatgcgtggcctcgctttgttc	Forward primer with BamHI for expression of RA92A in *E. coli*
GST-Ra92AR	ccgaattctaggcagcacttgactttcc	Reverse primer with EcoRI for use with GST-RA92AF

Note: The introduced restriction enzyme sites are underlined.
Abbreviations: DNA, deoxyribonucleic acid; PCR, polymerase chain reaction.

Purification and formulation of antigens

For purification of the antigens, 250 mL shaker cultures were used containing 50 mL Express Five® serum-free medium (Invitrogen) containing penicillin and streptomycin at final concentrations of 30 and 65 μg/mL, respectively. Heparin was used at a concentration of 10 units/mL to prevent cell aggregation. *Trichoplasia ni* High Five™ insect cells (originally supplied by Invitrogen and subsequently maintained by passage at the Laboratory of Virology in Wageningen) were infected at a density of 1.5–1.7 × 10^6 cells/mL with a MOI of 5 for Trp64 and a MOI 10 of Ra85A and Ra92A. The shaker culture was incubated for 72 hours at 120 rpm at 27°C. Purification of the recombinant RA86 proteins from both the culture medium and the cells was carried out using Talon immobilized metal affinity chromatography (BD Biosciences, East Rutherford, NJ). Cells were pelleted by centrifugation and resuspended in 1 mL equilibration/wash buffer (50 mM sodium phosphate and 300 mM sodium chloride; at pH 8.0) then disrupted by ultrasonication. The lysate was centrifuged at 12,000 rpm for 20 minutes at 4°C in a Sorvall SS-34 rotor to remove insoluble cell debris. The culture medium, containing secreted RA86 protein, was dialyzed overnight against the equilibration buffer before column loading. Complete protease inhibitor cocktail (Roche, Basel, Switzerland) was added to the culture medium and the cell lysate, according to the manufacturer's instructions. The protein bound to the column was eluted using 150 mM imidazole in equilibration buffer. The purified Ra86 proteins were dialyzed against phosphate buffered saline (PBS) and concentrated by dialysis against solid sucrose. Each recombinant Ra86 antigen (Ra85A and Ra92A) was formulated separately in the saponin-derived adjuvant Q-VAC at a concentration of 20 μg/mL. The recombinant Trp64 antigen was likewise formulated at a concentration of 25 μg/mL.

Immune-blotting with anti-His-tag and anti-Ra86 antibodies

The purified recombinant Ra85A, Ra92A, and Trp64 proteins were resolved in 12% SDS-PAGE under reducing conditions and transferred to Immobilon-P membrane (Millipore, Billerica, MA). The membranes were individually blocked in PBS with 5% skimmed milk, and incubated at 4°C overnight. After washing three times in PBS with 0.5% skimmed milk for 10 minutes each, the membranes were developed by addition of specific primary antibodies (from preliminary work) or mouse anti-His-tag (BD Biosciences, Breda, the Netherlands) diluted at 1:1000 in PBS with 1% skimmed milk and incubated for 1 hour at room temperature. After three washes in PBS, the membranes were incubated in 1:2000 of either rabbit anti-mouse or goat anti-rabbit IgG (Sigma, St Louis, MO) secondary antibodies conjugated to alkaline phosphatase. Color development was performed using BCIP/NBT (Roche) substrate in alkaline buffer using standard methods.[30]

