Comparison of the efficacy of *chitongo Theileria parva* sporozoites carried on ice to that carried in liquid nitrogen

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The University of Zambia

Lusaka

DECLARATION

I, **Chanda Chitala** do hereby declare that the contents of the dissertation being submitted herein are my original work and have not been previously submitted to any university for the award of a degree or any other qualification.

Signature…….

Date……

CERTIFICATE OF APPROVAL

This dissertation submitted by **Chanda Chitala** is approved as fulfilling the partial requirements for the award of the degree of Masters of Science in One Health Analytical Epidemiology by the University of Zambia.

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ABSTRACT

Theileria parva is a tick-transmitted protozoan parasite that causes an economically important disease of cattle called East Coast fever (ECF), in East, Central, and Southern Africa, including Zambia. Immunization against ECF by the infection and treatment method using the *T. Parva chitongo* strain in the Southern province has been found to be an efficient prophylactic technique to control the disease in the endemic areas. However, delivery of the vaccine to most rural areas is expensive because of the bulky transport medium which requires a four wheel drive vehicle to transport to the destination area. The aim of this study was to compare the efficacy of *Theileria parva chitongo* sporozoites carried on ice to that carried in liquid nitrogen in cattle.

The study was done in two phases, phase one was a field trial, while phase two was an indoor trial. In the field trial, 305 cattle were vaccinated with *T. parva chitongo* stabilate carried in liquid nitrogen (VCLf), while another 291 cattle were vaccinated with a stabilate carried on ice (VCIf). These were aged 3 months and above with no history of immunization. His was done in 5 districts in the southern province of Zambia.

The indoor trial, 17 male Friesian cattle aged between 3 months and 1 year, sourced from farms around Lusaka were used. Only cattle that tested negative for theileriosis on IFAT and microscopic examination of blood/lymph smears were included. The calves were randomly allocated to two treatment groups, one with calves vaccinated using conventional vaccine carried in liquid nitrogen (VGLi)(n_1=9) and the other group vaccinated with the vaccine carried on ice (VGIi)($n_{2=}$8). Dual blood samples were aseptically collected from the jugular vein and stored in well labelled plain and EDTA tubes. Thin blood smears, PCV determination, temperature and IFAT for both field and indoor trials were done. However, blood smears, temperature and PCV for the field trial were only done before immunization. Differential white blood cell count was determined only for the indoor trial.

The results of the study showed that, in the field trial, piroplasms on microscopy were detected in Choma, Kalomo, and Namwala districts before immunization with Namwala and Kalomo having the highest prevalence 16.67% (95% CI 9.96 - 23.38). The field trial also showed an overall seroprevalence on IFAT of 20% (95% CI 16.7 - 23.1) and 8.4% (95% CI 6.2 - 10.7) in the 1/40 and 1/80 dilution respectively before immunization. There was no significant difference in efficacy between *T. parva chitongo* stabilate carried on ice, and that carried on liquid nitrogen in both the 1/40 (p=0.450) and 1/80 (p=0.242) dilutions between group VCLf and group VCIf. In the indoor trial, the PCVs in both group VCLi and group VCIi were reducing as the trial went on, howevever, there was no significant difference in PCV (p=0.768). Differential white blood counts were done for both goups immunized wth VCLi and VCIi. The leucocyte count reduced as the study went on due to the reduction of the lymphocyte count. However, there was no significant difference between the two groups in lymphocyte counts (p=0.208). There was no significant in temperatures (p=0.0.248) between the group VCLi and group VCIi. There was also no significant difference in the probability of survival (p=0.829), the relative risk (p=0.889) and relative vaccine efficacy (95% CI -183 - 59) between group VCLi and group VCIi. These results show that *T. parva chitongo* stabilate carried on ice has the same efficacy as that carried on liquid nitrogen. Carrying of the vaccine stabilate on ice after removal from the liquid nitroge would greatly reduce the cost of ECF immunisation and make the procedure more adoptable to rural livestock keepers.

DEDICATION

This work is dedicated to my father Mbita Chitala for all the support given in order to help me be the best that I can be, my late Mother Esther Pepkai who never got the chance to see what I have turned out to be, my children Jennifer and Taizya who give me every reason to wake up each day and work hard, my siblings and finally my husband Eugene Bwalya who has always believed in me. Without these people I would not be who I am today.

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LIST OF ABBREVIATION

ANOVA	Analysis of Variance
CVRI	Central Veterinary Research Institute
CI	Confidence Interval
˚C	Degree Celsius
ECF	East Coast Fever
EDTA	Ethylenediamine Tetra-acetic Acid
ELISA	Enzyme-linked Immunosorbent Assay
GDP	Gross Domestic Product
IFAT	Indirect Fluorescent Antibody Test
ITM	Infection and Treatment Method
KG	Kilogram
LAMP	Loop-mediated Isothermal Amplication
MEM	Minimum Essential Media
n	Sample size
PCR	Polymerase Chain Reaction
PCV	Packed Cell Volume
RBC	Red Blood Cell
STATACorp	Stata Corporation Statistical Software
SELISA	Slide Enzyme-linked Immunosorbent Assay
VCLf	Vaccine Carried in Liquid Nitrogen -field
VCIf	Vaccine Carried on Ice -field
VCLi	Vaccine Carried in Liquid Nitrogen- indoor
VCIi	Vaccine Carried on Ice –indoor

CHAPTER ONE

1.0 Introduction

Theileria parva is a tick-transmitted protozoan parasite that causes an economically important disease of cattle called East Coast fever (ECF), in East, Central, and Southern Africa, including Zambia (Minjauw *et al*., 1997, Konnai *et al*., 2007). The parasite is transmitted by the three host tick, *Rhipicephalus appendiculatus* (Konnai *et al.,* 2007). Chizyuka and Mangani (1985) estimated that about 540,000 out of 1,115,000 cattle were at risk of the disease in the Eastern, Northern, Central and Southern provinces of Zambia by 1985. This estimate was adjusted upwards to 1,259,000 by Nambota (1988) for Eastern, Northern, Lusaka, Central and Southern provinces.

ECF has major economic implications especially when livelihoods depend on livestock rearing (Mbao *et al.,* 2006). The disease is a major constraint to development of the livestock industry in Zambia due to the high morbidity and mortality associated with it. Nambota *et al*., (1994), estimated the losses associated with the disease in the country to about 10,000 cattle deaths per annum. During the year 2010, it was estimated that a total of 18,073 cases of ECF and 4,911 deaths were reported in Zambia. The majority of these cases were reported from Southern, Eastern, Central, Copperbelt, Lusaka and Northern Provinces (Anon, 2010a). Thus, the control of ECF is a matter of utmost importance if the livestock industry has to gain its full potential, especially as a tool for poverty alleviation among the resource poor rural farmers.

Methods that have been used to control the disease include livestock movement control, tick control with acaricides, use of resistant breeds, chemotherapy and immunisation by the infection and treatment method (Radley, 1981). Prolonged use of chemicals to control ticks has resulted in various problems such as tick resistance, residues in animal products, environmental pollution and high costs (Ghosh *et al.,* 2007). Intensive use of acaricides interferes with enzootic stability, creating a susceptible population of animals which are liable to disease epidemics whenever there are disruptions to control programmes (Kocan *et*

al. 2000; Lawrence, 1985). In addition, drugs that are effective in treating the disease are expensive, making them unaffordable for most resource poor traditional farmers. These concerns have reinforced the need for alternative approaches to control tick infestations (Ghosh *et al.* 2007). Immunisation is a useful way of reducing reliance on acaricides to control tick-borne diseases.

Immunization against ECF by the infection and treatment method using the *T. Parva chitongo* strain in the Southern province has been found to be an efficient prophylactic technique to control the disease in the endemic areas (Marcotty *et al.* 2001, Anon. 2010a). This consists of the inoculation of live cryopreserved *Theileria parva* sporozoites (stabilate) and the simultaneous injection of a long-acting formulation of oxytetracycline (Radley *et al*. 1975). However delivery of the vaccine to most rural areas is expensive because of the bulky transport medium (liquid nitrogen) which requires a four wheel drive vehicle to transport to the destination area (Anon, 2011b). There is therefore a need to devise affordable ways of vaccine delivery to the resource poor farmers where it is desperately needed.

The purpose of this study was to determine whether vaccination with *T. parva chitongo* sporozoites transported on ice offer similar protection to that carried in liquid nitrogen in calves. These results of this study will help in deciding whether ice which is cheaper and easier to carry can be used as an alternative carriage material for *T. parva Chitongo* vaccine.

1.1 Problem statement and study justification

East Coast fever is assuming more importance as it continues to be a major economic problem not only in Zambia, but in many parts of Eastern, Southern and Central Africa (Makala *et al*., 2003). While the ITM offers hope in the control of the disease in the long run, it has a number of drawbacks. Stabilate vials should at all times be stored in either liquid nitrogen (-196 degrees Celsius) or ultra-freezer (-70 degrees Celsius). The diluent should be stored in a frozen state at -20 degrees celsius. The requirement for the maintenance of this cold chain up to the farm level makes the method less appropriate for rural use and it is expensive for livestock keepers (Mbao *et al*, 2007). There is therefore a need to devise ways in which the vaccine stabilate can be carried to the farms without a need for the liquid nitrogen. This will drastically reduce the cost of the infection and treatment method and improve its applicability in the immunisation of cattle against ECF. Such developments will

make the vaccine more accessible to rural areas (motorbike may be used), and immunization can be done in more than one place at the same time.

1.2 Objectives

1.2.1 General objective

To compare the efficacy of *chitongo theileria parva* sporozoites carried on ice to that carried in liquid nitrogen in cattle.

1.2.2 Specific Objectives

- To compare the proportions of the animals that seroconverted after vaccinating with *chitongo theileria parva* carried on ice and in liquid nitrogen.
- To compare the efficacy of the *T. parva* chitongo stabilates in calves vaccinated with sporozoites carried on ice and those carried on liquid nitrogen.
- To compare hematological profiles in calves vaccinated with *T. parva* chitongo stabilates carried on ice and to those carried on liquid nitrogen.

CHAPTER TWO

2.0 Literature review

2.1 Livestock production system in Zambia

The livestock sector has continued to play an important role in socio-economic development of the country and contributes towards household food security and wellbeing. It accounts for about 35% of total agricultural production (Anon, 2011b). During the year 2011, the livestock sector contributed about 8.9% to the national gross domestic product (GDP) (Anon, 2011b). With an estimated population of 3.9 million (Anon, 2011b), cattle are the most important type of livestock in Zambia and can be divided into three breeds or: European (*Bos taurus*), indigenous (*Bos indicus*) and the crosses of indigenous and taurine breeds. There are three main types of indigenous traditional cattle: (i) Barotse, a longhorned Sanga type found in Western Province, (ii) Tonga, a medium-horned Sanga type found in Southern and Central Provinces and (iii) Angoni, a short-horned Zebu type in Eastern Province (Pegram *et al.* 1986).

