

Occurrence of *Theileria parva* infection in cattle on a
farm in KwaZulu-Natal, South Africa

by

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Submitted in partial fulfilment of the requirements for the degree

Master of Science

in the Faculty of Veterinary Science,

University of Pretoria

8th January 2007

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Acknowledgements

I would like to thank my supervisor, Professor Banie Penzhorn (Department of Veterinary Tropical Diseases, University of Pretoria), and co-supervisors, Dr Marinda Oosthuizen (Department of Veterinary Tropical Diseases, University of Pretoria) and Dr Abdallah Latif (Agricultural Research Council-Onderstepoort Veterinary Institute) for their guidance and support during my research. I would also like to thank Ms Milana Troskie (DVTB, UP), Ms Anne-Mari Bosman (DVTB, UP), Ms Kgomotso Sibeko (DVTB, UP) and Ms Ronel Volschenk (ARC-OVI) for their technical support and assistance in teaching me techniques necessary for completing my project. Thank-you to the Department of Veterinary Tropical Diseases, University of Pretoria, and the Agricultural Research Council-Onderstepoort Veterinary Institute for allowing me to use the facilities and equipment in completing my project. Thank-you to the BioPAD Consortium for permitting me the opportunity and funding as part of the umbrella BioPAD Project BP040, Development of a Molecular Diagnostic Test Kit for Tick-borne Pathogens.

Thank-you to Mr A. Green and his staff, Dr H. Muller and the Ladysmith State Veterinary Office, especially Mr Q. Doidge, for their assistance in providing clinical histories, sample collection and tick feedings.

Thank-you to Prof. L. Prozesky for performing the post-mortem examination of bovine 9446/6.

Thank-you to Ms Rina Owen and Mr Schalk Human for providing statistical and data analysis support.

Thank-you to the staff at the research unit at Hluhluwe-iMfolozi Park, Ezemvelo KZN Wildlife.

Thank-you to Professional Zoolife Consultants for providing me the time to undertake this study.

And lastly, thank-you to my husband, Grant Bollaert, for his constant support and encouragement.

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Summary

Theileriosis is a tick-transmitted protozoal disease caused by several *Theileria* species. The most virulent species affecting cattle are *T. parva* and *T. annulata*. *Theileria parva* is responsible for causing East Coast Fever (ECF), Corridor Disease and January Disease. *Theileria parva* is endemic in certain areas of South Africa, Zambia, Zimbabwe and elsewhere. In South Africa these areas are controlled, include mostly game reserves containing infected African buffaloes and are in declared Corridor disease infected districts. African buffaloes are natural reservoirs of the *T. parva* parasite and if suitable tick species (*Rhipicephalus appendiculatus* and *R. zambeziensis*) are present, cattle may become infected. Currently, *T. parva* is diagnosed by conducting an array of tests including Giemsa-stained blood and lymph node smears, indirect immunofluorescent antibody (IFA) test, standard PCR and PCR/DNA-probe test. Recent progress in the diagnosis of *T. parva* has involved the development of a Reverse-Line Blot (RLB) technique and a real-time PCR for detecting *T. parva*. Over the last decade, several outbreaks of *T. parva* have occurred in cattle in the province of KwaZulu-Natal. Between 2002 and 2004, Mr A. Green, a Red Brangus stud farmer in the Ladysmith district, lost a total of 42 cattle to *T. parva* infection. An investigation into the epidemiology of *T. parva* in KwaZulu-Natal was structured in three parts: (i) a survey of Red Brangus on Mr Green's farm in order to gain insight into the current prevalence of theileriosis and other tick-borne diseases in the herd; (ii) transmission experiments from *T. parva*-infected cattle on Mr Green's farm to susceptible cattle to demonstrate vector transmission as well as replicate the disease process and pathology; (iii) tick transmission experiments from persistently *T. parva* positive-testing buffalo and persistently *T. parva* negative-testing buffalo, to susceptible bovines to demonstrate suitability of tick transmission methods and compare pathology and clinical signs seen on the farm and in the tick transmission experiments. The survey demonstrated that several factors may interact in determining the number of infected animals, the period that they remain infective to ticks as well as the susceptibility of the rest of the herd to *T. parva*. Sampled bovines did not show clinical signs of *T. parva*-associated disease and with test results indicated that the number of subclinical carriers may be high. Attempts to transmit *T. parva* from infected cattle on Mr Green's farm to susceptible cattle were not successful. Transmission from an infected buffalo to a susceptible bovine was successful and transmission of *T. parva* from a negative-testing buffalo to a susceptible bovine could not be demonstrated. These experiments give some confidence that the tick-transmission methods and diagnostic tests used were reliable. It remains important to consider African buffalo as a possible source of *T. parva* infection in a cattle herd even when a direct link cannot be established.

1 Introduction

1.1 General Introduction

Theileriosis is a tick-transmitted protozoal disease caused by several *Theileria* species, belonging to the family Theiliidae, order Piroplasmia, class Sporozoa, phylum Apicomplexa. Different *Theileria* species affect and are carried by various wild and domestic animals. The most virulent species affecting cattle are *Theileria parva* and *Theileria annulata*. During investigations into these most pathogenic of species, several others have been detected, including *Theileria mutans*, *Theileria taurotragi* and *Theileria velifera*. Although the taxonomy of *T. parva* at sub-species level is not yet resolved, advances have been made in characterising different strains.

Theileria parva is responsible for causing East Coast fever (ECF), Corridor disease and January disease in cattle. East Coast fever generally occurs in East African and some Southern African countries where it is endemic and responsible for widespread losses in susceptible cattle. Transmission from infected cattle to susceptible cattle is by *Rhipicephalus appendiculatus*, a three-host ixodid tick. Corridor disease occurs when the *T. parva* parasite is spread from asymptomatic, carrier African buffalo (*Syncerus caffer*) to susceptible cattle by the same vector as for ECF, as well as by *Rhipicephalus zambeziensis*. Corridor disease can also result in widespread losses and occurs in areas bordering game parks or conservancies accommodating infected buffalo. January disease is usually considered a milder form of the disease (Lawrence, de Vos and Irvin, 1994^c) and transmission via *R. appendiculatus* occurs between infected cattle and susceptible cattle. January disease occurs in Zimbabwe.

Clinical manifestations of these infections vary and may be peracute, acute, subacute or chronic and in various animals show a spectrum of clinical signs from no apparent reaction to severe reactions. Animals that recover are regarded as permanent carriers of the infection.

Theileriosis is a source of continuous economic importance in affected countries. It is difficult to control and once endemic, virtually impossible to eradicate.

The objectives of this study include (i) the investigation of the prevalence of infection of *T. parva* in cattle on a farm in KwaZulu-Natal, (ii) to evaluate the risk of *T. parva* transmission from carrier cattle to susceptible cattle on this farm and (iii) to verify that the tests currently in use are suitable for detecting *T. parva* in carrier buffalo and cattle.

1.2 Literature Review

Theileria parva and *T. annulata* are the species considered most pathogenic in cattle, whereas *Theileria* sp. (sable), *Theileria sylvicapræ* and *T. taurotragi* appear to be pathogenic in sable (*Hippotragus niger*) and roan (*Hippotragus equinus*) antelope, grey duiker (*Sylvicapra grimmia*) (Neitz, 1957; Neitz and Thomas, 1948) and greater kudu (*Tragelaphus strepsiceros*), respectively (Nijhof, Pillay, Steyl, Prozesky, Stoltz, Lawrence, Penzhorn and Jongejan, 2005). *Theileria annulata* causes tropical theileriosis in cattle and is found in tropical and subtropical countries of southern Europe, northern Africa, Sudan, Eritrea, the Near and Middle East and Central Asia, including southern parts of the former USSR, India and southern China (Allsopp, Baylis, Allsopp, Cavalier-Smith, Bishop, Carrington, Sohanpol and Spooner, 1993; Pipano, 1994). The extent of distribution of *T. annulata* in the Far East is not yet known and is still to be established (Pipano, 1994). *Theileria parva* occurs in southern, eastern and central Africa, and causes East Coast fever, Corridor disease and January disease. The sources of *T. parva* infections and outbreaks have been found to be carrier cattle and carrier buffalo. Other *Theileria* spp. found in domestic stock appear to be less virulent, but may also cause disease and loss of production. *Theileria lestoquardi* is found in sheep, *T. taurotragi* in eland (*Taurotragus oryx*), and *T. velifera*, *Theileria serpentii*/ *Theileria orientalis*/ *Theileria buffeli* complex and *T. mutans* in cattle (Norval, Perry and Young, 1992).

The focus in this study was on *T. parva* diseases affecting southern Africa. For descriptive purposes and convenience, *T. parva* was previously divided into three subspecies: *T. parva parva* (cause of ECF), *T. parva lawrencei* (cause of Corridor disease) and *T. parva bovis* (cause of January disease or Zimbabwean theileriosis). Molecular evidence suggests that although *T. p. bovis* and *T. p. parva* appear to be very closely related, there is tremendous genetic variation between these isolates and *T. p. lawrencei* isolates (Collins and Allsopp, 1999). Presently, this trinomial classification has been dropped and the causative organisms are simply referred to as cattle-associated and buffalo-associated *T. parva* (Perry and Young, 1993). Some consider *T. parva* strains to consist of a continuum of subspecific types found to occur in different hosts rather than being separated by geographical distribution (Collins, Allsopp and Allsopp, 2002). Others have found that different strains of *T. parva* are responsible for disease in different areas (Uilenberg, Perie, Lawrence, de Vos, Paling and Spanjer, 1982). Animals that are immune to one strain from a particular area are not necessarily immune to strains from other areas (Latif, Hove, Kanhai and Masaka, 2001).

1.2.1 Disease Syndromes:

East Coast fever (ECF)

In 1901, a shipment of cattle from Dar-es-Salaam via Beira, was involved in introducing ECF to Mozambique, Zimbabwe and South Africa. These cattle were being introduced to southern

African countries because the Rinderpest pandemic had resulted in devastation of the cattle population. East Coast fever was extremely virulent to all types of cattle and resulted in heavy mortalities. It was finally brought under control between 1946 and 1955, by implementing a rigorous programme of intensive dipping, quarantining infected farms and compensated slaughtering. Although ECF was eradicated in southern Africa, the vector, *R. appendiculatus*, is still widespread.

Although ECF, Corridor disease and January disease all share similar clinical signs (see section 1.2.4), the course and duration of disease may vary. East Coast fever has an incubation period of eight to 25 days. The course of the disease is usually shorter if the challenge is severe (Lawrence, de Vos and Irvin, 1994^a). The mortality rate in fully susceptible cattle can be nearly 100%. In indigenous zebu cattle in endemic areas, however, the mortality is low even though morbidity is nearly 100% (OIE and Centre for Food Security and Public Health, Iowa State University, 2004). About five percent of animals may undergo a prolonged recovery. Pyrexia and enlarged lymph nodes are consistent features, even if infected animals are partially immune or have inherent resistance (Lawrence *et al.*, 1994^a)

Corridor disease

African buffalo are asymptomatic carriers of *T. parva*. Waterbuck (*Kobus ellipsiprymnus*) are also considered natural reservoir hosts (Ogden, Gwakisa, Swai, French, Fitzpatrick, Kambarage and Bryant, 2003). *Rhipicephalus appendiculatus* and related species of ticks (*R. zambeziensis*) become infected with *T. parva* after feeding on buffalo. Once these ticks have moulted, they are capable of transmitting the parasite to susceptible cattle, which then contract Corridor disease. The disease is usually fatal. It was first diagnosed in the ‘corridor’ between Hluhluwe and iMfolozi game reserves in KwaZulu-Natal, South Africa (Uilenberg, 1999). This buffalo-associated *T. parva* remains endemic in certain buffalo herds in South Africa and Zimbabwe, and Corridor disease still occurs in both countries.

Clinical signs are similar to those seen in ECF, but the course of disease is usually shorter, with deaths occurring within four days of the onset of disease. Mortality is usually in the region of 80% (Lawrence, de Vos and Irvin, 1994^b).

January disease

Once ECF had been eradicated in Zimbabwe, another form of theileriosis emerged – January disease. The disease is maintained in the cattle population and transmitted predominantly by *R. appendiculatus* adult ticks (Lawrence *et al.*, 1994^c). For this reason, the highest number of clinical cases occurs during the summer months when adult *R. appendiculatus* ticks are plentiful. It is not known whether this disease came about as a result of a transformation of ECF strains or buffalo-associated *T. parva* strains (Norval, Fivaz, Lawrence and Brown, 1985);

by selection of a sub-population from a group of *T. parva* strains; or as a result of diapausing *R. appendiculatus* strains being less efficient at transmitting the *T. parva* parasite than the more tropical non-diapausing *R. appendiculatus* strains (Norval *et al.*, 1992).

January disease involves clinical signs similar to those seen in ECF, but the course of the disease may be shorter, with deaths occurring within four days of the onset of disease. Serological evidence and experimental infections indicate that this form of disease is commonly subclinical or mild (Lawrence *et al.*, 1994^c).

1.2.2 *Theileria parva* life cycle

When a suitable tick vector (for example *R. appendiculatus* larva or nymph) feeds on an infected animal, the tick ingests *T. parva* merozoites present in the host's erythrocytes. These merozoites undergo gametogenesis and fertilization in the tick's gut lumen. The resulting zygote invades an epithelial cell of the tick's gut and remains there until the tick has undergone a moult. Thereafter, the zygote develops into a motile kinete that migrates to the tick's salivary glands. Once the post-moult nymph or adult tick starts feeding on a new host, the kinete develops into a sporozoite. Sporozoites are then released in the tick's saliva into the host animal during the later stages of feeding (Lawrence *et al.*, 1994^a).

The sporozoites penetrate lymphocytes and develop into schizonts. This results in a lymphoproliferative disorder and lymphadenomegally. Some of the schizonts undergo asexual division and later differentiate into merozoites. Merozoites invade the host's erythrocytes, and develop into piroplasms, which are infective for ticks.

Very little asexual reproduction of *T. parva* piroplasms occurs in the erythrocytes of bovines, unlike species such as *T. annulata* (Norval *et al.*, 1992).

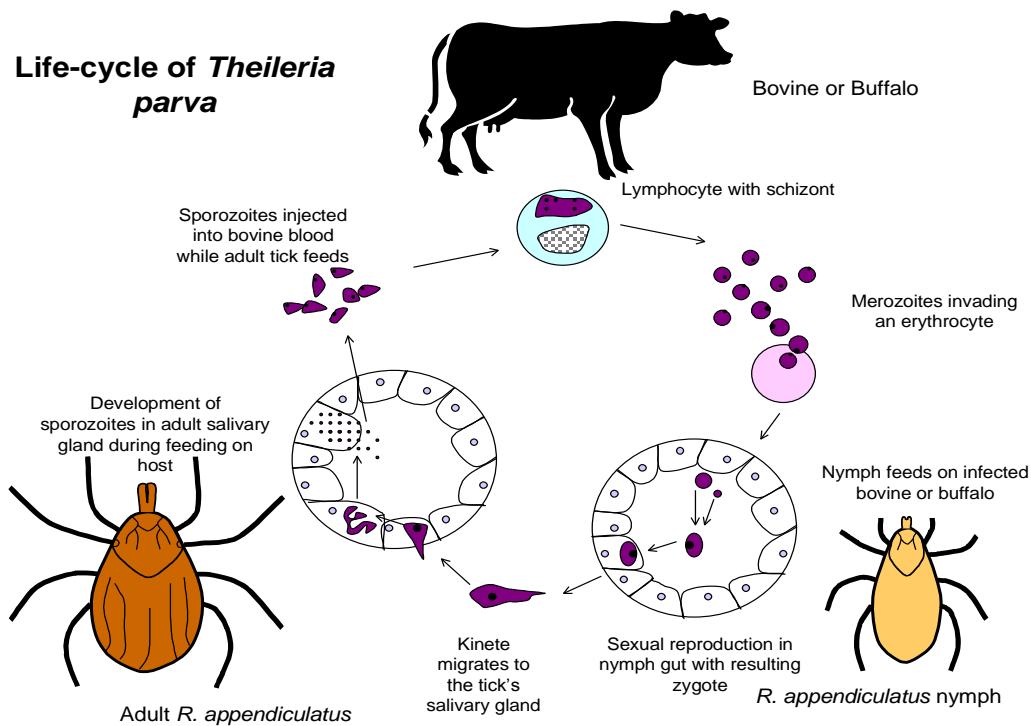


Figure 1.1: Life-cycle of *Theileria parva*.

