EFFECT OF EAST COAST FEVER MARIKEBUNI VACCINE ON FEEDING AND REPRODUCTIVE SUCCESS OF *Rhipicephalus appendiculatus*

BY

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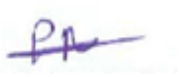
I56/31845/2019

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE IN APPLIED PARASITOLOGY OF THE UNIVERSITY OF NAIROBI

2022

DECLARATION

This thesis is my original work and has not been submitted elsewhere for examination or award of a degree. Where other people's work or not my own work has been used, this has been properly acknowledged and referenced in accordance with the University of Nairobi requirements.

Signature  Date 7th November 2022

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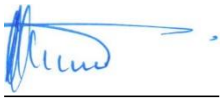
Signature  Date 13th November 2022

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DEDICATION

I dedicate this thesis to the Lord Almighty, who gave me knowledge and wisdom to come up with the idea behind this work.

ACKNOWLEDGMENT

I am thankful to God Almighty for his constant support throughout the entire writing of this thesis. My special appreciation goes to my supervisors: Professor Florence A. Oyieke, Professor Richard W. Mukabana, and Doctor Monicah Maichomo for their guidance, support, wise suggestions, and corrections throughout the entire writing of this thesis. My gratitude goes to Professor Ndegwa, Doctor Mutahi, Doctor Owino and Doctor Odongo for their kind advice. May the Lord bless you.

My utmost appreciation goes to the Institute Director of Kenya Agricultural and Livestock Research Organization-Veterinary Sciences Research Institute (KALRO-VSRI) Doctor Monicah Maichomo, for providing calves and other inputs that were used in this study and access to the VSRI laboratories. The Institutional Animal Care and Use Committee (IACUC) at Veterinary Sciences Research Institute-VSRI approved this on-station research activity, which is greatly acknowledged. My special appreciation goes to all the team, Protozoology Division at VSRI for their support, useful suggestions, and guidance during the collection of data for this thesis.

I want to specifically thank my employer, the Teacher's Service Commission (TSC) for granting me the study leave to pursue my studies. Not forgetting Mr. Morgan for aiding me during the study leave session. God bless you abundantly.

Finally, I thank God for using my family and friends as a blessing to me through prayers and words of encouragement throughout the research study. God showers you with blessings in abundance.

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LIST OF ABBREVIATIONS AND ACRONYMS

AIC	Akalke's information criterion
APCs	Antigen presenting cells
ANOVA	Analysis of variance
CTL	T lymphocytes
DNA	Deoxyribonucleic acid
DVS	Director of Veterinary Services
ECF	East Coast Fever
ELISA	Enzyme-linked immunosorbent assays
GADM	Global Administrative Area
G.L.M	Generalized linear model
IACUC	Institutional Animal Care and Use Committee
IFAT	Indirect Fluorescent Antibody Test
ILRI	International Livestock Research Institute
ITM	Infection and Treatment Method
KALRO	Kenya Agricultural and Livestock Research Organization
LAMP	Loop-mediated isothermal amplification
OIE	World Organization of Animal Health (WOAH)
Pan-FRET	Pan-forster resonance energy transfer
PBI	Peripheral blood leukocytes
PBS	Phosphate buffered saline
PCR	Polymerase chain reactions
PCR-RFLP	Polymerase chain reaction-Restriction Fragment Length Polymorphism
PIM	Polymorphic immunodominant molecule

RPM	Revolution per minute
SYBR	Synergy Brands
TSC	Teachers Service Commission
Var	Variance
Vif	Variance inflation factor
VSRI	Veterinary Science Research Institute

ABSTRACT

East Coast Fever is a tick-borne disease of economic importance in bovines caused by *Theileria parva*, a protozoan parasite transmitted transstadially by a three-host tick, *Rhipicephalus appendiculatus*. The control of ECF requires integrated pest management, including vaccine development. *Theileria parva* Marikebuni vaccine is a live parasite vaccine used to immunize cattle against ECF. At present, there is no information on the effect of this vaccine on the feeding and reproductive success of *R. appendiculatus*. This study investigated the interaction between the *T. parva* vector and its immunized host,cattle. Three groups of ECF naïve calves aged between 3-12 months were selected for the study, each having six calves. Calves in the first group, the "immunized group", received 1 ml of Marikebuni vaccine and a long-acting oxytetracycline blocking agent at 30ml/kg body weight. The 2nd group, "Control Group 1" received uninfected tick material and the blocking agent while the 3rd group, "Control Group 2" received the uninfected tick material only. Ticks were counted and weighed before and after application to calves. Incubation of ticks was done at 27-28 ºC optimum temperature and 80% -85% humidity. The number of ticks that fed successfully, the mean blood meal weight, and the number of successfully molted nymphs were analyzed to indicate the feeding success of *R. appendiculatus*. The mean egg mass weight, number of egg batches and clutch sizes of eggs indicated oviposition success. The number of live larvae hatched determined the egg viability. Data were compared using linear regression, an ANOVA test, and a binary logistic regression model using R statistical software Version 4.1.3 (2022-03-10), at a $p<0.05$ significance level. The outcomes of this study showed that the number of fed nymph ticks (OR = 0.996; p-value = 0.29); nymph blood meal weight (OR = 0.712; p-value = 0.34); number of successfully molted nymphs (OR = 1.004, p value = 0.36); number of successfully fed adult female ticks (OR = 1.05; p-value = 0.93); and the adult female tick blood meal weight (OR = 0.32, p value = 0.48; F = 3.26, p-value = 0.11)did not differ significantly between *R. appendiculatus* that fed on immunized calves and those that fed on controls. The egg mass weight differed significantly between the ticks that fed on immunized calves and controls (F = 7.993; p-value = 0.023), and a significant pairwise average difference of 1.34 was detected between the ticks that fed on immunized and control group 2 calves and between those that fed on control groups 1and 2 (adjusted p-value =0.03). Other parameters determining oviposition success did not differ significantly between the ticks fed on the immunized group and the controls: clutch sizes of eggs (OR = 1.0, P-value = 0.40); number of egg batches (OR = 0.08, p-value = 0.35). The number of live larvae that hatched successfully did not differ significantly between the immunized and control groups (OR = 0.99; p-value = 0.33). In conclusion, the blood feeding success of *Rhipicephalus appendiculatus* was not significantly altered by the vaccine, despite the observed differences ($p >0.05$). The oviposition success of the tick vector was altered by the vaccine, reducing the egg mass weight in ticks that fed on the immunized group significantly ($p<0.05$). The viability of eggs from *R. appendiculatus* ticks was not significantly impacted by the vaccine, despite the observed differences ($p > 0.05$). The findings of this work shed light on the efficacy of the Marikebuni vaccine against *R. appendiculatus*. Significantly reduced egg mass weight on ticks fed on cattle immunized with Marikebuni vaccine shows that the vaccine indirectly leads to some degree of vector control. The production and distribution of this vaccine in ECF endemic regions of sub-Saharan Africa should therefore be increased.

CHAPTER 1

GENERAL INTRODUCTION

1.1. Background information on the importance of East Coast Fever Disease in Sub-Saharan Africa

East Coast Fever (ECF) is a tick-borne protozoal ailment that occurs in cattle and domesticated buffaloes in East, Central and South Africa and is caused by the *Theileria parva* parasite (Morrison *et al.,* 2020). The three-host tick vector, *Rhipicephalus appendiculatus,* transmits this parasite transstadially. The cyclical change of this parasite occurs within the larvae, nymphs and adult stages during the development of the vector (Nene *et al.,* 2016).

Exotic cattle are the major source of dairy milk in Kenya, but their production is challenged by their high susceptibility to ECF, which causes high morbidity and mortality and is expensive to treat (Behnke and Muthami, 2011). Available data indicates an annual mortality of approximately one million cattle, equivalent to a financial loss of approximately 300 million USD (International Livestock Research Institute, 2014; GALVmed, 2019).

Acaricide is the major control strategy for the ECF tick vector (Abbas *et al.,* 2014). Although effective to some extent, this control method has significant drawbacks, such as high costs running into a million dollars per year, development of relative resistance, and being environmentally unfriendly (Kivaria 2006; Singh *et al*., 2022). The high cost of control is also attributed to tick resistance developed with continued use of the limited range of acaricide (Abbas *et al*., 2014). Besides, acaricide residues also find their way into the environment and animal products like meat and milk and pose a high risk to human health.

To reduce reliance on acaricide, research on alternative control options, specifically the use of vaccines, has made good progress. Infection and Treatment Method (ITM) has been ongoing in the East African region since the 1970s (Perry, 2016). Based on the available clinical data, the vaccine was approved by the governments of Kenya, Malawi, and Tanzania in May 2010. In Kenya, there are two such types of ITM vaccines: the Marikebuni vaccine and the ‘‘Muguga Cocktail’’ vaccine. The Marikebuni vaccine is based on live parasites of *T. parva* and is manufactured and marketed by the Kenya Agricultural and Livestock Research Organization (KALRO annual report, 2015-2016). At present, there are no documented findings showing the impact of this vaccine on *Rhipicephalus appendiculatus*. Blood feeding in the tick vector is vital, as it is required for molting to the next stage. The blood obtained by adult male and female ticks is utilized to produce sperm and egg respectively, and also carry out other physiological processes. Hindrances to proper blood feeding has a detrimental effect on the reproductive success of *R. appendiculatus* ticks (Kaufman, 2007). The main objective of this work was to evaluate the effect of the *Theileria parva* Marikebuni vaccine on the vector tick after feeding on immunized cattle. This was done by comparing the blood feeding success, oviposition success and viability of eggs laid between vectors that fed on immunized and non-immunized cattle.

1.2: Problem Statement

The dairy industry is impacted by ECF which causes high morbidity and mortality in cattle, leading to low production. In endemic regions, ECF kills at least one million cattle per year (ILRI, 2014). In addition to the deaths caused by the disease, approximately 28 million more cattle are at risk of infection with ECF. With these statistics seemingly increasing every year, this translates to huge economic losses, approximating 300 million USD. This is disruptive to small scale farmers, who comprise the majority of dairy producers and whose livelihood

depends on livestock. The disease causes a reduction in production rates (Behnke and Muthami, 2011; International Livestock Research Institute, 2014). The main control strategy, the chemical control method, is costly and environmentally unfriendly, and vectors have increasingly developed resistance to acaricide. Better control strategies for ECF are required, such as the use of vaccines. The purpose of this study was to determine the effect of the Marikebuni vaccine on the blood feeding and reproductive success of *Rhipicephalus appendiculatus*. The results would hopefully contribute to a refined ECF immunization strategy and subsequently to increased cattle productivity in Kenya and Sub-Saharan Africa.

1.3: Justification of the study

According to the literature, the existence of parasites in both the livestock host and the transmitter tick may have an impact on the tick vector (Labruna, 2011**;** Benelli, 2020). However, at present, there is no information on any documented trial that shows the impact of live booster injection on blood feeding success, oviposition success and viability of eggs of *R. appendiculatus*. This research investigated the *T. parva* Marikebuni vaccine's effect on the blood feeding success, oviposition success and viability of eggs of *Rhipicephalus appendiculatus* fed on cattle immunized with Marikebuni vaccine. The findings of this study will lead to a refined ECF control strategy in ECF endemic regions of sub-Saharan Africa.

1.4: Hypotheses

1.4.1: Null hypotheses

i. East Coast Fever Marikebuni vaccine does not affect the blood feeding success of the tick vector *Rhipicephalus appendiculatus.*

ii. East Coast Fever Marikebuni vaccine does not affect the oviposition success of *Rhipicephalus appendiculatus.*

iii. East Coast Fever Marikebuni vaccine does not affect the viability of eggs of *Rhipicephalus appendiculatus.*

1.4.2: Alternative hypotheses

i. East Coast Fever Marikebuni vaccine affects the blood feeding success of *Rhipicephalus appendiculatus.*

ii. East Coast Fever Marikebuni vaccine affects the oviposition success of *Rhipicephalus appendiculatus.*

iii. East Coast Fever Marikebuni vaccine affects the viability of eggs of *Rhipicephalus appendiculatus.*

1.5: Study Objectives

1.5.1: General objective

To determine the effectiveness of the *Theileria parva* Marikebuni vaccine on the blood feeding and reproductive success of *Rhipicephalus appendiculatus* in Kenya.

1.5.2: Specific Objectives

i. To assess the blood feeding success of *R. appendiculatus* that feeds on cattle immunized using the Marikebuni vaccine.

ii. To determine the oviposition success of adult *R. appendiculatus* that feeds on cattle immunized using the Marikebuni vaccine.

iii. To determine the viability of eggs laid by *R. appendiculatus* that feeds on cattle immunized using the Marikebuni vaccine.

CHAPTER 2

LITERATURE REVIEW

2.1: Tick –borne diseases

The tick vector comes second to mosquitoes in the transmission of infections and is thus significant in transmitting various infections in livestock (Raboloko *et al*., 2020; Chiuya *et al.,* 2021). *Theileria annulata*, which causes Mediterranean theileriosis, is transmitted by *Hyalomma* species (Bishop *et al.,* 2004; Zaeemi *et al.,* 2011). Rickettsial infections of *Ehrlichia ruminantium* cause heart water disease and are transmitted by the bont tick of the genus *Amblyomma* (Faburay *et al.,* 2007; Tumwebaze *et al.,* 2020). It is an important and severe disease that kills animals very quickly after the first clinical signs are detected. This genus of ticks also transmits benign *T. mutans* and *T. velifera* (Bishop *et al.,* 2004; Tumwebaze *et al.,* 2020). *Babesia bigemina* and *Babesia bovis* are other infections transmitted by *Rhipicephalus decoloratus* and *R. microplus* ticks, respectively (Yabsley and Shock, 2013; Tumwebaze *et al.,* 2020). These infections result in a protozoan disease called Babesiosis. Additionally, *Anaplasma marginale,* which causes anaplasmosis is transmitted by *R. decoloratus* and sometimes biting flies (Tumwebaze *et al.*, 2020). This is a rickettsial disease whose principal symptoms include high fever, anemia, jaundice, and sometimes constipation. Older cattle are more susceptible to anaplasmosis than calves, which mostly develop the mild disease, recover, and become carriers. Reports have shown that an animal with coinfections of anaplasmosis and ECF is likely to show severe reactions even in very low *Anaplasma* parasitemia (Zaeemi *et al.,* 2011; Ringo *et al.,* 2018).

In cattle hosts, tick bites cause cutaneous effects such as irritation, inflammatory response, hemorrhage, focal dermal necrosis, and wounds that get secondary infections with bacteria such as *Staphylococcus* and screwworm myiasis (Taylor *et al.,* 2016). There is significant blood loss due to tick infestation, which compromises production and productivity (Jongejan

and Uilenberg, 2004; Taylor *et al.,* 2016). It also causes the death of calves due to chronic and acute anemia. Adhering sites of *R. appendiculatus* on cattle are damaged by the tick, resulting in poor hide and skin quality (Taylor *et al.,* 2016; Jaime Betancur Hurtado and Giraldo-Ríos, 2019).

Rhipicephalus appendiculatus transmits several species of *Theileria,* such as *Theileria parva bovis* and *T. parva lawrencei,* that cause January and corridor disease, respectively. *Theileria parva taurotragi,* on the other hand, causes benign infections (Bin Tarif *et al.,* 2012; Latif *et al.,* 2019). Thogoto and Dhori viruses are also transmitted by this vector to animals and humans (Gong, 2015). This rhipicephalid tick also transmits *T. parva* parasites that cause ECF. This is an ailment of bovines that results in increased morbidity and mortality in livestock, leading to huge productivity losses in endemic regions. It is therefore rendered to be the most financially significant tick species in Sub-Saharan Africa (Walker *et al.,* 2003; Morrison *et al*., 2015).

2.2: East Coast Fever disease

East Coast Fever (ECF) infects cattle and domesticated buffaloes across East, Central, and Southern Africa. This disease, also known as theileriosis, is linked to a protozoan parasite, *Theileria parva,* that is carried transstadially by a three-host tick, *Rhipicephalus appendiculatus* (Bishop *et al.,* 2004; Olds *et al*., 2018). The African buffalo (*Syncerus caffer*) is known to be a reservoir for *T. parva* infections, as are the water bucks (*Kobus* spp) (Morrison *et al.,* 2020). In Kenya, ECF is prevalent in many areas of the coastal and highland regions, and its distribution corresponds to the presence of the tick vector *R. appendiculatus* as well as livestock hosts (Chenyambuga *et al*., 2010). The vector is found at elevations up to 8000 feet (2,438.4 m) above sea level and annual rainfall above 500 mm (Gachohi *et al*., 2012). ECF kills approximately one million cattle per year (ILRI, 2014). Of the various livestock products, milk is a particularly frugal and significant animal product (Behnke and Muthami, 2011). In Kenya, the bulk of milk production is by exotic cows,

which are highly susceptible to ECF infection (Wangila, 2016). Naïve cattle are susceptible to ECF infection, which can cause 100% mortality in both indigenous and exotic breeds if untreated, hence posing a threat to the livestock sector (Wesonga *et al*., 2010).

2.3: East Coast Fever (ECF) causative agent

Theileria parva is the causative agent of ECF in cattle and is transmitted by the *Rhipicephalus appendiculatus* tick vector, which primarily prefers to reside in the ears of cattle (CDC, 2017). Research has shown that the lifecycle of the parasite involves developmental stages in the mammalian host and tick vector, where the parasite goes through sexual advancement in the vector tick and asexual advancement in the cattle host (Nene *et al.,* 2016). The distribution of *Theileria parva* infections in sub-Saharan Africa is mostly in East, Central and South Africa (Figure 2.1). This distribution corresponds with that of *Rhipicephalus appendiculatus* (Erasmus *et al*., 2002; Olwoch *et al*., 2008; Gachohi *et al.,* 2012).

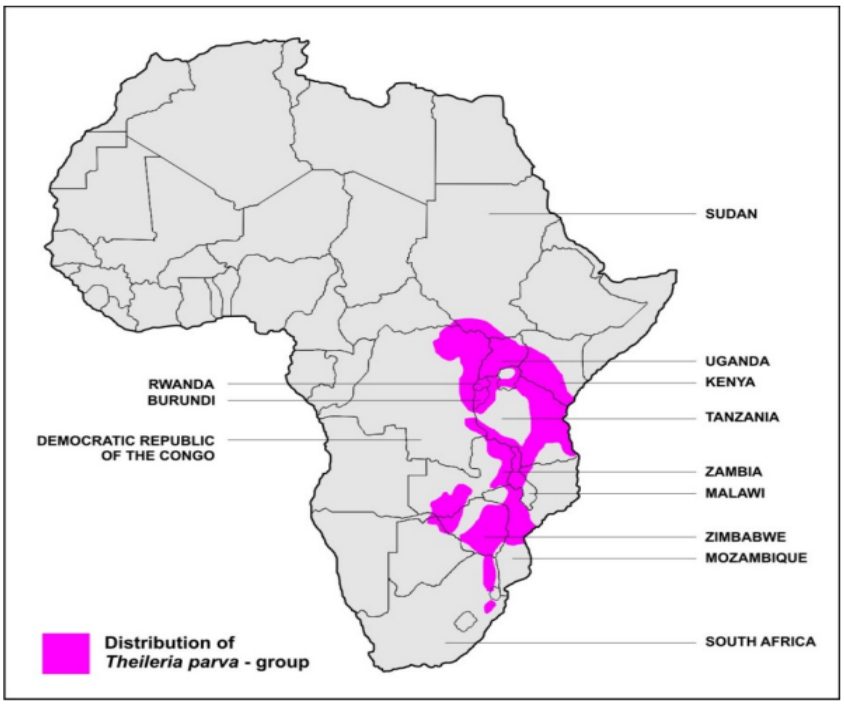


Figure 2.1: Distribution of *Theileria parva* infections in Africa (Lawrence *et al*., 2005).

