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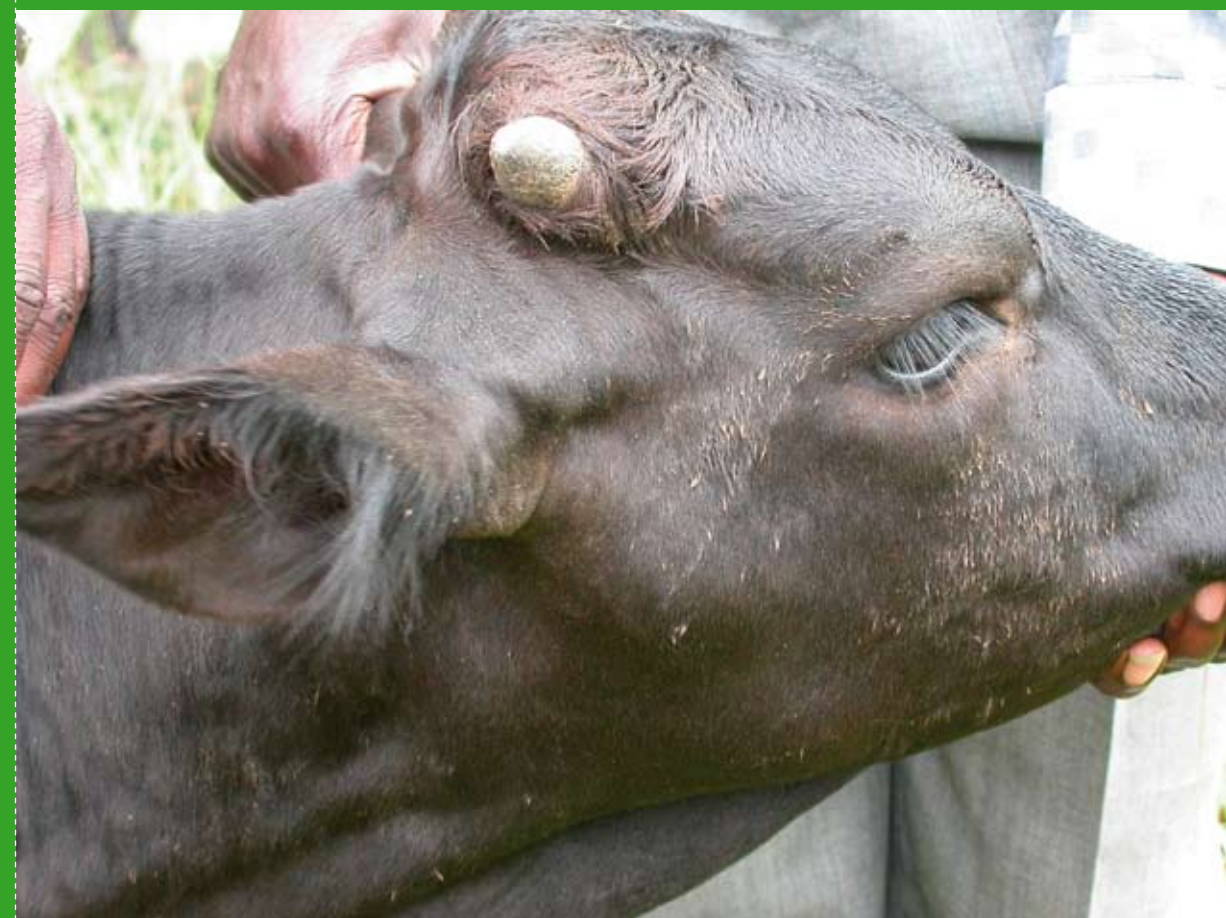

IN VITRO QUANTITATION OF *THEILERIA PARVA* SPOROZOITES FOR USE IN VACCINATION AND SPOROZOITE NEUTRALISATION ASSAYS

Victor MBAO

Proefschrift voorgedragen tot het behalen van de graad van
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To Caroline Mulenga

and our children Josiah and Tasheni.

Nine kapoli mukokota nsono, imibanga mise

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General Introduction

"Victory is won not in miles but in inches. Win a little now, hold your ground, and later, win a little more." Louis L'Amour

East Coast fever (ECF) is an acute and often lethal tick-borne disease of cattle presently endemic in eastern, central and southern Africa. The disease is caused by the protozoan apicomplexan parasite, *Theileria parva*, closely related to the malaria-causing *Plasmodium* species, with a high degree of specificity for both its vertebrate and invertebrate hosts. It is transmitted by the ixodid tick *Rhipicephalus appendiculatus* complex, also known as the "African brown ear-tick".

There are very few livestock diseases in the world today as devastating for the livestock farmer as East Coast fever. The disease is characterized by high morbidity and mortality, resulting in an estimated loss of about US$160 million per year in cattle deaths and lost production. About 20 million cattle are at risk in the endemic regions and one million die each year. It has major economic implications especially when livelihoods depend on livestock rearing. Consequently, research into the control of the disease has been prolific since the identification of the causal organism and vector in the early 20th century.

Theileria parva has a typical apicomplexan obligatory intracellular lifecycle infecting successively bovine lymphocytes, bovine erythrocytes, tick gut epithelial cells and salivary gland cells. The parasite replicates by schizogony and merogony in the lymphocyte and sporogony in the tick salivary gland. Ticks become infected when they feed on infected hosts and ingest infected erythrocytes. The parasite develops in the tick and is transmitted to the vertebrate host by the next tick instar by injection of sporozoites (infective stages for mammals) into the feeding site. Within a few minutes, the sporozoites invade lymphocytes in which they differentiate into schizonts and transform the host cell into a cancer-like phenotype. The transformation results in massive proliferation of infected cells and invasion of organs and tissues, which causes the clinical and pathological signs of the disease. These include fever, swollen lymph nodes, focal necrosis of lymphoid organs, lymphomata in the kidneys, interstitial pneumonia and froth in the lungs, and ulcers and hemorrhagic changes along the membranes of the digestive tract. Some of the schizonts undergo merogony and the resulting merozoites escape by rupture of the lymphocytes and invade erythrocytes, which are in turn ingested by ticks.

The severity of the disease depends on the numbers of infective sporozoites injected. This dose is in turn dependent on several factors: the numbers of ticks infesting the host, the proportions of ticks

infected and the infection levels within the ticks. The latter two parameters depend on the density of infected bovine hosts as sources of infection and the susceptibility of ticks to infection. Climatic conditions also determine the levels and distribution of the disease because they either favour or limit the survival of the tick vector. Another major determinant of the disease presence is the type of farming system, for example, herds that are trekked long distances in search of feed and water may be exposed to a higher risk of contact with infected ticks. Depending on the disease occurrence, areas are classified as either endemic or epidemic. An area is termed epidemic if disease appears for the first time and endemic if it is established. The classification of the degree of endemicity and the criteria used to define it are currently important topics of much discussion but generally, endemicity is classified as either stable or unstable.

Cattle that have contracted the disease and survive become solidly immune to that particular parasite antigenic type. The protective immunity generated against *T. parva* is mediated by cytotoxic lymphocytes rather than antibodies. However, antibodies directed against sporozoites are generated although they do not confer protection. Recovered cattle become parasite reservoirs and remain a source of infection for ticks.

East coast fever can be controlled by different methods or combinations of these methods: tick control, livestock movement restrictions, treatment and immunisation. Tick control works well when it is well managed with careful acaricide applications, adequate water and good waste disposal. It may also be the strategy of choice when there is a high risk of other tick-borne diseases. However, it is expensive and the acaricides may contaminate the environment. The risk for development of acaricide resistance by the tick can not be ignored and a single break in the tick control regimen can result in disease and death of animals. Livestock movement restrictions are difficult to enforce in most African countries due to limited resources and the sheer expanse of farming areas. Treatment is effective if instituted early in the course of the disease and a number of highly efficacious drugs are available although they are expensive and often beyond the capacity of the livestock owner to pay.

Among the control strategies, immunisation by the "infection and treatment" (I & T) method has great potential and is being applied by an increasing number of countries and farmers. It is currently the only method available for protecting cattle against lethal infections from parasites of the same antigenic type or strain as those in the vaccine. Immunisation involves inoculating cattle with a defined dose of live parasite sporozoites and simultaneously injecting a long acting formulation of tetracycline. The infective material is prepared as suspensions of triturated infected ticks and

cryopreserved as stabilates. The presence of several antigenic types in a locality complicates both the epidemiology and the control of the disease by immunisation.

Before field use, stabilates are titrated to assess their efficacy and to determine the optimal immunising doses. The principle objective is to calculate a dose that gives maximum protection with minimum clinical effects. Determining the actual number of viable sporozoites contained in a stabilate dose is difficult and cumbersome for routine dose determination. The method used is to inoculate groups of cattle with stabilate at different dilutions and evaluate clinical and parasitological reactions. While the major demand for quantitation of *T. parva* stabilates is dose determination, it is also required in research to compare diverse stocks as well as in improving vaccine production, storage and distribution. In improving stabilate production protocols, quantitation is necessary to prove that proposed modifications do not alter significantly the efficacy of the stabilate. Evaluating these effects *in vivo* is at great cost and is not precise due to variations in reactions of individual animals. In addition, it raises animal welfare concerns. It is for these reasons that less expensive, more reliable and ethically acceptable substitute techniques are being sought for quantitating sporozoite concentrations and studying immunological mechanisms.

In vitro techniques have been shown to offer a possible solution. They are relatively cheap, ethically acceptable and give repeatable results. In addition, results are obtained in a fraction of the time that *in vivo* procedures take. In this thesis, we explore the optimization of two such techniques, *in vitro* titration and Sporozoite Neutralisation Assays (SNA) and use them to quantitate several variations of stabilates. The work attempts to show that viable sporozoites in stabilates can be quantitated relative to a control stabilate and SNA's can be adapted to approximate the *in vivo* conditions thereby making them more accurate. The statistical analyses required to analyse the two procedures are also discussed and improvements proposed that take into account random factors which may result in erroneous interpretation of results if ignored. This is more critical for experiments that require many repetitions in order to generating adequate sample sizes.

Using these *in vitro* procedures we first evaluate several critical processes in stabilate production and storage as they affect the survival of sporozoites and consequently stabilate efficacy. Tick homogenisation, medium composition and cryoprotection, freezing rates, storage on ice or in a domestic freezer and lyophilisation are investigated. Secondly, we use the refined Sporozoite Neutralisation Assay to screen sera obtained from cattle that had been inoculated with various forms of *T. parva* parasite material: live sporozoites, dead sporozoites and live schizont-infected

lymphocytes. The sporozoite neutralising capacity of sera raised against these materials is compared to that of anti-serum from a recombinant p67 immunised animal.

Chapter 1 – Quantitation of Theileria parva *sporozoites for use in vaccination and Sporozoite Neutralization Assay: Literature review*

"There came large, handsome oxen from the interior to Sena on the River Cuama, sixty miles form the coast. The oxen died suddenly, though fine and in good condition, and were given to the soldiers for food" - Father Monclaro in 1569

(Theal, 1916) cited by Henning (1932) who wrote that this was *"probably the first record of a disease that can be identified as East Coast fever"*

1.1. Introduction

This chapter reviews available literature on aspects of quantitating *Theileria parva* sporozoite stabilates for immunisation and related fields of research. Immunisation by the Infection and Treatment (I & T) method is currently the only technique for protecting susceptible cattle against East Coast fever, a disease of economic importance in eastern central and southern Africa. The history, epidemiology and control options of the disease are presented in brief.

Survival of *T. parva* in the natural hosts and *in vitro* is re-visited as it pertains to quality of immunising stabilates. The survival of the parasite is currently assessed by titrating selected doses of stabilates in groups of cattle. Available information is examined on other techniques of quantitating this survival and their suitability for vaccine dose determination reviewed.

1.1.1. The history of East Coast fever

East Coast fever (ECF) is considered the most economically important tick-borne disease of cattle in affected areas (McInerney *et al.*, 1992). The disease was endemic along the coast of East Africa "for generations" (Mettam and Carmichael, 1936 - cited by Norval *et al.* (1992f) and is believed to have originated in buffalo populations (Norval *et al.*, 1992f). Notwithstanding the opening quote to this chapter, which refers to a "probable" outbreak of the disease, scientific records describe the disease as having been introduced into southern Africa through Zimbabwe (then Southern Rhodesia) in 1902 by a consignment of cattle shipped from Tanzania to Beira, Mozambique for restocking after the rinderpest epidemic of 1896-97 (Gray and Robertson, 1902 - cited by (Dolan, 1999). High mortalities in both local and imported cattle ensued and the disease was initially wrongly diagnosed as Rhodesian redwater (babesiosis) by Theiler (1903). From this focus, the disease spread to other parts of central and southern Africa where the vector was already present.

A causative agent for East Coast fever was first described by Koch in 1898 (Norval *et al.*, 1992e) who mistook it for young forms of *Babesia bigemina* while investigating Texas fever in East Africa. The organism was first named *Piroplasma kochi* by Stephens and Christophers (1903) and a few years later called *Piroplasma parvum* by Theiler (1905), both cited by Norval *et al*. (1992e).

It was subsequently called *Theileria parva* when Bettencourt, Franca and Borges (1907) cited by Norval *et al*. (1992d), created a new genus comprising 26 species of *Theileria*.

1.1.2. Biology of T. parva

1.1.2.1. Classification of *Theileria* species

The taxonomy of *T. parva* according to Levine (1988):

Kingdom: **Protista**

Subkingdom: **Protozoa**

Phylum: Apicomplexa

Class: **Sporozea**

Subclass: **Piroplasmia**

Order: Piroplasmida

Family: Theileriidae

Genus: ***Theileria***

The species of economic importance that infect domesticated animals are: Theileria parva, Theileria annulata, Theileria taurotragi, Theileria mutans, Theileria velifera, Theileria orientalis in cattle; Theileria ovis, Theileria hirci in sheep and goats and Theileria equi in horses. Two former Babesia spp are now commonly described as T. equi and Theileria microti (Uilenberg, 2006). The latter causes human theileriosis (= human babesiosis) and is therefore of zoonotic importance.

Based on clinical manifestations and for convenience, *T. parva* was classified trinomially in the 1980's as 1) *T. parva parva*, that causes classical ECF, 2) *T. parva lawrencei* responsible for "Corridor disease" transmitted from buffalo to cattle and 3) *T. parva bovis* implicated in "January disease" unique to Zimbabwe (Uilenberg *et al.*, 1982). With improved molecular characterization techniques, it was decided to drop this form of classification and revert to single taxonomy as *T. parva* since there was no molecular basis for the distinction (Anon.1989).

It is known that within *T. parva*, stocks with distinct antigenic characteristics exist (Radley, 1981; Irvin, 1987; Nambota *et al.*, 1997; Geysen *et al.*, 1999). This poses complications for immunisation as protection is strain specific (Goddeeris *et al.*, 1986) and therefore, vaccine stocks have to be carefully matched to prevailing local stocks in particular regions. A few examples of stocks with antigenic differences that are relevant to immunisation are given in Table 1.

1.1.2.2. Hosts

T. parva is totally dependent on its hosts and cannot survive as a free living organism. Buffalo and cattle are the intermediate hosts while the sexual stages occur in three-host ixodid ticks *Rhipicephalus appendiculatus* and *Rhipicephalus zambeziensis*, which are principal field vectors of the parasite (Theiler, 1904; Lawrence *et al.*, 1983). The tick is considered the definitive host because the parasite undergoes a sexual propagation in this host. *R. appendiculatus* and *R. zambeziensis* are three-host ticks meaning that each instar feeds on a separate host and in between drops to the ground where moulting takes place (Figure 1). It does not infect goats or sheep under natural conditions though experimentally, ovine lymphocytes pre-treated with proteases are susceptible *in vitro* but the internalised sporozoite does not differentiate into the schizont stage (Syfrig *et al*., 1998). *T. parva* infected bovine lymphoblasts can also be established experimentally in severe combined immunodeficiency (SCID) mice where they develop into tumours (Fell and Preston, 1993).

The parasite is transmitted transstadially, meaning infection picked up in one instar is transmitted at the next instar (larva to nymph or nymph to adult) (Lawrence *et al.*, 2004). Transovarian transmission of *Theileria* spp does not occur in ticks (Theiler, 1904) and neither will infected larva transmit infection as adults if the intervening nymphal stage feeds on uninfected hosts (Lawrence *et al.*, 2004).

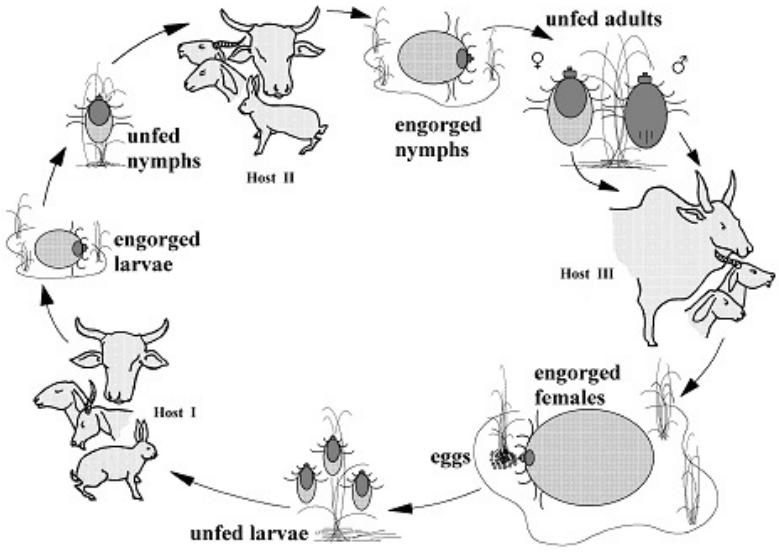

Figure 1: Life cycle of *Rhipicephalus appendiculatus*

Table 1: Some stocks of *Theileria parva* used for immunizing cattle against ECF.

Stock	Origin	Country	Reference
Katete	Katete	Zambia	(Geysen *et al.*, 1999)
Chitongo	Namwala	Zambia	(Geysen *et al.*, 1999)
Muguga	Kikuyu	Kenya	(Brocklesby *et al.*, 1961)
Marikebuni	Coast Province	Kenya	(Irvin *et al.*, 1983)
Buffalo 7014	Laikipia	Kenya	(Morzaria *et al.*, 1990)
Kiambu	Kiambu	Kenya	(Radley *et al.*, 1975b)
Kasoba	Karonga	Malawi	(Musisi *et al.*, 1996a)
Serengeti	Serengeti	Tanzania	(Radley *et al.*, 1975b)
Boleni	Boleni	Zimbabwe	(Lawrence and Mackenzie, 1980)
Uganda	Kigungu	Uganda	(Minami *et al*., 1983)
Nyakizu	Nyakizu	Rwanda	(Paling and Geysen, 1981)

1.1.2.3. Cycle of *Theileria parva* in the tick host

Tick instars feeding on an infected bovine ingest erythrocytes infected with piroplasms. The piroplasms differentiate into micro and macro-gametes in the gut lumen subsequently fusing to produce zygotes that invade the epithelial cells (Watt and Walker, 2000). This is the sexual reproductive stage of the parasite. Motile kinetes are formed from zygotes and migrate through the haemolymph to the salivary glands about the same time as the tick undergoes moulting (Mehlhorn *et al*., 1978). They invade the "e" cells of the type III acini and develop into multinucleated sporoblasts (Fawcett *et al.*, 1982a). Type III acini are responsible for fluid transport as the tick concentrates blood meals (Bowman and Sauer, 2004) and Binnington (1978) described the role of "e" cells as one of secreting granules that may be used in cementing the tick to the host upon attachment. When the tick starts feeding, the sporoblast syncytium undergoes cytoplasmic fission resulting in mature uni-nucleated sporozoites (Fawcett *et al.*, 1982a), which are the infective stage for cattle. Although experimental induction of sporogony by exposing the ticks to a temperature of 37°C has been described (Young *et al.*, 1979), in nature it is generally assumed that the tick needs

to feed for this process to occur. Sporozoites are about 0.75 – 1.5 µm in diameter and have a surface coat that is 20 – 25 nm thick (Shaw *et al.*, 1991; Shaw and Young, 1994)

Transmission to the bovine host occurs when the tick injects sporozoites suspended in the saliva as it feeds. Purnell and Joyner (1967) demonstrated that infected ticks feeding on blood products presented in glass capillary tubes salivated infective stages of *T. parva* into the blood meal in the tube. The tick's lifespan may be reduced by virtue of being infected (Watt and Walker, 2000). Similarly, reduced viability in *Boophilus* species heavily infected with *Babesia* has been observed (Ouhelli *et al.*, 1987; De Vos *et al.*, 1989).

1.1.2.4. Cycle of *Theileria parva* in the bovine host

Theileria parva successively invades lymphocytes and erythrocytes. It differs in a number of respects from other apicomplexans in that it does not have a well defined apical complex (Fawcett *et al.*, 1982b) and the secretory products of rhoptries and microspheres are ejected after internalisation (Shaw *et al.*, 1991). The sporozoite attaches to the lymphocyte and is internalized by active endocytosis within three minutes of attachment (at 37°C) (Shaw *et al.*, 1991). *In vitro* studies have shown that attachment is a random event and not energy dependent, whereas internalization is energy dependent and optimised at 37°C (Shaw *et al.*, 1991). It begins with close approximation of the parasitic and host cell membranes most possibly by legand-receptor mechanisms. *T. parva* differs from its apicomplexan counterparts in that it can attach to the target cell in any orientation (Fawcett *et al.*, 1982b). The sporozoite is then progressively internalised by becoming tightly apposed to the host membrane, in a "zippering" phenomenon described by Fawcett *et al*. (1982b) (Figure 2). It sheds its own surface coat (Webster *et al.*, 1985) and then dissolves the host cell membrane to escape into the host's cytosol (Fawcett *et al.*, 1982b) possibly as a defensive mechanism against the host cell's lysozymes. The internalised sporozoite quickly becomes associated with microtubules of the host cell, stabilises them (Shaw, 2003) and in the first few days following infection, develops into a pyriform shape that resembles *Babesia* piroplasms (Stagg *et al*., 1981).

From about day five post infection, development into a multinucleated syncytium, i.e. a macroschizont, takes place. This stage transforms the host cell and the lymphoblast starts to divide exponentially in synchrony with the schizont's own divisions so that both daughters cells usually inherit parasite material (Hulliger *et al.*, 1964; Stagg *et al.*, 1980; Stagg *et al.*, 1981). Rocchi *et al.* (2006) showed that not all originally infected cells are transformed and that even among transformed cells, there is sometimes failure for some daughter cells in receiving progeny

schizonts. The schizont is pulled apart on the microtubule spindle as the cells divide and sometimes one of the daughter cells is not infected or receives more schizont material than the other. This seems the most plausible explanation for the presence of uninfected cells in continuous cell lines of dividing lymphoblasts.

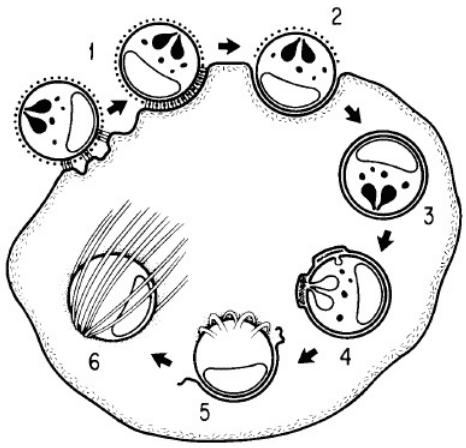

Figure 2: Drawing illustrating the sequence of events during the entry and establishment of *Theileria parva* in bovine lymphocytes. Reproduced from Shaw *et al.* (1991). (1) Binding of sporozoite to lymphocyte surface membrane (2) a very close apposition of parasite and lymphocyte membranes forms (3) "zippering" effect of the two membranes progressively internalises the sporozoite (4) the enclosing lymphocyte membrane separates from the sporozoite as contents of microspheres and rhoptries are released (5) lymphocyte membrane is dissolved and the sporozoite escapes into the host cell's cytoplasm (6) lymphocyte microtubules surround the sporozoite.

A proportion of these schizonts undergoes merogony and the merozoites are released into the general circulation upon rupture of the host cell. Merozoites infect erythrocytes in which they become piroplasms, the end stage of the cycle in the bovine host. Merogony at very low frequency may be the main mechanism by which infection is maintained in the carrier status (Maritim *et al.*, 1989; Dolan, 1999).

The mechanism bringing about lymphocyte transformation is not completely understood but several theories have been proposed. Some pieces of research implicate suppression of the lymphocyte's programmed cell death (PCD) by transcriptional factors or induction of heat shock proteins (Heussler *et al.*, 1999; Heussler *et al.*, 2001). This process would support the phenomenon of immortalisation. Other research pieces propose that oncogenes are switched on or the parasite inactivates pathways that prevent uncontrolled cell replication or both through transcription factors as reviewed by Chaussepied and Langsley (1996) to explain the cancerous

behaviour. It would not be farfetched to speculate that a combination of all or some of these factors and others yet to be elucidated could be at play simultaneously (Shiels *et al*., 2006). Figure 3 illustrates the parasite's developmental cycle in the bovine and tick hosts.

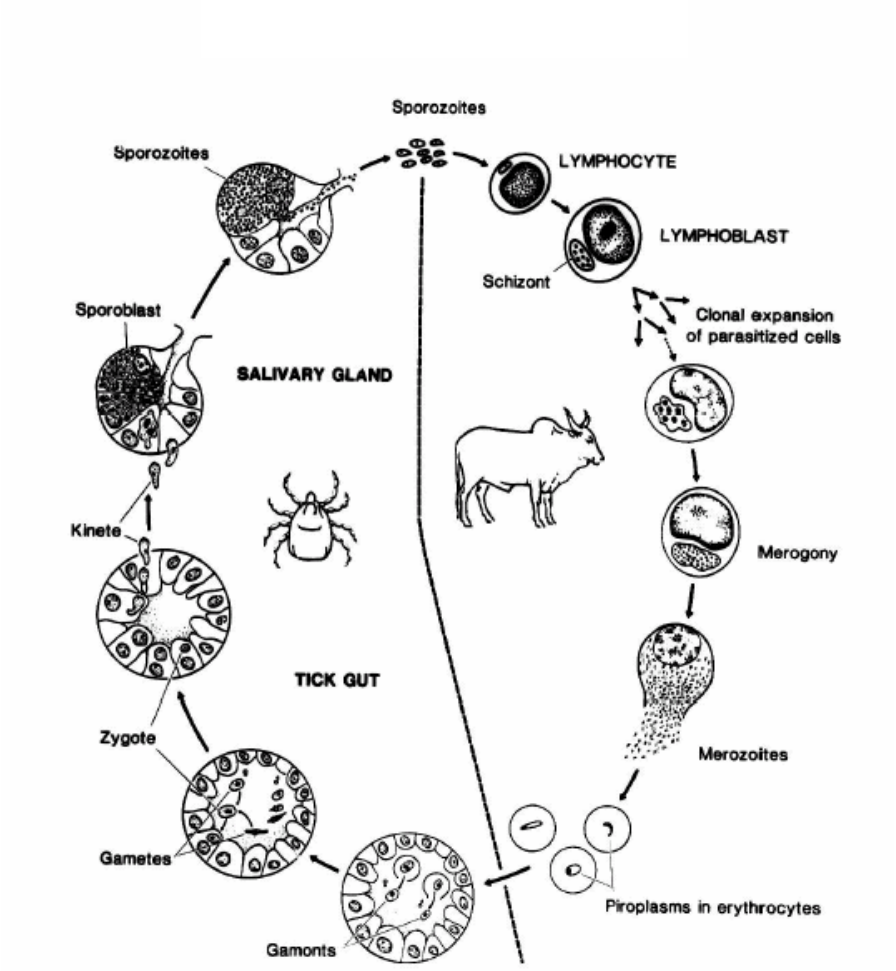

Figure 3: *Theileria parva* in the bovine and tick hosts (Bishop *et al.*, 2004).

1.1.3. Pathogenesis and pathology of T. parva

ECF is a lymphoproliferative condition and the pathology is attributed to massive invasion of lymphoid tissues, lungs and other organs by proliferating transformed lymphocytes (Jarrett *et al.*, 1969; Radley *et al.*, 1974; Dolan *et al.*, 1984b; Norval *et al.*, 1992b). Infected cells release and induce release of cytokines causing inflammation of the tissues. Destruction of infected lymphocytes releases enzymes and proteins enhancing inflammation. Infiltration of the lungs by proliferating cells induces interstitial pneumonia and exudative interlobular edema, which is the cause of death in acute cases. The condition is also characterized by multifocal necrosis of lymphoid tissue giving a picture of multi-centric lymphoma, which coincides with the onset of

fever. There is significant leucopoenia in lethal cases (Irvin and Mwamachi, 1983). In contrast to tropical theileriosis, caused by *Theileria annulata*, anaemia is not a common feature of East Coast fever (Wilde, 1967; Maxie *et al.*, 1982). However, Mbassa *et al*. (1994) claim that a Tanzanian stock of *T. parva* causes severe anaemia by merozoites invading erythroid precursors. Fandamu *et al*. (*in press*) also reported significant decrease in the PCV of cattle undergoing lethal reactions after infection with *T. parva* Katete. Therefore, it is apparent that some *T. parva* stocks exceptionally induce anaemia.

The severity of disease is dependent upon *T. parva* stock (Morrison, 1996), quantum of infection (Radley *et al.*, 1974; Dolan *et al.*, 1984b) and bovine host factors (Morrison *et al.*, 1996; Fandamu *et al.*, *in press*). For instance, the Boleni strain from Zimbabwe is known to be mild enough for cattle to control the infection without chemotherapy (Kanhai *et al*., 1997). Exotic breeds (*Bos taurus*) are generally more susceptible to developing clinical disease than African indigenous (*Bos indicus*) cattle (Ndungu *et al.*, 2005).

1.1.4. Immunity

Cattle surviving infection develop a solid immunity to the homologous strain of the parasite for at least three years (Burridge *et al.*, 1972). The role of humoral responses is not considered significant in ECF immunity as evidenced from the failure of protection against lethal challenge after transfer of immune sera or colostrum to susceptible animals (Muhammed *et al.*, 1975). In addition, Creemers (1982) has shown that anti-sera from lethally infected cattle were negative in antibody-dependent cytotoxic assays indicating that they did not initiate antibody mediated cytotoxicity. The most studied sporozoite antigenic determinants inducing detectable neutralising antibodies against sporozoites are the Polymorphic Immunodominant Molecule (PIM) (Toye *et al.*, 1996) and the 67kD circum-sporozoite protein termed p67 (Nene *et al.*, 1992). Specific antibodies directed against sporozoites do have the capacity to neutralise the parasite and consequently control infection but high titres are required for a full effect as the time sporozoites spend in the extra-cellular environment is very short. It could also be envisaged that specific antibodies directed against merozoites could play a role on the number of infected erythrocytes.

It can be deduced from the foregoing that long lasting protective immunity is not humoral mediated. Cell-mediated immunity has been shown to be the major protective mechanism by demonstration of adoptive transfer of immunity between twins (Emery, 1981; McKeever *et al.*, 1994). Infected cells express mostly MHC class 1 molecules on their surface. The immune response, mediated by cytotoxic T-lymphocyte (CTL) of the $BoT8^{+}$ subset, is directed at these

T. parva infected lymphoblasts (Morrison *et al.*, 1987; Goddeeris *et al.*, 1990; Taracha *et al.*, 1992; Taracha *et al.*, 1995). These CTL are parasite strain specific and restricted to the MHC class 1 markers (Goddeeris *et al.*, 1986).

1.1.5. Epidemiology

The disease is generally endemic in the eastern, central and southern regions of Africa within the geographic distribution of the tick vector (Figure 4). Tick numbers, tick infection and susceptibility of bovine hosts are the main determinants of risk of infection (Medley *et al*., 1993). Climatic factors, particularly rainfall, affecting tick distribution and survival have been found to play a major role in the occurrence and seasonality of the disease (Branagan, 1973a; Branagan, 1973b; Short *et al.*, 1989; Berkvens *et al.*, 1995; Mulumba *et al.*, 2000; Mulumba *et al.*, 2001; Fandamu *et al.*, 2005). Transmission and occurrence of disease may be seasonal or not depending on the rainfall patterns that determine the tick instars and population densities on the host (Norval *et al.*, 1991; Mulumba *et al.*, 2000; Mulumba *et al.*, 2001). The main bovine host factor complicating the epidemiology of the disease is the carrier state in recovered or immune animals (Neitz, 1964; Young *et al.*, 1986; Dolan, 1986a; Bishop *et al.*, 1992; Latif *et al.*, 2001), which serve as infection sources for field ticks. Other bovine host factors include susceptibility to *T. parva*, resistance to ticks, breed and age (Fivaz *et al.*, 1989; Koch *et al.*, 1990; Latif *et al.*, 1991; Ndungu *et al.*, 2005). It has been shown recently that the mean volume of erythrocytes is correlated to the fatality of cases with smaller sizes predisposing the symptomatic animal to death (Fandamu *et al.*, *in press*).

The degree of disease establishment is broadly classified as epidemic or endemic. Endemic stability is a state in which all calves come in contact with the disease within a few months of age and morbidity is low (Moll *et al*., 1986). This situation is observed in East Africa where climatic conditions allow the all year round possibility of the tick to infect the bovine host and where innate resistance of the local cattle to the prevailing parasite stocks is known to occur (Moll *et al*., 1984).

In contrast, Billiouw *et al.* (2002) have shown that this is not the case for eastern Zambia where the disease was introduced more recently, i.e. during the early 20th century, and tick presence and therefore transmission pressure is seasonal.

