

**Theileriosis in roan antelope (*Hippotragus equinus*):  
identification of vectors and experimental  
transmission using a tick-derived stabilate**

by

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## SUMMARY

Theileriosis in roan antelope (*Hippotragus equinus*): identification of vectors and experimental transmission using a tick-derived stabilate

by

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Four hand-reared, naïve roan antelope 6 months of age were exposed to naturally infected pasture on a game farm in Mpumalanga where roan are known to die from theileriosis. The predominant ticks parasitising these animals at this time (during the months of January and February) were *Rhipicephalus appendiculatus* and *R. evertsi evertsi* adults. After a period of 5 weeks the animals developed signs of clinical theileriosis, characterized by anorectal lymphadenomegaly, shortly followed by pyrexia and generalised peripheral lymphadenomegaly. Around the time of pyrexia, peripheral blood smears showed severe mononuclear leukocytosis, with some leukocytes containing cytoplasmic theilerial schizonts (Koch's bodies). This was followed by erythrocytic piroplasmaemia. Animals were treated successfully using buparvaquone.

Primary hyperplasia of the local draining lymph nodes (*Lnn. anorectales*) of adult *R. e. evertsi* feeding sites indicated vector status of theileriosis for this tick species. After recovery from theileriosis, these animals were confirmed carriers of *Theileria* sp. (sable) by PCR and DNA probe analysis.

Laboratory-bred larvae and nymphae of *R. e. evertsi* and *R. appendiculatus* respectively were fed on the ears of these roan antelope, now known to be carriers. Engorged nymphae were recovered from the roan. A cryopreserved stabilate was

produced from each species, using an adapted protocol for *Theileria parva* tick stabilate preparation. Both stabilates were tested for infectivity in *T. sp.* (sable) negative (PCR analysis) roan antelope calves that were intensively hand reared under tick-free conditions. The stabilate derived from *R. e. evertsi* induced clinical theileriosis at doses between 5 and 60 tick equivalents per calf. The *R. appendiculatus* stabilate did not induce clinical disease or infection; the calves remained PCR negative throughout the trial period. The calves that survived the *R. e. evertsi* stabilate trial showed no clinical reaction to field exposure, in contrast to the controls and *R. appendiculatus* stabilate inoculated calves which all exhibited clinical theileriosis.

The stabilate has the potential to be used as the infection source in an infection-and-treatment method of immunising roan antelope against theileriosis.



Roan (*Hippotragus equinus*) antelope. Mpumalanga lowveld, Malelane, South Africa.

## Chapter 1

### INTRODUCTION

The genus *Theileria* is a large group of tick-borne protozoal apicomplexan parasites classified in the family Theileriidae, order Piroplasmida (Levine *et al.* 1980).

Theileriosis is the disease caused by pathogenic *Theileria* in susceptible host species. This organism parasitizes mononuclear leukocytes (most often lymphoblastic) resulting in mononuclear leukocytosis followed by erythrocytic piroplasmaemia (Lawrence, Perry & Williamson 2004). In contrast to *Theileria* affecting domesticated production animals which has been studied intensively for over a century, this group of organisms and the disease (theileriosis) occasionally associated with it has been widely reported but not studied in detail in wildlife.

In roan and sable antelope a particular *Theileria* species that causes mortality has been identified and because it was recovered first from a sable antelope that died from theileriosis it was provisionally named *Theileria* sp. (sable) (Stoltz & Dunsterville 1992). Theileriosis is the single most important fatal infectious disease of roan, and to a lesser degree sable antelope, contributing to decreasing population numbers throughout southern Africa. The highest mortality rate is encountered amongst calves younger than 3 months of age and newly introduced naïve animals. Mortality ranging between 10% and 90% is regularly recorded on game ranches in South-Africa (personal observation). The conventional approach of acaricide application to decrease tick numbers allowing low-dose natural exposure to *T.* sp (sable) has failed to decrease mortalities in roan but has been partially successful in sable antelope.

In an attempt to identify which control measures were likely to succeed, it was important to determine basic epidemiological facts about this disease in roan antelope. The aim of this study was:

- Hand rearing of naïve (PCR negative for *T. sp* (sable)) day-old roan antelope calves to serve as experimental animals.
- Infection via natural exposure with close clinical observation.
- Vector identification to study host-disease-vector relationship.
- Artificial infection of naïve roan antelope calves with *T. sp* (sable) using a tick-derived stabilate.
- Testing an infection and treatment method of immunisation of roan antelope calves against theileriosis, to provide a practical tool for theileriosis control during re-introduction and breeding projects for roan antelope in South Africa in areas where the disease is known to be prevalent. This is the first attempt at investigating the use of a tick-derived stabilate as a method of theileriosis infection in a wildlife species.

## Chapter 2

### LITERATURE REVIEW

Roan antelope (*Hippotragus equinus*) (Geoffroy Saint-Hilaire 1803) is the second largest antelope on the African continent belonging to the family Bovidae. At the turn of the previous century their distribution range stretched from western and central Africa to eastern Africa, reaching as far south as the northern parts of South Africa. In recent times the population of roan antelope has decreased in all parts of its former range and today it occurs mainly in conservation areas (Van der Horst 2005). Morphological taxonomy divides roan antelope in six subspecies i.e. *H. e. equinus*, *langheldi*, *bakeri*, *cottoni*, *charicus* and *koba* (Ansell 1971). Molecular phylogenetic studies have shown that only two distinct subspecies occur. Western roan (*H. e. koba*) has been found to differ significantly enough from the other five roan subspecies. The other subspecies are rather subpopulations of *H. e. equinus* with genetic exchange that occurs (Alpers *et al.* 2004).

The species is listed in the IUCN red list of threatened species as a conservation-dependent species due to general population decline. If this trend continues the species may be listed as vulnerable or threatened in the near future. Habitat destruction and poaching are the most important negative impacting factors (Anon 2008).

The reason for population decline of roan antelope in the Kruger National Park, South Africa, has been speculated to be artificial water hole management, resulting in increased predation due to prey species encroaching on roan territories within the park (Harrington 1999, Grant *et al.* 2002) and competition for grazing (Kroger & Rogers 2005, Kroger & Rogers 2006). Mortality from disease conditions such as anthrax has been implicated but not enough supportive evidence for its role in population decline could be proven (Harrington 1999). On private game ranches in

South Africa and the occasional nature reserve, higher stocking rates have contributed to increased prevalence of many disease conditions, especially tick-borne disease, which under natural stocking rates may not have been present. Relocation of naïve animals into infected areas also plays a significant role. With theileriosis in roan and sable antelope this certainly appears to be the case (Wilson *et al.* 1974, Wilson & Hirst 1977, Meltzer 1993). The number of cases of this disease has also increased due to escalating game ranching (personal experience). In 1960, South Africa had in the region of 10 privately owned game ranches. This number had increased to 5000 in 2000 at an annual rate of 7% between 1993 and 2000. Nearly 50% of the total number of game ranches is in the Limpopo province of South Africa (Child 2004), a region known for high prevalence of tick-borne diseases among livestock, hence the tendency to move away from livestock to game ranching.

The genus *Theileria* is a large group of tick-borne, apicomplexan protozoal organisms with a complex life cycle consisting of many developmental stages in the tick and host. When an infected tick feeds on a suitable mammalian host, sporozoites from infected tick salivary glands injected in the skin infect lymphocytes and develop into cytoplasmic schizonts (Koch's bodies) resulting in local, followed by systemic, lymphoid proliferation. Erythrocytes are infected by the next stage, known as piroplasms. Feeding ticks ingest the piroplasm-infected erythrocytes, and sexual replication takes place in the intestinal lumen of the tick, with zygotes invading the intestinal cells as kinetes. During and after moulting of the tick, the kinetes migrate to and infect tick salivary glands to form sporozoites, completing the life cycle. Asexual replication takes place between every developmental stage, except in the intestine of the tick (Shaw 2002, Lawrence, Perry & Williamson 2004).

Numerous species and subspecies of *Theileria* exist, of which most are apathogenic. Some may however cause disease in certain animal hosts. In cattle, significant pathogenic species include *T. parva* (Theiler 1904) and *T. annulata* (Dschunkowsky &

Luhns 1904). In small domestic ruminants, *T. lestoquardi* (Lawrence 2004) and in sheep *T. sp* (China) (Yin *et al.* 2007) are of economic importance.

The presence of theilerial piroplasms in random blood smears of East African wild ungulates is a common feature (Brocklesby & Vidler 1966, Oosthuizen *et al.* 2009) and is most often a sign of a carrier state rather than disease (Grootenhuis & Young 1981). In recent times PCR screening of random blood samples collected from a number of healthy individuals from a variety of wildlife species in South Africa supported these findings and suggested widespread carrier states (sometimes more than one species per animal) in the general African wildlife population (Nijhof *et al.* 2005, Pfitzer *et al.* 2011,)(Steyl 2005, unpublished data).

In wild antelope, natural cases of clinical theileriosis (also known as cytauxzoonosis, a term occasionally used for a theileriosis-like syndrome in certain species (Grootenhuis & Young 1981), differing only in that schizonts develop in cells of the histiocytic series as opposed to lymphocytes (Neitz 1956)) have been reported in a number of species, i.e. roan antelope in West Africa in 1912 (Todd & Wolbach 1912) and in South Africa since 1974 (Wilson *et al.* 1974); sable antelope (*Hippotragus niger*) in Southern Africa (Neitz 1957, Wilson *et al.* 1974); eland (*Taurotragus oryx*) (Brocklesby 1962, Grootenhuis *et al.* 1980); grey duiker (*Sylvicapra grimmia*) (Neitz & Thomas 1948); kudu (*Tragelaphus strepsiceros*) (Neitz 1957); giraffe (*Giraffa camelopardalis*) (McCully, Keep & Basson 1970); tsessebe (*Damaliscus lunatus*) (Jardine 1992); waterbuck (*Kobus ellipsiprymnus*)(Steyl 2011, personal observation) and white tail deer (Robinson *et al.* 1967). Clinical and often fatal cytauxzoonosis has also been reported in wild felids, i.e. tigers (*Panthera tigris*)(Garner *et al.* 1996, Jakob & Wesemeier 1996) and a captive lion (*Panthera leo*)(Peixoto *et al.* 2007). None of these cases was followed up with transmission studies except for experimental infection of theileriosis in eland with *T. taurotragi* using infected *R. appendiculatus* ticks (Grootenhuis *et al.* 1977, Grootenhuis, Young & Kanhai 1978, Grootenhuis *et al.* 1980).

Gene sequencing and comparison of isolates from recent cases in western roan antelope (*H. e. koba*) in Malelane, Republic of South Africa, confirmed, as had been suspected previously, that the same *Theileria* species is involved in southern Africa in roan and in sable (Nijhof *et al.* 2005). This *Theileria* species is unique, and naming it is currently under discussion. It was isolated in lymphoblast cell culture from a sable that died of theileriosis in South Africa and referred to as *Theileria* sp. (sable) (Stoltz & Dunsterville 1992). From the known *Theileria* species, phylogenetic analysis shows that *T.* sp. (sable) is closest related to *T. separata* (Uilenberg & Schreuder 1976), a benign *Theileria* species of sheep (Lawrence, de Vos & Irvin 2004), and a *Theileria* species isolated from a grey duiker, *T.* sp. (grey duiker). Within the genus *Theileria*, *T.* sp. (sable) is separated the farthest from *T. equi* and the *Cytauxzoon* group of parasites (Nijhof *et al.* 2005).

*Theileria* sp. (sable) is responsible for roan and sable mortalities in Mpumalanga and elsewhere in South Africa. This was confirmed by diagnosis of theileriosis at necropsy in these species over a wide area of the northern and eastern parts of RSA (cases recorded at the Section of Pathology, Dept. of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria). Identification of the *Theileria* species involved and confirmation of *T.* sp. (sable) as the pathogen was performed using a recently developed PCR and DNA probe on reverse line blot (RLB) assay (Nijhof *et al.* 2005). Currently, no serological test exists that can be utilized in screening for *T.* sp. (sable) exposure or status.

The significant impact of East Coast fever (*T. parva* infection) on cattle herds in Africa has prompted intensive research in understanding and controlling the disease for decades. In the mid-1930s an integrated, state-funded program, involving movement control, short-interval dipping, quarantine and intensive surveillance was successful in eradicating *T. parva* from the *Rhipicephalus appendiculatus* tick population in RSA

(Lawrence 1992). Only localised areas in and around the Kruger National Park and Hluhluwe-iMfolozi Park, which keep African buffalo (*Syncerus caffer*), the only known wild reservoir of *T. parva*, remained infected. In the case of *T. sp.* (sable), randomly collected blood samples from various other species in the Malelane area tested positive by PCR. These included nyala (*Tragelaphus angasii*), blue wildebeest (*Connochaetes taurinus*), reedbuck (*Redunca arundinum*), blesbok (*Damaliscus pygargus phillipsi*) and bushbuck (*Tragelaphus sylvaticus*). No mortality due to theileriosis has been described in these species. Eradication of *T. sp.* (sable) from the tick vector population as a method of control of theileriosis in roan is not an option, as numerous other wild antelope are carriers and movement control would be an impossible task.

*Rhipicephalus*, *Amblyomma*, *Hyalomma* and *Haemaphysalis* species (21 species in total) have been identified as vectors for various *Theileria sp.* of livestock in eastern, central and southern Africa (Lawrence, Perry & Williamson 2004). Of these, *R. appendiculatus* (brown ear tick) is a well-known vector of *Theileria* species in cattle in southern Africa (Norval 1992) and is also one of the more dominant species and widely distributed throughout the northern and eastern parts of RSA. In African antelope, it infests a wide range of species including roan and sable. These factors have led to the suspicion that *R. appendiculatus* should be considered a strong contender as a vector for theileriosis in roan antelope. However, early field observations during outbreaks of the disease in roan also supported the possibility of *R. e. evertsi* as a vector for *T. sp.* (sable) (personal observation). No scientific study has ever been done to establish the vector/s for theileriosis in roan antelope.

Tick control through the use of acaricides has been an important prophylactic approach to *T. parva* infection in cattle, although sustainability remains questionable (Norval 1992, Pipano, Morzaria & Spooner 2008). Trigger applicators (Tick-off®) containing a residual pyrethroid, Drastic Deadline® (Flumethrin 1%) or Tick-off® acaricide dip (Cypermethrin 0.4% & Permethrin 0.8%) for adult animals and manual

application of Frontline® (Fipronil) for neonates were used to control tick load in animals on a property in Malelane, RSA, experiencing a high incidence of theileriosis. Although reducing tick numbers significantly, this approach failed to control theileriosis in roan antelope. Clinical and necropsy data showed that roan antelope calves with minimal tick infestation still developed fatal theileriosis (personal observation).

