DIAGNOSIS OF TICK-BORNE DISEASES IN CATTLE IN BUSHBUCKRIDGE, MPUMALANGA, SOUTH AFRICA AND IDENTIFICATION OF *THEILERIA PARVA* CARRIERS

By

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Dedication

I dedicate this dissertation to the memory of my late father

Mr. Jimmy Josita Hamooya Coopa

who inspired me to aim for greater things in life, without whose encouragement and role in shaping who I am, I would not have achieved this dream; I will always be thankful for who he has been to me.

Declaration

I hereby declare that this dissertation, which I submit in fulfillment of the degree of Master of Science at the University of Pretoria, South Africa, is my own work. This work has not been previously submitted by me for a degree at another university.

……

Chimvwele Namantala Choopa

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List of Abbreviations

ARC-OVI	: Agricultural Research Council – Onderstepoort Veterinary Institute
B catch-all 1&2	: *Babesia* genus-specific probes 1 and 2
bp	: base pairs
CA	: Capillary tube agglutination
C-ELISA	: competitive enzyme-linked immunosorbent assay
Cp	: crossing point
DNA	: deoxyribonucleic acid
E/A catch-all	: *Ehrlichia/Anaplasma* group-specific probe
ECF	: East Coast fever
ECL	: enhanced chemiluminescence
EDAC	: 1-ethyl-3-(-3-dimethylamininopropyl) carbodimide
EDTA	: ethylene diamine tetra-acetic acid
EIR	: Entomological Inoculation Rate
ELISA	: enzyme-linked immunosorbent assay
FMD	: Foot-and-mouth disease
HDSS-Live	: Health and Demographic Surveillance System in Livestock
HHWRS	: Hans Hoheisen Wildlife Research Station
IFAT	: indirect fluorescent antibody test
IHA	: indirect hemagglutination assay
MCP	: Mnisi Community Programme
PCR	: polymerase chain reaction
PIM	: polymorphic immunodominant molecule
qPCR	: quantitative real-time PCR
RFLP	: restriction fragment length polymorphism
RLB	: reverse line blot
rRNA	: ribosomal ribonucleic acid
SDS	: sodium dodecyl sulphate
SSPE	: sodium chloride-sodium phosphate EDTA
T catch-all	: *Theileria* genus-specific probe
T/B catch-all	: *Theileria/Babesia* group-specific probe
TBDs	: tick-borne diseases
UDG	: uracil DNA glycosylase
VNTRs	: variable number tandem repeats

SUMMARY

DIAGNOSIS OF TICK-BORNE DISEASES IN CATTLE IN BUSHBUCKRIDGE, MPUMALANGA, SOUTH AFRICA AND IDENTIFICATION OF *THEILERIA PARVA* CARRIERS

by

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The Mnisi community is in the north-eastern corner of the Bushbuckridge Municipal Area, Mpumalanga Province, South Africa. This community is located at the livestock/wildlife interface sharing borders with several game reserves, and livestock are likely to be exposed to diseases with a wildlife reservoir, such as Corridor disease. Known tick vectors of important diseases such as Corridor disease, redwater, heartwater and anaplasmosis are present in the area. Although the farmers frequently dip their cattle in acaricide-filled dip tanks to control the tick burden, tick-borne diseases (TBDs) are still a major problem. This study was undertaken to determine if the symptoms of cattle in poor health in the Mnisi community could be attributed to TBDs. Corridor disease has previously been identified in cattle in the Mnisi community. Recent experimental studies have shown that *T. parva* DNA can be detected in infected cattle that survive the disease in the field. An additional aim of the study was therefore to identify *T. parva* carrier cattle in the area, and to search for evidence of selection of cattle-adapted *T. parva* parasites in carrier cattle.

The study was conducted from July 2012 to June 2013. During the study period, samples from clinically sick cattle suspected of TBDs were collected to determine the cause of their symptoms. Blood smears from the clinically sick cattle were analysed using light microscopy while some cases were subjected to histopathology and *T. parva*-specific quantitative real-time polymerase chain reaction (qPCR). DNA extracted from blood samples and in some cases tissue samples collected from clinically sick cattle (n=137) was tested for the presence of haemoparasite DNA

using the reverse line blot (RLB) hybridization assay. To identify *T. parva* carrier cattle, records from Hluvukani Animal Clinic and Bushbuckridge State Veterinary office were scrutinized to identify herds that may have been exposed to *T. parva* infection. Blood samples (n=670) were collected from herds that had recorded Corridor disease cases in the past three years, as well as herds that may have shared grazing with buffalo from the Kruger National Park and surrounding private game reserves. The indirect fluorescent antibody test (IFAT) was used to check for *T. parva* antibodies. Seropositive herds were revisited, as well as herds that had confirmed Corridor disease cases during the study period, and blood samples were collected (n=432). DNA extracted from these samples was screened for the presence of *T. parva* DNA using the *T. parva*-specific qPCR. In an attempt to find evidence of selection of cattle-adapted *T. parva*, the p67, p104 and PIM parasite genes were amplified from qPCR positive samples, and the amplicons were cloned and sequenced.

Out of the 137 clinical disease cases examined from the study area, 24 cases of TBDs were diagnosed, of which 19 were *Theileria* related. The RLB hybridization assay confirmed the presence of tick-borne haemoparasites in the Mnisi community: 89 of the 137 clinical disease cases (65.0%) were found positive for one or more haemoparasite (*Theileria*, *Babesia*, *Anaplasma* and/or *Ehrlichia* species) while 48 (35.0%) were negative or below the detectable limit of the test.

IFAT results indicated that there is a high seroprevalence of theileriosis (63.6%) in the Mnisi community area, but this may be due to cross reactions with other *Theileria* parasites known to be present (e.g. *T. taurotragi*). Fewer cattle (13.4%) were seropositive at the highest titre tested (160), and these are most likely to be associated with *T. parva*. In DNA extracted from blood samples from these seropositive herds, the *T. parva*-specific qPCR detected *T. parva* in eleven samples (2.6%). Eight of the eleven cattle were re-sampled six months later, but only one was still qPCR positive. All of the p104 and PIM sequences and two of the three p67 sequences were characteristic of buffalo-type *T. parva* alleles previously identified, implying that the *T. parva* infections in the cattle were transmitted directly from buffalo to cattle, and providing no evidence of selection of cattle-type alleles in the carrier animals.

The study revealed that TBDs are a problem in the Mnisi community and surrounding area. Most important of the TBDs identified was Corridor disease, a notifiable disease in South Africa, which was the cause of most deaths among the cattle that were sampled. There was no evidence for the selection of cattle-derived *T. parva* alleles in any of the samples from *T. parva* carrier

cattle, but a p67 sequence obtained from a clinical case was closely related to previously-identified alleles from cattle-derived isolates. *Theileria parva* DNA could only be detected in carrier cattle for a limited time post-exposure, suggesting that the infection will be cleared in infected animals before larvae or nymphs are available to pick up infections the following season. However, one bovine was still qPCR positive six months post-exposure, albeit with a very high Cp value (indicating a very low parasitaemia). The selection of *T. parva* parasites in cattle from the diverse *T. parva* population in African buffalo, therefore, remains a concern in the Mnisi community area, and at other livestock/wildlife interfaces in South Africa, but the risk is probably very low.

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Ticks have direct detrimental effects on cattle (de Castro, 1997) but more importantly, they transmit various pathogenic micro-organisms from infected cattle to healthy ones (Jongejan *et al*., 2007). Tick distribution and occurrence differs with locality and vegetation type (Mtshali *et al*., 2004). Tick-borne diseases cause serious economic losses amongst ruminants in southern Africa through mortality, reduction in meat and milk yield and through the institution of control measures (Makala *et al*., 2003). The tick-borne diseases of economic significance in the communal areas of South Africa are babesiosis, anaplasmosis and heartwater (Dreyer *et al*., 1998; Mbati *et al*., 2002) while theileriosis is a notifiable disease.

Theileria parva, an apicomplexan protozoan parasite transmitted by the three-host ticks *Rhipicephalus appendiculatus, R. zambeziensis* and *R. duttoni,* causes the most economically important tick-borne disease of cattle in eastern, central and southern Africa (Mukhebi *et al*., 1992; Norval *et al*., 1992). *Theileria parva* is an intracellular parasite that infects bovine lymphoid cells in its schizont stage and erythrocytes in its piroplasm stage. The piroplasm stage of *T. parva* is infective to *R. appendiculatus* ticks. The infection is acquired by immature ticks during blood feeding. Transmission is strictly transstadial: the parasite is transmitted only by the nymphal and adult stages after acquiring infections during feeding as larvae or nymphs, respectively. In tick-borne haemoprotozoan parasite infections, the level of parasitaemia in the hosts at the time of tick feeding is critical for efficient acquisition of the pathogens by the ticks (Eriks *et al*., 1993; Ochanda, 1994). In nature, the African buffalo (*Syncerus caffer*) is the reservoir host of *T. parva* (Dolan, 1989; Barnett & Brocklesby, 1966).

Theileria parva infection in cattle (theileriosis) may manifest as three disease syndromes. *Theileria parva parva* was historically considered the causative agent for East Coast fever (ECF) while *T. p. lawrencei* was considered to be the cause of Corridor disease and *T. p. bovis* the cause of January disease (Lawrence, 1979; Uilenberg, 1981a). Researchers have however found no biological grounds to justify the three subspecies. Instead *T. parva* parasites are now classified as “buffalo-derived” or “cattle-derived”, with buffalo-derived *T. parva* causing Corridor disease and cattle-derived *T. parva* causing ECF and January disease.

Since the eradication of ECF in South Africa in 1954 (Anonymous, 1981), Corridor disease has become the most important form of theileriosis in the country. A difference between Corridor disease and ECF is that Corridor disease cases exhibit low schizont parasitoses and piroplasm parasitaemias. The parasites are only transmitted directly from buffalo to cattle as affected cattle usually die before piroplasms appear (Neitz, 1955; Neitz *et al*., 1955). However, recent reports suggest that *T. parva* DNA is detectable in cattle at the livestock/wildlife interface in South Africa (Yusufmia *et al*., 2010; Mbizeni *et al*., 2013). The adaptation and the maintenance of buffalo-derived *T. parva* parasites in cattle therefore cannot be ruled out in South Africa.

1.2 Tick-borne diseases in South Africa

Agricultural productivity and the economic well-being of commercial and communal farmers can effectively improve through improved animal health. Cattle contribute significantly to the livelihoods of communal farmers. They are a source of food, they provide draught power for crop production, hides, manure and are a source of savings (Sansoucy, 1995). Ticks cause production losses in cattle. Significantly, they transmit diseases from infected cattle to healthy ones. Ticks transmit a great variety of pathogenic micro-organisms and are among the most important vectors of diseases affecting animals (Jongejan *et al.*, 2007). Tick-borne diseases cause high morbidity and mortality, decreased meat and milk production and loss of draught power, manure and financial resources through the institution of control measures (Makala *et al*., 2003). The most economically important genera of tick-borne prokaryotic and eukaryotic haemoparasites infecting cattle in communal areas are the rickettsiae, *Anaplasma* and *Ehrlichia* (Bell-Sakyi *et al*., 2004), and the protozoan parasites, *Babesia* and *Theileria*. In South Africa, anaplasmosis, babesiosis and heartwater (caused by *Ehrlichia* infection) are the most important constraints to improved productivity and health of cattle (Mtshali *et al*., 2004), while theileriosis or Corridor disease, is a notifiable disease. A better understanding of the epidemiology of these diseases will enable animal health workers to design better disease control strategies.

1.2.1 Anaplasmosis

Of the three *Anaplasma* (Rickettsiales: Anaplasmataceae) species that are recognised in cattle, *A. marginale* and *A. marginale* subsp. *centrale* are known to infect cattle in southern Africa (Potgieter & Stoltsz, 2004). The intra-erythrocytic organisms appear as dense, deeply purple,

roundish inclusions in Giemsa-stained smears. *Anaplasma marginale* organisms are located predominantly on the margin of the erythrocyte while the majority of *A. marginale* subsp. *centrale* organisms are located in the centre of the erythrocyte. The principal biological vectors of anaplasmosis are the ixodid ticks but argasid ticks can also transmit *A. marginale* (Raja e*t al*., 1986; Potgieter, 1981). The five tick species implicated in the transmission of *A. marginale* in South Africa are *Rhipicephalus (Boophilus) decoloratus*, *R. (B.) microplus*, *R. evertsi evertsi*, *Hyalomma marginatum rufipes* and *R. simus* (Potgieter, 1981; Potgieter & Stoltsz, 2004). Transmission mainly takes place transstadially although intrastadial transmission has also been shown (Stiller *et al*., 1980). Anaplasmosis is also easily transmitted mechanically by needle passage of infected blood but this is dose dependant (Ristic, 1968).

The complete developmental cycle of *A. marginale* occurs in the mature erythrocyte after the initial bodies released from parasitized erythrocytes invade other erythrocytes by penetrating the cytoplasmic membrane (Ristic, 1968; Francis *et al.*, 1979). Although the complete life cycle of the parasite and its mechanism of transmission by the ticks are not fully understood (Kocan, 1986), five morphological colony types that appear to represent stages in a developmental sequence of *A. marginale* in the midgut epithelial cells of adult *Dermacentor andersoni* have been categorized (Kocan *et al*., 1982).

The clinical symptoms of bovine anaplasmosis include fever, weight loss, abortion, lethargy, icterus, and occasionally death, ususally in animals older than two years (De Waal, 2000). Lifelong immunity is acquired by persistently infected or "carrier" cattle which are resistant to clinical disease on challenge exposure. European *Bos taurus* breeds (Holstein, Brown Swiss, or Hereford) are more likely to develop acute anaplasmosis than the indigenous Zebu cattle and their crossbreeds. Most cattle farming areas in South Africa occur in the endemic and epidemic areas of anaplasmosis (De Waal, 2000).

1.2.2 Babesiosis

There are four known *Babesia* species that infect cattle in southern Africa namely, *Babesia bovis*, *B. bigemina* (De Vos, 1979), *Babesia occultans* (Gray & De Vos, 1981) and another species yet to be named (De Waal *et al*., 1990). In South Africa, economically significant cases of bovine babesiosis, also known as redwater, are caused by *B. bovis* and *B. bigemina*. Although the diseases caused by *B. bovis* and *B. bigemina* are clinically similar, it is important to separate them for a number of reasons, hence the common names Asiatic (or European) and

African redwater respectively. Even though *B. bovis* is the more virulent of the two parasites, *B. bigemina* is probably more important in southern Africa because of its wider distribution (De Vos *et al*., 2004).

The only known vector for *B. bovis* in southern Africa is *R. (B.) microplus* (Potgieter, 1977). *Rhipicephalus (Boophilus) microplus* is also a confirmed vector of *B. bigemina* (Buscher, 1988). Other vectors for *B. bigemina* are *R. (B.) decoloratus* (Potgieter, 1977) and *R. evertsi evertsi* (Buscher, 1988). *Babesia bovis* is usually small, measuring up to 2 μm in diameter while *B. bigemina* is larger and can extend to the diameter of an erythrocyte, although large forms of *B. bovis* are found (Potgieter, 1977).

Initial development of *Babesia* spp. in the ticks is in the basophilic epithelium cells of the gut where schizogony occurs after fusion of the gametes in the intestinal lumen. The *Babesia* spp. parasites are transmitted transovarially with engorging adult ticks becoming infected when they ingest the parasites, and the ticks of the next generation transmit the infection to the host. Only *R. (B.) microplus* larvae transmit *B. bovis* and the ensuing stages are not infective, while only nymphal and adult *R. (B.) microplus* transmit *B. bigemina* to the bovine host (Friedhoff *et al*., 1988). Transmission by *R. e. evertsi* is transovarial and only the nymphal stage infects the bovine host (Buscher, 1988).

In cattle, development is only in erythrocytes after the merozoites penetrate the cell membrane with the aid of a specialized apical complex (Potgieter & Els, 1977). Parasitaemias of up to 1% in *B. bovis* and 5% in *B. bigemina* are common on stained smears from acutely affected cattle. The percentage can be more than 20% at the peak of the infection (Mahoney, 1969).

An animal with an acute form of babesiosis manifests anaemia, fever, haemoglobinuria, ataxia, high parasitaemia, and sometimes dies. Cattle serve as reservoirs for transmission if they recover from primary acute infection, either naturally or after chemotherapy, as they remain persistently infected (Bock *et al*., 2004). Though the clinical signs are similar in both infections, *B. bovis* infections can develop very rapidly with sudden and severe anaemia, icterus and death which will occur with little warning (Callow *et al*., 1981). Parasitaemia in recovered animals is virtually undetectable on microscopy and these become carriers.

Cerebral babesiosis is caused by *B. bovis*. It is restricted to the higher rainfall areas where *R.* (*B.*) *microplus* is prevalent, which include Eastern Cape, KwaZulu-Natal and Mpumalanga

provinces of South Africa (De Vos, 1979). *Babesia bovis* infection can be confused with other nervous conditions in cattle such as heartwater, cerebral theileriosis, sporadic bovine encephalomyelitis, plant poisonings, chlorinated hydrocarbon pesticide poisoning, vitamin B_1 deficiency and bacterial meningitis and meningoencephalitis. Signs of acute redwater may or may not accompany the manifestation of a variety of central nervous system symptoms involved in cerebral babesiosis. The outcome of the disease is almost invariably fatal but the course is usually short (De Vos *et al*., 2004). At post-mortem, the cerebrum and cerebellum have a characteristic cherry-pink colour (Callow, 1984; Callow *et al*., 1993; Rodgers, 1971).

1.2.3 Heartwater

Heartwater is caused by the rickettsial organism *Ehrlichia* (formerly *Cowdria*) *ruminantium* (Dumler *et al*., 2001) which is transmitted by ticks of the genus *Amblyomma*. It causes heavy losses in cattle in southern Africa. Exotic and naïve indigenous cattle are more severely affected by heartwater than cattle from endemic areas.

The occurrence of the tick vector limits the distribution of heartwater (Allsopp *et al*., 2004; Maillard & Maillard, 1998). *Amblyomma variegatum* and *A. hebraeum* are the major vectors of heartwater, with the latter being the main vector in southern Africa (Maillard & Maillard, 1998). When the larvae and nymphs of *Amblyomma* spp. feed on infected hosts at the time when *E. ruminantium* is circulating in the blood, they become infected. When they subsequently feed on susceptible hosts, they infect the hosts but do not lose the infection (Andrew & Norval, 1989). The parasites are transmitted to the vertebrate host probably by regurgitation or through the saliva of the tick while feeding (Allsopp *et al*., 2004). Replication of *E. ruminantium* initially seems to take place in macrophages and reticulo-endothelial cells in the regional lymph nodes. Thereafter, they are disseminated via the blood stream and invade the endothelial cells of blood vessels in various organs and tissues where further replication occurs. *Ehrlichia ruminantium* most readily infects endothelial cells of the brain in domestic ruminants and this coincides with the onset of the febrile reaction (Du Plessis, 1970).

Heartwater manifests in peracute, acute, subacute and clinically inapparent forms and as a result, the incubation period ranges from 9 to 29 days with an average of 18 days. The peracute infection kills the animal within the first few hours of development of fever, either with or without any clinical signs having been manifested. Acute heartwater is characterised by a high fever of 40°C which persists for three to six days showing small fluctuations, before

the body temperature falls to subnormal shortly before death. Nervous signs occur during the later stages which range from mild incoordination to pronounced convulsions. Fever remains high for a period of up to ten days in the subacute form. Animals with previous exposure to heartwater are particularly prone to develop the clinically inapparent form of heartwater (Alexander, 1931).

Animals which show clinical signs usually die if they are not specifically treated for heartwater. Traditionally, diagnosis of heartwater is demonstrated by making a brain smear at post-mortem for examination by light microscopy. The *E. ruminantium* organisms can be seen in the cytoplasm of endothelial cells of blood vessels in brain tissue. Brain smears (hippocampus or cerebral grey matter) are prepared in such a way that segments of capillaries remain intact for examination after staining (Purchase, 1945). In South Africa, heartwater occurs wherever the vector tick, *A. hebraeum*, is present (Allsopp *et al*., 2004).

1.2.4 Theileriosis

Protozoan parasites of *Theileria* species infect wild and domestic animals in most tropical and subtropical regions of the world (Dolan, 1989). One of the two economically important species in cattle, *T. annulata*, causes tropical theileriosis in large areas of North Africa, southern Europe, the Middle East and Central Asia. The other, *T. parva*, the causative agent of East Coast fever (ECF), Corridor disease and January disease occurs in east, central and southern Africa. A group of relatively benign *Theileria* parasites, *T. velifera*, *T. orientalis*/*T. buffeli* complex and *T. mutans*, mainly located in Africa, may cause disease and loss of production in cattle. *Theileria lestoquardi* infects sheep while *T. taurotragi* infects eland (*Taurotragus oryx*) (Norval *et al*., 1992) and cattle (De Vos & Roos, 1981).

Transmission of *T. parva* only occurs through the medium of the tick vector. The three natural tick vectors capable of transmitting *T. parva* are *R. appendiculatus*, *R. zambeziensis* and *R. duttoni* (Norval *et al*., 1992). *Theileria parva* is transmitted transstadially i.e. larval or nymphal ticks become infected when they take a blood meal containing piroplasms and in turn infect susceptible cattle when they have their next meal as nymphs and adults respectively. The African buffalo (*Syncerus caffe*r) is the reservoir host of *T. parva* (Dolan, 1989; Barnett & Brocklesby, 1966).

1.2.4.1 History of theileriosis in South Africa

The ravages of the rinderpest epidemic of 1896 and the South African War (1899 to 1902) dramatically reduced the cattle population in southern Africa. In order to restock the region there was importation of cattle from all over the world, including German East Africa (now Tanzania) and Kenya. East Coast fever, an endemic cattle disease in eastern Africa, was introduced south of the Zambezi river during the restocking period between 1901 and 1903. It is estimated that from 1901 to 1960 the total mortality in the region due to ECF was 1,400,000 head with over 100,000 slaughtered in control operations (Norval *et al*., 1992). The disease was eradicated after a prolonged campaign which included movement control, tick control, destocking of infected pastures and slaughter. East Coast fever was eradicated from southern Mozambique by 1917, from Zimbabwe by 1954 and from South Africa by 1955. Swaziland had their last case in 1960.

In South Africa today, Corridor disease is the most important theilerial infection which poses a threat to cattle farmers since the eradication of ECF in the 1950s (Stoltsz, 1989).

1.2.4.2 Disease syndromes

The parasites of the *T. parva*-group are morphologically and serologically indistinguishable (Lawrence, 1979; Burridge *et al.*, 1974). Historically they were grouped on the basis of the clinical and epidemiological features of the diseases they caused. A trinomial nomenclature was used to distinguish the different types (Lawrence, 1979; Uilenberg, 1981b): *T. parva parva* for parasites causing ECF, *T. parva lawrencei* for those causing Corridor disease and *Theileria parva bovis* for those causing January disease (which has only been reported in Zimbabwe; it resembles ECF more closely but infections are milder and occur only in the summer months). There is a gradual range of parasites between two extremes: the *lawrencei*-type with few schizonts and very few piroplasms on one hand and the *parva*-type with numerous schizonts and high piroplasm parasitaemias on the other. Later studies revealed that there is a great deal of antigenic and genetic variation between *T. p. lawrencei* isolates, while there are small differences between *T. p. bovis* and *T. p. parva* parasites, but there is currently no way to distinguish between the subspecies (Irvin *et al*., 1983; Minami *et al*., 1983; Conrad *et al*., 1987; Irvin *et al.*, 1989; Collins & Allsopp, 1999). Since there is a lack of biological evidence for discrimination of the subspecies, the trinomial naming system was discarded and the parasites are now referred to as cattle-derived or buffalo-derived according to their host of

origin (Perry & Young, 1993). Thus, ECF and January disease result from cattle-to-cattle transmission of cattle-derived *T. parva*, while Corridor disease results from buffalo-to-cattle transmission of buffalo-derived *T. parva* (Neitz, 1957; Koch *et al*., 1988).

1.2.4.2.1 East Coast fever

East Coast fever is characterized by proliferation of lymphoblasts infected with theilerial schizonts throughout the body, especially in the lymph nodes, spleen, kidneys, liver and lungs (Lawrence *et al.*, 2004b). Sick cattle exhibit enlarged lymph nodes and pulmonary oedema, and intermandibular subcutaneous oedema may also be seen. The disease can result in mortalities as high as 90% (Uilenberg, 1981b; Neitz, 1957). The main vectors are the brown ear ticks, *R. appendiculatus* and *R. zambeziensis* (Lawrence *et al.*, 1983).

1.2.4.2.2 Corridor disease

Corridor disease is the most important theilerial infection posing a threat to the cattle farming industry in South Africa since the eradication of ECF in the 1950s (Stoltsz, 1989). In South Africa, buffalo-derived *T. parva* remains a threat in areas where there are common grazing grounds with cattle and infected buffalo and where the tick vectors occur. Periodic Corridor disease outbreaks occur on farms bordering the game reserves where infected buffalo are present, namely Kruger National Park and Hluhluwe-iMfolozi Park (Bigalke *et al*., 1976).

There are similarities in the pathogenesis and pathology of Corridor disease and ECF. The main feature that distinguishes Corridor disease from ECF is the characteristic low schizont parasitosis and piroplasm parasitaemia. Death usually occurs within three to four days after the onset of the first signs (Lawrence *et al.*, 2004a). The disease is considered self-limiting because most of the cattle die before the parasites develop to the tick-infective stage, the piroplasm (Neitz *et al*., 1955). Blouin and Stoltsz (1989) state that, although *R. appendiculatus* and *R. zambeziensis* are both vectors, the latter is the preferred vector.

1.2.4.2.3 January disease

January disease, a virulent form of theileriosis, was identified after the eradication of ECF in Zimbabwe (Matson, 1967; Koch, 1990). The disease has a strict seasonal occurrence which is from January to March. This coincides with the activity of the adult *R. appendiculatus*, the

natural vector of cattle–derived *T. parva* (Matson, 1967). *Rhipicephalus zambeziensis* is also a known vector (Koch, 1990).

The pathogenesis and pathology of January disease are very similar to that of ECF and the clinical features are the same (Lawrence *et al*., 2004c). The frequency of schizonts in the lymph nodes of affected cattle varies but there is always a low percentage of erythrocytes infected with piroplasms (Koch, 1990).

1.2.4.3 *Theileria parva* life cycle

Figure 1-1 shows the complex life cycle of *T. parva*, which involves several morphologically distinct developmental stages in the tick and mammalian host cells (Shaw, 2003). Infected nymphal or adult ticks transmit *T. parva* parasites when they feed on mammalian hosts. The sporozoites follow no specific orientation in binding and entering the host cells. The presence of sporozoites in the lymphoid cells causes these cells to proliferate in an unregulated manner leading to a rapid clonal expansion of parasitized cells in the lymphoid tissues. Sporozoites then develop into multinucleate syncytial schizonts. At the beginning of merozoite formation the schizonts appear to become microschizonts (Mehlhorn & Schein, 1984). The development of the merozoites destroys the host cells and the merozoites become free to infect the next host cell, the erythrocyte. At this stage the piroplasms that develop are variable in size.

Ticks become infected by ingesting infected erythrocytes. In the tick, gametogenesis and fertilization takes place in the gut lumen and the resulting zygotes invade the gut epithelial cells. The motile kinetes leave the gut cells and invade the salivary gland. Finally, the tick feeding initiates the rapid development of the sporozoites in the salivary glands. The infective sporozoites are emitted during the final stage of feeding.

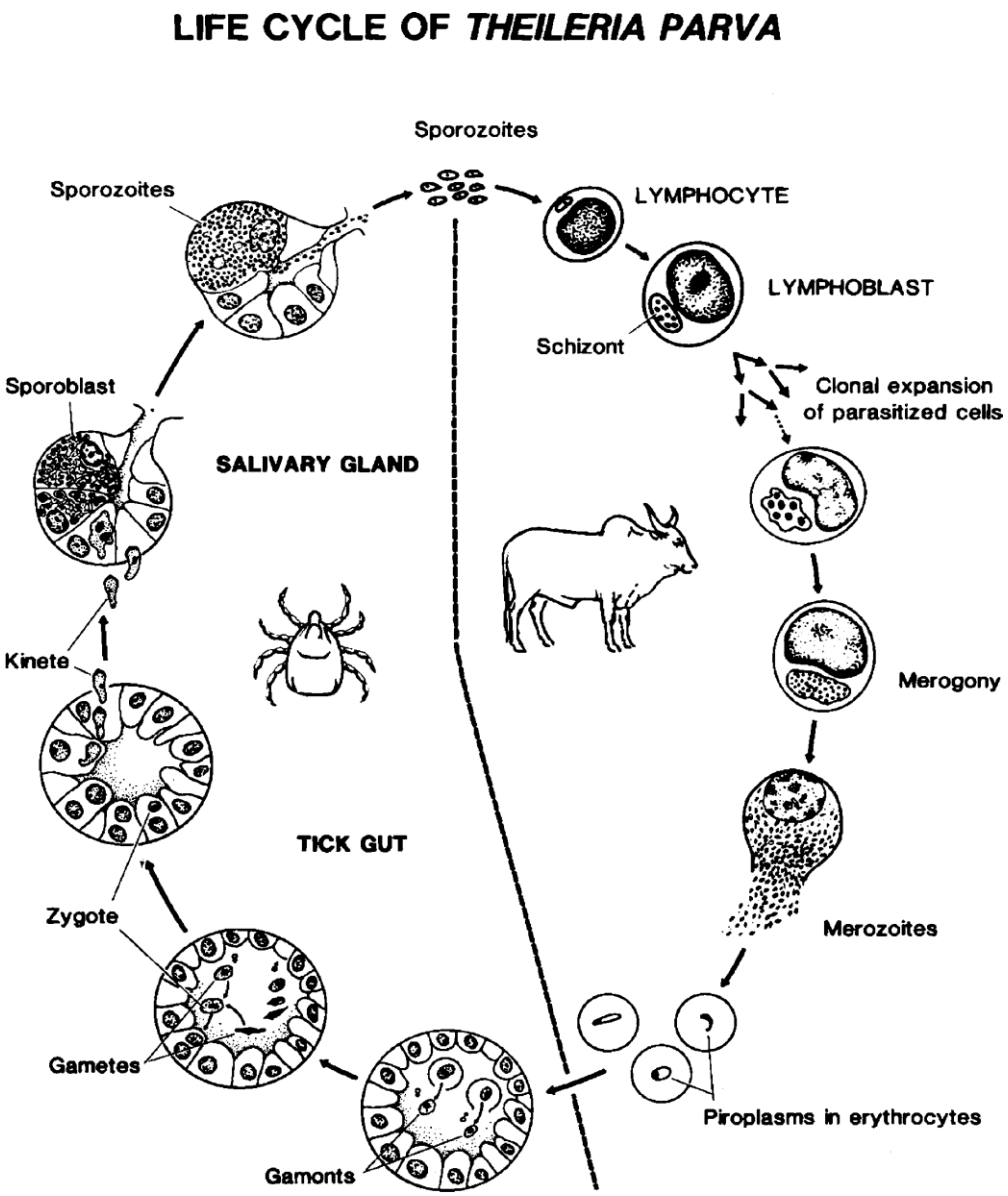

Figure 1-1: Life cycle of *Theileria parva* (from Norval *et al.*, 1992).

1.3 Treatment and control of tick-borne diseases

In other parts of Africa, integrated methods are used to achieve meaningful control of tick-borne diseases; these include a combination of tick control, control by immunisation and treatment by chemotherapy. In South Africa, buffalo are physically separated from cattle, as Corridor disease outbreaks are always associated with the presence of African buffalo (Bigalke *et al.*, 1976).

1.3.1 Tick control

South Africa first achieved tick control by combining pasture spelling, control of cattle movement and acaricide application (Dolan, 1999). As a result of rising acaricide costs, acaricide resistance and environmental considerations, other control methods were developed which included vaccines against ticks, slow-release acaricide devices, more efficient means of topical application of acaricides, manipulation of hybrid sterility between closely-related tick species and the use of pheromones to disrupt mating or to attract ticks and so improve the efficiency of the acaricide treatments. Biological tick control has been discussed by veterinary scientists and in some instances tested but has not as yet been implemented deliberately on a large scale (Norval *et al*., 1992). Development of resistance by ticks to acaricides is considered a major concern in maintaining effective tick control (Dolan, 1999). Although the importance of tick control has been recognised, it is also important to consider endemic stability, an epidemiological state of a population in which clinical disease is scarce despite high levels of infection (Norval *et al*., 1992), as a result of the abundant presence of infected vector ticks. Disruption of endemic stability, for example by vigorous tick control measures, can result in an increase of clinical disease cases. Instability can occur as a result of reduction in the number of infected ticks [Entomological Inoculation Rate (EIR) i.e. the number of infected tick bites per day per animal of less than 0.01] on animals less than nine months of age and this is before colostral and innate immunity disappear (http://www.afrivip.org/sites/default/files/Ticks_control/endemic_stability.html, accessed on 22/09/2015).

1.3.2 Chemotherapy

Several drugs have been used and/or developed to treat clinical cases of anaplasmosis, babesiosis, heartwater and theileriosis.

Specific treatment early in the course of anaplasmosis or prior to development of high parasitaemia is essential. The only effective chemotherapeutic agents for its treatment in many countries are tetracyclines (Kuttler, 1980; Mishra & Sharma, 1979; Ristic, 1981).

Although a number of compounds have been used to treat babesiosis, none has been found to be ideal for the purpose. Some diamidine compounds have proven to be both safe and effective but have residue effect problems (Anonymous, 1984; Kuttler, 1988; Kuttler & Aliu,

1984).

Tetracyclines, especially oxytetracycline, are used for the treatment of heartwater. At a dosage of 10 to 20 mg/kg, short-acting oxytetracycline formulations are most commonly used. The treatment of animals with long-acting oxytetracycline formulations is equally effective (Uilenberg, 1983).

Several drugs have been developed that are very effective in treating clinical theileriosis in cattle (Dolan, 1986; Morgan & McHardy, 1986). It was seen that prolonged treatment with chlortetracycline prevented the development of clinical disease following *T. parva* infection (Neitz 1953; Brocklesby, 1964) as tetracyclines have a suppressive effect only in the early stages of *Theileria* infections. After screening many compounds, naphthoquinone was identified as having theileriacidal activity (McHardy *et al*., 1976). Naphthoquinone was, however, not developed further because it was too expensive to synthesize (McHardy & Rae, 1981). After the demonstration that parvaquone was effective against tick-induced *T. parva* infections as well as sporozoite stabilate-induced infections, extensive trials were conducted in which susceptible cattle were exposed to natural theileriosis challenge (Dolan *et al*., 1984). Treatment was recommended at a two treatment dose of 10 mg/kg at 48 hour intervals.

Another naphthoquinone, buparvaquone, is 20 times more effective than parvaquone *in vitro* and eight times more effective *in vivo* against *T. parva* infection (McHardy *et al*., 1985). Halofuginone, unlike parvaquone was found to be ineffective when used early in the infection (Chema *et al*., 1987). For chemotherapy to be useful, early and rapid diagnosis is required so that treatment can be given in the early stages of clinical disease.

None of the drugs has proven effective in sterilizing *T. parva* infections in carrier cattle, even in combination (Stoltsz, 1989; Potgieter *et al*., 1988). It is therefore prohibited to use chemotherapy to control Corridor disease in South Africa as the treatment after an outbreak could establish carrier states in cattle (Potgieter *et al*., 1985).

1.3.3 Immunisation

While vaccines have been developed for many of the tick-borne diseases, most of these are blood vaccines containing live organisms. Cold storage, limited shelf life of the vaccine, the possibility of causing morbidity and mortality in vaccinees and the risk of attenuated

organisms reverting to a pathogenic state are limitations of the use of live vaccines (Jenkins, 2001).

Several immunisation methods have been used to protect cattle against anaplasmosis in countries where the disease is endemic, but none is ideal (McHardy, 1984). Although it is not used in North America, due to differences in veterinary policies and vaccination strategies (Potgieter & Stoltsz, 2004), a blood vaccine containing the less pathogenic *A. marginale* subsp. *centrale* which gives partial cross-protection against *A. marginale*, is the most widely accepted method of immunisation (http://www.oie.int/fileadmin/Home/fr/Health_standards/tahm/2.04.01_BOVINE_ANAPLASMOSIS.pdf, accessed on 09/09/2015).

Some countries have exploited the long-lasting immunity after a single infection with *B. bovis*, *B. divergens* or *B. bigemina* to immunise cattle against babesiosis (Bock *et al*., 2008; Mangold *et al.*, 1996; Pipano, 1997). Specifically selected strains of *Babesia*, mainly *B. bovis* and *B. bigemina* are used to manufacture most of these live vaccines.

