CHAPTER 1

General Introduction

And God said, Let us make man in our image, after our likeness: and let them have dominion over the fish of the sea, and over the fowl of the air, and over the cattle, and over all the earth, and over every creeping thing that creepeth upon the earth. ***Gen 1:26***

1.1 Background

Cattle theileriosis caused by *Theileria parva* infections is associated with high mortality, primarily in exotic and crossbred cattle, but also in indigenous calves and adult cattle in endemically unstable areas (Perry and Young, 1995). This results in major constraints on cattle production and the expansion of the dairy industry. In 1989 the cost of cattle theileriosis, East Coast fever (ECF), was estimated at $186 million (Mukhebi *et al*., 1992) in 11 affected countries in the African region. Following the introduction of ECF to South Africa in 1902, an estimated 5.5 million deaths of cattle were attributed to ECF. The control and the ultimate eradication of the disease cost the country R100 million (Anonymous, 1981).

Theileria parva infections in cattle manifest in three different disease syndromes, namely, ECF, Corridor disease and January disease. East Coast fever was introduced into southern Africa at the turn of the 19th century and was eventually eradicated after a costly campaign involving quarantine of infected farms and compensated slaughter of infected cattle herds (Anonymous, 1981). After the eradication of ECF, Corridor disease became the most important form of theileriosis in South Africa. Corridor disease is still a serious threat in areas where there are common grazing grounds between cattle and infected buffalo and where the tick vectors, *Rhipicephalus appendiculatus* and *R. zambeziensis* occur (Uilenberg, 1999). Since the South African cattle population is completely naïve to *T. parva*, it should be protected against exposure. Therefore, in South Africa today, cattle theileriosis is a controlled disease and authorities try to keep infected buffalo separated from livestock to prevent the spread of the disease.

Theileria parva has existed in Cape buffalo (*Syncerus caffer*) for thousands of years (Uilenberg, 1981; Young, 1981) and the parasite still circulates in the buffalo population in South Africa. Consequently, in South Africa, infections by *T. parva* parasites do not only impact on the cattle industry, but also affect the game farming industry. Buffalo are natural reservoirs of the parasite, and when infected by *T. parva* their value significantly decreases to ten times less than their "clean" counterparts. The financial implication of this extends to the loss of revenue if the game properties lose their attraction for tourists or hunters due to the absence of buffalo.

Corridor disease is still endemic in buffalo populations in some parts of South Africa. As a result, buffalo are required to be tested at least five consecutive times before they can be relocated to a disease-free area, as a means of controlling of the spread of the parasite to Corridor disease-free areas.

1.2 Thesis rationale

Corridor disease is a controlled disease in South Africa. The Agricultural Research Council-Onderstepoort Veterinary Institute (ARC-OVI) is the only institution in the country with a mandate from the South African government to test for *T. parva* infections in cattle and buffalo. Previously, a package of tests including microscopic examination of blood smears, the indirect fluorescent antibody test (IFAT) and a convetional PCR/probing test was used for this purpose. These tests, particularly the microscopic examination of blood smears and IFAT, lacked the desired sensitivity and specificity to detect *T. parva* infections which often occur in the presence of other *Theileria* species, as mixed infections. The application of the PCR/probing test improved the sensitivity and specificity that conventional diagnostic tests suffered over the years. However, because this assay is both time- and labour-intensive, it was no longer an ideal test for processing large numbers of samples resulting from the increasing demand for disease-free buffalo in South Africa. The ARC-OVI needed a more sensitive, more specific and less time-consuming diagnostic technique to detect *T. parva*-positive animals. Therefore, the use of real-time PCR technology to improve molecular diagnostics of *T. parva* infection was evaluated in this study.

Theileria parva is also the causative agent of ECF, a fatal disease of cattle which devastated the cattle population in South Africa in the early 1900s. Although ECF was eradicated in South Africa, there is a concern that it could recur. The principal vector for *T. parva*, the three-host ixodid tick *R. appendiculatus* is still widespread and the cattle population would be highly vulnerable should the parasite be introduced from an endemic area. Similarly if there was a recrudescence of the ECF-causing parasite from the existing population of parasites in buffalo, the cost of treatment and control would be enormous. It is not known whether the parasite that caused ECF was transmitted to buffalo during the ECF epidemic, or whether there exists in buffalo an ancestral subpopulation of *T. parva* parasites that could become adapted to cattle. Therefore, three *T. parva* genes coding for the antigenic proteins p67, p104 and polymorphic immunodominant molecule (PIM) were characterized in order to investigate the presence of cattle-type parasites in buffalo in South Africa.