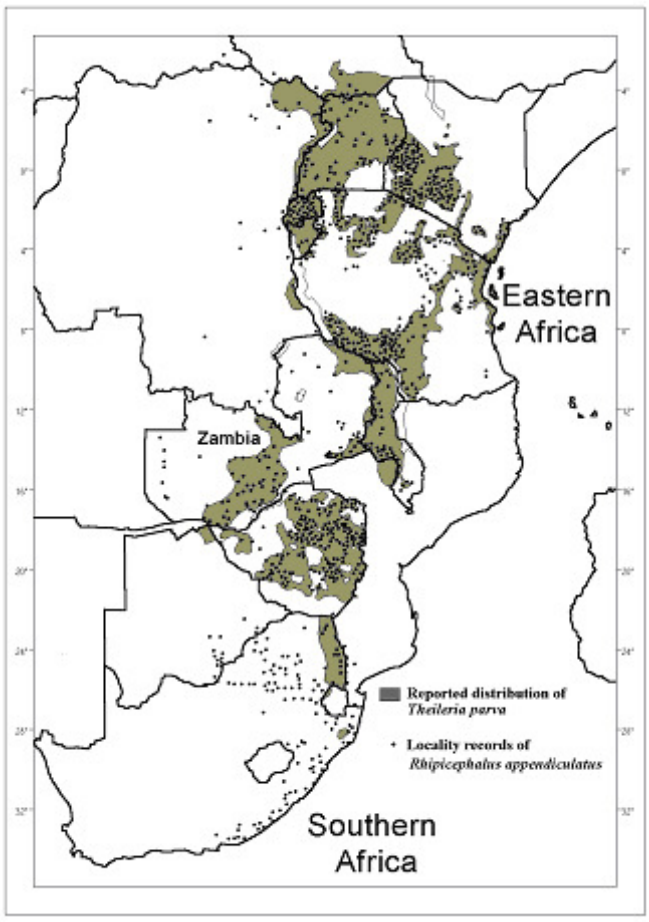

Figure 4: Distribution range of *Theileria parva* (adapted from Norval *et al*., 1992e; Speybroeck *et al*., 1999 and Chaka, 2001) and the distribution of *Rhipicephalus appendiculatus* (adapted from Berkvens *et al*., 1998; Speybroeck *et al*., 1999; Walker *et al*., 2000 and Chaka, 2001).

Some authors have graded the degrees of endemicity from very stable to very unstable (Norval *et al.*, 1992c) depending on factors such as age at first contact of calves, sero-prevalence in the adult population and case fatalities. Billiouw (2005) suggests a reclassification of the states into epidemic, first level endemic stable, second level endemic stable and ultimate endemic stable states. The criterion is mostly based on stability of infection prevalence and age at first contact. As such, he classifies the state in eastern Zambia as first level endemic stability.

1.1.6. Diagnosis

1.1.6.1. Clinical signs

Cattle undergoing a clinical manifestation of the disease present with a high fever, which may reach 42°C. They develop enlarged superficial lymph nodes especially the parotidian (ln.

parotideus superficialis) and prescapular as they are near the predicted tick feeding sites, standing coat, lacrimation, anorexia and dyspnoea in acute cases. Diarrhoea may also be present in more chronic cases. Corneal opacity and nervous signs may be observed in some cases, the latter being commonly misdiagnosed as cowdriosis.

1.1.6.2. Laboratory

East Coast fever is diagnosed by detection of piroplasms in the blood, schizonts in lymph node biopsies, antibodies by the Immuno-Fluorescent Antibody Test (IFAT) (Burridge and Kimber, 1972) and PCR techniques (Bishop *et al.*, 1992; Ogden *et al.*, 2003). PCR, though highly sensitive, is not suitable for routine field use due to high cost. A slide enzyme-linked immunosorbent assay (SELISA) described by Kung'u and Goodger (1990) for *Babesia* has been adapted to use schizont-infected lymphoblasts as antigen for ECF diagnosis.

1.1.6.3. Post-mortem

Most of the pathology observed at post-mortem examination results from effects of tissue infiltration by proliferating lymphoblasts. The signs include ulceration of the abomasal mucosa, multifocal necrosis of liver and kidneys, froth in the trachea, enlarged and hemorrhagic lymph nodes, hemorrhagic enteritis of the caecum (Zebra stripping) and enlarged spleen. Lymphoid tissues may be atrophic if the animal has passed through a chronic course of the disease.

Field-use positive diagnosis consists of presence of clinical signs and demonstration of schizonts. In the absence of detectable schizonts, clinical signs with or without detectable piroplasms only constitute a suspected case (Yeoman, 1966).

1.1.7. Control

The control of ECF is a matter of utmost importance for the livestock industries of the region. Controlling the disease has evolved from livestock movement control and slaughter, to tick control with acaricides, to immunisation by the infection and treatment method (Radley, 1981; Norval *et al.*, 1992e). Effective chemotherapy has also been developed but tick control has been the most widely implemented strategy (Berkvens, 1991; Pegram *et al.*, 2000). Integrating the various methods, depending on epidemiological status, among other considerations, is currently the policy of choice in many affected regions (Uilenberg, 1996). To a large extent, uncontrolled livestock movement is responsible for spread of both vector and parasite to new areas (Berkvens *et al.,*1988; Billiouw *et al.*, 1999). However, limiting the movement of cattle from infected areas is largely unsuccessful in countries where the informal farming sector is difficult to police, or where

nomadic pastoralism is the main livelihood of the people, due to sheer expansiveness of the areas involved.

1.1.7.1. Chemotherapy

From the early 1980's, research was directed at evaluating the efficacy of a myriad of compounds previously identified as having some antitheilerial activity including chloroquine, tetracycline, halofuginone, menoctone and parvaquone (Dolan, 1981). The ones that were efficacious and became commercially available were based on three compounds namely, quinazolinones (halofuginone) and the naphthoquinones (parvaquone and buparvaquone) (Dolan *et al.*, 1984a; Kiltz and Humke, 1986; Chema *et al.*, 1987; Dolan *et al.*, 1992). The main constraints with chemotherapy are that the drugs are expensive and treatment must be instituted early in the course of disease to be effective. Paradoxically, the advent of chemotherapy complicated the epidemiology of the disease in that recovered cattle become carriers (Dolan, 1986a; Dolan, 1986b). Carriers are then sources of *T. parva* for further transmission to susceptible hosts. Dolan (1999) has postulated that, deducing from evidence that *T. parva* is surviving chemotherapy, drug resistance may already be in place. Therefore, recovered animals may be transmitting selected resistant parasite populations. It is unlikely that resistance will favour more virulent strains. Studies of the closely related organisms, *Plasmodium* and *Eimeria* spp., have shown that there is no correlation between drug resistance and occurrence of virulent stocks (Williams, 2006; Giha *et al.*, 2006). Giha *et al*. (2006) found no statistically significant differences in the frequencies of *P. falciparum* resistant to chloroquine between severe and acute uncomplicated malaria patients. Olumese *et al*. (2002) working with Nigerian stocks proposed that "chloroquin-resistant parasites are more virulent and thus will naturally progress to severe disease" after observing an association between *in vitro* resistance to chloroquine and clinical outcome of the disease. This seems to be rather the exception than the rule as observed by Peters (1968) who found that, out of nine *P. falciparum* tested, only the Nigerian strain scored high virulence at moderate sensitivity to the drug.

1.1.7.2. Immunisation

The "infection-and-treatment" (I & T) method of immunisation developed by Radley *et al*. (1975a) is currently the only available technique for immunoprophylaxis against homologous challenge. The method is widely used in many of the affected regions of eastern and southern Africa (Berkvens *et al.,*1988; Uilenberg, 1999; Marcotty *et al.*, 2001; Fandamu *et al.*, 2006). It is executed by simultaneous inoculation of tick derived live sporozoites of *T. parva* as stabilates and a long acting tetracycline formulation. Stabilates are produced by triturating adult

R. appendiculatus ticks, artificially infected as nymphs, in suitable media. The method is described in full detail in the appropriate chapters. Given its importance for the present work, the major steps of I & T are herewith outlined in Figure 5.

Major components of freezing media used to preserve tick derived *T. parva* sporozoites are: cryopreservative agent (CPA), amino acids, buffers, antibiotics.

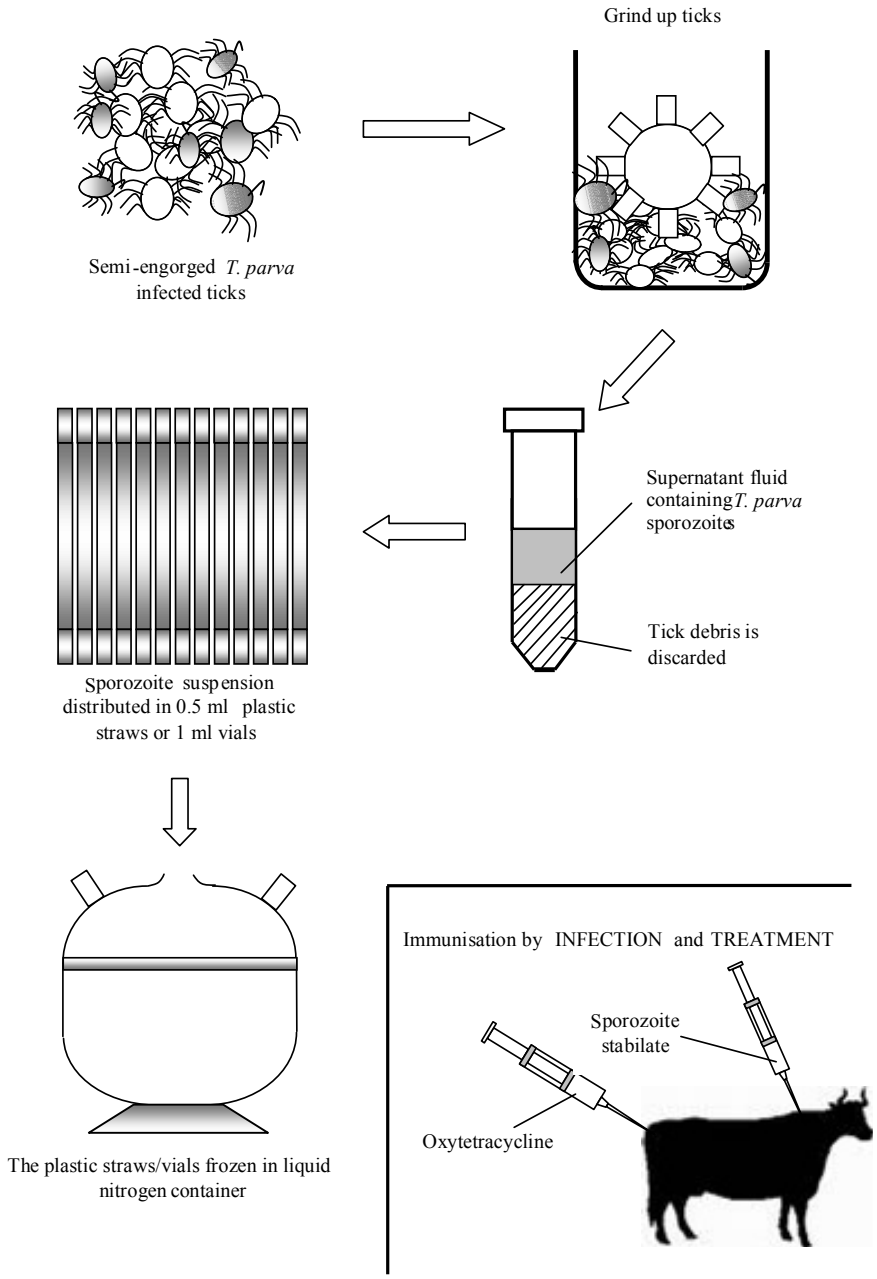

Figure 5: A diagrammatic representation of stabilate production and the Infection and Treatment method of immunisation against *Theileria parva.* Adapted from Norval *et al.* (1992a).

After extraction, the stabilates are purified by centrifugation to precipitate tick debris, and inert debris in case of manual extraction using crushed glass (or sand). A CPA is then added and the suspension adjusted to an equivalent concentration of 10 ticks/ml before cooling to -196 ºC by a standard slow freezing two-step procedure (OIE, 2005).

Quality control of stabilates is critical and focuses on determination of immunizing doses and efficacy. In principle, it is aimed at quantitating infective sporozoites in a stabilate. Quantitation is routinely done using naïve cattle. Groups of susceptible cattle are inoculated with a number of titrated doses and a number of clinical responses such as days of fever, parasitosis and parasitemia are evaluated. The serological response is monitored and the animals are challenged with a potentially lethal dose 35 days post-infection to assess the protection. Presently, this is the only method of quantitating infection potential for field use stabilates. Quality control involves estimating infectivity of the stabilates, which is directly related to the quantity of infective sporozoites (Radley *et al.*, 1974; Dolan *et al.*, 1984b), and testing so that the immunizing dose does not cause overt disease but also protects.

The Achilles' heel of the I & T method is the strain specificity of the immunity generated. In addition, immunized animals become carriers of the parasite (Kariuki *et al.*, 1995; Marcotty *et al.*, 2002). As an effort at producing a broad spectrum vaccine, a polyvalent vaccine termed Muguga cocktail, was developed in the early 1980's (Radley *et al.*, 1975a; Radley, 1981). It comprised three stocks: Muguga , Kiambu 5 and Serengeti-transformed (Radley *et al.*, 1975b), all isolated from eastern Africa and has been used widely in east and central African countries (Radley, 1981).

However, controversy arose concerning epidemiological implications of introducing exotic parasite stocks in regions far removed form the polyvalent-vaccine stock origins. Since it is possible to experimentally hybridise stocks during sexual reproduction in the tick, it is possible that this occurs in nature though the extent is unknown (Bishop *et al*., 2004). Therefore, exotic stocks may undergo genetic recombination with local stocks further complicating the epidemiology. Geysen *et al*. (1999) strongly suspects that one of the Muguga cocktail components is now widely distributed among the local stocks isolated from southern Zambia where the cocktail was used in immunisation trials. To circumvent the possible risks of introducing exotic strains, some countries opted to use locally isolated stocks. Such univalent vaccines are currently used in different regions such as the Katete and Chitongo stocks in Zambia (Berkvens *et al.,*1988; Geysen *et al.*, 1999), Boleni in Zimbabwe (Lawrence and Mackenzie, 1980; Hove *et al*., 1995) and Marikebuni in Kenya (Irvin *et al.*, 1983)

Since the mid 1970's, there has been a concerted effort at developing sub-unit vaccines that have the advantage of not inducing a carrier state and are not strain-specific. Studies have shown that cattle receiving repeated *T. parva* challenge mount a humoral response against the sporozoite stage (Musoke *et al.*, 1982). A circum-sporozoite antigenic protein (p67) was identified in such animals and recombinant forms of this protein have been shown to induce high antibody titres in cattle (Musoke *et al.*, 1992; Kaba *et al.*, 2005). Recombinant p67 proteins, though promising, have only offered partial protection under field conditions (Heussler *et al.*, 1998; Kaba *et al.*, 2004; Musoke *et al.*, 2005) and a correlation between antibody titres and degree of protection is yet to be shown (Wagner *et al.*, 1974; McKeever *et al.*, 1999; Nene *et al.*, 1999).

Concurrently, research is focussed on identification and characterization of antigenic determinants that provoke cellular immunity and the dynamics of the intra-lymphocytic stage of the parasite, which may be used in sub-unit or DNA vaccines (Taracha *et al.*, 1997; Heussler *et al.*, 1998; Graham *et al.*, 2006) and models for vaccination regimens (Hall *et al*., 2002). This approach aims to induce cell mediated immunity which is much more efficient than antibody responses as reviewed in section 1.3. The search also continues for sporozoite neutralising vaccine candidate antigens (Skilton *et al*., 2000). It is envisaged that combining anti-sporozoite and anti-schizont sub-unit vaccines, could confer a more solid immunity. The anti-sporozoite immunity would serve to reduce the parasite challenge, by neutralising a proportion of the inoculated sporozoites, as the severity of disease is dose dependent.

1.2. The parasite survival

1.2.1. Sporozoites

Studies of infected ticks show that parasite survival depends on climatic factors (humidity, temperature) and on abundance of infection (Watt and Walker, 2000; Ochanda and Young, 2003; Ochanda *et al.*, 2003). Theiler (1905) reported that in the absence of feeding, adult *R. appendiculatus* lose their infection within 15 months after their last meal as nymphs while in a similar study, starved adult ticks were still infected with a non-pathogenic *Theileria* spp. after a period of 20 months (Newson *et al*., 1984). Young *et al*. (1983) reported the survival time of *T. parva* in adult ticks to be up to 547 days in quasi-natural conditions. In nymphs maintained in quasi-natural conditions, survival of infection can be up to 50 weeks and in laboratory conditions 26 weeks (Ochanda and Young, 2003).

It is postulated from *in vitro* observations that in the bovine host, the sporozoite becomes associated with target cells and is internalized in a matter of minutes or seconds (Fawcett *et al.*, 1982b; Webster *et al.*, 1985). Thus, its lifespan as an extra-cellular organism once injected by the feeding tick is very short. The short time in which it attaches and enters target cells is most likely a mechanism that ensures its evasion of the hostile extra-cellular environment.

Being an obligate intra-cellular organism, survival of *T. parva* outside the vertebrate and invertebrate host is only possible through suspension of the metabolic reactions by cryopreservation. The preparation of cryopreserved stabilates made the I&T method of immunisation possible and in the search for effective preservation of live sporozoites, a technique adapted from preservation of trypanosomes was developed by Cunningham *et al*. (1973a). In principle, organisms preserved at -196 ºC remain viable virtually indefinitely as biochemical reactions are suspended due to insufficient thermal energy (McGee and Martin, 1962). It is the transition from ambient temperature to storage temperature and back that is deleterious and the degree of damage depends on the cryo-preservative agents (CPA's) used and the rates of cooling and thawing. During stabilate production, sporozoites are subjected to stressful conditions between extraction from tick salivary glands and freezing as well as the subsequent thawing process. In this period, survival is particularly affected by the type of suspending medium and its associated osmolarity and pH, the rates of cooling/thawing and the holding temperatures and periods (Kimbita *et al.*, 2001; Kimbita *et al.*, 2004).

1.2.2. Schizonts

Theileria parva schizonts have the ability to transform the host cell to a lymphoblast state with resultant repeated mitotic divisions (Stagg *et al*., 1981). Maritim *et al*. (1989) showed that in the recovered bovine host, schizonts infected lymphocytes persist though at a reduced proliferation rate. However, some strains are known to induce a sterile immunity. In the laboratory, cultures of infected lymphoblasts can be propagated indefinitely from the immortalized cells (Hulliger *et al.*, 1966).

1.3. Quantitation of *T. parva*

Stabilates of *T. parva* sporozoites are produced by homogenising infected adult *R. appendiculatus* ticks in suitable media to which cryopreservatives are added for low temperature storage (Cunningham *et al.*, 1973a) as described in chapter 3. These stabilates are used for immunisation

against East Coast fever and for *T. parva* research. Quantitation of infectivity is critical for immunisation and research purposes in general, and in particular, when the effects of different preparation procedures, cryopreservatives (Cunningham *et al.*, 1973b; Kimbita *et al.*, 2001), composition of media and storage conditions (Musisi *et al.*, 1996b; Marcotty *et al.*, 2001; Kimbita *et al.*, 2004) are being explored in the development of improved storage and cryopreservation methods. Another important use for stabilates is in sporozoite neutralisation assays that are used to quantitate the degree of inhibition of sporozoite infectivity exerted by various immune sera *in vitro* (Musoke *et al*., 1982).

It is critical to have a measure of the infectivity of newly produced *T. parva* stabilates in order to determine immunizing doses and also to make comparisons between stabilates originating from different tick batches. Quantitating the potency of a stabilate based on the number of sporozoites per dose is difficult. Apart from the strain-related variability in virulence, the technical difficulties in assessing the number of viable sporozoites are considerable. Two quantitations are usually done during the course of stabilate production: one on the infected tick batches and the other on the resulting stabilate. The first one is mainly to exclude poorly infected batches from production.

Assuming that an infected acinus contains 100,000 sporozoites (Fawcett *et al.*, 1982a), the first step in quantitation involves screening tick batches to determine the average number of infected acini per tick (abundance). Tick batches from the field or laboratory will have varying degrees of infection depending on a number of factors that include piroplasm parasitemia, host susceptibility to ticks, tick stock, susceptibility of tick stock to particular *T. parva* parasite stock, duration of infection in ticks, ambient and or moulting temperatures (Young *et al.*, 1984; Ochanda *et al.*, 1988; Young *et al.*, 1996). Quantitation is done by histological examination of stained salivary glands for presence of sporozoites (Büscher and Otim, 1986).

To determine the efficacy of a stabilate for use in immunisation, a second quantitation is made by inoculating groups of cattle with a selection of doses of stabilate. This is necessitated because irrespective of the tick infection level, the recommended OIE standard is 10 ticks/ml (OIE, 2005). Secondly, because of the many factors influencing infection in ticks, stabilates of a stock of *T. parva* from different tick batches are likely to have varying degrees of infectivity. In addition, there may not be a direct correlation between tick infection and concentration of infective sporozoites in the stabilate (Marcotty *et al.*, 2004).

1.3.1. Direct quantitation

1.3.1.1. Histological

Light microscopy allows for the quantitation of infected acini in dissected salivary glands of ticks (Büscher and Otim, 1986). Briefly, the procedure involves dissecting the dorsum off partially engorged ticks to expose the viscera. The salivary glands are then separated from other organs, excised and placed in a drop of normal saline on a microscope slide. The acini are spread out with a teasing needle to obtain a monolayer after which the slide is air dried and fixed in pure methanol. Staining is commonly done with Methyl Green Pyronine (Walker *et al*., 1979) or Feulgen's reaction as described by Blewett and Branagan (1973). Infected acini stain a grainy pink with Feulgen's reaction. The parameters of importance arising from counting of infected acini are: **abundance,** which is the mean number of infected acini per tick, or **intensity,** which is mean number of infected acini per infected tick or **prevalence,** which is the proportion of infected ticks in the batch.

Determining the number of sporozoites in ticks involves estimating the number of sporozoites per infected acinus. Jarrett *et al*. (1969) estimated that one infected salivary gland would contain about 5 x 10^4 sporozoites and, therefore, an infected tick would contain 10^5 sporozoites. This was an underestimation as Fawcett *et al*. (1982a) found about 10^5 sporozoites per acinus using low power electron microscopy. Some workers have used this latter estimate e.g. Musoke *et al*. (1992) when they reported concentrations of 5 x 10^4 sporozoites in SNA's. Rocchi *et al.* (2006) put the number at 10^4 sporozoites per acinus but did not explain how they arrived at this estimate.

1.3.1.2. Molecular

Because of the potentially high sensitivity of the Polymerase Chain Reaction (PCR), molecular techniques have been used to detect and quantitate *T. parva* sporoblasts and sporozoites in ticks (Chen *et al.*, 1991; Watt *et al.*, 1997). Watt *et al*. (1997) used a direct visual grading (0 to 5) of the brightness of PCR bands on a 1.6 % agarose gel to quantitate infection in ticks and compare these with histological techniques. However, this is quite a subjective method and although sufficient for their objectives, may not be suited for stand-alone routine quantitation. Moreover, it may be unsuitable for quantitating infectivity of stabilates because it does not discriminate between sporoblasts and sporozoites.

1.3.1.3. Flow cytometry

Goddeeris *et al.* (1991) and Yagi *et al*. (2000) used flow cytometry to quantitate purified *T. parva* schizonts and *Theileria sergenti* piroplasms, respectively. This technique is highly precise with

high through-put as it counts thousands of individual organisms per second as they pass down a fluid stream oblique to a laser beam. Further, it can sort the particles based on size or other set parameters. Nevertheless, the technique only works well for purified cells, otherwise there is a lot of "noise" and it has a restriction on minimum particle size of about 300-400 µm. This would render flow cytometry unsuitable for sporozoites quantitation because stabilates contain a lot of tick debris and individual sporozoites are between 0.75 and 1.5 µm (Shaw, 2003), too small for reliable detection by flow cytometry.

1.3.2. In vivo *quantitation*

1.3.2.1. Principles

Viability or infectivity of *T. parva* has been quantitated in cattle using either tick-derived sporozoites (Radley *et al.*, 1974; Cunningham *et al.*, 1974) or macroschizont infected lymphocytes (Büscher *et al.*, 1984). Assessment using macroschizont infected lymphocytes is cumbersome in that it is restricted to inoculation of autologous *T. parva* infected lymphoblasts (Teale, 1983; Morrison *et al.*, 1986). It is easier to assess sporozoite infectivity by inoculating cattle with serially diluted stabilates and analyzing clinical and parasitological parameters. This method is used routinely in determining immunizing doses. The method is constrained in that for the same dose, reactions of individual animals are variable (Cunningham *et al.*, 1974) and secondly, because of economical, ethical and humane restrictions on the use of experimental cattle. Consequently, a sample size limitation is imposed which inevitably widens confidence interval of the estimates in regression analyses.

1.3.2.2. Analysis of data

Various ECF reaction classifications have been proposed based on different clinical and parasitological parameters including pre-patent period to detection of schizonts, macroschizont index (MSI), piroplasm parasitemia, time to and duration of febrile response, degree of lymphocytopaenia and others (Dolan *et al.*, 1984b; Anon.1989; Morzaria *et al.*, 1990; Kung'u and Goodger, 1990; Norval *et al.*, 1992g; Rowlands *et al.*, 2000). These have served as the response variable in a number of dose-response models. It is not the intention of this work to present a detailed study of these models but a few are mentioned here as examples.

One method of analysis has been to calculate the T_{10} (Jarrett *et al.*, 1969), which is the time in days from the first MSI to ten-fold multiplication of infected lymphoblasts detected in the contra-lateral prescapular lymph node. Usually inoculation in bovines is made on the parotid lymph node (ln. parotideus superficialis) on one side and the time taken for the infection to be detected in the

prescapular lymph node on the opposite side is a measure of infectivity. By extrapolation of the resulting plots and using the assumption that parasite replication rate was constant, initial quantities of infecting sporozoites could be estimated.

Dolan and colleagues (1984b) used parasitological and clinical reactions as the response variable in the independent action model that described the dose dependency of disease reactions, although infectivity appeared as a threshold character. Basically, this model compares the probabilities of the responses (effect predictions) in function of the dose. The predictor variables included time to onset of parasitemia, macroschizonts, fever, recovery and death. Some workers have found that the complexity of the computations involved in the independent action model, although applicable by professional statisticians, are a burden for field workers (Chen and Pounds, 1998).

1.3.3. In vitro *quantitation*

1.3.3.1. Principles

The macroschizont stage of *T. parva* induces transformation of infected lymphocytes both *in vivo* and *in vitro* as reported by Stagg *et al.* (1981) and Dobbelaere *et al.* (1988). This unique characteristic enables the setting up of immortalized cell lines and *in vitro* cultivation systems for studying certain characteristics of the parasite outside the vertebrate host. *In vitro* infection of bovine lymphocytes by *T. parva* sporozoites was first described by Brown *et al*. (1973). Many workers have since developed and used the technique in various protocols to study *T. parva* (Danskin and Wilde, 1976; Emery and Kar, 1983; Dolan *et al.*, 1985; Conrad *et al.*, 1989; Chaussepied and Langsley, 1996; Knight *et al.*, 1996; Kimbita *et al.*, 2004). *In vitro* systems have many advantages over *in vivo* systems in being cheaper and easily repeated, statistical variation is reduced as cells originating from a single donor may be used for series of investigations and by minimizing animal suffering, they are ethically more acceptable. Repeated testing of the same stabilate give close ranging results (Wilkie *et al.*, 2002) as opposed to wide variations seen in *in vivo* quantitation (Cunningham *et al.*, 1974; Dolan *et al.*, 1984b).

A few *in vitro* infection systems for quantitation of sporozoites have been documented. The underlying principle is that since *T. parva* induces a dose dependent reaction (Dolan *et al.*, 1984b; Mutugi *et al.*, 1988), the higher the number of viable sporozoites inoculated, the higher the number of lymphocytes infected and transformed within respective parasite stocks. Broadly, the models for *in vitro* quantitations are based on the proportion of infected target cells or on the proportion of infected culture wells in given stabilate dilution series:

a) Proportions of infected cells

Comparison of proportions of schizont infected lymphoblasts from pools of bovine peripheral blood mononuclear cells (PBMC) incubated with different *T. parva* stabilates or dilutions of the same stabilate (Gray and Brown, 1981) are made. A variation of this is practiced in SNA's in which stabilate concentration is kept constant and anti-sera are diluted serially. Neutralisation is then calculated from percentages of schizont bearing cells (Musoke *et al*., 1992). The main drawbacks are that the distribution of the counts is not clear and depending on time of reading, relative proportions of infected cells may vary.

b) Proportion of infected culture wells

This involves calculating and comparing Effective or Infective Doses (ED or ID, respectively) of serially diluted stabilates that result in a given proportion of lymphocyte-plated wells being infected in microtitration plates (Wilkie *et al.*, 2002; Marcotty *et al.*, 2004). This has the advantage of presenting a known distribution (binomial) and the effect of time is insignificant because wells are either negative or positive at time of reading.

1.3.3.2. Analysis of *in vitro* data

Statistical rendering of the data has been varied. Various analytical studies have been published, based mostly on analysis of variance (ANOVA) and regression analyses. Wilkie and colleagues (2002) compared effective dilutions in tick equivalents (ED_{50}) calculated by the Spearman-Karber method. Marcotty *et al*. (2004) using a stratified or a robust logistic regression model compared effective doses (ED_{50}) in a manner similar to Wilkie *et al*. (2002) but developed a multi-sessional approach in which more repetitions (sessions) of each experiment were done. Each session was then treated as a primary sampling unit in the analysis. This approach not only reduces confidence intervals when the data are stratified but captures the clustering effect of the sessions which may be overlooked in other models. For instance, classical ANOVA may not adequately take into account this inter-sessional variation and the associated standards errors.

Other weaknesses of fixed models include a lack of consideration for interactions between variables and for random factors. Explanatory variables are simply assumed to be independent i.e. there is no covariance between them. Random factors that may be present due to stratifying or multistage sampling as the case may be in *in vitro* titrations, can give erroneous results if not taken into account (Speybroeck *et al.*, 2003). These can be addressed by more robust mixed effect modelling such as Generalized Linear Latent and Mixed Models (GLLAMM) (Rabe-Hesketh *et al.*, 2002), which capture these effects.

1.3.4. Applications of in vitro *quantitation*

1.3.4.1. Stabilate production and storage parameters

The detrimental effects of various handling and storage processes on the infectivity of stabilates have been an area of much interest. These have been investigated by evaluating the alteration in infectivity of sporozoites for lymphocytes *in vitro*. Kimbita *et al.* (2004) used *in vitro* systems to quantitate effects of holding temperature and compared various media as candidates for stabilate production. They found L-15 medium stabilates to be superior in conserving infectivity over MEM, Optimem, Iscoves modified MEM and RPMI 1640. They also evaluated infectivities of sporozoites harvested from ticks at various stages of maturation (Kimbita and Silayo, 1997) and the effects of various cryoprotectants (Kimbita *et al.*, 2001). Marcotty *et al*. (2004) investigated the effect of high speed centrifugation on sporozoites. These studies helped in identifying some of the processes and reagents that influence sporozoite survival during stabilate production and that are critical for optimization of production parameters.

1.3.4.2. Sporozoite Neutralisation Assays

Based on the evidence that serum from theileriosis recovered calves neutralises sporozoite infectivity *in vitro* (Gray and Brown, 1981), techniques have been developed to quantitate titres of antibodies against native and recombinant sporozoite antigenic proteins (Musoke *et al.*, 1984; Musoke *et al.*, 1992; Kaba *et al.*, 2004). Briefly, the techniques involve a short incubation of sporozoite suspensions with the test sera prior to introduction of PBMC. This is then followed by a single or serial sampling for schizont scoring. Another application is the study of cross reactivity of inter-species sporozoite antigens as used by Knight *et al.* (1996).

However, these techniques may give antibodies more time to bind to epitopes than actually happens *in vivo* in which the parasite has only a few minutes before being internalised (Shaw *et al.*, 1991). A better approximation would be to introduce the sporozoites into a mixture of cells and antiserum thus simulating the competition that ensues *in vivo*. Sporozoite Neutralisation Assays will become important tools in the current efforts to develop and assess efficacy of potential sub-unit vaccines against ECF that elicit a humoral response against sporozoites. It is therefore important to explore and implement assay systems that are standardised, reproducible, robust and easy to evaluate.

Chapter 2 - Objectives

If A is success in life, then A equals x plus y plus z. Work is x; y is play; and z is keeping your mouth shut. Albert Einstein (1879 - 1955)

2.1. General Objective

To develop and to use the *in vitro* tool in view of improving *Theileria parva* live vaccine production, storage and immunisation against East Coast fever.

2.2. Specific Objectives

To quantitate *T. parva* (types Katete and Chitongo) stabilate infectivity loss due to storage conditions. The conditions to be investigated are storage at 4ºC (on ice) for up to 24 h and at -20ºC for up to four weeks using glycerol and sucrose as cryoprotecting agents. It was envisaged that if stabilate could be stored for at least a week in domestic freezers without appreciable infectivity loss, it would reduce the cost of storing it in liquid nitrogen during immunisation campaigns.

To quantitate and compare infectivity of *T. parva* stabilates produced by manual and homogenizer methods of sporozoite extraction. Though OIE recommends use of homogenizers, different labs prefer one or the other method. However, no comprehensive study has been conducted that quantitatively compares the infectivities of the resultant stabilates *ceteris paribus*. It is envisaged that using a homogenizer would ease and improve the production of stabilate for immunisation purposes.

To quantitate infectivity of *T. parva* stabilates after snap freezing, multiple refreezing and lyophilisation attempts. It is envisaged that snap freezing of stabilates would ease stabilate production. Determining the infectivity loss of refrozen stabilates would allow for adjustment of doses during the combination stage of polyvalent vaccine production. Lyophilised stabilate would be less expensive to store and therefore cheaper to deliver to farmers.