Preparation of antigen for enzyme-linked immunosorbent assay (ELISA) assay

To analyze serum samples from the immunized rabbits by ELISA, recombinant Ra85A, Ra92A, and Trp64 proteins were produced in *E. coli* BL21 (DE3) cells. The antigen for ELISA was prepared in bacteria to exclude any cross-reactions with residual insect cell material potentially resulting from the insect expression system. To this aim, their nucleotide sequences were amplified by PCR using primers as indicated in Table 1, and cloned as BamHI/EcoRI fragment into pGEX-2T (Amersham Biosciences, Piscataway, NJ) as a C-terminal fusion to glutathione-S-transferase (GST). After induction of protein expression, the bacteria were solubilized in 8 M urea or 0.03% SDS and incubated for 1–2 hours at room temperature before sonication to obtain soluble protein, which was then batch purified using GST sepharose (Amersham Biosciences). The protein was eluted from the column using 10 mM of reduced glutathione in 50 mM Tris-HCl, pH 9.5. The GST tack was clipped off with 80 units/mL thrombin in PBS, which was removed by incubation with p-bimidizine (Sigma). The protein concentration was estimated using the BioRad protein assay.

Analysis of antibody responses by ELISA

Serum samples were collected from all experimental and control rabbits before immunization and fortnightly thereafter, and the antibody response monitored using an indirect antibody-capture ELISA as described.[31] Wells of Maxisorb 96-micro-well plates (Nalge Nunc International, Roskilde, Denmark) were coated overnight at 4°C with 50 μL containing recombinant Ra86 (5 μg/mL) or Trp64 antigen (2.5 μg/mL) in PBS buffer pH 7.2. The wells were blocked with 1% sodium caseinate (Sigma) in PBS containing 0.1% Tween® 20. Doubling dilutions of serum were done serially starting at 1:100 in 1% skimmed milk in PBS containing 0.1% Tween 20. Horseradish peroxidase-conjugated anti-rabbit IgG (Pharmacia Amersham) was used at a dilution of 1:5000 to detect rabbit anti-Ra86 and anti-Trp64 antibodies. The diammonium salt of 2,2′-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) was used as chromogen and hydrogen peroxide as substrate for peroxidase, and the optical density values determined at a wavelength of 405 nm.

Tick biological parameters and statistical analyses

The effects of immunization on adult female ticks, engorged nymphs, and engorged larvae were determined by measuring the parameters in ticks fed on vaccinated rabbits and comparing the results with those from ticks fed on control rabbits. All engorged adult female ticks naturally detached from the immunized rabbits were collected daily, counted, weighed, and examined. The percentage reduction of mean weight of adult females (DR %) was calculated for Ra86 or Trp64 vaccines as described previously,[32] as $100 \times (1 - \text{PMTV/PMTC})$ where PMTV is the mean weight of adult females dropped from vaccinated rabbits with either Ra86 or Trp64, and PMTC is the mean weight of adult females dropped from control rabbits. The mean quantity of eggs laid by ticks was used to express their reproductive capacity, and hatchability was expressed as the percentage of viable eggs. The data obtained using these parameters were summarized as mean ± standard error. Statistical differences between the immunized group and the corresponding control were assessed using analysis of variance, and significant differences were indicated by a probability of $P < 0.05$.

Results

Expression and immuno-blot analysis of Ra86 and Trp64 proteins

Both Ra86 variants were expressed in high levels as soluble recombinant proteins in the insect cell lysate and culture media and were clearly detectable by immuno-blot analysis using anti-Ra86 antibody and anti-His-tag antibody (Figure 1). Following Western blot analysis, proteins with a molecular mass of approximately 77 kDa and 89 kDa corresponding to Ra92A and Ra85A, respectively, were detected. These sizes were higher than the molecular mass predicted from the coding sequence and could be due to post-translational modification during secretion. N-Glycosylation (Asn-Xaa-Ser/Thr) sequences were predicted in positions 179, 276, 349, 383, and 475, with a threshold above 0.5 and a jury agreement of 7/9, 8/9, and 9/9, respectively, for Ra85A, and four potential N-glycosylation sites and several phosphorylation sites were predicted for Ra92A. The Trp64 cement protein homolog was expressed in the cytoplasm as inclusion bodies and migrated with a molecular mass of ~18 kDa on SDS-PAGE and Western blot, also higher than the predicted size of 15.5 kDa (Figure 2).

