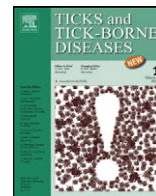




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Original article

Immunization of cattle with Ra86 impedes *Rhipicephalus appendiculatus* nymphal-to-adult molting

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ABSTRACT

Commercial vaccines based on the tick gut protein Bm86 have been successful in controlling the one-host tick *Rhipicephalus (Boophilus) microplus* and provide heterologous protection against certain other non-target ixodid tick species. This cross protection, however, does not extend to the three-host tick *R. appendiculatus*, the vector of the protozoan parasite *Theileria parva*. When transmitted to cattle, *T. parva* causes the often fatal disease East Coast fever. Here, we used insect cell-expressed recombinant versions of the *R. appendiculatus* homologs of Bm86, named Ra86, to vaccinate cattle. We measured multiple fitness characteristics for ticks that were fed on cattle Ra86-vaccinated or unvaccinated. The Ra86 vaccination of cattle significantly decreased the molting success of nymphal ticks to the adult stage. Modeling simulations based on our empirical data suggest that repeated vaccinations using Ra86 could reduce tick populations over successive generations. Vaccination with Ra86 could thus form a component of integrated control strategies for *R. appendiculatus* leading to a reduction in use of environmentally damaging acaricides.

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Introduction

The brown ear tick, *Rhipicephalus appendiculatus*, is the vector of the apicomplexan parasite *Theileria parva* (Lounsbury, 1904; Theiler, 1908). The adult and nymphal stages of *R. appendiculatus* feed preferentially on bovines (cattle or buffalo), but can also infest large antelopes, sheep, and goats. The natural host of *T. parva* is the African buffalo (*Syncerus caffer*), which is an asymptomatic parasite carrier, and this host–pathogen combination is likely to have co-existed in Africa for centuries (reviewed in Norval et al., 1992). However, when transmitted to cattle, particularly *Bos taurus* breeds, *T. parva* causes a rapid and fatal lympho-proliferative disorder known as East Coast fever, first reported in Zimbabwe in 1902 (Gray and Robertson, 1902).

R. appendiculatus acquires *T. parva* through the uptake of a blood meal from a *T. parva*-infected bovine. Piroplasms in erythrocytes are liberated into the tick gut lumen during digestion of the blood meal and once free in the gut, they differentiate into male and female gametes. The gametes fuse to produce a diploid zygote able

to penetrate the gut wall. Motile kinetes exit the gut wall into the hemolymph and then enter the salivary glands. Within the salivary glands, sporozoite development occurs specifically within the 'e' cell of the type III acinus (Fawcett et al., 1982a,b, 1985). After receiving a yet unknown stimulus at the beginning of tick attachment and feeding, sporozoites undergo a final differentiation step enabling them to infect bovine host cells.

After entering the vertebrate host, sporozoites invade lymphocytes and develop into the schizont stage. During the schizont stage, the host cells undergo uncontrolled cell proliferation synchronous with parasite division. Some schizonts differentiate to the merozoite stage infecting erythrocytes. These merozoites develop into piroplasms that are taken up by the *R. appendiculatus* vector, thereby closing the life cycle. Transmission of *T. parva* is strictly transstadial and for the parasite life cycle to perpetuate, the tick vector must feed successively on a bovine host infected with *T. parva* and then on a second bovine host susceptible to infection.

Adult *R. appendiculatus* have been regarded as the main tick stage responsible for *T. parva* transmission in the field, with the role of nymphs being largely overlooked until recently (Purnell et al., 1971; Ochanda et al., 1996). Field surveys show that *R. appendiculatus* feed on cattle in a ratio of 10 nymphs to 1 adult (Short and Norval, 1981), and the cumulative effect of nymphs may be

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equal to that of adults, making them an important developmental stage for disease transmission dynamics. Infective sporozoites are produced at least one day earlier in nymphs compared to adults (Sonenshine, 1993). Importantly, *T. parva* can survive in nymphs under field conditions for up to one year (Ochanda et al., 2003).

Effective tick control is currently the mainstay for limiting tick-borne diseases worldwide. Tick control has largely relied on acaricide application, but the search for alternative integrated methods has intensified due to the evolution of acaricide resistance and mounting environmental concerns (reviewed by George et al., 2004). One promising alternative is vaccination of cattle, which has been effectively implemented for control of the cattle tick *R. microplus* (reviewed by Willadsen, 2004). To date, 2 commercial vaccines have been employed [TickGARD Plus™ (Intervet) and Gavac™ (Heber Biotech)], both based on the *R. microplus* gut antigen Bm86. The decrease of the reproductive capacity of ticks feeding on vaccinated animals (de la Fuente et al., 1998) led to a decline in tick-borne disease incidence (de la Fuente et al., 1999). Cross protection following Bm86 vaccination of cattle has been demonstrated against a number of other tick species including *R. (Boophilus) annulatus* (Pipano et al., 2003), *R. (Boophilus) decoloratus*, *Hyalomma anatolicum anatolicum*, and *H. dromedarii* (de Vos et al., 2001; Odongo et al., 2007). However, no cross protection was seen against *Amblyomma variegatum* and *R. appendiculatus* adult ticks (de Vos et al., 2001; Odongo et al., 2007). When the *H. a. anatolicum* homolog of Bm86, Haa86, was used for the vaccination of cattle, a significant reduction of the reproductive capacity of *H. a. anatolicum* ticks was observed (Azhahianambi et al., 2009). Similarly, the *R. annulatus* homolog, Ba86 was able to control both *R. annulatus* and *R. microplus* (Canales et al., 2009). These studies highlight the potential use of Bm86 homologs for the development of effective anti-tick vaccines.