The use of a tick-derived stabilate is an underutilised but effective method of establishing immunity for the control of East Coast fever in cattle in East and Central Africa (Cunningham *et al.* 1973, Radley 1981). For the prophylactic control of tropical theileriosis due to *T. annulata*, cattle can be vaccinated using a culture-derived schizont-infected stabilate (Pipano & Shkap 2000). The use of an infective stabilate can cause clinical disease, which is controlled by prophylactic treatment with oxytetracyclines or buparvaquone at strategic times during disease incubation (McHardy *et al.* 1985, Norval 1992). This is known as an infection-and-treatment method of immunisation. In the case of *T. parva* and *T. annulata*, oxytetracycline is administered at the same time as infection (day zero) or alternatively on day zero and 3 or 4 days later (Radley 1981). Tetracyclines have been shown to have a suppressive effect if administered during the incubation period (Neitz 1953), but were less successful when treating clinical theileriosis (Lawrence, Perry & Williamson 2004). In the case of clinical disease the drug of choice is buparvaquone (McHardy 1989).

There is no record of any attempt having been made to immunise wild antelope against theileriosis by any method.

## Chapter 3

### MATERIALS AND METHODS

#### 3.1 Experimental design

A farm in the summer rainfall region of RSA known to suffer regularly from roan mortalities due to theileriosis was selected in the Mpumalanga province. The farmer, a breeder of endangered antelope species, was keeping the roan breeding stock grouped in camps ranging from 20 to 30 ha in size.

Hand-reared roan calves known to be PCR negative for *T. sp. (sable)* were exposed to ticks in a camp with a recent history of mortality due to theileriosis. Constant monitoring of various clinical parameters indicative of theilerial infection was performed on these animals. Calves were treated when they became infected.

After recovery, these calves were confirmed carriers of *T. sp. (sable)* by PCR testing of blood samples. Laboratory-bred immature stages of the predominant tick species (*R. appendiculatus* and *R. e. evertsi*) collected from cattle in the area were fed on these calves, removed and left to mature to adults. After they had fed on laboratory rabbits, salivary gland dissections, staining and PCR screening for *Theileria* species (also *Babesia* and *Ehrlichia* species) were performed on a subset of these ticks. The bulk of the ticks were used to prepare ground-up tick stabilates. Salivary gland dissection and PCR screening were repeated for field-collected ticks from camps where roan were known to die from theileriosis.

Hand-reared roan calves raised under tick-free conditions were randomly selected and PCR tested for *T. sp. (sable)*, with negative results. These animals were used for stabilate infection and titration trials. Various clinical parameters detecting theilerial

infection were monitored. Constant monitoring for ticks and regular dipping with a residual acaricide was maintained throughout the trials.

Following the stabilate-infection trials, all animals were exposed to natural infection by release into tick-infested veld where roan were known to contract theileriosis. Animals were monitored and treated on confirmation of clinical theilerial infection.

### **3.2 Hand rearing of roan antelope calves**

In setting up an experiment to identify the possible tick vectors, it was decided to hand rear all roan calves born during the 2002 calving season under tick-free conditions. Calves were collected from the pasture 24 hours after birth. This timeline was based on ensuring passive colostral immunity transfer, while forestalling *Theileria* sporozoite maturation and infection from attached ticks (Shaw 2002).

On arrival at the hand-rearing facility all calves were thoroughly dipped with a contact pyrethroid dip (Bayticol® Flumethrin) and clearly identified using plastic ear tags with a sequential numbering system. This facility was rectangular in shape, about 2000 m<sup>2</sup> in area, divided into three devegetated runs and fenced off with 1.8m high wire mesh. In one corner, 3 infrared heated rooms with concrete floors were built. *Eragrostis curvula* hay (believed to be free from ticks) on river sand was used as bedding in these rooms. River sand was used to cover the surface of the outer runs. The bedding material in the rooms was replaced weekly and in the runs as needed. The calves were kept in the heated rooms until strong and well adapted to bottle feeding. Before entering the facility, handlers were sprayed using a pyrethroid aerosol (Bayticol aerosol®). Regular interval dipping (fortnightly with a long-acting pyrethroid, Deadline® or Tick-off®) continued during the hand-rearing process and no other mammals had access to the unit. Roughage feed available to the calves consisted of *Eragrostis tef* sourced from cultivated land and pelleted game cubes (in-house formulation). All these steps were implemented as precautionary measures in

ensuring a tick-free hand-rearing unit. All calves were hand reared until 4 months of age with weaning over a period of 2 weeks. Calves were fed a milk formulation (at body temperature) consisting of the following ingredients: 1 litre of non-pasteurised, full cream, dairy cow milk (the Jersey breed was preferred for its higher fat content) + 1 large chicken egg yolk + 5ml cream + 5ml Biorem® (ruminant probiotic). The allocated quantity per calf was calculated, using weekly measured body weight, as:  $[10\% \times \text{body weight (kg)} = \text{litres per day}]$ , divided into three feeds (6-hour intervals starting at 6:00 AM). The volume of milk was increased according to body weight up to a maximum of 4 litres/calf/day.

During hand rearing of the roan antelope calves (44 calves in total), no disease or mortality as a result of theileriosis was documented. A few animals died from dietary and/or viral diarrhoea (*Rotavirus* diarrhoea diagnosed from faeces submitted to Lancet Laboratories) during the first 6 weeks of age. No disease occurrence of any kind was documented after 8 weeks of age.

### **3.3 Polymerase chain reaction test (PCR) and reverse line blot assay (RLB)**

All submitted blood samples were analysed by the molecular laboratory in the Dept. of Tropical Diseases, Faculty of Veterinary Science, University of Pretoria. PCR product from the blood samples was applied to a reverse line blot assay (Gubbels *et al.* 1999) for the detection of a wide range of haemoprotozoal co-infections (Addendum A) including *T. sp* (sable) (Nijhof *et al.* 2005).

### **3.4 Selection of trial animals and preparation for natural exposure**

Four male roan calves (numbers 210, 218, 222 and 242) were selected randomly at 4 months of age. The animals were physically restrained and blood was collected in EDTA, stored at -20°C, and submitted for PCR (RLB).

On receipt of the PCR results, confirming that they were not infected, the four roan males were chemically immobilized using a drug cocktail consisting of 20 - 35µg/kg Etorphine HCl and 0.3mg/kg Azaparon. While anaesthetised where they were translocated over a short distance to a vegetated camp on the farm where *Theileria*-infected ticks were known to occur. Mortality in roan due to theileriosis had occurred in this camp no longer than 8 months prior to this experiment, and it had since been accommodating a roan herd. This herd was transferred to an adjacent camp 2 weeks prior to the experiment. The four males were purposely left undipped in an attempt to expose them to ticks as soon as possible after arrival. This field trial was conducted during the months of January and February 2003 (mid-summer in the summer rainfall area of RSA) with tick numbers close to a peak.

### **3.5 *Theileria* infection monitoring**

As the animals were still being bottle fed, it proved very easy to habituate them to handling, thus facilitating a wide spectrum of possible monitoring parameters. The following parameters were monitored over a period of 6 weeks:

#### **3.5.1 Tick infestation**

Time of noticeable tick infestation, species present, attachment sites and subjective assessment of tick load were recorded.

#### **3.5.2 Rectal temperature**

Rectal temperature was measured 2X/day at morning and afternoon feeding times using a calibrated digital rectal thermometer. A rectal temperature higher than 40°C (especially if ambient temperature was below 28°C) was considered a fever reaction.

#### **3.5.3 Habitus**

Subjective scoring of feeding behaviour. Scoring values from 1 to 4 were applied, with 4 indicating good general health and behaviour progressively deteriorating to 1 which implied clinical illness.

#### **3.5.4 Peripheral lymph node palpation**

Scoring of lymph node size (0 – 4) with 4 being visibly enlarged without palpation and 0 normal on palpation.

#### **3.5.5 Lymph node aspiration**

Fine needle aspiration using a sterile 38mm 20G hypodermic needle was conducted simultaneously on the superficial cervical and other lymph nodes if they were visibly enlarged at times of blood collection. The same procedure as for peripheral blood smear staining and examination was followed (see below).

#### **3.5.6 Peripheral blood smears**

Peripheral blood smears were obtained every second day by pricking of the tail tip using a 19mm 21G needle. The sample was immediately transferred to a glass slide, fixed and stained using the Diff Quik® staining method. The sample was examined using a standard light microscope for lymphoblastic transformation and theilerial schizont/piroplasm presence. Blood smears were also examined for other vector-borne haemoparasitic infections such as *Anaplasma* and *Babesia* species.

#### **3.5.7 Mucous membrane examination**

Colour of the conjunctival mucosae was assessed subjectively at regular intervals when animals allowed ocular manipulation and also at every chemical immobilization opportunity.

### **3.6 Blood samples for PCR detection of *T. sp.* (sable)**

Blood was collected in EDTA every 7 days during chemical immobilization. The

samples were clearly identified, dated and transferred within 2 hours to a -20°C freezer for later PCR testing.

### **3.7 Treatment of clinical theileriosis**

A clinical case of theileriosis was defined as: pyrexia above 40°C shortly followed, preceded or accompanied by theilerial schizonts in circulation detectable by blood smear examination. These findings were the criteria for initiating anti-theilerial therapy using buparvaquone (Butalex®) once at 2.5mg/kg intramuscularly. If fever had not abated 48h later, a second injection was administered. A staggered treatment regimen was applied to the different animals in an attempt to determine efficacy of anti-theilerial therapy in relation to the first day of fever. The following regimen was applied: Animal 218 was treated on the first day of pyrexia. Animal 210 was treated one day, 222 two days and 242 three days post-pyrexia.

### **3.8 Laboratory breeding and preparation of ticks for tick feeding**

Engorged adult females of the two dominant tick species present during field exposure, i.e. *R. e. evertsi* and *R. appendiculatus*, were collected from cattle in the Malelane area and transferred to the laboratory for egg laying using standard operating procedures (Heyne, Elliott & Bezuidenhout 1987). Larvae of *R. appendiculatus* (3-host tick) were fed on rabbit ears and backs after hatching and left to engorge. These engorged larvae were collected and stored in batches of 250 in ventilated plastic vials to moult and mature to nymphae under controlled laboratory conditions. Eggs from *R. e. evertsi* (2-host tick) were weighed and divided into batches containing an estimated 500 eggs each. These eggs were left to hatch and mature to larvae, ready for feeding on *T. sp.* (sable) carrier hosts.

### **3.9 Vector identification and stabilate preparation: tick feeding on *T. sp.* (sable) carrier animals**

Upon PCR confirmation of carrier status for *T. sp.* (sable) and RLB screening for other blood-borne protozoal organisms (Addendum A), these naturally exposed four animals were relocated to tick-free boma conditions.

The confined carrier roan calves were prepared for tick feeding by applying linen ear-bags to their ears (Heyne, Elliott & Bezuidenhout 1987). A few modifications to the described method entailed the following:

- To prevent animals from shaking the ear-bags loose, a “head bandage” was applied with adhesive bandage (Elastoplast®) and strategically placed temporary nylon dermal sutures. The latter fixed the bag to underlying skin.
- It was necessary to chemically immobilize (see above) the animals for fitting of ear-bags and placement of ticks.

The four animals were divided into pairs. One pair received the larvae of *R. e. evertsi* and the other nymphae of *R. appendiculatus* applied in batches of 500 per ear per animal.

After 6 days, the animals infested with the *R. appendiculatus* nymphae were immobilized and the bags with engorged nymphae removed. It was necessary to scrape engorged, but still attached, nymphae off the skin using the shaft of a scalpel handle. The ear-bags of the animals containing the *R. e. evertsi* larvae were removed after 14 days and engorged nymphae collected.

### **3.10 Salivary gland investigation of fed ticks**

The engorged nymphae of both tick species were housed under laboratory conditions until moulted to adults. For their mouth parts to harden and appetite to increase, these adult ticks were left to “mature” for 2 weeks under laboratory conditions. Matured adult ticks were then fed on laboratory rabbits for a period of 4

days to allow salivary gland maturation to occur (Cunningham *et al.* 1973, Morzaria *et al.* 1999, Pipano, Morzaria & Spooner 2008). The rabbits were euthanised using Sodium Pentobarbitone (Eutha-Naze®) and ticks immediately removed with curved forceps taking care not to put pressure on the anterior salivary region. These ticks were placed in a tick-proof ventilated container and, within 4 hours, individually embedded (with the scutum facing up) in a Petri dish containing paraffin wax. Ticks, 100 of *R. e. evertsi* and 150 of *R. appendiculatus*, equal numbers of males and females, were then dissected (Blewett & Branagan 1973) in phosphate-buffered saline (PBS), using a standard stereomicroscope. Salivary glands were removed and transferred to a glass microscope slide to dry. The slides were then processed by staining with methyl-green/pyronin (Walker 1979) and examined for *Theileria* spp. infected salivary acini using a standard light microscope.

A representative sample of about 50 ticks for each species was dissected, and the salivary glands removed and suspended in 2ml vials containing isotonic saline solution for PCR (RLB) detection of *T. sp.* (sable) and other possible protozoal organisms.

### **3.11 Infection of field ticks**

Approximately 160 adult *R. appendiculatus* ticks were randomly hand collected from grass stalks during February from various roan camps on the farm. No significant number of adult *R. e. evertsi* could be found at this time using the same method of collection. The *R. appendiculatus* ticks were fed on rabbits and included in a separate sample. These camps had experienced mortalities due to theileriosis no longer than 6 months earlier. One hundred ticks were dissected and stained (as above – 3.10). Thirty additional dissected salivary glands were submitted for PCR. A screening profile that included a wide range of vector-borne haemoparasites (*Theileria*, *Anaplasma*, *Ehrlichia* and *Babesia* species; see Addendum A for details) was applied to these samples using the RLB method.

### 3.12 Preparation and cryopreservation of tick stabilates

The method of stabilate preparation described below conforms, in general, to the proposed OAU/FAO/ILRI Standards for *Theileria parva* stabilate preparation (Pipano, Morzaria & Spooner 2008).

Moulted adults of the two *Rhipicephalus* spp. were prefed on the ears of rabbits for 4 days in order for sporozoite maturation to occur (Blewett & Branagan 1973, Morzaria *et al.* 1999). Once prefed, the ticks were carefully removed from the ears of the rabbits after euthanasia (Eutha-Naze®: Sodium Pentobarbitone) and placed in a ventilated plastic jar. Preparation of ticks and stabilate production proceeded as follows:

- Ticks were thoroughly washed:
  - a. Under running tap water in a fine-meshed sieve for 5 minutes to remove rabbit debris, and subsequently transferred to a glass beaker.
  - b. Rinsed with 70% ethanol 3 times (1 minute each) in a glass beaker.
  - c. Rinsed with sterile distilled water 3 times (1 minute each) and drained.
- Ticks were placed on filter paper in a dry glass beaker and transferred to a –20°C freezer for a maximum of 5 minutes to immobilise them.
- Ticks were removed from the freezer and stored on a sterile stainless steel tray and counted to determine the volume of grinding medium needed for the tick-derived suspension. Counting of all the ticks was made simple by keeping them relatively immobile on a chilled stainless steel tray in crushed ice. [NOTE: For larger tick batches, a manageable subset of ticks is weighed and counted separately. The total number of ticks can then be calculated using the following formula: Total number of ticks = (total weight of ticks / weight of counted fraction) X number of ticks in counted subset].

