RESPONSES OF CROSSBRED CALVES TO *THEILERIA PARVA* INFECTION FOLLOWING TARGETED STIMULATION WITH TOLL-LIKE RECEPTOR 7 (TLR7) AGONIST

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ABSTRACT

Control of East Coast fever (ECF) depends on the use of acaricides against ticks, chemotherapy and vaccination. ECF vaccination is based on an infection and treatment method (ITM), which induces life-long immunity if cattle are exposed to mild tick infestation. However, ECF vaccination has several limitations, such as high cost of simultaneously used antibiotics. The primary objective of this study was to determine whether Toll-like receptor 7(TLR7) agonist could effectively induce strong immunity in calves prior to ECF vaccination and replace the use of long acting tetracycline. Twenty crossbred calves were split into 4 groups; adjuvant (N=10), vaccinated (N=4), infected (N=4) and control (N=2). Stimulation of calves with TLR7 agonist induced a strong innate response in terms of a rapid rise in temperature, skin inflammatory response and pronounced swelling of the lymph node in calves. TLR7-priming induced a significant impact on the response of the calves to subsequent *T. parva* infection. In comparison to the infected group, lymphocyte counts were higher in calves which received the adjuvant. Furthermore, the combined effect of TLR7 agonist stimulation and *T. parva* parasite challenge induced a high level of IFN-gamma response almost similar to the response shown by ECF vaccinated calves. Similarly, the adjuvant group attained higher antibody level earlier than the infected control calves. Based on the clinical signs observed from day 14 onwards, calves which received TLR7 agonist developed milder disease signs compared to the infected control calves, which were all treated against ECF on day 18. Only one calf out of ten TLR7-primed calves developed clinical disease signifying a potential adjuvant role of the agonist in ECF vaccination. Possibly the adjuvant acted in this mechanism. This study has demonstrated a practical application TLR7 agonist in ECF vaccination, without simultaneous use of antibiotics.

DECLARATION

I, **Kelly Owusu Sarfo**, do hereby declare to the Senate of Sokoine University of Agriculture that this dissertation is my own original work done within the period of registration and that it has neither been submitted in any other institution.

_____ _____

Kelly Owusu Sarfo Date

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The declaration is hereby confirmed by;

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Prof. Paul Simon Gwakisa Date

(Supervisor)

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AIDS	Acquired Immune Deficiency Syndrome
APCs	Antigen Presenting Cells
BCG	Bacille Calmette Guerin
CD4+	Cluster of Differentiation-4
CD8+	Cluster of Differentiation-8
CTL	Cytotoxic T-lymphocyte
DCs	Dendritic Cells
DNA	Deoxyribonucleic acid
ECF	East Coast Fever
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme Linked Immunosorbent Assay
FAO	Food and Agriculture Organization
GMP	Good Manufacturing Practices
HIV-1	Human Immunodeficiency Virus-1
IDRI	Infectious Disease Research Institute
IFAT	Immunofluorescent Antibody Test
IFN-γ	Interferon-gamma
IL-2	Interleukin-2
Inos	Inducible Nitric Oxide
ITM	Infection and Treatment Method
kDA	Kilodalton
LAMP	Loop-mediated isothermal amplification
LAOTC	Long Acting Oxytetracycline
MC	Muguga Cocktail

MCHC	Mean corpuscular haemoglobin concentration
MHC	Major Histocompatibility complex
mRNA	Messenger Ribonucleic Acid
Mtb	*Mycobacterium tuberculosis*
P67	Protein-67
PAMPs	Pattern Associated Membrane Proteins
PCR	Polymerase Chain Reaction
PIM	Polymorphic Immunodominant molecule
PP	Percent Positivity
PRRs	Pattern Recognition Receptors
RFLP	Restriction Fragment Length Polymorphism
RLB	Reverse line blot
rRNA	Ribosomal ribonucleic acid
TB	Tuberculosis
TBDs	Tick-borne diseases
TH1	T-helper cell-1
TH2	T- helper cell-2
TLR11	Toll-like Receptor-11
TLR2	Toll-like Receptor-2
TLR3	Toll-like Receptor-3
TLR4	Toll-like Receptor-4
TLR5	Toll-like Receptor-5
TLR6	Toll-like Receptor-6
TLR7	Toll-like Receptor-7
TLR8	Toll-like Receptor-8
TLR9	Toll-like Receptor-9

TLRs	Toll-like Receptors
TNF-α	Tumor Necrosis Factor- Alpha
WHO	World Health Organization

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

East Coast fever (ECF), a tick-borne disease of cattle caused by a protozoan parasite*Theileria parva* and transmitted by the brown ear tick *Rhipicephalus appendiculatus* is one of the major limitations to the development of cattle industry in the eastern, central and southern Africa including Tanzania (Bazarusanga, 2008).

Clinical symptoms of the disease commences in about 10 to 14 days after infection of the animal. The first symptom is swelling of lymph node which is usually accompanied by fever (40-42°C), loss of appetite (anorexia) and lacrimation. ECF also causes lymphodestructive disease those results in severe leucopoenia in fatal cases. Anaemia is uncommon. In the later stages of infection, interstitial pneumonia and pulmonary oedema are seen clinically as acute dyspnoea and frothing at the nostrils, are often the predominant sign. Corneal opacity, sometimes resulting in blindness can develop. Widespread subcutaneous oedema and diarrhoea are not unusual and in some cases central nervous system involvement is observed. The case fatality can be as high as 95%. Animals that recover from infection become immune for life against homologous challenge and become carriers, able to transmit the infection to ticks (Di Giulio *et al.,* 2009).

Recent statistics have shown that over 20 million cattle are at risk and the disease causes major financial losses of up to hundreds of millions of dollars per year to small scale farmers and pastoralists whose source of income depend on these animals (GALVmed newsletter, 2010). ECF still remains the most important tick-borne disease in eastern and

central Africa. At present, the practical method of control of the disease combines methods of tick control, calf immunization and treatment of clinical cases (Billiouw, 2005).

ECF has also been reported to cause significant productivity losses associated with the reduced lactation of recovering cattle (Dolan, 1999). The mortality rate has been reported to be as high as 90% in susceptible animals. It also causes high mortality especially in exotic and crossbred cattle, as well as indigenous calves below 6 months of age (Minjauw and Mcleod, 2003; Minjauw *et al.,* 1999; Minjauw *et al.,* 1998).

Vaccination presently remains the best strategy available to effectively control infectious diseases. For instance, the eradication of several infectious diseases accompanied with lessening morbidity and mortality rate of others can currently only be achieved by vaccination strategies. Nonetheless, in the case of tuberculosis (TB), the development of a vaccine (*Mycobacterium bovis* BCG) did not have the ability to eradicate the illness(Reyn, 2012). Therefore, efforts are underway to develop vaccines that will improve or replace BCG, with the ability to avoid infection and prevent the development of any of its disease forms that is sound among immunocompromised individuals and capable of eliciting a protective immune response by several cellular populations (Orme, 2013).

Vaccine adjuvants are possibly the bestwidelydiscovered applications for Toll-like receptor (TLR) agonists. The logical design of specific TLR agonists with reduced toxicity but increased potency, as compared to adjuvant candidates from only a decade ago, offers the opportunity to meet the stringent safety criteria required for prophylactic vaccines. Antigen presenting cells (APCs),primarily dendritic cells, are the key cellular

targets, as they create the link between innate and subsequent adaptive immune responses (Steinman, 2008; Pulendran and Ahmed, 2006).

Toll-like receptors (TLRs) have been reported to work based on the appliance that upon identification of a pathogen or its mechanismsthroughTLRs, immature antigen-presenting cells such as dendritic cells (DCs) undergo maturation, depart the site of infection, and travel to the draining lymph node. The developed DCs present their acquired antigens and stimulate antigen-specific T-cells, leading to antigen-specific acquired immunity along with immunological memory. The kind of acquired immunity establishedmight be induced by which TLR(s) is stimulatedalong with the specific subset of DC activated.The employment of TLR agonists as vaccine adjuvants mightsignify an effective strategy for the improvement of vaccines with better protective immunity(Iwasaki and Medzhitov, 2004).

Conceding that there is overabundance of natural pathogen originated products adeptin activating innate immune mechanisms, attempts have driven on expanding small synthetic molecules for treatment in humans as well as animals that might have benefits with value to reliableproductionin addition todispensation by particularpaths. This is an important reflection with respect to the development of safe vaccine adjuvants. Toll-like receptor 7 and/or 8 agonist when used as vaccine adjuvant has been reported to increases protective immunity against *Leishmania major* in BALB/c Mice (Zhang and Matlashewski, 2008).

1.2 Research Problem Statement and Justification

The high rates of mortality and morbidity of ECF and the outlay of current methods of control place a high economic burden on small scale cattle farmers, pastoralists and the countries of Africa in which the disease is prevalent.

The present method of immunization involves the simultaneous use of a lethal dose of cryopreserved sporozoites and a long-acting oxytetracycline. Since the immunity spawn is often parasite stock specific, the method poses major limitations (Nene *et al.,* 1995).

The infrastructure in ECF endemic areas which involves the requirement of a liquid nitrogen cold chain is rarely adequate to fosterefficient delivery of cryopreserved sporozoites to the field and the subsequent treatment with oxytetracycline makes it expensive. Furthermore, animals vaccinated by the infection and treatment method remain life-long carriers of the parasite, which poses risk for spread of the disease (Dinga *et al.,* 2015; Musoke *et al.,* 1992). Additionally, the high production cost of the three independent stocks that are combined to produce the *T. parva* Muguga Cocktail makes the acquisition of the vaccine expensive to farmers (Norling *et al.,* 2015).

Vaccines that are presentlyoperational have been established by a realisticmethod. Nonetheless, newrealisticapproaches are now required to improve vaccines against more intricate pathogens including *T. parva*. Imminent vaccines might be spawned on the basis of moderndevelopments in our understanding in what manner the innate immune response guides the acquired immune response and immunological memory (Pulendran and Ahmed, 2006). The innate immune response might be triggered by varied pathogen-associated molecular patterns through the Toll-like receptor (TLR) family of receptors, and this plays a vital role in leading the acquired immune response.

Currently, available vaccines have limited protective efficacy against disease progression and does not prevent spread of infection among animals because of their inability to elicit wide-spectrum immune responses. At present, the infection and treatment method (ITM) of vaccination is the practical method available for the prevention of the disease in cattle.

However, the method of vaccination only provides limited protection against ECF. Therefore, the development of alternative vaccines and strategies for increasing the efficacy of vaccination against ECF are urgently required. The main objective of this study was to determine whether TLR7 agonist could effectively induce strong innate immunity in calves and replace the use of long acting oxytetracycline (LAOTC), which is usually injected simultaneously with live *T. parva* sporozoites during the infection and treatment method for ECF vaccination.

1.3 Objectives

1.3.1 Overall objective

To investigate the responses of crossbred calves to *Theileria parva* infection following targeted stimulation with Toll-like receptor 7 (TLR 7) agonist.

1.3.2 Specific objectives

The study had the following specific objectives:

1. To investigate whether Toll-like Receptor 7 (TLR7) agonist can induce a strong innate immunity in crossbred calves
2. To determine immunological,haematological and parasitological responses to *T. parva*in calves pre-stimulated with TLR7 agonist, prior to infection.
3. To determine if stimulation with TLR7 agonist can induce significant protection of calvesagainst clinical ECF disease, without use of antibiotics.

1.4 Hypothesis/Research Questions

1. Does TLR7 induce a strong innate immunity in crossbred calves?
2. What are the immunological,haematological and parasitological responses to *T. parva*in calves pre-stimulated with TLR7 agonist, prior to infection?
3. Does stimulation with TLR7 agonist induce significant protection of calves against clinical ECF disease, without use of antibiotics?

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 East Coast Fever (ECF)

East Coast fever, caused by the protozoan parasite *Theileria parva,* ranks first in tick-borne disease constraints of cattle in sub-Saharan Africa.It is transmitted principally by the African brown ear tick *Rhipicephalus appendiculatus* and is characterized by the proliferation of lymphoblasts infected with *Theileria* schizonts throughout the body, particularly in the lymph nodes, spleen, kidneys, liver and lungs (Minjauw and Mcleod, 2003; Norval *et al.,* 1992). ECF has been reported to be the most important tick-borne disease in eastern and central Africa with over 20 million cattle being at risk and losses of up to hundreds of million US dollars per year (GALVmed, 2015).

ECF has an overwhelming impact on pastoralists and smallholder farmers because it can kill within 3-4 weeks of infection and it is present in 11 countries where roughly 28 million cattle are at risk, but has the potential to spread with the uncontrolled movement of infected cattle as the distribution of the tick vector and suitable tick habitats is wider than that of the parasite. Over one million cattle die of ECF each year resulting in annual losses exceeding $300 million (Malak *et al.,* 2012; McLeod and Kristjanson 1999; Norval *et al.,* 1992).

Tick-borne diseases (TBDs) - Anaplasmosis, Babesiosis, Cowdriosis and Theileriosis cause major economic losses to the livestock sector in tropical and subtropical regions of the world (Jongejan and Uilenberg, 2004; Okello-Onen *et al.,* 2003). In Tanzania, the annual losses to the livestock sector from TBD have been calculated as US$ 364 million, including mortality of 1.3 million cattle. Theileriosis was estimated to account for 70% of

these losses while Anaplasmosis, Babesiosis and Cowdriosis accounted for the remaining 30% (Kivaria, 2006a). Due to high mortality and morbidity rates, livestock diseases are an impediment to the livelihoods of poor farmers in Africa, who struggle to attain food, nutritional and economic security.

2.2 Pathogenesis of *Theileria parva*

Theileria parva infects cattle and African buffalo (*Syncerus caffer*) and undergoes successive development in lymphocytes and erythrocytes, the latter being infective for the tick vector. Parasite multiplication occurs predominantly within lymphocytes and this is the pathogenic stage. Sporozoites taken up by lymphocytes rapidly escape from the endocytic vacuole into the cytosol where development to the schizont stage results in transformation and proliferation of the host cells. Division of the parasite is synchronized with that of the host cell, as parasite multiplication occurs by clonal expansion of the infected cells. In susceptible animals, parasitized lymphocytes undergo uncontrolled proliferation and disseminate throughout the lymphoid system, eventually leading to destruction of lymphoid tissues and death within three to four weeks of infection (Dobbelaere and Rottenberg, 2003).

