

**Immunization of roan antelope (*Hippotragus equinus*)
using *in vitro* cultured *Theileria* species (sable) schizonts**

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IMMUNIZATION OF ROAN ANTELOPE (*HIPPOTRAGUS
EQUINUS*) USING *IN VITRO* CULTURED *THEILERIA* SPECIES
(SABLE) SCHIZONTS

by

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Declaration

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With the exception of the abovementioned assistance this thesis is the candidate's own original work. It is not submitted concurrently in candidature for any other degree.

Summary

IMMUNIZATION OF ROAN ANTELOPE (*HIPPOTRAGUS EQUINUS*) USING *IN VITRO* CULTURED *THEILERIA* SPECIES (SABLE) SCHIZONTS

By

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Theileria species (sable) causes significant mortalities in roan (*Hippotragus equinus*), and to a lesser extent, sable antelope (*Hippotragus niger*) yearly. Treatment of the condition and an 'infect and treat' vaccination method using a tick-derived stabilate both rely on the availability of buparvaquone, a naphthoquinone with anti-theilerial activity. As buparvaquone is a controlled drug which is not commercially available in South Africa, a viable commercial alternative prevention or treatment method is necessary to control this disease. This study explores the effectiveness of an alternative vaccination method using *Theileria* sp. (sable) infected *in vitro* cultured leukoblasts.

A *Theileria* sp. (sable) containing cell line was initiated from lymph node biopsy material of an infected roan antelope and the parasite was successfully propagated *in vitro*. Attenuation is believed to have been achieved by 16 cycles of passage.

Real time PCR suggests that the parasite was successfully transmitted via subcutaneous inoculation with this cell line to two naïve roan antelope. These two inoculated animals remained clinically unaffected by challenge with a tick stabilate used in the 'infect and treat' vaccination method. In contrast, the two

unvaccinated control animals became clinically ill and required buparvaquone treatment after challenge.

This pilot study provides enough evidence to encourage further investigation in the use of *Theileria* sp. (sable) infected cells as a potential vaccine. A field study involving more animals which are challenged by natural infection after inoculation is the proposed next step.

Introduction

The *Theileria* genus consists of a group of tick-transmitted blood parasites found in many wild and domestic animals. While most *Theileria* species are considered benign, some can cause diseases of varying severity collectively called theileriosis. The more malignant theilerioses are severe lymphoproliferative diseases with high morbidities and mortalities characterised by leukocytosis, generalised lymphadenomegaly, bleeding disorders, anaemia and generalised oedema.

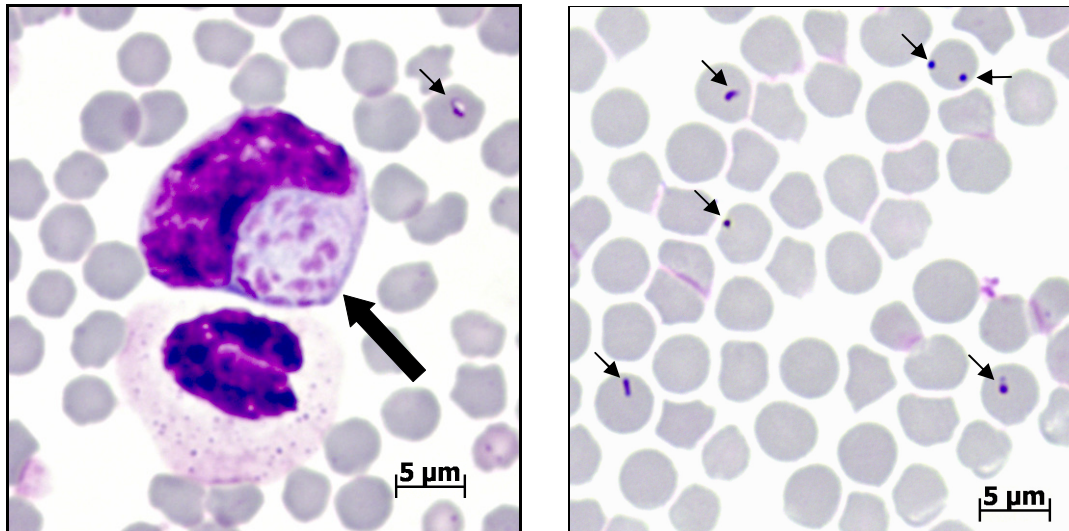
A *Theileria* species (then named *Theileria hippotrangi*) was first detected in a roan antelope in 1912 and what is presumed to be the same species has subsequently been found to cause significant mortalities in roan (*Hippotragus equinus*) and to a lesser extent, sable antelope (*Hippotragus niger*) yearly. Roan and sable antelope are rare, being two of the most valuable antelope species at game auctions annually and the last two remaining antelope from the genus *Hippotragus* after the extinction of the blue buck (*Hippotragus leucophaeus*). Recently the roan antelope's survival has been classified by the World Conservation Union Red List as 'dependant on ongoing active conservation efforts'.

Game farmers across southern Africa have attempted to build up the numbers of roan antelope. Despite very successful breeding under intensive and semi-intensive conditions, attempts to establish breeding herds and reintroduce animals into some environments, e.g. savannah bushveld, have resulted in high mortalities of especially calves as a result of theileriosis. With this disease further threatening the survival of roan antelope, an effective control method is essential.

Currently only 'infect and treat', including 'vaccination with a tick stabilate and treat' techniques have been successful preventative measures. The drug used in both these methods is buparvaquone, a controlled drug which is not commercially available in South Africa, necessitating the development of an alternative preventative measure not requiring access to buparvaquone. This study aims to determine if a *Theileria* species isolated from roan antelope in

South Africa can be transmitted from a schizont infected *in vitro* cultured leukoblast cell line, and if so, if immunity against subsequent challenge could be induced safely, determining the feasibility of pursuing attenuation for a future effective vaccine.

Figure 1: Blood smears (x1000 magnification) showing *Theileria* sp. (sable) piroplasms (small arrows) and a schizont (large arrow)



Literature review

Theileriosis

Theileriosis is a broad term for disease caused by parasites from the genus *Theileria*, tick-transmitted intra-cellular protozoa that also include organisms previously classed under the genera *Cytauxzoon*, *Gonderia* and *Haematoxenus* (Dolan 1989). Many *Theileria* species have been discovered in a wide variety of herbivores. In cattle alone, in eastern, central and southern Africa, 6 species have been identified, being harboured by up to 14 species of ticks. Most of these *Theileria* are benign or usually benign, in that infections do not normally result in morbidity or mortality of the host animals. The malignant species however cause severe financial losses of livestock yearly in many countries across the world (Mukhebi, Perry & Kruska 1992). Clinical signs can vary greatly depending mainly on the species of parasite and host, but are mostly as a result of haemolysis and lymphoid hyperplasia, resulting in especially pulmonary oedema, anaemia, icterus, lymphadenomegaly, diarrhoea, emaciation and even corneal oedema and central nervous symptoms (Lawrence, Perry & Williamson 2004). Morbidity and mortality are often high.

While the vast majority of *Theileria* species infecting wildlife are considered to be asymptomatic, resulting mortalities have been reported in various antelope, including the grey duiker (*Sylvicapra grimmia*) (Neitz & Thomas 1948), greater kudu (*Tragelaphus strepsiceros*) (Neitz 1957), eland (*Tragelaphus oryx*) (Grootenhuis, Morrison, Karstad, Sayer, Young, Murray & Haller 1980), tsessebe (*Damaliscus lunatus*) (Jardine 1992), sable antelope (*Hippotragus niger*) (McInnes, Stewart, Penzhorn & Meltzer 1991) and roan antelope (*Hippotragus equinus*) (Wilson, Bartsch, Bigalke & Thomas 1974).

