Towards integrated control of East Coast fever, a devastating disease of cattle

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Summary

Infection of cattle with the tick-borne apicomplexan parasite *Theileria parva* results in the fatal lympho-proliferative disease known as East Coast fever (ECF). ECF is considered to be one of the most devastating diseases of livestock in sub-Saharan Africa affecting all sectors of livestock production. The three-host brown ear tick, *Rhipicephalus appendiculatus* is the primary vector of *T. parva* with all tick life stages adapted to feeding on cattle. Currently, there is no effective vaccine against ECF and control of *R. appendiculatus* and *T. parva* occurs primarily through the use of disease tolerant breeds, chemotherapy and acaricide application. Widespread application of acaricides for tick and tick borne-disease control is becoming less viable due to rapid development of acaricide resistance. In addition concerns regarding environment and product contamination due to long-term persistence of acaricide residues and high costs associated with infrastructure maintenance exist. Alternative measures for tick control based on integrated and sustainable methods are urgently needed.

Control of the one host tick *Rhipicephalus microplus* through anti-tick vaccination has been shown to be viable culminating in the commercial products Gavac™ and TickGard™. Both vaccines are based on the recombinant protein Bm86 expressed as concealed antigen in the *R. microplus* midgut. Vaccination elicits humoral immune response targeting Bm86 during tick feeding resulting in extensive gut damage. In this thesis, anti-tick vaccines were evaluated for their ability to control *R. appendiculatus* tick populations and to interfere with transmission of *T. parva* in their natural host-pathogen-vector system.

The *R. appendiculatus* homologue of Bm86, named Ra86, was examined for of its impact on nymphal and adult *R. appendiculatus* ticks after feeding on Ra86 vaccinated cattle. The molting success of nymphal ticks to the adult stage was significantly reduced in ticks feeding on Ra86 vaccinated animals in comparison control animals. Simulations based on our empirical data suggest that repeated Ra86 vaccinations would reduce tick populations over successive generations. This experiment showed for the first time that Ra86 based vaccination could play a part in integrated pest management and control strategies for *R. appendiculatus*. Reducing the nymphal population molting to adult instars has implications for
ECF clinical disease severity. *T. parva* transmission by adult ticks is commonly associated with more severe ECF disease symptoms when compared to nymph mediated transmission. Reduction of the number of adult ticks before they can transmit *T. parva* may lead to diminution of the negative impact of ECF on animal productivity. Additionally, Ra86 vaccination lowered *T. parva* infection levels in ticks that fed on vaccinated cattle indicating that targeting the tick gut could affect the uptake of *T. parva* from infected cattle and/or further development within the vector.

Vaccines that block parasite transmission either to- or from- the vertebrate host have been proposed for the control of numerous vector-borne diseases including ECF. Cattle were vaccinated with a multivalent recombinant antigen cocktail containing *R. appendiculatus* antigens TRP64, histamine binding protein (male and female variants) and subolesin. Included in this cocktail was the *T. parva* protein p67, located on the sporozoite surface. Transmission of *T. parva* sporozoites from infected ticks to the vaccinated cattle was compared to control cattle. Establishment of ECF was 20 % lower in the vaccinated animals indicating that this multivalent vaccine partially prevented disease establishment. Importantly, animals that showed no or only mild ECF symptoms after cocktail vaccination and infected tick exposure were solidly protected from lethal needle challenge with the homologous *T. parva* stock. This work demonstrated for the first time that transmission of *T. parva* can be reduced by vaccination resulting in lowering of ECF clinical cases while still advantageously enables establishment of immune protection.

Current experimental infection of animals with *T. parva* involves the needle administration of cryo-preserved isolated sporozoites. This highly artificial infection method differs from natural tick based *T. parva* infection in both the delivery route and the number of parasite injected over time. A reliable tick-based infection method for ECF resembling natural field situations is essential for future intervention studies. Here we show for the first time that the *R. appendiculatus* RAM-L tick line is a suitable tool that reproducibly delivers *T. parva* infectious sporozoite doses to cattle resembling endemically stable field situations. Our newly developed RAM-L tick based infection model can provide information on the potential protective capacity of experimental subunit *T. parva* vaccines requiring substantially smaller animal numbers than conventional field trials. Additionally, combination vaccines targeting
both tick-vector as well as the parasite can now be evaluated.

At the recent Seventh International Conference on Ticks and Tick-Borne Pathogens, 2011, in Zaragoza, Spain, over half of the presentations given in the vaccine session focused on anti-tick vaccination. While historically the main focus of anti-tick vaccination has been on the control of the *R. microplus*, the application of similar approaches to other tick species is being pursued. This work adds novel information on anti-tick and transmission blocking vaccine testing for control of *R. appendiculatus* and *T. parva*. 
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ATV</td>
<td>Anti-tick vaccine</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocytes</td>
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<tr>
<td>ECF</td>
<td>East Coast fever</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbant assay</td>
</tr>
<tr>
<td>ILRI</td>
<td>International Livestock Research Institute</td>
</tr>
<tr>
<td>IPM</td>
<td>Integrated pest management</td>
</tr>
<tr>
<td>ITM</td>
<td>Infection and treatment method</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>RAM-L</td>
<td><em>Rhipicephalus appendiculatus</em> Muguga Low Line</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
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<tr>
<td>SwissTPH</td>
<td>Swiss Tropical and Public Health Institute</td>
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<tr>
<td>TCGF</td>
<td>T cell growth factor</td>
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<td>TBV</td>
<td>Transmission blocking vaccine</td>
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CHAPTER 1: INTRODUCTION

**History of East Coast fever**

East Coast fever (ECF) is a tick-borne disease caused by the apicomplexan parasite *Theileria parva*. “ECF has undoubtedly had more impact on the development of the beef and dairy cattle industries, on veterinary infrastructure, legislation and policies, and on veterinary research in Africa than any other livestock disease complex” (quote from P.J. McCoster, 1991).

ECF was first described officially in 1902 after the importation of cattle into southern Africa from East Africa to restock cattle herds after a devastating rinderpest epidemic. ECF is a major disease of cattle in 11 countries in eastern, central and southern Africa. Affected countries are Burundi, Kenya, Malawi, Mozambique, Rwanda, Sudan, Tanzania, Uganda, Zaire, Zambia and Zimbabwe. (Brown et al., 1990; Norval et al., 1992). The relatively high cost of treatment coupled with poor veterinary services available, especially to smallholder farmers, imply that only a small proportion of infected animals have access to proper treatment. Considerable economic losses are caused by ECF with annual estimates of US$168 million and mortality of 1.1 million cattle (Mukhebi et al., 1992).

*T. parva* has likely co-evolved in East Africa with its natural reservoir, the African buffalo (*Syncerus caffer*) (Norval et al., 1992). Transmission of buffalo-derived *T. parva* stocks to cattle results in severe forms of ECF (Corridor disease) associated with high mortality rates while circulation of *T. parva* within the cattle population results in milder forms of disease (Norval et al., 1991). Clinically, infection of cattle with *T. parva* is characterized by a persistent high fever, lymphadenopathy, infiltration of infected lymphocytes into lymphoid tissue, oedema and towards the end, leucopenia. Infection of exotic *Bos taurus* breeds, introduced to increase milk and meat production, results in high morbidity and mortality. Indigenous *Bos indicus* breeds show higher disease tolerance although morbidity and mortality may still be a major concern, particularly in calves (Norval et al., 1992; Kivaria et al., 2004). Younger animals are generally more tolerant to infection due to a combination of protective maternal antibodies and lower tick burdens (Norval et al., 1992; Marcotty et al., 2002). Endemic stability is observed in regions where repeated exposure of young animals through natural field tick challenge induces and boosts protective immunity. Adult animal populations in these areas generally show no clinical signs of infection (Norval et al., 1992).
**Rhipicephalus appendiculatus as vector of ECF**

The primary vector for *T. parva* transmission is the three-host tick *Rhipicephalus appendiculatus* (Lessard et al., 1990, Lawrence et al., 1994). The distribution of *R. appendiculatus* over Africa is shown in figure 1. The preferred feeding hosts for all tick stages are cattle/African buffalo; additional hosts may include dogs and other small mammals, particularly for immature larval and nymphal stages (Norval et al., 1992; Walker et al., 2003). All three stages of the tick develop over a feeding period of four to seven days. After feeding, the tick detaches from the host and undergoes molting or egg laying (in the case of engorged adult females). Following this period of inactivity, ticks will find a suitable host and resume feeding or die in the case of spent adult females. The entire life cycle of *R. appendiculatus* can be completed within a minimum of three months but may take up to a year. Each adult female can produce between 3000 and 5000 eggs (Norval et al., 1992; Walker et al., 2003). *R. appendiculatus* population growth is strongly dependent on climatic conditions. In southern Africa where *R. appendiculatus* completes one generation a year, ECF disease incidents are seasonal. Adults occur from December to March coinciding with the onset of the rainy season and will only begin questing for a host after the onset of the rains. Larvae hatch late in the summer towards the cooler winter period (April-August) and molt into nymphs in winter to early spring (July-October). In regions where rain is less seasonal, multiple tick generations can overlap each other and disease incidents may occur throughout the year (Norval et al., 1991). In addition to *T. parva*, *R. appendiculatus* serves as vector for *Theileria taurotragi* (causing benign bovine theileriosis); *Anaplasma bovis* (causing bovine ehrlichiosis) and *Rickettsia concorii*, (causing tick typhus in humans and Nairobi sheep disease virus) (Walker et al., 2003).
**T. parva life cycle**

*T. parva* belongs to the sub-phylum Apicomplexa and genus Theileria. The *T. parva* genome has been sequenced and it is distributed over four chromosomes with a total size of 8.3 Mbp (Gardner et al., 2005). In contrast to *Plasmodium*, not all components of the apical complex are present in *T. parva* and entry into host cells is not orientation specific as with other apicomplexan parasites. Rhoptries and micronemes are not involved in the entry process but are involved in parasite establishment in the host cell after entry (Shaw et al., 1991). Maintenance of *T. parva* populations occurs through developmental stages alternating between the bovine host and tick vector (Figure 2). During feeding, *R. appendiculatus* acquires *T. parva* piroplasms contained within erythrocytes from an infected animal. Most piroplasms are destroyed by acid phosphatases secreted by gut epithelial cells but remaining cells differentiate into male and female gametes. Fused gametes form a diploid zygote are able to penetrate the gut wall through an unknown mechanism. Intracellular parasites remain free within the cytoplasm and are not contained within a parasitophorous vacuole (Shaw and
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Young, 1994). With tick molting, motile kinetes exit the gut wall into the hemolymph which provides a pathway to salivary glands. Within the salivary glands, sporozoite development occurs specifically within the ‘e’ cell of the type III acinus (Fawcett et al., 1982a, Fawcett et al., 1982b, Fawcett et al., 1985). Parasite multiplication through sporogony is induced at beginning of tick feeding although it can be artificially induced by elevated temperatures (Young an Leitch, 1981).

Sporozoites are transmitted to the cattle host through saliva pumped into the feeding site during feeding. Components of tick saliva promote T. parva transmission, facilitating the entry of parasites into lymphocytes (Shaw et al., 1993). Multinucleated schizont parasites contained within the cytoplasm of host lymphocytes undergo proliferation resulting in synchronous division of parasite and host cell. The schizont appears to interact with host cell nuclear spindle ensuring that each daughter cell receives a T. parva parasite after division (Hulliger et al., 1964; Hulliger, 1965; Stagg et al., 1980; Dobbelaere and Heussler, 1999). Uncontrolled proliferation of T. parva transformed lymphocytes followed by infiltration of host tissue results in the major pathology of theileriosis (Norval et al., 1992). In vitro, T. parva sporozoites infect all lymphocytes apart from monocytes and neutrophils, although in vivo only infected T lymphocytes with α/β T cell receptors are observed (Baldwin et al., 1988; Morrison et al., 1996). A proportion of schizonts differentiate to merozoite stages resulting in the destruction of the host cell and liberation of parasites (Melhorn and Schein, 1985). Merozoite invasion of erythrocytes seems to occur in a similar manner to the invasion of lymphocytes (Shaw and Tilney, 1992). Merozoites develop into piroplasm stage, infectious to the tick vector required for the completion of the life cycle. Intra-erythrocytic multiplication of piroplasms does not occur in Theileria species and transmission of T. parva within the tick is strictly trans-stadial. For the cycle to perpetuate, a tick must feed successively on a bovine host infected with T. parva and then on a second bovine host that is susceptible to infection. Feeding on an alternative host results in loss of infection by the tick (Norval et al., 1992; Bishop et al., 2004).
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Figure 2. Life cycle of *T. parva* alternating between the tick *R. appendiculatus* and the bovine host (Bishop et al., 2004).

**Control of *R. appendiculatus* and *T. parva***

1. **Disease tolerant breeds**

   Control of *R. appendiculatus* and *T. parva* occurs primarily through the use of disease tolerant breeds, chemotherapy and acaricide application (Norval et al., 1992). The continued use of acaricides for tick control has resulted in large-scale resistance development observed in a number of tick species such as *Rhipicephalus microplus* and *Rhipicephalus decoloratus*. Although resistance occurs more rapidly in one-host ticks, it is still a concern to three-host ticks such as *R. appendiculatus* (George et al., 2004). Additional concerns relating to acaricide use include environmental and product contamination as well as prohibitively high cost of infrastructure maintenance and acaricide purchase (Kocan, 1995; George et al., 2004). The use of tick-tolerant animals is the most viable method of risk reduction for small-
scale farmers however the use of such animals is invariably associated with a reduction in animal productivity (Minjauw and McLeod, 2003). Certain cattle breeds such as Zebu and Sanga are known to be more resistant to tick infestations and better able to tolerate *T. parva* infections. The use of either full or crossbred cattle allows survival of animals using minimal tick control strategies. As severity of East Coast fever is dose dependent, animals able to deter tick infestations are less likely to be exposed to high levels of *T. parva* challenge (reviewed in Norval et al., 1992).

2. Vaccination

The development of live vaccines has occurred for most livestock tick-borne diseases of significance with various levels of efficacy achieved (reviewed by Pipano, 1995). Attempts to produce live blood and *in vitro* culture sporozoite based vaccines for *T. parva* were generally unsuccessful (Pipano, 1995). Development of the current Infection-and-Treatment Method (ITM) of immunization technique occurred in 1977 involving the inoculation of a lethal dose of isolated and cryopreserved *T. parva* sporozoites simultaneously with long acting oxytetracycline formulations (reviewed by di Giulio et al., 2009). Such vaccination is possible due to the ability to harvest metabolically active *T. parva* sporozoites from infected tick salivary glands. Harvested sporozoites remain infective to susceptible cattle after cryopreservation and thawing, although, the quantitative effect on sporozoite viability is not known (di Giulio et al., 2009). After inoculation, protective immunity is induced, based mainly on MHC class I restricted cytotoxic T cells (McKeever, 1990; Morrison and Goddeeris, 1990; McKeever and Morrison, 1994) and vaccinated animals are protected against lethal challenge with autologous parasites (Taracha et al., 1995.). Antigenic variation between *T. parva* strains exist and cross protection between strains is variable (Taracha et al., 1995). Currently, three parasite strains are incorporated into the vaccine stabilate known as the Muguga Cocktail, namely Kiambu 5, Serengeti-transformed and Muguga (Radley, 1981; Dolan, 1987) and contributing genotypes within each stabilate determined (Patel et al., 2011).

Although the vaccine has shown to be generally effective in terms protection, extensive adoption in the field has been low due to a number of reasons. Vaccination results in a carrier state in cattle and parasite transmission to non-vaccinated cattle may introduce new strains to
endemically stable regions (Oura et al., 2004; Oura et al., 2007). Due to antigenic diversity between parasite stocks, breakthrough infections can occur resulting in losses of animals despite vaccination. Cold chain delivery and storage is required to maintain parasite viability meaning that vaccination in remote areas is problematic. Due to vaccine inoculation of live parasites, adverse reactions can occur despite treatment with oxytetracycline and animal monitoring after vaccination is recommended, which, depending on herd size, may be extensive (Minjauw and McLeod, 2003, di Giulio et al., 2009). Production of the stabliate is complex and requires extensive in vivo testing for each batch to determine dose levels. As a result, production of a million vaccination doses requires a staggering 130 head of cattle, 500 rabbits and the application of at least 600 000 nymph *R. appendiculatus* ticks as well as skilled manpower for the dissection and harvesting of salivary glands (Radley et al., 1975; di Giulio et al., 2009).

Intensive research into sub-unit vaccine development for *T. parva* has been carried out for the last 20 years. A 67 kDa protein located on the surface of sporozoites (p67) is conserved among cattle-derived isolates (Musoke et al., 1992; Nene et al., 1996). Vaccination with p67 showed promise under laboratory needle challenge conditions although it was ultimately abandoned after repeated poor field performance (Musoke et al., 1992; Honda et al., 1998; Bishop et al. 2003; Kaba et al., 2005; Musoke et al., 2005).

The major protective immune effector mechanism is thought to be mediated by a MHC Class I restricted CD8+ cytotoxic T cell response directed towards the schizont infected cell (Morrison and McKeever, 2006) and is discussed further in chapter 7. A schizont-stage specific subunit vaccine has been proposed to induce this protective response. Five antigen candidates were identified based on recognition by MHC Class I restricted CD8+ cytotoxic T cell responses in cattle. Development further than initial evaluation has not taken place mostly due to lack of appropriate antigen delivery systems for induction of cytotoxic T cells (Graham et al., 2006; Graham et al., 2007; Akloo et al., 2007).
**Conclusion**

Currently, no method of ECF control which is both effective and meets the needs of diverse livestock production systems exists. Acaricide-based methods of ECF control are not sustainable and alternative control methods are desperately needed. With continuously increasing demands on milk and meat production, the use of low production *T. parva* resistant cattle breeds is no longer economically viable. Globally, acaricide use as the sole measure of tick control is problematic and integrated control strategies are generally seen as the way forward. The immunological control of ticks through anti-tick vaccination will likely represent a component of integrated control strategies for ticks and tick-borne diseases. Proven effective for the control of the cattle tick *Rhipicephalus microplus*, immunization with anti-tick vaccines has resulted in effective tick and tick-borne disease control and reduced acaricide use (de la Fuente et al., 2007). Through the work reflected in this thesis, an extensive evaluation of anti-tick vaccination for control of *R. appendiculatus* and *T. parva* was undertaken. A review on the history and current status of anti-tick vaccination in livestock is presented in chapter 2. In chapter 3, the evaluation of the *R. appendiculatus* antigen Ra86 for its potential to control *R. appendiculatus* populations and the effect on *T. parva* transmission is discussed. Chapter 4 focuses on the development and evaluation of a tick-based cattle infection model for *T. parva*. This tick-based model of *T. parva* infection was imperative for testing of a multivalent transmission blocking vaccine (chapter 5). Chapter 6 describes the adaptation of the *R. appendiculatus* Muguga tick stock to feeding in vitro using a silicone membrane system. Lastly, a preliminary investigation into the immune response of calves infected with tick-transmitted *T. parva* is shown in chapter 7.
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Anti-tick vaccines for the control of ticks affecting livestock

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Accepted book chapter for Molecular Vaccines: From Prophylaxis to Therapy
Abstract

Ticks are obligate hematophagous arthropod parasites affecting most terrestrial vertebrate species. Their importance as disease vectors is due to the abundance and diversity of organisms they transmit to their vertebrate host. In addition, secondary infections of the attachment site, direct toxicosis and paralysis can occur due to tick feeding. The impact of tick-borne diseases is most heavily felt in the livestock sector, where production is limited in many areas due to high tick infestations and tick-borne disease prevalence. Currently, large-scale tick control is achieved through acaricide application but continued use has resulted in resistance to several active ingredients. As an alternative, the immunological control of ticks through vaccination has been proposed. Proof of concept has been shown culminating in commercial vaccines for the control of the cattle tick *Rhipicephalus microplus*. Despite this initial success the development of anti-tick vaccines faces a number of unique obstacles due to complex interactions between tick and vertebrate hosts. This complexity does however allow novel areas of vaccine development to be followed that are unavailable to other vector-borne diseases. Tick antigens localized in the tick gut are concealed from the host immune response during normal tick feeding. With the uptake of the blood meal from a vaccinated host, damage to the gut wall occurs which may result in death or decreased reproductive capacity. The use of ‘concealed antigen’ candidates has been successful in effectively reducing tick populations with successive generations. Furthermore, a number of ‘exposed antigen’ candidates, present in the saliva or cement cone, have also been investigated for their ability to interfere with tick feeding and block parasite transmission. With the completion of tick genome sequencing projects the number of candidate antigens for evaluation as vaccine candidates increasing. In this chapter the history and potential future of anti-tick vaccine development is outlined with particular reference to control of ticks and tick-borne diseases affecting livestock. Anti-tick vaccine advances for the major tick species affecting livestock, *Rhipicephalus*, *Amblyomma* and *Hyalomma* will be discussed.
**Introduction**

Ticks are widely distributed throughout the world affecting 80% of the world’s cattle population\(^1\). The economic importance of ticks and tick-borne diseases (TBD) has been estimated by a number of studies however they most likely represent an under estimation of the real impact of these arthropod vectors and their transmitted diseases. This is partly due to studies having a single focus such as direct losses, cost of control etc. and partly because they often focus only on a single disease or tick species\(^2,3,4\). To further complicate estimations, losses are measured differently for the livestock sector compared to human/companion animals. Tick feeding has devastating effects including disease transmission, paralysis, toxicosis and secondary infections of the tick-feeding site\(^4,5\). The effect of ticks and tick-borne diseases is particularly pronounced in the livestock sector where it is repeatedly rated highly for its impact on the livelihood of farmers, particularly in countries of the South which are heavily dependent on agricultural production\(^3,4\). Heavy tick infestations in livestock can result in anemia, reduction of body weight, loss of body condition and hide damage, an important factor in leather production industries\(^2,6\).