Livestock production system in the country is divided into commercial and traditional sectors. In the traditional sector, three main breeds types of cattle are the Tonga, the Baroste (Sanga types) and the Ngoni (Zebu types) (Aregheore, 1994). Traditional livestock farming is the preserve of rural families (Chilonda *et al.* 1999) and is conducted mostly in Western, Southern, Central and Eastern Provinces. Besides being used as a source of food and draught power, cattle serve many other functions in the traditional sectors. They are regarded as a symbol of wealth, are used in marriage ceremonies, are used in setting court fines, and provide income for the family. Cattle dung is used as inorganic fertilizer in the crop fields. Small scale cattle farms are family holdings characterized by low input and low output (Aregheore, 1994). Disease control is limited and in most cases tick control is intermittent or absent. Because of the extensive production system, herd intermixing and poor disease control, a variety of cattle diseases, including TBDs, are prevalent in the traditional sector (Chilonda *et al.* 1999)

Commercial farms are large undertakings, both beef ranches and dairy farms. They require big capital investment but also have large economic returns. Commercially, cattle ranching

and dairy farming provide a good source of employment to many unskilled people. The main commercial livestock areas are Southern province, Central province, Lusaka province, Copperbelt province and the Eastern province (Aregheore, 1994). The main breeds of cattle in the commercial sector are Boran, Afrikander, Sussex, Gelbvieh, Brahman, Friesian and Simental. Tick control is practised intensively leaving the animals highly susceptible to TBDs if challenged. In contrast to the traditional sector, these diseases can be clinically important and can reach epidemic proportions when intensive control measures break down (Chilonda *et al.* 1999).

2.2 Aetiology of ECF

Theilerioses are protozoan infections of wild and domestic *Bovidae* which occur throughout much of the world. *Theileria parva*, a protozoan parasite of domestic cattle and wild buffalo is the most important *Theileria* species in Africa, south of the equator (Fandamu, 2005; Mtambo, 2008; Norval *et al*., 1992). The classification of *Theileria parva* has for a long time been controversial (Irvin and Mwamachi, 1987). However Levine *et al.* (1980) classified the parasite as follows; Phylum: Apicomplexa, Class: Sporozoea, Subclass*:* Piroplasmia, Order: Piroplasmida, Family: Theileriidae, Genus: *Theileria*, Species: *Theileria parva*

Other species under the genera *Theileria* are *T. annulata*, *T. taurotragi*, *T. mutans, T. velifera* and *T. orientalis*. Of the six identified *Theileria* spp. that infects cattle, the two most pathogenic and economically important are *T. parva* and *T. annulata*. *Theileria parva* occurs in Eastern and Southern Africa and causes East Coast fever (ECF or Corridor disease) in East, Central and Southern Africa, while *T. annulata* causes tropical theileriosis (TT), also known as Mediterranean theileriosis and occurs in North Africa, southern Europe and Asia (OIE, 2008)

ECF was first recognized in Southern Africa as East Coast fever when it is was introduced at the beginning of the 19th century with cattle imported from East Africa where the disease is believed to have been endemic for several centuries. This was after the cattle population was devastated by Rinderpest (Norval *et al*., 1992) and this new disease caused dramatic losses with high mortalities and was initially confused with red water. This virulent cattle disease later known as ECF was identified for the first time at several locations south of the Zambezi

River in 1901 with its principal vector the brown ear tick, *Rhipicephalus appendiculatus*, which was already widespread in the region but previously not associated with any disease (Norval *et al*., 1992).

2.3 Vectors of *Theileria parva*

The ticks *Rhipicephalus appendiculatus* and *Rhipicephalus zambeziensis (*Walker *et al*, 1981, Mtambo, 2008) and to a lesser extent *Rhipicephalus duttoni (*Neumann 1907, Mtambo 2008) are the only known field vectors of *Theileria parva.* In Zambia collections of *R. appendiculatus* have been reported in all provinces (Akafekwa, 1976, Mtambo 2008), while *R. zambeziensis* has been reported in the major depressions of the Luangwa and Zambezi river valley systems (MacLeod, 1978) and in Southern province (Speybroeck *et al*., 2002). In the Southern province of Zambia *R. appendiculatus* and *R. zambeziensis* co-occur in the same areas and on the same host at the same time (Speybroeck *et al*., 2002).

2.4 Distribution

The factors known to affect the occurrence of *T. parva* include the distribution of *R. appendiculatus*, the presence of cattle and wild bovidae, resistance of the host cattle to ticks and tick-borne diseases and tick control policies that affect tick populations (Lessard *et al*., 1990). The distribution of *R. appendiculatus* is influenced by several factors, the most important of which are climate, vegetation and host availability (Fandamu, 2005). *Theileria parva* is currently distributed within eleven countries in Eastern, Central and Southern Africa, where it is a major constraint to cattle production (Mukhebi *et al.*, 1992, Fandamu 2005). The affected countries include Burundi, Democratic Republic of Congo, Kenya, Malawi, Mozambique, Rwanda, Sudan, Tanzania, Uganda, Zambia and Zimbabwe as shown in Figure 2.1. In Zambia Theileriosis is currently reported in Southern, Eastern, Lusaka, Central, Copperbelt, Muchinga and Northern Provinces. Western Province is the only major cattle rearing province that has been spared from the disease to date. The disease has also not been reported from Luapula and North-western provinces that have least cattle population (Anon, 2010b).

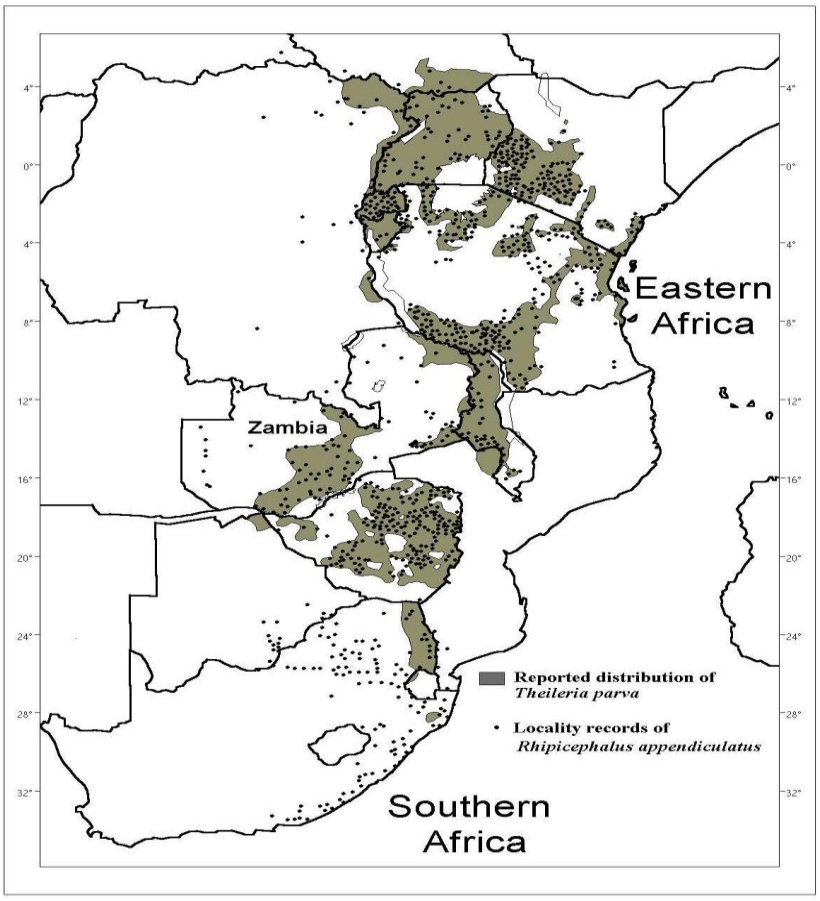


Figure 2.1: Distribution range of *Theileria parva* (adapted from Norval *et al.*, 1992; Speybroeck *et al.*, 1999, Chaka, 2001 and Fandamu, 2005) and the distribution of *Rhipicephalus appendiculatus* (adapted from Speybroeck *et al.*, 1999; Walker *et al.*, 2000, Chaka, 2001 and Fandamu, 2005).

2.5 Life cycle and transmission

Theileria parva is trans-stadialy transmitted by the nymphal and adult stages of the three-host tick, *Rhipicephalus appendiculatus* after acquiring infections during feeding as larvae or nymphs, respectively (Konnai *et al*., 2005). It undergoes developmental cycles in both the bovine host and the tick vector (Fandamu, 2005) (Figure 2.2). During feeding, the infected tick will pass on sporozoites to a susceptible bovine through its saliva. Following infection, the sporozoites invade the T and B-lymphocytes of the host animal (FAO, 1984), where they develop into schizonts by a process known as schizogony. This process is associated with transformation of the infected host cells to a state of uncontrolled proliferation. By associating with the mitotic spindle, the parasite divides in synchrony with the host cell,

resulting in each daughter cell inheriting the infection (Rocchi *et al.*, 2006). This stage of host and parasitic cell division is associated with the severity of the pathology and clinical signs seen in ECF (Rocchi *et al*., 2006).

Later in infection, some of the schizonts undergo merogony with the resultant merozoites giving rise to piroplasms that infect red blood cells (Mtambo 2008). It is the presence of these piroplasms in the infected animal's blood that provide the source of infection for other ticks when they feed at the time of parasitaemia. After being taken up by the feeding tick, piroplasms differentiate into micro and macro-gametes in the tick mid-gut, subsequently fusing to produce zygotes that invade the epithelial cells (Watt and Walker, 2000).The zygotes transform into motile kinetes and migrate into salivary glands where they invade the "e" cells of the type III acini and develop into multinucleated sporoblasts (Fawcett *et al*., 1982).When the tick starts feeding, the sporoblast syncytium undergoes cytoplasmic fission resulting in mature uni-nucleated sporozoites (Fawcett *et al.*, 1982) that are infective to cattle.