Theileriosis in roan antelope

Piroplasms resembling *Theileria* parasites were first noticed in roan antelope in 1912 in West Africa (Todd & Wolbach 1912). In South Africa however their

presence was first recorded in 1974 on nature reserves in the area then known as the Transvaal, some losing up to 57% of the resident roan population over a period of 1 year with 80% of the mortalities occurring during the first 12 weeks of age (Wilson *et al.* 1974). Theileriosis, recorded at that stage as cytauxzoonosis, was discussed as a possible contributing cause to mortality in roan and sable antelope, especially in young calves. Though most of the calves that were available for postmortem examination seemed to have evidence of the disease, the authors were uncertain of the significance of this parasite's presence.

In 1977 cytauxzoonosis was confirmed as the cause of mortality in some young roan and sable, with the outstanding pathology being severe anaemia, ascites, hydrothorax, fibrinous pericarditis and hydropericardium (Wilson & Hirst 1977). High parasitaemias were evident on blood smears and histopathology revealed parasites and mononuclear cell infiltrates in various organs, especially affecting the myocardium and adrenals. Some roan and sable calves died as early as 2 and 5 days of age respectively, suggesting possible transplacental transmission. The authors confirmed this suspicion by placing heavily pregnant roan cows in small enclosures and finding parasites on blood smears made from their calves immediately after birth before exposure to ticks was possible.

The most common ticks found on confirmed cases were the brown ear tick (*Rhipicephalus appendiculatus*), the red-legged tick (*Rhipicephalus evertsi evertsi*) and the blue tick (*Rhipicephalus [Boophilus] decoloratus*) (Wilson & Hirst 1977). Feeding trials with *R. appendiculatus* and *R. evertsi evertsi* suggested that *R. evertsi evertsi* is a more efficient vector than *R. appendiculatus* (Steyl, J.C.A., Lawrence, J.A., Prozesky, L. & Penzhorn, B., Faculty of Veterinary Science, University of Pretoria, unpublished work). The role of *R. decoloratus* is unclear.

DNA sequencing of the 18S rRNA gene suggests that the *Theileria* species causing mortalities in both roan and sable antelope is the same parasite (Nijhof, Pillay, Steyl, Prozesky, Stoltsz, Lawrence, Penzhorn & Jongejan 2005). It was also found to be identical to the *Theileria* species sequenced by Allsopp

et al. (Allsopp, Cavalier-Smith, De Waal & Allsopp 1994) (Genbank accession number L19081) when it was provisionally named *Theileria* sp. (sable) and seems to be present in various healthy Bovidae, including the African buffalo (*Syncerus caffer*), klipspringer (*Oreotragus oreotragus*), reedbuck (*Redunca arundinum*), blue wildebeest (*Connochaetes taurinus*), blesbok (*Damaliscus pygargus*) and cattle (*Bos indicus*) (Nijhof *et al.* 2005). Samples were from various regions in southern Africa and Tanzania, also indicating a wide distribution. The same parasite was also found in red hartebeest (*Alcelaphus buselaphus caama*) (Spitalska, Riddell, Heyne & Sparagano 2005).

Current control methods

The high mortalities in roan antelope caused by *Theileria* sp. (sable), and this parasite's ubiquitous distribution, necessitate effective control methods if conservation efforts and farming with roan antelope are to be successful.

No control methods for *Theileria* sp. (sable) are described in the literature. Control measures for cattle-derived *Theileria parva* infection in cattle are however well documented and are listed by Lawrence *et al.* (2004) as isolation of naïve animals from potential vectors, destocking, tick control, treatment of affected cases and vaccination of susceptible animals. These measures and their applicability to *Theileria* sp. (sable) are discussed below:

- Isolation of susceptible animals from ticks is unlikely to be practical or successful when dealing with wild animals. The stress of confinement and absence of a natural extensive habitat are unlikely to result in a thriving breeding herd.
- In the case of East Coast fever, an area needs to be cleared of all potential hosts for 15 to 18 months (Lawrence *et al.* 2004). Even if an area can be cleared of all hosts harbouring *Theileria* sp. (sable), subsequent restocking is extremely likely to result in the reintroduction of the parasite because of the large number of herbivore species that can potentially carry it (Nijhof *et al.* 2005).

- Tick control is likely to be the most effective method until a practical and viable vaccine becomes available. Strategic tick control should result in natural 'vaccination' with a low dose of parasite. Once again, in the case of wild animals, especially if managed extensively, developing a successful tick control program can be difficult if not impossible.
- It is likely that successful treatment of clinically affected animals will result in immunity to the parasite. No published treatment regimes are available, but Steyl *et al.* (Steyl, J.C.A., Lawrence, J.A., Prozesky, L. & Penzhorn, B., Faculty of Veterinary Science, University of Pretoria, unpublished work) have reported on various supportive treatment options including intravenous colloids, immunosuppressive doses of corticosteroids, vitamins and even blood transfusion from a domestic goat. The mainstay of the treatment is however considered to be buparvaquone, an analogue to a derivative of the naphthoquinone named menoctone. Buparvaquone is also considered to be the most effective drug against other *Theileria* species (Muraguri, Kiara & McHardy 1999).
- Vaccination as a method of control will be considered in the following sections.

Successful vaccines against various other theilerioses

The most extensively studied and economically important theilerioses which occur in cattle are East Coast fever, caused by *Theileria parva*, and Tropical theileriosis, caused by *Theileria annulata* (Morrison & McKeever 2006).

East Coast fever (Theileria parva)

The most widely used and currently only proven successful way of vaccinating cattle against *Theileria parva* is the 'infect and treat' method (Morrison & McKeever 2006). Infection is normally achieved by injecting live cryopreserved sporozoites produced from passage through ticks fed on infected cattle while at the same time treating with a long-acting oxytetracycline formulation.

Oxytetracycline treatment suppresses development of the parasite without eliminating it, thus preventing clinical disease but allowing the development of immunity. This method of vaccination using the most common combination of isolates, called the Muguga cocktail (containing Muguga, Kiambu 5 and Serengeti isolates) resulted in immunity to challenge by up to a thousand times the lethal dose of sporozoites under experimental conditions (Morzaria, Irvin, Voigt & Taracha 1987) with immunity seemingly lasting for up to 3 to 5 years (Burridge, Morzaria, Cunningham & Brown 1972).

Problems and limitations with this method of vaccination include the necessity of using live animals for vaccine production as well as the need to titrate the effective dose of every new batch of sporozoite vaccine, as oxytetracycline fails to protect cattle against higher doses of parasites (Morrison & McKeever 2006). Any animals that fail to respond to oxytetracycline treatment may need other theilericidal drugs to prevent them from succumbing to the disease induced by the vaccine. These other theilericidal drugs (e.g. buparvaquone) can be difficult to obtain or be too expensive for developing farmers. Strict quality control of each batch of vaccine is essential to prevent the transmission of other bovine pathogens. As this vaccine needs to be stored in liquid nitrogen and used immediately after thawing, maintaining the cold chain can be a challenge in remote areas lacking appropriate infrastructure. Another important limitation is the immunogenic variation between isolates in different regions, resulting in variable cross immunity.

Currently a similar experimental sporozoite vaccine in roan against *Theileria* sp. (sable) has been produced and used successfully in South Africa and the adjacent Swaziland by Steyl *et al.* (Steyl, J.C.A., Lawrence, J.A., Prozesky, L. & Penzhorn, B., Faculty of Veterinary Science, University of Pretoria, unpublished work). It has however been found that co-administration of oxytetracycline seems to be ineffective in preventing clinical disease and mortality, necessitating buparvaquone administration 2 weeks after infection as treatment. Buparvaquone is not commercially available to veterinary practitioners in South Africa, because of its effectiveness against and induction of a carrier state in Corridor disease (Buffalo-derived *Theileria parva* infection), currently listed as a controlled disease. Identification of an alternate drug or

development of a vaccine not requiring buparvaquone treatment is therefore essential if a commercial preventative measure is to become available.

