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FULL PAPER

Effects of anti-tick cocktail vaccine against *Rhipicephalus appendiculatus*

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Abstract

***Rhipicephalus appendiculatus* serpin-3 (RAS-3), *R. appendiculatus* serpin-4 (RAS-4) and a 36-kDa immuno-dominant protein of *R. appendiculatus* (RIM36) were reported as candidate antigens for the anti-tick vaccine to control ixodid ticks. In the present study, we generated recombinant proteins of RAS-3 (rRAS-3), RAS-4 (rRAS-4) and RIM36 (rRIM36), and assessed their potency as an anti-tick cocktail vaccine in cattle model. RT-PCR analysis showed that RAS-3, RAS-4 and RIM36 transcripts were detected in both adult male and female ticks during feeding. Immunization of cattle with the combination of rRAS-3, rRAS-4 and rRIM36 had raised antibodies against all recombinants and anti-sera had reacted with the molecules from the tick salivary gland extract. Tick infestation challenge demonstrated protective immunity against female ticks, resulting in mortality rates of 39.5 and 12.8 % for the vaccinated and control groups, respectively. Moreover, the mortality rate of *Theileria parva*-infected female ticks was 48.5 and 10.8 % in the vaccinated and control group, respectively. In order to evaluate the levels of pathogen transmission capacity by *T. parva*-infected ticks fed on immunized cattle, the occurrence of *T. parva* in the bovine parotid lymph node and peripheral blood was also determined and quantified by real-time PCR. Although the infection with *T. parva* could not be protected by the vaccine, the occurrence of pathogen in peripheral blood was delayed 1 to 2 days after the infestation challenge in vaccinated group. These results suggest that this cocktail vaccine plays a role in the prevention of tick infestation.**

Key Words: anti-tick vaccine, serpin, cement, *Theileria parva*

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Introduction

Ticks are distributed widely around the world, and are found in all terrestrial and non-terrestrial regions. Ticks surpass all other arthropods in numbers and variety of pathogens that they can transmit to mammals, and are ranked second to mosquitoes as vectors of human diseases[27]. The economic impact of ticks and tick-borne diseases, together with costs of control measures, has been estimated at 7 billion dollars globally in the livestock sector[23]. Among of tick species, *Rhipicephalus appendiculatus* (*R. appendiculatus*) is one of the most important species infesting domestic and wildlife animals in Eastern, Central and Southern Africa. They play as vectors of cattle- and buffalo-derived *Theileria parva* (*T. parva*), which is the causative agents of East Coast fever and Corridor diseases[31]. The diseases are fatal in cattle, particularly in exotic *Bos taurus* animals, with major economic impact. *R. appendiculatus* is also a vector of several viral diseases of livestock and humans, and has been extensively studied concerning the promotion of the transmission of tick-borne viruses by salivary gland products[32, 33].

Currently, the mainstream approaches for tick control measures heavily rely on the use of chemical acaricides. However, the use of acaricides is associated with a number of disadvantages such as chemical contamination of the food chain and environmental pollution as well as the rapid development of acaricides-resistant ticks[9]. Acquisition of drug resistance by the tick is a problem in particular, because rapid emergence of tick resistant to acaricides has discouraged the efforts to develop new drugs due to the high research, development and registration costs. In addition, this method requires frequent cost- and labor-intensive applications of acaricides, especially in the rainy season in tropical and subtropical climates[26, 46]. These aspects taken into consideration, alternative measures for tick control become necessary. Other approaches proposed for tick control have included the use of hosts with natural resistance to ticks, pheromone-impregnated decoys for attracting and killing ticks, biological control agents and vaccine[8, 41, 46, 50]. These control methods were proved effective, but most of them have been discontinued for falling short from being practical in tick control programs. In contrast, the advantages of anti-tick vaccines include specificity of target species, environmental safety, absence of human health risk, residue-free food products, ease of administration and lower costs[45]. Immunological protection is based on the fact that the host can naturally develop resistance against tick infestation after repeated of ticks. Acquired immunity to tick infestation is expressed as an increase in feeding duration, engorgement weight, inhibition of molting, high tick mortality and impaired reproduction and viability of ova[48, 52]. Intensive efforts to develop an anti-tick vaccine against the cattle tick *Boophilus microplus* (*B. microplus*) have resulted in the commercialization of the first anti-arthropod vaccine in Australia[51], which gives a practical example that tick infestations can be controlled by immunological strategy. Despite the development of anti-*B. microplus* vaccine in 1986 and its commercialization in 1994, alternative and more effective anti-tick vaccines for *B. microplus* and other ticks are not yet available[21]. Therefore, further efforts are in progress to identify candidate antigens for the development of an anti-tick vaccine against other economically important ticks such as *Rhipicephalus spp., Amblyomma spp., Ixodes spp. and Haemaphysalis spp.*[1, 2, 10, 11, 38, 42, 44].