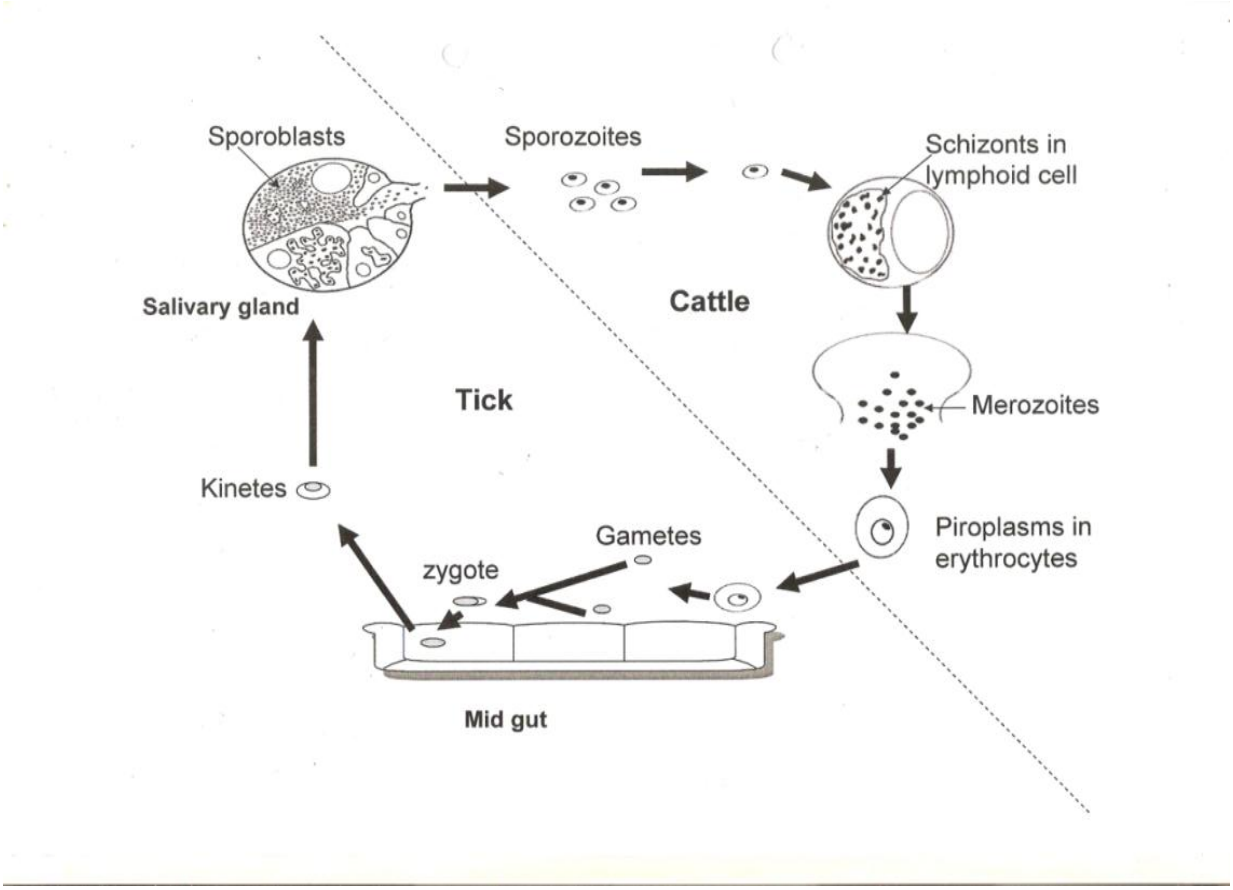


Figure 2.2*:* Schematic diagram of the life cycle for *Theileria parva*: Adopted from Simuunza, 2009).

2.6 Clinical signs

The incubation period of *T. Parva* ranges from 8-12 days under experimental conditions (Fandamu, 2005), but may be much more variable in the field. Schizogony is associated with transformation of the infected cells to a state of uncontrolled proliferation. This stage of parasitic division is associated with the severity of the pathology and clinical signs seen in *T. parva* infections (Rocchi *et al*., 2006). An occult phase of 5-10 days follows before infected lymphocytes can be detected in Giemsa-stained smears of cells aspirated from lymph nodes (Kahn *et al*, 2005). Piroplasms can be detected 5-8 days after the detection of schizonts. Fever occurs 7-10 days after parasites are introduced (inoculated) in the host (Fandamu, 2005). Death usually occurs within 30 days after infection of susceptible cattle by infected ticks (Irvin and Morrison, 1987).

East Coast Fever is characterized by lymphadenopathy, pyrexia, dyspnea and frothing due to interstitial pneumonia and pulmonary oedema. Other signs may include subcutaneous oedema, diarrhoea, lacrimation and mortality. Petechiation on mucous membranes, inappetance, ceasation of rumination, salivation, serous and nasal discharge, rapid and weak heartbeat, and intestinal ulceration may be exhibited (Mtambo *et al*., 2008).

2.7 Diagnosis

ECF is initially suspected from the presence of clinical signs described above. Confirmative diagnosis will require demonstration of the presence of or evidence of exposure to the parasites. In the Laboratory, East Coast fever is diagnosed by either detection of piroplasms in the blood, schizonts in lymph node biopsies, antibodies by the Immuno-Fluorescent Antibody Test (IFAT) (Burridge and Kimber,1972) or polymerase chain reaction (PCR) (Ogden *et al.,* 2003). PCR, although highly sensitive, is not suitable for routine field use due to high cost. It however is described as the best test for discriminating *T. Parva* from acute infections caused by other *Theileria species*. The disease can also be diagnosed using loop-mediated isothermal amplification (LAMP) (Skilton *et al*., 2002) and reverse line blot assay which is based on hybridisation of PCR products to specific oligonucleotide probes immobilised on a membrane (Gubbels *et al*., 1999).

A slide enzyme-linked immunosorbent assay (SELISA), described by Kung'u and Goodger (1990) for *Babesia cacacahas* has also been adapted to use schizont-infected lymphoblasts as antigen for ECF diagnosis.

The most widely used field diagnostic test for *Theileria* species is the indirect-fluorescent antibody (IFA) test. For the IFA test, both schizont and piroplasm antigens may be prepared on slides or in suspension and preserved by freezing at ≤ –20°C, except in the case of the piroplasm suspension, which is stored at 4°C (OIE ,2008). Test sera are diluted with bovine lymphocyte lysate and incubated with the antigen in suspension, and anti-bovine immunoglobulin conjugate is then added. Using the test as described, the fluorescence is specific for the causative agent. The IFA test is sensitive, fairly specific, and usually easy to perform (OIE terrestrial manual, 2008).The major drawback of the IFA test is its lack of specificity as *T. Parva* cross reacts with other *Theileria* parasites like *T. Taurotragi* and *T.*

Annulata (Burridge *et al.*, 1974; Norval *et al.*, 1992) and it is also often difficult to assess the degree of fluorescence.

PCR and ELISA are the most sensitive tests followed by IFAT and then blood smear. PCR is also the most specific test and blood smears the least specific. The comparison of the sensitivity, specificity, cost and throughput of four methods used in the diagnosis of TBDs of cattle is shown in table 2.1.

Table 2.1: Comparison of methods for detection of ECF. +=very low, ++=low, +++=high and ++++=very high (adapted from Simuunza, 2009)

Method	Sensitivity	Specificity	Cost	Throughput
PCR	++++	++++	++++	++++
Blood smear	+	+	+	++
IFAT	++	++	++	++
ELISA	+++	++	++	+++

2.8 Epidemiology

Risk factors that have been identified to influence the occurrence of TBDs include the distribution and abundance of tick vectors, the abundance and movement of cattle populations and the host resistance to both the tick and the responsible pathogen (Bakheit *et al*, 2002; Norval *et al.*, 1988; Salih *et al*., 2007, Simuunza *et al.*, 2010). The ability of larval and nymphal *R. appendiculatus* ticks to pick-up *T. parva* from carrier cattle and to transmit it to naive animals after moulting into nymphs and adults, respectively, was assessed (Marcotty *et al*., 2002). Although both instars were capable of picking up infection and subsequently transmitting the sporozoites to induce clinical and lethal ECF in susceptible cattle, it was observed that the prevalence of *T. parva* infection in nymphs was much lower than in adult ticks, confirming the primary role of adults in the transmission of ECF in endemic conditions. Similar results were obtained from the field where by the increase in the incidence of ECF clinical cases corresponded with the peak of adult *R. appendiculatus* activity (Marcotty *et al*., 2002). Natural infection rates found in vector ticks were much lower than that observed in experimental conditions. These findings indicate that an important proportion of the vector

ticks in the field tend to feed on alternative hosts other than cattle. It has also been reported that older ticks seem to lose part of their infectivity (Marcotty *et al*., 2002).

Carrier status in animals exists for *T. parva.* A carrier animal is one that has survived the primary infection and afterwards maintains the infectious parasite stages (piroplasms) in blood at levels high enough to infect ticks but often too low to be detected by normal parasitological investigations (Medley *et al*., 1992). Animals that recover are immune to subsequent challenge with homologous strains, but may be susceptible to some heterologous strains. Most recovered or immunized animals remain carriers of the infection (Kahn, 2005)

Epidemiological state of theileriosis can be determined in a population of cattle, by using five indicators: herd antibody prevalence, disease incidence, age group of cattle affected by the disease and case-fatality (Norval *et al.*, 1992). These indicators can be used to classify ECF affected areas into endemic, epidemic and free areas.

- The **Endemic areas**: These are areas where the parasite (*T. parva*) and the disease (ECF) are established and only calves are affected by the disease and all older animals have been exposed to the disease and are considered to be immune. Case fatality rate is low.

- **Epidemic areas:** These are newly ECF infected areas where conditions for disease transmission exist. In these areas, all age groups of cattle are affected and the case fatality ratio is close to 100%.
- **Free areas:** These are areas where the parasite (*T. parva*) is absent and the disease is not reported.

2.9 Control and treatment

East Coast fever can be controlled by different and/or a combination of these methods: tick control, livestock movement restrictions, chemotherapy and immunisation (Mbao *et al*., 2007).

2.9.1 Tick control

Vector control includes application of acaricides, immunisation and selection of tick resistant cattle. Acaricides need prolonged application to be effective but with time. However,

prolonged use of chemicals to control ticks has various problems which includes: the tick developing resistance, accumulation of residues in animal products, environmental pollution and the high cost of arcaricides (Ghosh *et al.* 2007). Intensive use of acaricides may also interfere with enzootic stability, hence creating a susceptible animal population which are liable to disease epidemics whenever there are disruptions to control programmes (Kocan *et al.* 2000; Lawrence *et al.* 1980). Tick resistant breeds of cattle and anti-tick vaccines have also been used. At the moment no vaccine is available for the control of the vectors of *T. parva.*

2.9.2 Use of resistant Breeds

Genetically resistant animals, i.e. animals which show a heritable ability to become immunologically resistant to tick infestation, are a vital component of many tick control strategies (Willadsen 2004). The Zebu (*Bos indicus*) breeds are generally more resistant than the European *Bos Taurus* cattle (De Castro, 1997). However it may be difficult to breed for tick resistance while at the same time preserving some of the desirable traits or, resistance may simply fail to develop in some hosts (Simuunza *et al*., 2011; Wilkinson *et al*., 1962).

2.9.3 Livestock movement control

The movement of livestock in Zambia is controlled by cattle movement permits issued by Government Veterinary Officers from the Department of Veterinary Services (Anon, 2010c). Whilst the restrictions on cattle movements can restrict trade, they are necessary to limit the spread of cattle diseases in general and ECF in particular.