Herein, we describe for the first time the use of 2 variants of *R. appendiculatus* Bm86 homolog, Ra85A and Ra92A, together collectively as Ra86 (Saimo et al., 2011) as an anti-tick vaccine in cattle. We assessed its effect on a range of biological parameters of nymphal and adult *R. appendiculatus* stages. We also tested the impact of Ra86 vaccination on *T. parva* development in the tick vector, which has not been previously reported to our knowledge. Finally, we used a model of *R. appendiculatus* population dynamics to simulate the long-term effects of multiple vaccinations on tick populations in the field.

Materials and methods

Protein production

The 2 variants Ra85A and Ra92A will be referred to collectively as Ra86, following the nomenclature of Saimo et al. (2011). In instances where they were treated differently or separately, they will be referred to individually. Recombinant Ra86 was produced using the Bac-to-Bac Baculovirus Expression System (Invitrogen, Carlsbad, CA, USA). This approach was used because constructs from expressing both variants have been previously described (Saimo et al., 2011). Recombinant baculovirus constructs encoding the sequences for the expression of the 2 variants of Ra86 (Kamau et al., 2011; Saimo et al., 2011) were donated by Wageningen University, Dept. of Virology, and expressed according to standard protocols. Briefly, *Trichoplusia ni* (Tni) High Five cells (Invitrogen) were infected at a multiplicity of infection (MOI) of 5 with recombinant baculovirus. Virus was combined with Grace's Incomplete media (Invitrogen) in a total volume of 1 ml and added to a 75-ml tissue culture flask containing a confluent layer of Tni High Five cells and incubated for 1 h at 27 °C with gentle agitation. Following incubation, the supernatant containing virus was removed and

replaced with Express High Five Media (Invitrogen) and cultures incubated at 27 °C for 72 h for recombinant protein expression. Recombinant protein was harvested from cell cultures by collecting cells and media from culture flasks, transferring to 50-ml sterile tubes (Sterilin). The contents were centrifuged at 3500 × g for 10 min at 4 °C to separate supernatant and cell fractions. The supernatant fraction was treated with a 25× stock solution of Complete Protease Inhibitor Cocktail tablets (EDTA-free) (Roche, Mannheim, Germany) resulting in 1× concentration in the final supernatant volume. The cell pellet was resuspended in 500 μl of Complete, EDTA-free working solution. All Complete, EDTA-free solutions were made according to manufacturer's instructions. The control vaccine preparation consisted of uninfected Tni High Five cells treated as described above.

Protein isolation and concentration

To isolate the recombinant protein, cells were sheered using agitation with silica beads (0.1 mm) in a FastPrep-24 machine (MP Biologicals). A cycle of 30 s agitation followed by cooling on ice for 5 min was repeated 3 times. Centrifugation at 10,000 × g for 5 min at 4 °C in a bench top centrifuge removed cellular debris, with the resulting supernatant being pooled with the original culture supernatant. Recombinant protein was recovered by bulk precipitation with using 80% ammonium sulphate saturation and centrifugation (3000 × g, 30 min, 4 °C) (TOMY MRX-150). The resulting protein pellet was suspended in 5 ml of phosphate buffered saline (PBS) and dialyzed overnight at 4 °C against PBS. Expression of Ra86 and recovery after ammonium sulphate precipitation was confirmed using SDS-PAGE and immune-blotting with anti-Ra86 sera, generated in a previous study (Saimo et al., 2011). The anti-Ra86 sera used recognized both Ra85A and Ra92A proteins. Protein quantification of Ra85A, Ra92A, and control preparation was carried out separately using the Pierce BCA assay (Thermo Scientific, Rockford, Illinois) according to the manufacturer's instructions.

SDS-PAGE and immunoblotting

Resolution of Ra86 and control protein was carried out by SDS-PAGE. Proteins resolved on SDS-PAGE gels were transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). All incubation periods were for 1 h at room temperature. Immunoblots were blocked using 5% skimmed milk in Tris-buffered Saline with Tween 20 (TBST) (10 mM Tris, 150 mM NaCl, and 0.05% Tween 20), pH 8.0. Following blocking, the membrane was washed 3 times in TBST, then incubated for 1 h at room temperature in anti-Ra86 rabbit polyclonal sera (Saimo et al., 2011) diluted 1:500 in TBST. After washing 3 times with TBST, blots were incubated with 1:5000 with anti-rabbit whole Ig linked to peroxidase conjugate (Amersham Biosciences) diluted in TBST. Following incubation, blots were washed 3 times with TBST and developed using diaminobenzidine (DAB) with H₂O₂ added as the substrate.

Vaccine formulation

Samples prepared for the Ra86 vaccination group included 50 μg of the preparation for Ra85A and 50 μg of the preparation for Ra92A for each animal. Animals were vaccinated 3 times separated by 5-week intervals. To evaluate the possible effect of Tni High Five cell debris on ticks, a control preparation of 50 μg of precipitated protein from Tni High Five cells only was treated in an identical manner to Ra86-expressing cells. Antigen preparations were adjusted to a volume of 1 ml with PBS and emulsified in an equivalent volume of Montanide ISA 50 V adjuvant (Seppic) according to manufacturer's instruction.