The present study proceeds with the identification and characterization of serine protease inhibitors (serpins) as vaccine candidates from hard tick strains[10, 11, 42]. Serine proteases are generally regulated by serpins which target the enzyme active site or the enzyme active loop[6]. Serpins play an important role in the homeostasis of organisms. In vertebrates, serine proteases have been proved to play important roles in blood coagulation, fibrinolysis, complement activation and tissue modeling[15, 16, 39, 40]. In invertebrates, these proteases are involved in fundamental physiological roles in the limulus hemolymph clotting cascades[12, 13, 25, 30], innate immune responses, and in molting and pro-

phenoloxidase cascade[37]. Some of the tick serpins play regulatory roles in tick-host interactions[38]. Thus, they could theoretically be considered potential candidates as vaccinal immunogens[28].

In the present study we have extended our research on the use of serpins as vaccine immunogen for an effective control strategy against *R. appendiculatus* tick infestation in cattle and infectious diseases transmitted by ticks. The data obtained in this study show that a cocktail including recombinant proteins of RAS-3 (rRAS-3), RAS-4 (rRAS-4) and RIM36 (rRIM36) was able to induce a partially protective effect against *R. appendiculatus* infestation.

Materials and Methods

Animal: Friesians, a common breed of cattle in Zambia, were used in the tick challenge experiments at the Experimental Animal Facility of School of Veterinary Medicine of the University of Zambia. This breed is susceptible to *T. parva* infection and *R. appendiculatus* tick infestation, and thus they were maintained under a strict acaricidal control regime upon experiments.

T. parva-uninfected and -infected R. appendiculatus tick: The ticks (*R. appendiculatus*) used in this study were originally collected from vegetation by dragging with cotton flannel at the Shybuyunji region in the central part of Zambia. A laboratory colony of *T. parva*-uninfected ticks was established following feeding on rabbits. *T. parva*-uninfected ticks were used in the tick challenge experiment to examine the effects of anti-tick vaccine on tick feeding. To examine the influence of vaccination in the capacity of *R. appendiculatus* ticks fed on cattle to transmit *T. parva,* infected ticks were prepared as previously described[17,18]. *T. parva*-uninfected cattle were experimentally inoculated with 5 ml of a 50-fold dilution of the *T. parva* stabilate. The *T. parva* stabilate was obtained from the Center for Ticks and Tick-borne Diseases, Malawi, but the material was originally obtained from NVRC Muguga, Kenya. Five hundred *T. parva*-uninfected nymphal ticks were introduced and maintained on calf ears with the help of ear bags for 9 days after inoculation of *T. parva.* Five days after the introduction the engorged ticks were collected and allowed to molt. These *T. parva*-infected ticks were able to transmit *T. parva* by natural feeding as previously confirmed[17,18], and used for the tick challenge experiment to examine the effects of anti-tick vaccine on *T. parva* transmission.

RNA extraction and reverse transcription (RT)-PCR analysis: In order to determine mRNA expression profiles of RAS-3, RAS -4 and RIM36, total RNAs were extracted from partially fed (4 days after the start of feeding) ticks (both male and female) using the TRIZOL reagent according to the manufacturer's instructions (Invitrogen, CA, USA). The oligo-dT primed first strand cDNA was synthesized from 5 μg total RNA in 10 μl of a standard RT reaction mixture. A 1-μl aliquot of the RT product was used as template in 50 μl of a standard PCR reaction mixture with gene-specific primers of open reading frames (ORFs) for RAS-3 (sense primer; 5'-ATGCTCGCCAAATTTCTCTTTC-3', anti-sense primer; 5'-TCATAGTGTGTTAACCTCTCC-3'), RAS-4 (sense primer; 5'-ATGAAGTTTAATCATCTGC-3', anti-sense primer; 5'-AAAAATACCCCGTACATCCCAA-3'), which were designed as reported by Mulenga *et al.*[29], and RIM36 (sense primer; 5'-GGATCCATGAAGGTCTTCGTCGCTGTC-3', anti-sense primer; 5'-CTCGAGTTAGATTGCAACGTGTTCCTGTACTTG-3'), which were designed as reported by Bishop *et al.*[4]. Tick actin primers (sense-primer; 5'-TGTGACGACGAGGTTGCCG-3' and anti-sense primer; 5'-GAAGCACTTGAGGTGGACAATG-3') and bovine β-actin primers, (sense-primer; 5'-CTACCTCATGAAGATCCTCA-3' and anti-sense primer; 5'-TCGTTGCCGATGGTGATGA-3') were used as controls[54]. Negative controls for the RT reactions (RNA without RT) and PCR amplifications were always included in the assay. Ten μl of the PCR products were electrophoresed on agarose gels stained with 1 μg/ml ethidium bro-

mide.

DNA sequencing analysis of the cloned cDNAs: To confirm the nucleotide sequence of RAS-3, RAS-4 and RIM36, amplified PCR fragments were cloned into the pGEM-T easy vector (Promega, WI, USA). Nucleotide sequencing was performed an 8-capillary Beckman CEQ2000 automated sequencer (Beckman coulter, CA, USA) with vector-specific primers, SP6 and T7 (Promega), as well as gene-specific primers. DNA sequence analysis was carried out using a software package, GENETYX-WIN version 4.04 (Software development Co. Ltd., Tokyo, Japan), and sequence fragments were compared to those in the non-redundant protein database for homology using the NCBI blastx server program (http://www.ncbi.nlm.gov/BLAST). The secretory signal sequence was searched using the SignalP server program (http://www.cbs.dtu.dk/services/SignalP).