Tropical theileriosis (Theileria annulata)

Theileria annulata and *T. parva* have both been successfully attenuated by a number of cycles of schizont infected leukoblast replications *in vitro* (Morrison & McKeever 2006). Sufficient attenuation of *T. annulata* was achieved after 10 months of passage (Pipano, Irvin, Cunningham & Young 1981). Inoculating susceptible animals with this *T. annulata* cell culture seems to induce an effective immunity without causing any clinical disease and without co-administration of oxytetracycline or post-vaccination buparvaquone treatment being necessary. Only the *T. annulata* vaccine can however be used commercially, as the required cell culture dose in the case of *T. parva* is too high (100mℓ of cell suspension) to make this method of vaccination practical (Lawrence *et al.* 2004). In the case of *T. annulata*, an inoculum of 1×10^6 cells consistently resulted in parasite transmission (Pipano 1989), while *T. parva* required doses in excess of 1×10^8 cells (Pirie, Jarrett & Crichton 1970).

The reasons why transfer of *T. parva* through inoculation of infected cells into a host is more difficult than *T. annulata* are not completely understood. To date there are no reproducible systems for studying this transfer process *in vitro* (Morrison & McKeever 2006). One suggested explanation is that *T. annulata* targets macrophages, which are phagocytes, providing a natural and more efficient route of entry into host cells, whilst *T. parva* parasites prefer the non-phagocytic T lymphocytes (Boulter & Hall 1999). Other studies suggest genotype similarity between recipient and infected inoculated cells are of primary importance (Eugui & Emery 1981).

The use of *in vitro* live attenuated cell cultures overcomes many of the shortfalls of sporozoite vaccines in that live animals are not required on an ongoing basis, maintaining the cold chain is less essential (can remain viable for several days at 4°C) and the vaccine can be standardised with fewer quality control issues (Morrison & McKeever 2006). There is however some evidence that continual passage can result in incomplete protection against heterologous isolates, suspected to be as a result of a reduction in genetic

diversity of parasites with an increase in the number of cell cycles (Dargouth, Ben Miled, Bouattour, Melrose, Brown & Kilani 1996). Though preserving large amounts of parent stock from earlier passages may to some extent overcome this limitation, it still implies that vaccine production from a single tissue sample will be finite as all cells from parent stock will also eventually be used up in culture.

Concern exists that live vaccines may spread a parasite strain foreign to local strains for which local animals have insufficient immunity. Some attenuated cell lines have however been reported to lose their ability to differentiate into piroplasms, suggesting transmission through ticks may become impossible (Pipano 1974).

Subunit vaccines

Subunit vaccines promise to overcome many of the limitations of using live parasites (Morrison & McKeever 2006). Many antigens have been identified and suggested further routes of investigation, but to date no consistent successful results have been obtained. The use of live vaccines still offers the best option in the short to medium term.

***In vitro* cultured *Theileria* species (sable)**

Stoltz and Dunsterville (1992) managed to propagate the *Theileria* sp. (sable) parasite in a leukoblast cell culture obtained from an infected sable antelope's spleen cells using standard lymphoid cell culture methods. All material from this cell line was subsequently lost and no experiments were performed with these cells investigating attenuation or the possibility of transferring the parasite or immunity by inoculation into a naïve host.



Objectives and hypotheses

To establish a *Theileria* species (sable) schizont infected *in vitro* cultured leukoblast cell line

To ascertain if the parasite can be transmitted by administering these cells subcutaneously to a susceptible host

To evaluate the safety of using these cells for immunisation

To determine if inoculation with these cells will result in immunity to subsequent challenge

H^1_A : A roan antelope held in a tick-free environment testing negative for *Theileria* species on polymerase chain reaction (PCR) test will test positive a month after inoculation with the *in vitro* cultured schizont infected leukoblasts

H^1_0 : A roan antelope held in a tick-free environment testing negative for *Theileria* species on PCR will still test negative a month after inoculation with the *in vitro* cultured schizont infected leukoblasts

H^2_A : After inoculation with the *in vitro* cultured schizont infected leukoblasts, a roan antelope will not require buparvaquone treatment within a month of being challenged with a *Theileria* species (sable) infected tick stabilate

H^2_0 : After inoculation with the *in vitro* cultured schizont infected leukoblasts, a roan antelope will require buparvaquone treatment within a month of being challenged with a tick stabilate

Materials and methods

Model system, justification of model and experimental design

This study was a controlled prospective two-group parallel experimental study. It was not possible to approach this project as a blind study as the researcher was the person most qualified of all available personnel to prepare and administer the vaccine.

As there is no reproducible system for studying parasite transfer *in vitro* (Morrison & McKeever 2006), live animals had to be used to determine infectivity and resultant immunity after inoculation with infected leukoblasts.

Observational/analytical procedures and definitions

Animals

All animals used in this study were raised in a tick-free quarantine facility on Mauricedale Game Farm after being removed from their mothers within 24 to 48 hours after birth and hand reared on calf milk formula starting with 3 times daily feeds at 6am, 12pm and 6pm. The quarantine facility consisted of a boma with no vegetation other than trees for shade. The boma itself was situated a number of metres away from any natural vegetation and the walls of the boma consisted of wire fencing covered with thatch grass. Under this zero-grazing system teff hay was especially brought in from the Free State where at least one of the currently suspected *Theileria* sp. (sable) vectors, *Rhipicephalus appendiculatus*, does not occur. Grass is harvested from cultivated pastures where no livestock or game graze, further reducing the potential risk of tick infestation. Quarantine measures included designated feeders/handlers wearing overalls and gumboots with no duties which could result in exposure to tick-infested natural veld. On any suspicion of exposure handlers were instructed to use a flumethrin acaricidal spray (Bayticol Aerosol, Bayer) on clothing and boots. Any new calves entering the boma were thoroughly soaked with a flumethrin containing dip (Bayticol Cattle Dip, Bayer) and all animals in the facility were treated with a long-acting permethrin

containing dip (Tick Off Superdip, Tick Off) every 3 weeks. The study animals were further treated with another acaricidal spray, fipronil (Frontline Spray, Merial), at every immobilisation for further protection against ticks.

The initial donor animal, a ten month old female roan antelope, was purchased from J.F. Hume¹ and transported from the quarantine facility to bomas at the University of Pretoria's Veterinary Faculty before being infected with *Theileria* sp. (sable). Four out of 16 calves (from the following year's calving) of 4 to 6 months age were selected in the quarantine facility on the farm by convenience sampling according to temperament and randomly assigned into two pairs forming the vaccinated and control groups and assigned the identifications 'Vac 1 & Vac 2' and 'Con 1 & Con 2' respectively. All four selected calves were females as no males displayed suitable temperaments.

Time scale

The time scale has been labelled from 'Day 0_i' to 'Day 30_i' (abbreviated as D 0_i to D 30_i) for the inoculation section of this study and 'Day 0_c' (equal to D 30_i) to 'Day 28_c' (abbreviated as D 0_c to D 28_c) for the challenge part, with inoculation and challenge taking place on D 0_i and D 0_c respectively.

Rectal temperatures

All animals' deep rectal temperatures were taken in °C by obtaining 2 to 3 readings at a time. The highest result was recorded. Temperatures were recorded at least once daily (except for Con 2 on D 23_i) and twice or even three times daily when any suspicion of pyrexia developed. These times were labelled 'am' for during the 6am feeding session and 'pm' for the 6pm feed. When taking a 6pm rectal temperature was impossible for whatever reason the reading obtained at the 12pm feed was used for plotting rectal temperatures graphs.

Blood samples and smears

All peripheral blood smears were prepared by clipping the hair on the tip of the animal's tail with a pair of curved scissors and pricking the skin with a 20

¹ Mauricedale Game Ranch, P.O. Box 427, Malelane 1320, South Africa

gauge (G) 1 inch (") hypodermic needle. Lymph node fine needle aspirates were obtained using a 20G 1½" needle attached to a 5mℓ syringe. Venous blood samples were collected in 5mℓ EDTA blood tubes from the cephalic veins using an 18G needle. Central blood smears were prepared from these EDTA blood samples.