Immunization of cattle with Ra86

Sixteen *Bos taurus* (Friesian) cattle one year of age, free from tick-borne infections, were raised and maintained under strict tick-free conditions at the International Livestock Research Institute (ILRI). Animals were randomized into 2 groups of 8 animals representing the Ra86 vaccine and control group. All vaccinated animals received 3 vaccine doses separated by 5-week intervals and administered subcutaneously at the prescapular region. Injections were split equally between the left and right sides. To monitor the humoral immune response, serum was collected before the first vaccination and subsequently at 2-week intervals until the end of experiment.

Monitoring immune response to vaccination by indirect ELISA

We observed a high background reaction in non-immune bovine sera and Ra86 using ELISA. Hence, an adapted form of ELISA was developed for immune response monitoring which reduced the background interference. Protan BA85 cellulose nitrate membranes (Schleicher & Schuell) were sectioned and numbered to produce a grid of 0.5×1 cm blocks. Ra86 protein (1 μ g) in 50 mM Tris pH 8.0 was spotted onto each block in the grid and incubated at 4 °C overnight. The following morning, the membrane was blocked with 2.5% (w/v) casein in PBS with 0.05% Tween 20 (PBST), pH 7.4 for 2 h at room temperature with gentle agitation. The membrane was washed 3 times for 5 min in PBST at room temperature. The blocks were cut from each other and placed individually into an ELISA plate (Polysorb, Nunc) which had been blocked overnight using 1% (w/v) casein in PBST. Collected serum [1:1000 dilution in 0.1% (w/v) casein in PBST] was applied to each well corresponding to time points 0 (before vaccination), 1 (2 weeks after inoculation 1), 2 (2 weeks after inoculation 2), 3 (2 weeks after inoculation 3). Positive control samples consisted of the purified monoclonal antibody 12.1 (Kopp et al., 2009), and the negative control was an unrelated bovine serum used at a starting dilution of 1:500. After incubation, the strips were washed 3 times for 5 min each in PBST and a secondary antibody (anti-bovine IgG, whole molecule, peroxidase conjugate, Sigma) or in the case of the positive control anti-mouse Ig whole molecule, peroxidase conjugate (Amersham Biosciences) added and incubated for 30 min at room temperature. Following incubation and washing, the strips were placed into a fresh ELISA plate and developed used SIGMAFAST OPD (Sigma) following instructions. Strips were removed and plates were read at OD_{405 nm}. Mean readings (with standard error) were calculated for each time point by grouping all animals from Ra86 and control-vaccinated groups.

Infection of cattle with *T. parva*

Two weeks following the final vaccination, all animals were infected with an estimated 5.9×10^4 sporozoites (1:20 dilution of *T. parva* Muguga stabilate 3087) subcutaneous injection at the right parotid lymph gland (Brown et al., 1977; Di Giulio et al., 2009) and treated simultaneously with a long-acting oxytetracycline. On a daily basis, beginning day 5 postinfection, we recorded rectal temperature, obtained blood smears from ear vein punctures, and performed lymph node biopsies from each animal. Reaction to infection and disease monitoring was assessed as outlined in Rowlands et al. (2000). All animal experiments and associated procedures were carried out with the approval of the Institutional Animal Care and Use Committee at ILRI.

Tick feeding on *T. parva*-infected cattle

The *R. appendiculatus* Muguga tick strain was collected from the field in the central highlands of Kenya in the 1950s and propagated

at the East African Veterinary Research Organization-Kenya Agricultural Research Institute (EAVARO-KARI). It was subsequently maintained as a laboratory stock at ILRAD/ILRI (Bailey, 1960). Before application onto cattle, ticks were maintained in BOD incubators at 28 ± 1 °C. Ticks harvested from both control and Ra86-vaccinated cattle were kept at 24 ± 1 °C, 80% relative humidity. Ten days following infection with *T. parva* sporozoites, 100 male and 100 female adult *R. appendiculatus* ticks were applied to each cattle, secured in tick feeding bags placed on the back of animals. Twelve days postinfection with *T. parva* sporozoites, 3000 nymphal ticks (measured by weight of 3 g average for 3000 engorged larvae) were applied to each animal. Nymphs were isolated in a separate bag secured to the back of each animal. Ticks were allowed to feed until fully engorged and naturally detached. After detachment, the ticks were collected, counted, weighed, and placed in incubators. The effect of Ra86 vaccination was assessed using the following parameters: tick mortality after collection from host, engorgement weight of adult females, nymphal engorgement weight, nymphal-to-adult molting success, egg laying capacity of adult females, egg hatching capacity, and effect on uptake of *T. parva* parasites by nymphal ticks.

Effects of vaccination on *T. parva* uptake by *R. appendiculatus* ticks

To evaluate effect of Ra86 vaccination in cattle on the ability of ticks to acquire *T. parva* infections, engorged nymphs were collected from Ra86 vaccinated and control animals and placed in incubators to allow them to molt into adults. To facilitate sporozoite maturation within the salivary glands of the adults (Kimbita et al., 2004; Howell et al., 2007), 60 males and 60 females from each of the 16 cattle were fed for 4 days on the ears of adult rabbits. Ticks were manually removed, dissected, and the salivary glands collected. One salivary gland of each tick was fixed to a microscope slide, stained with Feulgen stain, and examined using light microscopy (Büscher and Otim, 1986) for sporozoite detection. Abundance of infection was determined as the average percentage of infected acini per tick. The infection rate was determined as the number of ticks with infected acini out of the total number of fed ticks on each animal and the intensity of infection as the abundance of infection divided by the infection rate expressed as a percentage.