For heartwater immunisation, a vaccine comprising cryopreserved preparations of blood from sheep infected with virulent *E. ruminantium* organisms of the Ball 3 isolate is currently commercially available. This infection and treatment method involves the infection of the animal and treatment is administered at the proper time following rectal temperature monitoring (Allsopp *et al*., 2004).

In South Africa, immunisation against *T. parva* was first attempted in the early 20th century (Lawrence *et al.*, 2004b; Cunningham, 1977). *Theileria parva* immunisation is based primarily on the cellular mechanisms directed at the schizont infected cells (Eugui & Emery, 1981). The dose of *T. parva* sporozoites required for vaccination needs to be carefully chosen: too high a dose will break the protection while too low a dose will not produce immunity to the spectrum encountered in the field (Cunningham *et al*., 1973; Ngumi *et al*., 1992). The immunization method presently used involves the inoculation of a live, potentially lethal dose of parasite and simultaneous treatment with a formulation of a long-acting oxytetracycline (Radley, 1981).

Presently, immunisation of cattle against *T. parva* can only be achieved by infection of cattle with infective sporozoites derived from ticks and subsequent chemotherapy to control infection (Dolan, 1989; Young *et al*., 1990). Since there is no single drug or a combination of drugs which have proven effective in sterilising infection in carrier cattle (Stoltsz, 1989;

Potgieter *et al*., 1988), it means that vaccinated cattle become carriers as infective sporozoites are used in immunisation. Thus South Africa prohibits immunisation as this would establish carrier states in cattle.

1.3.4 Control of buffalo movement

A variety of African game species including African buffalo (*Syncerus caffer*) are reservoirs for anaplasmosis and heartwater (Potgieter & Stoltsz, 2004). African buffalo (*Syncerus caffer*) and Asiatic buffalo (*Bubalus bubalis*) can develop latent infections of *Babesia* and therefore serve as reservoirs for babesiosis (De Vos *et al*., 1987). African buffalo are the major wildlife reservoir for Corridor disease. Transmission occurs when infected buffalo share grazing grounds with cattle in the presence of the tick vectors *R. appendiculatus* and *R. zambeziensis* (Uilenberg, 1999). In South Africa there is strict physical separation of cattle and buffalo. Before relocation can be authorised, buffalo must be tested for four controlled diseases that are transmissible to cattle, namely, foot-and-mouth disease, bovine tuberculosis, bovine brucellosis and Corridor disease. Restriction of buffalo movement together with the enforcement of movement control measures and vigorous tick control in areas bordering Corridor disease endemic areas has probably contributed greatly to the control of Corridor disease (Stoltsz, 2011).

1.4 Diagnosis of tick-borne diseases

For meaningful assessment of the presence or absence of tick-borne diseases, and the degree of endemic stability to them, an effective reporting system is necessary. The diagnostic techniques to help achieve this goal include clinical examination, microscopy, serology and molecular methods. Sensitive and specific tests are required for the detection of tick-borne infections.

1.4.1 Clinical diagnosis

Symptoms of anaplasmosis include fever, weight loss, abortion, lethargy, icterus, and often death in animals older than two years (De Waal, 2000), while those of babesiosis are anaemia, fever, haemoglobinuria, ataxia, high parasitaemia, and sometimes death (Bock *et al*., 2004). In natural infections, the average incubation period for heartwater is 2–3 weeks; thereafter the animal has fever which may exceed 41°C within 1–2 days after onset of symptoms. During the

latter stages of heartwater, nervous signs occur which range from mild incoordination to pronounced lateral recumbency, pedalling and exhibition of opisthotonos, nystagmus, hyperaesthesia, chewing movements, and frothing at the mouth (Allsopp *et al*., 2004).

East Coast fever is a proliferative disease in its early phases accompanied by enlargement of several lymph nodes, particularly those draining the head. Animals have difficulty breathing and froth and diarrhoea are often present. Sometimes there is development of corneal opacity which sometimes results in blindness. Case fatality proportions can be as high as 90%. In Corridor disease, the lympho-proliferative stage is very marked but the lympho-destructive phase is less prominent than in ECF. Pulmonary oedema may or may not develop but subcutaneous oedema, particularly under the jaw, is common (Norval *et al*., 1992).

1.4.2 Microscopy

The preparation, staining and examination of thick and/or thin blood and lymph node biopsy smears, is one of the most effective methods available for the diagnosis of tick-borne diseases. This is a direct method of diagnosing infections. Care is taken when preparing smears where anaplasmosis is suspected: the rather indistinctive morphology of *Anaplasma* organisms means that foreign matter or specks of debris can confuse diagnosis. *Anaplasma marginale* organisms appear as dense, rounded and deeply stained intraerythrocytic bodies located on or near the margin of erythrocytes. These bodies are approximately 0.3–1.0 µm in diameter. *Anaplasma marginale* is distinguished from *A. marginale* subsp. *centrale*, as most of the organisms in *A. marginale* subsp. *centrale* have a more central location in the erythrocyte (Kocan *et al.*, 2004).

Babesia bovis, unlike the other *Babesia*, *Anaplasma* and *Theileria* spp., accumulates in peripheral blood vessels. Where babesiosis is suspected, it is therefore important to collect blood for making smears from the tip of the ear or tip of the tail. *Babesia bovis* is a small parasite which measures approximately 1.0–1.5 µm long and 0.5–1.0 µm wide and is often found as pairs that are at an obtuse angle to each other, usually centrally located in the erythrocyte. *Babesia bigemina*, typically pear-shaped, is usually a much bigger parasite (3.0–3.5 µm long and 1.0–1.5 µm wide), and is often found as pairs at an acute angle to each other (Bock *et al.*, 2004).

The best samples to collect when heartwater is suspected are well vascularised portions of the

brain such as the cerebrum, cerebellum or hippocampus. *Ehrlichia ruminantium* organisms are often found close to the nucleus and occur as clumps of reddish-purple to blue, coccoid to pleomorphic organisms in the cytoplasm of capillary endothelial cells (Allsopp *et al*., 2004) .

Theileria parva schizonts appear in lymphocytes seven days after the attachment of infected ticks and sometimes persist throughout clinical illness. Piroplasms appear in erythrocytes five to eight days after the detection of schizonts in new infections. This method is used for early and rapid diagnosis and treatment of *Theileria* infection. However, the difficulty of differentiating the schizonts and piroplasms from those of other *Theileria* species is a major limitation. Additionally, the sensitivity of microscopic examinations is very low such that it is difficult to detect piroplasms in carrier cattle (Norval *et al*., 1992).

1.4.3 Serology

A number of serological tests have been developed with the aim of detecting persistently infected animals. Serological methods used include the card agglutination test, complement fixation test, indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA).

In general, unless animals have been treated or are at a very early stage of infection (<14 days), serology using the competitive enzyme-linked immunosorbent assay (C-ELISA) or card agglutination test may be the preferred methods of identifying animals infected with *Anaplasma*. It should be noted that there is a high degree of cross-reactivity between *A. marginale* and *A. marginale* subsp. *centrale*, as well as cross-reactivity with both *A. phagocytophilum* and *Ehrlichia* spp. in serological tests (Al-Adhami *et al.*, 2011; Dreher *et al.*, 2005).

Cross-reactions with antibodies in areas where *B. bovis* and *B. bigemina* coexist are a particular problem with the IFAT. Antibodies against *B. bovis* and *B. bigemina* can be detected by the complement fixation test (Anonymous, 2006). Low sample throughput and subjectivity is the reason why ELISAs have largely replaced the IFAT (Anonymous, 1984).

Serological diagnosis of heartwater should only be used as a tool of investigation rather than for definitive diagnosis, as it is subjective and cross-reactions with related *Anaplasma* and *Ehrlichia* species are common (Allsopp *et al*., 2004).

Indirect methods based on serology have been developed for the diagnosis of Corridor disease; these include the capillary tube agglutination (Ross & Lohr, 1972), conglutination (Cawdery *et al*., 1968), IFAT (Burridge, 1971), indirect hemagglutination assay (Duffus & Wagner, 1974) and ELISA (Katende *et al*., 1998). The IFAT has limitations for large scale serological surveys in field situations where several *Theileria* species co-exist. Cross-reactions between *T. parva* and *T. taurotragi* can occur, especially when antibody titres are low in samples from areas where the distribution ranges of *T. parva* and *T. taurotragi* overlap (De Vos, 1982; Norval *et al*., 1992; Lawrence *et al*., 2004b). The ELISA technique has advantages such as the ability to analyse a large number of samples in a shorter time and to discriminate positive from negative sera without subjectivity (Madruga *et al*., 2000). However, the IFAT remains widely used in the diagnosis of *Theileria* parasites even though ELISA surpasses it in terms of sensitivity and specificity (Katende *et al*., 1998). ELISA and IFAT do not detect the parasites and at times the animals may remain seropositive even when the pathogens have been cleared (Dolan, 1986; Bishop *et al*., 1992).

1.4.4 Molecular methods

The conventional and serological diagnostic techniques for the detection of haemoparasites have limitations when used to test carrier animals. Other tests involving nucleic acid probes or the polymerase chain reaction (PCR) are more sensitive and can be used in the diagnosis of tick–borne diseases (Calder *et al*., 1996; Reghu *et al*., 2006). Such tests are far more sensitive than conventional techniques, but require specialized equipment and well-trained personnel.

1.4.4.1 Conventional polymerase chain reaction (PCR) assays

The PCR is a test tube system for replication of DNA which allows a “target” DNA sequence to be selectively amplified using a pair of oligonucleotide primers, each complementary to one end of the DNA target sequence. All components of the reaction buffer, nucleotides, primers, DNA and enzyme are heat stable and therefore the cycles can be performed by only changing the temperature. The repeated cycles consist of denaturation, annealing and extension. In all PCR experiments, great care must be taken to prevent contamination by previously amplified amplicons.

Theileria parva DNA was successfully amplified in experimentally infected animals but not in

field cattle with the use of primers derived from a repetitive sequence (TpR) in a PCR (Bishop *et al*., 1992; Watt *et al*., 1998). Due to lack of specificity, however, TpR primers also amplify other closely related non-pathogenic *Theileria* parasites (Watt *et al*., 1997). A PCR method based on detection of the p104 gene (Odongo *et al*., 2009; Skilton *et al*., 2002) has also been developed. PCR tests have also been used to detect *A. marginale* (Torioni De Echaide *et al*., 1998), *B. bovis* and *B. bigemina* (Buling *et al*., 2007; Costa-Junior *et al.*, 2006; Criado-Fornelio, 2007) and *E. ruminantium* (Little *et al*., 1997).

1.4.4.2 PCR–based hybridisation assays

More sensitive and specific diagnostic tests are required to detect low level infections or carrier states. To address this problem in the detection of *T. parva*, PCR amplification of the *Theileria* 18S ribosomal RNA (rRNA) gene followed by probing with radioactively labelled species-specific oligonucleotide probes was developed (Allsopp *et al.*, 1993). Amplification primers were designed in conserved regions of the 18S rRNA gene to amplify the gene from related organisms and species-specific probes were developed in the variable regions and used to differentiate among the different species, *T. parva, T. mutans, T. annulata, T. taurotragi*, *Theileria* sp. (buffalo) and *T. buffeli* (Allsopp *et al*., 1993). The pCS20 PCR and probe test (Waghela *et al*., 1991) which has been improved (Steyn *et al*., 2003; van Heerden *et al*., 2004) has been used to detect *E. ruminantium* both in ticks and ruminants. However, the additional hybridisation step required to confirm positive cases in PCR-based hybridisation assays make them lengthy and laborious methods, which can only be carried out by skilled laboratory personnel.

The reverse line blot (RLB) hybridisation assay is based on simultaneous PCR amplification of the 18S rRNA gene of related species with a single set of primers, followed by hybridisation to species-specific probes, making specific PCR reactions for individual species unnecessary. A reverse line blot hybridisation technique was initially developed for the simultaneous detection of bovine tick-borne protozoan parasites in the genera *Theileria* and *Babesia* (Gubbels *et al*., 1999). Subsequently, primers and probes were developed for the detection of *Ehrlichia* and *Anaplasma* species in domestic ruminants and ticks (Bekker *et al*., 2002). The two sets of primers required for the amplification of *Ehrlichia/Anaplasm*a and *Babesia/Theileria* small subunit rRNA genes have matching melting temperatures and therefore the same PCR amplification programme can be used for both of them. For detection and identification of the amplified PCR products, biotin-labeled species-specific

oligonucleotide probes are covalently attached to a nylon membrane. The PCR products are applied to the membrane using a miniblotter so that the direction of the PCR products is perpendicular to the direction of the oligonucleotide probes. In this way, the different pathogen species simultaneously amplified by PCR can each hybridize specifically at the cross-sections of the line containing the specific biotin-labeled oligonucleotide probe. These oligonucleotide-PCR product complexes are detected with enhanced chemiluminescence (ECL), which results in a reaction producing light and can be detected on an X-ray film (Gubbels e*t al*., 1999).

1.4.4.3 Quantitative real-time PCR (qPCR) assays

Quantitative real-time PCR (qPCR) has brought great improvement to molecular diagnosis (Nicolas *et al*., 2002; Kares *et al*., 2004). At the annealing steps during qPCR, a third oligonucleotide (a probe that contains a fluorescent group) binds downstream of one of the amplification primers. During the extension steps the probe generates a fluorescent signal. The intensity of this signal depends on both the fidelity of the PCR and the amount of authentic amplification product and this can be monitored throughout the process.

It is possible to genotype as well as quantify the amount of template molecules at the start of the amplification using qPCR. The amplified sequences can be characterised by melting-curve analysis while quantification is done by making use of the amplification curves. Among the sequence-specific probes used are hybridisation probes, hydrolysis probes and light up probes (Wilhelm *et al*., 2003). The major advantage of qPCR is that it is relatively fast and easy to perform in comparison to other diagnostic methods.

A sensitive and specific qPCR for the detection of *T. parva* in cattle and buffalo has been developed (Sibeko *et al*., 2008). Initially the test used *Theileria* genus-specific primers and a pair of *T. parva*-specific hybridisation probes, but the sensitivity of this assay may be compromised when *Theileria* genus-specific primers are used in samples containing mixed infections. The specificity and sensitivity of the test was therefore improved by designing a *T. parva*-specific forward primer but since the *Theileria* sp. (buffalo) 18S rRNA gene sequence is very similar to that of *T. parva*, it is impossible to design an amplification primer that does not also amplify *Theileria* sp. (buffalo) DNA. Even though an amplification is generated from *Theileria* sp. (buffalo) DNA, the test still remains specific for *T. parva* as only a *T. parva*-specific melting curve is generated (Sibeko *et al*., 2008).

Real-time PCR has also been described for the identification of *A. marginale* (Carelli *et al*., 2007; Decaro *et al*., 2008; Reinbold *et al*., 2010). Criado-Fornelio *et al*. (2009) developed two quantitative PCR methods that enabled the simultaneous detection of multiple *Babesia* species, even in carrier state infections. A qPCR assay based on pCS20 has been developed for the detection of *E. ruminantium* (Steyn *et al*., 2008).

1.5 Molecular characterization of *T. parva* parasites

There are antigenic and genetic similarities between cattle-derived *T. parva* parasites but there is a great deal of variation amongst buffalo-derived *T. parva* isolates (Irvin *et al*., 1983; Minami *et al*., 1983; Conrad *et al*., 1987; Conrad *et al*., 1989; Collins & Allsopp 1999). Several genes and antigens have been investigated in search of discriminatory differences between *T. parva* isolates. Among these are the genes coding for the sporozoite antigens, p67 (Iams *et al*., 1990; Nene *et al*., 1996), p104 (Skilton *et al*., 2002) and the polymorphic immunodominant molecule (PIM) (Baylis *et al*., 1993; Toye *et al*., 1996; Geysen *et al*., 2004). These surface proteins (PIM, p67 and p104) are capable of inducing antibodies and are exploited in discriminatory assays for *T. parva* isolates. The PIM, p67 and p104 proteins are encoded by single copy genes which share certain structural characteristics; they contain a polymorphic central amino acid sequence flanked by conserved amino and carboxyl termini (Toye *et al*., 1995; Skilton *et al*., 1998). The PIM gene is expressed in both sporozoite and schizont stages of the parasite while p67 and p104 genes are only expressed in the sporozoite stage (Katende *et al*., 1998; Shapiro *et al*., 1987).

1.5.1 p67 gene

Upon characterisation of the p67 gene sequence in East Africa, it was revealed that a 129 bp deletion in the central region of the gene was characteristic of cattle-derived *T. parva* isolates (allele 1) while no deletion was observed in buffalo-derived isolates (allele 2) (Nene *et al*., 1996; Nene *et al*., 1992). However, both p67 alleles were obtained in South African buffalo from the Kruger National Park, although it was not established whether the strain containing the p67 allele with a deletion could cause ECF (Collins, 1997). Subsequently, variants of both p67 alleles were identified in other buffalo samples in South Africa, as well as two additional p67 alleles (alleles 3 and 4) (Sibeko *et al*., 2010). Of concern, p67 allele 1, which is characteristic of cattle-derived *T. parva* isolates in East Africa, was identified in a sample from a bovine that tested *T. parva* positive on a farm near Ladysmith in the KwaZulu-Natal

Province of South Africa (Sibeko *et al*., 2010).

1.5.2 PCR-based RFLP assays

Based on restriction fragment length polymorphism (RFLP) profiles, the variable region of parasite antigen genes has been exploited in attempts to discriminate between buffalo-derived and cattle-derived *T. parva* isolates (Geysen *et al*., 1999; Bishop *et al*., 2001). The RFLP profiles that have been developed include PIM-based and p104-based semi-nested PCR-RFLP assays (Geysen, 2000).

The gene coding for the p104 antigen has been selected for PCR-RFLP analysis since there is limited polymorphism in the gene amongst cattle-type alleles, allowing for the distinction of buffalo-type from cattle-type alleles (Geysen *et al*., 1999).

The PIM gene has previously been used successfully to differentiate between *T. parva* stocks. Since there is variation in the central regions of the PIM gene, the polymorphism has been exploited for discrimination between *T. parva* stocks (Geysen *et al*., 1999; Bishop *et al*., 2001).

The PIM and p104 RFLP profiles from buffalo-derived *T. parva* stocks are more polymorphic than those from cattle-derived stocks and at the same time the profiles obtained from buffalo stocks are heterogeneous whereas the cattle-derived *T. parva* stocks are often homogeneous (Geysen *et al*., 1999). These methods were used to characterise *T. parva* field samples from cattle and buffalo in South Africa (Sibeko *et al*., 2011). The p104 and PIM alleles obtained from *T. parva* samples from cattle on a farm near Ladysmith in KwaZulu-Natal in which p67 allele 1 was identified, were either buffalo-type or “novel” (or mixed type in the case of PIM) (Sibeko *et al*., 2011). No p104 or PIM alleles identical to *T. parva* cattle-type alleles previously reported (Iams *et al*., 1990; Toye *et al*., 1995; Skilton *et al*., 2002) were identified in South African buffalo (Sibeko *et al*., 2011), although variants of p104 allele 1 (a cattle-type allele) were obtained. The sequence identities of p104 sequences obtained from buffalo (Sibeko *et al*., 2011) when compared with published sequences (Skilton *et al*., 2002) suggest that *T. parva* p104 alleles in South African buffalo are more diverse and that variations in the p104 gene may not be as limited as in cattle (Geysen *et al*., 1999).

1.5.3 Microsatellites

Microsatellites and minisatellites are locus-specific genetic markers based on short, or quasi-tandem repeats, known as variable number tandem repeats (VNTRs). These are powerful tools for investigating population structure in a range of organisms (Odongo *et al*., 2006). Micro- and mini-satellite markers have been developed for characterizing *T. parva* stocks and enable detection of higher levels of polymorphism than PCR-RFLP methods (Oura *et al*., 2005).

1.6 The Mnisi community

The Mnisi community, located at the livestock/wildlife interface, is in the north-eastern corner of the Bushbuckridge Municipal Area, Mpumalanga Province, South Africa. This community, with a population of about 40,000 people living in 8,500 households, shares borders with several game reserves: Andover to the north-west, Manyeleti to the east (Mpumalanga Tourism and Parks Agency reserves), Timbavati to the north and Sabi Sands to the south (private game reserves). The only barrier between livestock and the abundant wildlife populations is a fence, which is not impermeable. Foot-and-mouth disease (FMD) and Corridor disease are endemic in the area. The main agricultural activity of the community is livestock farming, with cattle being by far the most important animals. Although comprehensive disease surveillance measures are implemented through interaction between animal health technicians and farmers mainly in the form of cattle dip tanks, which every cattle herd must visit for dipping and FMD inspection once a week, tick-associated diseases and problems are still common (http://www.up.ac.za/en/mnisi-community-programme/article/275823/the-study-area, accessed on 09/09/2015). The University of Pretoria is one of the stakeholders in the Mnisi Community Programme (MCP), a multidisciplinary platform for research, teaching, learning and community engagement in the area.

1.7 Problem statement and hypothesis

Despite frequent dipping of cattle in the Mnisi Community Programme area, ticks are frequently found on cattle and tick-associated problems are a major concern. Tick-borne diseases are therefore also likely to be present. In southern Africa, anaplasmosis is most commonly confused clinically and pathologically with bovine babesiosis because many epidemiological factors of the two diseases are similar (Potgieter & Stoltsz, 2004). Nervous signs occur in most animals suffering from heartwater and they must be distinguished from

rabies, the nervous form of malignant catarrhal fever, cerebral babesiosis, cerebral theileriosis, chlamydiosis and other causes of meningitis and encephalitis.

Cattle infected by buffalo-derived *T. parva* can recover after treatment by chemotherapy and can be carriers of the parasite (Potgieter *et al*., 1988), but carrier states are not thought to occur naturally (Neitz *et al*., 1955). However, recent reports suggest that *T. parva* DNA is detectable in cattle at the livestock/wildlife interface in South Africa (Yusufmia *et al*., 2010; Mbizeni *et al*., 2013). The adaptation and the maintenance of buffalo-derived *T. parva* parasites in cattle in South Africa therefore cannot be ruled out. It is therefore hypothesised that:

1. There are clinical cases of tick-borne diseases, including Corridor disease, in the Mnisi Community Programme area.
2. *Theileria parva* carrier cattle are present in Corridor disease-endemic areas.
3. There is selection of a subpopulation of *T. parva* parasites in carrier cattle from the genetically diverse *T. parva* population in buffalo.

1.8 Objectives

The objectives of the study in the Mnisi community and surrounding area are therefore to:

1. Confirm clinical cases of tick-borne diseases;
2. Confirm diagnosis of Corridor disease cases;
3. Attempt to identify *T. parva* carrier cattle through a retrospective study;
4. Characterize the p67, p104 and polymorphic immunodominant molecule (PIM) genes from parasites present in clinical Corridor disease cases and in carrier cattle and compare to isolates from buffalo.

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CHAPTER 2

CONFIRMATION OF TICK-BORNE DISEASES FROM CLINICAL CASES IN NORTH-EASTERN BUSHBUCKRIDGE (MNISI COMMUNITY PROGRAMME AREA), WITH AN EMPHASIS ON CORRIDOR DISEASE

2.1 Introduction

Ticks are among the most important vectors of diseases affecting animals (Jongejan *et al.*, 2007). In southern Africa, tick-borne diseases (TBDs) cause serious economic losses of ruminants through mortality, reduction in meat and milk production and through the application of control measures (Makala *et al.*, 2003). Babesiosis, anaplasmosis and heartwater are the TBDs of economic significance in the communal livestock farming areas of South Africa (Dreyer *et al*., 1998, Mbati *et al.*, 2002) while Corridor disease (resulting from infection with buffalo-derived *Theileria parva*) is a notifiable disease.

The livelihoods of communal farmers are supported by cattle rearing, as cattle are a source of food and hides and provide draught power and manure for crop production. Furthermore, cattle are a form of savings (Sansoucy, 1995), a source of collateral and income, and a measure of status and wealth (Dovie *et al*., 2006; Shackleton *et al*., 1999). The economic well-being of both commercial and resource-poor farmers in South Africa is therefore substantially impacted by TBDs.

Anaplasma marginale is an intra-erythrocytic rickettsial parasite that infects cattle and causes bovine anaplasmosis (Potgieter & Stoltsz, 2004). *Anaplasma marginale* organisms are located predominantly on the margins of the erythrocyte. *Anaplasma marginale* subsp. *centrale* causes a milder form of anaplasmosis, and is used in a live blood vaccine in many countries, including South Africa (Potgieter & Van Rensburg, 1983). *Anaplasma marginale* subsp. *centrale* can be distinguished from *A. marginale* microscopically, as the majority of organisms are located in the centre of the erythrocyte. Ixodid ticks are the principal biological vectors of anaplasmosis although argasid ticks can also transmit *A. marginale* (Raja *et al*., 1986; Potgieter, 1981). The five tick species implicated in the transmission of *A. marginale* in South Africa are *Rhipicephalus (Boophilus) decoloratus*, *Rhipicephalus (Boophilus) microplus*, *Rhipicephalus evertsi evertsi*, *Hyalomma marginatum rufipes* and *Rhipicephalus simus*

(Potgieter & Stoltsz, 2004; Potgieter, 1981). Although transmission mainly takes place transstadially, intrastadial transmission also occurs (Stiller *et al.*, 1980). Mechanical transmission of anaplasmosis also readily occurs by needle passage of infected blood, although this is dose dependant (Ristic, 1968). Bovines with anaplasmosis exhibit fever, weight loss, abortion, lethargy and icterus, and death often occurs in animals older than two years (De Waal *et al*., 2000). Lifelong immunity is acquired by persistently infected or "carrier" cattle which are resistant to clinical disease on challenge exposure.

Babesia bovis and *Babesia bigemina* (De Vos, 1979*)* are apicomplexan protozoan parasites that cause bovine babesiosis. Asiatic redwater, caused by *B. bovis*, and African redwater, caused by *B. bigemina*, both occur in South Africa. Although the diseases are clinically quite similar, *B. bovis* is more virulent than *B. bigemina* while the latter is more widely distributed in southern Africa (De Vos *et al.*, 2004). *Babesia bovis* is transmitted by *Rhipicephalus (Boophilus) microplus*, which is the only known vector (Potgieter, 1977). *Rhipicephalus (Boophilus) microplus* is also a confirmed vector of *B. bigemina* (Buscher, 1988). Two other ticks species, *R. (B.) decoloratus* (Potgieter, 1977) and *R. evertsi evertsi* (Buscher, 1988) are also vectors for *B. bigemina*. Transmission of *Babesia* spp. is transovarial. Clinical signs of babesisosis in cattle are anaemia, fever, haemoglobinuria, ataxia and high parasitaemia, and death sometimes occurs. Cattle that recover from an infection of babesiosis have parasitaemia which is virtually undetectable on microscopy; these animals become carriers.

Ehrlichia (formerly *Cowdria*) *ruminantium* (Dumler *et al*., 2001) is an obligate intracellular rickettsial parasite that causes heartwater in ruminants. It causes heavy losses in cattle in southern Africa and disease is more severe in exotic and naïve cattle than indigenous ones (Maillard & Maillard, 1998). *Ehrlichia ruminantium* is transmitted by ticks of the genus *Amblyomma* and heartwater is limited to the distribution areas of the tick vector (Allsopp *et al.*, 2004). *Amblyomma hebraeum* is the main vector of heartwater in southern Africa, while *Amblyomma variegatum* is the major vector in other parts of Africa (Maillard & Maillard, 1998). Heartwater manifests in peracute, acute, subacute and clinically inapparent forms. Peracute infection kills the animal within the first few hours of development of fever, while the acute form is characterised by a high fever of 40°C which persists for three to six days, showing small fluctuations before the body temperature falls to subnormal shortly before death. Animals with heartwater show neurological signs during the later stages and usually die if they are not specifically treated for the disease.

Theileria parva is a tick-borne apicomplexan parasite that causes theileriosis in cattle in sub-Saharan Africa. The African buffalo (*Syncerus caffe*r) is the natural reservoir host of *T. parva* (Dolan 1989; Barnett & Brocklesby, 1966). Three disease syndromes (Uilenberg 1981; Lawrence, 1979) caused by *T. parva* have been recognized: East Coast fever (ECF), Corridor disease and January disease. These syndromes were originally thought to be caused by three different subspecies of *T. parva* (Norval *et al.*, 1992), but it has now been shown that the *T. parva* parasites that cause the different diseases are morphologically and serologically indistinguishable. Today two groups of *T. parva* parasites are recognised: 1) cattle-associated *T. parva* which causes ECF and January disease and results from cattle-to-cattle transmission of the parasite, and 2) buffalo-associated *T. parva* which causes Corridor disease and results from buffalo-to-cattle transmission (Neitz, 1957; Koch *et al.*, 1988). The tick species, *Rhipicephalus appendiculatus*, *R. zambeziensis* and *R. duttoni* are natural vectors capable of transmitting *T. parva* (Norval *et al.*, 1992). Corridor disease has a strict seasonal occurrence, from January to March, which coincides with the activity of the adult *R. appendiculatus*, the natural vector of *T. parva* (Matson, 1967). The adult *R. appendiculatus* emerge in the summer months of January to March while the larvae are most abundant in the post-rainy season (April to June). The nymphs are abundant in the dry months of July to October (Norval *et al*., 1992). *Theileria parva* is transmitted transstadially. An animal infected with *T. parva* is characterized by pyrexia, enlargement of superficial lymph nodes, severe pulmonary oedema and wasting (Lawrence *et al.*, 2004b).

Since ECF was eradicated from South Africa in the 1950s, Corridor disease has become the most important form of theileriosis in South Africa. Sporadic outbreaks of Corridor disease occur in cattle that come into contact with infected buffalo within the distribution range of the vector ticks (Stoltsz, 2011). Cattle infected with buffalo-derived *T. parva* can recover after treatment by chemotherapy and can become carriers of the parasite (Potgieter *et al*., 1988), but a carrier state in cattle is not thought to occur in South Africa, since most infected cattle die of Corridor disease. However, recent reports suggest that *T. parva* DNA is detectable in cattle at the livestock/wildlife interface in South Africa (Yusufmia *et al*., 2010; Mbizeni *et al*., 2013). It is thought that ECF was finally eradicated from South Africa only once all of the carrier cattle were eradicated. In the event that cattle can become *T. parva* carriers after infection with buffalo-derived parasites, then cattle-to-cattle transmission and the selection of cattle-adapted *T. parva* might become a possibility.

Theileria taurotragi was confirmed to be present in South Africa in 1981 (De Vos & Roos, 1981) and is believed to occur quite commonly in cattle within the distribution range of *R. appendiculatus* and *R. zambeziensis.* The eland (*Taurotragus oryx*) is the natural reservoir of *T. taurotragi* although it can also be infected by other *Theileria* parasite species. The main, if not the only cause of cerebral theileriosis in cattle in South Africa is *T. taurotragi* (De Vos, 1982).

Many tick species flourish under the climatic conditions in the Mnisi Community Programme (MCP) study area, and TBDs are thought to be endemic in livestock species in the area. Due to the abundant wildlife populations surrounding the Mnisi community, livestock are likely to be exposed to diseases with a wildlife reservoir, including Corridor disease from buffalo. Known tick vectors of important diseases such as Corridor disease, redwater, heartwater and anaplasmosis are present. Although the farmers frequently dip their cattle in acaricide-filled dip tanks to control the tick burden, a practice supported by government, and interact with animal health technicians in the area, tick-associated diseases are still a major problem (from http://www.up.ac.za/mnisi-community-programme/article/275823/the-study-area, accessed on 09/09/2015).

In an effort to determine if the symptoms of cattle in poor health in the MCP area could be attributed to TBDs, blood samples were taken from cattle suffering from suspected TBDs. The study involved contacting farmers at the dip tanks, and in a number of cases visiting them at their homesteads, where the cattle were sampled. Blood samples from sick cattle were collected and examined for the presence of haemoparasites by microscopy, histopathology, reverse line blot (RLB) hybridization and *T. parva*-specific quantitative real-time polymerase chain reaction (qPCR).

2.2 Materials and methods

2.2.1 Study site

The Mnisi community is situated in the north-eastern part of the Bushbuckridge Municipal Area, Mpumalanga Province, South Africa (Figure 2-1). The study area falls within the savannah ecosystem and is surrounded by wildlife reserves: Andover to the north-west, Timbavati to the north, Manyeleti to the east and Sabi Sand to the south. Agriculture is the main activity in the area and livestock farming, in particular cattle, is very important. The Hluvukani Animal Clinic, a satellite facility of the Onderstepoort Veterinary Academic Hospital of the University of Pretoria, is situated in the centre of the study area and services the needs of animal owners in the area.

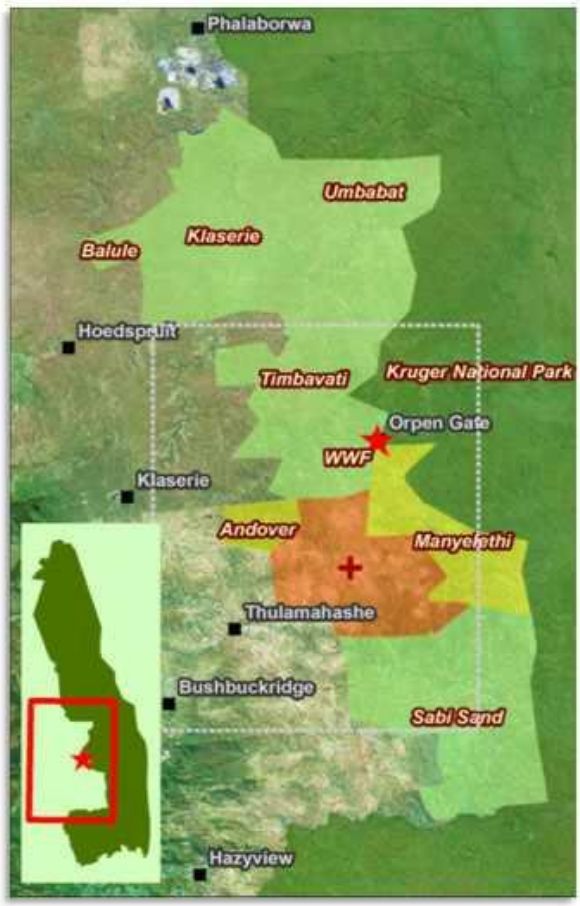

Figure 2-1: The study area in the Mnisi community (orange area) relative to the surrounding wildlife reserves and the Kruger National Park, with Hluvukani Animal Clinic in the centre (indicated by the red cross) (from http://www.up.ac.za/mnisi-community-programme/article/275823/the-study-area, accessed on 09/09/2015).

2.2.2 Target cattle

The study area has established systems in place to monitor the health of the cattle in the area. Sampling was done in collaboration with the Health and Demographic Surveillance System in Livestock (HDSS-Live) and the Hluvukani Animal Clinic projects within the same study area. Most cattle were uniquely and permanently identified by eartags and tattoo through the HDSS-Live project, which also conducts passive surveillance for disease syndromes in cattle at dip tanks through weekly interviews with all farmers. Animal Health Technicians of the Mpumalanga State Veterinary Services were available and assisted in communicating with the farmers and in some cases helped with sample collection. Dip tanks in the study area were visited once per week during the study period between September 2012 and May 2013. Where the study team was not present, the Animal Health Technicians or the Hluvukani Animal Clinic staff collected samples from identified cattle. Cattle showing TBD symptoms were sampled when they were attended to at the dip tanks or at Hluvukani Animal Clinic. One or a combination of clinical signs indicative of TBDs were considered, including pale mucous membranes, lethargy, laboured breathing, froth from the nostrils, constipation, jaundice, weight loss, abortion, fever, red discolouration of the urine, ataxia, nervous signs, enlarged superficial lymph nodes, corneal opacity, diarrhoea and, in some cases, death.

2.2.3 Sample collection

2.2.3.1 Consent form

A consent form (Appendix 1) was presented to the farmers before the study team collected samples from the identified cattle. Since most farmers did not have a good command of English, research assistants interpreted the information in the local language to help the farmers understand the purpose of the study.

2.2.3.2 Blood and lymph node smears

A drop of blood from a prick of the ear vein was placed on a clean glass slide. A second glass slide was used as a spreader. The spreader was placed at an angle of approximately 30º making contact with the blood. The blood was allowed to run to each end of the spreader and was then spread along the slide in a smooth rapid motion. The blood smear was quickly dried by moving the slide in the air.

In cattle with reactive lymph nodes (enlarged), lymph node fluid/tissue was drawn from the pre-scapular lymph node by piercing it with an 18-gauge hypodermic needle while holding it firmly to squeeze out the fluid. The fluid was expelled on to a clean glass slide and spread evenly using another glass slide.

The glass slides were labelled using a pencil (animal identity, animal name, location and date). In addition to this information, the age and sex of the animal was recorded. Care was taken to protect the smears from flies by placing them in a slide box and keeping them covered. The slides were transported to the laboratory at Hluvukani Animal Clinic where they were stained and examined.