2.4: Diagnosis of East Coast Fever

East Coast Fever disease requires proper diagnosis to ensure the survival of infected cattle. The disease diagnosis is based on characteristic clinical signs and can be confirmed by the demonstration of schizonts. The presence of antibodies against *Theileria parva* schizonts confirms cattle exposure to the ECF infection. Microscopy and molecular analysis confirm the presence of the infectious agent. Serological tests confirm the exposure of cattle and also determine the prevalence, incidence, and seasonality of ECF infection (GALVmed, 2019).

2.4.1: Clinical signs

ECF is characterized by an enlarged lymph node, a rise in temperature above 39.5 °C, anorexia, loss of condition, lacrimation, corneal opacity, coughing, terminal dyspnea, diarrhea, nasal discharge, and petechial hemorrhages on the mucus membrane. Calves are susceptible to the disease, with high mortality being recorded (World Organization for Animal Health (OIE), 2015).

2.4.2: Microscopy

Thick and thin blood smears are prepared by taking blood from the marginal ear vein on a slide or capillary tube. The slides are air-dried, and a thin smear is fixed in methanol. Thick and thin blood smears are dried and stained with Giemsa stain at 1:10 dilution with distilled water for half an hour and allowed to dry up before being examined (Paparini *et al*., 2012; World Organization for Animal Health (OIE), 2015). The smears are examined using a microscope with x40 objective lenses, followed by oil immersion at x100 to check for piroplasms in erythrocytes. The presence of piroplasms in the blood suggests exposure to ECF parasites but does not confirm ECF infection. Aspirate from the prescapular lymph node is used to make a thin smear, which is air-dried, fixed with methanol and Giemsa stained for examination, as described by OIE (2015). Examination using a microscope with x40 and x100 objective lenses will identify the presence of schizont-contaminated cells, which confirms the presence of ECF

infection (Paparini *et al*., 2012). Light microscopy has several disadvantages. These include the inability to detect the carrier state of the animals and the lower sensitivity compared to the polymerase chain reaction (Wangai *et al*., 2011).

2.4.3: Serological test

The indirect fluorescent antibody test (IFAT) is a gold standard serological test used to identify exposure of cattle to *Theileria parva* (OIE, 2014). Serological tests are useful in detecting any changes in the immune status of the recovered animal (OIE, 2014, 2015). However, various drawbacks are associated with this diagnostic method, such as cross reactivity observed between closely related species like *T. taurotragi* and *T. parva* antigen (Pienaar *et al.,* 2014).

Enzyme-linked immunosorbent assays (ELISA) utilize a select number of antigens responsible for the dominant immune response against most *Theileria* parasites. The P32 antigen of *T. mutans* and the polymorphic immunodominant molecule (PIM) were used to develop ELISAs that were more sensitive than IFAT (Mans *et al.,* 2015; OIE, 2014). The ELISA diagnostic method is cheap and fast for screening large numbers of samples. Both methods continue to be used in research, especially where molecular infrastructure does not exist (Kiara *et al.,* 2014).

2.4.4: Molecular diagnosis

Molecular diagnostics target specific genes and species, and they include the following: conventional polymerase chain reactions (PCR) followed by agarose gel electrophoretic analysis (Pienaar *et al*., 2011a), PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) methods (Zaeemi *et al.,* 2011), nested PCR (Odongo *et al.,* 2010), dot blotting and hybridization (Bhat and Rao,2020). An improvement on the latter method involves the use of a non-radioactive reverse line blot method or a DNA bead-based suspension array (Ros-Garcia *et al*., 2013). Others include: probe-based real-time PCR (Sibeko *et al*., 2008; Pienaar *et al*., 2014), Synergy Brands (SYBR) green real-time PCR assay (Pienaar *et al*., 2013), loop-mediated isothermal amplification (LAMP) assays (Salih *et al.,* 2008; Xie *et al.,* 2013), Pan-

forster resonance energy transfer (pan-FRET)-based assays (Perera *et al*., 2014), and high-resolution melt analysis (Salim *et al*., 2013). These molecular methods confirm the presence of parasite genomic material. It's possible to apply LAMP under field conditions, whereas many of these other assays require specialized equipment.

2.5: ECF disease control

Control of ECF is mainly achieved through tick control, treatment of the clinical disease, and the use of vaccines (GALVmed, 2023). An integrated management of the disease that combines any of the methods is also applied.

2.5.1: Chemotherapy

Treatment of East Coast Fever requires accurate diagnosis and prompt treatment, including of any concurrent infections such as anaplasmosis, for maximum cure rates. The disease is fatal if untreated, especially in exotic breeds of cattle. Some drugs used to treat ECF effectively include Buparvaquone and parvaquone (Musoke *et al*., 2004). The treatment with these drugs is limited by the fact that it's normally expensive for most African farmers, and the drugs do not eliminate the carrier state induced by the *Theileria* species (GALVmed, 2023).

2.5.2: Tick control

The key tool in tick-borne disease management is tick control. Some tick control measures currently being employed include hand-picking (Isihaka *et al*.,2022), host grooming (Hart, 2000), biological control (Kar *et al*., 2022), use of anti-tick plants (Wanzala, 2017), use of tick-resistant hosts such as zebu and sanga breeds (Shyma *et al*., 2015), and acaricide application (Muhammad *et al*., 2008; Kemunto *et al*., 2014). In Kenya, these tick control measures are implemented through weekly dipping of cattle in communal dips or hand spraying using a knapsack by individual farmers. In Kenya's highland and coastal endemic regions, acaricide use is the maintay control strategy used, though it's expensive and environmentally unfriendly. The ticks also develop some resistance (Abbas *et al*., 2014). The high cost of controlling ticks

using acaricide and treating ECF has led to an alternative, safer, cheaper and more effective method: the use of vaccines. These are cost-effective and environmentally friendly; therefore, they emerge as better control strategies for controlling the disease (De la Fuente *et al.,* 2007).

2.5.3: Infection and treatment method (ITM)

Currently, ITM is the technique being employed to vaccinate cattle against ECF in Africa. (Perry, 2016). This practice was advanced over 40 years ago at the East African Veterinary Research Organization, now the Veterinary Science Research Institute (VSRI), in KALRO Muguga (Radley *et al*., 1978; perry,2016). The system is based on evidence that cattle infected with ECF develop solid or lifelong immunity against subsequent infection after recovery (Norval *et al.,* 1992; Kivaria *et al.,* 2007).

The method involves administering a potentially lethal live parasite dose to cattle and at the same time inoculating them with a therapeutic dose of long-acting oxytetracycline, which blocks the establishment of the infection (Vercruysse *et al*., 2007). One of the ITM vaccines used to immunize cattle against ECF is the *Theileria parva* Marikebuni vaccine (Radley *et al*., 1975; Mutugi *et al*., 1989; Wanjohi *et al*., 2001). The vaccines yield reduced schizont parasitosis with no or minimal clinical response by the vaccinated cattle. The Marikebuni vaccine strain has been authorized by the director of veterinary services (DVS) to be used in dairy areas, and it is produced and marketed by KALRO (KALRO annual report, 2015-2016). The vaccine can reduce morbidity and mortality in cattle caused by ECF. Immunity developed due to infection with a single strain may not offer full protection against other strains. The Marikebuni vaccine, which was originally isolated from the Kenyan coast, was carefully chosen since it gave a broad spectrum of protection than any other strain (Irvin *et al.,* 1983; Mutugi *et al*., 1989; KALRO annual report, 2015-2016).

2.6: ECF vector *Rhipicephalus appendiculatus*

East Coast Fever is transmitted by a three-host brown ear tick, *Rhipicephalus appendiculatus*. This vector transmits *Theileria parva* parasites transstadially. The infective stages of *T. parva* in the tick vector are the sporozoites, which are injected into animals through the saliva of the feeding tick (Nene *et al*., 2016; GALVmed, 2023).

2.6.1: Geographical distribution of *Rhipicephalus appendiculatus*

Research has shown that about 15 rhipicephalid species occur in North Africa and Eurasia, and about 55 species occur in sub-Saharan Africa (Walker *et al.,* 2003; Tan *et al.,* 2021). The availability of these species is influenced by climate, vegetation, and the availability of a host (Norval *et al.,* 1992; Walker *et al.,* 2000; Gachohi *et al*., 2012). The distribution of *R. appendiculatus* in sub-Saharan Africa is mostly in East, Central and South Africa. Changes in the future dynamics of the vector distributions may be due to varying environmental conditions as human population growth increases, which leads to changes in land use and urbanization. Different farm management systems, cattle breeds, grazing systems, tick control methods, and rainfall availability may also affect future changes in vector distribution, which consequently affect ECF disease epidemiology (Erasmus *et al*., 2002; Olwoch *et al*., 2008).

2.6.2: Biology and behavior of *Rhipicephalus appendiculatus*

Rhipicephalus appendiculatus is an obligate hematophagous ectoparasite of vertebrate animals. The main host of *R. appendiculatus* is cattle, but it also parasitizes goats, dogs, and sheep. Wild animals, such as buffaloes, water bucks, elands, antelopes and great kudus, are also parasitized. It is a three-host tick with four phases of development: egg, larva, nymph, and adult. The larva, nymph, and adult stages of the tick are blood-feeders that feed on different hosts, thus showing teletropic behavior (Walker, 2003; CDC, 2017). *Rhipicephalus appendiculatus* can become well adapted to domestic cattle, with all blood-feeding stages feeding on the cattle host; immature stages attaching to the neck, eyelids, ears, muzzle, cheeks, anal area and dewlap. The

predilection site of the adult tick is the ear's pinna in bovines, but in heavy infestation, they can attach to the upper neck, and around the horn, eyelids, and anus (Walker, 2000; CDC, 2017)). The life cycle of the ECF vector *Rhipicephalus appendiculatus* is shown in Figure 2.3 below (CDC, 2017).

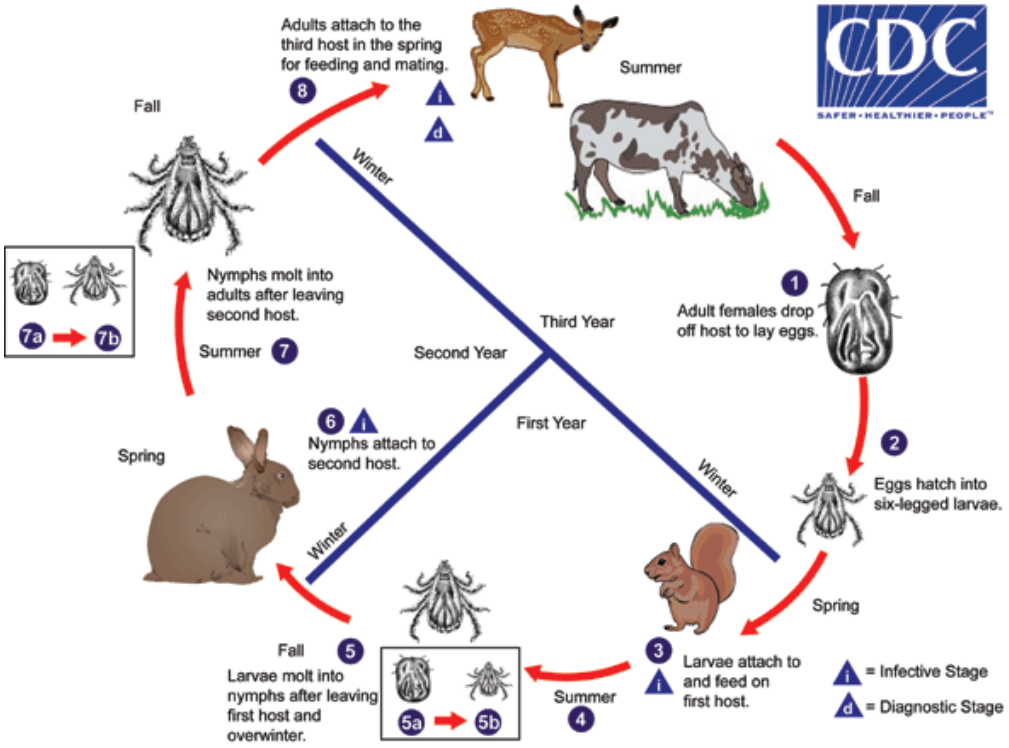


Figure 2.2: Life Cycle of the ECF vector *Rhipicephalus appendiculatus* (CDC, 2017).

Host-seeking behavior in ticks is reported to be influenced by various attractive host-derived stimuli such as host skin humidity, texture, and body temperature. Chemical stimuli of kairomones such as urine, feces, breath, and skin emanations also impact the host-seeking manner of ticks (Sika, 1996; Mordue and Mordue, 2003). The environmental factors also determine the host-seeking behavior of the ticks (Gilbert *et al*., 2014). For example, they actively search for a blood meal from the host under specific environmental conditions such as temperature, humidity, rainfall, day length, and duration of the rainy season (Pegram *et al*.,

1989). Host-derived odors influence the selection of the attachment site of the tick. These include carbon (IV) oxide emanating from host breath, skin emanations and volatile from excretory products of the host such as urine and feces (McMahon and Guerin, 2002). Research has found that upon finding the preferred host, the tick moves to the predilection site, where it is not at risk of being eliminated from the host. This serves to avoid competition among closely related tick species on the same host animal. It also enhances survival (Chilton *et al.,* 1992). *Rhipicephalus appendiculatus* adult ticks are observed to prefer feeding in the bovine ear pinna, while the larva and nymph stages are less selective, hence feeding on several other segments of the host apart from the pinna of the ear (Service, 2012).

The blood meal of *Rhipicephalus appendiculatus* ticks lasts for hours and even days and does not detach quickly from the host. Their bites are therefore not painful due to immunomodulatory hormones in their salivary glands (Sonenshine, 1991; Service, 2012). Their specialized mouth parts are placed through the skin of the host, and they anchor themselves with cement, penetrating the host epidermis superficially (Sonenshine, 1991). *Rhipicephalus appendiculatus,* a hard tick, feeds only once in each developmental stage (Sonenshine, 1991; Service, 2012). Prolonged periods of feeding are observed in *Rhipicephalus appendiculatus* ticks, where the nymph ticks take 5-7 days to complete feeding and the adults 7-9 days, during which the tick is capable of carrying infections to the host. For example, *Theileria* parasites are inoculated into the host 2-4 days after *Rhipicephalus appendiculatus* tick attachment (Mbogo *et al*., 1995; Ebel and Kamer, 2004; Konnai *et al*., 2007).

The amount of blood ingested by the tick depends on its developmental stage and species (Sauer *et al*., 1995). For instance, a fully engorged female tick weighs 50-100 times the unfed tick's body weight. Hard female ticks require a large blood meal, which they use for physiological processes and the production of thousands of eggs during oviposition. The eggs laid depend on the species and environmental and physiological conditions. Between 2,000 and

20,000 eggs are laid. After consumption, the females separate from the host and fall on the land, ready to oviposit. Oviposition can last for several days. The female empties her body, after which she dies. *Rhipicephalus appendiculatus* is recorded to complete oviposition after 21-28 days and even up to 60 days based on the physiological and surrounding conditions, such as humidity and temperature. During favorable conditions, the tick can lay up to ten thousand eggs (Kaufman, 2007).

The eggs produced by the tick are shown to be directly proportional to the size of the tick, whereas small ticks produce a smaller number of eggs compared to large ticks (Kaufman,2007). After oviposition, the larvae require a few days or weeks to hatch, depending on environmental conditions. The hatching process is fast in hot and humid weather. After hatching, the larvae climb to the tip of the vegetation around them as they wait for a suitable host to attach to (Kitaoka and Yajima, 1958; Kaufman, 2007; CDC, 2017).

2.7: Immune response of cattle to *Theileria parva* and *Rhipicephalus appendiculatus*

Following the infection of cattle with *Theileria parva*, the schizont that matures from the sporozoites induces multiple immune cell responses. In a similar process, following the infection of cattle with the vaccine, macro schizont move and reside inside the cytoplasm of lymphocytes that have been transformed. The infection and treatment method induce strain-specific insusceptibility mediated mainly by histocompatibility complex class 1-restricted CD8+ T cells which kill *T. parva* infected host cells as demonstrated by Morrison *et al.,* (2015). In addition, cattle subjected to *T. parva* infections develop humoral immune responses to a lot of parasite proteins, such as antibodies to polymorphic immunodominant molecules. This molecule has been outlined as the most reliable for measuring *T. parva* exposure in cattle (Toye *et al*., 1996; Morrison *et al.,*2015).

Cattle host non-specific immune responses, and the complement system is said to be the first line of defense against any invasion of disease-causing microorganisms. The complement

systems consist of a category of serum proteins that can be triggered by various progressions. When the complement system is initiated, molecules with several biological actions in inflammation and opsonization, as well as the cell death of invading disease-causing microorganisms, are generated. An adaptive immune reaction occurs when triggered antigen-presenting cells (APCs) move to the lymphoid tissues to present the antigen to T cells. The T cells are instrumental in the cellular immune response in the area of infection or aid in the triggering of B cells and the production of antigen-specific humoral immune reactions (Janeway *et al.,* 1999, 2001; Andrade *et al.,* 2005; Warrington *et al*., 2011).

Cattle immunized using the *T. parva* Marikebuni vaccine acquire cell-mediated immunity involving cytotoxic T cells and the immunity is lifelong, especially if there are more infections from ticks that boost the immunity (Di Giulio *et al*., 2009). Cell-mediated immunity is specifically developed for the *T. parva* parasite infection (Mckeever, 2001; Di Giulio *et al*., 2009). The infection also leads to the growth of humoral immune reactions in the immunized cattle. After immunization, a reduction in acaricide usage is advised as infected tick bites boost the immunity of the immunized cattle, as reported by Kivaria *et al*., (2007).