Although argasid ticks of veterinary importance exist, this review will focus solely on Ixodidae ticks. There are six genera of ixodid ticks of importance, namely *Amblyomma*, *Dermacentor*, *Haemaphysalis*, *Hyalomma*, *Rhipicephalus* and *Ixodes* (summarized in table 1). *Ixodes* genus ticks are most well known for their transmission of *Borrelia*, the agents of Lyme disease common in Europe, Asia and North-America. As this species is not of concern in the livestock sector, it will not be discussed further.

Ticks can be tolerated on livestock if numbers remain low and the distribution does not overlap with its associated tick-borne disease. In some instances, near complete eradication of ticks and tick-borne disease have been achieved, complete elimination of tick species from a geographic area is often not feasible\(^7,8\). Historically, tick and tick-borne disease control has focused on the control of ticks at tolerable levels through acaricide use and treatment of disease with appropriate drugs. In some cases acaricide-based tick control is often the only method of reducing tick populations without sacrificing productivity\(^8,9\). Acaricides are
commercially available in a number of formulations that are applied either directly onto livestock or in dipping vats where multiple animals can be passed through at regular time intervals. Acaricide application relies heavily on correct formulation and administration to be effective. A large number of chemical compounds have been found to be effective against ticks including arsenic (introduced ≈1983), DDT (≈1946), cyclodienes and Toxaphene (≈1947), organophosphates-carbamate group (≈1955), formamides (≈1975) and macrolides lactones (≈1981). The potency and usefulness of many of the above-mentioned compounds is gradually eroding with resistance developing in many tick species of *Rhipicephalus*, *Amblyomma* and *Hyalomma*. Multiple acaricide resistant tick stocks have been identified, limiting or entirely excluded the use of many acaricides. In addition to resistance, chemical control through acaricide application results in environmental pollution and residue tainting of meat and milk products. In economically weak countries, use of acaricides for tick control may be even less feasible due to the high cost involved in infrastructure maintenance and product purchase. For small-scale farmers, the formation of co-operatives may be an alternative to allow acaricide-based tick control. Where ticks have been removed by systematic acaricide application, collapse of protocols and infrastructure due to political unrest has resulted in the re-establishment of tick populations and high livestock mortality associated with disease outbreaks.

**Vaccination against ticks**

The guiding principle for anti-tick vaccination stems from early studies conducted on acquired host resistance to tick infestations. Repeated exposure of hosts to ticks or tick organ homogenates induced resistance to tick re-infestation. While the degree of resistance may vary between different tick and host species, evidence strongly suggests that natural resistance against tick infestation develops based on adaptive immune response mechanisms. Ticks feeding from hosts vaccinated with tick components take up effector molecules during feeding that mediate deleterious effects on the ticks. This effect manifests as reduction of feeding time, tick mortality (during or after feeding), reduced engorgement weights and reduced reproductive capacity of adult females. Eggs laid from ticks fed on
vaccinated hosts may also show reduced hatching rates. The overall result culminates in reduction of tick populations and tick-borne diseases.

There are several advantages of anti-tick vaccination over acaricide use for tick control. These include, reduced concerns regarding long-term contamination of animal products and environment with harmful residues and increased sustainability in terms of required infrastructure. Additionally, the resistance development to anti-tick vaccines is likely to be slower than that against acaricides and the costs of anti-tick vaccine development, production and registration is thought to lower than for a new acaricidal compound (estimated at US $ 100 million)\(^\text{5,16,17}\). Although anti-tick vaccination is often viewed as an alternative to acaricide usage, it can also be seen as a complementary approach within the framework of integrated pest management approaches (IPM). Where used appropriately, anti-tick vaccination results in reduction of acaricide use and potentially decreases acaricide resistance development\(^\text{5,17-20}\).

**Anti-tick vaccine candidates**

Many of the anti-tick vaccine targets have been identified using conventional immune-screening techniques. Immunization of vertebrate hosts with tick homogenates or purified tick extracts generates immune sera. These sera are used to screen for tick antigens detected by the host. The identification of tick proteins essential for tick survival is a useful method for more targeted antigen discovery, which is made increasingly possible as information is gathered on tick biology. With the availability of genome sequences for a number of tick species, the number of candidates for discovery is expanding through reverse vaccinology. A repository of the *R. microplus* genome data is a valuable resource for candidate mining\(^\text{21}\) and hopefully, such repositories for other important tick species will follow. The use of other techniques such as RNA interference (RNAi) has been useful in confirming the importance of anti-tick vaccine candidates and is likely to play a role in future anti-tick vaccine antigen discovery\(^\text{22}\).
Anti-tick vaccine candidates have been classified into two categories: exposed or concealed antigens. Exposed antigens are secreted in tick saliva during attachment and feeding on a host while concealed antigens are normally hidden from the host immune response. While Willadsen proposed that antigens should rather be classified according to structure or function, the main advantage of using the exposed vs. concealed distinction is that it has obvious important implications for potential immune evasion mechanisms by the tick. Molecular mimicry by ticks of host components has been observed and vaccination may induce host sensitivity and auto-immune reactions when exposed antigens are used. One advantage of using exposed antigens is that natural boosting occurs through tick feeding. Mechanistically, vaccination with exposed antigens is thought to induce a focal hostile environment unsupportive for tick attachment and feeding. Concealed antigens do not come into contact with the host immune response during natural tick feeding. Although often contained within the thoracic cavity of the tick, some salivary gland proteins can be characterized as concealed if they are not secreted into the tick-feeding site. One difficulty in the development of concealed anti-tick vaccines is that the antigen must be accessible to the induced humoral vaccine response. This often limits the number of candidates to those coming into prolonged and direct contact with the blood meal or where the humoral response can be transported over the gut barrier into the haemolymph. The second limitation of concealed antigens relates to natural boosting of the immune response. As the antigens do not come into contact with the immune response within the host, sufficiently high antibody levels must be induced through repeated vaccination. On the positive side, there is low natural selection pressure on these antigens and emergence of resistance is not of major concern. As the blood meal acts as the carrier for the effector immune responses, the anti-tick effect can take place over a longer period of time compared to exposed antigens. This effect may even extend beyond the mere feeding period into the inactive stages where digestion and molting/egg laying takes place.

**Review of successful anti-tick vaccine candidates**

The Bm86 based anti-tick vaccine remains the only anti-tick vaccine commercially produced and has become the benchmark for future anti-tick vaccine development and
evaluation. The gut-associated Bm86 glycoprotein was first identified in *R. microplus* although homologues in other tick species have since been identified\(^{27,28,30,31,32,33}\). The biological function of Bm86 remains unknown although it is thought to play a role in the digestion of the blood meal\(^ {34}\). In *R. microplus*, expression of Bm86 is increased during embryogenesis reaching the highest level in unfed larvae. Expression decreases during feeding and molting with lowest levels of expression detected during the resting stages of the tick\(^ {35}\). Bm86 has a translated coding sequence of 650 amino acids and a size of 71.7 kDa. The protein contains four potential N-linked glycosylation sites and a leader peptide suggesting transport to the cell surface\(^ {28}\). Localization studies have shown the molecule is located predominantly on the microvilli of gut epithelial cells\(^ {29,36}\). A single C terminal transmembrane sequence is present in the unprocessed protein which is replaced by a glycosyl-phosphatidyl inositol anchor in the mature protein. The protein also contains multiple predicted EGF repeats rich in cysteine residues\(^ {28,34}\).

Vaccination has been performed mostly with the whole molecule and protective epitopes for Bm86 have not been well determined. The site of a protective B-cell epitope was defined and additional epitopes are likely to exist\(^ {37}\). Overlapping cross-reactive immune-reactive epitopes have been found between Bm86 and the *R. decoloratus* homologue, Bd86\(^ {36,38}\). Vaccine efficacy is directly related to anti-Bm86 antibody titre and ability to control tick populations is directly related to achieving a strong antibody response\(^ {39-42}\). Substantial animal-to-animal variation has been observed in the ability to generate anti-Bm86 antibody titres which is likely related to the MHC Class II haplotypes expressed\(^ {42}\). Antibodies to Bm86 and cattle complement system are taken up during the blood meal. Antibody binding results in lysis of the gut epithelial cells culminating in impaired blood meal digestion. Strong antibody responses may induce tick mortality due to blood leakage from the gut into the hemolymph and ticks may turn reddish instead of grey\(^ {34}\).

Recombinant expression of Bm86 has been attempted in several expression systems including *Escherichia coli*\(^ {34}\), *Aspergillus nidulans*\(^ {43}\), *Aspergillus niger* and *Pichia pastoris*\(^ {45-48}\). Vaccine trials showed that Bm86 vaccination targeted mainly the adult stage of *R. microplus*,...
particularly, the number of adult females fully engorging and post-engorgement mortality. Reproductive capacity of adult *R. microplus* females was affected in terms of egg laying capacity and hatching of eggs\(^5,49\).

Under field situations, vaccination of cattle reduced tick numbers by 56% within a single generation and reduced the reproductive capacity by 72%\(^50\). Reversal of negative effects of tick feeding on live weight of vaccinated animals by an average increase in live weight of 18.6 kg over a six-month period was observed\(^40,41\). Extensive field trials in Cuba, Brazil, Argentina and Mexico showed between 55 and 100% control of *R. microplus* ticks within a 36-week period\(^42,51\). Importantly, complete control of acaricide resistant ticks could be accomplished by integrating Bm86 vaccination with acaricide use\(^52\) showing that integrated control systems are effective in controlling tick populations. Vaccination also decreased the amount of acaricides required to control tick populations and prolonged the time interval between cattle dipping\(^5\). Bm86 vaccination has been extensively evaluated for its ability to control other tick species. Almost complete cross-protection against *Rhipicephalus annulatus* has been reported\(^53,54\). Significant protection against *Hyalomma anatolicum*, *H. dromedarii* and *R. decoloratus* has been observed however no cross-protection however was seen against *R. appendiculatus* or *Amblyomma variegatum*\(^38,55\).

Amino acid sequence variations in Bm86 exist between different stocks of *R. microplus* and this was thought to contribute to differences in vaccine effectiveness over different geographic locations. Thus far, no definite correlation between vaccine effectiveness and Bm86 sequence homology has been shown\(^5\). A laboratory *R. microplus* tick strain from Argentina was shown to be resistant to Bm86 vaccination\(^47,51\) and the nucleotide sequence of the Bm86 gene showed substantial differences including 21 amino acid substitutions (out of 610 amino acids in the mature protein)\(^56\). The protein was subsequently renamed to Bm95 although whether it should be separately classed remains debatable. Bm95 has been successfully produced in *P. pastoris*\(^48\) and incorporated in a number of vaccine trials. Effects of Bm95 vaccination are similar to Bm86 vaccination with tick rejection, damage and death. Decreases in engorgement weights, oviposition and egg hatching are also demonstrated\(^57\). A
high level of cross-protective vaccine efficacy against Indian *Rhipicephalus haemaphysaloides* with Bm95 vaccination has been illustrated in cattle\(^{58}\). Using a novel production technique, fusion of immunogenic Bm95 peptides and the *A. marginale* MSP1 N-terminal region was protective against *R. microplus* infestations in rabbits. The reduction of tick oviposition and fertility was comparable to the commercial Bm86 vaccine\(^{59,60}\).

**Commercialization of Bm86 derived vaccines**

Commercialization of the Bm86 anti-tick vaccine in Australia under the name TickGard™ was undertaken by Commonwealth Scientific and Industrial Research Organization (CSIRO) in collaboration with Biotech Australia Pty. Ltd., and released by Hoechst Animal Health in 1994. Within four years, the commercial success of TickGard™ grew till it became the highest value tick treatment product in Australia. Unfortunately, a series of changes within the commercial partners involved in TickGard™ production and distribution resulted in discontinuation of production. The vaccine was briefly re-introduced by Intervet Australia Pty., Ltd. (Bendigo, Australia) but it currently not available for sale\(^{20}\). The Gavac Bm86 based vaccine has been far more successful in areas of Latin America. It was developed by the Center for Genetic Engineering and Biotechnology (Havana, Cuba) and released by Herber Biotec S.A. (Havana, Cuba) in 1993. The vaccine has been extensively used in Cuba where it has resulted in reduction of babesiosis and anaplasmosis as well as a dramatic reduction in required acaricide use\(^{20,42,61}\). Due to state mandated control programs, Cuba illustrates a best-case scenario outcome for anti-tick vaccine deployment\(^{20}\). The vaccine was registered in 1994, 1995 and 1997 for Colombia, Brazil and Mexico, respectively, with these countries reporting on similar control levels achieved\(^{20}\).

**Bm86 homologues of other tick species and their vaccine potential**

Homologues of Bm86 from a range of other tick species have shown promise as tools for tick control. Bm86 vaccination of cattle induces cross-reacting antibodies binding to gut sections of *R. decoloratus* although its anti-tick effect has not been determined. Mouse monoclonal antibodies induced against a synthetic Bd86 derived peptide recognized Bm86 homologues in *R. microplus*, *R. decoloratus*, *H. anatolicum anatolicum* and *R.*
appendiculatus\textsuperscript{35}. Identification and RNAi silencing of the *Haemaphysalis longicornis* Bm86 homologue showed a significant reduction in tick engorgement weight\textsuperscript{62}. The *R. annulatus* homologue of Bm86, Ba86 has been successfully expressed in *P. pastoris* and evaluated in cattle. Vaccination significantly affected tick infestations, oviposition and egg fertility. Additionally, cross protection occurred between Ba86 and *R. appendiculatus* ticks. Interestingly, the efficacy of both Ba86 and Bm86 was higher for *R. annulatus* than for *R. microplus* indicating that Bm86-like effects may be tick species dependent rather than sequence homology dependent\textsuperscript{60}. Vaccination of cattle with *H. anatolicum anatolicum* homologue, Haa86, resulted in significant increase in tick rejection, reduction of tick engorgement weights and egg mass\textsuperscript{32}. The effect of Bm86 homologue vaccination on the transmission of tick-borne diseases has been rarely studied. In one study, Haa86 vaccination reduced transmission of *Theileria annulata* parasites to susceptible calves\textsuperscript{63}. The expression pattern of the *R. appendiculatus* Bm86 homologue, Ra86, is notably different to the Bm86 expression\textsuperscript{35}. In a laboratory tick stock of *R. appendiculatus*, Ra86 homologs exist as two highly divergent allelic variants. Nucleotide sequence similarity between Ra86 and Bm86 is 73\% and 74\% for the two variants Ra85A and Ra92A, respectively\textsuperscript{33}. Vaccination of rabbits with baculovirus expressed Ra86 variants significantly affected engorgement weight and egg weight\textsuperscript{64}. None of these effects could be replicated in the bovine host using the same baculovirus construct for expression of Ra86 homologues. However, a previously unreported significant effect on the molting of nymph ticks to the adult stage occurred after Ra86 vaccination of cattle\textsuperscript{65}.

**Other anti-tick vaccine candidates**

A number of other anti-tick vaccine candidates have shown promise for control of both homologous and heterologous tick species, although none have been taken beyond initial proof-of-concept studies. These antigens are summarized in table 2.

**Dual action anti tick vaccines**

A dual action anti-tick vaccine candidate represents a novel concept in that exposed antigen share antibody binding epitopes with a concealed antigen. The advantage of this
group of antigens lies in the fact that natural boosting occurs through exposed epitopes while targeting concealed antigens. The best studied of these dual action candidates is the 64P protein also known as TRP64 and 64TRP. The protein identified in *R. appendiculatus* is 15k Da and appears to form part of the cement cone. Cross-reactive epitopes were found within the salivary glands, hemolymph, midgut of adult females and whole body extracts for larvae and nymph *R. appendiculatus*. One of the striking features of this antigen is that vaccination of truncated versions affected different stages of ticks. Vaccination mediated effects manifested as engorgement weight and egg mass reduction and direct tick mortality. Inflammatory and adaptive immune responses induced by vaccination with TRP64 disrupt tick feeding. Where feeding does occur antibodies taken up bind to cross-reactive midgut epitopes, rupture of the tick midgut and death of engorged ticks occurs. A broad, cross-protective effect was seen against *I. ricinis, R. sanguineus, A. variegatum* and *R. microplus*. This cross-reaction may be based on strong epitope conservation in the different tick species to maintain molecular mimicry of host collagen and keratin and natural boosting of antibody titres clearly occurs after tick infestation.

While anti-tick effects have been demonstrated in mice and guinea pigs, it failed to show an effect in rabbits using a baculovirus prepared antigen. Furthermore, no tick effect could be established using a bacterial expressed TRP64 as part of a multivalent vaccine in cattle (Olds et al., manuscript in preparation). The potential of TRP 64 as anti-tick vaccine candidate was substantiated after transmission blocking of tick-borne encephalitis virus to susceptible mice by *I. ricinus* ticks. The protective effect of the 64TRP vaccine was comparable to that of a single dose of a commercial tick borne encephalitis vaccine.