2.2.3.3 Whole blood samples

Blood was collected from the cattle in 10 ml EDTA vacutainer tubes by jugular or caudal vein puncture. Labelling of the tubes was done as for the smears. After collection, the blood samples were stored in cooler boxes until they were moved and placed in a -20°C freezer at the Hans Hoheisan Wildlife Research Station (HHWRS) awaiting DNA extraction. In accordance with the Animal Diseases Act No. 35 of 1984, a transport permit (Appendix 2) was issued each time blood samples were collected and moved from the MCP area (an FMD control area with vaccination) to HHWRS (an FMD infected area).

2.2.4 Staining and microscopic examination of blood and lymph node smears

The slides were processed for microscopic examination at the laboratory at Hluvukani Animal Clinic. They were fixed for 3 minutes in absolute methanol, then stained in 10% Giemsa stain (Merck Millipore) for 30 minutes in the case of fresh smears and 45 minutes in the case of smears prepared more than 48 hours earlier. The stain was washed off gently under tap water and the slides were then placed in an upright position to dry. A microscope (Primo star, Zeiss) was used to examine blood and lymph smears using 10x objective lens and 100x oil immersion lens. Photographs were taken using a 14.1 megapixel Cyber-shot camera (Sony).

2.2.5 Additional diagnostic tests

In cases where animals had died, tissue samples such as lymph nodes, spleen, liver and heart were taken (it was possible to collect post-mortem samples up to 24 hours after death) and

sent to the Pathology Section, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria for histopathology examination. In cases where Corridor disease was suspected, the State Veterinarian, Dr Bjorn Reininghaus, sent blood and/or tissue samples to the Agricultural Research Institute – Onderstepoort Veterinary Institute (ARC-OVI) for detection of *T. parva* by quantitative real-time PCR (qPCR). In cases where babesiosis or ehrlichiosis was suspected, brain impression smears were prepared within 24 hours of the animal's death and examined.

2.2.6 Extraction of DNA from blood samples

DNA was extracted at HHWRS from 200 µl of each blood sample using the QIAamp® DNA Mini Kit (Qiagen, Whitehead Scientific, South Africa). The extraction was done according to the method described by the manufacturer as follows: QIAGEN Protease (or proteinase K), (20 µl) was pipetted into a 1.5 ml microcentrifuge tube and 200 µl sample added to it. Thereafter 200 µl of Buffer AL was added to the sample and mixed by pulse-vortexing for 15 seconds. The mixture was incubated at 56°C for 10 minutes and then briefly centrifuged. Ethanol (96–100%), 200 µl, was added to the sample, pulse-vortexed for 15 s and then briefly centrifuged. The mixture was carefully applied to the QIAamp Mini spin column and centrifuged at 6,000 x g (8,000 rpm), after which the lysate was discarded. The spin column was placed in a clean 2 ml collection tube and centrifugation performed at 6,000 x g (8,000 rpm) to remove the remaining lysate, which was also discarded. Buffer AW1 (500 µl) was added and centrifuged at 6,000 x g (8,000 rpm) for 1 minute. The filtrate was discarded. The next step was to add 500 µl Buffer AW2 and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 minutes. Finally the spin column was placed in a clean 1.5 ml microcentrifuge tube, 100 µl Buffer AE added and incubated at room temperature (15–25°C) for 1 minute, and then centrifuged at 6,000 x g (8,000 rpm) for 1 minute to elute the DNA. The DNA was stored in the freezer in HHWRS at -20°C until the time the DNA was moved to the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria for analysis. Transport permits (Appendix 3) were issued for transport of the DNA samples to Pretoria.

2.2.7 Reverse line blot (RLB) hybridization assay

The RLB hybridization assay was performed as described previously (Gubbels *et al.*, 1999, Bekker *et al.*, 2002, Nijhof *et al.*, 2003, Nijhof *et al.*, 2005). The RLB test involves a number

of steps including covalent linking of genus- and species-specific probes to a biodyne C membrane, polymerase chain reaction (PCR) amplification of target sequences from DNA samples, hybridization of PCR products to the probes, and detection of hybridized products. The procedure is described below.

2.2.7.1 Membrane preparation

A biodyne C membrane, supplied by Separations, South Africa, was prepared by measuring a piece of membrane according to the size of the mini blotter apparatus (Isogen, Life Sciences) support cushion. It was then activated by incubating in 10 ml freshly prepared 16% EDAC (1-ethyl-3-(-3-dimethylamininopropyl) carbodimide) for 10 minutes. Each probe was diluted in 0.5 M $NaHCO_3$, pH 8.4 to a final concentration of 2 pmol/µl, and 200 µl was loaded onto the membrane using the miniblotter. *Anaplasma*, *Ehrlichia*, *Theileria* and *Babesia* genus- and species-specific probes that were included on the membrane are shown in Table 2-1. The membrane was incubated for 2 min at room temperature and inactivated with 100 mM freshly made NaOH for 8 min at room temperature on a shaker. Sodium chloride/sodium phosphate /EDTA (SSPE) buffer contains 0.02 M EDTA and 2.98 M NaCl in 0.2 M phosphate buffer (pH 7.4). The membrane was washed in 100 ml 2 X SSPE/0.1% SDS at 60°C for 5 minutes.

Table 2-1: Genus and species-specific RLB oligonucleotide probes that were used in this study. The degenerate position R denotes either A or G, W denotes either A or T and Y denotes either C or T.

Pathogen	**Sequence (5'- 3')**
Ehrlichia / Anaplasma group-specific probe	GGG GGA AAG ATT TAT CGC TA
Anaplasma bovis	GTA GCT TGC TAT GRG AAC A
Anaplasma marginale subsp. *centrale*	TCG AAC GGA CCA TAC GC
Anaplasma marginale	GAC CGT ATA CGC AGC TTG
Anaplasma phagocytophilum	TTG CTA TAA AGA ATA ATT AGT GG
Anaplasma (formerly *Ehrlichia*) sp. Omatjenne	CGG ATT TTT ATC ATA GCT TGC
Ehrlichia canis	TCT GGC TAT AGG AAA TTG TTA
Ehrlichia chaffeensis	ACC TTT TGG TTA TAA ATA ATT GTT
Ehrlichia ruminantium	AGT ATC TGT TAG TGG CAG
Theileria / Babesia group-specific probe	TAA TGG TTA ATA GGA RCR GTT G
Babesia genus-specific probe 1	ATT AGA GTG TTT CAA GCA GAC

Babesia genus-specific probe 2	ACT AGA GTG TTT CAA ACA GGC
Babesia bicornis	TTG GTA AAT CGC CTT GGT C
Babesia bigemina	CGT TTT TTC CCT TTT GTT GG
Babesia bovis	CAG GTT TCG CCT GTA TAA TTG AG
Babesia caballi	GTG TTT ATC GCA GAC TTT TGT
Babesia canis	TGC GTT GAC CGT TTG AC
Babesia divergens	ACT RAT GTC GAG ATT GCA C
Babesia felis	TTA TGC GTT TTC CGA CTG GC
Babesia gibsoni Japan	TAC TTG CCT TGT CTG GTT T
Babesia gibsoni USA	CAT CCC TCT GGT TAA TTT G
Babesia leo	ATC TTG TTG CTT GCA GCT T
Babesia major	TCC GAC TTT GGT TGG TGT
Babesia microti	GRC TTG GCA TCW TCT GGA
Babesia rossi	CGG TTT GTT GCC TTT GTG
Babesia vogeli	AGC GTG TTC GAG TTT GCC
Theileria genus-specific probe	ATT AGA GTG CTC AAA GCA GGC
Theileria annae	CCG AAC GTA ATT TTA TTG ATT TG
Theileria annulata	CCT CTG GGG TCT GTG CA
Theileria bicornis	GCG TTG TGG CTT TTT TCT G
Theileria buffeli	GGC TTA TTT CGG WTT GAT TTT
Theileria equi	TTC GTT GAC TGC GYT TGG
Theileria lestoquardi	CTT GTG TCC CTC CGG G
Theileria mutans	CTT GCG TCT CCG AAT GTT
Theileria parva	GGA CGG AGT TCG CTT TG
Theileria sp. (buffalo)	CAG ACG GAG TTT ACT TTG T
Theileria sp. (kudu)	CTG CAT TGT TTC TTT CCT TTG
Theileria sp. (sable)	GCT GCA TTG CCT TTT CTC C
Theileria taurotragi	TCT TGG CAC GTG GCT TTT
Theileria velifera	CCT ATT CTC CTT TAC GAG T

2.2.7.2 Polymerase chain reaction (PCR)

Primers RLB-F2 (5'-GAC ACA GGG AGG TAG TGA CAA G-3') and RLB-R2 (5'-biotin-CTA AGA ATT TCA CCT CTG ACA GT-3'), specific for *Theileria* and *Babesia* species (Nijhof *et al*., 2003), and primers EHR-F (5'-GGA ATT CAG AGT TGG ATC MTG GYT CAG-3') and EHR-R (5'-biotin-CGG GAT CCC GAG TTT GCC GGG ACT TYT TCT-3'),

specific for *Anaplasma* and *Ehrlichia* species (Bekker *et al*., 2002), were used to amplify respectively, the V4 and V1 hypervariable region of parasite small subunit rRNA genes. In each case, the reverse primer was labelled with biotin to allow for detection of the PCR product during the hybridization process.

Separate PCR master mix reactions were prepared for amplification of the *Theileria* and *Babesia* species, and for amplification of the *Ehrlichia* and *Anaplasma* species. For the *Theileria/Babesia* PCR master mix (18S rRNA), RLB-F2 and RLB-R2 primers were used, while for the *Ehrlichia*/*Anaplasma* PCR master mix (16SrRNA) EHR-F and EHR-R primers were used. The PCR reaction mixture was prepared using Platinum® Quantitative PCR SuperMix-UDG (Invitrogen, Celtic Molecular Diagnostics, South Africa). Reactions were performed in a 25 µl volume with a final concentration of 3 mM $MgCl_2$, 0.2 pmol of each primer, 0.5 U UDG, 200 mM dNTPs, 0.75 U Platinum® *Taq* DNA polymerase and 5 µl of template DNA (containing between 50 and 100 ng DNA). A touchdown thermal cycling programme (Table 2-2) was used (Nijhof *et al*., 2005). Mastermix with no DNA template (negative control), and known *A. marginale* subsp. *centrale* and *B. bigemina* DNA samples (positive controls) was included to monitor the occurrence of false positive or false negative results.

2.2.7.3 Hybridization

Hybridization was performed as described previously (Nijhof *et al*. 2005). The Biodyne C membrane containing genus- and species-specific oligonucleotide probes was activated in approximately 50 ml 2 X SSPE/0.1% SDS in a plastic container at room temperature for 5 min. The PCR products were prepared for hybridization by adding 130 µl of 2 X SSPE/0.1% SDS to a 25 µl aliquot of each PCR product, denaturing for 10 min at 99.9°C on a thermal cycler machine and cooling on ice immediately. Denatured PCR products were applied to the activated membrane using a miniblotter apparatus. Hybridization was done at 42°C for 60 min, after which samples were removed by aspiration and the membrane was removed from the blotter. The membrane was then washed twice in preheated 2 X SSPE/0.5% SDS for 10 min at 50°C, then incubated with 10 ml 2 X SSPE/0.5% SDS and 12.5 µl streptavidin-POD (peroxidase labelled) conjugate (1.25 U) for 30 min at 42°C. The membrane was washed twice in preheated 2 X SSPE/0.5% SDS for 10 min at 42°C and twice with 2 X SSPE for 5 min at room temperature. All incubations and washes were performed in the incubator with gentle shaking.

For detection of hybridized PCR products, enhanced chemiluminiscence (ECL), 10 ml of ECL (5 ml ECL1 + 5 ml ECL2) was added onto the membrane and mixed by shaking the container gently and keeping the membrane covered with ECL for 1 min at room temperature. The membrane was exposed to an X-ray film. Once the X-ray film had been developed, dark spots that appear as a result of a chemiluminescence reaction were identified where hybridization had occurred (Gubbels *et al.* 1999).

2.2.7.4 Stripping of the membrane

The membrane was stripped immediately after use, by washing twice with 1% SDS preheated to 80ºC for 30 minutes with gentle shaking. This was followed by one wash with 20 mM EDTA (pH8) at room temperature for 15 minutes with gentle shaking. After stripping, the membrane was stored at 4ºC in 20 mM EDTA (pH8) in a plastic container.

Table 2-2: Thermocycling programme for *Babesia/Theileria* and *Ehrlichia/Anaplasma* touchdown PCR.

Cycle	Time	Temperature	Purpose
1 cycle	3 min	37°C	Activate UDG
1 cycle	10 min	94°C	Inactivate UDG & activate *Taq* polymerase
2 cycles	20 sec	94°C	Denature double stranded DNA template
	30 sec	67°C	Anneal primers
	30 sec	72°C	Extension of PCR products by *Taq* polymerase
2 cycles	20 sec	94°C	
	30 sec	65°C	
	30 sec	72°C	
2 cycles	20 sec	94°C	
	30 sec	63°C	
	30 sec	72°C	
2 cycles	20 sec	94°C	
	30 sec	61°C	
	30 sec	72°C	
2 cycles	20 sec	94°C	
	30 sec	59°C	
	30 sec	72°C	
40 cycles	20 sec	94°C	
	30 sec	57°C	
	30 sec	72°C	
1 cycle	7 min	72°C	Final extension

2.3 Results

A total of 137 clinical cases of suspected TBDs were seen between September 2012 and May 2013. Samples collected were analysed by microscopy; in cases where cattle died, histopathology and a post-mortem examination were done, and for those suspected of Corridor disease, samples were sent to ARC-OVI for testing using a *T. parva*-specific qPCR assay. Of the 137 cases, 24 (17.5%) were positive for TBDs (anaplasmosis, babesiosis or theileriosis) on one or more of the tests done excluding RLB (Table 2-3). No heartwater cases were detected by microscopy and no cases of mixed infections were identified.

As a result of similarities in the clinical signs for a number of diseases, few cases had specific diagnoses made at the time of sampling. Samples were also sent to the University of Pretoria for the diagnosis of non-TBDs. Among the non-TBDs that were confirmed were lumpy skin disease, salmonellosis, tuberculosis and foot-and-mouth disease. A specific diagnosis could not be arrived at for many of the cases.

The samples from all the 137 clinical cases were later examined using the RLB assay (see section 2.3.4). For the 24 TBD cases, the overlap between the RLB results and the results of the other tests are highlighted in Table 2-3.

Table 2-3: Diagnosis of TBDs by microscopy, histopathology and *T. parva*-specific qPCR in cattle showing clinical signs in the Mnisi Community Programme area between September 2012 and May 2013. Results of tests done at the time of sampling were compared with RLB results (correlating RLB results are highlighted in blue). There were six cases where schizonts or piroplasms were seen on the blood smear but *T. parva*-specific qPCR was not performed. In five of these cases, schizonts and piroplasms seen by microscopy could be attributed to other *Theileria* spp. identified on RLB (highlighted in yellow). In the remaining case, histopothology examination indicated it was Corridor disease (highlighted in red), although both microscopy and RLB were negative.

ID	Date sampled	Sex	Age (years)	Dip Tank	Death	Microscopy	Histopathology	*T. parva* qPCR	RLB Results
C6	30.10.2012	F	8	Clare A	Yes	Negative (bs)[b]	*T. taurotragi*	ND[a]	*E/A* catch-all[e], *T/B* catch-all[f], *T* catch-all[g], *T. mutans*, *T. taurotragi*, *T. velifera*
C10	06.11.2012	F	0.1	Clare A	Yes	*Babesia bovis* (brs)[c]	ND	ND	*E/A* catch-all, *A. marginale* (f)[i], *T/B* catch-all, *T* catch-all, *B* catch-all 1[h], *B. bovis*, *T. parva*
C14	14.12.2012	F	0.3	Seville B	No	*A. marginale* (bs)	ND	ND	*E/A* catch-all, *A. marginale* (f), *T/B* catch-all, *T* catch-all, *B* catch-all 1, *T. velifera*
C28	14.12.2012	F	0.3	Athol	Yes	*Babesia bovis* (bs)	ND	ND	*T/B* catch-all, *B* catch-all 1, *B. bovis* (vf)[j]
C47	11.01.2013	F	5	Gottenburg	Slaughtered	Piroplasms (bs)	ND	ND	*E/A* catch-all, *A. marginale* (vf), *T/B* catch-all, *T* catch-all, *B* catch-all 1 (f), *B. bovis* (f), *T. mutans*, *T. taurotragi*, *T. velifera*
C57	31.01.2013	F	0.5	Welverdiend B	No	*A. marginale* (bs)	ND	ND	*E/A* catch-all, *A. marginale* (f)
C58	31.01.2013	F	0.5	Welverdiend B	No	*A. marginale* (bs)	ND	ND	*E/A* catch-all, *E. ruminantium*, *T/B* catch-all, *T* catch-all, *T. mutans*
C64	06.02.2013	F	0.8	Hlalakahle	No	Schizonts (ls)[d]	ND	ND	*E/A* catch-all, *A. marginale* (vf), *T/B* catch-all, *T* catch-all, *B* catch-all 1, *B. bovis*, *T. velifera*
C65	06.02.2013	F	3	Hlalakahle	Yes	Schizonts (ls)	ND	ND	Blood: Negative. Tissue: *E/A* catch-all (f), *T/B* catch-all, *B* catch-all 1, *T. mutans*, *T.velifera*
C66	06.02.2013	F	2	Hlalakahle	Yes	Schizonts (ls) & piroplasms (bs)	ND	Positive	*E/A* catch-all, *T/B* catch-all, *T* catch-all, *T.* sp. (sable) (f), *T. mutans*, *T. parva*, *T. velifera*
C70	14.02.2013	F	1.5	Burlington	Yes	Piroplasms (bs)	ND	ND	*T/B* catch-all (f)

C71	15.02.2013	F	8	Hlalakahle	Yes	Schizonts (ls)	Corridor disease	Positive	*E/A* catch-all, *A. marginale*, *T/B* catch-all, *T* catch-all, *T. parva*
C73	15.02.2013	F	0.3	Islington	No	Piroplasms (bs)	ND	ND	*E/A* catch-all, *A. marginale* (vf), *T/B* catch-all, *T*. catch-all, *B* catch-all 1
C80	19.02.2013	F	4	Welverdiend B	Yes	Piroplasms (bs)	Corridor disease	Positive	Blood: *E/A* catchall, *A. marginale*, *T/B* catch-all, *T*. catch-all, *B* catch-all 1. Tissue: *T/B* catch-all, *T*. catch-all, *T. parva*
C81	22.02.2013	F	8	Thlavekisa	Yes	Schizonts (ls)	Corridor disease	Positive	*E/A* catch-all, *A. marginale* (vf), *T/B* catch-all, *T* catch-all, *T. mutans*, *T. parva*
C84	26.02.2013	M	4	Islington	Slaughtered	Negative (bs)	ND	Positive	*E/A* catch-all, *A. marginale*, *T/B* catch-all, *T* catch-all, *T. mutans*, *T. parva*, *T. taurotragi*, *T. velifera T*. sp. (sable) (f)
C89	01.03.2013	F	7	Hlalakahle	Yes	Schizonts (ls) & piroplasms (bs)	Corridor disease	Positive	*E/A* catch-all, *T/B* catch-all, *T* catch-all, *T. parva*
C90	04.03.2013	M	5	Buffelshoek	Slaughtered	Negative (bs)	Corridor disease	ND	*T/B* catch-all, *T*. catch-all, *B*. catch-all 2 (vf), *T*. *mutans*, *T. taurotragi*, *T. velifera*, *Theileria*. sp. (sable) (f), *B*. catch-all 1, *B. bovis*
C108	12.03.2013	F	7	Hlalakahle	Yes	Schizonts (ls)	ND	Positive	*E/A* catch-all
C109	12.03.2013	F	6	Hlalakahle	Yes	Schizonts (ls)	ND	Positive	*E/A* catch-all
C110	13.03.2013	F	0.5	Seville B	Yes	Schizonts (ls)	ND	Positive	Tissue: *E/A* catch-all, *A. marginale*, *T/B* catch-all, *T* catch-all, *T. parva*
C123	02.04.2013	F	4	Welverdiend B	Yes	Schizonts (ls)	ND	Positive	Blood: *E/A* catch-all, *A. marginale*, *T/B* catch-all, *T* catch-all, *Theileria* sp. (sable), *T. mutans*, *T. velifera*. Tissue: *T/B* catch-all, *T* catch-all, *T. mutans*, *T. parva*, *T. velifera*
C129	09.02.2013	F	8	Hlalakahle	Yes	Negative (bs)	ND	Positive	Tissue: *T/B* catch-all, *T* catch-all, *T. parva*
C133	26.04.2013	F	7	Hlalakahle	Slaughtered	Schizonts (ls)	ND	Positive	*T/B* catch-all, *T* catch-all, *T. parva*, *T. velifera*

[a] ND: not done

[b] (bs): blood smear

[c] (brs): brain smear

[d] (ls): lymph smear

[e] *E/A* catch-all: *Ehrlichia/Anaplasma* group-specific probe

[f] *T/B* catch-all: *Theileria/Babesia* group-specific probe

[g] *T* catch-all: *Theileria* genus-specific probe

[h] *B* catch-all: *Babesia* genus-specific probe

[i] (f): faintly positive

[j] (vf): very faintly positive

More theileriosis cases were diagnosed in the MCP area than the other TBDs combined during the study period (Figure 2-2) as determined by microscopy, histopathology and *T. parva*-specific qPCR.

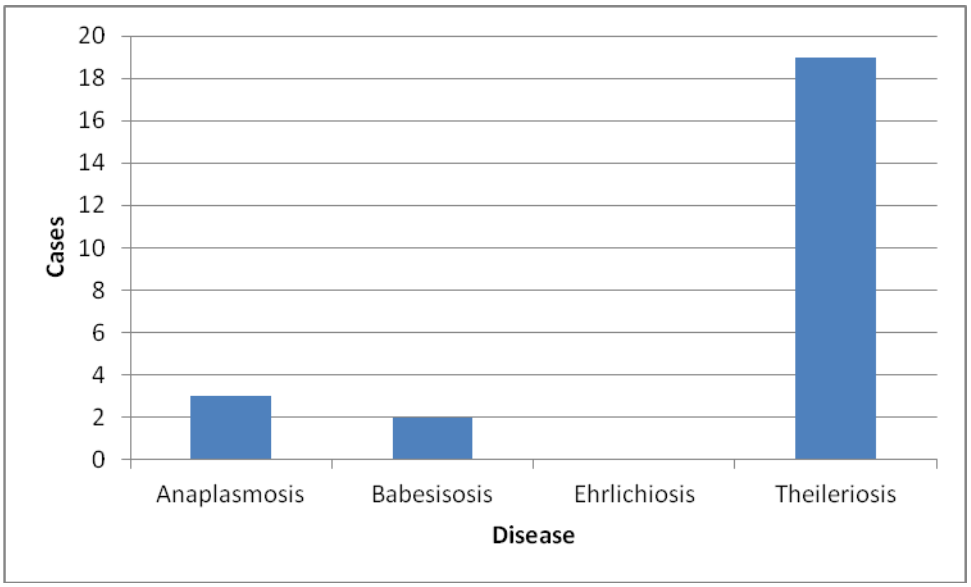

Figure 2-2: Number of clinical cases attributed to TBDs as determined by microscopy, histopathology and *T. parva*-specific qPCR.

2.3.1 Clinical and post-mortem examinations

Detailed symptoms for all 24 clinical cases examined are given in Appendix 4.

The clinical signs observed for the *T. taurotragi* case were inappetance, cycling and paddling movements in the animal's late stage of illness. The signs presented in cases of cattle with anaplasmosis were constipation and anaemia. Anaemia, neurological signs, lethargy and dehydration were observed in the case of a calf diagnosed with cerebral babesiosis. The calf later died and a brain crush smear was made in addition to the blood smear.

Twelve Corridor disease cases confirmed by *T. parva*-specific qPCR were encountered, from February to April. One Corridor disease case was confirmed on histopathological examination but no *T. parva*-specific qPCR was performed on this sample. The cattle with Corridor disease had anaemia, presented neurological signs, were lethargic and in some cases were dehydrated. Additionally, the cattle had enlarged lymph nodes, fever and labored breathing (Figure 2-3 A).

The case-fatality rate for Corridor disease cases confirmed by *T. parva*-specific qPCR was 83.3% (10/12), and the remaining two animals were slaughtered. On necropsy, it was observed that most of these cattle had enlarged lymph nodes, liver and spleen. Petechial haemorrhages were also observed on the heart, liver, kidneys and on the mucosa of the intestines of most of these cattle. One of the typical post-mortem characteristics seen in Corridor disease cases was the froth in the trachea which should be the cause of the labored breathing (Figure 2-3 B).

Figure 2-3: A cow with a fever and laboring to breath (A) and a post-mortem case where the lungs were swollen and froth was seen in the trachea (B).

2.3.2 Microscopy examination

Blood, lymph and brain samples for making smears were collected and kept in a dust-free environment to ensure good results. Matchstick-shaped piroplasms in blood smears and schizonts in lymph node biopsy smears were seen in cattle with Corridor disease (Figure 2-4 A & B), but these were scanty. Small leaf-like paired objects were seen in blood smears made from *B. bovis* infected cattle (Figure 2-4 C). The objects looked more like *Theileria* piroplasms but were slightly bigger, although they were smaller than those of *B. bigemina*. In fatal cases where babesiosis was suspected, brain impression smears were made. Paired dot-like structures were seen on examination of brain impression smears of cattle with cerebral babesiosis (Figure 2-4 D). Blood smears from cattle infected with *A. marginale* had small dot-like structures on the edges of erythrocytes (Figure 2-4 E).

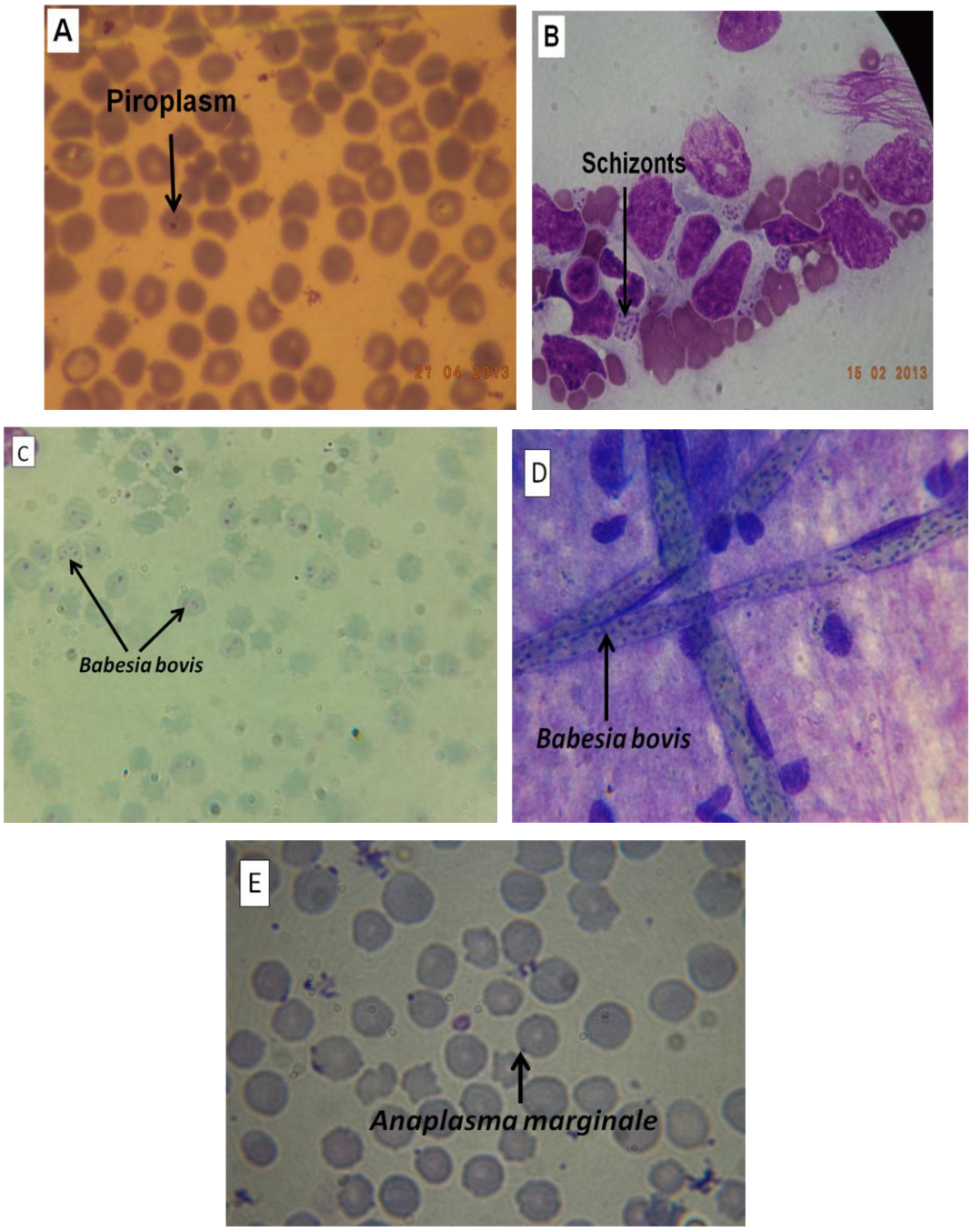

Figure 2-4: Microscopic examination of smears using 10x objective lens and 100x oil immersion lens. Blood (A) and lymph node (B) smears from Corridor disease infected cattle showing piroplasms and schizonts respectively. Blood (C) and brain (D) smears from cattle with *B. bovis* infections. Dot-like structures on the margin of red blood cells on blood smear (E) made from *A. marginale* infected cattle.

2.3.3 Histopathology and qPCR

More than one type of sample was taken from some of the 24 cases (Table 2-3, Appendix 4), particularly in cases where animals died or were slaughtered. Lymph node, heart, liver, lung and spleen tissue samples from six cases were sent for histopathological analysis (Pathology Section, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria) while one or a combination of EDTA blood, lymph node and spleen samples from 12 cases were sent for *T. parva*-specific qPCR analysis (ARC-OVI). Blood and lymph node smears were made and EDTA blood was collected from cattle that did not die. Not all the 24 cases had post-mortem examinations done.

The histopathology findings of the *T. taurotragi* case showed that the lumens of many meningeal arterioles and the hepatic blood vessels contained large numbers of lymphoblastic cells. Koch's bodies (schizonts) of *Theileria* spp. could not, however, be demonstrated because often, in cerebral theileriosis, it is difficult to demonstrate schizonts and piroplasms. In *T. parva* cases, the pathology findings were that the heart showed mild to moderate perivascular lymphocytes, plasma cells and some macrophages while most lymph node sections showed reactivity mostly as expanded interfollicular zones and marked sinus histiocytosis. Amongst the lymph node tissue cells were large lymphoblastic cells, some of which had intracytoplasmic *Theileria* spp. morulae. The lungs had moderately acute diffuse interstitial pneumonia with severe oedema and the spleen had marked red pulp congestion and lymphocyte depleted follicles.

The results of the smear examinations, histopathological findings and *T. parva*-specific qPCR are presented in Table 2-4. Of the two *B. bovis* positive cases, parasites were found on blood smears in one while parasites were found in capillaries of the brain impression smear in the other. Some samples were positive for particular parasites in more than one test.

Table 2-4: The occurrence of 24 confirmed TBD infections from 137 cattle with clinical signs identified in the Mnisi Community Programme area as determined by examination of blood and lymph node biopsy smears by light microscopy, histopathology examination and *T. parva*-specific qPCR assay.

Dip Tank	**Microscopy**				**Histopathology**	**qPCR**	**Cases**
	A. marginale	***B. bovis***	**Piroplasms**	**Schizonts**	**Theileriosis**	***T. parva***	
Athol		1					**1**
Buffelshoek					1		**1**
Burlington			1				**1**
Clare A		1			1		**2**
Gottenburg			1				**1**
Hlalakahle			2	8	2	7	**9**
Islington			1			1	**2**
Seville B	1			1		1	**2**
Thlavekisa					1	1	**1**
Welverdiend B	2		1	2	1	2	**4**
Total positive	**3**	**2**	**6**	**11**	**6**	**12**	**24**

2.3.4 Reverse line blot hybridization

DNA extracted from blood samples from all 137 clinical cases were examined using the RLB hybridization assay. Representative RLB results, showing hybridization of amplicons from clinical samples with the group-, genus- and species-specific probes, are shown in Figure 2-5.

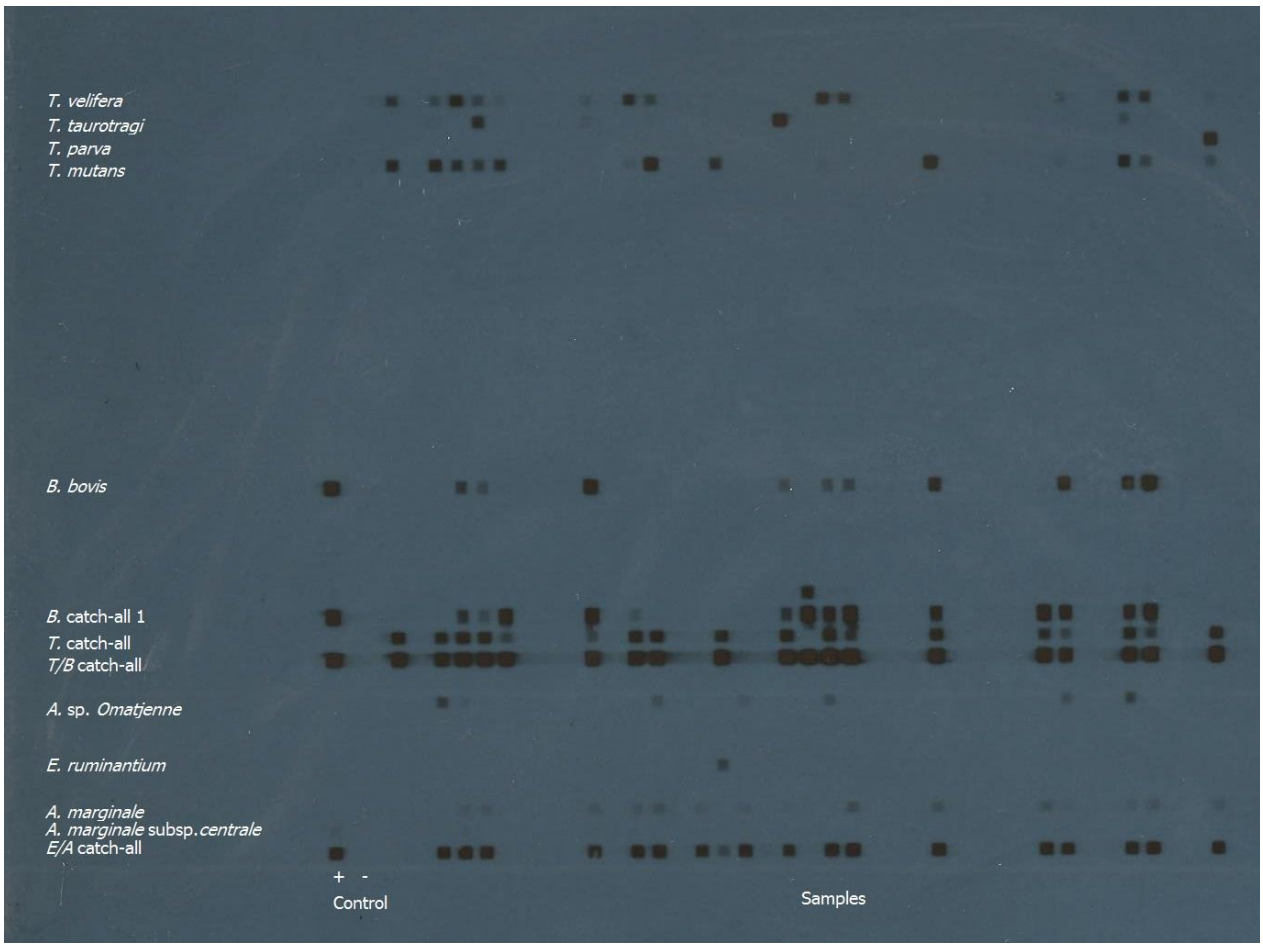

Figure 2-5: Analysis of *Theileria, Babesia, Anaplasma* and *Ehrlichia* PCR products, amplified from genomic DNA extracted from infected blood or lymph node samples, using the RLB hybridization assay. Probes were loaded in horizontal lanes and samples were loaded in vertical lanes. *E/A* catch-all: *Ehrlichia/Anaplasma* group-specific probe; *T/B* catch-all: *Theileria/Babesia* group-specific probe; *T* catch-all: *Theileria* genus-specific probe; *B* catch-all: *Babesia* genus-specific probe.