After the infective bite, the parasites localize and multiply in lymph nodes. The first clinical sign of ECF in cattle appears 7 to 15 days after attachment of infected ticks. This is seen as a swelling of the draining lymph node, usually the parotid, for the ear is the preferred feeding site of the vector. A few days later, fever develops and other superficial lymph nodes get enlarged. Over the next week or so, other clinical signs start to show. Fever ensues and continues throughout the course of infection. The rise in temperature is rapid and is usually in excess of 39.5°C but may reach 42°C. Anorexia develops and loss of condition follows. Other clinical signs may include lacrimation, corneal opacity, nasal

discharge, terminal dyspnea, and diarrhea. Before death, the animal is usually recumbent, the temperature falls, and there is a severe dyspnea due to pulmonary edema that is frequently seen as a frothy nasal discharge, a soft cough due to fluid in the lungs, difficulty in breathing, diarrhoea sometimes with blood tinged, muscle wasting and white discolouration of the eyes and gums. The parasite then invades red blood cells at which point the animal is infective to ticks. Sometimes the parasite can invade the central nervous system resulting in a fatal condition called “turning sickness and paralysis" which is associated with the blocking of brain capillaries by infected cells and results in neurological signs. If not treated, the affected animals can collapse and die within three or four weeks of the infective tick bite.

The severity and time course of the disease depend on, among other factors, the magnitude of the infected tick challenge, for ECF is a dose-dependent disease, and on the strain of parasites. Mortality in fully susceptible cattle can be nearly 100 percent. A frothy exudate is frequently seen around the nostrils of an ECF-infected animal. Signs of diarrhea, emaciation, and dehydration may be seen. Lymph nodes are greatly enlarged and may be hyperplastic, hemorrhagic and edematous. In acute cases of ECF, lymph nodes are edematous and hyperemic but often become necrotic and shrunken in more chronic disease.

Generally, muscles and fat appear normal but, depending on relative acuteness of infection, fat may become greatly depleted. Hemorrhages and ulceration may be seen throughout the gastrointestinal tract, particularly in the abomasum and small intestine, where necrosis of Peyer's patches can be observed. Lymphoid cellular infiltration appears in the liver and kidney as white foci. The most striking changes are seen in the lungs. In most cases of ECF, interlobular emphysema and severe pulmonary edema appear, the

lungs are reddened and filled with fluid, and the trachea and bronchi are filled with fluid and froth (Fever, 2004).

2.3 ECF Control

Control measures against ECF fall into three categories: tick control, treatment of acute clinical cases and immunization by the infection and treatment method. Control of ECF is feasible but it requires a good plan and any tick control measures must consider other local tick-borne diseases in that particular area (Kivaria *et al.,* 2006b).

2.3.1 Tick control

Tick control with acaricides is the most prevalent method of tick-borne disease control in livestock (Di Giulio *et al.,* 2009). Acaricides are applied to the skin of the animal using a dip tank, spray race, manual (hand) sprayer, or other method. While acaricide use has continually been called unsustainable because of the expense, infrastructure, and organization required for regular application, as well as the potential for the development of resistance, the method has been in use since 1909 (Morrison and McKeever, 2006; Perry and Young, 1995). Acaricide use also fundamentally alters the ecology of tick populations, which has important ramifications for the risk of tick-borne infection in both cattle and buffalo (Randolph and Rogers, 2006).

The application of acaricides means that domestic animals act as a sink for ticks, leading to a reduction of the effective carrying capacity of ticks in the environment where acaricides are used (Petney and Horak, 1987). If the tick population is severely reduced by the practice of acaricide use on cattle, it could have a strong effect on the transmission and persistence of a tick-borne pathogen even in a multi-host system (Perry and Young, 1995).

Dipping of cattle in arsenic preparations was widely used in the first half of the century to control ECF. These were replaced with the organochlorines, organophosphates, carbamates and pyrethroids as resistance emerged. Techniques of application including spray races, hand-operated sprays, pour-on formulations and impregnated ear tags have been developed as alternatives to dipping. Cattle must be treated strictly according to manufacturer's instructions, with some products two times a week where the challenge is heavy. There are many obstacles, which hamper the use of tick control in the African situation such as lack of water, unavailability of acaricides, cost, toxicity and lack of co-operation among farmers.

2.3.2 Chemotherapy

Clinical cases of ECF can be treated by chemotherapy. The majority of developments in chemotherapy have occurred in the last twenty years (Norval *et al.,* 1992). There are presently three effective drugs for treatment of ECF: Halofuginone lactate (Terit), parvaquone (Clexon and Parvaxone) and buparvaquone (Butalex). Halofuginone is easy to administer but is ineffective when used in early infection. The recommended dose is 1.2 mg/kg orally at 48h intervals. However the high toxicity and its long waiting period for milk and meat consumption have limited its use in many countries (Norval *et al.,* 1992). Subsequently, a very safe and effective parvaquone compound was developed. This drug is effective against schizont and sporozoite stages of *T. parva* but requires two treatments at a dosage of 10mg/Kg at 48h intervals and do not require milk withdrawal after administration.

In many endemic ECF settings, the most commonly used anti-theilerial drug is buparvaquone, a parvaquone derivative. McHardy *et al.* (1985) have shown that buparvaquone is 8 times more effective than parvaquone in vivo against *T. parva*

infection but butalex is 5 times more expensive and had a relatively longer waiting period for meat consumption. In the field, a single treatment at a dosage of 2.5 mg/kg is effective, but a second injection may be required at 48h interval, as some strains of *T. parva* are not fully controlled by one dose.

Buparvaquone is the most commonly used for treatment against ECF on smallholder farm in most endemic areas. The availability of therapeutic means is a significant development in the control of ECF. However, there are two constraints to the widespread use of medication: the drugs are too expensive for most African farmers and rapid, accurate diagnosis and administration of the drug are required for effective therapy (Norval *et al.,* 1992). In addition, the widespread use of chemotherapy has increased the number of carriers over the past years. Treated and recovered animals become carriers of infection and parasites surviving treatment will be transmitted by recovered cattle (Dolan, 1999).

Development of these theilericidal compounds, parvaquone and subsequently, its derivative buparvaquone ensure the survival of cattle with clinical *T. parva* or *T. annulata* infection. Treatments with these agents do not completely eradicate theilerial infections leading to the development of carrier states in their hosts. These compounds are highly effective when applied in the early stages of clinical disease but they are less effective in the advanced stages in which there is extensive destruction of lymphoid and hematopoietic tissues (Sibeko, 2009). Each of these drugs has been introduced to the market within the last 20 years (Norval *et al.,* 1992). Apart from theilericidal chemotherapeutics available for controlling the disease, scarce resources among the farmers, poor diagnosis and untimely administration of drugs remains as a significant constraint.

2.3.3 Immunization

Naturally acquired immunity in infected animals has led to the development of Infection and Treatment Method of immunization (ITM), using live *T. parva* sporozoites (Radley *et al*., 1975). Immunization of cattle against theileriosis by the infection and treatment method (ITM) (Radley, 1978), offers the prospect of a less costly and more effective control of the disease without continued reliance on expensive acaricides. The vaccination regime involves inoculation of cattle with sporozoites of the original stabilate, at the same time treating with long-acting formulation of oxytetracycline (Di Giulio *et al*., 2009). The infection and treatment method provides life-long immunity against the homologous immunizing strain. For a number of reasons this control method has not been adopted in many ECF affected areas. The method depends on liquid nitrogen for storage and maintenance of live parasites and immunized animals are often susceptible to differing immunological strains.

Benefits for immunization process against ECF are increasing the survival rate of calves in which the mortality rate decreases down to 2% annually among pastoralists (Lynen *et al.,* 2006). Also with vaccination, tick resistance to acaricides has been reduced, with reduced frequency of using acaricides and lower tick control costs by up to 50% (Lynen *et al.,* 1999). A shortcoming of the infection and treatment method of immunization procedure which has limited its practical application is that, immunization with one stock of the parasite does not provide protection against all other stocks of *Theileria* parasite (Radley *et al.,* 1975; Irvin and Mwamachi, 1983). Vaccine production involves different processes such as passage of the parasite through ticks and cattle, which may result in recombination which affects stabilates composition, therefore extensive infectivity testing and titration in cattle, to determine the safety and efficacy of the immunizing dose is very important (Di Giulio *et al*., 2009).

Protection engendered by this method of immunization is 'strain' specific; as a result attempts have been made to identify single stocks, or combinations of several parasite stocks that provide broad immunological protection (Bishop *et al*., 2001). Combination of several *T. parva* stocks in the vaccine to induce a broad protection was planned to be implemented in Eastern Africa where there is heterogeneity in the parasite populations (Odongo *et al*., 2006; Oura *et al*., 2005; Radley *et al*., 1975).

The most widely used ITM vaccine stabilate in Eastern Africa is a trivalent vaccine ('Muguga Cocktail'), which includes parasites from three stocks: Kiambu 5, Muguga and Serengeti-transformed. This trivalent vaccine has been used to immunize cattle in Malawi, Tanzania, Uganda and Zambia (Musisi *et al.,* 1992). Theileriosis cannot completely be isolated from other tick-borne diseases and immunization against ECF should be considered as only part of an integrated control of the whole package and it has to be cost effective and sustainable. Risk from other tick-borne diseases such as Anaplasmosis and Babesiosis limits adoption of reduced acaricide tick control practices following ITM, hence farmers do not see significant reduction in cost after adoption of the method (Kivaria *et al*., 2007).

Most of the animals at risk are maintained in extensive management systems and traditional tick control and chemotherapy alone hardly represent efficient methods for preventing damage caused by ECF. The limitations associated with these control methods and the shift away from intensive acaricide use in Africa have prompted a search for new, more effective and sustainable control strategies. It was generally recognized that cattle in enzootic areas could become immune after surviving a *T. parva* infection. Neitz (1953) found that cattle exposed to tick infection and given oxytetracycline during the incubation period could survive and develop immunity. This knowledge was used to develop an

immunization method based on a tick stabilate inducing infection followed by concomitant treatment (Radley *et al.,* 1975).

This method of vaccination commonly known as "Infection and Treatment Method (ITM)" has been tested in the field in several countries in eastern and southern Africa. Results demonstrated that it is very effective in a given area if properly administered (Bazarusanga, 2008). Nevertheless, the method was found to be insufficient for the ECF region-wide application. The immunity conferred by one strain affords protection against only a limited number of different *T. parva* stocks (Cunningham *et al.,* 1973; Irvin *et al.,* 1983). Failure to protect fully has been attributed to the existence of variability in the parasite population. Determining live parasite immunisation approach must be based on the parasite immunological diversity found in an area (Geysen *et al.,* 1999).

2.3.3.1 Delivery of ITM immunization to pastoralist cattle in Tanzania

The United Nations (UN) has estimated the world population to reach 9.6 billion by 2050 with the highest increase occurring in developing countries (UN, 2015). This radical population growth will therefore lead to high demand for livestock products, thereby creating wealth and livelihoods for the rural poor (UN, 2015). For instance, in Tanzania cattle production has increased over the years(Mashingo *et al.* 2014). Mashingo *et al.* (2014) reported that human population in 1961 was 9 million with 3 million cattle, and in 2012, it increased to 45 million and 28 million respectively.

After Ethiopia and Sudan, Tanzania is one of the largest cattle producers in Africa, which supports about 37% of the rural community (Mashingo *et al*., 2014). The livestock subsector therefore contributes immensely towards the economy of Tanzania with 13% of the Agricultural Gross Domestic Product (AGDP) and 3.8% to the National GDP, helping

towards the attainment of the development goals set out in the National Shared Growth and Reduction of Poverty (NSGRP), MKUKUTA of Vision 2025 (URT, 2010).

The agro-pastoral and pastoral livestock sector accounts for 70% of the national herd. In 1997, the overall costs of tick and tick-borne diseases amounted to US$ 64.7 million and mortality associated with ECF resulted in losses of US$ 35.1 million (Mcleod and Kristjanson, 1999). ECF is responsible for up to 70% of deaths in 6–8 month old calves (Homewood *et al.,* 2012; Lynen *et al.,* 2006) and represents a major constraint to the livelihoods of pastoralists and agro-pastoralists. After limited evaluation of live immunization during 1989–1992), Tanzania authorized deployment of ITM using the Muguga cocktail (MC). Until 1998, the major drawback of ITM was the percentage of animals that developed clinical ECF caused by the immunization. This resulted in a need to monitor vaccinated animals from days 14 to 26 post-immunization and treat those that reacted severely. Whereas the average percentage of severe reactors after ITM was 3.9%, between 15% and 44% immunization reactors were recorded in the Kilimanjaro region. The use of an OTC formulation with an increased concentration of 20–30 mg per kilogram (Lynen *et al.,* 1999) and the availability of two standardized batches of *T. parva* Muguga Cocktail stabilate (FAO-1 and FAO-2) (Morzaria *et al.,* 1999) virtually eliminated the problem of severe reactors. Currently, less than 0.05% severe reactors are recorded (Lynen *et al.,* 2006). This enabled immunization of large numbers of animals, which increased the acceptance of ITM by extension workers and livestock owners (Homewood *et al.,* 2006) and created the momentum for rapidly increased adoption of ITM in pastoral and agro-pastoral areas. Between 1998 and 2005, more than 130 000 animals were immunized in Tanzania using the improved 30 mg kg^{-1} OTC regime. Calves between one and four months of age in the pastoral livestock sector accounted for more than 95% of the animals immunized (Lynen *et al.,* 2006). By October 2008, the number

of animals immunized had increased to 361 000. ITM immunization has been privatized and delivered on a commercial basis since 2003.