To quantitate and compare the effects of suspending sporozoites in MEM (the standard stabilate medium), RPMI 1640, PBS and FCS as stabilate media. RPMI 1640 is more commonly used in cell culture laboratories than MEM whereas PBS is the most common buffer. FCS is a potentially cheaper alternative to the routinely used MEM complemented with bovine serum albumin.

To optimize a Sporozoite Neutralisation Assay protocol and validate it by assessing neutralisation potential of various immune sera originating from cattle inoculated with recombinant p67 antigen, live sporozoite stabilate, dead sporozoites and schizont-infected lymphoblasts. The assumption was that cattle immunized with schizonts or dead sporozoites could induce production of anti-p67 antibodies detectable by the assay.

Chapter 3 –Comparison of two Theileria parva *extraction methods*

Based on:

Mbao, V., Speybroeck, N., Berkvens, D., Dolan, T., Dorny, P., Madder, M., Mulumba, M., Duchateau, L., Brandt, J. and Marcotty, T. (2005) Comparison of manual and homogenizer methods for preparation of tick-derived stabilates of *Theileria parva*: equivalence testing using an *in vitro* titration model. *Parasitology* **131**, 45-49

"To know how to hide one's ability is great skill" François de la Rochefoucauld

3.1. Introduction

Theileria parva is a protozoan parasite that is transmitted to cattle by the 3-host tick *Rhipicephalus appendiculatus* and causes East Coast fever, a disease of major economic importance in Eastern, Central and Southern Africa (Young *et al.*, 1988). Its control is achieved mainly by vector control but also by livestock movement control and immunisation. The infection-and-treatment method of immunisation (Radley *et al.*, 1975a) is the only available means of conferring immunity to cattle against homologous challenge (Uilenberg, 1999). The process involves simultaneous inoculation of tick-derived sporozoites and a long-acting tetracycline. Production protocols for *T. parva* sporozoite stabilates have been documented for both manual-extraction by mortar and pestle (Farrant, 1970; Purnell *et al.*, 1973; Cunningham *et al.*, 1973a; Cunningham *et al.*, 1973b) and for tissue homogenizers (Kimbita *et al.*, 2001; Kimbita *et al.*, 2004), the latter being recommended by OIE (2000).

Although the use of homogenizers is considered the standard method for sporozoite extraction, no comprehensive study comparing the yield of sporozoites by each method has been published. Homogenization has advantages in the production of large volume stabilates because large numbers of ticks can be processed fairly quickly and the stabilate diluted, cryoprotected, aliquoted and stored in a much shorter time following their removal from animals. The drawbacks of this method include possible overheating of stabilates and occasional lack of complete disintegration of tick material. The determination of actual numbers of live sporozoites in stabilates has been a challenge for routine production but the introduction of an *in vitro* titration technique (Marcotty *et al.*, 2004) in which an effective dose (ED) is determined and taken as the unit of sporozoite concentration has improved quality assessment in the production process. The present study describes attempts to quantify the difference between the two methods in terms of sporozoite yield. From the disadvantages of homogenisation, it was hypothesized that manually ground stabilates, taken as the standard in this study, would produce a higher sporozoite yield than machine-homogenized stabilates, and we set out to quantify this difference by equivalence testing. Equivalence testing

goes a step further than significance testing as it can quantify a predicted maximum difference between 2 parameters that are assigned the null hypothesis of non-equivalence. In our study, the multiplicative model (Diletti *et al*., 1991) was used to focus on the ratio of the sporozoite yields of the manual to the homogenizer methods.

3.2. Materials and Methods

3.2.1. Preparation of T. parva *sporozoite stabilates*

The *T. parva* Katete stock that had been isolated at Kalapula village in Katete, Zambia in 1984 (Geysen *et al*. 1999) was used. *T. parva* sporozoites were extracted from infected adult *R. appendiculatus* ticks following their infection as nymphs as described by FAO (1984). Briefly, susceptible Friesian cattle were inoculated with *T. parva* Katete subcutaneously next to the pre-parotid lymph node (ln. parotideus superficialis). When infection developed, *R. appendiculatus* nymphs, collected originally from the vegetation near Wafa village (13° 35' S, 32° 30' E, 980 m) in Eastern Zambia were applied to their ears. The engorged ticks were collected and allowed to moult in an incubator at 22°C and 80–90% relative humidity. Six weeks after moulting, they were fed on rabbits for 4 days to induce sporogony of the parasite (Kimbita and Silayo, 1997). Following removal from the rabbits, 800 ticks were split randomly into 4 groups of 200. Two groups were homogenized using an Omni-mixer Homogeniser1 (Omni International, USA, model 17106) following the OIE protocol (OIE, 2000) and labelled H1 and H2. The other 2 groups were ground manually using a mortar and a pestle (M1 and M2). Sporozoites were extracted in 20 ml of cooled (4°C) Minimum Essential Medium (with Hank's salts), 35 g/l BSA (Acros organics # 240401000 and #240400100), PenicillinG (Sigma # P3032), Streptomycin sulfate (Sigma # S 9137) and Kanamycin monosulfate (Sigma # K 1377).

For homogenization, a large aperture head (shaft Ø 20 mm, rotor Ø 15 mm) was used at low speed (mark 3) for 2 min followed by 3 min of a small aperture head (shaft Ø 10 mm, rotor Ø 7.5 mm) at the same speed. The receptor (Nalgene1 wide mouth plastic bottle, 60 ml) containing the ticks was kept in an ice bath throughout. The groups for manual extraction were ground separately for 15 min with a pestle in a mortar containing glass fragments. Extraction was performed by two persons in turn to assure continuous and intense crushing. For both extraction methods, a sample was examined under a stereoscopic microscope at the end of the extraction period to check the quality of tick disintegration. The resulting tick material was made up to 25 ml in medium obtained from rinsing the tools and receptor used for extraction. The suspensions were then centrifuged separately

at 50 *g* for 5 min in 10 ml centrifuge tubes in a cooled (4°C) centrifuge. Supernatant fractions were harvested using sterile Pasteur pipettes, leaving the large tick debris behind, and transferred to a beaker to which an equal volume of cold glycerol (150 g/l) MEM/BSA was added drop-wise: first 5 min 1 drop every 2 sec and thereafter 1 drop/sec. The stabilates were stirred continuously in an ice bath throughout this process. Four batches of 50 ml stabilate each, giving a 4 tick-equivalent (t.e.) per ml concentration were produced. They were aliquoted into appropriately labelled 1.5 ml cryogenic vials (Nalgene1). The tubes were placed in an ultra-freezer (-80°C) for 24 h and then transferred to permanent storage in liquid nitrogen.

3.2.2. Isolation and activation of PBMC

The method of Peripheral Blood Mononuclear Cells (PBMC) isolation is based on Goddeeris and Morrison (1988) modified by Marcotty *et al*. (2004). In brief, blood from the jugular vein was collected in Alsever's solution (Alsever and Ainslie, 1941) from a Friesian heifer. PBMC were isolated by density gradient and suspended at $6x10^6$/ml in culture medium comprising RPMI-1640 (Life Technologies # 52400-025) with 25 mM HEPES, FCS (15%), gentamycin (50mg/ml), 2-mercaptoethanol (0.5 M) and L-glutamine (0.2 M). The cells were kept at 37°C in a CO_2 incubator overnight (see Appendix I.A).

3.2.3. In vitro *titration*

The *T. parva* stabilates were titrated *in vitro* as described by Marcotty *et al*. (2004), with some modifications. In brief, *T. parva* stabilates were thawed and diluted serially (6 series by 1.5-fold dilution) in 96-well flat-bottomed microplates across columns. Then 50 µl of PBMC were added to 50 µl of sporozoite suspension in each well. The plates were incubated for 1 h at 37°C in a CO_2 incubator (5% in air), centrifuged (210 *g* for 10 min) and the excess medium was decanted. Fresh culture medium (150 µl per well) was added to the plates which were then re-incubated for 10 days under the same conditions.

On day 10, cyto-centrifuged samples were prepared and stained with Giemsa's stain. Wells positive or negative for schizonts were scored 1 or 0 respectively. A total of 6 sessions were set up as shown in Table 2.

Table 2: Titration sessions for comparison of extraction methods. M-manual, H-homogeniser

	Stabilate			
Session	**M1**	**M2**	**H1**	**H2**
1	48		48	
2	71		72	
3		72		72
4	93			91
5		88	95	
6		48		48
TOTALS	212	208	215	211

3.2.4. Statistical analysis

For the comparison of extraction methods, the binary results were analysed using a random effect logistic regression in Stata® (Stata/SE 8.0 for Windows Statistical Software. Stata Corporation, Texas). The proportion of positive wells was the response variable and the logarithm of the stabilate concentration and the method of extraction were used as explanatory variables.

The test session was taken as a random effect given the importance of the clustering effect within test sessions (Marcotty *et al.*, 2004). The model can be written as follows:

$$\log\left(\frac{\pi}{1-\pi}\right) = \alpha + \beta_{\ln(t.e.)} \times \ln(t.e.) + \beta_{method} \times method + \nu + \varepsilon$$

where π is the proportion of positive wells, α a constant, *ln(t.e.)* the natural logarithm of the sporozoite dose in tick-equivalents, $\beta_{ln(t.e.)}$ the coefficient of the stabilate dose, *method* the method used (0 for homogenizer and 1 for hand-extraction), β_{method} the coefficient of the method, n the cross-sectional random effect of the session and e the residual error.

The coefficients of the model were used to estimate ED_{50}, the dose that is effective in 50% of the cases, for the two methods:

$$\ln(ED_{50}) = -\frac{\alpha + \beta_{method} \times method}{\beta_{\ln(t.e.)}}$$

A comparison of the sporozoite yield by the multiplicative model of the ratio $ED_{hand}/ED_{machine}$ was then made by non-linear combinations of estimators:

$$\ln(ED_{hand}) - \ln(ED_{machine}) = -\frac{\beta_{method}}{\beta_{\ln(t.e.)}} \Rightarrow \frac{ED_{hand}}{ED_{machine}} = \exp\left(-\frac{\beta_{method}}{\beta_{\ln(t.e.)}}\right)$$

In the first step, data were regressed independently for the 4 stabilates (2 methods X 2 replications). In the second step, the data were pooled and average estimates generated to obtain a global model per method. The maximum expected ratio was calculated using the delta method (Oehlert, 1992).

3.3. Results

The regression model showed a highly significant effect of the natural logarithm of the dose expressed in tick-equivalents, *ln(t.e.)*, on the predicted proportion of positive wells (odds ratio=16, $P<0.001$). Figure 6 plots the predicted proportion of positive wells against the stabilate dose for each of the 4 stabilates i.e. 2 that were manually extracted (m) and 2 obtained by homogenization (h). Whereas the curves of the manually produced stabilates overlap, the difference between the ED_{50} of the 2 homogenized groups was 0.01 t.e. and their ratio 1.9 (95% CI: 1.5–2.5).

In the global model (log likelihood= -334, $\chi^2<0.001$), the difference between the two methods was marginally non-significant (odds ratio=1.45, $P=0.058$). Figure 7 shows the estimates and confidence intervals of logistic regression model of the pooled data (appendix II includes observed averages). The ED_{50} of the two methods are 0.13 t.e. and 0.15 t.e. for the manually ground and the homogenized stabilates respectively.

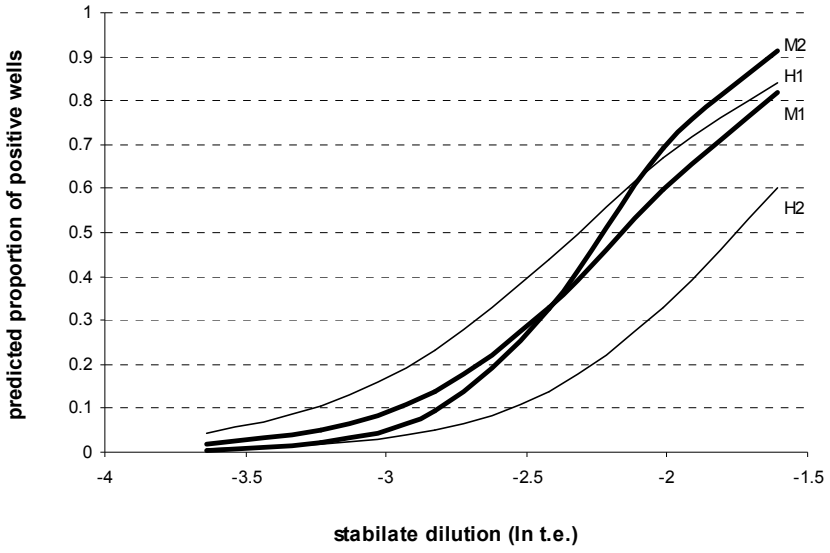

Figure 6: Variability within and across methods (Manual = thick lines, Homogeniser = thin lines).

The ratio $ED_{(hand)}/ED_{(machine)}$ was estimated as 1.14 (95% CI: 0.99–1.30). The average curves of the two regressions are horizontally equidistant and the dilution factor corresponds to the difference of

stabilate potency between the two methods. In other words, machine-ground stabilate was estimated to correspond to a dilution of the manually ground stabilate by 1.14.

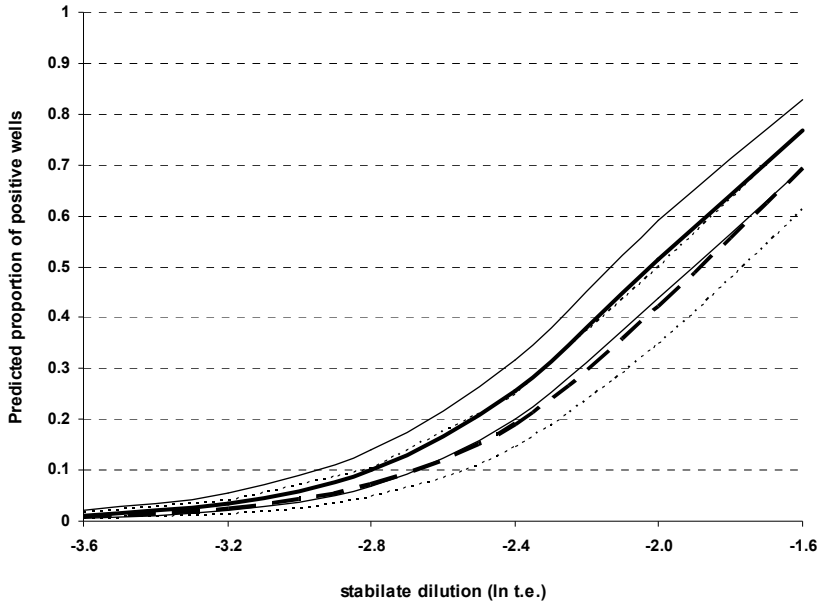

Figure 7: Titration curves of manually and homogeniser extracted stabilates: estimates (Manual = bold solid line, homogeniser = bold dashed line) and 95% confidence intervals (thin lines). Upper limit for the Homogeniser curve is partly obstructed by the average of Manual curve. See appendix II for data points.

3.4. Discussion

The manually produced stabilate had a marginally higher yield of sporozoites than the homogenized stabilate, as was hypothesized. Our interest was also to see, not just the significance of the difference but 'how excessive ' the difference might be. Previously, aspects of *T. parva in vitro* infections have been compared by analysis of variance and statistical significance ((Kimbita and Silayo, 1997; Kimbita *et al.*, 2001; Wilkie *et al.*, 2002; Kimbita *et al.*, 2004; Marcotty *et al.*, 2004). Here we show the usefulness of equivalence testing; it gives a quantitative measure of how inferior or superior the test parameter (homogenization) is compared to a reference method (manual extraction). The results show that the difference between manual and homogenizer extraction is minimal, in terms of viable sporozoite yield, as the ratio of the ED does not exceed 1.3 (95% confidence interval upper limit) and it is concluded that both methods have equivalent efficiency. It is likely that, if the analysis had been based solely on significance testing, more repetitions of *in vitro* comparisons would have narrowed the confidence intervals and yielded a significant

difference. It is for this reason that equivalence testing is recommended as it can show that certain factors, in this case extraction method, do not yield very different results.

Manual-extraction presents the advantage of a much reduced risk of overheating and is easier for monitoring thorough extraction. In addition, the equipment is readily available in most laboratories. However, it is labour intensive, more time consuming and carries a higher risk of contaminating the stabilate. This is more so with *T. parva* stocks like Chitongo (Geysen *et al.*, 1999), a stock used in southern province of Zambia for which the immunizing dose is 20 times higher than that of Katete. For this stock, tens of thousands of ticks are ground per batch of stabilate. These disadvantages are reduced when using a homogenizer.

Homogenized stabilates showed a relatively higher variability in their titration curves than the manually produced batches. This reflected a higher repeatability for the manual method. The observation was contrary to the expectation that homogenization would give a higher repeatability. It is possible that in the manual method, the ticks were more thoroughly ground. This could also explain the lower ED_{50} for the manual method. This observation might indicate that machine homogenization requires further standardization. However, only 2 stabilates per method were produced and this could be a case of random variation rather than a trend. Furthermore, the difference between the two homogenization repetitions (corresponding to a dilution of 1.9 times) is still rather small, considering other sources of variation such as tick infection rates, stabilate production and/or storage.

Therefore, it would be important to study within method variability based on a greater number of repetitions and also to test different brands of homogenizer and homogenizing heads in different laboratories (reproducibility). The use of a single homogenizing head could greatly increase the undesirable proportion of whole ticks and tick tissues that are still intact (Berkvens, unpublished observations 2004). It is postulated that the larger head disintegrates the ticks while the smaller head opens up the tick salivary gland acini to release more sporozoites, making the use of the two heads essential for good yields. Furthermore, over-heating of the stabilate could result from excessive speed and/ or prolonged homogenization due to blunt homogenizing heads. The effects of prolonged homogenization, while keeping the suspensions at low and preferably constant temperature, have not been studied. This could pose a further risk, if indeed, ‘over-homogenizing’ induced a mechanical stress for the sporozoites. For these reasons, it might be worthwhile to develop ‘homogenizing indicators’ to evaluate the extraction quality. Examples could include *in vitro* titrations and monitoring the proportion of uncut ticks. Such indicators could be used to calibrate tick densities, time of extraction, speeds, and other factors for optimum stabilate

production. These indicators would also be useful in evaluating the repeatability and reproducibility of the two methods.

Finally, it should be noted that the batches of ticks were ground using a homogenizer and container with only 200 ticks in 20 ml of media while OIE (2000) recommended batches of 1000 in 50 ml. This was limited by the small numbers of ticks available and the need to have sufficient volume to cover the homogenizing heads. These deviations from the standards could have resulted in the higher variability observed among the machine ground stabilates. Differences in terms of shapes of machine receptacles, volume and tick density might also have important effects on the extraction quality and on the optimal extraction speed and time.

Chapter 4 - Infectivity of Theileria parva *sporozoites following cryopreservation in four suspension media and multiple freezing*

Based on:
Mbao, V., Berkvens, D., Dolan, T., Speybroeck, N., Brandt, J., Dorny, P., Van den Bossche, P. and Marcotty, T. (2006) Infectivity of *Theileria parva* sporozoites following cryopreservation in four suspension media and multiple refreezing: Evaluation by *in vitro* titration. *Onderstepoort Journal of Veterinary Research* **73**, 207-213

" In a sense, words are encyclopedias of ignorance because they freeze perceptions at one moment in history and then insist we continue to use these frozen perceptions when we should be doing better." - Edward de Bono

4.1. Introduction

The inoculation of *Theileria parva* sporozoites into cattle usually causes East Coast fever (ECF), an acute and often fatal lymphoproliferative disease of major economic importance in Eastern, Central and Southern Africa (Young *et al.*, 1988). This obligate intracellular parasite is transmitted mainly by the three-host tick, *Rhipicephalus appendiculatus*, from which sporozoites can be extracted. These sporozoites extracts are then cryopreserved as stabilates and used for immunisation by the infection and treatment (I & T) method, challenge of immune or vaccinated animals, *in vitro* testing and research investigations. The I & T method of immunisation (Radley *et al.*, 1975a) is the only means currently available for immunizing cattle against homologous challenge (Uilenberg, 1999). The technique requires simultaneous inoculation of *T. parva* sporozoites and a long acting tetracycline. It is widely used in several provinces of Zambia and other countries in the region (Uilenberg, 1999; Marcotty *et al.*, 2001; Fandamu *et al.*, 2006). Univalent stabilates are used in Zambia but stabilates containing several *T. parva* stocks are required elsewhere e.g. in Tanzania (Morzaria *et al.*, 2000). The extraction of *T. parva* sporozoites into different media has been described for both experimental and field-use stabilates (Purnell *et al.*, 1973; Cunningham *et al.*, 1973a; Cunningham *et al.*, 1973b; Kimbita *et al.*, 2004). These media include bovine serum and Eagle's Minimum Essential Medium (MEM) supplemented with bovine serum albumin (BSA). The stabilates for field immunisation are routinely produced using MEM supplemented with BSA.

Well-characterized and homogeneous stabilates need to be available for immunizing cattle against East Coast fever, use in *in vitro* Sporozoite Neutralisation Assays and research in general. For developing countries, they should be cheap and easy to produce. The powdered formulation of MEM is much cheaper to import into Africa than the liquid form but is not always readily available. Therefore, other media for their efficiency in maintaining sporozoite infectivity were evaluated. Two media to test and compare with MEM were selected, namely the powder formulation of

Roswell Park Memorial Institute (RPMI 1640) and phosphate-buffered saline (PBS). RPMI 1640 is the most common medium used for cell culture while PBS, which does not contain nutrients, is a very basic buffer solution found in most laboratories. BSA, on the other hand, is expensive. Hence, the possibility of using FCS instead of MEM with BSA was investigated.

The objective of the second study was to quantify the loss of infectivity for stabilates undergoing a refreezing step after production. This technique is used in the production of polyvalent ECF vaccines to allow titration of individual components before mixing them. It is also envisaged that, with the onset of veterinary services privatization in Zambia and several other countries in the region, stabilate refreezing may be considered by some animal health service providers in an attempt to salvage left-over doses after an immunisation campaign. Refreezing may also be useful in cases where homogeneous stabilate needs to be used at different time periods for research work. Ensuring homogeneity of the stabilates by pooling and refreezing aliquots for use in particular sets of experiments is one practical method of removing variability in infectivity seen in stabilate taken from different storage vials (own unpublished observations 2005). From an immunisation protocol using refrozen stabilates (Njuguna and Musisi, 1996), it is known that *T. parva* sporozoites do survive refreezing cycles and it is expected that they lose some viability at each cycle. It is, however, not known to what extent. There is therefore need to have empirical data on the effect of such a process for quality assurance. In this study, sporozoite infectivity was evaluated after single and multiple refreezing cycles. Multiple cycles were included to amplify any effects there might be, and to aid in calculating the average loss per cycle.

Titration of stabilates was done *in vitro*. Equivalence testing (see chapter 3) was used to calculate the effect of alternative media on sporozoite viability compared with the standard medium (MEM/BSA) and quantify loss of sporozoite infectivity due to refreezing. A random effect model was applied in view of the levels of confounders at tick batches, grinding pools and storage vials.

4.2. Materials and Methods

4.2.1. Media

All media and additives were obtained from Invitrogen (Carlsbad, California), unless otherwise stated. The following media were used for preparation of the sporozoite suspensions: MEM (with

Earle's salts), RPMI-1640 (powder formulation), PBS and heat inactivated FCS. The MEM, RPMI and PBS solutions were supplemented with BSA (Acros organics # 240401000 and #240400100) (Acros Organics, Belgium) at 35 g/l and all of them, including FCS, with L-Asparagine (BDH Biochemical, United Kingdom) at 100 mg/l, HEPES (25 mM/l), Penicillin-Streptomycin at 100 iu/ml and Kanamycin at 100 µg/ml. The pH of media was adjusted to 7.0-7.2 using sodium bicarbonate.

4.2.2. Stabilate preparations

Three batches of nymphal *R. appendiculatus* ticks were infected at different times and locations with the *T. parva* Katete stock and allowed to moult to adults in an incubator at 22°C and 85% relative humidity. Six to 8 weeks after engorgement as nymphs, the adult ticks were fed on rabbits for 4 days to induce sporogony of *T. parva* (FAO, 1984) and removed. The ticks in Batches 1 and 2 were divided randomly into groups as shown in Table 3 and ground separately in each of the four media in the volumes shown.

Table 3: Number of ticks ground per batch per medium

	Batch 1		***Batch 2***	***Batch 3***
Vol[a] (m/)	20	20	25	50
Final conc (t.e./m/)	5	5	10	10
MEM	200	200	500	1000
PBS	200	200	500	
RPMI	200	200	500	
FCS	200	200	500	

For Batch 1, eight groups were ground (Table 3) using an Omni-mixer Homogeniser® (Omni International, USA, model 17106) following the standardized international protocol (OIE, 2005) with some modifications (see chapter 3). The Batch 2 ticks were ground using an Ultraturax® tissue homogeniser (Janke & Kunkel KG, Staufen, Germany, model TP18/2). Batch 3 ticks were ground manually using a mortar and pestle for 15 min (Cunningham *et al.*, 1973a). The different methods of grinding were used due to different laboratory set-ups in the three places where the stabilates were produced.

An equal volume of chilled medium with 15 % (w/v) glycerol was added drop-wise to the ground up tick supernatant (FAO, 1984). The extracts were stirred continuously in an ice bath. All suspensions from the 13 tick groups were aliquoted into 1.5 ml cryogenic vials (Nalgene®) (1 ml/vial), cooled in an ultra freezer at -80°C for 24 h and then stored in liquid nitrogen.

4.2.3. Refreezing cycles

The stabilate from Batch 3 was used for the stabilate re-freezing experiment. A 'Cycle' was defined as a refreeze and subsequent thaw process. Vials were thawed by placing them in a water bath at 37°C for 5 min. Before the first refreeze cycle, thawed vials were pooled to ensure homogeneity, centrifuged (400 *g* for 10 min) to remove fungi and yeasts (Marcotty *et al.*, 2004) and supernatants re-aliquoted. In a fist step, two titration sessions were set up to compare control and single-refreeze stabilates. Aliquots of the thawed, pooled and centrifuged stabilate material were kept on ice (control material, not for re-freezing) while the rest was refrozen. Re-freezing was for 1.5 h in a -80°C freezer. In a second step, five groups of vials from the same homogeneous pool were subjected to several cycles. All groups were refrozen once. After 1.5 h, four of these groups were thawed and refrozen. After a further 1.5 h, three groups from the previous four were refrozen and this continued until the last group had undergone a fifth cycle. Each group had been subdivided into three subgroups and each of these subgroups was held on ice for 5, 30 and 60 min before a subsequent re-freezing.

4.2.4. In vitro *titrations*

The protocol for *in vitro* titration of *T. parva* tick-derived stabilates that was used is that described by Marcotty *et al*. (2004) and modified as described in chapter 3. Briefly, test stabilate is diluted serially in 96-well microtitration plates and then bovine Peripheral Blood Mononuclear Cells are added to the wells. The plates are incubated for 10 days at 37 ºC in 5 % CO_2 after which cyto-centrifuged samples are taken, stained with Giemsa's stain and microscopically examined for *T. parva* macroschizonts.

Titrations for media comparison were conducted in two stages. The first stage comprised six sessions in which all four media were titrated in parallel. There were four sessions for tick Batch 1 stabilates and two for tick Batch 2 stabilates. In the second stage, only MEM and RPMI 1640 stabilates were compared in six sessions: four sessions for tick Batch 1 stabilates and two for tick

Batch 2 stabilates. The total number of microtitration wells read for the first and second stages were 855 and 574, respectively. Stabilates from Batch 1 were diluted serially 6 times (1.5 times per dilution step) and those from the second (Batch 2) 12 times (1.5 times per dilution step). This was necessitated because the first batch had lower infectivity, as determined by a preliminary *in vitro* titration.

The multiple frozen and thawed stabilates (Cycles 1 to 5) were titrated in parallel a day after the refreezing cycles. Six and two titration sessions for the multiple freezing and control experiments, respectively were conducted. A total number of 1,115 and 335 microtitration wells were read for multiple freezing and control experiments, respectively.

4.2.5. Statistical analysis

Data from the two experiment stages of media comparison (i.e. all four media and MEM vs. RPMI) were pooled. Wells either scored positive or negative. The binary results were analysed by logistic regression using Generalized Linear Latent and Mixed Models (GLLAMM) in Stata8/SE® (StataCorp, 2003). The proportion of positive wells was the response variable. The three explanatory variables were: the natural log of the stabilate concentration in tick equivalents (*ln* t.e.), the experiment stage and the stabilate medium. Several random effects that could affect the model were identified, namely the tick batch, grinding pools and stabilate storage vials. Random effects are factors or hidden variables that do not interest us but still have an effect on the variability of the data and as such should not be ignored during the analysis.

Comparison of respective infectivities of test stabilate media to those of MEM stabilates were conducted by calculating the ratios of effective doses $\left[ED_{MEM} / ED_x\right]$ where *ED* = the Effective Dose, in tick equivalent, of stabilate that results in a given proportion of wells to be positive and *x* = test media. The ratios were calculated by use of non-linear combination of estimators (nlcom) in Stata8/SE® (see chapter 3), which also fits 95 % confidence intervals around the estimates (ratios). The level of significance was set at 5 %.

The same model was used for the assessment of the effect of multiple freezing. The natural log of the stabilate concentration (*ln* t.e.), the Cycle number and the holding time were continuous explanatory variables. The Cycle was regarded as a continuous variable so as to enable estimation

of a loss of infectivity per cycle. The response variable was Proportion of positive wells and 'session' of titration was a random effect. Ratios of effective doses $\left[EDx \big/ EDx+1 \right]$, where x = Cycle number or holding time, were calculated to compare sporozoite infectivities at each cycle and holding time. To express this as infectivity losses between subsequent cycles, the calculated ratios were subtracted from unit (1-ratio) e.g. a ratio of 0.99 per cycle is actually 1 % loss of infectivity per cycle.

4.3. Results

Differences in the infectivity of stabilates prepared and stored in PBS (n=215), RPMI (n=502) and FCS (n=212) were not statistically significant (P>0.05) when compared to MEM (n=500). Estimates of ED ratios were 0.59, 1.03 and 0.67 for PBS, RPMI and FCS, respectively (Table 4). The regression curves of predicted values, comparing infectivities, were very close, with MEM and RPMI showing a superimposition of their curves (Fig. 8 and Appendix III). While the dose (*ln* t.e.) was significant (P<0.001), the experiment stage did not significantly influence the outcome (P=0.10).

Table 4: Ratios of effective doses (EDMEM/EDx) with lower and upper 95% confidence limits. MEM = Minimum essential medium and X = test stabilate medium (PBS, RPMI or FCS).

Medium	Estimate*	Lower limit	Upper limit
PBS	**0.59**	0.31	1.14
RPMI	**1.03**	0.63	1.67
FCS	**0.67**	0.35	1.29

*This ratio is an indicator of the relative infectivity of stabilates in comparison to the reference (MEM)

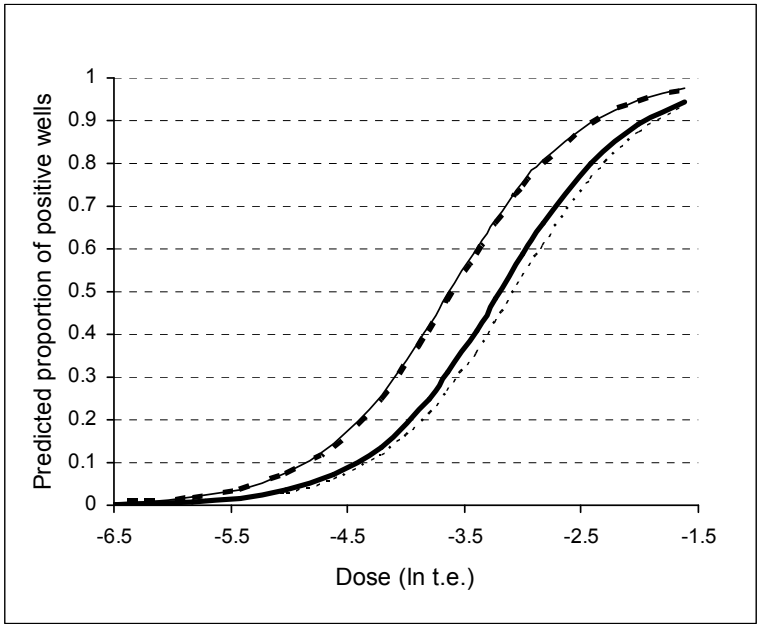

Figure 8: Titration curves of RPMI (thin continuous line), MEM (thick broken line), FCS (thick continuous line) and PBS (thin broken line) based sporozoite stabilates.

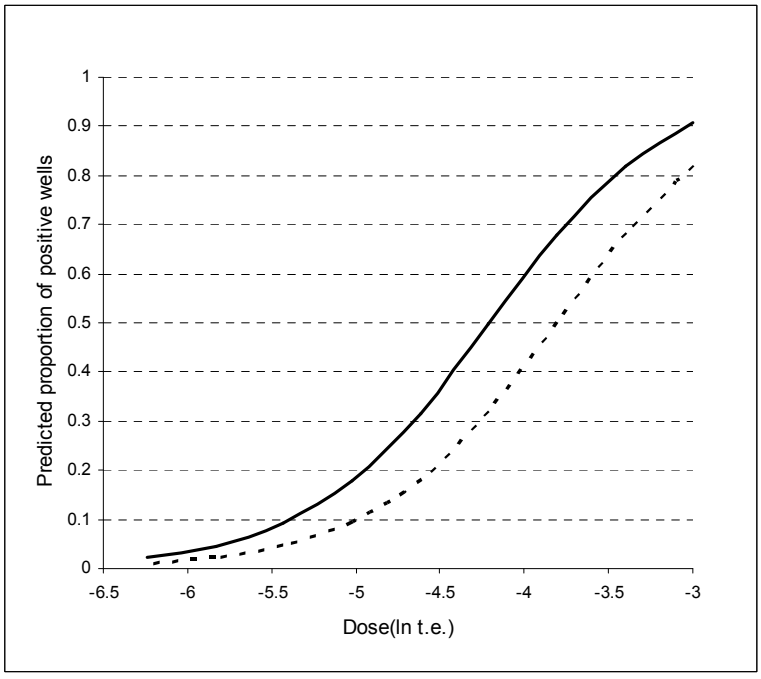

Figure 9: Titration curves of stabilate that underwent a single refreeze/thaw cycle (broken line) and control stabilates (1.5 h storage on ice) (solid line).