Positive RLB results for samples from all 137 clinical cases are presented in Figure 2-6 and the RLB raw data are shown in Appendix 5. Of the 137 clinical cases, 89 (65.0%) were found to be positive for one or more haemoparasite species (*Theileria, Babesia, Anaplasma* and/or *Ehrlichia*) and 48 (35.0%) were negative or below the detection limit of the test. One sample (0.7%) hybridized to the *Theileria/Babesia* group-specific probe only and not to any of the species-specific probes, as compared to 17 samples (12.4%) that hybridized only to the *Ehrlichia/Anaplasma* group-specific probe.

Six *Theileria*, four *Anaplasma*, two *Babesia*, and one *Ehrlichia* species were identified. In decreasing order of occurrence, the *Theileria* parasites identified were *T. velifera* (35.8%), *T.*

mutans (29.2%), *T. taurotragi* (11.7%), *T. parva* (10.2%) and *Theileria* sp. (sable) (7.3%). *Babesia bovis* (27.7%) was detected but no *B. bigemina* positive samples were identified. A few samples were positive for *B. rossi* (1.5%). *Anaplasma* parasites identified were *A. marginale* (35.0%), *Anaplasma* sp. *Omatjenne* (6.6%), *A. bovis* (2.2%) and *A. marginale* subsp. *centrale* (0.7%). *Ehrlichia ruminantium* was detected in 5.8% of the samples. Many samples were RLB-positive for the causative agents of TBDs although for most of these, no specific diagnoses of TBD were made at the time of sampling.

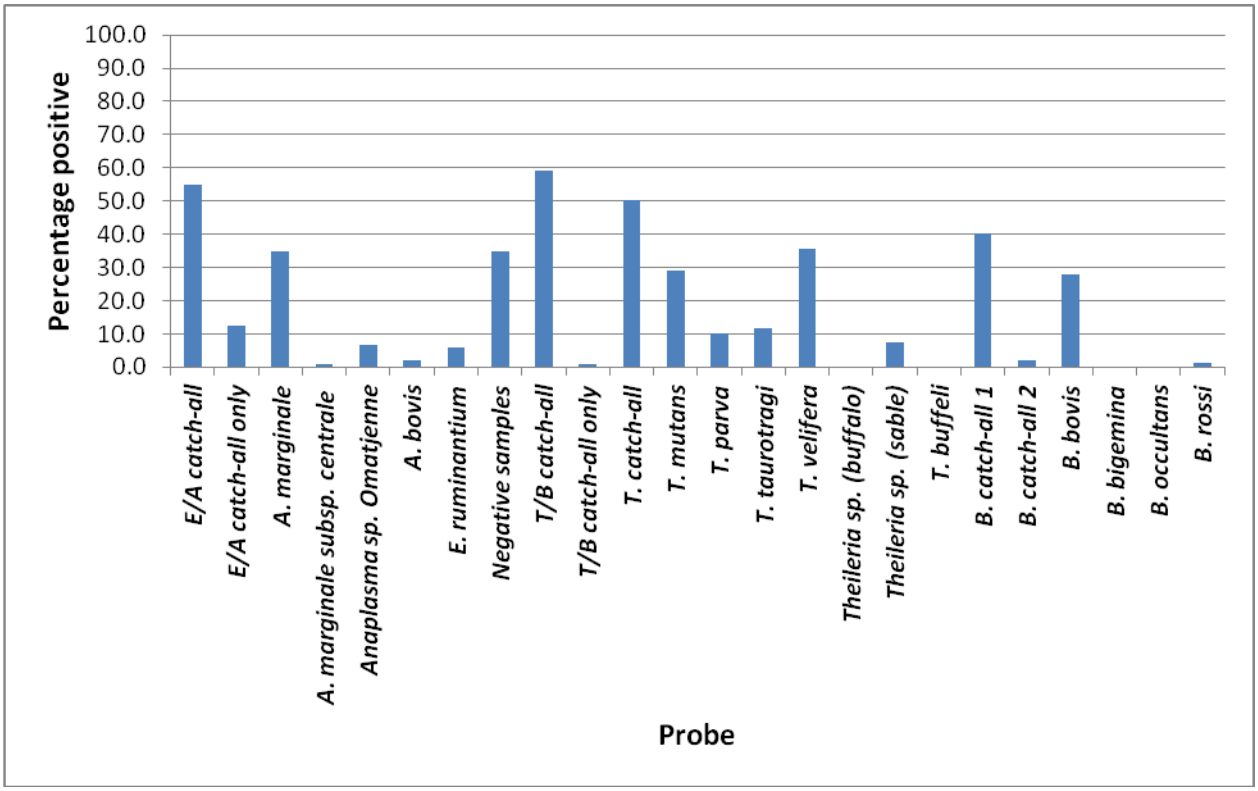

Figure 2-6: The occurrence of *Theileria/Babesia* and *Ehrlichia/Anaplasma* species in 137 cattle samples (clinical cases) from Mnisi community and surrounding areas as determined by the RLB hybridization assay. *T/B* catch-all: *Theileria/Babesia* group-specific probe; *E/A* catch-all: *Ehrlichia/Anaplasma* group-specific probe.

DNA extracted from blood samples from 14 (10.2%) of the clinical cases tested positive for *T. parva* using the RLB assay. Ten of these were also *T. parva* qPCR-positive (Table 2-3). Sample C10, the case of cerebral babesiosis, was also positive for *T. parva* on RLB, but the qPCR was not done. Three samples that tested positive for *T. parva* on RLB (C3, C82 & C91) were not among the 24 samples that were diagnosed with TBDs. Samples C3, C10, C82 and C91 may therefore have come from cattle that may have been *T. parva* carriers. Two samples that had

tested positive using the *T. parva*-specific qPCR (C108 and C109) did not test *T. parva*-positive using RLB.

2.3.5 Confirmed Corridor disease cases

The map below (Figure 2-7) shows the spatial distribution of the 12 *T. parva* cases confirmed using *T. parva*-specific qPCR plus the one *T. parva* case diagnosed only by histopathology. All the Corridor disease cases were from dip tanks located close to the game fences indicating that the infections may have come from the natural reservoir, the African buffalo.

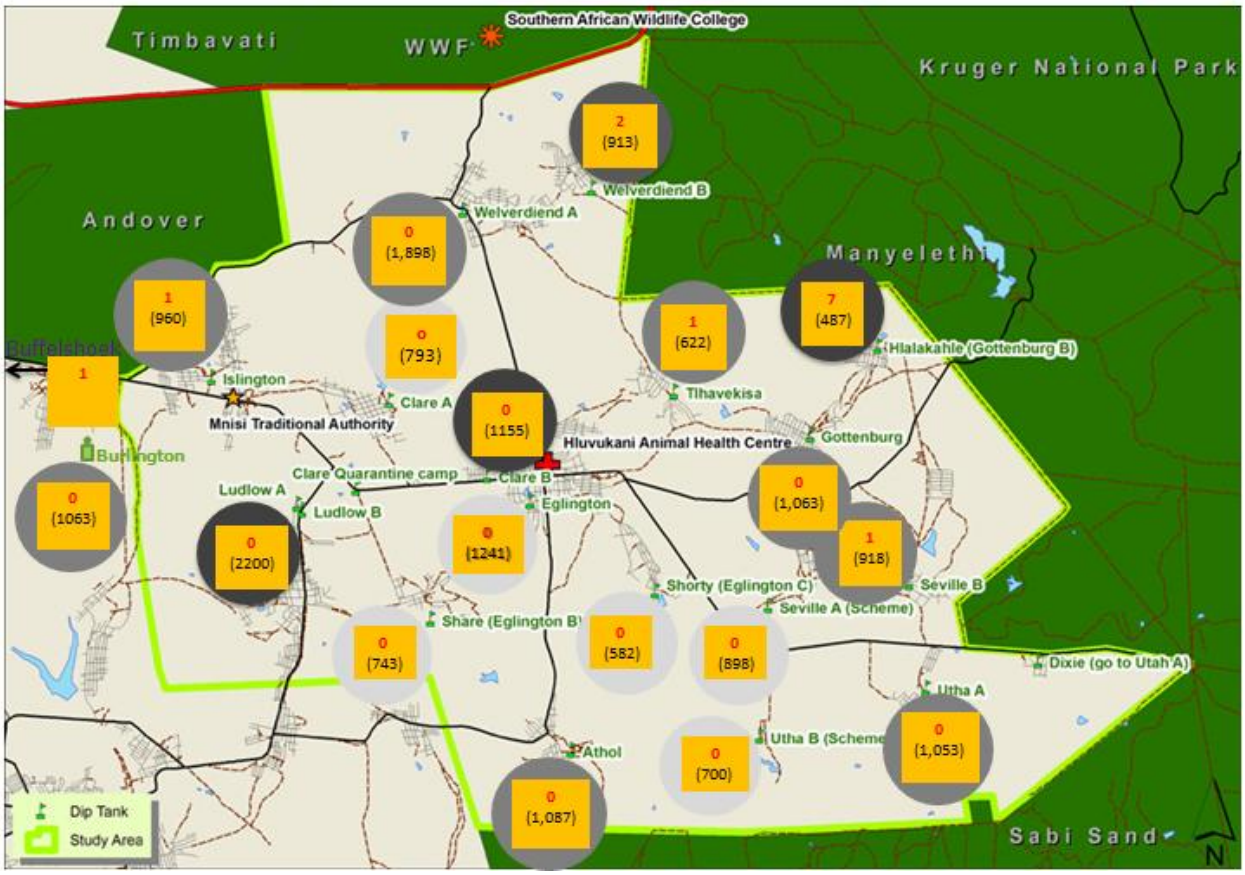

Figure 2-7: Spatial distribution of clinical cases. Homesteads are indicated by matrices while the remaining areas are either arable lands or grazing areas. More than 10 clinical cases were identified at four dip tanks (deep grey circles) while at eight, 6-10 clinical cases were identified (medium grey circles). The light grey circles represent the six dip tanks where fewer than 5 clinical cases were identified. The top figure in the orange rectangles indicates the number of *T. parva* cases confirmed by qPCR, as well as the one positive *T. parva* case from Buffelshoek confirmed by histopathology, while the bottom figure in brackets indicates the cattle population at each dip tank where these data were available.

The Corridor disease cases were experienced during mid to late summer (Figure 2-8) when the adult tick vectors are known to emerge (Norval *et al.*, 1992).

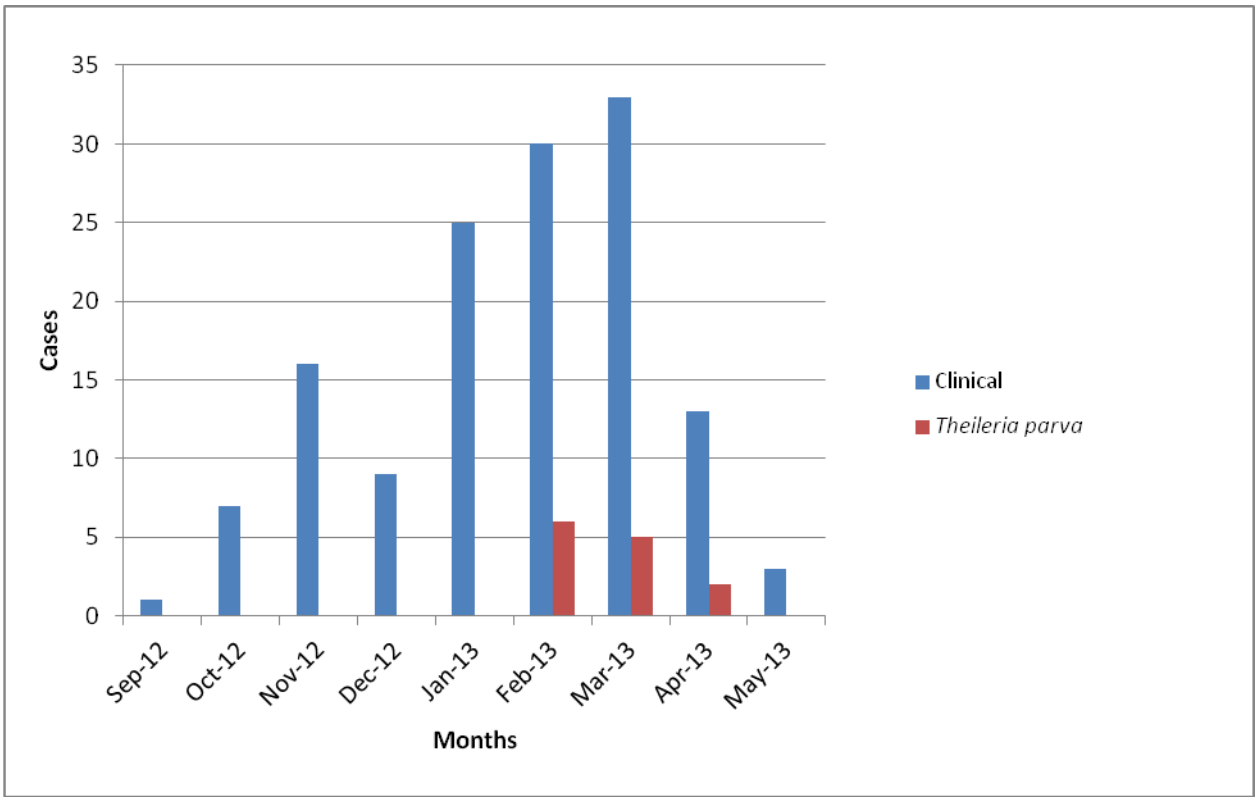

Figure 2-8: The temporal distribution of the 12 *Theileria parva* cases confirmed by *T. parva*-specific qPCR plus one diagnosed by histopathology examination in relation to the clinical cases seen during the study period in Mnisi Community Programme area.

2.4 Discussion

In South Africa, anaplasmosis, babesiosis and heartwater are amongst the most important constraints to improved productivity and health of cattle (Mtshali *et al*., 2004), while Corridor disease is a notifiable disease. Of all cattle mortalities in South Africa, 18% are caused by the TBDs babesiosis, heartwater and anaplasmosis (De Waal, 2000). A total of 137 cattle showing symptoms that could be attributed to TBDs were sampled in the Mnisi Community Programme (MCP) area during the one year study period. More clinical cases could have been handled, but due to the poor awareness of the farmers on animal management and health, sick animals were often left behind when the rest were taken to the dip tanks for inspection, and many clinical cases were therefore not examined. Microscopic examinations of both blood and lymph node biopsy smears combined with histopathology and qPCR results confirmed the presence of TBDs in cattle in the Mnisi community and surrounding area. In addition, parasites that cause babesiosis, heartwater, anaplasmosis and Corridor disease were detected by RLB hybridisation in the samples that were collected and examined in the study.

Of the 137 clinical cases seen, 26 (18.0%) died. Mortalities attributed to TBDs were identified in this study, but the number of confirmed TBD cases was low. The actual percentage of TBD cases could not be ascertained accurately because some deaths were reported too late and the carcasses had decomposed too much to allow a conclusive examination, or else the carcasses had already been disposed of. In addition, a number of clinical Corridor disease cases handled were not confirmed with the more sensitive tests because it was not always possible for the state veterinarian to send the samples for either histopathological or qPCR diagnosis and the cost of these tests was a prohibiting factor. Not all of the deaths were as a result of TBDs, as the clinical signs observed in the 137 cattle examined in this study were often non-specific and could be attributable to other diseases. Furthermore, a number of cases had no conclusive diagnosis and the real cause of death could not be ascertained. Other diseases which may have accounted for death or clinical signs in cattle in the area include brucellosis, tuberculosis and lumpy skin disease, which have been detected in research projects done by other University of Pretoria students in the area. Of the clinical cases examined, 24 (17.5%) could be attributed to TBDs (anaplasmosis, babesiosis or theileriosis) by microscopy, histopathology, post-mortem examination, or *T. parva*-specific qPCR assay at the ARC-OVI. By contrast, the RLB hybridization assay revealed that most cattle sampled were infected with one or more haemoparasite, notably *A. marginale* and *B. bovis*. The scarcity of clinical disease, together with the high levels of infection of cattle with *A. marginale* and *B. bovis*, suggest endemic

stability of bovine anaplasmosis and babesiosis in the MCP area. By contrast, the relatively large numbers of Corridor disease cases, coupled with a lack of *T. parva* infection in cattle that were not diagnosed with the disease, suggest endemic instability of Corridor disease in the area.

Anaplasmosis appears to be endemic in most of the cattle-farming areas in southern Africa (Krystynak, 1986). The presence of bovine anaplasmosis in the MCP area was confirmed by the presence of *A. marginale* in blood smears from three cases. The RLB assay results also confirmed the presence of *Anaplasma* spp. in many of the samples (35.0% *A. marginale*, 6.6% *Anaplasma* sp. Omatjenne, 2.2% *A. bovis* and 0.7% *A. marginale* subsp. *centrale*). Development of naturally-acquired immunity occurs in cattle reared in endemic areas (Neitz & Alexander, 1945). It is probable that the MCP area is an endemically stable area as very few cattle had severe clinical disease even though vector tick burden was high and *A. marginale* was detected in 35.0% of the samples. In South Africa, the one-host tick, *R. (B.) decoloratus* has been incriminated, together with four other ticks, *R. (B.) microplus, R. simus, R. evertsi evertsi* and *H. marginatum rufipes*, as being the vector for *A. marginale* (Bigalke *et al*. 1976; Potgieter, 1981). The presence of *Rhipicephalus* (*Boophilus*) tick vectors in the study area (Hein Stoltsz, University of Pretoria, personal communication) thus also suggests the occurrence of anaplasmosis in the area. A variety of African game species such as giraffe (*Giraffa camelopardalis*) (Brooklesby & Vidler, 1966), sable antelope (*Hippotragus niger*) (Thomas *et al.*, 1982), African buffalo (*Syncerus caffer*) and black wildebeest (*Connochaetes gnou*) (Potgieter, 1989) have been shown to harbour *Anaplasma* spp. The game species could therefore be a source of infection in the MCP area, as many cattle graze at the livestock/wildlife interface.

Babesia bovis was first reported in southern Africa in 1941 (Neitz, 1941). It probably came with the Asian blue tick *R. (B) microplus* introduced in the late nineteenth century. *Babesia bigemina*, an endemic disease in the African subcontinent, is transmitted by both *R. (B) microplus* and *R. (B) decoloratus* (Potgieter, 1977). Distribution of *B. bovis* is restricted to areas where *R. (B) microplus* is prevalent and this includes the Eastern Cape, KwaZulu-Natal and Mpumalanga provinces of South Africa. The two bovine babesiosis cases identified on smear examination and 27.7% RLB assay positive samples indicate the presence of *B. bovis* in MCP area. The tick vectors are present in the MCP area (Hein Stoltsz, University of Pretoria, personal communication). No *B. bigemina* cases were identified by either microscopy or RLB. This could be as a result of the absence of the parasite in the area or the parasites were present

at levels that were undetectable by both tests. Alternatively, it is also possible that the RLB may not detect all field strains of *B. bigemina* in South Africa as it has previously been observed that few or no *B. bigemina* positive samples are detected by RLB in cattle from areas where this parasite is expected to be present (Milana Troskie, University of Pretoria, personal communication). The actual occurrence of babesiosis in the MCP area may have been underestimated as farmers frequently treat their animals with babesiacides to which they have access (personal observation of farmers), often within 24 hours of showing symptoms. This would result in the degeneration of the parasites and it would not be possible to detect them using microscopy or even the more sensitive RLB. The use of blood of general circulation was avoided in making blood smears as such specimens contain up to 20 times fewer *B. bovis* than capillary blood (Callow *et al*. 1993). This was however unavoidable when collecting whole blood in vaccutainer tubes for DNA extraction.

Heartwater occurs wherever the vector ticks (ticks of the genus *Amblyomma*) are present. *Amblyomma variegatum* and *A. hebraeum* are the two major vectors in Africa, and *A. hebraeum* is the main vector of heartwater in southern Africa (Maillard & Maillard, 1998). It has previously been reported that mortalities from heartwater in endemic areas of South Africa are three-fold higher than babesiosis and anaplasmosis combined (Neitz, 1968; Uilenberg, 1983). It is therefore a major obstacle to the introduction of high-producing animals in sub-Saharan Africa to upgrade local stocks. However, no heartwater cases were seen among clinically ill cattle in the MCP area. This result may have been due to the lack of awareness by the farmers of the need to have post-mortem examination done on all cattle that die. The diagnosis of heartwater is made by examining a brain smear by light microscopy, taken on necropsy. A number of *E. ruminantium*-positive samples (5.8%) were detected on RLB hybridization assay, indicating the presence of this haemoparasite in the MCP area. It was noted that cattle in the study area have suffered udder damage due to infestation by ticks with long mouth pieces such as *A. hebraeum*, the vector of heartwater (personal observation). Sick cattle were usually treated with antibiotics upon presumptive diagnosis. This therefore means cases of heartwater may have been treated even without definitive diagnosis. Since animals that have recovered from infection are typically immune to re-infection for six months to four years but may be carriers of the disease for eight months or longer (Bell-Sakyi *et al*., 2004), *E. ruminantium* is probably circulating in the MCP area. Through personal observations and discussions with local state veterinarians, *E. ruminantium* is a major problem in goats in the MCP area, especially exotic breeds that have been introduced into the area for breeding.

Corridor disease is the most important theilerial infection posing a threat to cattle farmers in South Africa today, since the eradication of ECF in the 1950s (Stoltsz, 1989). Periodic Corridor disease outbreaks occur on farms bordering the game reserves where infected buffalo (natural reservoir host) and the tick vector, *R. appendiculatus*, are present. This includes the Kruger National Park which borders the study site (Bigalke *et al*., 1976). The seasonal appearance of the various stages of the tick vector influences the seasonality of outbreaks (Brocklesby *et al*., 1966). This was reflected in the cases that were seen during the study. All Corridor disease cases diagnosed occurred later in the summer (February to April), a period which coincides with the emergence of the adult *R. appendiculatus* ticks (Norval *et al.*, 1992). The dip tanks that were affected were those closer to the game fences (Figure 2-8). The farmers interviewed revealed that Corridor disease cases were most often seen in herds that occasionally found their way into the game reserves whenever fences were down. Other positive cases were from herds that grazed in areas where buffalo had strayed when they broke out of the game reserves.

Out of the 24 cases of TBDs diagnosed in the study area, 19 were *Theileria*-related. More than 80% of cattle in susceptible herds die of Corridor disease (Lawrence *et al*., 2004a). This was evident in cases that were handled during the study. Ten of the twelve Corridor disease cases confirmed by qPCR were post-mortem cases and the two that were still alive when a diagnosis was made, as well as the case identified as Corridor disease by histopathology, were earmarked for slaughter at the advice of the veterinarian. Necropsies of the Corridor disease cases handled reflected classical Corridor disease findings, such as congested lungs and trachea, which would cause laboured breathing while the animal was still alive. Another element that was noted was the short period between the time the animal was first identified with symptoms and the time of death. Few schizonts and scanty piroplasms were seen in blood smears even with prolonged searches. All 19 samples were taken from animals that had high probability of contact with African buffalo and exhibited typical Corridor disease symptoms.

Out of the 19 *Theileria* related cases, 12 were confirmed as *T. parva*-positive using *T. parva*-specific qPCR while one was shown to be *T. taurotragi*-positive on histopathology. A *T. parva*-specific qPCR result was not obtained for the remaining six cases. Histopathology findings indicated that one of these cases was Corridor disease although both microscopy and RLB were negative. Schizonts and piroplasms were seen in blood and lymph smears from the other five cases but these samples were *T. parva* negative on RLB assay. The parasite life stages seen in these five cases could be attributed to other *Theileria* spp. detected by RLB

(these samples were RLB-positive for *T. taurotragi*, *T. mutans* and/or *T. velifera*) as other *Theileria* spp. have piroplasm and schizont stages in their life cycles (El Sawalhy, 1999). Piroplasms and schizonts of the different *Theileria* spp. are difficult to differentiate solely on the basis of their morphology, and this is even more complicated if mixed infections occur (Morzaria, 1989). However, it also is possible that all five could have been Corridor disease cases, since Corridor disease was confirmed histopathologically in at least one case where *T. parva* could not be detected by RLB, and, in addition, in two cases which were *T. parva*-qPCR positive, RLB was not able to detect the infection. The qPCR has previously been reported to be more sensitive than the RLB assay in detecting *T. parva*; the analytical sensitivity of the qPCR assay for the detection of *T. parva* was determined as 100% at parasitaemia of $8.79x10^{-4}$% (Sibeko *et al*., 2008). The type of sample collected for analysis also has an impact on the diagnosis of *T. parva*. The DNA extracted from some blood samples was found to be negative for *T. parva* on qPCR and RLB while DNA from lymph node tissue from the same animal was positive. This could have been as a result of the stage of the infection at the time of sampling. Schizonts in the lymphatic system appear earlier after an animal is infected as compared to piroplasms in the blood stream, and the negative RLB results were therefore probably due to very low parasitaemia in the blood samples.

More parasites were identified by the RLB hybridization assay than by microscopy, and both pathogenic and nonpathogenic haemoparasites could be detected and distinguished by the RLB. The detection of large numbers of haemoparasites in the clinical samples was expected since the RLB is much more sensitive than microscopy and allows specific identification of parasites present (Calder *et al*., 1996; Reghu *et al*., 2006). The sensitivity of the RLB assay enables the detection of carrier state in cattle (Gubbels *et al*., 1999) and RLB results therefore do not necessarily indicate that the animals that were positive were clinically sick from the infection. Other haemoparasites that were detected by RLB included *T. taurotragi*, *T. mutans*, *T. velifera*, *Theileria* sp. (sable), *B. rossi* and *Anaplasma* sp. Omatjenne. Except for *B. rossi* all of these haemoparasites are known to occur in cattle and usually do not cause disease. We expected to identify many of these haemoparasites since they share tick vectors with parasites that cause TBDs such as babesiosis, anaplasmosis and heartwater (Dreyer *et al*., 1998; Mbati *et al*., 2002), known to occur in the MCP area. The *B. rossi* positive finding could be due to an incidental infection (when a non-host is accidentally infected with a parasite, it is usually eliminated, but can sometimes remain in the incidental host for a short while, but does not cause disease), or due to cross-reaction of the RLB probe with a previously unknown target. The clinical signs shown by animals which tested RLB-negative may have been due to other

diseases with symptoms similar to those of TBDs.

The RLB hybridization assay yielded 10.2% *T. parva* positive samples. Among these, three samples were *T. parva* positive on RLB but the parasites were not detected on microscopy. The first case was a cow which was not seen sick but was found dead in the kraal the following morning. At post-mortem examination it was observed that it had slightly enlarged superficial lymphnodes and the respiratory system was slightly congested. The second case was a cow which was only lethargic while the third one had diarrhoea, depressed appetite and was anaemic. No definitive diagnoses were arrived upon for these three cases. The samples were therefore not sent for further *T. parva* tests. Upon follow up, it was noted that the two surviving cattle had been slaughtered. Since the RLB was positive, it is possible that these cattle were infected with *T. parva*. In addition, although all 13 cattle that were diagnosed with Corridor disease died or were slaughtered, two of the five cases in which piroplasms and schizonts were observed but Corridor disease could not be confirmed, survived. If these cattle were misdiagnosed, they could have become reservoirs for *T. parva*. Our results suggest that it is possible that cattle infected with *T. parva* could be missed during a Corridor disease outbreak, and this could result in cattle that test positive for *T. parva* as shown by Yusufmia *et al*. (2010) and Mbizeni *et al*. (2013). If any of these animals developed a high enough piroplasm parasitaemia, it is possible that they could pass the infection to the tick vector. The possibility of the presence of *T. parva* carrier cattle and cattle-to-cattle transmission of *T. parva* parasites in South Africa cannot be ruled out.

With regard to *Ehrlichia* and *Anaplasma* infections alone, the RLB results revealed that there were more single infections (33.6%) than mixed infections (6.6%) while in the case of *Theileria* and *Babesia* infections alone, mixed infections were more common (38.7%) than the single infections (11.7%). Out of the 48 *A. marginale* positive samples and 38 *B. bovis* positive samples, 20 of them had mixed *A. marginale*/*B. bovis* infections; we would expect to detect many mixed *A. marginale* and *B. bovis* infections, since these haemoparasites share a common tick vector.

The RLB hybridization assay yielded more *A. marginale* and *B. bovis* positive samples than microscopy as RLB is more sensitive than microscopic examination (Calder *et al*., 1996; Reghu *et al*., 2006). Some of these may have been carriers of the parasite, but others may indeed have had clinical symptoms of bovine anaplasmosis or bovine babesiosis. Since a number of cattle owners have some knowledge of treating their cattle, it was learnt that they

would give blanket treatment with oxytetracycline to their cattle at the first indication of any clinical sign of disease. Some farmers would only ask for help from the state veterinarian or Hluvukani Animal Clinic after their own treatment had failed. This practice has a negative impact on diagnosis especially with the use of microscopy as some parasites would degrade due to the prior treatment.

The study revealed that tick-borne pathogens are common in cattle in the Mnisi community and surrounding area and cases of anaplasmosis, babesiosis and Corridor disease were identified. Although the physical damage the ticks cause to the animal was not part of the study, it was observed that this was a big problem as well. This was seen from the number of cows that had abscesses and sores on the skin and especially the udders causing severe damage to the teats (personal observation). Results from samples analysed using RLB hybridization show that only 35% of the cattle were negative for tick-borne parasites. Among the TBDs confirmed by the tests done on the samples were anaplasmosis and babesiosis which are two of the TBDs of economic significance in the communal areas of South Africa (Dreyer *et al*., 1998, Mbati *et al.,* 2002). However, clinical disease was rare (only three cases of anaplasmosis and two of babesiosis were confirmed), despite the high incidence of *A. marginale* and *B. bovis* infection revealed by RLB in the cattle sampled, suggesting endemic stability of bovine anaplasmosis and babesiosis in the MCP area. Management practices in the area, such as weekly dipping and treatment with tetracycline on presenting with a fever, may contribute to endemic stability of TBDs in the MCP area. The conditions in the area, including the presence of vector ticks and high incidence of infection, are such that calves are exposed to a regular challenge at a young age, when the disease is often mild, resulting in functional immunity to challenge. Cattle are dipped regularly, reducing the tick challenge but not removing it completely, and adult cattle that present with a fever are usually treated with antibiotics allowing them to survive the infection and boosting their immunity. Most important of the TBDs detected in cattle during field diagnosis in the Mnisi community was Corridor disease, a notifiable disease, which had the highest number of cases confirmed (13/24) among the cattle that were sampled during the research period. *Theileria parva* was detected by RLB in very few cattle that were not diagnosed with Corridor disease. Therefore, in contrast to anaplasmosis and babesiosis, there is endemic instability of Corridor disease in the MCP area. The cattle in the area are therefore naïve to *T. parva* infection and they are highly susceptible to the disease when the parasite is transmitted from buffalo to cattle.

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CHAPTER 3

IDENTIFICATION OF *THEILERIA PARVA* POSITIVE CATTLE FROM NORTH-EASTERN BUSHBUCKRIDGE MUNICIPALITY

3.1 Introduction

Ticks transmit various pathogenic microorganisms from infected cattle to healthy ones (Jongejan *et al.,* 2007). Babesiosis, anaplasmosis and heartwater are the tick-borne diseases of economic significance in the communal areas of South Africa (Dreyer *et al.,* 1998; Mbati *et al.,* 2002) while Corridor disease, caused by *Theileria parva*, is a notifiable disease.

Theileria parva, an apicomplexan protozoan, is transmitted by three natural tick vectors: *Rhipicephalus appendiculatus, R. zambeziensis* and *R. duttoni* (Norval e*t al.,* 1992). The parasite is strictly transmitted transstadially. The African buffalo (*Syncerus caffer*) is the natural reservoir of *T. parva* and is also a carrier of the relatively benign *T. mutans* and the apathogenic *T. velifera*, which also infect cattle. The *T. buffeli*/*T. orientalis*/*T. sergenti* complex is another group of relatively benign organisms that can cause disease and loss of production in cattle under certain circumstances (Norval *et al.,* 1992). *Theileria taurotragi*, which infects eland (*Taurotragus oryx*), can also infect cattle and has been implicated in cases of cerebral theileriosis in South Africa (De Vos, 1982).

Theileria parva is the causative agent of East Coast fever (ECF), Corridor disease and January disease. The parasite occurs in parts of east, central and southern Africa (Norval *et al.,* 1992). Previously, the organisms which caused the different disease syndromes were distinguished as subspecies of *T. parva*: *Theileria parva parva* was considered the causative agent of ECF and *T. p. bovis* of January disease while *T. p. lawrencei* was considered to be the cause of Corridor disease (Uilenberg 1981, Lawrence 1979). The lack of biological evidence for discrimination of the subspecies led to the discarding of the trinomial naming system and the parasites are now referred to as “cattle-derived” or “buffalo-derived” according to their host of origin (Perry & Young, 1993). Thus, ECF and January disease result from cattle-to-cattle transmission of cattle-derived *T. parva* while Corridor disease results from buffalo-to-cattle

transmission of buffalo-derived *T. parva* (Neitz, 1957; Koch *et al.* 1988).

The parasites of the *T. parva*-group, which are morphologically and serologically indistinguishable (Burridge *et al.*, 1974; Lawrence, 1979), remain a major constraint to the development of the livestock industry of many developing African countries (Dolan, 1989). Corridor disease, the most important theilerial infection in South Africa since the eradication of ECF in the mid-1950s, continues to pose a threat to cattle farmers (Stoltsz, 1989).

Although there are similarities in the pathogenesis and pathology of Corridor disease and ECF, there is a distinct difference in that Corridor disease cases exhibit low schizont parasitoses and piroplasm parasitaemias. Corridor disease is considered a self-limiting disease as parasites are only transmitted directly from buffalo to cattle: infected cattle usually die before the parasite reaches the tick-infective stage, the piroplasm (Neitz, 1955, Neitz *et al.*, 1955).

Corridor disease occurs where cattle graze in veld that has been used by infected African buffalo and the tick vector is present, which results in buffalo-to-cattle transmission. Stoltsz (1989) states that this is the reason there is strict control of movement of infected buffalo from infected game reserves by veterinary authorities to avoid contact with naïve South African cattle. Over the many decades since ECF was eradicated from South Africa, sporadic outbreaks of Corridor disease have occurred in cattle that have come into contact with infected buffalo within the distribution range of the vector ticks (Stoltsz, 2011). Cattle infected by buffalo-derived *T. parva* can recover after treatment by chemotherapy and can be carriers of the parasite (Potgieter *et al*., 1988) because none of the drugs have proven effective in sterilizing *T. parva* infections in carrier cattle, even in combination (Stoltsz, 1989; Potgieter *et al*., 1988). It is therefore prohibited to use chemotherapy to control Corridor disease in South Africa because of the risks of establishing carrier states in cattle (Potgieter *et al*., 1985). Although a carrier state is not thought to occur naturally (Neitz *et al*., 1955), recent reports suggest that *T. parva* DNA is detectable in cattle at the livestock/wildlife interface in South Africa (Yusufmia *et al*., 2010; Mbizeni *et al*., 2013). In South Africa, the adaptation and maintenance of buffalo-derived *T. parva* parasites in cattle is of great concern. It is thought that ECF was finally eradicated from South Africa in the 1950s once the carrier cattle were eradicated. In the event that cattle can become *T. parva* carriers, as suggested by the findings of Sibeko (2009), Yusufmia *et al*. (2010), Sibeko *et al*. (2011) and Mbizeni *et al*. (2013), then cattle-to-cattle transmission and the selection of cattle-adapted *T. parva* might become a reality. This study was therefore initiated to see if *T. parva* carrier cattle could be identified in

the MCP area, and if so, to look for evidence for selection of cattle-adapted types.

A number of diagnostic techniques can be employed to determine if an animal is infected with *T. parva*. Investigating the animal's history and analyzing its clinical signs are of great importance. The clinical signs include enlargement of several lymph nodes especially those draining the head, difficulty breathing, frothing, diarrhoea and development of corneal opacity, sometimes resulting in blindness. The clinical signs can then be confirmed by microscopy (Norval *et al.*, 1992). Microscopy involves the visualization of the schizonts in the early stages of the disease and in the later stages, piroplasms can be seen. This is demonstrated in blood smears from cattle that have been in contact with buffalo or where contact is presumed to have occurred (Lawrence *et al.*, 2004a). The parasites of the *T. parva*-group are however morphologically indistinguishable (Burridge *et al*., 1974; Lawrence, 1979). Infection with buffalo-derived *T. parva* can also be demonstrated by tick transmission (xenodiagnosis) but this method is impractical on a large scale (Lawrence *et al*., 2004b).

