Investigations of the *Theileria parva* carrier-state in cattle at the livestock/wildlife interface of the uPhongolo-Mkuze area in KwaZulu Natal, South Africa

by

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DECLARATION

I (Sikhumbuzo Mbizeni) declare that the dissertation I hereby submit for the degree Magister Scientiae at the University of Pretoria is my own work and where assistance was received, it has been reported as such in the text and the acknowledgements. The investigations reported in this dissertation have not been submitted by me for a degree at other University.

Signature……………………………   Date…………………….

ACKNOWLEDGEMENTS

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Acronyms and Abbreviations

$	Dollar
%	Percentage
ARC	Agricultural Research Council
DNA	Deoxyribonucleic Acid
CA	Capillary agglutination
CFT	Complement fixation test
CTL	Cytotoxic T Lymphocytes
EDTA	Ethylene diaminetetra acetic acid
KZN	KwaZulu-Natal
et al.	and others
ECF	East Coast fever
GIT	Gastrointestinal tract
GIS	Geographical information system
IFA	Immunofluorescent antibody
IHA	Indirect haemaglutinations
IT	Infection and treatment immunization
MAb	Monoclonal antibody
MHC	Major histocompatibility complex
OVI	Onderstepoort Veterinary Institute
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PIM	Polymorphic immunodominant
rRNA	Ribosomal ribonucleic acid
RT	real-time
SANAS	Southe African National Acreditation System
TpR	*T. parva* repetitive gene sequence
USD	United States Dollar
µl	microlitre

SUMMARY

Corridor disease (*Theileria parva* infection in cattle associated with carrier buffaloes) was not reported to cause serious outbreaks prior to 1994. From 2002-2004, outbreaks in cattle have increased in the areas where the disease is endemic in buffalo populations. In this study, the occurrence of Corridor disease outbreaks in the Zululand district municipality was closely monitored from 2004-2009. The observations included the number of cattle involved in the outbreaks, clinical signs, parasitological and post-mortem examinations while blood for serum and in EDTA were collected for serological (IFA test) and molecular (real-time PCR) tests specific for *T. parva*. Samples were collected from cattle involved in the outbreak, the sick and presumed recovered cattle. Recovered cattle from the farms were brought to the laboratory at the Onderstepoort Veterinary Institute for further investigations. This included tick pick-up and transmission attempts to demonstrate their carrier status as well as assessing their immunity to further experimental challenge using virulent *T. parva* stabilate.

Results were obtained on Corridor disease outbreaks in the study area and *ad hoc* locations comprising a total of 15 commercial farms and community diptanks in the district from 2004 to 2009. A total of 31 outbreaks were recorded during the study period. The number of outbreaks per year was stable, being 3 or 4 from 2004 to 2007. A 100 percent increase was recorded in the subsequent years, 2008-2009. In one location, Morgenzon farm comprising a commercial and community farmers, had experienced regular outbreaks from 2004-2009. It is also noted that some farms experienced outbreaks for three consecutive years. Three other farms had experienced outbreaks for the first time in either 2008 or 2009. The most severe outbreak occurred in Nyalisa in 2009 where the disease was experienced for the first time in one herd in which 202 cattle were involved and 57 died within 30-40 days after the onset of the disease. Using all the tools mentioned above, the cause of death was confirmed to be due to *T. parva* infection.

The Corridor disease outbreaks that were investigated, have mostly been reported during the months from March-May (88 %) but some (8 %) were encountered during the winter months (June-August). The distribution of outbreaks mainly coincided with the activity period of adult *R. appendiculatus*.

During the investigation period, a total of 846 cattle were tested for Corridor disease and the prevalence was found to be 27 %. The percentage of cattle which were found positive by PCR was 16.5. Seven percent were found positive on both PCR and IFA tests, an indication of the development of a carrier state. However, 10 % of the cattle remained sero-positive with no indication of being parasite-carriers (real-time PCR negative).

Five cattle which recovered from an apparent severe *T. parva* infection in the field and confirmed to be positive by PCR, all became negative before they were used in the transmission experiments. Ticks derived from these cattle were used to infect susceptible bovines but only *T. taurotragi w*as transmitted. The xeno-diagnosis failed to demonstrate the carrier state in these field cattle.

 The five Corridor disease recovered cattle obtained from different study locations mentioned above, received lethal challenge using *T. parva* buffalo-derived stabilate. All challenged animals, including the susceptible control, showed schizont parasitosis as detected by the *T. parva* real-time PCR test starting day 11 to 23. All animals also developed significant antibody titer to *T. parva* by day 28. Of the field cattle, only one bovine which showed mild reactions manifested by high temperature on day 11 for two consecutive days and schizonts parasitosis in lymph nodes on day 15 for only two days and recovered. The rest of the field cattle did not show any clinical or parasitological reactions during the observation period (103 days). The control bovine had high fever and showed schizonts parasitosis by day 11 for seven consecutive days. The reaction was classified as severe and had to be treated.

Unfed *R. appendiculatus* collected off grass from one of the study sites were applied to feed on a susceptible bovine and only *T. taurotragi* was transmitted. There were no apparent clinical signs and the animal behavior kept normal during the observation period (60 days).

This study suggests that Corridor disease should be considered as an "emerging disease" and more stringent control methods should be implemented.

CHAPTER 1
General introduction

1.1   Background

Buffalo-associated theileriosis, commonly known in South Africa as Corridor or buffalo disease, is a fatal disease of cattle caused by buffalo-derived *Theileria parva* stocks transmitted by the African brown ear ticks, *Rhipicephalus appendiculatus* and *Rhipicephalus zambeziensis*. Corridor disease outbreaks are generally encountered when there is contact between carrier African buffalo (*Syncerus caffer*) and susceptible cattle or when cattle graze pastures where carrier buffaloes have been. In the face of an outbreak mortality rate can exceed 90 % (Neitz *et al*., 1955; Potgieter *et al*., 1988).

In recent years, there has been an increase in interest on game farming as part of the growing and lucrative ecotourism industry in the country including KwaZulu-Natal Province (KZN) (Stoltsz, 1996a). Buffalo is one of the most sought-after game animals, the so-called "Big-Five", to be introduced into private game reserves. More cattle farms in the area of Madugu, Mkhuze, uPhongolo in the Northern KZN have been converted into game farms or game ranching enterprises.

Consequently, there has been an increase in the introduction of *Theileria parva*-infected buffaloes onto private game farms in the above-mentioned and other areas of KZN province. National legislation on keeping of buffaloes prescribed that *Theileria parva*-infected buffaloes can only be kept on registered game farms and those farms must be within the designated corridor line of the province. One of the requirements for registration is that *T. parva*-infected buffaloes must be kept on farms adequately enclosed with game proof fence which are electrified on the inside with minimum voltage of 5500V at all times (Animal Diseases Act 984, Act No. 35).

Prior to 2002 Corridor disease outbreaks were reported only on cattle farms neighboring game reserve in the Hluhluwe area. It was believed that the control measures were adequate. In most instances the source of outbreaks would be traced back to the buffalo that had been seen wandering around the cattle grazing areas (Annual report of Directorate of Veterinary Services: Allerton reports No: 02/2000; 02/2001 and 01/2002).

In the following years there was an increase in Corridor disease outbreaks that were reported in Vryheid and Nongoma State Veterinary areas (Annual report of the Directorate of Veterinary Service KZN, No. 01/2003 and 01/2004; Latif *et al*., 2005; Thompson *et al*., 2008). Most of the outbreaks investigated were confirmed by Onderstepoort Veterinary Institute (OVI) to be *T. parva* infections using serology and molecular diagnosis. The course of the disease recognized from these outbreaks seemed to be different from what is believed to occur in the epidemiology of Corridor disease in South Africa, in which case the disease was considered to be invariably fatal in cattle. In some outbreaks not all cattle that showed clinical signs had died but some fully recovered. Several factors have been shown to have contributed to cattle to recovery from the disease ranging from a low dose infection with *T. parva* parasites to a possibility of treatment with oxytetracyclines during early stages (incubation) of the disease (Dolan, 1981; Dolan *et al*., 1984).

1.2   Justification

If Corridor disease is not adequately controlled, it poses a serious threat to cattle-farming communities in the area. The Corridor disease designated area which allows keeping of infected buffaloes is inhabited by more than 40 000 head of cattle belonging to communal communities. These cattle are at risk of contracting Corridor disease (Annual report of the Directorate of Veterinary Service KZN - Allerton report No. 01/2003 – Annual Stock census; Latif *et al*., 2005). For control measures to be effective, legislation has to be derived from informed latest research on the epidemiology of the disease.

The introduction of East Coast fever (ECF, caused by *Theileria parva*) from East Africa in the year 1902 resulted in the death of about one million cattle (Potgieter *et al.,* 1988). It was eradicated in the mid 1950s through very expensive measures including the introduction of chemical tick control, quarantine measures and slaughter policies. However, the great

abundance of the vector ticks poses the risk of the reintroduction of ECF. On the other hand, Corridor disease remains to be a problem of great concern. The cattle-derived *T. parva* stocks which cause either ECF or January disease and maintained by cattle to cattle transmissions have not been reported to occur or to cause carrier state in cattle population in South Africa (Potgieter *et al.*, 1988). It has however been demonstrated in East Africa that buffalo-derived *T. parva* strains after serial passage in cattle by means of a vector tick can behaviorally change and become parasitologically indistinguishable from cattle-adapted *T. parva* stocks (Grootenhuis *et al.*,1987) .

Many of the commercial farmers who have suffered serious losses in the face of Corridor disease outbreaks have sold their farms and moved to other areas where there are no *T. parva*-carrier buffalo. Other substantial economic losses include the cost of dipping during the outbreak, the loss of valuable cattle, loss of the source of income and the risk of re-infections. The already crippled cattle-farming community due to Corridor disease cannot survive the advent of cattle-adapted *T. parva,* which is causing a lot of economic losses in the countries in which they occur.

1.3 General Objectives:

The main aim of this study was to establish the possible *T. parva* carrier status of the cattle which are observed to have recovered from the disease and their possible role in the maintenance or spread of the disease. The study will provide information on the magnitude of the problem (as numbers of outbreaks) and also help to better understand the epidemiology of Corridor disease in the livestock/game interface in uPhongolo-Mkuze area.

CHAPTER 2
Literature Review

2.1 Brief introduction on the epidemiology of bovine theileriosis in Southern Africa

Bovine theileriosis is a tick-borne disease associated with infection by one of various *Theileria* species. *Theileria* species are obligate intracellular protozoan parasites that infect a wide range of both wild and domestic animals. They are transmitted by ixodid ticks, and have complex life cycle in both the mammalian and tick hosts. The most pathogenic and economically important *Theileria* spp. of cattle in Africa are *Theileira parva* and *Theileria annulata,* as they are responsible for almost all cattle mortalities caused by theileirosis in countries where they occur (Norval *et al*., 1992). *Theileria parva* parasites are responsible for a variety of disease syndromes known as Corridor disease, East Coast fever (ECF) and January disease in central, eastern and southern Africa. *Theileria annulata* is the causative agent for Tropical theileriosis in the northern and the Mediterranean Region of Africa, southern Europe, Middle East and central Asia (Norval *et al*, 1992; Lawrence *et al*., 2004a, b, c).

Several other *Theileria* species are regarded to be of low or no pathogenicity in cattle. These are *Theileria mutans, Theileria taurotragi, Theileria velifera* and *Theileria sergentii/ Theileria orientalis/ Theileria buffeli* group. *Theileria mutans* is usually considered to be of low pathogenicity but sporadic fatal infections have been reported. *Theileria taurotragi* is also usually considered to be of low pathogenicity in cattle but severe cases had been reported and it has also been implicated as the possible cause of cerebral theileriosis disease (Lawrence and Williamson, 2004). *Theileria taurotragi* infections in eland have been reported to be fatal (Norval *et al*., 1992). In South Africa, *Theileria* species sable is considered of economic importance, causing serious mortalities in roan antelope (*Hippotragus equinus*) and sable antelopes (*Hippotragus niger*) (Stoltsz, 1996b)

2.1.1 East Coast fever (ECF) (cattle-derived *T. parva* infection)

East Coast fever (ECF) is a fatal lymphoproliferative disease of cattle caused by cattle-derived *T. parva* (formerly *T. parva parva)* parasites. ECF was introduced in the region south of Zambezi in the period 1901 to 1903 with the cattle that were imported from East Africa for restocking after a devastating epidemic of rinderpest in 1896 (Lawrenc*e et al.,* 2004a). It was subsequently eradicated from South Africa by 1955, from Zimbabwe by 1954 and southern Mozambique by 1917. Eradication of ECF in these countries was through implementation of stringent control measures such as quarantine, intensive dipping, and slaughter of recovered cattle and where possible resting of infested pastures for 15-18 months (Lawrenc*e et al.,* 2004a). The disease was believed to be one of the economic disasters occurring in the country over the past century. ECF is still prevalent in certain areas of eastern, central and southern Africa and is regarded as the most economically important tick-borne disease in those areas. The economic impact of the disease has been estimated at $USD 169 million per year (Mukhebi *et al*., 1992).

Pathogenesis of this disease may be divided into three stages: the pre-patent stage, the lympho-proliferative stage and the lympho-destructive stage. The prepatent stage is characterized by the inoculation of *Theileria* sporozoites by a tick and demonstration of schizonts in the draining lymph nodes in about two to three days. This is accompanied by onset of fever (Lawrence *et al.,* 2004a). Once inside the lymphocyte, the sporozoites develop into schizont stages while at the same instance transforming the host cell into a lymhoblastoid type with the capacity to divide repeatedly (Irvin, 1985; McKeever *et al*., 1999; Lawrence *et al.,* 2004a). Parasitized lymphoblasts stimulate active lymphoid proliferation and dissemination of large numbers of infected and non-infected lymphoblasts throughout the body, which invade other lymphoid and non-lymphoid tissue. Lymphoid proliferation is followed by extensive lymphocytolysis associated with non-specific activation of the immune system, particularly the cellular response mediated by cytotoxic T lymphocytes (CTLs) (McKeever *et al.,* 1999; McKeever, 2001; Rocchi *et al*., 2006). A

characteristic feature of this disease in its advanced stages is a pronounced leukopenia which is due to massive depletion of lymphocytes in the lymphoid organs. The disease is known to be highly fatal in cattle but the outcome of infection is also influenced by various factors such as the dose of the transmitted sporozoites, breed and susceptibility of the individual animal. Incubation period is generally about 15 days but may range between 8 and 25 days. Susceptible cattle usually succumb to severe pulmonary oedema (McKeever *et al*., 1999; Lawrence *et al*., 2004a). *Theileria parva* carrier state is well established and maintained by cattle that have either spontaneously recovered or those that have been treated and subsequently recovered from *T. parva (parva)* infections (Dolan *et al.,* 1984).

2.1.2   Corridor disease or buffalo disease (buffalo-derived *T. parva* infection)

Corridor disease is an acute mostly fatal disease of cattle that is caused by infection with buffalo-derived *T. parva* parasites, formerly known as *T. parva lawrencei*. The disease was first reported in South Africa with an outbreak that occurred in the corridor between Hluhluwe and Umfolozi game reserves in the Northern KwaZulu-Natal (Neitz *et al*., 1955). Prior to this outbreak a similar disease syndrome had been recognized in Zimbabwe in 1934 and was associated with buffaloes, hence the name buffalo disease (Lawrence, 1979). Corridor disease outbreaks in cattle populations can result in a mortality rate of more than 90 % (Neitz *et al*., 1955; Potgieter *et al.,* 1988). It is believed to be self-limiting in cattle populations, however, once cattle are removed from the source of infection, even in the presence of vector tick, *R. appendiculatus,* meaning there was no development of a carrier state in recovered cattle. Buffalo-derived *T. parva* infections in cattle are also characterized by low schizont parasitosis and very low piroplam parasitaemia, which are the infective stages to vector ticks (Neitz *et al*., 1955; Potgieter *et al.,* 1988*;* Uilenberg, 1999; Lawrence *et al.,* 2004b; Thompson *et al*., 2008).

In South Africa, Corridor disease carrier buffalo populations are found in the greater Kruger National Park and registered adjacent buffalo farms in the Mpumalanga and Limpopo provinces. They are also found in Hluhluwe-iMfolozi, Eastern Shores, Ndumu and Tembe game reserves as well as on registered private buffalo farms in the Corridor disease controlled area of KwaZulu-Natal (Animal Diseases Act 1984, Act No. 35). To ensure that infected buffaloes do not come into contact with susceptible cattle, buffaloes are kept

separately on registered and appropriately fenced game reserves. Corridor disease outbreaks are usually encountered whenever cattle graze pastures where infected buffaloes are or have been within a distribution range of a brown ear ticks (Lawrence *et al.,* 2004b).