In vitro expression of recombinant RAS-3, RAS-4 and RIM36: The ORFs encoding RAS-3, RAS-4 and RIM36cloned in pET-32a were expressed in *E. coli* strain AD494 (DE3) pLysS (Novagen, WI, USA). The coding sequences were initially generated by PCR using the cloned full-length cDNA of RAS-3, RAS-4 and RIM36 as a template, and gene-specific primers in which the *Eco*RV /*Sac*I (New England Biolabs Inc. MA, USA) for RAS-3 and RAS-4, and *Bam*HI / *Xho*I for RIM36 restriction enzyme sites were added for the unidirectional cloning. The resulting plasmids were amplified in *E. coli* strain DH5α (Promega) and purified using QIAprep Spin Miniprep kit (Qiagen, Hilden, Germany). The purified plasmid was digested with appropriate restriction enzymes to obtain the RAS-3, RAS-4 and RIM36 inserts, which were subsequently ligated into corresponding cloning sites of the pET-32a expression vector. The plasmids were used to transform AD494 (DE3) pLysS cells. The induction of expression of recombinant proteins was carried out by the addition of 0.9 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubation for 6 hr at 37℃. Expressed insoluble rRAS-3, rRAS-4 and rRIM36 were affinity-purified on nickel-charged columns under the denaturing conditions according to the manufacturer's instructions (Novagen). As a control, the protein histidine-tagged TRX was expressed in the *E. coli* carrying the intact pET32a vector and purified under similar conditions.

Immunization of cattle with recombinant proteins: One-year-old Friesian cattle were used for the immunization and tick challenge experiments. Five cattle were immunized with the combination of rRAS-3, rRAS-4 and rRIM36 for the vaccinated group, while five other cattle were immunized with rTRX protein for the control group. Each dose contained 1 mg recombinant protein (about 300-350 μg of each tick recombinant protein) mixed with Freund's complete adjuvant for the first immunization and Freund's incomplete adjuvant for the subsequent two-booster injections, at 14-day intervals. The control group received 1 mg rTRX protein, under the same immunization protocol. Serum samples, collected from cattle after the immunization, were analyzed by Western blot to confirm the presence of antibodies against recombinant proteins.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis: SDS-PAGE under reducing conditions was performed according to the conventional method. For the Western blot analysis, purified recombinant proteins and tick crude proteins prepared by homogenizing 10 pairs of partially fed adult ticks were electrophoresed on a 10% polyacrylamide gel and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, MA, USA). The membrane was incubated with the sera from cattle pre- and post-immunized with the combination of rRAS-3, rRAS-4 and rRIM36 or with rTRX protein at 25℃ for 1 hr and washed 3 times with 3% Tween 20 in phosphate-buffered saline (PBST). Subsequently, the membrane was incubated with peroxidase-conjugated mouse anti-bovine IgG (ICN Biomedicals ins., CA, USA) at 25℃ for 1 hr

and washed 3 times with PBST. Positive signals were visualized by using 3, 3-diaminobenzidine tetrahydrochloride and cobalt chloride. The sera were co-incubated with *E. coli* strain AD494 (DE3) pLysS extract for 1 hr prior to use, in order to adsorb the antibodies raised against *E. coli* proteins and sera from cattle immunized with rRAS-3, rRAS-4 and rRIM36 were incubated with rTRX protein.

Tick challenge experiment: effects of anti-tick vaccine on tick feeding and T. parva transmission: *T. parva*-uninfected adult ticks were used for the tick challenge experiment to examine the effects of anti-tick vaccine on tick feeding. Cattle were infested with 10 adult ticks in ear bags for two weeks after the last booster dose, and visual examination was performed daily for all bags. The biological parameters analyzed were attachment rates, number of engorged ticks, weight of ticks at fall-off and mortality rates for female ticks, and attachment rates, number of survived ticks and mortality rates for male ticks. Egg weight laid by fed female ticks was not monitored. Death of ticks was confirmed by observing all detached and moribund, partially fed or engorged ticks at room temperature for about 10 to 20 minutes. Within this period all ticks that did not show any mobility were confirmed dead. The mortality rate was calculated as the total number of dead ticks on the host and dead ticks without oviposition divided by the total number of ticks that attached on the host. Two immunized and two control cattle were challenged with *T. parva*-infected ticks to examine the effects of the anti-tick vaccine on *T. parva* transmission. The cattle were subsequently infested with 10 adult ticks in ear bags for one week after the first tick infestation experiment. Blood was collected from each animal every day after infestation. On day 7, needle biopsies from the parotid lymph node were conducted. Biopsy of the parotid lymph node was performed with a help of 18-gage needle (Terumo, Tokyo, Japan) and 50 ml syringe (Terumo). Tick parameters were measured as described in the first challenge experiment.