During the uptake of the blood meal, the amount of iron increases dramatically in the tick gut where tick ferritins act as iron-storage proteins. Ferritin 2 (Fer2) is a gut-specific protein secreted into the tick hemolymph where it acts as an iron transporter. It is expressed in all tick developmental stages and silencing by RNAi of Fer2 has an adverse impact on tick feeding, oviposition and larvae hatching. Vaccination of cattle with recombinant homologues of Fer2 in *Ixodes ricinus* and *R. microplus* showed excellent control of *R. ricinus* and *R. microplus*. 
and *R. annulatus* for the *R. microplus* homologues\(^71\). Notably, the vaccine efficacy of recombinant Fer2 was comparable to the benchmark obtained with Bm86 vaccination against *R. microplus*\(^71\).

An antigen that received much attention is subolesin. Initially termed 4D8, the antigen was first discovered in *Ixodes scapularis*\(^72\) but it has been characterized in several other tick species\(^73-75\). Targeted reduction of subolesin mRNA using RNAi resulted in degeneration of tick gut, salivary glands, reproductive tissues and embryos\(^74-78\). Immunization of cattle with recombinant subolesin resulted in reduced *R. microplus* survival and reproductive capacity\(^79,81\). Additionally, subolesin was found to control tick gene expression and impact the tick innate immune response to pathogens reducing tick infection by tick-borne diseases *Anaplasma marginale*, *Anaplasma phagocytophilum* and *Babesia bigemina*\(^77,80-86\). Most recently, immunogenic peptides of subolesin were fused to the *A. marginale* MSP1a N-terminal region, named SUB-MSP1a\(^30,59,77,87\). Vaccination of cattle showed high efficacy for control of *R. microplus* and *R. annulatus* through negative impact on tick reproduction and egg fertility\(^87\).

**Transmission blocking anti-tick vaccines**

An anti-tick vaccine able to reduce or block transmission of pathogens from the tick vector to the vertebrate host would aid in TBD control. Transmission blocking vaccines would be ideal in allowing endemic stability development by reducing clinical disease incidence in vaccinated herds. It is well known that saliva aids the transmission of many tick-borne diseases. Saliva-activated-transmission (SAT) is termed as ‘the indirect promotion of tick-borne pathogen transmission via the actions of bioactive tick saliva molecules on the vertebrate host’\(^88\). *Ixodes scapularis* salivary gland protein P11 facilitates migration of *Anaplasma phagocytophilum* from the tick gut to salivary glands\(^89\). The rate of *in vitro* infection of lymphocytes by *Theileria parva* is increased in the presence of *R. appendiculatus* salivary gland extracts\(^90\). Targeting these SAT components seems to be ideal for transmission blocking however, more research is required to identify suitable non-redundant candidates.
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The future of anti-tick vaccine development

The ongoing genome projects of several tick species will provide the community with a wealth of potential novel candidates to be tested as anti-tick vaccines\textsuperscript{17,21}. The use of RNAi experiments can help to functionally prioritize candidates that will be taken further for the study through genetic manipulation of ticks\textsuperscript{22}. Ideally, future anti-tick vaccines should simultaneously target a range of tick species since several tick species may co-feed on livestock. A broad action anti-tick vaccine may be developed by identification of antigens with common, conserved and essential functions across several tick species or by incorporation of multiple antigens into a cocktail.

After the identification of novel candidates, evaluation in the suitable animal model is of paramount importance. Testing of candidates in small animal models is accessible to many research laboratories around the world. Large animal facilities handling livestock experiments however are expensive and rare. Additionally, for many ticks and TBD, appropriate \textit{in vivo} challenge models have not been developed yet. However, it has been shown several times that sera generated from tick immune animals through repeated tick infestation detect different spectra of tick antigens depending on the animal species used. This difference in immune-dominance of tick components is most likely a consequence of long-term host pathogen co-evolution\textsuperscript{23}. One clear example is seen when \textit{Rhipicephalus sanguineus} ticks are fed on non-natural hosts like guinea-pigs. Resistance to tick infestation is developed even after a low number of tick-bites manifested as high tick mortality, reduced female tick weight and reproductive capacity\textsuperscript{91}. In contrast, when \textit{R. sanguineus} ticks are fed on dogs (their natural host), the reaction is significantly reduced, and mainly restricted to immediate inflammatory responses in the skin with a reduced delayed hypersensitivity response\textsuperscript{91}. Molecules present in tick saliva are thought to inhibit the host immune response and in non-host species, repeated tick exposure results in immune responses not present in the natural host systems\textsuperscript{23}. When testing the identical vaccine candidates for efficacy in different host-pathogen interaction systems conflicting results are often observed. Therefore, evaluation of novel candidates should be carried out at an early developmental step in the relevant natural host.
The commercialization of novel anti-tick vaccines should also take into account the lessons learned from Bm86 development\textsuperscript{20}. Despite being commercially available, the vaccine would not be deemed a commercial success. The only two commercial products based on Bm86, Gavac and TickGard are either limited to used in Latin America or removed from the market, respectively. This is striking for a product that targets a highly economically important tick species with a global distribution. Expectations regarding the performance of an anti-tick vaccine should be explained thoroughly to consumers. Since the eradication of distinct tick species from the globe is unrealistic, anti-tick vaccines will be most likely play a part in integrated pest control strategies. These strategies have shown to reduce both tick load and TBD transmission and have the potential to fulfill farmer and government requirement for disease control\textsuperscript{20,92,93}. Early integration of testing anti-tick vaccines together with acaricide treatment schedules will probably pave the way for efficient commercialization of novel products in conjunction with better farmer product information.

In conclusion, the need for novel methods of tick and TBD control is growing and there seems to be acceptance of farmers willing to use anti-tick vaccination\textsuperscript{94}. Development of novel commercial anti-tick vaccine products is beyond the possibilities of most academic institutions and novel public-private partnerships could speed up substantially the establishment of sustainable, cheap and environmentally friendly tick and TBD control strategies globally\textsuperscript{17}. 
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65. Olds, C., Mwaura, S., Crowder, D., Odongo, D., van Oers, M., Owen, J., Bishop, R., Daubenberger, C. Immunization of cattle with Ra86 impedes *Rhipicephalus appendiculatus*...
nymphal-to-adult molting. Ticks Tick-borne Dis 3, 170-178 (2012),


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Figure 1: Due to their large mouthparts, feeding by Amblyomma ticks may result in severe secondary infections of the tick feeding site A: *Amblyomma variegatum* adult male tick, B: *Amblyomma variegatum* adult female tick
Figure 2: Adult *Rhipicephalus appendiculatus* tick. Most known for the transmission of *Theileria parva* to cattle (East Coast fever), the tick species also transmits *Theileria taurotragi* (benign bovine theileriosis), *Anaplasma bovis* (bovine ehrlichiosis), *Rickettsia conori* (tick thyphus) and Nairobi sheep disease virus.
Figure 3: Immature *Rhipicephalus* ticks feeding on the ear of cattle (courtesy of S. Mwaura)

Figure 4: Immature *Rhipicephalus* ticks feeding around the eye of cattle (courtesy of S. Mwaura)
Figure 5: Collection of acaricide resistant adult *Rhipicephalus* ticks in Kenya (courtesy of S. Mwaura)
Table 1: A summary of important tick and tick-borne diseases of livestock\(^4\).

<table>
<thead>
<tr>
<th>Genus</th>
<th>Distribution</th>
<th>Tick borne diseases</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Amblyomma</em></td>
<td>Widely distributed through Africa (<em>A. variegatum</em>), South East Africa (<em>A. hebraeum</em>)</td>
<td>Cowdriosis of ruminants [<em>Ehrlichia (Cowdria) ruminantium</em> (Rickettsia)]</td>
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<td></td>
<td></td>
<td>Benign theileriosis of cattle [<em>Theileria mutans</em> (Protozoa)]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Severe secondary infections of tick feeding site in cattle</td>
</tr>
<tr>
<td><em>Dermacentor</em></td>
<td>Europe, Asia, North America, Africa(^a)</td>
<td>Bovine anaplasmosis [<em>Anaplasma marginale</em> (Rickettsia)]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Equine babesiosis [<em>Babesia caballi</em> (Protozoa)]</td>
</tr>
<tr>
<td><em>Haemaphysalis</em></td>
<td>Asia, Europe and lesser extent in Australia</td>
<td>Bovine babesiosis [<em>Babesia ovata</em> (Protozoa)]</td>
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<tr>
<td></td>
<td></td>
<td>East Asian bovine theileriosis [<em>Theileria buffeli</em> (Protozoa)]</td>
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<td></td>
<td></td>
<td>Babesiosis of small ruminants [<em>Babesia motasi</em> (Protozoa)]</td>
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<tr>
<td></td>
<td></td>
<td>Bovine babesiosis [<em>Babesia major</em> (Protozoa)]</td>
</tr>
<tr>
<td><em>Hyalomma</em></td>
<td>Asia, Europe, Africa</td>
<td>Bovine tropical theileriosis [<em>Theileria annulata</em> (Protozoa)]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Theileriosis of small ruminants [<em>Theileria lestoquardi</em> (Protozoa)]</td>
</tr>
<tr>
<td><em>Rhipicephalus</em>(^b)</td>
<td>America (North and South), Africa, Asia, Australia</td>
<td>Bovine babesiosis [<em>Babesia bovis</em> and <em>Babesia bigemina</em> (Protozoa)]</td>
</tr>
</tbody>
</table>
Equine piroplasmosis [Theileria equi and Babesia caballi (Protozoa)]
Bovine anaplasmosis [Anaplasma marginale, Anaplasma centrale (Protozoa)]
Babesiosis of small ruminants [Babesia ovis (Protozoa)]
Anaplasmosis of small ruminants [Anaplasma ovis (Protozoa)]

\[a\] Although present in Africa, not considered important

\[b\] The Rhipicephalus genus now includes tick species formerly known as Boophilus. Most important tick genus and includes cattle tick Rhipicephalus microplus, the main vector for bovine babesiosis and Rhipicephalus appendiculatus the vector for East Coast fever (bovine theileriosis) in cattle.
Table 2: List of anti-tick vaccine candidates evaluated for the control of ticks on livestock

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Location and function</th>
<th>Effect of vaccination</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMA7</td>
<td>63 kDa membrane bound glycoprotein widely distributed in tick tissues. Biological function unknown</td>
<td>Vaccination with <em>R. microplus</em> BMA7 showed significant protection against tick infestation. Effect was less than the Bm86 mediated vaccination</td>
<td>95</td>
</tr>
<tr>
<td>SBm7462</td>
<td>The synthetic peptide SBm7462 derived from Bm86</td>
<td>Cattle vaccination resulted in a efficacy above 80%</td>
<td>37</td>
</tr>
<tr>
<td>Bm91</td>
<td>Membrane-bound carboxydipeptidase Identified in <em>R. microplus</em> Located in the tick salivary gland</td>
<td>Increases the effect of Bm86 vaccination</td>
<td>96,97</td>
</tr>
<tr>
<td>Serine proteinase inhibitors</td>
<td>The serine proteinase inhibitors (serpins) have been the focus of a number of promising anti-tick vaccine studies.</td>
<td>Vaccination of rabbits with recombinant HLS1 resulted in 44% and 11% mortality in nymph and adult ticks, respectively. Vaccination of rabbits with <em>H. longicornis</em> serpin-2 resulted in a mortality rate of 45% and 43% in nymph and adult ticks, respectively. Four serpins have been identified in <em>R. appendiculatus</em>, named RAS1-4. Vaccination of cattle with recombinant RAS1 and RAS-2 conferred protective immunity with 61% reduction in nymphal engorgement weight, 28% and 43% adult female and male mortality rate, respectively.</td>
<td>98-101</td>
</tr>
<tr>
<td><strong>Troponin I-like protein</strong></td>
<td>Isolated from <em>H. longicornis</em> and induced specific antibody responses in both rabbits and mice. Located in muscle, cuticle, gut, and salivary glands.</td>
<td>Vaccination of mice and rabbits resulted in significant extension of feeding times for larvae and adult ticks, low larval engorgement rates and reduction in egg weights.</td>
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<tr>
<td><strong>Vitellin</strong></td>
<td>Most abundant yolk protein of <em>R. microplus</em> eggs. Antibodies raised to vitellin also recognized a 200 kDa polypeptide in the hemolymph of adult tick.</td>
<td>In its native form induced an immune response protecting sheep against infestation with <em>B. micrurus</em>.</td>
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</tr>
<tr>
<td><strong>Voraxin</strong></td>
<td>Engorgement factor</td>
<td>Rabbits immunized against the <em>A. hebraeum</em> voraxin, engorgement was seen to be reduced by 74% while all ticks fed on control rabbits fully engorged.</td>
<td></td>
</tr>
<tr>
<td><strong>Glutathione S-transferases</strong></td>
<td>Glutathione S-transferases (GST) are a family of enzymes involved in detoxification of xenobiotics and endogenous compounds and they have been identified in a number of tick species.</td>
<td>Recombinant <em>H. longicornis</em> GST-Hl as an anti-tick vaccine candidate has been evaluated in cattle for cross protection against <em>R. microplus</em> where an overall vaccine efficacy of 57% was shown against <em>R. microplus</em>.</td>
<td></td>
</tr>
<tr>
<td><strong>RIM36</strong></td>
<td>Isolated from <em>R. appendiculatus</em> and it is present in salivary glands (type III salivary gland acini) and the cement cone</td>
<td>Immunodominant, under natural infestation vaccination of cattle did not induce a clear protective effect although strong antibody responses were induced.</td>
<td></td>
</tr>
<tr>
<td><strong>HL34</strong></td>
<td>Saliva protein identified in <em>H. longicornis</em> through tick saliva immune rabbit serum immuno-screening of an adult tick cDNA library.</td>
<td>Vaccination of rabbits resulted in increased morbidity and mortality in nymphal and adult stages of <em>H. longicornis</em> although the effect did not reach statistical significance.</td>
<td></td>
</tr>
<tr>
<td><strong>p29</strong></td>
<td>29-kDa extracellular matrix-like protein from <em>H. longicornis</em> is thought to be a component of the cement cone.</td>
<td>Recombinant p29 immunization of rabbits resulted in mortality rates of 40% and 50% of <em>H. longicornis</em> larve and nymphs,</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER 2: ANTI-TICK VACCINES

<table>
<thead>
<tr>
<th>Protein</th>
<th>Identification</th>
<th>Function/Effect</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histamine binding protein</td>
<td>Identified in <em>R. appendiculatus</em> and has a role in itch response</td>
<td>No anti-tick effect in multivalent vaccination. Mild transmission blocking effect</td>
<td>117</td>
</tr>
<tr>
<td>Male Immunoglobulin-binding protein</td>
<td>Identified in <em>R. appendiculatus</em></td>
<td>Vaccination of guinea pigs resulted in slight female feeding impairment</td>
<td>24</td>
</tr>
<tr>
<td>β-N-acetylhexosaminidase</td>
<td>Identified in <em>R. microplus</em></td>
<td>Direct injection of rabbit polyclonal antibodies into replete females resulted in a 26% reduction in oviposition. No evaluation as a recombinant vaccine</td>
<td>118,122</td>
</tr>
<tr>
<td>5'-nucleotidase</td>
<td>Identified in <em>R. microplus</em></td>
<td>Inactive, truncated form of the protein produced a significant effect in sheep only where high antibody titres were induced. No effect in cattle</td>
<td>119,120</td>
</tr>
<tr>
<td>Calreticulin</td>
<td>Identified in <em>A. americanum</em>, <em>R. microplus</em></td>
<td>Poorly Immunogenic in cattle, no effect</td>
<td>121</td>
</tr>
<tr>
<td>Yolk pro-Cathepsin</td>
<td>Identified in <em>R. microplus</em></td>
<td>Antigenic in cattle, vaccination resulted in a protection rate of 25%</td>
<td>123-126</td>
</tr>
</tbody>
</table>

respectively. Reduction in engorgement weight of adult female ticks of 17% was noted.
Aims and Objectives

General Aim

Novel approaches to integrated control of ECF and its tick vector, *R. appendiculatus*, are of paramount importance for sustainable meat and milk production in East and southern Africa. This thesis aimed to develop novel approaches for the development and evaluation of anti-tick and ECF transmission blocking vaccines in the natural host – pathogen – vector system.

Specific Objectives

I. Evaluate the concealed tick gut antigen Ra86 as vaccine candidate for control of *R. appendiculatus* and *T. parva*

II. Develop a controlled tick infection model for *T. parva*

III. Asses a multivalent transmission blocking vaccine for ECF

IV. Establish an *in vitro* feeding system for *R. appendiculatus*
Immunization of cattle with Ra86 impedes *Rhipicephalus appendiculatus* nymphal-to-adult molting

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CHAPTER 3: EVALUATION OF CONCEALED R. APPENDICULATUS ANTIGEN RA86

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 Ra86 have been successful in controlling the one-host tick Rhipicephalus (Boophilus) microplus and provide heterologous protection against certain other non-target 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 Ra86, named Ra68, 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 Ra68 could thus form a component of integrated control strategies for R. appendiculatus leading to a reduction in use of environmentally damaging scaricicides.

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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 (Grey and Robertson, 1902). R. appendiculatus acquires T. parva through the uptake of a blood meal from a T. parva-infected bovine. Proplasmids 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 proplasmids 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 nymph 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, HaaB6, was used for the vaccination of cattle, a significant reduction of the reproductive capacity of H. a. anatolicum ticks was observed (Azahalianambi et al., 2009). Similarly, the R. annulatus homolog, RaB6, was able to control both R. annulatus and R. microplus (Canale 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, RaB6A and RaB9A, together collectively as RaB6 (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 RaB6 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 RaB6A and RaB9A will be referred to collectively as RaB6, following the nomenclature of Saimo et al. (2011). In instances where they were treated differently or separately, they will be referred to individually. Recombinant RaB6 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 RaB6 (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 μl and added to a 75-cm² 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 (Sterlin). The contents were centrifuged at 3500 x g for 10 min at 4°C to separate supernatant and cell fractions. The supernatant fraction was treated with a 25 x stock solution of Complete Protease Inhibitor Cocktail tablets (EDTA-free) (Roche, Mannheim, Germany) resulting in 1 x 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 sheared using agitation with silica beads (0.1 mm) in a FastPrep-24 machine (MP Biomedicals). A cycle of 30 s agitation followed by cooling on ice for 5 min was repeated 3 times. Centrifugation at 10,000 x 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 x 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 RaB6 and recovery after ammonium sulphate precipitation was confirmed using SDS-PAGE and immune-blotting with anti-RaB6 sera, generated in a previous study (Saimo et al., 2011). The anti-RaB6 sera used recognized both RaB6A and RaB9A proteins. Protein quantification of RaB8A, RaB9A, 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 RaB6 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 (TBS) (10 mM Tris, 150 mM NaCl, and 0.05% Tween 20), pH 8.0. Following blocking, the membrane was washed 3 times in TBS, then incubated for 1 h at room temperature in anti-RaB6 rabbit polyclonal sera (Saimo et al., 2011) diluted 1:500 in TBS. After washing 3 times with TBS, blots were incubated with 1:5000 with anti-rabbit whole Ig linked to peroxidase conjugate (Amersham Biosciences) diluted in TBS. Following incubation, blots were washed 3 times with TBS and developed using diaminobenzidine (DAB) with H2O2 added as the substrate.

Vaccine formulation

Samples prepared for the RaB6 vaccination group included 50 μg of the preparation for RaB6A and 50 μg of the preparation for RaB9A 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 RaB6-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 instructions.

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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 East African Veterinary Research Organization-Kenya Agricultural Research Institute (EAVARO-KARI). 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 the 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. Proton BABS cellulose nitrate membranes (Schleicher & Schuell) were sectioned and numbered to produce a grid of 0.5 x 1 cm blocks. Ra86 protein (1 µg) 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 (Polyisorb, 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 (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 using SIGMAFAST OPD (Sigma) following instructions. Strips were removed and plates were read at OD490 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 x 10^5 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 post-infection, 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 IUR.