The pathogenesis and clinical features of this disease are very similar to that of ECF. A distinguishing feature of Corridor disease is that its course is usually shorter: infected cattle can die within four days of the onset of clinical signs. Secondly, schizont parasitosis and piroplasm parasitaemia are not as pronounced as with ECF. Although buffalo-derived *T. parva* infections are considered to be lethal in cattle, severity of the disease is also dependent on other factors, such as the dose of infective sporozoites and the virulence of the strains (Mutugi *et al*., 1988).

Buffalo-derived *T. parva* carrier state in the cattle populations has been demonstrated in other African countries such as Zimbabwe, Kenya, Tanzania (Grootenhuis *et al.,* 1987; Kariuki *et al*., 1995; Latif *et al*., 2001a; Latif *et al*., 2002; Kivaria *et al*., 2007). In South Africa, carrier state in cattle has only been demonstrated under experimental conditions with splenectomized recovered animals and also in cattle that were treated with anti-theilerial drugs such as halofuginone after infection with buffalo-derived *T. parva* (Neitz, 1958; De Vos, 1982; Potgieter *et al*., 1985; Potgieter *et al.,* 1988).

Recently, Thompson *et al.,* (2008) carried out epidemiological investigations into Corridor disease outbreaks on a cattle farm in the Ladysmith area of KwaZulu-Natal. They failed to demonstrate the occurrence of the carrier state by tick transmission experiments although molecular and serological tests had confirmed the presence of *T. parva* parasites in recovered cattle. It should be noted these cattle became real-time PCR negative to *T. parva* prior to the transmission experiment being carried out. Research work carried out at the communal cattle herds neighboring the Hluhluwe-iMfolozi game park demonstrated the occurrence of sub-clinically *T. parva* infected animals (Yusufmia *et al*., 2010).

2.1.3   January disease or Zimbabwean theileriosis (cattle-derived *T. parva* infection)

January disease is a tick-borne disease caused by an infection with cattle-derived *T. parva* parasite formerly knows as *T. parva bovis*. The *T. parva* (*bovis*) parasites are distinguished from the *T. parva* stocks that cause ECF mainly on biological and epidemiological features (Uilenberg *et al*., 1982). This disease is mainly reported during rainy season (January to April) which coincides with the activity of adult *R. appendiculatus* and has only been diagnosed in Zimbabwe hence the name Zimbabwean theileriosis (Lawrence, 1979; Lawrence *et al.,* 2004c).

Eradication of ECF from Zimbabwe in 1954 was associated with the simultaneous elimination of non-diapausing eastern African strains of *R. appendiculatus*. This epidemiological feature has led to a notion that nymphal stages (June–October) under field conditions are not significant in the occurrence of Zimbabwean theileriosis *(*Koch *et al*., 1993).

Recently, Latif *et al*., (2001b) carried out epidemiological studies on this disease and actually demonstrated that nymphal stages of *R. appendiculatu*s under natural conditions can transmit *T. parva* parasites resulting in fatal disease. They also showed that Zimbabwean theileriosis transmitted by the adult stages of *R. appendiculatus* can be as pathogenic as ECF. They believed that various management conditions, such as keeping of cattle with game animals and grazing cattle in crop fields during dry months (June–October), limit contact between cattle and ticks (larvae). These management practices consequently reduce the incidences of the disease during nymphal active season (Latif *et al.,* 2001b).

The pathogenesis of January disease is also similar to that of ECF, but was regarded by Irvin *et al*., (1989) to be of lower virulence than the latter. A characteristic feature of Zimbabwean theileriosis is that *T. parva* (bovis) stocks produce very few schizonts and

piroplasms are infrequent, which is comparable to that of buffalo-derived *T. parva* (*lawrencei*) parasites (Lawrence, 1979; Lawrence *et al.,* 2004b).

2.2   Transmission and life cycle: *Theilera parva* parasites

Theileria parva are obligate intracellular protozoan parasites of the African buffalo known to cause ECF, Corridor disease and January disease in cattle. It has recently been discovered that buffalo-derived *T. parva* parasites can also infect waterbuck, which may be a reservoir host (Lawrence *et al.,* 2004b). Under natural conditions *T. parva* is mainly transmitted by the three-host brown ear ticks, *R. appendiculatus,* but in more arid areas of southern Africa, *R. zambeziensis* becomes the principal vector (Norval *et al*., 1992; Lawrence *et al*., 2004b). Natural transmission of *T. parva* parasites by the vector tick is solely transstadial. This means that the parasites can only be transmitted by either the nymphal or adult stages of the tick after being acquired by preceding stages during feeding.

In its life cycle, *T. parva* (Figure 2.1*)* undergoes various distinct and complex developmental stages in the mammalian host and in the vector tick *R. appendiculatus.* It is influenced by other various intrinsic factors, such as vector competence (Young and Purnell, 1973) and also extrinsic factors like temperature (Young *et al*., 1984) that determine the success of parasite acquisition and transmission between the host and the vector.

The main distinct growth stages known to occur in the mammalian host are the macro- and micro-schizonts, the merozoites and the piroplams. In the vector, the main developmental stages are gametocytes which develop into gametes (sexually differentiated into macro or micro-gametes), zygote, kinete and eventually into sporozoites in the salivary glands, which are the infective stages for the mammalian host (Norval *et al*., 1992; Lawrence *et al*., 2004a). It is believed that genetic recombination between different *T. parva* strains occurs during the sexual stage which contributes to the wide antigenic diversity of *T. parva* parasites in the field (Katzer *et al*., 2009).
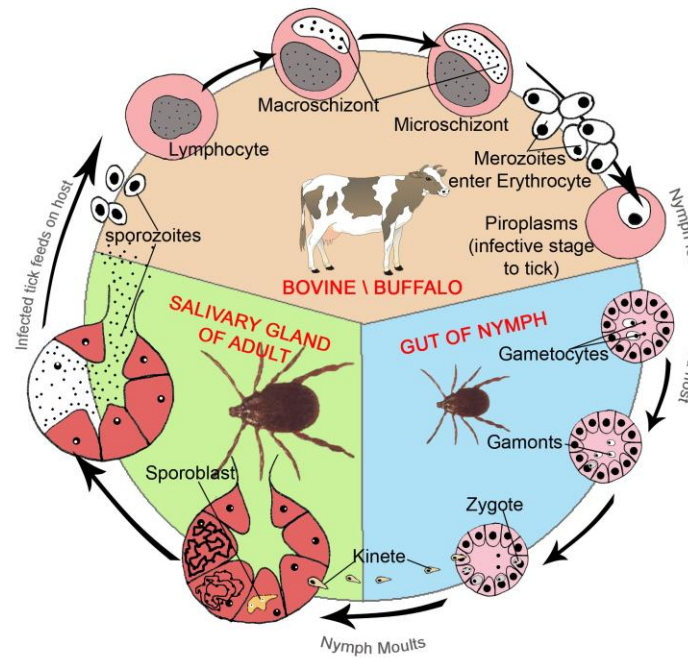
Figure 2.1: Life-cycle of *T. parva* in bovine and ticks. Modified from a figure prepared by A.S. Young (Norval *et al.*, 1992)

2.3. Transformation of buffalo-derived *T. parva* into cattle-adapted *T. parva*

In experimental serial cattle-tick transmission attempts of a buffalo-derived *T. parva* isolate in East Africa, it was found to have transformed into a form behaviorally indistinguishable from a cattle-adapted *T. parva* type (Barnett and Brocklesby, 1966; Maritim *et al*., 1992; Grootenhuis *et al.,* 1987). In South Africa, attempts to transform a buffalo-adapted *T. parva* into cattle–adapted type have not been successful (Neitz, 1957; De Vos, 1982; Potgieter *et al*., 1988). However, the South African isolate from buffalo was apparently transformed into cattle-adapted strain in Kenya (Potgieter *et al.,* 1988; Lawrence *et al.,* 2004b). The phenomenon of "transformation" of buffalo-derived *T. parva* into cattle-adapted is still a controversial research subject. There is a view that parasite population selection might have occurred rather than parasites transformation. This is based on the fact that buffalo-derived *T. parva* infections are associated with transmission of several or a mixture of stocks of the *Theileria* parasite, and during the process of continual infection of cattle through the vector tick, a *T. parva* subpopulation that is adapted to cattle is selected for (Collins *et al*., 2002).

2.4   Immunity and Diagnosis

2.4.1 Immunity

There is a wealth of literature in the subject of immunity and in the understanding of immunobiology (Irvin, 1985; McKeever *et al.,* 1999; McKeever, 2001; Kratzer *et al*., 2007; Morrison, 2009). The following paragraph is only a resumé which is also important to our understanding of the diagnosis of the *T. parva*. Infection with *T. parva* parasites either by feeding of infected ticks or by the inoculation of sporozoites stabilate elicits both cellular and humeral immune response in the mammalian host. It has been established that cell-mediated responses play a significant role in the protection and recovery of animal after an infection with *T. parva*. The cell-mediated responses are directed against the schizont-

infected lymphoblasts. It has been demonstrated that although all lineages of bovine lymphocytes are susceptible to infection by *T. parva*, the T-cell lineage is mostly infected (McKeever *et al.,* 1999). The key mechanism responsible for elimination of infection is the activation of cytotoxic T lymphocytes (CTLs) of CD8$^+$ subsets of T cells, which have the capacity to lyse autologous parasitized cells. The parasite specificity of CTLs has been shown to be mediated by genetically-restricted major histocompatibility complex (MHC) class I on the surface of the schizont-infected lymphoblasts (McKeever *et al.,* 1999; McKeever, 2001).

Immunity developed to one parasite stock does not necessarily confer protection against other stocks. Cattle that have recovered from *T. parva* infections either spontaneously or after treatment develop strong immunity against a homologous stock, but might still be susceptible to a different stock of the same parasite (Morrison, 1984; Irvin, 1985; McKeever *et al.,* 1999; Lawrence *et al.,* 2004a).

Although humoral response to *T. parva* infections does not seem to be significant in the protection and recovery of infected cattle, anti-sporozoite antibodies have demonstrated capacity to neutralize infectivity of sporozoites, not only to homologous strains but also to heterologous stocks (McKeever *et al.,* 1999). Anti-schizont antibodies are not thought to be important in recovered cattle but only as a basis for serological diagnostic test for *T. parva* infections (Lawrence *et al.,* 2004a).

2.4.2 Diagnosis

2.4.2.1 Clinical and parasitological manifestations

Provisional diagnosis of any of the theileriosis disease syndromes can be made on the following observations: characteristic clinical signs, demonstration of schizonts and piroplasms in the lymph node aspirates and smears of peripheral blood. Reactions to infection with *T. parva* can be classified according to a number of parameters. The recommended classification, according to the ECF workshop held in Lilongwe, Malawi (Anon., 1989) is as follows:

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

This classification assists to describe the clinical presentation of the particular strains of *T. parva* in individual animals in different herds and regions. For instance, infection with certain *T. parva* strains has been observed to result in milder clinical reactions than others. However, definitive diagnosis cannot be made by clinical and parasitological findings as different *Theileria* species or sub-species may be indistinguishable on these characteristics but might provide a useful indication of the possible diagnosis (Lawrence, 1979; Lawrence *et al*., 2004a**)**

Other epidemiological observations such as prevalence of the tick vectors, *R. appendiculatus* and *R. zambesiensis,* in the locality of the outbreak and also the presence of Corridor disease carrier-buffalo on the same vicinity also assist with the correct diagnostic decision.

2.4.2.2 Xenodiagnosis

Xenodiagniosis has been used for years as definitive method to determine a *T. parva* carrier state (Young *et al*., 1981). This method involves experimental tick pick-up and transmission from an infected animal to susceptible host. Usually the nymphal stage of *R. appendiculatus* is applied to feed and the subsequent adult stage is used for transmission. The drawback with this method is that it is expensive, time consuming and the results are intermittent. The *T. parva* carrier animal might harbour only scant *Theileria* piroplasms and the transmission attempt fails to demonstrate the status of the animal (Latif *et al.*, 2001a). It is also not

practical to apply this diagnostic method in large scale in the field (Potgieter *et al.,* 1988; Lawrence *et al.,* 2004a).

2.4.2.3   Post-mortem examination

Theileria parva infections produce various pathological features which are almost similar in all three disease syndromes, i.e. ECF, Corridor and Zimbabwean theileriosis. The most prominent autopsy finding is severe pulmonary oedema which is normally associated with a copious amount of froth in the upper respiratory tract. General petechiation and massive ulceration occur in the abomasum and other parts of the gastrointestinal tract. Hydropericardium, hydrothorax ascites, epi- and endocardial haemorrhages are also found in some cases. Opacity of the cornea of the eyes, emaciation and white-spotted kidney (multifocal lymphoid hyperplasia) are also observed (Neitz *et al.,* 1955; Norval *et al.,* 1992*;* Lawrence *et al.,* 2004a). In the dead animal demonstration of schizonts and piroplasms on impression smears of lymph nodes, spleen, liver, lungs and kidneys are also characteristic. Smears should be stained with Giemsa's or any Romanowsky stains and are then examined under compound light microscope.

2.4.2.4   Serological Methods

Various serological tests have been used to demonstrate antibodies to *T. parva.* These include tests such as indirect haemaglutination tests (IHA), capillary agglutination (CA) and complement fixation test (CFT). These test methods had limited diagnostic value as they lacked specificity (Norval *et al.,* 1992).

Indirect Immunofluorescent Antibody Test (IFA)

The IFA is a serological test that is most widely used in the diagnosis and field surveys of bovine theileriosis. The diagnostic principle of the test is based on the detection of

antibodies using cultured *T. parva* schizont infected lymphoblasts as the antigen (Lawrence *et al.,* 2004a; OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 2008). The IFA test is able to detect *T. parva* antibodies in animals at about 30 days post-infection. Antibodies continue to be detectable for about 6 months after recovery in the absence of challenge. The main disadvantage with this test is that it is laborious to carry out and the fluorescent interpretations can be subjective (Burridge and Kimber, 1972; Norval *et al.*, 1992; Collins *et al.*, 2002; OIE, 2008). At low antibody titres, especially in the absence of continuous challenge, mixed infections can result in cross-reaction with *T. taurotragi* in areas where the distribution ranges of these two parasites overlap (De Vos, 1992*;* Norval *et al.,* 1992*;* Lawrence *et al.*, 2004).

Enzyme-linked immunosorbent assay (ELISA)

ELISA is another serological assay that has been developed to detect *T. parva* antibodies using recombinant polymorphic immunodominant molecules (PIM) found in both sporozoite and schizont lysates. PIM is a surface protein highly expressed by both schizonts and sporozoites and is considered to be the most immunogenic antigen of *T. parva* for induction of antibodies. PIM-based ELISA has been shown to be better than other ELISA using other recombinant proteins (p67 and p104 antigens) as well as than IFA, as it has a higher degree of sensitivity and specificity than the latter (Katende *et al.*, 1998).

Monoclonal antibody screening assays (MAb)

Serological characterization assays make use of a panel of anti-schizont monoclonal antibodies (MAbs) that recognize epitopes on the PIM of *T. parva* schizonts. The reactions to the panel of MAbs against schizont-infected cells derived from *in vitro* cultures are determined by an indirect fluorescent antibody (IFA) test. Extensive studies with several schizont MAbs of *T. parva* stocks have demonstrated that there is wide antigenic diversity in *T. parva* stocks and the diversity is more on buffalo-derived isolates than on cattle-derived isolates (Norval *et al.,* 1992). Using MAb and molecular characterization of 40 *T. parva* schizont-infected lymphocyte culture isolates of cattle originating mostly from northern and eastern Zimbabwe, Bishop *et al.*, (1994) showed relative homogeneity of the cattle-derived parasite population.

2.4.2.5 Molecular Methods

The advent of molecular diagnosis has led to the development of molecular techniques ranging from conventional PCR and DNA probes to the new most sophisticated techniques such as real-time PCR (RT-PCR). Molecular diagnostic techniques have improved sensitivity and specificity in the detection of DNA of organisms that cause theileriosis that previous diagnostic tests lacked (Collins *et al.,* 2002; Sibeko *et al*., 2008).