In the pastoralist sector, in which a large number of cattle (mainly calves) can be vaccinated simultaneously, livestock owners pay US$ 6–7 per calf, whereas for smallholders, the immunization cost is US$ 12–14 per animal (Lynen *et al.,* 2006). The increased price for small-scale dairy farmers is due to reduced economies of scale through immunization of fewer cattle per visit and the fact that these are usually adult animals, requiring higher doses of OTC. Many pastoral communities live in border areas, where grazing and water resources are accessed flexibly. Disease intervention programmes, such as ITM, in these areas have been adopted by related communities across borders (Lynen *et al.,* 2006). Several analyses of ITM adoption and environmental impact in pastoral communities in northern Tanzania have been conducted (Homewood, *et al.,* 2006; Lynen *et al.,* 2006). These included long-term longitudinal studies in pastoral communities in the Ngorongoro Conservation Area.

2.3.3.2 The Infection and Treatment Method for ECF vaccination: past, present and future perspectives

In view of the development of ITM immunization in the early 1970s, there has been considerable debate concerning the advantages of the technology among scientists and veterinary policy makers.Regardless of the effectiveness of the vaccine in stimulating protection experimentally, until recently, acceptance of ITM has been limited due to the concerns of veterinary authorities, the private sector and scientists on technical and policy issues. These included complexity of stabilate production, stabilate variability, the need for extensive *in vivo* testing in cattle and widespread field use of OTCs. There is also problemsconcerning the carrier state which is incessant, potentially tick-transmissible

infection that is typically induced by ITM and by natural infection (Bishop*et al.,* 1992; Geysen *et al.,* 1999).

Consideringthe widespread use of ITM which plays a significant role in ECF control, practical improvements to the current ITM technology at several levels has been a priority. Based on the drawbacks of ITM, conclusions from a stakeholder consultation held in August, 2014 recommended the most desirable improvements in the ITM vaccine or manufacturing process as follows:

1. Improving the cold chain. There were several aspects to this:
   - Removing the need for liquid nitrogen to store the vaccine
   - Developing a simpler diluent which can be kept at room temperature
   - Improving the post-reconstitution viability to allow for a longer duration between administration and delivery especially important in smallholder areas with straws of 40 doses or more.
   - Providing better storage equipment for transport of the vaccine
2. Removing the need for oxytetracycline
3. Removing the need for live animals during the production process
4. Developing ticks which can yield greater number of sporozoites
5. Eradicate the vector
6. Produce one vaccine which can be used in all countries
7. Provide the vaccine in a smaller dose packaging
8. Decrease the cost of the vaccine
9. Develop a sub unit vaccine
10. Develop an ECF vaccine which can be administered as an aerosol
11. Validate the use of other antibiotic formulations (ECF ITM report, August 2014).

The infection and treatment method (ITM) of vaccination plays a vital role in ECF control. However, this method ofvaccination has several limitations, such as high cost of simultaneously used antibiotics as well as delivery issues.Therefore, research in the direction of development of a recombinant vaccine that is equallyless expensive to manufacture and simpler to deliver should be aimed at which might ultimately result in an improved vaccine (Di Giulio *et al.,* 2009).

2.4 Diagnosis of *Theileria parva* infections

For routine diagnosis, conventional methods are used, whereas serological and molecular methods are utilized for research purposes and epidemiological studies. Conventional methods involve microscopic examination of Giemsa stained thin/thick blood films for detection of piroplasm and lymph node biopsy smears for detection of schizont. The mostly used serology tests are Indirect Immunofluorescent Antibody Test (IFAT) and Enzyme Linked Immunosorbent Assay (ELISA). Several molecular biology techniques have been employed as well. These include; conventional PCR assay, PCR-based hybridization assay, PCR-based RFLP assays, Real time PCR assays and Loop-mediated isothermal amplification (LAMP) assay.

2.4.1 Conventional methods

The common field diagnosis for theileriosis is based on clinical signs of the disease and microscopic examination of blood and lymph node smears for the presence of piroplasms and schizonts respectively. This is a method of choice for early and rapid diagnosis and treatment of the disease. These blood films are fixed in methanol and stained in 10% Giemsa stain for 30 minutes. Since piroplasms can be detected in clinically normal carrier animals, these should not be used to confirm the positive case during diagnosis unless the schizonts are seen (Norval *et al.,* 1992). At these stages the parasites can be differentiated

from other blood parasites by morphological appearance and staining properties, but the disadvantage of that method is that, *T. parva* schizonts and piroplasms are difficult to differentiate from those of other *Theileria* parasites (Sibeko, 2009, Morzaria *et al*., 1999). In dead animals, impression smears from cut lymph nodes or other lymphoid organs like spleen can be prepared, fixed, stained with Giemsa and examined under microscopy. Normally, piroplasms appear 5-8 days following the detection of schizonts, and their detection can be effected through thin blood film preparations.

2.4.2 Serological methods

Serological tests are reliable methods for detection of low grade or previous infections where measurement of antibody levels of a cattle herd is used for assessing the response to natural infection and also to vaccination for the purpose of disease control (Thrusfield, 2000). Serological methods such as the indirect fluorescent antibody test (IFAT) and enzyme linked immunosorbent assay (ELISA) tests are available for the detection and quantification of antibodies to tick-borne parasites.

The most widely used serological assay for detection of *T. parva* antibodies is indirect fluorescent antibody test (IFAT) although it has cross-reaction with other *Theileria* species (Burridge and Kimber, 1972, Goddeeris *et al*., 1982). Indirect ELISA has been used as highly specific and sensitive test for detection of *Theileria* spp. (*T. parva* and*T. mutans*) infection antibodies (Katende *et al*., 1998). The Polymorphic Immunodominant Molecule (PIM) based ELISA is highly sensitive and specific and is used for the screening of large number of bovine sera antibodies against *T. parva* in epidemiological studies. The principle behind the ELISA technique is based on PIM-base antigen expressed as recombinant fusion protein glutathione S-tranferase (Katende *et al.,* 1998). To detect *T. parva* antibodies both schizonts and piroplasms can be used, although the schizonts antigen is preferred as it confers a long duration of a serological response.

Evidence of *Theileria parva* infection is assessed by increased antibody levels as measured in an indirect ELISA test by the percent positivity (PP) of serum samples relative to a strong positive reference serum (Katende *et al*., 1998). Although ELISA is more sensitive (>99% sensitivity) and specific (94%-98% specificity) than IFAT, it has the same problem as IFAT since an animal may remain positive while it has already cleared the parasites (Bishop *et al*., 1992 and Sibeko, 2009). Other serological tests for diagnosis of theileriosis include coagulation test, capillary tube agglutination, indirect hemagglutination assay, complement fixation, and immunodiffusion test. Assessment of stable and unstable epidemiological states has been based on the prevalence results of serological diagnostic tests in extensive field survey (Norval *et al.,* 1992; Perry *et al.,* 1996).

2.4.3 Molecular techniques

Molecular tools can be used to differentiate *Theileria* species. The tests have proved to be highly sensitive and specific for detecting parasite DNA in blood. These molecular techniques range from the classical single polymerase chain reaction (PCR) to more advanced techniques based on the use of DNA probes (Collins *et al*., 2002). Early molecular detection techniques involved the use of probes to detect repetitive regions in parasite genomic deoxyribonucleic acid (DNA). With PCR, it is not always possible to detect mixed infections, but reverse line blot (RLB) hybridization assay which target the 18S ribosomal ribonucleic acid (rRNA) gene has been developed for identification and differentiation of distinct piroplasm species present in the same sample and therefore can detect subclinical infections (Sibeko*et al*., 2008).

2.5 Immunity to East Coast fever

Cattle exposed to *T. parva* infection develop an antibody response to several parasite proteins. Among these specificities, antibodies to the polymorphic immunodominant

molecule (PIM) have proved to be the most reliable in measuring exposure to *T. parva*. PIM can be polymorphic in size between different *T. parva* strains (Toye *et al.,* 1995a), but there is sufficient conservation of sequences between the variants for PIM to function as a diagnostic antigen (Katende *et al.,* 1998; Toye *et al.,* 1996). Hence, an ELISA assay based on recombinant PIM from the Muguga stock of the parasite is routinely used to determine prevalence and sero-conversion to the ITM vaccine (Patel *et al.,* 2015).

Early studies on passive transfer of sera from immune cattle failed to protect against challenge. In addition, immunization with parasite antigen failed to protect against ECF (Wagner *et al.,* 1974), leading to a consensus in the ECF literature that antibodies did not play a major role in mediating immunity to the disease. However, indirect evidence for a role for antibodies in mediating immunity to ECF was derived from the observation that cattle can develop sporozoite neutralizing antibodies (Musoke *et al.,* 1982). However, this activity is seen after multiple sporozoite exposure, suggesting weak immunogenicity of the relevant targets.

Animals that recover from infection with *T. parva* are solidly protected against homologous challenge (Burridge and Kimber, 1972). The serum from immune cattle contains antibodies against all stages of the parasite (Burridge and Kimber, 1972). The parasite is antigenically exposed to the immune system of the animal at the sporozoite surface, the lymphocyte surface, the merozoite surface and the infected red cell surface. The most relevant immune responses against *T. parva* are those against the sporozoite surface and those against the schizont.

Musoke and colleagues claim in two studies (Musoke *et al.*, 1982) that a role for serum antibody in recovery and protection should not be dismissed. The authors' reports that serum with high levels of antibody could neutralize the infectivity of sporozoites for cattle

and that, after repeated challenge with sporozoites of *T. parva* stock, cattle were immune to challenge with the homologous parasite and to two additional stocks. These humoral responses appear to recognize a wide range of *T. parva* isolates. Studies using a panel of monoclonal antibodies have identified neutralizing antigens on the sporozoite surface. A 67-kDA protein (p67) has been identified as the major neutralizing antigen (Nene *et al.,* 1992). The immunizing potential of p67 was evaluated in cattle, using an *Escherichia coli*-expressed recombinant form (Musoke *et al.,* 1992). The results seem promising but protection is not yet satisfactory. Immunized animals generated high titres of specific antibody and six of nine were protected against severe disease following homologous challenge.

Most of the information on immune mechanisms against *T. parva* has been obtained from studies of cattle immunized by the infection and treatment method. Several features of the immunity indicate that there is a lack of effective protection from antibody and that the immune mechanisms are cell-mediated and targeted against the schizont-infected cell. Transfer of immune serum to naïve cattle fails to protect against the disease and immune animals invariably develop a schizont parasitosis following challenge (McKeever, 2006). These animals are also protected against challenge with large numbers of autologous parasitized lymphoblasts propagated *in vitro*. Also, the lack of correlation between schizont-specific antibody and protection (Emery, 1981) suggested the operation of cell-mediated immune responses.

Further elucidation of the mechanisms responsible for clearance of infection was facilitated when techniques were developed for the culture of *T. parva*-infected bovine lymphocytes. Emery (1981) showed that cultures from naïve cattle generate only non-specific killing activity when cultured with infected cells but that killing is parasite

specific and restricted to autologous targets in cattle undergoing immunization or challenge with *T. parva*. Emery (1981) demonstrated later that the effector cells responsible for this activity reside in the T-cell compartment and that the killer cells belonged to the CD8+ T-cell population. Killing activity is present in the immune lymph node from day 7 after challenge, and in the blood between days 8 and 12 after challenge, coinciding with the emergence of parasitosis and its elimination (McKeever and Morrison, 1998).

McKeever and Morrison, (1998) shed more light on the nature of genetically restricted cytotoxic T-lymphocyte (CTL) responses in immune cattle undergoing challenge with *T. parva*. They established that these CTL were parasite-strain specific and were restricted by the MHC class I phenotype. The basis of the MHC restriction of T cell responses has since been established to be the association of foreign antigenic peptides with a groove on the outer regions of the class I MHC protein. The efficacy and specificity of *T. parva*-specific CTL responses depends therefore on selection of parasite peptides by host MHC antigens (Goddeeris, 1990). This class I MHC haplotypes has different ability to restrict in this regard.

McKeever, (1995) endorsed the role of CTL in immunity to the parasite. The first study established that transfer of responding CD8+ T-cells between immune and naïve identical twins conferred protection against infection with the immunizing stock of the parasite. The second study demonstrated that strain specificity of CTL responses correlates with the ability of immunized cattle to resist heterologous challenge.

Other cellular mechanisms may also contribute to protection of immune cattle against challenge. Baldwin *et al.* (1987, 1992) report parasite-specific responses in class II MHC-

restricted CD4+ T cells derived from immune cattle after challenge, some of which show killing activity. In short, there is sufficient evidence that immune cattle protect themselves against *T. parva* challenge by deploying parasite-specific class I MHC-restricted CTL. The response is vigorous and effective. Homologous challenge is quickly eliminated. The response is apparently dependent on the input of CD4+ T-cells. It may also be that the parasite is capable of evading the response through antigenic diversity, which is maintained through sexual recombination in the vector (McKeever, 1995).

2.6 The Role of Cytokines in Infections

Cells from both the adaptive and innate arms of the immune system secrete small proteins known as cytokines. These proteins are essential to the function of the human immune system. Cytokines perform their actions by binding to cell surface receptors, triggering a signal cascade leading to the modification of gene expression. Thus, the release of cytokines can have direct action on cells. Cytokines can stimulate or inhibit cell activation, they can promote proliferation, direct migration, induce death, and promote cell maturation (Lai and Yap, 2010). Berthoud *et al.* (2011) have shown that understanding the type and quantity of cytokine present during an infection can help understand important mechanisms involved in immunity and can give clues as to the type of cells present.

The evidence of infections is stored within memory immune cells that arise during cellular immune response and remain in memory in case disease reappears at a later time. Hypothetically, all infections eliciting cellular immunity leave behind disease (antigen) specific T and B-lymphocytes. These cells may therefore be queried to test for previous exposure to infections. The presence of disease-specific lymphocytes is most frequently determined by monitoring production of cytokines, small signaling proteins released by

immune cells in response to antigenic stimulation. The best example of this concept is monitoring IFN-γ for diagnosis of tuberculosis (Kwa and Revzin, 2012).