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/ILR (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 post-infection 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 (Rübsch & Ottin, 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 parameters 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 analyses 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
Chapter 3: Evaluation of Concealed R. Appendiculatus Antigen Ra86

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

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unvaccinated cattle</th>
<th>Vaccinated cattle</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infestation rate (IR)</td>
<td>0.65</td>
<td>0.65</td>
<td>1</td>
</tr>
<tr>
<td>Fecundity per adult (Fec)</td>
<td>1395</td>
<td>1395</td>
<td>1</td>
</tr>
<tr>
<td>Egg hatch rate (Egg H)</td>
<td>0.83</td>
<td>0.83</td>
<td>1</td>
</tr>
<tr>
<td>Larval mortality (Lar Mort)</td>
<td>0.98</td>
<td>0.98</td>
<td>2</td>
</tr>
<tr>
<td>Generation 1</td>
<td>0.99</td>
<td>0.99</td>
<td>2</td>
</tr>
<tr>
<td>Nymphal-to-adult molting (Nym-Molt)</td>
<td>0.10</td>
<td>0.094</td>
<td>c,b,c</td>
</tr>
</tbody>
</table>

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 = 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 (IR), fecundity (Fec), egg hatching rates (Egg H), and nymphal-to-adult molting success (Molt) are shown in Table 1. Larval mortality (Lar.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 0% 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 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 50V 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 50V. 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 2688 (SE = 127)
CHAPTER 3: EVALUATION OF CONCEALED R. APPENDICULATUS ANTIGEN RA86

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Fig. 2. Mean OD in ELISA to monitor the RA86-specific humoral immune response in vaccinated (●) and control-vaccinated (▲) ticks. 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

<table>
<thead>
<tr>
<th>Trait</th>
<th>Control-vaccinated cattle, mean (±SE)</th>
<th>RA86-vaccinated cattle, mean (±SE)</th>
<th>t₁,₁ (Z)²</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult engorgement weight (g)</td>
<td>0.44 (0.010)</td>
<td>0.46 (0.012)</td>
<td>1.31</td>
<td>0.21</td>
</tr>
<tr>
<td>Adult mortality (%)</td>
<td>35.0 (5.0)</td>
<td>34.0 (7.5)</td>
<td>0.78</td>
<td>0.47</td>
</tr>
<tr>
<td>Nymphal mortality (%)</td>
<td>11.1 (4.0)</td>
<td>4.3 (4.2)</td>
<td>1.24</td>
<td>0.24</td>
</tr>
<tr>
<td>Nymphal molting (%)</td>
<td>95.8 (55-97.5)</td>
<td>89.3 (52-95)</td>
<td>2.91</td>
<td>0.0036</td>
</tr>
</tbody>
</table>

* 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.

² Two sample t-tests were used to analyze mortality and adult engorgement.

Table 3

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vaccination status</th>
<th>Detachment day</th>
<th>One</th>
<th>Two</th>
<th>Three</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight of eggs laid per calf (g)</td>
<td>RA86</td>
<td>9.01 (1.33)</td>
<td>4.87 (0.93)</td>
<td>2.53 (0.30)</td>
<td></td>
</tr>
<tr>
<td>Weight of eggs unhatched per calf (g)</td>
<td>Control</td>
<td>7.92 (1.19)</td>
<td>5.11 (0.60)</td>
<td>3.02 (0.65)</td>
<td></td>
</tr>
<tr>
<td>Percentage of eggs failing to hatch</td>
<td>RA86</td>
<td>0.03 (0.15)</td>
<td>0.02 (0.30)</td>
<td>1.22 (0.23)</td>
<td></td>
</tr>
<tr>
<td>Percentage of eggs failing to hatch</td>
<td>Control</td>
<td>0.68 (0.12)</td>
<td>0.74 (0.15)</td>
<td>0.56 (0.16)</td>
<td></td>
</tr>
</tbody>
</table>

Values given represent means (±SE).

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

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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 (TickGARDPLUS) 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; Azthahianambi 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 (Wallace 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 (8 M 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 become imbedded and inaccessible. This observation was supported by similar findings with attempts to...
purify yeast-produced H. o. anatolicum Bm86 homolog, Haa86 (Ashahianambé 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 (Kanau 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 TickGARDTM 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 parasitism 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., 2005). 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.

Acknowledgements

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Appendix A.

Fig. A1

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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.

References


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Controlled tick transmitted *Theileria parva* infection confers protection against lethal needle challenge in calves

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Abstract

The study of tick-borne diseases in livestock typically employs needle administration of pathogens as an experimental challenge approach, although this does not accurately reflect the parasite challenge encountered under field conditions. We demonstrate that *Theileria parva* can be transmitted to cattle by a distinct *Rhipicephalus appendiculatus* tick line selected to deliver a *T. parva* challenge reproducing that encountered in the field. Interestingly, these animals were protected against a subsequent lethal sporozoite needle challenge indicating induction of protective immune responses. This model for East Coast fever can serve as a defined autologous challenge model for vaccine trials and infection studies in cattle.

Keywords

*Theileria parva*, *Rhipicephalus appendiculatus*, tick-cattle challenge model, tick-borne disease

Main text

The tick-borne apicomplexan parasite *Theileria parva* causes East Coast fever (ECF) in cattle, which is one of the most devastating diseases of livestock in sub-Saharan Africa. ECF results in high mortality and morbidity affecting all sectors of livestock production, particularly exotic *Bos taurus* dairy cattle and Zebu (*Bos indicus*) calves where *T. parva* infection may result in up to 50% of calf mortality in affected areas (Kivaria et al., 2004; Norval et al., 1992). The three-host brown ear tick, *Rhipicephalus appendiculatus* is the primary vector of *T. parva* with all tick life stages adapted to feeding on cattle. East Coast fever transmission is strictly trans-stadial with larval or nymphal tick stage infection occurring during feeding, and transmission to susceptible cattle occurring during feeding of nymphal and adult stages, respectively (Norval et al., 1992). Transmission efficiency of *T. parva* differs between tick life stages and adult male and female ticks. Development of infective sporozoites occurs in the ‘e’ cells of type III acini of tick salivary glands. Adult females contain the highest levels of ‘e’ cells followed by adult males with nymphal ticks having the lowest levels. Despite their abundance of ‘e’ cells, adult male ticks are not considered efficient vectors due to irregular rates of sporogony and intermittent feeding (Ochanda et al., 1996). In addition to transmission...
differences between sexes and life stages, it has been shown that tick stocks vary in their competence as vectors of *T. parva* (Ochanda et al., 1998; Odongo et al., 2009). Young et al. demonstrated that susceptibility of ticks to *T. parva* infection represents a heritable trait that can be manipulated to produce high- or low-level transmitting stocks by selective breeding of siblings families that exhibit high or low levels of vector competence (Young et al., 1995).

The severity of ECF is directly related to the dose of *T. parva* sporozoites delivered (Norval et al., 1992). Experimental infection of cattle with *T. parva* is currently performed using needle-based administration of cryo-preserved sporozoites harvested from homogenized *R. appendiculatus* salivary glands. Simultaneous inoculation of cattle with an estimated $5.9 \times 10^4$ live sporozoites and a long acting formulation of tetracycline form the basis of the ‘infection and treatment’ method of vaccination against ECF (reviewed by Di Giulio et al., 2009). Bovine Lymphocyte Antigen (BoLA) class I restricted, CD8+ cytotoxic T cells are currently the only well characterized effector mechanism for host control of *T. parva* elicited by the ‘infection and treatment’ live immunization method (Di Giulio et al., 2009). During tick feeding, sporozoites trickle into the cattle host over a period of several days and the simultaneous inoculation of high parasite numbers is unlikely to occur naturally as with needle challenge (Konnai et al., 2007). In addition, ticks inject a cocktail of molecules that modulate host immune and inflammatory responses and may also directly influence the ability of pathogens to infect host cells (reviewed by Wikel, 1999; Schoeler and Wikel, 2001).

Despite these essential differences, needle-based experimental infection of cattle with *T. parva* has, to date, been the only mechanism used to assess *T. parva* vaccine candidates for induction of protective immune responses (reviewed by Morrison and McKeever, 2006). We report here a novel, defined experimental tick-challenge model for *T. parva*. A selected *R. appendiculatus* tick stock named RAM-L, experimentally infected with *T. parva*, reliably transmitted *T. parva* sporozoites to naïve cattle. Following single exposure to tick delivered *T. parva*, all cattle were solidly protected against homologous lethal needle challenge.

The *R. appendiculatus* Muguga ‘low-line’, RAM-L, used for this study was developed and maintained at the International Livestock Research Institute (ILRI) Tick Unit. The differentiation of the original Muguga tick strain into distinct high- and low-transmitting tick lines involved the cross-breeding of siblings with greater or lesser susceptibility to *T. parva*
infection (Young et al., 1995). Uninfected ticks were maintained in Biological Oxygen Demand (BOD) incubators at 28 ± 1 °C and *T. parva* infected ticks at 24 ± 1 °C, 80% relative humidity. The *T. parva* Muguga strain was originally isolated from the Kiambu District of Kenya and has been maintained as laboratory stock through a series of tick passages (S. Mwaura, unpublished data).

To infect approximately 2000 RAM-L ticks with *T. parva*, a Friesian calf (*Bos taurus*), four months of age and free of detectable tick-borne pathogens was infected with a lethal dose of *T. parva* Muguga (ILRI stabilate 3087, 1 mL dose of 1:20 dilution) by subcutaneous injection under the right parotid lymph gland. Treatment with a short acting oxytetracycline formulation was administered on days 11, 12, 13 and 15 post-infection to control ECF symptoms and allow tick feeding over the period of patent piroplasm parasitemia. Rectal temperature was recorded daily and from day 5 post-infection, ear vein smears and lymph node biopsies were taken daily. Two thousand RAM-L nymphs were applied in a bag secured to the back of the animal on day 12 post infection, two days prior to the onset of piroplasm parasitemia. Ticks fed over a maximum period of seven days, and detached over a period of four days. Ticks from each day of detachment were pooled and designated as batch 1 – 4. Detached nymphs were placed into BOD incubators for six weeks to allow molting to adults and sorted into male and female ticks. Thirty adult male and thirty adult female ticks from each batch were pre-fed for four days on the ears of rabbits to induce sporozoite maturation within tick salivary glands. The infection rate was determined as the percentage of ticks with infected acini and abundance as the average number of infected acini per tick. Infection levels were determined by microscopic examination of Feulgen’s stained salivary glands.

*T. parva* infection parameters for the four RAM-L tick batches collected are shown in Figure 1. Overall infection rates were similar to natural field occurring tick infection rates in areas of endemic stability (Norval et al., 1992). Infected male ticks were not used for transmission experiments as male ticks are not considered to be effective vectors. Female ticks from Batch 3 were selected for experimental challenge of cattle because they represented the largest proportion of the experimentally infected tick batch and exhibited the lowest infection levels among the female batches. All experiments were carried out in
accordance with procedures approved by ILRI’s Institutional Animal Care and Use Committee, which insures adherence to internationally approved standards of animal welfare.

Ten Friesian (Bos taurus) bull calves (six months of age), serologically confirmed to be free of infection with Babesia bigemina, Anaplasma marginale, T. parva and Theileria mutans according to ELISA were reared under tick-free conditions. Calves were split into two groups of five animals each, due to space constraints within the tick unit animal containment facility. Identification of bovine classical class I alleles (BoLA class I alleles) of each animal was determined by PCR amplification using Bov14 and Bov7 primers followed by cloning and Sanger Sequencing (described by Birch et al., 2006). Classical class I alleles were identified by BLAST algorithm search against the cattle section of the Immuno Polymorphism Database (http://www.ebi.ac.uk/ipd/mhc/bola). Results shown in Table 1 emphasize that a broad range of different BoLA class I alleles were expressed in experimental cattle. Evidence suggests that the peptide-specificity of the CTL response to T. parva infected lymphocytes depends on the MHC genotype of the animal (reviewed in Norval et al., 1992).

Thirty female T. parva infected RAM-L adults from Batch 3 were applied in a bag attached to the right ear of each calf. Thirty T. parva uninfected male Muguga ticks were placed in the same bag to facilitate the feeding of female ticks. Female ticks were allowed to feed to repletion and detach after which they were collected and removed. Blood samples from both the jugular and ear vein were taken daily following tick application. Daily lymph node biopsies were taken from day ten after RAM-L application from the local lymph node draining the site of infection (right ear), and from day 15 from the contralateral pre-scapular lymph node (left shoulder). Rectal temperature was recorded daily from the onset of the experiment. Transmission of T. parva sporozoites to calves was evaluated using microscopy, PCR and serology. Microscopy was performed using Giemsa stained lymph node aspiration smears and blood smears for the detection of macroschizonts and piroplasms, respectively. Macroschizont infected cells were graded as I, II or III with increasing levels of infection designated by a higher number. Piroplasms were counted as the number of infected erythrocytes in a total of $1 \times 10^3$ erythrocytes screened. PCR based detection of low schizont parasitosis/piroplasm parasitemia in peripheral blood was performed in duplicate targeting the p104 gene of T. parva (Skilton et al., 2002, Odongo et al., 2010). Antibodies binding to the T.
parva polymorphic immunodominant molecule (PIM) antigen were detected using ELISA (Katende et al., 1998). Cattle serum samples for use in the PIM ELISA were taken 12 and 10 weeks post tick challenge for groups 1 and 2, respectively.

In nine out of ten animals, schizonts were detected in Giemsa stained regional lymph node biopsy smears. In animal BF019, no schizont parasites were detected however, this animal was tested positive using PIM ELISA and p104 PCR. In animal BF033, schizont parasites were detected in both regional and contra-lateral lymph nodes but duplicated p104 PCR results were negative although anti-PIM antibodies were detected in this animal (Table 2). Two animals were treated once with a short acting tetracycline formulation on days 20 and 22 to reduce discomfort caused by a persistent fever. The schizont parasitemia in the regional and contra-lateral lymph nodes had already declined by days 20 and 22.

The ability to withstand a homologous lethal challenge has been observed in animals recovered from primary *T. parva* infections (Norval et al., 1992). To verify that *T. parva* sporozoites had successfully been delivered by the RAM-L ticks and that *T. parva* specific immunity was induced, all animals were needle-challenged with a lethal homologous dose of *T. parva* Muguga stabilate. Two *T. parva* naïve calves of the same age and breed served as a control group for this experiment. Twelve (group 1) or ten weeks (group 2) after tick infestation, all animals were challenged with a 1 mL dose of 1:20 dilution *T. parva* Muguga (ILRI stabilate 3087) administered subcutaneously at the right parotid lymph node. The animals were monitored daily as described above for the RAM-L tick transmission experiment.

Control group animals underwent severe ECF reactions requiring repeated oxytetracycline treatment to prevent death. One control animal was euthanized after the completion of the experiment (day 23 post challenge) due to persistent infection that did not resolve after treatment. Hence, the parasite dose delivered was lethal to naïve cattle, characterized by persistent high fever, presence of *T. parva* schizonts in regional and contra-lateral draining lymph nodes followed by high piroplasm parasitemia. Control animals were classified as severe reactors to needle challenge (Table 2). In contrast, the ten *T. parva* infected RAM-L exposed animals were immune to this lethal challenge including animal BF019 in which no schizonts were detected after tick challenge. Six and four animals were graded as non-
reactors and mild to mild/moderate reactors, respectively, using the ECF index (Rowlands et al., 2000). Schizonts were observed in the parotid lymph node draining the site of infection in two animals and were detected for one and two days, respectively. Schizonts were not detected in the contralateral lymph node of any of these animals and none required drug treatment. These data clearly show that the immune response(s) induced by infection of cattle with *T. parva* infected RAM-L were capable of preventing subsequent ECF development. Schizonts were controlled at early stage and spreading to the contra-lateral lymph node was not observed (Table 2).

This is to our knowledge the first study undertaken to evaluate feasibility of using a laboratory tick strain, RAM-L, to reproducibly deliver *T. parva* sporozoites to a significant number of cattle. After tick challenge, all animals were exposed to *T. parva* sporozoites as indicated by the detection of anti-PIM antibody titers in serum and resistance to homologous lethal challenge. Importantly, the RAM-L ticks used here mimic the field situation of *T. parva* infection by *R. appendiculatus*, where the majority of individual ticks are uninfected or only lowly infected (Norval et al., 1992). Host-parasite interactions during and after delivery of *T. parva* by RAM-L can now be dissected at the molecular level, providing novel information with relevance to immune effector mechanisms induced in cattle following tick-mediated *T. parva* exposure in the field.

The effects of exposure to the tick-vector on tick-borne pathogen transmission and parasite development have been investigated in other systems (reviewed by Wikel, 1999; Schoeler and Wikel, 2001). Transmission of pathogens should ideally be performed using the tick-vector, since needle challenge excludes the potential impact of the tick on the route and quantity of infective pathogens introduced. Additionally, the potential modulatory effects of tick salivary gland molecules on transmission and disease progression are also eliminated (Wikel, 1999). Using the RAM-L challenge model, infectious sporozoites are transmitted to cattle over several days commencing roughly 72 hours after tick attachment (Konnai et al., 2007). This is in sharp contrast to one-time needle inoculation using a large number of cryopreserved sporozoites. Additionally, most needle delivery experiments have also involved administration of oxytetracycline in the ‘infection-and-treatment’ procedure with unknown
effects on the development of *T. parva* and modulation of immune responses induced in the host (Di Giulio et al., 2009).

When RAM-L tick infested animals were needle challenged with a potentially lethal needle dose of *T. parva* sporozoites, all animals were protected with no clinical signs of ECF detected. These data confirmed that all animals had been exposed to sporozoites and that a strong protective immune response against autologous re-challenge had been induced. It is well documented that under conditions of endemic stability *T. parva* does not result in high mortality rates (Norval et al., 1992). Calves are believed to be partially protected by maternal antibodies with the recruitment of a cellular effector mechanisms resulting in strong immunity to re-challenge. Repeated exposure to different parasite genotypes results in broadening of the spectrum of immunity and as a result, most adult cattle are immune to ECF under conditions of endemic stability (Norval et al., 1992).

There are a number of novel ECF vaccine candidate antigens that require evaluation for their ability to induce protection using defined delivery systems. However, testing of subunit ECF vaccine candidates for ECF control, such as the major sporozoite surface antigen p67 in the field is costly and results are difficult to interpret being prone to a number of unpredictable confounding factors (Musoke et al., 2005). Natural tick population density varies considerably throughout the year and *T. parva* parasite genetic diversity circulating at field sites is considerable but not fully defined at vaccine trial sites prior to immunization. The general health status of the vaccinated cattle also influences the testing outcome (Norval et al., 1992, Musoke et al., 2005). The system currently employed for evaluation of vaccine candidate antigens uses a high number of sporozoites delivered simultaneously by needle challenge. This may result in prematurely discarding candidates that could be effective under field conditions due to unrealistic experimental conditions. This is particularly important given that theoretical analyses suggest that vaccine effectiveness in the field may be achieved with lower levels of laboratory efficacy than has traditionally been believed (Woolhouse, 1995). Further development of the RAM-L model, will allow the number of infected ticks applied to each animal to be adjusted to reach doses of sporozoites that are useful for vaccine testing. Although it was not possible to directly determine the absolute number of sporozoites delivered to each animal by RAM-L, the average exposure can be calculated statistically.
based on the infection rates in the salivary glands of a selection of ticks derived from the challenge population. It is important to note that when using cryo-preserved sporozoites, the infective dose delivered is calculated prior to freezing of stabilates and the effect of freezing and thawing on both the number and metabolic activity of sporozoites remains unknown (Di Giulio et al., 2009).