Convenctional PCR assays make use of primers derived from a repetitive gene sequence (TpR) to amplify *T. parva* DNA in experimentally infected animals but fail to do so in field infections. The main drawback of this technique is that the TpR locus used exhibits some degree of polymorphism among *T. parva* stock and was not suitable for amplification of DNA from all field isolates including ECF-causing *T. parva* stocks such as *T. parva* "Muguga" (Bishop *et al*., 1992; Bishop *et al*., 1995). The TpR also lacked specificity as they amplified related *Theileria* species.

PCR/Probe assays were developed which make use of *Theileria*–specific primers for amplification of small subunits of ribosomal RNA (18S rRNA) gene to detect any *Theileria* parasites and specific oligonucleotide probes which detect different *Theileria* species. This technique has been used to differentiate *Theileria* spp. such as *T. parva*, *T. taurotragi*, *T. mutans*, *T. velifera* and *T. buffeli* as well as to discover previous unknown species such *Theileria* sp. (buffalo) (Collins *et al.,* 2002). This technique has improved sensitivity and specificity but is cumbersome and time consuming (Sibeko *et al.,* 2008).

Real-time PCR assays are newly developed molecular techniques that have recently been adopted by OVI as a diagnostic test for *T. pava* infections in buffaloes and cattle (Sibeko *et*

al., 2008). This test is highly sensitive and can detect *T. parva* with almost 100% certainty in carrier animals with very low piroplasm parasitaemia. The *T. parva*-specific RT-PCR is based on oligonucleotide primers and hybridization probes designed from 18S ribosomal RNA (rRNA) gene. The primers amplify both *T. parva* and the closely related *Theileria* sp. (buffalo) which can compromise the specificity of this assay but the hybridization probe specifically detects *T. parva* amplicons. This drawback has been rectified by the use of melting curve analysis after PCR amplifications to discriminate between *T. parva* and *Theileria* sp. (buffalo) with the use of RT-PCR hybridization probe chemistry (Sibeko *et al*., 2008). Recently, the problem of the previous test has been rectified by using a different RT-PCR hydrolysis assay which detected *T. parva* only (Papli *et al*., 2011).

2.5 Control of Bovine Theileriosis

2.5.1   Tick control

The method of control of ticks practiced in most of African countries is by the application of chemical acaricides to kill ticks through various ways such as plunge dipping, spraying, pour-on and the selective application on animals by hand-dressing. The introduction of chemical control of ticks originated in South Africa to eradicate the newly introduced ECF into the country (Norval *et al.,* 1992). This control method also resulted in the eradication of ECF in Swaziland, Mozambique and Zimbabwe (Norval *et al.,* 1992). It was also the main method of control of ECF in the central and central Africa before the development of the infection and treatment vaccines in the 1970s and anti-theilerial drugs (Norval *et al*., 1992; Lawrence *et al*., 2004a).

In spite of over 100 years of intensive dipping of cattle in South Africa, ticks have not been eradicated, nor has their population abundance been reduced. This dipping policy resulted in ticks developing resistance to acaricides and South Africa has acquired a bad reputation (Mekonen *et al*., 2002). On the other hand, in areas where dipping policy has been relaxed and there is no enforcement, endemic stability to babesiosis and anaplasmosis became evident (Tonnesen *et al*., 2006).

After the eradication of ECF in South Africa in the 1950s, the only theileriosis disease syndrome of major concern is Corridor disease caused by buffalo-derived *T. parva*. The main control strategy for Corridor disease is to prevent contact between infected buffalo and cattle which is achieved by keeping buffalo in securely fenced game ranches (Lawrence *et al*., 2004b). In the face of an outbreak, cattle are immediately removed from infected pastures and a regular tick dipping programme instituted. The infected pastures are then rested for approximately 15 to 18 monthly where practically possible. This control measure is not always practical to apply and it might be advisable to graze other animals such as sheep and goats that would help to clear up the infection from the vector ticks (Lawrence *et al*., 2004b).

2.5.2 Chemotherapy

Extensive research on effective anti-theilerial drugs started with the use of tetracycline compounds. It has been shown that treatment with tetracyclines during the early stages of the disease suppresses the development of *T. parva* infections (Neitz, 1958). The therapeutic activity of tetracycline was limited by the fact that infections have to be detected at early stages, at the pre-patent period, for the therapeutic regimen to be effective. This practice is not always practical in the field. Oxytetracyclines have been reported to have an effect on the early development of sporozoites into macroschizonts and the rapidly multiplying infected lymphoblasts and, as such, can prevent the onset of clinical disease (Young *et al*., 1990; Norval *et al.,* 1992)

Significant advances in the development of effective therapeutic compounds came with the discovery of the naphthoquine compound, menoctone. It was demonstrated to be very effective against *T. parva,* even when administered later during clinical theileriosis, but the compound was not further developed because it was expensive (McHardy and Rae, 1981). Another compound that had been shown to be effective against *Theileria* infections is halofuginone, a quinazoline compound. Halofuginone was formulated for oral administration as it was found to be highly irritant for parenteral route of administration. Its therapeutic activity is effective against the schizont stages but not the piroplasm of *T. parva* infections (Norval *et al.,* 1992).

Parvaquone (Clexon), also a naphthoquine compound, was then developed and shown to be effective against wide variety of *T. parva* stocks. It was formulated to be administered by intramuscular injection, at a recommended therapeutic dose of 10 mg/kg repeated after 48 hours. It was demonstrated to be effective against both the schizont and piroplasm stages of the parasites (Norval *et al.,* 1992).

Pursuit for more potent and safer antitheilerial drugs led to the development of Buparvaquone (Butalex, Coopers Animal Health), which is a second-generation hydroxynaphthoquine related to parvaquone. It is believed to be much more potent than parvaquone, particularly more effective against clinical theileriosis in the field. Buparvaquone is also compounded as an intramuscular formulation administered at a recommended dose of 2.5 mg/kg for the treatment of clinical theileriosis. A two-dose regimen is advisable with doses given 48 hours apart in cases where the disease is detected late (McHardy, 1989).

Because of fear of development of carrier state (section 2.6), treatment of bovine theileriosis is prohibited by law in South Africa (Animal Diseases Act 1984, Act No. 35). According to the regulations, animals that recovered from clinical Corridor disease should be sent for slaughter. f

2.5.3 Vaccination

Immunization of cattle against *T. parva* infection was first attempted in South Africa in 1911 (Theiler, 1911 cited in Norval *et al.,* 1992). Immunization involved intravenous injection of infected cells obtained from spleen and lymph nodes of sick cattle into susceptible cattle. This attempt was not successful. It resulted in lots of cattle dying after vaccinations and was discontinued.

Infection and treatment immunization (IT) is the method that is currently in use in central, eastern and southern African countries where *T. parva* infections are prevalent in cattle populations. Infection and treatment was started with the early observations by Neitz (1953 and 1957) that tetracylines has chemo-prophylactic effect against *T. parva* infections when administered during the incubation period of the disease (Brown *et al*., 1977). This

observation was later confirmed and utilized as basis for more research work focusing on way to improve and perfect this method for immunization purpose.

Infection and treatment method is basically the infection of susceptible cattle with viable *T. parva* cryopreserved sporozoites and simultaneously treating the recipient with short or long-acting formulation of oxytetracyclines (Radley *et al*., 1975). Long-acting formulations of oxytetraxyclines have a practical advantage over short-acting ones as they require less handling of animals. Protective immunity with this method is achieved on the principle that an animal gets a mild or asymptomatic body reaction and in the same process its body elicits immune responses (cell-mediated) that will ensure protection on subsequent infection. A major challenge with the development of IT vaccines has been the fact that different *T. parva* stocks have different immunogenic properties and may not cross-protect. It is therefore essential that vaccine stabilates are made from stocks that are endemic in that particular area.

The IT vaccine that underwent intensive field trials and is currently in use in East Africa is the trivalent *T. parva* "Muguga Cocktail" containing three strains: Kiambu 5, Muguga, and Serengeti transformed *T. parva* (Dolan *et al.,* 1980; Uilenberg, 1999). Local *T. parva* Katete stocks are utilized for immunization field trials in eastern Zambia and the Mandali isolates in the southern Zambia (Marcotty *et al*., 2002; Uilenberg, 1999). In Zimbabwe, *T. parva* Boleni stocks are used for large-scale cattle immunizations. Cross-immunity experiments have shown that the *T. parva* Boleni isolate confers protection against all tested Zimbabwean *T. parva* stocks (Koch *et al*., 1988) and also against other African *T. parva* stocks (Irvin *et al.,* 1989; Hove *et al*., 1995). *Theileria parva* Boleni based vaccines have been developed to be used for IT and for immunization without the concurrent use of oxytetracycline (Pegram *et al*., 1996; Kanhai *et al*., 1997).

The use of vaccination stocks in areas outside their area of origin (where endemic) have been associated with the possible risk of introducing foreign *T. pava* strains, which might even create new antigenic types through genetic recombination (Uilenberg, 1999; Di Giulio *et al*., 2008*)*. The main disadvantage with the IT method is that it very expensive and producing the vaccine is laborious. It also requires a liquid nitrogen cold chain to maintain the viability of the parasite stabilates which can be a challenge in some pastoral areas

(Norval *et al.,* 1992; Di Giulio *et al.,* 2008). Carrier state associated with IT method has been regarded as disadvantageous in areas where the vaccine stocks are not endemic, whereas it can be of advantage in endemic areas in maintaining enzootic stability (Young *et al.*, 1986).

Development of a new generation of vaccines that cost less to produce and are easier to deliver than the current IT is an everyday challenge for most research scientists. Recent studies have demonstrated that antibodies to a surface protein (p67) of *T. parva* sporozoites can neutralize infectivity of sporozoites to lymphocytes. The anti-sporozoite neutralizing antibodies provided protection in limiting the severity of infection by reducing the number of sporozoites capable of infecting lymphocytes. Field evaluation of recombinant p67 sporozoite vaccine has shown some degree of efficacy and it may present a promising candidate to be further exploited in pursuit of a subunit vaccine against *T. parva* (Musoke *et al.*, 1993; Honda *et al.*, 1998; McKeever *et al.*, 1999). More studies are focussing on the antigens targeted by *T. parva*-specific CD8+ CTLS which seem to play a fundamental role in recovery from and protection to *T. parva* infections as the vaccine candidates. Recent studies on PIM, the schizonts and sporozoite surface antigen have provided evidence that a PIM-based vaccine can induce protection (Ververken *et al.*, 2008). Morrison and McKeever (2006) reviewed the status of the current development in vaccines against *Theileria* parasites.

2.6 *Theileria parva* carrier state

Theileria parva carrier state in cattle is defined as the ability of the animal that has recovered from *T. parva* infection to infect vector ticks which subsequently transmit the parasite to a susceptible host (Maritim *et al.*, 1988*)*. The ability of the *T. parva* carrier animal to infect ticks during feeding is the determining factor in the epidemiology of theileriosis. There are two main ways by which a mammalian host can become a *T. parva* carrier: spontaneous recovery from an infection without treatment or a recovery after treatment. The IT method against *T. parva* infections has been very effective in rendering the cattle immune but these cattle remain carriers.

Evidence suggests that *T. parva* parasites in the mammalian host are maintained by either slow proliferation of macroschizont-infected lymphocytes which develop to merozoites infective to erythrocytes or by regular division of intra-erythrocyte piroplasms (Norval *et al.,* 1992). It is therefore possible that an animal that is infected with *Theileria* parasite to act as a carrier only initially, i.e. develop schizont parasitosis and piroplasm parasitaemia which are later cleared by the immune responses leaving the host immune but with no parasites producing the state of sterile immunity. An animal can also become an intermittently or sporadic carrier or become a persistent carrier (Maritim *et al.*, 1988; Norval *et al*., 1992*).*

Carrier state with cattle-derived *T. parva* stocks that cause either ECF or Zimbabwean theileriosis is prevalent in cattle populations where these diseases are endemic (Young *et al*., 1986; Koch *et al*., 1992; Latif *et al*. 2001a,b). In Kenya, the carrier state of *T. parva* strains in endemic ECF areas is believed to be approaching 100% in adult animals (Young *et al*., 1981; Maritim *et al.,* 1988). Buffalo-derived *T. parva* carrier state in cattle under natural conditions has been demonstrated in other African countries but not in South Africa (Grootenhuis *et al.,* 1987; Kariuki *et al.,* 1995; Latif *et al.,* 2001a; Latif *et al.,* 2002; Kivaria *et al.,* 2007). In South Africa, the carrier state has, however, been demonstrated under experimental conditions using splectomised cattle and after treatment with a curative antitheilerial drugs such as halofuginone (Neitz, 1958; De Vos, 1982; Potgieter *et al.,* 1988). Recent attempts to demonstrate a carrier status in cattle that have recovered from clinical theileriosis in KwaZulu-Natal have failed (Thompson *et al.,* 2008).

There are postulations with regard to the inability of buffalo-derived *T. parva* stock to produce a carrier state in cattle. There is empirical evidence to suggest that the *T. parva* stocks that are circulating in South African buffaloes have similar biological features to *T. parva* "Muguga" stocks of East Africa which are not known to produce carrier state in cattle (Young *et al.,* 1986; Sibeko *et al*., 2010).

Maintenance of *T. parva* in cattle populations requires a continuous circulation of the parasite between the mammalian host and the vector ticks (Norval *et al.,* 1992). This requires that an infected mammalian host, either a buffalo or cattle, should be able to infect ticks which subsequently transmit the parasites to a new host. The above scenario is possible when there is constant contact between infected mammalian hosts and susceptible

cattle, which is not the case in South Africa since infected buffaloes are kept separately in fenced game reserves (Maritim *et al.,* 1989; Norval *et al.,* 1992). Physical separation limits contact between known infected buffalo and susceptible cattle which might also contribute to inability for buffalo-derived *T. parva* to establish carrier state in cattle populations. One other reason has been that in tropical East Africa all stages of *R. appendiculatus* occur throughout the year which makes continuous host-vector *T. parva* circulation possible. In South Africa, there is only one rainy season (January to April) and very cold winter season. Different stages of *R. appendiculatus* have strict seasonal occurrences and mostly one tick generation is completed per year (Maritim *et al.,* 1989; Norval *et al.,* 1992).

2.7. Specific project objectives:

- To determine the number of Corridor disease outbreaks in cattle from 2004-2009 and to establish the prevalence of *T. parva* carrier-state in cattle-farming communities bordering buffalo farms.

- To monitor the course of the disease in introduced sentinel cattle on selected farming communities.

- To attempt *T. parva* infection in susceptible cattle by transmission from adult *R. appendiculatus* ticks collected from cattle grazing pastures under controlled conditions at OVI.

- To attempt *T. parva* parasites tick pick-up and transmission from Corridor disease recovered cattle through xenodiagnosis experiments under controlled conditions at OVI.

- To assess the immune status of the recovered cattle (from above) to experimental challenge using *T. parva* sporozoites stabilate under controlled conditions at OVI.

CHAPTER 3
MATERIAL AND METHODS

3.1 Study Sites

The project was conducted on cattle farms that are located in the Zululand district municipality of KwaZulu-Natal. With the exception of Reebokfontein communal diptank, this area falls within the Corridor disease designated area of the province that permits for keeping of *T. parva*-infected buffalo. The selected cattle farms (Table 3.1) in both commercial and communal (diptanks) farming areas adjoin game reserves (Figure 3.1) with *T. parva*-carrier buffaloes and had a known history of Corridor disease. The geographic coordinates of the Corridor disease outbreak study sites and other locations within the District where the disease was confirmed were obtained. Information on the numbers and locations of the buffalo reserves, including privately owned and Ezemvelo KZN wildlife nature reserves was generated from various Veterinary State offices, the Ezemvelo KZN wildlife and the Directorate of Veterinary Services in KZN. For each reserve the coordinates were obtained and a specialist in GIS was consulted to develop a Corridor disease-risk map (Figure 4.2).

3.2 Monitoring of cattle at sites

Cattle owners from communal diptanks and commercial farms were told to call the State veterinarian (the author) or the animal health technician of the area when they suspect any cases of cattle sick from Corridor disease. From 2004 to 2009, the State veterinarian and the

animal health technicians made regular visits to the sites between December and the end of July. The selected farms and other farms which had experienced Corridor disease outbreaks were closely monitored for Corridor disease and relevant samples collected from suspected cattle. Follow-up sampling was carried out on the sites where outbreaks had occurred. A close working relationship with the cattle famers was developed to allow early reporting of any suspected cases and by regular inspections of cattle by veterinary officials at dip tanks/farms. Routine cattle inspection and livestock census at the diptanks and farms are part of the duties of state veterinary officials.