A single re-freezing cycle resulted in a significantly lower infectivity (P=0.03) than the control kept on ice for 1.5 h (Fig. 9). The ED ratio was 0.68 (95 % CI: 0.48 – 0.95) and the estimated loss in infectivity was 32 %. The loss during multiple freeze cycles was also significant (P<0.001). On average, the ED ratio was 0.65 (95 % CI: 0.60 – 0.72) giving an estimated loss of 35 % per cycle (Fig. 10). Observed averages for figure 10 are plotted in Appendix IV. In both single and multiple re-freezing cycles, dose effect was significant (P<0.001). These results are shown in Table 5. Holding times were not significant (P=0.88) nor was the random effect of session (P=0.34). A stabilate kept on ice for 1 h did not lose more than 30 % of its infectivity.

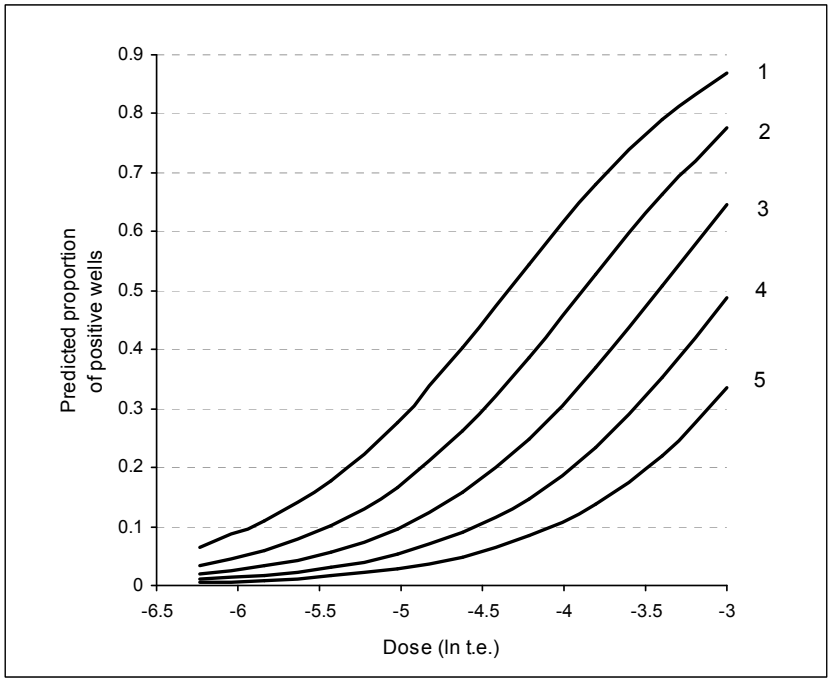

Figure 10: Titration curves of multiply frozen stabilates (Cycles 1 to 5)

Table 5: Percentage loss of sporozoite infectivity (1- ED ratio) after several cycles of refreezing with lower and upper 95 % confidence limits. ED (effective dose) ratio = EDcycle/EDcycle+1

Cycle	Estimate	Lower	Upper
single	**32%**	5%	52%
multi	**35%**	28%	40%

4.4. Discussion

Sporozoite infectivities for stabilates prepared in PBS, RPMI and FCS when compared to MEM were not statistically significantly different. However, the RPMI stabilates were closest in infectivity to MEM stabilates as shown by the equivalence test estimate (minimum 0.63 times). This observation agrees with the findings of Kimbita *et al*. (2004) when they compared different media including Leibovitz-15, Optimem and Iscove's MEM. In their study, RPMI and MEM stabilates showed similar infectivity when compared to L-15. Gray & Brown (1981) showed that neat serum could have inhibitory effects on sporozoite infectivity and this could explain why the ED of FCS was lower than that of MEM. However, the effect may not be very pronounced as the confidence interval is wide. FCS is expensive (about €107/l) due to the stringent standards required for international distribution. If it could be produced locally in existing regional laboratories with nationally or regionally accepted quality control standards from known naïve dams, it could be very much cheaper than international grade FCS. Newborn calf serum might also be used (€67.4/l) and would be much cheaper from local sources. The cost of using locally produced reagents would then be lower than commercial media supplemented with BSA (1 litre of RPMI with BSA at 3.5 % costs €72).The PBS stabilates were also less infective than MEM stabilates but still showed a reasonably high infectivity (59 % of that of MEM). It could be that the nutritive value of the defined medium components may not be as critical as their buffering and cryoprotective qualities during storage. If this is the case, PBS supplemented with BSA has the potential to be the cheapest alternative medium (€62/l). Although PBS and FCS were found to maintain a high proportion of the infectivity of the sporozoite dose, their suitability needs further investigation.

One refreezing cycle significantly decreased the infectivity of sporozoites in comparison to storage for 1.5 h on ice. The latter should not affect the infectivity as Marcotty *et al*. (2001) found that keeping a stabilate on ice for up to 6 h did not reduce sporozoite infectivity significantly. Although the actual freezing temperature was not measured in these experiments, 1.5 h was found to be sufficient to freeze the vial contents to -60^{o}C (Njuguna and Musisi, 1996). This is below the critical temperature range (-20^{o}C to -50^{o}C) at which ice crystals form and raise extra-cellular solute concentrations, a process that is detrimental to cell integrity in cryopreservation (Farrant, 1970). Subsequent re-freezing cycles caused similar losses in infectivity.

Refreezing of stabilates seems to induce considerable loss in infectivity. Attempts to refreeze stabilates left over from field immunisation would result in low quality stabilates that may not be protective upon inoculation. It would be helpful to have this cautionary information in the extension packages for stabilate delivery especially for the private sector involved in ECF immunisations. A re-titration of such stabilate may be necessary to determine appropriate immunising doses. This would be beyond the expertise of the service provider. Moreover, they may not have appropriate equipment to undertake a standard refreezing process. In unavoidable situations e.g. polyvalent ECF vaccine production, it would be advisable that the infectivity loss is considered and immunising doses adjusted accordingly. The process of refreezing may be useful in research work for reducing variability arising from different storage vials. This involves thawing the stabilate, pooling the contents of vials and refreezing for later use.

Holding the stabilates for up to 1 h on ice did not reduce the quality or infectivity of stabilates significantly. This is important as the process of preparing stabilates, particularly centrifuging and aliquoting, takes time before the stabilate is ready for freezing. In addition, including "batch" as a random effect not only takes into account the tick batches but the different methods of grinding (Omni-mixer Homogeniser®, Ultraturax® and manual) used in these experiments.

In conclusion, the study confirms the findings of Kimbita *et al*. (2004) that RPMI 1640 is as effective as MEM in supporting sporozoite infectivity. Therefore, it is recommended that RPMI 1640 that is properly supplemented can be used as an alternatively cheaper freezing medium in *T. parva* stabilate production where MEM is either too costly or not available. We also showed that there is an estimated loss in sporozoite infectivity of 35 % when stabilates are refrozen. This loss needs to be adjusted for in both research and field use stabilates.

Chapter 5 - Snap freezing of Theileria parva *stabilates*

"Success is to be measured not so much by the position that one has reached in life... as by the obstacles which he has overcome while trying to succeed" - Booker T. Washington

5.1. Introduction

East Coast fever (ECF), is a lymphoproliferative disease of cattle that is often fatal and causes high morbidity. It is caused by *Theileria parva*, an obligate intra-cellular Apicomplexan protozoon that is transmitted by the three-host ticks *Rhipicephalus appendiculatus* and *Rhipicephalus zambeziensis.* The disease causes major economic losses in the livestock industries of eastern, central and southern Africa (Young *et al.*, 1988). One of the control options is immunisation of susceptible young cattle stock by the Infection-and-Treatment (I & T) method (Radley *et al.*, 1975a) using cryopreserved stabilates of live sporozoites of the parasite. Immunisation is achieved by inoculation of a dose of stabilate with concurrent injection of a long acting tetracycline. The technique is applied widely in areas where the disease is endemic (Berkvens *et al.,*1988; Uilenberg, 1999; Marcotty *et al.*, 2001; Fandamu *et al.*, 2006).

Currently, freezing of triturated tick stabilates at the end of production is a two-step slow freezing procedure; first cooling in ultra freezers to -80°C and then transferred into liquid nitrogen (-196°C) (OIE, 2005). The method was adapted from low temperature storage of trypanosomes as described by Cunningham *et al.* (1963), cited by Cunningham *et al*. (1973a). In this work, we tested the effect of skipping the first step and directly plunging the vials into liquid nitrogen. The rationale was to approximate vitrification, a process that prevents intra-cellular crystallisation of water during cooling (James, 2004). Vitrification is widely used in cryopreservation of some helminth species and insect embryos, and has the potential to be adapted for parasitic protozoa with the advantage of achieving higher survival rates at recovery from cryo-storage (James, 2004). On the other hand, fast freezing is expected to be detrimental to the cells when vitrification is not achieved as the exchange of water and cryoprotectants through the cytoplasmic membrane might not be completed before the crystallisation of the water (Whittingham, 1980). Snap freezing would have the advantage of requiring less equipment and speed up stabilate production.

5.2. Materials and Methods

5.2.1. Stabilate preparations

Three stabilates of *T. parva* were produced as described in the OIE manual (2005). Briefly, *R. appendiculatus* ticks were infected as nymphs with the *T. parva* Katete stock (Berkvens *et al.,*1988; Geysen *et al.*, 1999) and allowed to moult in an incubator at 22°C and 85% relative humidity. Eight weeks later, the resultant adult ticks were fed on rabbits for four days to induce sporogony of the parasite. The ticks were ground for 15 min in a mortar and pestle using crushed glass in RPMI 1640 (25 mM HEPES), at a concentration of 20 ticks/m*l* supplemented with 3.5% (w/v) BSA. The resulting material was centrifuged and the supernatant divided into three lots. To each lot was added an equal volume of chilled RPMI 1640/BSA containing glycerol 15% (w/v), sucrose (0.6 M) or trehalose (0.2 M). Glycerol solution was added drop-wise. The extracts were stirred continuously in an ice bath during addition of cryopreservatives. Three stabilate suspensions with final concentrations of 7.5 % glycerol, 0.3 M sucrose and 0.1 M trehalose were prepared. Suspensions were diluted to a final concentration of 1 tick/m*l*, keeping cryoprotectant concentrations constant and aliquoted into 1.5 ml Nalgene® cryogenic vials (1 ml/vial). Vials were then placed in an ultra-freezer for 24 h before plunging in liquid nitrogen until use. The different freezing protocols were applied on the stabilate after thawing from cryopreservation. Such stabilates have been found to lose some infectivity but the procedure is a good way of reducing variability in infectivity stemming from different stabilate storage vials (see chapter 6).

5.2.2. Freezing

Vials of each of the three stabilates were thawed at 37°C for 5 min, pooled and centrifuged in a 50 ml Cellstar® tube (Greiner Bio-One, Frickenhausen, Germany) at 400 *g* for 10 min. This was to remove any yeast and fungi that could be present (Marcotty *et al.*, 2004). The supernatants were then re-aliquoted into fresh Nalgene® vials at 0.5 ml/vial and refrozen by the following methods: (a) placed in an ultra freezer for 24 h before plunging in liquid nitrogen (current method used in stabilate production), (b) controlled freezing in a programmed freezer (Minicool® 40 PC, Air Liquide, France) at 1 ºC/min till -40 ºC and then at 5 ºC/min till -100 ºC before plunging in liquid nitrogen and (c) snap freezing by plunging the vials directly in liquid nitrogen. All vials were kept on ice before freezing. There was an equilibration period of 10 min between aliquoting and freezing. The stabilates were held in liquid nitrogen for 24 h.

5.2.3. In vitro *titrations*

Titrations of the stabilates were as described by Marcotty *et al.* (2004) and modified as described earlier (chapter 3). Briefly, stabilates were thawed rapidly in a water bath at 37°C for 5 min. They were then diluted serially eight times (2-fold) and mixed with bovine Peripheral Blood Mononuclear Cells (PBMC) in 96-well flat-bottom microtitration plates (Nunc®, Roskilde, Denmark). The plates were incubated for 10 days at 37°C in 5% CO_2 in air. After incubation, cyto-centrifuged samples of each well were made on microscopy slides. The cells were stained with Giemsa's stain and examined for the presence of schizonts.

5.2.4. Statistical analysis

Data were analyzed in a generalised linear latent and mixed model in Stata® 9 (StataCorp., *Stata Statistical Software: Release 9.* College Station, TX: StataCorp LP). The proportion of positive wells was the response variable (binomial) and explanatory variables were the natural logarithm of the stabilate concentration expressed in tick equivalents (*ln* t.e.), the cryoprotectant and the freezing method. The two sessions during which stabilates were titrated were entered as random effects.

To compare the individual infectivities of the stabilates, ratios of their effective doses (ED) $\left[ED_{control}/ED_x\right]$ where x = a stabilate undergoing a particular freezing method, were calculated by non-linear combination of estimators [nlcom] in Stata® 9 (chapter 3). The ED was taken as a stabilate concentration giving a particular proportion of positive wells e.g. ED_{50} was the dose at which 50% of the wells were positive. The [nlcom] process also fits 95% confidence intervals of the estimates. The ratios quantified the sporozoite infectivities with respect to the infectivity of the control stabilate (sucrose stabilate frozen by programmed freezing).

5.3. Results

Numbers of wells read for sucrose, trehalose and glycerol were 189, 191 and 152, respectively. Sucrose stabilate undergoing the snap-freezing retained 27% of infectivity compared to the same stabilate in the control programmed freezing procedure. There was no significant difference in infectivity between sucrose stabilates from programmed freezing and those from 2-step slow freezing (P=0.88). Table 6 summarizes the results of the titrations and the titration curves are illustrated in Figure 11.

Table 6: *Theileria parva* sporozoite infectivity, expressed as Effective Dose (ED) ratios, after 2-step slow freezing, snap-freezing and programmed freezing (control) in glycerol, sucrose and trehalose

Method	CPA	ED ratio	Lower CL	Upper CL
2-step	Glycerol	0.09	0.04	0.22
	Sucrose	1.1	0.67	1.85
	Trehalose	1.05	0.53	2.09
Snap freezing	Glycerol	0.02	0.007	0.05
	Sucrose	0.24	0.13	0.43
	Trehalose	0.22	0.11	0.47
Control	Glycerol	0.08	0.04	0.17
	Trehalose	0.94	0.58	1.53

This table derives from the model described in the Materials and Methods and presented in figure 11. CPA = Cryoprotectant agent; ED ratio = EDsucrose-control/EDx where x = stabilate undergoing a particular method of freezing; CL = 95% Confidence Limit.

On comparing the methods, there was no difference in the efficiency of the two-step slow freezing from the control (P=0.68). The snap-freezing was significantly less efficient (P<0.001) than the control method. Stabilate infectivities were 1.1 (95% CI: 0.66 – 1.8) and 0.24 times (95% CI: 0.13 – 0.43) those of controls for 2-step and snap-freezing methods, respectively.

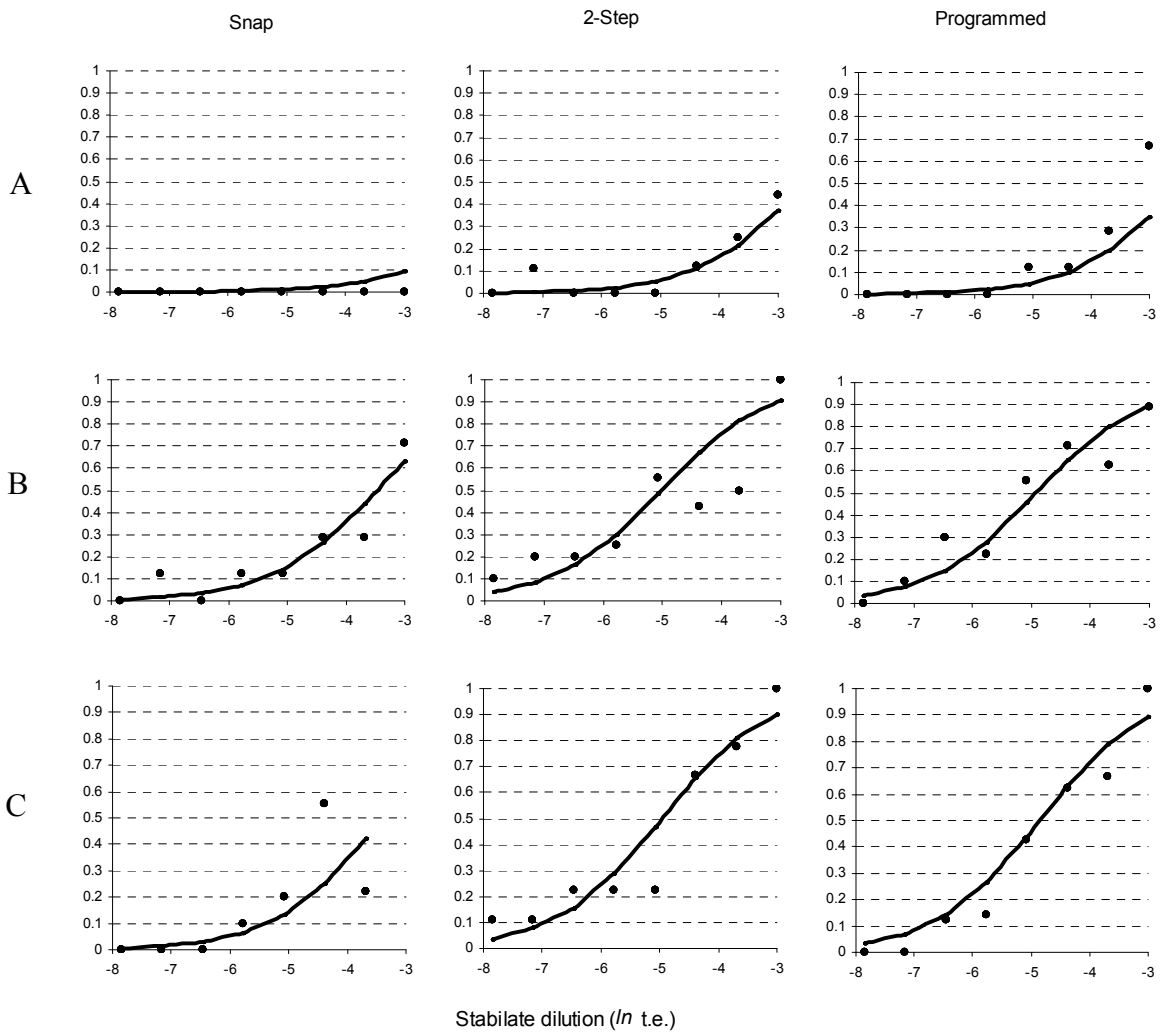

Figure 11: Titration curves (logistic prediction) of *Theileria parva* stabilates cryoprotected with (A) glycerol, (B) sucrose, (C) trehalose and frozen by snap-freezing, 2-step slow freezing and programmable freezer (control). Y-axis is the proportion of positive wells. The points are observed averages

5.4. Discussion

Snap-freezing of stabilates in the cryoprotectant formulations used induced significant infectivity losses, especially in the glycerol stabilates. It is speculated that the low recovery of viable sporozoites could be due to the packaging of the stabilates in 1.5 ml vials. This may have resulted in a non uniform cooling due to the diameter of the column with the material at the periphery of the tube freezing first and protecting the deeper material, that cooled more slowly from outside in. Ideally, straws should be used to increase the surface area to volume ratio. Also, a limited range of

cryoprotectant concentrations were used and the concentration of sucrose and trehalose could have been too low for snap-freezing. Solutions used for vitrification are at least 1 M (James, 2004). The loss of infectivity of the glycerol stabilate could have been either due to low concentration or the low starting infectivity of the stabilate. It is worth exploring the use of different concentrations and combinations of cryopreservatives.

This was a preliminary investigation that followed protocols of cryopreservative concentration and stabilate packaging that are used currently in *T. parva* stabilate production in our laboratory and internationally (OIE, 2005). Stabilate infectivities obtained for sucrose and trehalose of 27% and 23%, respectively of that of the control stabilate show that snap-freezing results in the recovery of viable *T. parva* sporozoites. This is a valuable finding suggesting that further exploration and optimisation of the snap-freezing process could yield an effective and economical method for cryopreservation of *T. parva* stabilates. It has been shown that when slow freezing of parasites is successful, vitrification has increased the surviving proportion at recovery from storage (James, 2004). We therefore conclude that, with improvements in the protocol, snap-freezing offers a potentially cheaper and easier means of cryopreserving *T. parva* stabilates.

Chapter 6 - Storage of Theileria parva *stabilates at 4 and -20°C*

Based on:
Mbao, V., Berkvens, D., Dorny, P., Van Den Bossche, P. and Marcotty, T. (2006) Comparison of the survival on ice of thawed *Theileria parva* sporozoites of different stocks cryoprotected by glycerol or sucrose. *Onderstepoort Journal of Veterinary Research* (Accepted for publication)

"In skating over thin ice our safety is our speed" - Ralph Waldo Emerson

6.1. Introduction

East Coast fever, an often fatal disease of cattle in eastern, central and southern parts of Africa is caused by *Theileria parva,* an obligate intracellular protozoan parasite. It is mostly transmitted by the three-host ixodid ticks *Rhipicephalus appendiculatus* and *Rhipicephalus zambeziensis.* The disease is a major constraint to livestock development in the affected regions (Young *et al.*, 1988). Control methods include immunisation by the Infection-and-Treatment Method (I&T) (Radley *et al.*, 1975a) in which doses of *T. parva* cryopreserved stabilates (Cunningham *et al.*, 1973a) are inoculated simultaneously with a long acting tetracycline. The method is widely applied in the field (Uilenberg, 1999; Marcotty *et al.*, 2001; Fandamu *et al.*, 2006).

Currently, stabilates are stored in liquid nitrogen. The maintenance of the cold chain up to the farm level is complicated which makes the method less appropriate for rural circumstances and is expensive for livestock keepers. This problem was partly alleviated in the Eastern Province of Zambia by storing the stabilate on ice for a few hours between thawing and inoculation. This method allows the distribution of the service using bicycles instead of motorcars. Studies conducted in this region using a glycerated *T. parva* Katete stock stabilate (Geysen *et al.*, 1999) showed that after 6 hours of storage on ice stabilates could still protect 90% of the immunized animals (Marcotty *et al.*, 2001). In the southern province of Zambia, the I&T immunisation against ECF makes use of the milder Chitongo strain (Geysen *et al.*, 1999). There is no information on how long stabilates of this *T. parva* strain can be stored on ice without appreciable loss in their potency.

It was envisaged that short-term storage of *T. parva* stabilates in domestic freezers would also reduce liquid nitrogen dependency. Stabilates could be stored possibly at the immunisation centres in freezers instead of liquid nitrogen containers during immunizing campaigns. In this study, the loss of infectivity of stabilates due to storage on ice and in domestic freezers was assessed by *in vitro* titration. The performance of the Chitongo stock was compared with that of the Katete stock with the aim of determining the suitability of the deferred immunisation technique using the Chitongo stock. Glycerol and sucrose cryoprotected stabilates were used as to evaluate effect of storage time on type of cryopreservative.

Various studies indicated that sucrose is a promising *T. parva* stabilate cryoprotectant. It is cheap and more user friendly than glycerol. Ongoing work seems to indicate that sucrose stabilates have higher infectivity, and therefore better infectivity, on recovery from cryopreservation compared to glycerol counterparts (own unpublished data, 2005). Since it is not known whether stabilates cryoprotected by sucrose show similar survival when stored on ice as glycerol-cryoprotected stabilates, the infectivities during ice storage of sucrose and glycerol Katete stabilates were compared.

A considerable amount of work on *in vitro* assessment of *T. parva* stabilates has been done (Wilkie *et al.*, 2002; Marcotty *et al.*, 2004). This has shown the potential of the technique as an economical and ethical way of assessing stabilate infectivity for determining immunizing doses, comparison of stocks and evaluating effects of various processes during stabilate production and cryopreservation. However, a relationship between *in vitro* and *in vivo* assays to predict actual immunisation potential of stabilates is yet to be made. In the present work, we evaluated this link by establishing a graphical relationship between effective doses that give 50% infectivity (ED_{50}) (*in vitro*) to proportions of animals successfully immunized (*in vivo*). When stabilate is stored on ice, its ED for respective levels of infectivity increases with time. These doses can easily be assessed *in vitro* and compared to the proportions of animals successfully immunized with similar stabilate stored in similar conditions.

6.2. Materials and Methods

6.2.1. Stabilate preparations

6.2.1.1. Animals

Two Friesian heifers kept in a tick proof stall were inoculated with 1 ml of *T. parva* Katete subcutaneously below the right parotidian lymph node (ln. parotideus superficialis). They were checked daily for rectal temperature and once the parotidian nodes were palpably swollen, biopsies were aspirated and smears prepared for calculating percentages of schizont-infected lymphoblasts. Thin jugular blood smears were made to check for parasitemia. Nymphal *Rhipicephalus appendiculatus* ticks were applied on day 10 post inoculation. The moulted ticks (adults) were pre-fed for 4 days on New Zealand white rabbits kept at the Institute of Tropical Medicine animal quarters. These ticks were used to produce stabilates K2g and K2s as described below. Another Friesian heifer was used similarly to infect a batch of ticks with *T. parva* Chitongo for the production of stabilate C1g.

6.2.1.2. Stabilates used

One Chitongo and three Katete stabilates were used for storage on ice. Katete stabilates were all produced from batches of *R. appendiculatus* adult ticks, infected as nymphs with the same *T. parva* seed stabilate. The first glycerated Katete stabilate (K1g) that was used in this study had been produced and tested *in vivo* by Marcotty *et al.* (2001). The two other Katete stabilates, K2g and K2s, were produced at the same time and from the same batch of ticks (abundance of infection - 15.7 acini/tick) for the present work but were cryoprotected using 7.5% (w/v) glycerol and 0.3 M (10% w/v) sucrose respectively. K2g and K2s were used for the -20°C storage experiment. The Chitongo stabilate (C1g) was cryoprotected with 7.5% glycerol.

6.2.1.3. Production

For all stabilate productions, infected nymphs were allowed to moult in an incubator at 22°C and 85 % relative humidity. Eight to 12 weeks after engorgement, the resulting adult ticks were fed on rabbits for 4 days to induce sporogony of the parasite. Harvested ticks were manually ground in a mortar using a pestle for 15 min in Minimum Essential Medium supplemented with Bovine Serum Albumin (MEM/BSA) (Purnell *et al.*, 1973) at a concentration of 20 ticks/ml. For glycerol stabilates, an equal amount of chilled MEM/BSA with glycerol at 15% (w/v) was added drop wise (OIE, 2005). For the sucrose stabilate, an equal amount of 0.6 M sucrose/MEM/BSA solution was added to give final concentration of 0.3 M. The 0.6 M sucrose medium had been prepared by dissolving 30.8 g of sucrose grains (Sigma #S1888) in 150 ml of MEM/BSA solution. Medium was buffered with HEPES. The extracts were stirred continuously in an ice bath. K2g and K2s stabilates were prepared in a single session and from a single batch of ticks, the only difference being that at point of cryoprotectant addition, half the extract was mixed with glycerol and the other with sucrose as described above. Some portions of the stabilates were further diluted to a final concentration of 1 tick/ml. All suspensions were aliquoted into 1.5 ml Nalgene® cryogenic vials (1 ml/vial). Vials were then placed in polystyrene boxes and transferred to a -80°C freezer for 24 h before being plunged into liquid nitrogen for storage.

6.2.2. Peripheral Blood Mononuclear Cells (PBMC)

PBMC were isolated as described in Appendix I.A.

6.2.3. Ice bath storage

Storage on ice meant keeping stabilate vials in a polystyrene box filled with water and pieces of melting ice (about 3°C). Groups of six vials of each stabilate were thawed at 37ºC for 5 min and stored on ice for different periods. The K1g stabilate was stored on ice for 3, 8 or 24 h. The C1g stabilate storage times were 1, 3, 6, 12 or 24 h. Finally, the thawed K2g and K2s stabilates were kept on ice for 6, 12 or 24 h before the titration. Stabilate for *in vivo* titration had been stored on ice for 8, 12, 16, 24 or 32 h as reported by Marcotty *et al*.(2001).

6.2.4. Storage at -20°C

A group of six vials of each stabilate was transferred from liquid nitrogen storage to a -20ºC freezer at 4, 2 or 1 week(s) before titration. The freezer temperature was recorded twice daily.

6.2.5. In vitro *titrations*

The K2g and K2s stabilates stored on ice including a control group (thawed at titration) were transferred to separate falcon tubes and centrifuged in 25 ml Cellstar® tubes (Greiner Bio-One, Frickenhausen, Germany) at 210 *g* for 10 min. The supernatants, being arbitrarily allocated to separate rows in a 96-well microtitration plate (12 rows by eight columns), were then diluted serially eight times (two-fold dilution) in four microtitration plates. PBMC were then added to the wells and the plates incubated at 37°C in a CO_2 incubator for ten days All stabilate groups were titrated in parallel in three sessions resulting in a total of 12 plates. A session was taken as a titration at a given time, sharing a batch of PBMC, culture media and stabilate diluents; thereby forming a cluster for purposes of statistical analyses (Marcotty *et al.*, 2004). For controls, stabilate freshly thawed from liquid nitrogen storage was used. C1g and K1g stabilates were titrated in single sessions. Stabilate was diluted serially 12 times (1.5 fold dilution). Table 7 presents the various sessions and microtitration plate set up.

For storage at -20ºC, all vials were thawed at 37ºC for 5 min together with a group of controls for either stabilate, four weeks after the first two groups of stabilates vials had been frozen. Stabilate from each vial was separately centrifuged. The rest of the procedure was as described for ice storage. Table 8 illustrates the sessions and numbers of wells read fro the experiment.

On day ten, cytocentrifuged smears of each well were prepared on microscope slides. To avoid cross contaminations between various stabilates, each row (corresponding to a different stabilate) was assigned to a particular cyto-centrifuge block and sampling was done from the lowest to the

highest stabilate concentration. The samples were stained with Giemsa's stain and examined with a light microscope. The presence of schizont-infected lymphoblasts was a positive score.

Table 7: Sessions and number of microtitration plate wells read for stabilates stored on ice.

	K1g	K2g	K2s	C1g
Number of sessions	1	3*	3	1
Plates/session	2	4*	4	2
Wells read	188	569	575	120

*same sessions and plates as for K2s. K: Katete, C: Chitongo, g: glycerol and s: sucrose

Table 8: Sessions and number of microtitration plate wells read for stabilates stored at -20ºC

	K2g	K2s
Number of sessions	3*	3
Plates/session	4*	4
Wells read	576	576

*same sessions and plates as for K2s. K: Katete, g: glycerol and s: sucrose

Experimental animals were maintained and treated humanely according to the guidelines laid down by the Ethics Commission of the Institute of Tropical Medicine of Antwerp, Belgium (DG003-MM-K-Rip). Details of animals used for production of K1g by Marcotty *et al*. (2001) are fully described in the given reference.

6.2.6. In vivo *titrations*

The *in vivo* titrations data used to compare with *in vitro* were obtained from Marcotty *et al*. (2001). Figure 12 illustrates the relationship between storage on ice and proportions of successfully immunized animals.

6.2.7. Statistical analysis

6.2.7.1. Infectivity losses of Katete and Chitongo (K1g & C1g) in function of time of storage on ice

Data were analysed by logistic regression in Stata9® (Stata Corporation, Texas). The proportion of positive wells was the response variable and explanatory variables were the dose (natural log of tick

equivalents [*ln* t.e.]) and storage time on ice. Storage time was considered both as a discrete variable and, in a simplified model, as a continuous variable. The two models were compared by means of a likelihood ratio test. The level of significance was set at 5%.

Estimation of residual infectivity was conducted by using ratios of effective doses $\left[ED_{control} / ED_x\right]$ where x = time of storage on ice. This was calculated using a non-linear combination of estimators which also calculates the ratios' respective confidence intervals (see chapter 3).

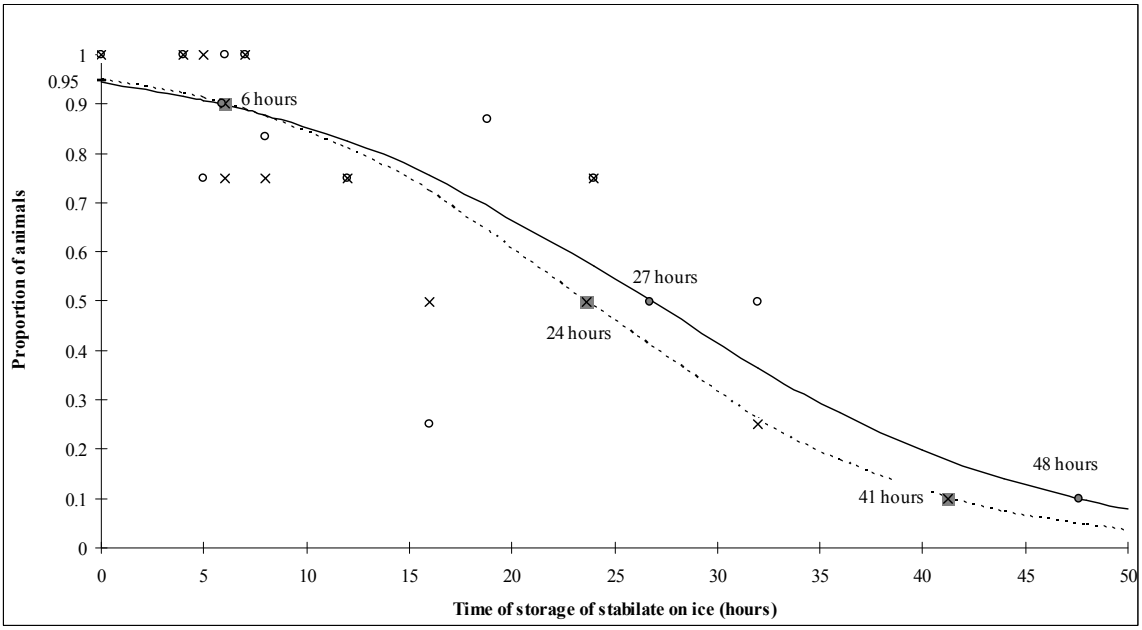

Figure 12: Effect of storing *Theileria parva* stabilates on ice on the ability to induce immunity in cattle (Marcotty *et al.* 2001). × Seroconversion (observed), ○ Resistance to lethal challenge (observed), ... Seroconversion (best fit): Logit (y) = 3.0 - 0.12 * x, ▬ Resistance to challenge (best fit): Logit (y) = 2.8 - 0.11 * x, ⊠ T0.1, T0.5 & T0.9 on seroconversion ● T0.1, T0.5 & T0.9 on resistance to lethal challenge.