The leading malaria vaccine candidate (RTS,S) currently undergoing Phase 3 testing, underwent extensive preclinical and clinical evaluation prior to the current formulation of the vaccine. The development of a controlled human malaria infection model (CHMI) at Walter Reed Army Institute of Research (WRAIR)/Naval Medical Research Center (NMRC) was an integral and essential part of the RTS,S vaccine development program. The CHMI model facilitated step wise development of optimal dosing, adjuvant formulation and immunization regimens before moving RTS,S into costly field trials. Importantly, protection data derived from CHMI were highly predictive for results obtained later in field studies (reviewed by Ballou, 2009 and Sauerwein et al., 2011). Results using experimental vaccine candidates that showed little or no protection in CHMI have been very helpful in guiding the subsequent research agenda and for terminating projects that are unlikely to achieve field success in a timely and appropriate manner (Ballou, 2009). Similar approaches are currently actively developed and pursued in other infectious diseases including human influenza (Carrat et al., 2008), tuberculosis (Minassian et al., 2011), Salmonella typhi (Levine et al., 2001) and dengue virus (Gunther et al., 2011).

The newly developed RAM-L model can provide information under controlled conditions that mimic the field relating to the protective capacity of experimental subunit T. parva vaccines using substantially smaller animal numbers than conventional field trials. Additionally, the system will enable evaluation of combination vaccines (transmission blocking vaccines) targeting both the tick-vector and the parasite.
References


Goddeeris, B.M., Morrison, W.I., Toye, P.G., and Bishop, R. (1990). Strain specificity of bovine Theileria parva-specific cytotoxic T cells is determined by the phenotype of the restricting class I MHC. Immunology 69, 38-44.


Parasitology 113 (Pt 3), 243-253.


Vaccine 13, 1045-050.

Figure 1: Distribution of *T. parva* infection in RAM-L. The parasites are present at low levels and occur in a minority of ticks within the population. Infection rate is determined as the number of ticks of the sample population (n=30) containing *T. parva* infected acini. Abundance is the average number of infected acini per tick examined.
Table 1: MHC classical class I allele typing of ten *T. parva* immune RAM-L tick challenged calves that were re-challenged with a lethal sporozoite dose demonstrating heterogeneity within the population.

<table>
<thead>
<tr>
<th>Animal Number</th>
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<th>Allele Group</th>
<th>Allele Group</th>
<th>Allele Group</th>
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</thead>
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Table 2: Response to RAM-L and subsequent lethal needle challenge based on clinical observations combined with classification using the ECF index (Rowlands et al., 2000).

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>Reaction to RAM-L delivered sporozoites</th>
<th>Reaction to lethal needle challenge</th>
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<td>Mild reactor</td>
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</tr>
<tr>
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<td>Severe reactor</td>
<td>Non reactor</td>
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<td>Severe reactor</td>
<td>Mild/Moderate reactor</td>
</tr>
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<td>Mild reactor</td>
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<td>Mild reactor</td>
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<tr>
<td>BF027</td>
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<tr>
<td>BF018¹</td>
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<td>Severe reactor</td>
</tr>
</tbody>
</table>

¹ Needle challenge control animals naïve to challenge
Subunit transmission blocking vaccine approach reduces *Theileria parva* transmission to cattle

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CHAPTER 5: TRANSMISSION BLOCKING VACCINES FOR ECF CONTROL

Abstract

Transmission blocking vaccines have been proposed for the control of many vector-borne diseases and a commercially available transmission blocking vaccine for the control of the tick-borne human Lyme disease has been developed. Infection of cattle with *Theileria parva* results in the lymphoproliferative disease, East Coast fever which is associated with high morbidity and mortality rates in susceptible cattle. A number of tick antigens have been proposed as vaccine candidates for the control of the tick vector of *T. parva*, *Rhipicephalus appendiculatus*. In this study, experimental cattle were vaccinated with a multivalent antigen cocktail containing the *R. appendiculatus* candidate antigens TRP64, histamine binding protein (male and female variants) and subolesin. Also included in the vaccine cocktail was the *T. parva* antigen p67, which is located on the surface of sporozoite cells. Using a tick-transmitted parasite model RAM-L, the effect of vaccination on the transmission of parasites to naïve susceptible calves was evaluated. All seven antigens in the cocktail induced a detectable antibody response. Establishment of disease in cattle was 20% lower in the vaccinated group indicating that the multivalent vaccine had a partial transmission blocking effect. Importantly, vaccinated cattle that did not show clinical symptoms of infection were protected from homologous lethal re-challenge. This indicates that immunity to disease was induced. These results suggest that transmission blocking vaccines for the control of East Coast fever should be further evaluated.

Keywords

Transmission blocking vaccine, *Theileria parva*, *Rhipicephalus appendiculatus*, p67, TRP64, subolesin, histamine binding protein, cattle

Introduction

Transmission blocking vaccines (TBV) aim to interfere with or block pathogen development within the vector responsible for pathogen transmission. As a result of vaccination pathogen transmission to the vertebrate host is reduced. Transmission blocking vaccines are principally based on immunization of vertebrate hosts with antigenic structures derived either from the pathogen or the transmitting vector. Transmission blocking vaccines can either block transmission from the vector to a vaccinated host or vaccination may inhibit
uptake of pathogens from an already infected host. In the case of the latter, while the vaccinated host is not protected from pathogen infection no parasite transmission to additional susceptible hosts can occur. Either approach may be a feasible strategy to reduce pathogen burden in endemic areas. An effective TBV may need to combine different antigens as the cumulative effect of antibodies targeting several antigens may produce more efficient transmission-blocking effects (Duffy and Kaslow, 1997; Gozar et al., 1998; Kongkasuriyachai et al., 2004).

During and after blood feeding, ticks are susceptible to antibodies and other modulatory molecules imbibed during the blood meal. If these antibodies induce disruption of gut integrity, interference of digestion may occur and the passage of infectious agents across the gut wall may also be affected. It is well known that repeated tick infestation results in local inflammation in vertebrate tissues that mediates resistance against ticks, probably through the pathways of delayed-type hypersensitivity. At the tick attachment site, a number of proteins molecules are secreted through the saliva into the skin of the host, including agents mediating host immune response suppression, blood anticoagulation and anti-inflammatory agents (Wikel, 1999; Bowman and Sauer, 2004).

East Coast fever (ECF) caused by the protozoan parasite *Theileria parva* is considered the most devastating of tick-borne diseases affecting cattle in sub-Saharan Africa. Transmitted by the three-host tick, *Rhipicephalus appendiculatus*, infection results in high mortality and morbidity rates (Norval et al., 1992; Minjauw and McLeod, 2003; Bishop et al., 2004). Currently, as with other tick-borne diseases of cattle, control of ECF relies primarily on acaricide treatment and the use of disease tolerant breeds. However, the emergence of acaricide resistance, environmental contamination concerns and costly infrastructure requirements pose significant challenges to their continued use. Although the infection-and-Treatment immunisation method (ITM), involving a potentially lethal injection of live sporozoites in combination with a long acting formulation of oxytetracycline is available, its large-scale deployment has been limited (Di Giulio et al., 2009). The development of a
sustainable control method for ECF remains critical for increased production in affected regions, particularly the small-holder cattle production system (Minjauw and McLeod, 2003).

Immunization of cattle with recombinant subolesin protected cattle from *Rhipicephalus (Boophilus) microplus* infestation by decreasing tick survival and reproductive capacity (Almazán et al., 2010; Merino et al., 2011). Additionally, subolesin was found to control tick gene expression, impact the tick innate immune response and decrease tick infection by tick-borne disease pathogens *Anaplasma marginale*, *Anaplasma phagocytophilum* and *Babesia bigemina* (de la Fuente et al., 2006; Kocan et al., 2009; Galindo et al., 2009; Zivkovic et al., 2010, Merino et al., 2011). The effect of subolesin on the biology of ticks has been demonstrated using RNAi resulting in degeneration of tick gut, salivary glands, reproductive tissues and embryos (de la Fuente et al., 2008; Nijhof et al., 2007; Almazán et al., 2005, Kocan et al., 2007; Almazan et al., 2010; de la Fuente et al., 2010). A dual-action vaccine target TRP64, identified as a *R. appendiculatus* cement cone protein, acts both as an ‘exposed’ antigen present in the saliva, as well as sharing ‘concealed’ antigenic epitopes within the tick midgut (Havliková et al., 2009). Vaccination with TRP64 has been shown to inhibit tick feeding as well as reduce levels of transmission of tick-borne encephalitis virus in mice (Labuda et al., 2006). Histamine binding proteins contained within the saliva are thought to play a role in controlling the itch-response of the host by sequestering histamine at the wound site competing with histamine receptors (Paesen et al., 1999). Although not previously evaluated as an anti-tick vaccine candidate, *R. appendiculatus* histamine binding protein was able to prevent murine allergic asthma (Couillin et al., 2004).

The *T. parva* antigen p67 is the major protein expressed on the sporozoite surface and has been implicated in the entry of parasites into lymphocytes. Results obtained from experimental p67 trials showed that vaccination could protect between 60 % and 70% of cattle from lethal sporozoite needle challenge (Musoke et al., 1992, Nene et al., 1996, Nene et al., 1999) however, evaluation under field challenge only resulted in a reduction of severe ECF cases by 30% (Musoke et al., 2005).
Herein, we describe the evaluation of a multivalent transmission blocking vaccine composed of a recombinant antigen cocktail of tick and parasite origin for control of ECF. The multivalent antigen cocktail included two female-specific and one male-specific *R. appendiculatus* histamine binding protein, an *R. appendiculatus* subolesin homologue, and the TRP64 cement protein (both full length and truncated 18-89 amino acids). Vaccinated animals were challenged using a selected tick line, RAM-L, which characteristically harbors low levels of *T. parva* infection similar to those found in areas of endemic stability (Young et al., 1995, Olds et al., manuscript submitted). This tick based experimental challenge was used to establish under simulated field conditions the *T. parva* transmission blocking effect and the anti-tick effect on a population of nymphal and adult *R. appendiculatus* ticks.

**Materials and Methods**

**Vaccine antigen expression and purification**

Antigens selected to form part of the cocktail included p67C (Musoke et al., 1992), histamine binding proteins male (HBPM, AAC63108.1, Mr 22 kDa), female one (HBPF1, AAC63106.1, Mr 48 kDa) and female two (HBPF2, AAC63107.1, Mr 70 kDa) (Paesen et al., 1999), TRP full length (TRPFL, AF469170.1, Mr 64 kDa), TRP truncated amino acids 18-89 (TRP18-89, Mr 58 kDa) (Trimnell et al., 2002) and Subolesin (4D8, ABA62331.1, Mr 47 kDa). Subolesin was expressed with a GST tag, which was removed prior to vaccination of cattle. Recombinant tick antigens were expressed and purified by GenScript Corp., New Jersey, USA. The production of p67 c-terminal (p67C) in pQE30 was performed as described in Bishop et al (2003). Due to the small size of p67C (80 amino acids corresponding to approximately 10 kDa), size exclusion chromatography was used for purification. Briefly, the cell pellet was resuspended in 10 ml of LEW buffer (Pierce) with 1mg/ml lysozyme added, and sonicated. The supernatant fraction was loaded onto a CentriPor column (Spectrum Medical Industries) with a cut-off of 10 000 Da and treated according to manufacturers instructions. The resulting protein was quantified using the Bradford protein assay with BSA as a standard.
SDS PAGE and immuno-blotting

Protein expression and purification was confirmed by SDS-PAGE and immuno-blotting. Proteins resolved on SDS-PAGE gels were transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) for immune-blotting. All incubation periods were for one hour at room temperature. Immuno-blots 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 and all washing steps were carried out using TBST. Following blocking, the membrane was washed, then incubated for one hour at room temperature in anti-his tagged antibody (Sigma). Following a further washing step, blots were incubated with 1:5000 with anti-rabbit whole Ig linked to peroxidase conjugate (Amersham Biosciences) diluted in TBST. After a final wash step, blots were developed using TMB substrate (Ingenasa) and the reaction stopped by rinsing the membrane in water. Antigens were determined to have a purity of 75% and above by SDS PAGE and coomassie blue staining.

Vaccine formulation

The seven antigens were split into two groups for vaccination to avoid antigenic competition between protein variants. Group A contained HBPM, HBPF1, TRPFL and Group B included 4D8, HBPF2, TRP18-89 and p67C. Inoculation preparations contained 50 µg of each antigen with the final volume adjusted to one ml with phosphate buffered saline (PBS) pH 7.4. Preparations were emulsified in an equivalent volume of Montanide ISA 50 V adjuvant (Seppic) mixed according to manufacturer’s instructions. Control preparations consisted of one ml PBS emulsified in an equivalent volume of Montanide ISA 50 V adjuvant.

Vaccine administration

Thirty Bos taurus (either Friesian or Friesian/Ayeshire cross) calves, 3 months of age, were serologically tested to be free of tick-borne infections. Calves were raised and maintained under strict tick-free conditions. Calves were randomized into two groups (Group 1 and Group 2), each containing ten vaccinated and five control animals. The two groups
CHAPTER 5: TRANSMISSION BLOCKING VACCINES FOR ECF CONTROL

were treated in an identical manner with a two-week separation between the two groups. Vaccinations were administered subcutaneously at the pre-scapular region with Group A antigens administered on the left shoulder and the Group B antigens on the right shoulder. Control preparations were split equally over the left and right sides. Each animal received three inoculations separated by four-week intervals.

Monitoring immune response to vaccination by indirect ELISA

Immune responses directed against individual antigens were monitored for each animal. Ninety six-well plates (Polysorp, Nunc) were coated with 0.5 µg recombinant protein per well and incubated for one hour at 37 °C. Plates were washed three times with PBS containing 0.5% (v/v) Tween 20 (PBST) and blocked with 1% casein in PBST for 30 minutes at 37 °C. All wash steps were carried out using PBST. Serum from trial animals was applied at an initial concentration of 1:500 and incubated for one hour at 37 °C. After washing, secondary antibody (anti-bovine IgG, whole molecule, peroxidase conjugate, Sigma) was added at a dilution of 1:10 000 in PBST for one hour at 37 °C. After the final incubation, plates were washed three times in PBST and given a final rinse in PBS. Plates were developed using SIGMAFAST OPD (Sigma) following instructions and signals were evaluated at optical density (OD) 405 nm. Mean readings (with standard error) were calculated for each time point by grouping control and vaccinated animals. Endpoint titers were determined as the last serum dilution where the OD of test sera was 2≥OD of negative control bovine serum donor. Time points evaluated were before vaccination (day 0), two weeks after inoculation one (week 2), two weeks after inoculation two (week 6), two weeks after inoculation three (week 10).

Theileriaparva tick challenge model: RAM-L

Tick strains

The *R. appendiculatus* Muguga ‘high-line’ and *R. appendiculatus* Muguga ‘low-line’ tick strains used for this study are both maintained at the International Livestock Research Institute (ILRI) Tick Unit. The Muguga tick strain was collected from 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). The differentiation of the Muguga strain into separate high- and low-lines involved the pooling of siblings from family lines with high or low susceptibility to infection (Young et al., 1995) and subsequent maintenance as separate stocks. Uninfected ticks were maintained in Biological Oxygen Demand (BOD) incubators at $28 \pm 1 \degree C$ and ticks infected with *T. parva* at $24 \pm 1 \degree C$, 80% relative humidity.

*Theileria parva* strain

*T. parva* Muguga (stabilate no.3087) was originally isolated from the Kiambu District of Kenya after which it was maintained as a laboratory stock through tick passage (Bailey, 1960) and as tick derived stabilates (Radley et al., 1974; Dolan et al., 1984).

**Challenge of vaccinated calves with RAM-L challenge model**

Thirty adult *T. parva* infected RAM-L females were secured using an ear bag attached to the right ear of each calf. Thirty uninfected male Muguga high-line ticks were placed in the same bag to facilitate the feeding of female ticks. Within the population of ticks, 20% of ticks contained infected acini with the average number of infected acini per tick being 1.16. Using 30 female ticks, the probability of parasite transmission to a susceptible calf was $p=0.99$ (Survival curve analysis, probability of transmission $p=0.999$, Therneau, 2012). Ticks fed till repletion and detached, at which point they were collected and the animals removed from the facility. Two weeks after the application of ticks to Group 1 calves, the Group 2 calves were treated in an identical manner. The anti-tick effect of the vaccine preparation was measured on uninfected adult Muguga high-line female ($n=50$) and male ($n=50$) applied to the left ear. Additionally, 200 nymphal ticks were applied in a bag attached to the back of each animal. Adult female engorgement weight, adult female egg laying capacity, nymph engorgement weight and molting efficiency were measured.

**Animal monitoring and recording**
Serum and peripheral blood mononuclear cells (PBMC) samples were taken every fortnight for the duration of the study. Blood samples from both the jugular and ear vein were taken daily after tick application. Daily lymph node biopsies were taken from day ten after tick challenge in the local lymph node draining the site of infection (right ear), and day 15 in the contralateral pre-scapular lymph node. Calves were treated as required with short- (Copermycin) or long-acting oxytetracycline (Butalex). Rectal temperature was recorded daily from the onset of the experiment.

Detection of *T. parva* transmission

Transmission of *T. parva* parasites and the establishment of infection was evaluated using a combination of microscopy, PCR and serology. Serology was performed daily on Geimsa stained lymph node aspiration smears as well as on blood smears for the detection of piroplasms. Macroschizont infected cells were graded as I, II or III with an increasing intensity designated by a higher number according to Rowlands et al. (2000). Piroplasm parasitemia was quantified as number of infected erythrocytes in a population of $1 \times 10^3$ viewed and expressed as a log value.

Blood samples taken from each calf two weeks after infection were used for DNA extraction using the QiagenDNeasy Blood and Tissue kit according to instructions. The p104 gene PCR parameters and the primers used in the primary PCR were as described previously (Skilton et al., 2002, Odongo et al., 2009, Odongo et al., 2010). All reactions were preformed in 15µl volumes using 5 µl of DNA extracted from blood for the primary reaction or 5 µl of the primary PCR reaction for the nested PCR reaction with 0.25 units of Taq DNA polymerase, (Promega), 1X PCR buffer (Promega), 200 mM of each dNTP (Promega) and 25 ng of primers and 1.5 mM of MgCl$_2$. The final PCR products were visualized by UV trans-illumination.

Antibodies directed against the *T. parva* PIM antigen were detected using the PIM-ELISA (Katende et al., 1998). Serum samples used for detection were taken 12 or 10 weeks post
tick challenge for Groups 1 and 2 respectively. In cases where animals died or were euthanized, the last sample before death was taken.

Following recovery from primary tick-delivered infection, all antigen-vaccinated calves from Group 1 and 2 were challenged with a lethal homologous dose of *T. parva* Muguga (stabilate no.3087). Two naïve susceptible calves served as controls in order to confirm lethal dose delivery. Animal monitoring was carried out as described infection delivered using infected ticks. All experiments were performed in accordance with procedures approved by ILRI’s Institutional Animal Care and Use Committee.