The observations made included:

Clinical examination: Clinical signs that are characteristics of *T. parva* infection syndromes to be observed during examination are the following: increase in temperature to above 40ºC, difficult and laboured breathing (dyspnoea), froth from the nostrils, lachrymation, enlargement of superficial lymph nodes, particularly superficial cervical lymph nodes, and in some cases diarrhoea and opacity of the eyes were observed (Norval *et al.*, 1992).

Parasitological examination: Thin blood smears were prepared from peripheral blood and biopsy smears prepared from enlarged superficial lymph nodes (superficial cervical). The smears were fixed in absolute methanol, stained in Giemsa's stain and examined under 100 X magnification using compound microscope.

Post mortem examination: Whenever possible, full post-mortem examination was performed to confirm the cause of death. Various organ impression smears were prepared from dead animals.

Serological and molecular examination: Blood for serum and whole blood in ethylene diamine tetra-acetic acid (EDTA) were collected using vacutainers by venipunture of caudal or jugular veins. Blood in the EDTA and the serum tubes was stored at temperature of about 4ºC immediately after collection and transported on ice to Onderstepoort Veterinary Institute for serological and molecular examination (see sections 3.3).

Table 3.1: Cattle farm/diptanks selected as study sites.

Farm/diptank name	Communal/commercial	Number of cattle (approximate)
Reebockfontein diptank	Communal	700
Morgenzon farm	Communal	100
Naaukloof/Mgambu	Commercial	50
Sweethome	Commercial	500
Kortnek	Communal	600
Magut/Panbult	Commercial	100
Nyalisa/Tokazi	Commercial	200
Llawarne/Magut	Commercial	200
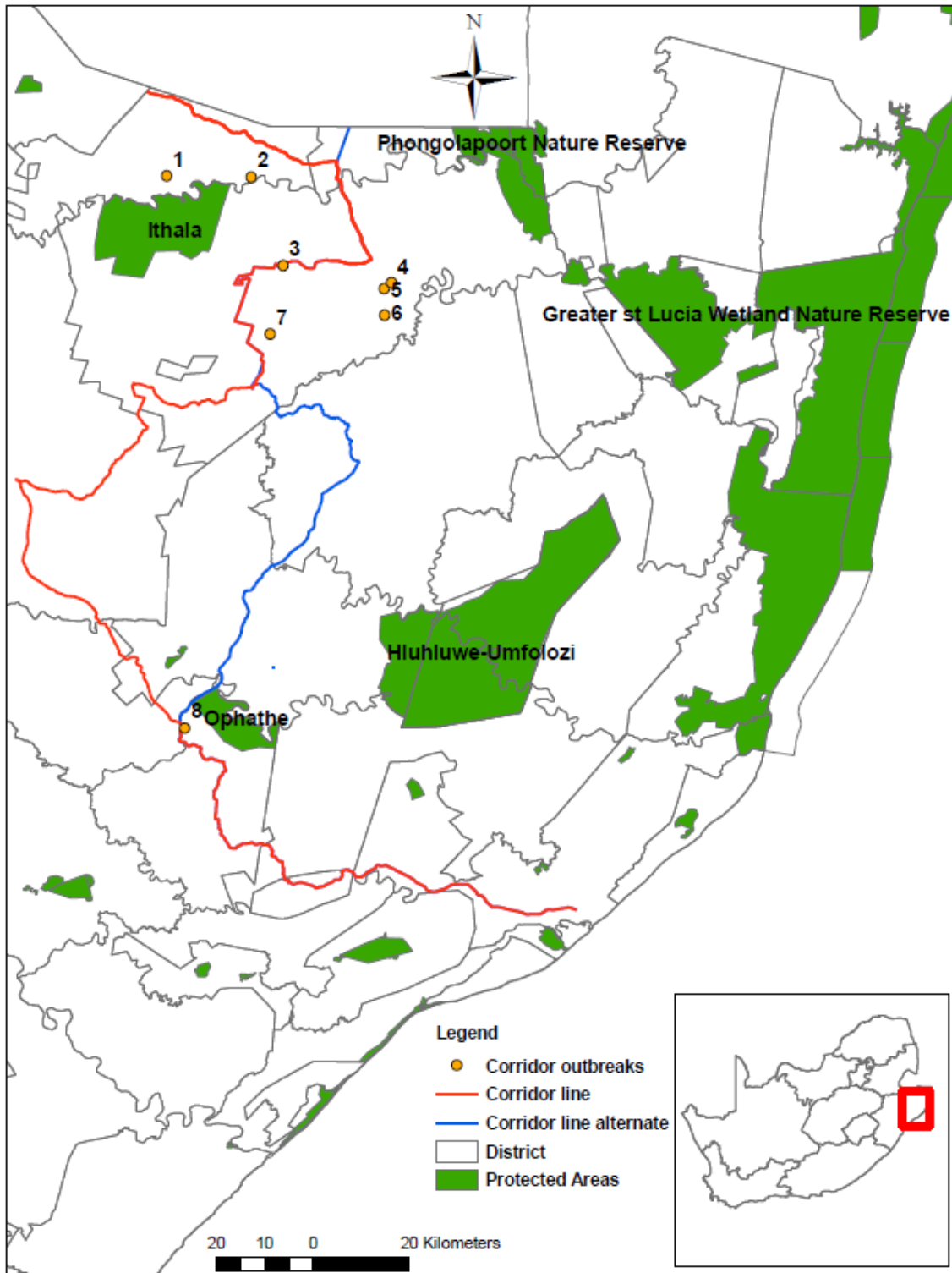
Figure 3.1: Distribution of study locations within the Corridor disease endemic zone in KZN. 1 (Kortnek), 2 (Reebockfontein diptank), 3 (Nyalisa), 4 (Llawarne/Magut), 5 (Magut/Panbult), 6 (Naaukloof/Mgambu), 7 (Morgenzon), 8 (Sweethome)

3.3   Diagnostic tests methods used at Onderstepoort Veterinary Institute

The indirect immuno-fluorescent antibody test (IFA)

The IFA is the standard serological diagnostic test used by OVI for demonstration of *T. parva* antibodies. The test is SANAS accredited test procedure and it is perfomerd by accredited scientist at OVI. Antigens used for the IFA test are prepared from *T. parva* schizonts derived from infected lymphoblastoids grown and maintained in tissue culture. Sera dilution of 1:40, 1:80 and 1:160 were used in the routine testing of the samples. The prepared samples were examined under a fluorescent microscope. The dilution of 1:80 is considered the standard cut-off titre for a positive reaction.

IFA test procedure: OVI

- The test serum collected from the field.
- The antigen slides in the plastic pockets, test and control sera are taken from storage in the freezer and placed on the workbench to thaw completely or can be thawed in the incubator at approximately 37ºC for 10 minutes. Reagents were allowed to warm to room temperature, 18 to 25ºC.
- A standard Dulbecco phosphate-buffered saline solution (PBS) was used to make dilutions of test sera at 1:40, 1:80, and 1:160. Negative and positive control sera were made up to dilutions of 1:80 with PBS.
- Antigen (*T. parva* schizonts grown and maintained in tissue culture medium) slides were fixed in cold acetone for 1 min and allowed to air dry.
- The diluted serum was drawn up as 24 ul and placed on the antigen slide, starting with the most dilute and then the more concentrated serum samples. Dilutions for each sample were placed in neighboring wells so as to allow for comparisons of fluorescence of each dilution. Each slide contained a positive and negative control sample.
- The slides were incubated in a humid chamber at 37ºC for one hour.

- Serum was removed from the slide by flicking it off swiftly. The slide was rinsed by dipping into PBS. The slide was immersed in a washing container containing PBS, placed on a magnetic stirrer at low revolutions and washed for 10 min.

- The washing was repeated using distilled water for five minutes. The slide was then air dried.

- Twenty-four microlitres of conjugate (diluted to 1:8 in PBS) was added to each well. The slide was incubated to humid chamber for one hour at 37ºC.

- The conjugate was flicked off and the slide briefly rinsed in PBS. The slides were washed in PBS for 10 min. on the magnetic stirrer as described above.

- The slides were allowed to air dry.

- A drop of 50 % glycerine/PBS was placed on each well and the slide covered with a coverslip.

- The slides were then examined under a fluorescent microscope by placing a drop of water on the coverslip and using the 50x water objective lens.

Real-time PCR protocol: OVI

The blood specimens in EDTA tubes were used for extraction of DNA for use in the detection of *T. parva* infections using real-time PCR assay (Sibeko *et al*., 2008). DNA was extracted from 200 μl whole blood in EDTA using MagNa Pure LC instrument (Roche Diagnostics, Mannheim, Germany). Extracted DNA was eluted at 100 μl buffer and stored at -20ºC. Real-time PCR assay was performed making use of Roche LightCycler instrument (Roche Diagnostics, Mannheim, Germany) which uses *Theileria* sp. specific primers and *T. parva* specific probes manufactured by TIB Molboi (Berlin, Germany) for amplification of extracted DNA. The test is SANAS accredited test procedure and it is perfomerd by accredited scientist at OVI.

Primer and Probe Preparation

- Sequence of *Parva* R, *Theileria* R, *Theileria* and *T. parva* probes were checked on receipt.

Table 3.2: Primers, probes and reagents used for *T. parva* PCR

NAME	SEQUENCE
Parva F	CTGCATCGCTGTGTCCCTT
Theileria R	ACCAACAAAATAGAACCAAAGTC
T. parva probe set	**ThPr640** 5'- LC Red640-TCG GAC GGA GTT CGC T – PH **Th.Anch.probe** 5' –GGGTCTCTGCAT GTGGCTTAT - FL
Theileria probe set	**Th Positive** 5' –AGAAAATTAGAGTGCTCAAAGCAGGCTTT – FL **PosLC705** 5' –LC Red705-GCCTTG AATAGTTTAGCATGGAAT--PH
Theileria parva probe	5'-Fam-ACG GAG TTC GCT TTG TCT GGA T –TAMRA-3'
Faststart Taq DNA Polymerase	
LC Uracil DNA Glycosylase	
PCR-grade water	
Reaction Mix	

- Each primer and probe was separately dissolved in the PCR-grade water to a volume indicated in the synthesis report to make up 100pmol/ul solution and 20pmol/ul solution respectively.
- 50ul of *Parva* F, *Theileria* R *Theileira* and *T. parva* were separated aliquoted into labeled Eppendorf tubes and store at -70° to -80° for long term storage or -20°C.
- Primers/probes and reagents from the lightCycler FastStart DNA MasterPlus HybProbe Kit were thawed at room temperature gently mixed and then centrifuged for 5 seconds.
- Reagents were then placed in tube compartments in cooling block.
- Primers R, Primer S and *T. parva* probe were made up to a 20 uM solution, i.e. 4 ul Primer/Probe to 16 ul of PCR grade water and then centrifuged for 5 seconds.
- 10 ul of enzyme was added to reaction mix and centrifuged for 5 seconds.

- The mix was then made up in an Eppendorf tube according to the corresponding number of samples being tested.
- Once the master mix was prepared it was vortex briefly for 3 seconds and then centrifuged for 5 seconds.
- The required number of lightCycler Capillaries was placed in pre-cooled centrifuge adapter in cooling block.
- 17.5 ul of the master mix was pipeted into each capillary.
- 2.5 ul of PCR-grade water was added into negative PCR control.
- DNA samples were incubated in a 90°C water bath/heating block for 5 seconds.
- They were then removed from the water bath/heating block and placed on ice for 5 seconds.
- 2 ul of DNA templates were added into the capillaries and adapters containing capillaries were placed into a standard benchtop microcentrifuge and centrifuged at 700 xg for 5 seconds (3000rpm). Two known positive samples were also included in the reaction.
- Capillaries were then inserted into the LightCycler sample carousel and placed into the lightCycler instrument and then run.

Quality Control of the Test
- The known negative should show no fluorescence, i.e. a flat line and the positive control should fluoresce, i.e. curve upwards

3.4 Description of the study sites

Reebockfontein diptank/farm

Cattle owners at this communal diptank farm mainly with Nguni and mixed beef cattle breeds. The cattle owners in this community normally graze their cattle on the camps bordering the game reserve during summer months. Tick control is mainly by government supported dip tanks. Communal farmers are supplied with amidines-based chemical acaricides such as amitraz. The frequency of dipping is every two weeks during summer and once a month in winter seasons. Investigations on this diptank started during the rainy seasons (January-April) of the years 2007, 2008 and 2009.

Morgenzon/Dlomodlomo farm

This farm has various small-scale farmers farming extensively and communally with Nguni and other mixed beef breeds. Tick control is fairly good supplied by cattle owners themselves and partially by government. Cattle are dipped weekly or bi-weekly during summer months and every three to four weeks during the winter months or strategically, depending on the tick burden on cattle. The acaricides used are mainly amidines (Amitraz) and synthetic pyrethroids-based dips. Some cattle owners also apply topical acaricides or pour-on dips on their own to supplement the dipping material supplied by government. The Corridor disease investigations on this farm started in April of 2005.

Naaukoof/Mgambu farm

This is a commercial farm with farm labourers also keeping cattle extensively on the farm. Cattle are dipped weekly to bi-weekly in summer months and/or strategically depending on the tick burden and monthly in winter months or strategically as mentioned above. The acaricides used are mainly amidines (Amitraz) and synthetic pyrethroids-based dip. The Corridor disease investigations on this farm started in May of 2004.

Sweethome farm

This is a commercial beef herd farming with Drakensberger cattle which are kept extensively. Cattle management and tick-control program on the farm is fairly good. Cattle are dipped strategically depending on the tick burden on the animals or farm. Investigations on this farm started in May of 2006.

Kortnek farm/diptank

This diptank has various small-scale famers on communal land, farming extensively with mainly Nguni, Brahmans and mixed beef breeds. Tick control is mainly by government supported dip tanks, which is by amidines (Amitraz) based dips applied bi-weekly during summer seasons and monthly during winter months. Some cattle owners also apply topical

acaricides and pour-on to supplement the dipping material supplied by government. The Corridor disease investigations on this farm started in March of 2008.

Panbult/Magut farm

This is the commercial farm of Brahman cattle which are reared extensively. Cattle management and tick-control program on the farm is also fairly good. The cattle are dipped strategically depending on the tick burden on the animals. Cattle are dipped more frequently during summer months and the less during winter season. The Corridor disease investigations on this farm started in March 2008.

Nyaluza/Tokoza

This farm was not initially chosen as one of the study sites but investigations on clinical disease and follow-ups were conducted after as huge outbreak that was encountered during March of 2009. Cattle were moved from a farm which is outside the Corridor designated area onto a rented cattle farm bordering a game reserve with *T. parva* infected buffaloes. A total of 202 cattle were moved into the farm on the 17th of February 2009 and they started dying on the 23rd of March 2009. Blood samples were collected as described above from six (6) clinically sick cattle in March 2009. Thirteen (13) more clinically sick cattle from the same herd were sampled and tested as above during April, 2009.

Other farms of interest:

Llawarne/Magut farm

This is a commercial cattle farm keeping mixed beef breeds and also keeps few wild animals on the farm, mainly small antelopes and warthogs. Cattle management and tick-control program on the farm is also fairly good. The cattle are dipped strategically depending on the tick burden on the animals or farm. Cattle are dipped more frequently during summer months and much less in winter.

3.5 Introduction of sentinel cattle into study sites with the history of Corridor disease outbreaks

3.5.1 Source of sentinels

Sentinel cattle were sourced from various areas outside the KwaZulu-Natal designated Corridor disease control area and on farms with fairly good tick control practices. These farms had no known history of Corridor disease outbreaks. These cattle were sourced from farms in Vryheid and Newcastle Local Municipalities.

3.5.2 Distribution to farms

The sentinel cattle were distributed to areas with known history of Corridor disease outbreaks to be kept and grazed with cattle of the local farmers. Certain farmers were selected to kraal them during the night and herd them with their cattle to grazing fields. Five Nguni and Brahman-cross breed cattle were distributed to Reebockfontein diptank in May 2007.

Ten Nguni cattle (yearlings) sourced from the Vryheid area were distributed to Morgenzon/Dlomodlomo farm in March of 2007. Six Bonsmara calves (yearlings) were distributed to Naaukloof/Mgambu farm and another six cattle of the same breed sourced from the same farm in the area of Newcastle were sent to Kortnek diptank.