Beyond the detection of IFN-γ, there is a strong interest in profiling other cytokines to discriminate between active and latent stages of TB (Casey *et al.,* 2010). Recent studies have suggested that TNF-α secretion by CD4+ T cells can be used to discriminate active and latent TB. In the case of viral infection, multiple studies have described cytokines as correlates of HIV progression or suppression (Pantaleo and Harari, 2006). In these studies, presence of Th1 cytokines, IFN-gamma and IL-2 was associated with suppression of HIV and was observed in long-term non-progressors, whereas Th2 cytokines were associated with rapid disease progression.

Monitoring the dynamics of pro-inflammatory and anti-inflammatory cytokines may also be important for cytokine interference therapy for HIV infection, assisting in lowering the viral set-point in patients (Katsikis *et al.,* 2011). For parasite infection, cytokines also have an appreciable impact. For instance the polarization to Th2 response in severe malaria infection usually results in higher serum levels of TNF-α in conjunction with lower IL-12 and IFN-γ levels. Therefore, serum TNF-α level can assist in predicting fatal outcomes (Angulo and Fresno, 2002).

The clinical signs of ECF emerge during the schizont stage of the parasite and host death can occur before the appearance of piroplasm. Thus, acute immune-response to *T. parva* infection aiming at the proliferating schizonts might heighten the disease progression. It has been reported that the clinical symptoms in acute infection in other protozoan diseases such as malaria and trypanosomiasis, are coupled with pro-inflammatory cytokines (Hirunpetcharat *et al.,* 1999).

Dinarello, (1996) reported that the acute inflammatory response is arbitrated through the fueled expression of pro-inflammatory cytokines, mostly tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β). These cytokines are known to be crucial mediators of the responses to microbial infection and induce fever, hypotension, and the production of other cytokines, chemokines and adhesion molecules. Hence, dysregulated activation of pro-inflammatory cytokines can lead to various inflammatory disorders.

Furthermore, experimental dispensation of both IL-6 and TNF-α to cattle stimulated the systemic inflammation, comparable to the clinical sign to ECF (Jongen-Lavrencic *et al.,* 1996). This raises the likelihood that the pro-inflammatory cytokines could be involved in the severity of the disease in *T. parva*-infected animals. *T. parva* transforms bovine lymphocytes, which can proliferate continuously without any exogenous growth factors and the parasite activates the host cell signal pathways including the nuclear factor-κB (NF-κB) and the activator protein-1 (AP-1). These transcriptional factors seem to control the expression of numerous cytokines controlling cell proliferation and anti-apoptosis in parasitized cells (Dobbelaere and Heussler, 1999). Through *in vitro* studies, cytokine profiles are well known for their roles in upsetting the proliferation of *T.parva*-transformed cell lines.

McKeever *et al.* (2006) showed that a number of *T. parva*-infected cell lines demonstrated a variation in the mRNA expressions of IL-1β, IL- 2, IL-4 and IL-6. Moreover, *T. parva*-infected T-cell clone provoked the up-regulation of IL-6, IL-10 and TNF-α mRNA, which are not uttered before the infection. However, these cytokines does not appear to restrain the cell growth of all cell lines explored and it is not clear whether cytokines are involved in ECF pathogenesis.

Yamada *et al.* (2008) reported that the upsurge of pro-inflammatory cytokines, IL-1β and IL- 6, account to a certain extent for the clinical signs of ECF. These cytokines form a part of the acute-phase response in cattle. The examination of cytokine profiles in *T. parva*-infected cattle would help the understanding of the disease pathogenesis. The elucidation of the mechanism of the pathogenesis of ECF may help developing methods needed to control the disease.

2.7 Toll-like Receptors (TLRs)

Toll-like receptors (TLRs) are pattern recognition receptors that recognize a wide variety of microbial molecules, called pathogen-associated molecular patterns (PAMPs).The innate immune response can be activated by diverse pathogen-associated molecular patterns through the Toll-like receptor (TLR) family of receptors, and this plays an important role in directing the acquired immune response (Iwasaki and Medzhitov, 2004). Among the 11 mammalian TLRs, TLRs 3, 7, 8, and 9 are present in the endosomes of cells and detect nucleic acids of intracellular DNA and RNA pathogens (Takeda *et al.,*2003).

It has been reported that upon the recognition of a pathogen or its components by way of TLRs, immature antigen-presenting cells such as dendritic cells (DCs) undergo maturation, exit the site of infection, and migrate to the draining lymph node. The mature DCs present their “acquired” antigens and stimulate antigen-specific T-cells, leading to antigen-specific acquired immunity along with immunological memory. The type of acquired immunity developed may be influenced by which TLR(s) is activated as well as the particular subset of DC activated. The use of TLR agonists as vaccine adjuvants may represent an effective strategy for the development of vaccines with improved protective immunity (Zhang and Matlashewski, 2008).

TLRs are important elements for host defense against every known category of human microbial pathogens, including protozoan parasites (Schulz*et al.,* 2005). TLR ligands stimulate innate, adaptive, and regulatory immune responses and as vaccine adjuvants represent a promising approach to stimulating strong immune responses and enhancing vaccine-induced protection(Schulz*et al.,* 2005).

Vaccine adjuvants are vital for effective development of defensive responses to antigens. Toll-like receptor (TLR) agonists are particularly becoming a promising prospect, as they employ the innate immune system to stimulate a more racy or robust and durable adaptive immune response (Hoeven *et al.,* 2016). Current knowledge on immune response has shown that most vaccines induce antibody responses that correlateto induce protective immunity against most pathogens. Further studies have also shown that antibody titers for most vaccines persistently increase which continues for decades. Vaccines which engage the use of live attenuatedstrains of pathogens are frequently potent by themselves,but majority subunit or killed immunogens are used as adjuvants which are capable of enhancing and shaping antigen-specific immune responses thereby rendering a delivery formulation by presenting vaccine antigens to the immune system in a more efficient way thereby increasing vaccine induced protective antibodyresponses (Plotkin, 2010; Amanna *et al.,* 2007).

Recent researches on the development of vaccine adjuvants have demonstrated that they could allow antigen sparing (e.g., novel influenza vaccines that would necessitate the rapid distribution to fight new pandemics and could enhance the strength and breadth of antibody responses). It has also been shown that adjuvants have the ability to subdue the challenges of inducing broadly neutralizing antibodies against both HIV-1 and influenza virus (Karlsson *et al.,* 2008).

Further studies have also shown that adjuvants can mediate their effects on humoral immunity by several mechanisms. These include enhancing uptake of antigen and transporting antigen at the site of immunization. Moreover, adjuvants can activate distinct innate immune pathways that profoundly alter both humoral and cellular immunity. Accordingly, the addition of TLR agonists have been used to boost vaccine responses and has been suggested as one means of enhancing the response to HIV-1 immunogens (Moody *et al.,* 2014).

The identification of the antigen recognition receptors for innate immunity, most notably the Toll-like receptors, has sparked great interest in therapeutic manipulation of the innate immune system. Toll-like receptor agonists are being developed for the treatment of cancer, allergies and viral infections, and as adjuvants for potent new vaccines to prevent or treat cancer and infectious diseases. As recognition grows of the role of inappropriate Toll-like receptor stimulation in inflammation and autoimmunity, significant efforts have begun to develop antagonists to Toll-like receptors as well (Kanzler *et al.,* 2007).

- **TLR7 agonist**

Synthetic TLR7 agonists are being tested in clinical trials to improve the efficacy of vaccines, reduce viral load, redirect allergic responses, and treat cancer (Kirschman *et al.,* 2012). The TLR7/8 ligand R848 has been approved by the U.S. Food and Drug Administration for use as a stand-alone entity, and has been proven to enhance the immune response to co-administered antigens as a vaccine adjuvant (Zhang and Matlashewski, 2008).

Toll-like receptors on immune cells are part of the innate or immediate immune response, but they promote adaptive immunity, or recognition of and response to specific viruses

and other pathogens. TLR7 activation leads to increased antigen presentation and enhanced activity of natural killer cells, antibody-producing B-cells, and CD4 and CD8 T-cells. A recent research finding, presented at CROI 2016, show that repeated dosing with a toll-like receptor 7 (TLR7) agonist suppressed viral replication for between three and four months in a small study of animals infected with an HIV-like virus. TLR7 agonists have previously shown promise in shock and kill strategies that might be part of a functional HIV cure (Whitney, J. and others. Repeated TLR7 agonist treatment of SIV+ monkeys on ART can lead to viral remission. CROI 2016, Abstract 95LB).

The concept that multiple TLR–ligand interactions are required for the induction of effective host resistance to pathogens has important implications for the design of improved strategies for vaccination and immunotherapy against infectious diseases. Individual TLR7, TLR8 and TLR9 agonists have already been used successfully as adjuvants to boost CD4+ and CD8+ T-cell responses to candidate microbial vaccine antigens. These agonists seem to be particularly effective when they are covalently conjugated to the immunogens (Trinchieri and Sher, 2007).

Therapeutic targeting of innate immunity with Toll-like receptor agonists or vaccine adjuvants and for that matter TLR7 agonist duringclinical and advanced preclinical programs represent only a fraction of the current efforts to clinically translate the current understanding of TLR and innate immunity. Many other strategies and tactics to stimulate or inhibit TLRs are being developed, and similar studies with other pattern recognition receptors (PRRs) are just beginning. The list of disease states for which one or more TLR represent a reasonable target is growing rapidly. This will surely continue to be a productive field for drug development in the future (Kanzler *et al.,* 2007).

The relevance of TLR stimulation in the induction of immunity to pathogens set the stage now for major research effort to examine the protective efficacy of TLR7 in vaccines against other tick-borne diseases.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Toll-like Receptor 7 (TLR7) agonist

The Toll-like receptor 7 agonist was delivered as a water-in-oil emulsion. The water-in-oil base enabled long-acting stimulation of the host innate immunity to provide protection of sufficient duration. The slow release is critical to allow stimulation of the innate response over a period of months during which naïve calves are first exposed to pathogens, to emulate natural field settings. The emulsion was obtained from IDRI (Seattle, WA USA). IDRI is a non-profit institution that develops adjuvants under Good Manufacturing Practice (GMP) criteria. Initially, the TLR7 agonist was tested in calves to identify the optimal dose and approximately 5μg (1ml) of the TLR7 agonist was used for testing immune stimulation in crossbred calves.

3.2 Calves and Experimental Design

Twenty F1 crossbred calves (Zebu x Sahiwal), aged 3-5 months old were purchased from a commercial Mruazi Heifer Breeding Unit near Tanga, Tanzania. These were used to test the protective capacity of stimulation of the innate immune system with TLR7 agonist. The calves were *T. parva* parasite free at the time of purchase, although they all had low level of maternal antibodies to *T. parva* as detected by an indirect ELISA. The basal antibody level was expected for calves grazed in ECF endemic areas. To test protection against *T. parva*, the calves were split into four groups: group 1 (n=10) were inoculated with the TLR agonist on day 0, followed by *T. parva* parasites 24 hours later (day 1), without oxytetracycline (TLR7-agonist / adjuvant group); group 2 (n=4) were immunized with *T. parva* by infection and treatment on day 1 (ECF-vaccination control group); group 3 (n=4) were infected with *T. parva* parasites on day 1 (Infection control

group) and group 4 (n=2) were naive calves not given anything (negative control). A group size of n=4 was proposed based on our prior individual studies with *T. parva* that showed a statistically significant difference in onset of parasites in blood and clinical signs. ECF vaccination entailed inoculation with the Muguga cocktail which contains three strains of *T. parva* sporozoites. The standard dose of Muguga cocktail of *T. parva* is generally fatal to 100% of naive cattle within 18 to 20 days of inoculation. The study applied ethical principles ensuring its quality and integrity and was conducted along ethical regulations of the Sokoine University of Agriculture (SUA), Morogoro, Tanzania.

3.3 Monitoring Parameters

To track innate responses after TLR7 agonist stimulation, we measured body temperature andskin thickness at injection site every 6 hours in the first 2 days and every day thereafter. Following *T. parva*infection, complete physical examination, including rectal temperature, palpation of peripheral lymph nodes was performed on each animal at least once per day, for 21 days.Blood was collected from the calves daily to monitor white blood cell (WBC) counts, percent lymphocytes, mean corpuscular haemoglobin concentration, specific antibody responses, level of interferon gamma,and onset of *T. parva* parasites. Detailed laboratory methods were performed as described hereinafter.At the onset of pyrexia (rectal temperature >39.5°C) and peripheral lymph node enlargement, needle aspirates were collected from affected nodes once daily, and Giemsa-stained smears of aspirates examined for schizont-infected lymphocytes. Calves proven with clinical ECF during the monitoring period were treated (2.5mg buparvaquone/kg body weight) based on a set of a priori criteria: fever >41°C for more than 48hand clinical signs consistent with *T. parva*.

3.4 Blood Samples

Blood samples were taken from each calf by jugular venipuncture using 5-ml vacutainer tubes (BectonDickson Vacutainer Systems, England) either with or without EDTA anticoagulant. Blood samples were kept in cool boxes with ice for not more than 30 minutes before processing in the laboratory. The bloodsamples were centrifuged at 3000×g for 20 min in the laboratoryand the serum or plasma aliquots were stored in a freezer at −20°C until ELISAtests were done. Blood smears were prepared, fixed and stained for parasitological examination. Blood samples for DNA extraction were kept frozenat −20°C until day of analysis.

3.5 Haematological Parameters

Blood parameters were measured on anMS4s automated haematology counter (Melet Schloesing Laboratories, France) using protocols approved by the manufacturer. The parameters and their normal ranges in bovines are as follows: white blood cell count, WBC (4.0-12.0m/mm³), hemoglobin, Hb (8.0-15.0g/dl), lymphocyte count (45.0-75.0%), mean corpuscular hemoglobin, MCH (11.0-17.0pg) and mean corpuscular hemoglobin concentration, MCHC (30.0-40.0g/dl).

3.6 DNA Extraction

Genomic DNA was extracted from whole blood using a commercial extraction kit (Zymoresearch, USA) according to the manufacturer's instructions.Briefly, DNA was extracted from 100ul of each blood sample. DNA templates were eluted in 50µl elution buffer provided with the kit, and stored at −20°C until analysis.The DNA yields were determined using a spectrophotometer (Jenway, Genova, UK).