The following procedures were carried out wearing face masks and protective gloves:

- Ticks were ground in MEM/3.5% BSA (bovine serum albumin) (Addendum B no: 1), using a pestle and mortar so that the concentration after grinding was less than 20 ticks/ml. The grinding vessel was placed in a tray of crushed ice during the grinding process. Grinding of the ticks was continued until no whole tick scutae or appendages could be seen with the naked eye. This process never took longer than 20 minutes.
- The ground-up tick suspension was adjusted to 20 ticks/ml by adding MEM/3.5% BSA and was centrifuged in 50 ml conical centrifuge tubes at 50g for 5 min at 4°C in an automated refrigerated centrifuge (Thermo Heraeus Mega 40R®).
- The ground-up tick supernatant (GUTS) was decanted and pooled, and the volume measured in a measuring cylinder. The suspension was placed into a suitable large flask, containing a magnetic follower, and placed in a tray of crushed ice with the ice level above that of the suspension.

At this stage, each stabilate was sampled for PCR (RLB) screening of *T. sp.* (sable) and other tick borne protozoa/rickettsia (Addendum A).

- An equal volume of MEM/3.5% BSA/15% glycerol (Addendum B no: 2) was added to the GUTS, at 4°C, from a separating funnel as a slow trickle with constant stirring, but without frothing. Equilibration occurred during the addition of the cryoprotectant over a period of not less than 45 minutes. This gave a final GUTS concentration of 10 ticks/ml.
- When the addition of cryoprotectant was completed the suspension was dispensed into pre-labeled 1 ml cryovials, and the vials were placed in storage racks on crushed ice. The vials were initially packed loosely in storage racks on crushed ice to ensure even freezing and then transferred to a refrigerator at 4°C. When filling was completed, the racks, well separated, were transferred to a –70°C freezer.

At this stage representative samples were collected for fungal and aerobic and anaerobic bacterial culture.

- After overnight freezing (at  $-70^{\circ}\text{C}$ ) the vials were packed more tightly into the storage racks and transferred to canisters in a permanent liquid nitrogen storage container.
- Stabilate preparation details were recorded and compared for both tick species.

### 3.13 Infection trials with tick-derived stabilates

Roan calves born during the 2003 season were removed from their mothers within 24 hours after birth for hand rearing under tick-free conditions. Once feeding strongly, blood was collected for PCR testing for *T. sp.* (sable). Calf ages at the start of the trials varied between 10 days and 3 weeks. After PCR confirmation that the calves were not already infected, they were randomly allocated in pairs for stabilate infection trials. One pair received the *R. e. evertsi* stabilate, another the *R. appendiculatus* stabilate, and the third pair served as negative controls, receiving an isotonic saline placebo (equivalent in volume to the stabilate). Based on salivary gland infection rates, it was decided to use a high dose, 60 tick equivalents (6 ml x [10 ticks/ml]) per animal.

Prior to inoculation, the stabilate vials (2 additional in case of spillage or vial leakage) were recovered from the permanent liquid nitrogen container, taking care to maintain the cold chain (for handling of vials, temporary liquid nitrogen containing beakers were used), and transferred to a portable liquid nitrogen flask. At inoculation, the required number of vials was removed from the nitrogen flask and immediately thawed in a water bath at  $38^{\circ}\text{C}$  while the trial animals were manually restrained. Once the stabilate was thawed, the correct amount was drawn up in a syringe and the animals were inoculated immediately. All animals were injected subcutaneously in the left side of the neck, dorso-cranial to the superficial cervical lymph node. Clinical monitoring was carried out by the same person and started 3 days prior to inoculation to determine baseline values. Monitoring parameters included the following:

### **3.13.1 Rectal temperature**

Rectal temperatures were measured with a calibrated digital thermometer and recorded at the same times in the mornings and afternoons. Ambient temperatures and animal activity were taken into account in rectal temperature interpretation. For this trial, two consecutive measurements exceeding 39.5°C were interpreted to be a fever reaction.

### **3.13.2 Lymph node palpation and fine needle aspiration**

Palpation of the superficial cervical lymph node, draining the inoculation site, was performed, and the size compared to the contralateral lymph node. The lymph nodes were scored (0–4) with 4 being visibly enlarged without palpation and 0 normal on palpation. Fine needle aspiration using a sterile 38mm 20G hypodermic needle was conducted on the superficial cervical and other lymph nodes if they were visibly enlarged at times of blood collection. The same procedure as for peripheral blood smear staining and examination was followed (see below).

### **3.13.3 Peripheral blood smears**

Peripheral blood smears were obtained every second day by pricking the tail tip with a 19mm 21G needle. The sample was immediately transferred to a glass slide, spread, fixed and stained using Diff Quik®. The smear was examined as soon as possible using a standard light microscope for lymphoblastic transformation and presence of theilerial schizonts/piroplasms. Blood smears were also examined for other vector-borne haemoparasites such as *Anaplasma* and *Babesia* species.

### **3.13.4 Blood samples**

Blood was collected in EDTA prior to and 3 weeks after stabilate inoculation for determination of *T. sp. (sable)* transmission success. The samples were clearly identified, dated and transferred within 2 hours to a -20°C freezer for subsequent

PCR testing.

A serum and duplicate EDTA blood sample was opportunistically, and during a pyrexia event, collected for haematology and serum chemistry (Lancet Laboratories).

### **3.13.5 Mucous membrane colour**

Colour of the conjunctival mucosae was assessed subjectively daily during feeding times.

### **3.13.6 Habitus**

Subjective scoring of feeding behaviour was performed. Scoring values from 1 to 4 were applied, with 4 indicating good general health, behaviour and feeding, progressively deteriorating to 1 which implied clinical illness.

A positive reaction to the stabilate infection was defined as pyrexia above 39.5°C on 2 consecutive occasions, together with theilerial schizonts in circulation detectable by blood smear examination. These findings served as the criteria for initiating anti-theilerial therapy using buparvaquone (Butalex®) once at 2.5mg/kg intramuscularly. If fever did not abate within 48h, a second injection was administered. Anti-theilerial therapy was performed at the request of the owner to minimise experimental losses and did not interfere with the results and the purpose of the study.

### **3.13.7 Post-mortem examination of mortalities**

In the event of mortality, macroscopic post-mortem examination was performed and abnormalities recorded. Organ samples were collected for histopathological examination.

## **3.14 Titration of *Rhipicephalus evertsi evertsi* stabilate**

As a dose of 60 tick equivalents of *R. e. evertsi* stabilate was found to be highly infective, the stabilate was titrated at 10 ticks (1ml stabilate) and 5 ticks (0.5ml stabilate) per animal. Randomly selected, *T. sp.* (sable) PCR negative, 4 to 6 week old animals were divided in pairs for each dose rate. A control pair was inoculated with an equivalent volume of isotonic saline placebo. The same therapeutic approach and clinical parameters used for the infectivity trials were applied in this study. In the event of mortality, complete anatomical necropsy was performed.

### **3.15 Post-infection field exposure**

Animals that survived the infection and titration trials, together with the controls, were kept under tick-free conditions until weaning at 4 to 5 months of age. At that stage the animals were chemically immobilized and blood was collected for *T. sp.* (sable) PCR testing. They were relocated to a vegetated, tick-infested camp where roan antelope were known to have become infected and had died from theileriosis 9 months earlier. For the following 6 months, the habitus (mainly behaviour and appetite) of the animals was monitored carefully for signs of illness. No acaricide treatment was applied for the period. Having been hand reared, the animals tolerated routine visual inspection, but would not allow handling without sedation. If any signs of illness occurred, animals were sedated and clinically examined. If clinical theileriosis was diagnosed, the animals were treated with a single dose of buparvaquone.

## Chapter 4

### RESULTS

#### 4.1 Natural exposure trial: *Theileria* infection monitoring

Hand-reared roan calves known to be PCR negative for *T. sp.* (sable) were exposed to ticks in a camp with a recent history of mortality due to theileriosis. Constant monitoring of various clinical parameters indicative of theilerial infection was performed on these animals. Calves were treated when they became infected.

##### 4.1.1 Tick infestation

Larval infestation occurred after about 2 days and adult infestation 5 days after introduction of the roan into the camp. This was marked by increased scratching activity, especially of the ears. Adult *R. appendiculatus* ticks were the predominant tick species and stage feeding around the head, neck and outer surface of the pinnae. Its numbers ranged from about 40 to 80 at a time per animal. Immatures were also present in low numbers on the inner peripheral area of the pinnae. Around the same time, the adult stage of *R. e. evertsi* was observed feeding in the peri-anal and inguinal regions. Its numbers never increased to more than 20 per animal. *R. e. evertsi* immatures (larvae and nymphae) were present in low numbers in the outer part of the inner ear canal.

Occasionally other species could be found, either singly or in very low numbers (less than 5). These included *Rhipicephalus (Boophilus)* species around the neck and shoulder and *Amblyomma hebraeum* in the inguinal and peri-anal region.

##### 4.1.2 Rectal temperature

Ambient temperature in Malelane during summer is very high and the evaporation factor low due to high humidity. This often led to very high rectal temperatures on hot and humid afternoons. These were, as expected, on average higher than morning

temperatures. As morning temperatures were most indicative of true pyrexia, only these are recorded here (see Figures 2 to 5). The earliest true pyrexia (higher or equal to 40 °C) was observed in animal 218 at 33 days post-release into the tick-infested camp. The days to first pyrexia for the other animals were as follows: 210 on day 35; 222 on day 36 and 242 on day 37. The fever reactions in the animals had specific temporal relationships with blood smear and lymph node size changes (see Figures 2 to 5). Rectal temperatures remained abnormally high for 24 to 48 hours after anti-theilerial therapy.

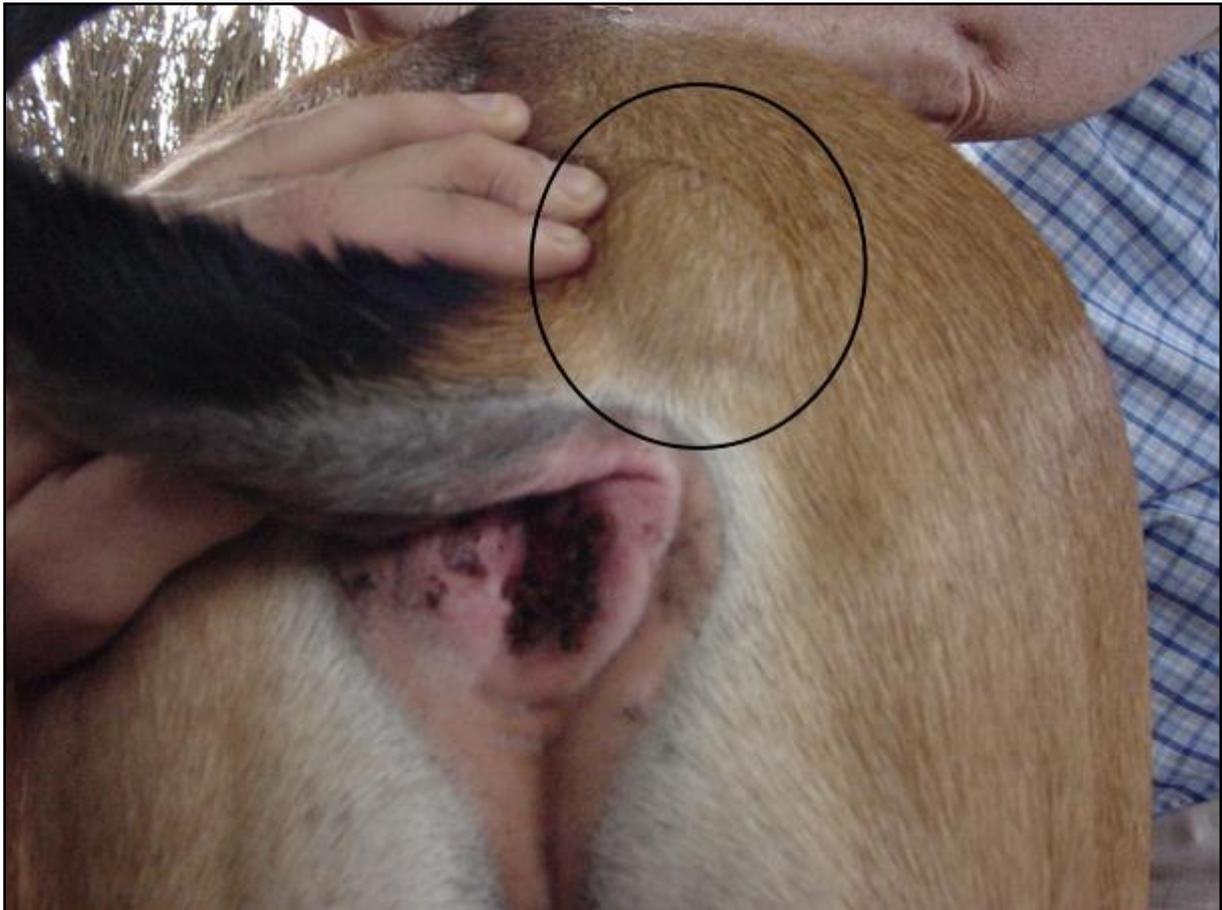
#### **4.1.3 Habitus**

Initially (2 weeks into the trial) the animals showed slight tick worry but they soon became habituated. All animals were feeding well and scored 4 continuously until the day of pyrexia. Although still drinking milk, enthusiasm decreased and all animals lost their appetite within 24h after the first fever. All scored around 1 to 2 at this stage. The duration of poor habitus was 2 and 3 days longer for animals 222 and 242 respectively and continued while their rectal temperatures remained high. Within 24h after the rectal temperatures dropped to within the normal range, habitus steadily improved over 3 days. Grazing activity was poor for all animals during the first 10 days after treatment for theileriosis. Around 2 weeks after treatment for theileriosis all animals showed significant loss in body condition, which relates to poor habitus during recovery from the infection.

#### **4.1.4 Peripheral lymph node palpation**

The superficial cervical lymph node (SLn) was the easiest to palpate and score. As the tick infestation increased (see tick infestation), a small increase in size of all four animals' SLn's could be detected, all scoring around 2 from 2 weeks after introduction to pasture. Five days prior to the first animal (218) showing pyrexia, the anorectal lymph nodes (ALn), bilateral to the tail base, were visibly enlarged without the need of palpation (score 4 ) (Figure 1a). At the time of first fever, marked general

enlargement of peripheral lymph nodes could be detected, scoring between 3 and 4 (Figure 1b). This temporal pattern was the same for all four animals. Within 7 days after treatment for theileriosis the lymph node size decreased considerably back to a score of 2, with the anorectal nodes becoming visibly and palpably undetectable 10 days after treatment.