3.5.3 Monitoring

The sentinel cattle were inspected at weekly to bi-weekly intervals by the State veterinarian (the author), assisted by the local animal health technician. The State veterinarian also responded to any suspected cases reported by the farmers. Sentinel cattle were grazed and herded together with cattle of the community or commercial farmers and only dipped when the tick infestation was becoming a nuisance. They were also examined and necessary samples collected as mentioned above (section 3.2).

3.6 Infectivity of *Rhipicephalus appendiculatus* collected from cattle grazing areas

Collections were made at Morgenzon/Dlomodlomo in 2008 and Naaukloof/Mgambu farms/diptanks in 2009 which are part of the selected project site. Unfed *R. appendiculatus* adults were collected from the cattle pastures during the summer season (January to April) and were taken to OVI for infectivity testing. Ticks were allowed to feed on a susceptible Hereford bovine obtained from the OVI disease-free herd. Tick application, clinical and parasitological monitoring were carried out daily after tick application (as explained in the coming section 3.71, 3.7.2). The adult brown ticks from the second collection from Naaukloof/Mgambu were dissected and salivary glands used for extraction of DNA. The extracted DNA was tested for the presence of *Theileria* parasites using real-time PCR.

3.7 Xeno-diagnosis (*Theileria parva* parasites pick-up and transmission using
**   *R. appendiculatus* nymphs)**

This was an attempt to assess the infectivity of ticks fed on Corridor disease-recovered cattle obtained from the different study sites to susceptible cattle. The work was conducted at OVI under controlled conditions (ECF quarantine stables). The pick-up was done by feeding clean laboratory-reared *R. appendiculatus* nymphs on five recovered carrier-cattle (Nos. KZN1, KZN2, N9, G8, and 8198) that were moved to OVI.

3.7.1 Tick feeding on cattle

The ears of the cattle were cleaned with soap and water for three consecutive days to ensure that there were no acaricide residues that might interfere with tick attachment. Ear-bags were attached to the base of the ears using an adhesive shoe-maker's glue. *Rhipicephalus appendiculatus* nymphs were placed inside the ear-bag and allowed to feed until engorgement. The engorged ticks that had dropped off were collected and counted into groups. Engorged ticks were then maintained at a relative humidity of 75% and temperature of 28ºC. They were allowed to moult and harden over a period of about 8 weeks. The ensuing adult ticks from each carrier animal were fed as described above on intact, susceptible bovines obtained from OVI disease-free stock.

3.7.2   Monitoring of animals

Before conducting the experimental infection of cattle, approval was obtained from the OVI-Animal Ethics Committee as a requirement to perform studies on animals. Cattle were examined and observed on daily basis for the development of *T. parva* parasites and clinical signs. These included: daily body temperature palpation of superficial lymph nodes and preparation of thin blood smears. If the lymph nodes, parotid and superficial cervical, were found enlarged, then a needle aspirate biopsy was performed and the aspirate smear prepared. Blood smears and lymph node smears were stained with Giemsa and examined under light microscope for the presence of schizonts and piroplasms. Bovines showing severe clinical signs of Corridor disease were treated with Buparvaquone (Butalex, Coopers Animal Health). Animals that did not respond were then euthanized for humane reasons using a captive bolt and exsanguination. Post-mortem examinations were done on all animals that died during the experiments to confirm the cause of death. Impression smears were made from various internal organs, fixed in ethanol and stained with Giemsa for parasitological examination.

3.8 Challenge of Corridor-recovered cattle obtained from different sites under controlled conditions.

The Corridor-recovered cattle obtained from the different study sites (KZN1, KZN2, N9, G8, 1202 and 0605) were also challenged with a buffalo-derived *T. parva* stabilate to assess their level of immunity to Corridor disease infection. The stabilate, provided by the Programme Parasites, Vectors and Vector-borne Diseases, OVI, had been tested and proved to be lethal in susceptible cattle. These cattle were inoculated with a volume of 2 ml each (equivalent to 20 ticks) subcutaneously over the parotid lymph node area. An intact, susceptible bovine (No: 9374/4) was also injected as mentioned above and was used as a control animal. All cattle were observed daily and sampled as mentioned above. Any animal showing severe body reaction to Corridor disease received treatment or, if required, was euthanized following the procedure stipulated above under tick transmission.

CHAPTER 4
RESULTS

4.1 MONITORING OF CORRIDOR DISEASE OUTBREAKS: 2004-2009

4.1.1 Occurrence of confirmed outbreaks per locality at the study sites and other recorded outbreaks

A summary of the confirmed Corridor disease outbreaks in the study area and from other locations comprising a total of 15 commercial farms and community diptanks in the district from 2004 to 2009 is shown in Table 4.1. A total of 31 outbreaks were recorded during the study period. The number of outbreaks was stable from 2004 to 2007, ranging from 3-4 but doubled in the subsequent years, i.e. 2008/2009 (Figure 4.1). At one location, Morgenzon farm comprising a commercial and community farmers, regular outbreaks occurred from 2004-2009. It is also noted that some farms experienced outbreaks for three consecutive years. Three other farms had experienced outbreaks for the first time in either 2008 or 2009. The localities of Corridor disease outbreaks are illustrated in Fig. 4.2 in relation to the buffalo game reserves.
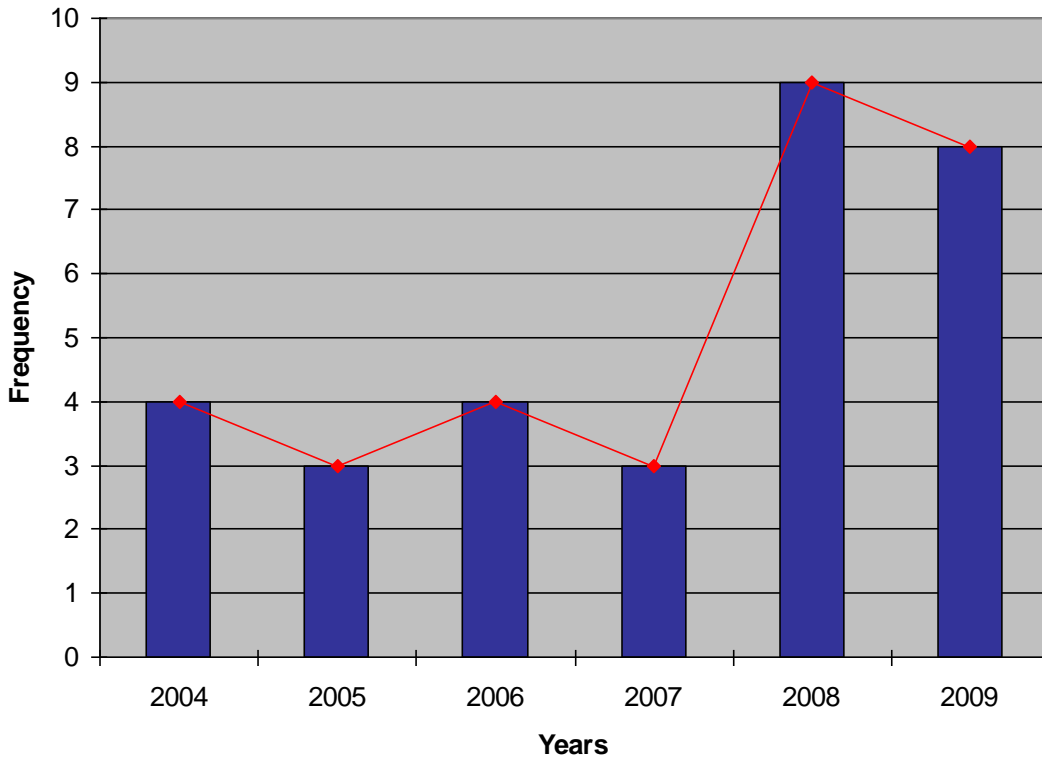
Figure 4.1: Number of Corridor disease outbreaks at the study area per year

Table 4.1: Occurrence of confirmed outbreaks per locality in the study sites and other recorded outbreaks

Farm/dip tank	2004	2005	2006	2007	2008	2009
Reebockfontein	-	-	-	+	+	+
Morgenzon farm	(+)	+	+	+	+	+
Naaukloof/Mgambo	+	-	-	+	+	+
Sweethome	-	-	+	-	+	-
Kortnek – Diptank	-	-	-	-	+	+
Panbult	-	-	+	-	+	-
Nyalisa	-	-	-	-	-	+
Magut	-	-	-	-	+	+
Goedgetrofen	-	+	-	-	-	+
Bedrog	-	+	-	-	+	-
Veeplaas	-	-	+	-	-	-
Smaldeel	+	-	-	-	-	-
Morgenzon	+	-	-	-	-	-
Bedrog- Goss	-	-	-	-	+	-
Sekane/Ntabayezulu	-	-	-	-	-	+
Total of Outbreaks	**4**	**3**	**4**	**3**	**9**	**8**
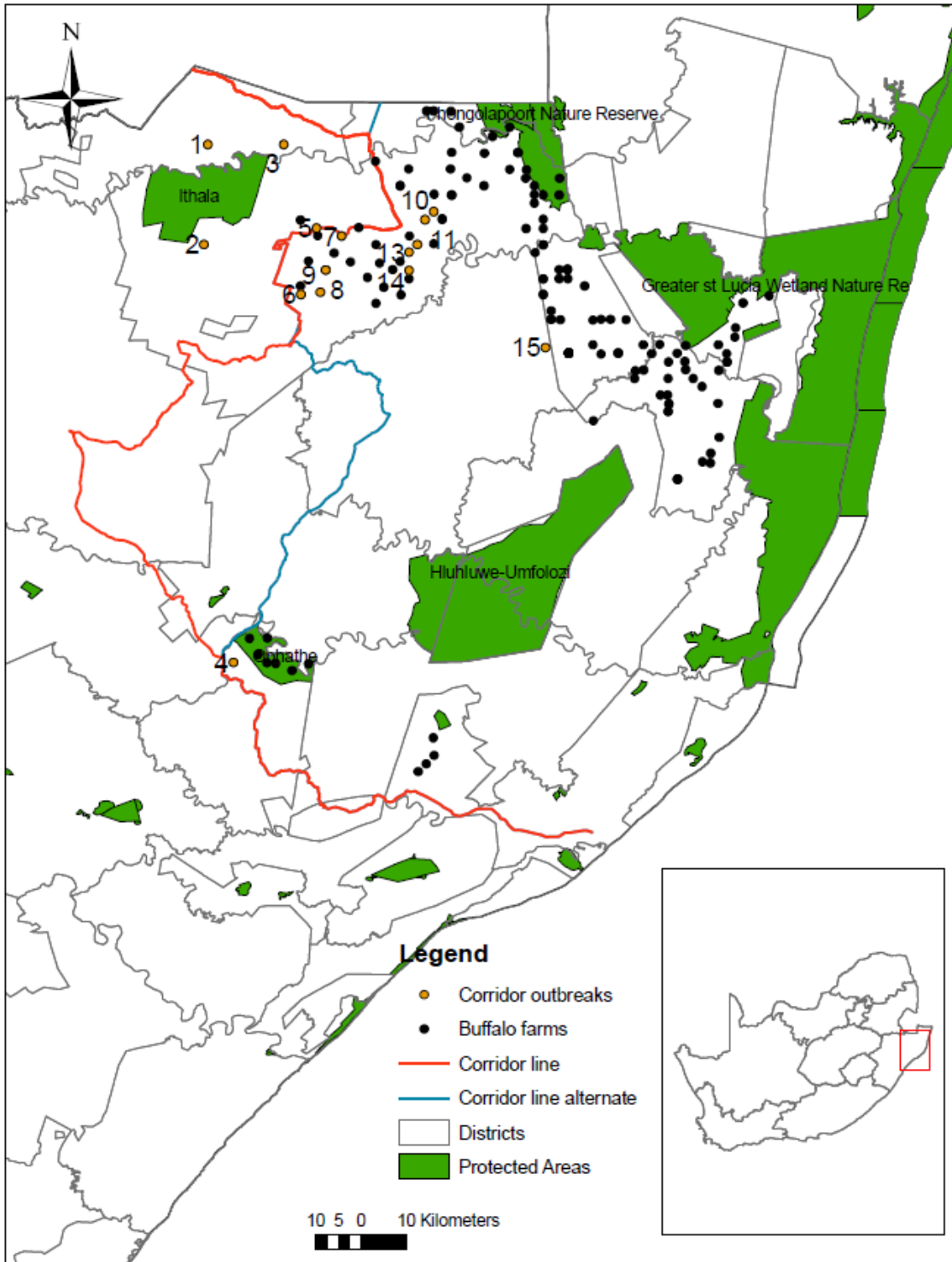
Figure 4.2: Distribution map of confirmed Corridor disease outbreaks in the study area (15 locations, Table 4.1), in relation to game reserves harbouring buffaloes.

Reebockfontein diptank

During February/March of 2007, four sick bovines showing typical clinical signs indicative of Corridor disease were sampled for serological and molecular tests. In January 2008, five cases showing clinical signs were examined and sampled. Only one suspected case was investigated and sampled in 2009.

All four bovines sampled during February and March of 2007 were found positive for *T. parva* parasites on real-time PCR but only one had significant antibodies on IFA with the titre of 1:80. The farmers lost 20 cattle during this outbreak. A total of 129 cattle were randomly sampled in May and July 2007: 19 (14.7 %) and 10 (7.7 %) were found positive by IFA and PCR, respectively.

Three of the five cattle that were sampled during the investigation of January 2008 tested positive for *T. parva* parasites on real-time PCR and only two were found positive for *T. parva* antibodies on IFA test, with titres of 1:80 and 1:160. Some of the cattle reported during this outbreak only had mild clinical signs and recovered. A total of 27 cattle were randomly sampled during the season: 12 (44.4 %) were found to have significant antibody titres while 4 (14.8 %) were positive by PCR.

The single suspected case investigated during January 2009 gave negative results on both real-time PCR and IFA tests.

Morgenzon/Dlomodlomo farm

This farm was closely monitored for outbreaks of Corridor disease from the summer season of 2005. The first outbreaks that were investigated were in April and May of 2005 where various communal famers lost a total of 25 cattle to Corridor disease. On the first investigation, carried out in April, relevant samples were collected from five cattle showing clinical signs. The second outbreak was reported in May 2005 and was suspected to be a continuation of the first outbreak. Five cattle were sampled during this second episode for testing as mentioned under Materials and Methods. All five cattle were found positive on

the IFA test, with high titres of 1:80 and 1: 160. Two of these cattle were also positive on real-time PCR for *T. parva* parasites.

During June 2006, two cattle which were suspected to have recovered from Corridor disease were sampled for serological and molecular tests. The two bovines were found to have positive reactions on the IFA giving titres of 1:80, but both were negative on real-time PRC test for *T. parva* parasites. Only one suspected case was investigated in May of 2007 and gave negative results on both tests.

The samples taken from five clinical cases during April 2007 were all positive for *T. parva* parasites on both real-time PCR and on IFA test, with titres ranging between 1: 40 and 1: 80.

In April 2008, five confirmed cases of Corridor disease on clinical and post-mortem examinations were sampled and tested. Blood samples were also collected from three other clinically sick animals and sent for confirmatory tests. Post-mortem examinations were conducted on carcasses from two cattle that had died and the findings were characteristic of Corridor disease, i.e. severe lung oedema, widespread haemorrhages of the trachea and ulcerations of the abomasal musosa. The three clinically sick cattle that were sampled during the outbreak, all tested positive on the real-time PCR for *T. parva* parasites. One of the three bovines was positive on the IFA test for *T. parva* antibodies as well as on PCR. The farmers lost five cattle in this outbreak.

In April 2009, two suspected outbreaks were investigated. On the first investigation five cattle with moderate classical signs of Corridor disease were sampled and three cattle were also sampled and tested on the second outbreak. All five cattle sampled during the first outbreak in April 2009 were confirmed by real-time PCR to have been infected with Corridor disease. These cattle did not develop antibodies to *T. parva,* as the IFA test gave negative results. On the second outbreak during the same month, two of the three cattle tested, were positive on real-time PCR only and one was found positive on the IFA.

Naaukoof/Mgambu farm

Monitoring of outbreaks on this herd started in May 2004 after the farmers had lost five cattle suspected to be of Corridor disease. Two suspect cattle on clinical examination were sampled for serological and molecular tests. Corridor disease was confirmed on PCR, but the IFA test was negative.

There were no suspect cases reported or investigated in the following seasons of 2005 and 2006. In May 2007 the farmers lost cattle showing classical signs of Corridor disease. Four suspected cases of clinical signs were sampled to confirm the diagnosis on serological and molecular tests. All four cattle investigated were confirmed to be infected with Corridor disease: positive on real-time PCR for *T. parva* parasites and only one which was also positive for antibodies of *T. parva* on IFA test, with a titre of 1:160.