In Zambia, movements within ECF endemic areas are allowed. However, movements from endemic areas to non-endemic areas are only allowed on the following conditions (Anon, 2010b):

- Animals to be moved must test negative by immunofluorescent antibody test, and lymph and blood smear microscopy.
- Cattle can only be allowed to move within seven days after the test. If they overstay for more than seven days, they should be subjected to a new set of tests;

- Animals are treated with acaricides before they are moved to ensure that they are tick free;

- Animals are treated with Buparvaquone before they are moved to ensure increased probability of freedom from parasite;

- Animals are subjected to compulsory quarantine at destination under close veterinary supervision for a minimum period of 26 days (The incubation period for East Coast fever is 7-25 days) and are checked for any signs of ECF by the local veterinary officer before they could be allowed to mix with other animals; and,

- Animals for slaughter must be branded with slaughter brands and must be slaughtered under veterinary supervision within 24 hours of their arrival at destination.

2.9.4 Chemotherapy

There are three effective drugs for the treatment of ECF: parvaquone (Clexon™ and Parvaxone™®), buparvaquone (Butalex™®), and halofuginone lactate (Terit™®). Each of these drugs was introduced to the market within the last 20 years (Norval *et al*., 1992). 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 immediate administration of the drug are required for effective therapy (Norval *et al.*, 1992). Halofuginone is toxic and is no longer available on the markets.

2.9.5 Immunisation

Theiler (1911) demonstrated that cattle could be infected with *T. parva* by transfer of infected cells from tissues of clinically affected animals, and that recovered animals were immune to subsequent challenge (Theiler, 1911). These results by Theiler and other observations, led to Spreull taking a field trial in which he vaccinated 283,000 cattle with spleen and lymph node cell suspensions from *T. parva* infected cattle (Spreull 1914). Unfortunately, about 25 % of the animals died as a result of infection established by the immunisation procedure. However, 70 % of the cattle that survived were immune to subsequent challenge. This led to the development of the infection and treatment method (ITM) which is currently the only available technique for immunoprophylaxis against homologous challenge (Radley *et al.*, 1975). A long-acting tetracycline and defined doses of cryo-preserved sporozoites given to

cattle was found to induce immunity against *T. parva*. This protocol results in solid immunity to challenge with only homologous parasite. A polyvalent vaccine termed Muguga cocktail, was developed in the early 1980's (Radley et al., 1975a; Radley, 1981).this was known as the Muguga cocktail, and caused immunity against a range of heterologous isolates (Radley *et al.* 1975b). It contained a mixture of three selected parasite isolates (Muguga, Kiambu 5 and Serengeti). This vaccine has been deployed extensively in Tanzania and Uganda and to a lesser extent in Malawi and Zambia (McKeever 2007). In addition, experimental immunisation with a single parasite isolate (Marikebuni) provided protection against challenge from a number of heterologous isolates (Morzaria *et al.* 1987). This parasite stock has been used to successfully vaccinate cattle in the field in Kenya (Morrison & McKeever 2006). In the Eastern province of Zambia, the local *T. parva* Katete stock is used to immunize cattle, while in the Southern province, the local Chitongo strain has been used. Major histocompatibility complex (MHC) class I-restricted cytotoxic T lymphocytes are believed to be the major protective immune effector echanism in cattle (McKeever *et al.* 1999; Preston and Jongejan 1999). For *T. parva* sporozoit antigens and schizont antigens were used in vaccine development. These provoke MHC class I- and class II-restircted T cells (Preston and Jongejan 1999).

Theileria parva is an obligate intra-cellular organism and needs its vertebrae and invertebrate host to survive. It can only survive outside these hosts through suspension of the metabolic reactions by cryopreservation. The preparation of cryopreserved stabilates in the I&T method of immunisation has been adapted from preservation of trypanosomes was developed by Cunningham et al. (1973) (Mbao,2006).

The maintenance of the cold chain in liquid nitrogen up to the time of inoculation and the cost of the reference long-acting oxytetracycline (Terramycin LA, Pfizer) have been the main drawbacks of the method (Marcotty *et al*. 2001). In addition, there are occasional difficulties arising from either failure to achieve immunisation due to cold chain breakdown and sporozoites inactivation or severe, possible fatal, clinical reaction due to inadequate doses or quality of tetracycline used to attenuate pathogenic effects of administration of live parasites (Minjauw and Mcleod, 2001). Other draw backs are that immunized animals become carriers of the parasite (Kariuki *et al*., 1995) and most vaccines can only offer protection to homologous challenge, limiting wider use of this method in a country. No literature could be

found about why it was previously assumed that it was better to keep the stabilate in liquid nitrogen rather than ice.

CHAPTER THREE

3.0 Materials and Methods

3.1 Study Design

The study was carried out in two phases. Phase one was a field trial and the second phase was an indoor trial. The study was done to compare the efficacy of *Theileria parva chitongo* sporozoites carried on ice to that carried in liquid nitrogen in cattle. The indoor trial was also done to compare haematological profiles in calves vaccinated with *T. parva chitongo* stabilates carried on ice and to those carried in liquid nitrogen. The vaccine used in both the field and indoor trial was purchased from Centre for tick and tick borne diseases (CTTBD) in Malawi.

3.2 Field trial

The field trial was similar to that conducted by Marcotty *et al.*, (2001). It was conducted in southern province of Zambia. Five districts, selected purposively, were included in this study, namely Mazabuka, Choma, Monze, Namwala and Kalomo. In each district, herds were randomly selected. All animals in each herd aged three months and two years that had no history of vaccination against ECF or were not ear tagged were included in the trial. Each herd was then divided into two groups. Cattle in the first group were vaccinated with the conventional vaccine carried in liquid nitrogen (VGLf) and the second group was vaccinated with the vaccine carried on ice (VGIf). Blood samples in EDTA tubes were collected and temperature taken before immunisation on day zero and 35 days after immunisation for each animal. The temperature of the animals was also taken once prior to vaccination. This study was comparing the efficacy of *theileria parva chitongo* sporozoites carried on ice to that carried in liquid nitrogen in the field. This was done by comparing seroconversion ratios between group vaccinated with *T. parva* carried on ice and the group vaccinated with T. parva carried in liquid nitrogen.

3.2.1 Sample Size calculation for field trial

This was determined using stataSE12®. In calculating the sample size, the significance level (α) was set at 0.05, the study power at 0.95, the proportion of animals to seroconvert with

VGLf was 0.9 and with VGIf was 0.78 and $n_1/n_2=1$. This gave us a sample size of $n_1=256$ and $n_2=256$. In this study $n_1=302$ were vaccinated with *T. parva* chitongo carried in liquid nitrogen and $n_2=291$ cattle were vaccinated with *T. parva chitongo* carried on ice. The basic rules considered for sample size calculation for clinical trials were as described by Tushar (2010).

Thus a total of 593 from 29 herds in 5 districts were sampled at the beginning of the field trial. Choma, Kalomo and Namwala had each 120 cattle included, while Mazabuka had 116 and 117 from Monze.

3.2.2. Sample collection for the field trial

Whole Blood samples in EDTA coated vacutainer tubes were collected from all cattle included in the study, in the five different districts prior to immunisation. These animals' ages included in the study were approximately from three months to two years with no history of vaccination. However, the animals were vaccinated without knowing their *T. parva* antibody status. The blood was checked later on. Blood smears on blood slides were also prepared. In addition, rectal temperatures to ensure that only animals that are generally healthy are vaccinated and packed cell volume (PCV) were also recorded prior to vaccination. Whole blood in EDTA coated vacutainer tubes was collected from the same animals after 35 days to check whether the animals had seroconverted.

3.2.3 Vaccination of calves in the field trial

For the VCLf (*Theileria parva Chitongo* vaccine carried in liquid nitrogen), the vaccine vials were removed from liquid nitrogen then thawed in water at 37°C for 5 minutes. The stabilate was then diluted with a diluent which contained Eagle's minimum essential medium (MEM) and antibiotics (Penicillin, streptomycin and kanamycin). One ml of stabilate was mixed with nine mls of diluent for a final dilution of 1:10. The diluted vaccine was left at ambient temperature for five minutes for stabilization of the stabilate in the diluents before immunization could be done. The diluted stabilate was then given to the first group of animals from the same herd. All animals were immunized within 30 minutes after dilution.

For the VCIf (*Chitongo Theileria parva* vaccine carried on ice) the vaccine vials were removed from liquid nitrogen thawed in water at 37°C for five minutes. The vaccine was then diluted with a diluent as described above and transferred onto ice bath (ice cubes + water in a

small cooler box). This was done from the respective district veterinary office. The diluted stabilates were then transported to the field in the cooler box. The animals were brought to a central place and vaccination took place four to six hours after thawing. This was because previous studies reported that immunisation performed with a stabilate kept on ice for up to 4- 6 hours had an efficiency of 90% (Marcotty *et al* ., 2001). Animals in each vaccination group were tagged with ear tags of different colours after vaccinating.

These were injected subcutaneously over the pre-parotidian lymph node and injecting a long acting oxytetracycline intramuscularly. All the Immunised animals were closely monitored for 2 weeks post-vaccination for side effects (anaphylactic shock and dysimmunisation,). The animals were also monitored for fever (>40) and presence of schizonts on lymph node smear. The rectal temperatures were recorded and the parotid glands palpated for any signs of swelling once a week.

In the field, animals showing signs of illness from diseases other than tick borne diseases (TBDs) during the monitoring period were treated with drugs that would not have an effect on development *T. parva* (penicillin or penicillin based drugs).

3.3 Indoor trial

3.3.1 Study animals

A parallel group study design that involved the use of male Friesian cattle aged between 3 months and 1 year was done. The cattle were sourced from farms around Lusaka which practiced strict tick control. The sourced cattle were only included in the study after they tested negative for theileriosis on IFAT and microscopic examination of blood/lymph smears. The calves were assigned identity numbers after which they were randomly allocated to two treatment groups, one with calves vaccinated using conventional vaccine carried in liquid nitrogen (VCLi)(n_1=9) and the other group vaccinated with the vaccine carried on ice (VCIi)(n_2=8). This study was comparing efficacy of *theileria parva chitongo* carried in liquid nitrogen to that carried on ice between two groups and further to see whether temperature and haematological parameter results could be replicated under controlled conditions. It was also done to compare the survival chance or probability after challenging between the group VCLi and the group VCIi.

3.3.2 Sample Size determination

For the experimental study the sample size was determined using sigmaXL® (version 6.1), considering the basic rules for sample size calculation for clinical trials as described by Tushar (2010), with the following assumptions:

Alternative proportion (P0): The proportion of surviving animals after challenge with a neat stablilate among those that were vaccinated with the vaccine carried in liquid nitrogen was taken as 0.97

Hypothesised proportion (P1): The proportion of surviving animals after challenge with neat stabilate among those that were vaccinated with the vaccine carried on ice was taken as 0.7

Statistical power (1-Beta) used was 0.95.