**FIGURE 1a:** Marked anorectal lymphadenomegaly – Score: 4



**FIGURE 1b:** Marked superficial cervical lymphadenomegaly – Score: 4

#### **4.1.5 Lymph node aspiration**

At introduction of the roan to tick-infested pasture, lymph node aspirates showed predominantly small lymphocytes, with few blast-transformed lymphocytes and an occasional macrophage. At first enlargement of the SLn. (score 2) there was an increase in the blast-transformed lymphocyte component and eosinophils. This finding continued until the first anorectal lymph node impression smear was performed in reaction to marked enlargement (score 4 on animal 218, 5 days prior to pyrexia). At this stage, no equivalent enlargement could be detected in the SLn's nor any other lympho-centers. Initially, on cytology of ALn aspiration, blast-transformed lymphocytes predominated, some of which were atypical and resembled neoplastic cells. The cytological features in these cells were large, round to ovoid, often indented to bean-shaped nuclei, containing 1 to 2 large nucleoli, and increased

amounts of amorphous basophilic cytoplasm. Regular mitotic figures also featured. It sometimes proved difficult to differentiate between these “neoplastic” lymphoblasts and immature monocytes. No typical theilerial schizonts (Koch’s bodies) could be detected at this stage on the ALn impression smears. At the time of pyrexia, all peripheral lymph nodes scored 4 and impression smears showed marked lymphocytic blast-transformation, in addition to cytoplasmic protozoal inclusion bodies characteristic of theilerial schizonts. Within 1 week of treatment for theileriosis, the impression smears were characterised by decreased lymphoblast numbers, large numbers of pyknotic and karyorrhectic lymphocytes and nuclear debris in the background. Theilerial schizonts were difficult to find from 72 h post-buparvaquone therapy and when present showed marked pyknosis.

#### **4.1.6 Peripheral blood smears**

In comparison to blood smears performed prior to natural exposure, peripheral smears showed increased numbers of eosinophils from about 1 week into the trial that coincided with slight lymph node enlargement (score 2). From 3-5 days prior to pyrexia a progressive atypical lymphoblastosis could be detected. This change continued, with the first cytoplasmic theilerial schizonts demonstrable in lymphoblasts 24 hours prior to the first fever reaction. The first intra-erythrocytic theilerial piroplasms could be seen from the day of fever with numbers increasing as the disease progressed. The numbers of theilerial schizonts and piroplasms decreased over 4–6 days after therapy until only the occasional piroplasm could be detected. Examination for other microscopically detectable haemoparasites was negative.

#### **4.1.7 Mucous membrane examination**

Mucous membrane colour was pink at the start of the trial and remained so until around 5 days after the first fever reaction. Mucous membrane colour at this stage varied from pale pink at first, progressively turning pale yellow around 2 days later.

Animal 242 showed more intensely pale yellow mucous membranes in comparison to the others. Normal pink mucous membrane colour returned within 2 weeks after treatment.

FIGURE 2 - 5: Blood smear, lymph node findings and day of treatment in relation to rectal temperature.

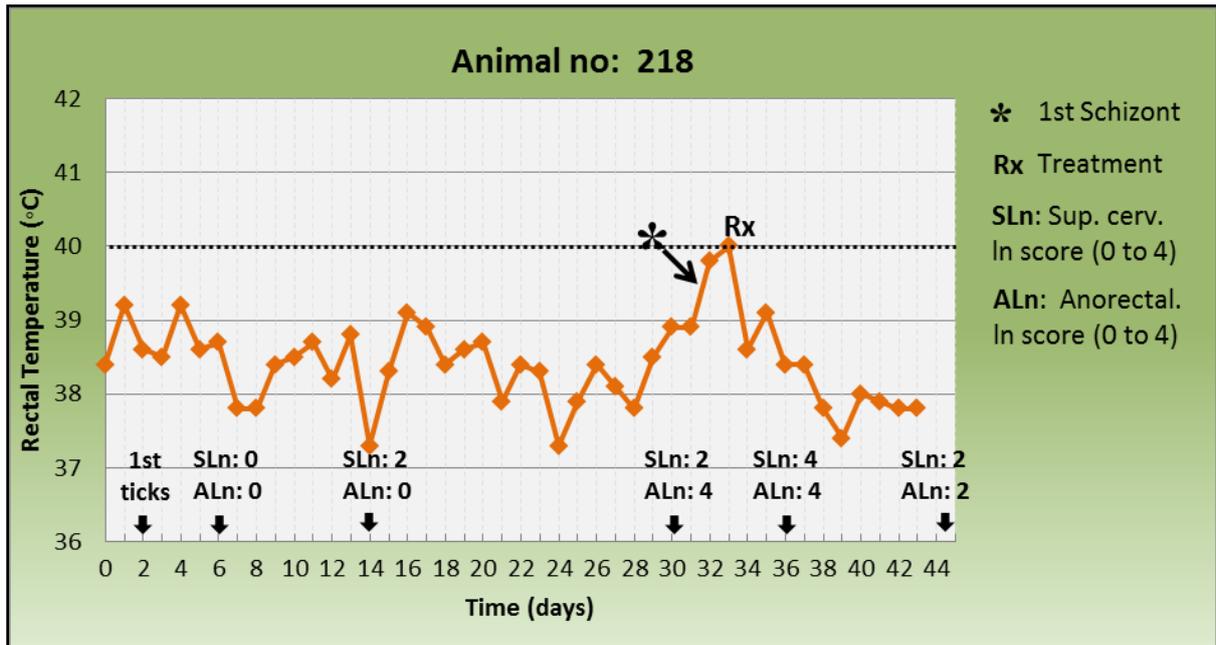


FIGURE 2

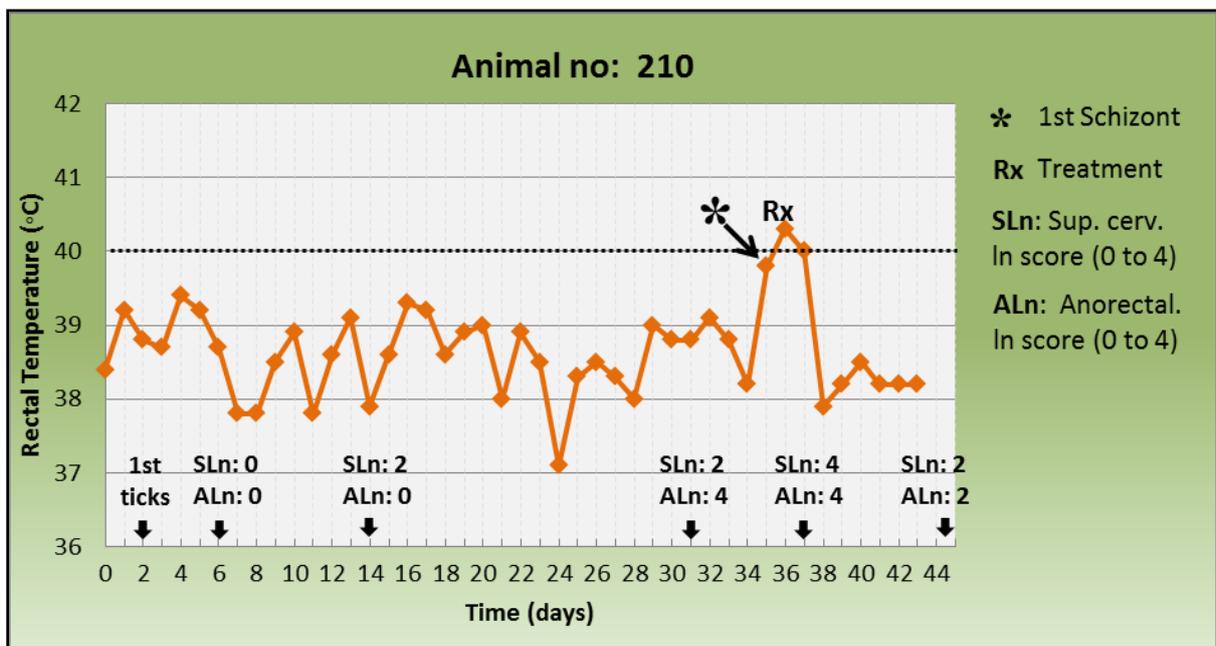


FIGURE 3

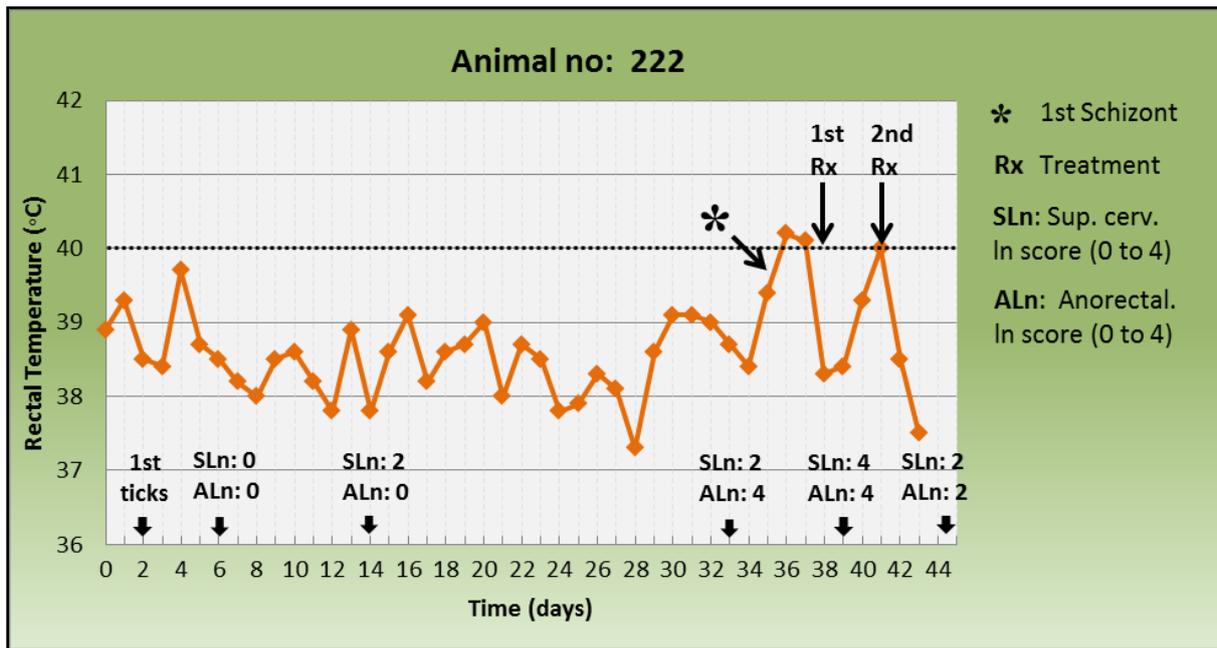


FIGURE 4

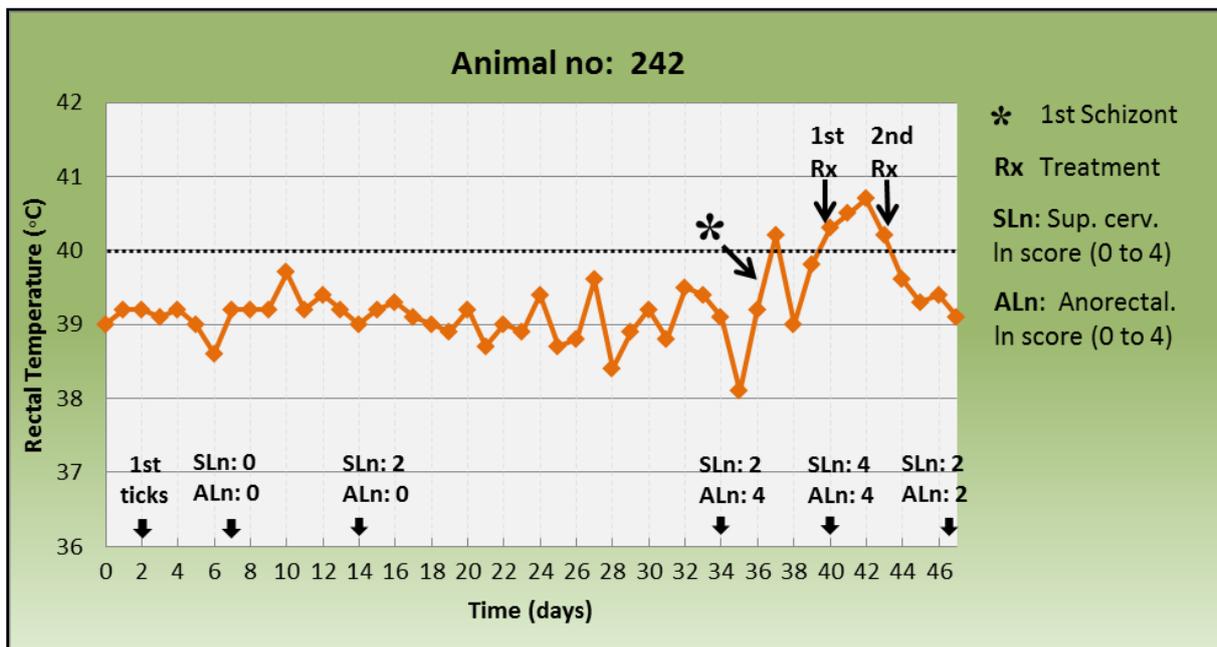


FIGURE 5

#### 4.1.8 Blood samples for PCR detection of *T. sp.* (sable)

PCR (RLB) results for the natural exposure trial showed that all four animals were negative prior to natural exposure. This negative status changed to positive for *T. sp.* (sable) in all four animals at the first fever reaction and remained as such 6 weeks

later.

#### **4.1.9 Treatment of clinical theileriosis**

Animals responded well to treatment with buparvaquone with clinical parameters improving and returning to normal at varying intervals (see above). Animals 222 and 242, which received delayed treatments 2 and 3 days respectively after the first pyrexia, required a second injection of buparvaquone as their rectal temperatures returned to fever range within 48 hours after the initial treatment. In comparison, the other two animals showed a significant drop in rectal temperature within 48 hours, and did not require a second injection of buparvaquone.

#### **4.2 Vector identification and stabilate preparation: tick feeding on *T. sp. (sable)* carrier animals**

After recovery from theileriosis, the naturally exposed roan calves were confirmed to be carriers of *T. sp. (sable)* using PCR screening and used as hosts for tick feeding in investigation of tick infectivity and preparation of tick-derived stabilates.

Apart from *T. sp. (sable)*, no other blood-borne protozoa or rickettsiae in the RLB screening panel (Addendum A) was detected in the blood samples. Boma relocation and adaptation occurred uneventfully.

Ear-bag fixation and tick activity inside resulted in considerable discomfort initially, and the animals spent a lot of time attempting to remove the bags. However, no sedation was necessary to limit ear-bag damage. Appetite decreased for the first 2 days, after which it normalised.

At recovery of ticks from the ear-bags, reasonable numbers of engorged *R. appendiculatus* nymphae (approximately 690 (34.5%) in total) were recovered from the

total of 2000 applied to the pair of animals. In comparison, the *R. e. evertsi* infested bags yielded only about 480 (24%) in total from the 2000 larvae applied. Although engorged, many nymphae were still attached at the time of recovery and needed manual removal using a curved forceps, taking care not to injure the mouth parts (Figure 6a & b).