In May 2008, three suspected Corridor disease cases were also sampled and tested as mentioned above. Another one suspected case was investigated and sampled in July 2008. All three cattle that were tested during an outbreak investigated in May 2008 were positive on real-time PCR for *T. parva* parasites, but all were negative for *T. parva* antibodies on IFA test. The other suspected case investigated during July was also confirmed to be Corridor disease; positive on real-time PCR test but negative on IFA test. This animal had recovered from the disease.

In March of 2009 only one suspected clinical case was investigated and sampled as previously mentioned. Again, the results had demonstrated the presence of *T. parva* infection in the bovine: both real-time PCR and IFA (titre of 1:80) were positive.

Sweethome farm

Close monitoring started during the summer season of 2006 when Corridor disease was suspected on the farm. Six clinically sick cattle were sampled in May for serological and molecular tests. All of the six cattle were confirmed to have been infected with *T. parva* as they all tested positive on the real-time PCR test, but only one showed a positive result on IFA test, with titre of 1:40. The farmer lost 25 cattle during this outbreak.

The farmer had several grazing camps on his farm, but the Corridor disease outbreak was confined to one camp which bordered a game reserve with *T. parva* infected buffaloes. The farmer was then advised by the state veterinary officials to rest the camps where the outbreaks were encountered for approximately 18 months. These camps were then rested as from June of 2006 and the whole of 2007 until the beginning of 2008.

In March 2007, three suspected cases with mild clinical signs was investigated and sampled. Whole blood in EDTA tubes and serum were collected from the suspect cattle. Corridor disease could not be confirmed on either of these bovines: negative results on both real-time PCR and IFA tests.

After about 20 months of resting, cattle were re-introduced to this camp, during the summer months of 2008. An outbreak was reported again on the same camp that year. Blood samples were collected from two suspect bovines showing clinical signs of the disease. One of the two cattle tested positive on real-time PCR and the other was only positive on the IFA test with a titre of 1:80.

Post-mortem examination was also conducted on the carcass of the cow that had died on the farm in 2008. The following characteristic lesions were observed: Severe pulmonary oedema, lung emphysema, widespread haemorrhages on the parenchymatous organs, especially on the trachea and the gastro-intestinal tract mucosa including the abomasal mucosa. Impression smears from different internal organs were positive for schizonts and piroplasms of *Theileria* spp on microscopic examination. The owner lost five cattle during this outbreak.

Kortnek farm/diptank

Investigations of clinical cases at this diptank started in March 2008 after five cattle suspected to be infected with Corridor disease had died. Two of the clinically sick cattle were examined and sampled. One of the two bovines tested positive for *T. parva* parasites on real-time PCR test but negative on IFA test. The other animal was negative on both tests.

During January 2009 only one Corridor disease suspected case was sampled and tested after the owner had lost three cattle with similar signs. Corridor disease was confirmed in this bovine: positive on real-time PCR for *T. parva* parasites but negative on IFA test. Three cattle died during the course of this outbreak.

Panbult/Magut farm

The first Corridor disease investigation on this farm, where the famer had lost five cattle, was conducted in June 2006. On this investigation only one cow that was clinically sick was sampled as previously mentioned and tested. The disease was confirmed to be Corridor disease as the samples from the animal all came positive for both *T. parva* parasites and antibodies on real-time PCR and IFA tests with a titre of 1:80.

In June 2007 one suspect case was investigated and sampled, but Corridor disease could not be confirmed. Negative results were obtained on both real-time PCR and IFA tests.

In April 2008, 12 cattle showing classical clinical signs of Corridor disease were sampled for parasitological, serological and molecular tests. During this outbreak, post-mortem examinations were also conducted on three carcasses of cattle that had died and organ impression smears were prepared. All of the cattle were confirmed to have been infected with *T. parva*: eleven out of the 12 cattle tested positive for *T. parva* parasites on RT-PCR, 8 were positive on both RT-PCR and IFA tests and only one on IFA only. The IFA titres were highly significant, ranging between 1: 80 and 1: 160. Lymph nodes aspirates smears were also prepared from some animals and *Theileria* spp. schizonts were demonstrated on some smears. Post-mortem examinations were conducted on three carcasses of cattle that had died and organ impression smears were also prepared. The following characteristic lesions were observed: Severe pulmonary oedema, widespread hemorrhages on the parenchymotous organs especially in the gastro-intestinal tract mucosa including the abomasal mucosa which also showed necrotic foci. Schizonts and piroplasms of *Theileria* spp were demonstrated on the impression smears on microscopic examination. The owner lost 20 cattle in a period of three weeks on this outbreak.

During July 2009, only one suspected case was investigated and sampled for further testing. Post-mortem examination was also conducted on a cow that had died and organ impression smears were made and sent for further examination. Corridor disease could not be demonstrated in this animal as results from both IFA and real-time PCR tests were negative.

Nyalisa/Tokazi

In the third week of February 2009 a herd of 202 cattle was moved to Nyaluza from Vetspruit farm (outside the Corridor disease control area). All cattle were believed to have been dipped. On 23rd March cattle became sick with signs characteristic of Corridor disease (34 days after introduction). On 27th March six sick cattle were sampled: 6/6 positive by real-time PCR (37 days after introduction). On 3rd April, further thirteen sick catlle were also sampled. The results confirmed the cause of disease to be *T. parva*: 12/13 were positive by real-time PCR and three were positive on both real-time PCR and IFA, with titres of 1: 80. All sick cattle showed signs indicative of Corridor disease and characteristic gross lesions on postmortem examination. Post-mortem examinations were done on a total of 11 carcasses. The farmer lost a total of 58 (40 days after introduction). The herd of cattle was then moved out of Nyalisa to Tokazi.

One of the communal farmers on the same farm lost five cattle due to Corridor disease during the same period. Three cattle from this farmer were sampled and tested but corridor disease could not be confirmed. There was no evidence of buffaloes that might have broken at any time through the fence and strayed into those cattle grazing area.

Llawarne farm/Magut

Monitoring on this farm started in March 2008, with investigations on an animal that had died suspected to be due to Corridor disease. Blood and serum samples were collected as required from one cow showing clinical signs characteristic of Corridor disease. The blood and serum tested positive for *T. parva* parasites on real-time PCR and for *T. parva* antibodies on IFA test, with a titre of 1:160.

Another suspected outbreak was also investigated during May 2008, i.e. two months after the first confirmed outbreak. One clinically sick animal suspected to have been due to Corridor disease was sampled and tested. This animal also tested positive for *T. parva* parasites on real-time PCR and for *T. parva* antibodies on IFA test with the titre of 1:80. Post-mortem examination was also conducted on a carcass of a cow that had died during this outbreak on the farm. Whole blood in EDTA vacutainers and organ impression smears were collected to confirm the cause of death. The post-mortem examination findings from the carcass of a cow showed the following characteristic lesions indicative of Corridor disease: severe pulmonary oedema, lung emphysema, widespread haemorrhages on the gastro-intestinal tact mucosa and ulceration of the abomasal mucosa (Fig. 4.3). The whole blood collected from this carcass tested positive on real-time PCR for *T. parva* parasites and some organs were positive for *Theileria* spp. schizonts on microscopic examination. The owner lost six cattle during the course of the outbreaks of 2008.

The on-farm monitoring continued for the following year. In March 2009, five Corridor disease suspected cases were investigated and three of them were sampled and tested. Corridor disease was confirmed in two of the three animals: they tested positive on both real-time PCR test and IFA tests for *T. parva* parasites and *T. parva* antibodies, respectively, with titres of 1:80. The tests on the third animal were negative on both tests. Furthermore, post-mortem examination was conducted on the carcass of a cow that had died during this outbreak on the farm. Characteristic lesions indicative of Corridor disease were observed.
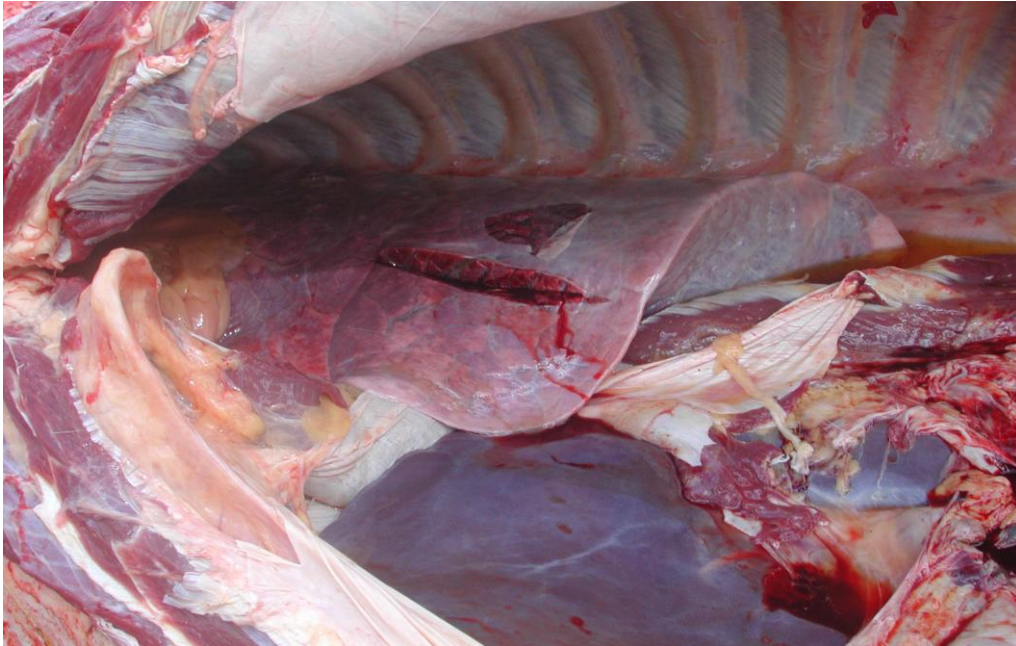
Figure 4.3: Severe pulmonary oedema and emphysema - Observation on the post-mortem examination conducted on the field.

Figure 4.4: Abomasal ulcerations and widespread petechiations - Observation on the post-mortem examination conducted on the field.

4.1.2 Seasonality of Corridor disease outbreaks

The Corridor disease outbreaks that were investigated, have mostly been reported during the months from March-May (88 %) but there were some (8 %) encountered during the winter months (June-August). The distribution of outbreaks mainly coincided with the activity period of adult *R. appendiculatus*. The frequency distribution of Corridor disease positive cases that were confirmed on Real-Time PCR at different study sites (total number 105) is shown in Figure 4.5.
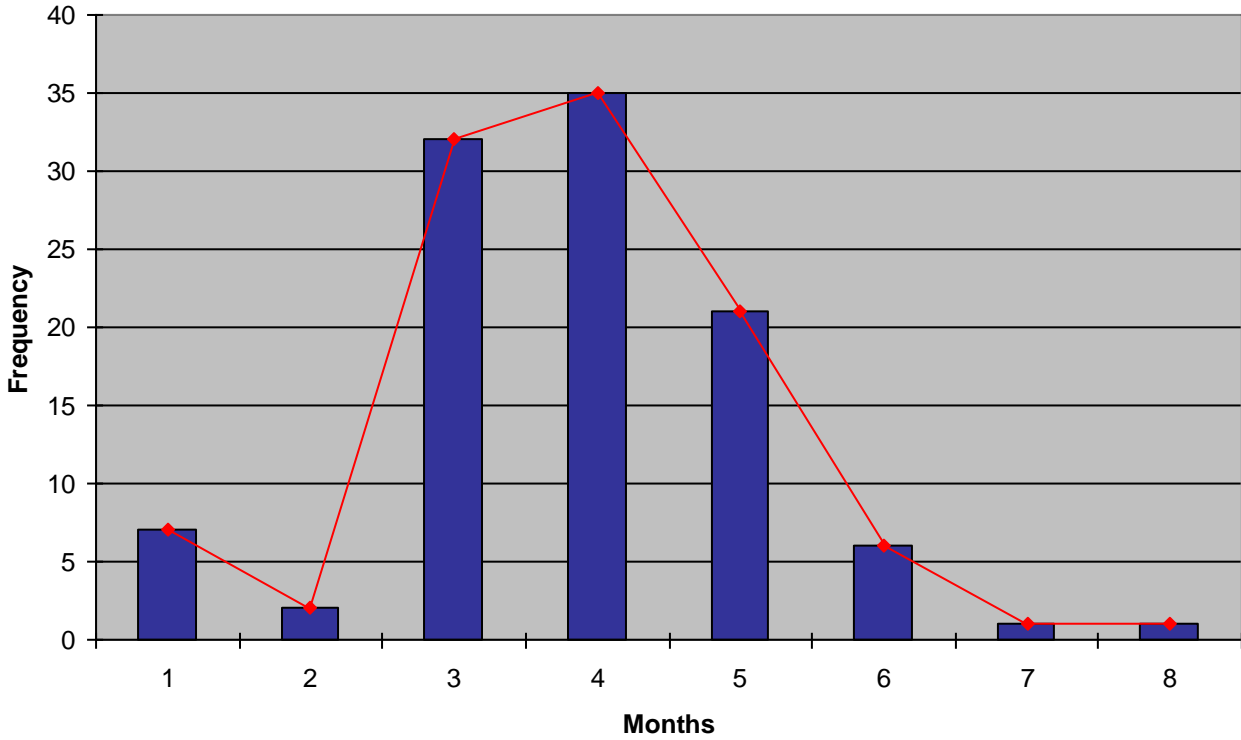
Figure 4.5: Frequency distribution of confirmed Corridor disease cases (n-105) at the study sites

(X-axis: 1 = January, 8 = August)

(Y-axis: f = frequency)

4.1.3. Follow-up of Corridor disease outbreaks in cattle at different sites

The cattle herds from the above-mentioned selected sites and other farms with interesting outbreaks were followed up. Cattle reported to have recovered by the owners and other cattle on the herds were randomly sampled in order to identify animals that might be potential carriers of *T. parva*. Blood was collected in EDTA and serum tubes from a total of 599 cattle to test for *T. parva* parasites (real-time PCR) and antibodies against *T. parva* (IFA). The results for individual locations are shown in Table 4.2. Overall, 20.5 % and 7.3 % of tested cattle were positive in the IFA and real-time PCR, respectively.

- **Reebockfontein diptank**

The cattle from various farmers on this diptank were randomly sampled in April 2007. Blood samples in EDTA and serum vacutainers were collected from 80 cattle. Ten of the 80 cattle tested positive on IFA test for *T. parva* antibodies with titres of 1:80 (4) and 1: 160 (6). Only 5 of the 80 tested positive on real-time PCR for *T. parva.*

A follow-up sampling of the same communal herd was conducted on July of 2007 with all sampled animals being ear-tagged and numbered for identification. A total of 49 cattle were sampled from various farmers as mentioned above. Twelve of the 49 cattle tested positive on IFA test for *T. parva* antibodies with titres of 1:80 (1) and 1:160 (11). Five of the 49 cattle sampled tested positive on real-time PCR for *T. parva* parasites.

In May 2008, 11 cattle including those that had recovered were re-sampled. Four of the 11 cattle tested positive on IFA test with titres of 1:80 (2) and 1: 160 (2). Three of the 11 cattle sampled tested positive on real-time PCR for *T. parva* parasites. In June 2008, 13 of recovered cattle that had tested positive were re-sampled and tested. Six of the 13 animals tested positive on IFA test with titres of 1:80 but all tested negative on the real-time PCR for *T. parva* parasites. Five more animals which were sick and reported to have recovered were also sampled in early June 2008. Two of the 5 animals tested positive on IFA test but all tested negative on real-time PCR. One animal (Figure 4.6) (KZN2) from the community farmers that had tested positive on real–time PCR for *T. parva* parasites and recovered was sent to OVI for more experiments (Materials & Methods Sections 3.9, 3.10).

In summary, a total of 156 cattle were tested for Corridor disease: 20 % and 9 % were positive in the IFA test and real-time PCR, respectively (Table 4.2).

- **Morgenzon**

During May 2007, 92 cattle from various farmers were sampled for serological and molecular tests. Twenty-one animals tested positive on IFA with titres of 1:160 and six were found to be parasitaemic on real-time PCR.

The herd was randomly re-sampled in May 2008 and a total of 52 cattle were tested. Out of these, 15 tested positive on IFA with titres of 1:80 (11) and 1:160 (4) and three on real-time PCR.