1.2.3 Epidemiology of theileriosis in southern Africa

East Coast fever was introduced to southern Africa from eastern Africa in 1901/02 with the importation of infected cattle. East Coast fever was eradicated; the vector, *R. appendiculatus*, still remains and Corridor disease is still present in South Africa (Lawrence *et al.*, 1994^b).

Theileria parva infection does not always result in disease. Buffaloes are able to carry the parasite without becoming ill and show few, if any, clinical signs. Once infected with *T. parva*, buffalo are considered to be persistently infected with the parasite (Lawrence *et al.*, 1994^b). Cattle usually present with obvious clinical signs and either die from the associated disease or become persistently infected with the parasite (Lawrence *et al.*, 1994^b). These permanent carriers, whether buffaloes or cattle, remain a source of infection to susceptible animals that share the same habitat, provided that there is a suitable vector to transmit the parasite.

Theileria parva is endemic in certain areas of South Africa, Zambia and Zimbabwe. In South Africa these areas are controlled and are declared Corridor disease infected districts. Areas found within these districts include the Kruger National Park (KNP) and Hluhluwe-iMfolozi and their neighbouring game parks and farms in the north-eastern parts of South Africa. Corridor disease outbreaks occur in areas bordering on or near where infected buffalo herds graze. Buffaloes are natural reservoirs of the *T. parva* parasite and if suitable tick species (*R. appendiculatus* and *R. zambesiensis*) are present, cattle may become infected. Corridor disease has been regarded as self-limiting since cattle usually die before the parasite develops

into the piroplasm, which is infective to the tick (Norval *et al.*, 1992). However, some experimental evidence suggests that some bovines survive the disease and may be capable of passing it on to other cattle (Lawrence *et al.*, 1994^b).

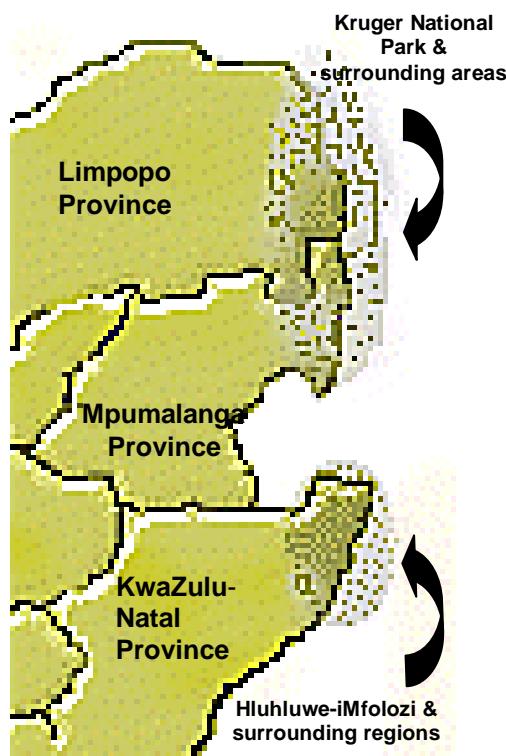


Figure 1.2: Areas where Corridor disease occurs endemically in South Africa.

Cattle that survive a *T. parva* infection become permanent carriers of the parasite. Furthermore, carriers may arise from being immunised (Bishop, Sohanpol, Kariuki, Young, Dolan and Morzaria, 1992; Irvin, Morzaria, Munatswa and Norval, 1989; Maritim, Kariuki, Young and Mutugi, 1989) or after being treated with anti-theilerial drugs (Dolan, 1986). In this way one can foresee that it is possible that new strains may be introduced into new areas or that a carrier state may develop which could interfere with control programmes already in place.

In South Africa, the greatest threat of *T. parva* disease outbreaks comes from infected buffaloes coming into contact with susceptible cattle. Legislation and registration of farms and movement of buffaloes within South Africa are currently controlled by the National Directorate of Animal Health (2002).

- Buffaloes from the KNP and neighbouring reserves are categorised as “Foot and Mouth Disease (FMD) carrier buffaloes”. These buffaloes are considered to be carriers of FMD and Corridor disease, and are infected or potentially infected with bovine brucellosis and bovine tuberculosis.

- Buffaloes from Hluhluwe-iMfolozi and neighbouring reserves are categorised as “Corridor disease carrier buffaloes”. These buffaloes are considered to be carriers of Corridor disease and are potentially infected with bovine brucellosis and bovine tuberculosis.
- “FMD/Corridor disease free buffaloes” are those buffaloes considered to be free of FMD and Corridor disease and may be kept on farms or reserves registered for FMD/Corridor disease free buffaloes.
- Owners of farms who wish to accommodate buffaloes on their properties are required to apply for registration with the National Director of Animal Health. The requirements that the applicants need to adhere to involve keeping of classified buffaloes in the specific region, strict fencing requirements and regular inspections by state veterinarians.
- “Red cross” movement permits strictly control all buffalo movements after appropriate disease testing. The buffalo disease-testing schedule for FMD/Corridor disease-free buffaloes include the blocking ELISA test for FMD; smear examination, IFAT and real-time PCR assays for Corridor disease; intradermal tuberculosis (TB) test for bovine TB and complement fixation test for brucellosis. Buffaloes from the KNP or Hluhluwe-iMfolozi are automatically considered Corridor disease infected.
- Should a buffalo have a positive result for Corridor disease, it must be translocated to a registered Corridor disease farm or destroyed under veterinary supervision. The remaining negative animals are kept under quarantine and retested twice. The first of the two tests is done a minimum of 30 days after the removal of positive animals, and the second within a three-month interval while under tick-free conditions, to confirm their negative status. The farm or used camps must be rested of cattle and buffaloes for a minimum period of two years.

Barnett and Brocklesby (1966) demonstrated that buffalo-associated *T. parva* could transform into cattle-associated *T. parva* (Norval *et al.*, 1992). This transformation would allow the parasite to become established in a cattle herd. Transformation has not been observed in southern Africa. Nevertheless, as long as suitable physical separation of buffaloes and cattle is maintained, there are no ‘incidental’ mechanical vectors of infected ticks, a strict rule of quarantining affected farms is enforced, positive cattle are slaughtered and susceptible cattle are prevented from being ‘immunised’ by treating affected herds with anti-theilerial drugs, it is unlikely to occur.

If, however, there is continuous contact between infected buffaloes and susceptible cattle, if *R. appendiculatus* ticks are present throughout the year and if many of the cattle become carriers of buffalo-associated *T. parva*, it is possible that buffalo-associated *T. parva* may become established in cattle (Norval, Lawrence, Young, Perry, Dolan and Scott, 1991; Lawrence *et al.*, 1994^b) in South Africa. It has also been shown that a low innate susceptibility of cattle, continuous exposure to all instars of *R. appendiculatus* and sufficient infection prevalence of *T. parva* in *R. appendiculatus*, all contribute to the development of endemic stability (Norval *et al.*,

1992; Perry, Lessard, Mukhebi and Norval, 1992). This is certainly not the case in South Africa, where most cattle on commercial farms are exotic breeds or crosses and where *T. parva* only occurs in pockets in buffalo areas and the surrounds. This indicates that the South African National Herd would be at a high risk and susceptible to *T. parva* epidemics, should the disease not be confined to designated Corridor disease areas.

As seen in ECF outbreaks in eastern Zambia and Malawi, peaks of disease incidence of *T. parva* are expected in the rainy season (December to March, when adult ticks are most abundant) and the dry season (May to July, when nymphs are most abundant) (Latif, Hove, Kanhai, Masaka and Pegram, 2001). The highest numbers of cases are seen during the summer, when nymph-to-adult transmission dominates the infection mechanism (Billiouw, Vercruyse, Marcotty, Speybroeck, Chaka and Berkvens, 2002; Chinombo, Mzoma and Musisi, 1989).

Particular strains of ticks also influence the epidemiology of the disease. Scientists in South Africa failed to transform a buffalo-associated *T. parva* isolate that was subsequently transformed to a cattle-adapted *T. parva* in Kenya. This may have been as a result of the use of different tick stocks in which the parasite behaved differently (Norval *et al.*, 1992). Furthermore, *R. appendiculatus* populations from eastern Africa tend to become more highly infected with *T. parva* than those originating from southern Africa, resulting in a more severe disease state in affected cattle (Norval *et al.*, 1992). A reason for this may be that infectivity of unfed adults declines with time: non-diapausing adult ticks, as found in East Africa, become active soon after moulting, when infectivity is highest, whereas diapausing adult ticks from southern Africa may remain inactive for several months and in so doing lose infectivity (Norval *et al.*, 1992).

It has also been found that the prevalence of infection in ticks that have fed on *T. parva*-infected buffaloes seem to have a higher prevalence than ticks that fed on infected cattle; i.e. carrier buffaloes are much more infective to ticks than carrier cattle (Norval *et al.*, 1992; Young and Grootenhuis, 1985). Thus, *T. parva* transmitted by ticks from buffaloes to cattle is likely to be more severe than *T. parva* transmitted by ticks from cattle to cattle.

1.2.4 Clinical Signs and Pathology

A distinction between *T. parva*-carrier animals and clinical cases of disease must be made. Carriers are either cattle or buffaloes that become permanently infected after transmission of the parasite. An infection may result in the manifestation disease or be subclinical and asymptomatic. Usually cattle will show some reaction, even if it is mild, whereas infections in buffaloes are asymptomatic. Reactions to infection with *T. parva* can be classified according to

a number of parameters. The recommended classification, according to an ECF workshop held in Lilongwe, Malawi (Anon., 1989), is as follows:

- No reaction or no apparent reaction: no parasites are detected and no clinical signs are apparent.
- Mild reaction: few schizonts are detected, no fever occurs or fever persists for less than four days. The animal is otherwise clinically normal and recovers.
- Moderate reaction: schizonts are detected, fever persists for longer than four but less than nine days. The animal shows mild and transient clinical signs and recovers.
- Severe reaction and recovery or death: schizonts are detected, fever persists for eight days or longer and the animal has obvious signs of theileriosis. The animal may recover from a severe reaction but usually dies.

This classification aids in describing the clinical manifestation of particular strains of *T. parva* in individual animals, particular herds and in particular regions. For example, some strains are found to result in mild reactions in indigenous cattle herds under constant tick challenge, but severe reactions in exotic or cross-breeds under low tick challenge (Irvin *et al.*, 1989; Perry *et al.*, 1992).

Another factor that may contribute to the clinical manifestation of a *T. parva* infection is the age of the cattle being exposed. In Zimbabwe it was found that the mortality attributed to January disease in young calves (less than seven months old) is lower than that in adult cattle in the same herd (Koch, Kambeva, Norval, Ocama, Masaka, Munatswa, Honhold and Irvin, 1990). This resistance seems to be short-lived, however, and may be related to the infective dose received. The reason given is that calves tend to carry far fewer ticks than adult cattle and therefore the total number of parasites transmitted may be lower in calves than adults.

The clinical signs associated with a severe *T. parva* infection are: pyrexia, enlargement of superficial lymph nodes, severe pulmonary oedema, wasting and death. Other clinical signs include lacrimation, corneal opacity, diarrhoea and occasionally neurological signs ("Turning Sickness"). The incubation period varies between eight and 25 days and the illness may be prolonged for up to 25 days. Terminally, dyspnoea develops with an increasing respiratory rate, watery cough and discharge of frothy fluid from the nostrils. A small number of animals may survive, but convalescence is prolonged. Affected adult cattle may remain unproductive for months (Lawrence *et al.*, 1994^a) and calves may experience stunted growth (Moll, Lohding, Young and Leitch, 1986).

Clinical signs can easily be linked to the pathology seen in animals that have died or been slaughtered as a result of *T. parva* infection. Characteristically on post-mortem examination, lymph nodes are enlarged, hyperplastic and oedematous and/or haemorrhagic. Serous atrophy of fat may be marked. Serosal surfaces may show petechiae and ecchymoses. Ulcers and

haemorrhages are often seen throughout the gastro-intestinal tract, especially in the abomasum. Lymphocytic infiltrates are often seen in the liver and kidneys. The lungs are most severely affected, showing severe oedema, interlobular emphysema and froth-filled trachea and bronchi (Lawrence *et al.*, 1994^a).

1.2.5 Diagnosis

Investigation of *T. parva* outbreaks is complicated by the occurrence of carrier cattle or buffaloes that are infected but do not show clinical signs. Therefore, it remains important to determine whether the parasite is present in a herd by conducting an array of tests that will diagnose the disease in ill animals as well as detect subclinical carriers. These assays include Giemsa-stained blood and lymph node smears, indirect immunofluorescent antibody test (IFAT), PCR/DNA-probe test and most recently a real-time PCR assay. In cattle that have died as a result of this disease, a gross post-mortem examination with characteristic pathology, supported by histopathology and other techniques discussed below, would be suitable for diagnosing the disease in a herd.

- Blood smear and lymph node smear examination

Smears are examined for schizonts and piroplasms. Some animals may have low parasitaemias, and therefore infection may not be detected on blood smear examination. Thus, a negative result should not be regarded as confirmation that infection is absent but rather that the parasitaemia, if present, was too low to be detected microscopically. Different *Theileria* species' piroplasms do differ morphologically, but their morphology also varies during the course of infection, rendering this method diagnostically fallible (Norval *et al.*, 1992).

- Xenodiagnosis

This process involves allowing laboratory-reared nymphal ticks to engorge on an animal believed to be infected with *T. parva*. The nymphs are collected and allowed to moult. Once the adult ticks have hardened, they are placed on a susceptible animal and allowed to feed. This susceptible animal is observed for clinical signs, including fever, and regular blood smears and lymph node aspirate smears are made to observe for the occurrence of *T. parva* schizonts. Should the animal become very ill, it is usually euthanised and a full post-mortem examination performed. The advantages of performing a xenodiagnosis include being able to prepare tick stabilates; follow the course of disease from infection to recovery or death and correlate that with the clinical process observed in the field; and to collect multiple blood samples which may allow for isolation of the particular strain causing disease in a specific area. The disadvantages include that once a susceptible animal is infected, it may undergo undue suffering if not euthanised timeously, and in comparison with other diagnostic methods, it is expensive. Xenodiagnoses are usually only used in a situation where *T. parva* has never occurred in a particular area and it is essential to

confirm that the clinical process occurring in the field is associated with a *T. parva* infection in the herd.