3.7 Nested PCR for Detection of *T. parva*

Primers derived from the *T. parva*-specific104-kDa antigen (p104) gene were used in the PCR amplification aspreviously described by Odongo *et al*. (2010) and Iams *et al*. (1990).The sequences of the forward and reverse primers were 5'ATT TAAGGA ACC TGA CGT GAC TGC 3' and 5'TAA GAT GCC GAC TAT TAATGACAC C 3', respectively, for first round and 5'GGC CAA GGT CTCCTT CAG AAT ACG3'and 5'TGG GTG TGT TTC CTC GTC ATC TGC3', respectively, for the second round. The nested polymerase chainreaction (nPCR) amplifications were performed in a total volume of10 ul that included 6.25ul master mix (Quick-Load Taq 2X Master Mix, New England Bio Labs Inc.), 10 pmol of each forward and reverse primers, 2.5 ulDNA template and nuclease free water. The second round amplification used 0.25 ul of the first round products. Amplification conditions involved an initial denaturation step of 94^{o}C for five minutes, followed by 35 cycles of denaturation at 94^{o}C for 60 seconds, annealing step at 60^{o}C for 60 seconds and extension at 72^{o}C for 60 seconds in the first round. The second round amplification conditions were the same as for the first round except that an annealing temperature of 50^{o}C was used(Kabi *et al*., 2014).The reactions were carried outin a thermocycler (ProFlex PCR system, Applied Biosystems, USA). PCR products were separated on 1.5% agarose gels and images visualized and documented on a Gel Doc^{TM}(Bio Rad, USA). Positive nPCRproducts were identified as 277 bp DNA fragments.

3.8.1ELISA for Antibodies (Indirect ELISA)

The PIM-based enzyme-linked-immunosorbent assay (ELISA) described by Katende *et al*. (1998) was used to measure specific antibodies to *T. parva*. Optical density (OD) values were measured at 450nm on an Erba LisaScan II ELISA reader (ERBA diagnostics, Mannheim GmbH, Germany). The ELISA results were expressed as percent positivity (PP) values. The ELISA has been estimated to have a sensitivity of 99% and a specificity of 97% (Katende *et al.,* 1997). The OD readings were used to compute percent

positivity (PP) for each sample using the formula: Percent Positivity (%PP) = Mean OD (sample or negative control) divided by mean OD of positive control multiplied by 100. Any reading of 20 percent positivity or higher was considered positive.

3.8.2 ELISA for IFN-gamma

A bovine interferon gamma-specific ELISA assay kit was used as per manufacturer's specifications (Biorad, USA). Briefly, the plates were coated with 100 ul coating antibody and incubated at room temperature for one hour. The plates were then washed three times with wash buffer (0.05% Tween 20 diluted in 50mM TBS, pH 8.0), blocked with 200ul of buffer (2% Fish skin gelatin)and incubated for one hour at room temperature. The plates were washed three times and 100ul of diluted IFN-gamma standards as well as plasma samples (diluted) were added in duplicate wells. The plates were further incubated for 1h at room temperature and washed. Then 100ul of pre-diluted HRP-conjugated streptavidin was added to each well followed by a 1h incubation at room temperature. The plates were again washed, and 100ul of HRP substrate (TMB) was added to each well. The plates were agitated for a minute followed by incubation for 15 minutes. The reaction was stopped by addition of 100ul of 2M H_2SO_4, and absorbance was read at 450 nm on an Erba LisaScan II ELISA reader (ERBA diagnostics, Mannheim GmbH, Germany). The concentration of IFN-gamma in plasma was calculated by extrapolating from the respective standard curves, and the values were expressed as biological units of activity in nanogram per milliliter.

3.9 Data Analysis

Data were entered, checked and cleaned using Microsoft excel. Microsoft Excel was also used to compute descriptive statistics and graphical representations. Analytical statistical procedures (ANOVA and Chi-square) were carried out using Stat View (Version 5.0.1). Statistical significance was tested at the 95% confidence level.

CHAPTER FOUR

4.0 RESULTS

4.1 Responses of Calves Following TLR7 Agonist Stimulation and Parasite Challenge

The 20 calves split into 4 groups as described above, were monitored for up to 36 days following either simultaneous TLR7 agonist stimulation followed by *T. parva* parasite challenge, ECF vaccination (usually done as simultaneous *T. parva* inoculation with long acting oxytetracycline) or following infection with *T. parva* parasites. Group 4 calves were a blank control which was not given any thing. Monitoring was done by daily measurement of rectal temperature, skin thickness at injection site, parotid lymph node width, white blood cell count, percent lymphocytes and mean corpuscular haemoglobin concentration.

4.1.1 Rectal temperature

Calves which received TLR7 agonist showed a sharp increase of body temperature soon after administration of the adjuvant and 24h later their mean rectal temperature rose from 38.5°C to 40.7ºC. Mean rectal temperature of calves in this group 48h later was significantly higher compared to that of calves in the vaccinated (39.23±0.08) (P = 0.0024) and infected (38.93±0.11) (P = 0.0003) groups (Figure 1a). Following inoculation of *T. parva*, 24h later (day 2) the adjuvant group as well as the vaccinated group showed significantly higher temperature (39.23±0.08) compared to that of the calves in the infected group (38.93±0.11) (P=0.0058). By day 3, highest temperature was recordedamong vaccinated calves, while infected calves remained with normal (<39°C) temperature and calves in the adjuvant group retained intermediate temperature (39°C).

Figure 1a demonstrates the trend of body temperature of the calves during the first 3 days of the study and figure 1b shows temperatures of the calves from day 8 to day 21.

a. b.

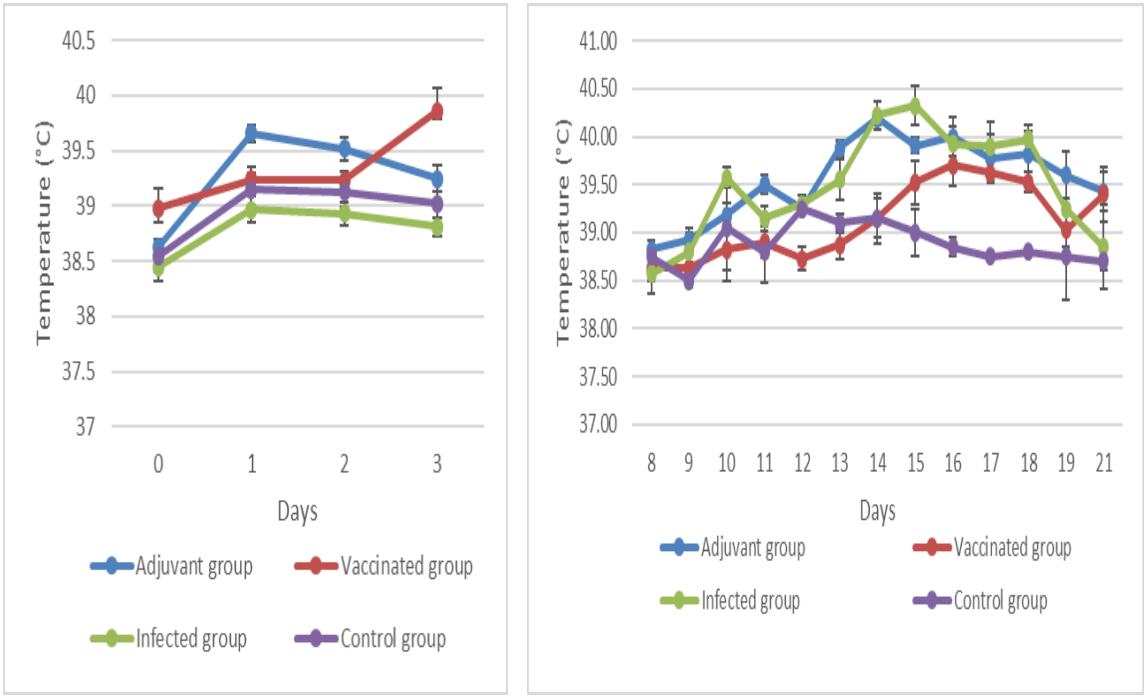

Figure 1: Rectal temperature of calves from day 0 to day 21

Rectal temperature was measured in the morning daily at the same time. Results presented are the mean temperatures with standard errors of the means.

Monitoring of temperature of the calves beyond the first 3 days of the study showed that although the temperatures went down between days 5 and 8, however starting from day 9, temperatures incrementally increased in calves of adjuvant and infected groups and reached 40°C on day 14. While the adjuvant group calves were fairly quick to lower their temperature from 40.2°C back to just below 40°C, the infected group calves sustained a high fever (40-40.5°C) for 72 hours (days 14-16). During days 15-17, all 4 calves in the infected group showed typical signs of ECF (Table 1) and were thus treated with buparvaquone at 2.5mg/kg body weight on day 18 and they all successfully recovered.

Out of the 10 calves in the adjuvant group, only one of them developed ECF-like signs (Table 1) without severe temperature and was treated on day 23 using buparvaquone as described above. Although calves in the vaccinated group also developed temperature up to 39.5°C between days 15 and 18, however their temperature gradually decreased thereafter.

4.1.2 Inflammation of the skin

Injection of the TLR7 agonist on the side of the neck led to a rapid thickening of the skin within the first 6h post administration and reaching a maximum skin thickness at the injection site 48h later (Figure 2). Mean skin thickness of calves in the adjuvant group (37.72±1.94 mm) at 48h post adjuvant administration was significantly higher compared to that of calves in the vaccinated (8.38±0.55 mm) ($P = 0.0408$) and infected (8.29±0.83 mm) ($P = 0.0355$) groups.

Skin thickness of calves in the other groups, on the other hand, remained unchanged post infection or vaccination. Further monitoring of skin thickness at injection site up to day 21 showed that calves which were stimulated with TLR7 agonist retained an inflammatory skin response, and although their skin thickness gradually decreased, however these calves showed a prolonged and heightened skin thickness compared to the other calf groups.

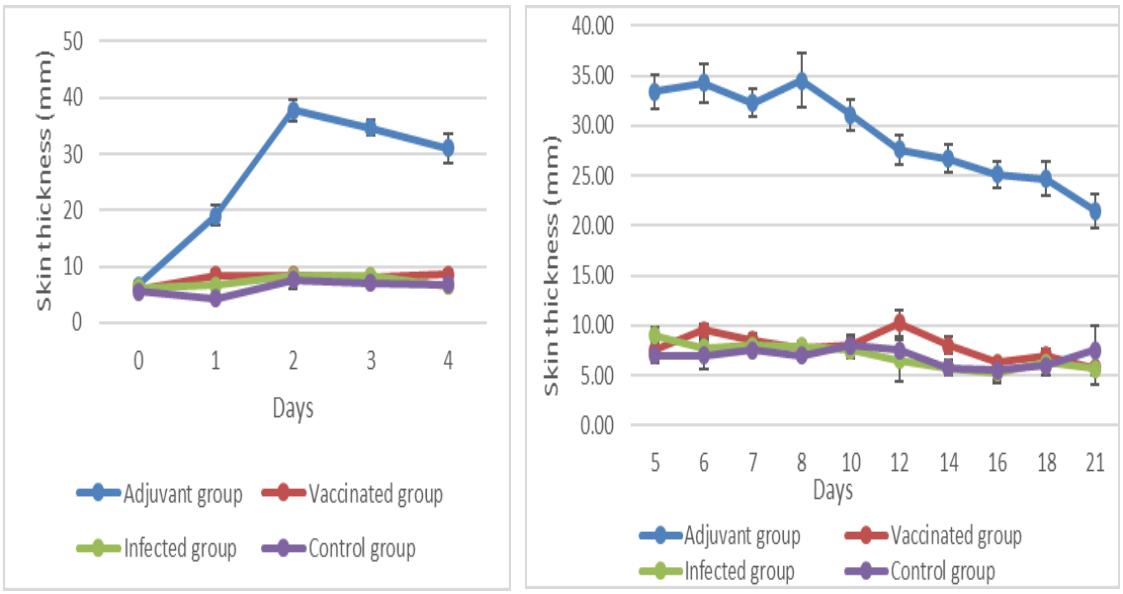

Figure 2: Skin inflammation in calves under different experimental treatments

Skin thickness was measured on day 0 and subsequently every 24h during the study. Results presented are the mean skin thickness (mm) with standard errors of the means at each time point.

4.1.3 Lymph node swelling

The width of prescapsular lymph node was measured to compare the progression of *T. parva* multiplication in calves of the different groups. Figure 3 demonstrates the trends of lymph node size development of calves in the different groups measured prior to injection of parasites (i.e., day 1) and subsequently days 4, 8, 12, 16 and 21. Swelling of lymph node was documented in calves of the adjuvant, vaccinated and infected groups, but the swelling was more pronounced among calves in the adjuvant group.

The lymph node of calves in the adjuvant group enlarged from 35.2mm on day 0 to 39.4mm on day 4 with a maximum size on day 8 (43.5mm). Maximum lymph node width was recorded on day 16 in calves of the infected group and on day 21 in calves of the vaccinated group. Interestingly, the lymph node size of calves in the adjuvant and vaccinated groups was almost the same (39 mm) on day 21.