Theileria parva infection is also detectable retrospectively through demonstration of antibody titres using serological techniques. Capillary tube agglutination (CA) (Ross & Lohr, 1972), conglutination (Cawdery *et al.*, 1968), immunofluorescent antibody test (IFAT) (Burridge, 1971; Lawrence *et al.*, 2004a), indirect hemagglutination assay (IHA) (Duffus & Wagner, 1974) and enzyme-linked immunosorbent assay (ELISA) (Lawrence *et al*., 2004a; Katende *et al.*, 1998) are some of the serological tests that have been developed. The IFAT uses cultured *T. parva* schizont-infected lymphoblasts as antigen (Allsopp *et al.*, 1985). Although the IFAT is still widely used, it has the disadvantage of being subjective in interpretation and lacks specificity as it cross-reacts with other *Theileria* species, mostly *T. taurotragi* (Burridge *et al.*, 1974). The cross-reaction occurs when antibody titres are low in samples with *T. parva/ T. taurotragi* mixed infections (De Vos, 1982; Norval *et al*., 1992; Lawrence *et al*., 2004b). The *T. parva*-specific antibody detection ELISA is based on the use of a recombinant polymorphic immunodominant molecule (PIM) and is the serological test of choice (Katende *et al.*, 1998; Toye *et al.*, 1991). Although the ELISA is highly sensitive and does not cross-react with *T. taurotragi*, *T. mutans*, *T. annulata* or *T. buffeli* (Burridge *et. al.*, 1973), the IFAT is still the most widely used test in many countries.

Due to the difficulty of detecting *T. parva* in carrier cattle, a number of PCR techniques have been developed that use *T. parva*-specific primers based on single copy genes (Toye *et al.*, 1991; Iams *et al.*, 1990). *Theileria parva* DNA has been successfully amplified from experimentally

infected animals (Bishop *et al*., 1992; Watt *et al*., 1998). Since more sensitive and specific diagnostic tests are required to detect low level infections or carrier state, *T. parva* PCR amplification of the *Theileria* 18S ribosomal RNA (rRNA) gene followed by probing with radioactively labelled species-specific oligonucleotide probes was developed (Allsopp *et al*., 1993). Subsequently, a sensitive and specific quantitative real-time PCR (qPCR) for the detection of *T. parva* in cattle and buffalo was developed (Sibeko *et al*., 2008). It should be noted however, that since the *Theileria* sp. (buffalo) 18S rRNA gene sequence is very similar to that of *T. parva,* an amplification product is generated from *Theileria* sp. (buffalo) DNA, but the test still remains specific for *T. parva* as only a *T. parva*-specific melting curve is generated (Sibeko *et al*., 2008).

The aim of Chapter 3 was therefore to identify *T. parva* carrier cattle by (1) testing cattle from herds with previously confirmed or suspected cases of Corridor disease over the past three years using IFAT to identify seropositive herds, and then (2) testing the *T. parva* seropositive herds and herds which had Corridor disease cases during the study period using the *T. parva*-specific qPCR to identify *T. parva* positive cattle.

3.2 Materials and Methods

3.2.1 Sampling strategy

Since the presence of subclinical *T. parva* carrier state is expected to be low in cattle, having previously been measured at 6.7% and less in cattle at the livestock/wildlife interface in KwaZulu-Natal (Yusufmia *et al*., 2010; Mbizeni *et al*., 2013), sample collection was targeted at herds that would offer a higher chance of identifying more positive cases, i.e. those with suspected and/or confirmed cases of Corridor disease or those that had shared grazing lands with buffalo, in the previous three years. Information on Corridor disease cases in the study area from the three years prior to the study (2009 to 2011) was sourced from the Bushbuckridge State Veterinary office, since Corridor disease is a notifiable disease under Animal Diseases Act No. 35 of 1984. Information on Corridor disease cases was also obtained from the records at the Hluvukani Animal Clinic. Information on cattle sharing grazing lands with buffalo and some of the previous Corridor disease outbreaks was obtained through verbal communication with the local farmers at dip tanks.

3.2.2 Study area

The study area, Mnisi Community Programme (MCP) area, is situated in the north-eastern part of Bushbuckridge Municipal Area, Mpumalanga Province, South Africa. This community, located at a wildlife interface, has livestock farming as its main agricultural activity and cattle by far as its most important animals. High host densities together with the sub-tropical climate provide suitable conditions for known vectors of important diseases such as Corridor disease, redwater, heartwater and anaplasmosis in the MCP area. Cattle in the study area were all maintained under traditional husbandry systems. They were grazed in the bush during the day and penned in kraals situated at the homesteads at night. The tick challenge on the cattle varied throughout the year although cattle herds visited dip tanks (Figure 3-1) for dipping and inspection by animal health technicians once a week.

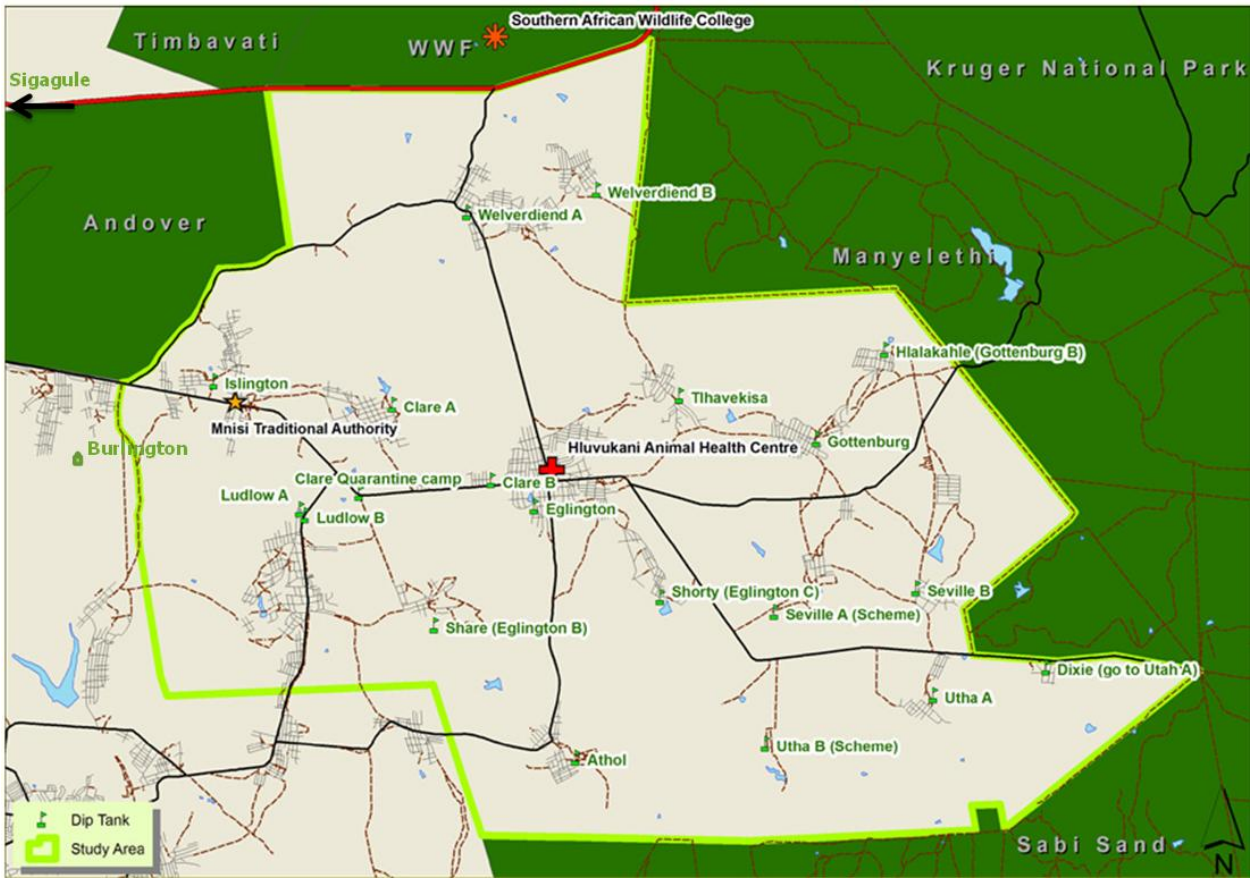

Figure 3-1: Map of the Mnisi Community Programme area showing the location of dip tanks in the study area (http://www.sanparks.org/parks/kruger/conservation/scientific/noticeboard/sciencenetworkmeeting2009/Presentations/kriek.pdf, accessed on 09/09/2015).

3.2.2.1 Sampling sites, collection of blood samples and IFAT to identify seropositive herds

A total of 670 blood samples were collected from cattle in 101 herds at 11 dip tanks (Figure 3-1). Dip tanks chosen for sampling were: Athol (n=33 from 12 herds), Gottenburg (n=90 from 28 herds), Hlalakahle (n=51 from 22 herds), Seville B (n=106 from 4 herds), Share (n=6 from one herd), Welverdiend A (n=44 from one herd), Welverdiend B (n=80 from two herds), Utha A (n=80 from 12 herds) and Utha Scheme (n=60 from three herds). Although they were outside the study area, Sigagule (n=30 from four herds) and Burlington (n=90 from 12 herds) dip tanks were also sampled, as relevant information on past cases of theileriosis in these herds was available from Bushbuckridge State Veterinary office and Hluvukani Animal Clinic. Samples were collected from all cattle in the identified herds that were presented at the dip tank on the day of collection. The samples were collected from apparently healthy cattle from July 2012 to November 2012.

The study received clearance from the University of Pretoria Animal Ethics Committee (Protocol: V047/12). Ethical procedures when collecting samples from the farmers' cattle were followed and a consent form (Appendix 1) was presented to the farmers as described in Chapter Two. Blood was collected under clean conditions in 10 ml plain Vacutainer tubes by jugular or caudal vein puncture. The tubes were labeled using a pencil (animal identity, location and date). In addition to this information, the record sheet included more information such as age and sex of the animal. After collection, the blood samples were stored in cool boxes until they were transported to the laboratory at Hans Hoheisen Wildlife Research Station (HHWRS) with the necessary movement permits (Appendix 2). The blood samples were allowed to stand overnight at room temperature and centrifuged the following day at 2,500 rpm for five minutes, in readiness to separate the serum. The serum was pipetted into 2 ml serum vials and stored in a household freezer (-20°C).

A transport permit (Appendix 6), for the movement of the sera to Pretoria, was issued by the State Veterinarian Orpen-Bushbuckridge North which indicated that the sera be sent first to the Agricultural Research Council-Transboundary Animal Diseases (ARC-TAD) laboratories for heat inactivation. After the heat inactivation, the sera were sent to ARC-Onderstepoort Veterinary Institute (ARC-OVI) laboratories for serological diagnosis using IFAT. The IFAT was performed at two different dilutions, 1:80 and 1:160. The Statistical Package for Social Sciences (SPSS) was used to analyse the results and descriptive statistics used to determine the prevalence and 95% confidence intervals. The chi-square test was employed to compare the IFAT results between sexes and among the age groups.

3.2.2.2 Collection of blood samples for qPCR screening and DNA extraction

The IFAT results were used to identify seropositive herds, which were revisited to collect further blood samples. Blood samples were also collected from herds that may have shared grazing grounds with African buffalo and herds that had confirmed *T. parva* cases during the study period (Chapter Two). A total of 432 samples were collected from apparently healthy cattle from 17 herds in 11 dip tanks (Figure 3-1). Dip tanks chosen for sampling were: Athol (n=8), Burlington (n=49), Clare A (n=41), Hlalakahle (n=94), Islington (n=15), Seville B (n=29), Thlavekisa (n=19), Utha A (n=17) and Utha Scheme (n=9), Welverdiend A (n=50) and Welverdiend B (n=111). Samples were collected from all cattle in the herds identified that were presented at the dip-tank on the day of collection.

Ethical procedures when collecting samples from the farmers' cattle were followed and a consent form (Appendix 1) was presented to the farmers as described in Chapter Two. Blood was collected from the cattle in 10 ml EDTA Vacutainer tubes by jugular or caudal vein puncture. Labelling, care and storage of blood samples was done as described in Chapter Two. The necessary permits for movement of samples from Hluvukani Animal Clinic to HHWRS were also obtained as in Chapter Two (Appendix 2).

DNA was extracted from the 432 blood samples at HHWRS from 200 µl of each blood sample using the QIAamp® DNA Mini Kit (Qiagen, Whitehead Scientific, South Africa). Extraction of DNA was done according to the method described by the manufacturer and as described in Chapter Two. The DNA was eluted in 100 µl of elution buffer and stored at -20°C until it was transported to the University of Pretoria, with the necessary permit (Appendix 3).

3.2.2.3 Screening of DNA for *T. parva* using *T. parva*-specific qPCR

A *T. parva*-specific quantitative real-time PCR (qPCR) hybridization assay (Sibeko *et al*., 2008) was used to screen the DNA samples. The primers and probes used are given in Table 3-1.

Table 3-1: *Theileria parva* probes and primer sequences for the *T. parva*-specific qPCR assay.

Probe / Primer name	**Sequence (5'-3')**
T.parva Sensor 640	LC Red640 – TCG GAC GGA GTT CGC T – PH
T.parva Anchor	GGG TCT CTG CAT GTG GCT TAT – FL
Parva-F (*T. parva*-specific forward primer)	CTG CAT CGC TGT GTC CCT T
Theileria-R (*Theileria* genus-specific reverse primer)	ACC AAC AAA ATA GAA CCA AAG TC

The reaction was performed in a 20 µl volume with a final concentration of 2x LightCycler FastStart DNA Master^{Plus} Hybridization Probes mix[1] (Roche Diagnostics, Mannheim, Germany), 0.5 µM of each of the primers (*Parva*-F *T. parva*-specific forward primer and *Theileria*-R *Theileria* genus-specific reverse primer), 0.1 µM of each of the hybridization probes, 1U uracil deoxy-glycosylate (UDG) (Roche Diagnostics, Mannheim, Germany) and 2 µl of template DNA. Thermal cycling was performed in a LightCycler® v2 (Roche Diagnostics, Mannheim, Germany) and consisted of 40°C for 10 minutes (initial activation of UDG), 95°C for 10 minutes (activation of FastStart *Taq* DNA polymerase), followed by 45 cycles of 95°C for 10 seconds (denaturing), 58°C for 10 seconds (primer annealing) and 72°C for 15 seconds (product extension). To analyse the melting curve, the reactions were heated from 40°C to 95°C at a heating rate of 0.2°C per second. Measurement of the fluorescence values was done at 640 nm (*T. parva*-specific signal). For each run, a positive control containing *T. parva* Muguga DNA was included, and a negative control containing water instead of a DNA template was used.

[1] 10X LightCycler FastStart DNA Master^{Plus} Hybridization Probes mix contains FastStart *Taq* DNA polymerase, reaction buffer, dNTP mix (with dUTP instead of dTTP) and 10 mM $MgCl_2$. The final concentration of $MgCl_2$ in the reaction mix is therefore 2 mM. FastStart *Taq* DNA polymerase is a chemically modified *Taq* DNA polymerase which is inactivated at room temperature and becomes activated at high temperatures (i.e. during the preincubation step at +95°C for 5-10 minutes)

3.3 Results

3.3.1 IFAT results

The seroprevalence of antibodies to *T. parva* infection in herds at dip tanks with a history of previous cases of Corridor disease and in herds that had apparently shared grazing with African buffalo in the MCP area in Mpumalanga province of South Africa was determined by IFAT (Table 3-2). The samples were collected over a period of four months. The samples without any detectable *T. parva* antibodies at any dilution were categorized as negative, while those with detectable fluorescence at a 1:80 or a 1:160 dilution were considered positive. Samples found positive at a 1:80 dilution were further divided into two categories: weakly positive and positive differentiated by the intensity of fluorescence. The highest overall *Theileria* seropositive cases in cattle were recorded in Utha Scheme (78.3%) and Burlington (70.0%) while the lowest were recorded in Share (50.0%) and Welverdiend A (52.3%) (Table 3-2). The other dip tanks had seroprevalences ranging from 52.5% to 66.7% (Table 3-2).

Table 3-2: Detection of bovine antibodies to *T. parva* by IFAT from cattle sampled at selected dip tanks in the Mnisi Community Programme area.

Dip Tank	Negative (%)	Weak Positive 1:80 (%)	Positive 1:80 (%)	Positive 1:160 (%)	Total Positive (%)
Athol (n=33)*	11 (33.3)	7 (21.2)	10 (30.3)	5 (15.2)	22 (66.7)
Burlington (n=90)**	27 (30.0)	18 (20.0)	25 (27.8)	20 (22.2)	63 (70.0)
Gottenburg (n=90)*	31 (34.4)	6 (6.7)	41 (45.6)	12 (13.2)	59 (65.6)
Hlalakahle (n=51)**	19 (37. 3)	14 (27.5)	14 (27. 5)	4 (7.8)	32 (62.8)
Seville B (n=106)*	41 (38.7)	9 (8.5)	53 (50.0)	3 (2.8)	65(61.3)
Share (n=6)*	3 (50.0)	0 (0.0)	3 (50.0)	0 (0.0)	3 (50.0)
Sigagule (n=30)*	11 (36.7)	5 (16.7)	10 (33.3)	4 (13.3)	19 (63.3)
Utha A (n=80)*	29 (36.3)	7 (8.8)	35 (43.8)	9 (11.3)	51 (63.8)
Utha Scheme (n=60)**	13 (21.7)	6 (10.0)	24 (40.0)	17 (28.3)	47 (78.3)
Welverdiend A (n=44)*	21 (47.7)	1 (2.3)	18 (40.9)	4 (9.1)	23 (52.3)
Welverdiend B (n=80)*	38 (47.5)	12 (15.0)	18 (22.5)	12 (15.0)	42 (52.5)
Total (n=670)	**244 (36.4)**	**85 (12.7)**	**251 (37.5)**	**90 (13.4)**	**426 (63.6)**

* Herds with a history of Corridor disease cases

** Herds that had apparently shared grazing with African buffalo

The overall detection of antibodies to *T. parva* was 63.6% (95% confidence [CI]: 57.0-68.2%), suggesting that the seroprevalence of *T. parva* antibodies in cattle from the selected dip tanks in the MCP area was high (Figure 3-2). However, many samples were positive or weakly positive at a 1:80 dilution (37.5%, 95% CI 32.8-44.1% and 12.7%, 95% CI 6.7-18.1%, respectively), while at a 1:160 dilution, 13.4% (95% CI 7.2-18.0%) of the samples were positive (Figure 3-2). Since cross-reactions occur between *T. parva* and other *Theileria* species, especially *T. taurotragi*, when the antibody titres are low (De Vos, 1982; Norval *et al*., 1992; Lawrence *et al*., 2004b), the samples that were positive at a 1:160 dilution are most likely to be the true *T. parva* positives. It is therefore more likely that the seroprevalence of *T. parva* antibodies in cattle from the selected dip tanks in the MCP area was low (13.4%, 95% CI: 7.2-18.0%).

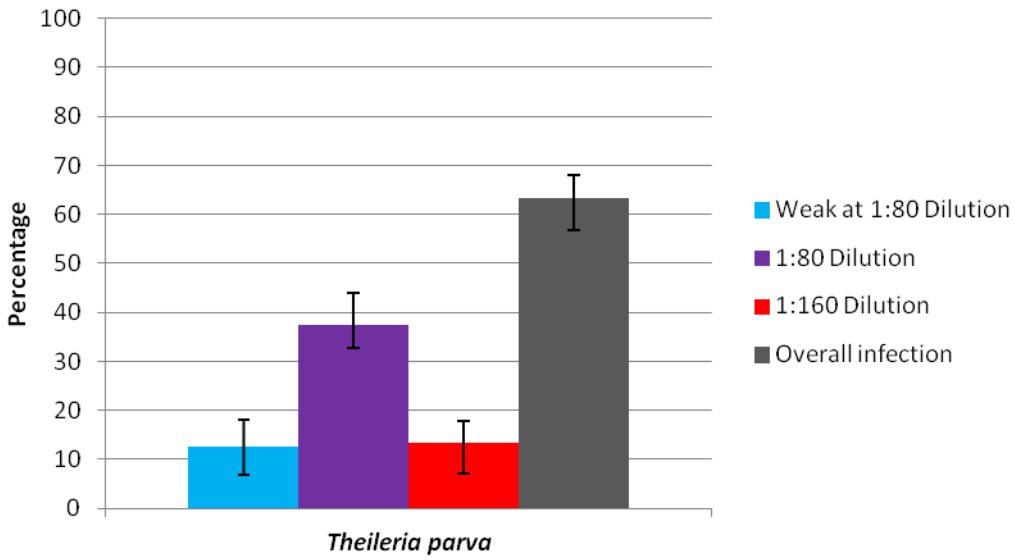

Figure 3-2: Serological detection of bovine antibodies to *T. parva* as determined by IFAT from herds at selected dip tanks in the Mnisi Community Programme area. The bars indicate 95% confidence intervals.

The prevalence of antibodies to *T. parva* in female and male cattle and among different age groups in herds from selected dip tanks in the MCP area are shown in Table 3-3. There was no significant difference in the prevalence of antibodies to *T. parva* between female and male cattle ($p>0.05$). The prevalence of antibodies to *T. parva* in cattle under 1 year old was significantly lower than the rest of the age groups ($\chi^2=12.202$, $p = 0.007$).

Table 3-3: The seroprevalence of *T. parva* in different sex and age groups of cattle from selected dip tanks in the Mnisi Community Programme area.

Variable	Category	Number of cattle sampled	Number of positive cattle	Percent prevalence (95% confidence interval)	χ^2 - value	p-value
Sex	**Female**	570	363	63.7 (56.8-67.9)	0.017	0.911
	Male	100	63	63.0 (56.0-71.4)		
Age group	**<1 year**	87	41	47.1 (37.4-58.0)	12.202	0.007*
	1-2 years	239	156	65.3 (58.5-76.7)		
	3-4 years	162	105	64.8 (50.5-73.0)		
	>4 years	182	124	68.1 (60.7-73.4)		

* a significant difference is indicated by $p<0.05$.

3.3.2 *Theileria parva*-specific quantitative real-time polymerase chain reaction (qPCR) results

DNA extracted from a total of 432 blood samples collected from seropositive herds, herds that may have shared grazing grounds with African buffalo and herds that had confirmed Corridor disease cases during the study period were screened using the *T. parva*-specific qPCR (Sibeko *et al.*, 2008). A positive qPCR result was indicated by an increase in fluorescence as PCR products accumulated during the reaction (Figure 3-3). The presence of *T. parva* was confirmed by melting curve analysis which yielded *T. parva*-specific melting peaks at 63±0.62°C in all samples for which increased fluorescence was observed (Figure 3-4). The melting curve analysis revealed small shoulder peaks at 51.0 – 55.0°C as well as the main peak at 63±0.62°C (Figure 3-4); these shoulder peaks have previously been observed in *T. parva* positive samples (Sibeko *et al.*, 2008).

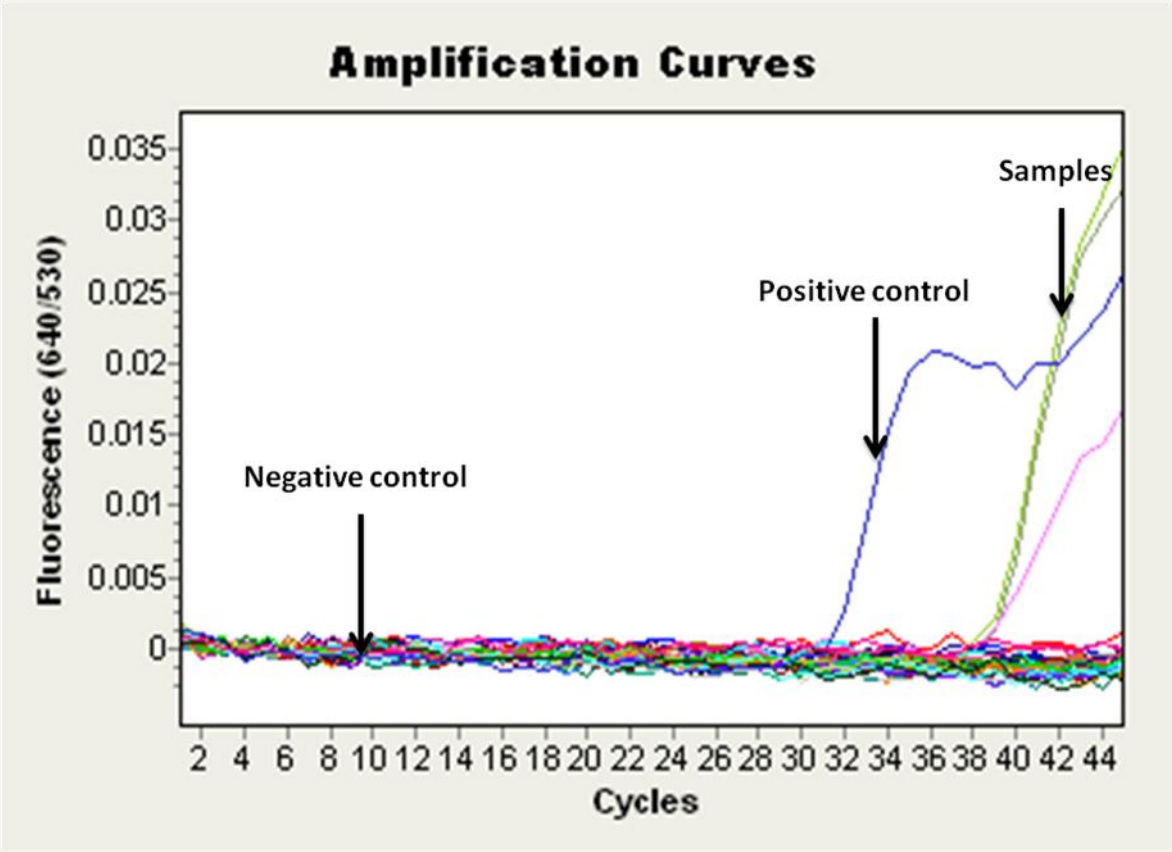

Figure 3-3: Representative amplification curves showing an increase in fluorescence at 640 nm in *T. parva* positive samples from the study area.

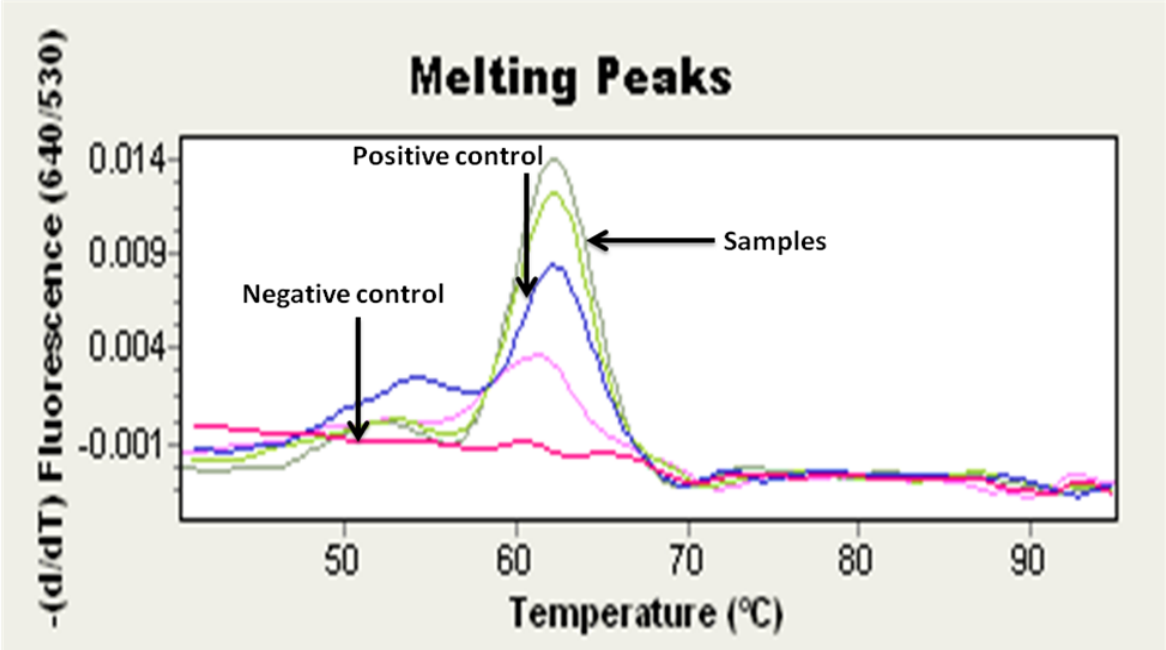

Figure 3-4: Melting curve analysis at 640 nm, showing the *T. parva*-specific melting peak at 63±0.62°C for four positive *T. parva* DNA samples examined.

Theileria parva DNA was detected in 11 out of the 432 samples (2.6 %) using the *T. parva*-specific qPCR assay (Table 3-4). Positive results are indicated by the Cp value (the crossing point), which is the cycle at which fluorescence from amplification exceeds the background fluorescence. A lower Cp correlates with a higher starting concentration of target DNA in a sample. Most of the Cp values were greater than 30 with one exception, (F369, with a Cp of 27.0), indicating a very low parasitaemia in most samples (Table 3-4).

Mbizeni *et al*. (2013) recently reported *T. parva* carrier state (as indicated by qPCR) in cattle from KwaZulu-Natal, but the carrier state was short-lived, lasting only 30-50 days post-infection. The 11 qPCR-positive cattle were therefore revisited after six months, but only eight animals could be located on the follow-up visit (Table 3-4). Of these, only one was still qPCR-positive, but with a very high Cp value (36.4).

Table 3-4: Field samples that tested positive using the *T. parva*-specific qPCR assay. The final two columns show qPCR results of the eight cattle that were resampled after six months.

Sample no.		Dip tank	Collection Date	Cp[a](640/530)	Collection Date	Cp(640/530)
1	**F 25**	Welverdiend A	08.04.2013	34.13	13.11.2013	–[c]
2	**F 79**	Welverdiend B	10.05.2013	34.57	14.11.2013	36.4
3	**F 238**	Hlalakahle	09.05.2013	>40	15.11.2013	–
4	**F 246**	Hlalakahle	09.05.2013	37.24	15.11.2013	–
5	**F 268**	Hlalakahle	09.05.2013	36.77	15.11.2013	–
6	**F 287**	Hlalakahle	03.04.2013	37.08	15.11.2013	–
7	**F 300**	Hlalakahle	03.04.2013	31.69	ND[b]	ND
8	**F 312**	Hlalakahle	09.05.2013	37.14	ND	ND
9	**F 328**	Seville B	08.05.2013	36.03	13.11.2013	–
10	**F 369**	Islington	10.04.2013	27.01	ND	ND
11	**F 376**	Islington	10.04.2013	33.83	13.11.2013	–

[a] Cp = The crossing point, or the cycle at which fluorescence from amplification exceeds the background. A lower Cp correlates with a higher starting concentration of target DNA in a sample.

[b] ND = Not done

[c] – = Negative

3.3.3 Spatial distribution of *T. parva* positive cattle

The spatial distribution of cattle with *T. parva* antibodies as determined by IFAT from the dip tanks that were selected for sampling in the MCP area is shown in the map below (Figure 3-5). The data indicates that exposure to *T. parva* (or related *Theileria* species) is widespread in cattle at the dip tanks examined which were almost all at the livestock/wildlife interface. Samples that were positive at a 1:160 dilution (most likely to be true *T. parva* positives) were found at all dip tanks sampled, except Share. The map also shows the location of apparently healthy cattle that were shown to be *T. parva*-positive by qPCR; these were found at dip tanks in the northern part of the MCP area (Figure 3-5).

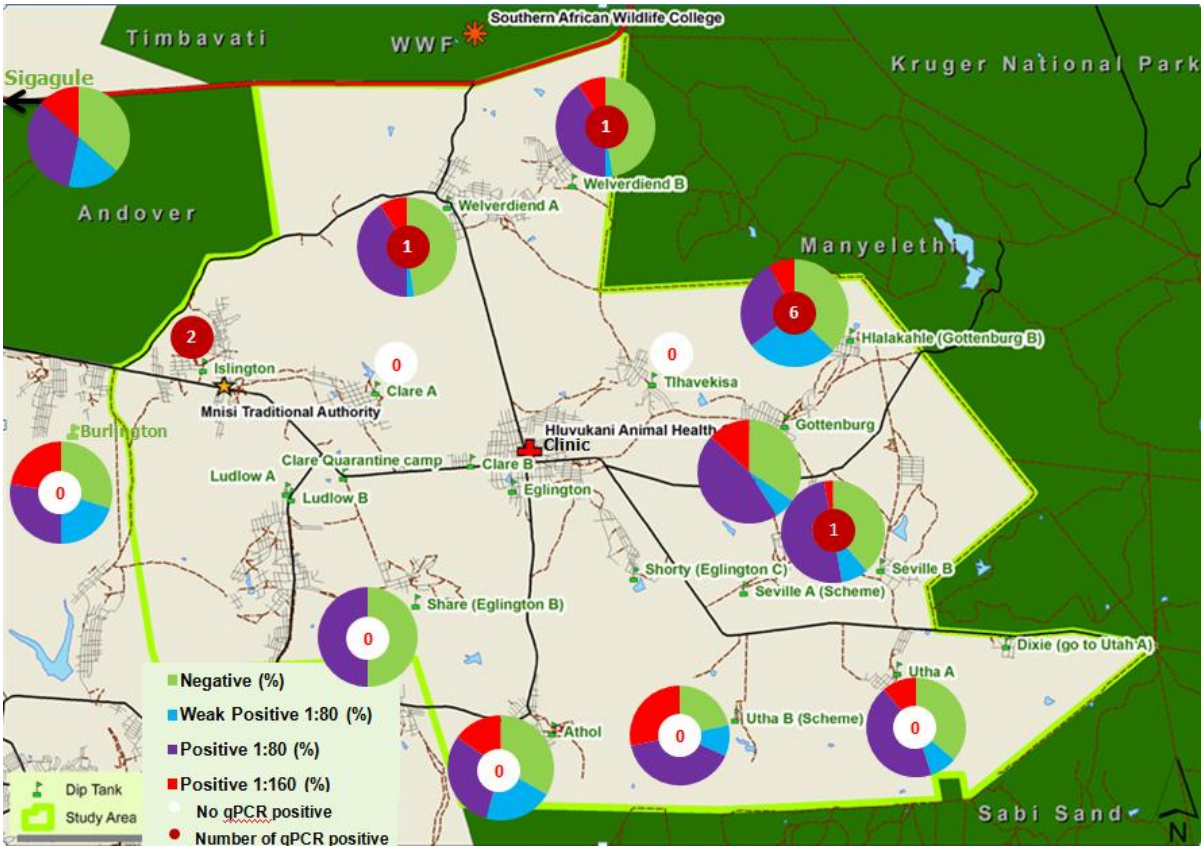

Figure 3-5: Map of the Mnisi Community Programme area (http//web.up.ac.za) showing seroprevalence of *T. parva* antibodies in cattle sampled from selected dip tanks (pie charts). Colours in square blocks in the key refer to IFAT results in the pie charts. The number of *T. parva* qPCR-positive samples from apparently healthy cattle at dip tanks is indicated in brown circles inside the pie-charts, while dip tanks at which no cattle tested positive by qPCR are indicated by white circles. There were no previous cases of Corridor disease or information on cattle sharing grazing grounds with African buffalo available for Islington, Clare A and Tlhavekisa dip tanks at the time seroprevalence sampling was done.

3.4 Discussion

The study conducted in the MCP area used IFAT and qPCR for the identification of *T. parva* carrier cattle. The IFAT, which is widely used to determine the seroprevalence of *T. parva*, and the *T. parva*-specific qPCR, which detects parasite DNA, were used to analyse blood samples from apparently healthy cattle which may have been exposed to *T. parva* infections in the three years prior to the study. The results from both the IFAT and qPCR suggest the presence of subclinical *T. parva* carrier cattle in MCP area, albeit at a very low prevalence. Levine (1973), Schwabe *et al*. (1977), Maritim *et al*. (1989) and Young *et al*. (1986) defined *T. parva* carrier state as the ability of animals recovered from *T. parva* infection to infect vector ticks which subsequently transmit the parasite to susceptible hosts. Mbizeni *et al*. (2013) were not able to transmit the infection from the *T. parva*-positive cattle they identified. Whether the *T. parva*-positive cattle identified in our study are true carriers or not is thus debatable, but the identification of *T. parva* positive cattle in South Africa remains a concern.