- **Indirect Immunofluorescent Antibody Test (IFAT)**

This assay was designed to determine whether an animal has been exposed to *T. parva* and mounted an immune response, i.e. seroconverted. *Theileria parva*-infected cells (a tissue culture monolayer) are fixed on a glass slide. Sample serum is incubated on the slide and antibodies in the serum bind to the antigen. Thereafter the slide is rinsed to remove any unbound antibodies. Anti-bovine antibodies conjugated to a fluorescent compound are then incubated with the antigen-antibody complexes. The anti-bovine antibodies bind to the antigen-antibody complex and the excess is washed away. The slide is then examined for fluorescing complexes under a fluorescent microscope. A positive result shows fluorescence whereas a negative has no fluorescing complexes. Results may be subjective, difficult to interpret and may not be reproducible (Collins *et al.*, 2002). Difficulty interpreting IFAT results often involves cross-reactions between *T. parva* and other *Theileria* species (OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 2004; Morzaria, 1989).

- **PCR/DNA probe Test**

DNA is extracted from the blood or tissue sample. A universal set of primers is used to amplify a fragment of the 18S rRNA gene using PCR. PCR products are then visualised in ethidium bromide agarose gel, after electrophoresis, under ultra-violet light. This method is capable of detecting the presence of the *Theileria* genus. In order to detect *T. parva*, amplicons are blotted with the *T. parva* radioactively labelled probe (Collins *et al.*, 2002). Since parasitaemias fluctuate during an infection, should the parasitaemia in the infected animal or a blood sample be low, the parasite DNA may not be detected. This would then produce a negative result even though the animal is infected. A further disadvantage of this method is that it is extremely time consuming.

Recent progress in the diagnosis of *T. parva* involves the development of a Reverse Line Blot (RLB) Technique and a real-time PCR for detecting *T. parva*. The RLB technique is usually reserved for use in research and not for routine diagnostic tests. The real-time PCR has been validated for the diagnosis of *T. parva* in cattle and buffaloes in South Africa.

- **Reverse Line Blot (RLB) Technique**

This assay involves amplification of the V4 variable region of the 18S rRNA gene of *Theileria* and *Babesia* spp. The PCR products (which are biotin-labelled) are allowed to bind to oligonucleotides fixed to a membrane and specific for each *Theileria* species. These oligonucleotide-PCR product complexes are then exposed to peroxidase-labelled streptavidin and rinsed, and incubated with Electrochemiluminescent (ECL) detection fluid before exposure to ECL hyperfilm. This results in reactive areas on the membrane being represented on hyperfilm and corresponding to positive reactions for specific *Theileria* species. Gubbels, de Vos, van der Weide, Viseras, Schouls, de Vries and Jongejan (1999)

illustrated the application of RLB to detect and differentiate various *Theileria* and *Babesia* species. Subsequent to that, RLB has been developed in order to detect and differentiate *Anaplasma* and *Ehrlichia* species. RLB has been recommended for use in epidemiological monitoring of tick-borne diseases by Gubbels *et al.* (1999) and has been used in detection and differentiation of *Theileria* and *Babesia* species in small ruminants (Schnittger, Yin, Qi, Gubbels, Beyer, Niemann, Jongejan and Ahmed 2004; Taoufik, Sonneveld, Nijhof, Hamidjaja, Pillay, Oosthuizen, de Boer and Jongejan, 2004).

- *Theileria parva*-specific real-time PCR assay

Real-time PCR allows for visualisation of amplification of PCR products by fluorescence during the actual reaction. It is used for genotyping as well as quantifying the initial amounts of template molecules. Characterisation of amplified sequences can be done by melting-curve analysis and quantification is done by making use of amplification curves. Fluorescent signals are generated by double-stranded DNA (dsDNA)-specific dyes or by sequence-specific fluorescent oligonucleotide probes. SYBR Green I is the most commonly used dsDNA-specific dye. Sequence-specific probes may include hybridisation probes, hydrolysis probes and light-up probes (Wilhelm and Pingoud, 2003). Real-time PCR has been validated for diagnosing *T. parva* in cattle and buffaloes in South Africa at the Department of Veterinary Tropical Diseases, University of Pretoria (DVTD, UP). The real-time PCR method involves a PCR reaction in which primers are used to amplify the V4 variable region of the 18S rRNA gene to which the species-specific hybridisation probes hybridise. Donor probes are labelled at the 3' end with a reporter fluorescein and acceptor probes are labelled at the 5' end with an acceptor fluorophor (LC Red640). The donor fluorescein is excited by the blue light LED source and the acceptor is not, so that no acceptor fluorescence is observed from probes free in solution. During primer annealing, the donor and acceptor probes hybridise adjacent to each other on the DNA. In so doing, excitation energy is transferred from the donor to the acceptor probes. This results in emission of light of a different frequency and associated with excitation of the acceptor probe, which is then detected by the machine. This reaction will only occur if the probes anneal to the DNA within a short distance of each other on the DNA strand. Should the probes bind to another region of the DNA, there should not be any reaction occurring since the probes would be too far apart or one not binding at all. A considerable advantage of this assay is that it is relatively quick and easy to perform in comparison with other diagnostic methods.

1.2.6 Control and Treatment of Theileriosis

In areas bordering game farms in South Africa, buffalo-associated theileriosis has primarily been controlled by physical separation of buffaloes and cattle in accordance with the National Directorate of Animal Health. Tick control in these areas has to be strict. Intensive dipping programmes are required and this may be very expensive. Other control methods include

slow-release acaricide devices, more effective methods of application of topical acaricides, manipulation of hybrid sterility between closely related tick species and use of pheromones to disrupt mating and improve acaricide treatment (Lawrence *et al.*, 1994^a). Biological tick control methods have been studied, some tested but not yet implemented on a large scale (Norval *et al.*, 1992).

In countries such as Kenya, *T. parva* is endemic in cattle herds and responsible for losses in production. Cattle in these areas are not protected by twice weekly application of acaricides (Moll, Lohding and Young, 1984; Moll *et al.*, 1986). By using infection (either stabilates or natural) and treatment immunisation cattle farmers in these areas are able to markedly improve their production (Young, Leitch, Dolan, Mbogo, Ndungu, Grootenhuis and de Castro, 1990) as well as reduce long-term costs (Pegram, James, Bamhare, Dolan, Hove, Kanhai and Latif, 1996).

A similar strategy was adopted in order to try to control buffalo-associated *T. parva* on the Zimbabwean Highveld. Cattle were immunised with different *T. parva* stocks and then exposed to buffalo-associated *Theileria* challenge. It was found that immunised cattle survived the theileriosis challenge for at least 18 months whereas non-immunised cattle suffered severe and fatal theileriosis (Latif *et al.*, 2001).

It has also been established that following inoculation with a mild strain, such as *T. parva* (Boleni), immunisation may be achieved without having to treat the infection with anti-theilerial drugs (Irvin *et al.*, 1989). It is important to note, however, that cocktail stabilates such as the Muguga cocktail impart broad but not universal protection by immunisation (Musisi, Quiroga, Ngulube and Kanhai, 1989).

The International Livestock Research Institute (ILRI) in Nairobi, Kenya, is currently directing research into the development of a subunit vaccine (The *Theileria parva* Genome Database, no date). Identifying target antigens in the parasite is proving challenging, however, and more work is required in this area.

A recent study in the Southern Province of Zambia has provided another weapon in the arsenal against *T. parva*. Fandamu, Duchateau, Speybroeck, Marcotty, Mbao, Mtambo, Mulumba and Berkvens (2005) described how the incidence of *T. parva* disease increased with the presence El Niño, linking disease outbreaks with climatic phenomena. In this way, multiple El Niño Southern oscillation index (MEI) ranks can be used as an early-warning system to predict ECF outbreaks. This affords veterinary services an opportunity to prepare for, control and possibly prevent severe outbreaks.

Theileriosis can be treated. Tetracyclines were the first drugs used to treat this disease. They are only effective in the very early stages of infection, before clinical signs are present. Later, parvaquone and buparvaquone were developed – the most effective being buparvaquone. Early diagnosis is still essential for treatment to be effective. None of the drugs are effective once respiratory signs are present. By treating an affected herd with these drugs, animals that have not yet shown clinical signs but are incubating the disease are effectively immunised. This results in an increase in the number of carrier cattle in the herd. In South Africa the use of chemotherapy has been prohibited because of the danger of carrier status developing in cattle infected with buffalo-associated *T. parva* (Potgieter, Stoltz, Blouin and Roos, 1988).

1.3 Recent Outbreaks

According to Collins *et al.* (2002), huge profits can be made by unscrupulous operators who are tempted to circumvent regulations and move buffaloes from prohibited areas. Such an incident occurred near Bela-Bela in 1994, when buffaloes were moved from the FMD control area near the KNP (Collins *et al.*, 2002; Kotze, van Vuuren, Coetzee, Cook, le Roux, Lourens and Viljoen, 1994).

Several problems pertaining to Corridor disease have arisen over the last eight to nine years in KwaZulu-Natal, as reported at the Wildlife Theileriosis Workshop held at the Faculty of Veterinary Science, University of Pretoria (Penzhorn, 2003). In 1991, a farmer in the Ladysmith district lost 3-4 cattle to theileriosis (diagnosed on histopathology by Vetdiagnostix, South Africa). There were reportedly no buffaloes in the area. In 1998, assumed “Corridor disease-free buffaloes” were sold from Spioenkop, Weenen and Itala. Some of these buffaloes were moved to a cattle farm at Wasbank, where 210 cattle on a neighbouring farm subsequently died from *T. parva*. In 2002, there were several outbreaks of Corridor disease on cattle farms near Hluhluwe. These outbreaks usually occurred on farms adjacent to buffalo farms. Some of the cattle on these farms sero-converted without showing any clinical signs of disease i.e. became carriers. In 2003 there were a further three or four outbreaks on cattle farms in the KZN Corridor disease control area.

1.3.1 Case Study: Mr A. Green’s Farm, Ladysmith, South Africa

Between 2002 and 2004, Mr Alan Green, another farmer in the Ladysmith district, lost a total of 42 cattle to suspected *T. parva* infection. Most losses were confirmed by the ARC-OVI to be as a result of *T. parva* infection, although there had been some losses in the year prior to that which had not been explained. Originally it was thought that there was no possible connection to buffaloes. In subsequent communications with the farmer and his wife, however, it was established that his farm borders the Spioenkop Nature Reserve and that buffaloes had been seen in the area within the previous four to five years.

The first suspected outbreak occurred on the section of Mr Green's farm called Maria's Heuwel (Figure 1.3) in April 2001. Originally it was thought that the herd was suffering from a redwater outbreak, but the animals did not respond to treatment. Cows were sick for approximately 3 days with pyrexia and coughing. On post mortem examination the private practitioner, Dr H. Muller, found hydrothorax, hydropericardium, lung oedema and emphysema, as well as 'Clostridial-type' lesions around the kidneys. After another three animals died, samples were sent to Vetdiagnostix (Pietermaritzburg, South Africa). The diagnosis was acute fibrinous pneumonia. Treatment with 'Micotil 300' (300mg/ml tilmicosin, Elanco Animal Health), 'Nuflor' (300mg/ml florfenicol, Schering-Plough Animal Health), 'Tylo 200' (200mg/ml tylosin, Phenix, Virbac RSA), penicillins and tetracyclines was attempted. None of the affected animals recovered, but it was noted that those that had been treated with tetracyclines survived the longest.

In January 2002, the same clinical phenomenon occurred. After six deaths, a carcass was sent to Allerton Diagnostic Centre in Pietermaritzburg, from where a specimen was sent to the ARC-OVI. A positive PCR/DNA-probe result for *T. parva* was recorded. During the remainder of 2002 another two animals died. In the summer months Mr Green dipped his cattle every two weeks with an acaricide with a residual action, as well as applying pour-on between the ears to specifically try to control *R. appendiculatus*. This herd of animals was moved to another area of the farm, Harmony (Figure 1.3), during the winter.

On the 25th of January 2003, another animal died in the Harmony area of the farm. In January-February 2004, Mr Green lost another 17 animals on the Waverly and Easby sections of his farm (Figure 1.3). During the 2003 and 2004 outbreaks, the rest of the animals in the herds were treated with tetracyclines and regularly and intensively dipped for *R. appendiculatus* ticks. The number of deaths was reduced with this strategy.

In 2004, 60 of his young sale bulls were tested for *T. parva*. Of those 60 young bulls, 25 animals tested positive on one or more of the ARC-OVI's standard panel of tests (blood smear, IFAT, conventional PCR, and PCR/DNA-Probe).

Because of the increased number of Corridor disease outbreaks within South Africa, it was decided that these circumstances should be investigated. The outbreak on Mr Green's farm was especially perplexing since there was no known direct link with infected buffaloes.

The farms are located in the KwaZulu-Natal Midlands about 30km from Ladysmith. Three of the farm sections run adjacent to each other (Harmony, Waverly and Easby) and the fourth (Maria's Heuwel) is along the same road but separated by a distance of about five kilometres (Figure 1.3). Harmony borders on the Spioenkop Nature Reserve and all four farms

occasionally have antelope on them. The entire herd consists of about 2000 head of Red Brangus and about 200 head of Jersey. The Jerseys have been kept intensively and did not experience any losses due to theileriosis. The Red Brangus are kept extensively, except for young bulls that are kept in smaller pastures and fed concentrates in addition to grazing before the yearly sales. The animals are kept in herds according to age and gender and moved between the farms as the owner sees fit for pasture management.



Figure 1.3: General layout of farm sections in relation to surrounding areas and main roads.

Green Areas: Mr Green's farms.

Pink Area: Spioenkop Nature Reserve

Purple Area: Surrounding farmlands.

The investigation was structured in three parts:

- Survey of Red Brangus on Mr Green's farm in order to gain insight into the current prevalence of theileriosis and other tick-borne diseases in the herd.
- Transmission experiments from cattle on Mr Green's farm believed to be *T. parva*-infected, to susceptible cattle under controlled conditions in order to demonstrate vector transmission as well as replicate the disease process and pathology seen in affected cattle on Mr Green's farm.

Tick transmission experiments from persistently *T. parva* positive-testing buffalo and persistently *T. parva* negative-testing buffalo, as determined by real-time PCR assay, to susceptible bovines. This was done under controlled conditions, in order to demonstrate suitability of tick transmission methods and compare pathology and clinical signs with those

seen on the farm and in the tick transmission experiment from *T. parva* infected cattle to susceptible cattle.

2 Materials and Methods

2.1 Survey of Cattle on Mr A. Green's Farms

The aim of the cattle survey on Mr Green's farms was to gain insight into the potential number of *T. parva*-infected animals and in so doing gain an impression of the extent of the risk of infection to non-infected animals. Furthermore, this survey would provide base-line data that could be used in order to determine a rate of infection and incidence of disease if required later. Other tick-transmitted diseases were also to be surveyed to gain insight into the infectious-disease load on the herd. See Figure 2.1 for the schematic representation of the survey done.