**FIGURE 6a:** Calico cloth ear-bags protecting *R. appendiculatus* nymphae feeding on a roan antelope calf.



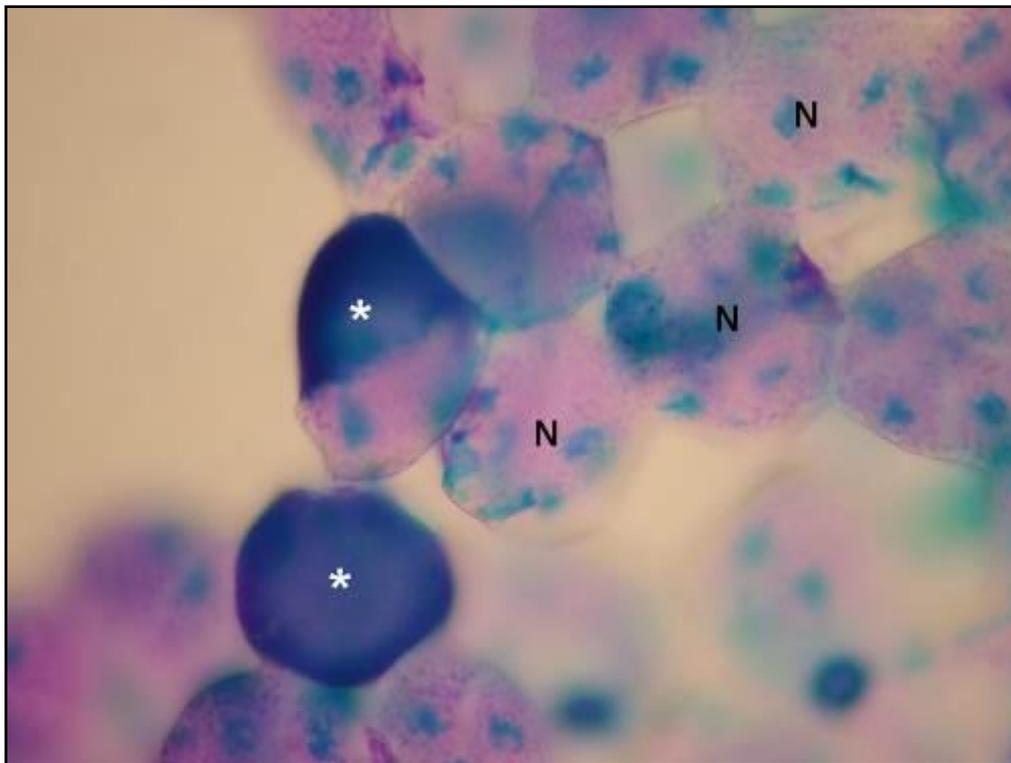
**FIGURE 6b:** Engorged *R. appendiculatus* nymphae ready to be collected.

#### **4.2.1 Salivary gland investigation of pre-fed ticks**

Feeding ticks of both species on rabbits was very successful, with more than 90% of placed ticks attaching after 12 hours, resulting in high recovery rates of ticks after 4 days of feeding. Methyl-green/pyronin tick salivary gland staining revealed infected acini to be enlarged and nuclei obscured by fine granular basophilic contents as described by Walker (1979) (Figure 7). In comparison, normal acini show clearly identifiable basophilic nuclei and pale globular eosinophilic cytoplasm. The quantitative results are illustrated in Table 1.

**TABLE 1:** Ear-bag fed ticks on *T. sp.* (sable) carrier roan antelope: Methyl-green/pyronin tick salivary gland staining.

Assessed parameters	<i>R. e. evertsi</i>	<i>R. appendiculatus</i>
Total ticks dissected	100	150
Total infected ticks	5 (5%)	5 (3.3%)
Infected salivary acini	9	6
Average infected acini /infected tick	1.8	1.2



**FIGURE 7:** Methyl-green/pyronin stain of infected tick salivary glands. \* – Infected tick salivary acini. N – Normal salivary acini

The PCR (RLB) results for 50 dissected salivary glands from *R. e. evertsi* and *R. appendiculatus* were positive for *T. sp.* (sable) in both species.

### 4.3 Infection in field ticks

Field-collected adult ticks (*R. appendiculatus*) recovered from grass in camps where *T.* sp. (sable) infected ticks were known to be present, were subjected to the same feeding, dissecting and staining procedures as above with the results presented in Table 2.

**TABLE 2:** Field collected adult *R. appendiculatus* ticks: methyl-green/pyronin tick salivary gland staining & PCR

Assessed parameters	Results
Total dissected	100
Total infected ticks	9 (9%)
Infected salivary acini	42 (11)†
Average infected acini/infected tick	4.6 (1.2)†
PCR (RLB) for <i>T.</i> sp. (sable) (30 ticks)	Positive

† One tick had 31 infected acini (lesser value indicate number excluding this tick)

### 4.4 Preparation and cryopreservation of tick stabilates

After pre-feeding and removal of ticks for salivary gland dissection, the remaining ticks were used to prepare tick-derived stabilates. The final number of ticks available for grinding was 290 and 450 for *R. e. evertsi* and *R. appendiculatus* respectively. From tick salivary gland dissection and staining results (see above), infection rates were found to be in the region of 5% for both tick species. Based on these data, both stabilates were stored undiluted at 10 ticks per ml, i.e. 0.5 infected ticks/ml.

Only the suspension prepared from *R. e. evertsi* was positive for *T.* sp. (sable) on PCR. No other haemoparasitic infections were demonstrated with RLB screening

(Addendum A).

Despite the addition of antibiotics during stabilate preparation, aerobic bacterial culture of the stabilates yielded *Pseudomonas stutzeria* and *Corynebacterium* species. Fungal culture revealed a *Mucor* species.

#### **4.5 Infection trials with tick-derived stabilates**

Randomly selected, hand-reared roan calves raised under tick-free conditions were used for stabilate infection and titration trials. Various clinical parameters detecting theilerial infection were monitored.

All calves tested PCR negative for *T. sp.* (sable) prior to stabilate inoculation. The stabilate thawing and inoculation procedures were uneventful. No adverse reactions to the inoculum were detected within 6 hours in any animal.

##### **4.5.1 Rectal temperature**

All animals allowed rectal temperature monitoring, with mild manual restraint necessary in some. Although afternoon rectal temperatures were recorded, rectal temperatures in the mornings reflected a more sensitive relationship to a true fever reaction and are illustrated for each animal (Figure 8 & 9). Day 0 represents the day of stabilate inoculation. In comparison with the controls, both animals inoculated with the *R. e. evertsi* stabilate (Figure 8) showed significantly increased rectal temperatures, exceeding 39.5°C, on day 11 and day 12 post-inoculation. Rectal temperatures of both animals inoculated with the *R. appendiculatus* stabilate (Figure 9) stayed within the same range as the average for the control animals (Ave. controls) throughout the 4 week monitoring period. Only the two animals inoculated with the *R. e. evertsi* stabilate required buparvaquone therapy. Animal no 304 was treated twice, 72h apart, as the fever reaction returned (Figure 8). Although both animals were treated at the first sign of fever, they died 19 days (no 303) and 21 days (no 304)

post-inoculation.

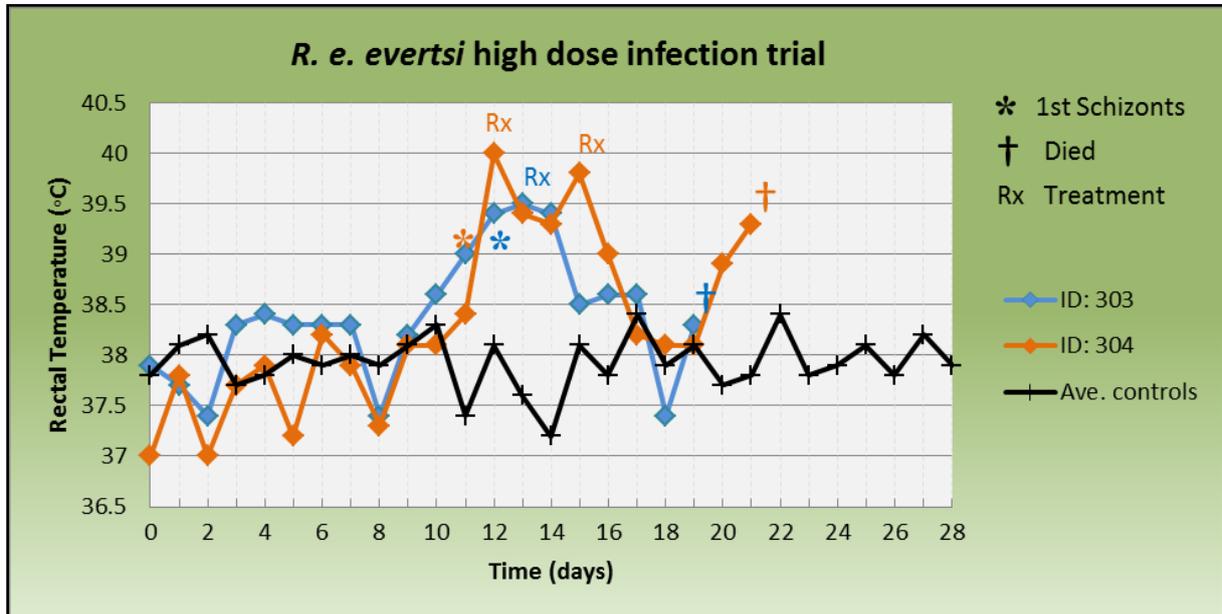


FIGURE 8: *Rhipicephalus evertsi evertsi* high-dose infection trial

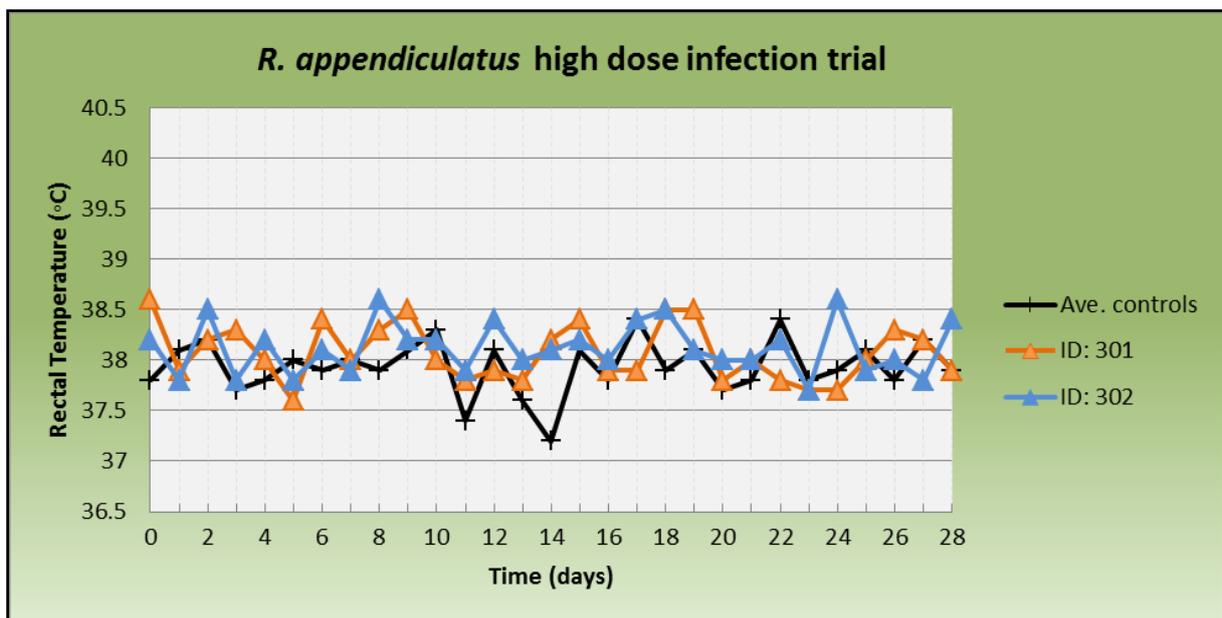


FIGURE 9: *Rhipicephalus appendiculatus* high-dose infection trial

#### 4.5.2 Lymph node palpation and fine needle aspiration

The left superficial cervical lymph nodes of all the stabilate-inoculated animals showed increased size to a score of 2 (2x normal) from day 2 to day 5 post

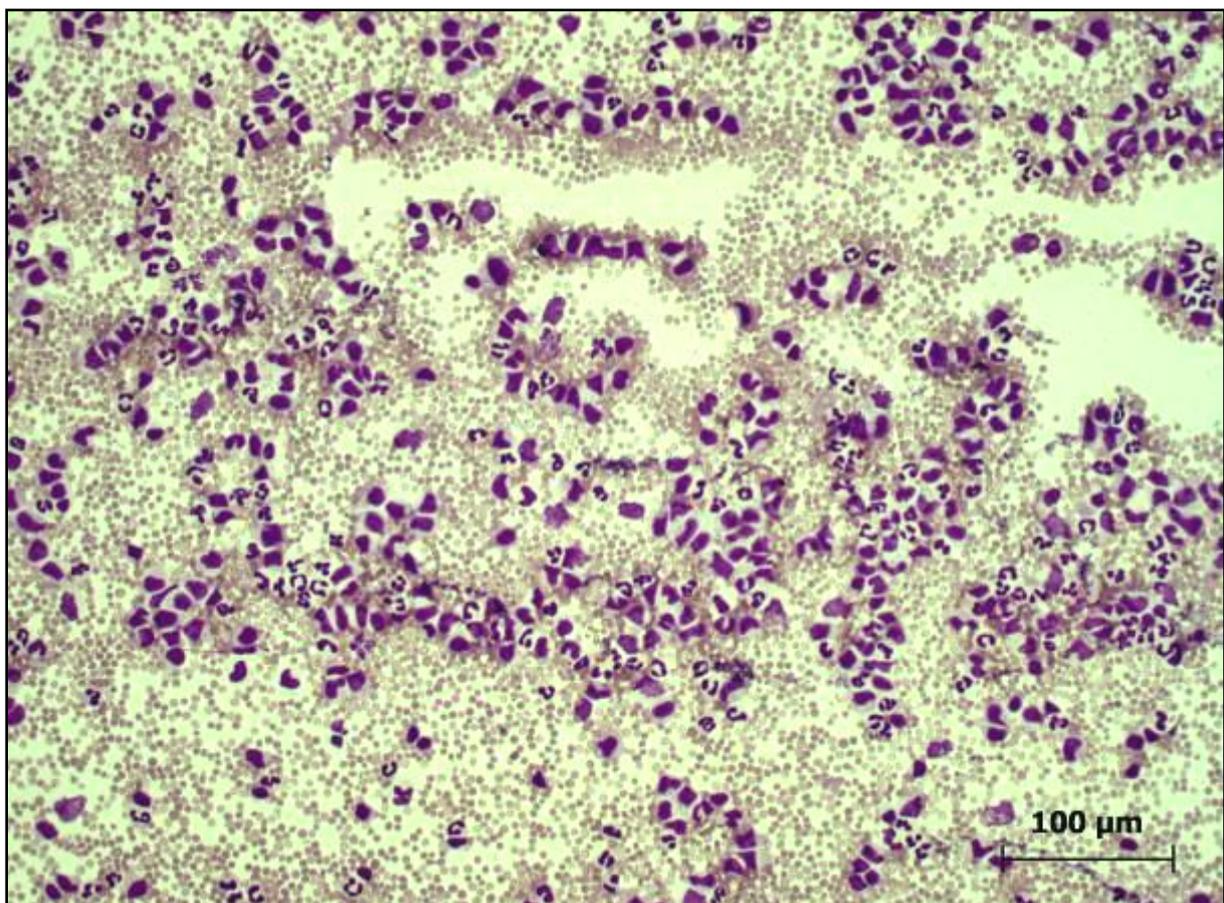
inoculation. The score increased to 3 (3x normal) from day 9, and to 4 (4x normal and visibly enlarged) from day 12 in the *R. e. evertsi* inoculated animals. In addition, a generalised lymphadenomegaly was detected from day 12 post-inoculation. No significant increase in size after the post-inoculation reaction was detected in the *R. appendiculatus* group. The control group lymph nodes remained at a score of 1 (normal) throughout the monitoring period.