The IFAT has been an essential tool that has contributed to an understanding of the epidemiological situation throughout Africa where *T. parva* is endemic (Norval *et al.*, 1992). The test is based on detection of antibodies using cultured *T. parva* schizont-infected lymphoblasts as the antigen (Lawrence *et al.*, 2004b; http://www.oie.int/international-standard-setting/terrestrial-manual/access-online/, accessed on 09/09/2015). The study of *T. parva* seroprevalence in the MCP area described here is the first intensive survey, to our knowledge, involving the serological diagnostic test in cattle at this wildlife interface. The study, conducted from July to November 2012, suggests that exposure to *T. parva* or related species is widespread in cattle at the dip tanks examined, most of which were close to the wildlife interface. These dip tanks were chosen for sampling because of a history of Corridor disease cases or because of exposure of the cattle to African buffalo. These results were therefore expected as the cattle at those particular dip tanks were at a higher risk of interacting with African buffalo and wild reservoirs of other *Theileria* spp. in the game parks and private game reserves that border the MCP area. The *T. parva* seroprevalence in cattle at these dip tanks is therefore probably not representative of all cattle in the MCP area, and a more comprehensive study would need to be done to determine the *T. parva* seroprevalence across the whole region.

In the Eastern Province of Zambia, the diagnostic accuracy of IFAT is said to be affected by epidemic periods during which the majority of cattle are infected and *T. parva* seroprevalence closely follows the buildup of herd immunity, while during endemic periods when disease

incidence is seasonal, *T. parva* seroprevalence fluctuations are considerable even though animals have been infected (Billiouw *et al.,* 2005). Therefore, the vector abundance, and consequently *T. parva* challenge, in the MCP area may affect the IFAT results since sensitivity to the IFAT varies with the presence and intensity of the *T. parva* transmission (Billiouw *et al.,* 2005). One of the factors that leads to such variation is the strict seasonal occurrence of different stages of the *R. appendiculatus* vector ticks (Norval *et al.,* 1992; Maritim *et al.,* 1989). The samples in this study were collected immediately after the activity period of the adult *R. appendiculatus* which is in mid-summer to the end of summer, January to April/May (Walker *et al*., 2000). This meant that the cattle that were infected during the summer would have developed the antibodies during the time the samples were collected. The IFAT results of the study are therefore highly likely from infections acquired in the 2011/2012 summer season, as *T. parva* antibody titres develop 14-28 days after the infection (Norval e*t al.,* 1992) and are detectable in animals for periods of up to six months after infection, in the absence of re-challenge (Burridge & Kimber 1973).

The IFAT detected *T. parva* antibodies in 63.6% of samples, but this seemingly high seroprevalence may be as a result of the varying degrees of serological cross-reactions that occur with other *Theileria* spp., especially *T. taurotragi* (De Vos, 1982; Stoltsz, 1989). Several lines of evidence suggest that *T. taurotragi* is present in the MCP area. The area is within the distribution range for both *R. appendiculatus* and *R. zambeziensis*, two of the known vectors of *T. taurotragi* (Young *et al.,* 1977; Lawrence *et al.,* 1983). *Rhipicephalus appendiculatus* and *R. zambeziensis* are also the vectors of *T. parva* which is known to occur in the MCP area (Chapter Two). Furthermore, at least one clinical case was attributed to *T. taurotragi* infection, and *T. taurotragi* DNA was detected using the RLB hybridization assay in 11.7% of clinical cases (Chapter Two). Thus, the apparent high *T. parva* seroprevalence in the study area may be due to cross-reactions between *T. parva* and *T. taurotragi.* The true *T. parva* seroprevalence is thus probably most accurately reflected by the highest dilution (1:160) indicating a low seroprevalence of 13.4%.

The detection of *T. parva* in 11 of the 432 DNA samples (2.6%) using the *T. parva*-specific qPCR assay suggests that not all cattle that were infected with *T. parva* died from Corridor disease. Cattle in the study area may not have received an infective dose, or they may have recovered from Corridor disease either naturally or due to the early interventions that farmers take to treat their animals. It was observed that farmers in the MCP area stock antibiotics, especially tetracycline, which they use whenever their cattle get sick. Tetracycline is used to control *T. parva* in the infection and treatment method of immunization (Norval e*t al.,* 1992).

Farmers in most cases called in the veterinary officials only when the treatments they tried did not succeed.

The scenario in this study is similar to the study by Mbizeni *et al.* (2013). In their study in Tokazi, uPhogolo-Mkuze area of South Africa, 54 cattle were sampled two months after a Corridor disease outbreak, and tested for *T. parva* parasites using the *T. parva*-specific qPCR. Of these, one animal tested positive (1.9%). Although they do not mention if this animal had also tested positive during the outbreak, their study shows that some cattle can test positive on qPCR as late as 103 days after the first detection of *T. parva*. The Cp value for the animal in question was very high (Abdalla Latif, ARC-OVI, personal communication). In our study, the amplification curves of all the *T. parva* positive samples, except one (sample F 369, Cp value 27.01), started rising after the 30th cycle. High Cp values in the qPCR are an indication of very low parasitaemias in the cattle.

Mbizeni *et al.* (2013) found that the carrier state as detected by qPCR is short-lived with very low parasitaemia which is undetectable within a period of 30 to 50 days after first detection. Our findings were similar, with only one out of the eight cattle resampled after six months still qPCR positive. Although there was no follow-up during the intervening six months, it is likely that the infection was the same one that had been detected earlier because the parasitaemia had declined from the initial results (as indicated by the Cp value which rose from 34.57 to 36.40). Mbizeni *et al.* (2013) fed nymphs on five recovered cattle, which became PCR-negative 30-50 days after they first tested positive, but the adult ticks did not transmit *T. parva* to naïve cattle, although *T. taurotragi* was transmitted. It is probable that transmission of *T. parva* did not occur in their study because the parasitaemia was very low in the recovered cattle such that the nymphs could not ingest sufficient parasites to cause disease when they were fed as adults on susceptible cattle.

Transformation of buffalo-derived *T. parva* to cattle-derived *T. parva* was first demonstrated by Barnett and Brocklesby (1966) in East Africa after serial tick passage of the parasite in cattle, but Potgieter *et al.* (1988) could not transform a South African buffalo-derived *T. parva* stock into a form of the parasite that could be transmitted between cattle through serial passages. Moll *et al.* (1984; 1986) suggested that transformation of buffalo-adapted *T. parva* would occur where there is continuous contact between cattle and buffalo where all stages of the vector tick, *R. appendiculatus*, are present throughout the year and where many cattle are carriers of buffalo-derived *T. parva*. This may be one of the reasons Corridor disease appears not to have transformed to ECF to date in southern Africa, unlike what is suspected to have happened in East

Africa (Barnett & Brocklesby, 1966; Young & Purnell, 1973; Maritim *et al*., 1989). With the seasonality of the vector ticks in South Africa, Corridor disease cases occur only when the adult ticks are active in the summer months of January to March. By the time the nymphs emerge in the dry months of July to October (Norval e*t al.*, 1992) our results and those of Mbizeni *et al*. (2013) suggest that it is unlikely that cattle infected the previous summer have sufficiently high *T. parva* parasitaemias to infect the nymphs. Therefore it is most unlikely that cattle-to-cattle transmission will occur under the present conditions in South Africa. However, global warming and climate change could alter the status quo. The South African veterinary authorities should be aware that *T. parva* carrier cattle do occur and future climate changes could result in climatic conditions conducive to all tick stages being present throughout the year, which could eventually lead to cattle-to-cattle transmission of *T. parva* in South Africa. It is therefore important to maintain and scale up the already existing measures to prevent the increasing number of Corridor disease outbreaks (Mbizeni *et al.*, 2013) to avoid the emergence of subclinical *T. parva* carrier cattle in South Africa and the chance of transformation in the future.

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CHAPTER 4

CHARACTERIZATION OF THE p67, p104 AND POLYMORPHIC IMMUNODOMINANT MOLECULE (PIM) GENES OF *T. PARVA*-POSITIVE CATTLE FROM NORTH-EASTERN BUSHBUCKRIDGE, MPUMALANGA

4.1 Introduction

Theileria parva is the causative agent of East Coast fever (ECF) Corridor disease and January disease which occur in east, central and southern Africa. The parasites of the *T. parva*-group, which are morphologically and serologically indistinguishable, remain a major constraint to the development of the livestock industry of many developing African countries (Dolan, 1989). Corridor disease, the most important theilerial infection in South Africa since the eradication of ECF by the mid-1950s, continues to pose a threat to cattle farmers (Stoltsz, 1989). In Chapter Three, we identified 11 samples from apparently healthy cattle in the Mnisi Community Programme (MCP) area, north-eastern Bushbuckridge, Mpumalanga, that were infected with *T. parva* (2.5%). Similar cases of subclinical *T. parva* carrier cattle have been reported in other recent studies in South Africa (Yusufmia *et al*., 2010; Mbizeni *et al*., 2013). The presence of *T. parva* positive cattle is a concern, since the selection of cattle-adapted *T. parva* could become a possibility resulting in cattle-to-cattle transmission

In order to identify cattle-adapted forms of *T. parva*, it is necessary to distinguish between cattle-derived and buffalo-derived *T. parva* isolates. Several authors have attempted to distinguish between buffalo-derived and cattle-derived *T. parva* parasites. Among genes with discriminatory sequence differences between *T. parva* isolates are the sporozoite antigen gene, p67, the polymorphic immunodominant molecule (PIM) gene which has been extensively characterised (Baylis *et al.*, 1993; Toye *et al.*, 1996; Geysen *et al*., 2004), and the p104 and p150 genes (Geysen *et al*., 1999; Bishop *et al*., 2001).

The characterisation of p67 gene sequences in East Africa revealed that a 129 bp deletion in the central region of the gene was characteristic of cattle-derived *T. parva* isolates (allele 1) while no deletion was observed in buffalo-derived isolates (allele 2) (Nene *et al.*, 1992; Nene

et al., 1996). However, both p67 alleles were obtained in South African buffalo from the Kruger National Park, although it was not established whether the strain containing the p67 allele with a deletion could cause ECF (Collins, 1997). Together with two additional p67 allele variants (alleles 3 and 4), both East African p67 alleles were identified in other buffalo samples in South Africa (Sibeko *et al.*, 2010). Of concern, the p67 allele 1, which is characteristic of cattle-derived *T. parva* isolates in East Africa, was identified in a sample from a bovine that tested *T. parva* positive on a farm near Ladysmith in the KwaZulu-Natal Province of South Africa (Sibeko *et al*., 2010).

Geysen *et al.* (1999) used restriction fragment length polymorphism analysis of PCR products (PCR-RFLP) to characterise the p104 gene of buffalo-derived and cattle-derived *T. parva* isolates. Limited polymorphism was found in the p104 gene of cattle-derived *T. parva*, while PCR-RFLP profiles from buffalo-derived *T. parva* were more polymorphic. The findings in the study by Sibeko *et al*. (2011) identified additional *T. parva* p104 alleles in samples from South African buffalo, suggesting that p104 is even more diverse than previously suggested (Geysen *et al*., 1999).

The PIM gene, although already expressed in the sporozoite stage, is expressed predominantly by the schizont stage of the parasite (Shapiro *et al.*, 1987; Toye *et al.*, 1991; Katende *et al.*, 1998; Graham *et al.*, 2007). The polymorphism in the central region of the PIM gene has been exploited for discrimination between *T. parva* stocks (Geysen *et al.*, 1999; Bishop *et al.*, 2001). The PIM PCR-RFLP profiles from buffalo-derived *T. parva* stocks are more polymorphic than those from cattle-derived stocks. The profiles obtained from buffalo stocks are heterogeneous whereas the cattle-derived *T. parva* stocks are often homogeneous (Geysen *et al.*, 1999). The QPEP tetrapeptide repeat in the variable region of the PIM (Toye *et al*., 1995) was found to be characteristic of cattle-derived *T. parva* PIM sequences (Geysen *et al*., 2004). Toye *et al*. (1995) reported a 20-amino acid insert (VDQQQPVQQPSQDQPSGPDS) as characteristic of buffalo-type PIM peptide sequences.

South African *T. parva* samples have recently been characterized using size differentiation and sequence variation of the p67, p104 and PIM *T. parva* antigen genes (Sibeko *et al.*, 2010; 2011). Among the samples characterized from an outbreak of theileriosis in Ladysmith (Thompson *et al*., 2008), a p67 allele (allele 1) was found, identical to the p67 allele of the ECF-causing Kenyan *T. parva* Muguga strain (Sibeko *et al.*, 2010). In addition, three cattle samples from the Ladysmith farm had almost identical p104 and PIM PCR-RFLP profiles and inferred amino acid

sequences to those of *T. parva* Muguga (Sibeko *et al.*, 2011). These results suggest that *T. parva* parasites with genetic characteristics of cattle-derived parasites can be transmitted from buffalo and might be selected in cattle in South Africa.

The aim of the current study was to characterize selected *T. parva*-positive samples collected from clinically sick cattle and from healthy cattle in the MCP area between September 2012 and May 2013 by sequence analysis of p67, p104 and PIM genes.

4.2 Materials and methods

4.2.1 DNA samples

The qPCR-positive DNA samples (Table 4-1) identified from clinical cases (Chapter 2) and from *T. parva* qPCR-positive carrier cattle (Chapter 3) were used in the characterization.

Table 4-1: List of samples from clinically sick (C) and apparently healthy (F) cattle that were analysed at ARC-OVI and DVTD using qPCR and found positive for *T. parva.*

	Animal ID	Collection Date	Dip Tank	qPCR test done at
1	**C66**	06.02.2013	Hlalakahle	ARC-OVI
2	**C71**	15.02.2013	Hlalakahle	ARC-OVI
3	**C80**	19.02.2013	Welverdiend B	ARC-OVI
4	**C81**	22.02.2013	Thlavekisa	ARC-OVI
5	**C84**	26.02.2013	Islington	ARC-OVI
6	**C89**	01.03.2013	Hlalakahle	ARC-OVI
7	**C108**	12.03.2013	Hlalakahle	ARC-OVI
8	**C109**	12.03.2013	Hlalakahle	ARC-OVI
9	**C110**	13.03.2013	Seville B	ARC-OVI
10	**C123**	02.04.2013	Welverdiend B	ARC-OVI
11	**C129**	09.02.2013	Hlalakahle	ARC-OVI
12	**C133**	26.04.2013	Hlalakahle	ARC-OVI
13	**F 25**	08.04.2013	Welverdiend A	DVTD
14	**F 79**	10.05.2013	Welverdiend B	DVTD
15	**F 238**	09.05.2013	Hlalakahle	DVTD
16	**F 246**	09.05.2013	Hlalakahle	DVTD
17	**F 268**	09.05.2013	Hlalakahle	DVTD
18	**F 287**	03.04.2013	Hlalakahle	DVTD
19	**F 300**	03.04.2013	Hlalakahle	DVTD
20	**F 312**	09.05.2013	Hlalakahle	DVTD
21	**F 328**	08.05.2013	Seville B	DVTD
22	**F 369**	10.04.2013	Islington	DVTD
23	**F 376**	10.04.2013	Islington	DVTD

4.2.2 PCR amplification and purification of amplicons for sequence analysis of the gene encoding p67

A standard PCR was used to amplify the gene encoding p67 (Nene *et al*., 1996) from DNA samples that were positive on *T. parva*-specific qPCR, using oligonucleotide primers IL 613 and IL 792 (Table 4-2). The primers amplify all four p67 alleles (Collins, 1997; Sibeko *et al*., 2010). The reaction was performed in a final volume of 25 µl, containing 1x MyTaq™ Mix[2] (Bioline, Meridian Life Science Company, United Kingdom), 0.16 µM of each primer and 2.5 µl extracted DNA. The amplification programme was as follows: an initial denaturation at 95°C for 10 min; 30 cycles of denaturing at 95°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min; and a final extension at 72°C for 7 min. Each run included a positive control containing *T. parva* Muguga DNA and a negative control containing water instead of a DNA template was used. PCR products were visualized by electrophoresis on a 1.5% agarose gel stained with ethidium bromide. PCR products were purified using a QIAquick PCR Purification Kit (QIAGEN group) and sent to INQABA Biotechnologies (South Africa) for cloning and sequencing.

4.2.3 PCR amplification and purification of amplicons for sequence analysis of the genes encoding p104 and PIM

The genes encoding p104 and the PIM were amplified using a nested PCR protocol as previously described (De Deken *et al.*, 2007).

4.2.3.1 PCR amplification of the p104 gene

In the first round p104 PCR, primers p104 F2 and p104 5 (Table 4-2) were used. The reaction was performed in a final volume of 25 µl, containing 1x MyTaq™ Mix (Bioline, Meridian Life Science Company, United Kingdom), 0.16 µM of each primer and 2.5 µl extracted DNA. The amplification programme was as follows: an initial denaturation at 94°C for 5 min; 35 cycles of denaturing at 94°C for 5 min, annealing at 58°C for 30 sec, extension at 72°C for 1 min; and a final extension at 72°C for 7 min. Reaction conditions for the second round PCR were the same

[2] MyTaq™ Mix is a ready-to-use 2x mix developed for fast and highly-specific PCR. It contains MyTaq DNA polymerase, reaction buffer, dNTPs, $MgCl_2$ and enhancers at proprietary concentrations formulated to deliver the best results.

as those for the first round, except that primers p104 2nF and p104 5 (Table 4-2) were used and 0.5 µl of the first round PCR product was added as template. The amplification conditions were the same as in the first round except the annealing temperature was raised to 60°C and 25 cycles of denaturation, annealing and elongation were done. Each run included a positive control containing *T. parva* Muguga DNA and a negative control containing water instead of a DNA template was used. PCR products were visualized by electrophoresis on a 1.5% agarose gel stained with ethidium bromide. PCR products were purified using a QIAquick PCR Purification Kit (QIAGEN group) and sent to INQABA Biotechnologies (South Africa) for cloning and sequencing.

Table 4-2: Sequences of primers used in the PCR assay for the amplification of the p67 locus and semi-nested PCR assays for the amplification of the p104 and PIM loci of *Theileria parva.*

Primers	Sequence (5'-3')	PCR Round
IL 613	ACAAACACAATCCCAAGTTC	
IL 792	CCTTTACTACGTTGGCG	
p104F2	CCACCATCTCCTAAACCACCGTT	1
p104 2nF	AACCACCGTTTGATCCATCATTCA	2
p104 5	TAAGATGCCGACTATTAATGACACCACAA	1 & 2
PIM1	GTGAATGTTGTGATCTTAATCC	1
PIMFm	ATTCCACTGGTTCTTCCGATSTA	2
PIMR4	CCCACAACCGTGGAATGGCGTA	1 & 2

4.2.3.2 PCR amplification of the PIM gene

In the first round PIM PCR, primers PIM1 and PIMR4 (Table 4-2) were used. The reaction was performed in a final volume of 25 µl, containing 1x MyTaq Mix (Bioline, Meridian Life Science Company, United Kingdom), 0.16 µM of each primer and 2.5 µl extracted DNA. The amplification programme was as follows: an initial denaturation at 94°C for 5 min; 35 cycles of denaturing at 94°C for 5 min, annealing at 60°C for 30 sec, extension at 72°C for 1 min; and a final extension at 72°C for 7 min. Reaction conditions for the second round PCR were the same as those for the first round, except that primers PIMFm and PIMR4 (Table 4-2) were used and 0.5 µl of the first round PCR product was added as template. The amplification conditions were the same as above (p104) except the annealing temperature was raised to 62°C and 25 cycles of

denaturation, annealing and elongation were done. Each run included a positive control containing *T. parva* Muguga DNA and a negative control containing water instead of a DNA template was used. PCR products were visualized by electrophoresis on a 1.5% agarose gel stained with ethidium bromide. PCR products were purified using a QIAquick PCR Purification Kit (QIAGEN group) and sent to INQABA Biotechnologies (South Africa) for cloning and sequencing.

4.2.4 Sequence analysis

To eliminate *Taq*-induced sequence errors, forward and reverse sequences for at least five clones per PCR product were assembled and edited using CLC Main Workbench version 7.0.2. In some cases, different sequences were obtained from different clones from the same product and these were grouped and assembled separately. The consensus of each group of sequences was considered as the sequence(s) for that PCR product from each animal sampled.

4.2.5 Phylogenetic analysis

The resulting consensus sequences of p67, p104 and PIM clone sequences were aligned with published sequences (Table 4-3) as well as sequences obtained from Dr Kgomotso Sibeko (University of Pretoria, personal communication) using ClustalX version 2.1 (Larkin *et al*., 2007). The sequences constituting the alignments were trimmed to equal nucleic acid length using BioEdit Sequence Alignment Editor (Hall, 1999). Prior to performing phylogenetic analysis, the amino acid sequences for each gene were obtained using ExPASy (Expert Protein Analysis System) translation tool (Gasteiger *et al*., 2005). Phylogenetic analysis was done by neighbor-joining, maximum-likelihood and maximum-parsimony methods using MEGA6: Molecular Evolutionary Genetics Analysis version 6.0 (Tamura *et al*., 2013) and phylogenetic trees were generated for p67 and p104 consensus sequences. The trees were generated using a few of the more closely related sequences to the consensus sequences generated in the study. The maximum-likelihood analysis was based on the JTT matrix-based model (Jones *et al*., 1992) while the maximum-parsimony tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm (Nei & Kumar, 2000). All bootstrap consensus trees were inferred from 1,000 replicates. It was not possible to perform phylogenetic analyses for the PIM sequences because the sequences are too polymorphic to allow reliable analysis.

Table 4-3: Previously published p67, p104 and PIM sequences

Isolate name	Accession number and reference
	p67
***T. parva* Muguga**	Allele 1: M67476 (Nene *et al*., 1992)
***T. parva* KNP2**	Allele 1: AF079177 (Nene *et al*., 1999)
***T. parva* Hluhluwe3**	Allele 2: AF079176 (Nene *et al*., 1999)
***T. parva* 7013**	Allele 2: AF079175 (Nene *et al*., 1999)
***T. parva* 7014**	Allele 2: U40703 (Nene *et al*., 1996)
***T. parva* Mar1 49**	Allele 3: JX442244 (Sibeko *et al*., 2010)
***T. parva* Ita8-13**	Allele 3: JX442245 (Sibeko *et al*., 2010)
***T. parva* HIP5 1-16**	Allele 3: JX442246 (Sibeko *et al*., 2010)
***T. parva* KNP102-9**	Allele 3: JX442247 (Sibeko *et al*., 2010)
***T. parva* Wel24 2-5**	Allele 3: JX442248 (Sibeko *et al*., 2010)
***T. parva* KNPW8 35**	Allele 3: JX442249 (Sibeko *et al*., 2010)
***T. parva* KNP102-26**	Allele 4: JX442250 (Sibeko *et al*., 2010)
***T. parva* KNPW8 48**	Allele 4: JX442251 (Sibeko *et al*., 2010)
	p104
***T. parva* Muguga**	Allele 1: M29954 (Iams *et al*., 1990)
***T. parva* Marikebuni**	Allele 2: AY034069 (Skilton *et al*., 2002)
***T. parva* Boleni**	Allele 3: AY034070 (Skilton *et al*., 2002)
***T. parva* 7014**	Allele 4: AY034071 (Skilton *et al*., 2002)
	PIM
***T. parva* Muguga**	L06323 (Baylis *et al*., 1993)

4.3 Results

The dip tanks at which clinical Corridor disease cases and carrier cattle were identified were situated along the immediate livestock/wildlife interface as shown in the map (Figure 4-1) below (see Chapters 2 & 3 for details).

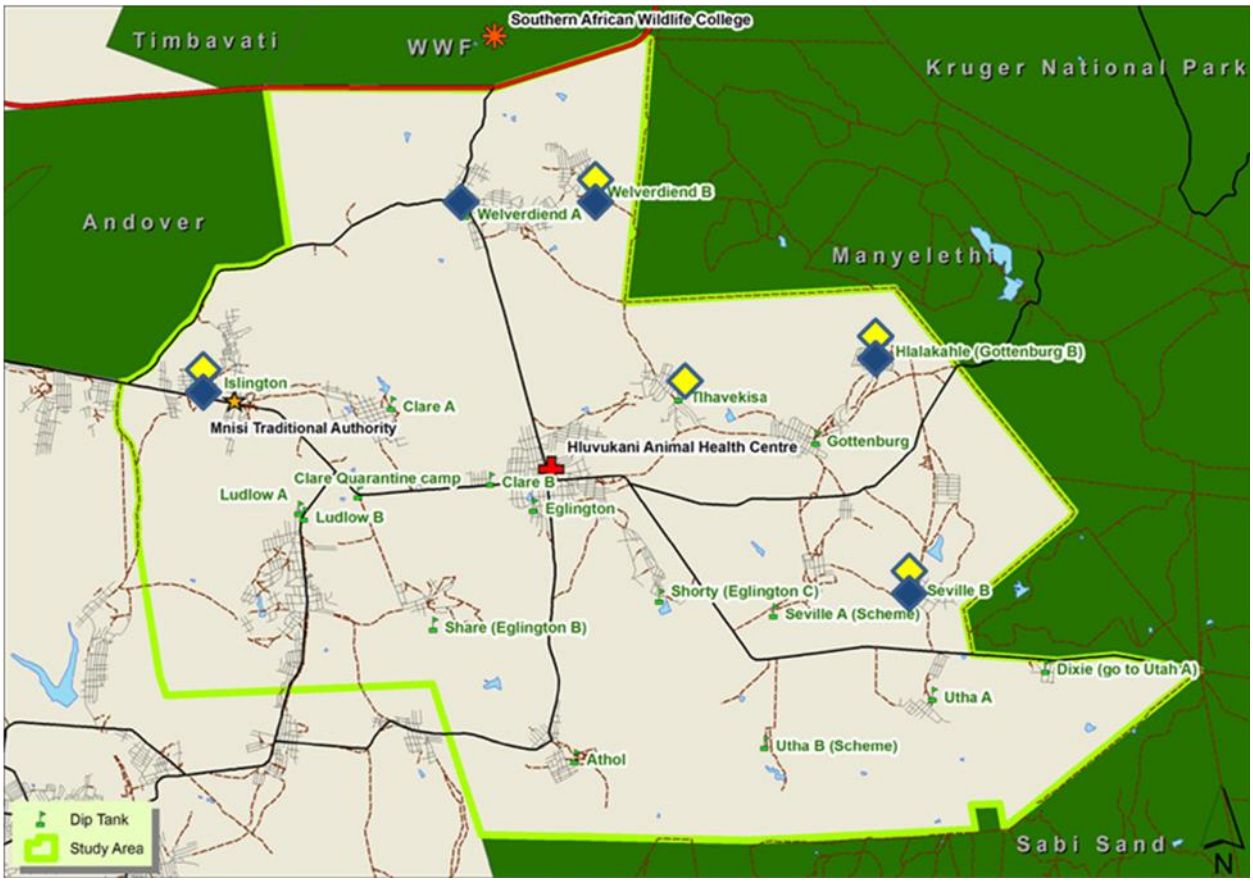

Figure 4-1: Map of Mnisi Community Programme area indicating dip tanks where *T. parva* cases from clinically sick cattle (yellow diamonds) and carrier cattle (blue diamonds) were found.

4.3.1 PCR amplification of p67, p104 and PIM genes

The p67, p104 and PIM loci were amplified from all samples shown in Table 4-1, although some did not yield any products. The PCR products amplified from each gene were run on a 1.5% agarose gel for the estimation of the length of the amplicons. Representative PCR products are shown in Figure 4-2 (p67) and Figure 4-3 A (p104) and B (PIM). No amplification was obtained from the water negative controls indicating that no contamination was introduced during the PCRs. *T. parva* Muguga DNA was included in each set of PCRs as a positive control and bands of the expected sizes were obtained for each set of amplifications.

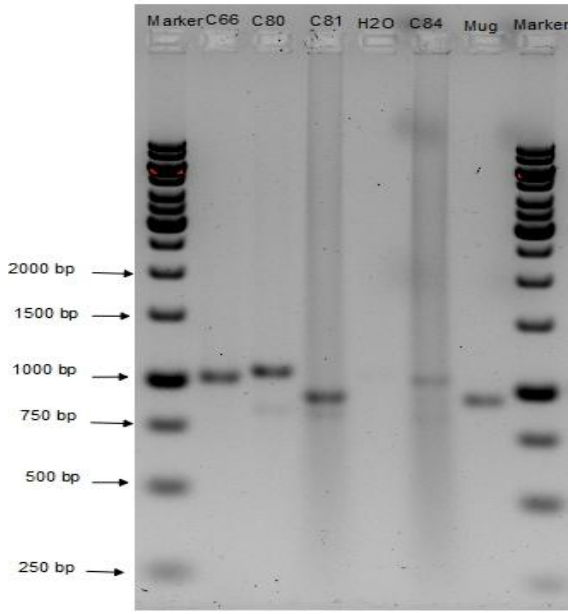

Figure 4-2: Amplicons obtained by PCR amplification of the central region of the p67 gene locus from *T. parva* qPCR positive samples collected from MCP area in South Africa. Marker: the 1 kb plus DNA marker (Fermentas Life Sciences), Mug: *T. parva* Muguga positive control (expected band size 900 bp), H_20: water negative control. See Table 4-1 for the dip tanks from which the isolates originated.

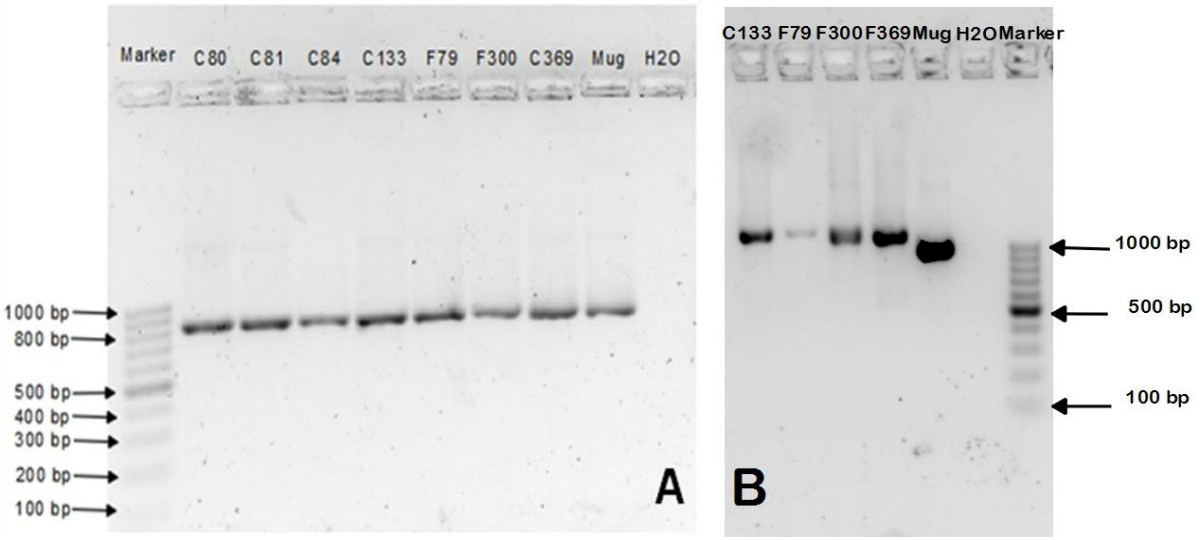

Figure 4-3: Amplicons obtained by PCR amplification of the p104 (A) and PIM (B) genes from *T. parva* qPCR positive samples collected from MCP area in South Africa. Marker: 100 bp DNA marker (Fermentas Life Sciences), Mug: *T. parva* Muguga positive control (expected band size approximately 800 bp for p104 and approximately 1000 bp for PIM), H_20: water negative control. See Table 4-1 for the dip tanks from which the isolates originated.

Approximate lengths of the PCR products of each of the three genes obtained from the samples that were *T. parva* qPCR positive are shown in Table 4-4. Sibeko *et al.* (2010) and Mwega *et al.* (2014) found p67 amplicons ranging from 0.8 to 1.1 kb. Samples analysed by Sibeko *et al.* (2011) yielded p104 PCR products of approximately 0.8 kb while the PIM gene PCR products obtained from the same study ranged in size from 0.7 to 1.2 kb. Products that were obtained in this study fell within the expected range. The PCR products were cloned and sequenced and sequence data were obtained from the PCR products shown in bold in Table 4-4.

Table 4-4: Amplicons obtained by PCR amplification of the p67, p104 and PIM genes of *T. parva* qPCR positive samples from the MCP area. Sequence data were obtained from the PCR products shown in bold.

Sample ID	Estimated length of PCR products (bp)		
	p67	**p104**	**PIM**
C66	**1050**	800	1000
C71	1100	800	1000
C80	800 (vf[a]) & 1100	**800**	1000
C81	800 (vf) & 900	**800**	1200
C84	800 (vf) & **1100**	**800**	**1000**
C89	900, 1000 & 1100	800	1000
C108	1000	800	1050
C109	1000	800	1000
C110	1100	800	1000
C123	1000	800	1000
C129	1000	800	1000
C133	1100	800	**1200**
F 25	– [b]	800	1200
F 79	–	**800**	1200
F 238	–	–	1000
F 246	–	800	700
F 268	–	–	1000
F 287	–	–	1000
F 300	900, 1000 & 1100	**800**	1100
F 312	–	–	1200
F 328	–	–	1000
F 369	**1000**	**800**	**1100**
F 376	–	800	1200

[a] vf: indicates a very faint band visible on the agarose gel

[b] –: indicates that no visible PCR products were obtained

4.3.2 Sequence and phylogenetic analysis of p67, p104 and PIM genes

4.3.2.1 p67

p67 sequences were obtained from samples from two clinical cases (C66 and C84) and a carrier (F369). Although two PCR products were obtained from sample C84, clones of only the larger product were obtained (the smaller amplicon was very faint). Two different p67 sequences approximately 1070 bp in length (C84.1 and C84.2) were obtained from clones of the larger PCR product from sample C84. The p67 sequences obtained from clones of samples C66 and F369 were homogeneous. The p67 sequences were translated to amino acid sequences and aligned with published sequences and the alignments were used to generate phylogenetic trees using the neighbor-joining, maximum-likelihood and maximum-parsimony methods. All methods yielded trees with similar topologies and good bootstrap support values. A representative p67 gene tree generated using the maximum likelihood method is shown in Figure 4-4 and two main groups are highlighted, A and B, as previously reported (Sibeko *et al*., 2010). The p67 sequence obtained from carrier animal, F369, grouped with other p67 allele 4 sequences obtained from buffalo from the Kruger National Park (Sibeko *et al*., 2010). Two of the p67 sequences obtained from clinical cases, C66 and C84.2, grouped with buffalo-type p67 allele 2 sequences. The other sequence obtained from a clinical case, C84.1, grouped with cattle-type p67 allele 1 sequences, although it did not contain the deletion characteristic of allele 1 sequences.

4.3.2.2 p104

p104 sequences were obtained from samples from three clinical cases (C80, C81 and C84) and three carriers (F79, F300 and F369). The p104 sequences were translated to amino acid sequences and aligned with published p104 sequences. The alignments were used to generate phylogenetic trees using the neighbor-joining, maximum-likelihood and maximum-parsimony methods. All methods yielded trees with similar topologies although the bootstrap support values were not strong. Figure 4-5 shows a representative p104 gene tree generated using the Maximum Likelihood method. Two main groups were seen, although these were not the same as those previously reported (Sibeko *et al.*, 2011). The four previously published alleles (1, 2, 3 and 4) (Table 4-3) clustered in Group A. The p104 sequences from clinically sick cattle (C80, C81G and C84) clustered in Group A with p104 sequences from buffalo while p104 sequences from the carrier cattle (F79, F300, F369) clustered in Group B with p104 sequences from buffalo from South Africa.