**Data Analysis**

Non-responding animals were defined as animals in which no symptoms of *T. parva* infection were observed. Mild-reactors were defined as animals in which no symptoms of infection were observed other than pyrexia. In Mild/Moderate-reactors, schizonts were observed in the regional lymph node of infection but not in contra-lateral lymph node and no piroplasms were observed in blood smears. Moderate-reactors included animals where schizonts were detected in both regional and contra-lateral lymph nodes but no piroplasms were detected in blood smears. Moderate to severe reacting animals had detectable schizonts in both regional and contra-lateral lymph nodes as well as piroplasm infected erythrocytes. Severe-reactors were defined as animals which died directly from infection or were euthanized (classification adapted from Rowlands et al., 2000). For each animal, symptoms were recorded for the day of onset, duration and intensity of symptom (Rowlands et al., 2000). Right parotid lymph node aspirates represented the regional lymph node draining the site of infection, the left pre-scapular lymph node represented the contra-lateral lymph node. Jugular vein blood was collected for piroplasm parasitemia. Differences between groups were analysed with One-way ANOVA if the data was normally distributed. Where the data was not-normally distributed and could not be transformed Kruskal-Wallis analysis of variance was carried out (STATA, StataCorp LP). Kaplan Meier survival curves were generated for time till onset of symptoms (GraphPad, GraphPad Software Inc.). Two
animals from Group 2 were removed due to concurrent lung-worm infections exacerbating East Coast fever reactions.

**Results**

*Vaccination and monitoring of animals*

Thirty *Bos taurus* calves, 3 months of age, were vaccinated three times at one monthly intervals with the antigen cocktail. In Figure 1, recombinant proteins used for vaccination expressed in *E. coli* separated by SDS-PAGE and stained with Coomassie blue are shown. The antigens were pure and exhibited at the expected molecular sizes. Protein expression and purification was confirmed using western blot analysis. Each animal received a total of 150 µg of each antigen over the three inoculations, formulated in Montanide ISA50V adjuvant. Serum samples were collected from the animals before vaccination and 2 weeks after each of the three vaccination events. All serum samples were stored at -20 °C until end of vaccination and then tested simultaneously in ELISA for reactivity against each individual antigen. Antibodies were induced in all vaccinated animals against all antigens inoculated while control animals remained negative (Figure 2).

*T. parva transmission by RAM-L tick line*

Two weeks after the final vaccination, the first group of 15 animals (ten vaccinated and five controls) was transferred into the tick unit animal containment facility. Thirty adult *T. parva* infected RAM-L females were secured using an ear bag attached to the right ear of each calf. Thirty uninfected male Muguga high-line ticks were placed in the same bag to facilitate the feeding of female ticks. Within the population of ticks, 20% contained infected acini with the average number of infected acini/dissected tick calculated as 1.16. The intensity of infection (percentage of infected ticks / average infection rate) was calculated as 5.8. As one infected acinus is sufficient to transmit *T. parva* to a susceptible *Bos taurus* animal resulting in ECF development (Young et al., 1983), using 30 RAM-L female ticks for each animal would be sufficient to transmit parasites to every calf in the study. Ticks fed till repletion and detached, at which point the ticks were collected and the animals removed from
Transmission of parasites and the establishment of *T. parva* infection was evaluated using a combination of microscopy, PCR and serology. The animals were monitored daily through lymph node needle biopsies starting from day ten after tick application (regional lymph node draining site of tick application). From day 15 onwards the contra-lateral pre-scapular lymph node aspirates were monitored by microscopy. Serum samples for anti-PIM ELISA were collected 12 and 10 weeks post tick application for Group 1 and 2, respectively. For *T. parva* DNA detection using p104 PCR, blood samples were taken from each calf two weeks after tick application. Parasite transmission occurred in all calves as indicated by detection of anti-PIM antibodies. In two animals (one control and one vaccinated animal), no parasites could be detected by p104 PCR analysis suggesting that even where parasites were not detected by PCR, exposure to *T. parva* had occurred based on anti-PIM antibodies (Table 1).

*Impact of vaccination of ECF development*

In comparison to the control group, the number of vaccinated animals developing schizonts in the regional lymph node was reduced (13 out of 18 animals compared to 9 out of 10 control animals). This difference is presented in Figure 3B which shows the number of days to onset of schizont parasitosis. A measurable although statistically not significant effect of the vaccination was observed. Two of the five animals not exhibiting parasite establishment did not develop pyrexia and were classified as non-responders. No non-responder animals were found in the control group. Interestingly, once schizonts were detected in animals, ECF progressed at equal rate and intensity irrespective of the vaccination status of animals. No difference was observed between vaccinated and control animals for the time of duration and intensity of other symptoms. The spread of infection measured by the presence of schizonts in the contra-lateral lymph node, occurred in 61.5% of vaccinated animals and 67.5% of control animals (difference of proportions test, p=0.751). In animals with schizonts in the
contra-lateral draining lymph node, 62% of vaccinated animals and 60% of control animals went on to develop piroplasm parasitemia (difference of proportions test, p=0.917). Following lethal challenge of vaccinated animals, only one animal exhibited the emergence of schizont parasites in the regional lymph node draining the site of infection (Table 2). Parasites were detected at a grade of I, the lowest possible grade, and persisted for one day. Roughly half of the animals showed no reaction to lethal needle challenge (non-responders) with remaining calves classified as mild-reactors showing only low grade, transient pyrexia. This demonstrates that a protective immune response was induced in all vaccinated calves, most importantly in non- and mild-reactors.

**Anti-tick effect of multivalent vaccination of cattle**

To determine the anti-tick effect of vaccination on the biological fitness of *R. appendiculatus* adult and nymphal ticks, 50 adult and 2000 nymphal ticks were applied to ear and back bags of each animal for adult and nymph ticks respectively. The results of vaccination on both adult and nymphal tick populations are shown in Table 3. No difference in adult female engorgement weight, egg-laying capacity, nymph engorgement weight or molting rates were seen indicating that vaccination had no effect on the feeding and reproductive capacity of *R. appendiculatus* ticks. No effect on direct mortality was noted between the two groups (analysis of variance, p= 0.2007) with an average of 47.8 adult females (SE=1.552) collected from vaccinated animals and 46.2 (SE=1.114) from control animals.

**Discussion**

Transmission blocking vaccines offer an avenue for vector-borne disease control in both humans and livestock. For control of *Ixodes*-transmitted Lyme disease, a TBV based on the Osp A protein of *Borrelia burgdorferi* has been developed and commercialized (LYMErix, SmithKline Beecham Biologicals). Osp A has been shown to be essential for tick gut colonization, survival and development of spirochetes (Yang et al., 2004). Vaccine induced antibodies against OspA results in transmission blocking to a future host. While this approach does not protect the vaccinated host, it does inhibit transmission to a future
susceptible host, hence these vaccines are described as ‘altruistic vaccines’. For control of *Plasmodium* infections, the development of altruistic TBVs has been identified as an essential tool for reduction of transmission leading to malaria eradication (The malERA Consultative Group on Vaccines, 2011). While a number of candidates have been evaluated, the furthest development has occurred with the Pfs25 protein homologues of *P. falciparum* and *P. vivax*, Pfs25 and PfV25, respectively (reviewed in Coutinho-Abreu and Ramalho-Ortigao, 2010). Pfs25 is a 25 kDa protein expressed on the surface of the zygote and ookinete stages (Kaslow et al., 1988). The antigen has been successfully expressed, purified and formulated in Montanide ISA51 adjuvant. Phase I clinical trials have been carried out with this Pfs25 formulation and the antibodies induced inhibited *P. falciparum* oocyst development in *Anopheles stephensi* by more than 90%, highlighting the feasibility of the approach. However, safety concerns prevented further development of this product (Wu et al., 2008).

Transmission-blocking activity has been demonstrated in dogs vaccinated with the Leishmune® [surface molecule (FML)-vaccine]. The vaccine containing the *Leishmania donovani* fructose-mannose ligand in a saponin adjuvant was not designed as a TBV. However vaccination inhibited *L. donovani* and *L. chagasi* procyclic promastigote-binding to dissected midguts of the *Lutzomyia longipalpis*. Both rate and intensity of infection were significantly reduced in the vector. Notably, the effect was still pronounced a year after vaccination (Saraiva et al., 2006).

To evaluate the potential of TBV for ECF control in cattle, a cocktail vaccine containing tick and parasite antigens was expressed, formulated for cattle vaccination and evaluated for efficacy under experimental tick challenge. Vaccination with the multivalent cocktail significantly increased the number of animals not exhibiting detectable clinical signs and symptoms of ECF. The response to infection with *T. parva* and ensuing disease severity is directly related to the sporozoite dose delivered (Jarrett et al., 1969; Radley et al., 1974; Dolan et al., 1984). Using the vaccination were describe here, the transmission of parasites
is likely to have been curtailed, resulting in a lower sporozoite dose delivered to vaccinated calves.

Increasing the proportion of mild-reactor animals is particularly attractive in areas where conditions of endemic stability persist or their creation is desired. This is particularly important for resource limited small-holder dairy farming systems, for example in Western Kenya and Uganda where there is currently little tick control. Reducing disease severity in such systems is of great socio-economic importance as they contribute up to 80% of livestock related products in developing countries (FAO, 2012). Complete prevention of *T. parva* transmission in such systems are not suitable since it does not enable establishment of appropriate immune responses to infection and increases risk of ECF outbreaks associated with high mortality rates in older animals (Norval et al., 1992; Billiouw et al., 2002). This is of particular concern where cattle are held in close proximity to the natural reservoir of infection, the African buffalo. Increasing the proportion of animals not developing disease symptoms while undergoing a primary infection allows animals to acquire resistance to future infection without experiencing the negative pathologic effects of disease. Cattle become resistant to subsequent challenge and gradually broaden their immune response through repeated exposure to heterologous parasite strains (Norval et al., 1992). In our experiments, vaccination of calves increased the number of animals undergoing primary infection without exhibiting clinical disease symptoms. Importantly, these calves developed an immune response equivalent in terms of protection from lethal challenge to calves that experienced moderate to severe symptoms of infection. It is common for animals with low schizont parasitosis to recover from infection and withstand lethal challenge (Eugui and Emery, 1981; reviewed in Norval et al., 1992). Here we show that vaccinated calves not exhibiting any parasitosis still develop an immune response able to withstand lethal homologous challenge.

None of the anti-tick effects demonstrated in small-animal models were mirrored in our experiment using the original host-vector system. The effect of vaccination with TRP64 was originally evaluated in Balb/c mice, in which transmission blocking activity was noted (Labuda
Vaccination with different length versions of TRP 64 resulted in a significant anti-tick effect on *R. appendiculatus* feeding from vaccinated guinea pigs. Mortality, mean engorgement weight and egg-laying mass were all significantly affected (Trimnell et al., 2002). Cross-reaction between TRP 64 and other tick species was also exhibited in guinea pigs (Trimnell et al., 2005). Despite the induction of high antibody titres to both variants of TRP64 in cattle, none of the above anti-tick effects could be reproduced. These findings were confirmed by a recently published data reporting the anti-tick effect of TRP64 in rabbits (Saimo et al., 2011).

The observed differences in protection between these host-tick systems may be partly related to tick-antigen recognition by the host species. Tick components detected by the non-natural host species may not be immunogenic in the natural host – tick system (reviewed by Steen et al., 2006). It is generally accepted that for anti-tick vaccination to be effective, high antibody titres should be induced by vaccination (Willadsen, 2004). It is equally important that the conformation of the recombinant antigen is maintained in such a way that protective epitopes are identified by the vaccinated host. It is also possible that we did not test the most effective tick antigen in this experiment, since there has been no systematic evaluation of all available tick derived vaccine candidates.

Endemic stability for ECF is defined as stage of host-tick-pathogen interaction in which there is a high level of pathogen challenge experienced by cattle via infected ticks with an absence of clinical disease despite constant pathogen exposure. In adults, a high level of protective immunity is observed in the absence of clinical disease (Norval et al., 1992). However, it is unknown what level of infected tick exposure and individual tick-infection rates are required to achieve this stability in absence of costly interventions such as acaricide treatments. Currently, the assessment of endemic stability in tick-borne diseases rests on sero-prevalence studies (Jonsson et al., 2012). The fact that all RAM-L exposed animals developed anti-PIM antibodies and vaccinated animals were solidly protected against lethal sporozoite needle challenge supports the use of this ELISA for measurement of ECF endemicity status.
In summary, this vaccine trial highlights the potential role of transmission reducing approaches for ECF control in cattle and is the first large-scale experimental evaluation of a TBV using a natural tick challenge system resembling field conditions. Results obtained also underscore earlier observations that it is difficult to extrapolate results from experimental model host species and that anti-tick vaccine evaluation should therefore to be carried out at an early stage in the target host species.

References


CHAPTER 5: TRANSMISSION BLOCKING VACCINES FOR ECF CONTROL


Minjauw, B., and McLeod, A. (2003). Tick-borne diseases and poverty. The impact of ticks and tick-borne diseases on the livelihood of small scale and marginal livestock owners in India and eastern and southern Africa. Research report, DFID Animal Health Programme, Centre for Tropical Veterinary Medicine, University of Edinburgh, UK


Tables and Figures

Figure 1: Coomassie blue stained gel showing SDS-PAGE analysis of the purified recombinant antigens incorporated into the multivalent vaccine. Lane 1: histamine binding protein male variant (22 kDa); Lane 2: histamine binding protein female variant one (48 kDa); Lane 3: histamine binding protein female variant two (70 kDa); Lane 4: TRP 64 truncated 18-89 (58 kDa); Lane 5: TRP 64 (64 kDa); Lane 6: Subolesin (47 kDa); Lane 7: p67C (10 kDa).
Figure 2: Development of antibody titres against vaccine antigens prior to and post vaccination. Time points are (day 0), two weeks after first immunization (week 2), two weeks after second immunization (week 6) and two weeks after third immunization (week 10).
Figure 3: Kaplan-Meier curves for time of first onset of symptoms in vaccinated and control groups of cattle. 
A shows the onset of pyrexia, B shows the detection of schizont parasites in the regional lymph node draining the site of infection (right parotid lymph node), C shows the detection of schizont parasites in the contra-lateral lymph node (left scapular lymph node) and D shows the detection of piroplasm infected erythrocytes in jugular vein blood.
Table 1: Development of East Coast fever symptoms in cattle vaccinated with the multivalent antigen cocktail after exposure to RAM-L *T. parva* infected ticks.

<table>
<thead>
<tr>
<th>Animal ID</th>
<th>Pyrexia(^1)</th>
<th>Regional lymph node parasitosis(^1)</th>
<th>Contra-lateral lymph node parasitosis(^1)</th>
<th>Piroplasm(^1)</th>
<th>p104 PCR</th>
<th>Positive PIM ELISA reading(^2)</th>
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<td>NEG</td>
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</table>

\(^1\) Values are displayed as the day of first detection (duration observed in days). Where symptoms were not observed in an animal, no values are reflected.
The average Percentage Positive (PP) value over duplicate samples. The PP is calculated as the (OD of test/OD of strong positive) x 100 (Wright et al., 1993; Katende et al., 1998).

* Animal BF035 died from sudden onset ECF symptoms on day 15 post challenge
Table 2: Comparison of ECF severity in vaccinated calves after primary exposure to *T. parva* infected RAM-L ticks and secondary exposure through lethal needle challenge. All calves are protected from lethal homologous challenge.

<table>
<thead>
<tr>
<th>Animal ID</th>
<th>Primary infection response</th>
<th>Lethal challenge response</th>
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</thead>
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<td>BF002</td>
<td>Mild-reactor</td>
<td>Mild-reactor</td>
</tr>
<tr>
<td>BF003</td>
<td>Non-responder</td>
<td>Non-responder</td>
</tr>
<tr>
<td>BF005</td>
<td>Mild/Moderate-reactor</td>
<td>Mild-reactor</td>
</tr>
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<td>BF006</td>
<td>Mild/Moderate-reactor</td>
<td>Non-responder</td>
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<td>Mild-reactor</td>
<td>Mild-reactor</td>
</tr>
<tr>
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<td>Mild-reactor</td>
<td>Non-responder</td>
</tr>
<tr>
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<td>Moderate/Severe-reactor</td>
<td>Non-responder</td>
</tr>
<tr>
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<td>Moderate/Severe-reactor</td>
<td>Mild-reactor</td>
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<tr>
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<td>Mild-reactor</td>
</tr>
<tr>
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</tr>
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<td>BF028</td>
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</tr>
<tr>
<td>BF029</td>
<td>Mild/Moderate-reactor</td>
<td>Mild-reactor</td>
</tr>
<tr>
<td>BF030</td>
<td>Non-responder</td>
<td>Non-responder</td>
</tr>
<tr>
<td>BF031</td>
<td>Mild/Moderate-reactor</td>
<td>Mild-reactor</td>
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<td>BF032</td>
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<td>Mild-reactor</td>
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<tr>
<td>Control 2</td>
<td>Severe</td>
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</table>

1 Response to *T. parva* infection determined by clinical observations in conjunction with ECF index (Rowlands et al., 2000). Non-responders showed no symptoms of infection while mild responders showed only mild fever and no visible parasitosis.

2 Animal BF035 died of severe East Coast fever after tick exposure.

3 Low grade schizont infected cells detected for one day
### Table 3: Evaluation of the effect of transmission blocking vaccination on the biological fitness of *R. appendiculatus* ticks. No effect on any of the tick biological parameters were seen.

<table>
<thead>
<tr>
<th></th>
<th>Vaccinated</th>
<th>Control</th>
<th>P value</th>
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<tbody>
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<td></td>
<td></td>
</tr>
<tr>
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<td>1.2 (SE=0)</td>
<td>1.0000</td>
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<tr>
<td>Molting success</td>
<td>5.1 (SE=3.45)</td>
<td>2.1 (SE=0.74)</td>
<td>0.3556</td>
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<tr>
<td><strong>Adult</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Number engorged</td>
<td>47.8 (SE=1.60)</td>
<td>46.2 (1.11)</td>
<td>1.0000</td>
</tr>
<tr>
<td>Average adult</td>
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<td>0.5477 (SE=0.0019)</td>
<td>0.1797</td>
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<tr>
<td>engorgement</td>
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<tr>
<td>weight total</td>
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<td>11.45 (SE=0.86)</td>
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<tr>
<td>Average egg</td>
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<td>0.2464 (SE=0.0304)</td>
<td>0.0679</td>
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<td>weight/tick</td>
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<tr>
<td>Un-hatched egg</td>
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<td>1.78 (SE=0.27)</td>
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<tr>
<td>weight total</td>
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<td>16.2 (SE=2.43)</td>
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<td>Un-hatched egg</td>
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<tr>
<td>weight/tick (%)</td>
<td>17.48 (SE=1.74)</td>
<td>16.2 (SE=2.43)</td>
<td>0.9649</td>
</tr>
</tbody>
</table>

1. Average weight of a population of 100 individuals
2. Percentage of ticks failing to molt in a population of 100 individuals
Parameters influencing *in vitro* feeding of *Rhipicephalus appendiculatus* adult female ticks

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Manuscript in preparation to be submitted to the *Journal of Medical Entomology*
Abstract

Ticks serve as efficient vectors for the transmission of disease causing pathogens to both humans and animals. As a result, significant efforts are made towards the control of ticks and tick-borne diseases. The feeding of ticks in vitro provides a platform for a number of standardized assays with diverse and far-reaching applications for tick and tick-borne disease research. Using silicone-based membranes, a number of tick species have been adapted to feeding in vitro including Rhipicephalus microplus, Amblyomma variegatum and Amblyomma hebraeum and Ixodes ricinus. The three-host tick Rhipicephalus appendiculatus is responsible for the transmission of Theileira parva to cattle resulting in the fatal lymphoproliferative disorder, East Coast fever. We report here for the first time the adaptation of R. appendiculatus adult females to in vitro-silicone based membrane feeding.