Buffy coat smears were prepared from the interface between plasma and the packed cell portion of spun down heparinised glass capillary tubes.

All smears were stained with RapiDiff[®] (Clinical Diagnostic Sciences) and examined under a microscope at 100x and 1000x (oil) magnification. The researcher examined routine blood smears randomly, without relating them to the animals from which they came until all the smears collected during that session were examined. In some instances, however, it was impossible not to be aware of the identity of the animal in advance, because the researcher himself made *and* examined the blood smears, with the shape of the film sometimes betraying which smear was being viewed. It was also impossible to be unaware of an animal's identity when examining the blood smear of a single animal collected during a particular feeding session.

Polymerase chain reaction (PCR)

Samples for polymerase chain reaction (PCR) evaluation were sent to the Molecular Biology Laboratory of the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, South Africa for confirmation of *Theileria* species (sable) or *Theileria* genus infection status.

Two PCR systems were used. The first was the reverse line blot (RLB) hybridization method with a *Theileria* species (sable) species-specific probe developed in collaboration with the University of Utrecht which could differentiate between different *Theileria* species (Nijhof *et al.* 2005). The second was the LightCycler[®] v2 (Roche Diagnostics, Mannheim, Germany) real-time PCR using a *Theileria* genus-specific probe which only identified *Theileria* parasites up to genus level (Sibeko, Oosthuizen, Collins, Geysen, Rambritch, Latif, Groeneveld, Potgieter & Coetzer 2008).

DNA extraction

DNA was extracted from 200µl of EDTA blood using the QIAamp® DNA Extraction Kit (QIAGEN, Southern Cross Biotechnologies) following the manufacturer's instructions. Extracted DNA was eluted in 100µl elution buffer and stored at 4°C until further analysis.

Reverse line blot (RLB) hybridization assay

The RLB hybridization assay was used to determine the calves' infection status, and to confirm the uninfected state of the donor animal before and infected state after inoculation. The parasite V4 hypervariable region of the parasite 18S rRNA gene was amplified using *Theileria* and *Babesia* genus-specific primers RLB F2 [5'-GAC ACA GGG AGG TAG TGA CAA G-3'] and biotin-labeled RLB R2 [5'-Biotin-CTA AGA ATT TCA CCT CTA ACA GT-3'] following the touchdown PCR programme as previously described (Nijhof *et al.* 2005). The PCR amplicons were analyzed using the RLB hybridization technique as described. A *Theileria* and *Babesia* genus-specific probe and 27 species-specific probes were included on the membrane (Table 1).



Table 1: Genus and species-specific RLB probes used in this study

| Oligonucleotide probe | Sequence (5' – 3')* |
|-----------------------------------------|--------------------------------|
| <i>Theileria/Babesia</i> genus-specific | TAA TGG TTA ATA GGA RCR GTT G |
| <i>Theileria</i> genus-specific | ATT AGA GTG CTC AAA GCA GGC |
| <i>Babesia</i> genus-specific 1 | ATT AGA GTG TTT CAA GCA GAC |
| <i>Babesia</i> genus-specific 2 | ACT AGA GTG TTT CAA ACA GGC |
| <i>Babesia bicornis</i> | TTG GTA AAT CGC CTT GGT C |
| <i>Babesia bigemina</i> | CGT TTT TTC CCT TTT GTT GG |
| <i>Babesia bovis</i> | CAG GTT TCG CCT GTA TAA TTG AG |
| <i>Babesia caballi</i> | GTG TTT ATC GCA GAC TTT TGT |
| <i>Babesia canis</i> | TGC GTT GAC GGT TTG AC |
| <i>Babesia divergens</i> | ACT RAT GTC GAG ATT GCA C |
| <i>Babesia felis</i> | TTA TGC GTT TTC CGA CTG GC |
| <i>Babesia gibsoni</i> | CAT CCC TCT GGT TAA TTT G |
| <i>Babesia major</i> | TCC GAC TTT GGT TGG TGT |
| <i>Babesia microti</i> | GRC TTG GCA TCW TCT GGA |
| <i>Babesia rossi</i> | CGG TTT GTT GCC TTT GTG |
| <i>Babesia</i> sp. (sable) | GCG TTG ACT TTG TGT CTT TAG C |
| <i>Babesia vogeli</i> | AGC GTG TTC GAG TTT GCC |
| <i>Theileria annulata</i> | CCT CTG GGG TCT GTG CA |
| <i>Theileria bicornis</i> | GCG TTG TGG CTT TTT TCT G |
| <i>Theileria buffeli</i> | GGC TTATTT CGG WTT GAT TTT |
| <i>Theileria equi</i> | TTC GTT GAC TGC GYT TGG |
| <i>Theileria lestoquardi</i> | CTT GTG TCC CTC CGG G |
| <i>Theileria mutans</i> | CTT GCG TCT CCG AAT GTT |
| <i>Theileria ovis</i> | TTG CTT TTG CTC CTT TAC GAG |
| <i>Theileria parva</i> | GGA CGG AGT TCG CTT TG |
| <i>Theileria separata</i> | GGT CGT GGT TTT CCT CGT |
| <i>Theileria</i> sp. (buffalo) | CAG ACG GAG TTT ACT TTG T |
| <i>Theileria</i> sp. (kudu) | CTC CAT TGT TTCTTT CCT TTG |
| <i>Theileria</i> sp. (sable) | GCT GCA TTG CCT TTT CTC C |
| <i>Theileria taurotragi</i> | TCT TGG CAC GTG GCT TTT |
| <i>Theileria velifera</i> | CCT ATT CTC CTT TACGAG T |

*Symbols used to indicate degenerate positions: R = A/G; W = A/T; Y = C/T

Theileria genus-specific real-time PCR

The *Theileria* genus-specific forward [5'-GGT AAT TCC AGC TCC AAT AG-3'] and reverse [5'-ACC AAC AAA ATA GAA CCA AAG TC-3'] primers were used for the amplification of a 230 bp fragment of the V4 hypervariable region of the *Theileria* 18S rRNA gene as previously described (Sibeko *et al.* 2008). For the detection of *Theileria* species present in a sample, the *Theileria* genus anchor (5'-AGA AAA TTA GAG TGC TCA AAG CAG GCT TT—FL) and *Theileria* genus sensor (5'-LCRed705-GCC TTG AAT AGT TTA GCA TGG AAT—PH) probes were included in the reaction. The amplification mixture and real-time PCR



reaction conditions were as described by Sibeko *et al.* (2008). Temperature cycling was performed in a LightCycler® v2 (Roche Diagnostics, Mannheim, Germany).

Stabilate

A tick-derived sporozoite vaccine (batch number '01/06/06 R.e. Mar') was used to infect the donor animal. Stock from this same batch was used to challenge the vaccinated and control animals.

This vaccine was developed by Steyl *et al.* as a crude suspension of sporozoites prepared from ground up adult *Rhipicephalus evertsi evertsi* ticks mixed with glycerol as a cryoprotectant and frozen in liquid nitrogen (Steyl, J.C.A., Lawrence, J.A., Prozesky, L. & Stoltsz, H., Faculty of Veterinary Science, University of Pretoria, unpublished work). As nymphs these ticks had been placed in canvas ear bags and applied to infected roan antelope before moulting and being fed on rabbits for the sporozoites to mature. Quality control included aerobic and anaerobic bacterial culture as well as light microscopy of ticks' salivary gland acini to confirm infection. The presence of *Theileria* sp. (sable) was also confirmed by RLB hybridisation PCR before adding the cryoprotectant.