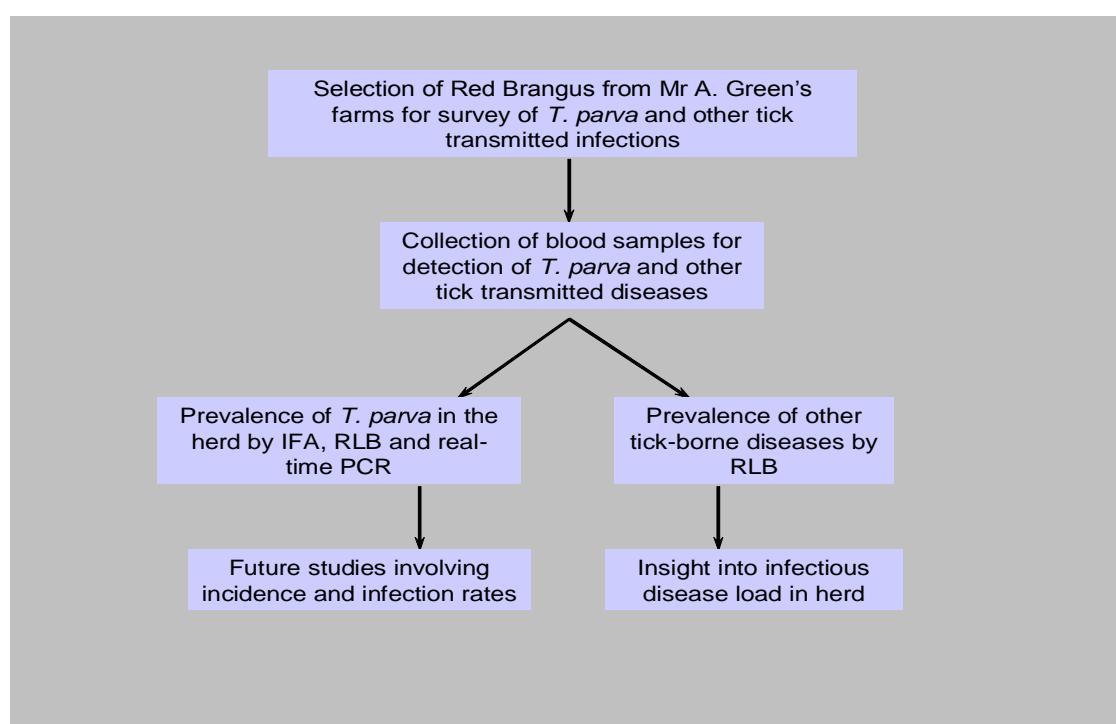


Figure 2.1: Schematic representation of the survey of Mr Green's farm.

Sample Collection:

During January 2005, 170 Red Brangus cattle on Mr Green's farm were sampled to determine prevalence of infection of *T. parva*. The number of cattle from each sex and age group were chosen in proportion to their representation in the Red Brangus population on the farms and a proportionate number of animals were chosen on each section. Within each of the smaller herds, they were chosen randomly, except for calves. Calves were chosen according to which cow had been selected in order to determine whether there was any relationship between dam and calf in disease transmission. Blood in EDTA (5 ml) and serum (5 ml) tubes was collected by venipuncture of the tail vein or jugular vein. The blood in the serum tubes was allowed to coagulate and both sets of samples were stored at ca. 4°C. The EDTA samples were used for real-time PCR and RLB assays and the decanted serum for IFAT assays.

2.1.1 DNA Extraction

DNA was extracted from 200 µl EDTA blood using the blood and body fluid spin protocol and reagents described in the QIAamp® DNA Mini Kit (QIAGEN, Germany). The DNA extracts were eluted in 100 µl buffer AE and stored frozen at -20°C.

2.1.2 *Theileria parva*-specific Real-Time PCR Assay

This test has been validated for use in diagnosing *T. parva* infections in cattle and buffalo using the LightCycler V2.0 (Roche Diagnostics, Mannheim, Germany). The LightCycler® FastStart DNA Master^{PLUS} HybProbe (Roche Diagnostics) was used for the PCR mix. The primers and probes were obtained from TIB Molbiol (Berlin, Germany) and are protected by intellectual property laws and therefore cannot be published currently. A positive (*T. parva*-carrier buffalo) and negative (PCR grade water) control were used in each run.

2.1.3 Reverse Line Blot method

The RLB PCR protocol, reagents, equipment and RLB membranes used were those recommended and produced by ISOGEN Life Science® (Maarsen, The Netherlands). In the ISOGEN RLB hybridisation kit, 36 probes for *Anaplasma*, *Ehrlichia*, *Babesia* and *Theileria* species are included (Table 2.1).

A positive (*Babesia felis* positive sample) and negative (PCR grade water) control were used in each run.

RLB PCR Reagents

Primers RLB-F2 and RLB-R2 (see Table 2.2) were used to amplify a ~400 bp region of the V4 variable region of the 18S rRNA gene.

Table 2.1: RLB species-specific probes.

Lane	Species	Probe Sequence ¹ (from 5'-3')
1	<i>Ehrlichia/Anaplasma</i> catch-all	GGG GGA AAG ATT TAT CGC TA
2	<i>Anaplasma centrale</i>	TCG AAC GGA CCA TAC GC
3	<i>Anaplasma marginale</i>	GAC CGT ATA CGC AGC TTG
4	<i>Anaplasma phagocytophilum</i>	TTG CTA TAA AGA ATA ATT AGT GG
5	<i>Anaplasma phagocytophilum</i>	TTG CTA TGA AGA ATA ATT AGT GG
6	<i>Anaplasma phagocytophilum</i>	TTG CTA TAA AGA ATA GTT AGT GG
7	<i>Anaplasma phagocytophilum</i>	TTG CTA TAG AGA ATA GTT AGT GG
8	<i>Ehrlichia ruminantium</i>	AGT ATC TGT TAG TGG CAG
9	<i>Anaplasma bovis</i>	GTA GCT TGC TAT GRG AAC A
10	<i>Ehrlichia chaffeensis</i>	ACC TTT TGG TTA TAA ATA ATT GTT
11	<i>Ehrlichia</i> sp Omatjenne	CGG ATT TTT ATC ATA GCT TGC
12	<i>Ehrlichia canis</i>	TCT GGC TAT AGG AAA TTG TTA
13	<i>Theileria/Babesia</i> catch-all	TAATGGTTATAAGGRCRGTG
14	<i>Babesia felis</i>	TTA TGC GTT TTC CGA CTG GC
15	<i>Babesia divergens</i>	ACT RAT GTC GAG ATT GCA C
16	<i>Babesia microti</i>	GRC TTG GCA TCW TCT GGA
17	<i>Babesia bigemina</i>	CGT TTT TTC CCT TTT GTT GG
18	<i>Babesia bovis</i>	CAG GTT TCG CCT GTA TAA TTG AG
19	<i>Babesia rossi</i>	CGG TTT GTT GCC TTT GTG
20	<i>Babesia canis canis</i>	TGC GTT GAC GGT TTG AC
21	<i>Babesia canis vogeli</i>	AGC GTG TTC GAG TTT GCC
22	<i>Babesia major</i>	TCC GAC TTT GGT TGG TGT
23	<i>Babesia bicornis</i>	TTG GTA AAT CGC CTT GGT C
24	<i>Babesia caballi</i>	GTT GCG TTK TTC TTG CTT TT
25	<i>Theileria</i> sp Kudu	CTG CAT TGT TTC TTT CCT TTG
26	<i>Theileria</i> sp Sable	GCT GCA TTG CCT TTT CTC C
27	<i>Theileria bicornis</i>	GCG TTG TGG CTT TTT TCT G
28	<i>Theileria annulata</i>	CCT CTG GGG TCT GTG CA
29	<i>Theileria buffeli</i>	GGC TTA TTT CGG WTT GAT TTT
30	<i>Theileria</i> sp Buffalo	CAG ACG GAG TTT ACT TTG T
31	<i>Theileria mutans</i>	CTT GCG TCT CCG AAT GTT
32	<i>Theileria parva</i>	GGA CGG AGT TCG CTT TG
33	<i>Theileria taurotragi</i>	TCT TGG CAC GTG GCT TTT
34	<i>Theileria velifera</i>	CCT ATT CTC CTT TAC GAG T
35	<i>Theileria equi</i>	TTC GTT GAC TGC GYT TGG
36	<i>Theileria lestoquardi</i>	CTT GTG TCC CTC CGG G

1= Symbols indicating degenerate positions: R = A / G, W = A / T, K = G / T

Table 2.2: Primers used for *Theileria/Babesia* PCR for 18S rRNA gene.

Primer	Sequence	Orientation	Tm (°C)
RLB-F2	5'-GAC ACA GGG AGG TAG TGA CAA G	+	57.9
RLB-R2	5'-Biotin-CTA AGA ATT TCA CCT CTG ACA GT	-	53.7

RLB PCR Protocol

1. The UDG Master Mix (Roche Diagnostics) was prepared for the required number of samples and negative and positive controls. Twelve and a half microliters of 0.1 U of UDG Master Mix, 0.25 µl of 25 pmol of forward and reverse primers with 9.5 µl of PCR grade water were used per reaction with a volume of 22.5 µl per reaction.
2. A volume of 2.5 µl of template DNA was added to each PCR tube and mixed by pipetting up and down. The positive and negative controls, as mentioned above, were used in order to ensure that the process was functioning correctly (positive control) and that there had been no contamination (negative control).
3. The thermocycler programme used is seen in Table 2.3.

Table 2.3: Thermocycler programme for *Theileria/Babesia* touchdown PCR.

1 cycle	3 min	37°C	Activation of UDG
1 cycle	10 min	94°C	Inactivation of UDG & activation of Taq
2 cycles	20 sec 30 sec 30 sec	94°C 67°C 72°C	
2 cycles	20 sec 30 sec 30 sec	94°C 65°C 72°C	
2 cycles	20 sec 30 sec 30 sec	94°C 63°C 72°C	
2 cycles	20 sec 30 sec 30 sec	94°C 61°C 72°C	
2 cycles	20 sec 30 sec 30 sec	94°C 59°C 72°C	
40 cycles	20 sec 30 sec 30 sec	94°C 57°C 72°C	
1 cycle	7 min	72°C	Final extension

RLB Hybridisation

1. The membrane was incubated for 5 min in ~10 ml 2 x SSPE/0.1% SDS at room temperature.
2. Ten microlitres of PCR product was diluted in 150 µl 2 x SSPE/0.1% SDS.
3. Diluted PCR products were denatured for 10 min at 100°C in a thermocycler and cooled on ice immediately after. The samples were centrifuged for a couple of seconds once cooled.
4. The membrane was placed in the miniblitter, with the slots perpendicular to line pattern of applied probes. The probes are listed in Table 2.3. Ink-lanes were found directly under the opening of the slots.
5. Residual fluid was removed by aspiration.
6. Slots were filled with diluted PCR product, avoiding air bubbles from entering the slots. Empty slots were filled with 2 x SSPE/0.1% SDS so as to avoid cross flow.
7. Hybridisation took place on a horizontal surface at 42°C for 60 min.
8. Samples were removed by aspiration and then the membrane was removed from the blotter.
9. The membrane was washed twice in preheated 2 x SSPE/0.5% SDS for 10 min at 50°C in an incubator under gentle shaking.
10. The membrane was incubated with 10 ml 2 x SSPE/0.5% SDS with 2.5 µl streptavidin-POD (peroxidase labelled) conjugate (Roche Diagnostics) (1.25 U) for 30 min at 42°C
11. The membrane was washed twice in preheated 2 x SSPE/0.5% SDS for 10 min at 42°C in an incubator under gentle shaking.
12. The membrane was washed twice with 2 x SSPE for 5 min at room temperature under gentle shaking.
13. Ten millilitres of premixed ECL solution (Perkin Elmer) (5 ml ECL1 and 5 ml ECL2) was spread over the membrane in a closed plastic container and incubated for 1min at room temperature.
14. The membrane was placed between 2 clean overhead sheets and placed in the exposure cassette with the radiography film.
15. The film was exposed for 10-20 min. The ECL reaction peaks after 20 min and quickly fades.
16. The film was developed and demonstrated the fluorescent reactions where the PCR products had bound to the probes on the membrane.
17. The membrane was stripped according to the instructions issued for care and maintenance of the RLB membrane.
18. The film was placed on a grid and each sample lane was correlated with the DNA probes.

2.1.4 Immuno-fluorescent antibody test

The method used was the standard IFAT test used at the ARC-OVI for diagnostic testing (Laboratory Manual Serology Volume II. Onderstepoort Veterinary Institute, Protozoology Division). Dilutions of 1:40, 1:80 and 1:160 were used. The dilution, 1:80, is considered the standard cut-off titre at the ARC-OVI for positive reactions. It was decided to do the 1:40 comparison since this would increase the specificity of the test (Minjauw, Otte, James, de Castro and Sinyangwe, 1998) and possibly aid in ruling out positives due to *T. taurotragi* cross-reactions (OIE, 2004; Morzaria, 1989). Samples that show strong positive fluorescence at 1:40, but weak at 1:80 or 1:160 are more likely to be *T. taurotragi* cross-reactions than those samples that react at all three dilutions since cross-reactions are more likely to occur at low titres (OIE, 2004).

IFAT protocol according to ARC-OVI standard procedures

1. Rabbit anti-bovine IgG conjugated to fluorescein thiocyanate (Sigma-Aldrich, South Africa), was used in the IFAT. It was stored at 4°C so as to avoid repeated freezing and thawing. The reactivity remains unchanged for up to 18 months, when stored at this temperature.
2. Antigen slides (containing *T. parva* in tissue culture monolayer), test and control sera were incubated at 37°C for 10 min. Reagents were allowed to warm to room temperature (18 to 25°C).
3. A standard Dulbecco phosphate-buffered saline solution (PBS) was used to make dilutions of test sera at 1:40, 1:80 and 1:160. Negative and positive control sera were made up to dilutions of 1:80 with PBS.
4. Antigen slides were fixed in cold acetone for 1 min and allowed to air dry.
5. The diluted serum was drawn up as 24 µl and placed on the antigen slide, starting with the most dilute and then the more concentrated serum samples. Dilutions for each sample were placed in neighbouring wells so as to allow for comparisons of fluorescence of each dilution. Each slide contained a positive and negative control sample.
6. The slides were incubated in a humid chamber at 37°C for one hour.
7. Serum was removed from the slide by flicking it off swiftly. The slide was rinsed by dipping into PBS. The slide was immersed in a washing container containing PBS, placed on a magnetic stirrer at low revolutions and washed for 10 min.
8. The washing was repeated using distilled water for five minutes. The slide was then air-dried.
9. Twenty-four microlitres of conjugate (diluted to 1:80 in PBS) was added to each well. The slide was incubated in a humid chamber for one hour at 37°C.
10. The conjugate was flicked off and the slide briefly rinsed in PBS. The slides were washed in PBS for 10 min on the magnetic stirrer as described above.
11. Slides were allowed to air dry.

12. A drop of 50% glycerine/PBS was placed on each well and the slide covered with a coverslip.
13. The slides were then examined under a fluorescent microscope by placing a drop of water on the coverslip and using the 50 x water objective.

2.1.5 Statistical analysis

In investigating the prevalence of infection in Mr Green's herd, it was also important to compare the results received from the different assays to determine the reliability and reproducibility of the results. Fisher's Exact Test was used to compare the results received from each assay, i.e. real-time PCR and RLB results were compared, as well as IFAT and RLB results. It was taken that if $P \leq 0.05$, then the difference was seen to be statistically significant.

2.2 Attempted transmission of *T. parva* from Mr A. Green's cattle to susceptible cattle

Transmission of *T. parva* by *R. appendiculatus* ticks from previously tested *T. parva*-infected animals on Mr Green's farm to susceptible animals, under controlled conditions, would demonstrate the vector responsible for the transmission of the infection, and replicate the clinical signs, disease processes and pathology seen on the farms previously. Susceptible cattle were kept under controlled conditions to exclude the contribution of compounding factors on the farms, including other infectious diseases, the role of inclement weather and stresses associated with competition in a herd. If the tick transmission experiments were to be unsuccessful, transmission by tick stabilate would be attempted. See Figure 2.2 for the schematic representation of the transmission experiments from cattle on Mr Green's farm to susceptible cattle.