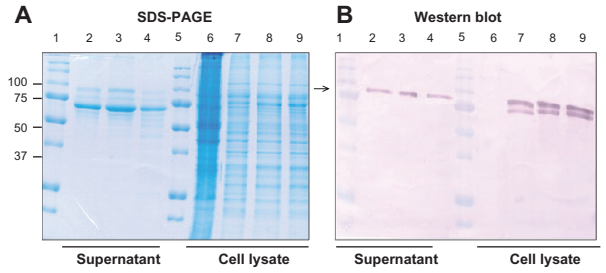

Figure 1 Ra86 recombinant proteins expressed in insect cells. Ra85A-infected Tni High Five and the culture medium were analyzed by (**A**) SDS-PAGE and (**B**) Western blotting. Lanes 1 and 5 contain a protein plus marker (BioRad, Hercules, CA) lanes 2–4 contain culture media of Tni-H5 cells infected with AcRa86 (initial and passage 1 infections); on probing with specific antibody, approximately a 90 kDa fragment was detected. Cell lysates of uninfected (lane 6) and infected cells (lanes 7–9) were also analyzed. No bands were detected in lane 6, whereas lanes 7–9 showed two Ra86-specific proteins of approximately 75 kDa and 90 kDa. The 90 kDa protein probably represents a glycosylated form. The expressed Ra92A also comprised two proteins with very similar molecular weights (results not shown).
Abbreviations: SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TniH5, *Trichoplusia ni* (High Five™; Invitrogen, Carlsbad, CA).

Antibody responses following vaccination

Specific rabbit antibody responses directed against the Ra86 and Trp64 recombinant antigens observed in immunized and control rabbits are shown in Figure 3. Anti-Ra86 and anti-Trp64 sera obtained from immunized rabbits reacted with the bacterial recombinant Ra86 and Trp64 antigens, in ELISA. Specific IgG antibody titers were initially detected in sera 2 weeks after the priming dose and were continually present throughout the experiment. The highest antibody titers were obtained 2 weeks after the booster innoculation, and the antibody response was relatively homogeneous among all Ra86 vaccinated rabbits, with no significant differences in individual antibody responses between the animals.

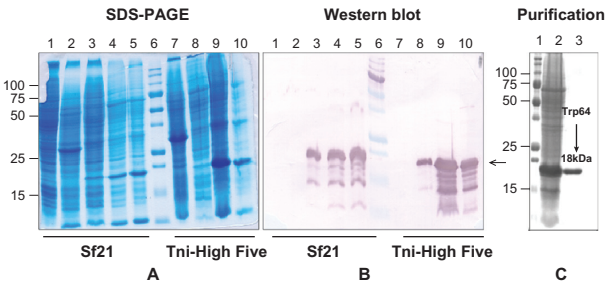

Figure 2 Trp64 recombinant protein in insect cells. Proteins were analyzed by (**A**) SDS-PAGE and (**B**) Western blot analysis, performed on a duplicate gel with anti-His antibody. Lane 1 contains uninfected Sf21 insect cells; lanes 2 and 7 Sf21 and Tni-High Five insect cells infected with wild-type AcMNPV. Sf21 (lanes 3–5) and Tni-High Five (8–10) cells were infected with AcTRP baculovirus and harvested at 24, 48, or 72 hours post-infection. Lane 6 contains a protein marker (sizes are indicated on the right in kDa). TRp64 protein was purified using (**C**) Talon-columns: and lane 1 contains a protein marker; lane 2, Tni-High Five cells infected with AcTRP; and lane 3, purified TRP.
Abbreviations: SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Tni-High Five, *Trichoplusia ni* (High Five™; Invitrogen, Carlsbad, CA).