Data analysis

We used two-sample *t*-tests to analyze the following measures from Ra86-vaccinated versus control cattle: adult tick engorgement weights, adult tick fecundity, adult and nymphal tick mortality rates, tick egg-hatching rates, and *T. parva* infection rates in host cattle. Each of these response variables met the assumptions of normality and homogeneity of variance. For nymphal-to-adult molting rates, we used two-sample Wilcoxon *z*-tests to compare control and vaccinated groups, as the data did not conform to a normal distribution and could not be transformed to meet assumptions of a parametric test. The above analyzes were performed in JMP (SAS Institute, 2010).

Population modeling

We developed a stage-structured Leslie matrix model, using data from the experiments and the literature relating to tick population parameters in the field, to simulate the effects of vaccination on tick population dynamics. The model had a generational time step, with 2 tick generations per year, which is typical in many areas of sub-Saharan Africa (reviewed in Norval et al., 1992). In each generation, events in the model occurred in the following

Table 1
Parameter values used in the population-modeling simulations used to extrapolate the effect of entire herd vaccination with Ra86 over time.

Parameter	Unvaccinated cattle	Vaccinated cattle	Source
Infection rate (IR)	0.65	0.65	1
Fecundity per adult (Fec)	1350	1350	1
Egg hatch rate (Egg.H)	0.83	0.83	1
Larval mortality (Lar.Mort)			
Generation 1	0.98	0.98	2
Generation 2	0.99	0.99	2
Nymphal-to-adult molting (Nym.Molt)	0.10	0.094	a,b,c

Nymphal-to-adult molting success on unvaccinated cattle represented the average of values (log-transformed) from our experiments and Randolph and Rogers (1997). Values for vaccinated cattle were reduced accordingly based on data from our experiments.

^a Data from current experiments.

^b Data from Randolph and Rogers (1997).

^c Data from current experiments and Randolph and Rogers (1997).

order: (i) Adults infested hosts; (ii) adults laid eggs; (iii) eggs developed into larvae; (iv) larvae developed into nymphs; (v) nymphs developed into adults. Values for infection rates, egg laying, and mortality were based on data from the experiments described (Table 1). The initial number of adult females per animal was 50, and the carrying capacity was 200, which represented the maximum number of ticks found on individual cattle in field surveys (Randolph, 1997). We assumed that either 100% or 0% of cattle in a herd was vaccinated, representing a single cattle herd that is managed uniformly. With each set of parameters, the model was simulated for 25 years (50 tick generations).

$$Ad_Host_t = Ad_Tot_{t-1} * IR$$

$$Eggs_t = Ad_Host_t * Fec$$

$$Nym_t = Eggs_t * Egg.H * (1 - Lar.Mort)$$

$$Ad.Tot_t = Nym_t * Molt$$

where Ad_Host_t is the number of adults that successfully infest a host in generation t and Ad_Tot_{t-1} are the number of total adults in the environment at the end of the previous generation. All adults that did not infest a host died. $Eggs_t$ and $nymphs_t$ were the number of eggs and nymphs on hosts in generation t . Values for infestation rates (Inf), fecundity (Fec), egg hatching rates (Egg.H), and nymphal-to-adult molting success (Molt) are shown in Table 1. Larval mortality (Larv.Mort) differed based on the generation of the year (first or second) with values shown in Table 1.

We conducted a sensitivity analysis to examine variation in the model output with different parameter values. In these analyses, we increased and decreased the fitness of individual ticks by 5 or 10% from our standard parameter values. For simplicity, we increased the number of eggs produced per adult from 1350 to 1418 or 1485 (increases of 5 and 10%), or decreased the number of eggs produced per adult from 1350 to 1283 or 1215 (decreases of 5 and 10%). We did not vary mortality, as varying mortality by 5 or 10% within our Leslie matrix model produced the same results as varying the fecundity by 5 or 10%. For each set of initial conditions, we ran the model for 25 years (50 generations).

Results

Vaccination of cattle with Ra86 preparation

Using the baculovirus system, recombinant Ra86 was expressed and purified. Western blot analysis using an anti-Ra86 rabbit

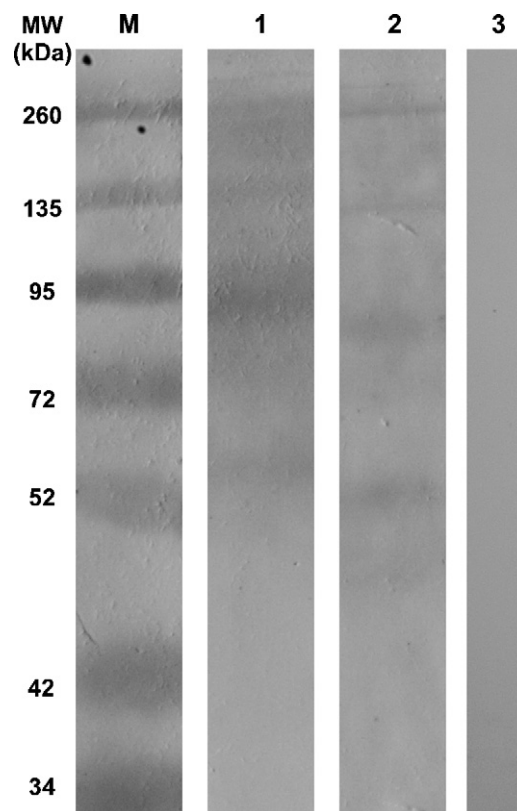


Fig. 1. Immunoblot analysis of baculovirus-expressed recombinant Ra86 showing the formation of Ra86 aggregates after ammonium sulphate precipitation to be used for vaccination of cattle. Anti-Ra86 antibodies (Saimo et al., 2011) were used to identify baculovirus-expressed recombinant Ra86. Lane M, Spectra lane™ Multi-color Broad Range Protein Ladder (Fermentas); lane 1, Ra85A; lane 2, Ra92A; lane 3, Control vaccine preparation.

polyclonal serum (Saimo et al., 2011) confirmed expression and purification (Fig. 1). The predicted molecular weights for Ra92A and Ra85A are 77 kDa and 89 kDa, respectively (Saimo et al., 2011). The Ra86 preparations obtained after purification contained aggregates ranging in size from 75 kDa (Ra92A) and 95 kDa (Ra85A) to more than 135 kDa (Fig. 1).