Research has shown that the host reacts to tick damage to the skin and tick presence by forming a hemostatic plug, activating coagulation cascades, vasoconstriction, and inflammatory responses, all of which are likely to disrupt the tick feeding. Supplement constituents, prostaglandins, leukotrienes, chemokines, and cytokines take part in inflammatory cell recruitment to the area of a bruise as the tick attacks (Janeway, 1999; 2001; Andrade *et al.,* 2005; Warrington *et al*., 2011). The skin is the primary organ in a tick-host interface that plays an important role in retaliation against tick invasion as well as pathogen transference by the vector. However, the ticks are normally successful in their feeding due to the availability of countless salivary compounds in their salivary glands, which reduces possible defensive mechanisms of the irritated host such as rubbing and scratching (Wikel, 1996; Kazimirova and

Stibraniova, 2003; Brossard and Wikel, 2008; Wikel, 2013). They have evolved various plans to modulate the innate immunity and cell-mediated immunity of the host, avoiding impaired feeding and subsequently, reproductive success (Brossard and Wikel, 2008). Due to to their defining characteristics the tick salivary compounds are concluded to act as immunomodulators of host immune reactions (Ribeiro *et al*., 1990, 2003**;** Nuttall, 2019). Tick-borne pathogens such as *T. parva* are reported to have evolved with their vectors and hosts and hence can survive in the two biological systems due to their adaptations. If the tick vector is not able to overcome the host barriers, its duration of attachment to the host is reduced. This also reduces pathogen transmission and the establishment of the parasite in the host (Labruna, 2011**;** Benelli, 2020).

Ticks require a blood meal for them to molt to the next growth stage. The blood meal is also utilized by the tick vector for the development of eggs and sperm during the adult feeding stage. In this study, the tick vector was applied to the cattle immunized with the *T. parva* Marikebuni vaccine and non-immunized controls, and its blood feeding success was assessed. This was followed by an assessment of oviposition success and the viability of egg oviposited.

CHAPTER 3

MATERIALS AND METHODS

3.1: The study site

This research was conducted at Kenya Agricultural and Livestock Research Organization - Veterinary Science Research Institute (KALRO-VSRI) laboratories located in Muguga, Kiambu County, Kenya. It is along the Nairobi-Naivasha highway, approximately 30 km north of Nairobi city. The site has an altitude of 1675M above sea level, at latitude 1^0 13'S, and a longitude 36^0 38’ E. Average annual rainfall is approximately 1200 mm with a mean humidity of 1716 mm. The climate in this region is quite favorable for the ticks to thrive.

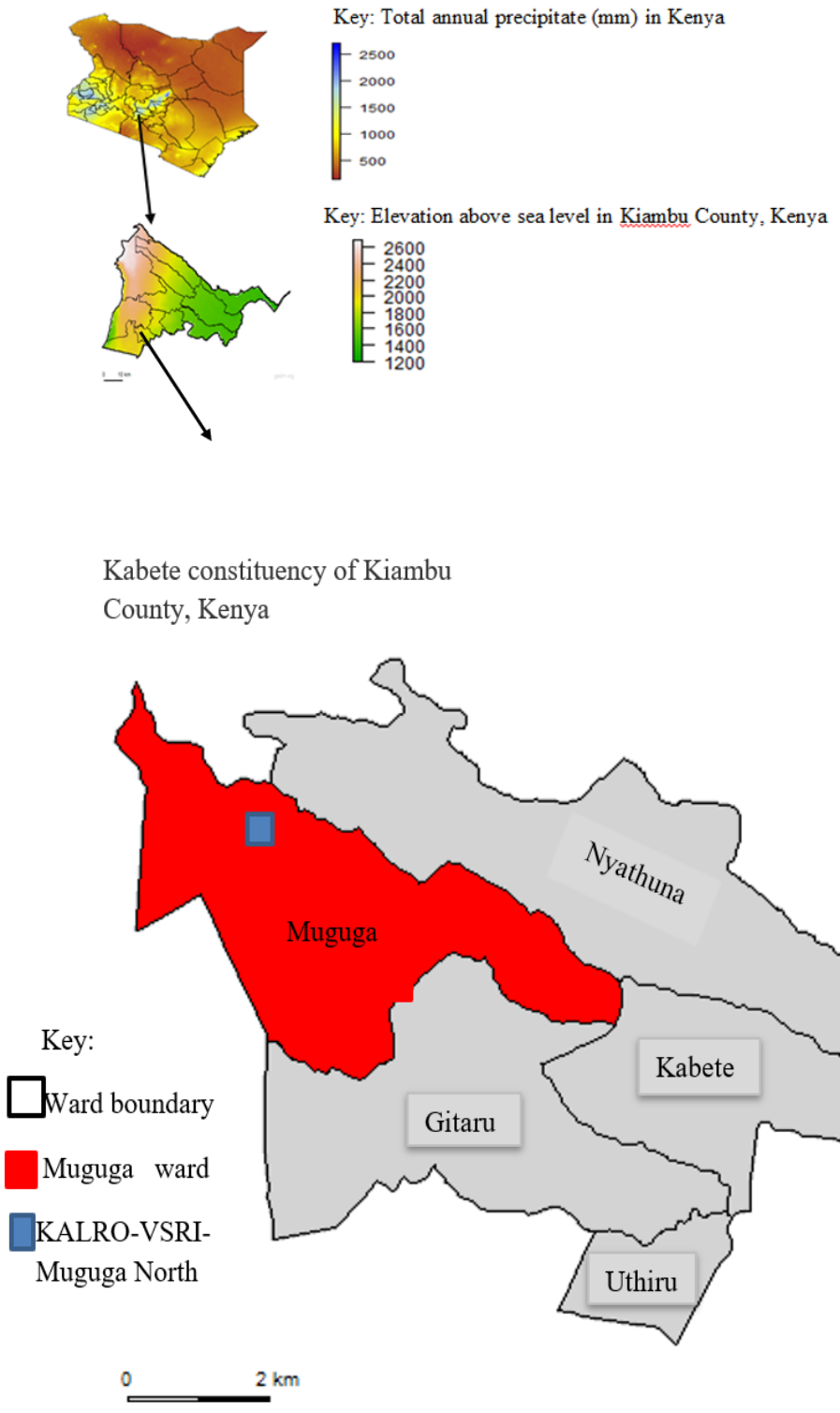


Figure 3.1: Location of the Study Site:-KALRO-VSRI-Muguga North.

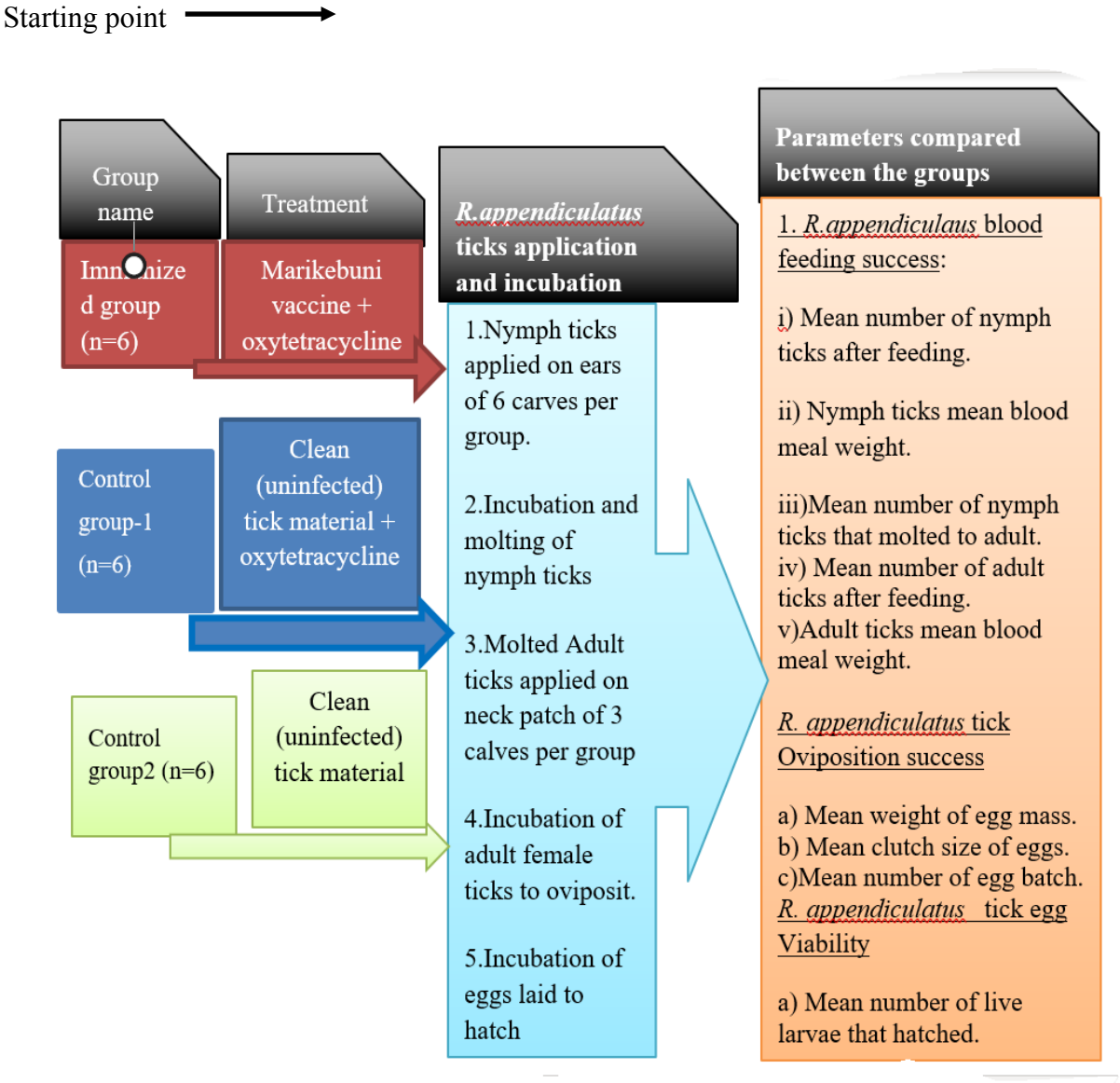
(Created by Author based on GADM version4.1)

3.2: Experimental study design

An after-only with control groups experimental research design consisting of three groups of ECF naïve calves aged between 3 and 12 months was used. The calves are recommended for use in veterinary vaccine efficacy challenges due to their susceptibility to ECF (World Organization for Animal Health (OIE), 2014). Each group had six calves, as shown in Table 3.0. Calves in the 1st group, referred to as the "immunized group", received 1 ml of Marikebuni vaccine and a long-acting oxytetracycline blocking agent at 30 ml/kg body weight. The 2nd group, "Control Group 1," received clean (uninfected) tick material and the blocking agent, while the 3rd group, "Control Group 2," received the clean (uninfected) tick material only.

Table 3.1: Treatment and control groups in the experimental study design used to test the effect of the T. parva Marikebuni vaccine on the feeding and reproductive success of Rhipicephalus appendiculatus

Group name.	Immunized group	Control group1	Control group2
Number of calves per group	6	6	6
Treatment	Marikebuni vaccine+ oxytetracycline	Uninfected tick material + oxytetracycline	Uninfected tick material only



Key: n=number of calves per group

Figure 3.2: Study design flow chart illustrating the variables assessed in the determination of the effect of the *Theileria parva* Marikebuni vaccine on feeding and reproductive success of *Rhipicephalus appendiculatus*.

3.2.1: The parasite stock used in the experiment

Theileria parva infectious tick material (Marikebuni vaccine) was obtained from KALRO-VSRI. The stabilate had been made ready for use, following the technique explained by Cunningham *et al.,* (1973). This technique utilizes Infectious particles of *Theileria parva* obtained from infected *Rhipicephalus appendiculatus*, either by using an in vitro feeding technique or by grinding the ticks in a suitable medium. The addition of fetal calf serum containing 15% glycerol (v/v) to the infective material is done, after which the infective material is distributed to glass capillary tubes. The tubes are then frozen to either -80 °C or -196 °C. (Cunningham *et al*., 1973; Patel *et al*., 2016). This technique was modified by Radley *et al.*, (1975) with the development and optimization of first short-acting and subsequently long-acting formulations of oxytetracycline to control the infections (Radley,1978). 30 mg/kg of long-acting oxytetracycline was reported to be the preferred dosage (Lynen *et al.*,1999; Di Giulio *et al.,* 2000).

Sporozoites stabilate stock of *Theileria parva* Marikebuni was used to immunize the experimental cattle (the immunized group). Irvin *et al.,* (1983) isolated this *Theileria parva* parasite stock from Kilifi County, Kenya, which was shown to provide good protection against severe challenge with other *Theileria* strains. A dose estimates of *T. parva* sporozoites using a 1:20 immunizing dilution of the original stabilate combined with treatment using long-acting oxytetracycline was used in the study.

3.2.2: Experimental Animals

New Zealand white rabbits were obtained from KALRO–VSRI for two purposes: propagating *R. appendiculatus* tick colonies from larvae to nymphs (section 3.3.1), and feeding the adult ticks during the preparation of uninfected tick material (section 3.2.4). The rabbits were properly housed in cages and given pellets, mineral supplements, and water throughout the study.

Thirty calves were purposely selected from KALRO herds. Before inclusion in the study, they were screened for the presence of *Theileria parva* parasites and any other tick-borne infections such as babesiosis and anaplasmosis (World Organization for Animal Health (OIE), 2014, 2015). Only healthy calves qualified to be enrolled in the study as per the inclusion criteria: naïve to ECF and aged between 3 and 12 months (Mbogo *et al*., 1995). Any calf exposed to acaricide treatment for 8 weeks before enrollment was excluded. Out of the thirty calves screened for parasites, twenty-four (24) calves were not infected with parasites. They were therefore included in the study. The calves comprised nine Boran and fifteen *Bos taurus* (6 Ayrshire and 9 Friesian breeds) of both sexes and between 3 and 12 months.

The calves were acclimatized, and their health was assessed. This allowed them to stabilize psychologically, nutritionally, and physiologically, thereby promoting reproducible experimental results (Obernier and Baldwin, 2006). All the calves were dewormed with Force Oneplus® (Highchem), a broad-spectrum anthelmintic containing 1.5% Levamisole hydrochloride and 3.0% Oxyclozanide with cobalt. Out of the twenty-four (24) calves enrolled in the study, eighteen (18) calves were randomly sampled and assigned to the three groups, as informed in Table 3.1 and Appendix iv. Each calf in each group occupied its own tick-free cubicle. They were allowed an additional 14 days to acclimatize to the cubicle environment. The remaining six calves were utilized in the determination of Marikebuni vaccine viability, as described in Section 3.2.3.

3.2.3: Marikebuni vaccine viability test

The Marikebuni vaccine viability test was conducted to check that the *Theileria parva* sporozoites in the Marikebuni vaccine were still alive and fit for use in the study. The procedure applied by Musisi *et al*., (1996) was used. Briefly, three samples of Marikebuni vaccine straws (M001, M002, and YP316B) were obtained from different liquid nitrogen canisters (CAN1, 2, and 8). They were thawed by rubbing the straws between the palms. One milliliter neat stabilate

was inoculated to two Boran calves per straw in each canister. The calves were monitored for development of clinical signs and blood screened for parasites up to day 28, to determine vaccine viability. The calves developed clinical ECF and were treated using Buparvaquone and long-acting oxytetracycline, thus concluding that the vaccine was viable and fit for use in this study. The results of the viability test are shown in appendices II and III. The vaccine kept in liquid nitrogen has a practically unlimited self-life (World Organization for Animal Health (OIE), 2014).

3.2.4: Preparation of uninfected tick material

Uninfected tick material was prepared using adult *Rhipicephalus appendiculatus* ticks fed on New Zealand white rabbits, as described by Mbao *et al.,* (2007) and OIE, (2014). Briefly, non-infected adult *R. appendiculatus* were fed on New Zealand white rabbits for 4 days. The non-infected ticks were harvested from the rabbit, counted and weighed. One hundred and twenty (120) ticks were used to make the tick material for 12 animals. The phosphate buffered saline/Minimum Essential Medium (PBS/MEM) grinding volume was approximately 10 ticks /ml. The ticks were cleaned with tap water, followed by 70% alcohol to kill bacteria on their surfaces. They were then rinsed with distilled water three times to remove the alcohol (Mbogo *et al.,* 1995). The ticks were then blot dried, put in a complex mixture of saline and culture medium 3.5% PBS/MEM in a beaker, and ground using a mortar and pestle. The supernatant was sieved in a beaker and spun at 750 revolutions per 5 minute (750RPM/ 5 min) at +4 °C. The supernatant's volume (total) was measured and 7.5% glycerol was added. It was then stirred for 45 minutes in an ice basin. Straws were filled using a machine (0.5ml volume), then transferred to an ultra-low freezer (-70 °C) overnight in cups. The straws were transferred to liquid nitrogen, ready for use.

3.2.5: Inoculation of calves using Marikebuni vaccine and uninfected tick material

Blood was drawn from the jugular vein of each calf in each group and taken to the VSRI laboratory to determine the initial immune status of the calves before vaccination. The *T. parva* antibody titre of all the calves in each group was < 40, hence naïve to ECF (Appendix IV). The vaccination of calves was done following the standard inoculation procedure as described by Mbogo *et al*., (1995). Briefly, the calves in each group were vaccinated as indicated in Table 3.0. Two vaccine straws were removed from the liquid nitrogen canister. Each straw consisted of 0.5 ml of *Theileria parva* parasite suspension. The straws were thawed by rubbing between the palms of warm hands, cut on one end with scissors, and the content emptied into a bottle of diluent containing 20 ml diluent. The bottle was gently shaken to allow the contents to mix. The diluted stabilate was then allowed to stand for 30 minutes to equilibrate in readiness for use. Daily rectal temperatures were recorded for each animal. The weight of each animal was determined using a weigh band. The long-acting oxytetracycline dose calculation involved 1 ml per 10 kg body weight Plus 10% more of long-acting oxytetracycline than the calculated dose due to risk of underestimation of animal body weight. The injection of oxytetracycline was done intramuscularly, followed by injection of vaccine subcutaneously close to the superficial lymph node. The injection of Marikebuni vaccine and uninfected tick material was done on the right prescapular lymph node for all animals in each group for purposes of monitoring the infection.

The vaccination was monitored using clinical observations, microscopy, and serology (World Organization for Animal Health (OIE), 2014; 2015). The clinical signs used to monitor the vaccination included: an enlarged lymph node, rise in temperature above 39.5^0C, anorexia, loss of condition, lacrimation, corneal opacity, coughing, terminal dyspnea, diarrhea, nasal discharge, and petechial hemorrhages on mucus membrane (OIE, 2015). Calves in the immunized group showed mild *T. parva* reactions with swelling of the lymph nodes on day 7-

14. The control group1 calves showed no apparent clinical reactions to inoculation with non-infected tick material, probably due to co-treatment with oxytetracycline. The calves in control group 2 were observed to be susceptible to other infections compared to the immunized and control group1 calves (Appendix V).