Significance level (alpha) used was 0.05.

From this, the sample size for the whole study including the control group was 20 (10 animals per group). However, due to financial challenges only 19 bull calves ranging from three months to eight months old were included in the study.

3.3.3 Sample collection

Before each sampling, the calves were examined clinically to determine their health status. From clinical examination, basic parameters for each subject such as the rectal temperature, presence or absence of lymphadenopathy were determined. The approximate age of the animals was obtained from the owners. Dual blood samples were aseptically collected from the jugular vein using vacutainer needles and adapter in EDTA and plain tubes. The Blood in the EDTA tubes were used to make blood slides and PCV determination. The Sera in the plain tubes was separated and stored at -20^0C until needed for laboratory analysis. The samples were collected twice before vaccinating, 41 days post vaccinating and 30 days after challenge. Sample collection was done every other day or after three days when it was not possible to do it every other day throughout the course of the study.

3.3.4 Vaccination of the indoor trial calves

For both the VCLi (*Theileria parva Chitongo* vaccine carried in liquid nitrogen) and the VCIi (*Chitongo theileria parva* vaccine carried on ice), the vaccine vials were prepared as in

section 3.2.3. The vaccines were administered subcutaneously over the pre-parotidian lymph node concurrently with a long acting oxytetracycline intramuscularly.

All the Immunised animals were closely monitored for 2 weeks post-vaccination for side effects (anaphylactic shock and dysimmunisation,) as described in section 3.2.3.

3.3.5 Challenge of animals in the indoor trial

The animals were challenged 41 days post vaccination. Neat stabilate of *T. parva chitongo* was used to challenge the animals. Each vial of the neat stabilate normally has a concentration of sporozoites which is equivalent to 10 ticks per ml but may have been adjusted depending on whether parasite infection rates in a particular batch were either very high or very low (OIE, 2008). This was given to the animals by injecting subcutaneously over the pre-parotidian lymph node

3.4. Lab analysis of samples

3.4.1 Thin blood slide examination

Thin blood smears were made for both field and experimental animals. Thin blood smears were made as described by Dacie (1984). Briefly, a drop of blood was placed on the centre line of a glass slide. Another slide, which was used as a spreader was placed at an angle of 45 degrees to the slide containing the blood drop and moved back to make contact with the drop. The drop was allowed to spread along the line of the spreader and the film was spread by a rapid, smooth, forward movement of the spreader. The smears were dried and fixed with 70% methanol and stained with Giemsa and examined at x100 under oil immersion for the detection of haemoparasites. A minimum of 100 fields were examined per slide to determine whether parasitaemia was present.

3.4.2 Determination of packed cell volume (PCV)

Determination of PCV was done for both field and indoor animals. The PCV was measured using the microhaematocrit method as described by Embert (1986). Briefly, blood was collected into heparinised capillary tubes. The blood was allowed to enter the tube by capillarity, leaving at least 15 mm of the tube unfilled. The tube was then sealed at one end using a sealant. The sealed tubes were placed in a microhaematocrit centrifuge and span at 9 000 revolutions per minute (rpm) for five minutes before measuring the PCV using a PCV reader.

3.4.3 Differential white blood cell counts

This was only done for the experimental animals. The resultant stained thin smears prepared for haemoparasite examination, were used for differential leukocyte counts as described by Embert (1986). Briefly, stained thin blood smears were examined using the oil immersion objective for accurate cell identification. The slides were examined starting with the thin end of the smear, and systematically traversing the slide. The fields that were used for examination were those in which erythrocytes were well separated and the leukocytes thinly spread. Cells were identified as previously described (Anon., 2012) and a record was made using a multiple tally counter. A total of 100 cells were counted per slide.

3.4.4 Indirect fluorescent antibody test

This was done for both field and experimental animals. Plasma for the field trial and sera for the indoor trial was used for this test. Prior to testing, if the plasma were frozen, it was thawed before proceeding. Using U- shaped micropipette plates 195 ul of phosphate buffer saline (PBS) were dispensed into all wells in column 1, 3, 5, 7, 9 and 11 using a multi-channel pipette. Five ul of test plasma was sensed into each well containing 195 ul of PBS. This was the 1:40 dilution. Each well corresponded to one particular animal's plasma. 100 ul of PBS was then dispensed into all wells in columns 2, 4, 6, 8, 10 and 12 using a multi channeled pipette. Then 100 µl of diluted plasma with PBS was transferred from column 2 into column 1, i.e. 100 µl from well A1 into A2 and B1 into B2 and so on. This is the 1:80 dilutions. The negative and positive controls were also diluted in a similar manner. Then The IFAT- *T. parva* antigen slides where then removed from the -20°C deep freezer and dried using a hair dryer. Then, ten circles were made on the antigen slides using nail polish. These dilutions were performed for each sample. Fifteen µls of each diluted test plasma was pipetted into each ring for the 1:80. For the field trial 1:40 and 1:80 dilutions were made were examined. This was to make sure that weak positives and weak negatives were also detected. The 1:40 dilution is more sensitive than the 1:80. For the indoor trial only 1:80 dilutions were examined, because this is what is normally used for routine diagnosis for animal movements at Central Veterinary Research Institute (CVRI) and has it been validated. Fluorescein labelled antiglobulin (conjugate) was placed on the preparation which gave a yellowish-green fluorescent on positive samples when visualised under a fluorescent microscope.

3.5 Data Analysis

Stata SE 12 was used as the data analysis software. Descriptive statistics were generated for each group of animals in both the field and indoor trials. The chi-square test was used to test for association among categorical variables measured between the groups and the student's t-test for association between continuous variables. The relative risk for the indoor trial and survival analysis was done and Kaplan-meier curves were generated for each group and a log-rank test carried out to check for statistical significant differences in survival time between animals immunized with *T. parva* chitongo carried on ice and those immunized with that carried in liquid nitrogen. The relative vaccine efficacy (VE) was calculated as described by Orientien *et al*., (1985):

VE(%)=(1-RR)*100, where RR is the relative risk

And the 95% Confidence interval for the vaccine efficacy was calculated as follows:

- For the lower limit of VE=(1-RR_u)*100, where RRu is the upper limit of the relative risk
- For the upper limit of VE=(1-RR_l)*100, where RR_l is the lower limit of the relative risk

CHAPTER FOUR

4.0 Results

4.1 Field trial

A total of 593 cattle comprising of 29 herds from five districts were sampled at the beginning of the field trial. Hundred and twenty animals in each district were sampled in Choma, Kalomo and Namwala, 116 from Mazabuka and 117 from Monze (Table 4.1). From the 593 samples 291 were from cattle vaccinated with *T. parva* Chitongo stabilate carried on ice (VCIf), while 302 were from cattle vaccinated with *T. parva* chitongo stabilate carried in liquid nitrogen (VCLf). These animals were vaccinated without knowing whether they were positive or negative for *T. parva*.

The temperature, weight and PCVs of the animals were taken before vaccinating. The temperature of the cattle before vaccinating ranged from 37.7 ^{0}C to 39.9 ^{0}C with a mean value of 39.1 ^{0}C (95% CI 39 - 39.1). The weight ranged from 33 kgs to 340 kgs, with a mean value of 120.1 kgs (95%CI =115.6 - 124.5) and standard deviation of 55.5Kgs. The PCVs ranged from 20% to 50%, with a mean value of 34.9% (95% CI=34.5 - 35.4).

From the 593 blood smears collected, No piroplasms were seen in 549 of the animals, while *Theileria* piroplasms were seen in 44 of them at the time of vaccination (this was known later in the study as the analysis could not be done immediately). Kalomo and Namwala districts had the highest number of cattle positive for *T. parva* piroplasms on microscopy, with 16.7% each (95% CI = 10 - 23.4) (Table 4.1). No *T.parva* piroplasms were observed from cattle from Mazabuka and Monze districts.

Table 4.1: Pre-immunization prevalence of *Theileria* piroplasms in the study districts.

District	**No. animals sampled**	**Prevalence (%)**	**95% Conf. Interval**
Choma	120	2.5	0 -5.3
Kalomo	120	16.7	10 - 23.4
Namwala	120	16.7	10 - 23.4
Mazabuka	116	0	-
Monze	117	0	-

The overall pre-immunization results for the IFA test showed that in the 1/40 dilution 20.0 % (95% CI=16.7% - 23.1%) were positive, while in the 1/80 dilution 8.4% (95%CI=6.2% to10.7% were positive. The IFAT pre-immunisation results showed that in the 1/40 dilutions Monze had the highest proportion of positives with 33.3% (95% CI= 24.7 -41.9) and in the 1/80 dilutions Choma had the highest proportions of positives with 12.5% (95% CI= 6.5 - 18.7) as shown in table 4.2. Furthermore, 3.39% (95% CI=0.10%-0.67%) of the samples collected on the day of vaccination were positive both on blood smears and IFAT (In the 1:40 dilution).

Table 4.2: Pre-immunization prevalence of *Theileria parva chitongo* on IFAT 1/40 and IFAT 1/80 in the study districts

District	**n**	**Proportion of animals that tested positive before immunizations in 1/40 (%)**	**95% Conf. Interval**	**Proportion of animals that test positive before immunization in 1/80 (%)**	**95% Conf. Interval**
Choma	120	13.3	7.2 - 19.5	12.5	6.5 - 18.5
Kalomo	120	11.7	5.9 - 17.4	5.8	1.6 - 10.1
Mazabuka	116	30.2	21.8 - 38.6	11.2	5.4 - 17
Monze	117	33.3	24.7 - 41.9	6.8	2.2 - 11.4
Namwala	120	11.7	5.9 - 17.4	5.8	1.6 - 10.1

Tables 4.3 shows the number of animals that were negative before immunization and were presented by owners in each district and tested positive after immunization. These post immunization results in table 4.3 show that Namwala had the highest proportion of positives in the 1/40 dilution with 95.1 % (95% CI=90.8 – 99.3) and Mazabuka had the highest proportion of positives in the 1/80 dilution with 93% (95% CI=86.9 - 99).

Table 4.3: Proportions of samples that tested positive after immunization from those that were negative before immunization per district in the 1/40 and 1/80 dilutions at 95% CI

District	n[1]	Proportion of animals that tested positive after immunization 1/40 (%)	95% Conf. Interval	Proportion of animals that test positive after immunization 1/80 (%)	95% Conf. Interval
Choma	97	84.5	77.3 - 91.8	81.4	73.6 - 89.2
Kalomo	91	89	82.5 - 95.5	84.6	77.1 - 92.1
Mazabuka	71	93	86.9 - 99	93	86.9 - 99
Monze	61	77.1	66.4 - 87.7	75.4	64.5 - 86.3
Namwala	101	95.1	90.8 - 99.3	58.4	48.7 - 68.1

The 1/40 dilution results showed that from the animals immunized with VCLf 89.6% (95% CI= 85.5 - 93.7) had seroconverted and from those immunized with VCIf 87.1% (95% CI= 82.5 - 91.7) had seroconverted (table 4.4). The 1/80 dilution results showed that VCLf 80.2% (95% CI= 74.8 - 85.6) seroconverted while in VCIf 75.1% (95% CI= 69.2 - 81) had seroconverted (table 4.4).