Twenty known recovered animals that had previously tested positive on real-time PCR were re-tested in June of 2008. Out of the 20 cattle sampled, 10 tested positive on IFA with the titres of 1:80 (7) and 1:160 (3) but all were found negative on real-time PCR.

In summary, a total of 164 cattle were tested for Corridor disease: 28 % and 5.5 % were positive in the IFA test and real-time PCR, respectively (Table 4.2).

Table 4.2: Follow-up of Corridor disease outbreaks in cattle at the study sites. Sampling was carried between January and August in each year.

Site	Year	Number of sampling	Number of cattle	I FA positives (%)	PCR positives (%)
Reebokfontein	2007	2	129	19 (14.7)	10 (7.7
	2008	3	27	12 (44.4)	4 (14.8)
		Total	**156**	**31 (19.9)**	**14 (9.0)**
Morgenzon	2007	1	92	21 (22.8)	6 (6.5)
	2008	2	72	25 (34.7)	3 (4.2)
		Total	**164**	**46 (28.0)**	**9 (5.5)**
Naaukloof	2007	2	21	4 (19.0)	6 (28.6)
	2008	1	32	2 (6.3)	4 (12.5)
		Total	**53**	**6 (11.3)**	**10 (19.9)**
Sweethome	2006	2	19	10 (52.6)	3 (15.8)
	2008	1	70	3 (4.3)	1 (1.4)
		Total	**89**	**13 (14.6)**	**4 (4.5)**
Kortnek	2008	1	13	1(7.7)	0 (0)
	2009	1	24	0 (0)	1 (4.2)
		Total	**37**	**1 (2.7)**	**1 (2.7)**
Panbult	2008	2	100	5 (5.0)	5 (5.0)
		Total	**100**	**5 (5.0)**	**5 (5.0)**
Nyalisa	2009	1	54	21 (38.9)	1 (1.9)
		Total	**54**	**21 (38.9)**	**1 (1.9)**
Overall Total			**599**	**123 (20.5)**	**44 (7.3)**

Figure 4.6: Recovered cow with bilateral corneal opacity **- KZN2**

Figure 4.7: Diarrhoea and emaciation **- KZN2**

- **Naaukloof/Mgambu**

A follow-up investigation was conducted in July 2004 on two cattle suspected to have recovered from clinical disease. The two cattle were sampled and tested; only one was found to be positive on IFA, with a titre of 1:80, but was negative on real-time PCR. This animal had tested positive on PCR the previous month.

The follow-up investigations were conducted in June 2007, when 17 cattle that were supposedly sick were also sampled and tested. Of these cattle, five tested positive on real-time PCR and all tested negative on IFA test. Four of the abovementioned recovered cattle that had previously tested positive on real-time PCR were re-sampled and tested in October 2007. Three of the four tested positive on IFA test with titres of 1:80 (1) and 1:160 (3). One of the three which had significance also tested positive on real-time PCR. The herd was re-sampled as a follow-up in January of 2008 and blood and sera of 32 cattle were tested. Of these 32, four tested positive on real-time PCR and two on IFA with titres of 1: 160 in both.

In summary, a total of 53 cattle were tested for Corridor disease: 11.3 % and 19.9 % were positive in the IFA test and real-time PCR, respectively (Table 4.2).

- **Sweethome**

The follow-up investigations on this herd started after the outbreaks of 2006. In June 2006, 15 of supposedly recovered cattle were sampled and tested. Of the 15 cattle sampled, nine tested positive on IFA with low titres of 1:40. Two of them were also found to be positive on real-time PCR for *T. parva* parasites. Four of the recovered bovines were re-sampled in August 2006; three showed weak reactions of 1:40 and one which was found positive in real-time PCR in the previous sampling was still positive. One (KZN1) of the recovered cattle that had tested positive repeatedly was sent to OVI for more experiments (Materials and Methods 3.9, 3.10).

This farm did not experience outbreaks in 2007, since the grazing camp that bordered the game reserve where the initial outbreaks occurred was rested for about 20 months. The follow-up on this farm was conducted again after the outbreak of 2008. All 70 cattle that

were kept on the camp that experienced the outbreak were sampled in May 2008. Of the 70 tested, four were positive on IFA, with titre of 1:40 (1) and 1:160 (3), and only one was found positive on real-time PCR as well.

In summary, a total of 89 cattle were tested for Corridor disease: 14.6 % and 4.5 % were positive in the IFA test and real-time PCR, respectively (Table 4.2).

- **Kortnek diptank and Llawarne (Magut) farm**

The follow up sampling was conducted in May 2008 from 10 cattle that were identified as "recovered". Only one of the ten cattle tested positive on IFA only, with a titre of 1:160. Three further cattle, reported to have recovered from the infection, were tested but gave negative results on all tests.

In January 2009, 24 cattle from various owners in the community were reported to have been sick of Corridor disease. The cattle were inspected. Some were confirmed to have clinical signs of Corridor disease and were sampled and tested. Four of the cattle were found positive on real-time PCR test only and all were negative on IFA test.

In summary, a total of 37 cattle were tested for Corridor disease: 2.7 % and 2.7 % were positive in the IFA test and real-time PCR, respectively (Table 4.2).

- **Panbult/Magut farm**

The follow-up investigation on this herd was conducted in May 2008 cattle, five of the 94 cattle sampled tested positive on both the real-time PCR and IFA test which gave high titres of 1:160. Six of the cattle identified as having recovered, were re-sampled and tested in June 2008 but all results came negative both on the IFA and real-time PCR. All five cattle were sent to OVI for more experiments (Materials and Methods 3.9, 3.10).

In summary, a total of 100 cattle were tested for Corridor disease: 5 % and 5 % were positive in the IFA test and real-time PCR, respectively (Table 4.2).

- **Nyalisa/Tokoza**

The follow-up sampling on the herd which was moved away from the infected grazing areas was conducted during August of 2009. Twenty-one (38.9 %) of the 54 tested cattle were found positive on IFA only while one (1.9 %) came positive on both real-time PCR and IFA tests.

4.1.4 Analysis of serological and molecular test results of field cattle, from 15 localities from 2004-2009

Below is the analysis of the follow-up of Corridor outbreaks in cattle at different sites. The total number of cattle tested for Corridor disease from case farms/communal dips was 846.

- The number of cattle which were found positive by real-time PCR, including those who were also positive by IFA, was 1:40 (16.5 %). This result demonstrated the infection with *T. parva* at the time of sampling, either with the *T. parva* schizont stage and/or the piroplasms.

- The number of cattle which were found positive by the IFA test, including those which were also positive by real-time PCR, were 141 (16.7 %)

- The number of cattle which were found positive by the IFA test but were tested negative by real-time PCR was 82 (10 %)

- The number of cattle which were found positive in both real-time PCR and IFA tests was 55 (7 %)

- The total number if infected animals, i.e. the sum of those testing positive in PCR including those which were IFA-test positive (140), and those which were IFA positive only (82), was 222.

- Prevalence of *T. parva* infection: 222/846 = 27 %

4.1.5. Monitoring of sentinel cattle introduced into different study sites.

Table 4.3: Infections with Corridor disease in sentinel cattle at different study sites.

Date	Locality	Number introduced	Number infected/died
2007/05	Reebockfontein	5	2/1
2007/02	Morgenzon	10	3/2
2008/04	Naaukloof/Mgambu	6	1/1
2008/04	Kortnek/Scheepers	6	4/4

- **Rheebokfontein diptank**

Five sentinel cattle were introduced to this diptank in May 2007 to graze with the community cattle. In May 2008 one sentinel (N9) had moderate clinical signs of Corridor disease and the infection was confirmed to be due to *T. parva* by the molecular test. The animal recovered spontaneously. A second sentinel animal was reported to be sick and died but post-mortem examination was not done to confirm the cause of death. Animal number N9 was retested in June 2008, and was found to be positive in the IFA test with titres of 1:80 but tested negative on real-time PCR for *T. parva*. However, the animal tested positive on real-time PCR on a third retest after two weeks. One animal (KZN2) from the community farmers which had severe Corridor disease reaction and recovered, confirmed through laboratory tests, and the sentinel (N9) were sent to OVI for further investigations (Materials & Methods Sections 3.9, 3.10).

- **Morgenzon**

Ten sentinel cattle obtained from an Nguni breeding farm situated outside the Corridor disease designated area were introduced into Morgenzon in March 2007. They all tested negative to *T. parva* in the IFA and PCR before being introduced on the farm.

The sentinels were sampled in January 2008 and one tested positive on IFA with a titre of 1:160 but negative on real-time PCR. Eight of the sentinels were re-sampled and tested in

March 2008. One more animal tested positive on the real-time PCR but negative on IFA. Two of the sentinels became sick and died during this period but post-mortem was not conducted. One of the sentinels (G8) that persistently tested positive for Corridor disease was sent to OVI for further experiments. All of the sentinels (7) were re-sampled and tested in January 2009. Four tested positive on the IFA with titres of 1:80 (2) and 1:160 (2) but all were negative on real-time PCR.

- **Kortnek diptank and Llawarne(Magut) farm**

Six sentinel cattle were introduced to this farm in April 2008. They were sampled twice in April 2008, with negative results on both tests. One of the sentinels died and post-mortem examination could not be conducted because it was reported too late. It was noticed that the local farmers at this diptank (Kortnek) were not co-operative. The remaining sentinel cattle (4) were moved in December 2008 to another farm (Llawarne) with known history of Corridor disease outbreaks. One of the sentinel animals died on the farm in January 2009. Blood sample in EDTA was collected before it died and the cause of death was confirmed as Corridor disease: positive on real-time PCR for *T. parva*.

- **Naaukloof/Mgambu**

Six sentinel cattle (Bonsmara breed) were introduced to the farm during February 2008 to run with the local cattle. They were first sampled in February on the day they were introduced and were re-sampled in March, May and June 2008 and were negative at all samplings. Four (4) of the sentinels were re-sampled and tested in March 2009 and were all negative. One of them died but post-mortem examinations could not be performed.

4.2   Tick pick-up and transmission attempts

The adult *R. appendiculatus* fed as nymphs on five Corridor disease recovered cattle obtained from different study locations were applied to feed on five susceptible bovines. The number of ticks applied per animal varied and ranged from 98–200 (Table 4.4). The results of tick pick-up and transmission attempts are shown in Table. Ticks from all the field recovered cattle failed to transmit *T. parva* to susceptible bovines. However, ticks from three cattle (KZN1, G8, and N9) transmitted *T. taurotragi* only (Table 4.5). No clinical reactions were observed in these animals and the body temperature remained normal although *Theileria* schizonts were seen in the parotid and superficial cervical lymph nodes for seven consecutive days.

Table 4.4: Ticks fed on infected carrier cattle and transmission attempts to susceptible bovines under controlled conditions.

Date of application: adult tick	Tick pick-up: Animal number – carrier bovines	Tick transmission: animal number – susceptible bovines	Number of ticks dropped (males & females): *R. appendiculatus*
09/March/2007	KZN 1	8395	200
06/September/2007	KZN2	8428	144
06/February/2009	8198	9448	156
23/June/2009	N9	0750	150
24/July/2009	N8	0749	98

Table 4.5: Theileriosis reactions in susceptible cattle from **Table 4.4** above.

Locality: Origin of animal	Animal Number: *T. parva* infected	Date tested positive on PCR: Clinically sick	Days when tested PCR negative: Follow-up test.	Xenodiagnosis (Infectivity)
Sweethome	KZN1	15/08/2006	31	*T. taurotragi*
Reebockfontein	KZN2	04/05/2007	50	No transmission
Morgenzon	G8 (Sentinel)	13/05/2008	41	*T. taurotragi*
Reebockfontein	N9 (Sentinel)	13/05/2008	41	*T. taurotragi*
Panbult/Magut	8198	12/05/2008	42	No transmission

4.3 Challenge of Corridor disease recovered cattle obtained from different study locations using *T. parva* buffalo-derived stabilates

A total of six cattle originating from different study farms and one control were challenged using a virulent *T. parva* stabilate. The results are shown in Table 4.6. All challenged animals, including the susceptible control, showed schizont parasitosis as detected by the *T. parva* RT-PCR test starting day 11 to 23. All animals also developed significant antibody titre to *T. parva* antigens (IFA test) by day 28. Of the field cattle, only bovine 0605 showed mild reactions manifested by high temperature (temperature above 39.5°C) on day 11 for two consecutive days and schizont parasitosis in lymph nodes on day 15 for only two days and recovered. The rest of the field cattle did not show any clinical or parasitological reactions during the observation period (103 days). The control bovine 9374/4 had high fever and showed schizont parasitosis by day 11 for seven consecutive days. The reaction was classified as severe and the animal had to be treated with Buparvaquone (Butalex, Coopers Animal Health).

Bovines N9, G8 , KZN1 and KZN2 became aparasitaemic (RT-PCR negative) by day 28, bovines 0605 and 1202 by day 73 and 103 respectively, but the latter became parasitaemic (RT-PCR positive) again by day 103.The control bovine 9374/4 remained parasitaemic throughout the observation period (103 days) (Table 4.6).

Table 4.6: Challenge of field carrier cattle and a susceptible bovine using a buffalo-derived *T. parva* stabilate.

Animal number	Period from first infection to challenge (month)	*Theileria parva* status after challenge: PCR (IFA titres)				
		11-23 days D*	28 days	56 days	73 days	103 days
G8	13	+ D17 (-)	- (+)	- (+)	- (+)	- (+)
N9	13	+ D17 (-)	- (+)	- (+)	- (+)	- (+)
1202	13	+ D11 (-)	+ (+)	+ (+)	- (+)	+(+)
0605	13	+ D11(-)	+ (+)	+ (+)	+ (+)	- (+)
KZN2	24	+ D23 (-)	- (+)	- (+)	- (+)	- (+)
KZN1	31	+ D23 (-)	- (+)	- (+)	- (+)	- (+)
9374/4 Control	0	+ D11 (-)	+ (+)	+ (+)	+ (+)	+ (+)

D* – Number of days after challenge.

4.4 Challenge of susceptible cattle with unfed adult *R. appendiculatus* collected off grass in the field.

Two collections of unfed *R. appendiculatus* adults collected off grass in the field were made between January and April 2008 and 2009 during the period coinciding with the adult brown ear tick high seasonal activity. Collections were made from selected project sites at Morgenzon/Dlomodlomo and Naaukloof/Mgambu farms/diptanks. Questing adult *R. appendiculatus* were easily spotted on the shafts and the tips of the grass on cattle grazing fields. The pasture was overgrazed and only 14 adult ticks were collected in 2008 at Morgenzon/Dlomodlomo farm after an hour of searching. Ticks were applied to feed on a susceptible bovine at OVI quarantine stables and only *T. taurotragi* was transmitted. There were no apparent clinical signs and the animal's behaviour remained normal during the observation period (60 days). However, *Theileria* schizonts appeared in the lymph node with high parasitosis for 6 days. It is noteworthy that a tissue culture isolate, confirmed to be *T. taurotragi* by molecular test, was successfully maintained from this animal.

Similarly, 16 unfed *R. appendiculatus* adults were collected off grass at Naaukloof/Mgambu in 2009. The ticks were dissected and the salivary glands were used for extraction of DNA. The extracted DNA was tested for the presence of *T. parva* using real-time PCR. The samples tested negative for *T. parva* but were positive for RT-PCR for *T. taurotragi* (OVI data; not shown).

CHAPTER 5
DISCUSSION AND CONCLUSIONS

5.1 Discussion

5.1.1   Monitoring of Corridor disease outbreaks

The field and laboratory investigations and findings have clearly demonstrated the occurrence of Corridor disease on a total of 15 commercial and communal diptanks at the cattle/game interface of the study area. It is also evident that these localities of disease outbreaks were on the increase from 2004-2009. It was noted that two new foci of outbreaks were reported in 2009 (Table 4.1). Most Corridor disease outbreaks were encountered during the months of March to June of the summer season, but a few were reported earlier or later during winter months (July-August). This higher period coincides with the high activity period of *R. appendiculutus* adults, which are known to be efficient in the transmission, as compared to the nymphal stage, of *T. parva* to susceptible cattle. This situation is similar to the seasonal occurrence of Zimbabwean theileriosis where outbreaks are reported during the summer rainy season each year, hence the name January disease (Lawrence, 1979; Lawrence *et al.,* 2004c). Most suspected outbreaks investigated have been confirmed in this study by close monitoring of clinical cases, using parasitological examination, by carrying post-mortem examinations, employing serological tests (IFA) and molecular tools (Real-time PCR).