The microscopy technique detected the presence of *Theileria parva* parasites (Paparini *et al*., 2012; Mans *et al*., 2015). The animals in each group were restrained before the collection of blood and lymph node aspirate on day 7,14, 21, 28 and 35 post immunization. The blood was taken from the vein of the ear after pricking with a lancet. Thin blood smears were prepared and observed using a microscope to identify the presence of *T. parva* parasites and other parasites such as *Anaplasma* and *Babesia.* Identification of white blood cells present in blood smear was also done. A lymph node smear was made by puncturing the right prescapular lymph node with a needle and exuding the aspirate on a slide to make a thin smear and examined for the presence of *T. parva* parasite. The slides were air dried, fixed with methanol and stained with giemsa stain before examination (OIE, 2014; 2015). Calves in the immunized group were observed to have macro-schizonts in their lymph node smears. The white blood cell count was observed to increase in immunized group calves. One calf in control group 3 showed an increase in white blood cell count on day 14 post inoculation, an indication of reaction to the non-infected tick material inoculated or infection (Appendix V).

The IFAT serological test assessed the exposure of the calves to the parasite by detecting antibodies against *T. parva* after vaccination (OIE, 2014, 2015; Mans *et al*., 2015). Briefly, the blood was collected from the animals on day 7, 14, 21, 28 and 35 using vacutainer tubes. The blood was left on the bench overnight to allow serum separation. The serum was then separated, put in micro vials, labeled and kept in an ultra-low freezer, ready for the test. The frozen serum was thawed and diluted using phosphate buffered saline (PBS) at dilutions of 1:40 and 1:160. The diluted serum was then transferred onto slides and incubated at 37 °C for

30 minutes. The 30-minute PBS immersion three changes helped wash the slides. 20 microliters of conjugate were added to the slides and incubated at 37 ℃ for 30 minutes. The slides were then washed three times by immersing them in PBS for 10 minutes. The slides were mounted under a cover slip with 50% glycerol and examined under a microscope. This test helped in detecting changes in the immune status of the animal after vaccination. Calves in the immunized group developed *T. parva* schizont antibodies by day 28 post-immunization, while those in the control groups did not show any exposure to *T. parva* parasites (Appendices IV and V).

3.3 Feeding success of *Rhipicephalus appendiculatus* on cattle immunized with the Marikebuni vaccine

The tick rearing unit was surrounded by a water moat to ensure no ticks entered or escaped to the surrounding (Troughton and Levin, 2007; Nuss *et al*., 2017). *Rhipicephalus appendiculatus* larvae were applied on the ears of New Zealand white rabbits. The nymph *R. appendiculatus* were applied on the ears of immunized and control calves, while the adult tick stages were applied on the neck region of the immunized and control calves.

3.3.1: Propagation and incubation of *Rhipicephalus appendiculatus* larvae

Clean *Rhipicephalus appendiculatus* ticks (Muguga tick colony) provided by KALRO-VSRI were used in this study. Approximately, 16000 larvae of *R. appendiculatus* were applied on ears of the New Zealand white rabbit after shaving (Troughton and Levin, 2007). The engorged larvae were incubated to molt to nymphs. Ten (10 ml) glass tubes plugged with cotton wool and kept in desiccators over saturated potassium chloride solution maintained each batch of fed larvae ticks at a relative humidity of 85%. The desiccators were incubated at 28 ± ºc and in complete darkness (Levin and Schumacher,2016).

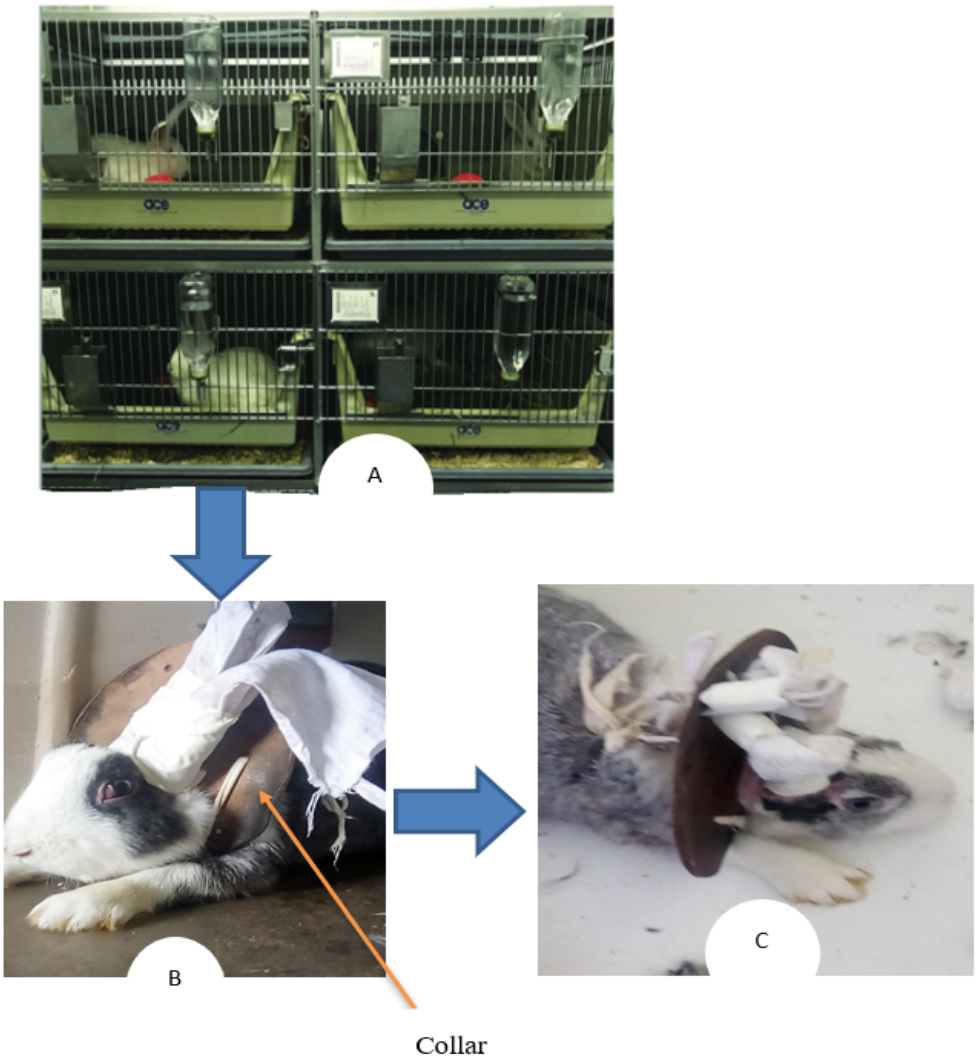


Figure 3.3: Application of *Rhipicephalus appendiculatus* on the rabbit ears.

Key: [A]. caged rabbits, [B]. rabbit with ear bag and collar ready for larvae application, [C]. rabbit with ear bag fastened on the collar after larvae application.

3.3.2: Application of nymph and adult *Rhipicephalus appendiculatus* ticks on immunized and control group 1 and 2 calves

The number of calves used to apply nymph ticks was six per group and of both sexes, except in control group2 where one of the calves died. The number of calves used to apply adult ticks were three per group and of female sex only. This application was done as indicated in table 3.2.

Table 3.2: Number of nymphs and adult ticks fed on immunized and non-immunized calves in the experiment to determine feeding success of *Rhipicephalus appendiculatus*

Group name	Immunized group	Control group1	Control group2
Treatment	Marikebuni vaccine+ oxytetracycline	Uninfected tick material + oxytetracycline	Uninfected tick material only
Application of nymph *R. appendiculatus*			
Number of calves	6	6	5
Nymph ticks applied per calf	12000	12000	12000
Application of adult *R. apendiculatus*			
Number of calves	3	3	3
Adult ticks applied per calf	60males:60females	60 males:60 females	60males:60females

Uninfected *Rhipicephalus appendiculatus* nymphs were counted and weighed using an analytical electronic balance accurate to 0.0001g and a four-digit tick counting device. After manual restraint of the immunized group and control group 1 and 2 calves, they were washed and shaved on the ears using an electric shaver (Figure 3.4a). Muslin bags were put on the clean

shaved regions using adhesive (Conta), which was allowed to harden and its odor disappear. The application of ticks was done by applying approximately 600 nymphs per ear of each calf to make a total of 1200 nymph ticks per calf that weighed approximately 0.1 mg. The ear bag was tied on the other end to restrict the ticks within the shaved region (Figure 3.1b). The nymph ticks that fed and dropped were harvested 7–8 days after application. The number and weight of nymph ticks that fed and dropped to molt were determined, as well as their blood meal. They were then incubated to molt into adult as described in section 3.3. 1. *Rhipicephalus appendiculatus* nymphs that successfully molted to adult in immunized, control1 and control2 groups were counted, weighed and applied to calves following the procedure described by Levin and Schumacher, (2016). Briefly, the adults *R. appendiculatus* were applied to three groups of female cattle consisting of 3 calves each as indicated in table 3.2. The calves in each group were washed and shaved on the neck region using an electric shaver (Figure 3.4: d). The muslin bags were glued on the neck patch using an adhesive (Conta) and allowed to dry as the strong odor disappeared (Figure 3.4e). A total of 120 adult ticks originating from immunized calves were applied to each immunized calf on the neck patch. Each calf received 60 males and 60 females. The muslin bag was tied on the other end to restrict the ticks on the shaved neck region (Figure 3.4e). This procedure was repeated for the control group 1 and 2 calves using the adult ticks originating from the control group 1 and 2 calves respectively. Full details of the application are provided in Appendices VI and VII. After feeding on immunized and non-immunized control calves, the engorged adult female ticks were harvested, enumerated, weighed, and their blood meal determined.

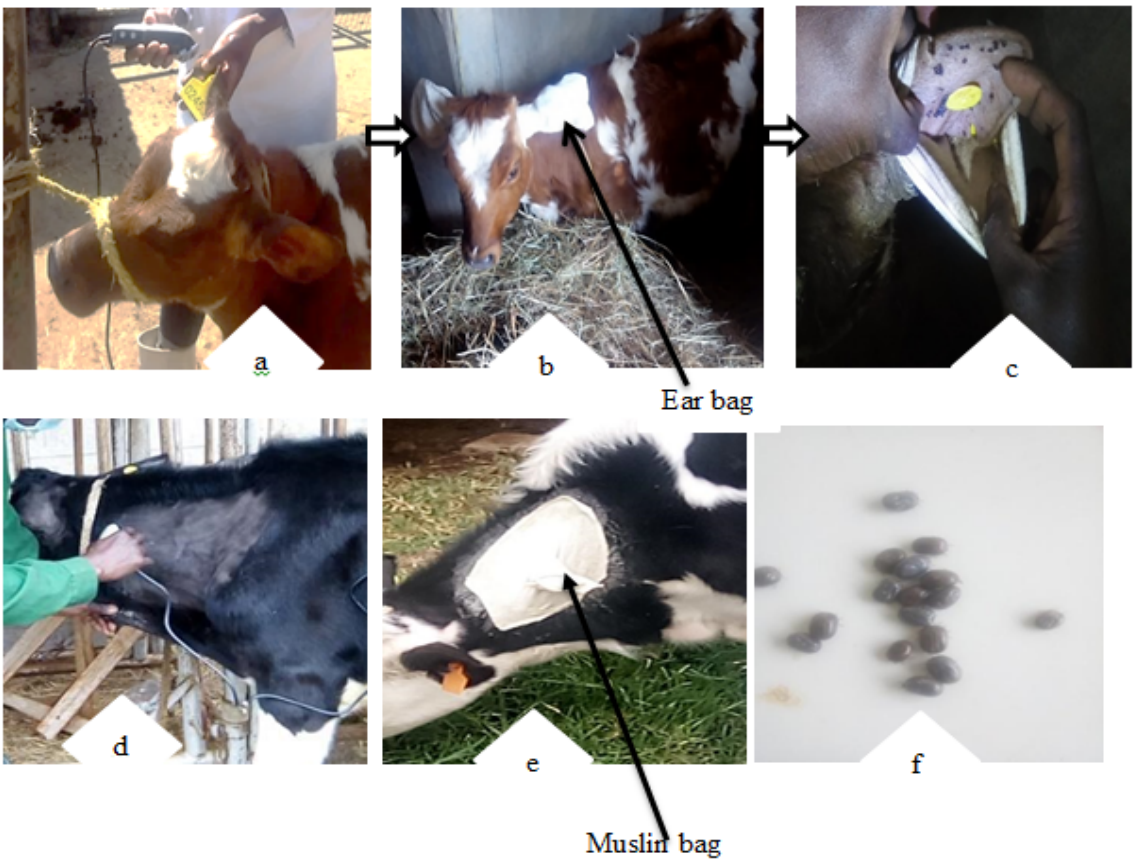


Figure 3.4: Application of *Rhipicephalus appendiculatus* nymphs on the ears and adults on the neck region of calves in an experiment to assess the feeding success of the tick on cattle immunized with Marikebuni vaccine.

Key: [a.] shaving a calf on ears, [b.] calf with ear bags, [c.] engorged nymph ticks on ears, [d.] shaving a calf on the neck region, [e.] muslin bag glued on the shaved region, [f.] replete female ticks after feeding.

3.4: Oviposition success of *Rhipicephalus appendiculatus* fed on cattle immunized with Marikebuni vaccine

To achieve this objective, adult *R. appendiculatus* female ticks that fed on immunized and control group 1 and 2 calves were harvested, counted and weighed (section 3.3.2). They were then placed in Petri dishes to lay eggs. The 90 mm diameter Petri dishes were labeled, sealed

with tape, and stored at an optimum temperature of 27°c-28°c and relative humidity of 80% - 85% (Pinter *et al.*, 2002; Levin and Schumacher, 2016). Each tick was put in its own Petri dish (Figure 3.5b). An electronic balance was used to weigh the eggs after incubating the female ticks for 21–28 days. The number of egg batches was counted and recorded. Observation under a stereoscope and counting using a handheld 4 -digit counting device determined the clutch sizes of the eggs (Dipeolu, 1991; Levin and Schumacher, 2016).

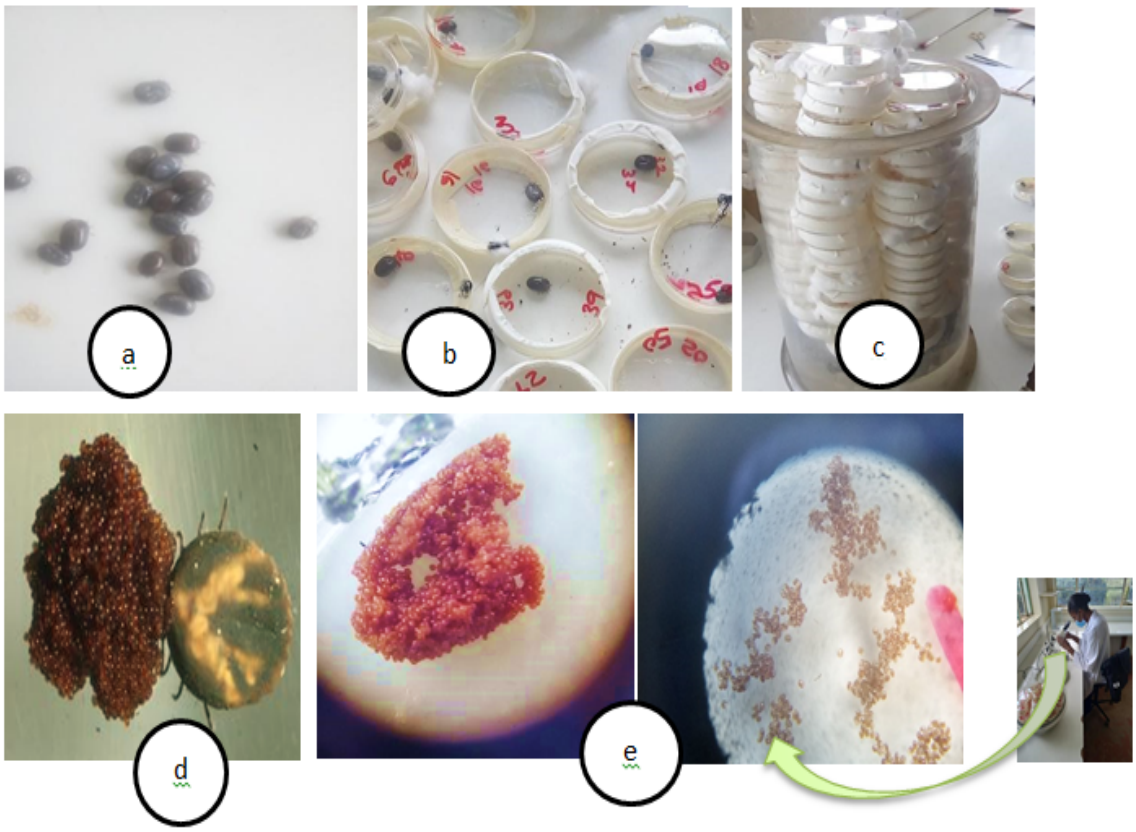


Figure 3.5: Illustration of adult female *Rhipicephalus appendiculatus* incubation in an experiment to determine the oviposition success of *Rhipicephalus appendiculatus* fed on cattle immunized with Marikebuni vaccine.

Key: [a.] engorged female tick, [b.] each tick was placed in a petri dish and sealed with tape, [c] petri dishes were placed in bowls ready for incubation, [d.] female *R. appendiculatus* tick laying eggs after incubation, [e.] eggs seen under a dissecting microscope.

3.5: Determination of the viability of eggs oviposited by *Rhipicephalus appendiculatus* ticks fed on cattle immunized with *Theileria parva* Marikebuni vaccine

Incubation of eggs to hatch to larva involved putting the eggs obtained in section 3.4 above into petri dishes sealed with tape at an optimum temperature of 27-28ºC and 80 -85% relative humidity to hatch. Live larvae that hatched were counted. The criteria for determining whether the larvae were alive or not involved the inability of the larvae to walk, and larvae that were able to walk were considered live. Breathing gently on the larvae stimulated them to move. A dissecting microscope was used to identify the larvae that could not walk. The live larvae were removed from the Petri dish with a paint brush and immobilized using cotton wool moistened with water. The live larvae trapped in the cotton wool were counted using the handheld 4-digit counting device. Counting was not done where all larvae were evidently alive. In this case, the number of eggs recorded in the petri dish served as the number of live larvae.

3.6: Data entry, management, and analysis

Data were entered and managed in Excel program. The analysis of the data utilized R statistical software version 4.1.3 (2022-03-10). The normality of data was assessed by carrying out the Shapiro-Wilk Normality Test (Patrick Royston, 1995; Mishra *et al*.,2019). Data were analyzed using ANOVA test, linear regression and binary logistic regression models (Chambers *et al.,* 1992; Andrew Gelman and Jennifer Hill, 2005; Bevans, 2022). AIC- (Akalke's information criterion) was used to compare models of different sizes, where the model with the lowest AIC was taken to better fit the data. Residual deviance compared models of the same sizes. Models with low residual deviance fitted the data. Variance inflation factor (Vif) assessed multicollinearity in the models. Models with Vif less than 5 were considered. The best fit model was determined using the goodness of fit test such as Hosmer and Lemeshow, Cox and Snell, Negelkerke, and chi-square. The baseline category in the model was non-immunized cattle host.