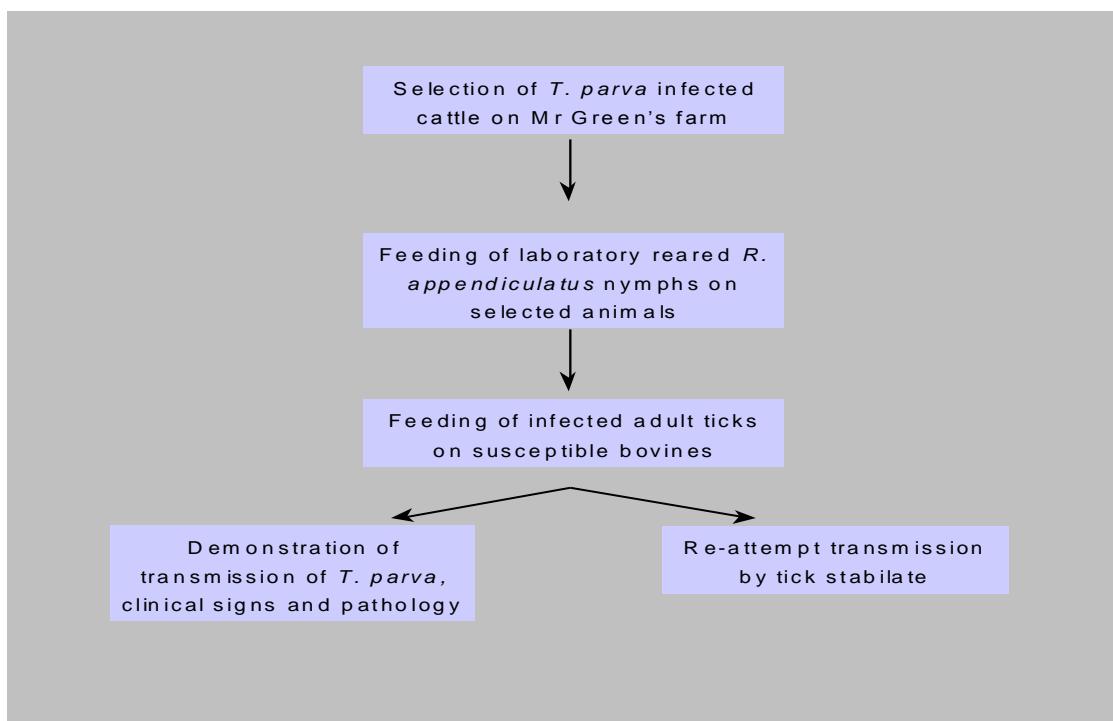


Figure 2.2: Transmission of *T. parva* from infected cattle on Mr Green's farm to susceptible bovines under controlled conditions.

2.2.1 Attempted tick transmission of *T. parva* from Mr Green's cattle to susceptible cattle

Several cattle on Mr Green's farm had previously been tested at the ARC-OVI for *T. parva* infection. Three young bulls were selected. All three had tested positive on the DNA-Probe PCR assay during 2004 (see Table 2.4).

Table 2.4: Bovines that tested positive at ARC-OVI for *T. parva* used for infecting laboratory-reared *R. appendiculatus* nymphs on Mr Green's farms.

Animal Number	Diagnostic methods for which the animal was positive at OVI during 2004
S24	<i>T. parva</i> DNA probe/PCR, conventional PCR, blood smear
316	<i>T. parva</i> DNA probe/PCR, conventional PCR
415	<i>T. parva</i> DNA probe/PCR

The infection pick-up process was attempted by feeding clean laboratory-bred *R. appendiculatus* nymphs on the selected *T. parva*-infected bovines on Mr Green's farm. The preparation for tick feeding involved cleaning the bovines' ears on three consecutive days with Sunlight® soap and water. This was done to ensure that there was no residual acaricide on the ears, which if present, would have interfered with tick attachment. The following day, cotton ear-bags were attached to the shaved skin around the ears. Genkem® adhesive was used to attach the ear-bags. *Rhipicephalus appendiculatus* nymphs (n=600) were placed in each ear-

bag and allowed to feed for four to six days (see Figure 2.3). On days 4, 5 and 6, the engorged ticks that had dropped off were collected out of the earbags.



Figure 2.3: Feeding of laboratory-reared *R. appendiculatus* ticks on infected bovine S24 on Mr Green's farm.



Figure 2.4: Ticks collected from bovines S24, 316 and 415 on day 5 of tick feeding.

The number of nymphs collected from each bovine was 400 from S24, 300 from 316 and 800 from 415 (see Figure 2.4). The engorged nymphs were counted into groups of 100 and placed in specially adapted pill-boxes in a portable acaridarium until transported back to the ARC-OVI. Once in the acaridarium, the ticks were maintained at a relative humidity of 75% and temperature of 26°C. After being transported back to the permanent acaridarium facility at the ARC-OVI, the ticks moulted and hardened over a period of eight weeks.

Thereafter, ticks from each bovine were fed on rabbits in plastic containers attached to their backs, at 60 ticks per rabbit for 3 days. This allowed for maturation of the *T. parva* parasites. The ticks were collected off the rabbits and used to determine *T. parva* infection prevalence in the tick's salivary glands from S24, 316 and 415, and to prepare a tick stabilate of ticks from S24.

Prior to preparing the stabilate, the ticks' salivary glands were dissected out and examined under a light microscope to determine infection prevalence. Thirty-three ticks originally fed on S24, 42 from 316 and 43 from 415 and pre-fed on rabbits were dissected out. The tick dissection method used is as follows: ticks were cut in half sagitally and placed under a stereo microscope in a drop of saline. The salivary glands were then removed from the body cavity with fine forceps and placed on a glass slide. The salivary glands were then teased out to prevent acini from obscuring each other. These acini were fixed and stained with Feulgens stain and examined under a light microscope for *Theileria* sp. infected acini. The percentage of infected ticks was determined by dividing the number of infected salivary glands (Feulgens positive) by the number of ticks dissected. The average number of infected acini per infected tick was determined by counting the individual positive-reacting acini divided by the number of infected ticks. A further small number of the ticks pre-fed on the rabbits were dissected and the salivary glands used for extraction of DNA. The QIAGEN QIAamp DNA Mini Kit (tissue protocol) was used for extraction of DNA from the salivary glands. The extracted DNA was tested for the presence of *T. parva* using real-time PCR.

Tick stabilate (OVI Ref: 1/2/3/752) was prepared by grinding the remaining ticks from S24, pre-fed on the rabbit, into a slurry of which the final concentration was 10 ticks/ml.

The rest of the adult ticks were divided into two groups and fed on intact, susceptible bovines 8182 and 8183 (see Table 2.5). These bovines were tested at the OVI prior to the experiment and were determined as being free of tick-borne diseases. These bovines were housed in the East Coast fever stables at the ARC-OVI under strict tick control and in accordance with ARC-OVI ECF stable procedures.

Table 2.5: Ticks fed on infected bovines on Mr Green's farm and the recipient animals.

Infected bovine on which colony ticks were fed	Susceptible bovine number
Bovines 316 and 415	Bovine 8182
Bovine S24	Bovine 8183

The same method as described above for feeding the nymphs was used for feeding the infected adult ticks, but only 150 adults were applied to each ear. These bovines were observed daily for clinical signs. These included fever, nasal discharge and lacrimation, lymph adenomegally, lethargy, depression, changes in appetite and loss of body condition. The normal temperature range was considered to be 37.5°C to 39.5°C. Furthermore, a blood smear and an EDTA blood sample (for RLB and real-time PCR assays) were taken daily up until day 35. Blood samples were taken by venipuncture of the jugular vein using Vacutainer® apparatus. Lymph node aspirates and smears were made when the lymph nodes became enlarged. Blood and lymph node smears were stained with Giemsa and examined for schizonts and piroplasms in lymphocytes and erythrocytes, respectively. These animals were to be euthanised for humane reasons, by captive bolt and exsanguination, should they have become seriously ill and as determined by a veterinarian, to be suffering. Post-mortem examinations were to be done by a specialist veterinary pathologist on all animals that died during the experiments.

2.2.2 Attempted transmission of *T. parva* in a tick stabilate to susceptible cattle

Subsequent to the tick transmission experiment, a stabilate transmission experiment was attempted. Stabilate, OVI Ref: 1/2/3/752, was used to attempt transmission of *T. parva* from S24 to splenectomised, susceptible bovine 9378/8.

A volume of 1.7 ml of stabilate was injected subcutaneously over the parotid lymph nodes of bovine 9378/8. The same observations and samples were taken as mentioned above, and the same policies regarding euthanasia and post-mortem examinations were adhered to.

2.3 Tick transmission of *T. parva* from buffaloes to susceptible cattle

Transmission of *T. parva* from buffalo to cattle was attempted in order to confirm that the tick transmission method used was efficacious as well as to gain confidence in the diagnostic tools being used and developed for diagnosing *T. parva* in South Africa. See Figure 2.5 for the schematic representation of transmission experiments from buffalo to susceptible cattle.

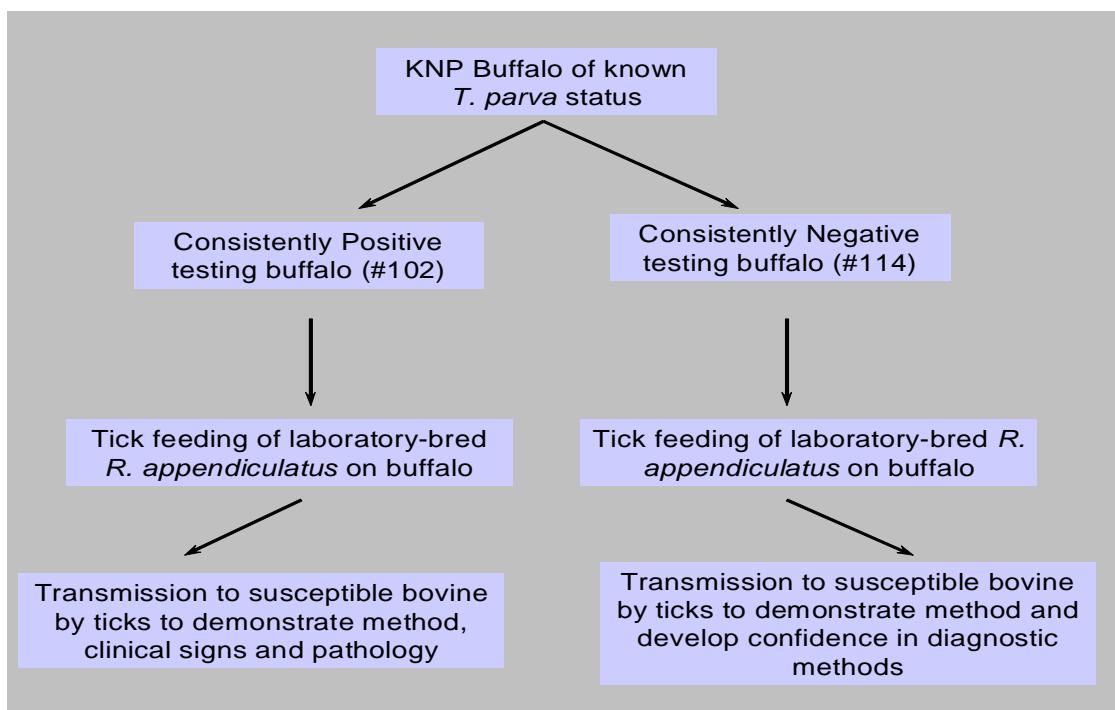


Figure 2.5: Transmission of *T. parva* infection from buffaloes to cattle.

Transmission of *T. parva* from two buffaloes to two susceptible bovines was attempted. The buffaloes originated from a breeding project in the Kruger National Park. The buffaloes were donated to BioPAD and were housed at the ECF stables at the OVI. The buffaloes were tested several times on real-time PCR prior to the tick feedings being attempted. Buffalo 102 tested positive for *T. parva* infection consistently on five occasions and buffalo 114 tested negative consistently on five occasions. These two buffaloes were also tested on RLB. Buffalo 102 was positive for *T. parva* but below detection limits for other *Theileria* spp.. Buffalo 114 was below detection limits for all *Theileria* spp.. Since these animals were not free-ranging, but rather captive-bred and accustomed to some handling, they were not excessively stressed while being housed at the ARC-OVI.

The buffaloes were immobilised for placement of ear-bags and *R. appendiculatus* nymphs. Etorphine (9.8 mg/ml) was used at dose rate of 0.01mg/kg with azaperone (40 mg/ml) at a dose rate of 0.25 mg/kg. Diprenorphine was used for reversal at twice the etorphine dose. The ticks were allowed to feed and engorge for 5 days and then the buffaloes were immobilised again to remove them.

The nymphs were allowed to moult and harden, before placement on bovines. Three hundred ticks were collected from buffalo 114 and 450 ticks were collected from buffalo 102.

Table 2.6: Results of previous assays on KNP donated buffaloes.

Buffalo Number	Results from Real-Time PCR (done 5 times)	Bovine on which ticks were then fed
114	Consistently below detection levels	9374/4
102	Consistently positive	9446/6

Because it was expected that bovine 9446/6 would become ill, 100 ticks were used to prepare a stabilate for future use (the same method as described above was used).

One hundred *R. appendiculatus* flat adults fed on the experimental buffaloes were placed on each ear of the susceptible bovines (see Table 2.6). The same methods for tick feeding were used as described above. The same observations and samples were taken as mentioned above, and the same policies with regard to euthanasia and post-mortems were adhered to.

3 Results

3.1 Survey of Cattle on Mr A. Green's Farm

The blood and serum samples taken from the Red Brangus cattle for the survey on Mr Green's farm were tested on RLB, real-time PCR and IFAT. The results of each group have been compared below. None of these animals sampled presented with any clinical signs indicating illness over the period that sampling took place on the farm.

None of the one-year-old (n=7) or mature bulls (n=4) tested positive for *T. parva* on RLB or real-time PCR. Three of the one-year-old bulls tested positive for *T. taurotragi* and one tested positive for *T. taurotragi* and *B. bovis* on the RLB. Only one animal (R25, mature bull) showed an inconclusive result for *T. parva* antibodies, i.e. IFAT positive at a titre of 1:40 only.

None of the one-year-old heifers (n=22) tested positive for *T. parva* on the RLB or real-time PCR, but two tested positive and one inconclusive for *T. parva* antibodies on IFAT. Furthermore, six animals tested positive for *T. taurotragi* on RLB.

The two-and-a-half-year-old heifers (n=18) all tested negative (below detection levels) for *T. parva* on RLB and real-time PCR. Five had inconclusive results for *T. parva* antibodies on IFAT. *Theileria taurotragi* (nine positives), *B. bigemina* (one positive) and *B. bovis* (two positives) were also detected on the RLB (see Table 3.1). Samples from animals T239, T261, T362, T379, T420 and T437 were all below detection limits for all three assays.

Table 3.1: Results of 2.5-year-old heifers from Mr Green's farm.

2.5 Year Old Heifers	RLB	<i>T. parva</i> real-time PCR	IFA
(HAOC) 518	<i>T. taurotragi</i>	Negative ¹	Negative ¹
T042	<i>T. taurotragi</i>	Negative	Inconclusive ²
T076	Negative ¹	Negative	Inconclusive
T237	<i>T. taurotragi</i>	Negative	Negative
T252	<i>T. taurotragi + B. bovis</i>	Negative	Negative
T271	<i>T. taurotragi + B. bovis</i>	Negative	Negative
T434	<i>T. taurotragi</i>	Negative	Negative
T478	Negative	Negative	Inconclusive
T528	<i>T. taurotragi</i>	Negative	Inconclusive
T566	Negative	Negative	Inconclusive
T576	<i>T. taurotragi</i>	Negative	Negative
T664	<i>T. taurotragi + B. bigemina</i>	Negative	Negative

1: Negative = below detection limit

2: Inconclusive = positive at a titre of 1:40 only

Five of the cows (n=61) tested positive for *T. parva* on real-time PCR and two of those five also tested positive on RLB. Three cows tested positive and four results were inconclusive for *T.*

parva antibodies on IFAT. Other infections that were detected were *T. taurotragi* (36 positives), *B. bovis* (six positives) and *B. bigemina* (one positive) (see Table 3.2).