Quantitative PCR for the quantification of T. parva: Primers targeting the highly conserved *TPR1* and *p67* genes within the *T. parva* strains were used to perform the quantitative PCR as previously described with minor modifications[17]. Bovine genomic DNA was purified from 0.5 ml whole blood samples and parotid lymph node biopsy using the Wizard™ Genomic DNA Purification kit (Promega) according to the manufacturer's instructions. Three µl (30 ng) of template DNA was added to a 17 µl reaction mixture containing PCR buffer, each of the oligonucleotide primers (to a final concentration of 0.2 µM) and LightCycler-FastStart DNA Master SYBR Green I (Roche Diagnostics, Mannheim, Germany). The *T. parva p67* primers were IL144; 5'-TCA GGC GCA GCA TCA ACA GGT-3' and IL145; 5'-GTT CTT TCC CCT TCA TAT GCC C-3'; which produce a 233-bp product[5]. The *T. parva TPR1* primers were IL 194; 5'-ATA TAT CCA GCC ATA GCT CCT GGA ATG ATT -3' and TPR1-180; 5'-TCC CCA ATT ACA TGT AGG AGA CAC G-3', which produce a 180-bp fragment[18]. Results were expressed as weight of the *T. parva* genome per 30 ng of template DNA derived from 0.5 ml of bovine whole blood. DNA quantification was accomplished by referring to a standard curve prepared from 10-fold serial dilutions ranging from 10^{1} to 10^{-7} pg of purified plasmids encoding amplicons derived from *p67* and *TPR1* genes. For the evaluation of *T. parva* in the biopsies, the bovine *β-globin* gene in each sample was also amplified using the primers β-globin (5'-ACA CAA CTG TGT TCA CTA GC-3') and β-globin anti-sense (5'-CAA CTT CAT CCA CGT TCA CC-3'). The relative amount of *T. parva* DNA present in each sample was determined as the ratio between the concentration of PCR products of *T. parva* gene and the bovine *β-globin* gene.

Accession Numbers: The Genbank accession numbers of genes characterized in this manuscript are *Rhipicephalus appendiculatus* serpin-3, AAK 61377; *Rhipicephalus appendiculatus* serpin-4, AAK61378; *Rhipicephalus appendiculatus* putative cement protein RIM36 mRNA, AY045761;

Haemaphysalis longicornis serpin-2, AB162827.

Results

Sequence analysis of cDNAs encoding RAS-3 and RAS-4

The nucleotide sequences of cloned cDNAs encoding RAS-3 and RAS-4 were confirmed by sequence analysis. The open reading frames for RAS-3 and RAS-4 encode 398 and 486 polypeptides, with predicted molecular masses of 43.2 and 53.9, respectively. Deduced RAS-3 and RAS-4 proteins have also been predicted to have 16-amino acid signal peptides and are likely to be secreted proteins. BLAST analysis demonstrated the significant similarity of the RAS-3 protein to *B. microplus* serpin and *H. longicornis* serpin-2. The RAS-4 protein also showed similarity to *Ixodes ricinus* (*I. ricinus*) serpin-2 precursor and *H. longicornis* serpin-2. Moreover, an in-depth comparison of RAS-3 and RAS-4 sequences to predicted open reading frames in known DNA sequences deposited in GenBank revealed significant similarity to serpins of other ticks.

Detection of cDNAs coding for RAS-3, RAS-4 and RIM36 in fed tick

To investigate the expression profiles of RAS-3, RAS-4 and RIM36, total RNA from male and female ticks were analyzed by RT-PCR. RAS-3, RAS-4 and RIM36 transcripts were not detected in pre-feeding ticks (data not shown). On the other hand, RAS-3, RAS-4 and RIM36 transcripts were detected in both partially fed male and female ticks (Fig. 1). The PCR products of RAS-3, RAS-4 and RIM36 were consistent with the expected size of 1.2, 1.5 and 1.0 kb, respectively. Their nucleotide sequences were confirmed by sequence analysis. Since the fed tick has considerable amount of host blood, RNA samples obtained from host blood cell were analyzed by RT-PCR under similar conditions, but no amplifications were detected (data not shown).

Reactivity of anti-sera from cattle immunized with recombinant RAS-3, RAS-4 and RIM36 with recombinant proteins and tick denatured proteins

Recombinant RAS-3, RAS-4 and RIM36 (rRAS-3, rRAS-4 and rRIM36) were expressed *in vitro*. Purified rRAS-3, rRAS-4 and rRIM36 proteins migrated as a single band on reduced SDS-PAGE with a calculated molecular weight of around 65.2 (rRAS-3), 75.9 (rRAS-4) and 54.8 (rRIM36) kDa, respectively. These molecular masses agreed with the protein sizes predicted from the DNA sequences and fused with the 22 kDa-rTRX protein (Fig. 2A, lanes 3-5). Prior to the tick challenge experiment, sera raised by immunization of the combination of rRAS-3, rRAS-4 and rRIM36 were tested by Western blotting to determine whether immunization of cattle with the cocktail vaccine induced antibodies that recognize all recombinant proteins and tick crude proteins. No specific signal against rRAS-3, rRAS-4 and rRIM36 and tick crude proteins was detected in the sera of pre-immunized cattle (data not shown). The sera after the immunizations recognized rRAS-3, rRAS-4 and rRIM36 (Fig. 2B, lanes 2-4) and denatured proteins in the tick crude proteins (Fig. 2B, lane 5). Mainly, three signals were de-