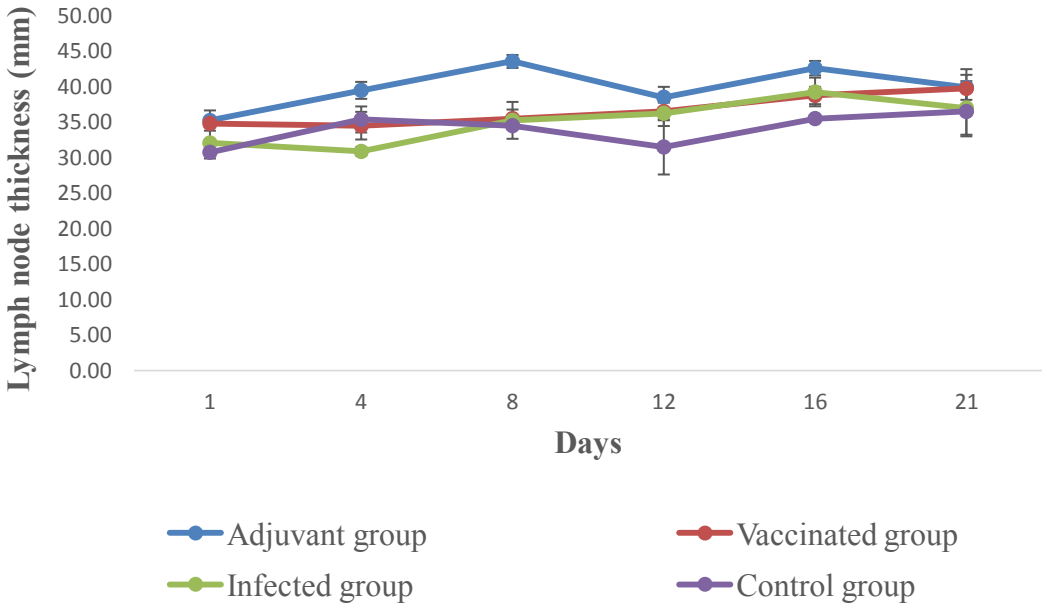

Figure 3: Lymph node width of calf groups from day 0 and beyond under different treatments

Lymph node width was measured on day 0 and subsequently every 24h during the study. Results presented are the mean lymph node thickness at each time point.

4.1.4 Haematological parameters

4.1.4.1 White blood cell counts

Following TLR7 agonist administration, calves in the adjuvant group showed significant elevation of white blood cell counts (14.96±1.13 m/mm³) compared to calves in the vaccinated (11.34±0.29 m/mm³)($P = 0.0444$) and infected (10.67±1.94 m/mm³) ($P = 0.0207$) groups 24h post adjuvant administration (Figure 4).

Mean white blood cell counts of calves in this group was highest (16.31±0.06 m/mm³) 48h post adjuvant administration and was at this time point also significantly higher compared to calves in the vaccinated (11.64±0.59m/mm³)($P = 0.0039$) and infected (10.67±1.94 m/mm³) ($P = 0.0008$) groups.Similarly calves in the control group had

significantly lower white blood cell counts (8.39±1.60m/mm³)compared to the experimental groups (P = 0.0413).

Figure 4 further demonstrates that following *T. parva* infection or vaccination, white blood cell counts of calves in groups 2 and 3 remained within physiological normal range (4.0-12.0 m/mm³), however the adjuvant group calves retained higher white blood cell count beyond the normal range (16.31±0.06 m/mm³) which resumed to normal level on day 3 and beyond.

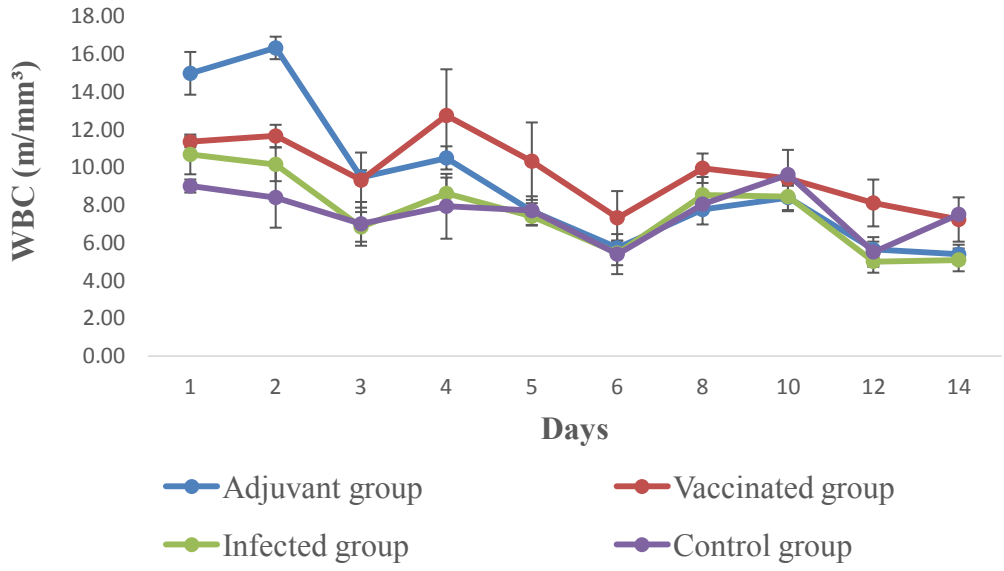

Figure 4: White blood cell counts of calf groups under different treatments

White blood cell counts were measured on day 0 and subsequently every 24h during the study. Results presented are the mean white blood cell counts with standard errors of the means at each time point.

White blood cell counts of calves in the adjuvant group decreased drastically after day 4 with a slight increase being recorded after day 6 which later decreased after day 10 (Figure 4). Similarly, calves in the infected group had initial decreased white blood cell counts (day 4 to day 6), which increased after day 6 but later declined after day 10 of the

study. Nevertheless, calves of the vaccinated group had higher white blood cell counts compared to calves of the other groups from day 4 onwards.

4.1.4.2 Mean corpuscular haemoglobin concentration

Mean corpuscular haemoglobin concentration (MCHC) was determined in order to indicate whether the calves became anaemic during the different experimental treatments. Results in Figure 5 show that the MCHC values for calves in the various groups was within physiological norm (30-40g/dl) during the first 4 days of the study. Similarly, no significant variation of the mean corpuscular haemoglobin (MCH), haemoglobin concentration (HB) as well as red blood cell counts (RBC) were observed within or between the calf groups from day 0 to day 4 (data not shown).

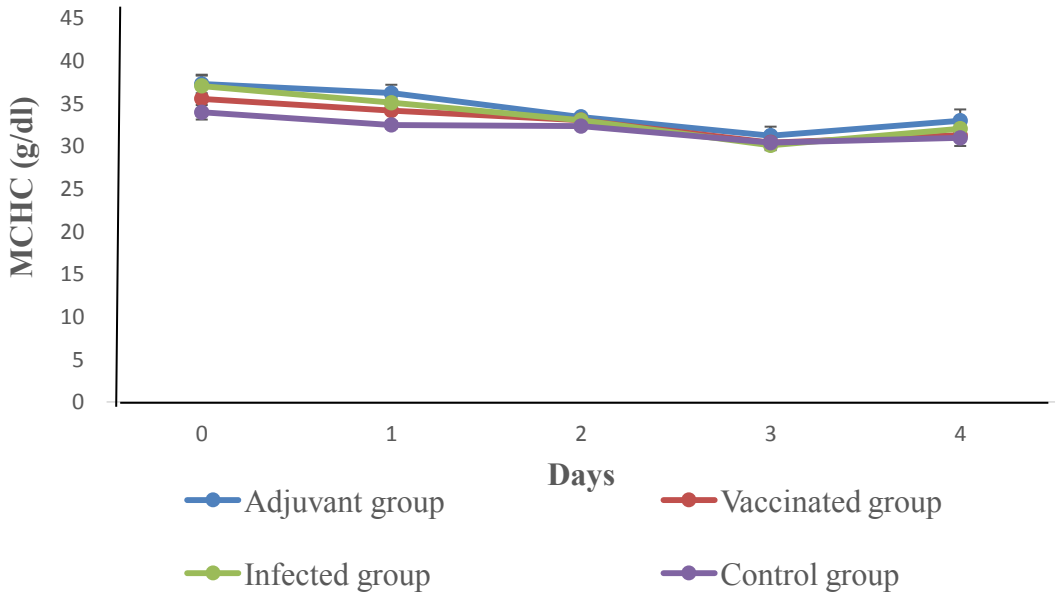

Figure 5: Mean corpuscular haemoglobin concentration of calf groups from day 0 to day 4 under different treatments

Mean corpuscular haemoglobin concentration was measured on day 0 and subsequently every 24h during the first 4 days. Results presented are the mean corpuscular haemoglobin concentration with standard errors of the means.

4.1.5 Interferon-gamma (IFN-gamma)

Interferon gamma was measured to monitor immunological progression following stimulation of calves with TLR7 agonist as well as post challenge with *Theileria parva* parasites using a specific ELISA assay. Figure 6 shows that plasma level of IFN-gamma of calves in the adjuvant and vaccinated groups rose sharply on day 4. Highest amplitude of IFN-gamma was observed in the vaccinated calves on day 8 (3.84±1.66 ng/ml) but the adjuvant group demonstrated longer persistence of plasma concentration of IFN-gamma (2.66±0.95 ng/ml) up to day 10. Calves in the infected group had lowest plasma IFN-gamma levels throughout the study, although this parameter was slightly elevated on day 10. A significant difference was shown between the infected (0.00±0.00 ng/ml) and adjuvant (2.85±0.89 ng/ml) groups (P = 0.004) on day 8. Similarly, a significant difference in levels of IFN-gammawas shown between calves in the vaccinated (3.84±1.66 ng/ml) and the infected groups (0.00±0.00 ng/ml) (P = 0.0015) on day 8. Figure 6 shows the trend of IFN-gamma in the various calf groups.

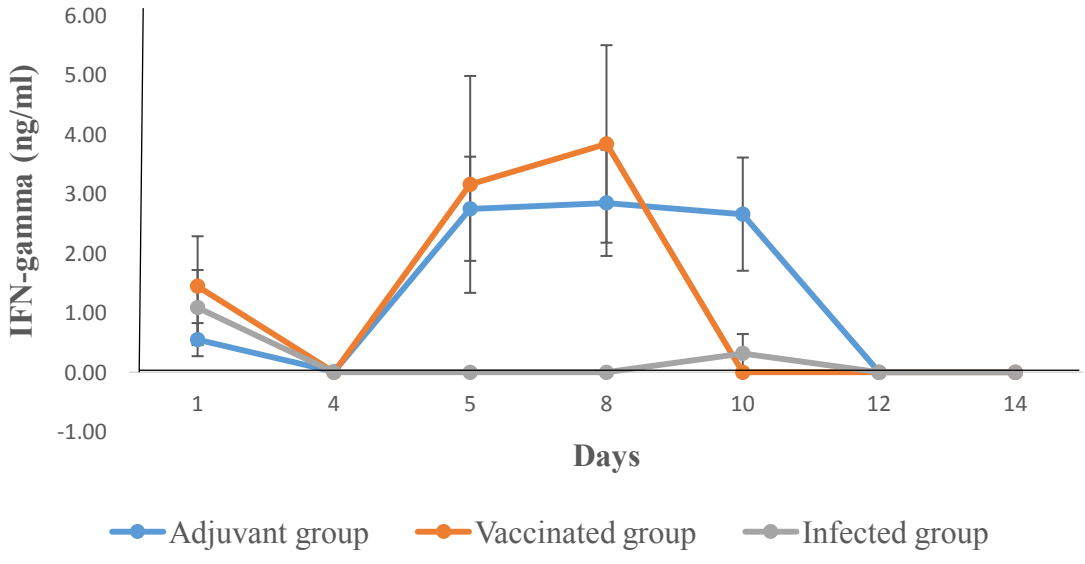

Figure 6: Quantification of interferon-gamma (IFN-gamma) in serum samples measured from calves following different treatments

Measurement of IFN-gamma levels was performed using an IFN-gamma ELISA and cross-reactive antibodies against bovine IFN-gamma, and quantified using a standard provided by the manufacturer. Results presented are the mean values with standard errors of the mean at each time point.

4.2 Appearance of T. parva Parasites and Specific Antibodies in Blood

Using the specific PCR method,no *T. parva* parasites were detected in blood of the calves in the various groups from days 0 to 13. Parasites were detected in calves of the various groups by day 14, when proportion of *T. parva* positive calves was 50% (N=2/4) in the infected group, 90% in the adjuvant group (N=9/10) and 100% in the vaccinated group (N=4/4). Further, by day 21 all calves in the three groups tested positive for *T. parva* (Figure 7).

Furthermore, all calves were tested for presence of *T. parva* specific antibodies at the beginning of the study. Based on the ELISA kit specification of a cut-off point of 20% as the measure of antibody positivity, all experimental calves had detectable levels of specific antibodies to *T. parva* (PP range 29-38) (Figure 7). The level of antibodies rose starting from day 14 and by day 16, a 55% rise was shown in the adjuvant group and a 53% rise in the vaccinated group but no rise of antibody level was noticeable on this day among the infected calves. Antibody level in this group as well as the adjuvant and vaccinated groups increased rapidly after day 16.

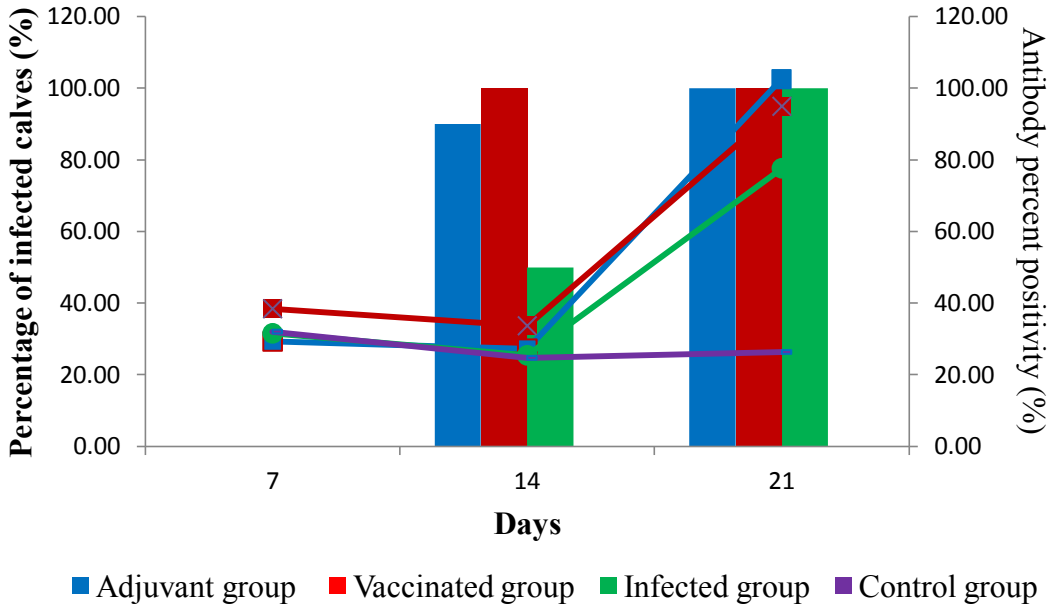

Figure 7: Sero-positivity and percentage of infected calves following different experimental treatments

Shown are results of percentage of infected calves (Bars) and antibody percent positivity (Lines) of calves during the study.