Fine needle aspirate results on day 3 revealed a mixed population of lymphocytes with a large number of lymphoblasts present in all the stabilate inoculated animals. No theilerial schizonts could be detected in any of the smears at this stage. On Day 9 lymphoblastic transformation of most lymphocytes, with large amounts of free nuclear and cytoplasmic debris indicative of accelerated apoptotic activity, was present in the *R. e. evertsi* inoculated group. No theilerial schizonts were detected. The first theilerial schizonts were detected in the *R. e. evertsi* group lymph node aspirates during the first fever reaction.

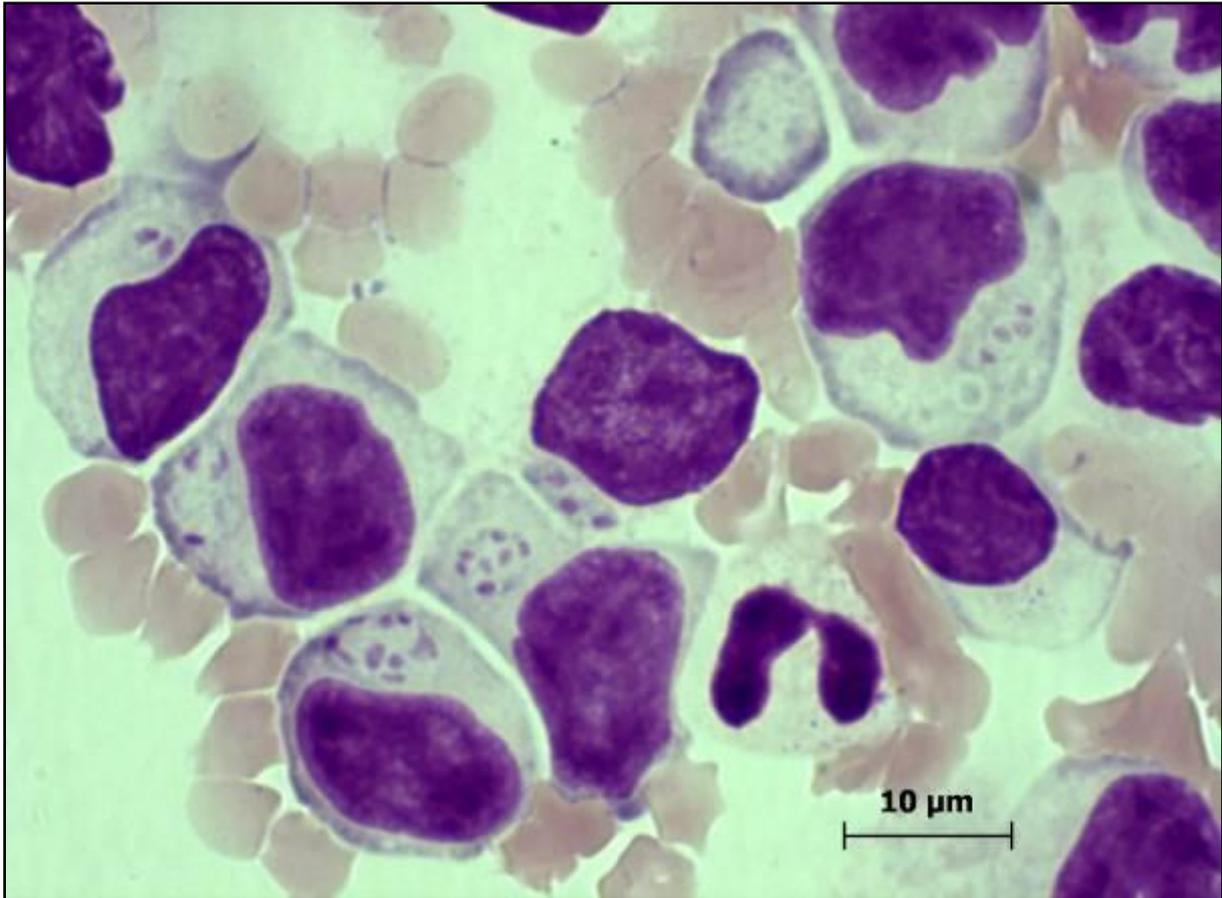
#### **4.5.3 Peripheral blood smears**

Peripheral blood smears of all animals at birth showed marked hypochromic poikilocytosis. Erythrocytes had normalised by the time the animals entered the stabilate inoculation trials. No abnormality on blood smears could be detected for the first 9 days post-inoculation. On day 10 after *R. e. evertsi* stabilate inoculation marked blast-transformed lymphocytosis could be demonstrated in both animals. Many of these cells were difficult or impossible to distinguish from monoblasts. On day 10 to day 11 the lymphoblastosis escalated to an atypical lymphoblastic/monoblastic leukaemoid reaction with scant *Theileria* schizonts demonstrable in the cytoplasm (Figure 10a & 10b). This finding slightly preceded the fever reaction detectable on day 11 to day 12 in this group. Lymphoblastosis persisted for 3 days post-pyrexia, and theilerial schizont numbers increased exponentially. Shortly after the first theilerial schizonts were observed the first theilerial piroplasms made their

appearance. Their numbers also increased dramatically over the next 3 days. Parasite degeneration could be demonstrated microscopically 2 to 3 days after buparvaquone therapy. Piroplasm numbers subsequently decreased significantly, but they never entirely disappeared from blood smears. The control and *R. appendiculatus* inoculated groups showed no significant haematological deviation from the norm. No other blood-borne micro-organisms were detected microscopically during the 4-week monitoring period.



**FIGURE 10a:** Mononuclear leucocytosis/atypical lymphoblastosis (Diff Quik® 100xmag.).



**FIGURE 10b:** *Theileria* schizont infected mononuclear leukocytes/lymphoblasts (Diff Quik® 1000xmag.).

#### 4.5.4 Blood samples

All animals were negative for *T. sp. (sable)* by PCR prior to stabilate inoculation. Blood samples collected at pyrexia (i.e. day 12 and day 13 for animals no 304 and 303, respectively) after *R. e. evertsi* stabilate inoculation were positive for *T. sp. (sable)* for both animals. Samples from the *R. appendiculatus* group and the controls remained negative throughout. No other haemoparasites were detected with the RLB method (Addendum A) in any of the trial animals.

#### 4.5.5 Mucous membrane colour

From day 14 post-inoculation, 3 days after the fever reaction, mucous membrane colour of the two calves inoculated with the *R. e. evertsi* stabilate faded to pale-pink,

gradually changing to pale-yellow with terminal multifocal conjunctival petechiae at around day 19 to day 21. No significant change could be detected in mucous membrane colour in any of the control and *R. appendiculatus* stabilate inoculated animals.

#### 4.5.6 Habitus

All animals scored 4 at the start of the trials. At onset of the fever reaction in the *R. e. evertsi* inoculated group, the calves' appetite decreased and their habitus deteriorated quickly from a score of 4 on day 9 to a score of 1 by day 14. Once they refused to drink milk, dehydration slowly set in and contributed significantly to clinical deterioration. Habitus never returned to normal for these animals, even after the fever reactions dissipated following buparvaquone therapy. The habitus of the control animals and *R. appendiculatus* inoculated group never dropped below a score of 4.

The control and *R. appendiculatus* trial calves were hand reared until weaning, from where they were released into camps where *T. sp.* (sable) infected ticks were known to be present, for a natural exposure trial (see below).

#### 4.5.7 Post mortem examination of mortalities

Macroscopical findings at necropsy were similar for both animals that died following the *R. e. evertsi* stabilate infection. General changes included severe anaemia, icterus (Figure 11), dehydration, multi-organ petechiation/ecchymosis (Figure 12), peripheral and visceral lymphadenomegaly due to cortical lymphoid hyperplasia (often difficult to distinguish from neoplastic lymphoid proliferation). Specific organ lesions identified in order of importance were: severe terminal pulmonary oedema (Figure 13); acute, peri-acinar hepatitis and cholestasis; multifocal, suggillative ("paint-brush") serosal and injection site intramuscular haemorrhage; mild to moderate enterorrhagia; moderate to severe splenomegaly due to red pulp

hyperplasia (Figure 14); moderate bilirubinuria.



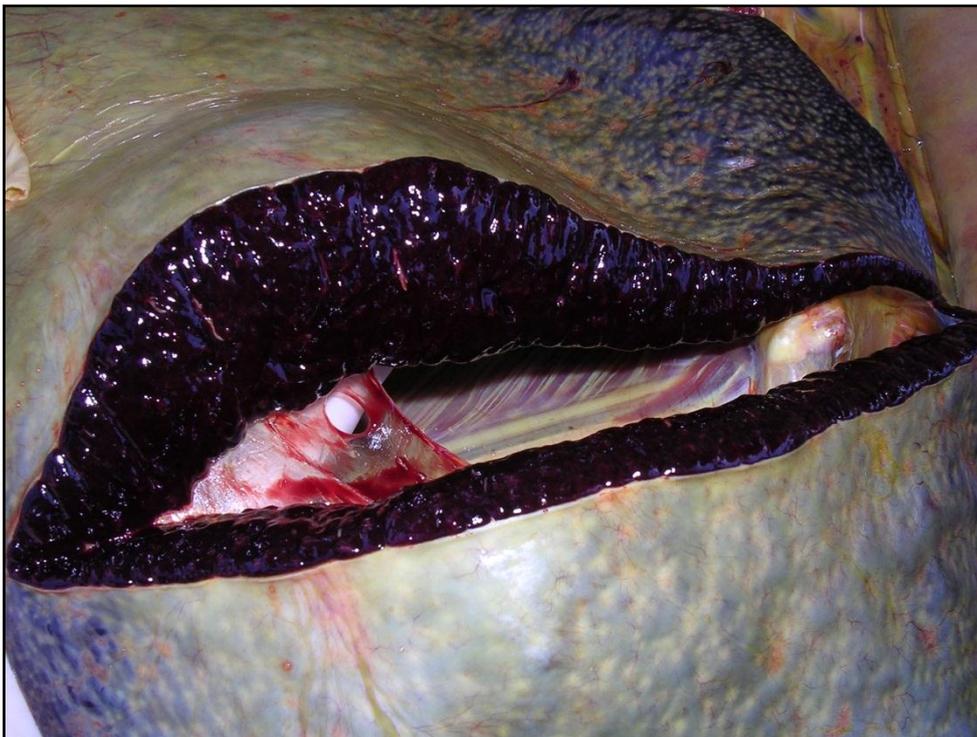
**FIGURE 11:** Conjunctival mucous membrane showing anaemia masked by intense icterus and anaemia.



**FIGURE 12:** Abdominal and thoracic evisceration. Intense generalised icterus, severe splenomegaly and serosal ecchymosis to "paint brush" suggillations.

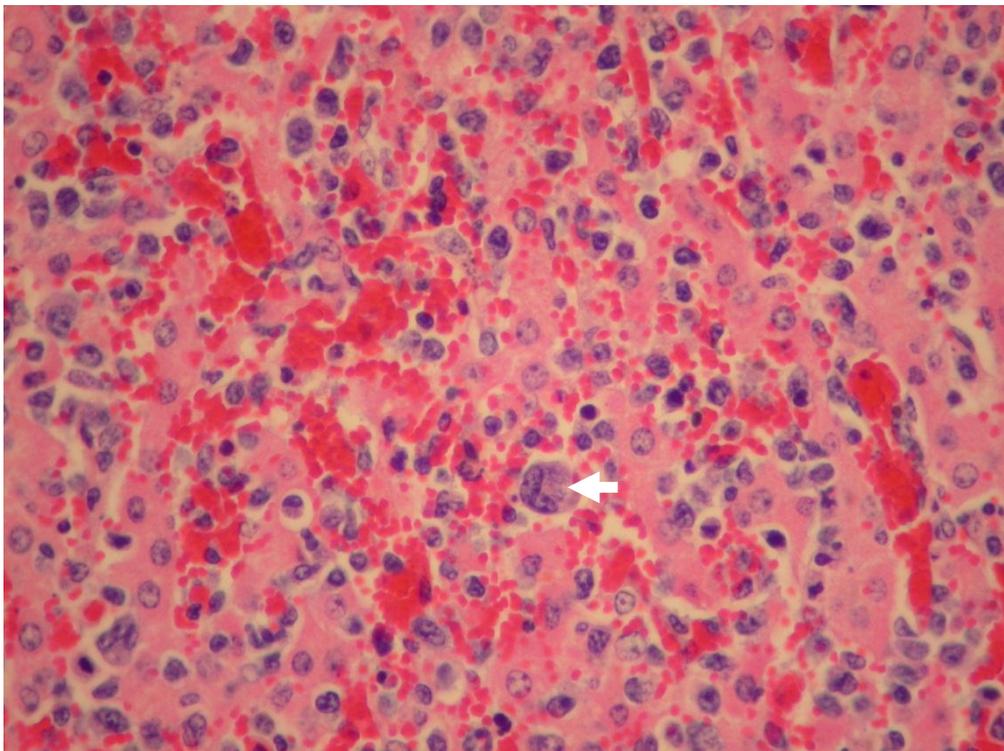


**FIGURE 13:** Sectioned lung and trachea showing intense icterus and terminal pulmonary oedema.



**FIGURE 14:** Sectioned spleen showing bulging red pulp indicative of red pulp hyperplasia due to erythrophagocytosis.

Histological examination revealed multi-organ, interstitial, blast-transformed mononuclear leukocyte (lymphoid/monocytic) infiltration often associated with haemorrhage and scant theilerial schizont infection. In lymph nodes, marked disturbance of architecture occurred due to blast-transformed mononuclear leukocyte proliferation, making it difficult to differentiate from neoplastic behaviour. Protein-rich fluid filled a large percentage (> 50%) of pulmonary alveoli, occasionally associated with haemorrhage. Hepatic sinusoids and portal triads showed marked infiltration and proliferation of blast-transformed mononuclear leukocytes with moderate sublethal vacuolar injury of hepatocytes in the peri-acinar region. Prominent bile pigment accumulation was present in inter-hepatocellular canaliculi. Apart from the interstitial changes (see above), bilirubin pigment could be demonstrated in renal tubules. Splenic changes included severe red pulp expansion due to erythrophagocytic red cell trapping in addition to mononuclear leukocytic proliferation.