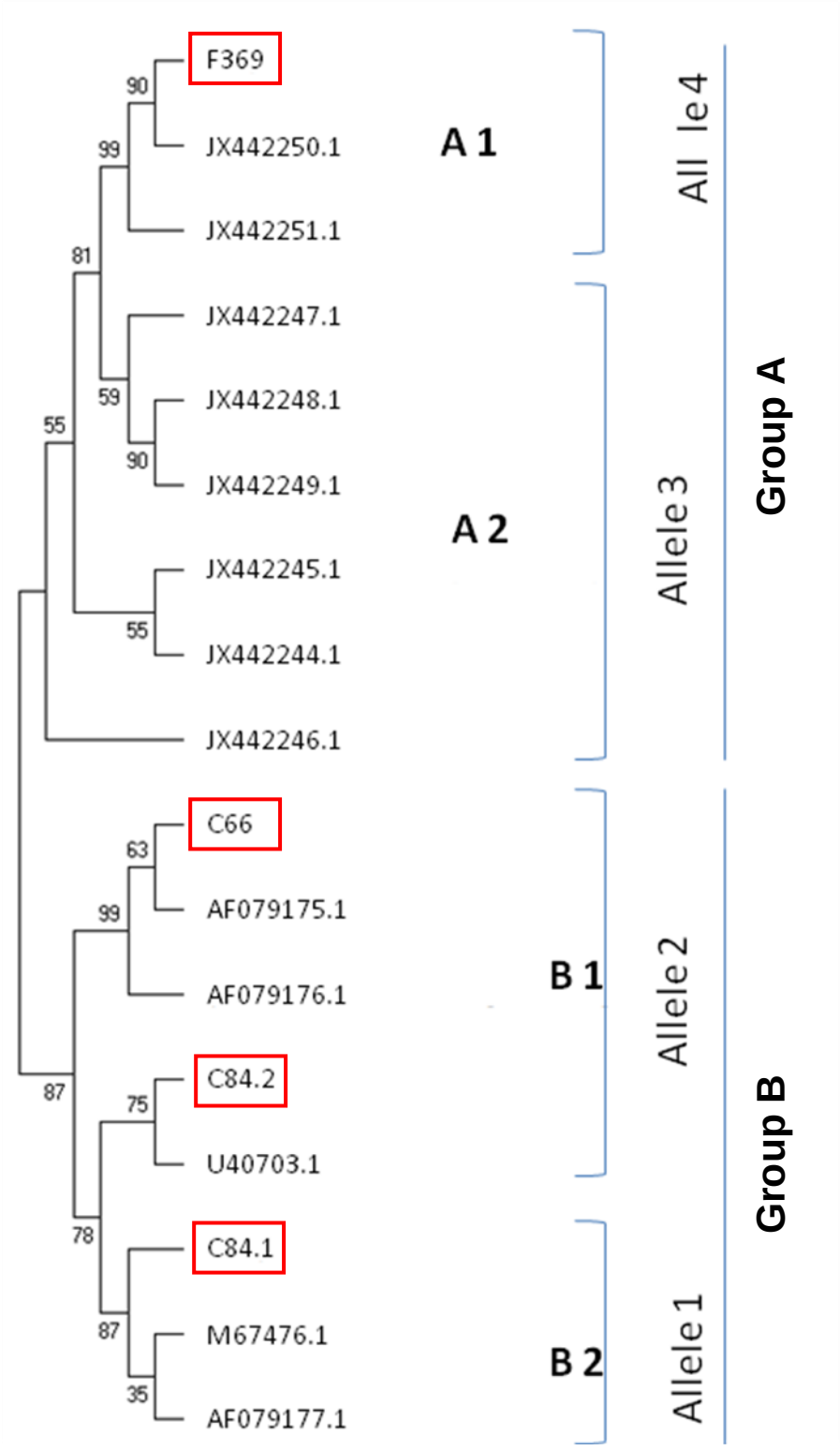

Figure 4-4: Phylogenetic relationship of *T. parva* strains from the Mnisi Community Programme area based on the p67 sequences as revealed by the maximum likelihood method. p67 sequences obtained in this study are shown in red boxes. The gene tree was inferred by using the maximum likelihood method using the JTT matrix-based model. The bootstrap consensus tree is inferred from 1000 replicates.

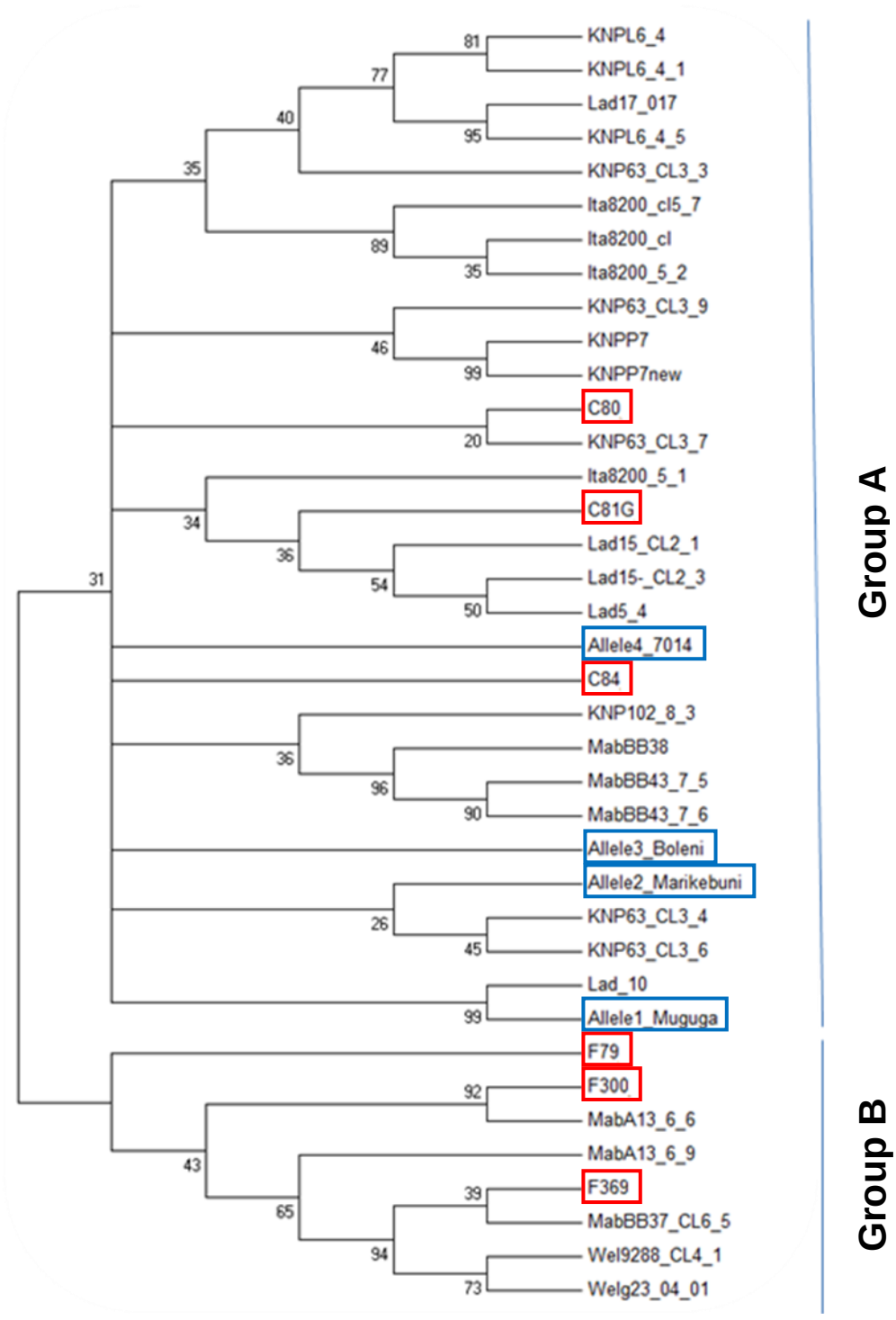

Figure 4-5: Maximum likelihood tree based on the p104 amino acid sequences depicting genetic relationships of *T. parva* strains from Mnisi Community Programme area (red boxes) and published p104 sequences (blue boxes, see Table 4-3 for accession numbers) as well as p104 sequences from the study by Sibeko (2009). The gene tree was inferred by using the maximum likelihood method using the JTT matrix-based model. The bootstrap consensus tree is inferred from 1000 replicates.