Table 3.2: Results of cows from Mr Green's farm

Cow	RLB	<i>T. parva</i> real-time PCR	IFA
AG	<i>T. taurotragi</i>	Negative ¹	Negative ¹
F701	<i>T. taurotragi</i>	Negative	Inconclusive ²
H127	<i>T. taurotragi</i>	Negative	Inconclusive
H394	<i>T. taurotragi + B. bovis</i>	Negative	Negative
I041	<i>T. taurotragi</i>	Negative	Negative
I146	<i>T. taurotragi</i>	Negative	Negative
I190	Negative ¹	Positive	Negative
I199	<i>T. taurotragi</i>	Negative	Negative
I215	<i>T. taurotragi</i>	Positive	Negative
I335	<i>T. taurotragi</i>	Negative	Negative
I438	<i>T. parva</i> + <i>T. taurotragi</i>	Positive	Negative
I512	<i>T. taurotragi + B. bovis</i>	Negative	Negative
J014	<i>T. taurotragi</i>	Negative	Negative
J187	<i>T. taurotragi</i>	Negative	Negative
K234	<i>T. taurotragi</i>	Negative	Positive ³
K298	<i>T. taurotragi</i>	Negative	Negative
K364	<i>T. taurotragi</i>	Negative	Negative
K453	<i>T. taurotragi + B. bovis</i>	Negative	Negative
L004	<i>T. taurotragi</i>	Negative	Negative
L264	<i>T. taurotragi</i>	Negative	Negative
M020	<i>T. taurotragi</i>	Negative	Negative
M119	<i>T. parva</i> , <i>T. taurotragi</i> , <i>B. bovis</i> + <i>B. bigemina</i>	Positive	Positive
M204	<i>T. taurotragi</i>	Negative	Negative
M394	<i>T. taurotragi</i>	Negative	Negative
P110	<i>T. taurotragi + B. bovis</i>	Negative	Negative
P607	<i>T. taurotragi + B. bovis</i>	Negative	Negative
Q180	<i>T. taurotragi</i>	Negative	Negative
Q385	<i>T. taurotragi</i>	Negative	Negative
R012	<i>T. taurotragi</i>	Negative	Negative
R086	Negative	Positive	Negative
R184	<i>T. taurotragi</i>	Negative	Negative
R215	Negative	Negative	Inconclusive
R234	<i>T. taurotragi</i>	Negative	Negative
R248	<i>T. taurotragi</i>	Negative	Negative
R260	<i>T. taurotragi</i>	Negative	Positive
R294	<i>T. taurotragi</i>	Negative	Negative
R311	<i>T. taurotragi</i>	Negative	Negative
R421	Negative	Negative	Inconclusive
R82	<i>T. taurotragi</i>	Negative	Negative
T682	<i>T. taurotragi</i>	Negative	Negative

1: Negative = below detection limits

2: Inconclusive = positive at a titre of 1:40 only

3: Positive = positive at a titre ≥ 1:80.

Samples from cows I431, J614, K128, K333, M163, M200, M250, M295, M320, M406, M516, Q498, R137, R166, R197, R204, R253, R358, R362, R434 and R528 were all below detection levels on all three assays.

One of the calves (n=58) tested positive for *T. parva* on real-time PCR, while 10 positive and 8 inconclusive results for *T. parva* antibodies were found on IFAT. *Theileria taurotragi* (30 positives) and *B. bigemina* (four positives) were detected on the RLB (see Table 3.3).

Table 3.3: Results of calves from Mr Green's farm

Calf	RLB	<i>T. parva</i> real-time PCR	IFA
X012	<i>T. taurotragi</i>	Negative ¹	Negative ¹
X035	<i>T. taurotragi</i>	Negative	Negative
X098	<i>T. taurotragi</i>	Negative	Positive³
X177	<i>T. taurotragi</i>	Negative	Positive
X184	Negative ¹	Negative	Inconclusive ²
X207	<i>T. taurotragi</i>	Negative	Negative
X213	<i>T. taurotragi</i>	Negative	Negative
X220	<i>T. taurotragi</i>	Negative	Positive
X239	<i>T. taurotragi</i>	Positive	Inconclusive
X241	Negative	Negative	Positive
X251	<i>T. taurotragi</i>	Negative	Negative
X256	<i>T. taurotragi</i>	Negative	Negative
X267	<i>T. taurotragi</i>	Negative	Negative
X282	Negative	Negative	Inconclusive
X285	<i>T. taurotragi</i>	Negative	Negative
X288	<i>T. taurotragi</i> (weak)	Negative	Negative
X291	<i>T. taurotragi</i>	Negative	Inconclusive
X305	<i>T. taurotragi</i>	Negative	Negative
X312	<i>B. bigemina</i>	Negative	Negative
X322	<i>T. taurotragi</i>	Negative	Positive
X324	<i>T. taurotragi</i>	Negative	Positive
X327	Negative	Negative	Positive
X348	<i>T. taurotragi</i> + <i>B. bigemina</i>	Negative	Negative
X350	<i>T. taurotragi</i>	Negative	Negative
X388	<i>T. taurotragi</i>	Negative	Inconclusive
X415	<i>T. taurotragi</i>	Negative	Positive
X422	Negative	Negative	Inconclusive
X426	<i>T. taurotragi</i>	Negative	Positive
X439	<i>T. taurotragi</i>	Negative	Inconclusive
X453	<i>T. taurotragi</i>	Negative	Negative
X463	<i>B. bigemina</i>	Negative	Positive
X483	<i>T. taurotragi</i>	Negative	Negative
X518	<i>B. bigemina</i>	Negative	Negative
X568	<i>T. taurotragi</i>	Negative	Negative
X593	<i>T. taurotragi</i>	Negative	Negative
X602	<i>T. taurotragi</i>	Negative	Negative
X605	<i>T. taurotragi</i>	Negative	Inconclusive
X637	<i>T. taurotragi</i>	Negative	Negative

1: Negative = below detection limit

2: Inconclusive = positive at a titre of 1:40 only

3: Positive = positive at a titre $\geq 1:80$.

Samples from calves X071, X072, X178, X183, X193, X254, X258, X259, X265, X297, X328, X394, X401, X419, X449, X488, X499, X501, X515, X566 were all below detection levels on all three assays.

None of the calves of the cows that tested positive for *T. parva* on RLB and real-time PCR tested positive on either test. Two of these calves had positive and one had inconclusive results on the IFAT. One calf (X239) tested positive for *T. parva* on real-time PCR, whereas its dam (J187) did not test positive on the real-time PCR, RLB or IFAT.

Almost all of the cattle that were IFAT positive for *T. parva* antibodies, tested negative or below detection levels on RLB and real-time PCR. The number of animals that tested positive increased when the cut-off was reduced to 1:40 (see Figure 3.1 below).

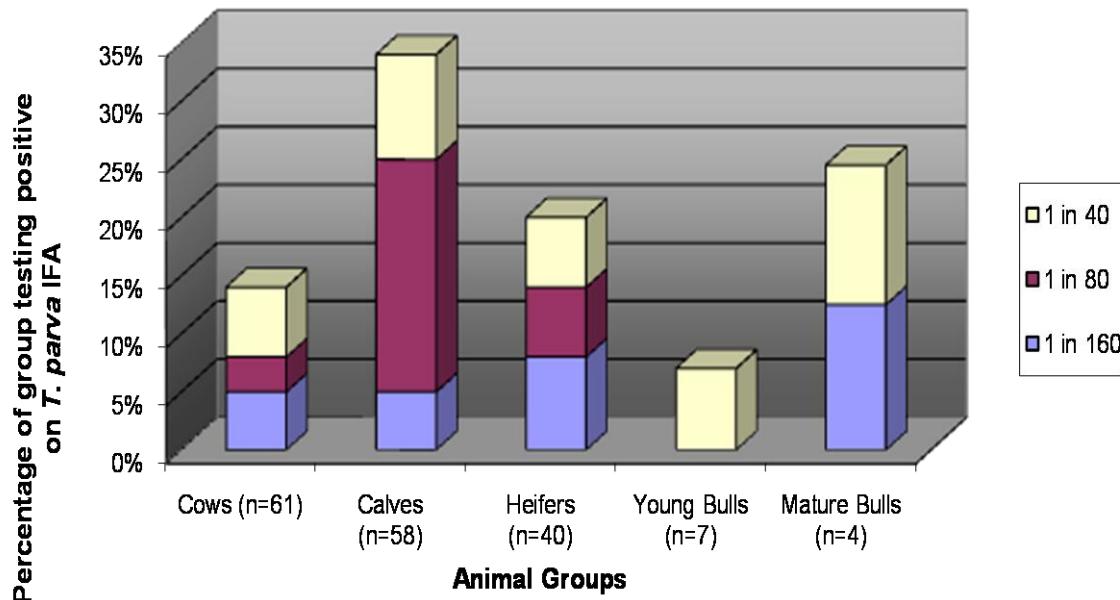


Figure 3.1: *Theileria parva* IFAT titres and specificity

Tick-borne diseases in the herd were compared between the various groups (see Figure 3.2 below).

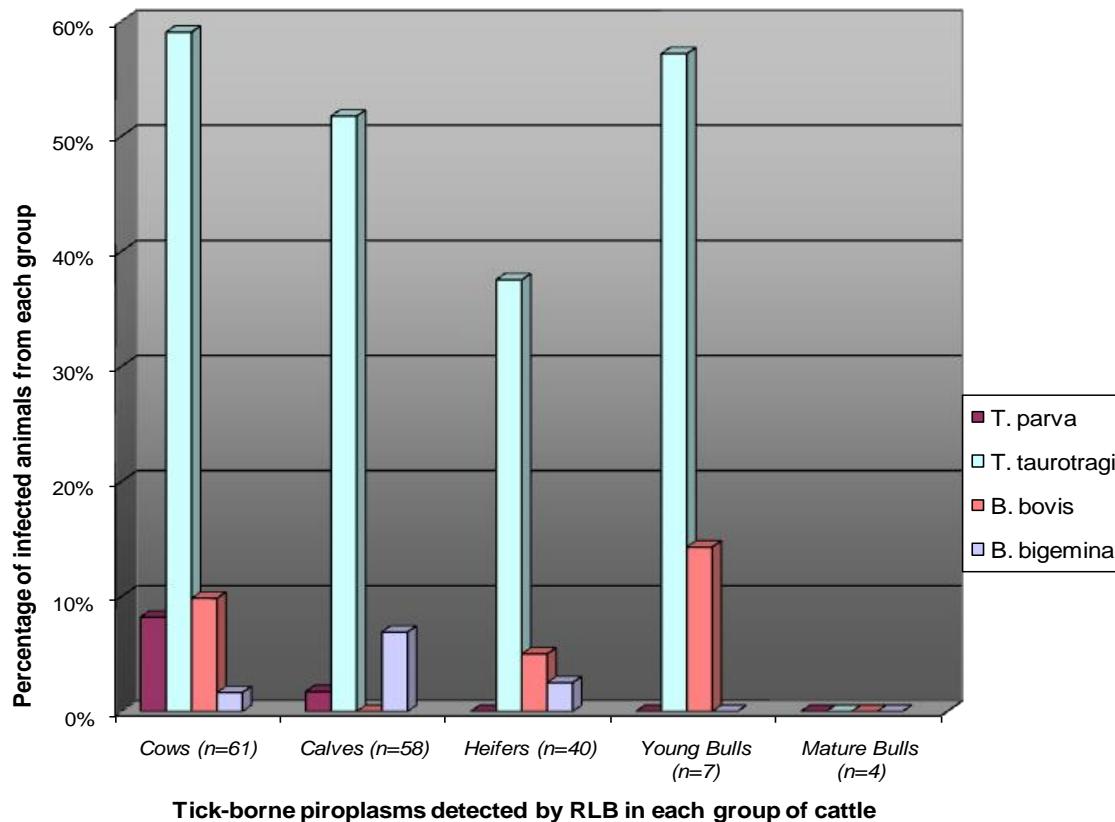


Figure 3.2: Tick-borne piroplasms detected by RLB in cattle from Mr Green's farm.

The highest percentage of animals infected with *T. parva* and *T. taurotragi* were the adult cows. By contrast, the young bulls had the highest percentage of animals infected with *B. bovis*. Calves had the highest percentage of animals infected with *B. bigemina*.

The results from the three assays were compared using the Fisher's Exact Test (see Tables 3.4 and 3.5 below).

Table 3.4: Fisher's exact test comparing real-time PCR and RLB for *T. parva*

		T. parva results on RLB →		
T. parva results on real-time PCR ↓	Negative	Negative	Positive	Total
	Frequency:	162	0	162
	Percentage:	96.43	0.00	96.43
	Row Percentage:	100.00	0.00	
	Column Percentage:	97.59	0.00	
	Positive	4	2	6
	Frequency:	2.38	1.19	3.57
	Percentage:	66.67	33.33	
	Row Percentage:	2.41	100.00	
	Column Percentage:			
	Total	166	2	168
	Frequency:	98.81	1.19	100.00
	Percent:			

Two-sided Pr ≤ P: 0.0011

In the comparison of the real-time PCR and RLB assays using the Fisher's Exact Test, $P \leq 0.0011$ is statistically significant. It is important to remember that the sample size of positive animals is fairly small and for this reason the results should be interpreted with caution. Tendencies do suggest that there is a significant difference in the sensitivities of the two tests, i.e. the real-time PCR method is more sensitive than the RLB.

Table 3.5: Fisher's exact test comparing IFAT and RLB for *T. parva*

		T. parva results on RLB		
		Negative	Positive	Total
T. parva results on IFAT	Negative	132	1	133
	Frequency:	89.19	0.68	89.86
	Percentage:	99.25	0.75	
	Row Percentage:	90.41	50.00	
	Column Percentage:			
	Positive	14	1	15
	Frequency:	9.46	0.68	10.14
	Percentage:	93.33	6.67	
	Row Percentage:	9.59	50.00	
	Column Percentage:			
	Total	146	2	148
	Frequency:	98.65	1.35	100.00
	Percent:			

Two-sided Pr ≤ P: 0.1931

In the comparison of IFAT and RLB results, this P value indicates that there appears to be no relationship between the results of two tests performed. This may indicate that one of the tests is unreliable. It may also indicate that there is no direct relationship because the two tests are measuring different substances, i.e. antibodies involved in the immune response of the animal versus the presence of parasite DNA in the animal.

3.2 Attempted transmission of *T. parva* from Mr A. Green's cattle to susceptible cattle

3.2.1 Attempted tick transmission of *T. parva* from Mr Green's cattle to susceptible cattle

Bovine 8182 received ticks that had been fed on bovines 316 and 415 on Mr Green's farm. The infection rates of the ticks dissected out to determine the infection prevalence in the ticks fed on bovines 316 and 415 are shown in Table 3.6.

Table 3.6: Prevalence of infection and average number of infected acini in dissected ticks fed on bovines 316 and 415

Bovine	Percentage of dissected ticks infected with a <i>Theileria</i> sp.	Average number of infected acini per infected tick
316	92%	18.08 acini/tick
415	100%	14.5 acini/tick

RLB and real-time PCR results are shown in Table 3.7.

Table 3.7: Results from tick transmission from Mr Green's bovines, 316 and 415, to susceptible bovine 8182.