6.2.7.2. Infectivity losses of glycerol and sucrose stabilates (K2g & K2s) in function of time of storage on ice

The comparison of the infectivity of stabilates cryoprotected with glycerol or sucrose was conducted by a logistic regression using the GLLAMM command (Generalised Linear Latent and Mixed Models) in STATA®, with vial and session as random effects. The explanatory variables were stabilate cryoprotectant (sucrose or glycerol), stabilate dose (*ln* t.e.) and time of storage on ice.

The response variable was the proportion of positive wells. Interactions between time and stabilate as well as dose and stabilate were tested. Initially, all variables except the dose were entered as discrete variables. This was the saturated model. The non-significant interactions were dropped and the analysis redone with time as continuous variable. This was the simpler model. When the two models were not statistically different (P>0.05), the simpler model was adopted.

6.2.7.3. Comparison of the viability estimations in *in vivo* and *in vitro* experiments (K1g *in vivo* and *in vitro*)

Results from the *in vivo* evaluation of the K1g stabilate were obtained from previous experimental work (Marcotty *et al.*, 2001). The best fit curve on observed proportions of protected animals was calculated in a logistic model.

Taking the time of storage as a common axis for the *in vivo* and *in vitro* infectivity loss evaluations, the *in vitro* ED50 estimates were plotted against predicted proportions of protected animals (in vivo) that had been inoculated with K1g.

6.2.7.4. Infectivity losses of K2g and K2s in function of time of storage at -20ºC

Analysis of -20ºC stored stabilates was as in 6.2.7.2 above.

6.3. Results

The time of storage on ice was taken as a continuous variable as the likelihood ratio tests comparing these models to the models using the time as a discrete variable were not significant (P>0.05).

Infectivity losses of Katete and Chitongo (K1g and C1g) in function of time of storage on ice

The two stabilates were kept in separate models as they were tested separately. For K1g, the residual infectivity after each hour of storage on ice was estimated to be 0.99 of the infectivity in the preceding hour (95 % CI: 0.96 - 1.02). The effect of storage time was not significant whether time was a discrete variable (P=0.99, P=0.24 and P=0.55 for times 3, 8 and 24 h, respectively) or continuous variable (P=0.45) (Figure 13.A and Appendix VIII.A).

The residual infectivity of C1g after storage on ice was 0.96 of the infectivity in the preceding hour (95 % CI: 0.93 - 1.00). Similarly, storage time was not significant for discrete time (P=1, P=1, P=0.17 and P=0.17 for times 3, 6, 12 and 24 h, respectively) or continuous time (P=0.07) (Figure 13.B and Appendix VIII.B).

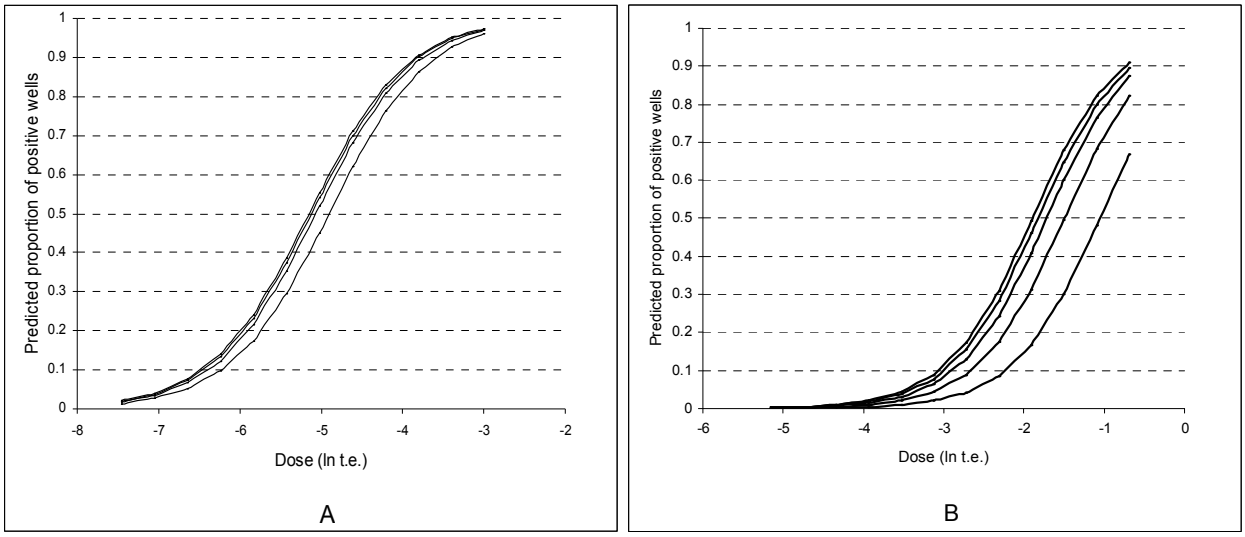

Figure 13: A) Titration curves of K1g (Katete) stored on ice for 0, 3, 8 and 24 h (from left to right). [ln t.e.] is the natural log of the stabilate dose expressed in tick equivalents, and B) C1g (Chitongo) stored on ice for 1, 3, 6, 12, 24 h (from left to right). (See Appendices VIII.A and B).

Infectivity losses of glycerol and sucrose stabilates (K2g and K2s) in function of time of storage on ice

Since the stabilates were tested in parallel, infectivity losses of the two stabilates were analysed in one model. The K2g had a residual infectivity of 0.99 of that in the preceding hour of storage. Effect of storage time was not significant (P=0.35) for the studied time periods. The K2s had 0.97 residual infectivity per hour of storage. Here, effect of storage time was significant (P=0.04). The interaction between stabilate and storage time was not significant (P=0.45). Base infectivity, i.e. infectivity after production and cryo-storage (before storage on ice) for sucrose was 10 times higher that of glycerol (95 % CI: 6.2 - 16.7) (Figure 14 and Appendix IX).

Comparison of the viability estimations in *in vivo* and *in vitro* experiments (K1g *in vivo* and *in vitro*)

The graphical representation shows that an increase of ED_{50} from 0.006 to 0.007 tick equivalents (*in vitro*) results in the protection proportion dropping from 92 % to 57 % (*in vivo*) (Figure 15).

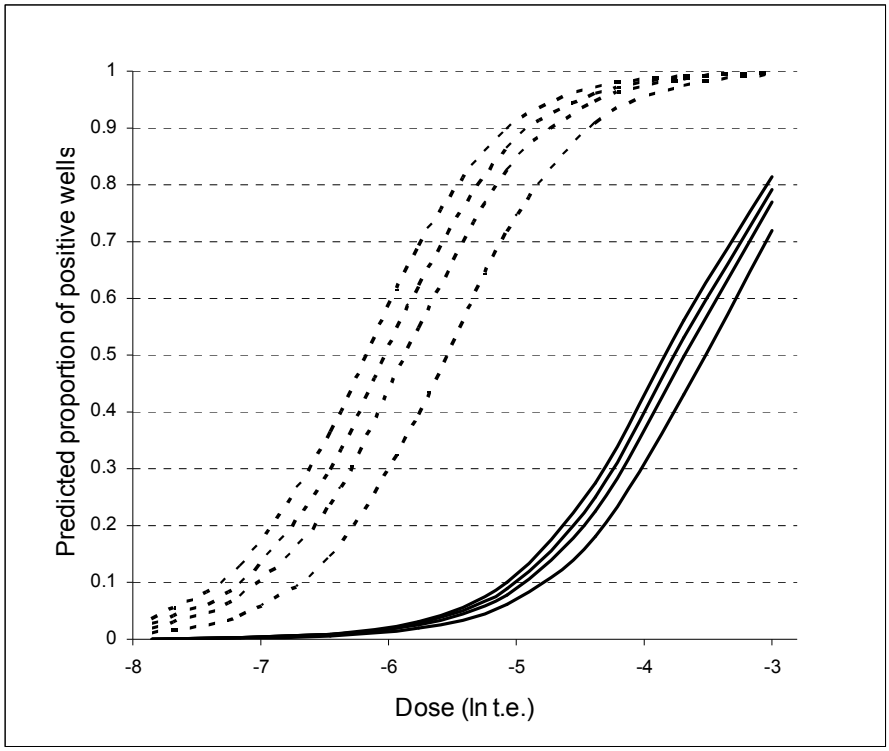

Figure 14: Titration curves of Katete-K2g (——) and K2s (-----) stabilates stored on ice for 0 (control), 6, 12 and 24 h (from left to right). [ln t.e.] is the natural log of the stabilate dose expressed in tick equivalents. Individual graphs and data points illustrated in Appendix IX.

Infectivity losses of glycerol and sucrose stabilates (K2g and K2s) in function of time of storage at -20ºC

Sucrose stabilates retained only 2.3, 2.7 and 0.7% of the infectivity of control stabilate after 1, 2 and 4 weeks of storage respectively (Figure 16). The effect of storage time on infectivity was significant at all storage periods (P<0.001).

Since all glycerol stabilate wells from week four were negative, the data were excluded from the logistic regression. Residual infectivities for glycerol stabilate were 39 and 4% of control for 1 and 2 weeks of storage respectively (Figure 17). Storage time was a significant predictor of stabilate infectivity after 2 weeks of storage (P=0.004). The random effect of storage vials was significant (P=0.016).

The interaction between stabilate and storage time was significant i.e. the effect of storage time was dependent on the type of cryoprotectant.

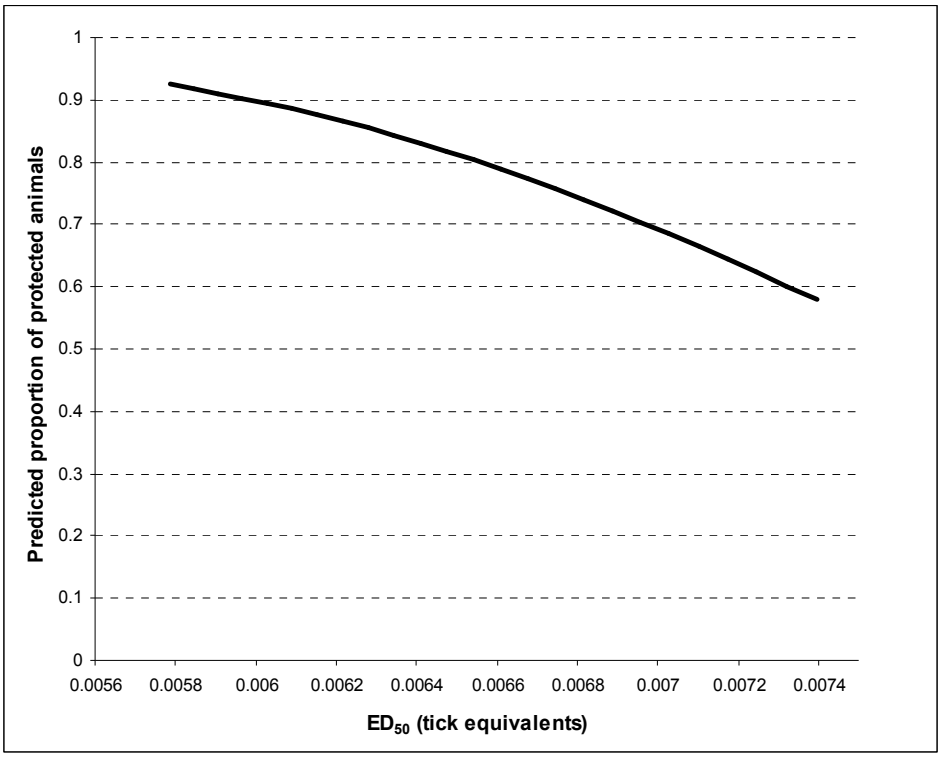

Figure 15: Correlation between proportions of protected animals against *in vitro* ED_{50}.

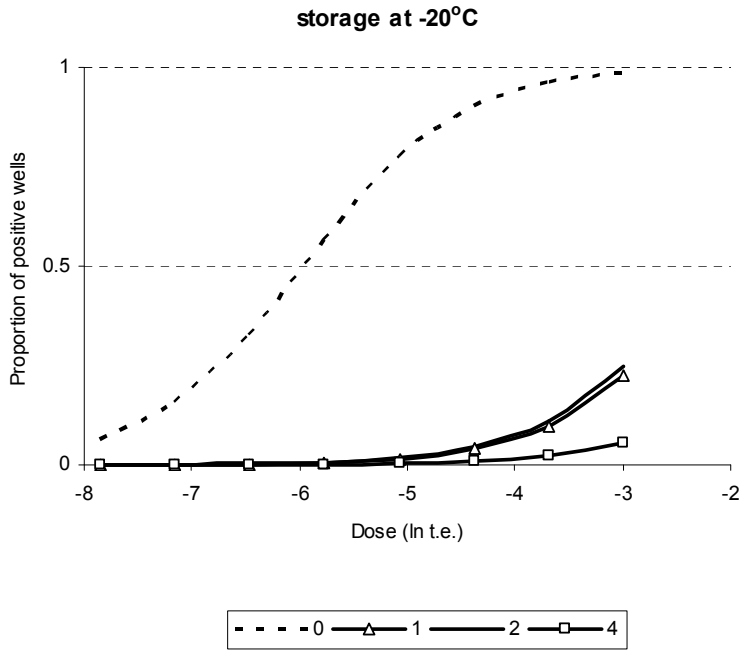

Figure 16: Titration curves for *Theileria parva* sucrose stabilate stored at -20ºC for 1, 2 and 4 weeks.

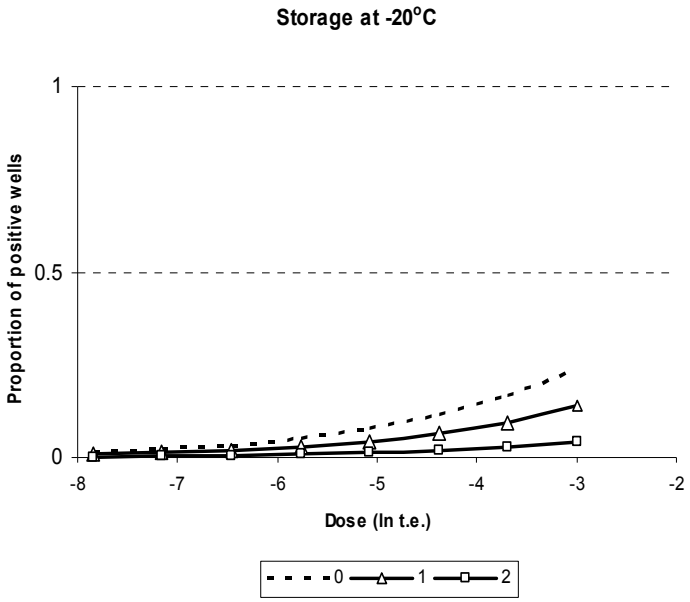

Figure 17: Titration curves for *Theileria parva* glycerol stabilate stored at -20ºC for 1 and 2 weeks.

6.4. Discussion

The loss of infectivity after short-term storage on ice (4°C) of Katete and Chitongo strain *T. parva* stabilates with glycerol or sucrose as cryoprotectant was minimal. This observation is in line with the one of Musisi *et al*. (1996b) who found that animals were protected with a trivalent stabilate stored on ice for 15 h. Similar results were obtained by Marcotty *et al.* (2001) who observed successful immunisation in 90% of cattle inoculated with stabilates stored on ice for up to 6 h. Except for the sucrose-stabilate (K2s), the effect of storage on ice for up to 24 h was not statistically significant. It is assumed that an effect of storage time on infectivity (reduced infectivity) will be observed for longer storage periods than investigated in this work. However, it was observed that keeping stabilate on ice for longer periods resulted in contamination of the cultures on several occasions. This was most likely due to fungal proliferation (from stabilate tick material) at this temperature.

The observed rates of infectivity loss for the Katete and Chitongo strains storage on ice were similar. This indicates that the deferred immunisation technique that has been applied in the Eastern Province of Zambia since 1996 could be used in the Southern Province where the Chitongo stock is used. This would greatly simplify the delivery of stabilate to the remote areas and make delivery much cheaper. Regarding the two different strains used here, the infectivity loss dynamics observed

may, therefore, be true for other *T. parva* stocks in other regions of Africa. However, it may be necessary to similarly test such stocks to confirm this assumption.

Since the interaction between stabilate and time was not significant when comparing Katete glycerol and sucrose stabilates, it is assumed that the stabilate cryoprotected with sucrose did not lose infectivity faster or slower than the stabilate cryprotected with glycerol. In terms of base infectivity (infectivity at time 0), sucrose stabilate appeared to have higher titres despite having been prepared from the same batch of ticks and during the same production session. This was also seen in comparisons of other glycerol/sucrose stabilates prepared with a similar protocol (own unpublished observation, 2005) and may be due to lower toxicity of sucrose for sporozoites. However, the observed difference in titre may also be due to an effect on the host cells, for instance, glycerol may be more toxic to lymphocytes. It would be necessary to conduct a more direct comparison (without use of lymphocytes) of the effects of these two cryoprotectants for a good conclusion e.g. by comparing the proportion of live sporozoites on recovery from cryopreservation using reverse transcription-polymerase chain reaction. Sucrose therefore remains a potentially cheaper and better candidate for field stabilates.

There is an apparent loss of infectivity in the order of 61% and 96% for glycerol and sucrose stabilates, respectively after one week storage at -20ºC. Sucrose stabilates exhibited a faster deterioration of sporozoite viability compared to glycerol as seen from the titration curves and significant statistical interaction between storage time and cryopreservatives. This could indicate a poor stabilisation of proteins by the sugar at relatively high temperatures. It is also possible that at this temperature, re-crystallisation of intracellular water occurs which is detrimental for frozen biological materials (Diller *et al.*, 1972; James, 2004). This degree of infectivity loss is too high to make this storage method suitable.

The attempt at relating *in vitro* to *in vivo* results revealed that proportion of protected cattle was reduced by 35% (92% to 57%) in going from an *in vitro* ED_{50} of 0.006 to 0.007 t.e. In terms of stabilate dilutions, this would be similar to diluting a stabilate by a factor of 1.2 times. Practically, this dilution is too small to account for the corresponding large loss in protection. Cunningham *et al*. (1974) observed a decrease in protection of only about 10% on diluting stabilate from 1/150 to 1/450 (3 times dilution). A possible explanation for the lack of reasonable agreement could be due to the small number of animals that had been used in the *in vivo* trial (about four animals per storage period) by Marcotty *et al*. (2001). The *in vivo* trial also lacked repetitions. These two factors could have resulted in an underestimation of residual infectivity of the stabilate, assuming that *in vitro*

results are more accurate. On the other hand, the *in vitro* model assumes that sporozoites are live or dead i.e. infective or not. However, some sporozoites might be weakened by storage on ice but remain infective *in vitro*. Sporozoites in a similar state may no longer be infective *in vivo* because of more hostile conditions like non-specific immune reactions. This would result in an overestimation of residual infectivity if we assume the *in vivo* result to be more accurate. In short, this lack of agreement between *in vivo* and *in vitro* observations could be explained by either the lack of accuracy of the *in vivo* trial or, more likely, by the different conditions to which weakened sporozoites are exposed in the two techniques. Such biases and limitations should be considered carefully when using the *in vitro* model.

Notwithstanding the considerations outlined in effecting a relationship between the techniques, the *in vitro* technique remains a valuable alternative to *in vivo* testing as it remains easier to carry out, humane and cheaper. Further, *in vitro* titration/evaluation has the advantage that sessions are easily repeated to offset random variation seen in *in vivo* titrations. Ideally, *in vitro* and *in vivo* trials using the same thawed stabilate and a larger number of animals should be set up in parallel, preferably with several repetitions to confirm the relation between *in vivo* and *in vitro T. parva* titration techniques.

In conclusion, storage at -20°C is not a feasible option for short-term storage of stabilates due to the considerable infectivity loss observed after one week of storage. When stored on ice, sucrose protected stabilates lose infectivity at the same rate as glycerol protected ones. Chitongo and Katete strains have similar infectivity losses when stored on ice. Therefore, Chitongo stabilates used in the Southern Province of Zambia can be delivered to the immunisation points in this way (up to 6 h). This would reduce the costs and complications associated with stabilate delivery in liquid nitrogen thus making the I & T immunisation option available and affordable to more cattle keepers. Further, this finding could be valid for other strains of *T. parva* in other regions affected by East Coast fever.

Chapter 7 - Lyophilisation of Theileria parva *sporozoites*

"Now faith is being sure of what we hope for and certain of what we do not see"
Hebrews 11:1

7.1. Introduction

East Coast fever (ECF), a disease of cattle caused by *Theileria parva* is of major economic concern due to direct losses incurred through its control and mortality (Mukhebi *et al.*, 1992). Among the control measures targeted at East Coast fever is immunisation by the "Infection and Treatment" (I & T) method. However, the method has a few constraints. Since the immunity generated is strain specific, it calls for isolation and judicial use of different *T. parva* stocks for specific localities. Secondly, it relies on a costly cold chain both for storage and delivery. *T. parva* stabilates have to be kept in liquid nitrogen to preserve parasite viability. Simplification of this cold chain by assessing viability of stored stabilates on ice (Musisi *et al.*, 1996b; Marcotty *et al.*, 2001) and attempts to freeze-dry the stabilate (Marcotty *et al.*, 2003) has been explored. Delivery of stabilate on ice is currently the method of choice in the eastern province of Zambia.

Successful resuscitation of freeze-dried sporozoites was demonstrated by Marcotty *et al*. (2003). However, studies to repeat this success or optimize it were not conclusive. As this is a potentially less expensive strategy for storage and delivery of immunising stabilates, we conducted further *T. parva* stabilates lyophilisation experiments using two lyoprotectants and several lyophilisation protocols.

7.2. Materials and Methods

7.2.1. Parasite material and cryoprotectants

Two stocks of *T. parva,* Katete and Chitongo, were used. The Chitongo and Katete stocks were isolated from Namwala and Katete districts of the southern and eastern provinces of Zambia, respectively in the early 1980's, (Berkvens *et al.,*1988; Geysen *et al.*, 1999). Parasite materials were suspended in RPMI 1640 (25 mM HEPES, Life Technologies # 52400-025) medium supplemented with BSA (Acros organics # 240401000 and #240400100) at 3.5% (w/v) and antibiotics. Two lyoprotectants were used: Sucrose (Sigma #S1888-500g) or Trehalose (Sigma # T-0167) at molar concentrations of 0.3 M and 0.1 M, respectively.

A first batch of Katete stabilate (AV) was prepared at a concentration of 10 ticks/ml. It was dispensed in 1 ml aliquots in 5 ml penicillin glass bottles giving a depth of about 5 mm. The second batch of Katete stabilate (KA) was similarly prepared from a separate batch of ticks. It was aliquoted into 5 ml and 25 ml penicillin bottles, 1 and 3 ml to a bottle, respectively. The 25 ml bottles were placed on their sides and frozen in that position to give a shallower depth of stabilate material of about 4 mm but larger surface area.

The *T. parva* Chitongo parasite material was prepared as: the normal stabilate (CA), sediment portion (CA-sed), whole infected salivary glands (CA-acini) and whole ticks (CA-tick). The sediment was collected at the interface of the supernate and debris after centrifugation of the ground up tick material. It was presumed this layer would contain a higher concentration of sporozoites than the supernate. Glands dissected from 50 female ticks were stored as 1 ml sucrose aliquots, five pairs to a vial. Fifty whole ticks, five to a vial, were aliquoted in 1 ml sucrose medium. Whole ticks and glands were used on the assumption that the tissue would provide extra protection for the sporozoites during drying.

7.2.2. Freezing protocol

The Katete stabilate was used in one set of experiments and the Chitongo material in another. Both had been pre-frozen at production to -80°C by placing the vials in polystyrene boxes that were then put in an ultra freezer. This is the first step in cryopreserving stabilates for field use (OIE, 2005).

7.2.3. Drying protocols

7.2.3.1. *Theileria parva* Chitongo stock

Chitongo parasite material (CA) was lyophilised using a Labconco® FREEZE DRYER 5 (Labconco Corp, Kansas City). Vials were weighed before and after drying. Materials were dried with several variations of a protocol as illustrated in Table 9.

Table 9: Lyophilisation protocols for *Theileria parva* infected material

Material	Shelf temp (°C)	P_c (hPa)	Time
CA	-35	0.02	11
CA	-20	0.02	11
CA-sed	-30	0.02	10
CA-sed	-18	0.01	2
CA-tick	-36	0.01	19
CA-acini	-10	0.02	2

The vials were introduced into the drying chamber when the condenser temperature had gone below -65ºC and the set shelf temperature had been reached. The ballast valve was left open during primary drying and only switched off to reduce pressure in the secondary drying. Temperatures of the condenser, shelf and vacuum (hPa) were recorded regularly during the drying process. Due to a non-functioning temperature sensor, product temperatures could not be monitored. Shelf temperature was left to equilibrate with ambient temperature during the course of drying. Vials were stoppered at atmospheric pressure and stored at -20°C.

7.2.3.2. *Theileria parva* Katete stock

Stabilates were dried in a Unitop 600 SL® freezer-dryer (Virtis, NY). Operating temperature and chamber pressures were carefully controlled based on several protocols.

Protocol 1 - Stabilate was dried for 17 h. The vials were labelled K0303 (Table 10).

Protocol 2 - Stabilate was dried for 20 h and labelled L9 (Table 11).

Protocol 3 - Stabilate was dried for 20 h and labelled L10. Table 12 shows the protocol used.

Vials were stored at -20°C after drying.

Table 10: Lyophilisation protocol for K0303. P_c – Chamber pressure

	Shelf temp (°C)	P_c (hPa)	Time
Prefreezing 1	-40		1 h
Put vials in lyophiliser			
Drying	-35	0.08	30 min
	-25	0.08	15 min
	-25	0.08	10 h
	-20	0.05	15 min
	-20	0.05	6 h
Close vials and stop the machine			

Table 11. Lyophilisation protocol for L9. P_c – Chamber pressure

	Shelf temp (°C)	P_c (hPa)	Time
Prefreezing	-40		1 h
Put vials in lyophiliser			
Hold	-40		7 h
Drying	-35	0.08	30 min
	-25	0.08	15 min
	-25	0.08	10 h
	-20	0.05	15 min
	-20	0.05	2 h
Close vials and stop the machine			

Table 12. Lyophilisation protocol for L10. P_c – Chamber pressure

	Shelf temp (°C)	P_c (hPa)	Time
Prefreezing	-40		1.5 h
Put vials in lyophiliser			
Drying	-35	0.04	30 min
	-25	0.03	19.5 h
	-25	0.08	10 h
Close vials and stop the machine			

7.2.4. Assessment of infectivity

7.2.4.1. *In vitro* assessment

Chitongo

For controls, un-lyophilised stabilate from the same batches was used. Stabilates CA and CA-sed were assessed in separate sessions. For each stabilate, 20 microtitre-plate wells were set up. About $3*10^5$ PBMC were incubated with 50 µl of stabilate in each well.

Whole ticks and tick glands were first reconstituted by adding sterile water equal to weight difference recorded after drying and gently mixing for half a minute. The tick materials were left to equilibrate for about 15 min before being homogenized using an Ultra-turrax® tissue grinder for 5 min followed by centrifugation at 400 *g* for10 min. Recovered supernatant was transferred in 200 µl aliquots to twelve 1.5 ml microtubes to which 500 µl PBMC suspension were added. The tubes were incubated for 40 min in an Eppendorf Thermomixer Compact ® (Eppendorf, Hamburg) at 37°C and 1000 rpm after which they were centrifuged and the pellets re-suspended in 1 ml aliquots of culture medium in 12-well plates. They were incubated at 37°C in a CO_2 incubator for 10 days.

The cultures were examined at the end of incubation and scored for presence of schizonts.

Katete

Stabilates were reconstituted by adding 0.8 ml of sterile water to each vial. After centrifugations and separations of supernates, they were distributed in 50 µl aliquots in microtitration-plate wells, 40 wells per cryopreservative type. An equal volume of PBMC suspension containing $3*10^5$ cells was then added to each well and the plates incubated for 10 days.

For control, cryopreserved (un-lyophilised) stabilate was assessed in the same *in vitro* sessions.

7.2.4.2. In vivo assessment

Lyophilised stabilate CA was appraised by injecting 20 ml of reconstituted material into a Friesian heifer 10 ml subcutaneously below the right ln. parotideus superficialis and 10 ml intravenously into the jugular vein.

7.2.5. Vital staining

Vital staining was performed using Ethidium monoazide (EMA, 3-amino-8-azido-5-ethyl-6-phenyl-phenanthridium bromide: $C_{21}H_{18}N_5Br$) (Sigma-Aldrich®, Germany). EMA penetrates damaged cell membranes (dead cells) to covalently bind with genomic DNA and prevents such bound DNA from being amplified by PCR. Thus, presence of a PCR product means the membranes are intact and therefore from viable cells (Nogva *et al.*, 2003; Rudi *et al.*, 2005). Samples of lyophilised and non-lyophilised stabilates (CA) were EMA stained to check viability by PCR.

Briefly, EMA was added to reconstituted and purified stabilate at a final concentration of 20 µg/ml in 1.5 ml microtubes. The mixtures were placed on ice and photo-activated by placing the tubes for 30 min under laboratory fluorescent lighting. The tubes were then centrifuged at 14,000 *g* for one minute, the pellet washed once in 400 µl PBS buffer and DNA extraction performed by the method of Boom *et al*. (1990; 1999). DNA extracts were then subjected to PCR and products resolved by electrophoresis through a 2% (w/v) Agarose gel.

7.3. Results

7.3.1. In vitro *and* in vivo *infectivity assessment*

Cultures of PBMC incubated with various *T. parva* lyophilised sporozoite materials were negative. The steer that had been injected with lyophilised stabilate did not show any clinical or parasitological reactions. PBMC incubated with control stabilates (cryopreserved) became infected and demonstrated schizonts.

7.3.2. Vital staining

DNA extracts from both the lyophilised and cryopreserved *T. parva* stabilates were amplified as shown by PCR bands on Agarose gel (Figure 18).

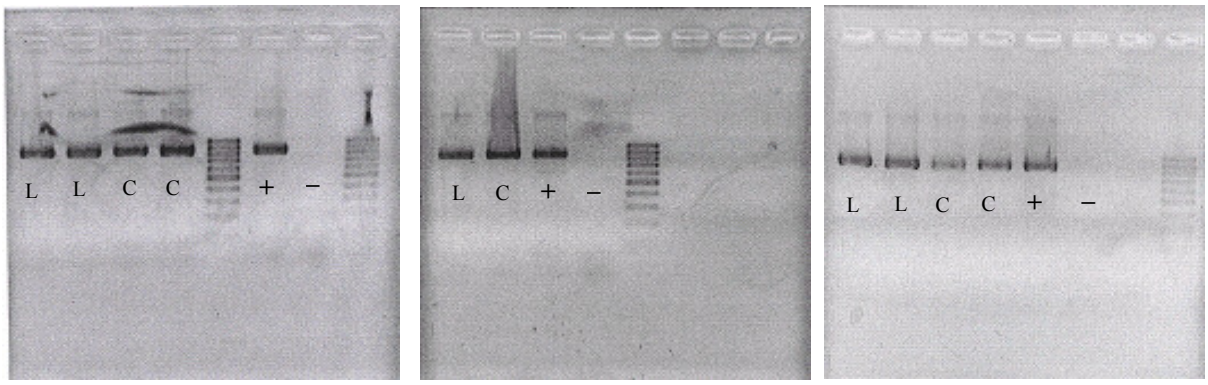

Figure 18: PCR bands of extracts of EMA treated (L) lyophilised and (C) cryopreserved *Theileria parva* stabilates with (+) positive and (-) negative controls.

7.4. Discussion

The various attempts at lyophilisation of *T. parva* stabilates and other infective materials were unsuccessful. It is evident that the deleterious stage is that of drying and/or resuscitation and not the freezing stage. The freezing stage is unequivocally less damaging as evidenced by the ability for the cryopreserved un-lyophilised stabilates to infect PBMC *in vitro* in this work and the largely successful cryopreservation of immunizing stabilates in the field. Vital staining with EMA showed that the integrity of the sporozoite wall is well preserved in spite of loss of viability. This is an encouraging finding and may be indicative of a denaturing process limited to functional proteins.