Introduction

The feeding of ticks in vitro from artificial skin-mimicking membranes has a number of potential uses. Currently, the tick colony maintenance requires regular tick feeding on experimental animals. This procedure is associated with pain for the animals. Using in vitro tick feeding the number of animals required for tick colony maintenance can be significantly reduced (Kröber and Guerin, 2007a). For the development of anti-tick vaccines, in vitro feeding allows initial investigations on the role of a potential anti-tick vaccine antigen in tick survival and reproduction. Titration of antibodies directed towards distinct tick antigens can be used to determine minimum levels required to induce tick damage. These assays could possibly lead to surrogate markers of protection for anti-tick vaccines. In addition, in vitro feeding enables antigen localization studies and elucidating the detailed mechanisms of protection through vaccination induced antibodies. High-throughput screening for acaricide resistance as well as identification of new acaricide compounds can be aided. Studies on transmission of tick borne diseases, tick saliva and cement cone constituents can be carried out more easily, cheaper and with more control compared to the expensive in vivo techniques.
Attempts to encourage ticks to feed from artificial membrane systems have been successful for a number of species. Soft ticks (Acarina: Argasidae) with their short feeding times were the first to be adapted to in vitro feeding through Parafilm® membranes (Hokama et al., 1987; Schwan et al., 1991; Rennie et al., 2000). Compared to soft ticks, hard ticks (Acarina:Ixodidae) feed for a longer time periods and therefore adaptations in the experimental setup are needed. Feeding of hard ticks has been attempted using membranes derived from a number of sources including embryonated hen egg (Pierce and Pierce, 1956), bovine and rabbit skin (Kemp et al., 1975; Voigt et al., 1993), glue-impregnated Baudruche membrane (Waladde et al., 1991, Waladde et al., 1993, Waladde et al.,1995) and silicone based membranes (Kuhnert, 1996, Kröber and Guerin, 2007b). The silicone membrane feeding method described by Kuhnert and later modified by Kröber and Guerin has been successfully used to feed *Rhipicephalus microplus*, *Amblyomma variegatum* and *Amblyomma hebraeum* and *Ixodes ricinus* ticks (Kuhnert et al., 1995; Kuhnert, 1996, Kröber and Guerin, 2007b). Successful artificial membrane feeding of *Rhipicephalus appendiculatus* has been described by Waladde et al. using a biodegradable glue-impregnated Baudruche membrane. The transmission of *T. parva* piroplasm to feeding ticks has also been achieved using this approach (Waladde et al., 1991; Waladde et al., 1993, Waladde et al., 1995). However, using this method it was difficult to get reproducible levels of tick feeding at the ILRI tick unit. Here we attempted to adapt the silicone membrane method described by Kröber and Guerin for feeding of larval, nymphal and adult stages of *R. appendiculatus* (Kröber and Guerin, 2007b). Essentially, this method employs silicone membranes attached to the base of a cylindrical feeding chamber. Ticks placed within the chambers are contained using a ventilated stopper. The membrane is then immersed in blood contained within commercial six-well plates. We report here for the first time, the successful feeding of *R. appendiculatus* adults from silicone membranes.

**Materials and Methods**

*Membranes*
The making of silicone membranes and feeding chambers was carried out as described previously by Kröber and Guerin (2007a; 2007b). Briefly, a silicone RTV-1 Elastosil E4 (Wacker) was mixed with silicone oil (Fluka) till a sticky 'frog grip' was achieved. To make the mixture more fluid for equal spreading, 150 g/kg\(^{-1}\) hexane was added. The mixture was spread over 70 × 120mm lens-cleaning paper (Kodak) attached with adhesive tape to kitchen plastic film. Silicone was spread thinly over the tissue by repeated scraping with a rubber ruler. Membranes were left to polymerize overnight at room temperature. The following day, membranes were measured and uniform selections between 60 and 120 µm were cut to fit the feeding chambers. Membranes were attached using Elastosil E4 silicone glue (Wacker). To provide additional attachment stimuli, glass fiber 1.4 mm mesh mosquito netting was glued to the membrane with silicone glue. A 2 mm thick tile spacer was placed inside the chamber resting above the membrane to create additional borders for tick attachment. The Perspex feeding chambers, netting and tile spacers were kindly donated by Patrick Guerin.

An alternative Biobrane (Smith & Nephew) membrane was evaluated for its ability to support tick feeding. The Biobrane membrane was created to mimic the elasticity of skin for the treatment of burn wounds and is made of silicone membrane bonded to a nylon mesh to which peptides from a porcine dermal collagen source have been bonded to the nylon membrane to form a flexible membrane (http://global.smith-nephew.com/master/BIOBRANE_27563.htm). No alterations to the membrane were made other than sealing perforations with silicone glue. Scent attachment stimuli were added and ticks covered with hair as before. Tile spacers were also added to the chambers before tick application.

**Bovine blood preparation**

Blood was collected from donor bovine maintained under strict tick-free conditions since birth. Blood was collected using three anti-coagulation methods to determine the effect of anticoagulant choice on tick feeding. Blood was collected in heparin or ethylenediamine tetraacetate (EDTA) vaccutainer tubes or through manual defibrination by stirring with a
stainless steel spoon. For manual defibrination, blood was stirred with a till a large blood clot was formed around the spoon. The blood clot was removed and remaining blood supplemented with 2 g/L glucose (Sigma) to stabilize erythrocytes and stored at 4 °C till use. A portion of the blood was stored at -20 °C to evaluate the effect of blood freezing on tick feeding. Prior to adding to feeding wells, gentamycin solution (5 µg/mL) and ATP (10^{-3} mM) was added to blood warmed to 37 °C. Blood was changed at 12-hour intervals where agitation was not employed and every 24 hours where agitation was employed during tick feeding. During blood changes, tick chambers were cleaned of execs fecal matter and the outside cleaned with saline solution. Feeding plates were placed in biological oxygen demand incubators at 37 ± 1°C with 80% relative humidity.

**Bovine blood mixing**

Blood contained within the feeding wells was either continuously mixed through stirring or left to settle by gravity. Where blood mixing was employed, a small magnetic stirrer bar was placed on the base of the well of the six well plate. The chamber was then suspended above the stirrer bar to allow free rotation of the stirrer bar. The conventional six well plate could not be used to feed more than one chamber where stirring was employed as magnetic bars interfered with each other. In this case, a new blood chamber was developed. Rectangular containers were made from Perspex by the ILRI engineering department with depth of 3 cm, length of 20 cm and width of 12 cm. A cover plate with 6 holes the diameter width of the feeding chamber was made to fit securely over the blood reservoir container. A maximum of six chambers were then immersed in the same blood reservoir. In cases where fewer feeding chambers were needed, additional holes were covered in Parafilm to prevent air contamination of the blood. A magnetic stirrer bar was then placed as centrally as possible between feeding chambers.

**Testing of tick feeding stimuli**

Bovine and rabbit hair was evaluated for its ability to facilitate tick feeding. Hair was clipped from animals not exposed to acaricides. Hair extracts were produced from bovine
and rabbit hair by chopping hair into 1 cm lengths. Methanol (200 mL) was added to hair and stirred for twenty minutes. After stirring, extract was removed and stored in aliquots at -80 °C. An African buffalo ear-wash scent extract (produced previously by S. Mwaura) was also evaluated in this context. The effect of tick fecal matter on attachment and feeding was evaluated through application of fresh fecal matter to the membrane immediately prior to tick application. This fecal matter was collected from adult *R. appendiculatus* ticks feeding on rabbit ears during routine colony maintenance.

*Rhipicephalus appendiculatus* ticks

*R. appendiculatus* ticks used for all feeding experiments were from the laboratory Muguga-high line stock. The Muguga tick strain was collected from 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, Young et al., 1995). Different adult male to female ratios were investigated with a maximum of ten adult ticks added to the chamber. Female and male ticks were always added simultaneously. Where larval or nympal stages were evaluated, storage vials containing ticks were placed at 4 °C for 2 minutes. Once chilled a spatula was used to remove and add a scoop of larval or nympal ticks to each chamber. Adult ticks were stimulated before application by gently breathing over the vial before addition to the chamber. Our Muguga tick lines no longer have a diapause cycle and can be maintained in incubators without light.

**Results and Discussion**

Membrane thickness and continuous mixing of blood were discovered to be essential to the feeding of *R. appendiculatus* ticks *in vitro*. When ticks were applied to the thicker membranes (120 µm), adult ticks were seen to probe the membrane repeatedly within the first hour of application. After this initial period no further attempts to attach and feed were observed and ticks quested to the top of the chamber. Membranes of 100 µm or less was determined as the best for supporting adult tick attachment and feeding. Commercial
membrane Biobrane was found to be too elastic to be attached to chamber with tightness sufficient for tick perforation. The shore hardness of membranes is known to play a role in successful tick feeding in vitro (Kröber and Guerin, 2007b) and it is possible that this parameter was not met by the commercial membrane. Although Waladde showed that ticks would feed from heparinized blood (Waladde et al., 1993), we found that R. appendiculatus ticks fed only from manually defibrinated blood. Heparinized blood has a strongly metallic smell after incubation and it is possible this smell was unappealing to ticks. Defibrinated blood from frozen stocks was also not accepted for feeding possibly due to a change in taste due to the rupture of erythrocyte with freezing. The ratio of male to female ticks was also seen to be important. An equal ratio of five males to five females per chamber was best for female feeding and engorgement.

Separation of blood into a bottom erythrocyte layer and a covering serum layer occurred within hours of application to the six-well plate. Once separation occurred, ticks stopped feeding and detached from the feeding site. Small cement cones were noted on the bottom of the membranes even though ticks were no longer feeding, indicating that attachment did take place for a short time. We have observed that one factor essential for tick feeding is the requirement to have blood continuously mixed for extended feeding to occur. However, the use of a magnetic stirrer is not very practical as it requires a magnetic stirrer apparatus to be placed within the incubator. Additionally, due to the magnetic interactions, only one stirrer can be used per plate. This does not allow multiple chambers with variations to be investigated simultaneously. An alternative method is to connect the blood chamber to a peristaltic pump which may better facilitate blood movement.

Using agitated blood, attachment and feeding occurred for all female adult ticks and a full engorgement in 40% of female ticks was observed. Although this is lower than would be achieved in vivo, it relates directly to the size of the feeding chamber. Two engorged female ticks fill the space of the feeding chamber and as a result, there is little space for remaining females to engorge. Importantly, female ticks that engorged fully weighed between 400 and
230 mg; this is comparable with tick feeding engorgement weight from a natural host. Female ticks engorging first were found to be up to double the weight of later engorging females, highlighting the possible effect of space constraints on female tick engorgement weight.

When adult ticks attached and feed, they were very sensitive to changes in the environment. Ticks showed signs of agitation with removal of the hair covering and cleaning of the chamber. Attached ticks showed leg movement and attempts to detach from the membrane. Free ticks began to quest to the top of the chamber. Adult male ticks could never be seen attached to the membrane and feeding even when continuous mixing of blood was employed. This is not unusual as it is known that adult male feeding is sporadic. Where female ticks did attach and feed, male ticks were observed in close proximity to female ticks. Male ticks died within a couple of days of addition to the chamber possibly after completion of mating. Interestingly, where no female feeding occurred, male ticks did not die within the week period of evaluation. The best ratio of five adult female to five adult male ticks resulted of full female engorgement. With a higher male to female ratio of eight males to two females, no female engorgement occurred although feeding did take place. Female ticks fed over a period of ten days and rapid engorgement occurred over the last 48 hours (over days 8-10). Interestingly, this in vitro feeding took longer than the average period in vivo that is usually between five and seven days. Figures 1 to 4 show adult tick feeding results.

Evaluation of feeding stimuli showed that bovine hair combined with bovine hair extract was superior to rabbit hair and rabbit hair extract for encouraging tick feeding. The application of fecal matter had no impact on the rates of tick attachment nor did buffalo ear wash scent extract. Larval and nymphal R. appendiculatus showed no interest in in vitro tick feeding. It is possible that even the thinner membranes were still too thick for them to successfully feed. Additionally, larval ticks could not be contained within the feeding chambers and escaped through the mesh stopper.
Conclusion

Although successful feeding of *R. appendiculatus* was ultimately achieved, the current technique is not as robust as has been reported by Körber et al (2007a, 2007b). The hand made production of membranes is difficult and membranes that are not uniform or do not have the desired thickness are unsuitable to support tick feeding. The current dimensions of feeding chambers do not allow for feeding more than five adult females with space for only two ticks to engorge fully at any given experiment making statistical analyses difficult. An option of making larger chambers was explored but has not been successful due to the uneven membrane thickness that was produced by us. The prevention of blood separation into its components due to gravidity has to be addressed.

We have explored here thoroughly the variables influencing *in vitro* feeding success of *R. appendiculatus*. Clearly, refinement of the current method will lead to improved success rates of *R. appendiculatus* feeding. This feeding technique will open avenues for vaccine development, acaricide testing, biological investigations and reduction of animal experimentation.
References


CHAPTER 6: IN VITRO FEEDING OF R. APPENDICULATUS


Figures

Figure 1. Feeding chamber after addition of fresh blood on day three after tick application.

The chamber on the left was modified to have a magnetic stirrer bar placed beneath the membrane. No questing ticks are seen on top of the chamber as attachment and feeding took place. On the right chamber, tick feeding without blood stirring is attempted. Questing ticks can be seen that move into the top part of the chamber. In the right chamber, no tick attachment and feeding occurred.
Figure 2: Top view onto a chamber in which successful tick feeding after three days of incubation.

After three days, if attachment was successful, ticks were disturbed as little as possible to allow for complete feeding and engorgement. Fresh fecal matter can be clearly seen particularly where feeding ticks are observed. Successful tick attachment and feeding occurred at the wall of the feeding chamber (upper and lower right quadrants). A dead tick can be seen at the bottom left quadrant (marked with a star).
Figure 3 Result of tick feeding when testing a 100 µm tick silicone membrane without blood stirring after three days.

One female tick showed partial feeding, shown attached in the upper right quadrant, although compared to stirred feeding females less advanced by the same time point. This tick was irritated by removal of hair cover and detached shortly hereafter. It failed to reattach and feed to repletion. Dead ticks can be seen in the lower left quadrant (marked with stars).
Figure 4: A 400 mg female tick engorged after feeding on 100 µm silicone membrane with blood stirring. Not all females within the chamber have space to fully engorge as this female did, most likely due to space constraints. This in vitro fed female tick went on to lay roughly 200 mg of eggs which hatched into viable larvae.
Evaluation of *Theileria parva* specific Cytotoxic T Lymphocytes in cattle after tick-delivered sporozoite inoculation.

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Introduction

Animals recovering from primary *T. parva* infection are immune to lethal autologous challenge with up to $5 \times 10^8$ schizont infected cells (Eugui and Emery, 1981). Antibody responses are induced upon *T. parva* infection to sporozoite parasites but their involvement in protection from infection is unclear. Transfer of sera from ECF immune to naïve animals does not protect against fatal ECF (Norval et al., 1992, Muhammed et al., 1975). This does not necessarily preclude a role for humoral immune responses in protection. Repeated infestation of cattle and rabbits with ticks infected with *T. parva* induced antibodies neutralizing sporozoite infectivity *in vitro* (Musoke et al., 1982). Antibodies targeting the sporozoite surface antigen p67 have been explored extensively as a potential subunit vaccine candidate against ECF (reviewed in McKeever and Morrison, 1998).

The effector mechanism for protection against *T. parva* infection is mediated through parasite-specific CD8+ T cell responses targeting schizont infected lymphoblasts (Eugui and Emery, 1981, Goddeeris et al., 1986, Morrison et al., 1987). This immune response has been studied in great detail and is reliably induced in cattle after inoculation of cryopreserved sporozoites. Large numbers of parasite specific cytotoxic T lymphocytes (CTLs) are generated in the lymph node draining the sporozoite inoculation site. At the height of the immune response, 1 in 30 T cells leaving the infected lymph node via the efferent lymph vessel are parasite specific CTLs (McKeever et al., 1994). These CTLs proliferate *in vitro* in the presence of autologous schizont infected cells and respond with parasite specific killing activity (Pearson et al., 1979, Eugui and Emery, 1981). While the initial CTL response observed in primary infection with *T. parva* requires input from activated parasite specific CD4+ T helper cells, in immune animals, the CTL response can be recalled *in vitro* in the presence of T cell growth factors or CD4+ T cells responding to unrelated antigens (Taracha et al., 1997).
Parasite specific CD8+ T cell responses are directed towards cells displaying *T. parva* derived peptides bound to Class I MHC molecules on the surface of schizont infected cells (Emery et al., 1981; Morrison et al., 1987). Evidence suggests that the peptide-specificity of the CTL response depends on the MHC genotype of the animal. In most cases, the response is restricted by one haplotype and certain biases for haplotypes exist (Goddeeris et al., 1986; Goddeeris et al., 1990, Taracha et al., 1995). As a result, it is thought that the immune response to *T. parva* is limited to a set of immune-dominant peptide-MHC determinants (MacHugh et al., 2009, Taracha et al., 1995). All experiments inducing experimentally *T. parva* specific immune responses have been performed with isolated sporozoite infected animals. However, *T. parva* specific immune responses elicited in cattle after exposure to *T. parva* infected ticks have not been studied in any detail. We have established recently a reliable tick-based infection model for *T. parva* in cattle. These animals developed strong ECF protective immunity and investigations into the nature of the elicited immune response and their comparison to needle challenged animals have become feasible. Peripheral blood mononuclear blood samples taken from cattle before and after exposure to *T. parva* infected ticks were evaluated for their parasite specific CTL activity.

**Materials and Methods**

**Animals**

Samples evaluated for the following study were taken during the RAM-L challenge model evaluation (Chapter 2). Animal BB007 was used as a positive control. This animal was infected with a lethal dose of *T. parva* Muguga coupled with oxytetracycline treatment seven years ago. Once recovered, BB007 has been repeatedly challenged to maintain immunity. This animal reliably exhibits CTL activity *in vitro*.

**Isolation of peripheral blood mononuclear cells (PBMC)**

Isolation of PBMC from whole blood was carried out for each animal before tick exposure, two months after tick exposure and 28 days post homologous lethal needle challenge. Animals BF007 and BF018 were sampled at day 28 post needle infection only.
Thirty mL of blood was bled from the jugular vein of each calf into an equivalent volume of Alsever’s solution. PBMCs were extracted using a process of Ficoll-Paque layering (Ficoll-Paque PREMIUM, GE Healthcare) and centrifugation. Erythrocytes were lysed by incubation with Tris-ammonium chloride buffer at 37 °C for 5 minutes. Remaining cells were washed a minimum of three times in 50 mL of Alsever’s solution and collected by centrifugation till a white pellet was formed. The final pellet of PBMCs were resuspended in 2 mL of 10% DMSO in HyClone Fetal Bovine Serum (Thermo Scientific) and stored under liquid nitrogen till used.

**Infection of PBMCs with T. parva sporozoites to create infected lymphoblast cell lines**

PBMCs taken before exposure of calves to either tick or needle delivered T. parva sporozoites were infected with Muguga 3087 dissected salivary gland stabilate 4240. Cells were infected using the method described by Goddeeris and Morrison, by incubation at 37 °C for 1.5 hours with agitation every 20 minutes (Goddeeris and Morrison, 1988). T. parva lymphoblast lines were maintained in tissue culture medium (HEPES buffered RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, 50 µg/ml streptomycin, and 5 x 10⁻⁵ M β-mercaptoethanol), incubated at 37 °C with 5% CO₂.