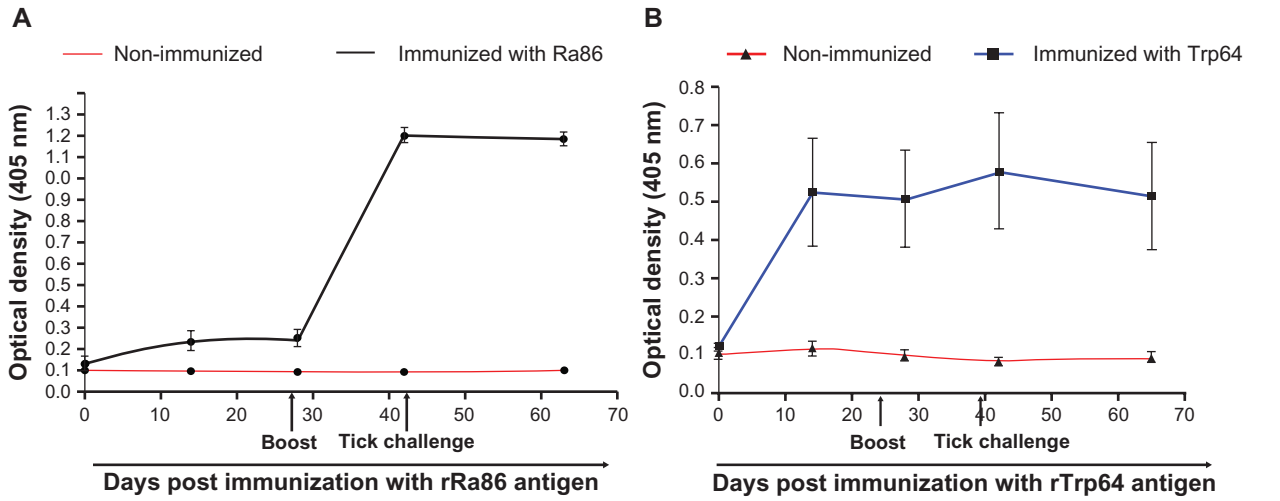

Figure 3 Immune response in rabbits immunized with (**A**) rRa86 gut and (**B**) rTrp64 cement protein, measured by ELISA. Day 0 is the day of immunization. The boost was given 28 days after the initial immunization. The rabbits were challenged with ticks on day 42. The plates were coated with Ra85A (A) or Trp64 (B) expressed in *Escherichia coli* (BL-21) cells. Optical density values were measured in serum samples diluted 1:100. Results show mean serum immunoglobulin G titers for each group (n = 7 or n = 6 vaccinated with ra86 and Trp64 proteins, respectively; n = 7 unvaccinated controls for a total of 21) + SD of 0.5118 ± 0.2440 for Ra86 and 0.1579 ± 0.08762 for Trp64. There were individual variations in the antibody responses in Trp64 vaccinated rabbits.
Abbreviation: ELISA, enzyme-linked immunosorbent assay.

Individual differences in antibody responses were observed in rabbits immunized with Trp64. No specific IgG antibodies were detected in sera from the seven control rabbits.

Effect of vaccination on *R. appendiculatus* infestation

Following tick infestation, the adult tick attachment failure was 14.1% and 11.6% for Ra86 and Trp64 immunized rabbits, respectively, compared with 1.9% for control rabbits. The mean engorgement weight (mg) per tick was 392.5 ± 122 mg and 447.6 ± 126 mg for Ra86 vaccine and Trp64, respectively, whereas control ticks had a mean weight of 545.9 ± 73 mg per tick (Table 2). A total of 17 ticks (23.3%) feeding on Ra86 immunized rabbits and 11 ticks (9.6%) that fed on Trp64 ticks died 1 week post-repletion; this compared to one dead tick feeding on nonimmunized rabbits. Inhibition of tick infestation was 60.1% for Ra86 and 37.7% for Trp64. A small proportion (5%) of ticks had a change in their normal gray color to a red coloration, an indication of gut damage. These ticks were counted among the 17 dead ticks that had fed on Ra86 immunized rabbits. There was no evidence of damage on the single tick that died following feeding on a control rabbit. The mean weight per engorged tick was significantly reduced in ticks fed on Ra86 immunized rabbits compared with controls ($P < 0.05$). However, there was a minor but not statistically significant effect on the engorged tick weight for ticks that fed on Trp64 immunized rabbits when compared with controls. No significant effects were observed on the weights of larvae and nymphs that had fed on Ra86 and Trp64 immunized rabbits, compared with the control rabbits.