This vaccine was successfully used for the 'infect and treat' vaccination method in roan antelope for the last 3 years (Steyl, J.C.A., Lawrence, J.A., Prozesky, L. & Penzhorn, B., Faculty of Veterinary Science, University of Pretoria, unpublished work).

Experimental procedures

Cell culture

Infection of donor with *Theileria* sp. (sable)

The donor animal was immobilised using 3.5mg thiafentanyl oxalate (A3080, Wildlife Pharmaceuticals) and 20mg azaperone (Stresnil, Janssen Animal Health) in a 2mℓ dart with a Pneudart® delivery system. The dart wound was treated by injecting 2mℓ of long-acting benzylpenicillin

(Duplocillin, Intervet) into the wound and applying a topical tetracycline spray (Terramycin Wound Spray, Pfizer) over the site. A 5mℓ EDTA blood sample was collected from the cephalic vein to determine if the animal had previously been exposed to *Theileria* sp. (sable) using the RLB PCR system.

Consistent with J.C.A. Steyl's current vaccination procedure, 0.6mℓ of the tick stabilate vaccine was injected subcutaneously in the left prescapular lateral neck region. The anaesthetic was reversed by administering 35mg naltrexone (Trexonil, Wildlife Pharmaceuticals) intravenously.

Confirmation of success of infection

Fourteen days later, the immobilisation was repeated as described above. An EDTA blood sample was again collected for RLB PCR to determine if the animal was successfully infected with *Theileria* sp. (sable).

Lymph node biopsy and blood sample collection for culture

The area over the left prescapular lymph node was shaved and prepared for surgery using chlorhexidine soap (Hibiscrub, Zeneca) and surgical alcohol spray. Lignocaine 2% (Lignocaine Injectable 2%, Bayer) local anaesthetic was injected subcutaneously in a line block over the planned incision site. A 6 cm skin incision was made, the lymph node exposed by blunt dissection and an excisional lymph node biopsy taken. Depth of anaesthesia was maintained using ketamine (Kyron) and diazepam (Kyron) administration intravenously to effect. Closure was routine using 2/0 monofilament nylon. The animal was treated for theileriosis with 4mℓ buparvaquone (Butalex, Coopers Animal Health) at 2.5mg/kg for an estimated 80kg bodyweight intramuscularly.

The lymph node biopsy material was transported to the Onderstepoort Veterinary Institute for *in vitro* culturing of the parasite.

***In vitro* culture**

Culture of the parasite was performed as described (Zweygarth, Benade, Steyl, Prozesky, Koekemoor & Josemans 2009). The prescapular lymph node biopsy material was chopped into small pieces. To break up the tissue clumps further, they were homogenized in a glass homogenizer. The resulting cell suspensions were centrifuged at 30 x g for 10 min. The cell pellets were resuspended in complete culture medium and put into 25 cm² culture flasks. The culture medium consisted of RPMI 1640 medium (Gibco 82400) containing 25 mM HEPES, 2 g/l sodium bicarbonate, 10% (v/v) heat-inactivated foetal bovine serum, 0.2 mM hypoxanthine, 100 IU/ml penicillin and 100 µg/ml streptomycin. Cultures were incubated at 37°C. The culture medium was changed twice a week. Cultures were monitored by examining cytocentrifuged aliquots of the cell suspensions. In order to visualize the schizonts, culture samples were air dried, fixed in methanol and stained with Giemsa (5%).

At the time of culture initiation, very few cells containing macroschizonts were detected in Giemsa-stained smears. The first subculture was carried out 16 days after culture initiation. Thereafter subcultures were carried out twice a week harvesting 2.5 - 5 x 10⁶ parasites per flask each time. Sixteen cycles were completed in 10 weeks.

DNA sequencing was performed on the 18S rRNA gene extracted from these *in vitro* cultured cells and compared with sequence data obtained from the *Theileria* sp. (sable) identified by Nijhof *et al.* in 2005 (Zweygarth *et al.* 2009).

Inoculation of experimental animals

A dose of 3.2 x 10⁶ cells per 1ml of suspension was used. This dose is comparable to the dose of 1 – 5 x 10⁶ cells per 1ml used with *Theileria annulata* (Morrison & McKeever 2006).

Preparation of inocula

Four 1ml aliquots of 3.2×10^6 schizont infected *in vitro* cultured leukoblasts from cycle number 16 were prepared by E.P. Zwegarth and frozen in 2ml plastic containers at -50°C . These doses were transported from the Onderstepoort Veterinary Institute to Mauricedale Game Ranch in liquid nitrogen. Each of 2 doses (other 2 doses kept as reserve) was thawed in water of between 37 and 38°C and drawn up in a 2ml syringe with a 20G needle immediately before inoculating calves Vac 1 and Vac 2. Calves Con 1 and Con 2's control doses of 1ml sterile water in 2ml syringes with 20G needles were prepared before any animals were immobilised to limit immobilisations' durations as far as possible.

Inoculation (Day 0_i)

The 4 roan calves were immobilised with 2mg thiafentanyl oxalate and 16mg azaperone each. Ketamine was given in 50mg and diazepam in 10mg boluses intravenously to effect to maintain anaesthesia when necessary. In all animals the dart wounds were treated as mentioned previously, peripheral blood smears were taken from the tips of the animals' tails, 5ml EDTA blood samples were taken from the cephalic veins for PCR and fine needle aspirates were prepared on both (left and right) anorectal lymph nodes.

The appropriate cell suspension or sterile water dose was injected subcutaneously in the right peri-anal area. This site was selected rather than the prescapular area as the animals tolerated having their perineum touched (the handlers stimulated defaecation from an early age) better than the prescapular site. This made monitoring lymphadenomegaly of the anorectal lymph nodes far easier.

The calves were treated with fipronil spray as a precaution to prevent any future tick attachment for 3 to 4 weeks in case of a tick breaching the quarantine measures. Anaesthesia was reversed with 20mg naltrexone intravenously.



Monitoring (Day 0_i to 30_i)

The calves' deep rectal temperatures were recorded once to twice daily at 6am and/or 6pm during bottle feeding. Peripheral blood smears were made daily, mostly during the midday bottle feed at 12pm from Day 0_i to Day 21_i. Any depression in habitus or appetite was noted. Any observed enlargement or asymmetries of anorectal lymph nodes were also recorded.

Treatment

As this cell culture had not been used in any animal before this study, it was decided to treat any inoculated animal(s) that developed pyrexia with buparvaquone (2.5mg/kg) intramuscularly to minimise the risk of a calf succumbing to the vaccine. Any sick animals needing supportive treatment received it as deemed necessary. An EDTA blood sample was collected at the same time for PCR and buffy coat smear and anorectal lymph node fine needle aspirates and peripheral blood smears were prepared. Buparvaquone treatment was repeated in any animal still or again displaying pyrexia 2 days after the first treatment.

Challenge

As cell mediated immunity via T-helper cells is normally mostly responsible for protection against blood parasites, the proliferation and maturation of this cell population was taken into account to calculate the day of challenge with the tick stabilate (Goldsby, Kindt, Osborne & Kuby 2000). These T-helper cells reach their maximum population numbers and are mature by day 4 to 5 after reacting to an antigen, with their numbers persisting at high levels for weeks to months thereafter.

In the case of *Theileria annulata*, an initial transient febrile response is seen between days 5 and 8 post-inoculation, during which time inoculated cells may be re-isolated from the regional lymph node (Nichani, Thorp, Brown, Campbell, Brown, Ritchie & Spooner 1999). This reaction is followed by a more prolonged pyrexia starting at about day 12, during which the parasites seem to be transferred to the recipient animal's cells. This latter reaction seems to be essential for the development of immunity. Challenge with the tick stabilate

was arbitrarily decided to take place 10 days after the second observed period of pyrexia *subsided*, which resulted in Day 30_i coinciding with Day 0_c.