3.6.1: Determination of the blood feeding success of *Rhipicephalus appendiculatus* fed on cattle immunized with the Marikebuni vaccine

The weight of the blood meal was determined by calculating the difference between the weight of the nymph and adult ticks before and after feeding. A scatter plot with a regression line was drawn using R statistical software to identify any relationship between the tick weight before feeding and the weight of the blood meal (Murrell, 2005; Fox and Weisberg, 2019). Data were fitted in a logistic regression model to predict the effect of the vaccine on the blood feeding success of *R. appendiculatus*. The predictor variables included: the number of nymph ticks after feeding, nymph blood meal weight, and the number of nymph ticks that molted to adults. The number of replete female ticks and their blood meal weight also predicted the effect of the Marikebuni vaccine on the blood-feeding success of the adult tick vector. An ANOVA test was also performed to compare the mean blood meal weight of adult female ticks between the groups.

3.6.2: Determination of oviposition success of *Rhipicephalus appendiculatus* fed on cattle immunized with Marikebuni vaccine

Parameters compared between the immunized and non-immunized cattle to determine the oviposition success of *R. appendiculatus* included the mean weight of egg mass, clutch sizes of eggs and number of egg batches. The mean weight of egg mass was compared using an ANOVA test. A binomial logistic regression model fitted the data to predict the effect of the vaccine on the number of egg batches and clutch sizes of the eggs. A linear regression analysis to determine the relationship between the egg mass weight and the female tick's weight after blood meal was done.

3.6.3: Determination of viability of eggs of *Rhipicephalus appendiculatus* fed on cattle immunized with Marikebuni vaccine

The viability of *R. appendiculatus* eggs was determined by fitting a binomial logistic regression model to predict the effect of the vaccine on the number of live larvae that hatched successfully.

3.7: Ethical clearance

The Institutional Animal Care and Use Committee of KALRO-VSRI approved this work upon compliance with all resources evaluated and coded: KALRO-VSRI/IACUC 023/04062021

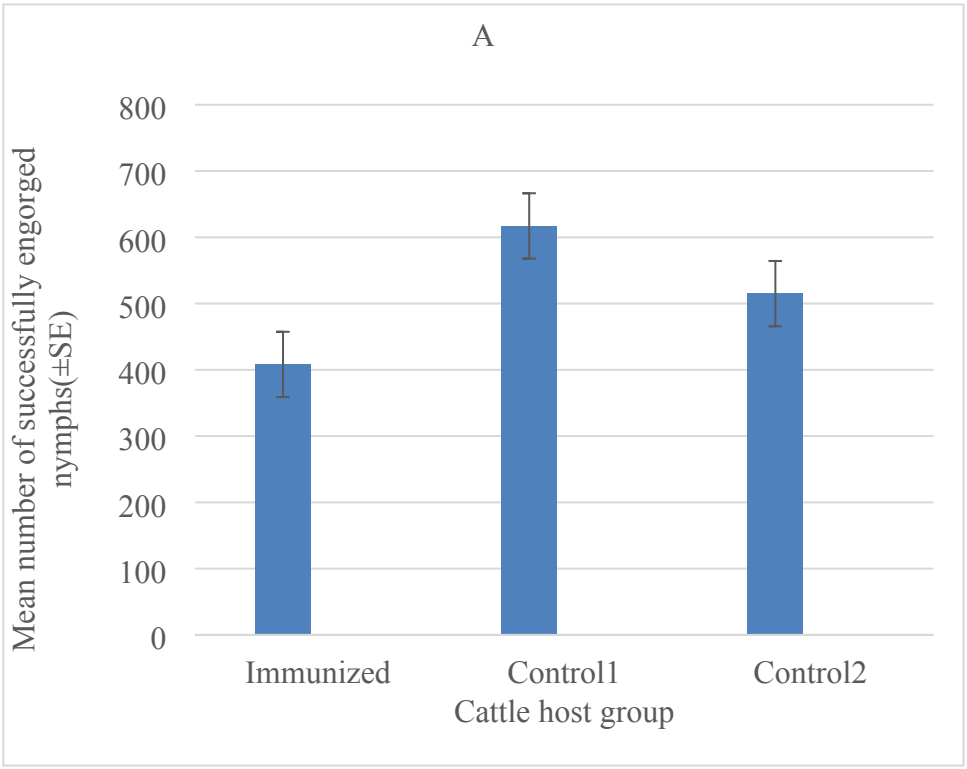
CHAPTER 4

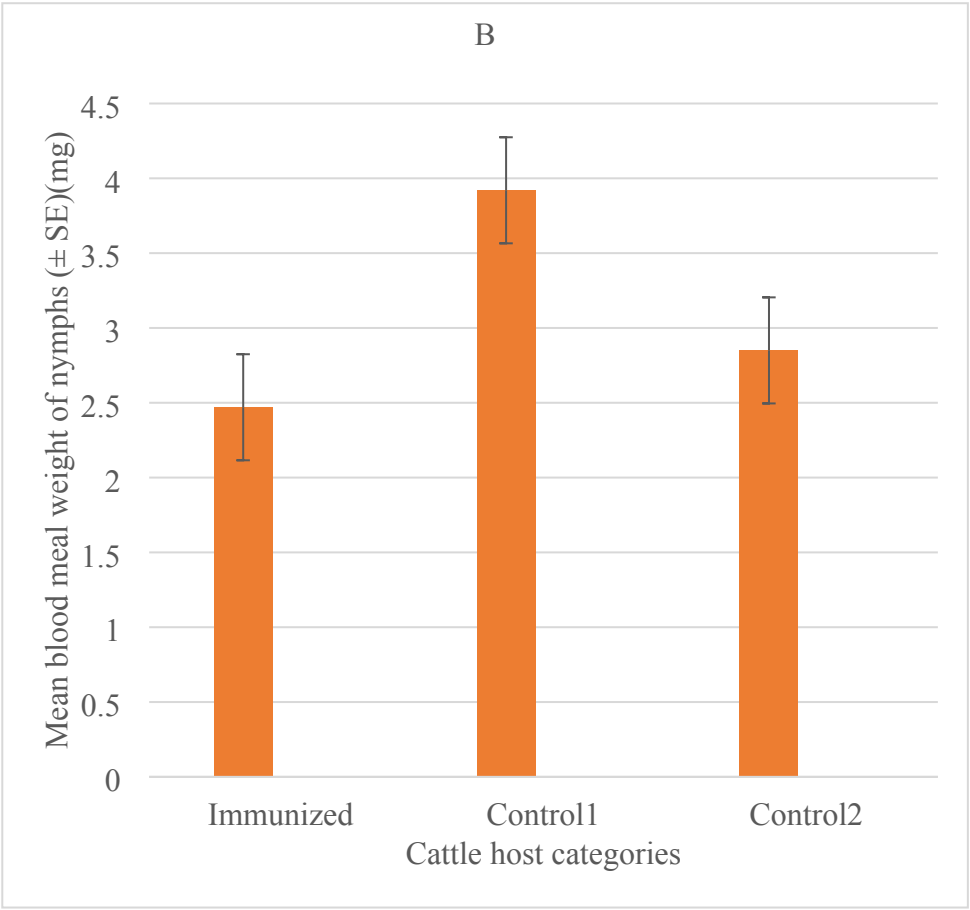
RESULTS

4.1: Determination of blood-feeding success of *Rhipicephalus appendiculatus* on cattle immunized with Marikebuni vaccine

4.1.1: *Rhipicephalus appendiculatus* nymph blood-feeding success

There was an observed difference in feeding success of *R. appendiculatus* nymphs between ticks that fed on immunized calves and those that fed on controls in all the parameters compared. The observed difference was, however, not statistically significant ($p > 0.05$) (Table 4.1). The mean number of nymphs that fed successfully was highest in nymphs that fed on control group 1 (617.17 ± 159.39), followed by those that fed on control group 2 (515 ± 239.70) and finally those that fed on immunized calves (408.17 ± 356.32) (Figure 4.1 A). The observed difference in mean number of fed nymphs was not significantly different between the ticks that fed on immunized calves and those that fed on controls (OR = 0.996; p-value = 0.29) (Table 4.1). The nymphs that fed on control group1 calves recorded the highest mean blood meal weight (3.92 mg ± 1.07), followed by those that fed on control group 2 calves (2.85 mg ± 1.44), and finally those that fed on immunized group (2.47 mg ± 2.44)(Figure 4.1B). The observed difference in mean blood meal weight of the nymphs was, however, not statistically significant (OR = 0.712; p-value = 0.34) (Table 4.1). The mean number of nymphs that molted successfully to adult was (82.3 ± 81.5), in those that fed on immunized calves. Those that fed on Control group1 and 2 calves had a mean molt of (114.8 ± 122.3), and (85.6 ± 141.3) respectively (Figure 4.1 C). This observed variation was, however, not statistically significant (OR = 1.004; p value = 0.36) (Table 4.1).


A
800
700
600
500
400
300
200
100
0
Mean number of successfully engorged nymphs(±SE)
Immunized
Control1
Control2
Cattle host group


B
4.5
4
3.5
3
2.5
2
1.5
1
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0
Mean blood meal weight of nymphs (± SE)(mg)
Immunized
Control1
Control2
Cattle host categories

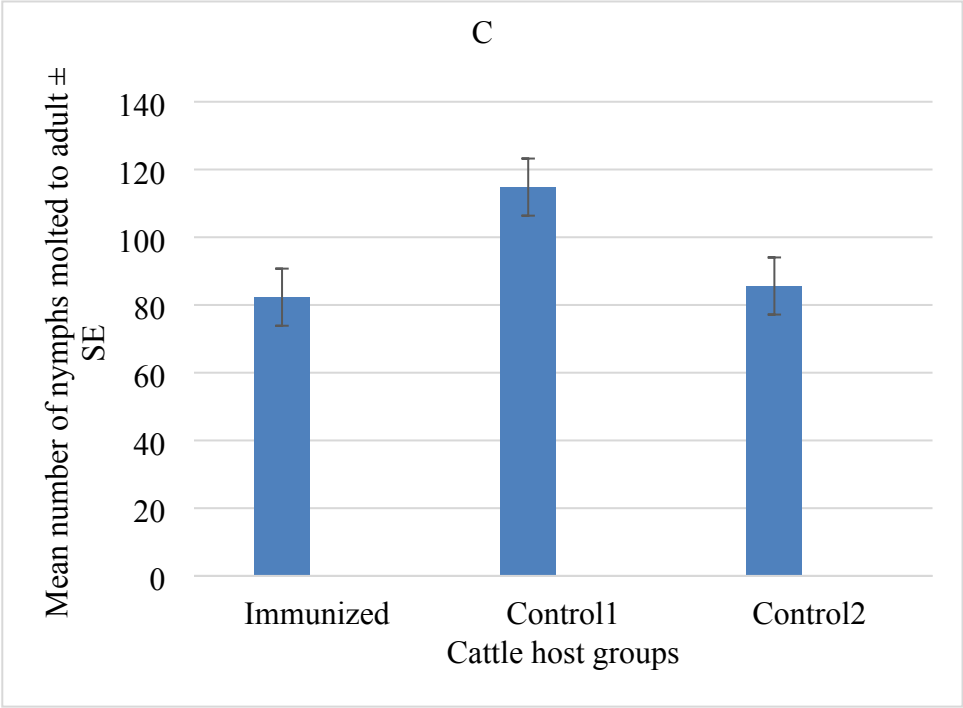


Figure 4.1: Feeding success of *Rhipicephalus appendiculatus* nymphs fed on calves immunized with *Theileria parva* Marikebuni vaccine.

Key: [A]. Mean number of successfully engorged nymphs fed on immunized, control 1, and control 2 calves; [B]. Mean blood meal weight of nymphs fed on immunized, control 1, and control 2 calves. [C]. Mean number of successfully molted nymphs in immunized, control 1, and control 2 calves.

The number of nymph ticks that successfully fed on immunized calves was 99.6% as many as those that fed non-immunized calves, and was not significantly different (OR = 0.996; p-value = 0.29). A positive constant (intercept) indicates that the probability of the ticks that are fed on immunized calves being affected by the vaccine is > 0.5 (Table 4.1). Although the nymphs that fed on immunized cattle imbibed 71.2% as much blood as those that fed on the non-immunized calves, the difference was not significant (OR = 0.712; p-value = 0.34). The number of nymph ticks that molted to adults had a positive coefficient (Table 4.1). This means that the molting of the nymphs that fed on immunized calves was more likely to be altered by the vaccine. The

number of nymphs that fed on immunized calves and successfully molted into adults was 0.04% as many as those that fed on non-immunized calves, though the difference was not significant (OR = 1.004; p-value = 0.36).

Table 4.1: Logistic regression analysis of the blood-feeding success of *Rhipicephalus appendiculatus* nymphs fed on cattle immunized with *Theileria parva* Marikebuni vaccine

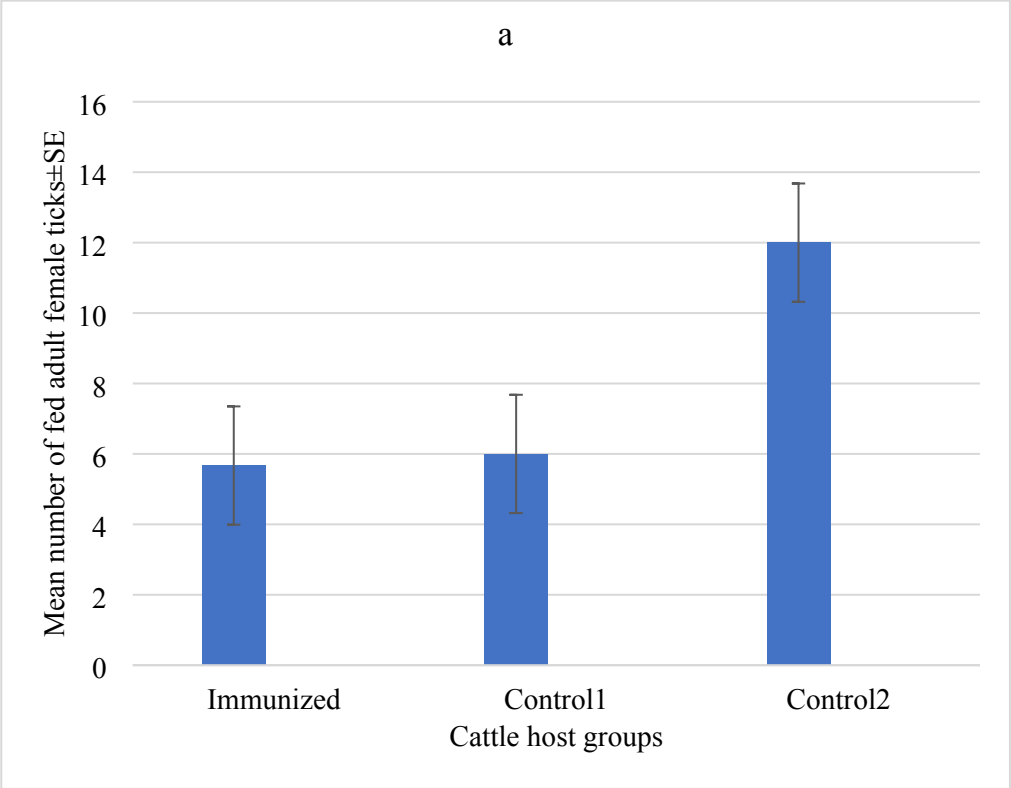
		97.5%Cl for Odds Ratios				
	COEF (SE)	Lower 2.5%	Odds Ratio	Upper 97.5%	z-values	p-value
Constant (intercept)	1.56(1.47)				1.1	0.29
#of engorged nymphs	-0.004(0.003)	0.99	0.996	1.00	-1.27	0.20
# of Molten nymphs	0.004(0.004)	0.99	1.004	1.01	0.91	0.36
Bloodmeal weight(g)	-0.34(0.35)	0.31	0.712	1.41	-0.95	0.34

Key: COEF-coefficient, SE-standard error, Cl-Confidence level, R^2=0.14 (Hosmer&Lemeshow), 0.17(Cox&Snell), 0.24(Negelkerke), Model χ^2 (3) = 3.18, p-value = 0.36, p > 0.05.

4.1.2: Blood-feeding success of *Rhipicephalus appendiculatus* adult females fed on cattle immunized with the *Theileria parva* Marikebuni vaccine

In this study, there was an observed difference in feeding success of the adult female ticks that fed on immunized calves compared to the control group 2 calves, though the difference was not statistically significant (p > 0.05) (Tables 4.2 and 4.3). The mean number of replete adult female ticks was highest in ticks that fed on control group 2 (12 ± 2.65), followed by those that fed on control group 1 (6 ± 3.61), and finally those that fed on immunized calves (5.67

$\pm$ 3.61) (Figure 4.2a), and was not significantly different (OR = 1.05; p-value = 0.93). Adult female ticks that fed on control group 2 calves recorded the highest mean blood meal weight (3.89 $\pm$ 1.67), followed by those that fed on control group 1 calves (1.73 $\pm$ 1.49). This was lowest in those ticks that fed on the immunized group (1.3321.76 $\pm$ 0.51) (Figure 4.2b). This observed variation was, however, not statistically significant (OR = 0.32, p-value = 0.48; F = 3.26, p-value = 0.11) (Tables 4.2 and 4.3).



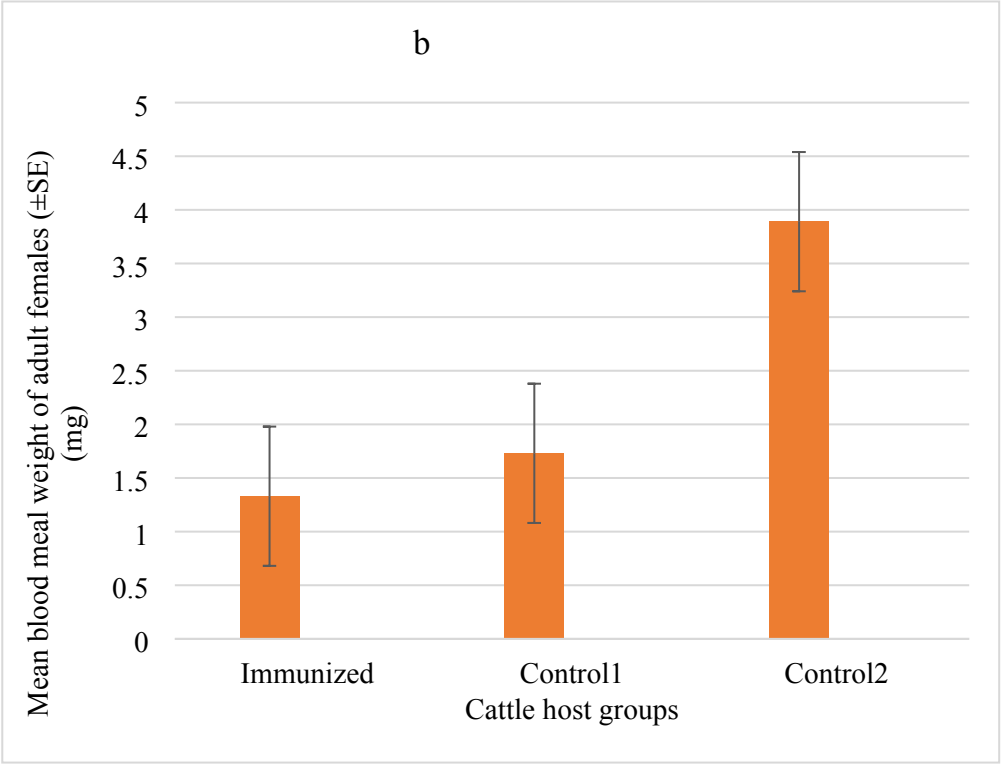


Figure 4.2: Feeding success of *Rhipicephalus appendiculatus* adult female fed on calves immunized with *Theileria parva* Marikebuni vaccine.