Table 2 Biological parameters of detached *Rhipicephalus appendiculatus* female ticks from rabbits immunized with Ra86 and Trp64 in relation to ticks detached from rabbits in control groups

Tick biological parameter	Ra86 (n = 7)	TRP64 (n = 6)	Control (n = 7)
Unattached ticks (%)	14.1	11.5	1.9
Total tick drop (all rabbits)	73	114	130
Mean engorgement weight per tick (mg)	392.5[a] ± 122	443.6 ± 126	545.3 ± 73
Number of dead/damaged ticks 1 week post-repletion	17	11	1
Mean egg weight per tick (mg)	183.8[a]	278.8	297.9
Egg hatchability (%)	91.7	87.6	93.5
Mean weight of engorged larvae (mg)	0.562	0.542	0.549
Mean weight of engorged nymphs (mg)	11.06	10.99	10.92

Notes: Figures in brackets refer to number of rabbits in each experimental group; [a]$P < 0.05$.

Efficacy of vaccination on tick egg weight and egg-laying capacity

To assess the effect of immunization on tick weight and egg-laying capacity, adult engorged females were taken from both groups of animals and studied. On average, 77% and 90% of ticks feeding on Ra86 and Trp64 immunized rabbits, respectively, survived and successfully laid eggs, compared with 98% of ticks fed on control rabbits. Egg laying commenced from the 6th day post-engorgement and continued to the 11th day for both immunized and control groups. The mean weight of eggs laid per surviving *R. appendiculatus* female adult tick was significantly reduced in ticks fed on Ra86 immunized rabbits relative to those fed on control rabbits ($P < 0.05$). Oviposition was reduced by 21% and 4% for ticks collected from Ra86 and Trp64 vaccinated rabbits, respectively. Inhibition of oviposition was 24% for Ra86 and 10% for Trp64. There were no significant differences in egg weights from ticks that had fed on Trp64 immunized rabbits compared with control rabbits. Overall, considering the combination of the reduction in tick infestation and oviposition, the efficacy of the vaccines on adult female *R. appendiculatus* was 69.7% for Ra86 and 43.9% for the Trp64 vaccine formulations.

Discussion

In the present study, Ra86 (Ra85A and Ra92A variants) and a Trp64-homologous antigen from the MUGUGA (ILRI) stock of *R. appendiculatus* were evaluated for their efficacy in inducing antibody responses and reducing tick infestation of adult *R. appendiculatus* in rabbits. Previous investigations have demonstrated the usefulness of the TickGARD and GAVAC recombinant Bm86 vaccines in protection of cattle against infestation with *R. microplus* by reduction of tick populations and to reduce use of acaricides in integrated control strategies,[9,33–37] with the mechanism being based primarily on antibody-mediated damage to tick gut cells. In the current study, rabbits immunized with insect cell-expressed recombinant Ra86 exhibited increased mortality and decreased engorgement weights in adult female ticks, resulting in a reduced overall number of progeny. This effect was also demonstrated, although to a lesser extent, for a homolog of the Trp64 protein, previously shown to induce broad-spectrum protection against a range of ticks in a guinea pig model.[22] The divergent Trp64 homolog used in this study was isolated from the ILRI Muguga tick stock. High mortality rates in female ticks, resulting in experimental reduction in tick numbers and in the amount of eggs produced, implying lower tick populations in the field, has been reported for *Rhipicephalus* and other tick genera by Imamura et al[38] and Nuttall et al[19] when rabbits were immunized with concealed and exposed tick antigens. An interesting observation is that significant vaccine protective effects were only observed in adult *R. appendiculatus* ticks and not in the larval or nymphal stages. This has also been suspected to be the case for Bm86 when used to control the one-host tick *R. microplus*,[8] but was difficult to directly prove experimentally due to the nature of the life cycle of *R. microplus* on cattle, in which all three instars molt in situ on the same mammalian host.