Marcotty *et al*. (2003) demonstrated a successful lyophilisation of *T. parva* stabilate. However, attempts to repeat this were not successful (Marcotty, personal communication, 2004). They reported that, even in the successful event, the majority of sporozoites could have been killed by the process putting the surviving proportion at less than 1% (Marcotty *et al.*, 2003). It is speculated that the stabilate used in their work contained a very high concentration of viable sporozoites consequently raising the number surviving lyophilisation

Possible reasons for failure include choice of lyoprotectants. Carpenter and Crowe (1988), suggest that the ability for a sugar to stabilise a protein during drying may depend on how it binds to the protein during the process. It may be that the sugars used in our studies did not bind sufficiently well to functional proteins. This would allow unfolding of the proteins during dehydration. Another

possible reason is lack of vacuum at sealing of vials. Some workers have proposed that cells stand the stress of desiccation better if stored under vacuum (Puhlev *et al*., 2001). Although Marcotty *et al*. (2003) sealed the vials at atmospheric pressure in their successful attempt, it can still be speculated that vacuum sealing could improved recovery of viable sporozoites.

From the negative results of the *in vitro* and *in vivo* infectivity assessment, it is evident that the product formulations and protocols explored are unsuitable and need further work. It is recommended to investigate combinations of lyoprotectants and modifications of freeze-drying protocols keeping in mind the cost-benefit ratios of such developments. In conclusion, the desiccation tolerance of the *T. parva* sporozoite cell seems to be extremely low. The cell membrane is however preserved and this observation, together with a previous successful event, justifies further work in this field.

Chapter 8 - Sporozoite Neutralisation Assays and refinement of titration protocol

"The man who is swimming against the stream knows the strength of it" - Woodrow Wilson (1856 - 1924)

8.1. Introduction

This chapter describes modifications to the Sporozoite Neutralization Assay (SNA), an *in vitro* technique used to assess the effects of neutralisation on *Theileria parva* sporozoites in general, and immunisation potential of p67 recombinant proteins in particular. The optimised protocol was then validated by use of a known anti-p67 serum, a negative control (FCS) and other sera collected from cattle immunised by various methods and preparations of *T. parva*.

In vitro systems are useful for studying biological processes outside the natural environment. They enable transplantation of organisms from their hosts to model systems while maintaining vital life processes by manipulating the environment artificially, usually in a liquid medium, to simulate natural conditions. When designed and executed properly, these systems offer the advantages of finer control, ethical acceptance and experiments are easily repeatable with less variation than obtained in *in vivo* trials.

Theileria parva, the apicomplexan protozoan that causes ECF in cattle, has been studied *in vivo* and *in vitro.* Since the successful *in vitro* infection of lymphocytes with *T. parva* by Brown *et al*. (1973), the technique has been applied in studying diverse aspects of the parasite (Gray and Brown, 1981; Fawcett *et al.*, 1984; Jongejan *et al.*, 1984; Goddeeris and Morrison, 1988; Brown *et al.*, 1989a; Brown *et al.*, 1989b; Aboytestorres and Buening, 1990; Dobbelaere *et al.*, 1991; Toye *et al.*, 1996; Kimbita and Silayo, 1997; Wilkie *et al.*, 2002; Bishop *et al.*, 2003; Madder *et al.*, 2003; Marcotty *et al.*, 2004; Rocchi *et al.*, 2006). Maintenance of *T. parva* cultures is relatively simple because the infected cells are immortalised by the parasite as described in chapter one.

Cattle infected with *T. parva* produce detectable antibodies against the parasite. The target antigenic proteins include p67 and PIM antigens. P67 is expressed in the sporozoite and PIM in both sporozoite and schizont stages of the parasite (Katende *et al.*, 1998). Although humoral response does not confer protective immunity against ECF in nature, the study of various bovine immune sera immunoglobulins and their respective target antigens is critical in the development of potential sub-unit vaccination strategies against East Coast fever. One technique used in such studies is the

SNA based on the observation that immune serum interferes with sporozoite infectivity *in vitro* as described by Gray and Brown (1981).

Typically, SNA's are carried out by incubating sporozoites with test sera (about 30 min) after which PBMC are introduced. In this system, antibodies have more time to interact with sporozoites before they contact and enter lymphocytes. This method is not very representative of *in vivo* events in which sporozoites are inoculated into a complex environment containing antibody and PBMC. Moreover, sporozoites invade target cells in a very short time (Shaw *et al.*, 1991) and therefore, indications are that the period of exposure to antibodies is relatively shorter *in vivo* than the present SNA prescribes. Therefore, it was desirable to attempt to approximate the natural process as closely as possible by introducing sporozoites into a culture of PBMC's containing test sera.

The modifications were aimed at optimising the protocol for use in quantitating *T. parva.* The modifications not only address the maintenance of the viability of cells, but also provide for more efficient management of the culture system and reduced variability. Full descriptions of the existing procedures are given elsewhere (Musoke *et al.*, 1984; Musoke *et al.*, 1992; Marcotty *et al.*, 2004) and a comparison of the original and refined protocols are presented in Appendices I and II. The stages that needed improvement were identified as: a) storage of PBMC, b) discontinuation of PBMC stimulation with Con-A, c) replacement of microtubes, which are cumbersome and limit the number of repetitions that can be done per session, with 96-well plates for incubation, d) counting of positive wells rather than cells, e) incubation of antibody with PBMC and f) PCR reading of cultures instead of microscopy.

8.2. Materials and methods

8.2.1. PBMC storage

Collection and isolation of PBMC were as described in chapter 3. Cells were stained with carboxyfluoroscein succinimidyl ester (CFSE) at a pre-titrated concentration of 0.56µl per $1*10^7$ PBMC (Appendix V). The cell suspension was split into two 15 ml lots in 50 ml culture flasks, one flask was stored overnight in a refrigerator (4°C). The other was stored overnight at 37°C in an incubator (5% CO_2 in air) with the cap loosened.

The following day, samples from the two flasks were analysed by flow cytometry (FACScan®, Becton Dickinson). Data were analysed in WinMDI® version 2.8 based on 10,000 events acquired without any gating. Mean CFSE fluorescence in log units were obtained and dot plots of forward

scatter (FSC) versus side scatter (SSC) constructed. FSC measures size of cells while SSC the complexity of intracellular contents.

On another occasion, PBMC were collected and portion of the suspension distributed to 96-well microtitration plates (Nunc®, Roskilde, Denmark) at 40 µl/well. The 96-well plates were incubated overnight at 37°C in an incubator (5% CO_2 in air). The remaining portion was stored in a 50 ml Cellstar® tube (Greiner Bio-One, Frickenhausen, Germany) in a refrigerator. This set was for demonstrating sporozoite neutralisation.

8.2.2. Exclusion of Con-A

A 96-well culture plate of *T. parva*-infected lymphoblasts was prepared as described in Appendix I.A with the activation step excluded. Another plate was set up with the original protocol (Appendix I.A without alterations) as a control. Cyto-centrifuge smears were made after 10 days of incubation and compared.

8.2.3. Use of 96-wellmicrotitration plates Vs *microtubes*

Two titrations, one with the original (Appendix I.A) and the other with the refined (Appendix I.B) protocols were conducted using glycerol and sucrose stabilates to assess the effect of using plates for dilutions and incubation. The PBMC were collected, isolated and stored as described in Appendix I.B. The titrations were done the following day and cultures scored from cyto-centrifuge smears after 10 days. The refined protocol included a 1 minute shaking of plates after 30 minutes of primary incubation instead of the 1 hour shaking in microtubes for the original protocol. Effects of this reduced period of shaking on the infectivity of stabilates were analysed.

8.2.4. Use of PCR for reading titration results

A titration of glycerol, sucrose and trehalose stabilates was set up as described in chapter 5. After removal of samples for cyto-centrifuge smears (100 µl from each well), the plates were treated as described in Appendix VI for DNA extraction and PCR reading. The PCR products were then run though 2 % (w/v) Agarose gel. Each well tested on PCR was scored positive or negative and compared to the corresponding result from Giemsa-stained smears (Figure 19).

Analysis of the agreement between the two scoring procedures was done by calculating Cohen's kappa and McNemar's chi-square. Landis & Koch (1977) proposed the following scale to describe the degree of agreement between tests: 0.21-0.40, "Fair"; 0.41-0.60,"Moderate"; 0.61-0.80,

"Substantial"; 0.81-1.00 "Almost perfect". A kappa greater than 0.7 is considered a satisfactory agreement between tests.

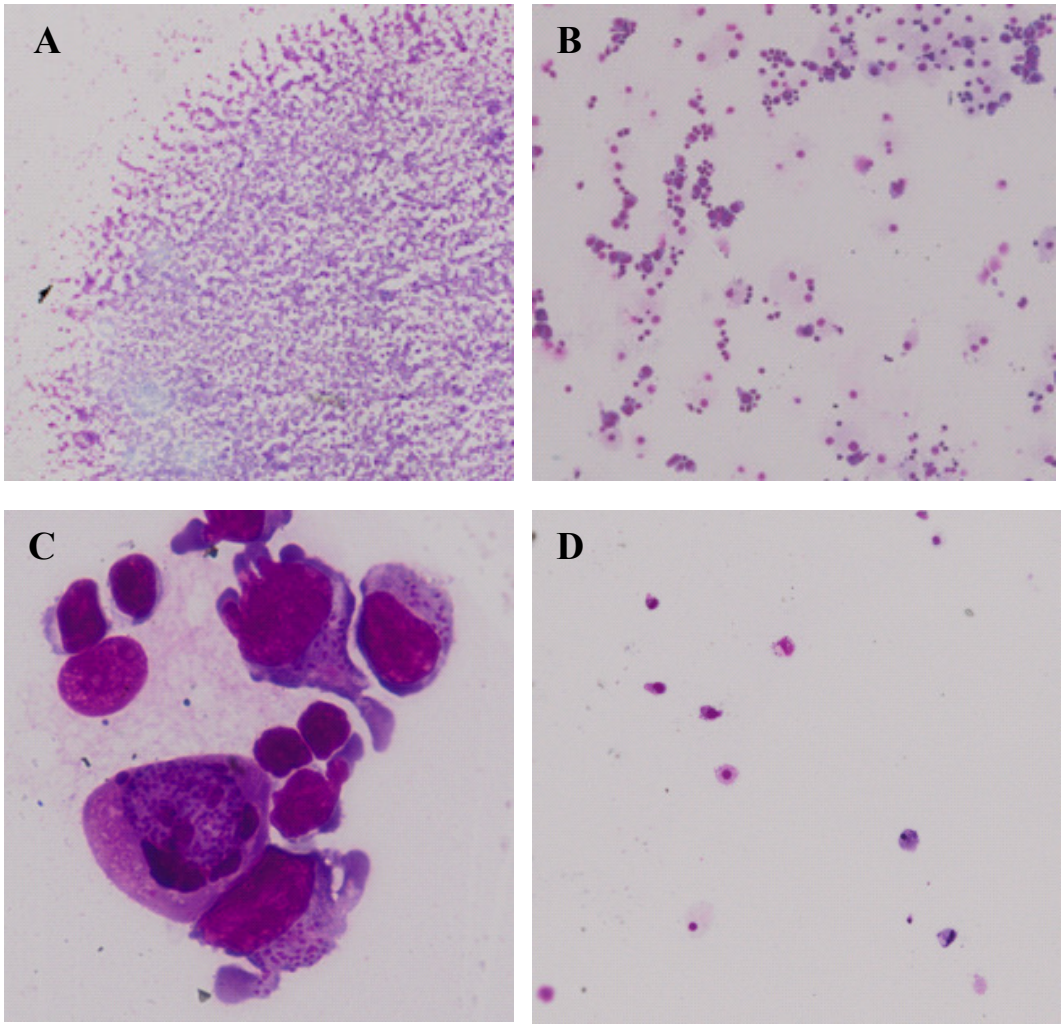

Figure 19: Cyto-centrifuge smears of PBMC at day 10 of culture: (A) smear at 20X, (B) positive smear at 400X, (C) Theileria parva schizont infected lymphoblasts at 1000X, (D) a negative smear at 400X.

8.2.5. Inoculations

8.2.5.1. Live sporozoite inoculation

Animal number 131 was inoculated with 1ml of a 1/100 *T. parva* (Katete) stabilate subcutaneously below the parotid gland (ln. parotideus superficialis). At three weeks post inoculation, it was injected with Butalex (Schering-Plough, Uxbridge) at 2.4 mg/kg. Serum and plasma were collected before inoculation and once every week thereafter until week six post inoculation.

8.2.5.2. Dead sporozoite inoculation

Animal number 6146 was inoculated with 20 ml of a lyophilised *T. parva* (Katete AV) stabilate. Lyophilisation of this stabilate is reported in chapter 7. The dose was divided into two with 10ml being injected subcutaneously above the left parotid gland (ln. parotideus superficialis) and the other 10 ml on the contra-lateral lymph node. The animal was inoculated with similar amounts two weeks later. Serum and plasma were collected before inoculation, at week four, week five and week six post inoculation and stored at -20°C.

8.2.5.3. Schizont inoculation

A culture of schizont-infected cells was established with PBMC collected from a Friesian heifer number 6148. Briefly, 2 ml of the stabilate were thawed and purified by centrifugation (Marcotty *et al.*, 2004). 200µl Aliquots were transferred into six 1.5 ml micro-tubes (Sarstedt, Numbrect) to which 500µl of PBMC at $6*10^6$/ml were added. Tubes were incubated for 20 min in a thermoshaker at 37°C (1000 rpm) after which the cells were spun down, pellets re-suspended in 2 ml fresh culture medium and transferred to each well of a 12 well culture plate. Medium was changed after 12 days and some cells were transferred to Petri dishes.

At weeks one, three and four after collection and infection of PBMC, cultures were centrifuged and re-suspended in fresh culture medium at a concentration of 100,000 cells/ml and 1 ml of this inoculated at each instance subcutaneously below the right parotid lymph node (ln. parotideus superficialis) of animal number 6148. Serum and plasma were collected before inoculation, at weeks three, four and five post first inoculation and stored at -20°C.

8.2.6. Sporozoite Neutralization Assay

The three sets of sera from animals 131, 6146 and 6148 were assessed in a Sporozoite Neutralization Assay as described in Appendix VII. Since the percentage inhibition was the unit of interest, sera dilutions were kept constant (1/25) and stabilate diluted serially as in *in vitro* titrations. P67-hyperimmune serum and plain FCS were used as positive and negative controls, respectively. The p67 serum had been obtained from animals inoculated with a recombinant p67 antigen (GFP:p67deltaSS) constructed by Kaba *et al*. (2004) and kindly provided by Intervet®. Two samples were assayed from each animal i.e. pre inoculation and post inoculation serum. The post inoculation serum was that which was positive in the SELISA. All six sera plus positive (anti-p67 serum) and negative (heat inactivated FCS) controls were assayed in triplicate rows in each of four sessions. A sucrose *T. parva* stabilate was used as source for sporozoites.

8.2.7. Serology

The three sets of sera were subjected to three serological tests to clarify their serology states.

8.2.7.1. Slide-Enzyme Linked Immunosorbent Assay (SELISA)

SELISA with peroxidase uses schizont-infected lymphoblasts as antigen attached to Teflon coated slides. Test serum samples were diluted to 1/500 and 1/1000 in serum buffer. Positive and negative control samples were diluted to 1/640. Aliquots of 15 µl of test and control sera were then added to wells and incubated in a moist chamber for 30 min at 37ºC. The slides were washed twice in PBS. Peroxidase conjugate (15 µl) was then added to each well and the slides incubated for 30 min at 37ºC. Slides were washed twice in PBS, 15 µl of substrate was added to each well and incubated in a moist chamber for 20 min in the dark at room temperature. They were then washed in distilled water for a minute before mounting with 1:1 water/glycerol. Examination was by standard light microscopy. The assay was used to screen sera for use in the Sporozoite Neutralisation Assay.

8.2.7.2. p67 ELISA

Sera were assessed by a direct ELISA with a purified *E. coli* expression p67 product (GST-p67C). Wells were coated with 50 ng/well of the purified protein. End titres were calculated with Multicalc® program.

8.2.7.3. Polymorphic Immunodominant Molecule (PIM) ELISA

Serum samples from the three animals were assayed in a PIM-ELISA as described by Katende *et al*. (1998). Plates were read by a Multiskan EX® reader (Thermolabsystems®, Vantaa, Finland) and results expressed as percentage positive of positive control serum. Cut off was set at 20%.

8.2.8. Statistical analysis

Analysis of binary data was carried out as described in chapter 2 for the titrations. For SNA's, the objective was to calculate stabilate effective doses for each serum. A logistic regression was used in Stata®9 (StataCorp, *Stata Statistical Software: Release 9.* College Station, TX) with proportion of positive wells as response variable and stabilate dose expressed as the natural logarithm (*ln*) of the tick equivalent (t.e.) was the predicting variable. The percentage inhibition was then determined by use of ED ratios $\left(\frac{ED_{control}}{ED_{test}}\right)$, where *control* = FCS and *test* = test serum. The ratio gives the relative

infectivity of the stabilate in test serum to that in FCS. The percentage of inhibition or neutralisation then becomes 1- $\left(\frac{ED_{control}}{ED_{test}}\right)$. Confidence intervals (95%) were fitted around the estimated ratios.

8.3. Results

8.3.1. PBMC storage

Figure 20 shows dot plots of PBMC cytometry. The highlighted area (red circle) indicates an

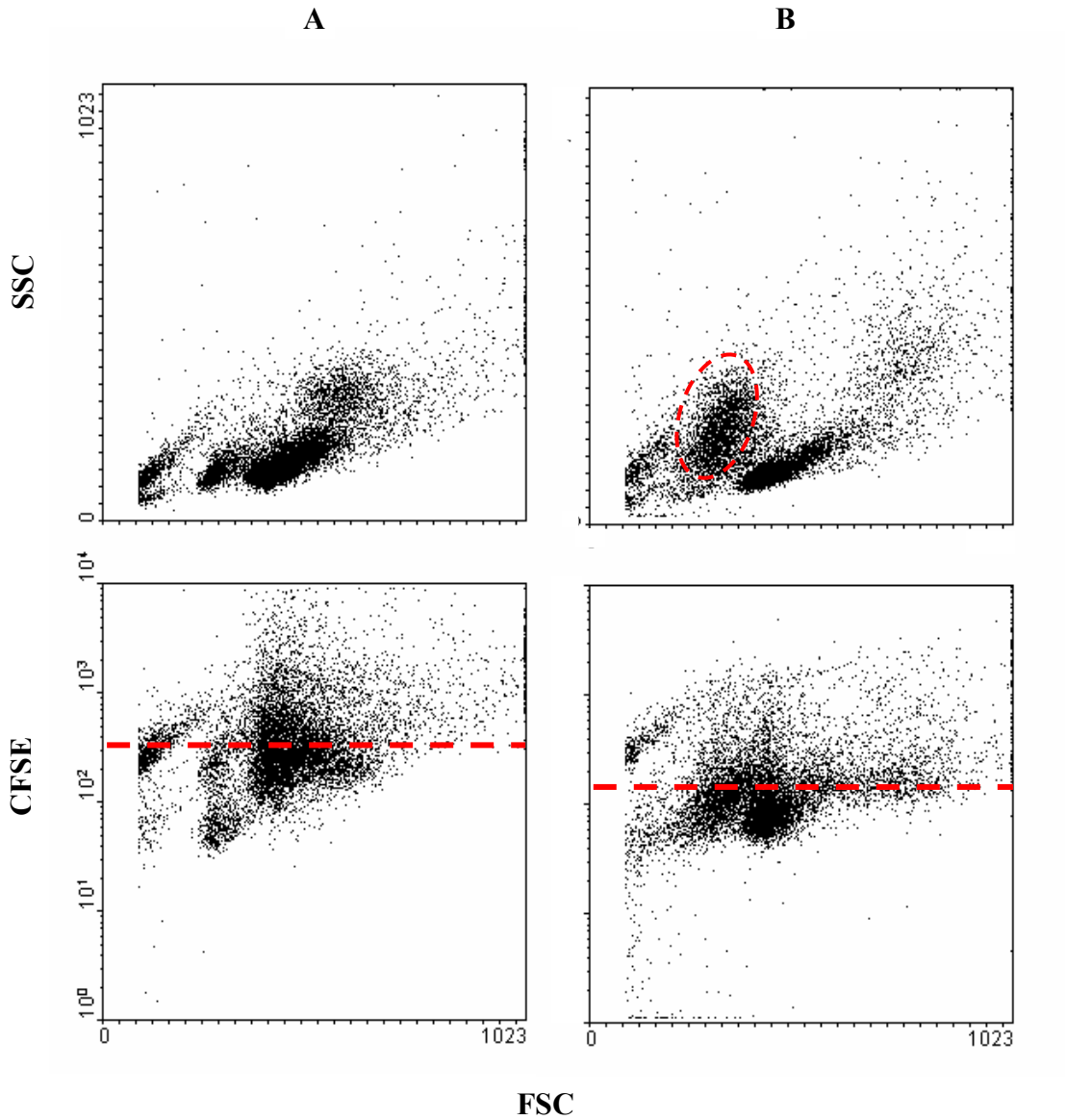

Figure 20: Dot plots of PBMC kept at (A) 4°C and (B) 37°C. Side scatter (SSC) and CFSE florescence were plotted against Forward scatter (FSC). Red dotted circle shows cells of reduced size and granularity and the lines indicate mean florescence.

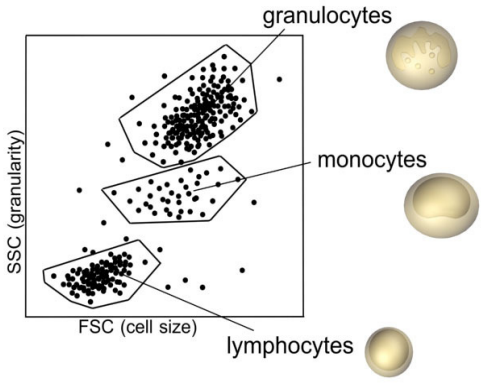

Figure 21: Diagrammatic representation of normal PBMC profile on cell size and granularity (Courtesy of http://www.uni-greifswald.de/~immuteach/methods/facs/images/02facs.jpg).

upward shift (cells becoming more granular) of a cluster of cells which could be indicative of apoptotic PBMC (Ormerod, 1994) in the population kept at 37°C. Figure 21 is a diagrammatic presentation of typical regions of PBMC subsets. It was further noted that the 37°C sample gave a lower mean fluorescence: 134 compared to 331.7 CFSE log units of the sample stored at 4°C.

8.3.2. Exclusion of Con-A

Examination of smears from the two procedures revealed a clearer presentation of cells when Con-A is excluded (Figure 22).

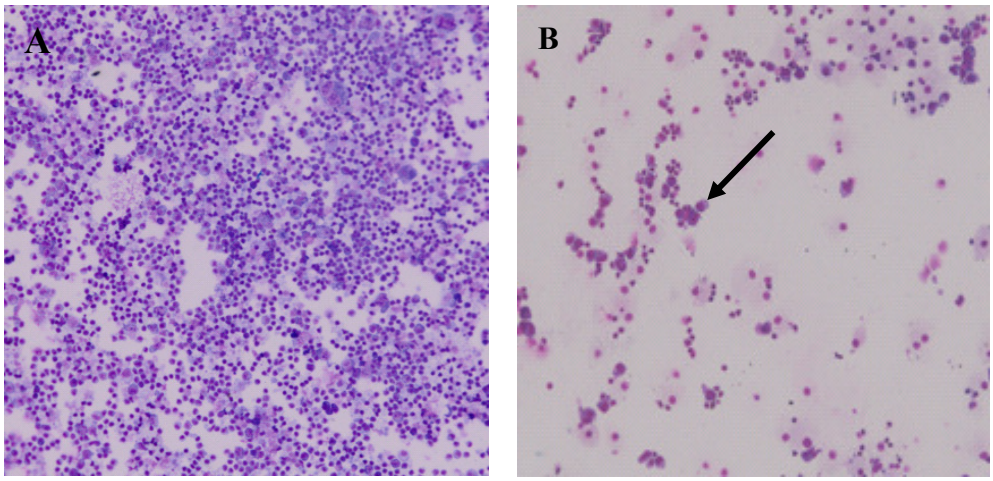

Figure 22: Cyto-centrifuged smears of *Theileria parva* infected lymphoblasts x400 (A) stimulated with Con-A and (B) not stimulated. Infected cells are easily spotted in (B) unlike in (A).

8.3.3. Use of 96-well microtitration plates Vs *microtubes*

Sucrose stabilates titrated with the refined protocol had a significantly higher infectivity compared to those in the original protocol (P=0.01). Interaction between dose and period of shaking was significant (P=0.03).

The effect of change in the period of shaking on the glycerol stabilates was non-significant (P=0.1) and neither was the interaction between shaking and the dose (P=0.2). Titration curves of glycerol and sucrose protected stabilates using the refined protocol compared to the original are illustrated in Figure 23.

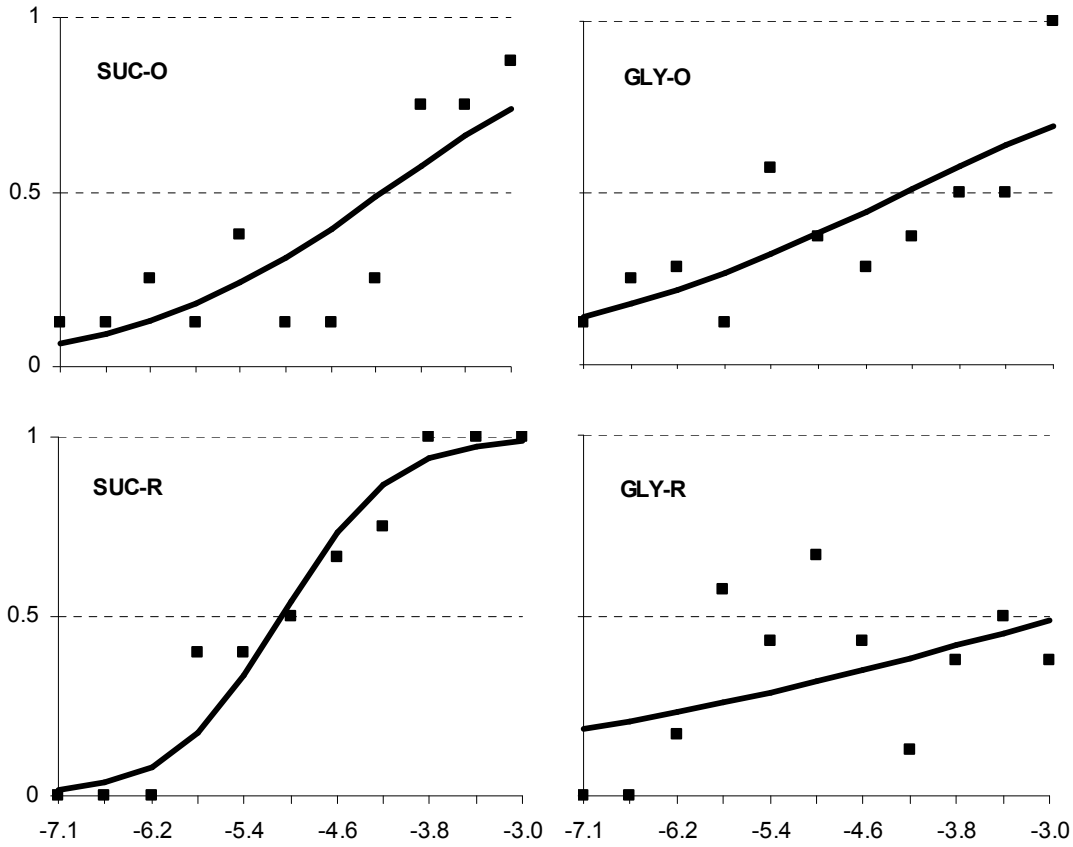

Figure 23: *In vitro* titration curves of (SUC-O) sucrose and (GLY-O) glycerol *Theileria parva* stabilates titrated using the original protocol and (SUC-R) sucrose and (GLY-R) glycerol stabilates with the refined protocol.

8.3.4. Use of PCR for reading titration results

Cohen's kappa test agreement for the two reading techniques was 0.76 and the McNemar's X^2 was 3.6 (Table 13).

Table 13. Comparison of PCR and smear reading for *in vitro* titrations.

	Smear	PCR		Total	Cohen's kappa
		+	–		
Sucrose	+	14	0	14	1
	–	0	10	10	
Trehalose	+	17	5	22	0.42
	–	0	2	2	
Glycerol	+	9	3	12	0.66
	–	2	18	20	
Global	+	40	8	48	**0.76**
	–	2	30	32	

Cohen's kappa	
<0.2	*slight*
0.2-0.4	*fair*
0.4-0.6	*moderate*
0.6-0.8	*substantial*
>0.8	*almost perfect*

8.3.5. Sporozoite Neutralisation Assay

No significant neutralisation relative to the control serum (FCS) was detected for all six test sera (P>0.05). The positive control serum (p67) was 100% inhibitory (P<0.001). Results are summarized in Table 14.

Table 14. Percent neutralisation of sporozoites with 95% confidence intervals. Post = post inoculation, pre = pre inoculation, live = live sporozoite, dead = dead sporozoite.

serum	estimate	lower 95% CL	upper 95% CL
Live post	0.22	0.54	-0.33
Live pre	0.04	0.43	-0.62
Dead post	0.26	0.53	-0.17
Dead pre	0.17	0.48	-0.31
Schizont post	0.25	0.52	-0.19
Schizont pre	0.05	0.40	-0.51
Control +	1.00	1.00	0.99

8.3.6. Serology

Serum from animals 6148 and 131 were positive at 1/500 at week 4 and week 5, respectively on SELISA. Only the animal inoculated with live sporozoites was positive on p67 ELISA at 1/125. The two animals inoculated with live sporozoites and schizonts, respectively were positive on PIM (Table 15).

Table 15: Antibody detection of post inoculation sera as detected by the three assays.

ANTIGEN	SELISA	P67	PIM
Live sporozoites	+	+	+
Dead sporozoites	-	-	-
Schizonts	+	-	+

8.4. Discussion

Storage of PBMC at 4°C seems to preserve cell vitality better than at 37°C. The comparison of cell samples kept at 4°C and 37°C showed that the refrigerated sample had a higher population of live cells. Hunt and her colleagues (2005) using rodent and human cells have shown that "pausing" of mammalian cells by storing at 4°C maintains functionality up to nine days. Our results are in agreement and indications are that, in the short term, refrigeration preserves cell vitality better than an overnight incubation at 37°C (5% CO_2). The lower staining of the 37°C sample could be due to CFSE leakage from a proportion of cells which may indicate cell deterioration because the stain does not leak, in appreciable amounts, from healthy cells (Vander Top *et al.*, 2006). Alternatively, the diluted stain in the 37°C sample could be due to a higher number of daughter cells. A shift towards higher granularity was evident in the cells kept at 37°C. Cells undergoing apoptosis become more granular because nuclear chromatin is condensed, the organelles are compacted and the cell shrinks (Ormerod, 1994). CFSE staining was preferred to labelling with monoclonal antibodies that discriminate PBMC subpopulations because the focus was on the effect of storage conditions on the whole system and not necessarily on a particular cell phenotype.

The exclusion of Con-A activation of cells shortened the procedure by 48 hours in addition to making slides easier to read. The density of cells when activated had made it difficult to spot the infected ones.

Replacing microtubes with microtitration plates makes the test more user friendly as it cuts out use of thermoshakers dilutions in separate sets of tubes and laborious transfer from these to plates for final. Shaking the plates for 1 minute outside the incubator appears to result in a higher proportion of positive wells for sucrose protected stabilates relative to glycerol stabilates. This could be due to conditions such as better pH maintenance because the plates are placed at 37°C in a CO_2 incubator for primary incubation. However, with the 1 min shaking, it was observed for the glycerol stabilates that some wells with higher sporozoite dose gave false negatives. The cause for these false negatives is not known but it was speculated that some factor in the glycerol stabilate negatively

interacted with target cells. The effect of this factor would be reduced as a consequence of dilution or prolonged shaking (as see in the wells with more diluted stabilate or the original protocol).

Sporozoite neutralising antibodies were not detectable in significant amounts from animals inoculated with live sporozoites, dead sporozoites and schizont infected lymphoblasts. This was despite having positive results on SELISA and PIM-ELISA for live sporozoite and schizont immune sera. Although on significance testing, sera were no different from negative control, quantitatively, the ratio estimates show an increasing order of neutralisation with all pre-inoculation sera being lower than post-inoculation sera. This may be an indication of inhibitory potential of post- over pre-inoculation sera or simply an artefact. The failure to detect any inhibition by serum from live-sporozoite inoculation may be due to lack of multiple boosters. It has been shown in related work that before detectable amounts of antibodies are produced, repeated challenge is required (Musoke *et al.*, 1982; Musoke *et al.*, 1984).

Despite several challenges with autologous schizont-infected cells and positivity on SELISA and PIM-ELISA, there was no detectable sporozoite inhibition with serum from this animal. It may be presumed that anti-PIM antibodies do not play a significant role in blocking sporozoites *in vitro* indicating that SNA is rather specific for anti-p67 immunoglobulins. The finding agrees with the observation that p67 expression is low in the schizont stage (Honda *et al.*, 1998).

For statistical analysis, reading proportions of infected wells instead of cells presents a known distribution for statistical analysis and is practically much easier. It is more objective because a well is either positive or negative. The dynamics of cell proliferation are subject to a lot of variation, including number of originally infected cells, rate of starvation as media nutriments are exhausted, suffocation due to increased acidity of the medium and manipulation during medium replenishment. For these reasons, counting of individual cells may be prone to a higher variation.

A drawback with this protocol is fixed sera dilutions. Low-titre sera may be over diluted and consequently be undetectable making the assay less sensitive. However, incorporating serial sera dilutions at the same time as stabilate dilutions complicates the procedure and reduces the number of replicates in a given microtitration plate.