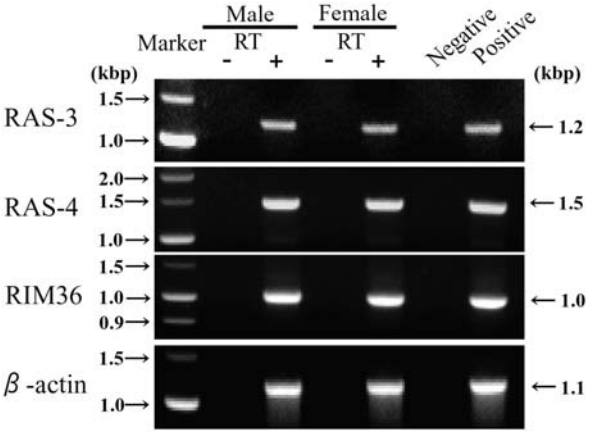

Fig. 1. Detection of RAS-3, RAS-4 and RIM36 cDNAs in feeding tick. Total RNAs extracted from adult male and female ticks were analyzed by RT-PCR using specific primers. Predicted product sizes of RAS-3, RAS-4, RIM36 and tick actin are 1.2, 1.5, 1.0 and 1.1 kb, respectively.

tected in tick crude proteins, with molecular weights of around 62 (rRAS-3), 42 (rRAS-4) and 40 (rRIM-36) kDa, respectively. Recognitions of recombinants proteins by anti-sera indicate that all recombinants have immunogenicity.

Effects of anti-tick vaccine on tick feeding

To assess the effects of immunization on female ticks fed on vaccinated and control cattle, biological parameters such as attachment rates, number of engorged ticks, mortality rates and engorgement weights were observed for 21 days (Table 1). Engorgement of female ticks started on day 6 and finished on day 15 after tick infestation challenge in both vaccinated and control groups. There was no apparent difference in the attachment rates (observed on day 5) between both groups (96.0% in control group and 98.3% in vaccinated group). The average number of fully engorged ticks per ear was 8.2 and 6.0 in the control and vaccinated groups, respectively. The ticks that continuously attach and feed until day 15 did not fully engorged and later died. The mortality rate of female ticks in vaccinated group (39.5%) was higher than in control group (12.8%, $P < 0.05$). The weight of engorged female ticks that fed on vaccinated animals was similar to that of the control group. Despite the fact that there were no differences in the weight of fully engorged ticks and the attachment rates between vaccinated and control groups, an apparent increase in the mortality rate indicated that vaccination of cattle with the combination of rRAS-3, rRAS-4 and rRIM36 had a protective effect against ticks. In order to assess the effect of

Table 1. Effects of the anti-tick vaccine on *T. parva*-uninfected female ticks

	Immunized group[a]	
	Control[b]	Vaccinated[c]
Attachment rates (%)	96.0±5.4	98.3±4.0
Number of engorged ticks	8.2±1.5	6.0±0.4
Tick weights (mg)	579.4±72.4	589.3±72.1
Mortality rates (%)[d]	12.8±9.1	39.5±22.9[e]

[a] Each group contains 5 cattle. Ten pairs of adult ticks were introduced into each ear bag (right and left). Results are expressed as the mean±S.D. for each ear bag.
[b] Animals in the control group were immunized with rTRX protein that was fused with rRAS-3, rRAS-4 and RIM36.
[c] Animals in the vaccinated group were immunized with a combination of rRAS-3, rRAS-4 and rRIM36.
[d] Mortality rate was calculated from the ticks that could not survive during and after feeding.
[e] $P < 0.05$ compared to the control (Student's t-test)

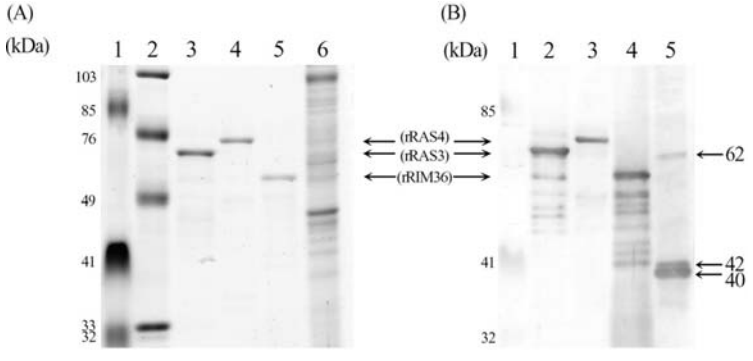

Fig. 2. Expression of rRAS-3, rRAS-4 and rRIM36 in *E. coli* and antigenicity of recombinant proteins. Panel (A) shows purified recombinant proteins and tick whole extracts electrophoresed on 10% polyacrylamide gel and stained with Coomassie brilliant blue R-250. Lane 1, Molecular marker (corresponding with the marker blotted on the PVDF membrane for Western blot analysis); lane 2, Molecular Marker (for SDS-PAGE); lane 3, rRAS-3; lane 4, rRAS-4; lane 5, rRIM36; lane 6, tick crude proteins. Panel (B) shows the membrane incubated with sera from cattle immunized with a rRAS-3, rRAS-4 and RIM36 cocktail vaccine. Lane 1, Molecular marker (for Western blot: pre-stained marker); lane 2, rRAS-3; lane 3, rRAS-4; lane 4 ,rRIM36; lane 5, tick crude proteins.