The effect of vaccination on tick infestation and oviposition were more pronounced for ticks that fed on Ra86 vaccinated than in the unvaccinated rabbits. The effects on weight and reproductive parameters in the present study are in accordance with what has been reported by Ghosh and Khan[38] and Andreotti[39] using the Bm86 antigen to control *R. microplus*. The protective mechanism in the host is likely to be at least partially a result of lysis of tick gut epithelial cells through the anti-Ra86 antibodies present in the blood meal taken from vaccinated rabbits, resulting in reduced tick survival and fertility. Such protective mechanisms have also been reported by de la Fuente et al[9] and Almazan et al.[33] Similar results were reported by You[40] and Kumar et al,[41] where antibodies were consumed by ticks together with the blood meal and were postulated to damage gut digestive cells, thereby affecting transport of nutrients and tick reproductive performance.

Marked inflammatory reactions were observed on the ears of the immunized rabbits where the larvae and nymphs were attached. Aggravated loss of hair and reddening of the skin were seen on the back of the rabbits where adult ticks were attached. Although cutaneous cellular responses were not measured in the present study, tick salivary gland proteins have been shown to induce cellular immune responses in previous studies.[19,22,42–44] Studies on the induction of host cellular immune responses by tick infestation have been reviewed in reference.[37]

With regard to the reduced efficacy of Trp64 as compared with Ra86, it is interesting to note that a glycine/proline-rich protein has resulted in a degree of protection against *Haempahysalis* ticks in a rabbit model.[24] However, both the recombinant *R. appendiculatus* glycine/proline-rich cement proteins that have previously been tested as candidate vaccines in cattle, RIM36[45] and Trp64[22], were ineffective in controlling tick infestations (unpublished data, AJ Musoke and R Bishop). It may also be the case that, as for the gut

antigen Bm86, tick cement antigens[46] are more effective against a heterologous species challenge than a homologous challenge. This hypothesis is consistent with the transmission blocking activity of recombinant *R. appendiculatus* Trp64 against a pathogen transmitted by a species in the completely different and distantly related tick genus *Ixodes ricinus*.[22] However, the authors of the present paper are not sure whether the fact that Trp64 is expressed as inclusion bodies in insect cells or its eventual route of immunization may have affected its immunogenicity.

In conclusion, the Ra86 variants have potential for inclusion in subunit multivalent vaccines for control of ticks, and through repeated application they may also be useful in reduction of tick-borne diseases such as East Coast fever. A logical future step would be to test the feasibility of controlling ticks through immunization with multiple tick antigens to provide proof-of-principle for the development of multivalent vaccines against a broad range of tick species. Such vaccines may also prevent transmission of pathogens[47] and could also be used in combination with recombinant pathogen antigens to improve the performance of subunit anti-pathogen vaccines, for instance against a *Theileria parva* challenge delivered by ticks in the field.[48]

Acknowledgments

The authors thank: the staff of ILRI, especially the tick unit, Dr Lucy Kamau, Naftaly Githaka, and Eunice Onyango; Ms Sabine van der Sanden and Ms Anu Kariath for their part in the project as MSc students at Wageningen University; Dr Anne Nanteza of Makerere University; and Dr Dick Schaap from Intervet International, Boxmeer, The Netherlands for his useful advice and for formulating the vaccines used in this study. The tick research experiments at ILRI were supported by the International Consortium on ticks and tick-borne diseases (ICTTD-III), EU INCO-DEV, 6th Framework. Margaret Saimo was supported by WOTRO grant WB 93-445 from the Netherlands Organisation for Scientific Research.

Disclosure

The authors have no conflict of interest with funding sources in this work.

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