There was no significant difference in seroconversion between those animals vaccinated with stabilate carried in liquid nitrogen and that carried on ice for both the 1/40 and 1/80dilutions (P=0.450, P=0.242, respectively)(Tables 4.4 and 4.5 respectively)

[1] Number of animals that tested negative before immunization

Table 4.4: Positive post sampling proportions of IFA Test of 1/40 and 1/80 dilutions, *T.parva* vaccine carried in liquid nitrogen and that carried on ice

Dilutions	**Vaccine type**	**n**	**Prevalence (%)**	**95% Confidence Interval**	**p-value**
1/40	VCLf	212	89.6	85.5 – 93.7	0.450
	VCIf	209	87.1	82.5 -91.7	
1/80	VCLf	212	80.2	74.8 – 85.6	0.242
	VCLi	209	75.1	69.2 - 81	

.

4.3 Indoor trial

Of the 19 animals that were procured, 2 animals died before the experiment begun due to causes not related to the study. The remaining 17 animals (Nine in the group immunized with vaccine carried in liquid nitrogen-VCLi and eight in group immunized with vaccine carried on ice-VCIi) were immunized on the 28th day of the study as shown by the red arrow (Figure 4.1) and challenged after 41 days post-immunization as shown by the green arrow. The first piroplasms were seen from the group immunized with stabilate carried on ice on day six post-immunization and were seen 12 days post-immunization in the group vaccinated with stabilate carried on liquid nitrogen. The first schizonts were detected 12 days post immunization in some animals of both groups. Antibodies to *T. parva* on IFAT were detected from day 8 post immunization in some animals in both groups (6 in group VCLi and 6 in group VCIi. According to the IFAT test all animals in the two groups had seroconverted by day 14 post-immunization. During the experiment 4 more animals had died due to causes unrelated to the disease under study. These were on the 1st, 8th, 20th and 23rd day post-immunization. These were not included in the analysis. This left 14 animals to follow-up during the experiment: six in the group immunized with the vaccine carried on ice (VCIi) and 8 in the group immunized with vaccine carried in liquid nitrogen (VCLi).

Mean PCVs in each group per week were calculated and the results are shown in Figure 4.1 for the two groups. The figure shows that both groups had similar PCVs during the period of

the trial. The PCVs of the animals reduced in both groups as the trial went on. In group VCLi the mean PCV before immunization was 33.2% (95% CI 31 – 35.5), after immunization it was 33.3% (95%CI 32.4 – 34.2) and after challenging it was 30.1% (95% CI 28.3 – 31.9). In group VCIi the mean PCV before immunization was 32.9% (95% CI 30.6 – 35.2), after immunization it was 34% (95% CI 32.9 – 35.2) and after challenging it was 29.8% (95% CI 28 – 31.6). The PCVs continued to reduce in both groups after challenging the animals. The overall mean PCV in group VCLi was 32.10% (95% CI 31.2 - 32.1), while it in group VCIi was 32.29% (95% CI 31.3 - 33.3). There was no significance difference in the change in PCV between the two groups (p = 0.768).

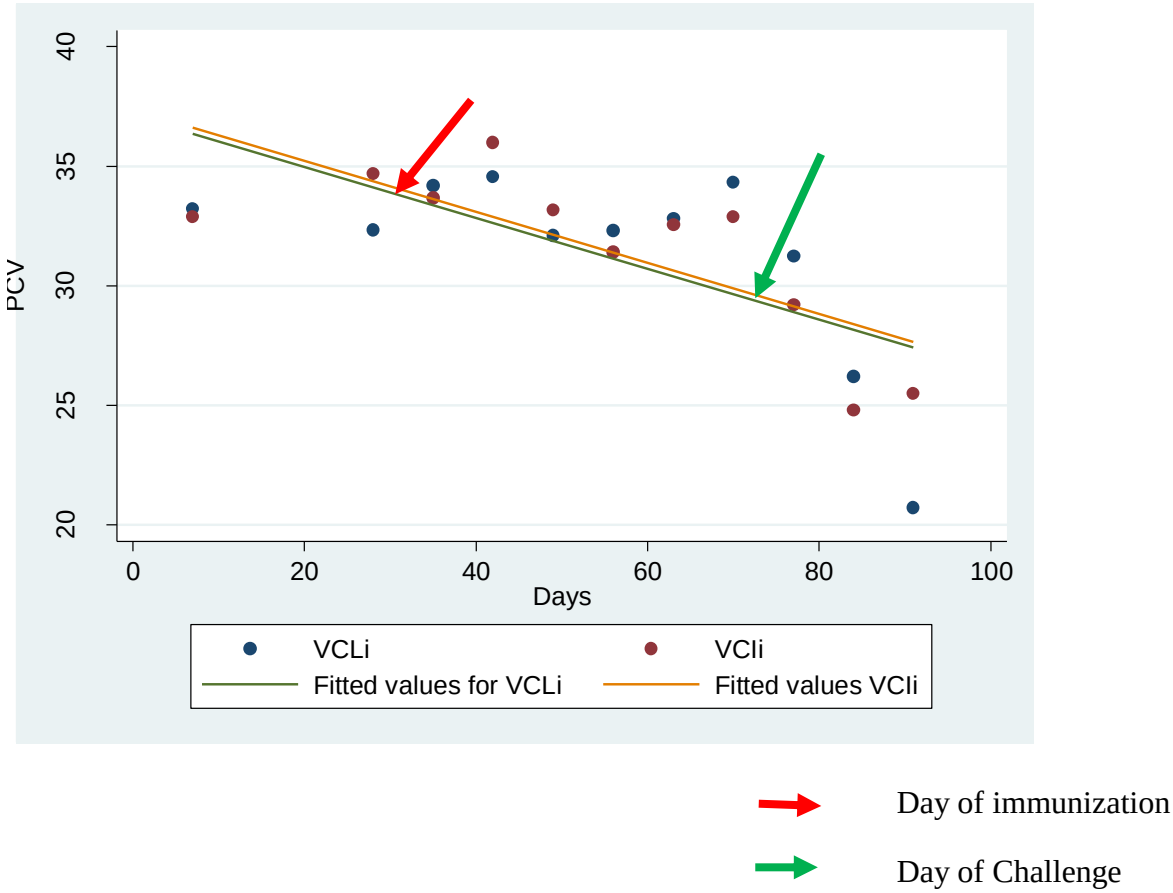


Figure 4.1. Weekly mean PCV of animals immunized with VCLi and VCIi

The mean temperature in each group per week was calculated and scatter plot and fitted lines are shown in Figure 4.2. The two groups also had similar mean temperatures during the

course of the experiment. The mean temperature in group VCLi before immunizations was 38.5˚C (95%CI 38.4 – 38.6), after immunization it was 38.6˚C (95%CI 38.4 – 38.7) and after challenging it was 38.7˚C (95%CI 38.4 – 38.9). The mean temperature in group VCIi before immunizations was 38.8˚C (95%CI 38.4 – 39.2), after immunization it was 38.4˚C (95%CI 38.3 – 38.7) and after challenging it was 38.4˚C (95%CI 38.1 – 38.6). Mean variations in temperature within the groups from before immunizations to after challenging were minimal. The overall mean temperature in group VCLi was 38.6˚C (95%CI 38.5 - 38.7) and that in group VCIi was 38.5 ◦ C (95%CI 38.3 - 38.6). There was no significant difference in the change in temperatures between the two groups (P=0.2484).

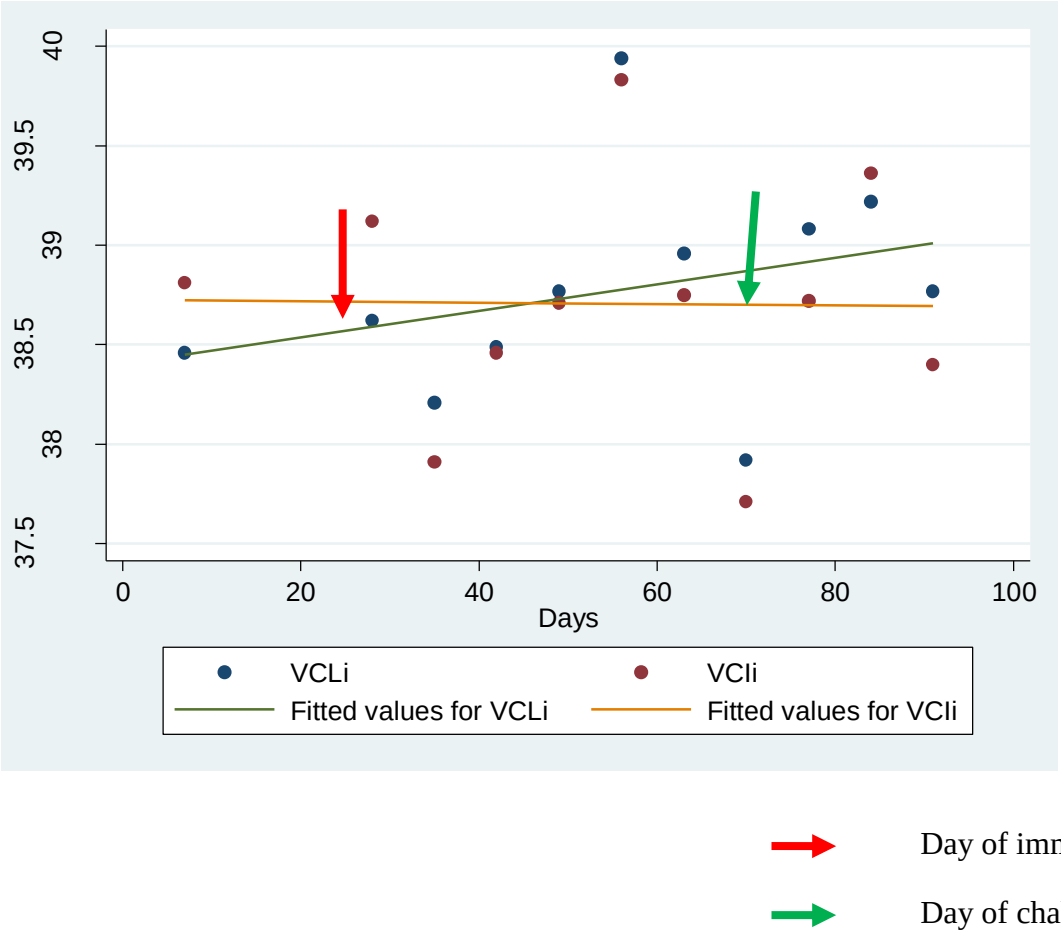
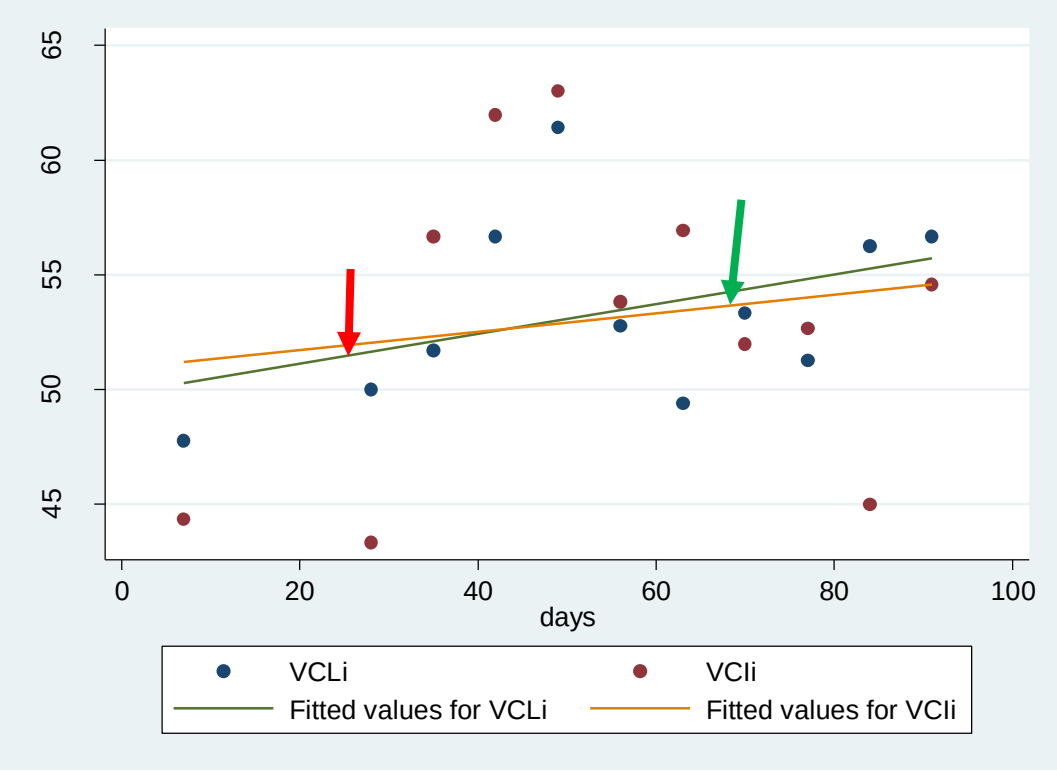