Table 2. Effects of the anti-tick vaccine on *T. pavra*-uninfected male ticks

	Immunized group[a]	
	Control[b]	Vaccinated[c]
Attachment rates (%)	94.0±0.9	95.0±0.8
Number of survived ticks[d]	7.2±3.1	5.0±3.1
Mortality rates (%)[e]	23.7±30.1	47.6±30.8

[a]Each group contains 5 cattle. Ten pairs of adult ticks were introduced into each ear bag (right and left). Results are expressed as the mean±S.D. for each ear bag. Ticks crushed by the host were not included.
[b]Animals in the control group were immunized with rTRX protein that was fused with rRAS-3, rRAS-4 and RIM36.
[c]Animals in the vaccinated group were immunized with a combination of rRAS-3, rRAS-4 and rRIM36.
[d]Number of survived ticks was counted on day 15 when all female ticks were engorged.
[e]Mortality rate was calculated from the ticks that could not survive during feeding.

Table 3. Effects of the anti-tick vaccine on *T. parva*-infected female ticks

	Immunized group[a]	
	Control	Vaccinated
Attachment rates (%)	97.5 (95, 100)	95.0 (90, 100)
Number of engorged ticks	8.5 (7,10)	5.0 (3,7)
Tick weights (mg)	560.1 (500.8, 619.4)	617.6 (638.6, 596.6)
Mortality rates (%)	10.8 (0,21.5)	48.5 (30.0, 66.6)

[a]Each group contains 2 cattle. 10 pairs of *T. parva*-infected female ticks were introduced into each ear bag (right and left ear). Data indicate the mean values obtained from two animals shown in parentheses.

immunization on male ticks, we analyzed attachment rates and number of the surviving ticks until day 15 or the end of the feeding period of female ticks (Table 2). There was no apparent difference in the attachment rates (observed on day 5 after the beginning of tick challenge infestation) between both groups (94.0% in control group and 95.0% in vaccinated group). The average number of surviving ticks enumerated on day 15 was 7.2 and 5.0 ticks in the control and vaccinated groups, respectively. The mortality rate of male ticks in the vaccinated group (47.6 %) was higher than that in the control group (23.7 %).

Table 4. Effects of the anti-tick vaccine on *T. pavra*-infected male ticks

	Immunized group[a]	
	Control	Vaccinated
Attachment rates (%)	100.0 (100, 100)	92.5 (95, 90)
Number of survived ticks	8.25 (6.5, 10)	4.75 (3,6.5)
Mortality rates (%)	17.5 (0,35)	48.1 (27.8, 68.4)

[a]Each group contains 2 cattle. Ten pairs of *T. parva*-infected male ticks were introduced into each ear bag (right and left). Data indicate the mean values for two animals, each of which is shown in parentheses.

Effects of anti-tick vaccine on T. parva transmission

To assess vaccine efficacy to interfere pathogen transmission, vaccinated and control cattle were subsequently challenged with *T. parva*-infected ticks after the un-infected tick infestation experiment. The effects of vaccination on *T. parva*-infected ticks (female: Table 3, male: Table 4) were mostly consistent with those observed in the un-infected tick experiment. Furthermore, we conducted these experiments to determine whether the immune responses against the tick are protective and whether this immunization is effective against the transmission of *T. parva.* The protection was determined by analysis of the biological parameters of ticks and presence of *T. parva* in bovine parotid lymph node and peripheral blood by quantitative real-time PCR. *T. parva* DNA was detected in the parotid lymph node samples on day 7 (Fig. 3), whereas bovine blood became PCR-positive on day 11 post-infestation (Fig. 4). *T. parva* DNA load in the biopsy samples derived from the control cattle was higher than in vaccinated cattle. Moreover, one of the vaccinated cattle was still negative for *T. parva* in the biopsy samples on day 7 post-exposure (Fig. 3). The PCR product of *p67* gene was detected on day 11 after tick infestation in the control group, while it was detected on day 12 or 13 post-infestation in the

vaccinated cattle (Fig. 4). However, a gradual increase in *T. parva* DNA load was observed during the infestation (Fig. 4), based on the quantified *p67* gene by real-time PCR. Levels of infection (*T. parva* DNA load) showed no difference between the groups. The clinical signs of the cattle became severe, and body temperatures were over 40℃ from day 14 post-exposure. The animals were treated with oxytetracyclines on day 15, following guidelines of the Institutional Animal Care and Use Committee of School of Veterinary Medicine, University of Zambia.