Key: [a]. Mean number of successfully fed adult females in immunized, control 1, and control 2 calves; [b]. Mean blood meal weight of adult females in immunized, control 1, and control 2 calves.

The number of replete adult female ticks that fed successfully had a positive coefficient, which means that the number of adult female ticks that fed on an immunized host were more likely to be impacted by the Marikebuni vaccine (Table 4.2). The blood meal weight had a negative coefficient, indicating that the blood meal weight of the adult female ticks that fed on immunized calves was less likely to be altered by the vaccine. The number of adult *R. appendiculatus* that successfully fed on immunized calves was 5% as many as those that fed on non-immunized calves, though the difference was not statistically significant (OR =1.05; p

-value = 0.93) (Table 4.2). Although the ticks that fed on immunized calves imbibed 32% as much blood as those that fed on non-immunized calves, the variation was not statistically significant (OR = 0.32; p = 0.48) (Table 4.2). Unless otherwise stated, blood meal weight (z-value = -0.71) was a stronger predictor of vaccine effect on tick blood feeding success compared to the number of replete female ticks (z-value = 0.09), as indicated by a large z-value (Table 4.2).

Table 4.2: Logistic regression analysis of the blood-feeding success of *Rhipicephalus appendiculatus* adult females fed on cattle immunized with *Theileria parva* Marikebuni vaccine

		97.5%Cl for Odds Ratios				
	COEF(SE)	Lower 2.5%	Odds Ratio	Upper 97.5%	z-value	p-value
Constant (intercept)	1.16(1.9)				0.61	0.54
# Of replete female ticks	0.05(0.52)	0.31	1.05	2.76	0.09	0.93
Blood meal weight	-1.14(1.6)	0.01	0.32	8.20	-0.71	0.48

Key: COEF-coefficient, SE-standard error, Cl-confidence level.

Note.R^2 = 0.21 (Hosmer& Lemeshow), 0.23 (Cox&Snell), 0.32 (Negelkerke), Model χ^2 = (2) = 2.38, p-value = 0.3, p > 0.01.

The blood meal weight of *R. appendiculatus* adult females was not significantly different between the female ticks that fed on immunized calves and those that fed on control calves, despite the observed variation. The p-value (0.11) of F-statistics (3.26), was more than 0.05 significance level (p < 0.05) (Table 4.3).

Table 4.3: ANOVA test on blood-feeding success of *Rhipicephalus appendiculatus* adult females fed on cattle immunized with *Theileria parva* Marikebuni vaccine

		DF	Sum of squares	Mean sum of squares	F- value	Pr(>F)
Weight of blood meal (g)	Ind	2	11.42	5.709	3.26	0.11
	Residuals	6	10.51	1.751		

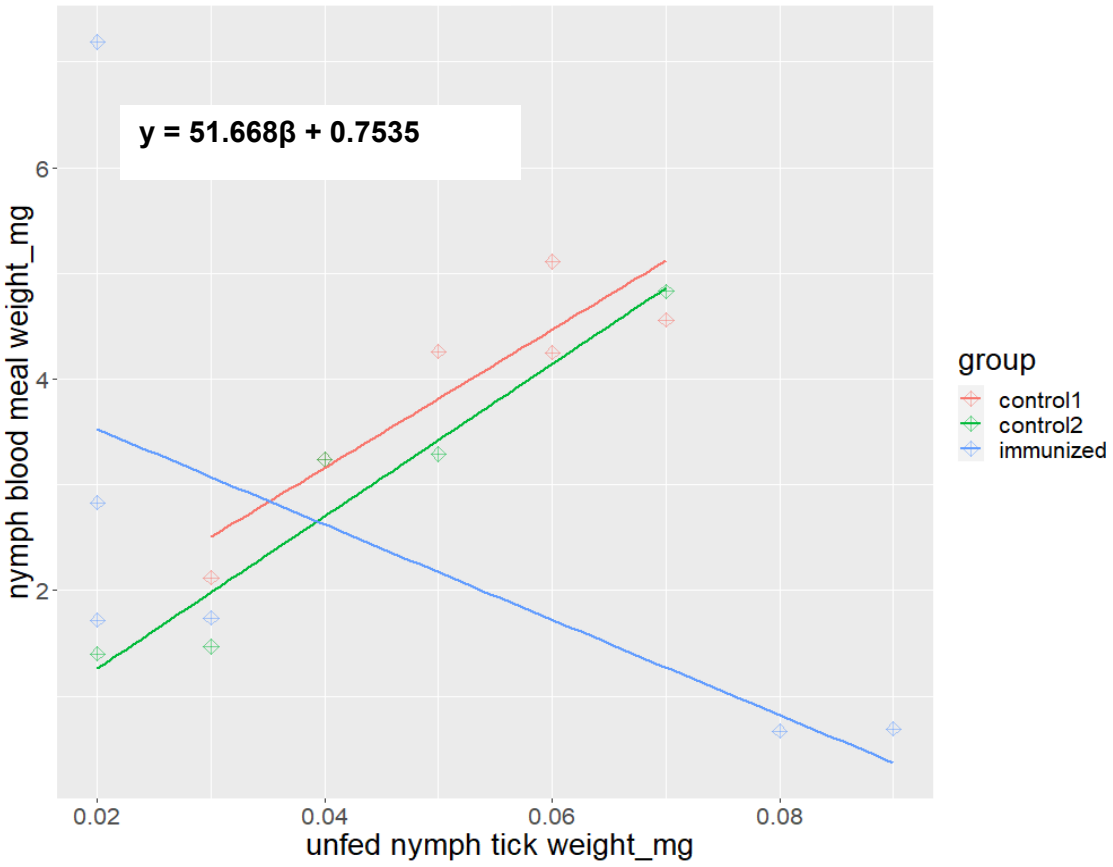
Key: Ind = independent variable, Residuals = all variation explained by the independent variable, sum of squares = variation between the group means and overall mean, F-value = test statistic from F test, Pr (>F) = p- value of F –statistic.

4.1.3: Relationship between *Rhipicephalus appendiculatus* weight before feeding on immunized and control calves and the weight of the blood meal (mg) after feeding

Simple linear regression was used to test if nymph and adult *R. appendiculatus* weight before feeding significantly predicted their blood meal weight. The fitted regression model for the nymphs was: blood meal weight =51.668+0.7535 * (unfed nymph tick weight). The overall regression was statistically significant ($R^2 = 0.425$; $F(1, 15) = 12.83$; $p < 0.05 = 0.00278$). It was observed that the unfed nymph weight (mg) significantly predicted the blood meal weight of the nymphs ($\beta = 0.7535$; $p < 0.05$). There was a significant positive correlation between the weight of *R. appendiculatus* nymphs before feeding and the blood meal weight after feeding on control group 1 and 2 calves. The blood meal weight was observed to increase as the size of the nymphs increased in control group 1 and 2 calves (Figure4.3A). However, a negative correlation was recorded by the nymphs that fed on immunized calves, with the blood meal weight decreasing as the size of the nymphs increased in the immunized group (Figure 4.3A).

The unfed adult female tick weight (mg) also significantly predicted the blood meal weight of the adult female *R. appendiculatus* ($\beta = 0.4918$; $p < 0.05$). The fitted regression model for the adult female *R. appendiculatus* was: adult blood meal weight (mg) = 91.3+0.4918 * (unfed adult female tick weight (mg)). The overall regression was statistically significant ($R^2 = 0.7261$; $F(1, 7) = 22.21$; $p = 0.$ 0.002176). A positive correlation between the weight of female ticks before feeding on control calves and the blood meal weight was observed (Figure 4.3B). The amount of blood meal weight increased as the size of the adult female ticks increased. The correlation between the blood meal weight and the weight of the female ticks fed on immunized calves was observed to be negative (Figure 4.3B).

A



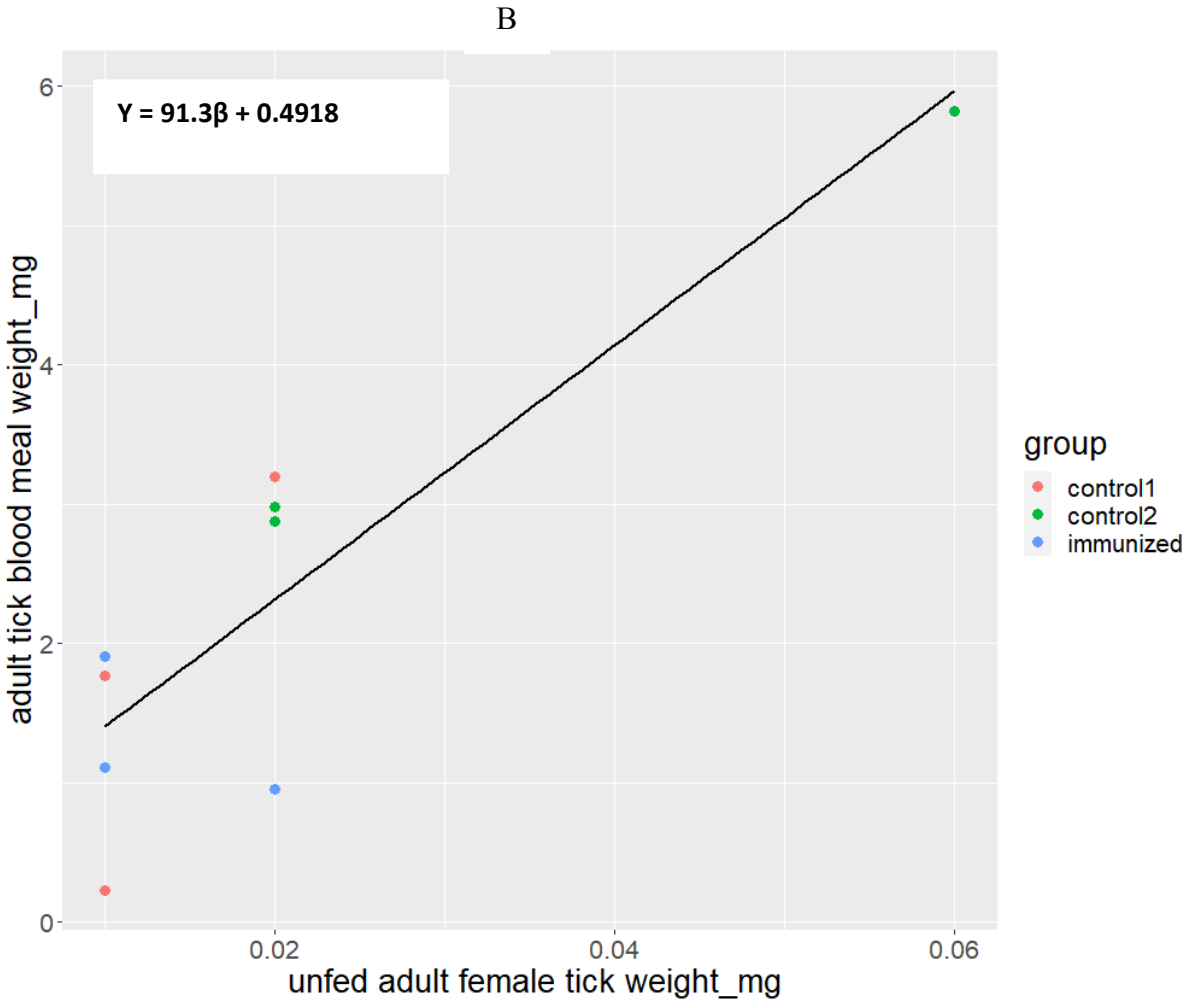


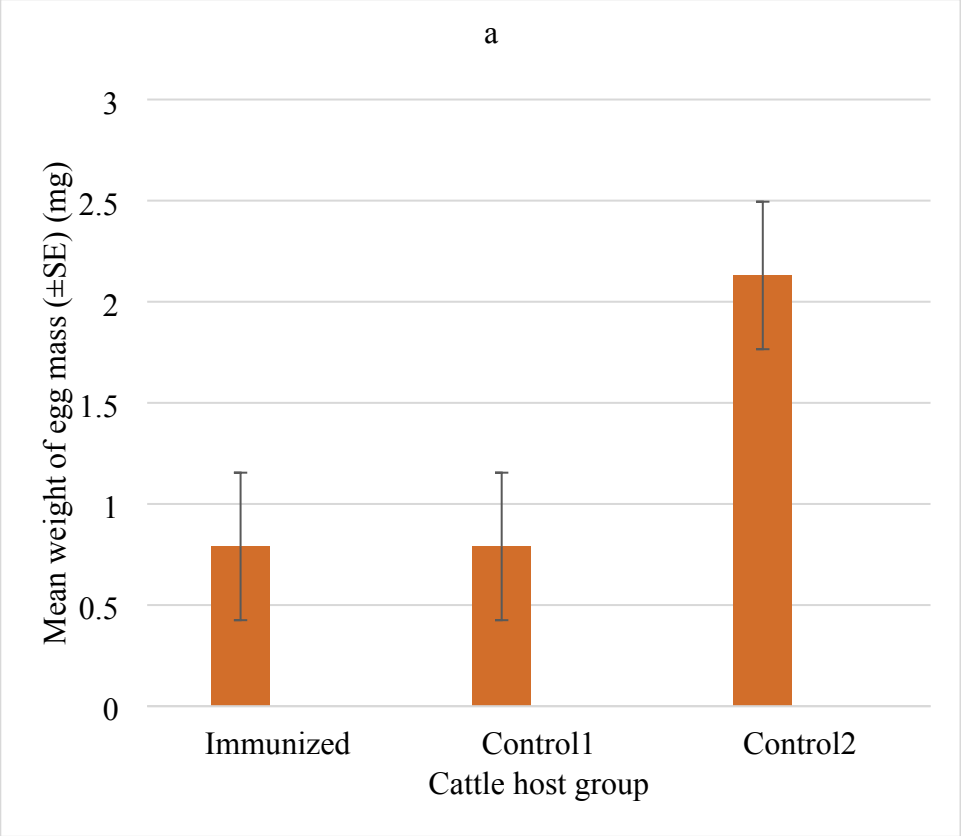
Figure 4.3: Relationship between *Rhipicephalus appendiculatus* weight before feeding on *T. parva* Marikebuni immunized calves and the controls and the weight of the blood meal (mg) after feeding

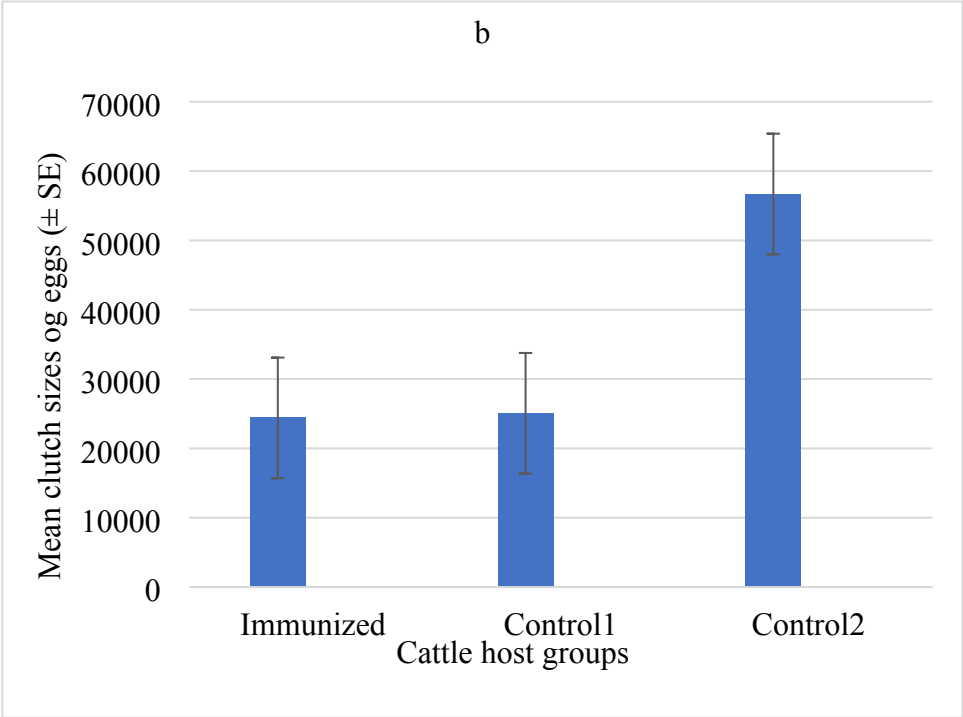
Key: [A.] Scatter plot of nymph means blood meal weight (milligrams) against nymph weight before feeding on calves immunized with Marikebuni vaccine, control1 and control2 calves; [B.] Regression analysis of adult female tick weight before feeding on cattle immunized with Marikebuni vaccine and control group 1and2 against the blood meal weight (mg).

4.2: Determination of Oviposition success of *Rhipicephalus appendiculatus* fed on cattle immunized with *Theileria parva* Marikebuni vaccine

The mean weight of egg mass laid by female ticks after feeding on control group2 calves was the highest (2.13±0.73) compared to that of ticks that fed on immunized calves (0.79±0.02) and

control group 1 calves (0.79±0.33) (Figure 4.4a). This observed variation in egg mass weight of *R. appendiculatus* was statistically significant ($p < 0.05$) (Table 4.4). The mean clutch size of eggs of *R. appendiculatus* that fed on immunized calves was observed to be lowest (24393±4911.23) compared to those that fed on control 1 (25063±16109.94) and 2 (56896.33±28263.08) calves (Figure 4.4b). The observed variation was however not statistically significantly ($p > 0.05$) (Table 4.6). *Rhipicephalus appendiculatus* that fed on immunized calves had the lowest mean number of egg batches (5.66±1.53) compared to those that fed on control 1(6±3.61) and 2 (14.66±7.23) calves. The mean number of egg batches did not differ significantly between the ticks that fed on immunized calves and those that fed on control calves, despite the observed variation ($p > 0.05$) (Figure 4.4c; Table 4.6).