As *in vitro* systems are developed to replace *in vivo* quantitation of *T. parva* sporozoites, it will be necessary to make them as simple as possible with results that are more precise. This objective is enhanced in this work by cutting down on time between infection and reading of results, use of 96-well plates in place of microtubes and by scoring wells instead of cell counting which is laborious. The ease of the tests allow for more repetitions to generate more data that in turn reduce confidence

intervals. Results can be presented more clearly and precisely because the analysis includes confidence intervals of estimates

Chapter 9 - General Discussion

"Even those who fancy themselves the most progressive will fight against other kinds of progress, for each of us is convinced that our way is the best way." — Louis L'Amour

9.1. Overview

This chapter presents the major findings of the study and discusses their respective implications for improving the methods of quantitating *Theileria parva* stabilates for East Coast fever immunisation and Sporozoite Neutralisation Assays. It also offers recommendations for further investigation in certain aspects of the present study that raise more questions than answers.

9.2. Quantitation of *T. parva* sporozoites in East Coast fever vaccine stabilates

The *in vitro* method of quantitating sporozoites has potential to offer a cheaper, easier and more ethical solution for quality control of vaccine stabilates. The present work displays this potential and goes further to quantitatively assess the impact of various processes on vaccine quality using a well defined statistical approach.

The original *in vitro* titration protocol has been improved in the following ways: Exclusion of the PBMC activation step not only reduces the time of the assay by 48 hours but also improves the presentation of the smears making reading easier. "In-plate" manipulations e.g. dilutions and incubation, remove the cumbersome use of microtubes and thermo-shakers. However, it was observed that for glycerol stabilates, the higher doses gave negative cultures with the refined protocol. This phenomenon could not be fully explained though it was speculated that some factors in the stabilate interacted with the target cells at these concentrations, which could have been moderated by the one hour shaking in the original protocol. This artifact was unique to glycerol stabilates as it was not observed with sucrose and trehalose protected stabilates. It would be interesting to investigate this phenomenon further in view of improving the protocol for glycerol stabilates.

Low temperature storage of PBMC overnight seems to maintain viability of cells better than incubation at 37°C. This exposes more live lymphocytes to sporozoites in the *in vitro* infection model raising the overall sensitivity of the system. Weak stabilates in particular require a "sensitive" system. The down side of lowering the threshold is that highly potent stabilates may

yield all-positive results across dilution ranges making logistic regression analyses complicated. However this would be limited only to situations where different stabilates are titrated in parallel. The use of rows instead of columns for stabilate dilutions series in microtitration plates presents more columns for test replications per microtitration plate. The overall range of stabilate dilutions was still preserved by increasing the dilution factor to two-fold instead of 1.5-fold.

The statistical analyses of data were made more robust by taking random factors and any interactions between variables into consideration. Multilevel logistic regression was introduced whenever hierarchical data were encountered as presented in chapter 4. Most commonly, "stabilate" and "session" of the experiment were used as random factors. GLLAMM® (Rabe-Hesketh *et al.*, 2002) was run in Stata® (StataCorp, *Stata Statistical Software: Release 9.* College Station, TX: StataCorp LP) for this procedure as it takes care of several levels of random factors (latent variables) which in this case mainly arise from experiment repetitions. Another source of random variation is the "tick batch" in comparison of stabilates derived from different batches of ticks. Repetitions of experiments narrows confidence intervals but concurrently adds the effect of a random variable of the "session" because of a clustering effect described by Marcotty *et al*. (2004). Such variables have potential of not only statistically interacting with the variables of interest but might also result in inference of statistical significance where none exist (Speybroeck *et al.*, 2003). Comparison of stabilates or effects on stabilates is made easy through calculating ratios of ED's. This approach approximates equivalence testing where the null hypothesis assumes a difference between the groups being compared. In addition, a confidence interval is fitted by a process called non-linear combination of estimators (NLCOM) in Stata (StataCorp, 2005). Therefore, factors by which stabilate infectivities differ can be obtained thereby going beyond simply stating significant differences.

Caution must be exercised in interpreting *in vitro* results as these conditions do not mimic exactly those encountered by sporozoites *in vivo*. It is not inconceivable, for instance, that weakened sporozoites may be rendered impotent by non-specific immune responses *in vivo* whereas *in vitro*, they may still be infective for PBMC due to a less hostile environment. This would raise the sensitivity of the *in vitro* assay in relation to *in vivo* titrations. Judicious interpretation of results is therefore required when attempting to infer relationships between the two systems. However, within the confines of *in vitro* quantitations, effects on sporozoite survival can be accurately compared within and across different stabilates in a standardized way and precludes the wide variations observed in *in vivo* titrations (Cunningham *et al.*, 1974).

There is presently no comprehensive study undertaken to link *in vitro* to *in vivo* titration results. The attempt at this feat and possible reasons for discrepant results are given in chapter 6. Possibly, a cascade of random errors arose from storage of stabilate on ice, comparison of "infectivity" *in vitro* to "protected proportion" *in vivo* instead of more direct "infectivity parameters" (e.g. parasitemia) and relatively small *in vivo* sample size. It is recommended that a more comprehensive study in this regard be undertaken excluding the preceding sources of error.

9.3. Vaccine production processes

The comparison between manual and homogenizer methods of *T. parva* sporozoite extraction from the tick revealed that the two methods yield similar quantities of viable sporozoites. This information enables the vaccine producer to exercise freedom in the choice of technique depending on stabilate volumes, convenience and costs. The ease of standardisation is another factor that could be used in such decisions. For the homogenisation method, it is critical to use a large and a smaller head in sequence for efficient extraction.

Studies on alternative media show that RPMI can be used where MEM is not available. Although there were no statistically significant differences between the performance of both PBC and FCS and the control (MEM), the lower 95% confidence limits were very low. From this observation, their use in stabilate production can not be recommended until more studies are done towards a better confidence. Refreezing *T. parva* stabilates as practiced in polyvalent vaccine production needs to be exercised with caution. The estimated loss of infectivity derived from this present study needs to be considered for efficacious vaccine production. On the other hand, certain experimental designs could benefit from a thaw-pool-refreezing cycle of stabilate vials. It would then be considered that such refrozen stabilates have reduced variability due to the original storage vials.

The rates at which stabilates are cooled directly affect viability of micro-organisms for reasons more fully described elsewhere (Diller *et al.*, 1972; Mazur, 1984; Nowshari and Brem, 2001). Each organism has specific requirements in this regard. Viable sporozoite recovery rates obtained in this work show that the two-step slow freezing protocol presently used in the field (OIE, 2005) is as efficient as programmed freezing. Snap-freezing is quite deleterious in the present formulation of the suspending media. From the relative quantitation results, indications are that increased concentrations of sucrose and trehalose and possibly their combinations would be better cryopreservatives than glycerol for this cooling procedure. Further experimentation with higher concentrations is strongly recommended to approximate vitrification which is a simpler freezing

procedure than two step slow freezing and has potential for even higher yields of viable sporozoites at resuscitation.

9.4. Storage conditions

The rate of stabilate infectivity loss upon storage on ice is minimal. This may allow delivery on this medium during immunisations. The loss rates were similar for glycerol and sucrose protected stabilates. In addition, we have shown that loss dynamics for Katete are similar to those of the Chitongo stock and can therefore speculate that ice storage can work for the southern province of Zambia where Chitongo is used. Caution should however be taken to include an *in vivo* assessment before implementation of the technique. Our attempt to store stabilates at -20°C resulted in high sporozoite infectivity loss. This method of short term storage therefore seems not to have a practical field application.

Some disparities between glycerol and sucrose stabilates extracted from single tick batches were observed. Sucrose seemed to give consistently higher infectivities compared to glycerol, except in the case of the storage at -20°C where sucrose stabilates deteriorated faster than glycerol stabilates. Though there is strong evidence that sucrose stabilates had relatively higher infectivity, this may be a result of an interaction between the cryopreservatives and the target cells. It was further observed that some wells of higher concentration of glycerol stabilates gave false negatives (chapter 8). For these reasons, it is recommended that the comparative effects of these cryopreservatives on PBMC be assessed *in vitro*. Sucrose stabilates are at least as infective as glycerol stabilates in vitro and should therefore be assessed *in vivo* with the view of replacing glycerol which requires drop-wise addition during vaccine production.

Resuscitation of lyophilised *T. parva* sporozoites was not achieved in this work. Although survival of lyophilisation is commonly limited to prokaryotic cells, this was once demonstrated for *T. parva* (Marcotty *et al.*, 2003). Since liquid nitrogen storage is very expensive and the I & T method is the only practical technique for immunising cattle at present, efforts at identifying cheaper storage methods should be continued. The various attempts made are a basis for trying other protocols and lyoprotectants. In this light, attempts at lyophilisation using cocktails of various lyoprotectants should be made, as long as the reagents do not make the vaccine too expensive for cattle keepers.

9.5. Sporozoite Neutralisation Assays

The technique described in this work is appropriate for screening sera for sporozoite neutralising antibodies for two reasons. Firstly, it seems not to pick up non-specific antibodies except anti-p67. This is illustrated by the positive PIM-ELISA and SELISA on some samples that were negative on Sporozoite Neutralisation Assay. Secondly, it simulates *in vivo* conditions more closely by introducing sporozoites into a mixture of antibody and target cells instead of pre-incubating sporozoites with antibody first. Further, the results confirm that unless an animal is repeatedly challenged, the humoral response with respect to p67 is undetectable and that PIM antibodies do not play a major role in *in vitro* neutralisation of sporozoites. It is also shown that expression of the p67 in the schizont stage is very low as the assay was unable to pick it up despite repeated challenge of the experimental animal with massive numbers of infected lymphoblasts. This low expression has been observed by other workers (Musoke, personal communication 2006). Adjuvanting the dead sporozoites could have probably elicited a higher antibody titre. However, since efficacy of vaccination regimens was not the object of the study, this was not tried.

This refined assay may also be used to assess effects of agents directed against the schizont stage e.g. chemotherapeutics. However, caution should be exercised as the effects may not be directed at the parasite *per se* but at the target host cell. Pre-evaluation of effect on target cells may therefore be necessary. The assay may equally be employed in elucidating the possible interference of maternal antibodies in calf immunisation (Marcotty *et al.*, 2002). If neutralisation of sporozoites by sera from calves can be demonstrated, implications are that calves suckled during periods of heavy ECF challenge may not be immunised as the sporozoite numbers presented to lymphocytes may be reduced severely.

9.6. Conclusions and recommendations

The *in vitro* titration has been optimised it takes 48 hours less and excludes difficulties associated with use of microtubes and shaker placed inside CO_2 incubators. Reading of resultant cultures can be done by PCR further cutting down on time spent processing cyto-centrifuge smears.

Stabilates extracted by homogenisers are not inferior to manually extracted ones. It is recommended that, for bulk stabilates, homogenisers are used whereas manual extraction can be employed for smaller batches.

RPMI is at least as good as MEM in supporting sporozoite infectivity in stabilates. As such it can be used in vaccine production centres where importation of MEM is restrictive.

Sucrose has potential to be a more user friendly cryopreservative. It exhibits comparatively better infectivities *in vitro* and should be investigated further possibly by directly determining amounts of live sporozoites on recovery from cryopreservation using reverse transcription-polymerase chain reaction or by *in vivo* comparison.

Refrozen stabilates lose 35% of initial infectivity. It is recommended that in procedures were refreezing is necessary e.g. polyvalent vaccine production, this factor be considered in determining immunising doses. Attempts of stabilate refreezing in field immunisation should not be made as this would result in poor quality vaccine.

Snap freezing in present stabilate formulation and packaging is not a feasible alternative to slow freezing. However, results indicate potential to improve the technique and it is therefore recommended that further studies be undertaken. Snap freezing or indeed vitrification would reduce on equipment needed during stabilate production further simplifying the protocol.

Short term storage of stabilates on ice does not result in significant infectivity loss rates. This is true irrespective of parasite stock (Chitongo or Katete) or cryopreservative (glycerol or sucrose). With this observation, it is recommended to deliver the Chitongo stabilate on ice during immunisation in the Southern Province of Zambia after an *in vivo* test. In contrast, trials to store in a domestic freezer failed to yield sufficient sporozoite viability upon thawing. This method is not recommended.

Attempts to repeat a documented occasion of *T. parva* lyophilisation failed. It may be worthwhile to test other lyoprotectants and combinations of such agents.

The Sporozoite Neutralisation Assay was refined and analysis of data made more precise by use of ED ratios with respective confidence intervals. The use of binary observations makes it easier to interpret than counting proportion of infected lymphoblasts as practised before. PCR reading of microplates further simplifies the procedure. It was observed that this assay is quite specific for anti-p67 antibodies.

The challenge of relating *in vitro* results to *in vivo* implications remains. Not until we can predict the proportions of protected animals from *in vitro* results can there be any meaningful use for *in vitro* titrations as regards determination of ECF immunising doses. This calls for a concerted research effort from all institutions, both private and public, that are involved in the control of this

deadly livestock disease because a breakthrough would benefit p76 sub-unit vaccine research and the tried and tested I & T method of immunisation.

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APPENDICES

APPENDIX I

In vitro titration of *Theileria parva* sporozoite stabilates

A. ORIGINAL PROTOCOL

Day -3

Peripheral Blood Mononuclear Cells isolation

- Fill aseptically two 20 ml syringes with 12 ml Alsever's solution
- Take jugular blood from donor in the Alsever-half-filled syringes to fill completely the syringes (~ 25 ml * 2)
- Keep the syringes at 37°C during transportation to the lab

- Use 50 ml Falcon tubes for the separation of PBMC's from blood by a density gradient
- Take 17 ml of Nycoprep (1.077 Animal®)
- Add very gently 17 ml of Alsever-blood on top of Nycoprep
- Centrifuge at 900 *g* for 15 min (no acceleration, no deceleration)
- Take PBMC off from the Nycoprep surface with an auto-pipette at slow speed (+/- 10 ml)
- Transfer PBMC into other 50 ml Falcon tubes
- Fill up Falcon with wash medium - RPMI 1640+Gentamycin (50mg/ml)
- Centrifuge at 800 *g* for 10 minutes
- Decant supernatant
- Resuspend cells in 50 ml wash medium
- Centrifuge at 800 *g* for 10 min
- Decant supernatant
- Resuspend cells in 10 ml culture medium (**RPMI** + **FCS** (15%) + **Gentamycine** (50mg/ml) + **L-glutamine** (200mM) + **2-mercaptoethanol** (0.5 M)
-

PBMC counting:

- Dilute cell suspension 1/100 in Türck's solution (i.e. 0,1 ml in 0,9 ml 2 times)
- Transfer to Neubauer's counting chamber - 0,100 mm * 0.0025 mm^2
- 1 counting area = 25 large squares = 400 small squares = 0.1 mm^3 = 0.1 µl.

  - Count in 10 counting areas
  - Number of PBMC/ml = average counted in counting areas * 10^6
- Add medium to obtain 3 *10^6 PBMC/ml
- Transfer 12 ml PBMC in 50 ml culture bottles (1 bottle will be for 1 plate)
- Keep the PBMC at 37°C in an incubator (5% CO_2 in air), the bottle lying on its large surface, with the stopper loose

Day -2

Stimulation with Concanavalin A

- Resuspend PBMC by shaking the bottle and leave the bottle standing up for a few hours
- Adjust the PBMC concentration (to 6 *10^6 PBMC/ml) by taking 6 ml of the supernatant off
- Add Con-A at 2.5 µg/ml of cell suspension
- Keep the PBMC at 37°C in a CO_2 incubator (5% in air), the bottle lying on its large surface, with the stopper loose

Day -1

Preparation of media and Vortex

- Prepare flucytosine media, transfer to CO_2 incubator
- Thaw stabilate diluent by storing in a CO_2 incubator until the following day
- Warm up vortex in a dry 37°C incubator to avoid condensation when transferring to CO_2 incubator

Day 0

***In vitro* titration**

(an example of comparing a glycerol with a sucrose stabilate is given here)

- Quantity of stabilates: 3 ml of dil 1 of each stabilate
- KA0507g 1/10 (7.5% glycerol)
- KA0507s 1/10 (0.3M sucrose)
- Thaw stabilates in a water bath (37°C) for 5 min (record time)
- Transfer stabilates in a 50 ml falcon tube
- Centrifuge at 400 g for 10 minutes
- Recover supernatants using pastets (=dil 1)

- Prepare 4 x 12 = 48 eppendorf tubes (4 rows x 12 columns)
- Fill columns 2-12 of two rows with 150 µl of stabilate diluent -7.5% glycerol
- Fill columns 2-12 of next two rows with 150 µl of stabilate diluent – 0.3M sucrose
- Fill first column with 450 µl of respective stabilate (dil 1)

- Make 1.5x serial dilutions of stabilate by transferring 300 µl from column to column
- Add 150 µl PBMC in each tube
- Let incubate the tubes for 1 h in thermoshakers (37°C, 1000 rpm) (record time)

- Centrifuge the tubes at 210 g for 10 min
- Decant supernatant
- Resuspend the cells in 0.6 ml (per tube) culture medium
- Transfer 0.2 ml from each tube in 2 wells of a 96-well plate

- Label the plates
- Transfer to CO_2 incubator (37°C) - (record time)
- Leave undisturbed for 10 days

Day 10

- Destroy fungi contaminated cells with 10N NaOH.
- Check cell cultures for clumps
- Take cytospins: mount slides with a Filter Card “Shandon”, centrifuge 50 to 100 µl at 215 *g* for 5 minutes.
- Stain with May-Grunwald / Giemsa’s stain
- Score cultures for the presence or the absence of schizonts

B. REFINED PROTOCOL

Day -1

Peripheral Blood Mononuclear Cells isolation

The same as in Appendix I.A

PBMC counting:

The same as in Appendix I.A

After counting,

- Add medium to obtain $6*10^6$ PBMC/ml
- Transfer 12 ml PBMC in 50 ml culture bottles (1 bottle will be for 1 plate)
- Keep the PBMC overnight in a refrigerator, the bottles lying on their large surface.

Day 0

***In vitro* titration**

(An example comparing glycerol and sucrose stabilates is given here)

- Fill columns 2-12 top half of the plate, with 50 µl of stabilate diluent (7.5% glycerol)
- Fill columns 2-12 bottom half of the plate, with 50 µl of stabilate diluent (0.3M sucrose)
- Fill first column with 150 µl of respective stabilate (dil 1) (4 replicates per stabilate)
- Make 1.5x serial dilutions of stabilate by transferring 100 µl from column to column (using a multichannel pipette).
- Pipette-mix about 7 times at each dilution step including dil 1.
- Discard 100µl from the last column

- Add 50 µl PBL in each well using a multichannel pipette.
- Pipette-mix about 3 times (Use a fresh set of tips for each column)
- Incubate the plate for 30 min at 37°C in CO_2 incubator (record time)
- Shake plate for 1 min using a Titertek® shaker (Flow Laboratories) at 10 Hz and return to incubator for another 20 min

- Centrifuge the microplate at 210 *g* for 10 min
- Decant supernatant

- Add 150 µl medium in each well using a multichannel pipette (Use a fresh set of tips for each column). Pipette-mix about 3 times at each step.

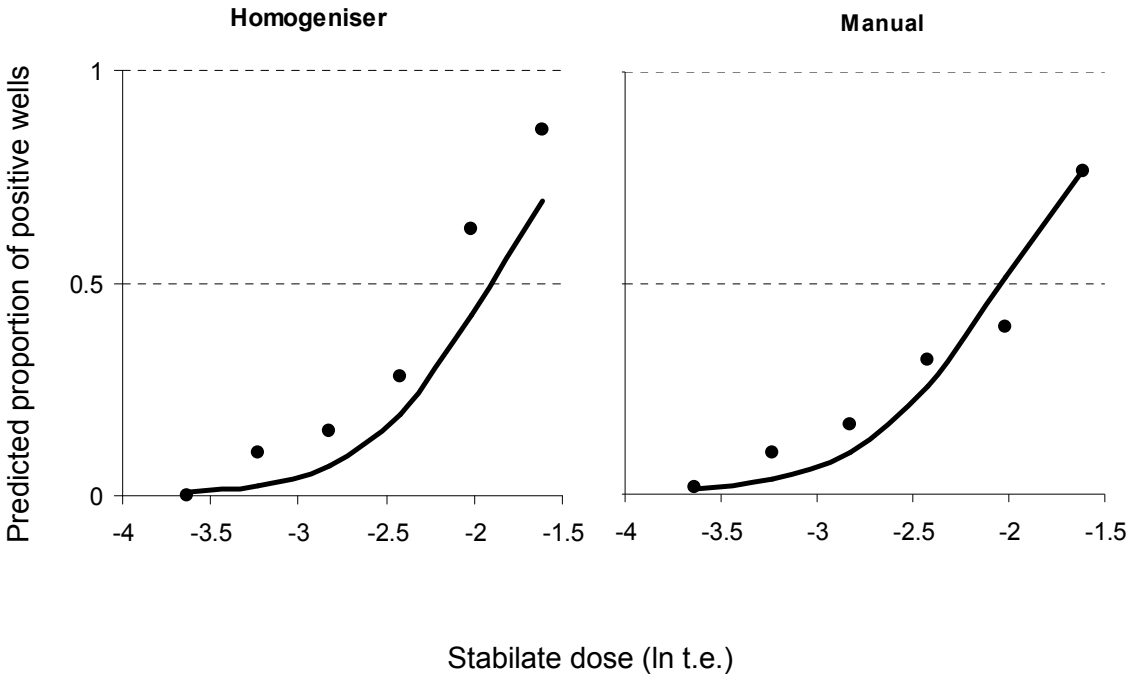
- Label the plate
- Transfer to CO_2 incubator at 37°C (record time)
- Leave undisturbed for 10 days

Day 10

- Destroy fungi contaminated cells with 10N NaOH.
- Check cell cultures for clumps
- Make cyto-centrifuge smears: mount slides with a filter card, centrifuge 50 to 100 µl at 215 *g* for 5 min
- Stain with 12.5% Giemsa's stain in Sörensen's phosphate buffer (0.008 M, pH 7.2)
- Score cultures for the presence or the absence of schizonts

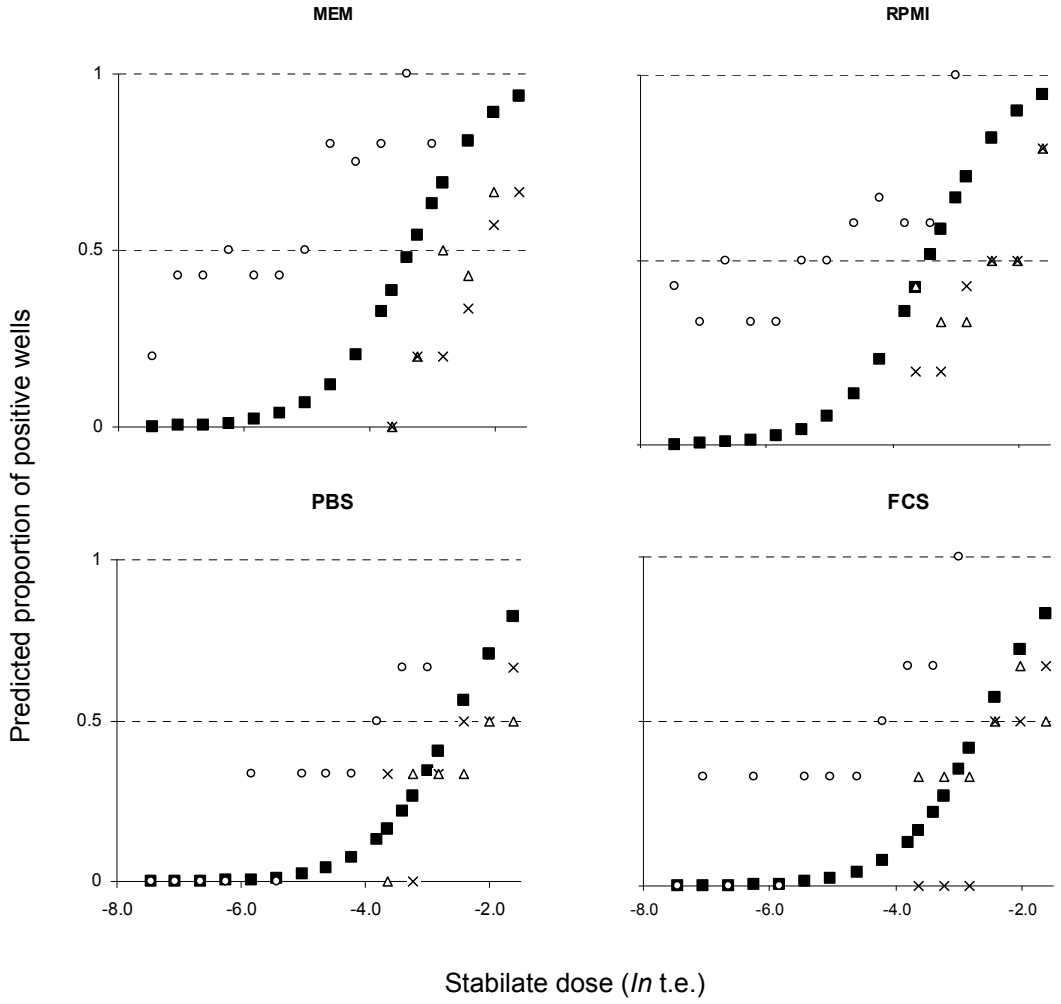
APPENDIX II

Titration curves of *Theileria parva* stabilates extracted by homogeniser and manually (as depicted in Figure 7 of chapter 3)



APPENDIX III

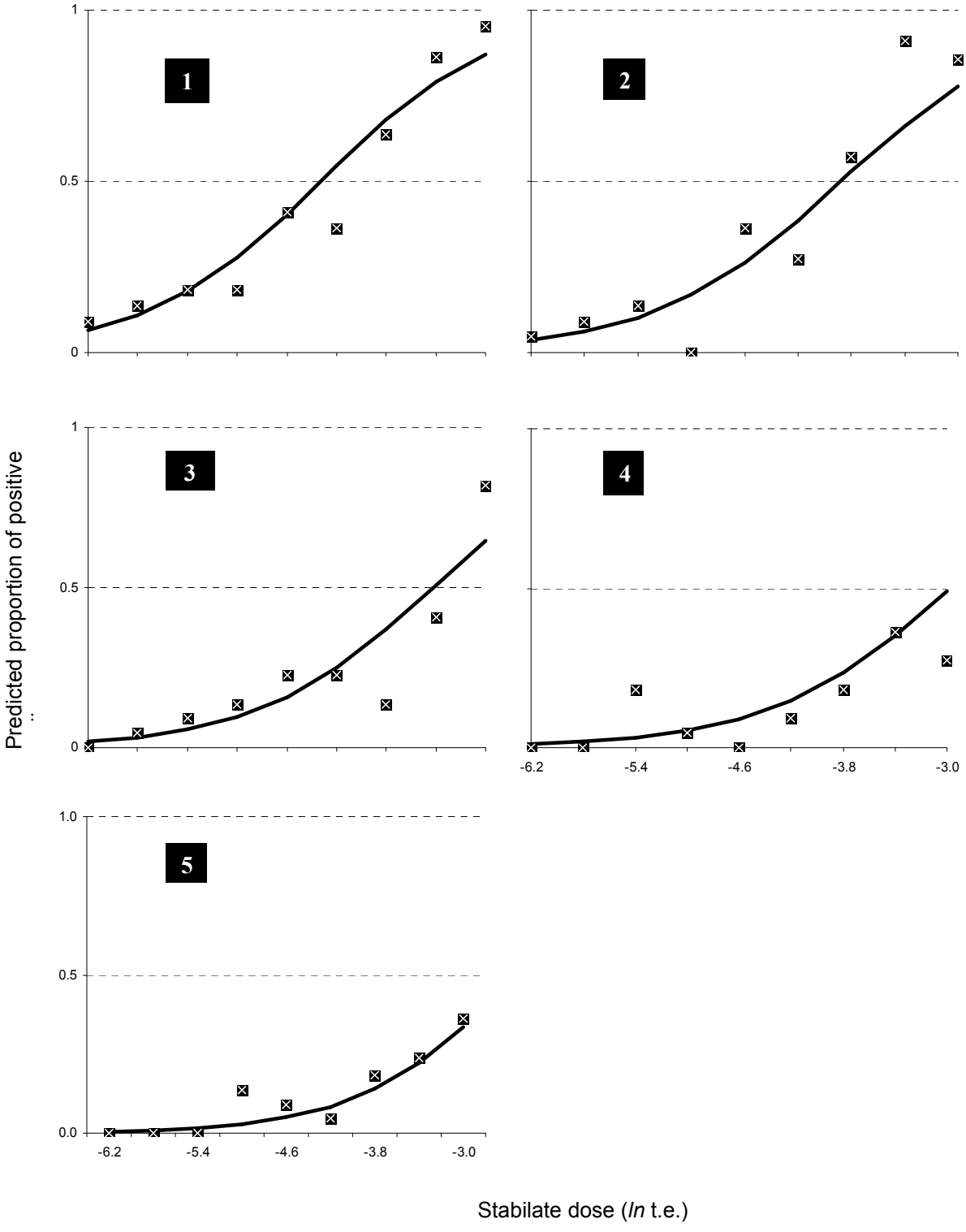
Titration curves for *Theileria parva* sporozoites suspended in four media: MEM, RPMI, PBS and FCS (as depicted in Figure 8 of chapter 4).



■-Predicted values, ○- Batch 2, ×-Batch 1(a) and ∆-Batch 1(b)

APPENDIX IV

Titration curves of multiply refrozen *Theileria parva* stabilates (Cycles 1 to 5) as depicted in Figure 10 of chapter 4.



APPENDIX V

CFSE staining

- Isolate PBMC and count (Appendix I)
- Spin down at 10,000 rpm for 5 minutes
- Resuspend in 1 ml ice-cold PBS and transfer to eppendorf tube
- Add CFSE at 0.56µl/1x10^7 PBMC to the suspension.
- Vortex gently
- Incubate at 37°C in water bath for10 min in the dark
- Centrifuge at 4°C (10,000 rpm for 5 min)
- Resuspend in cold RPMI (15% FCS)
- Wash 3 times to remove excess stain and any DMSO (10,000 rpm for 5 min)
- Resuspend in culture medium if required

APPENDIX VI

PCR reading of *in vitro* titration plates

- Prepare the titration plates and incubate for 10 days as described in Appendix I (B)
- Starting with 50 µl of cell culture material in each well, add 50 µl of 0.7M ammonium hydroxide per well
- Suspend plates over boiling water with covers off for 20 min
- Leave at room temperature for about 20 min to vaporise the Sodium hydroxide
- Store in refrigerator overnight
- Dilute extract 100 times (10 times twice) within 96-well plate (10µl extract + 90µl milli-Q® water) with 10 minutes of shaking (ELISA shaker) at each step.
- Take 10 µl from each well and add to 40 µl of Master mix
- Do PCR with *T. parva* Cox-primers (Cox F/R, 40 cycles)

APPENDIX VII

Sporozoite Neutralization Assay protocol

Day -1: PBMC isolation

- Prepare PBMC at $6*10^6$/ml as describes in Appendix I
- Aliquot in 2 flat-bottomed Nunclon® microtitration plates (Nunclon, Roskilde) (40 µl /well)
- Store at 37°C in a CO_2 incubator
- Store the rest of PBMC in culture bottles in the refrigerator

Day 0: *In vitro* infection

PLATE SETUP (an example comparing pre- and post-immune sera from three animals is given)

- Label the titration plates according to the test sera:
  Plate 1, three rows each for 131 pre, 6146 pre, 6148 pre and FCS.
  Plate 2, three rows each for 6146 post, 6148 post, 131 post and p67.