4.3 ECF Clinical Signs

The calves were monitored for clinical signs for *T. parva* infection (ECF). Any calf that showed any of the following signs; elevated temperature (>39.5°C), nasal discharge, lacrimation, coughing, diarrhoea, body weakness, loss of appetite and grinding of teeth was recorded and put under closer follow-up observation. Nasal discharge, lacrimation and cough appeared earliest among calves starting from day 15. While nasal discharge was first seen in a calf in the adjuvant group (Figure 9A), lacrimation and coughing were simultaneously observed in calves in the infected group.

While weakness and diarrhoea (Figure 9B) were recorded in one calf (ID 1195) in the adjuvant group on days 19-20, grinding of teeth was shown by one calf (ID 1176) in the vaccinated group on day 22. All four calves in the infected group showed rectal

temperature above 40ºC for more than 48h and were therefore treated using buparvaquone at 2.5mg/kg body weight on day 18. None of the vaccinated calves showed any signs of clinical disease, whereas 1 calf out of 10 in the adjuvant group developed fever for two days and was treated for ECF on day 23 (Table 1).

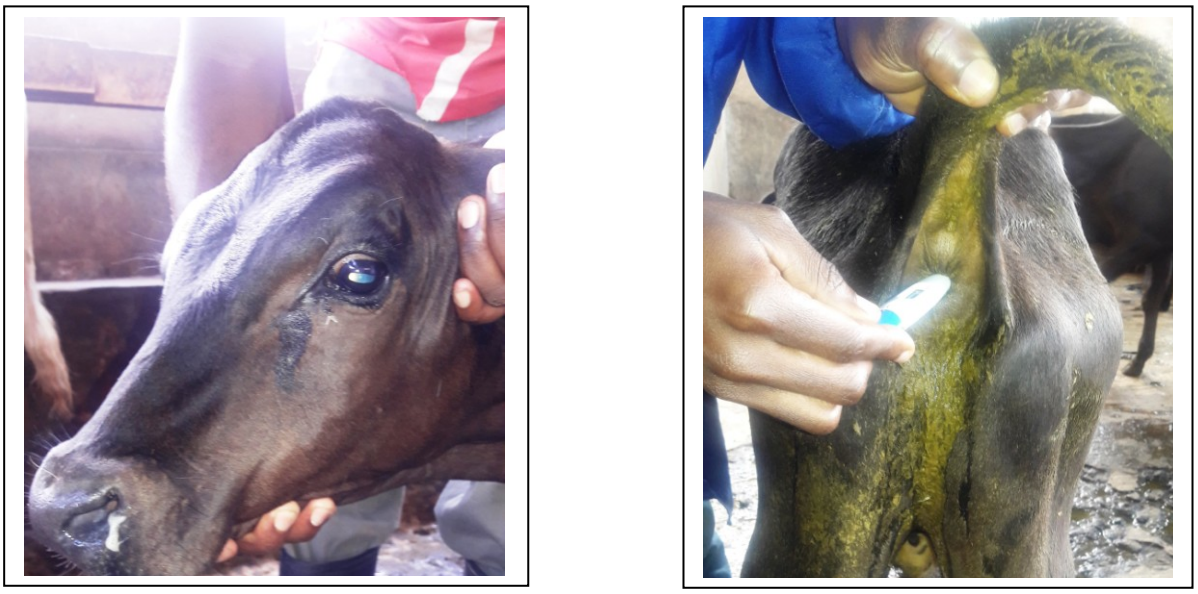

Figure 8: Clinical signs in a calf within the adjuvant group (ID 1195) following TLR7 administration and parasite infection

(A) Nasal discharge and lacrimation was observed in one calf in the adjuvant group after day 15. (B) Diarrhoea was observed after day 21 in one calf of the adjuvant group.

Table 1: **Day of appearance of clinical signs following TLR7 administration and parasite infection**

Groups	ND	LM	ECF Treatment	G T	CO	D	W
Adjuvant	15(1)	16(1)	23(1)	-	19(1)	21(1)	19(1)
Vaccinated	17(1)	17(1)	-	22(1)	22(3)	-	-
Infected	16(1)	15(1)	18(4)	17(1)	15(1)	-	-

Table 1: The numbers represent days when each clinical sign was first observed or when the animals were treated. Numbers in brackets show the number of calves showing the respective ECF sign. ND - Nasal discharge, LM – Lacrimation, GT- Grinding of teeth, CO – Coughing, D – Diarrhoea, W – Weakness.

4.4 Trends of Rectal Temperature, White Blood Cell Counts, and Percent Lymphocytes in Relation to ECF Clinical Signs

Figure 9 demonstrates trends of white blood cell counts relative to body temperature of calves during 21 days of monitoring following either, adjuvant stimulation prior to *T. parva* infection (Figure 9a), ECF vaccination (Figure 9b) or *T. parva* infection (Figure 9c). The figures indicate two high temperature peaks, with early temperature peak between days 1-3 and second temperature peak between days 14-16.

As reported above, TLR7 agonist induced a significant temperature rise in calves. Additionally, parasite challenge also induced a rise of temperature, although this was to a lesser extent, compared to the TLR7 agonist effect. Thus, temperature of calves in the infected group rose from 38.5°C to 38.9°C 24h following parasite challenge, whereas the temperature of calves in the vaccinated group rose 48h post vaccination from 38.9°C to 39.8°C. Highest temperatures were recorded in all calves during the second high peak, whereas calves in the adjuvant group recorded a temperature of 40.2°C on day 14, which later naturally declined to normal after day 18. Highest peak temperature for infected group calves was 40.3°C on day 15 and remained high for 72h, before declining after the 4 calves in this group were treated for ECF infection on day 18.

Highest temperature shown by ECF vaccinated calves was 39.7°C but this was transient between days 16 and 18. When the trend of white blood cell and lymphocyte counts was

examined at same time points used to describe trend of rectal temperature, it appeared that white blood cell counts gradually decreased 2-3 days after parasite challenge or vaccination and reached lowest level on days 14-16, a time point when the calves showed highest rectal temperature and most ECF disease clinical signs. Comparison of lowest WBC counts attained by the different calf groups shows that infected group calves had the lowest counts (4.26 m/mm^3), followed by adjuvant group calves (5.38 m/mm^3), whereas vaccinated calves had a slightly higher WBC counts (6.34/mm^3) when compared on same days.

Furthermore, data on lymphocyte counts provides evidence of lympho-proliferation during the first 6 days after parasite challenge or vaccination (Figure 10). As the data portrays, lymphocyte counts of calves in the infected, vaccinated and adjuvant groups significantly rose from day 1 to day 6 and highest lymphocyte counts were recorded on day 12. Thereafter, the trend of lymphocyte counts was similar to that of WBC, with a decrease on days 14-16, coinciding with elevation of rectal temperature. Lymphocyte counts, like WBC counts resumed to normal after day 18, when the infected calves were treated, whereas calves in the adjuvant and vaccinated groups passed through the critical period without adverse disease effects or requirement for ECF treatment, except for one calf in the adjuvant group.

a. Adjuvant group b. Vaccinated group c. Infected group

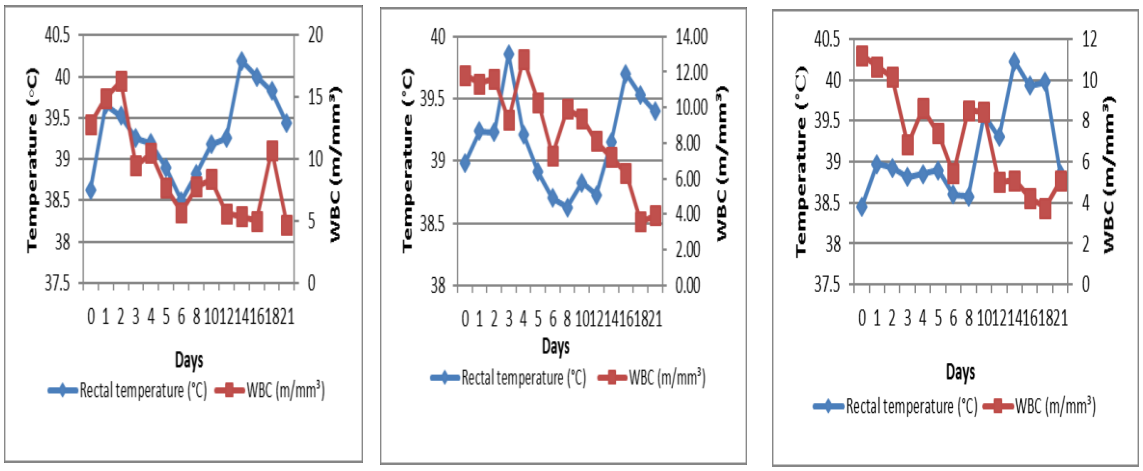

Figure 9: Comparison of temperature and white blood cell counts of calves following different experimental treatments

Shown are results of mean rectal temperature (blue line) and mean white blood cell counts (red line) of calves during the study.

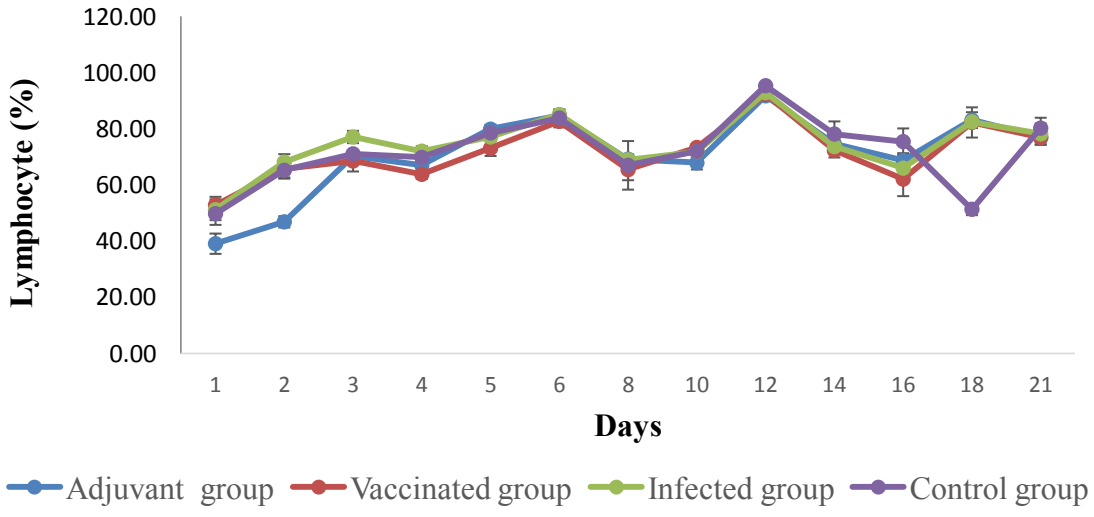

Figure 10: Percent lymphocyte of calves under different experimental treatments

Shown are results of mean percent lymphocyte of calves with standard errors of the means at each time point.

CHAPTER FIVE

5.0 DISCUSSION

The primary objective of this study was to determine whether TLR7 agonist could effectively induce innate immunity in calves and replace the use of long acting tetracycline, which is usually injected simultaneously with live *T. parva* sporozoites during the infection and treatment method for ECF vaccination. A major finding of this study is that for the first time it has been classically demonstrated that use of long acting tetracycline may be substituted by This study has shown that TLR7 agonist is a potent stimulator of innate immunity and can be used to induce immunity of calves prior to the infection and treatment method for ECF vaccination, without simultaneous use of long acting oxytetracycline. Results of the present study have revealed that 90% of calves stimulated with this agonist 24h prior to artificial infection with live *T. parva* parasites, did not develop clinical ECF disease. This study has demonstrated that the combination of TLR7 agonist and *T. parva* parasites, when the agonist was administered 24h earlier, was able to induce significant protection against *T. parva* infection in calves.the approach of prior innate stimulation of cattle with a potent immunological stimulator, such as the TLR7 agonist.

TLR7 agonists have demonstrated potential as vaccine adjuvants, since they directly activate antigen presenting cells (APCs) and enhance both humoral and cellular immune responses especially Th1 responses (Vasilakos and Tomai, 2013). Although the natural ligands for TLR7 are ssRNA, the vast majority of vaccines used thus far been performed with synthetic small molecule imidazoquinolines such as imiquimod and resiquimod. TLR7 agonist has been used in a vast majority of vaccine studies such as cancer, HIV, influenza, TB, tumor trials and treatment of genital warts associated with viral infection.

In this study, we showed, for the first time, that TLR7 plays a distinct role in *T. parva* infection.

Rectal temperature induced by TLR7 agonist stimulation was successfully used as an indicator for monitoring of the calves prior to parasite challenge. In this study, temperature measured 24h post TLR7 agonist stimulation was used as the decision point for infecting the calves with *T. parva* parasites or not.

The temperature aroused by the TLR7 agonist was sustained by the calves for more than 48h. The elevated temperature and associated innate stimulation was then investigated if the agonist can further boost adaptive immunity to *T. parva* without simultaneous use of antibiotics, as usually is the case during ECF vaccination. Daily monitoring of rectal temperature revealed two high temperature peaks over a 21-day period. The early peak, shown on days 1-3 was only recorded in calves from the adjuvant and vaccinated groups, but none of the calves in the infected group developed high temperature during this period. This suggests that TLR7 agonist, just like the way *T. parva* sporozoites given as a vaccine with antibiotics, may exploit similar mechanisms by which the immune system is induced to resolve the parasite infection.