After 3 Ra86 inoculations delivered in combination with Montanide ISA 50 V as adjuvant, the induced antibody levels were monitored in each animal using ELISA. The control animal group was inoculated similarly with a crude preparation of the uninfected Tni High Five cells in combination with Montanide ISA 50 V. Clearly, animals inoculated with Ra86 preparations developed antibody titers against the Ra86 preparation used for vaccination when compared to the control animal group (Fig. 2). The humoral immune responses of the Ra86-vaccinated group to the Tni High Five insect cell control preparation did not differ from the control-vaccinated group (data not shown).

Effect of Ra86 vaccination on tick biological parameters

Nymphs took 5–7 days to engorge after application onto cattle, and adult female ticks took 7–9 days to fully engorge. All ticks detached over a 3-day period, and detachment days for both nymphal and adult ticks were designated as days 1, 2, and 3 with a higher number indicating a later detachment date. No discoloration or morphological changes in nymphal or adult ticks were observed in ticks feeding on either Ra86-vaccinated or control cattle. On average, 66 (SE = 7.48) engorged females and 2668 (SE = 127)

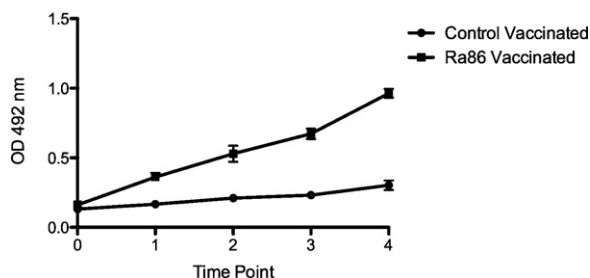


Fig. 2. Mean OD in ELISA to monitor the Ra86-specific humoral immune response in vaccinated (■) and control-vaccinated (●). Time points assessed were 0 (before vaccination), 1 (2 weeks following first inoculation), 2 (2 weeks following second inoculation), and 3 (2 weeks following third inoculation) as described in 'Materials and methods'.

engorged nymphs were collected from a control animal, while 65 (SE = 5.36) and 2871 (SE = 121), respectively, were collected from Ra86-vaccinated animals.

Ra86 vaccination did not significantly affect adult or nymphal mortality rates, or adult engorgement weights (Table 2). However, vaccination with Ra86 significantly (two-sample Wilcoxon z-tests, $p=0.0036$) reduced the molting success of engorged nymphs to adults (Table 2). In some cases, the ticks died as engorged nymphs, in others, the nymphs molted into adult ticks, but died before the removal of shed exoskeleton. The most dramatic effect was in the case of one animal, where 48% of nymphal ticks originally applied failed to molt.

For both control and Ra86-vaccinated groups, the egg laying capacity of engorged females decreased from detachment days 1–3 (Table 3). Females fed on Ra86-vaccinated and control-vaccinated animals did not differ significantly in total egg laying across all days (Table 3). The egg weights produced by females represented a 56% and 52% body-weight to egg-weight conversion for Ra86-vaccinated and control-vaccinated animals, respectively (Table 3). Interestingly, vaccination reduced the number of eggs hatched from

the female ticks that engorged on day 3. From Ra86-vaccinated animals, 49% of eggs failed to hatch compared to 27% of eggs from ticks that fed on control-vaccinated animals (Table 3).

Population modeling

Simulated tick populations on control animals increased from the initial density of 50 adult ticks to the carrying capacity of 200 in 19 years (38 generations), with an additional 2000 nymphs (Fig. 3). After 19 years, the tick population fluctuated between 150 and 200 adult ticks (1500–2000 nymphs), reflecting the differing levels of mortality in the first and second generation of each year (Randolph, 1997). On Ra86-vaccinated animals, however, tick populations declined over time from the initial value of 50 adult ticks, reaching 20 adult ticks and 225 nymphs by year 25 (Fig. 3). Thus, vaccination shifted tick populations into a negative growth pattern over time.

Sensitivity analyses indicated that tick populations on both Ra86-vaccinated and control-vaccinated cattle increased over time when biological fitness of individual ticks was increased by 5 or 10% compared with the standard model (Fig. A1(A) and (B)). However, tick population growth was substantially slower on vaccinated animals in both cases. In contrast, when fitness of individual ticks was decreased by 5 or 10% compared with the standard model, tick populations on both Ra86-vaccinated and control-vaccinated cattle declined over time (Fig. A1(C) and (D)). However, similar to previous results, Ra86 vaccination resulted in lower population densities over time compared to unvaccinated herds, particularly in the first 15 years of simulations (Fig. A1(C) and (D)), confirming the robustness of the model.