Figure 4.2 Mean daily temperatures of the calves in the two groups.

The mean numbers of leucocytes per week for each group were calculated (figure 4.4). Everything apart from the lymphocyte count was within normal range. There was a slight

increase in the leukocyte count probably due to the slight increase in the lymphocytes after immunizing the animals in both groups. A graph generated to compare the mean lymphocyte count between two groups (figure 4.3). The mean lymphocyte count (in percentages) for group VCLi before immunizations was 47.8% (95% CI 38.3 – 57.3), after immunization it was 54.9% (95% CI 52.3 – 57.4) and after challenging it was 52.7% (95% CI 49.7 – 55.7). The mean lymphocyte count (percentages) for group VCIi before immunizations was 44.3% (95% CI 34.7 – 53.9), after immunization it was 58.7% (95% CI 56.5 – 60.8) and after challenging it was 53% (95% CI 49.7 – 55.3). The increase in percentage count of lymphocytes in both groups was from the day of vaccination to the end of the experiment. The mean percentage of the lymphocytes in group VCLi was 53.4% (95% CI = 51.4 - 55.3) while in group VCIi was 55.3% (95% CI = 53.2 - 57.2 as shown in table 4.5. There was no significant difference in values of lymphocytes between the two groups as shown in table 4.5 (P=0.2081). There was no change in the number of Neutrophils, Eosinophils and Platelets in both groups during the course of the experiment as shown in Figures 4.6 and 4.7. These were within normal range of 2-7% for Monocytes, 2-20% for Eosinophils, 15-45% Neutrophils, and 0-2% basophils.



Day of immunization

Day of Challenge

Figure 4.3. Mean percentage of lymphocytes for animals vaccinated with VCIe and VCLe per week

Table 4.5 Mean number of lymphocytes for the two groups throughout the study

Group	mean	95% confidence interval	p-value
VCLi	53.4	51.4 55.3	0.208
VCIi	55.2	53.2 57.2	

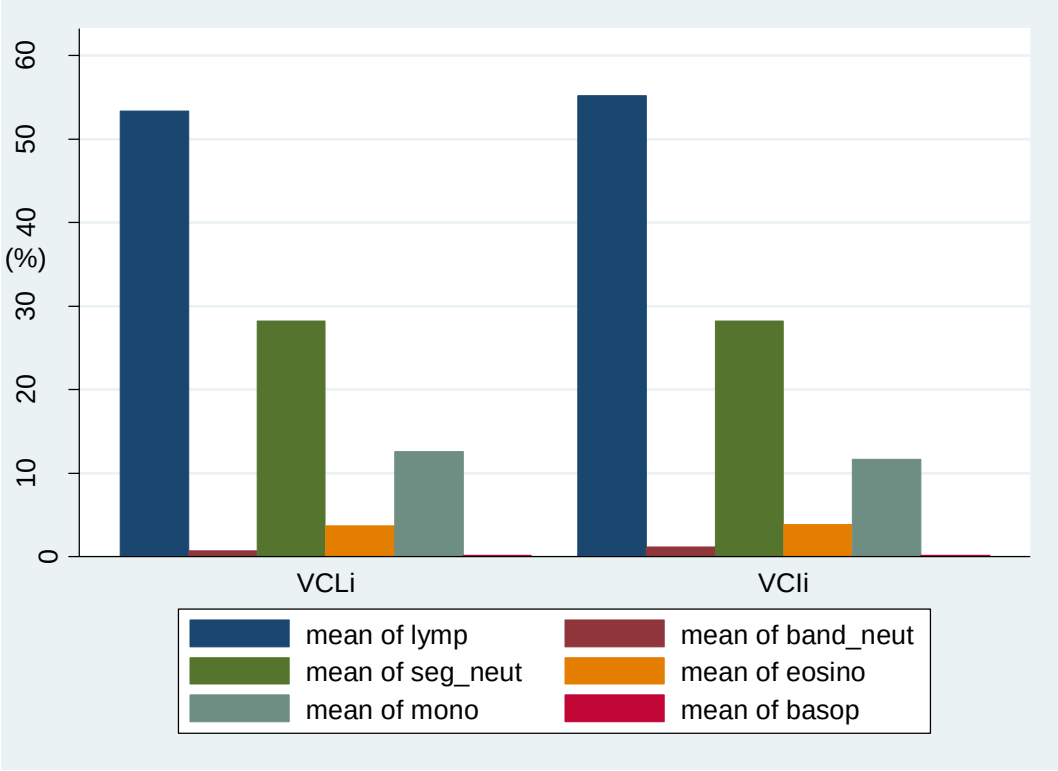


Figure 4.4. Differential blood cell count for animals immunized with vaccine carried on liquid nitrogen (VCLi) and vaccine carried on ice (VCIi)

Survival analysis was done on 14 animals from the group vaccinated with VCLi and VCIi. Only right censoring was considered in our study. Two animals in each group did not die during the study. These where put down at the end of the study time. The total time at risk was 663 animal days. The failure event was death of an animal. Table 4.6 below shows survival time by vaccine according to the carriage material. The calves in the VCLi group started dying on the 20th day after challenge, while the animals in VCIi group started dying 23 days after challenge. The column fail represents the number of animals that died during the time interval. At the end of 63 days post challenge only 2 animals were left in each group. The row function represents the probability that the subject will die in the given time interval, conditional upon surviving without the outcome up to the beginning of the time interval at 95% confidence interval for each time interval. A log-rank test was carried out (Table 4.7) to compare the survival probability between the group VCLi and group VCIi. The test showed that there was no significant difference between this two groups (p=0.8294)

Table 4.6. List of survival time by vaccine according to type of carriage material

Time in Days from post-immunisation	Total No. of animals at time t	Fail	Lost	Function	[95% CI of survival function]
VCLi					
20	8	2	0	0.7500	0.3148 0.9309
42	6	1	0	0.6250	0.2293 0.8607
51	5	1	0	0.5000	0.1520 0.7749
59	4	2	0	0.2500	0.0371 0.5581
63	2	0	2	0.2500	0.0371 0.5581
VCIi					
23	6	1	0	0.8333	0.2731 0.9747
29	5	1	0	0.6667	0.1946 0.9044
49	4	1	0	0.5000	0.1109 0.8037
59	3	1	0	0.3333	0.0461 0.6756
63	2	0	2	0.3333	0.0461 0.6756

Kaplan-meier graph (Figure 4.5) shows the probability of survival for animals immunized with VCLi and of animals immunized with VCIi. There was no significant difference in the survival time between the two groups (p=0.8294) (Table 4.8).