**FIGURE 15:** Liver. Marked sinusoidal mononuclear leukostasis. Arrow: intracytoplasmic theilerial schizont (400 x magnification Haematoxylin & eosin staining).

## 4.6 Titration of tick-derived stabilates

In an attempt to determine an infective dose with minimal or no clinical adverse effects, titration of tick stabilates was performed.

In the light of no significant clinical response in comparison to the control group during the infection trial, it was decided not to proceed with a titration trial involving the *R. appendiculatus* stabilate. The results here thus reflect only titration trials using the *R. e. evertsi* stabilate.

### 4.6.1 Rectal temperature

The two animals inoculated with a 10-tick equivalent dose of stabilate showed rectal temperatures above 39.5°C from day 15 to 16 post inoculation (Figure 16). Animal no 318 showed a secondary fever reaction on day 19. This animal died 7 days later. The group that received the 5-tick equivalent dose showed a considerably delayed response with fever reactions occurring around day 21 for animal no 324 (Figure 17). Animal no 320 never reached 39.5°C. However, a significant rising trend in temperature around day 26 to a maximum of 39.3°C, and correlation with other monitoring parameters that showed systemic theilerial development, confirmed a positive reaction in this animal.

Upon reaching the fever threshold of 39.5°C, all animals were treated with buparvaquone. Rectal temperatures dropped significantly to within normal range for all animals within 36 hours after treatment. Animal no: 318 in the 10-tick dose group received a second treatment 3 days after the first due to a secondary fever reaction. Animal no 320 was treated with buparvaquone on detection of theilerial schizont infection, even though it never developed a fever reaction.

Rectal temperatures of both animals in the control group remained within the normal range throughout the 4-week monitoring period.

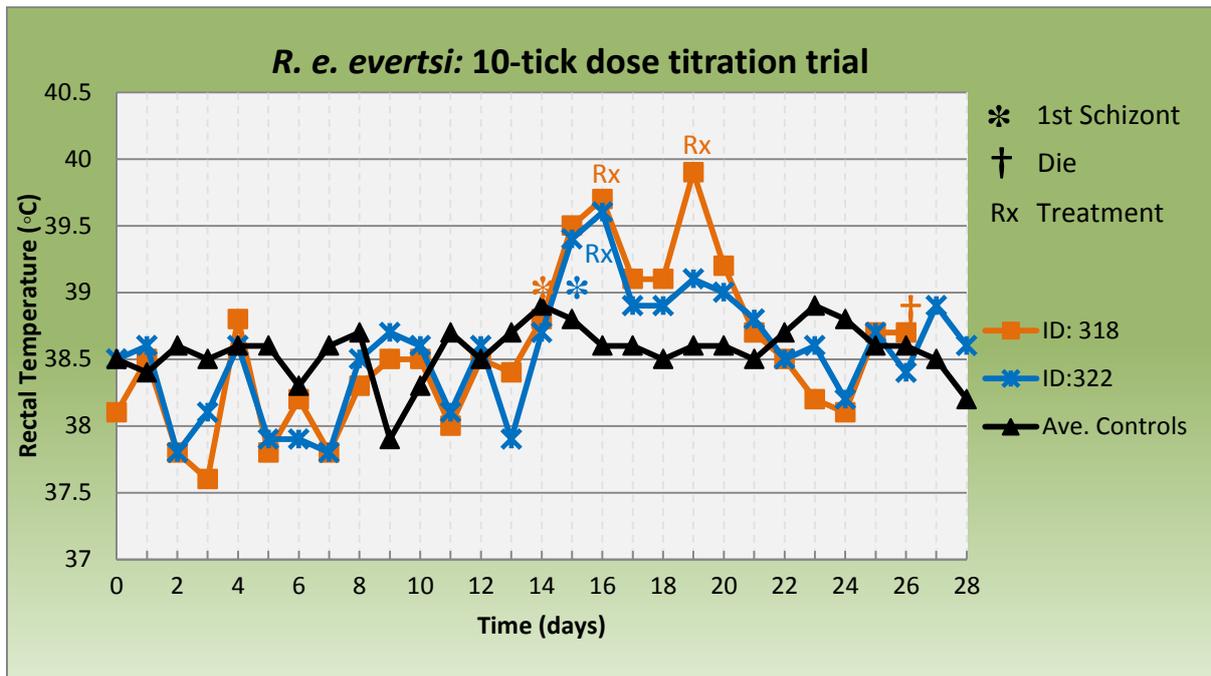


FIGURE 16: *Rhipicephalus evertsi evertsi* 10-tick dose titration trial

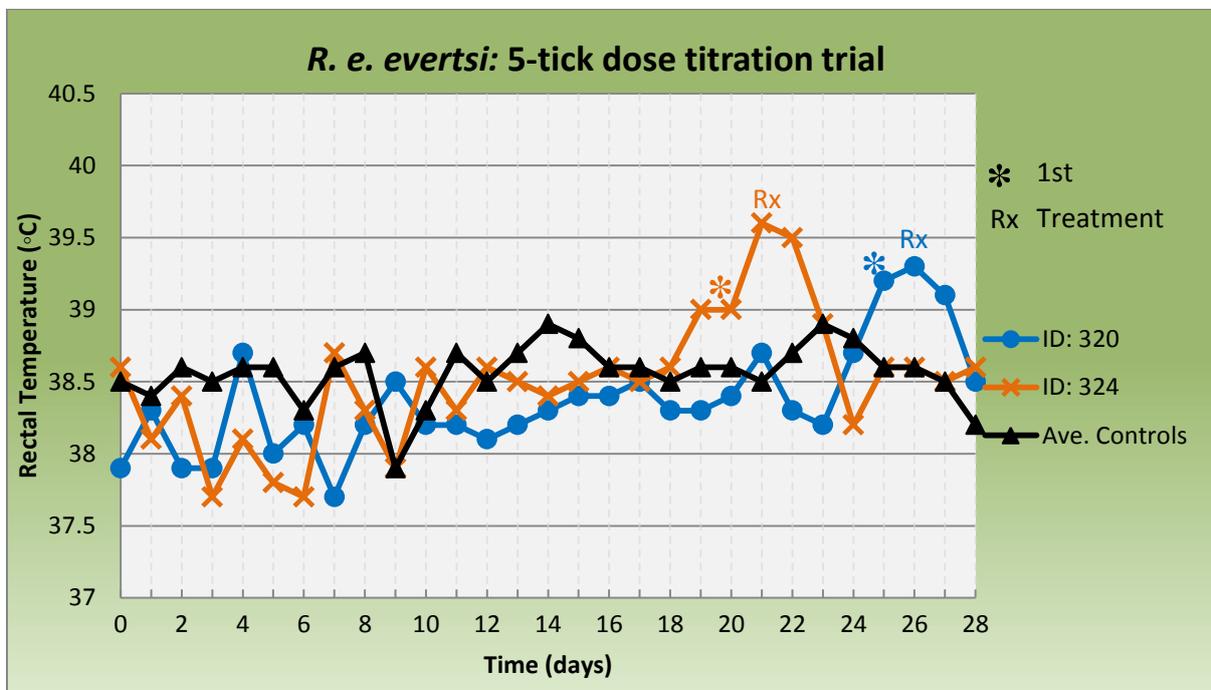


FIGURE 17: *Rhipicephalus evertsi evertsi* 5-tick dose titration trial

#### 4.6.2 Lymph node palpation and fine needle aspiration

The left superficial cervical lymph nodes of all inoculated animals in the titration trials showed a delayed but similar pattern of development in relation to the fever reaction as in the high dose infection trials. All animals in the stabilate titration groups showed significant increase in size of other peripheral lymph nodes around 3 days prior to their respective fever reactions. The control group lymph nodes remained normal throughout the monitoring period.

Fine needle aspirate results showed the same changes associated with accelerating lymphoblastic activity responsible for increasing lymph node sizes as in the infection trial. Theilerial schizonts could be detected only during and shortly after a fever reaction was recorded.

#### **4.6.3 Peripheral blood smears**

Blood smears remained normal until 24 hours prior to the fever reaction, when lymphoblastosis was detected, but at lower levels than in the infection trial. Theilerial schizont numbers were scant at the time of the fever reaction but increased significantly for two days after the fever reaction, followed by decreasing numbers and degeneration after buparvaquone therapy. Theilerial piroplasms occurred in low numbers from the first appearance of schizonts. Occasional piroplasms could be detected continuously in blood smears after anti-theilerial therapy.

For animal 320 in the 5-tick dose titration group, a very mild lymphoblastic response was detected around day 26. Theilerial schizonts were present but very difficult to find.

The control group showed no significant haematological deviation from the norm. No other blood-borne micro-organisms were detected microscopically during the 4-week monitoring period.

#### **4.6.4 Blood samples**

As with the infection trials, all animals tested PCR (RLB) negative for *T. sp.* (sable) at the start of the titration trials. Samples collected at the time of the fever reactions and subsequently for both the 10 and 5-tick dose groups tested PCR positive for *T. sp.* (sable). No other haemoparasites were demonstrated with the RLB method (Addendum A) in any of the trial animals.

#### **4.6.5 Mucous membrane colour**

Mucous membrane colour for both titration groups turned from pink to pale-pink between 3 to 5 days after their respective fever reactions. Normal pink colour returned for animal no 322 in the 10 tick dose group and both the animals in the 5-tick dose group. For animal no 318, however, the mucous membrane colour deteriorated to pale-yellow around day 20 and remained as such until it died on day 26 post-inoculation. No significant change could be detected in mucous membrane colour in the control animals.

#### **4.6.6 Habitus**

All animals selected for the titration trial were in good health with a habitus score of 4 at the start of the trials. As expected the habitus of the titration groups dropped to a mean score of 2.5 for 2 days, starting at the respective fever reactions. All animals kept suckling, albeit reluctantly, during this period. At the time of the second fever reaction, animal no 318 in the 10-tick dose group stopped drinking, and its habitus score dropped to 1 until it died 7 days later. Dehydration and starvation contributed significantly to this low score and ultimately death. The habitus for the control animals never dropped below a score of 4.

#### **4.6.7 Post-mortem examination of mortalities**

No significant difference in necropsy findings could be demonstrated in animal no 318 that died during the titration trial in comparison to the animals in the infection

trial. Histopathologically, however, a lesser degree of mononuclear leukoblastic infiltration occurred in organs.

#### 4.7 Post-infection field exposure

Following the stabilate-infection and titration trials, all animals were exposed to natural infection by release into tick-infested veld where roan were known to contract theileriosis. Animals were monitored and treated on confirmation of clinical theilerial infection.

At weaning, the 2 animals inoculated with the *R. appendiculatus* stabilate and the controls from the infection and titration trials all tested PCR negative for *T. sp.* (sable). Simultaneously, the 3 calves that survived from the 10 and 5-tick dose *R. e. evertsi* titration trials were still PCR positive. Immobilization and relocation of these animals to the vegetated camp where roan were known to have become infected and die from theileriosis occurred uneventfully. The trial was performed during the months of January and February, which is mid-summer in a summer rainfall area. All animals became tick infested within 1 week of relocation. As expected, the predominant tick species were *R. appendiculatus* and *R. e. evertsi*. A fair number of *Rhipicephalus decoloratus* and/or *R. microplus* and a few *Amblyomma* and *Hyalomma* species were also recorded.

Detection of illness was first recorded in one of the animals of the titration control group around 35 days post-relocation. Signs were lethargy, mildly listless gait, drooping of ears and lagging behind the other animals. On closer examination the anorectal lymph nodes were visibly enlarged bilaterally, and the respiration rate was increased in comparison with the others. The animal was sedated for further examination including rectal temperature, blood collection in EDTA, blood smear, lymph node palpation and mucous membrane inspection. The rectal temperature was 40.3°C; peripheral lymph nodes were 4x enlarged and mucous membrane colour

was pink. A preliminary diagnosis of theileriosis was made and the animal treated with buparvaquone. Microscopic examination of the peripheral blood smear confirmed the diagnosis of theileriosis through the identification of numerous blast-transformed lymphocytes that contained theilerial schizonts. Scant intra-erythrocytic theilerial piroplasms were also present at that stage. PCR testing revealed that the animal was infected with *T. sp. (sable)*.

In the following 10 days all the other control animals and the *R. appendiculatus* stabilate inoculated animals contracted clinical theileriosis due to *T. sp. (sable)* and were treated. All animals recovered uneventfully. The three animals from the *R. e. evertsi* titration trials showed no sign of clinical theileriosis throughout the monitoring period and beyond.

## Chapter 5

### DISCUSSION

No attempt has been made previously to identify the vector of theileriosis in roan, but field observations that outbreaks usually coincided with heavy infestations with *R. appendiculatus* led to the assumption that this species was the vector. *R. e. evertsi* has been viewed as an apparently incidental parasite, but was included in the investigation as it is known to transmit other *Theileria* species e.g. *T. equi* which is pathogenic in horses (Theiler 1906) and *T. separata* that is non-pathogenic in sheep and goats (Uilenberg 1997).

In ticks fed experimentally on carrier (low, non-clinical piroplasmaemia) animals, no statistically significant difference for infection level per infected tick and number of ticks infected with *T. sp.* (sable) could be demonstrated between *R. e. evertsi* and *R. appendiculatus*. From these findings, it can be concluded that both species become infected with *T. sp.* (sable) and are potential vectors.

The site of lymphadenomegaly was a very important finding in support of identifying a vector for theileriosis in roan exposed to natural tick infection. It is well known, from theilerial kinetic studies, that the local draining lymph node/s are first to enlarge during the initial stages of theileriosis in cattle (Morrison *et al.* 1981). During natural tick exposure the anorectal lymph node was the first to enlarge. The drainage area for this lymph node includes the peri-anal region and tail. The only ticks feeding at this site at the time of lymphoid hyperplasia were *R. e. evertsi* adults, suggesting that *R. e. evertsi* will under endemically stable field conditions transmit *T. sp.* (sable).

The epidemiological role of *R. appendiculatus* in theileriosis of roan antelope is not clear, however the fact that it becomes infected when feeding on carrier animals suggests an important role. Heavy salivary gland infection was shown to occur in field-collected *R. appendiculatus* ticks from camps where recent mortality due to theileriosis occurred in roan. This suggests that some field-collected ticks were feeding on highly piroplasmaemic hosts and were capable of picking up heavy infection rates. It indicates that *R. appendiculatus* has the potential to not only infect but also to expose animals to heavy infection, triggering and possibly perpetuating clinical disease within a population. Epidemiological surveys indicate that theileriosis in roan antelope occurs mostly in regions of South Africa where both tick species occur (personal observation). Infection without disease mostly occurs in regions where only *R. e. evertsi* is found. This suggests that *R. appendiculatus* may play an important role as co-vector with *R. e. evertsi* to exceed the subclinical threshold infection dose resulting in disease. In addition, it has been shown experimentally in rabbits that *R. appendiculatus* has immunosuppressive (lymphocytotoxic) effects on the host, increasing susceptibility to clinical disease (Fivaz 1989).