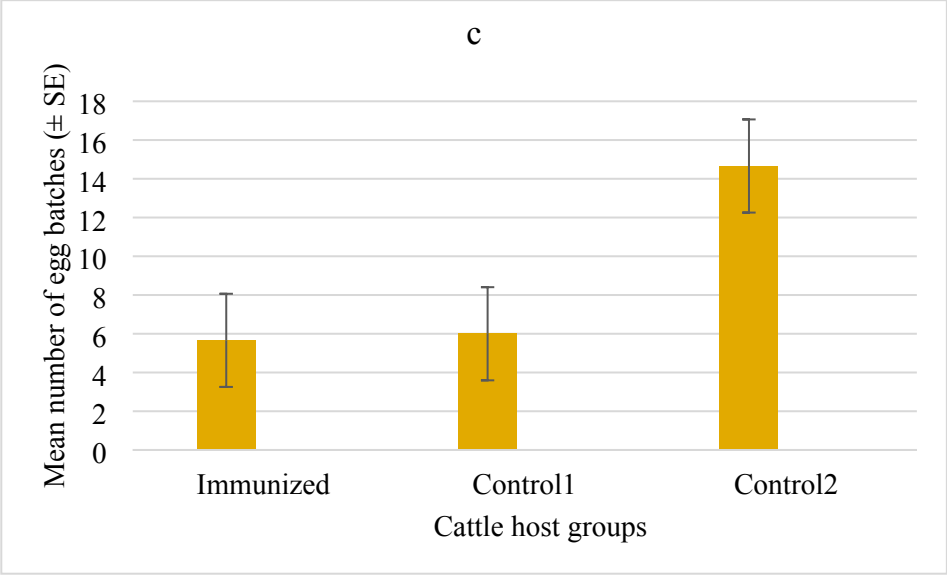


Figure4.4: Oviposition success of *Rhipicephalus appendiculatus* adult female fed on cattle immunized with *Theileria parva* Marikebuni vaccine.

Key: [a].mean weight of egg mass of *R. appendiculatus* fed on immunized, control1 and control2 calves; [b]. mean clutch sizes of eggs of *R. appendiculatus* fed on immunized, control1 and control2 calves; [c] .mean number of egg batches of *R. appendiculatus* fed on immunized, control1 and control2 calves .

The egg mass weight of *R. appendiculatus* differed significantly between the ticks that fed on immunized calves and those that fed on controls, as evidenced by the p-value (0.023) of F-statistics (7.993) that was less than 0.05 significance level (Table 4.4). Post-hoc Tukey HSD test on ANOVA results showed that there was a significant pairwise difference between weight of egg mass of ticks that fed on control group2 and immunized group calves, with an average difference of 1.34 (adjusted p-value= 0.031). Similarly, egg mass weight of ticks that fed on control group 1 & 2 differed significantly, with an average difference of 1.34 (adjusted p-value= 0.03). The Adjusted p- value was less than 0.05 significance level. However, the mean weight of egg mass of ticks that fed on immunized and control group 1 calves did not differ significantly, the adjusted p-value (1.0) being greater than 0.05 significance level (Table 4.5).

Table 4.4: ANOVA test on oviposition success of *Rhipicephalus appendiculatus* fed on cattle immunized with Marikebuni vaccine

Parameter		Degrees of freedom	Sum of squares	Mean sum of squares	F- value	Pr(>F)
Tick egg mass weight (mg)	Ind	2	3.609	1.8045	7.993	0.023*
	Residuals	6	1.355	0.2258		

Key: Ind=independent variable, Residuals=all variation not explained by independent variable, Sum of squares =variation between the group averages and overall average, df= degrees of freedom, F-value=test statistic from F test, Pr (>F) = p-value of F –statistic. p-value of F-statistics with asterisks * was significantly different.

Table 4.5: Post-hoc Tukey HSD multiple comparisons of means on the ANOVA test result in the determination of the oviposition success of *Rhipicephalus appendiculatus* fed on cattle immunized with Marikebuni vaccine.

Cattle host groups compared	Diff	95%family-wise confidence level		Adjusted p-value
		Lwr	Upr	
Immunized - control group2	1.34	0.15	2.53	0.031
Immunized -control group 1	0.0	-1.19	1.19	1.0
Control proup1-control group2	1.34	0.15	2.53	0.031

Key: diff=average difference, lwr =lower limit, upr=upper limit

The number of eggs laid by *R. appendiculatus* was further analyzed using logistic regression. A positive constant (intercept) signals that the probability of the ticks that are fed on immunized calves being affected by the vaccine is greater than 0.5. The number of egg batches had a negative coefficient, indicating that the egg batches of the ticks that fed on immunized calves were less likely to be altered by the vaccine. The clutch sizes of eggs had a positive coefficient, which means that the egg clutch sizes originating from ticks fed on the immunized host were more likely to be altered by the vaccine. The clutch sizes of eggs of the ticks that fed on immunized calves did not differ significantly (OR = 1.0; p-value = 0.40). Although the egg batches of the ticks that fed on immunized calves were 8% as many as those of the ticks that fed on control calves, the variation was not statistically significant (OR = 0.08; p-value = 0.35) (Table 4.6).

Table 4.6: Logistic regression analysis of oviposition success *Rhipicephalus appendiculatus* fed on cattle immunized with the Marikebuni vaccine

COEF(SE)		97.5%Cl for Odds Ratio			z-value	p-value
		Lower 2.5 %	Odds Ratio (OR)	Upper 97.5 %		
Constant (intercept)	1.184(2.14)				0.55	0.58
Egg batch	-2.53(2.73)	0.00006	0.08	3.2	-0.93	0.35
Egg clutch sizes	0.0005 (0.0006)	0.99	1.0	1.0	0.85	0.40

Key: COEF-coefficient, SE-standard error, Cl-Confidence level. Note: $_R^2$=0.27 (Hosmer& Lemeshow), 0.29 (Cox&Snell), 0.40(Negelkerke). Model $\chi^2_{=}$ (2) =3.04, p>0.05(p=0.23).

4.2.1: Relationship between female *Rhipicephalus appendiculatus* engorgement weight and weight of egg mass (mg) after feeding on Marikebuni vaccine immunized calves and control calves

There was a significant positive association between female *R. appendiculatus* engorgement weight after feeding on immunized and control group 1 and 2 calves and its egg mass weight (mg). Egg mass weight increased with an increase in replete female tick weight. Replete adult female tick weight (mg) significantly predicted the egg mass weight (mg) (β = -0.025; p < 0.05). The fitted regression model was: egg mass weight (mg) =91.3+0.4918*(replete female tick weight (mg)). The overall regression was statistically significant (R^2 = 0.643; F (1, 79) = 145.1; p = < 2.2 e -16) (Figure 4.5).

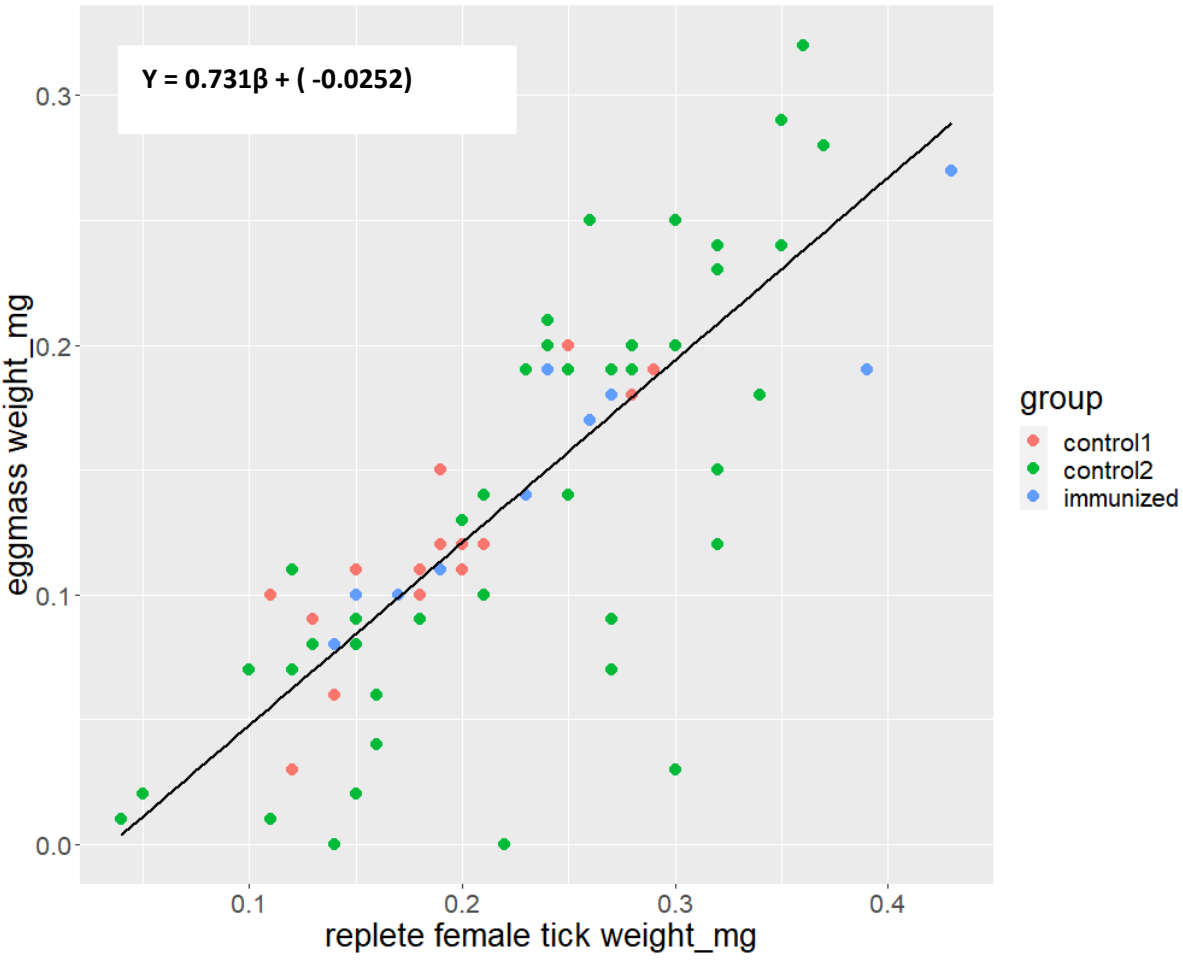


Figure 4.5: Regression analysis of *Rhipicephalus appendiculatus* egg mass weight (mg) against replete female tick weight (mg) after feeding on calves immunized with Marikebuni vaccine and the control calves.

4.3: Determination of Viability of eggs of *Rhipicephalus appendiculatus* fed on cattle immunized with Marikebuni vaccine

There was an observed difference in the mean number of live larvae that hatched between the immunized (24140±4916), control 1(24783.33±15946.87) and control 2 (56376.33±27162.81) calves though the difference was not significant ($p>0.05$) (Table 4.7; Figure 4.6)

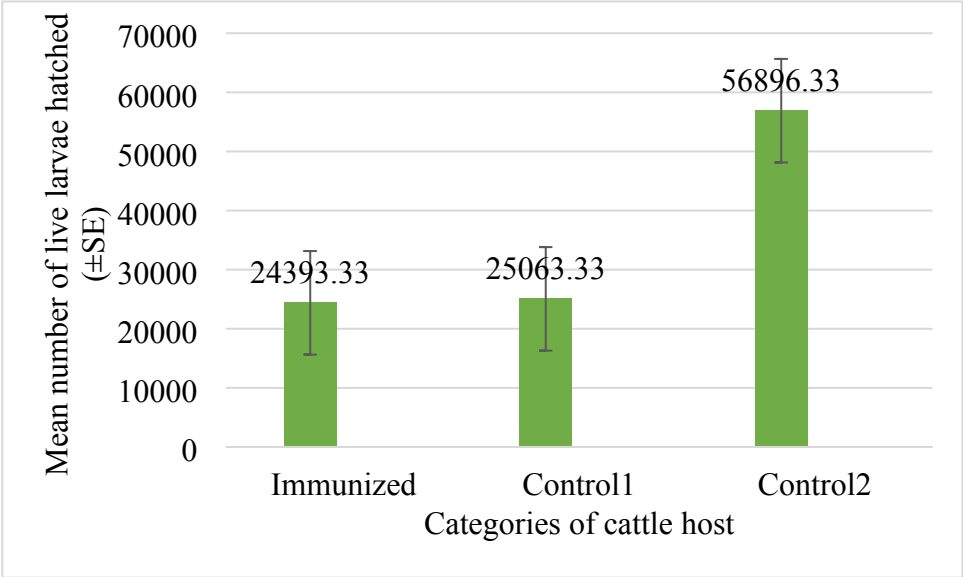


Figure 4.6: Mean number of live larvae hatched (± standard error) in immunized, control1 and control 2 calves in an experiment to determine the viability of eggs of *Rhipicephalus appendiculatus* fed on cattle immunized with *Theileria parva* Marikebuni vaccine

The number of live larvae that hatched successfully in eggs originating from ticks that fed on immunized calves was 99.9% as many as those originating from the ticks that fed on non-immunized calves. The observed variation was, however, not statistically significant (OR = 0.99; p-value = 0.33) (Figure 4.6; Table 4.7). The hatching of eggs originating from the ticks that fed on immunized calves was less likely to be affected by the vaccine, as indicated by a negative coefficient (Table 4.7).

Table 4.7: Logistic regression analysis of the viability of eggs of *Rhipicephalus appendiculatus* fed on cattle immunized with the Marikebuni vaccine

	COEF(SE)	97.5%Cl for Odds Ratio			z-value	p-value
		Lower 2.5 %	Odds Ratio (OR)	Upper 97.5 %		
Constant (intercept)	1.26(1.94)				0.65	0.52
#of Live larvae	-6.454e-05 (6.611e-05)	0.999	0.999	1.0	0.98	0.33

Key: COEF-coefficient, SE-standard error, Cl-Confidence level. Note. $_R^2$= 0.14(Hosmer& Lemeshow), 0.17 (Cox&Snell), 0.23(Negelkerke). Model χ^2= (1) =2.53 p>0.1 (p=0.11).

CHAPTER 5

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1: Discussion

The mean number of *Rhipicephalus appendiculatus* nymphs that imbibed blood successfully was lowest in nymphs that fed on immunized calves compared to the controls (Figure 4.1 A). The observed variation was, however, not statistically significant (OR = 0.996; p-value = 0.29) (Table 4.1). The nymphs that fed on immunized calves also recorded the lowest mean blood meal weight compared to the control groups, although the difference was not statistically significant (OR = 0.712; p-value = 0.34) (Table 4.1; Figure 4.1B). The mean number of nymphs that molted successfully into adults was lowest in nymphs that fed on immunized calves compared to those that fed on the control calves. The observed variation was, however, not statistically significant (OR = 1.004; p-value = 0.36) (Table 4.1; Figure 4.1C). These observations imply that the amount of blood imbibed by the nymph ticks contributes to the successful molting of the tick to the next developmental adult stage, as reported by Kaufman, 2007 and CDC,2020. With the amount of blood imbibed by nymphs that fed on immunized calves being low compared to the controls, their ability to molt into adults was more likely to be affected by the vaccine. This was confirmed by a positive coefficient on the number of molted nymphs in the fitted logistic regression model (Table 4.1).

The unfed nymphs' weight (mg) was observed to significantly predict the blood meal weight of the nymphs ($\beta = 0.7535$; $p < 0.05$). A significant negative correlation between the nymphs' weight before feeding and the blood meal weight in nymphs that fed on immunized calves was recorded (p-value = 0.0027) (Figure 4.3A). This means that they were not able to imbibe large amounts of blood despite being heavy, compared to those that fed on non-immunized calves. This observation could also imply that there was some degree of disrupted feeding on the nymphs that fed on immunized calves and were not able to counter the host immune response

compared to those that fed on control calves (Sonenshein, 1991; Wang *et al.,* 1999; Walker *et al*., 2003; Brossard and Wikel, 2008). The nymphs that fed on non-immunized calves, on the other hand, recorded a positive correlation, with larger ticks recording heavier blood meals than smaller ticks (Figures 4.3A). Similar positive association between the blood meal weight of the ticks and the size of the tick has been reported by other workers such as Kitaoka and Yajima, (1958); Sonenshine, (1991) and McCoy *et al.,* (2010).

Male *Rhipicephalus appendiculatus* plays a vital role in the engorgement mass and repletion time of female ticks. The proteins secreted from the male gonad speed up salivary gland degeneration and ovarian development. They also stimulate female engorgement. The full engorgement of female Ixodid ticks is attained after mating (Wang *et al*., 1998; Kaufman, 2007). The male ticks therefore only served to stimulate the female tick's feeding and were not part of the comparison in this study. The feeding success of the adult female *R. appendiculatus* was similar to that of the nymphs, with observed variations between the ticks that fed on immunized calves and those that fed on controls not being statistically significant.

The mean number of replete *Rhipicephalus appendiculatus* adult females was highest in ticks that fed on control group 2 calves, followed by those that fed on control group 1 and finally those that fed on immunized calves (Figure 4.2a). This observed variation was not statistically significant (OR = 1.05; p-value = 0.93). The mean blood meal weight of adult ticks was lowest in ticks that fed on immunized calves compared to those that fed on control calves, although the variation was not statistically significant (OR = 0.32, p-value = 0.48; F = 3.26, p-value = 0.11) (Figure 4.2b; Table 4.2 and 4.3). These observations imply that the ability of the tick to counter the host immune response might have contributed to the observed difference between the ticks that fed on immunized calves and those that fed on non-immunized calves (Sonenshine *et al.,* 2002; Kazimirova, 2008; Kazimirova and Stibraniova, 2013; Wikel, 2013). Substances in the tick saliva are reported to reduce the host's immune reaction (Chimelar *et*

al., 2015, 2016; Nuttall,2019). This means that the adult *R. appendiculatus* that fed on immunized calves were not able to counter the host immune response compared to those that fed on control calves.

Other factors such as host grooming could have also contributed to the observed difference between the ticks that fed on immunized calves and those that fed on non-immunized calves (Wang *et al*., 1999; Hart, 2000; Service, 2012).

The unfed adult female tick weight (mg) significantly predicted the blood meal weight of the adult female *R. appendiculatus* ($\beta = 0.4918$; $p < 0.05$). A significant negative correlation was recorded between the weight of female ticks before feeding and their blood meal weight after feeding on immunized calves, with larger ticks recording a low blood meal weight. However, the adult female ticks that fed on control non-immunized calves recorded a positive correlation, with larger ticks recording heavier blood meals than smaller ticks (p- value= 0.002176) (Figures 4.3B). Other workers, such as McCoy *et al.*, (2010), reported a positive association between the blood meal weight of the ticks and the size of the tick before feeding. This means that there was disrupted feeding in adult female ticks that fed on immunized calves compared to those that fed on non-immunized calves, reducing their vectorial capacity indirectly (Sonenshine, 1991; Wang *et al.,* 1999; Walker *et al*., 2003).