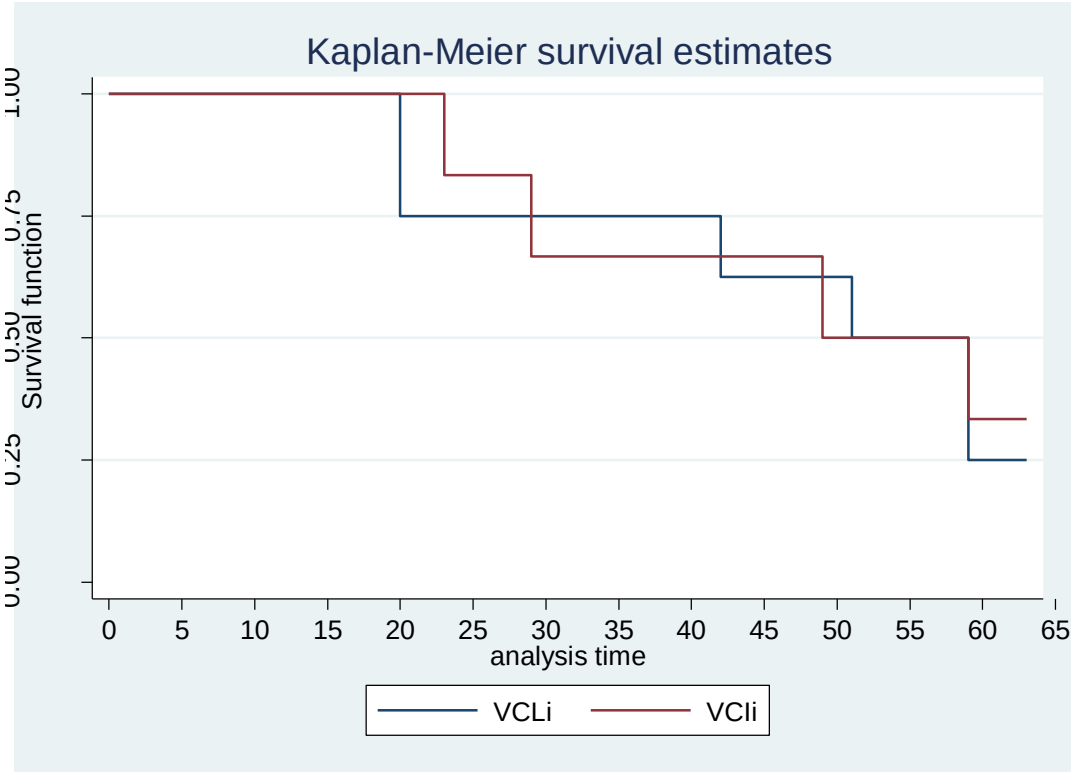


Figure 4. 5. Kaplan meier survival time estimates for group VCLi and group VCIi

Table 4.7.Log-rank test for equality of survivor functions

Vaccine	Events observed	Events expected	p-value
Stabilate in liquid nitrogen	6	5.68	0.829
Stabilate on ice	4	4.32	
Total	10	10.00	

In the group vaccinated with stabilate carried on liquid nitrogen, six out of eight calves died due to challenge with *T. parva*, while in the other group, four out of six calves died. Relative

risk was calculated 1.07(95%CI 0.41-2.83). There was no significant difference in relative risk between VCLi and VCIi (p=0.889).

The relative vaccine efficacy was estimated as 7% (95% CI = -183 - 59). Since the estimated 95% confidence interval included zero, it can be concluded that there was no significant difference in efficacy between the stabilate carried on ice and that carried in liquid nitrogen.

CHAPTER FIVE

5.0 Discussion

The aim of the field trial was to compare the efficacy of *Theileria parva chitongo* sporozoites carried on ice to that carried in liquid nitrogen. This was done by comparing seconversion ratios between the group vaccinated with stabilate carried carried on ice and the group vaccinated with stabilate carried in liquid nitrogen. The field study found no significant difference in seronversion rates between cattle immunized with stabilated carried in liquid nitrogen and that carried on ice for up to 4-6 hours in both the 1:40 and 1:80 dilutions. However, a similar study by Marcotty *et al*., (2001) in katete district using the katete strain found that immunisation performed with a stabilate kept on ice up to 6 hours was more efficient than the standard method (stabilate carried in liquid nitrogen). However, the difference in results could be due to many other factors that need further study. These factors may include the difference in stabilate used or regions where the immunization was carried out or difference in cultures.

In the field trial, animals positive for ECF on IFA test and blood smears examination were detected in some districts even though the animals were showing no clinical signs. This could be an indication of the endemic status of the disease in most plateau districts of Southern province of Zambia (Nambota *et al*., 1994). The IFA test may also have been less accurate because in endemic regions the seroprevalence fluctuates considerably although most animals have been infected (Billiouw *et al*., 2005). The detection of piroplasms from the blood smears could be due to the presence of carrier animals. These are animals that have recovered from the primary infection but maintain piroplasms circulating in the blood at levels that are high enough to infect ticks but not always detectable by routine diagnostic techniques (Medley *et al*., 1992). Given the low specificity of microscopy (Darghouth *et al* 1996, Simuunza, 2009), the detection of piroplasms only without schizonts, may also have been due to other non pathogenic *Theileria sp*. (Norval *et al.*, 1992). Mazabuka and Choma despite being endemic to *Theileria parva* showed 0% prevalence in piroplasms. This may have been due the low sensitivity of microscopy/blood smears (Darghouth *et al* 1996, Simuunza, 2009).

Generally from the pre-immunization results, the study showed that the IFA test was more sensitive than microscopy in detecting *theileria parva*. However, this detection of piroplasms without schizonts is inconclusive because this might be due to the presence of other theileria species. Blood smears are not the most accurate form of diagnosis for *T.parva* because accurate diagnosis relies on the training and experience of the laboratory personnel, and is considered to have low sensitivity and specificity (Darghouth *et al* 1996, Simuunza, 2009). This could explain the lower detection of positives from the blood slides as compared to the IFA test. The 1:40 is more sensitive than the 1:80 dilutions as demonstrated by Billiouw *et al.,* (2005). From our study both pre and post immunization results show that the 1/40 dilution detected more positives compared to the 1/80 dilution.

The mean temperatures of the animals at time of sampling were within the normal range, though on the high side. According to the Merck manual, (2005) the temperature range of beef cattle is from 36.7°C to 39.1°C. This may be because the sampling was done at a time of the day when tempratures were high. The body temperature of cattle increases as the day progresses and latter starts to reduce in the night and in addition, it may further increase depending on the activity and stress that is exerted on the animals (Parish *et al.,* 2009). The animals were being chased around in the kraal to be caught before vaccinating. However, it is important to note that some of the cattle sampled were positive for ECF. Therefore, it is possible that some of the higher than normal temperatures could have been due to the disease.

The weights of cattle included in the field study were very variable, with large standard deviations. The Cattle breed mostly found in southern province is the Tonga and its crosses (Aregheore 1994). Traditional herds normally reach weights of 500kgs for bulls and 300 kgs for cows (Aregheore 1994). Our study included all animals ranging from three months and above that were not ear tagged or had no history of vaccination. This partly explains the wide variations in the obtained weights of animals

The estimated mean PCVs of the study animals were slightly below range. According to the Navarre (2007), the PCV of beef cattle ranges from 24% to 46%. Navarre (2007) also observed that the PCV was usually on the upper 20s in adult cattle and slightly higher in calves. Our study animals consisted of both calves and adults as long as they had no history of vaccination. Anaemia has not been recognised as a major feature in classical *T. parva*

infections (Maxie *et al*., 1982) although it has been described in cattle infected with certain stocks of *T. parva* (Norval *et al*., 1992e4). From the pre-immunisation IFA test and blood smears, some of the cattle were infected with *Theileriosis.* These could explain the lower ranges in the PCVs, although the effect of other anaemia causing infectious and non-infectious diseases could not be ruled out. However from the mean value these results show that the animals were generally in good health before vaccinating.

The aim of the indoor trial was also to compare efficacy of *Yheileria parva chitongo* carried in liquid nitrogen to that carried on ice between two groups and further to see whether temperature and haematological parameter results could be replicated under controlled conditions. Only male dairy animals were included in this study due to financial contain. Male dairy animals tend to be cheaper than female dairy animals which are mainly used for milk production. Some study animals were lost before the experiment due to causes not related to the diseases under study. This could have most likely reduced the power of the study (Eng, 2003), although it is unlikely that it could have resulted in wrong conclusions.

The survival analysis data showed that there is no significant difference in survival time between the two groups hence the animals responded the same despite the difference in the carriage of the vaccines. The study also showed that the relative vaccine efficacy was not significantly different between VCLi and VCIi. Marcotty *et al.,* (2001), showed that there was a significant difference between *T. parva katete* strain vaccine carried in liquid nitrogen and that carried on ice. In their study *T. parva katete* strain carried on ice was better. However, Mbao *et al*., (2007) showed that Chitongo and Katete strains have similar infectivity losses when stored on ice.

The two groups had similar PCVs during the course of the trial. The PCVs started reducing after vaccinating the animals on the 28th day of the trial and reduced further after challenging the animals on the 69th day of the trial. This finding is similar to that reported in the study by Fandmu *et al*., (2007), where they found that anaemia occured in both lethal and non-lethal *Theileria parva* infected animals due to a reduction in PCV and Mean corpuscular volume (MCV). IIana (2012) also found that theileriosis caused a significant decrease in PCVs in cattle.

The average temperatures in both groups were within normal range apart from days six-eight after immunisation and days 10-12 after challenging. High temperatures were expected in theileriosis infection according to Fandamu, (2005). Variations in body temperature were observed throughout the study, mainly because they were not taken at the same time of the day. Temperature variations could have been further been attributed to environmental factors such as weather and also exercise of animals during the process of restraining.

The average number of lymphocytes in both groups per week was increasing from the day of immunization until the end of the experiment. However these were within the normal range of lymphocytes. The normal range of lymphocytes of dairy cattle is 45%-75% (Merck Manual, 2005), The slight increase of leukocytes in both groups was probably due to proliferation of lymphocytes in the lymphoid organs as defensive response to invading parasite. The sporozoites invade host lymphocytes where they rapidly differentiate into schizonts, a process associate with transformation of the infected cells to a state of uncontrolled proliferation (Fawcet *et al*. 1982). All other white blood cells were within normal range (Merck Veterinary Manual , 2005).

Blood and lymph smears showed that piroplasms and schizonts could be detected 6-12 days and 12 days post-immunisation respectively. According to the IFAT results, seroconversion of the animals had started by day 8 and all animals had seroconverted before challenge. According to Fandamu (2005), under experimental conditions, using either infected ticks or sporozoites stabilate, the incubation period ranges from 8 to 12 days.

It was observed that a number of animals had died during the experiment. Freisian cattle which are bos taurus were used in this experiment. *Bos taurus* breeds are very susceptible to *T. parva* infection (Norval *et al*., 1992). It should be pointed out that resistance can be reduced in situations where there is malnutrition and stress of the animal (Fandamu *et al.*, 2007). Housing the animals may have stressed them and also the experiment was carried out in the cold season of the year making it difficult to keep them warm.

CHAPTER SIX

6.0 Conclusion

The field and indoor trials were conducted to compare the vaccine efficacy of *Theileria parva chitongo* carried in liquid nitrogen to that carried on ice. In the field trial it was found out that the seroconvesion rates were not significantly for cattle vaccinated with stabilate carried on liquid nitrogen and that carried on ice. In the indoor trial it was also found that the probability of survival between the two groups after challenge was not significantly different time. Further, the efficacy, temperature and haematological profiles were also similar. It can therefore be concluded from these results that the *T. parva chitongo* stabilate carried on ice has the same efficacy as that carried in liquid nitrogen and that the hematological parameters were the same accross the two experimental groups.

6.1 Reccomendations

This study indicates that *Theileria parva chitongo* stabilates can be carried on ice for upto six hours in the districts where it is used. Therefore, the carrying of theileria parva chitongo stabilates up to six hours should be encouraged because this method is cheaper and logistically easier. However, there is need for further studies to compare why the katete stabilate in a previous study was more efficacious on ice while our study concluded no significant difference when they have the similar infectivity losses when stored on ice.

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