Effect of vaccination on acquisition of T. parva infections

The infection of cattle with *T. parva* sporozoites showed no difference in reaction indices (Rowlands et al., 2000) between vaccinated and control animals. Piroplasms were observed within the

Table 2
Effect of Ra86 vaccination of cattle on tick biological parameters, including adult and nymphal engorgement weight, mortality rate after feeding, and molting success of nymphal ticks to the adult stage.

Trait	Control-vaccinated cattle, mean (±SE) ^a	Ra86-vaccinated cattle, mean (±SE) ^a	t_{15} (Z) ^b	P
Adult engorgement weight (g) ^c	0.44 (0.010)	0.46 (0.012)	1.31	0.21
Adult mortality (%) ^c	35.0 (5.4)	34.0 (7.5)	0.78	0.47
Nymphal mortality (%) ^d	11.1 (4.0)	4.3 (4.2)	1.24	0.24
Nymphal molting (%) ^d	95.8 (95–97.5)	89.3 (52–95)	2.91	0.0036

^a For nymphal molting, the median and range are shown rather than standard error, as data were highly non-normal. Nymphal molting success was evaluated using two-sample Wilcoxon z-tests.

^b Two sample t-tests were used to analyze mortality and adult engorgement.

^c Number of observations was 516 for the Ra86-vaccinated group and 521 for the control-vaccinated group.

^d Number of observations was 22,970 for the Ra86-vaccinated group and 21,345 for the control-vaccinated group.

Table 3
Relationships between adult female egg laying and egg hatching capacities given for the 3 individual detachment days (means and standard errors are shown) after feeding till repletion on Ra86 and control-vaccinated cattle.

Parameter	Vaccination status	Detachment day		
		One	Two	Three
Weight of eggs laid per calf (g)	Ra86	9.01 (1.33)	4.87 (0.93)	2.53 (0.30)
	Control	7.92 (1.19)	5.11 (0.60)	3.02 (0.65)
Weight of eggs unhatched per calf (g)	Ra86	0.93 (0.15)	0.92 (0.30)	1.22 (0.23)*
	Control	0.68 (0.12)	0.74 (0.15)	0.56 (0.16)*
Percentage of eggs failing to hatch	Ra86	11.5 (2.20)	22.8 (7.64)	49.0 (8.59)*
	Control	10.4 (2.06)	16.6 (3.91)	20.7 (7.84)*

Values given represent means (±SE).

* Indicates a significant difference ($P < 0.05$) between vaccinated and control groups.

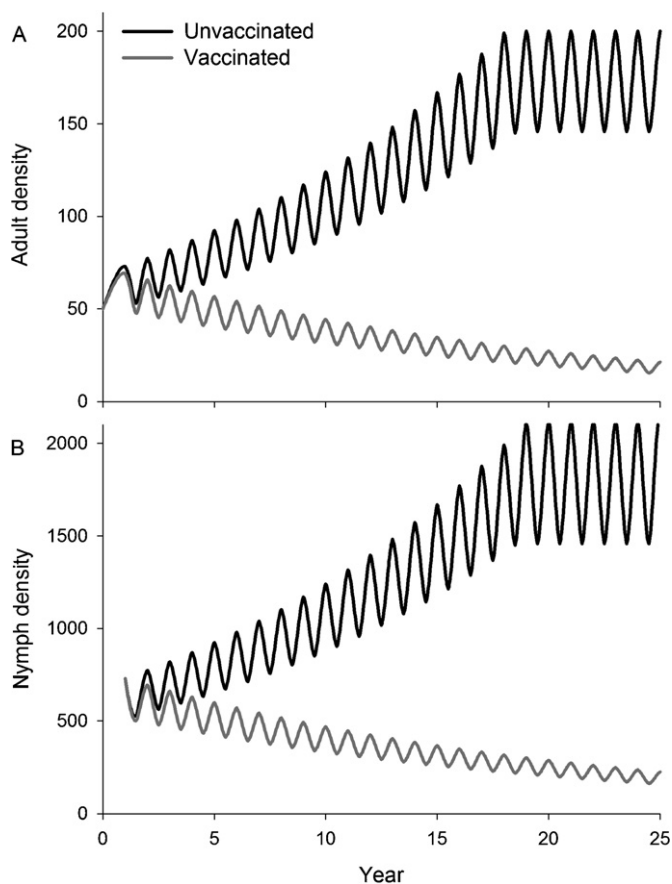


Fig. 3. Simulated (A) adult and (B) nymphal tick population densities over time with Ra86 vaccination (black) or control vaccination (grey) using stage-structured Leslie matrix tick population dynamics model showing a decreasing population densities over time with Ra86 vaccination.

same range in each animal over the tick feeding period ensuring that all ticks received exposure within the same range. *Theileria parva* uptake by ticks was measured as the infection rate, abundance of infection, and the intensity of infection for each tick. Interestingly, levels of sporozoite infection in the salivary glands had a tendency to be marginally lower in ticks fed as nymphs on Ra86-vaccinated animals (Table 4).

Discussion

Protection against ticks after vaccination of cattle using TickGARD™ (recombinant Bm86) is likely to be mediated through induction of antibodies binding to and damaging cells of the tick gut wall (Willadsen et al., 1995; Kopp et al., 2009). A strong correlation between Bm86 antibody titers and subsequent protection against tick infestation has been observed (Willadsen et al., 1995). For protection to persist, antibody titers should remain at a high level, which in the case of Bm86 requires a vaccination schedule of 4 consecutive inoculations, 12 weeks apart (Willadsen et al., 1995).