It is known that TLR agonists have a great potential as vaccine adjuvants, and one of their key features is that they induce strong immune responses (Pulendran and Ahmed, 2011). The second high temperature peak was shown by calves in all three experimental groups (adjuvant, vaccinated and infected groups) starting on day 10 with highest temperatures (>39.5°C) being on days 14-16. Fever recorded from day 10 was an expected sequel after parasite introduction and the highest temperatures recorded on days 14-16 in the different groups coincide with increased number of parasitized cells in blood, what was also

confirmed by PCR detection. It is generally known that elevated body temperature is associated with infection and inflammation and helps a range of immune cells to work better. Recent research has reported that the generation and differentiation of CD8+ cytotoxic T-cells is enhanced by mild fever-range hyperthermia. Specifically, elevated body temperature changes the T-cells' membranes which may help mediate the effects of micro-environmental temperature on cell function (Mace *et al.,* 2011).

Furthermore, elevated temperature has also been associated with enhanced phagocytic potential of dendritic cells, in addition to augmenting interferon-α production in response to viral infection; up-regulation of the expression of TLR2 and TLR4, suggesting a role for thermal signals in enhancing pathogen sensing by innate immune cells. Febrile temperatures further increase dendritic cell expression of MHC class I and class II molecules and co-stimulatory molecules, including CD80 and CD86, and can augment the secretion of the Th1 cell-polarizing cytokines IL-12 and TNF (Casadevall, 2016; Evans *et al.,* 2015). Findings from the present study therefore support the role of the TLR7 agonist in mobilization of innate and adaptive immune cells, potentially augmenting the migration of antigen-presenting cells, such as skin Langerhans cells, to draining lymph nodes (the site where the agonist as well as the parasites were inoculated).

In this study the effect of the TLR7 agonist was also shown through an innate inflammatory response following a subcutaneous injection of the adjuvant on the side of the neck of calves, close to the parotid lymph node. The rapid and significant increase of skin thickness evidenced 6h after TLR7 agonist injection, reflects not only accumulation of inflammatory cells at the injection site, but also the local recruitment of lymphocytes around the injection site.

Clinical ECF is classically associated with a leukopenia (Irvin, 1983). Accordingly, white blood cell count has been used as an indicator of prognosis in cases of ECF and animals that maintain their WBC during infection are more likely to recover from ECF (Irvin, 1983). Infection challenge with *T. parva* of calves in this study resulted in leukopenia. However, calves, which were immunized with the adjuvant showed increased white blood cell counts soon following TLR7-agonist priming and sustained high WBC counts up to 24h after challenge with *T. parva.*The initial WBC rise in this group implies that TLR7 agonist must have primed a massive immune response. These findings allow hypothesizing that the TLR7 agonist stimulated innate immune responses which subsequently directed acquired immune responses, predominantly a Th1 response, which was able to outpace the concurrent multiplication of parasites, thereby successfully protecting the calves.

Unlike the trend described for WBC, red blood cells as well as other erythrocytic parameters, including mean corpuscular hemoglobin concentration and haemoglobin concentration did not vary significantly over the study period within or between groups. The non-variation may be due to the brief observation period from the infection challenge to the time of active *T. parva* parasite division in RBCs, when piroplasm fully appear, although other studies have reported mild anaemia 11 days post *T. parva* infection (Fry *et al.,* 2016).Previous reports by Dolan *et al.* (1982) and Billiouw (2005) also collaborates these findings that anaemia may not be recognized as a major feature in classical *T. parva* infections with certain stocks of *T. parva* (Norval *et al.,* 1992).

Results from the IFN-gamma trends indicated that TLR7 agonist significantly boosted the response of calves to *T. parva* challenge. This study signified the possibility of protecting calves from clinical ECF disease by immune stimulation with TLR7 agonist prior to

infection challenge with *T. parva* sporozoites. The usual ECF vaccination entails simultaneous injection of a lethal dose of live *T. parva* sporozoites with long acting tetracycline. In the present study, oxytetracycline was not given to the TLR7 agonist primed calves. The TLR7 agonist primed a strong Th1 response in adjuvant group calves, whose plasma IFN-gamma level rose sharply 3 days after infection, and was comparable in kinetics with the levels shown by vaccinated calves. Furthermore, the TLR7 agonist priming provoked a longer IFN-gamma persistence which dropped to baseline plasma level on day 10 compared to day 8 in vaccinated calves. Calves which received *T. parva* challenge without the agonist, on the other hand, recorded low plasma IFN-gamma levels throughout the study. The primary objective of the present study was to determine whether TLR7-agonist could be effective as a *T. parva* vaccine adjuvant when injected 24h prior to the infection challenge with the Muguga cocktail. The results from this study demonstrated that the approach used here resulted in significant protection of 9 out of 10 calves against clinical ECF disease. The IFN-gamma analysis argued that the agonist in all likelihood primed a Th1 response as demonstrated from the data shown in Figure 6.

The TLR7 adjuvant ostensibly altered the quality and the magnitude of the acquired immune response to *T. parva*. In the approach by this study calves primed with the adjuvant were given a parasite infection challenge 24h after TLR7 agonist stimulation. The parasites were given in the same dose as approved for the standard ECF vaccination protocol using the live Muguga cocktail, but without simultaneous antibiotics. The 24h timing for challenge was based on a local inflammatory (skin thickness) and raised rectal temperature (>39.5°C). From a practical consideration, timing for the infection challenge has a major influence on the efficacy of the agonist.

Further study will however be required to optimize the timing for parasite challenge, beyond 24h used in this study, before this approach may be applied under field conditions, considering that grazing cattle may not get a natural infective tick bite 24h after TLR7 agonist stimulation. Furthermore, dose of the adjuvant is also of paramount importance. In this study, a single subcutaneous injection of 1ml was used. However, from a practical consideration, field application of the agonist will require a tailored design of booster priming with the adjuvant in order to stimulate an adequate level of innate immune and acquired responses. Previous studies have shown that imiquimod (TLR7 agonist) represents a potential vaccine adjuvant (Zhang and Matlashewski, 2008), including a live infection model for herpesvirus infection in guinea pigs (Harrison, *et al.,* 1994). The present work is the first study, however, to investigate the use of TLR7 agonist in calves prior to infection challenge with *T. parva* parasites, which cause ECF. The main objective of this study was to investigate whether the TLR7 agonist may be incorporated into the ECF live vaccine and replace use of antibiotics.

The significance of this work is in several-fold. First, cattle acquire life-long immunity following ECF-vaccination as well as following natural infection and recovery. Since the current live vaccine has several delivery limitations (cold chain, batch-to batch variation, cost of antibiotics) newer improvements will reduce cattle deaths and have a direct impact on livelihoods of millions of resource-poor livestock keepers, majority of who are pastoralists. Second, data from this study has demonstrated that calves were protected from clinical ECF disease without simultaneous use of antibiotics during a parasite challenge. The exclusion of antibiotics from the standard ECF vaccination protocol will make the vaccine cheaper and affordable to many more livestock keepers and most importantly will reduce use of antibiotics in livestock.

To further authenticate the effect of the TLR7 agonist, calves primed with the adjuvant were compared with their vaccinated or infected cohorts in terms of onset and severity of clinical disease signs, onset of parasites and antibody percent positivity. The sign that most distinguished the adjuvant-primed calves was a persistent high temperature during the entire observation period (36 days), but without crossing above 40°C for more than 48h. Furthermore, only one out of ten calves from this group showed clinical signs similar to the 4 infected cohorts. This calf was treated on day 23 compared to the 4 infected calves, which were treated on day 18 after manifestation of high temperature above 40°C for 3 consecutive days. A potential impact of the agonist was also remarked in terms of onset of parasites and specific antibodies in blood. Thus, by day 14, 100% of vaccinated calves and 90% of agonist-primed calves were detected with parasite DNA in their blood, compared to 50% of infected calves showing a positive PCR result on this day. Onset of *T. parva* in the blood of ECF-vaccinated calves signifies manifestation of mild infection, with the calves exhibiting a low level of parasitemia, accompanied by mild fever which is usually controlled by the host's immune response, leading to a carrier state. Essentially, 9 out of the 10 TLR7-primed calves exhibited on day 14 a similar clinical status leading towards development of a carrier state, signifying a strong immune response induced by the agonist.

On day 21, the 4 infected calves also showed a PCR-positive signal for *T. parva* providing evidence that calves of this group also acquired immunity following infection and recovery (these 4 calves were treated against ECF on day 18). Onset of most clinical signs as well as parasites on day 14 agrees with a previous study by Di Giulio *et al*.(2009) and Billiouw (2005), who reported appearance of severe ECF clinical signs between days 10 and 14 after natural infection. In those studies, the first symptoms seen on cattle were

swelling of lymph nodes, usually accompanied by fever (40-42°C), loss of appetite (anorexia) and lacrimation.

Antibody percent positivity was determined to provide an indication of extent of seroconversion of calves following challenge or vaccination. The data (Figure 7) suggest that the calves had detectable levels of specific antibodies prior to commencement of the study. The levels were however basal (ELISA test cut-off point PP=20), and this was expected since all the calves were weaned from their mothers just before being brought into the experiment, hence carried maternal antibodies. Further, the calves were screened and confirmed to be free from parasites (tick-borne or gastro-intestinal) at the beginning of the study. Following vaccination or infection with *T. parva* parasites, level of antibodies rose gradually beginning from day 12 and the antibody rise was steeper from day 14. This agrees with an induced antibody response following challenge or vaccination. Taken together, antibody and parasite onset detected by ELISA and PCR tests, respectively, have demonstrated an agreement in kinetics on day 14 post infection or vaccination.

Data generated in this study propose a prognostic value of body temperature, when taken together with WBC counts and percent lymphocytes for ECF monitoring. Rectal temperature of calves inversely varied with total white blood cell counts but percent lymphocytes increased with temperature, and this was more vivid 12 days after infection. The WBC count and temperature dynamics can be attributed to the dissemination of parasites in blood. Taken together, results of this work pave way to future studies to determine the detailed protocols for practical application of TLR7 agonist as an adjuvant in ECF vaccination. This study has clearly demonstrated the role of the TLR7 agonist in innate stimulation and its subsequent effect on the trajectory of the adaptive immune response in *T. parva* infection. These preliminary findings will aid not only in the

development of an improved delivery of the ECF vaccine, but also in eliciting an efficacious cellular immunity with the live sporozoites without simultaneous use of oxytetracycline.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

This investigative study has produced data which have clearly shown that TLR7 agonist can be effectively incorporated into ECF vaccination as an adjuvant, thereby omitting use of antibiotics. The results from this study demonstrated that TLR7 agonist stimulation followed by *T. parva* parasite challenge resulted in significant protection against *T. parva* infection in 9 out of 10 calves. Thus, calves in the adjuvant group did not develop clinical disease to *T. parva*, although like their infected cohorts, they showed similar clinical signs at reduced amplitude. Stimulation of calves with TLR7 agonist induced a strong innate response including a dramatic rise in temperature, increased skin thickness and pronounced swelling of the lymph node compared to calves of the other groups.

Data on haematological parameters of calves revealed that all experimental calves exhibited leukopenia and lymphocytosis. However, calves, which were immunized with the adjuvant showed increased white blood cell counts soon following TLR-agonist priming and sustained high WBC counts up to 24h after challenge with *T. parva* thereby priming a massive immune response.Furthermore, other erythrocytic parameters, including mean corpuscular hemoglobin concentration and haemoglobin concentration did not vary significantly over the study period within or between groups. Analysis of IFN-gamma indicated that TLR7 agonist significantly boosted the response of calves to *T. parva* challenge, which primed a strong and persistent Th1 response in adjuvant group calves and was comparable in kinetics with the levels shown by vaccinated calves.

Comparison of the effect of the TLR7 agonist with their vaccinated or infected cohorts in terms of onset and severity of clinical disease signs, onset of parasites and antibody

percent positivity, revealed a persistent high temperature as the sign that most distinguished the adjuvant-primed calves during the observation period. Furthermore, only one out of nine calves from this group showed clinical signs similar to the 4 infected cohorts.

A potential impact of the agonist was also remarked in terms of onset of parasites and specific antibodies in blood. Thus, by day 14, 100% of vaccinated calves and 90% of agonist-primed calves were detected with parasite DNA in their blood, compared to 50% of infected calves showing a positive PCR result on this day. Antibody percent positivity revealed that calves had detectable levels of specific antibodies prior to commencement of the study. However, following vaccination or infection with *T. parva* parasites, level of antibodies rose gradually beginning from day 12 and the antibody rise was steeper from day 14.

The implication/impact of this study is that it paves way for a practical application of the approach of innate stimulation of crossbred calves with TLR7 agonist as an adjuvant during ECF vaccination, without simultaneous use of antibiotics. TLR7 has previously been reported to have a significant value in vaccine studies such as cancer, HIV, influenza, TB, tumor trials and treatment of genital warts associated with viral infection, but the current study is the first classical demonstration of its potential application in vaccinating cattle against an economically important disease.

6.2 Recommendations

1. The significance of this study is in several-folds. First, cattle acquire life-long immunity following ECF-vaccination as well as following natural infection and recovery. Since the current live vaccine has several delivery limitations (cold

chain, batch-to batch variation, cost of antibiotics) newer improvements will reduce cattle deaths and have a direct impact on livelihoods of millions of resource-poor livestock keepers, majority of who are pastoralists. Second, data from this study has demonstrated that calves were protected from clinical ECF disease without simultaneous use of antibiotics during parasite challenge. The exclusion of antibiotics from the standard ECF vaccination protocol will make the vaccine cheaper and affordable to many more livestock keepers and most importantly will reduce use of antibiotics in livestock.

2. Furthermore, from a practical consideration, timing for the infection challenge has a major influence on the efficacy of the agonist. Further study will however be required to optimize the timing for parasite challenge, beyond 24h used in this study, before this approach may be applied under field conditions, considering that grazing cattle may not get a natural infective tick bite 24h after TLR7 agonist stimulation. Finally, dose of the adjuvant is also of paramount importance. In this study, a single subcutaneous injection of 1ml was used. However, from a practical consideration, field application of the agonist will require a tailored design of booster priming with the adjuvant in order to stimulate an adequate level of innate immune and acquired responses.

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