4.3.2.3 PIM

PIM sequences were obtained from two clinical cases (C84 and C133) and from a carrier (F369). All three PIM sequences aligned with other buffalo-type PIM sequences, even though the sequence from sample C84 was truncated (Figure 4-6). The PIM sequences from samples C133 and F369 had the 20 amino acid motif (VDQQQPVQQPSQDQPSGPDS) that has previously been identified as characteristic of buffalo-type PIM sequences (Toye *et al*., 1995); the C84 PIM sequence was not long enough to include this motif (Figure 4-6). None of the sequences contained the tetrapeptide repeat (QPEP) characteristic of cattle-type PIM alleles (Toye *et al*., 1995).

```
C133                    YSTGSSDLTHVDTEYNDDSSSSETSQQPQQPPDQPVDQQQPVQQPSQDQPSGPDSQDQPV
HIP32_1-8               DSTGSSDVTQVDSESNDTSSSSETSQQPQQPPDQPVDQQQPVQQPSQDQPSGPDSQDQPV
Wel2304-1               DSTGSSDVTQVDTESNDDSSSSETSQQP---PDQPVDQQQPVQQPSQDQPSGPDSQDQPV
KNP_O11-6-2             DSTGSSDVTQVDTESNDDSSSSETSQQP---PDQPVDQQQPVQQPSQDQPSGPDSQDQPV
F369                    -STGSSDVTQVDTESNDDSSSSETSQQP---PDQPVDQQQPVQQPSQDQPSGPDSQDQPV
C84                     ----------------------------------------------------------
KNP_O11_4-1             DSTGSSDVTQVDTESNDDSSSSETSQQP---PDQPVDQQQPVQQPSQDQPSGPDSQDQPV
T.parva_Schoonspruit    DSTGSSDVTQVDTESNDTSSSSETSQQGKPQPDQPQDQPDQHQQPTQGDTSGQQGPDTPQ
L06323                  DSTGSSDVTQVDTESNDTSSSSETSQQGKPQPDQPQDQPDQHQQPTQGDTSGQQGPDTPQ

C133                    DHQQPTQADSSGQQGQQQQPLDQPTGQPGPLEPVDQQQQPEPQPQPEPQPDQP-------
HIP32_1-8               DHQQPTQADSSGQQGQQQQPLDQPTGQPGPLEPVDQQQQPEPQPQPEPQPDQP-------
Wel2304-1               DHQQPTQADSSGQQGQQQQPLDQPTGQPGPLEPVDQQQQPQP------------------
KNP_O11-6-2             DHQQPTQADSSGQQGQQQQPLDQPTGQPGPLEPVDQQQQPQPQPQPQPEPDQPQDQQGQQ
F369                    DHQQPTQADSSGQQGQQQQPLDQPTGQPGPLEPVDQQQQPQPQPQP--EPDQPQDQQGQQ
C84                     ----------------------------------DQQQQPQDQPSQDDTS----------
KNP_O11_4-1             DHQQPTQADSSGQQGQQQQPLDQPTGQPGPLEPVDQQQQPQPQPQPQPEPDQPQDQQGQQ
T.parva_Schoonspruit    PIQEPSGPVQPDQTGQ------------GPVEPVDQQQQPTQGDTS--------------
L06323                  PIQEPSGPVQPDQTGQ------------GPVEPVDQQQQPTQGDTS--------------
                                                    ******

C133                    -------TGQQGQQPPIQPVDQQQPVQEPAKHDPTGQQQQPQP----QPEPEQTPETPPQ
HIP32_1-8               -------TGQQGQQPPIQPVDQQQPVQEPAKHDPTGQQQQPQP----QPEPEQTPETPPQ
Wel2304-1               ----------------------------------------------------------
KNP_O11-6-2             QQPLDQPTGQQGQQPPVQPVDQQQTPKQPAKDDPTGQQQQPQPKPEPEPEPEQTPETPPQ
F369                    QQPLDQPTGQQGQQQ--QPLDQPTG--QPGPLEPVDQQQQPQP--QPEPEPEQTPETPPQ
C84                     -----------GPETPVQPVDQKQTPQEPAKDEPTGQQQQ----PEPEPQPEQTPETPPQ
KNP_O11_4-1             QQPLDQPTGQQGQQPPVQPVDQQQTPKQPAKDDPTGQQQQPQPKPEPEPEPEQTPETPPQ
T.parva_Schoonspruit    --------GQQGQQPQDQPVQEQD-----------GQDSQGTP--------EQTPDQSGQ
L06323                  --------GQQGQQPQDQPVQEQD-----------GQDSQGTP--------EQTPDQSGQ

C133                    QEQPTPDDHPSGQQ--GQQPPVQGQGAQDSPTPD-DQPVDHQQPVQEPVQQEE-PQPEP-
HIP32_1-8               QEQPTPDDHPSGQQ--GQQPPAQGQGAQDSPTPD-DQPVDHQQPVQEPVQQEE-PQPEP-
Wel2304-1               ----------------------------------------------------------
KNP_O11-6-2             QEQPTPDDQPVDQQQPQDEPVQQEQGPQDSPTPD-DQPVDQQQPVQEPVQQEQPQPEPEP
F369                    QEQPTPDDHPSGQQ-PQDEPVQQEQGPQDSPTPD-DQPVDQQQPVQEPVDQQQ-------
C84                     QEQPTPDDQQSGQQ--PQDAPVQEQGAQDSPTPD-DQPVDQQQPVQEPEPSEEQPQPQP-
KNP_O11_4-1             QEQPTPDDQPVDQQQPQDEPVQQEQGPQDSPTPD-DQPVDQQQPVQEPVQQEQPQPEPEP
T.parva_Schoonspruit    QPGPDAPDQPVYQQQPVQQPSGQQQQPQPRPQPQPDQPVDQQQEPPTPEDQPS-------
L06323                  QPGPDTPDQPVYQQQPVQQPSGQQQQPQPRPQPQPDQPVDQQQEPPTPEDQPS-------
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C133                    -QPQPEPEPEQPQDQPVDQQQPV-----QDQPSGQETPQPIPDGQPVQEPPEQKPEPEPE
HIP32_1-8               -QPQPEPEPEQPQDQPVDQQQPV-----QDQPSGQETPQPIPDGQPVQEPPEQKPEPEPE
Wel2304-1               ---QPQPQPEQPQDQPVDHQQPS-----QDQPSGQETPQPIPEDQPVQEPTEQKPEPE--
KNP_O11-6-2             -EPQPQPEPEQPQDQPVDHQQPS-----QDQPSGQETPQPIPEDQPVQEPTEQKPEPE--
F369                    -QPQPQPEPEQPQDQPVDHQQPS-----QDQPSGQETPQPIPEDQPVQEPTEQKPEPE--
C84                     -EPQPEPEPEQPQDQPDQHQQPT-----QDQPSGQETPQPIPDGQPVQEPPEQKPEPEPE
KNP_O11_4-1             -EPQPQPEPEQPQDQPVDHQQPS-----QDQPSGQETPQPIPEDQPVQEPTEQKPEPE--
T.parva_Schoonspruit    -GPDSPDQPDQ-HHQPTPAAQ---------PQPEPQPEPQPEPQPEPVQEPPEQ-------
L06323                  -GPDSPDQPDQ-HHQPTPAAQPQTQPQPEPQPEPQPEPQPEPQPEPVQEPPEQ-------
                           :.  :*:* :.**    *          **. *  *** *: :*****.**

C133                    QTPEHTPSKDDLSGEELVKPSEGHMTGAAADGSGQPPDKKPDDDSKGKDGSKSDSGTPSK
HIP32_1-8               QTPEHTPSKDDLSGEELVKPSEGHMTGAAADGSGQPPDKKPDDDSKGKDGSKSDSGTPSK
Wel2304-1               QTPGHAPSKDDPTGEEPVKPSEGHMTGAAADGSGQPPDKKPGDKSKGKDGSKSDSGTPSK
KNP_O11-6-2             QTPEHAPSKDDPTGEEPVKPSEGHMTGAAADGSGQPPDKKPGDESKGKDGSKSDSGTPSK
F369                    QTPEHAPSKDDPTGEEPVKPSEGHMTGAAADGSGQPPDKKPGDESKGKDGSKSDSGTPSK
C84                     QTPEHTPSKDDLSGEEPFKPSEGHMTGAAADSSGQPPDKKPGDESKGKDGSKSDSGTPSK
KNP_O11_4-1             QTPEHAPSKDDPTGEEPVKPSEGHMTGAAADGSGQPPDKKPGDESKGKDGSKSDSGTPSK
T.parva_Schoonspruit    -TPEHTPSKDDASGEVPVKPSEGHMTGAAADGSGQPPDKKTDDDSKGKDGSKSGSGTPSK
L06323                  -TPEHTPSKDDASGEVPVKPSEGHMTGAAADGSGQPPDKKTDDDSKGKDGSKSGSGTPSK
                         ** *:***** :**  .*************.********..*.*********.******
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Figure 4-6: Clustal X alignment of PIM sequences obtained from clinical cases and carrier animals aligned with cattle-type PIM alleles (*T. parva* Muguga [accession number L06323] and *T. parva* Schoonspruit) and buffalo-type *T. parva* PIM sequences from buffalo from Hluhluwe-iMfolozi Park (HIP32_1-8), Kruger National Park (KNP_O11-6-2, KNP_O11_4-1), and Welgevonden Game Reserve (Wel2304-1). *Theileria parva* Schoonspruit and buffalo-type PIM sequences were obtained from the study by Sibeko (2009). The 20 amino acid motif characteristic of buffalo-type PIM sequences is shown in the red box. The tetrapeptide repeat characteristic of cattle-type PIM alleles is shown in the black box.

4.3.2.4 Summary of sequence analyses of p67, p104 and PIM genes from clinical cases and apparently healthy *T. parva*-positive cattle

All but one of the *T. parva* p67, p104 and PIM gene sequences obtained in this study were buffalo-type *T. parva* alleles (Table 4-5). A p67 allele that was similar but not identical to *T. parva* cattle-type p67 allele 1 was identified from a clinical case. No cattle-type *T. parva* alleles were found in any of the samples from carrier cattle.

Table 4-5: Sequences obtained from *T. parva* p67, p104 and PIM genes from clinical samples and carrier cattle and the type of alleles they clustered with

Clinical cases		
Gene	**Sample**	**Type of allele**
p67	C66	allele 2 buffalo-type
	C84.1	allele 1 cattle-type
	C84.2	allele 2 buffalo-type
p104	C80	buffalo-type
	C81 G	buffalo-type
	C84	buffalo-type
PIM	C84	buffalo-type
	C133	buffalo-type
Carrier cattle (*T. parva* qPCR-positive)		
Gene	**Sample**	**Type of allele**
p67	F369	allele 4 buffalo type
p104	F79	buffalo-type
	F300	buffalo-type
	F369	buffalo-type
PIM	F369	buffalo-type

4.4 Discussion

In this study, we identified samples that tested positive for *T. parva* using qPCR from both clinically sick cattle (identified in Chapter 2) and apparently healthy cattle at the livestock/wildlife interface in the MCP area, north-eastern Bushbuckridge, Mpumalanga (identified in Chapter 3). Yusufmia *et al*. (2010) and Mbizeni *et al*. (2013) have recently reported subclinical carriers of buffalo-associated *T. parva* in cattle along the livestock/wildlife interface areas in KwaZulu-Natal, South Africa. The identification of *T. parva* positive cattle in South Africa remains a concern and characterization of such samples might help us to evaluate their potential for cattle-to-cattle transmission.

In an attempt to characterise the *T. parva* parasites identified in cattle with clinical Corridor disease and in carrier cattle, the p67, p104 and PIM genes were amplified, cloned and sequenced. It was observed that some of the genes could not be amplified from some of the samples although all of the samples were qPCR positive. This was especially true for the samples from carrier cattle. This could be as a result of the samples having very low parasitaemia as evidenced by the high Cp values obtained with the qPCR assay for the carrier cattle samples. It is often difficult to amplify parasite DNA from carrier animals even using a nested PCR approach. Some samples from the clinically sick cattle also yielded products with very faint bands when visualized by electrophoresis. Faint PCR products or failure to amplify could also be due to differences in the primer target regions in some of the field samples. If there were sufficient differences, it would reduce the efficiency of primer annealing during the PCR. Purification of PCR products further reduces the quantity of the amplicons, therefore reducing the concentration of PCR products for cloning and sequencing. Thus, although PCR products were obtained from many of the samples, it was possible to sequence only a few of these.

Sibeko *et al*. (2010) compared the p67 sequences obtained in their study with previously reported sequences (allele 1 and 2) and two were novel (allele 3 and 4). In our study, sequence variants of p67 alleles 1, 2 and 4 were obtained. Three of the p67 sequences obtained, F369, C66 and C84.2, grouped with buffalo-type p67 sequences. One of the sequences obtained, C84.1, grouped with cattle-type p67 allele 1 sequences. The p104 sequences obtained in Sibeko *et al.* (2011) grouped into two main clades, clade A, which could be subdivided into A1 and A2 and clade B, which was subdivided into B1 and B2. The sequences obtained from clinical cases in this study grouped closely with sequences in clade A, while the sequences from carrier cattle grouped closely with sequences in clade B.

Although different groupings of p104 sequences were obtained in this study from those obtained by Sibeko et al. (2011), all the p104 sequences from both clinically sick cattle (C80, C81G, C84) and carrier cattle (F79, F300, F369) clustered with p104 sequences from buffalo from South Africa. The three PIM sequences obtained from clinically sick cattle (C84 and C133) and carrier cattle (F369) were more similar to buffalo-type PIM alleles than cattle-type PIM alleles as identified by Toye *et al*. (1995).

Our results suggest that the *T. parva* infections in the MCP cattle were transmitted directly from buffalo to cattle, since all of the p104 and PIM sequences and two of the three p67 sequences were characteristic of the buffalo-type *T. parva* alleles previously identified (Iams *et al*., 1990; Baylis *et al*., 1993; Nene *et al*., 1992; Nene *et al*., 1996; Nene *et al*., 1999; Skilton *et al*., 2002; Sibeko *et al*., 2010). Through communication with farmers it was noted that the *T. parva* infections were observed in herds that either strayed into the game reserves or grazed in areas where buffalo were noticed during the study period as well as in the past three years. Although contact between cattle and *T. parva* infected buffalo could not be confirmed, the findings in this study are similar to those of Sibeko (2009) as p67, p104 and PIM alleles obtained from the *T. parva* samples suggested that the parasite population circulating may have originated from buffalo. This is supported by the observation that the dip tanks at which clinical Corridor disease cases and carrier cattle were identified were situated along the immediate livestock/wildlife interface.

In the study by Mbizeni *et al*. (2013), it was noted that the *T. parva* infection detected in cattle lasts for a short time (30-50 days) and this was also seen in our study where only one out of eight *T. parva* qPCR positive cattle revisted after six months was still positive (Chapter 3). Therefore, as observed in Chapter 3, this is one of the reasons why cattle-to-cattle transmission is most unlikely to occur in the MCP area given the existing conditions. This correlates with the sequencing results which provide no evidence for selection of cattle-type *T. parva* alleles in either the clinically sick or carrier cattle samples (although it should be noted that limited numbers of p67, p104 and PIM sequences were obtained from only a small number of samples, and more sequences should be examined to give more confidence in our conclusion).

If climatic conditions in the area should change or if parasites with the ability to sustain infection in cattle should emerge in the future, this situation might change and it might become possible for cattle-to-cattle transmission to occur. One of the p67 sequences, C84.1, grouped with cattle-

type p67 allele 1 sequences, suggesting that parasites with characteristics of cattle-derived *T. parva* isolates might exist in buffalo in the area. There is the possibility that ECF-causing *T. parva* parasites might have been transmitted to the African buffalo during the ECF epidemic in the past and cattle-type alleles might still circulate in the African buffalo in South Africa. If this was the case, the spread of the cattle type might be possible when infected African buffalo share grazing with cattle. This is a risk factor that may still exist. Therefore, given appropriate conditions, the selection of cattle-type *T. parva* parasites in the MCP area might still become a possibility in the future and the existing parasite diversity needs to be investigated further. More samples need to be examined to confirm the existing sequence data and to confirm whether other cattle-type *T. parva* sequences are present in cattle in the MCP area. Given that the reasons for the lack of cattle-to-cattle transmission in South Africa are still not precisely known, it is important to maintain or even scale up the Corridor disease control measures in place. This will prevent the increasing number of Corridor disease outbreaks (Mbizeni *et al.*, 2013) currently being experienced and therefore avoid the emergence of subclinical *T. parva* carrier cattle in South Africa and the chance of transformation in the future. It is imperative that cattle in the area should be tested regularly for *T. parva* infection.

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CHAPTER 5

GENERAL DISCUSSION AND CONCLUSION

5.1 Introduction

The study done in the Mnisi Community Programme (MCP) area was conducted to determine the extent of the problem of tick-borne diseases (TBDs) and to investigate the importance of Corridor disease along the livestock/wildlife interface. Although there are interventions in place to control TBDs, clinical disease cases were detected during the study period. This chapter gives an overview of the study findings and their implications.

5.2 Tick-borne disease cases in Mnisi Community Programme area

Resource-poor farmers graze their cattle on communal rangelands (Masika & Mafu, 2004) which are generally characterized by poor management of cattle and low productivity. These farmers perceive ticks as the most important health constraint to their cattle (Dreyer *et al*., 1998; Dold & Cocks, 2001). The impact of TBDs on the livelihood of these resource-poor farming communities in developing countries has been ranked high (Minjauw, B. & McLeod, A. 2003; Perry, *et al*. 2002). The Nguni, an indigenous cattle breed of South Africa, is reported to be adapted to the harsh environments (Collins-Luswet, 2000). The indiscriminate crossing of Nguni cattle with exotic breeds to improve productivity (Dold & Cocks, 2001) is implicated in eroding the high tolerance to ticks and TBDs (Spickett *et al*., 1989; Mapiye *et al*., 2007; Muchenje *et al*., 2008). The breeding system may have had an impact on the clinical cases that were seen. The MCP area is in the vicinity of the Kruger National Park and shares borders with several game reserves: Andover to the north-west, Manyeleti to the east, Timbavati to the north and Sabi Sands to the south. These areas stock some game animals that are reservoirs of TBDs, and the cattle present in the MCP area are therefore at risk of exposure to diseases at the livestock/wildlife interface.

Combinations of diagnostic methods need to be used to determine the prevailing TBDs in an area. The process starts by looking at the clinical signs the cattle exhibit and then cattle are sampled. An array of samples ranging from blood and/or lymph smears and, in cases where cattle have died, organ samples are collected. Since each diagnostic test has its limitations, it is

necessary to have a combination of a number of them with as much background information available for a confirmatory diagnosis to be made. In this study microscopy, histopathology, the reverse line blot (RLB) hybridization assay and *T. parva*-specific quantitative real time polymerase chain reaction (qPCR) were used to assist with the diagnosis of TBDs in clinically sick cattle in the MCP area. Each time TBDs were suspected in clinically sick cattle, blood smears were made and microscopic examination was done. Additionally, other samples deemed necessary were collected for further confirmatory tests.

The tick vectors are abundant in the MCP area as could be seen on the cattle at the weekly inspections carried out by the animal health technicians (AHT) at the dip tanks. Although the cattle are regularly dipped, there are a number of challenges encountered with the vector control system in the area. One problem is that the system relies entirely on government support. Availability of acaricides was at times erratic due to procedural problems and this meant that cattle sometimes went without dipping. On many occasions not all cattle in herds would be taken to dip tanks for inspection. This happened when farmers were unable to gather all their cattle from the grazing areas. Since dip tanks are constructed away from homesteads, most of them are not connected to the water supply systems and this causes a challenge when refilling the dip tanks. At times animals were not dipped because there was not sufficient water available to enable the farmers to refill the dip tanks.

Anaplasmosis and babesiosis, two of the most important TBD constraints to improved productivity and health of cattle in South Africa (Mtshali *et al*., 2004), were diagnosed in the MCP area. Corridor disease, a notifiable disease by law (Stoltsz, 1989) was also diagnosed. Of all the 137 suspected TBD clinical cases handled, haemoparasites were detected in 20 (14.6%) of the samples on microscopic examination. This highlights how low the sensitivity of microscopy is because when the same samples were examined using the RLB hybridization assay, haemoparasites were detected in 89 (65.0%) of the samples either as single or mixed infections. Most of the positive samples detected (15 out of 20) on microscopy were *Theileria*-related and Corridor disease cases were high on the list. The piroplasms were very scanty and difficult to see, but schizonts were detected in the lymphocytes of most of those samples. The history of the cattle, the acute onset of the disease and knowledge of the seasonal emergence of the adult tick vector *R. appendiculatus* helped to diagnose Corridor disease. It was also observed that most of the mortalities were related to theileriosis. Thirteen of the 15 deaths recorded (Chapter 2) had single infections of theilerial parasites or theilerial parasites mixed with other haemoparasites.

In all, out of 137 clinical cases examined during the study period, 24 cases were attributed to TBDs based on microscopy, histopathology and *T. parva*-specific qPCR. Two cases of babesiosis and three cases of anaplasmosis were diagnosed; the remaining 19 cases were *Theileria*-related. Of these, one case was attributed to cerebral theileriosis caused by *T. taurotragi*. Twelve of the remaining cases were confirmed to be Corridor disease by *T. parva*-specific qPCR, and another case was diagnosed as Corridor disease based on the results of histopathology examination (Chapter 2).

The study confirms that tick-borne pathogens are common in the MCP area and TBDs do occur; therefore measures already in place need to be enhanced to reduce the clinical case incidences. The high incidences of *A. marginale* and *B. bovis* detected in cattle coupled with the abundance of vector ticks (personal observation) and the relatively low incidence of disease (three cases of anaplasmosis and two of babesiosis), suggest that the area is endemically stable for anaplasmosis and babesiosis. Most cattle-farming areas in southern Africa already appear to be endemic to anaplasmosis (Krystynak, 1986) and the study in MCP detected 35.0% of the samples positive for *A. marginale* on RLB. *Babesia bovis* was detected in 27.7% of the samples although *B. bigemina* was not detected on RLB hybridization assay. Heartwater was detected in 5.8% of the samples on RLB indicating its presence in the area. As in some other livestock/wildlife interface areas in South Africa, Corridor disease and other theilerial diseases were detected in the MCP area. As expected, the Corridor disease cases were diagnosed during the period coinciding with the emergence of the adult *Rhipicephalus appendiculatus* ticks (Brocklesby *et al*., 1966) towards the end of the summer season. More Corridor disease cases were confirmed among the cattle sampled during the research period (13/24) than any of the other TBDs. The very low *T. parva* infection in cattle that were not diagnosed with Corridor disease, along with the relatively high incidence of disease in cattle exposed to infection suggests that there is endemic instability of Corridor disease in the MCP area. The trend by some farmers of blanket treatment of cattle with oxytetracycline at the first sign of clinical disease can affect the epidemiology of diseases in an area. It is possible that the epidemiology of TBDs in the MCP area may have been influenced by this action.

Since microscopic examination of samples is not very sensitive but is one of the only readily available tests close to the farmers, it is important that the Department of Agriculture, Rural Development and Land Administration endeavours to invest in personnel and diagnostic equipment so that more sensitive diagnostic procedures can be brought closer to the farming communities. This would help in better diagnosing the TBDs within shorter periods unlike the

present situation where samples have to be sent to the Agricultural Research Council – Onderstyepoort Veterinary Institute (ARC-OVI) or to the University of Pretoria for confirmation of cases.

5.3 Identification of *Theileria parva* positive cattle in apparently healthy herds

The re-introduction or re-emergence of East Coast fever (ECF) in South Africa, either through re-introduction of the parasites from neighboring states such as Mozambique, or the transformation of buffalo-derived *T. parva* in cattle populations, has been a concern to veterinary authorities in South Africa ever since the eradication of the disease in the 1950s. Corridor disease has become the most important theilerial infection posing a threat to the cattle farming industry in South Africa since the eradication of ECF (Stoltsz, 1989). The sympatric occurrence of the vector ticks, *R. appendiculatus* and *R. zambeziensis* in many parts of Limpopo and Mpumalanga provinces (Norval *et al*, 1982; Stoltsz & Blouin, 1990) increases the possibility of Corridor disease outbreaks. Although Corridor disease is considered self-limiting because most cattle die before the parasites develop to the tick-infective stage (Neitz *et al*., 1955), and although control measures are in place to control the spread of Corridor disease, sporadic outbreaks still occur (Stoltsz, 2011).

Diagnostic tests used to detect cattle that have been exposed to Corridor disease include the indirect fluorescent antibody test (IFAT), a widely used serological test for the detection of antibodies to *Theileria* parasites (Katende *et al*., 1998), and a sensitive and specific qPCR for the detection of *T. parva* DNA in cattle and buffalo (Sibeko *et al*., 2008). Although the *T. parva* IFAT is highly sensitive for detection of antibodies in an epidemiological situation where only one species of *Theileria* exists, the specificity of the test reduces where there is a low level of cross-reactivity between *T. parva* and *T. taurotragi* (http//:www.oie.int/international-standard-setting/terrestrial-manual/access-online/, accessed on 09/09/2015). This can be overcome by testing at high dilutions. Cattle that have been exposed to *T. parva* infection develop antibody titres within 14 to 28 days (Norval e*t al.*, 1992) and the antibodies are detectable in the animal for as long as six months even in the absence of re-exposure (Burridge & Kimber 1973).

In order to detect low level infections or carrier state, Allsopp *et al.*, (1993) developed the *T. parva* PCR amplification of the *Theileria* 18S ribosomal RNA (rRNA) gene followed by probing with radioactively labelled species-specific oligonucleotide probes. Afterwards, a sensitive and specific quantitative real-time PCR (qPCR) for the detection of *T. parva* in cattle and buffalo

was developed (Sibeko *et al*., 2008). Although the *Theileria* sp. (buffalo) 18S rRNA gene sequence is very similar to that of *T. parva,* an amplification product is generated from *Theileria* sp. (buffalo) DNA, but the test still remains specific for *T. parva* as only a *T. parva*-specific melting curve is generated (Sibeko *et al.*, 2008).

In an attempt to identify *T. parva* carrier cattle in the MCP area, the IFAT was used to analyse serum samples from apparently healthy cattle which may have been exposed to *T. parva* infections in the past three years and the *T. parva*-specific qPCR was used to analyse DNA samples from seropositive herds (Chapter 3). The results from sera examined by IFAT revealed a seemingly high *T. parva* seroprevalence (63.6% of samples were positive for *T. parva* antibodies) but this result could be due to the varying degrees of serological cross-reactions that occur with other *Theileria* spp., especially *T. taurotragi* (De Vos, 1982; Stoltsz, 1989). It is important to note that cross-reaction occurs when antibody titres are low in samples with *T. parva/ T. taurotragi* mixed infections (De Vos, 1982; Norval *et al*., 1992; Lawrence *et al*., 2004), and this was probably the case in the cattle from the MCP area. The presence of *T. taurotragi* was indicated by the diagnosis of a clinical case of cerebral theileriosis caused by *T. taurotragi* together with *T. taurotragi* positive results (11.7%) of samples run on RLB (Chapter 2). The serum samples were run at different dilutions and results of the highest dilution (1:160) are thus probably the most accurate reflection of *T. parva* seroprevalence in the MCP area, at 13.4% (Chapter 3). It is therefore important to run samples at a high dilution to obtain more accurate results of *T. parva* seroprevalence in areas where *T. taurotragi* has also been detected. Both *T. parva* and *T. taurotragi* were present in the MCP area and highly likely at low antibody titres since samples were collected from apparently healthy cattle.

Theileria parva DNA was detected in 11 out of 432 samples (2.6%) using the *T. parva*-specific qPCR assay (Chapter 3). These cattle were found at four dip tanks in the northern part of the MCP area close to the livestock/wildlife interface. The notion that Corridor disease is a self-limiting disease needs more investigations as the results from this study as well as the study done by Mbizeni *et al*. (2013) (1.9% *T. parva* positive) indicate that *T. parva* positive cattle can be detected in areas where Corridor disease occurs. There may be a number of reasons for the detection of *T. parva* positive cattle: the animals may not have received an infective dose, the animals may have recovered from Corridor disease either naturally or due to the early interventions by farmers, or the animals may have been infected with a less virulent *T. parva* from the diverse population in infected buffalo. Whether the *T. parva* infection detected in the cattle in this study can be transmitted to other cattle is questionable, since the parasitaemias were

very low in these cases and the carrier state as detected by qPCR was short-lived with only one out of the eight cattle resampled after six months still qPCR positive. This, coupled with the seasonality of the vector ticks in the area, could limit the transmission of *T. parva* to susceptible cattle. Mbizeni *et al*. (2013) did a transmission experiment from cattle recovered from Corridor disease and no *T. parva* transmission took place, although *T. taurotragi* was transmitted. Even though it seems that *T. parva* transmission is unlikely to happen given current conditions, measures to reduce the ever-increasing number of Corridor disease outbreaks along the livestock/wildlife interface areas should be maintained and scaled up.

5.4 Molecular characterization of the genes coding for p67, p104 and the polymorphic immunodominant molecule (PIM) from *T. parva* positive cattle

The identification of *T. parva* positive cattle is a concern in South Africa, since it opens up the possibility for selection of cattle-adapted *T. parva*, which could eventually result in cattle-to-cattle transmission of the parasites and the re-emergence of East Coast fever. In order to characterise the *T. parva* parasites obtained in this study the sporozoite antigen gene, p67, the p104 gene and the polymorphic immunodominant molecule (PIM) gene were examined (Chapter 4). In East Africa p67 gene sequences revealed a 129 bp deletion in the central region of the gene characteristic of cattle-derived *T. parva* isolates (allele 1) and no deletion was observed in buffalo-derived isolates (allele 2) (Nene *et al*., 1996; Nene *et al*., 1992). Both p67 alleles were obtained in South African buffalo from the Kruger National Park (Collins, 1997). Sibeko *et al*. (2010) later identified variants of both p67 alleles in other buffalo samples in South Africa together with two additional p67 alleles (alleles 3 and 4). Sibeko *et al*. (2011) also characterised *T. parva* field samples from cattle and buffalo in South Africa using PIM and p104 RFLP profiles and sequence data.

The current study involved characterising *T. parva* qPCR positive samples from clinically sick and apparently healthy cattle. Not all the positive samples collected could be characterised as it was not possible to obtain PCR products from some of them. This was probably because the parasitaemias were low, especially in the samples from apparently healthy cattle. Another reason for lack of PCR products could be due to differences in the primer target regions in some of the field samples.

The gene coding for p67 was amplified and sequenced from three samples and four p67 sequences were obtained and compared with those previously reported by Sibeko *et al*. (2010).

Three of the p67 sequences grouped with other buffalo-type p67 sequences (alleles 2 and 4), and one (C84.1) grouped with cattle-type p67 alleles (allele 1). All the p104 sequences from clinically sick and apparently healthy cattle grouped with other p104 sequences from South African buffalo in clades A and B. All three PIM sequences obtained aligned with other buffalo-type PIM sequences. The results suggest that the infections of cattle in this study were caused by buffalo-associated *T. parva* because all the sequences obtained except for one grouped with buffalo-type alleles/clades. The p67 sequence that grouped with other cattle-type p67 alleles could suggest that parasites with characteristics of cattle-derived *T. parva* might exist in buffalo in the area. This finding like the case with Sibeko (2009) could indicate that p67 cattle-type alleles may not be exclusively associated with cattle-type *T. parva* parasites. In the cases of sequences from cattle along livestock/wildlife interface where contact with infected buffalo is highly likely, the presence of cattle-type alleles might indicate that parasites with characteristics of cattle-type *T. parva* could be present in the buffalo. If transmission of such parasites (cattle-derived type) was to occur, the selection of cattle-derived *T. parva* parasites in cattle in South Africa could become a possibility.

It should be noted that the sequences were obtained from a small number of samples, and it is therefore important that more sequences be analysed in the area to verify these findings.

5.5 Conclusion

The problem of TBDs in the MCP area, like in most resource-poor livestock farming areas, is real. This problem is compounded by the location of the area along the livestock/wildlife interface where reservoir hosts of TBDs are abundant. Although the game animals are fenced off from the communal grazing areas, some intermediate hosts are able to go through the fences and move the vector ticks to the communal grazing areas and finally to the cattle. Additionally there are times when the fences go down for various reasons and either the game animals invade the communal grazing lands or the cattle enter the game area. A lack of proper livestock management skills by many of the farmers worsens the situation. While there appears to be endemic stability of bovine anaplasmosis and babesiosis in the area, proper tick control would further reduce the incidence of these TBDs, but farmers are reliant on government taking the lead in tick control during the FMD weekly inspections at the dip tanks. Although government's provision of acaricides and some vaccines to the farmers is partly an incentive for the farmers to get the cattle to dip tanks, there are times when acaricides are not in stock. During such times, some farmers do not take the initiative of supplementing the government's efforts by buying

their own acaricides. In addition, it was observed that at times, not all cattle were presented at the dip tanks every week and therefore such cattle become reservoirs of ticks and more susceptible to TBDs.

Corridor disease cases in this study were diagnosed from samples that were from the immediate livestock/wildlife interface dip tanks, suggesting that the source of *T. parva* was the buffalo. This is supported by the sequence data which indicated that all but one of the sequences were characteristic of buffalo-associated *T. parva* parasites. Yusufmia *et al*. (2010) and Mbizeni *et al.* (2013) have previously confirmed the presence of subclinical carrier/*T. parva* positive cattle in areas along the livestock/wildlife interface in KwaZulu-Natal. Eleven *T. parva* qPCR positive cattle were also identified in the MCP area, but only one of these was still positive when it was examined six months later. Whether these animals are indeed *T. parva* carrier cattle or not is a research area that needs to be fully explored. In the study by Mbizeni *et al.* (2013), recovered cattle were unable to transmit *T. parva* infections to susceptible bovines, probably because the parasitaemia was too low to allow transmission. Corridor disease cases occur in South Africa only when the adult ticks are active in the summer months of January to March. Since the vector ticks in South Africa are seasonal, by the time the nymphs emerge in the dry months of July to October it is unlikely that cattle infected the previous summer would have sufficiently high *T. parva* parasitaemias to infect the nymphs. Therefore it is most unlikely that cattle-to-cattle transmission will occur under the present conditions in South Africa. Nevertheless, it is important to maintain and scale up the already existing measures to prevent the increasing number of Corridor disease outbreaks to avoid the emergence of subclinical *T. parva* carrier cattle in South Africa and the chance of transformation in the future.

5.6 References

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APPENDICES

Appendix 1: Consent form used to inform farmers about the study. Farmers signed the form to give permission to the study group to collect samples from their cattle

Appendix I: Consent form (adult)

Department of Veterinary Tropical Diseases
Faculty of Veterinary Science
P/Bag X04
Onderstepoort 0110
South Africa

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

CONSENT FORM FOR MNISI CORRIDOR DISEASE STUDY

UNIVERSITY OF PRETORIA

Faculty of Veterinary Science

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TITLE:

Confirmation of diagnosis of tick-borne diseases in the Mnisi area and identification of *Theileria parva* carrier cattle

INFORMED CONSENT (for persons >18 years old)

SECTION A: INFORMATION SHEET

Please read this consent agreement carefully before you decide to participate in the study.

You are invited to participate on a research study on tick-borne diseases focusing on Corridor disease that occurs in animals.

Corridor disease is a disease that is transmitted from buffalo to cattle by ticks. We would like to take blood samples from your animals so that we can look for signs of the disease in the blood. It will be of value to the community if we can understand this disease better and see if it is a problem in the community. Participation in this study is done through allowing staff and students of the University of Pretoria and Mpumalanga Veterinary Services to take blood samples from your animal and/or take lymph node biopsies and if they are positive for the disease on blood sample then re-sample the particular herd for farther sensitive tests. The time required is about a minute for each animal.

Participation is completely voluntary and you can stop being involved in the study at any time.

1

Appendix I: Consent form (adult)

There are no risks from participating in this study for you. Although taking blood samples from animals can cause minor trauma to the animal, all efforts will be made to cause as little distress as possible.

There are no direct benefits for participating, besides having gained knowledge into the Corridor disease status of your animals. You will be informed of the results of the sampling.

This study is supervised by the Animal Use and Care Committee, University of Pretoria (South Africa).

SECTION B: CONSENT FORM

I confirm that I have been informed about the study and that I have received a copy of the participant information sheet and the consent form. I have read and understood the information:

- o I consent voluntarily to participate in this study / discussion
- o I know that I will receive no payment for participating in the study
- o I know that I can stop my participation in this study at any time
- o I consent to having blood sample/s taken from my animals and lymph node biopsy if needed

I have had all my questions answered to my satisfaction.

(a)..... Name of respondent

(b)..... Signature or thumb print of respondent

(c)..... Signature of Researcher Or Clinician

(d)..... Signature of witness

(e)..... Place

(f)..... Date

2

Appendix 2: Permit for moving samples from Mnisi Community Programme study area to Hans Hoheisen Wildlife Research Station

Permit in	Permit out
	SAMPLES

Permit number: GN-BBR/3/Aug/2013

DEPARTMENT OF AGRICULTURE, CONSERVATION AND ENVIRONMENT

MPUMALANGA

DEPT. LANDBOU, BEWARING EN OMGEWING

LITIKO LETEKULIMA, KONGIWA NETEMVELO

MASTER MOVEMENT PERMIT

VETERINARY PERMIT TO MOVE ANIMALS / ANIMAL PRODUCTS
VEEARTSENYPERMIT VIR VERVOER VAN DIERE / DIEREPRODUKTE
IMVUMO YEKUHAMBISA TILWANE / TINTFO LETIHABULISAKO TETILWANE

In terms of the Animal Diseases Act No. 35 of 1984 permission is hereby granted to:
Kragtens die Wet op Dieresiektes Nr. 35 van 1984 word toestemming hiermee verleen aan:
Ngeligunya leMtsetfo weTifo teTilwane No. 35 wa 1984:

Name: Naam: Libito:	CHIMVUELE N. CHOOPA	ID No.:	
Address: Adres: Likheli:	HLUVUKANI ANIMAL CLINIC UNIVERSITY OF PRETORIA		
to move with: om te beweeg met: uvunyelwa yekuhambisa:	6 TWO HUNDRED	SPECIES: BOVINE	LINE / PRODUCT
identified as follows: geïdentifiseer as volg: letikhonjiswe nga:	BLOOD (SERUM) & LYMPH NODE BIOPSY SMEARS		
from the farm/diptank area/place: vanaf die plaas/dipbakgebied/plek: kusuka epulazini/edibhini/endzaweni:	MNISI AREA (1, 2, 3, 4, 5) HLUVUKANI CLINIC	in the district of in die distrik esigodzini sase	EHL NORTH
to the farm/diptank area/abattoir/place: na die plaas/dipbakgebied/abattoir/plek: kuya epulazini/edibhini/emadzeleni/endzaweni:	HANS HOHEISEN LAB	in the district of in die distrik esigodzini sase	EHL NORTH

On the following conditions:
1. This permit:
(a) is **valid for** 30 days from date of issue and for **one consignment only**
(b) must **accompany the animals** / products mentioned above;
(c) must be produced for **inspection** on demand by any land or animal owner or police or veterinary official;
(d) must be **kept** at a destination until collected by a veterinary official.
2. For **game** / game products a nature conservation permit is also needed.
3. Animals and products must be visibly free from **external parasites.**
4. Animals are to be transported in accordance with **animal welfare** guidelines.
5. **Other conditions:**

Op die volgende voorwaardes:
1. Hierdie permit:
(a) is **geldig vir 10 dae** vanaf datum van uitreiking en slegs vir **een beweging;**
(b) moet die diere / produkte hierbo vermeld **vergesel;**
(c) moet vir **inspeksie** getoon word op aanvraag van enige grond-of diereeienaar of polisie- of veeartsenybeampte;
(d) moet by bestemming gehou word totdat dit deur 'n veeartsenybeampte geneem word.
2. Vir **wild** / wildprodukte word 'n natuur-bewaringspermit ook benodig.
3. Diere en produkte moet sigbaar vry van **uitwendige parasiete** wees.
4. Diere moet volgens **dierewelsynsriglyne** vervoer word.
5. **Ander voorwaardes:**

Ngaphansi kwemibandzela lechazisiwe ngaphansi:
1. Lemvumo:
(a) **isebenta emalanga-lange 10** kusuka lusuku lwekukhishwa kwayo futsi **yekuhambisa kanye** kuphela;
(b) **Itawuhamba netilwane** / netintfo letihabulisako letibhaliwe ngenhla;
(c) itawukhombiswa **kuholwa** ngumuphi umnikati wendwazo kumbe umnikati wetilwane kumbe nguliphoyisa kumbe kusikhulu setekwelashwa kwetilwane, loyicelelako;
(d) **itawubhekwa** endzaweni yekufika khona tilwane / tintfo letihabulisako futsi itsatfwe ngesikhulu setekwelashwa kwetilwane.
2. **Kuhambisa kwetilwane tesiganga** / kwetintfo letihabulisako futsi kudzingwa imvumo yeTekongwa kweTemvelo.
3. Tilwane letintfo letihabulisako tetilwane tifanele kutsi tite **tidlangandvodza** letingabonakala.
4. Tilwane tifanele tihanjiswe ngaphansi kwetimiso **tekutiphilisa kahle.**
5. **Imibandzela leminye:**

* AS ON ATTACHED VETERINARY MOVEMENT MASTER PERMIT PROTOCOL & V. MOVEMENT FORM
* SAMPLE DETAILS TO BE CLEARLY INDICATED ON MOVEMENT FORM

6. Retention for / Quarantine for / Direct slaughter within: ..... days after arrival at destination

Seal no/s	NA	Place / Plek / Indzawo:	
Vehicle reg. no/s		Date / Datum / Lusuku:	26/07/2012

I hereby certify that all applicable control measures for issuing of this permit have been complied with.

PRINT NAME: AYANDA. MPEHLE — Issuing Veterinary Official:

STATUS: STATE VETERINARIAN — State Veterinarian / Staats-veearts / Dokotela weTilwane

26 JUL 2012

DR A MPEHLE
STATE VETERINARIAN
DATE STAMP
BUSHBUCKRIDGE

IMPORTANT NOTE / BELANGRIKE NOTA / SATISO LESIBALULEKILE
Permit holder who does not comply with this permit or any condition thereof is guilty of an offence.
Permithouer wat in stryd met hierdie permit of enige voorwaarde daarvan optree, is skuldig aan 'n misdryf.
Lonalemvumo longalandzela lemvumo kumbe muphi umbandzela wayo uphula umtsetfo.

DISTRIBUTION: VETERINARY OFFICIAL AT DESTINATION

BarPrint 221097 ☎ 013 712-2723

Appendix 3: Permit for movement of DNA samples from Hans Hoheisen Wildlife Research Station, Bushbuckridge to Department of Veterinary Tropical Diseases, University of Pretoria, Pretoria. The DNA was extracted from blood and in some cases it was also extracted from lymph nodes or spleen.

Permit in | Permit out

DEPARTMENT OF AGRICULTURE

MPUMALANGA

Permit number: Orpen sb/00013/2013

DEPT. LANDBOU
LITIKO LETEKULIMA

VETERINARY PERMIT TO MOVE ANIMALS/ ANIMAL PRODUCTS
VEEARTSENYPERMIT VIR VERVOER VAN DIERE / DIEREPRODUKTE
IMVUMO YEKUHAMBISA TILWANE / TINTFO LETIKHABULISAKO TETILWANE

In terms of the Animal Diseases Act No. 35 of 1984 permission is hereby granted to:
Kragtens die Wet op Dieresiektes Nr. 35 van 1984 word toestemming hiermee verleen aan:
Ngeligunya leMtsetfo we Tifo te Tilwane No. 35 wa 1984:

Name: / Naam: / Libito: C.N Choopa — ID No:

Address: / Adres: / Likheli: University of PTA DVTD

to move with: / om te beweeg met: / uvunyelwa yekuhambisa: Buchembrotherbacktray (S69) Bovine — Product/sample

identified as follows: / geïdentifiseer as volg: / letikhonjiswe nga: DNA Extraction

from the farm/diptank area/place: / vanaf die plaas/dipbak gebied/plek: / kusuka epulanzini/edibhini/endzaweni: Hlukukani / University PTA — in the district of / in die distrik / esigodzini sase: Bohlabela / TSHWANE

On the following conditions:
1. This permit:
(a) **is valid for 10 days** from date of issue and for **one consignment only**;
(b) must **accompany the animals / products** mentioned above;
(c) must be produced for **inspection** on demand by any land or animal owner or police or veterinary official;
(d) must be **kept** at a destination until collected by a veterinary official.
2. For **game / game products** a nature conservation permit is also needed.
3. Animals and products must be visibly free from **external parasites**.
4. Animals are to be transported in accordance with **animal welfare** guidelines.
5. **Other conditions:**

Op die volgende voorwaardes:
1. Hierdie permit:
a) is **geldig vir 10 dae** vanaf datum van uitreiking en slegs vir **een beweging**;
b) moet die diere / produkte hierbo vermeld **vergesel**;
c) moet vir **inspeksie** getoon word op aanvraag van enige grond-of diere eienaar of polisie- of veeartseny beampte;
d) moet by bestemming gehou word totdat dit deur 'n veeartsenydiens beampte geneem word.
2. Vir **wild / wildprodukte** word 'n natuurbewarings permit ook benodig.
3. Diere en produkte moet sigbaar vry van uitwendige parasiete wees.
4. Diere moet volgens dierewelsyn riglyne vervoer word.
5. **Ander voorwaardes:**

Ngaphansi kwemibandzela lechazisiwe ngaphansi:
1. Lemvumo:
(a) **isebenta emalanga-lange 10 kusuka** lusuku lwekukhishwa kwayo futsi **yekuhambisa kanye** kuphela;
(b) **itawuhamba netilwane / netintfo** letihabulisako letibhaliwe ngenhla;
(c) itawukhombiswa **kuhlolwa** ngumuphi umnikati wendwazo kumbe umnikati wetilwane kumbe nguliphoyisa kumbe kusikhulu setekwelashwa kwetilwane, loyicelelako;
(d) **itawubhekwa** endzaweni yekufika khona tilwane / tintfo letihabulisako futsi itsatfwe ngesikhulu setekwelashwa kwetilwane.
2. **Kuhambisa kwetilwane tesiganga / kwetintfo** lethibulisako futsi kudzingwa imvumo yeTekongwa kweTemvelo.
3. Tilwane letintfo letihabulisako tetilwane tifanele kutsi tile **tidlangandvodza** letingabonakala.
4. Tilwane tifanele tihanjiswe ngaphansi kwetimiso **tekutiphilisa kahle.**
5. **Imibandzela leminye:**

DNA samples to be send directly to UP (UH 2900, pbd)

6. Retention for / Quarantine for / Direct slaughter within: .... days after arrival at destination

Seal no/s		Place / Plek/ Indzawo:	HHWRC
Vehicle reg. no/s		Date / Datum / Lusuku:	13/06/2013

I hereby certify that all applicable control measures for issuing of this permit have been complied with.

Mathumane DH
Issuing Veterinary Official: — State Veterinarian / Staatsveearts / Dokotela weTilwane

IMPORTANT NOTE / BELANGRIKE NOTA / SATISO LESIBALULEKILE
Permit holder who does not comply with the permit or any condition thereof is guilty of an offence.
Permithouer wat in stryd met hierdie permit of enige voorwaarde daarvan optree, is skuldig aan 'n misdryf.
Lonalemvumo longalandzela lemvumo kumbe muphi umbandzela wayo uphula umtsetfo.

DISTRIBUTION: VETERINARY OFFICIAL AT DESTINATION

Ehl/N 00015
DEPARTMENT OF AGRICULTURE, RURAL DEVELOPMENT & LAND ADMINISTRATION
Chief Directorate: Veterinary Services
2013 -06- 13
State Veterinary Office: Orpen
Klaserie Ward
PROVINCE OF MPUMALANGA

Appendix 4: List of cattle sampled and the corresponding details

Sample ID	Date collected	Origin	Symptoms	Deaths	Sample	Results
C6	30.10.2012	Clare A	Loss of appetite, cycling movement & down with puddling movements	Yes	Heart, lung, kidney, spleen, brain and lymph node	Histopathology- Suspected cerebral theileriosis (*Theileria taurotragi*).
C10	06.11.2012	Clare A	Anaemic , neurological signs, lethargy and dehydrated	Yes	Blood and brain smears	Microscopy- Cerebral babesiosis
C14	14.12.2012	Seville B	Fever & lameness	No	Blood smear	Microscopy- *A. marginale*.
C28	14.12.2012	Athol	Calf found dead	Yes	Blood smear	Microscopy- *Babesia bovis.*
C47	11.01.2013	Gottenburg	Recumbent, congested nostrils & eye mucosa	Slaughtered	EDTA blood	Microscopy- Piroplasms seen.
C57	31.01.2013	Welverdiend B	Constipation and anaemic	No	EDTA blood and blood smear	Microscopy- *A. marginale.*
C58	31.01.2013	Welverdiend B	Constipation and anaemic	No	EDTA blood and blood smear	Microscopy- *A. marginale.*
C64	06.02.2013	Hlalakahle	Fever (39.9°C), enlarged lymph nodes and laboured breathing	No	EDTA blood and blood and lymph smears	Microcopy- Schizonts seen.
C65	06.02.2013	Hlalakahle	Fever (39.4°C), enlarged lymph nodes, limping for 3days	Yes	EDTA and blood and lymph smears	Microscopy- Schizonts seen.
C66	06.02.2013	Hlalakahle	Fever (41.6°C), enlarged lymph nodes & limping	Yes	EDTA blood, spleen, lymph node, blood and lymph smears	Microscopy- Schizonts & piroplasms seen. qPCR- *T. parva* positive.
C70	14.02.2013	Burlington	Lethargic and fever (40.1°C)	Yes	EDTA blood and blood smear	Microscopy- Piroplasms seen.
C71	15.02.2013	Hlalakahle	Loss of appetite/water intake & loss of condition	Yes	Lung, heart, liver, spleen lymph node	Microscopy- Schizonts seen. Histopathology- suspected Corridor disease. qPCR- *T. parva* positive.
C73	15.02.2013	Islington	Severe abnormal breathing	No	EDTA blood, blood and lymph smears	Microscopy- Piroplasms seen
C80	19.02.2013	Welverdiend B	Lethargic, loss of appetite, lacrimation & salivating	Yes	Heart, lung, kidney, spleen and lymph node	Microscopy- Piroplasms seen. Histopathology- Suspected Corridor disease. qPCR- *T. parva* positive.

C81	22.02.2013	Thlavekisa	Loss of condition, depressed & abnormal breathing & fever (41.1°C)	Yes	Heart, lung, kidney, spleen and lymph node	Microscopy- Schizonts seen. Histopathology- Suspected Corridor disease. qPCR- *T. parva* positive.
C84	26.02.2013	Islington	Collapsed, diarrhoea and increased inspiration	Slaughtered	EDTA blood	qPCR- *T. parva* positive.
C89	01.03.2013	Hlalakahle	Loss of appetite, collapsed, laboured breathing	Yes	Spleen and lymph node	Microscopy- Schizonts and piroplasms seen. Histopathology- Suspected Corridor disease. qPCR- *T. parva* positive.
C90	04.03.2013	Buffelshoek	Loss of appetite (3 days) and severe straining	Slaughtered	Heart, lung, kidney, spleen and lymph node	Histopathology- Suspected Corridor disease.
C108	12.03.2013	Hlalakahle	Sudden death	Yes	Heart, lung, kidney, spleen and lymph node	Microscopy- Schizonts seen. qPCR- *T. parva* positive.
C109	12.03.2013	Hlalakahle	Sudden death	Yes	Heart, lung, kidney, spleen and lymph node	Microscopy- Schizonts seen. qPCR- *T. parva* positive.
C110	13.03.2013	Seville B	Calf was lethargic in the evening and found dead in the morning	Yes	Spleen and lymph node	Microscopy- Schizonts seen. qPCR- *T. parva* positive.
C123	02.04.2013	Welverdiend B	Lethargic, anorexia, cornea eodema,bloody/mucoid diarrhoea with pangent smell	Yes	Blood and lymph smear, spleen and lymph nodes	Microscopy- Schizonts seen. qPCR- *T. parva* positive.
C129	09.02.2013	Hlalakahle	Circling movements, sudden death, cloudy eyes after death	Yes	Lymph node	qPCR- *T. parva* positive
C133	26.04.2013	Hlalakahle	High pulse, fever (40.8°C) & diarrhoea	Slaughtered	EDTA blood	Microscopy- Schizonts seen. qPCR- *T. parva* positive

Appendix 5: Results of the RLB hybridization assay for 137 cattle samples in the Mnisi Community Programme area.

Sample ID	*E/A* catch-all	*A. marginale*	*A. marginale* subsp. *centrale*	*Anaplasma sp. Omatjenne*	*A. bovis*	*E. ruminantium*	*T/B* catch-all	*T.* catch-all	*T. mutans*	*T. parva*	*T. taurotragi*	*T. velifera*	*Theileria* sp. (buffalo)	*Theileria* sp. (sable)	*T. buffeli*	*B.* catch-all 1	*B.* catch-all 2	*B. bovis*	*B. bigemina*	*B. occultans*	*B. rossi*
C1	+++	++					+++					++				+++		+++			
C2							+++	+++	+++			+++				+++		+++			
C3	++						+++	+++	+++	+++		+				++		++			
C4	+++																				
C5	+++					+++	+++	+++	+++			+				+++					
C6	+++						+++	+++	+++		+++	+++									
C7	+++						+++	+++	+++			+									
C8	+++				++		+++	+++	+++							+++		+++			
C9	+++						+++									+++		+++			
C10	+++	++					+++	+++		+++						+++		+++			
C11	++															++					
C12	+++	++					+++									+++		+++			
C13	+++	+																			
C14	+++	++					+++	+++				+++				+++					

C15	+++	++					+++	+++	+++		+++									
C16																				
C17																				
C18	+++	+					+++	+++	+		+++	+++				++		++		
C19	+++	+++					+++	++				+				+++				
C20	+++	++					+++	+++				+++								
C21	+++	++					+++	+++	+++		+++	+++								
C22																				
C23	+++	+		+			+++	+++	+		+++	+++				+		+		
C24	+++	++					+++	+++	+++			+++				+++		+++		
C25	+++	+					+++	+++	+++							+++				
C26	+++	+					+++	+++	+++		+++	+++								
C27	+++	+++					+++	+++				+++								
C28							+++									+++		+		
C29	+++	++					+++	+++	+++							+++		+++		
C30																				
C31	+++	+					+++	+++								+++		+++		
C32																				
C33	+++	+					+++	+++				+++				+++		+++		
C34	+++	+					+++	+++	+++			+++				++		++		
C35																				
C36							+++	+++				+++				+++	+++	+++		
C37	+++					++	+++	+++								+++		+		
C38	+++						+++	+++				+++				+		+		

C39	+++					+++	+++	+++	+++							++		+		
C40	+++						+++	+++								+++				
C41	+++	+			+		+++	+++	+++			+++				++				
C42																				
C43							+++	+++				+++								
C44																				
C45	+++			+++			+++	+++	+++			+++								
C46	+++	+					+++	+++	+++			+++				+++		+++		
C47	+++	+					+++	+++	+++		+++	+++				++		++		
C48							+++	+++	+++			+				+++				
C49																				
C50																				
C51																				
C52	+++	+					+++	+++			+	+				+++		+++		
C53																				
C54	+++	+					+++	+++	+			+++				++				
C55	+++	++		++			+++	+++	+++			+++								
C56																				
C57	+++	++																		
C58	+++					+++	+++	+++	+++											
C59	+++	+		+																
C60																				
C61	+++						+++	+++			+++					+++		+++		

C62							+++									+++	+++				
C63	+++			+			+++	+++				+++				+++		+++			
C64	+++	+					+++	+++				+++				+++		+++			
C65	++						+++		+++			+++				+++					
C66	+++						+++	+++	+++	+++		+++		++							
C67																					
C68	+++	+					+++	+++	+++							+++		+++			
C69																					
C70							++														
C71	+++	+++					+++	+++		+++											
C72																					
C73	+++	+					+++	+++								+++					
C74	+++			++			+++	+++				+				+++		+++			
C75																					
C76																					
C77	+++	+		+++			+++	+++	+++		++	+++				+++		+++			
C78	+++	+					+++	+++	+++			+++				+++		+++			
C79																					
C80	+++	+++					+++	+++		+++						+++					
C81	+++	+					+++	+++	+++	+++											
C82	+++	+++					+++	+++	+	+++		+++		++							
C83	+++	+++	+++	++	+		+++	+++	+++		+++	+++		+++		+++		+++			
C84	+++	+++					+++	+++	+++	+++	+++	+++		++							

C85	+++					+++	+++	+++				+++		+++						
C86																				
C87																				
C88	+++	+++		+			+++	+++				+++		+		+++		+++			
C89	+++						+++	+++		+++										
C90							+++	+++	+++		+++	+++		++		+++	+	+++			
C91	+++	+++					+++	+++		+++		+++								
C92	+++	+++					+++	++	++			++				++					
C93							++									+				
C94	+++					+++	+++	+++	+++							+++				
C95																				
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C97																				
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C100																				
C101	+++						++									++		++			
C102																				
C103	+++	+++				+++	+++	+++	+++			+				+++		+++			++
C104	+						++									+		+			
C105																				
C106																				
C107																				

C108	+++																			
C109	+++																			
C110	+++	+++					+++	+++		+++										
C111																				
C112	+++	+++				+++	+++	+++								+++				
C113	+																			
C114							+++									+++		+++			+
C115																				
C116																				
C117																				
C118																				
C119																				
C120																				
C121																				
C122																				
C123	+++	+++					+++	+++	+++	+++		+++		+++						
C124							+++									+++		+++		
C125																				
C126																				
C127																				
C128																				
C129							+++	+++		+++										
C130																				

C131																				
C132							+++	+++	+++		+++	+++		+++						
C133							+++	+++		+++		+++								
C134																				
C135	+++	+++					+++	+++	+++		+++	+++		+++		+++		+++			
C136	+++	+++					+++	+++												
C137	+++	+++					+++	+++			+++	+++								

+++	Positive
++	Faint positive
+	Very faint positive

Appendix 6: Permit for movement of cattle sera from Hans Hoheisen Wildlife Research Station, Bushbuckridge to Department of Veterinary Tropical Diseases, University of Pretoria, Pretoria.

Permit in	Permit out

Permit number: BR130131HS

DEPARTMENT OF AGRICULTURE, CONSERVATION AND ENVIRONMENT

MPUMALANGA

DEPT. LANDBOU, BEWARING EN OMGEWING

LITIKO LETEKULIMA, KONGIWA NETEMVELO

*, details of samples: as per attached list (initialled)

VETERINARY PERMIT TO MOVE ANIMALS / ANIMAL PRODUCTS
VEEARTSENYPERMIT VIR VERVOER VAN DIERE / DIEREPRODUKTE
IMVUMO YEKUHAMBISA TILWANE / TINTFO LETIHABULISAKO TETILWANE

In terms of the Animal Diseases Act No. 35 of 1984 permission is hereby granted to:
Kragtens die Wet op Dieresiektes Nr. 35 van 1984 word toestemming hiermee verleen aan:
Ngeligunya leMtsetfo weTifo teTilwane No. 35 wa 1984:

Name: / Naam: / Libito:	STOLTSZ, HEIN W.	ID No.:	
Address: / Adres: / Likheli:	UP, Vet Faculty, Vet. Trop. Diseases		
to move with: / om te beweeg met: / uvunyelwa yekuhambisa:	SIX-HUNDRED AND SEVENTY BOVINE SERUM SAMPLES		
identified as follows: / geïdentifiseer as volg: / letikhonjiswe nga:	packed in 7 trays with 96 ... *,		
from the farm/diptank area/place: / vanaf die plaas/dipbakgebied/plek: / kusuka epulazini/edibhini/endzaweni:	HHWRS	in the district of / in die distrik / esigodzini sase	EHLANZENI
to the farm/diptank area/abattoir/place: / na die plaas/dipbakgebied/abattoir/plek: / kuya epulazini/edibhini/emadzeleni/endzaweni:	OVI – REGISTRATION *	in the district of / in die distrik / esigodzini sase	TSHWANE

On the following conditions:
1. This permit:
(a) is **valid for 10 days** from date of issue and for **one consignment only**
(b) must **accompany the animals** / products mentioned above;
(c) must be produced for **inspection** on demand by any land or animal owner or police or veterinary official;
(d) must be **kept** at a destination until collected by a veterinary official.
2. For **game** / game products a nature conservation permit is also needed.
3. Animals and products must be visibly free from **external parasites.**
4. Animals are to be transported in accordance with **animal welfare** guidelines.
5. **Other conditions:**

Op die volgende voorwaardes:
1. Hierdie permit:
(a) is **geldig vir 10 dae** vanaf datum van uitreiking en slegs vir **een beweging;**
(b) moet die diere / produkte hierbo vermeld **vergesel;**
(c) moet vir **inspeksie** getoon word op aanvraag van enige grond-of diereeienaar of polisie- of veeartsenybeampte;
(d) moet by bestemming gehou word totdat dit deur 'n veeartsenybeampte geneem word.
2. Vir **wild** / wildprodukte word 'n natuurbewaringspermit ook benodig.
3. Diere en produkte moet sigbaar vry van **uitwendige parasiete** wees.
4. Diere moet volgens **dierewelsynsriglyne** vervoer word.
5. **Ander voorwaardes:**

Ngaphansi kwemibandzela lechazisiwe ngaphansi:
1. Lemvumo:
(a) **isebenta emalanga-lange 10** kusuka lusuku lwekukhishwa kwayo futsi **yekuhambisa kanye** kuphela;
(b) **Itawuhamba netilwane** / netintfo letihabulisako letibhaliwe ngenhla;
(c) itawukhombiswa **kuhlolwa** ngumuphi umnikati wendzawo kumbe umnikati wetilwane kumbe nguliphoyisa kumbe kusikhulu setekwelashwa kwetilwane, loyicelelako;
(d) **itawubhekwa** endzaweni yekufika khona tilwane / tintfo letihabulisako futsi itsatfwe ngesikhulu setekwelashwa kwetilwane.
2. **Kuhambisa kwetilwane tesiganga** / kwetintfo letihabulisako futsi kudzingwa imvumo yeTekongwa kweTemvelo.
3. Tilwane letintfo letihabulisako tetilwane tifanele kutsi tite **tidlangandvodza** letingabonakala.
4. Tilwane tifanele tihanjiswe ngaphansi kwetimiso **tekutiphilisa kahle.**
5. **Imibandzela leminye:**

* to be send directly for Reg. inactivation at OVI – TAD before testing at OVI; to be packed + labelled according to ... for infectious substance of Category B (UN 2900, P620)

6. Retention for / Quarantine for / Direct slaughter within: .... days after arrival at destination

Seal no/s	N/A	Place / Plek / Indzawo:	HHWRS
Vehicle reg. no/s	YCK 042 GP	Date / Datum / Lusuku:	13 / 01 / 31

I hereby certify that all applicable control measures for issuing of this permit have been complied with.

B. REININGHAUS — Issuing Veterinary Official:

State Veterinarian / Staatsveearts / Dokotela weTilwane

REPUBLIC OF SOUTH AFRICA
DEPARTMENT OF AGRICULTURE & LAND ADMINISTRATION
Directorate Veterinary Services
2013 -01- 31
312
DR. B. REININGHAUS
Reg. No: D06/7266
PROVINCE OF MPUMALANGA

IMPORTANT NOTE / BELANGRIKE NOTA / SATISO LESIBALULEKILE
Permit holder who does not comply with this permit or any condition thereof is guilty of an offence.
Permithouer wat in stryd met hierdie permit of enige voorwaarde daarvan optree, is skuldig aan 'n misdryf.
Lonalemvumo longalandzela lemvumo kumbe muphi umbandzela wayo uphula umtsetfo.

DISTRIBUTION: VETERINARY OFFICIAL AT DESTINATION

BarPrint 221097 ☎ 013 712-2723

Appendix 7: V047-12 Research Committee approval.

Ref: V047/12

University of Pretoria

16 October 2012

Faculty of Veterinary Science
Private Bag X04
Onderstepoort
0110

Tel: +27 12 529 8000
Fax: +27 12 529 8300

Dr NE Collins
Department Veterinary Tropical Diseases
(nicola.collins@up.ac.za)

Dear Dr Collins

PROTOCOL V047/12: CONFIRMATION OF DIAGNOSIS OF TICK-BORNE DISEASES IN MNISI AREA AND IDENTIFICATION OF *THEILERIA PARVA* CARRIER ANIMALS – *CN Choopa*

I am pleased to inform you that the abovementioned protocol was approved by the Research Committee.

Kindly note that, if there are animal ethical issues involved in the project, the protocol needs to be approved by the Animal Use and Care Committee as well before you may commence with the project.

Please take note of the attached document.

Kind regards

NIESJE TROMP
SECRETARY: RESEARCH COMMITTEE

Copy: Prof JAW Coetzer, Deputy Dean: Research (koos.coetzer@up.ac.za)
CN Choopa, Researcher (chimvwele@yahoo.co.uk)
Prof D Abernethy, HOD (darrell.abernethy@up.ac.za)
Prof M Oosthuizen, Departmental Research Coordinator (marinda.oosthuizen@up.ac.za)
Ms M Human, Student Administration (magda.human@up.ac.za)
Ms Elmarie Mostert, Animal Use and Care Committee (elmarie.mostert@up.ac.za)

Appendix 8: V047-12 Animal Use and Care Committee approval.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Confirmation of diagnosis of tick-borne diseases in Mnisi area and identification of Theileria parva carrier animals**
PROJECT NUMBER	**V047-12**
RESEARCHER/PRINCIPAL INVESTIGATOR	**CN Choopa**

STUDENT NUMBER (where applicable)	**UP_12377792**
DISSERTATION/THESIS SUBMITTED FOR	**MSc (Veterinary Tropical Diseases)**

ANIMAL SPECIES	**Clinical – cattle**	**Serum samples**	**EDTA blood**
NUMBER OF ANIMALS	**137**	**670**	**432**
Approval period to use animals for research/testing purposes		**October 2012 – October 2013**	
SUPERVISOR	**Dr. NE Collins**		

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date	October 2012
CHAIRMAN: UP Animal Ethics Committee	Signature	

S4285-15