***IN VITRO* TITRATION**

- Thaw stabilate diluent (15 ml) in a 37°C water bath
- Thaw 100 µl of each test serum
- Transfer 320 µl of refrigerator PBMC to each of eight Eppendorf tubes (labelled for each serum)
- Add 80 µl of test sera to each PBMC tube and suspend in a vortex mixer for a few seconds
- Transfer 10 µl of PBMC/serum mixture to each well of respective rows. Use separate tips for each PBMC/serum mix
- Place plates at 37°C in a CO_2 incubator

- Thaw 5 ml of *T. parva* stabilate in a water bath (37°C) for 5 min (record time)
- Transfer stabilate to a 50 ml Falcon® tube, merging the vials
- Centrifuge at 400 *g* for 10 min
- Recover supernatants in a sterile tube using a pastet (dilution 1)
- Prepare seven dilution tubes with 2 ml of diluent in each
- Make eight serial dilutions of stabilate (2 ml stabilate in 2 ml diluent) (dilutions 2 to 8)

- Transfer 50 µl of stabilate dilution series to respective columns
- Leave plates to incubate for 30 min at 37°C in a CO_2 incubator (record time)
- Shake both plates for 1 min on a Titertek® shaker and return to incubator for a further 20 min

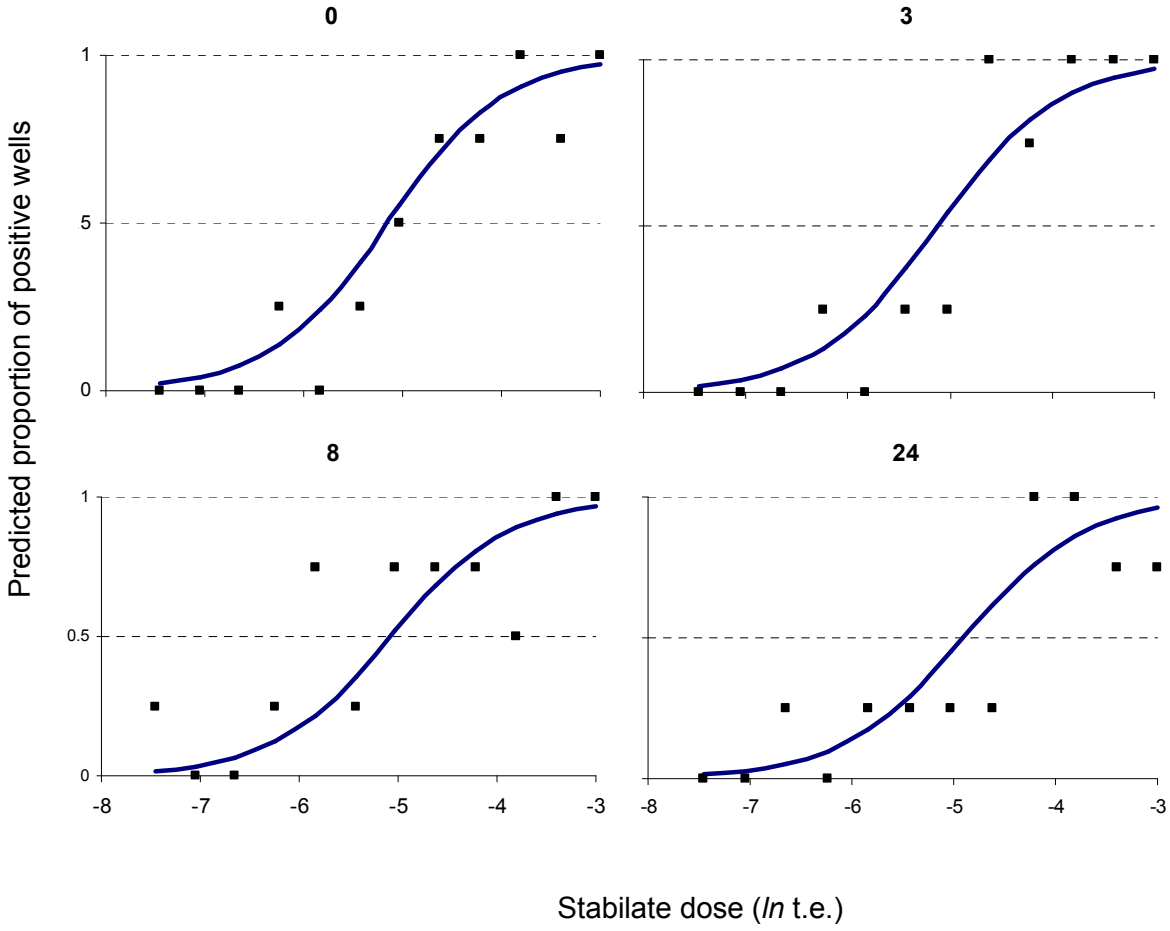
- Centrifuge the plates at 210 *g* for 10 min
- Decant supernatant
- Add 150 µl culture medium in each well using a multichannel pipette
- Transfer to CO_2 incubator (37°C) (record time)
- Leave undisturbed for 10 days
-

Day 10

- Make smears as in Appendix I (B)

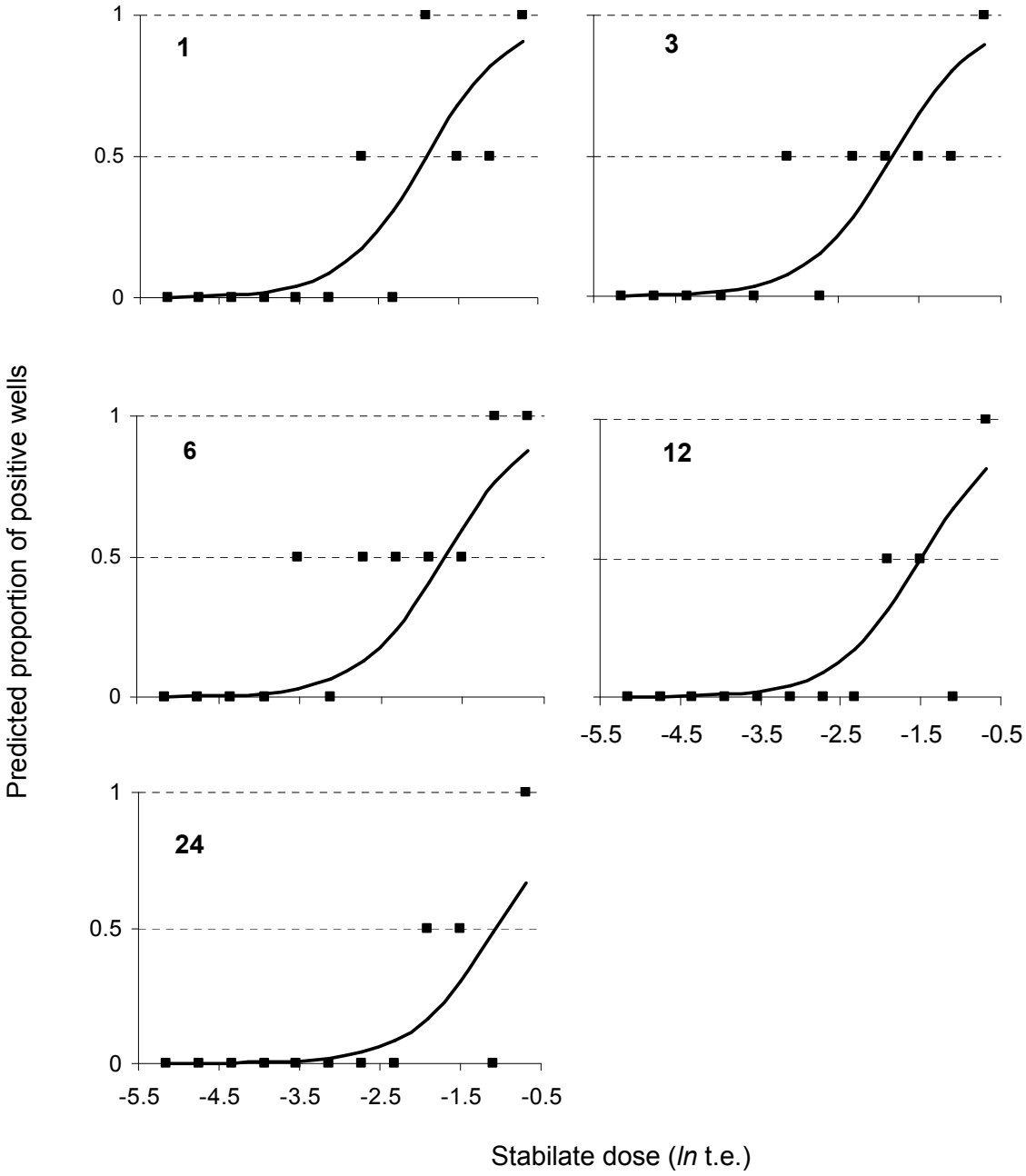
APPENDIX VIII. A

Titration curves of *Theileria parva* Katete (K1g) stabilate kept at 4°C for 0, 3, 8 or 24 hours. (Combined graphs are depicted in Figure 13.A of chapter 6).



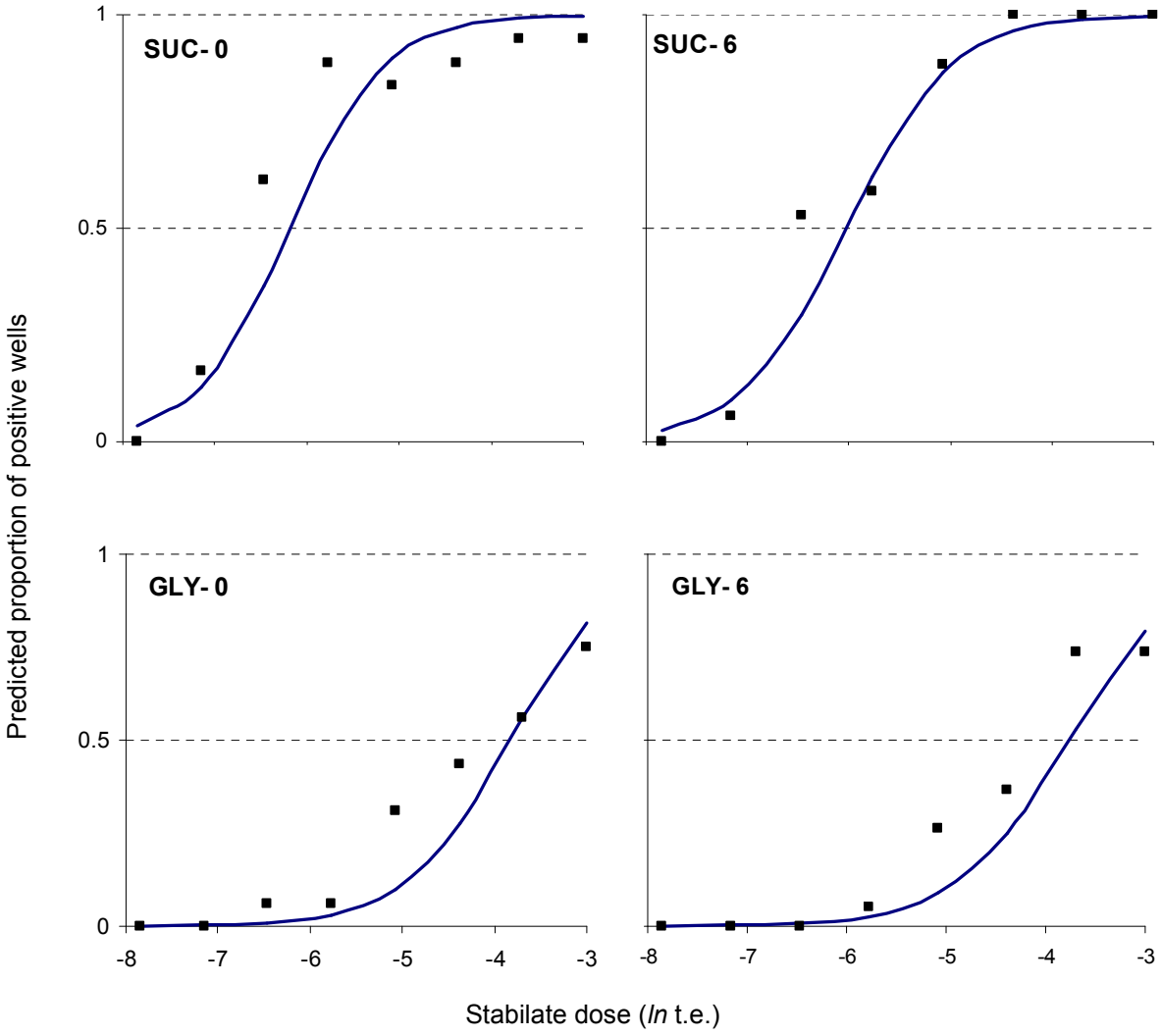
APPENDIX VIII.B

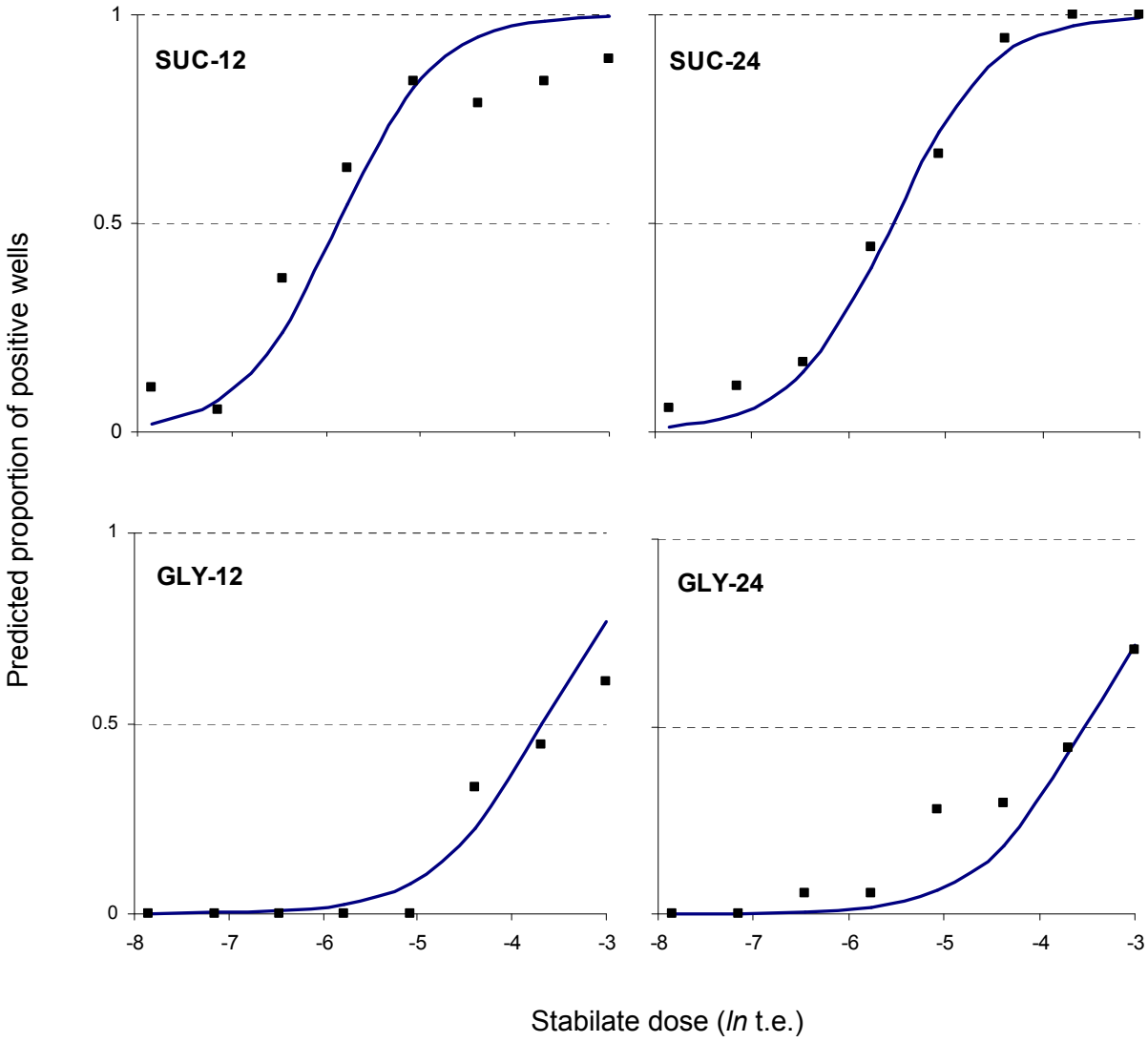
Titration curves of *Theileria parva* Chitongo (C1g) stabilate kept at 4°C for 1, 3, 6 or 12 or 24 hours. Combined graphs are depicted in Figure 13.B.



APPENDIX IX

Titration curves of Katete sucrose (SUC) and glycerol (GLY) *Theileria parva* stabilates (K2g and K2s) kept at 4°C for 0, 6, 12 or 24 hours. (Combined graphs depicted in Figure 14 of chapter 6).




SUC-12
SUC-24
GLY-12
GLY-24
Predicted proportion of positive wells
Stabilate dose (*ln* t.e.)
1
0.5
0
-8
-7
-6
-5
-4
-3

SUMMARY

But beyond this, my son, be warned: the writing of many books is endless...

Ecclesiastes 12:12

The disease, East Coast fever, and its control as it relates to the present work are set out in the General Introduction of this thesis. The introduction outlines briefly the economic implications of the disease, its epidemiology, diagnosis, control and usage of titrated sporozoite stabilates in immunising cattle. The first chapter introduces a brief history of the disease, discusses the classification of the causative agent, *Theileria parva*, its lifecycle within tick and mammalian hosts, disease epidemiology and control methods. Focus is placed on the Infection and Treatment (I & T) method of immunisation. Survival of the parasite in natural and experimental conditions is reviewed. The core of the chapter deals with the specifics of quantitating *T. parva* sporozoite stabilates used for vaccination. Although *in vitro* studies of theileriosis concerning characterization and immunology of the parasites abound, these techniques remain largely under-utilized in stabilate quantitation in preference for *in vivo* methods. Current *in vitro* and *in vivo* methodologies and their respective shortcomings are critically reviewed together with their respective data analysis methods.

The objectives of the thesis are presented in chapter two. The general objective was to develop and use the *in vitro* tool in view of improving vaccine production, storage and field immunisation against East Coast fever. More specifically, work was set out to 1) quantitate infectivity of stabilates stored at various temperatures, 2) compare stabilate quality from two extraction methods, 3) investigate feasibility of snap freezing stabilates, 4) evaluate potentially cheaper and more readily available stabilate media as alternatives to MEM and 5) refine the Sporozoite Neutralization Assay and validate it by assaying immune sera originating from cattle experimentally infected with recombinant p67, schizonts, live or dead sporozoites

In chapter three, a study set up to resolve the question "Are *T. parva* sporozoite stabilates produced by crushing ticks by mortal and pestle of better infectivity than those produced by tissue grinders or homogenizers?" is described. Observations were that manually extracted stabilates gave lower variation between production batches and the machine extracted ones were on average as infective as the manually extracted ones. Therefore, it was recommended that preference for either method should be based on cost, available infrastructure and conformity to standards.

Chapter four is an account of the quest for a cheaper and more readily available stabilate medium. The present combination of MEM/BSA is very expensive and so a few alternatives were investigated. These were: PBS, RPMI 1640 (Life Technologies # 52400-025) and plain FCS. All were supplemented with BSA except FCS. RPMI was not less than 0.63 times as efficient as MEM at 95% confidence level (0.63-1.67). PBS and FCS were 59% and 67% as efficient as MEM, respectively. The chapter also revisits refreezing of stabilates and its effect on stabilate quality. Refreezing is practiced in polyvalent stabilate production but the infectivity loss due to this process has not been quantitated. This study estimated the loss to be 35% of original infectivity. It is proposed to take into account this loss in constituting polyvalent vaccines.

Chapter five presents a preliminary attempt at snap-freezing of stabilates. This ultra-rapid cooling method could preclude the formation of intracellular ice crystals which is the main factor in freeze injury. As such, it would not only improve the sporozoite resuscitation rate but also cut down on the number of equipment used because stabilates would be directly plunged in liquid nitrogen after production. Test stabilates cooled this way were 24% as infective as controls. Although this recovery rate is low, the result shows that the technique is potentially feasible for *T. parva* stabilates. Some recommendations for optimizing the process are given, mainly targeted at adjustments in stabilate formulation and packaging that allows greater exposure of the formulation to the freezing agent (liquid nitrogen).

The sixth chapter documents trials to quantitate stabilate loss of infectivity due to storage in ice baths (4ºC) and in a domestic freezer (-20ºC). Glycerol and sucrose cryopreserved stabilates of two *T. parva* stocks (Katete and Chitongo) were assessed at the two temperatures. Stabilates were stored for up to 24 hours and four weeks at 4ºC and -20ºC, respectively. Storage at 4ºC resulted in infectivity loss for the two parasite stocks of 1 and 4% per hour for Katete and Chitongo, respectively. The hourly loss was not significant (P=0.45). Comparison of the effect of time on cryopreservatives showed that time was not a significant factor for glycerol (P=0.35) but so for sucrose (P=0.04). Freshly thawed stabilates indicated higher infectivity for sucrose, at 10 times that of glycerol protected stabilates (95% CI: 6.2 - 16.7). It was concluded that both Katete and Chitongo stabilates had similar loss rates when kept on ice. Sucrose could be a better cryopreservative as seen from the base infectivities. However, this needs *in vivo* confirmation as effects of glycerol on lymphocytes were not assessed and glycerol stabilates sometimes gave false negative results in the least diluted stabilate series.

For storage at -20ºC, sucrose and glycerol stabilates lost 98% and 61% of initial infectivity respectively, after one week of storage. It was speculated that intracellular water re-crystallisation

might have occurred as stabilates were raised to this temperature resulting in freeze-injury of the sporozoites. Due to the high infectivity loss, this method of storage is not recommended.

Chapter seven is an account of stabilate lyophilisation attempts aimed at repeating and improving a documented protocol. Lyophilisation is potentially a cheaper method than cryopreservation for stabilate storage. Authors of the documented attempt noted that although cattle inoculated with the product developed patent infections, less than 1% of sporozoites had survived the freeze-drying procedure. The rationale was that if it was repeatable, optimization of the procedure would raise the surviving proportion of sporozoites. Katete and Chitongo *T. parva* stabilates cryopreserved with sucrose or trehalose were tested. In addition, whole infected ticks, tick salivary glands and top layers of stabilate debris left after centrifugation at production were lyophilised. No sporozoite viability was evident in the lyophilised materials despite various modifications of the protocol and parasite presentations. Possible sources of failure are discussed.

In chapter eight, refinements to the Sporozoite Neutralization Assay are outlined and the improved protocol is used to assay various sera. Presently, SNA analyses are based on counting infected cells which is laborious and prone to high variability. The idea of scoring wells positive or negative in plates is proposed. It is more user-friendly and presents binary data that are easily analysed by logistic regression. The chapter also argues for the less cumbersome use of microtitration plates instead of microtubes for stabilate *in vitro* titrations. By shaking plates for a minute and keeping the target cells in the refrigerator before infection instead of 37ºC, the system as a whole was still as efficient as the original protocol. In addition, a quicker way of reading the culture plates by PCR was explored. The *in vitro* protocol was shortened by 48 hours by skipping the step of activating Peripheral Blood Mononuclear Cells with Con-A.

Sera collected at weekly intervals from cattle inoculated with schizonts and sporozoites were assayed in parallel with a positive (anti-p67) and negative (FCS) control. The tested sera were also subjected to PIM-ELISA, p67-ELISA and a SELISA using infected lymphoblasts as antigen. No significant sporozoite inhibition was evident from all three sera. Albeit, the assay demonstrated a clear distinction between the controls. Serum from the live sporozoite inoculation was positive on all three serology tests and that from the schizont inoculation was positive both on SELISA and PIM-ELISA. The lack of sporozoite neutralisation with serum from the animal receiving repeated schizont inoculation lends support to the documented observation of low p67 molecule expression in the schizont stage. This result also indicates inefficiency of sporozoite neutralisation by anti-PIM antibodies *in vitro* and that SNA is specific for anti-p67 antibodies.

Chapter nine summarizes and discusses the main results of the thesis with overall conclusions. It offers recommendations for further research in related areas to clarify some of the questions and speculations raised in the present work.

SAMENVATTING

De algemene inleiding situeert de ziekte Oostkustkoorts en haar controle in het kader van dit werk. De economische gevolgen van de ziekte, haar epidemiologie, diagnose en controle, meer bepaald het gebruik van sporozoiet stabilaten bij de immunisatie van runderen, worden uiteengezet. Hoofdstuk een begint met een kort overzicht van de geschiedenis van de ziekte en behandelt de classificatie en levenscyclus van het causale organisme, *Theileria parva*, evenals de epidemiologie en controlemethodes. Bijzondere aandacht wordt besteed aan de 'Infectie & Behandeling' immunisatie methode. Overleving van de parasiet wordt besproken onder natuurlijke en experimentele condities. Tenslotte worden de specifieke aspecten van de kwantificering van *T. parva* sporozoiet stabilaten belicht. Alhoewel *in vitro* methodes algemeen aangewend worden bij immunologische en taxonomische studies op gebied van theileriose, blijven deze technieken grotendeels ongebruikt bij de kwantificering van stabilaten, waar *in vivo* methodes het overwicht behouden. Voor- en nadelen van *in vivo* en *in vitro* technieken worden kritisch geëvalueerd tezamen met hun respectievelijke data analyse methodes.

De doelstellingen van deze thesis worden voorgesteld in hoofdstuk twee. De algemene doelstelling was de ontwikkeling en evaluatie van een *in vitro* hulpmiddel om stabilaat productie en opslag en immunisatie tegen Oostkustkoorts op het terrein te verbeteren. Meer specifiek werd getracht om (1) de besmettelijkheid te bepalen van stabilaten, bewaard bij verschillende temperaturen, (2) de kwaliteit van stabilaten te vergelijken na twee extractiemethodes, (3) de mogelijkheid te onderzoeken om stabilaten te snelvriezen, (4) goedkopere en meer beschikbare stabilaat media te evalueren, als alternatieven voor MEM en (5) de Sporozoiet Neutralisatie Test (SNT) aan te passen en te valideren door middel van immuun sera afkomstig van runderen, experimenteel geïnfecteerd met recombinant p67, schizonten of levende of dode sporozoieten.

In hoofdstuk drie wordt de vraag: "zijn *T. parva* sporozoiet stabilaten bekomen na het verbrijzelen van teken met mortier en stamper beter qua infectie dan stabilaten bekomen na het gebruik van weefselmolens en homogenisering?" beantwoord. De waarnemingen wezen uit dat manueel geëxtraheerde stabilaten een lagere variabiliteit vertoonden, maar dat machinale extractie niet resulteerde in lagere infectiviteit. De keuze van methode moet daarom gebaseerd zijn op kostprijs, aanwezige infrastructuur en conformiteit aan de gangbare standaarden.

Hoofdstuk vier behandelt de pogingen om een goedkoper stabilaat medium te vinden. De momenteel gebruikte combinatie van MEM/BSA is vrij duur en daarom werden enkele goedkopere en algemeen beschikbare alternatieven uitgetest: PBS, RPMI 1640 (Life Technologie # 52400-025) en zuiver FCS, allen behalve FCS aangevuld met BSA. RPMI was niet minder dan 0,63 maal zo efficiënt als MEM aan een 95% betrouwbaarheidsinterval (95% CI: 0,63 – 1,67). PBS en FCS

vertoonden respectievelijk 59% en 67% van de efficiëntie van MEM. Dit hoofdstuk bekijkt tevens opnieuw het herhaaldelijk invriezen van stabilaten en het effect hiervan op de stabilaat kwaliteit. Herinvriezen wordt aangewend bij de productie van polyvalente stabilaten, maar het effect ervan was nog niet nagegaan. Herinvriezen verminderde de oorspronkelijke infectiviteit met 35%. Voorgesteld wordt om dit verlies expliciet in rekening te brengen bij de productie van polyvalente stabilaten en bij het gebruik van stabilaten die accidenteel ontdooid en heringevroren werden.

Hoofdstuk vijf rapporteert een eerste poging om stabilaten te snelvriezen. Deze ultrasnelle manier van afkoelen kan mogelijk de vorming voorkomen van intracellulaire ijskristallen, de hoofdoorzaak van vriesschade bij stabilaat productie. Deze methode zou niet alleen sporozoiet reanimatie vergemakkelijken maar tevens de vereiste uitrusting vereenvoudigen aangezien stabilaten onmiddellijk in vloeibare stikstof zouden kunnen ondergedompeld worden. De infectiviteit van stabilaten, op deze manier afgekoeld, was 24% van stabilaten afgekoeld op de traditionele manier. Hoewel de efficiëntie heel wat lager ligt, tonen de resultaten aan dat deze techniek potentieel kan gebruikt worden voor *T. parva* stabilaten. Voorstellen om de productie te optimaliseren worden gegeven, meer bepaald voor de formulering van stabilaten en voor de verpakking om een zo groot mogelijk contact te verzekeren met het vriesmiddel (vloeibare stikstof).

Resultaten van experimenten om het verlies aan infectiviteit te onderzoeken na opslag in een ijsbad (4°C) en in een standaard huishouddiepvriezer (-20°C), worden gegeven in het zesde hoofdstuk. Daartoe werden glycerol en sucrose stabilaten van twee *T. parva* stammen (Katete en Chitongo) getest aan beide temperaturen. De stabilaten werden bewaard op 4°C en -20°C tot 24 uur en 4 weken respectievelijk. Verlies aan infectiviteit voor beide stammen waren respectievelijk voor Katete en Chitongo 1% en 4% per uur opslag, hoewel dit effect niet significant was (P=0,45). Een vergelijk van het tijdseffect op de bewaarmiddelen toonde aan dat tijd geen significante factor voor glycerol (P=0,35), wel voor sucrose (P=0,04). Bij pas ontdooide stabilaten was de infectiviteit van sucrose stabilaten tot 10 maal hoger dan deze van in glycerol-bewaarde stabilaten (95% CI: 6,2 – 16,7). Het besluit was dat beide stammen kunnen gehouden worden in ijsbad en dat sucrose betere resultaten geeft. Dit dient echter verder onderzocht in vivo aangezien de invloed van glycerol op lymfocyten niet werd onderzocht en dat glycerol-stabilaten soms vals-negatieve resultaten geven bij de minst verdunde stabilaten reeksen.

Bij bewaring op -20°C verminderde de infectiviteit van glycerol en sucrose stabilaten respectievelijk met 98% en 61% na een week opslag bij deze temperatuur. We veronderstellen dat toen de temperatuur steeg, vorstbeschadiging van de sporozoieten zich voordeed door intracellulaire herkristallisatie van water. Dit hoge verlies aan infectiviteit maakt deze methode is dus onbruikbaar.

Hoofdstuk zeven is een verslag van pogingen om stabilaten te vriesdrogen, meer bepaald om een beschreven protocol te herhalen en te verbeteren. Vriesdrogen is potentieel een goedkopere manier om stabilaat te bewaren. Bij een eerdere poging was men er in geslaagd om dieren te infecteren met gelyofiliseerd stabilaat, maar was tevens vastgesteld dat minder dan 1% van de sporozoieten de procedure overleefd hadden. Indien dit kon herhaald worden moest getracht worden om dit rendement te verhogen. Katete en Chitongo *T. parva* sucrose en trehalose stabilaten werden getest. Tevens werden gehele geïnfecteerde teken, speekselklieren van teken en stabilaatgruis gelyofiliseerd. In geen enkele combinatie werden leefbare sporozoieten gevonden. Mogelijke redenen van dit falen worden besproken.

In hoofdstuk acht wordt de optimalisatie van de Sporozoiet Neutralisatie Test (SNT) beschreven en getest aan de hand van verschillende sera. Momenteel is SNT gebaseerd op een telling van geïnfecteerde cellen, wat een tijdrovende en subjectieve bezigheid is. Voorgesteld wordt om testplaat wells in te delen in positieve en negatieve wells. Dit is een meer gebruikersvriendelijke methode met binaire resultaten die kunnen geanalyseerd worden in een logistische regressie. Dit hoofdstuk beargumenteert ook het gebruik van microtitratie platen in plaats van microtubes voor *in vitro* titraties. Door de platen gedurende één minuut te schudden en de target cellen in de koelkast te bewaren vóór infectie in plaats van bewaring bij 37°C, bleek het resultaat minstens zo efficiënt te zijn als datgene van de oorspronkelijke methode. Bovendien werd een snellere methode om platen te lezen door PCR geëvalueerd. Tenslotte werd de *in vitro* procedure ingekort met 48 uur door het gebruik van een mitogeen (Concavalin A) om PBM-celculturen op te starten, achterwege te laten.

Sera van runderen, experimenteel geïnfecteerd met levende sporozoieten, dode sporozoieten of lymfoblasten geparasiteerd door schizonten, werden wekelijks in parallel getest tegenover een positieve (anti-p67) en een negatieve (FCS) controle. De sera werden tevens getest in een PIM-ELISA, p67-ELISA en een SELISA met geparasiteerde lymfoblasten als antigen. Geen significante sporozoiet inhibitie werd vastgesteld bij de drie sera, ondanks dat een duidelijk verschil met de controles werd waargenomen. Serum van het dier, geïnfecteerd met levende sporozoieten, reageerde positief in de drie serologische testen. Serum van het schizont geïnfecteerde dier reageerde positief in de SELISA en PIM-ELISA. Het gebrek aan sporozoiet neutralisatie in serum van het rund, dat verschillende malen geïnoculeerd werd met schizonten, toont tevens de voorheen beschreven lage p67 expressie in schizonten aan. Anti-PIM sera blijken verder inefficiënt te zijn voor sporozoiet neutralisatie en de SNT is specifiek voor anti-p67 antilichamen.

De voornaamste resultaten worden samengevat en besproken in hoofdstuk negen met daarbij de algemene conclusies. Hierin worden eveneens aanbevelingen geformuleerd voor verder onderzoek teneinde vragen en veronderstellingen geopperd in dit werk op te helderen.

Curriculum Vitae

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Other training	2001. Epidemiology and data handling for state veterinarians. Centre for Ticks and Tick-Borne Diseases, Malawi.
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Secondary School	1987. General Certificate of Secondary Education (O levels).

Work experience

2001-2002	Assistance to the Veterinary Services of Zambia (ASVEZA) a joint Zambian-Belgian ECF control project, Lusaka. Counterpart team member to understudy project ECF epidemiologist.
1999-2001	Team Leader, ASVEZA South.
1997-1999	ECF control officer, (ASVEZA) South.
1997	Acting District Veterinary Officer, Ministry of Agriculture Food and Fisheries, Livingstone.

1995-1997 Veterinary Officer in charge of field data compilation and annual reporting at Provincial Veterinary Office, Southern province, Zambia.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
ASVEZA	Assistance to the Veterinary Services of Zambia
BSA	Bovine Serum Albumin
CFSE	Carboxyfluoroscein Succinimidyl Ester (Synonym CFDA-FE)
CI	Confidence Interval
CL	Confidence Limit
Con-A	Concanavalin A
CPA	Cryoprotectant Additive
CTL	Cytotoxic Lymphocyte
DNA	Deoxyribonucleic Acid
ECF	East Coast fever
ED	Effective Dose
ELISA	Enzyme-Linked ImmunoSorbent Assay
EMA	Ethidium Monoazide
FAO	Food and Agriculture Organisation
FCS	Fetal Calf Serum
FSC	Forward Scatter
GLLAMM	Generalised Linear Latent and Mixed Models
HEPES	4-(2-Hydroxyethyl)-1-PiperazineEthanesulfonic acid
I & T	Infection and Treatment Method
IFAT	Immunofluorescent Antibody Test
ITM	Institute of Tropical Medicine
L-15	Liebovitz medium
ln t.e.	Natural logarithm of the tick equivalent
MEM	Minimum Essential Medium
MHC	Major Histocompatibility Complex
MSI	Macro-schizont Index
OIE	International Office for Epizootics
p67	67kDa circum-sporozoite immuno-dominant protein
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCD	Programmed Cell Death

PCR	Polymerase Chain Reaction
RPMI 1640	Roswell Park Memorial Institute medium
SCID	Severe Combined Immuno-deficiency
SELISA	Slide Enzyme-Linked Immunosorbent Assay
SNA	Sporozoite Neutralization Assay
SSC	Side Scatter
t.e.	Tick Equivalent