Though there was an observed difference in the blood-feeding success of nymphs and adult *R. appendiculatus* that fed on immunized and non-immunized calves, the variation was not statistically significant ($p > 0.05$). This implies that an infected tick can feed on the immunized cattle without being significantly affected by the vaccine, hence transmitting *Theileria parva* parasites to the immunized cattle. Subsequently, the immunity of the immunized cattle is boosted (Wanjohi *et al.,* 2001**;** Kivaria *et al.*, 2007; Di Giulio *et al*., 2009).

The mean clutch size of eggs of *Rhipicephalus appendiculatus* that fed on immunized calves was low compared to those that fed on non-immunized control group 1and 2 calves (Figure

4.4b). The observed variation was, however, not statistically significant (OR= 1.0; P-value= 0.40) (Table 4.6). Although the egg batches of the ticks that fed on immunized calves were 8% as many as those of the ticks that fed on control calves, the variation was not statistically significant (OR= 0.08; p-value= 0.35) (Table 4.6). The observed variation in egg clutch sizes and number of egg batches could have occurred due to the fact that: the number of female adult *R. appendiculatus* that successfully imbibed blood was lowest in ticks that fed on immunized calves compared to those that fed on non-immunized control calves (Figure 4.2 a and b). The amount of blood imbibed was also lowest in ticks that fed on immunized calves compared to those that fed on non-immunized control calves.

The amount of blood imbibed by the tick impacts egg laying success, as reported by Kaufman (2007). Environmental factors and physiological conditions could also have contributed to the observed variation in mean clutch sizes and egg batches between the ticks that fed on immunized and control calves (Sonenshine *et al*., 2002; Service, 2012).

The weight of egg mass was significantly reduced in adult female ticks that fed on immunized calves compared to those that fed on non-immunized control group 2 calves ($p < 0.05$) (Figure 4.4a; Table 4.4). This could have been caused by the low blood meal weight recorded by the ticks that fed on the immunized calves compared to that of the ticks that fed on control group 2 calves (Figure 4.2b). The amount of blood imbibed by the tick has an impact on egg laying success of the tick, as reported by Sonenshine and Roe, (2014).

A significant negative relationship was observed between the weight of *Rhipicephalus appendiculatus* female adult ticks before feeding and the weight of blood imbibed by ticks that fed on immunized calves. On the other hand, the female adult ticks that fed on non-immunized calves recorded a significant positive correlation between the weight of unfed females and the amount of blood imbibed ($p < 0.05$) (Figure 4.3B). This means that the ticks that fed on immunized calves were not able to imbibe a large amount of blood in relation to their body

weight, compared to those that fed on controls. Replete adult female tick weight (mg) significantly predicted the egg mass weight in ticks that fed on immunized and non-immunized control group1 and 2 calves(mg) ($\beta = -0.025$; $p < 0.05$). The blood obtained by adult female ticks is utilized by the tick to produce eggs and carry out other physiological processes (Kaufman, 2007). Hindrances to proper blood feeding can have a detrimental influence on tick reproduction and viability (Wang *et al.*, 1999; Kaufman, 2007, 2010; Sonenshine and Roe, 2014). A large amount of blood meal is reported to cause a high number of eggs laid by the tick (Kaufman, 2007). The egg mass weight of *R. appendiculatus* that fed on both immunized and control group 1 and 2 calves was observed to increase significantly with increase in weight of replete female tick (p-value = <2.2 e -16; F=145.1 on 1 and 79 df) (Figure 4.5). Similar association between engorgement weight of female ticks and egg mass is reported by other researchers (Dipeolu, 1991; Wang *et al.,* 2002; Lopez-Arias *et al*., 2015). This implies that the ticks that fed on non-immunized control group 2 calves were able to feed successfully and therefore lay eggs successfully compared to those that fed on immunized calves. The *T. parva* Marikebuni vaccine therefore altered the oviposition success of *R. appendiculatus* significantly.

Although the number of *R. appendiculatus* live larvae that hatched was observed to be lowest in eggs originating from ticks that fed on immunized calves compared to those originating from control calves, the variation was not statistically significant (OR = 0.99, p-value = 0.33; $p > 0.05$) (Figure 4.6; Table 4.7). The low amount of blood meal recorded by the ticks that fed on immunized calves could have contributed to the reduced number of live larvae that hatched from eggs laid by these ticks compared to the controls (Figure 4.2b). A large amount of blood meal is reported to lead to a high number of eggs laid by the tick, which subsequently results in numerous hatched larvae (Kaufman, 2007). Environmental factors and physiological

conditions could also have led to the observed variation in the number of live larvae that hatched (Sonenshine *et al*., 2002).

5.2: Conclusion

Being hematophagous, blood feeding in *Rhipicephalus appendiculatus* is vital to the life of this tick. The blood obtained by nymph ticks is used to molt them into adults. Adult male and female ticks utilize this blood to produce sperm and eggs, respectively, and also carry out other physiological processes (CDC, 2020). The feeding success of the tick vector therefore impacts its reproduction and fecundity (Kaufman, 2007). In this study, the effect of the *Theileria parva* Marikebuni vaccine on *Rhipicephalus appendiculatus* blood-feeding success, egg-laying success, and viability of eggs laid after feeding were investigated for the first time. The findings of this study led to the following conclusions:

i. The amount of blood imbibed by the *R. appendiculatus* nymphs was not significantly altered by the *Theileria parva* vaccine. This was despite the observed low blood meal weight in nymphs that fed on immunized calves, compared to the those that fed on control calves.

ii. Although the molting from nymphs to adults in nymphs that fed on immunized calves was more likely to be influenced by the vaccine, the vaccine did not alter the molting significantly.

iii. The blood-feeding success of female adult *R. appendiculatus* was not significantly affected by the *Theileria parva* Marikebuni vaccine. This was despite the observed low blood meal weight and number of replete adult female ticks that fed on immunized calves compared to those that fed on non-immunized control group 1 and 2 calves.

iv. A significant negative relationship between the unfed *R. appendiculatus* weight and the weight of the blood meal imbibed was observed in ticks that fed on immunized calves. The ticks that fed on control calves, however, recorded a significant positive relationship with large ticks imbibing large amounts of blood.

v. *Theileria parva* Marikebuni vaccine influenced the oviposition success of *R. appendiculatus* significantly. The egg mass weight of the ticks that fed on immunized calves was significantly reduced compared, to that of ticks that fed on non-immunized control group 2 calves.

vi. The replete female *R. appendiculatus* weight significantly predicted the egg mass weight. The egg mass weight of *R. appendiculatus* that fed on both immunized and control group calves was observed to increase significantly with the increase in weight of the replete female tick.

vii. Immunizing calves using the *T. parva* Marikebuni vaccine did not influence the viability of eggs laid by *R. appendiculatus* significantly. This was despite the low number of live larvae originating from eggs of ticks that fed on immunized calves compared to those that originated from eggs of the ticks that fed on non-immunized calves.

viii. *Theileria parva* Marikebuni vaccine impacts the fecundity and fertility of *R. appendiculatus,* which indirectly amounts to some degree of vector control.

5.3: Recommendations

Theileria parva Marikebuni vaccine impacts the fecundity and fertility of *R. appendiculatus*, which indirectly amounts to some degree of vector control. This calls for an increase in Marikebuni vaccine production and distribution in endemic regions of sub-Saharan Africa.

5.3.1. Suggestions for further research

i. Further investigation of the vectorial capacity of *R. appendiculatus* that feeds on Marikebuni vaccine immunized calves should be pursued. This can be done by comparing the ability of infected *R. appendiculatus* to transmit the *T. parva* parasite to Marikebuni vaccine immunized cattle and the non-immunized cattle.

ii. Further studies should be conducted on *R. appendiculatus* eggs. This will establish the exact causal factor that led to the significant statistical difference in the egg mass weight between ticks that fed on Marikebuni vaccine-immunized calves and those that fed on non-immunized control calves.

iii. Establishment of the causal factor that led to the significantly reduced egg mass weight in *R. appendiculatus* fed on Marikebuni vaccine-immunized calves will provide an opening for further research. A new vaccine with a genetically modified causal factor can be developed. This will increase the chances of reduced egg-laying success for *R. appendiculatus*.

5.4: Limitations of the study

One of the challenges faced in this study was the lack of enough tick and *Theileria parva* naïve calves to be fed on the three blood feeding stages of *R. appendiculatus.* The study was therefore limited to the assessment of the blood feeding success of nymphal and adult stages of *R. appendiculatus*, with the exclusion of the larval stage. The number of calves on which the adult ticks were fed was reduced from six to three due to a shortage of experimental calves.

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8.0: APPENDICES

Appendix I: Letter of approval to conduct research

KENYA AGRICULTURAL AND LIVESTOCK RESEARCH ORGANIZATION (KALRO)
P.O. Box 32, KIKUYU 00902, KENYA
TEL: 020 – 2519769, 2524616, 2020512
Email: director.vsri@ kalro.org

Date: 7th June, 2021

When replying quote:
Our ref: COMTE/45/ (97)

Peninah Waithira Njoroge,
Msc. Student in Applied Parasitology,
School of Biological and Physical Sciences,
University of Nairobi,
P.O. Box ~~30197~~ 30456, Nairobi.

RE: Approval of your Master of Science research project entitled "Effect of East Coast Fever *Theileria parva* (Marikebuni) vaccine on reproductive performance of *Rhipicephalus appendiculatus* tick vector": KALRO-VSRI/ IACUC023/04062021

The Institutional Animal Care and Use Committee (IACUC) of Kenya Agricultural and Livestock Research Organization (KALRO) - Veterinary Science Research Institute (VSRI), Muguga North, met on 4th June, 2021 where the Masters of Science project proposal was presented by Peninah Njoroge and evaluated. The committee established that the work met the requirements needed to comply with animal welfare and use during its implementation. The presenter outlined an experimental protocol that had inbuilt animal welfare at the core of the experimental design and assured that welfare of ~~goats~~ cattle will be protected during implementation. The committee approved the project as stipulated to be implemented on-station at KALRO-VSRI, Muguga and the approval Code Number given is: **KALRO-VSRI/ IACUC023/04062021.**

This statement of KALRO-VSRI IACUC approval is included in all publications and any other work as may be required **"This research was approved by the Institutional Animal Care and Use Committee of KARLO-Veterinary Science Research Institute, Muguga North upon compliance with all provisions vetted under and coded: KALRO-VSRI/ IACUC023/04062021".**

Yours faithfully,

Dr. J.M. Nginyi, PhD
Chairperson, KALRO-VSRI, Institutional Animal Care and Use Committee (IACUC)

Appendix II: *Theileria parva* Marikebuni vaccine viability test (parasitological parameters

# of animals	Breed	nitrogen can #	Straw	Mean number of days to observation of schizonts	degree of schizont parasitosis	mean number of days to detection of piroplasms	piroplasm parasitemia
2	Boran	8	M001	11	Ma+++	11	Piro+++
2	Boran	1	M002	10.5	Ma+++	13	Piro+++
2	Boran	2	YP316B	8.5	Ma+++	9.5	Piro+++

Key: Ma+++ indicate macro schizonts observed in most of the field (>5% parasitosis), Piro+++indicates piroplasms parasitemia

Appendix III: *Theileria parva* Marikebuni vaccine viability test (clinical parameters and serology)

# of Animals	nitrogen can #	Stabilate straw used	mean number of days to Onset of fever	mean peak of fever (ºC)	mean duration of fever (days)	lymph node enlargement	mean PCV	general appearance	mean respiratory rate	serology (IFAT)
2	8	M001	no fever	_	_	Swollen lymph node	45	Anorexia	68 beats/min	Positive
2	1	M002	11	40.5	5	Swollen lymph node	42.5	Anorexia	66 beats/min	Positive
2	2	YP316B	8	39.9	4	Swollen lymph node	48	Anorexia	78 beats/min	Positive

Appendix IV: Data on calves used in determination Marikebuni vaccine effect on feeding and reproductive success of *R. appendiculatus*

Group name	Animal tag #	breed	Age (months)	sex	weight of animal (kg)	Treatment (marikebuni vaccine)	Amount of 30% long-acting oxytetracycline)	Amount of tick material	IFAT antibody titre (Dilition1:40 and 1:160)	
									Before treatment	After treatment
Immunized	A108 KL	Boran	4	f	67	1ml	8ml		<40	160
Immunized	2491	Friesian	7	f	92	1ml	10ml		<40	40
Immunized	2490	Friesian	13	f	146	1ml	16ml		<40	160+
Immunized	2469	Ayrshire	13	f	182	1ml	20ml		<40	160
Immunized	2441	Ayrshire	5	m	96	1ml	10ml		<40	40
Immunized	2480	Friesian	4	f	92	1ml	10ml		<40	160+
Control1	2462	Friesian	13	f	96	-	10ml	1ml	<40	<40
Control1	A118 KL	Boran	4	f	73	-	8ml	1ml	<40	<40
Control1	2453	Ayrshire	4	f	103	-	11ml	1ml	<40	<40
Control1	2467	Ayrshire	7	m	127	-	14ml	1ml	<40	<40
Control1	2489	Friesian	13	f	178	-	20ml	1ml	<40	<40
Control1	2472	Friesian	7	f	109	-	12ml	1ml	<40	<40
Control2	2447	Ayrshire	7	m	109	-	-	1ml	<40	<40

Control2	2460	Friesian	7	f	156	-	-	1ml	<40	<40
Control2	2494	Friesian	13	f	202	-	-	1ml	<40	<40
Control2	2597	Ayrshire	13	f	166	-	-	1ml	<40	<40
Control2	A119 KL	Boran	4	f	77	-	-	1ml	<40	<40

Key: IFAT antibody titre <40= negative ;40= Weakly positive;160=Positive;160+= Strongly positive.

f= female animal; m= male animal

Appendix V: *Theileria parva* Marikebuni vaccine post-immunization monitoring

# of animals	Day	clinical parameters observed	parasitological parameters	Hematology	serology (IFAT-*T.parva* schizont antibody titres)
Immunized group					
6	7	swollen lymph node (6)	lymphoblasts (2)	_	<40(6),40(0),160(0) ,160+(0)
	14	swollen lymph node (6)	Ma+++(1) Ma++(4) Ma+(1)	increased white blood cell count (6)	<40(5), 40(1), 160(0), 160+(0)
	21	_	_	white blood cell count increase (6)	<40(2), 40(2),160(1), 160+(1)
	28	_	_	white blood cell count increase (6)	<40(0), 40(2), 160(2), 160+(2)
	35	_	_	White blood cell count increase (6)	<40(0)40(2) 160(2) 160+(2)

Control group1					
6	7 14 21 28 35	_ _ _ _ _	_ _ _ _ _	_ _ _ _ _	_ <40(6) <40(6) <40(6) <40(6)
Control group 2					
5	7 14 21 28 35	_ _ _ _ fever (1)	_ _ _ _ strongyle (100) in stool sample	_ white blood cell count increase (1) _ _ increased white blood cell count (1)	_ <40(5) <40(5) <40(5) <40(5)

Key: Ma+++ indicates macro schizonts observed in most of the examined field (>5% parasitosis) Ma++indicates macro schizonts observed in50% of the microscope field of view (1-5%. Parasitosis) Ma+ indicates macro schizonts observed in few microscopes field (<1% parasitosis), Number in parenthesis=number of animals.

Appendix VI: Application of nymph *R. appendiculatus* to calves in an experiment to assess the blood feeding success of *Rhipicephalus appendiculatus* ticks fed on cattle immunized with Marikebuni vaccine

Group Name n=6	Number of nymphs before Feeding	Number Of nymphs After feeding	weight of nymphs Before Feeding	Weight of nymphs After Feeding	Number of nymph ticks molted to adult
Immunized group	1200	98	0.01	0.68	79
Immunized group	1200	1108	0.09	7.28	420
Immunized group	1200	282	0.02	1.74	181
Immunized group	1200	297	0.02	1.76	110
Immunized group	1200	399	0.03	2.41	137
Immunized group	1200	265	0.02	1.74	52
Control group1	1200	614	0.05	4.31	574
Control	1200	355	0.03	2.15	71

group1					
Control group1	1200	532	0.04	3.26	187
control group 1	1200	734	0.06	5.17	287
control group 1	1200	663	0.06	4.31	27
control group 1	1200	805	0.07	4.63	116
Control group2	1200	512	0.04	3.28	47
Control group2	1200	608	0.05	3.35	23
Control group2	1200	222	0.02	1.42	49
Control group2	1200	377	0.03	1.5	56
Control group2	1200	856	0.07	4.9	503

Key n= number of cattle hosts used to apply nymph *R. appendiculatus* ticks

Appendix VII: Application of adult *R. appendiculatus* on calves in an experiment to assess the blood feeding success of Rhipicephalus *appendiculatus* fed on cattle immunized with Marikebuni vaccine

Group name n=3	Number of Female Ticks Applied	Number of Male Ticks Applied	Number of Female ticks after feeding	Weight of female ticks before feeding	Weight of Female Ticks after feeding
Immunized group	60	60	4	0.01	1.12
Immunized group	60	60	6	0.01	1.92
Immunized group	60	60	7	0.02	0.98
Control group1	60	60	3	0.01	0.24
Control group1	60	60	5	0.01	1.78
Control group1	60	60	10	0.02	3.22
Control group2	60	60	25	0.06	5.88
Control group2	60	60	11	0.02	3
Control group2	60	60	9	0.02	2.9

Key: n= number of cattle host used to apply the adult *R.appendiculatus* ticks

Appendix VIII: Total weight of egg mass, clutch sizes of eggs, number of egg batches and number of live larvae of *Rhipicephalus appendiculatus* in an experiment to determine the oviposition success and viability of eggs of *Rhipicephalus appendiculatus* fed on cattle immunized with Marikebuni vaccine

Group name n=3	Number of female ticks that laid eggs	Number of egg batches per tick	Total number of tick egg batches	Total weight of tick egg mass	Total clutch sizes of eggs	Total number of live larvae hatched
Immunized group	4	1	4	0.59	18800	18550
immunized group	6	1	6	0.79	26380	26080
Immunized group	7	1	7	0.99	28000	27790
Control group1	3	1	3	0.56	11000	10760
control group1	5	1	5	0.64	21550	21460
control group1	10	1	10	1.17	42640	42130
control group2	10	1	10	1.56	35999	35819
control group2	23	1	23	2.95	88910	87170

control group2	11	1	11	1.89	46380	46140

Key: n= the number of cattle hosts used to determine reproductive success of *R.appendiculatus*; Immunized group=cattle immunized with *T.parva* Mrikebuni vaccine; Control group 1=unimmunized cattle inoculated with uninfected tick stabilate and 30% oxytetracycline; Control group2= unimmunized cattle inoculated with uninfected tick stabilate