PCR and challenge (Day 30_i/0_c)

All 4 animals were immobilised (with 3mg thiafentanyl oxalate, 16mg azaperone and ketamine and diazepam to effect) and blood samples collected for PCR and blood smears. Each animal was injected with 0.8ml of the tick stabilate in the left lateral neck area. This dose of tick stabilate is considered to be lethal when not followed by buparvaquone treatment (Steyl, J.C.A., Lawrence, J.A., Prozesky, L. & Penzhorn, B., Faculty of Veterinary Science, University of Pretoria, unpublished work). It was decided to use a site far removed from the initial vaccination site in order to evaluate systemic rather than local immunity. Fipronil treatment was repeated.

Monitoring (Day 0_c to 28_c)

The calves' deep rectal temperatures were again recorded once to twice daily. Peripheral blood smears were made daily from any animal developing even a mild increase in rectal temperature and from all animals from Day 14_c until Day 22_c for comparative purposes. Any change in habitus or appetite was again noted. Attempts were sometimes made to palpate the left prescapular lymph node, which was not tolerated by the calves and was consistently unsuccessful.

Treatment

Any challenged animal that developed pyrexia in conjunction with parasites (piroplasms or schizonts) observed on blood smear was immobilised and treated with buparvaquone (2.5mg/kg i/m). Any supportive treatment indicated was also administered, including dexamethasone (Kortico, Bayer) at 0.2mg/kg i/m and long-acting benzylpenicillin (26.5mg/kg i/m) injections. An EDTA blood sample was collected for PCR and buffy coat smear, and left prescapular lymph node fine needle aspirates and peripheral blood smears were prepared. Buparvaquone treatment was repeated in any animal still or again displaying pyrexia 2 days after first treatment.

Results

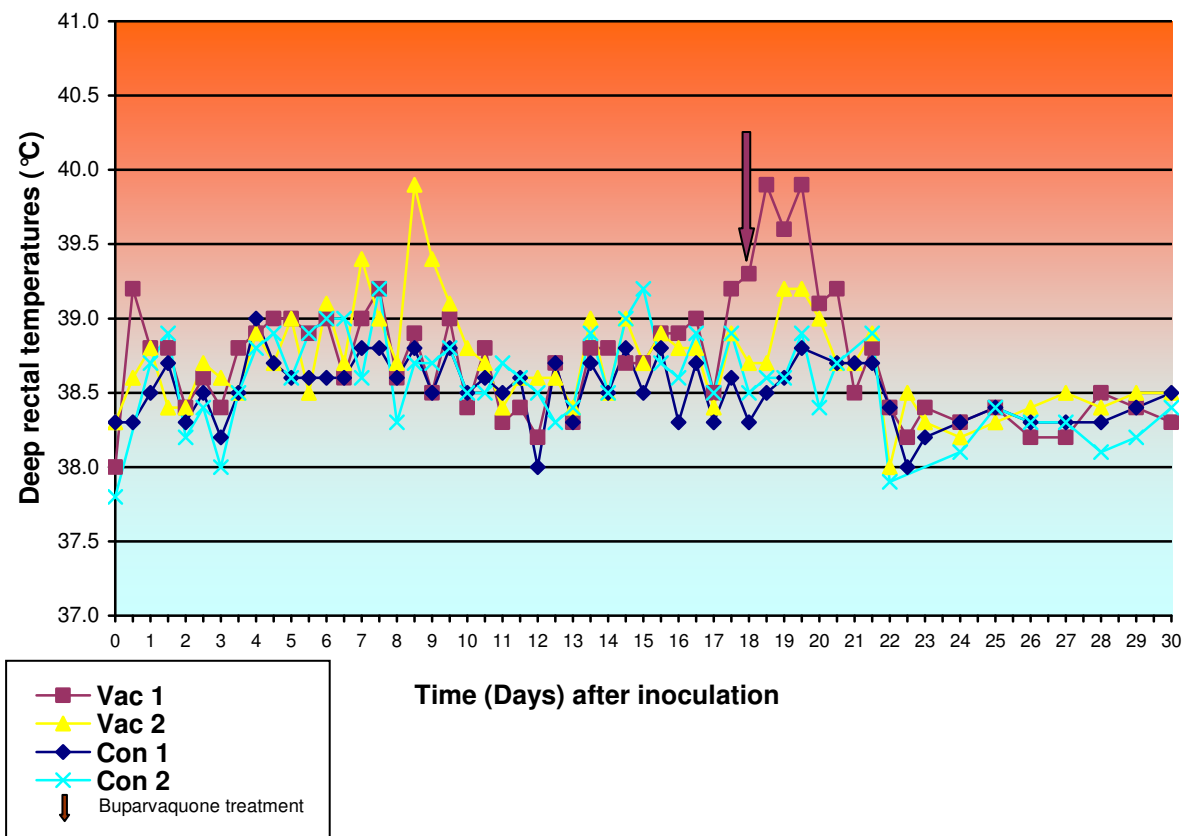
Inoculation

Habitus and appetite

There was no change in the habitus or appetite of any animal for the duration of the inoculation phase.

Temperatures

Figure 2: Inoculation stage deep rectal temperatures



Vac 1 developed a moderate pyrexia from D 18_i pm until D 19_i pm. Vac 2 displayed a similar spike in rectal temperature around D 8_i pm of even shorter duration. The control animals' temperatures remained normal during this stage of the study.



Blood samples and smears

Peripheral blood smears were performed (or at least attempted) daily on all animals from D 0_i to D 21_i. These blood smears' quality was extremely variable with good quality slides being obtained only infrequently. Factors preventing preparation of good smears were the presence of a liquid paraffin based dip on animals' coats and outdoor conditions like wind and rain. As a result emphasis was placed on evaluating the presence or absence of schizonts or piroplasms rather than trying to evaluate more subtle changes like the development of a mild leukocytosis. Lymph node aspirates performed during immobilisations were also not considered of sufficient and consistent quality for evaluation with results repeatedly being 'inconclusive'.

Six piroplasms were noticed on a buffy coat smear of Vac 1 on the day of treatment (D 18_i) with the central and peripheral blood smears remaining negative for parasites. No schizonts were seen. No parasites were noticed on peripheral blood smears of any of the other 3 calves for the duration of this inoculation stage of the study.

Lymphadenomegaly

A local swelling of the right anorectal lymph node of Vac 1 was first noticed on the morning of D 6_i and of Vac 2 that same evening. Lymph node enlargements were at first only detected by palpation in comparison with the unaffected left side, progressing to protruding very visibly craniolaterally to the base of the tail over the following 2 days. These lymphadenomegalies seemed to persist for some weeks before subsiding slowly until being recorded as absent on D 23_c (45 days later). The control animals showed no lymph node enlargements during the inoculation phase of this study.

Treatment

On D 18_i calf Vac 1 was treated with 187.5mg of buparvaquone even though it showed only a mild increase in rectal temperature. No other animals received treatment during the inoculation period.