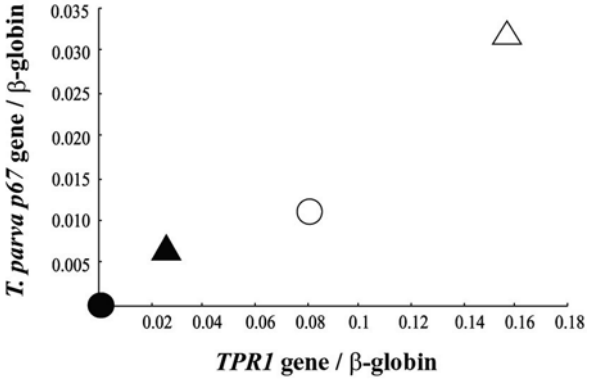

Fig. 3. Quantification of the *T. parva* DNA in the parotid lymph node of cattle challenged with *T. parva*-infected ticks. The relative *T. parva* DNA values were worked out as ratios by dividing the concentration of the real-time PCR products from the *T. parva* by that from the bovine β-globin. Symbols indicate a sample from control (open circle and triangle) or immunized cattle (closed circle and triangle).

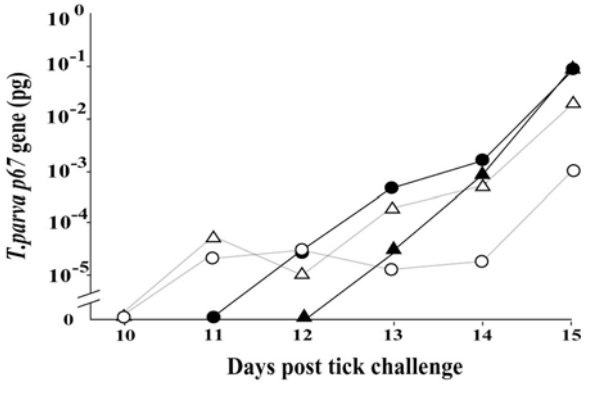

Fig. 4. Monitoring of the *T. parva* in peripheral blood of the cattle infested with *T. parva*-infected ticks. The *T. parva* DNA values were determined by real-time PCR. DNA load of the control (open circle and triangle) or immunized cattle (closed circle and triangle).

Discussion

In our previous study, we evaluated the efficacy of cocktail vaccine combined with two serpins (rRAS-1 and rRAS-2) from *R. appendiculatus,* and verified that the number of engorged nymphal ticks was significantly reduced and the mortality rate of adult ticks fed on immunized cattle was higher than that of ticks fed on the control group[10]. In the present study, we evaluated a cocktail vaccine of *R. appendiculatus* antigens, rRAS-3, rRAS-4 and rRIM36, and have analyzed the immune responses, tick infection and the protection from tick-borne transmission of *T. parva* elicited by these antigens. One purpose of these series of studies is to try a method to control ticks. Although knowledge of the tick biology is incomplete, research has allowed to gain insight into the development of an anti-tick vaccine strategy and the evasion of parasite from host immunity.

The experimental design limits the evaluation of benefits of using a cocktail of 3 different tick proteins, since the proteins were not evaluated separately. The selection of three tick proteins used in the immunization was based on their importance in tick physiology. Given that the two proteins are protease inhibitors and the other is a cement-like protein, therefore, it is considered that these are important for the feeding success of ticks. RAS-3 and RAS-4 are candidate vaccine immunogens, since HLS-2 was shown to be a promising antigen for vaccine development[11]. Furthermore, many data obtained from various tick species support that serpins are potential candidates as tick vaccine[1, 2, 10, 11, 38, 42]. Additionally, RAS-3 and RAS-4 are both predicted as extracellular proteins, which are key immunoprotective molecules that increase the probability of antibody binding *in vivo*[35]. The RAS-3 and RAS-4 transcriptions were detected in fed ticks regardless of sex and tissues analyzed[29],

which raises interest in these characteristics, since the antibodies against these proteins could be effective against adult ticks regardless of tick sex, even though the male takes relatively little amount of blood. Moreover, Nuttal *et al.*[35] suggests that proteins involved in some vital functions and present in the gut (or hemolymph) could be potential vaccine candidates. RIM36 is an immunodominant protein and likely to be a protein component of the cement cone[4]. We selected this protein as one component of the cocktail vaccine in the combination with RAS-3 and RAS-4, expecting some special effects against pathogen transmission. The RIM36 has originally been cloned and characterized by Bishop *et al.*[4]. The RIM36 contains two classes of glycine-rich repeats, a GL [G/Y /S/F/L] tri-peptide and GSPLSGF septa-peptide. Although the authors did not examine the protective effect against tick infestation, RIM36 was shown to be highly immunogenic as determined by antibody responses[4]. It is interesting to note that another similar protein, a secreted 15-kDa protein named 64TRP, was isolated from *R. appendiculatus*[43]. This keratin- and collagen-like protein appeared to be a component of the cement cone that anchors and seals the tick mouthparts in the host skin. Recently, it was shown that the 64TRP protein is able to induce a protective immune response against an infection of the tick-borne encephalitis virus (TBEV) transmitted by infestation with *I. ricinus*[21].