Results from Bovine 8182 (Intact; ticks from 316 and 415)		
Day	RLB	<i>T. parva</i> real-time PCR
Day 0	Negative ¹	Negative ¹
Day 5	Negative	Negative
Day 10	Negative	Negative
Day 15	<i>T. taurotragi</i>	Negative
Day 20	<i>T. taurotragi</i>	Negative
Day 25	<i>T. taurotragi</i>	Negative
Day 30	<i>T. taurotragi</i>	Negative
Day 34	<i>T. taurotragi</i>	Negative

1: Negative = below detection limits

Theileria parva was not transmitted during this experiment. *Theileria taurotragi* was detected on RLB. Bovine 8182 did not show any clinical signs associated with a *T. parva* infection and its body temperature remained within the normal range. Schizonts were noted on the blood smear from day 11 to day 18, and piroplasms were seen on the blood smear from day 15 onwards.

The experiment was replicated in bovine 8183, which received ticks that had fed on bovine S24.

Table 3.8: Prevalence of infection and average number of infected acini in dissected ticks fed on bovine S24

Bovine	Percentage of dissected ticks infected with a <i>Theileria</i> sp.	Average number of infected acini per infected tick
S24	57%	8.8 acini/tick

A small sample of these ticks' salivary glands was also tested on real-time PCR and was found to be positive for both *T. taurotragi* and *T. parva*.

The results of the RLB and real-time PCR assays are shown in Table 3.9 below.

Table 3.9: Results from tick transmission from Mr Green's bovine, S24, to susceptible bovine 8183.

Results from Bovine 8183 (Intact; ticks from S24)		
Day	RLB	<i>T. parva</i> real-time PCR
Day 0	Negative ¹	Negative ¹
Day 5	Negative	Negative
Day 11	<i>T. taurotragi</i>	Negative
Day 16	<i>T. taurotragi</i>	Negative
Day 21	<i>T. taurotragi</i>	Negative
Day 25	<i>T. taurotragi</i>	Negative
Day 30	<i>T. taurotragi</i>	Negative
Day 35	<i>T. taurotragi</i>	Negative

1: Negative = below detection limits

Theileria parva was not transmitted during this experiment either. *Theileria taurotragi* was detected on RLB, however. No clinical signs associated with a *T. parva* infection were noted and the bovine's body temperature remained within the normal range. Schizonts were seen on the blood smears from day 11 to day 19, and piroplasms were seen from day 17 onwards.

3.2.2 Attempted transmission of *T. parva* in a tick stabilate to susceptible cattle

Due to neither of the tick transmission experiments being successful in demonstrating transmission, a tick stabilate transmission was attempted. The tick stabilate used for infecting bovine 9378/8 was prepared from the ticks that were prefed on a rabbit, i.e. nymphs from bovine S24. Thirty-three of these ticks were dissected out, with 57% having infected salivary glands and of those, 8.8 infected acini per infected tick (as seen in Table 3.8). As already mentioned, a small sample group of these ticks was used for detection of *T. parva* on real-time PCR. The ticks were found to be positive for *T. parva* and *T. taurotragi*. The results of the RLB and real-time PCR assays performed on the bovine samples collected during the experiment are shown in Table 3.10.

Table 3.10: Results of tick stabilate transmission to susceptible bovine 9378/8

Results from Bovine 9378/8 (Splenectomised; stabilate from S24)		
Day	RLB	<i>T. parva</i> real-time PCR
Day 0	Negative ¹	Negative ¹
Day 5	Negative	Negative
Day 10	<i>T. taurotragi</i>	Negative
Day 15	<i>T. taurotragi</i>	Negative
Day 20	<i>T. taurotragi</i>	Negative
Day 24	<i>T. taurotragi</i>	Negative
Day 31	Negative	Negative
Day 35	<i>T. taurotragi</i>	Negative

1: Negative = below detection limits

Transmission of *T. parva*, by stabilate injection, to the susceptible bovine 9378/8 was not demonstrated. However, *T. taurotragi* was detected on the RLB. This animal's body temperature remained within the normal range throughout the experiment. No schizonts were seen on the blood smears, but piroplasms were detected on day 35.

3.3 Tick transmission of *T. parva* from buffaloes to susceptible cattle

In these two experiments, the attempt was made to demonstrate transmission of *T. parva* by *R. appendiculatus*, clinical signs associated with *T. parva* infection and to gain some indication as to the reliability of RLB and real-time PCR in detection of *T. parva*.

3.3.1 Tick transmission from Buffalo 102 to Bovine 9446/6

In the first of the two experiments, ticks that had been fed on a consistently real-time PCR positive testing buffalo (102) were allowed to feed on a susceptible bovine (9446/6).

Transmission of *T. parva* from buffalo 102 to bovine 9446/6 by *R. appendiculatus* was demonstrated. *Theileria buffeli*, *T. sp. (buffalo)* and *B. bigemina* were also detected on the RLB.

The demonstrated transmission would be regarded as a severe reaction according to the classification recommended in a workshop on ECF immunisation held in Malawi (Anon., 1989). The results of the RLB and real-time PCR assays are shown in Table 3.11.

Table 3.11: Results of tick transmission experiment from buffalo 102 (consistently positive) to susceptible bovine 9446/6.

Results from Bovine 9446/6 (Intact; ticks from buffalo 102)		
Day	RLB	<i>T. parva</i> real-time PCR
Day 0	Negative ¹	Negative ¹
Day 1	Negative	Negative
Day 2	Negative	Negative
Day 3	Negative	Negative
Day 4	Negative	Negative
Day 5	Negative	Negative
Day 6	Negative	Negative
Day 7	Negative	Negative
Day 8	Negative	Negative
Day 9	<i>Theileria sp.</i>	Negative
Day 10	Negative	Negative
Day 11	<i>T. parva</i>	Negative
Day 12	<i>T. parva</i>	Positive
Day 13	<i>T. parva</i>	Positive
Day 14	<i>T. parva</i>	Positive
Day 15	<i>T. parva</i> + <i>B. bigemina</i>	Positive
Day 16	<i>T. parva</i> + <i>B. bigemina</i>	Positive
Day 17	<i>T. parva</i> + <i>B. bigemina</i>	Positive
Day 18	<i>T. parva</i> , <i>T. buffeli</i> , <i>T. sp. Buffalo</i>	Positive
Day 19	<i>T. parva</i> , <i>T. buffeli</i> , <i>T. sp. Buffalo</i>	Positive
Day 20	<i>T. parva</i> , <i>T. buffeli</i> , <i>T. sp. Buffalo</i>	Positive
Day 21	<i>T. parva</i> , <i>T. buffeli</i> , <i>T. sp. Buffalo</i>	Positive

1: Negative = below detection limits

Blood smear examinations revealed the presence of schizonts from day 12 until the end of the experiment.

Clinical signs noted for Bovine 9446/6 during the experiment:

- Enlarged lymph nodes (especially the prescapular and submandibular lymph nodes) were noted from day 13 onwards.
- A mild, watery nasal discharge was noted on day 16. The discharge got progressively worse and became copious in the last 2 to 3 days. It remained watery and did not become purulent at any stage (Figure 3.3).



Figure 3.3: Watery nasal discharge

- The bovine's habitus was normal until day 17, whereafter it became progressively depressed (Figure 3.4).



Figure 3.4: Poor habitus noted from day 17 onwards

- The bovine's appetite remained normal until day 20, whereafter it stopped eating.
- There was a marked degeneration in the animal's general body condition from being at a condition score of four out of five at the beginning of the experiment, to a score of two and a half out of five at the end.
- The body temperature fluctuated between 38.3°C and 41.8°C (see Figure 3.5 below).
- On day 21, the bovine showed severe respiratory distress and was euthanised by captive bolt and exsanguination.

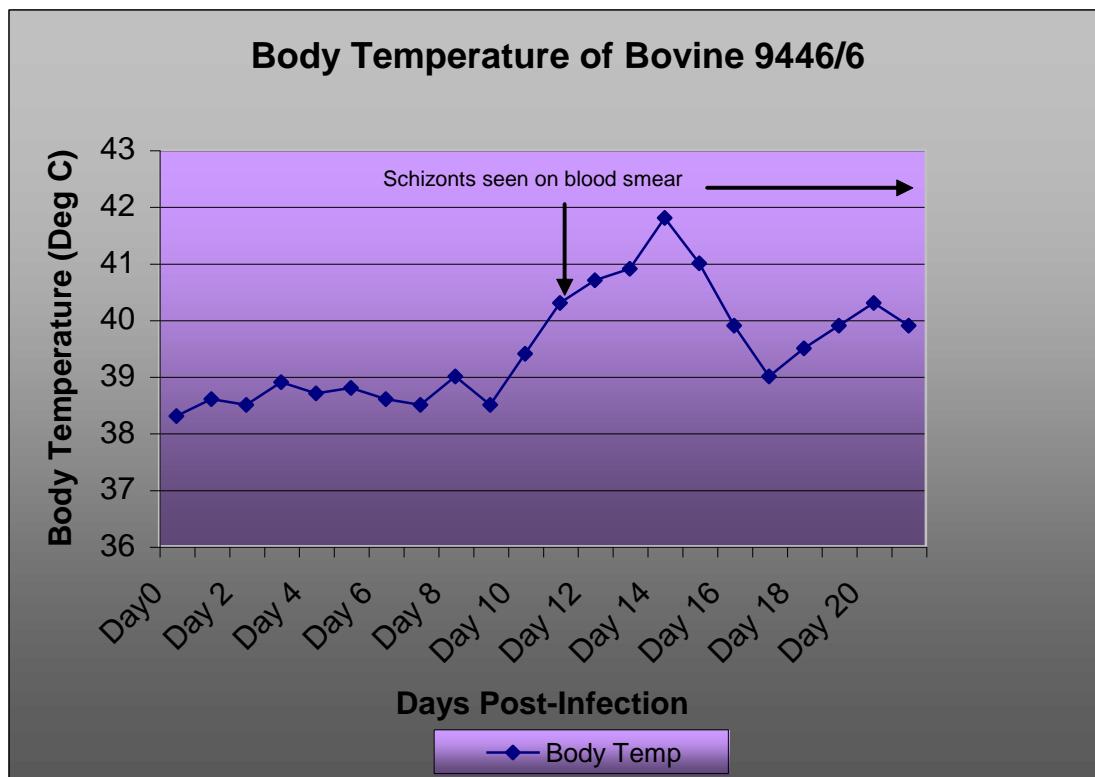


Figure 3.5: Body temperature of bovine 9446/6 for the duration of the experiment.

Post-Mortem Report for Bovine 9446/6:

Professor L. Prozesky of the Pathology Section, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, performed the post-mortem examination.

Six year-old, female, Hereford cross bovine.

Macroscopical Examination:

- Severe diffuse lung emphysema with severe, alveolar and interstitial lung oedema and scattered haemorrhages (Figures 3.6, 3.7, 3.8 and 3.9).



Figure 3.6: Tracheal froth, indicative of severe pulmonary oedema.



Figure 3.7: Severe pulmonary oedema and emphysema.



Figure 3.8: Severe pulmonary emphysema.



Figure 3.9: Severe pulmonary emphysema, oedema and suggilations.

- Multiple ulcerations of the abomasum with prominent oedema of the abomasal wall (Figures 3.10 and 3.11).



Figure 3.10: Abomasal ulcers. Subacute ulcer on left and healing chronic ulcer on right.



Figure 3.11: Multifocal ulceration and oedema of the abomasum.

- Moderate, multifocal, chronic, interstitial nephritis (Figure 3.12).



Figure 3.12: Multifocal, interstitial nephritis. White nodules are indicative of lymphocytic infiltration.

- Moderate hepatosis with oedema of the gall bladder wall
- Moderate splenic atrophy (Figure 3.13).



Figure 3.13: Splenic atrophy.

- Moderate generalised lymph adenopathy (Figure 3.14).



Figure 3.14: Hyperplastic lymph node

- Slight increase in the amount of joint fluid in larger joints.
- Petechial and ecchymotic haemorrhages in subcutaneous tissue, especially in the axillae (Figure 3.15).



Figure 3.15: Subcutaneous petechiae and ecchymoses.

Histopathological Examination:

- Lungs: Prominent, diffuse, mononuclear, interstitial pneumonia with emphysema and multifocal fibrinous pneumonia, haemorrhages and accumulation of alveolar macrophages.
- Abomasum: Multifocal ulcerative abomasitis with oedema.
- Kidneys: Prominent multifocal lymphocytic interstitial nephritis.
- Liver: Mild hepatosis with a mild portal lymphocytic triaditis.
- Spleen and Lymph Nodes: Generalised lymphoid hyperplasia of spleen and lymph nodes with multifocal, locally extensive, cortical necrosis of lymph nodes affecting lymphoid follicles and interfollicular tissue.
- Heart: Multifocal lymphocytic interstitial myocarditis.

Diagnosis: Theileriosis

Comments: The macroscopic and microscopic lesions are representative of theileriosis as described in cattle with special reference to East Coast fever. Prominent lesions were evident in most of the organs, particularly the respiratory tract.

3.3.2 Tick transmission from Buffalo 114 to Bovine 9374/4

In this experiment, transmission of *T. parva* from a consistently negative-testing buffalo to a splenectomised susceptible bovine was attempted. No infectious agents, including *T. parva*, were detected on RLB or real-time PCR. No parasites were seen on the blood smears. This bovine did not display any clinical signs that could be associated with *T. parva*. Its body temperature remained within the normal range for the duration of the experiment.

4 Discussion

4.1 The survey on Mr A. Green's farm and the significance of the results

In the survey of Mr Green's herd, there were no animals showing any clinical signs of illness during the period that sampling took place. Five of the six *T. parva* positive cattle were adult cows. The only exception is the calf X239. The possibility of the calf having been infected in utero is highly unlikely since the dam (J187) tested negative on the RLB, real-time PCR and IFAT. This would indicate that this calf became infected during the summer season of 2004/2005. Furthermore, none of the infected cows (I190, I215, I438, M119 and R86) had calves that tested positive for *T. parva* on the RLB or real-time PCR. On IFAT, though, two of these calves were positive, two negative and one had an inconclusive result for *T. parva* antibodies. See Table 4.1 for a summary of the survey results.

Table 4.1: Summary of *T. parva* survey results from Mr Green's farm

Assay used:	Number of animals positive or with inconclusive results for <i>T. parva</i> :
Real-time PCR	6
RLB	2
IFAT	34

Positive and inconclusive IFAT results of calves may have been as a result of maternally derived antibodies. Billiouw *et al.* (2002) suggested that maternal antibody prevalence in calves may indicate the immune status of the cow population. If one were to agree with this suggestion, the IFAT results from these calves would indicate that the prevalence in the cow population is even higher than the cows' IFAT results illustrate. In order to determine whether these calves do possess a level of immunity, one could perform a challenge experiment in which the calves testing positive on IFAT are exposed to a tick challenge and observed for clinical reactions. A control using a calf that was consistently negative for *T. parva* antibodies would be used for comparison.