Odongo et al. (2007) reported that after vaccination with TickGARD™, no effect on *R. appendiculatus* adult female tick mortality and fecundity was observed. In contrast, de Vos et al. (2001) showed an effect on *R. appendiculatus* adults fed on animals vaccinated with Bm86 with a 74% reduction in total egg weight due to a 25% reduction in individual tick egg production. However, due to the small sample size (2 animals per group) and low numbers of ticks applied onto each animal, the results should be interpreted with caution (de Vos et al., 2001). We show that Ra86 vaccination resulted in an improved anti-tick effect compared to what had been previously demonstrated using Bm86 delivered in an optimized commercial vaccine formulation. The main effect of Bm86 vaccination (TickGARD™) is described as a decrease in tick reproductive capacity and direct tick mortality (Jonsson et al., 2000). Here, the main and unexpected effect of Ra86 vaccination was on the nymphal stage. Vaccination significantly affected the ability of nymphal ticks to molt into the adult stage, an effect not previously reported for Bm86 or its homologs in other tick species (Willadsen, 2004; Liao et al., 2007; Azhahianambi et al., 2009; Canales et al., 2009). Interestingly, ticks that detached on day 3 from Ra86-vaccinated animals produced about 50% fewer eggs that were able to hatch compared to ticks fed on control-vaccinated animals. A possible interpretation could be that due to the longer feeding period on Ra86-vaccinated animals, higher levels of tick gut damaging antibodies accumulated in these ticks compared to ticks that detached on day 1. The observed differences between Bm86 vaccination on feeding *R. microplus* ticks and Ra86 vaccination on the feeding of *R. appendiculatus* may be explained in part by the different feeding behavior of the 2 tick species. *R. appendiculatus* is a three-host tick with each life stage feeding 4–7 days before detaching with the full cycle being completed in 3 months. In contrast, *R. microplus* feeds continuously on a host throughout all life stages without any rest period between life stages, the entire feeding cycle can be completed in 3 weeks (Walker et al., 2003). This prolonged feeding period in *R. microplus* allows for continuous exposure to anti-tick vaccine-induced antibodies, whereas in *R. appendiculatus*, the exposure is intermittent. Damage to the *R. microplus* gut induced by antibodies can be reversed (Kemp et al., 1989). If the same mechanisms exist in *R. appendiculatus*, the prolonged rest period between feedings may facilitate gut repair thereby reducing the effect of Ra86 vaccination. Additionally, if the amount of gut antigen present differs between tick species, different levels of protection induced by vaccination could be achieved (de Vos et al., 2001).

All animals in our study responded with development of Ra86 antibodies after 3 inoculations of the experimental vaccine formulation (Fig. 2). It is likely that that the aggregation of the insect cell-produced Ra86 may have contributed to the limited immunogenicity observed (Fig. 1). Despite the presence of an expressed C-terminal histidine tag, purification using Nickel affinity chromatography could not be successfully accomplished despite the use of denaturing conditions (8M urea). In addition, the protein was not detected with anti-histidine antibodies leading to the hypothesis that, through the folding of the protein and the formation of aggregates, the tags became imbedded and inaccessible. This observation was supported by similar findings with attempts to

Table 4
Theileria parva infection levels in adult male and female ticks fed as nymphs on *T. parva*-infected cattle that were either Ra86 or control preparation vaccinated.

Parameter	Male ticks		Female ticks	
	Ra86-vaccinated	Control-vaccinated	Ra86-vaccinated	Control-vaccinated
Infection rate	50.84 (12.94)	56.26 (10.94)	71.04 (10.88)	80.00 (8.06)
Abundance of infection (%)	6.80 (4.12)	20.80 (4.14)	27.80 (13.30)	34.05 (11.84)
Intensity of infection	11.03 (4.38)	16.59 (5.58)	23.97 (9.34)	37.22 (14.38)

All values represent means (±SE).

purify yeast-produced *H. a. anatolicum* Bm86 homolog, Haa86 (Azhahianambi et al., 2009). The efficacy of Bm86-based vaccination has been improved by the employment of different production systems with the best results achieved through the use of eukaryotic expression systems (reviewed in Willadsen, 2004) although, both the production of Haa86 and Ra86 made use of eukaryotic systems. The basic biochemical properties of the variants of Ra86, including the presence of multiple EGF-like domains and the overall organization of hydrophobic and hydrophilic stretches within the protein is similar to that of Bm86 based on the nucleotide sequence (Kamau et al., 2011). We postulate that by changing the recombinant antigen production system, higher antibody levels could be achieved that then translate directly into increased protection against ticks. Additionally, inclusion of a saponin-based adjuvant as used in TickGARD™ instead of the oil in water emulsion-based adjuvant used here may also lead to increased production of protective antibody levels.

Population modeling indicated that this observed effect on nymphal-to-adult molting could be sufficient to result in a gradual decrease in tick population densities over time (Fig. 3). The life cycles of *R. microplus* and *R. appendiculatus* vary greatly, with *R. microplus* feeding continuously on a single host (one-host tick) while *R. appendiculatus* feeds on 3 separate hosts (three-host tick) with periods of molting and digestion spent in vegetation. Despite less contact with the vaccinated host through the life cycle of *R. appendiculatus*, the projected effect of herd vaccination on tick population density was visible within 5 years. However, the full effect would take longer to be established in these vaccinated herds. This suggests that repeated vaccination over a long period of time would be required to significantly reduce tick populations. With improved production and formulation of the Ra86-based vaccine, however, the persistence of the effect may be lengthened. The effect of Ra86 vaccination in rabbits was found to significantly reduce adult female reproductive capacity (Saimo et al., 2011) further suggesting that a change in formulation may improve the impact in cattle.