Challenge

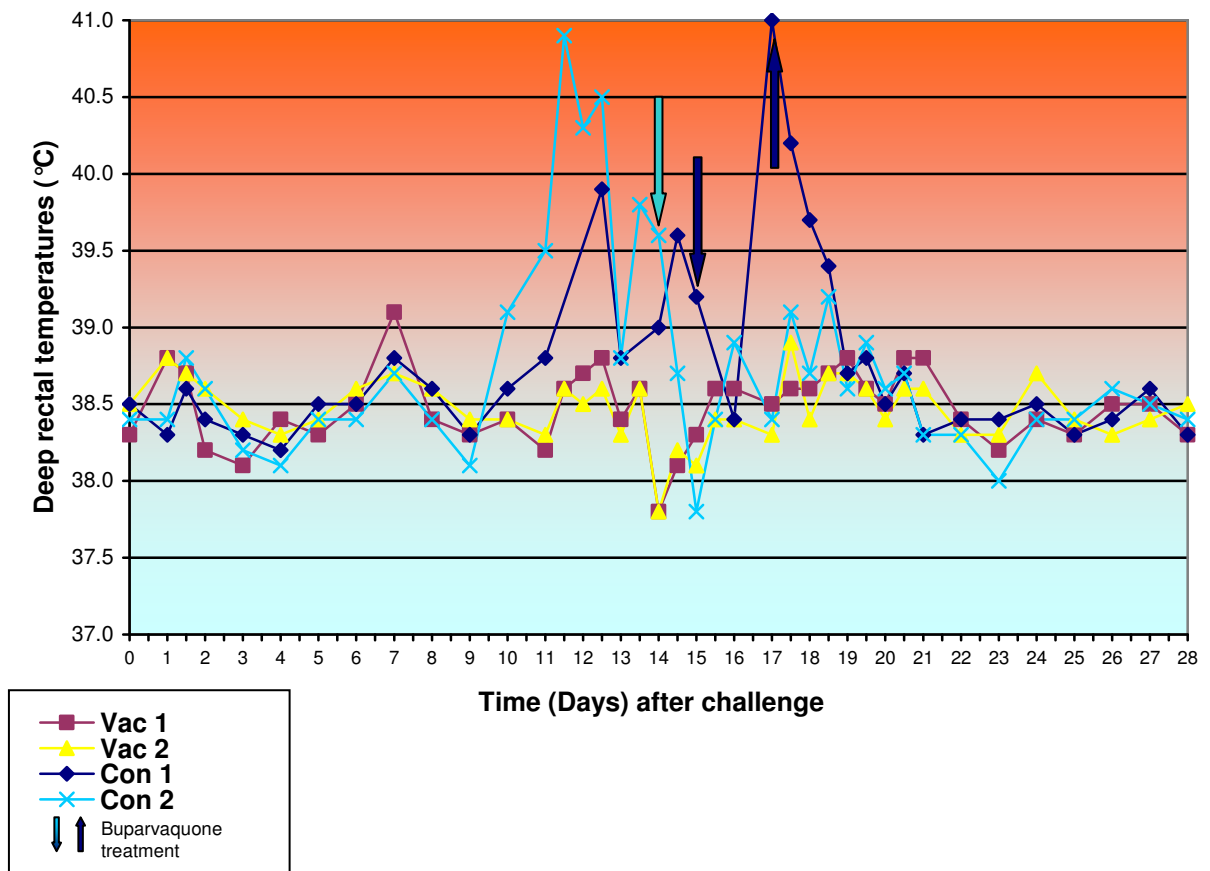
Habitus and appetite

Both Con 1 and Con 2 fed normally until D 12_c, when both calves refused a bottle feed. Con 2 seemed to have recovered by the next day while Con 1 continued to refuse at least one meal daily until D 15_c, after which she continued to have varying degrees of inappetance until her appetite returned to and remained normal from D 19_c. Con 1's habitus was also noticeably albeit intermittently affected from day D 12_c to day D 17_c with the calf seeming depressed and reluctant to get up when approached. Con 2's habitus remained normal throughout.

The vaccinated animals suffered no noticeable abnormalities of habitus or appetite.

Temperatures

Figure 3: Challenge stage deep rectal temperatures



Con 1 developed intermittent episodes of pyrexia from D 12_c pm, reaching a severe peak pyrexia on D 17_c pm with the temperature only returning to normal after treatment by D 19_c. Con 1 was treated on D 15_c and D 17_c.

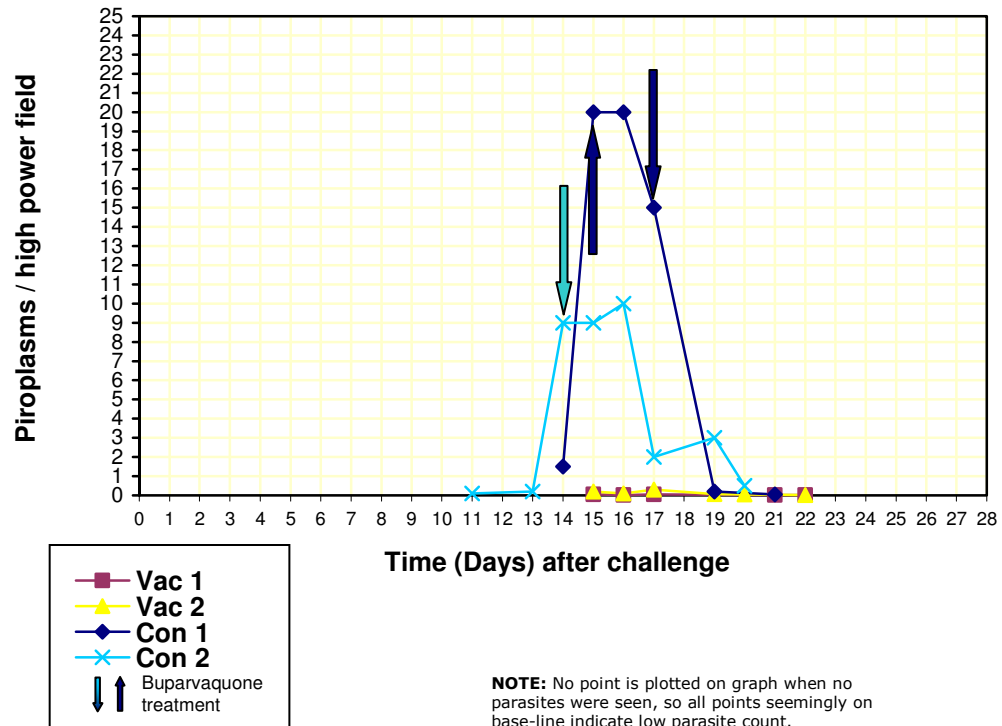
Con 2 showed an increase in rectal temperature from D 10_c am which progressed to a severe peak pyrexia by D 11_c pm, only returning to normal after treatment on D 14_c pm.

Neither vaccinated calf experienced pyrexia during this challenge stage of the study.



Blood samples and smears

Figure 4: Piroplasms count per microscopic high power field during challenge stage



A peripheral blood smear on D 0_c revealed no piroplasms in any of the four animals. Peripheral blood smears were again resumed from between D 11_c and D 14_c and continued, mostly daily, for all animals until D 22_c. Piroplasms were noticed only in very low numbers in Vac 1 and Vac 2 at a maximum of 1 parasite per 3 and 20 high power (1000x magnification) fields respectively, but were present in high numbers in Con 1 and Con 2, reaching up to 9 and 20 parasites per single high power field respectively.

Schizonts were seen in low numbers in Con 1 from D 15_c to D 17_c and in Con 2 from D 14_c to D 16_c. No schizonts were seen in Vac 1 and Vac 2.

Treatment

On D 15_c 212.5mg buparvaquone, 2.65g benzylpenicillin and 16mg dexamethasone were administered intramuscularly to Con 1. On D 17_c treatment was repeated with 225mg buparvaquone.



On D 14_c 200mg buparvaquone was administered to Con 2 as well as 2.12g benzympenicillin and 14mg dexamethasone given intramuscularly.

There was no indication for buparvaquone treatment for calves Vac 1 and Vac 2 during the challenge phase of this study.

Polymerase chain reaction (PCR)

Reverse line blot (RLB)

The RLB hybridization method confirmed that the donor animal became infected with *Theileria* sp. (sable) after being inoculated with the tick stabilate. When the RLB system was later used on samples collected during the inoculation and challenge phase of this study, inconsistent results were obtained in spite of repeating the tests on the same samples a number of times.