Subsequent to the 2003/2004 outbreak, Mr Green has not experienced anymore Red Brangus losses due to *T. parva* illnesses. He has kept up an intensive dipping routine and has slaughtered animals that tested positive on DNA probe PCR (ARC-OVI previous tests). With the high numbers of animals testing positive on IFAT (19 with inconclusive and 15 positive i.e. 20%), however, this could suggest that new infections are still occurring. It is possible that many of these IFAT positives are as a result of cross-reactions with *T. taurotragi* (OIE, 2004). Fifteen of the 34 animals that tested positive or had an inconclusive result on IFAT, were negative on RLB for *T. taurotragi*, however. Billiouw *et al.* (2002) showed that seroconversion

after a booster challenge is “immediate and short-lived” especially in situations where there is a vast difference in the challenge in different seasons. This may very well be the case in infected animals on this farm and would also explain why the number of infected animals detected on real-time PCR and RLB was so low – parasitaemias may be extremely low, resulting in few positives for parasite detection and confusing IFAT titres. This means that true results can occur where an animal has antibodies for *T. parva* (as detected by IFA) but no *T. parva* DNA (detected by RLB or real-time PCR) is detected because of an extremely low parasitaemia. This would explain why there appears to be no relationship between the RLB and IFAT results in the Fisher’s exact test. Another possible reason why there is a discrepancy is that one of the assays may be unreliable. Most likely, this would be the IFAT, since this has been alluded to by other researchers (Collins *et al.*, 2002; Norval *et al.*, 1992). One way in which one can attempt to resolve this dilemma is by challenging a sample group of these animals that have tested positive on the IFAT, with the *T. parva* strain occurring on the farm. This may help in determining whether these animals have built up immunity to the parasite and in so doing, give a clearer picture of the prevalence of infection.

Furthermore, with the entire herd having been treated with tetracyclines in the 2003/2004 summer season, as well as having been exposed to a low tick challenge due to stringent dipping and tick control, the herd was in effect vaccinated against *T. parva*. These measures reduced the risk of transmission from infected carriers to susceptible cattle. This ultimately complicated the investigation of the epidemiology of the disease in this herd. In other words, had the herd not been treated with tetracyclines, nor undergone such strict tick control, one would expect that there would have been many more deaths and fewer carrier animals. It is also worth considering that the predictive values of assays are low when the population that is tested has a low patent infection prevalence. In follow-up investigations to determine the rate of infection, it is essential that this factor be kept in mind when deciding on sample sizes and groups to be tested.

Since there were no animals showing clinical illness during the period of sampling, the *T. parva* positive results on IFAT, RLB and real-time PCR, indicate that most infections in this herd are subclinical and that many may be carriers, often with very low parasitaemias. This indicates that susceptible animals may still be at risk of becoming infected and may show the associated long-term effects of chronic *T. parva* infection, i.e. poor production. It remains to be seen if these herds show the expected reduced production.

Positive results for known cattle protozoal parasites, *T. taurotragi*, *B. bovis* and *B. bigemina*, provide insight as to the infectious challenges that the herd faces (see Table 4.2). *Theileria taurotragi* infection usually only results in a mild febrile reaction; however, occasionally an infected bovine may develop cerebral theileriosis or “turning sickness” (Lawrence, de Vos and Irvin, 1994^d). No clinical cases reported by the farmer have shown clinical signs consistent with

those of cerebral theileriosis. *Babesia bovis* and *B. bigemina* are responsible for causing redwater in cattle. Morbidity and mortality as a result of redwater varies according to the epidemiological state of these parasites in a herd. Severe losses, where the mortality rate is between five and ten percent, occur when *Bos taurus* breeds are brought into an area where *Boophilus* spp. are prevalent (de Vos and Potgieter, 1994). Since bovines on Mr Green's farm have previously been diagnosed with redwater, this mortality rate and high numbers of *Boophilus* ticks, would be a reason why it was considered a differential diagnosis when the *T. parva* outbreak first occurred. These infections may also play a role in the susceptibility of the cattle on the farm, to *T. parva* challenge. That is, bovines on the farm may have reduced immunity due to other infections that may make them more vulnerable to developing a *T. parva* infection than would be expected under other circumstances. There does not appear to be a particular reason for the differences in prevalence of the various parasites in the different groups.

Table 4.2: Summary of positive RLB survey results for parasites other than *T. parva*

Parasite:	Positive RLB results in each group:					
	Mature Bulls	1-year old Bulls	1-year old Heifers	2½-year old Heifers	Cows	Calves
<i>T. taurotragi</i>	0	4	6	9	36	30
<i>B. bovis</i>	0	1	0	2	6	0
<i>B. bigemina</i>	0	0	0	1	1	4

4.2 Transmission experiments from infected cattle on Mr Green's farm to susceptible cattle

Transmission of *T. parva* from the infected bovines (S24, 316, 415) on Mr Green's farm, to susceptible cattle at the ECF stables at the ARC-OVI by *R. appendiculatus* live ticks and stabilate, could not be demonstrated during these experiments. The salivary glands of some of the ticks that had fed on S24 were found to be positive for *T. parva* and *T. taurotragi* on real-time PCR. This would indicate that at least a small number of the ticks may have been infected with *T. parva*.

A reason for the transmission not having been successful may be that the infected cattle on Mr Green's farm had a very low parasitaemia during the tick feeding, resulting in very few of the ticks becoming infected. It is clear from the transmissions and subsequent assays done on the susceptible cattle that ticks did feed well on the infected bovines, since *T. taurotragi* parasites were transmitted. This occurrence would exclude the possibility of poor tick-feeding on infected or susceptible bovines being the cause for failure of transmission of *T. parva*.

A different reason for failure of the transmission attempt is that the *T. taurotragi* parasitaemia in the bovines on Mr Green's farm may have been much higher than that of *T. parva*. This may have resulted in competition between the parasites resulting in a *T. taurotragi* infection being successful in out-competing *T. parva*. This would also explain why there were schizonts seen on the blood smears of the susceptible animals, but the parasite DNA in their blood samples being below detection levels for *T. parva* on real-time PCR and RLB. The schizonts seen were most likely those of *T. taurotragi* and not *T. parva*.

4.3 Transmission experiments from buffaloes to susceptible cattle

Theileria parva, *T. buffeli*, *Theileria* sp. (*buffalo*) and *B. bigemina*, were detected on RLB and real-time PCR (*T. parva* only) from blood collected from bovine 9446/6 post tick transmission from buffalo 102 (persistently positive for *T. parva* on real-time PCR and RLB). This experiment confirmed that the method used for the tick transmission experiments was suitable and that the susceptible animal demonstrated typical clinical signs and pathology associated with *T. parva* infection. These clinical signs and pathology are very similar to those seen in affected and deceased cattle on Mr Green's farm. Samples from this experiment can be used for molecular characterisation and comparison with the *T. parva* strain found on Mr Green's farm. One would look specifically at the parasite antigenic genes p67, polymorphic immunodominant protein (PIM) and p104 of each strain and correlate them. By analysing these particular genes one would be able to determine whether a strain is most likely to be bovine- or buffalo-associated (personal communication with Ms Kgomotso Sibeko, Department of Veterinary Tropical Diseases, University of Pretoria). In so doing, one may be able to elucidate whether the infection originally came from buffaloes.

It is also important to remember, when comparing transmission experiments where the carrier animals are bovines versus buffaloes, that ticks become more heavily infected with *T. parva* parasites after feeding on buffaloes than on cattle (Norval *et al.*, 1992). Should the strain responsible for the outbreaks on Mr Green's farm have originated from buffaloes, a very low piroplasm parasitaemia in carrier bovines would be expected (Norval *et al.*, 1992). This would mean that the infection rates in the ticks' salivary glands would be very low and thus require far higher numbers of infected ticks to feed on a susceptible bovine in order to transmit the parasite. This would support the reason suggested earlier, that low parasitaemia in the carrier bovines on Mr Green's farm would result in failure of transmission.

Babesia bigemina was detected on the RLB during this experiment. *Rhipicephalus appendiculatus* is not normally regarded as a vector of *B. bigemina*. It is possible to infect buffaloes experimentally with *B. bigemina* but they do not appear to become carriers (personal communication with Prof. Banie Penzhorn, Department of Veterinary Tropical Diseases,

University of Pretoria). Since the samples were positive for *B. bigemina* for only a few days, it is most likely as a result of experimental error during the RLB assays.

Theileria buffeli and *T. sp. (buffalo)* were also detected on the RLB. Since the ARC-OVI had tested the buffalo for tick-borne diseases prior to the experiment and had not detected these parasites, it is questionable as to whether these parasites were transmitted or not.

In the experiment where a buffalo that consistently tested below detection levels for *T. parva*, was used, there was no evidence to demonstrate that *T. parva* was transmitted. This gives some confidence that if a bovine were to test below detection levels for *T. parva* on real-time PCR, on at least five consecutive occasions, it is unlikely that it would be able to transmit the infection. The timing of consecutive tests is important, however, because it is well known that the parasitaemia in *T. parva* infections waxes and wanes (Stoltz, 1996). Should an infected animal with a very low parasitaemia be tested five times within a short space of time, it is possible, although unlikely, that all five results would be below detection levels. In such a case, the risk of releasing infected buffalo still remains.

As discussed by Meltzer and Norval (1993), in a review on “Mathematical Models of Tick-borne Disease Transmission”, controlling *T. parva* can become an extremely complicated disease problem. Variations in cattle breed, eco-climatic zones, the proportion of infected ticks, tick-control measures used and immunisation-and-treatment method all affect the disease scenario on a particular farm in a particular herd. The mathematical model by Medley, Perry and Young (1993), discussed by Meltzer and Norval (1993), “is the best model to date describing the transmission of *T. parva*, and it illustrates how useful a reliable model can be”. In order to determine the long-term effects of this outbreak, as well as improving control of the disease on this farm and surrounding areas, one should consider employing such a model.

4.4 Conclusions

With the development of the eco-tourism industry, many farmers are converting cattle ranches to game farms or parks as it is believed it may be a more profitable venture. By so doing, the interface between wildlife and livestock is increasing at a rapid rate. These circumstances are conducive to diseases being spread from wildlife to livestock and vice versa. Many owners of game parks believe that by introducing the “Big Five” to their farms, the number of visitors will increase substantially. Thus the pressure on regulatory bodies by game park owners to introduce buffaloes into areas previously used for livestock farming will simply continue to increase. And as already mentioned, Collins *et al.* (2002) stated that huge profits can be made by unscrupulous operators who are tempted to circumvent regulations and move buffaloes from controlled areas. Under such circumstances, the risk of infected buffaloes being introduced to areas outside of the declared Corridor disease areas is high. Should outbreaks of

T. parva occur in many of these areas simultaneously, they would become extremely difficult to contain and may result in the rapid and widespread establishment of the parasite in cattle and wildlife populations throughout South Africa.

As evidenced by the survey of cattle on Mr Green's farm, there are several factors that may interact in the epidemiology of *T. parva* on his farm. It was originally thought that buffaloes could not possibly be involved in the occurrence of the disease (Penzhorn, 2003). With infected buffaloes being the major *T. parva* threat in South Africa, however, it would be naïve to exclude this source of infection without weighing up the possibility of infected buffaloes or ticks from infected-buffalo areas being considered. One should remember that buffaloes from Spioenkop, Ithala and Weenen, were sold at an auction which, after translocation, led to an outbreak of Corridor disease on a neighbouring cattle farm at Wasbank. Because the group of buffaloes sold at the auction had not been tested individually for *T. parva*, it is possible that buffaloes from Spioenkop may have been infected with *T. parva*, although Ezemvelo-KZN Wildlife considered them to be "Corridor disease free". It is also important to note that tenants renting the Maria's Heuwel property were involved in game capture operations and the potential of infected ticks coming onto the farm in that manner is also possible (personal communication with Mr Green and farmers in the surrounding area). A lone buffalo bull was also seen and shot dead in the area within the last 10 years. What is noted when studying the RLB and real-time PCR results, is that the prevalence of *T. parva* appears to be very low. In contrast, IFAT results indicate that *T. parva* exposure may be high. Extremely low parasitaemias may be the cause of few animals testing positive on RLB and real-time PCR, while high numbers are testing positive on IFAT. When cattle are infected with a buffalo-associated *T. parva* strain, they typically show an extremely low parasitaemia (Norval *et al.*, 1992). The general indication from the results is that there may be a relatively high proportion of carrier animals in these herds, most likely with low parasitaemias, and that susceptible animals within these herds are still at risk of becoming infected. In all, provided the tick control remains strict and that Mr Green undertakes to slaughter out infected animals, the risk of transmission appears to be low. Should control on the farm become less stringent, the risk of transmission of infection will be substantially increased.

When evaluating the tick-transmission experiments, it is also important to notice that although some of the adult ticks (fed on S24) were found to be *T. parva* positive on real-time PCR, they failed to transmit the *T. parva* infection. The most likely reason for this failure of transmission occurring is that the *T. parva* parasitaemias of the infected cattle (S24, 316 and 415) on which laboratory-reared nymphs were fed on Mr Green's farm, were probably very low. Very low schizont and piroplasm parasitaemias in cattle are characteristic in both Corridor disease and January disease (Lawrence *et al.*, 1994^b; Lawrence *et al.*, 1994^c). In Zambia, in 1970 and 1972, outbreaks of buffalo-associated *T. parva* disease occurred and were reported as Corridor disease (Nambota, 1989). From 1977 onwards, *T. parva* infections have been

reported consistently and it is suspected that buffalo-associated *T. parva* may have transformed to cattle-adapted *T. parva* (Norval *et al.*, 1992). Thus it is a possible indication that the source of the infection in the herd may have originally been associated with buffaloes but has now adapted to the cattle host.

Sensitive and specific diagnostic methods are of vital importance in establishing prevalence of disease in a herd. In this project, RLB and real-time PCR, relatively new diagnostic techniques, have been used along with IFAT, to determine the prevalence of infection in Mr Green's herd. RLB and real-time PCR were used, with the aid of blood smears, lymph node aspirates, clinical signs and post-mortem examination, for diagnosing *T. parva* infections in transmission experiments. In the survey, it was found that high numbers of animals were found to be positive on IFAT for *T. parva* antibodies. Real-time PCR detected six positive bovines and RLB detected two of those positive for *T. parva*. Although the comparative sample size was relatively small, using the Fisher's Exact Test, tendencies indicated that the real-time PCR method was more sensitive than the RLB. There did not appear to be a high correlation between bovines testing positive on IFAT and bovines testing positive on real-time PCR and RLB. There may be several reasons for this, including waxing and waning parasitaemias, cross reactions of *T. taurotragi* antibodies in the IFAT test and small sample volume used for real-time PCR and RLB assays. It is important to note, however, that *T. parva* was successfully transmitted via laboratory-reared ticks to a susceptible bovine (9446/6) from buffalo 102, which tested positive on real-time PCR on five consecutive occasions. This bovine went on to develop classical Corridor disease clinical signs and pathology, and also tested positive for *T. parva* on real-time PCR and RLB. Surprisingly, the RLB detected *T. parva* the day before the real-time PCR did. This is unusual since it has previously been found that real-time PCR detects infection earlier (personal communication with Dr Marinda Oosthuizen, Department of Veterinary Tropical Diseases, University of Pretoria). It is important to note that no infections, that could be detected by these methods, were transmitted to bovine 9374/4 from buffalo 114, which tested below detection limits on five consecutive occasions. This provides one with some confidence that should a buffalo test below detection levels for *T. parva* on real-time PCR on five consecutive occasions, one should be reasonably confident that *T. parva* will not be transmitted.

Theileriosis is a controlled disease in South Africa, and historically, government, veterinary services and farmers all went to great lengths and expense to eradicate ECF. South Africa is considered free of *T. parva*, except in designated Corridor disease infected areas including and bordering the Kruger National Park and Hluhluwe-iMfolozi. Therefore, the national herd is essentially naïve and completely susceptible to *T. parva*. It is imperative that outbreaks, as occurred on Mr Green's farm, be controlled and contained, regardless of the source of infection. In this project, it has been demonstrated that elucidating sources of infection, confirming modes of transmission and determining prevalence of infection in infected herds

can be tricky at best. As is seen in other African countries, theileriosis, once endemic, remains a difficult and expensive disease to manage.

5 References

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