When *T. parva* infection levels in ticks were analyzed, those fed on Ra86-vaccinated animals exhibited marginally lower levels of infection in all parameter assessed. The number of infected ticks of any stage required to effectively transmit *T. parva* under field conditions remains unknown. There are data indicating that persistently infected cattle with low levels of schizont parasitosis and piroplasm parasitemia (carrier animals) are able to infect ticks (Young et al., 1986) and ticks that have fed on animals with low levels of infection are in turn able to transmit *T. parva* to a

susceptible host while feeding (Konnai et al., 2006). This implies that neither high levels of *T. parva*-infected erythrocytes in cattle nor large numbers of ticks feeding on infected cattle are necessarily required for a disease state to persist in an area (Konnai et al., 2006). However, it is still necessary to determine the density of ticks required to maintain transmission in the field. The data reported here suggest multiple vaccinations with Ra86 would reduce tick densities over time. This raises the possibility that this vaccine could be used to push tick densities below a transmission threshold, as has been observed following repeated Bm86 vaccination (de la Fuente et al., 1999).

Vaccination with Bm86-based vaccines reduced the requirement for acaricide applications by 60% in Cuba (de la Fuente et al., 1998). In Mexico, the interval required for acaricide application was decreased from once every 14 days to 64 days after vaccination (Redondo et al., 1999). Population modeling highlighted the potential for Ra86 vaccination to serve as one component of an integrated tick-borne diseases control program. The modeling data suggested that Ra86-based cattle vaccination has the potential to slow, or even reverse tick population establishment and growth (Fig. 3). In our model, tick populations increased without vaccination, but declined in vaccinated herds. Thus, vaccination could significantly slow the growth of tick populations in the field, or even result in their gradual eradication from areas where vaccination is employed on a regular basis. In turn, these results indicate strongly that Ra86 vaccination in combination with acaricide application could have a profound influence on East Coast fever epidemiology in the field. In summary, our data indicate for the first time that Ra86 vaccination could become one component of integrated tick-borne disease control measures in East Africa, including the sustainable control of East Coast fever.

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Appendix A.

Fig. A1

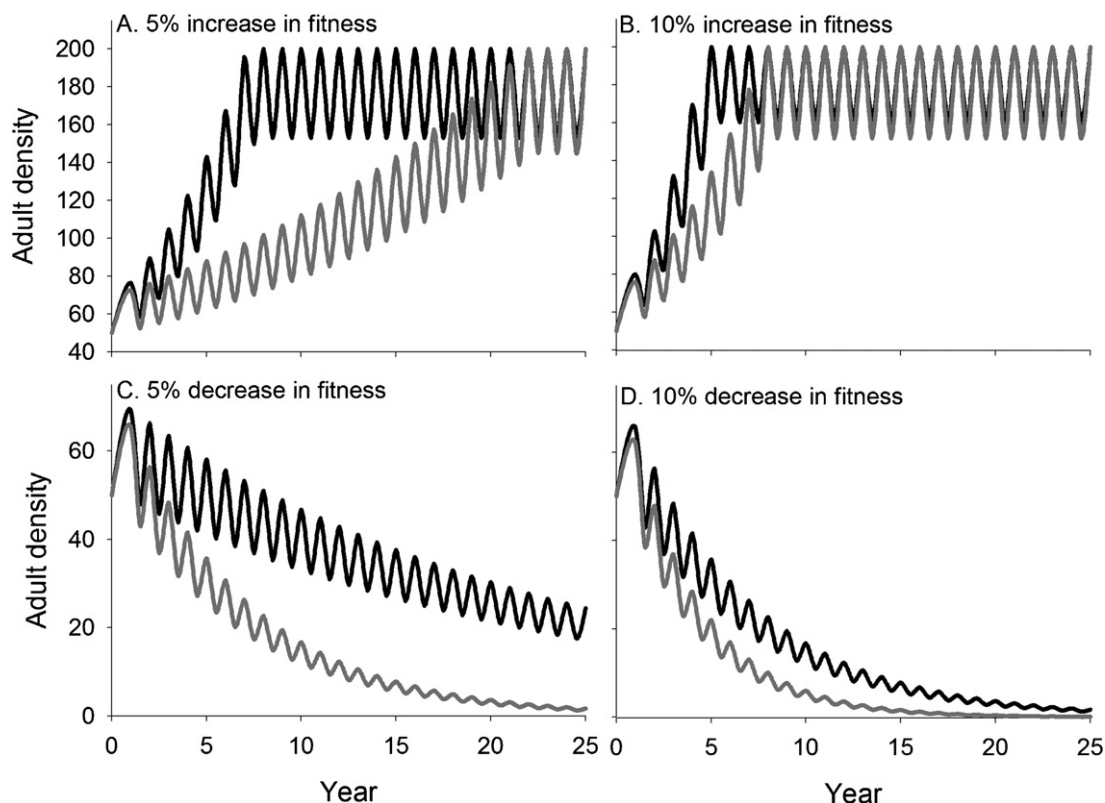


Fig. A1. Adult tick population densities on unvaccinated (black lines) and vaccinated (grey lines) herds with (A) 5% increase in fitness compared with the standard model, (B) 10% increase in fitness compared with the standard model, (C) 5% decrease in fitness compared with the standard model, and (D) 10% decrease in fitness compared with the standard model.

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