**Concavalin A (Con A) stimulated lymphoblast cell lines**

To establish continually growing Con A autologous cultures to serve as negative controls, pre T. parva challenge PBMCs were thawed and seeded at a density of 2 x 10⁶ / mL into 24 well plates with tissue culture media supplemented with 10 µg/ml Con A. Cultures were left to proliferate and maintained through splitting and feeding with tissue culture media supplemented with 5% T cell growth factor (TCGF).

**Cytotoxic T Lymphocyte assay**

Potential killing activity was investigated for two time points in RAM-L exposed calves BF011 and BF012, after tick infection and day 28 post lethal needle challenge. In animals BF007 and BF018, one time point, 28 days post needle challenge was evaluated. CTL
activity was measured using the standard 4 h-51Cr-release assay (Goddeeris and Morrison, 1988). Effector cells underwent two rounds of stimulation with Cesium-137 irradiated autologous TpL lines separated by seven-day intervals. Primary and secondary bulk stimulations were cultured in RPMI 1640 media supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, 50 ug/ml streptomycin, and $5 \times 10^{-5}$ M $\beta$-mercaptoethanol with the addition of 5% TCGF. One week following the second stimulation, chromium release assays were carried out with the highest starting dilution feasible. Positive control for the assay was represented by *T. parva* Muguga immune animal BB007 which has shown reproducible killing activity in this assay. Negative controls for each animal were represented by autologous ConA lymphoblast cultures. After incubation of effectors with Cr labeled targets, supernatants were transferred to Lumaplates (PerkinElmer, Waltham, MA, USA) and counted using a TopCounter machine (PerkinElmer, Waltham, MA, USA). The cytotoxicity was calculated as experimental release-spontaneous release/total release-spontaneous release.

**Flow cytometry**

To identify cell populations after stimulation with autologous TpL lines, samples were stimulated twice, seven days apart as for CTL assay preparation. Time points analyzed were after tick infection and 28 days post lethal needle challenge from animals BF019, BF025, BF031 and BF033 were used for the analysis. To determine the cell populations present after two rounds of stimulation, flow cytometry was carried out on a FACSCanto flow cytometer (Becton Dickinson). Cell populations were labeled by incubation on ice for one hour with monoclonal antibodies able to identify different cell populations. Primary antibodies included MM1A (CD3), IL-A11 (CD4), IL-51 (CD8), GB21A (Gamma Delta T cells), (T cells) and IL-A30 (B cells) (Neassens et al., 1997). Following washing, cells were incubated with appropriate FITC conjugated secondary antibodies.

**Results and discussion**
The CTL activity in calves was evaluated at two time points. The primary infection was induced through the feeding of RAM-L ticks (described in chapter 4). Two months post tick infection, after calves had fully recovered from infection, PBMC samples representing ‘after tick infection’ were collected. Calves were then challenged with a lethal needle sporozoite dose (1:20 T. parva Muguga, stabilitate 3087). PBMC samples were taken 28 days following lethal needle challenge representing the ‘after needle challenge’ time point. PBMC samples were isolated, frozen and stored in liquid nitrogen until animal experiment had been finalized. PBMC of all freezing time points were thawed and re-stimulated in vitro using autologous T. parva infected lymphoblast cell lines. The expansion of effector cells was unreliable and required supplementation of cultivation media with T cell growth factor. In vitro proliferation of re-stimulated cultures to reach sufficient cell numbers for CTL assays was still rarely achieved. Where sufficient cell numbers were produced as in animals BF007, BF011, BF012 and BF018, no specific chromium release was measurable (Figure 1, rows 1-3).

This negative result led us to determine the effect of PBMC storage in liquid nitrogen on performance of cells in CTL assays. Therefore, PBMC from animal BB007 were isolated freshly and re-stimulated in vitro. Aliquots of the same PBMC were frozen down in liquid nitrogen. The negative impact of freezing on in vitro cell expansion and CTL activity is shown in Figure 1 row 4. Killing activity was observed in freshly isolated and re-stimulated PBMC but not in recovered frozen cells. Hence, no conclusion on the induction of CTL activity in animals exposed to tick-delivered T. parva sporozoites can be drawn at the moment. Currently, novel cattle experiments are planned specifically addressing this question.

To determine the phenotype of cells proliferating after in vitro re-stimulation in the presence of TCGF, flow cytometry analysis after antibody staining was performed. After two weeks of cultivation, the number of viable cells varied between different samples. No single lymphocyte subset dominated the cell culture as shown in Figure 2.
Conclusions

Studies of the effector mechanisms and the antigens targeted in protective immune responses to *T. parva* are based on animals treated with needle administered sporozoites. This artificial method whereby a lethal dose of parasites is administered in a single inoculation may not represent an accurate reflection of the field situation, particularly in endemic regions where young calves experience low level primary parasite inoculations. The antigens targeted in these animals are most likely good subunit vaccine candidates since they exist under natural re-challenge situation. In our view, it is imperative to analyze the cattle immune response developed against *T. parva* under natural tick challenge.

References


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CHAPTER 7: CELLULAR IMMUNITY AFTER TICK TRANSMITTED T. PARVA INFECTION

1

2

3

4
Figure 1: CTL from tick- and needle-based *T. parva* challenge in animals tested in $^{51}$Cr-release assay. Effector: Target (E:T) ratios are indicated for Muguga infected autologous PBMC.
Figure 2A: Proportion of stained B cells of total population. A larger proportion of B cells were noted for animal BF031 both after tick and needle challenge.
Figure 2B: Proportion of stained Gamma Delta T cells of total population.

After tick infection  
After lethal needle
Figure 2C: Proportion of stained CD4+T cells of total population. BF033 showed a higher proportion of CD4+T cells.
After tick infection

BF019

After lethal needle challenge

BF025

BF031

BF033
Figure 2D: Proportion of stained CD8+T cells of total population. BF033 showed a higher proportion of CD8+T cells similar with CD4+ T cells.
Livestock have been intricately tied to the livelihoods of humans since their domestication eight to ten thousand years ago (Mignon-Grasteau et al., 2005). Nowhere is this connection currently stronger than for the rural small-holder, cash impoverished farmers and pastoralists who produce up to 90% of livestock products in countries of the South (FAO, 2012). Food and nutritional security are provided by livestock, which are able to convert vegetation from non-arable lands, crop residues and other household byproducts to high nutrient density food products. Livestock can increase crop production by acting as a source of manual labor and by providing manure-based fertilizer. They serve as a cash reserve able to mitigate fluctuations in crop production, unfavorable weather conditions and pricing. If allowed to breed, where sufficient grazing is available, livestock may even serve as an investment strategy. Increasing the herd or flock size allows extra animals to be sold and the proceeds funneled into areas such as healthcare and education of children. Livestock are considered gender equalizers as women who are not allowed to own or inherit land may in many cases be permitted to benefit from owning and selling livestock. Livestock also form an important part of many ceremonies such as marriage and coming of age celebrations where they may be used as exchange of property and status symbol (Minjauw and McLeod, 2003; IUCN, 2011; FAO, 2012).

Increasing livestock productivity is a key component of improving the quality of life of the poor and extremely-poor populations globally (FAO, 2012). Additionally, in global agriculture, a livestock revolution is underway that has profound implications for human health, livelihoods and the environment worldwide. Population growth, urbanization and income growth in developing countries are driving a massive increase in demand for animal based food products. Ticks and tick-borne diseases are considered to be the largest impediment to increased livestock production (Minjauw and McLeod, 2003; Jongejan and Uilenberg, 2004). Ticks affect roughly 80% of the world’s cattle population and effective tick and tick-borne disease control is imperative for increased and sustainable livestock production to take place (McCrosker, 1979; Minjauw and McLeod, 2003; Jongejan and Uilenberg, 2004). Of the tick-
borne diseases affecting cattle, East Coast fever (ECF), is the most devastating. ECF is caused by *T. parva*, transmitted by the brown ear tick *R. appendiculatus* and affects regions in East and Southern Africa. It has been estimated that ECF results in the death of over a million animals annually (Mukhebi et al., 1992). Current methods of ECF control are far from adequate and as with other tick species, the control of *R. appendiculatus* ticks through acaricides is becoming less and less sustainable. Although the Infection-and-Treatment method of live vaccination against ECF exists, adoption has been poor and is geographically limited. Sub-unit vaccines, particularly those targeting the pathogenic schizont stage of the parasite have been proposed, although, they are unlikely to be delivered to farmers in the immediate future.

One important factor that should be taken into account is that ECF is distributed widely throughout East Africa, simultaneously affecting markedly different types of agricultural production systems. These systems have different needs for tick and tick-borne disease control and as a result, effective control strategies may differ substantially. Large-scale farming systems would benefit from an integrated pest management approach of anti-tick vaccination coupled with limited acaricide application. Such farming systems have the financial capacity to control cattle grazing environments to a large extent and individual animal movement can be restricted. Through diagnostic testing, herd composition and disease status can be monitored and controlled. Near complete focal eradication of ticks under these conditions could be achieved through vaccination using concealed tick gut antigens such as Ra86 in combination with controlled acaricide use. With decline in tick populations, incidents of tick-borne diseases are likely to decrease as seen with Bm86 based vaccination (de la Fuente et al., 2007). In contrast to large-scale farming systems, small-scale farmers have limited cash resources and control over the grazing environment of their cattle is problematic since they may rely on small farming units (less than two hectares) or larger, shared, communal grazing areas. Of primary concern for small-holder farming systems is reducing the deleterious effects of ECF disease. The whole-herd vaccination coverage and acaricide employment required for effective tick control through concealed gut antigens is not
necessarily feasible under these conditions. Additionally, if communal grazing is employed, mixing of animals of different origins, disease status and vaccination histories occurs. In areas such as these, reducing the severity of ECF may be successfully achieved through transmission-blocking vaccines. Through a substantial reduction of the sporozoite parasite dose received by calves, natural immunity can be developed without animals experiencing severe disease symptoms. This protection should extend throughout the lifetime of an animal if natural low-level exposure is maintained.

The potential for tick and tick-borne disease control through anti-tick vaccination has been clearly demonstrated by the Bm86 based commercial products, Gavac™ and TickGard™ which were used widely to control *R. microplus*. Through the development of this vaccine, valuable lessons have been learned which should make second-generation anti-tick vaccine development more successful (Guerrero et al., 2012). Through the work performed within the framework of this PhD thesis and presented here, the potential of various anti-tick vaccine candidates were evaluated in their natural host-pathogen-vector system for their ability to control *R. appendiculatus* populations and interfere with *T. parva* transmission to the cattle host.

Anti-tick vaccination has been studied primarily for the control of the one host tick, *R. microplus*. Substantial differences in biology between *R. microplus* and *R. appendiculatus* will most likely translate into modifications of anti-tick vaccine development approaches necessary to successfully tackle *R. appendiculatus*. *R. microplus* feeds on cattle over a prolonged period of time which may take up to a month. This allows a continuous binding of vaccine-induced host anti-tick antibodies to the tick target antigen. In contrast, *R. appendiculatus* feeds between five and seven days on cattle. Within this relatively short time frame the full impact of the host immune system has to be delivered. Alternatively, targeting of novel anti-tick antigens with essential functions during the periods when *R. appendiculatus* ticks digest the blood meal, molt into the next instar or lay eggs could prolong the anti-tick effect of host antibodies. All life stages feed of *R. microplus* feed from the same bovine host.
and whole herd vaccination has been shown to be effective for Bm86 (de la Fuente et al., 2007). In contrast, with the three-host life cycle of *R. appendiculatus*, a single tick may feed on up to three separate cattle during its lifetime. In cases where all animals in the herd are vaccinated, maximum anti-tick effect is still obtained. However, if the immature stages feed on non-vaccinated cattle or alternative hosts, vaccine effects may potentially be lost.

Vaccination of cattle with Bm86 (TickGard™) had no measurable effect on feeding of adult female *R. appendiculatus* ticks (de Vos et al., 2001; Odongo et al., 2007). Inoculation of mice with synthetic peptides derived from the *R. decoloratus* homologue of Bm86, Bd86, induced antibodies binding to midgut sections of *R. appendiculatus* and *R. microplus* (Kopp et al., 2009). This clearly shows that certain epitopes are at least conformationally shared between the Bm86 homologs in these tick species. After vaccination of rabbits with baculovirus produced Ra86 (variants Ra85A and Ra92A), reduction of engorgement weight and egg production of adult female *R. appendiculatus* ticks was observed (Siamo et al., 2011). These effects resembled the Bm86 vaccination induced effects against *R. microplus* (Willadsen, 2004; de la Fuente et al., 2007). No effect on engorgement weights of either larval or nymphal populations of *R. appendiculatus* was observed (Siamo et al., 2011). However, after vaccination of cattle with baculovirus produced Ra86 (variants Ra85A and Ra92A), the Ra86 induced effect in rabbits was not reproducible (Olds et al., 2012 Chapter 3). Female adult tick engorgement weight and reproductive capacity was not affected by Ra86 vaccination of cattle. Surprisingly, a significantly higher proportion of nymphal ticks failed to molt to the adult stage after feeding from calves vaccinated with Ra86. This effect has important implications for the transmission of *T. parva* and ECF severity. Modeling experiments confirmed that through nymphal tick population reduction a general decrease in the *R. appendiculatus* tick population would occur over time. The transmission of *T. parva* by adult ticks has been generally associated with more severe ECF compared to nymphal transmission due to the higher parasite loads in adult tick salivary glands (Norval et al., 1992; Ochanda et al., 1996). Removal of nymphal ticks before molting to the adult stage will result in lowering of the overall ECF disease burden. Additionally, in this experiment, Ra86
vaccination reduced *T. parva* infection levels in nymphal ticks that did successfully molt into adult ticks. Currently, we hypothesize that improvements in recombinant Ra86 vaccine production and formulation will result in a more substantial negative impact on *R. appendiculatus* tick populations.

In summary, these results showed that vaccination with tick gut antigen homologs of Bm86 show potential for *R. appendiculatus* control. Recombinant Ra86 vaccination could serve as a component of successful tick population control and may also form a useful part of an ECF transmission-blocking vaccine. The use of Bm86 gut homologues for tick-borne disease control is underscored by a recent report by Jeyabel et al., 2010. Vaccination of cattle with Haa86, the Bm86 homologue from *Hyalomma anatolicum anatolicum* reduced the transmission of *Theileria annulata* from infected ticks to naïve cattle and an overall lower mortality rate was observed in vaccinated calves (Jeyabal et al., 2010).

Parasite transmission blocking as a method of ECF control can occur at two points within the *T. parva* parasite life cycle, either from an infected cattle host to the tick vector or from an infected tick vector to a susceptible cattle host. To evaluate vaccines designed to interfere with parasite transmission and/or development within the cattle or tick hosts, experiments should be performed in the natural host – tick system (Wikel, 1999). Currently, the widely used method for experimental infection of cattle with *T. parva* is through needle administration of high numbers of cryopreserved sporozoites (Di Giulio et al., 2012). This clearly does not reflect the situation in the field where sporozoites are injected together with saliva over a period of several days into the cattle host (Konnai et al., 2007).

Here we evaluated a novel tick line, RAM-L, for its ability to serve as an experimental tool for reproducibly delivering sporozoites to susceptible calves. The aim of this experiment was to develop a cattle – tick – pathogen system mimicking natural infection that takes place under field situations. This model should serve as an evaluation tool for novel candidates at an early stage of vaccine development. In Chapter 4, we demonstrate that the RAM-L line is
able to deliver sporozoites to all calves exposed to RAM-L tick challenge. RAM-L closely resembles ticks collected from areas where *T. parva* is endemically stable in the terms of infection levels and distribution within the RAM-L population. Interestingly, tick-mediated sporozoite delivery induced a fully protective immune response in cattle against lethal sporozoite needle challenge. To our knowledge, this is the first autologous tick–cattle infection model that enables novel and essential investigations into *T. parva* biology, tick–pathogen interaction and dissection of immune response against tick-borne diseases of cattle in the field. The strategies developed here can be extrapolated to study other cattle tick-borne diseases including babesiosis and anaplasmosis.

Next, using this RAM-L line, a multivalent transmission blocking vaccine designed to inhibit *T. parva* transmission from an infected tick vector to susceptible cattle was evaluated (Chapter 5). This vaccine contained several recombinant tick antigens in conjunction with a single parasite sporozoite component aimed to inhibit parasite entry into the bovine vascular system and lymphoid cells, respectively. In comparison to the control group, vaccination increased the number of animals undergoing primary *T. parva* infection without developing any (non-responder) or only mild clinical symptoms of ECF (mild-reactors). Mild reactions were characterized by the presence of a low-grade fever and no detectable sporozoite or piroplasm parasites while non-reactors showed no symptoms of infection. Importantly, these non-responder and mild-reactor calves fully withstood a subsequent lethal homologous needle-based sporozoite challenge. Hence, this multivalent vaccination reduced clinical ECF symptoms while at the same time allowed the establishment of a protective immune response through the very low levels of sporozoites injected. We show here that transmission-blocking vaccination for the control of ECF is feasible and, of paramount importance, that this vaccination does not prevent the development of protective ECF immunity. Clearly, the efficacy of the multivalent transmission-blocking vaccine described in chapter 5 has to be improved, possibly through novel vaccine formulations and/or better selection of vaccine candidates. The development of potential transmission-blocking vaccines interfering with *T. parva* macrogamete and zygote binding/invasion of tick gut cells has not been evaluated for
T. parva. The molecular mechanisms involved in sexual recombination and gut binding/invasion are essentially unknown and further research in basic parasite biology is fundamental. A similar vaccine approach has been shown to be viable for preventing Lyme disease transmission and in our opinion warrants further investigation for T. parva.

Identification of new targets for effective R. appendiculatus and ECF vaccination-based control is essential. This will require in-depth investigations studying tick biology at genomic, regulatory and proteomic levels. Although a complete R. appendiculatus genome is not available, substantial gene sequences are already known. A better understanding of the molecular mechanisms governing T. parva sporozoite entry into bovine cells will support selection of suitable candidates. With advances in genome sequence analysis (Patel et al., 2012, manuscript submitted), this resource for T. parva candidate selection is being actively pursued. A multi-stage, multi-pathogen vaccine incorporating essential and conserved antigens from both R. appendiculatus and T. parva might provide the best option for an efficient sub-unit vaccine approach.

As seen with Ra86, recombinant tick antigen production and correct folding of conformational epitopes can be difficult. Additionally, there is no easy way to evaluate these recombinant antigens for preservation of natural protein conformation present within the tick. Recently published papers describing a novel method of bacterial membrane bound Bm95 and Bm86 production may be useful for the production of Ra86 (Canales et al., 2008; Canales et al., 2009; Almazán et al., 2012). It is well known that the antigenic epitopes recognized by different vaccinated vertebrate host species may not necessarily overlap, resulting in different anti-tick effects when testing an identical product in various host species. None of the antigens evaluated in our study have been previously investigated in the autologous cattle - R. appendiculatus - T. parva system. The results obtained here differ from previous reports testing these antigens in mice, rabbits or guinea pigs. With the novel, defined tick challenge model based on RAM-L, future anti-tick vaccine evaluation under field-like conditions is now possible. In summary, this work shows that anti-tick vaccination has strong
potential to play a key role in novel, integrated control strategies for *T. parva* and its vector, *R. appendiculatus*.

**References**


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