Real-time PCR

Table 2: Inoculation and challenge phase real-time PCR results

| <u>Day:</u> | D 0 _i | D 18 _i | D 30 _i (D 0 _c) | D 14 _c /15 _c | D 63 _c |
|--------------------------|------------------|-------------------|---------------------------------------|------------------------------------|-------------------|
| | (Inoculation) | (Treatment) | (Challenge) | (Treatment) | (Release) |
| <u>Control group:</u> | | | | | |
| Con 1 | + | | + | + | + |
| Con 2 | - | | - | + | + |
| <u>Inoculated group:</u> | | | | | |
| Vac 1 | - | +/- | + | | + |
| Vac 2 | - | | + | | + |

Key to Table 1:

- +** Positive *Theileria* spp. result
- Negative *Theileria* spp. result
- Samples expected to be positive if transmission or challenge were successful during the study
- Samples expected to be negative if animals were unexposed to the parasite before the study

Real-time PCR performed on samples collected during the inoculation and challenge phase of this study showed both vaccinates were free from infection initially and became infected after vaccination. One control animal (Con 1) was positive from the beginning; the other (Con 2) became infected after challenge.

Discussion

Cell culture

This study confirmed the findings of Stoltsz and Dunsterville (1992) that *Theileria* species (sable) can be established in a leukoblast culture. This time the culture material was collected from a roan antelope.

Polymerase chain reaction (PCR)

The species-specific RLB probe for *Theileria* sp. (sable) was used during the cell culture phase of this study. When this probe was relied on to determine transmission during the inoculation and challenge phases, results became very erratic and paradoxical. The tests were repeated a number of times on a number of samples, with no consistent results. Suggested reasons for this included contamination and possible too low or too high parasitaemias.

On the laboratory's advice, a real-time LightCycler[®] v2 (Roche Diagnostics, Mannheim, Germany) PCR system was used to re-test these samples, as this was considered a more sensitive test which was less susceptible to error (Sibeko *et al.* 2008). The LightCycler[®] allows amplification and detection in a closed capillary tube, minimizing contamination problems.

The *Theileria* genus-specific probe available for the LightCycler[®] only detected *Theileria* species on a genus level, without being specific for *Theileria* sp. (sable) (Oosthuizen, Zweygarth, Collins, Troskie & Penzhorn 2008). DNA sequencing from the 18S rRNA gene extracted from this study's *in vitro* cultured cells however showed high levels of nucleic acid identity with sequence data obtained from the *Theileria* sp. (sable) identified by Nijhof *et al.* in 2005 (Zweygarth *et al.* 2009), confirming the presence of this specific parasite in the cell culture. Subsequently the increased sensitivity and resistance to error of the LightCycler[®] were considered more important than the RLB system's specificity for *Theileria* sp. (sable) in confirming transmission during the inoculation and challenge phases of this study.



Transmission of infection

Real-time PCR results show that both vaccinated calves developed an infection after vaccination.

One of the vaccinated calves (Vac 1) had both a positive and negative result 18 days after vaccination, suggesting that the parasitaemia may have been too low at that stage to detect consistently. The facts that the subsequent test on this animal remained positive rather than negative and that piroplasms were noticed on a buffy coat smear, albeit in small numbers, confirm the infection and validity of the positive test result. No piroplasms were noticed in the other vaccinated animal (Vac 2), but the positive PCR result confirmed transmission.

Successful transmission is also supported by the presence of clinical episodes of pyrexia and a marked persistent local lymphadenomegaly in both vaccinated calves following inoculation which were absent in the control animals.

One of the control animals (Con 1) was found by PCR to be infected with a *Theileria* sp. at the onset of this entire study. Consistent positive PCR results thereafter further substantiate prior infection.

This obviously raises the question if the two vaccinated animals could have contracted a *Theileria* sp. infection through natural tick transmission during this study rather than as a result of inoculation with the cell culture. There have been two isolated incidents on the farm in previous years when it was suspected that a tick may have evaded quarantine measures and transmitted the disease to individual animals. The carers reported no such incidents in the year of this study and no buparvaquone treatment other than that administered by the author was used during the year. The author also applied fipronil spray every 2 to 4 weeks and supervised quarantine measures and monitored pyrethroid dip application during the course of this study, making tick transmission highly unlikely. The lack of protective immunity to subsequent challenge of Con 1 also supports the unlikelihood of immunogenic natural infection as a result of a tick evading quarantine measures.

The explanation for Con 1's early *Theileria* spp. infected state is open for speculation. Transplacental transmission of other *Theileria* spp. has been found in horses (Allsopp, Lewis & Penzhorn 2007) and cattle (Baek, Soo, Kim, Hur, Lee, Jung, Onuma, Oluoch, Kim & Kakoma 2003) and congenital transmission of specifically *Theileria* sp. (sable) has been described in roan antelope (Wilson & Hirst 1977). It is therefore possible that congenital infection may have taken place. Mortalities as a result of theileriosis have been found in roan calves as early as 2 days of age, suggesting transplacental infection can also result in disease. The question if such an infection could in some instances result in immunity to theileriosis, or alternatively even increased susceptibility to the disease at a later stage following reinfection, remains unanswered. If Con 1 had contracted a congenital infection, if anything it would seem likely that it resulted in suppression of immunity to theileriosis, as Con 1 was clinically more severely affected by subsequent challenge than the other control animal.

As African wildlife, and even domestic animals, can host a variety of benign intra-erythrocytic organisms (Nijhof *et al.* 2005), another possibility would be that a *Theileria* species *other* than *Theileria* sp. (sable) may have been transmitted transplacentally and detected by the *Theileria* genus-specific real-time PCR, thus offering no immunity to subsequent challenge by *Theileria* sp. (sable).

Either way, though it cannot be ruled out, it would be considered likely that natural infection of Con 1 with *Theileria* sp. (sable) by tick transmission would have resulted in clinical signs and/or a protective immunity, neither of which was the case.

Safety

Only one of the two vaccinated animals (Vac 1) was treated with buparvaquone. This was more a precaution than a necessity in retrospect, as this calf was clinically only mildly affected. This calf experienced no decrease in appetite or habitus and a moderate pyrexia only developed after treatment.

Except for very few piroplasms noticed on buffy coat smear, parasites were not numerous enough to pick up on blood smears.

The other vaccinated animal (Vac 2) remained clinically healthy apart from a very early short duration moderate pyrexia after vaccination. It was elected not to administer any buparvaquone with no ill consequences. The fact that the reaction was so mild may have been the result of attenuation of the parasite over 16 subcultures.

Induction of immunity

Clinical results in inoculates and controls show that vaccination with the cell culture protected against clinical infection.

The two unvaccinated animals became clinically ill after challenge with the tick stabilate. These animals both experienced a marked protracted, albeit intermittent, and severe pyrexia with a decrease in appetite and, in one animal, habitus. The vaccinated group remained clinically completely unaffected following challenge. Though piroplasms were visible in vaccinated and control animals, their presence was noticed in far fewer numbers in the vaccinated animals. The unvaccinated animals also required buparvaquone administration as opposed to the vaccinated individuals which remained untreated during the *challenge* phase of this study (as mentioned previously, one of the vaccinated animals was treated as a precaution during the *inoculation* phase).

Conclusion

A *Theileria* sp. (sable) infected leukoblast cell line was successfully initiated and the parasite successfully propagated *in vitro*. The parasite was successfully transmitted via subcutaneous inoculation with this cell line to two naïve roan antelope. Inoculation with the cell line appears to be safe in that only one of the two vaccinated animals was treated with an anti-theilerial drug after vaccination. This treatment was a precaution and may not have been necessary.

After challenge with a tick stabilate containing *Theileria* sp. (sable) these vaccinated animals remained clinically unaffected and untreated. In contrast, the two unvaccinated control animals became clinically ill and required treatment after challenge.

Though the sample size is too small to provide statistically significant data on the safety, success of transmission and subsequent immunity to theileriosis, this pilot study delivers enough evidence to encourage further investigation. A field study involving more animals with natural challenge after inoculation should be the next step. The difficulty of obtaining sufficient numbers of experimental animals, because of the value of these antelope and the high mortality rate of this disease, is the main limiting factor for further research.

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