One locality of interest is the communal Reebockfontein diptank, which experienced severe losses during three consecutive years. Cattle at this communal diptank were normally grazed on the pastures neighboring Ithala game reserve/ranch during the rainy season of the year. This is the period when the community also plant their crop lands and to avoid cattle getting into crops field, they are put on distant camps. Ithala game reserve has buffaloes that were regarded as disease free, but in 2006/7 were found to be infected with *T. parva* (Vryheid State Veterinary Annual report, 2007). Since these buffaloes have all along been regarded as disease free the northern section of the game reserve is not fenced and has never posed a serious disease risk to the neighboring cattle farming community. Buffaloes do

stray out of the game reserve into cattle grazing areas and cattle may also stray into the reserve.

5.1.2 Corridor disease surveillance

This study is the first intensive effort to investigate the extent of the Corridor disease problem in KZN. The surveillance carried from 2004-2009 included a total of 846 cattle originating from 15 localities where outbreaks had been reported. The overall prevalence was found to be 27 %. A 16.5 % of cattle tested *T. parva* positive on real-time PCR and this included those which were also positive in the IFA test. These were the animals that became sick and might have died and those that had recovered. Blood samples from these cattle were possibly collected during either the schizont parasitosis stages (acute stage) and/or the piroplasm parasitaemia (chronic or carrier) stage of the disease. Interestingly, a similar percentage (16.7) of cattle which were seropositive also tested positive by the real-time PCR.

The serological and molecular investigations for potential *T. parva* carrier cattle have revealed valuable information Firstly, 10 % of cattle continued to be seropositive (IFA positive) and lost their circulating *T. parva* parasites as detected by the real-time PCR. Thus, they can not be described as *T. parva* carriers at that time. Secondly, 7 % of cattle were seropositive and parasitaemic (PCR positive). These were the animals which can be defined as *T. parva* carriers since it is known that significant antibody titres and the piroplasms stage develop 4 weeks after the feeding of infected ticks.

5.1.3 Tick pick-up and transmission experiments

Before going on discussing the present data, it is of importance to refer to the original work of Neitz *et al.* (1955) on the Corridor disease transmission experiments and the carrier state in cattle. The origin of the naming of the disease as "Corridor" came from these workers (Neitz *et al.*, 1955). As early as 1955, they fed adults *R. appendiculatus* obtained as nymphs fed on Corridor disease reacting bovines, onto susceptible cattle to demonstrate the carrier state. The ticks transmitted *Theileria* species resembling Tzaneen disease; most probably *T. taurotragi* infection. After recovery from mild infections all of the nine cattle succumbed to

lethal Corridor disease challenge using infected ticks. However, in separate experiments they were able to transmit Corridor disease using adult *R. appendiculatus* collected from the vicinity of buffalo in Hluhluwe game reserve. They concluded that: *R. appendiculatus* adults are the vectors, the disease maintains itself in buffalo populations in complete absence of cattle and that cattle do apparently not act as "reservoirs", which was confirmed by monitoring of previously infected herds of cattle which moved to other sites and the disease never re-appeared and by results obtained from laboratory transmission experiments. Neitz (1958) added that "apparently in cattle, *Gonderia lawrencei* (*T. parva* buffalo-associated*)*, was unable to complete its life cycle to the final stage of the development of the erythrocytic parasites necessary for the infection of ticks" and he described it as a "self-limiting infection in cattle in the absence of premune African buffalo".

Attempts on the transmission of *T. parva* using *R. appendiculatus* from infected and recovered cattle (5) obtained from different study sites failed to infect susceptible cattle. Three of the bovines (KZN1, G8 and N9) were able to transmit *T. taurotragi*, which might rule out the possibility of poor feeding of ticks on the animals as the cause of transmission failure of *T. parva* parasites. These results support the findings obtained by Neitz *et al.* (1955) and recently by Thompson *et al.* (2008).

Neitz (1958) reported on the "interference phenomenon" between vector-borne haemoprotozoan parasites in ticks fed on cattle with mixed infections. It can also be assumed that the presence of large numbers of *T. taurotragi* in these bovines could have out-competed *T. parva* parasites during transmission experiments. In the present study questing adult *R. appendiculatus* collected from the cattle grazing fields/camps only transmitted *T. taurotragi* to a susceptible bovine. Moreover, the salivary glands of another batch of adult ticks tested by molecular tools revealed the presence of *T. taurotragi* only. Thompson *et al.* (2008) obtained similar results from attempting transmission from previously *T. parva* infected cattle from a farm in KZN with previous history of Corridor disease outbreaks. These authors failed to demonstrate the carrier state in these cattle by xeno-diagnosis and only *T. taurotragi* was transmitted in three cases. It is noteworthy to mention that the adult ticks used in their transmission experiments had extremely high infection rates with *Theileria* parasites in their salivary glands, up to 90 %. The

"interference phenomenon" in cattle with mixed *Theileria* spp. infections had been considered to play a vital role in the biological control of ECF (Neitz, 1963).

The other reason for the failure of transmission experiments could be because of the very low parasitaemia during the period of tick feeding or as demonstrated in this study that 10 % of animals lose their circulating *T. parva* piroplasms as early as 41 days after infection (Table 4.6). It is known that buffalo-derived *T. parva* carrier state in cattle and as shown by Latif *et al.* (2001) for Zimbabwean theileriosis is not always continuous, which could influence the ability to transmit infection to ticks (Norval *et al.*, 1992).

Previous research work on transmission of buffalo-derived *T. parva* from intact carrier bovines to susceptible bovines have not been successful in South Africa but the carrier abilities were shown after these cattle were splenectomized (Potgieter *et al.,* 1988). The inability to successfully transmit buffalo-derived *T. parva* parasites from intact bovines can partly be attributed to the intrinsic biological features of these parasites. The biological features or behavior of buffalo-derived *T. parva* in cattle population is characterized by the scarcity of schizonts and piroplasm stages of the parasite. This would negatively affect the ability of recovered cattle to act as source of infection for vector tick (Latif and Hove, 2011).

5.1.4 Challenge of recovered cattle with buffalo-derived *T. parva* stabilate.

This experiment clearly demonstrated that although these cattle were aparasitaemic on real–time PCR test to *T. parva* parasites and could not transmit the infection to vector ticks, they had solid immunity against buffalo–derived *T. parva* challenge. It is quite interesting that these cattle demonstrated solid immunity to a buffalo-derived stabilate (from buffalo 39, Kruger National Park, see Materials and Methods) which had not been sourced from the localities where the cattle originated. Recent research studies have shown wide antigenic diversity in *T. parva* stocks derived from buffaloes but their antigenicity has not been tested in cattle (Mans *et al.*, 2011). This observation could imply that the *T. parva* stock in the stabilate used to challenge these cattle was antigenically similar to the *T. parva* isolates that had infected them on the field.

This experiment clearly demonstrated, again, the ability of cattle recovered from Corridor disease to clear their piroplasm parasitaemia. Four of the six carrier animals became negative for *T. parva* parasites, as detected by the real-time PCR, by day 31 post the challenge infection, the 3rd and 4th animals by day 73 and the 103 respectively but the former bovine (number 1202) became parasitaemic again by day 103. These findings support early research work that the carrier state in cattle is not always continuous, and that an animal can either act as initial carrier, sporadic or a continuous carrier (Norval *et al.*, 1992). These outcomes on *T. parva* challenge also support the surveillance data obtained in the present study that cattle that had recovered from the disease would become aparasitaemic in about two months post infection. Furthermore, the experimental challenge confirms that these cattle remain seropositive for extended periods of time with significant antibody titres for up to 103 days.

5.2   General discussion and conclusions

The present study is the first intensive surveillance for Corridor disease involving monitoring clinical cases, carrying out parasitological investigations and post-mortem examinations, and employing serological and molecular tools to clarify the epidemiology of the disease in South Africa. However, the discussion on the epidemiology of *T. parva* in South Africa can not be adequate without understanding the history of the occurrence and eradication of the disease. Just after two years of the start of the ECF pandemic in cattle, Theiler (1904) changed the original name of the disease from Rhodesian redwater to East Coast fever. In this original review which was read at the meeting of the South African Association for the Advancement of Science, he also summarized the current distribution and nature of the disease, the course of the disease and its pathology, susceptibility of different breeds of cattle and its transstadial transmission by its vector, the brown ear tick. The disease caused a historical devastation in the economy of the country in which about one million cattle died before it was eradicated after 50 years of tireless scientific and administration efforts (Lawrence, 1992). Neitz *et al.* (1955) described for the first time another form of a disease in cattle transmitted by the brown ear tick and closely related to East Coast fever which they named Corridor disease. The disease, which was associated with the presence of the infected buffalo population, caused high mortality approaching 95 % in the two cattle herds which had experienced the outbreak. In this original work they also described the course of the disease, its clinical signs, parasitological picture, its

pathology, and carried out some experimental work on its transmission. They stated that the organisms causing Corridor disease can be maintained in buffalo populations, in complete absence of cattle. They then concluded that "cattle do apparently not act as reservoirs", an observation they made from their continued monitoring of the two affected herds and confirming this statement by the failure to transmit the infection by means of nymphae and adults of *R. appendiculatus* which had fed in their preceding stages on cattle reacting to Corridor disease.

Theileria parva buffalo-associated carrier state in cattle has been a subject of controversy over time. As mentioned above, Neitz *et al.* (1955) stated that cattle do not play a role in the transmission. However, Neitz (1958) conducted series of transmission experiments and he concluded that his results "leave no doubt that recovered Corridor disease cattle can serve as reservoirs of this infection after having been splenectomised". This work should receive some criticism by looking carefully to the results of the tick pick-up and transmissions experiments presented in their Table 1. A total of seven bovines were used. Two bovines received an infection derived from a carrier buffalo, both reacted, one recovered without treatment and one received treatment during the patent period. One bovine was immune to a homologous challenge and this animal did not show patent piroplasm parasitaemia. However, the second bovine received a heterologous challenge using ticks infected with the Schoonspruit isolate of *T. parva* producing classical East Coast fever in cattle. This animal was found to be partially immune to "East Coast fever" when challenged. Interestingly, this bovine showed typical symptoms of ECF, i.e. as described in the text "schizonts and endoglobular (piroplasm) could be demonstrated in fairly large numbers in lymphatic gland and blood smears". This form of the disease is different from the classical Corridor disease as described by Neitz. A tick pick-up was made from this animal and used, together with ticks derived from the animal which received the homologous challenge, in the subsequent xeno-diagnosis experiments. Four susceptible cattle which had received infections from ticks derived from the two bovines, all experienced severe ECF reactions and died. Interestingly, one bovine (number 7) which had received infected ticks from the Corridor disease infected bovines, at the time when the parasitaemia was low, failed to react to Corridor disease. It is clear from this analysis that Neitz (1958) transmission experiments involved cattle which were carriers to ECF (Schoonspruit isolate) and not carriers to Corridor disease in which case the carrier state could not be demonstrated.

Thirty years after the work of Neitz (1958), attempts to study the Corridor disease carrier state in cattle came from the work of Potgieter *et al.* (1988). Their study on Corridor disease included chemotherapy, transmission and xeno-diagnosis and attempting "transformation" of the biological behaviour of the causative organism similar to results obtained from Kenya (Barnett and Brocklesby, 1966). They demonstrated the occurrence of Corridor disease carrier state in their transmission experiments. However, these workers used "un-natural mode of transmission" to carry their experiments. They used splenectomised cattle which were injected by the intravenous route with infected blood drawn from an infected bovine to make sure of the *Theileria* piroplasms presence and seen in the blood smears. These results obtained do not confirm or demonstrate to what happens in the natural situations.

Again, 20 years later, another study attempting the demonstration of the Corridor disease carrier state in cattle came from the work of Thompson *et al.* (2008). The work was a follow-up of a serious outbreak of the disease on one farm during 2002-2004. Infected and recovered cattle were identified by PCR *T. parva* specific and *R. appendiculatus* were put to feed on these animals. The transmission experiments failed to transmit *T. parva* parasites.

It can be concluded from the above discussion that there is no convincing study which had demonstrated the occurrence of Corridor disease carrier state developing in cattle. The present results also failed to obtain successful tick transmission. However, this does not mean that cattle to cattle tick transmissions do not exist. This is supported by the fact that 7 % of cattle from the Corridor disease study sites were found to be parasitaemic (Real-time PCR positive) and had high antibody titres which develop 28 days after the introduction of infection. This finding is circumstantial evidence of the presence of the carrier state in cattle which exists for short duration. It is not impossible that the nymphal ticks feeding at the right time may be infected and able to infect susceptible cattle. The present results obtained from the surveillance and from monitoring infected cattle under laboratory conditions also showed that 10 % of cattle lose their circulating parasites as early as 28 days after infection. It is most probable that the strict seasonality of *R. appendiculatus* population plays the major role in limiting cattle to cattle transmission. At the time when the cattle are parasitaemic, the nymphal stage of the tick would not be available. When this stage of the tick starts to appear, i.e. during late winter most of the cattle may have lost their infective stage to ticks, the piroplasm stage. It is still required to attempt tick transmission experiments on field cattle that had recovered from clinical disease and/or those that show

positive results in both the PCR and IFA tests. It is also of importance to challenge those cattle which have high antibody titres to *T. parva* after Corridor disease season to confirm the occurrence of the disease in specific cattle populations.

Implementation of Corridor disease control measures as dictated by the Veterinary Authorities include keeping of buffalo strictly separate from cattle, the immediate removal of cattle in case of outbreaks from such tick-infested pastures for a minimum period of 18 months to limit further spread of the disease, and embarking on stringent tick-control measures. However, the Veterinary Authorities have a concern over the increased buffalo breeding projects and the commercialization of buffalo for conservation, eco-tourism and hunting purposes resulting in the increase in the number of Corridor disease outbreaks. Thompson *et al.* (2008) reported recently *T. parva* outbreaks occurring in KwaZulu-Natal in the apparent absence of buffalo, which added more weight on the magnitude of disease. This non-buffalo associated outbreaks of disease remains a major concern as it indicates possible cattle to cattle transmission of the infection, similar to the Zimbabwean theileriosis and ECF syndromes. Zimbabwean theileriosis has never been diagnosed in the country. Moreover, Corridor disease was diagnosed at a buffalo breeding project in the Free State Province, where the presence of the tick vectors had not been recorded (Sibeko *et al*., 2010). Currently, the Veterinary Authorities of the Department of Agriculture, Fisheries and Forestry (DAFF) generously support research projects with three objectives: to study and clarify the epidemiology of the disease in buffalo and cattle, to come up with specific and sensitive molecular diagnostic tests and to study the genetic diversity of the causative agent/s and closely related species. The outcome is to provide them with information and required scientific support to execute their mandate of regulation and control of this important disease. The current study was one of the research projects aimed at elucidating the epidemiology of the disease in cattle populations.

Research on diagnostics provided tests which are sensitive and specific to reliably diagnose the carrier state in buffalo and cattle of extremely low parasitaemia and of mixed infections in buffalo (Sibeko *et al*., 2008; Papli *et al.,* 2011; Pienaar *et al*., 2011). The work on the genetic diversity of *T. parva* and closely-related *Theileria* species circulating in buffalo and cattle populations also revealed a wealth of information. Sibeko *et al.* (2010) reported the presence of genetic similarities in the p67 sequence in the South African *T. parva* isolate

from an outbreak farm near Ladysmith to be identical to that of *T. parva* Muguga the cause of ECF in Kenya. Mans *et al.* (2011) found 26 different genotypes circulating in buffalo and cattle and they raised questions whether these constitute different species, subspecies or are only variants of the major groups. They stated that there is not yet a consensus on the degree of variation within a genotype that would define a "species". A breakthrough was made on "*Theileria* species buffalo", closely related genetically to *T. parva* infecting buffalo, when Zweygarth *et al.*, (2009) were able to obtain an infected cell-line in tissue culture. This *Theileria* species of the buffalo was problematic in the diagnosis of *T. parva* in the buffalo but fortunately this species, recently confirmed to be a distinct species, does not infect cattle (Zweygarth *et al.*, 2009; Mans *et al.*, 2011).

The current study has provided valuable information with regard to the changing Corridor disease occurrence in the Zululand District of KwaZulu-Natal. More research work is still needed to be conducted on other important epidemiological factors that contribute to the rise in Corridor disease outbreaks in this area such as the issue of the adequacy of single game proof fence to keep buffaloes out of cattle grazing field and the possible role of other small game animals in the mechanical transfer of infected tick.

CHAPTER 6
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