Sera from the cocktail-immunized cattle recognized recombinant proteins, and there are three signals (one strong reaction against a 40 kDa-protein; a fainter reaction against a 42 kDa-protein and a faintest reaction against a 62 kDa-protein) in the tick crude proteins determined by Western blot analysis. Our data in this study did not confirm whether the recognized bands correspond to native RAS-3, RAS-4 and RIM36 proteins, because of impossibility to determine the exact molecular weights of native proteins based from the amino acid sequences. Nevertheless, positive signals on the tick crude proteins support the presence of antigenic epitopes in tick, which could be recognized through antibodies raised by cocktail immunization. Thus, Western blot results may help to validate the vaccine effects observed in this study. Anti-tick immunity induced by the combination of rRAS-3, rRAS-4 and rRIM36 immunization in cattle was shown to apparently damage the physiology of ticks as evidenced by mortality rates of ticks fed on cocktail-immunized cattle. The effect observed in the present study is similar to the effect on adult tick feeding after the immunization with the rHLS2 antigen[11]. The apparent increase in the mortality rate in female ticks directly reduces the number of ticks and ultimately leads to a reduced amount of egg production, and result in decrease tick population[49]. It is interesting to note that the amount of *T. parva* in the parotid lymph node determined by real-time PCR was lower in the vaccinated group, and the appearance of pathogen in blood was delayed 1 to 2 days after tick infestation in the vaccinated group. Though the response of vaccinated cattle against tick proteins did not completely inhibit the pathogen transmission, it delayed the transmission of tick-borne pathogens. Since there are few numbers of animals per group used in the trial with *T. parva*-infected ticks, it was not possible to do statistical analysis. At least, the results suggest a potential efficacy of the cocktail vaccine to protect against tick infestation.

The power of vaccine to decrease vector capacity has attracted significant attention, but the number of the reports is limited[3, 14, 24, 47]. For example, Pippano *et al.*[36] reported the immunity induced by the Bm86 (Tick-GARD) vaccine against *B. annulatus, B. bovis* and *B. bigemia*. Boue *et al.*[7] reported an assessment of Bm86 vaccination on tick transmitted diseases. Apart from a Bm86 vaccine, a tick cement protein (64TRP) of *R. appendiculatus* protected mice against TBEV transmission by *I. ricinus* ticks[21]. Despite the fact that ticks induce immunosuppression in the tick-feeding site and produce saliva-activated transmission (SAT) factors that promote pathogen transmission[19, 20, 34], an immune response against the tick can disrupt these mechanisms[21]. Disrup-

tion of immunomodulation in the tick-feeding site was caused by the induction of the clear cellular response in 64TRP-immunized mouse, and this disruption would also counteract the activity of SAT factors present in *I. ricinus* saliva that promote TBEV transmission[21]. The protective effects against TBEV observed in 64TRP-immunised mouse most likely result from humoral and cellular responses to tick protein rather than specific antiviral or SAT factor immunity. Therefore, these results afford to conjecture that the immunization with immuno-dominant cement proteins (e.g. 64 TRP, RIM36 and others) may have similar potential to reverse immune-modulation on tick-feeding site and affect the capacity to transmit pathogens. It is important to observe that animals inoculated with rRIM36 cement alone developed strong antibody responses, but the result of tick challenge infestation has not been reported[4,21]. We evaluated vaccine efficacy with a mixture of three antigens, not rRIM36 alone, therefore it is difficult to discuss whether the partial inhibition of *T. parva* transmission observed in this study was due solely to rRIM36.

Extensive studies have led to the identification of numerous tick antigens that can generate partial protective immune responses in immunized animals. However, despite the advancements in the identification of tick-protective antigens, the number of antigens evaluated as recombinant proteins in vaccines against tick infestations is limited. Evaluation and assessment of these antigens is required to characterize each antigen; together with that, an approach that administers several antigens simultaneously has been proposed and considered as one strategy to increase vaccine efficacy against ticks[8]. In particular, the reports of simultaneous administration of tick antigens are very few in number. In 1996, Willadsen *et al.*[53] reported that the addition of the Bm91 antigen enhanced the efficacy of the vaccination over that with Bm86 alone, to a statistically significant degree. Moreover, co-vaccination with two antigens did not impair the response of cattle to the Bm86 antigen[53]. Two years later, McKenna *et al.*[22] reported that co-vaccination with Bm86 and BMA7 enhanced immunity over that seen with a commercial vaccine based on Bm86 alone. These data also support the potential of cocktail immunization of tick antigens. In conclusion, although we recognized the crudity of the experimental design, our findings offer valuable information covering different aspects of tick vaccine strategy, which open new possibilities for the development of an anti-tick vaccine. This discovery is vital contribution, along with proper vaccine formulations, field trial evaluation and commercialization which are all steps required for development of anti-tick vaccines.

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