In the natural infection trial higher numbers of *R. appendiculatus* ticks attached around the same time as *R. e. evertsi*. With evidence showing that *R. appendiculatus* can become infected with *T. sp* (sable), questions pertaining to its ability to transmit this *Theileria sp.* arose. An important factor to consider, in assessing this ability of *R. appendiculatus* under natural conditions, is a diapause stage that exists in its life cycle in southern African populations. The diapause stage has been shown to decrease transmission efficiency of *T. parva* and may also be true for *T. sp* (sable) (Norval *et al.* 1991). Other factors to consider that may influence transmission success of *Theileria* species in the field include sex ratio of the ticks (Young & Leitch 1981) and host resistance to the specific tick species (Fivaz 1989).

Tick-derived stabilates prepared from *R. appendiculatus* and *R. e. evertsi* tested PCR

negative for *T. sp.* (sable) in the case of *R. appendiculatus*, but positive in *R. e. evertsi*. The infection trials also supported these results, indicating that *R. e. evertsi* is an important vector of theileriosis in roan antelope. In the case of *R. appendiculatus*, the stabilate could not induce infection, although the ticks were infected with *T. sp.* (sable). Technical error during stabilate preparation may have resulted in theilerial denaturation.

In the *R. e. evertsi* infection and titration trials rectal temperature measurements showed that the incubation period is dose dependent, with the incubation period being inversely proportional to the infection dose. At high dose rates the incubation period was between 11 and 12 days. At lower dose rates it could be extended up to 26 days. This finding is consistent with what was recorded in other theilerial infections (Jarrett, Crichton & Pirie 1969, Radley *et al.* 1974). The shorter the incubation period the higher is the probability of fatalities, even when animals were treated timeously with an effective anti-theilerial drug such as buparvaquone. Clinical disease has been reported to be proportional to sporozoite infection dose for *T. parva* infection in cattle (Wilson 1950, Radley *et al.* 1974, Dolan *et al.* 1984). Predictability of expected fever reactions seems to decline with lower infection doses, as reflected in significantly longer incubation periods between the animals in the 5-tick equivalent stabilate dose titration trial. This may be an indication of individual variation in development of immunity to theilerial infection.

During the natural infection study, animals started showing signs of theilerial infection between day 33 and 37 after field introduction. From the stabilate titration trials the longest incubation period was with the 5-tick dose infection at between 21 and 26 days. Adding the number of days (5 days) until field ticks were observed to attach for the first time on the animals, and allowing for tick salivary maturation of about 3-4 days before sporozoite inoculation occurs, it totals around 34-35 days. It can be estimated that the field tick exposure was equivalent to or possibly less than

in the 5 tick equivalent (0.25 infected ticks) stabilate exposure trial.

The use of buparvaquone as anti-theilerial chemotherapy is highly effective against theileriosis in roan antelope, but a delay (2 to 3 days) in treatment in relation to the first day of pyrexia resulted in most animals requiring a second injection. With high dose experimental infection, timeous treatment at first fever could not prevent eventual mortality. These findings revealed the importance of administering buparvaquone as close as possible to or even prior to the initial fever reaction. The efficacy of buparvaquone decreases considerably if administered after the fever reaction and if the primary sporozoite exposure dose is high (the threshold dose was not determined). PCR positive results for *T. sp.* (sable) weeks after buparvaquone therapy indicates that buparvaquone did not sterilize *T. sp.* (sable) infection. This is an important characteristic required from a drug that is to be used in an infection-and-treatment immunisation strategy against theileriosis, as sterilisation will render the animal susceptible to reinfection.

It was the first time that theileriosis, which has been well studied in cattle, has been followed clinically in detail under field conditions and following experimental infection in a wild antelope species. Previously, limited observations were recorded with experimental and natural *T. taurotragi* infection in eland antelope (Grootenhuis *et al.* 1980). Careful monitoring of various clinical parameters revealed many similarities in the clinical development and progression of theileriosis (especially East Coast fever) in cattle and in roan antelope.

Post-pyrexia changes in the mucous membrane colour of the trial animals were indicative of anaemia (pale pink) progressing to anaemia with icterus (pale yellow). These changes are consistent with signs occasionally observed in cattle suffering from East Coast fever (*Theileria parva*) (Lawrence, Perry & Williamson 2004) or *T. annulata* infection (Pipano & Shkap 2004).

Loss of body condition during the post-therapeutic recovery phase in the natural infection trial was mostly likely due to calves spending less time grazing and more time lying down and resting, although they still reluctantly drank milk. Loss of red cell mass and systemic inflammation also accounted for additional nutritional demand on the animal.

Peripheral blood smears of all animals at birth showed marked hypochromic poikilocytosis, reminiscent of sideroblastic anaemia, but speculated to be normal physiology of roan antelope (Parsons *et al.* 2006). This trait may have an important role to play in susceptibility to theilerial infection. Any condition adversely affecting erythrocyte count (even if physiological) will seriously limit the host's ability to respond to, and survive a haemolytic disease such as theileriosis. Eosinophilia detected on peripheral blood smears 1 week after field exposure to ticks was an expected finding with first-time exposure to ectoparasites (ticks).

In the infection trials, blood smear findings showed predictive value for pyrexia, with a lymphoblastic leukaemoid reaction occurring within 1 to 2 days prior to a fever reaction. There was a correlation between the infection dose and lympho-/monoblastic leukaemoid response in blood smears, where higher infection doses showed a comparatively stronger leukaemoid reaction. This finding may prove to have predictive value regarding clinical prognosis, where all animals with severe reactions died. Lymphocytolysis/monocytolysis in theilerial infections could result in fatal generalised vascular injury due to systemic (esp. pulmonary) cytokine liberation (Brown *et al.* 1995). Hence, the more severe the leukaemoid reaction, the higher likelihood that systemic threshold for free cytokine capacity could be exceeded, followed by multi-organ injury and failure. This pathomechanism was clearly observed at necropsy with generalized petechiation indicative of terminal vascular injury. This contributed to systemic coagulopathy resulting from thrombocytic

consumption and suboptimal hepatic clotting factor production due to hypoxic (anaemia) and endogenous cytotoxic (leukocytolysis) sublethal hepatocellular injury (Maxie *et al.* 1982). Vascular injury also caused proteinaceous pulmonary alveolar oedema and terminal pulmonary failure. Theilerial piroplasmaemia resulted in severe anaemia due to extravascular erythrolysis (splenic erythrophagocytosis) as has been observed with *T. annulata* infection in cattle (Sandhu *et al.* 1998). This also caused hyperbilirubinaemia (icterus) with bilirubinuria. The absence of haemoglobinuria indicates that intravascular haemolysis was not a significant feature in this disease. Pseudoneoplastic lymphoid proliferation in all lymph nodes was comparable to findings of theileriosis in domestic species.

No animal in the field and stabilate titration trials needed supportive therapy for opportunistic infections like pneumonia that are known to be a complication in cattle recovering from East Coast fever due to immunosuppression (Wagner *et al.* 1975, Muraguri 1999). Animals that died during high dose *R. e. evertsi* infection trials also did not show evidence of secondary infections.

In the stabilate infection trials, fine needle aspirates and palpation of lymph nodes showed that the reaction detected from day 2 to 5 after stabilate inoculation was the result of reactive hyperplasia, most probably in response to non-specific antigen present in the stabilate (low-pathogenic bacteria and fungi that were cultured from the stabilate and tick-derived somatic antigen). In the natural infection exposure trial, opportunistic lymph node aspiration may have given a false impression of the time of appearance of theilerial schizonts in the local draining lymph nodes, as they may have been present earlier than the sampling event. In the experimental infection trials schizonts could only be detected around the day of fever in the lymph nodes. In kinetic studies with *T. parva* infection in cattle, schizonts could be detected as early as day 7 post-infection in the primary draining lymph node (Morrison *et al.* 1981).

The young age (as young as 10 days) at which the animals reacted to the theilerial infection, even to the very low stabilate dose of 5 tick equivalents (0.25 infected ticks), emphasized that the non-specific neonatal resistance present in most ruminants against many tick-borne protozoal diseases and passive/maternal immunity do not play a significant role in protecting against clinical theileriosis (Levy, Clabaugh & Ristic 1982, Cunningham *et al.* 1989, Marcotty *et al.* 2002).

These may be some of the reasons for mortality being heaviest in young roan antelope calves as reported from many South African game ranches.

No animals that contracted clinical theileriosis in the field exposure trial died after being treated with buparvaquone. This was in contrast to deaths experienced in the high dose infection and 10-tick equivalent dose titration trials despite treatment. Possible explanations are that the animals in the field exposure trial were much older and heavier (around 3 months older) than the animals in the infection and titration trials, which may have diluted the effective primary sporozoite exposure dose. Older calves might have a stronger, more mature cellular immune system (Marcotty *et al.* 2002) and, lastly, the field exposure may have been much lighter and more spread out.

The production of *R. appendiculatus* derived stabilates from domestic animals such as cattle has many practical challenges. These and more were highlighted during attempts at producing tick stabilates from roan antelope, especially using a two-host species such as *R. e. evertsi* that needs an extended immature feeding stage.

Several factors could be considered as reasons for the relatively poor yield of engorged nymphae at tick recovery from ear-bags. Firstly, the animals, not being sedated, used every opportunity to remove the ear-bags with the hind hooves. Large numbers of ticks died during these attempts. Secondly, the duration of feeding for *R. e. evertsi* provided more opportunity for tick loss and injury. Lastly, "bag fatigue"

which started to set in after about 5 days resulted in ticks being lost through openings at the base of the bags. Nevertheless, tick feeding in ear-bags was still a fruitful exercise and can be applied practically on wild antelope.

Although fungal and aerobic bacterial organisms were isolated from the stabilates prior to use as inoculums, no complication involving any of these organisms was encountered. This may be attributed to low pathogenicity, subclinical exposure doses and optimal passively derived (colostrum intake at birth) anti-fungal/bacterial immune system defences in the trial animals.

In using roan antelope from semi-intensive ranching conditions as donors, a risk of contamination of the tick stabilates with other blood-borne, tick-transmitted organisms was an important factor to consider. However, from PCR screening of the trial animals and tick stabilates for many African haemoprotozoa and rickettsia, it would appear that no such contamination took place. Testing for other tick-transmitted organisms, i.e. viral and bacterial, was not performed. Ways to reduce contamination risk of *T. sp.* (sable) stabilates should be investigated (Pipano, Morzaria & Spooner 2008).

## Chapter 6

### CONCLUSION

Valuable information gathered during field tick exposure demonstrated that *R. e. evertsi* is a vector of *T. sp* (sable) and that *R. appendiculatus* seems to play an important role as co-vector of theileriosis in roan antelope. This was supported in the stabilate infection trials where infection using *R. e. evertsi* was possible even when ticks were fed on subclinical carrier hosts instead of clinically piroplasmaemic hosts. To determine the role of both species in the epidemiology of theileriosis outbreaks in roan, both tick species should be compared separately in tick infection and transmission trials involving carrier vs. piroplasmaemic animals.

This is the first time a wildlife species was experimentally infected with clinical theileriosis using a tick-derived stabilate. The use of a *T. sp* (sable) infected *R. e. evertsi* tick-derived stabilate succeeded in transmitting *T. sp* (sable) to roan antelope and ultimately provided immunity during field exposure. The value and rarity of roan antelope made the use of statistically significant animal numbers in stabilate infection trials impossible.

Clinical theileriosis in roan antelope responds favourably to buparvaquone treatment provided it is administered as close as possible and preferably prior to the primary pyrexia phase. Treatment with buparvaquone did not sterilize the infection in these animals, as persistence of infection was demonstrated by PCR. For more exact pathogenesis studies, parasite transmission and disease monitoring will have to be repeated in a controlled environment with known infection times and dose rates.

The feasibility of making a sporozoite-infected stabilate opens the door to numerous experimental options (*in vitro* and *in vivo*) in further studies of this disease in roan antelope. Drawbacks to large studies and immunization programs will be the

difficulty in producing large quantities of stabilate and the low availability of experimental animals.

The data obtained from the experiments in this study can be regarded as useful preliminary evidence to spark future application of the *R. e. evertsi* tick-derived stabilate under field conditions to determine the practicability of infection and treatment as an immunisation method for roan antelope against theileriosis. The early reaction of the calves to infection provides an ideal window for infection and treatment while still young and separated from the herd (roan antelope individually hide their calves in brush or tall grass up to an age of 2 weeks). Immunization through infection and treatment becomes a viable option for reintroduction programs e.g. captive-bred animals relocated to infected field conditions.

## ADDENDUM

### Addendum A

List of haemoparasites included in PCR (Reverse line blot (RLB) method performed by the Dept. of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria )

<i>Ehrlichia /Anaplasma</i> catch all	<i>Theileria /Babesia</i> catch all	<i>Theileria spp.</i> catch all
<i>A. centrale</i>	<i>B. catch all 1</i>	<i>T. sp. (kudu)</i>
<i>A. marginale</i>	<i>B. catch all 2</i>	<i>T. sp. (sable)</i>
<i>A. phagocytophilum</i>	<i>B. felis</i>	<i>T. bicornis</i>
<i>E. ruminantium</i>	<i>B. divergens</i>	<i>T. annulata</i>
<i>A. bovis</i>	<i>B. microti</i>	<i>T. buffeli</i>
<i>E. chaffeensis</i>	<i>B. bigemina</i>	<i>T. sp. (buffalo)</i>
<i>E. sp. (Omatjenne)</i>	<i>B. bovis</i>	<i>T. mutans</i>
<i>E. canis</i>	<i>B. rossi</i>	<i>T. parva</i>
	<i>B. canis</i>	<i>T. taurotragi</i>
	<i>B. vogeli</i>	<i>T. velifera</i>
	<i>B. major</i>	<i>T. equi</i>
	<i>B. bicornis</i>	<i>T. lestoquardi</i>
	<i>B. caballi</i>	<i>T. annae</i>
	<i>B. gibsoni</i>	
	<i>B. sable</i>	
	<i>B. leo</i>	
	<i>B. occultans</i>	

## **Addendum B** (Pipano, Morzaria & Spooner 2008)

### 1. MEM / 3.5% BSA

3.5g bovine serum albumin (BSA - Cohn fraction V) was dissolved in 100ml Eagles minimum essential medium with Earl's salts – pH 7.2 (MEM). The solution was stirred using a magnetic follower and maintained at 37°C until the BSA was dissolved. Penicillin and Streptomycin were added to give final concentrations of 100units and 100µg/ml respectively. The solution was transferred to a sterile glass bottle. Once the numbers of ticks were known, this solution was made up fresh, stored at 4°C and used within 24 hours.

### 2. MEM / 3.5% BSA / 15% Glycerol

Once the required volume was determined during GUTS preparation, this solution was freshly prepared as follows:

The required volume of Glycerol (analar) was added to MEM / 3.5% BSA at 37°C to give a final concentration of 15%. Due to its viscosity, the measuring cylinder containing glycerol was rinsed several times with the solution. Once dissolved the solution was kept at 4°C until used.

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