1.3 Thesis objectives

In view of the above, the principal objectives of this study were:

1. To develop a real-time PCR assay for specific detection of *T. parva* infections in cattle and buffalo blood samples and:
    - To evaluate the sensitivity and specificity of the real-time assay in comparison with other molecular assays.
    - To assess the applicability and reproducibility of the real-time PCR assay for routine diagnostics.
2. To discriminate between cattle- and buffalo-derived *T. parva* isolates by fragment size analysis and sequence analysis of the variable region of the *T. parva* p67 gene.
3. To investigate the presence of cattle-type *T. parva* parasites by PCR-RFLP and sequence analysis of the variable region of the *T. parva* p104 and PIM genes.

1.4 References

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CHAPTER 2

Literature Review

"The diversity of the phenomena of nature is so great, and the treasures hidden in the heavens so rich, precisely in order that the human mind shall never be lacking in fresh nourishment."
Johannes Kepler

2.1 Introduction

The two most pathogenic and economically important *Theileria* species, *T. parva* and *T. annulata,* are responsible for nearly all the cases of bovine theileriosis in most parts of the world (Mukhebi *et al*., 1992). *Theileria parva*, the causative agent of East Coast fever (ECF), Corridor disease and January disease, occurs in eastern, central and southern Africa, while *T. annulata*, which causes tropical theileriosis, occurs in large areas of southern Europe, northern Africa, Middle East and Central Asia. Under certain circumstances a group of relatively benign *Theileria* parasites, *T. velifera, T. orientalis/ T. buffeli* complex and *T. mutans*, mainly located in Africa, also cause disease and loss of production (Norval *et al*., 1992). Other *Theileria* species such as *T. lestoquardi* and *T. taurotragi*, cause disease in sheep and eland, respectively and the latter can cause mild infections in cattle (Norval *et al*., 1992). Thus, the genus constitutes an important class of tick-borne organisms causing disease in ruminants.

2.2 The parasite: *Theileria parva*

Theileria parva is an apicoplexan protozoan parasite known to cause East Coast fever, January disease and Corridor disease in cattle (Uilenberg *et al.,* 1982; Perry *et al*., 1991). By far it is the most pathogenic and economically significant *Theileria* sp. in eastern, central and southern Africa (Norval *et al*., 1992). In the field, transmission of *T. parva* occurs only through the medium of the tick vector. Its principal vector is the brown ear tick, *Rhipicephalus appendiculatus*, and other members of the genus *Rhipicephalus* and several *Hyalomma* spp. have been shown to be capable of transmitting *T. parva* in laboratory conditions (Neitz, 1957; Brocklesby, 1965; Lawrence *et al*., 1983). The Cape buffalo (*Syncerus caffer*) is the natural reservoir host of *T. parva* (Barnett and Brocklesby, 1966a). The waterbuck has been experimentally infected with *T. parva* and is thus thought to be a possible reservoir host (Stagg *et al*., 1994).

2.2.1 The life cycle of *T. parva*

Theileria parasites have complex life cycles that involve several morphologically distinct developmental stages in the tick and mammalian host cells (Figure 2.1). The transmission and survival of the parasite depend on the ability of various invasive stages, the sporozoite and merozoite in the mammalian host, the zygote and kinete in the tick vector, to recognize and invade specific host cells (Shaw, 2003).

2.2.1.1 In the mammalian host

Transmission of *T. parva* is only achieved by infected nymphal or adult ticks. The infected nymph or adult transmits infection during feeding when the sporozoites in its salivary glands have matured (Stagg *et al.*, 1981). Sporozoites enter lymphocytes and differentiate into schizonts inducing a lymphoproliferative disorder (Stagg *et al.*, 1980). The sporozoite entry process is not orientation-specific as has been observed with other apicomplexan parasites (Shaw, 2003). In the lymphocytes, the schizonts later differentiate into merozoites that invade the erythrocytes. In *T. parva*, there is little or no multiplication in the erythocytes, multiplication occurs exclusively in lymphocytes. In contrast, multiple rounds of asexual division have been observed to occur in both the erythrocytes and lymphocytes from species like *T. annulata* (Norval *et al.*, 1992). Once in the erythrocytes, merozoites develop into piroplasms, the stage of the parasite infective to ticks.

2.2.1.2 In the vector tick

Piroplasms in the erythrocytes of the mammalian host are ingested during tick feeding. The sexual stage of development of *T. parva* occurs in the gut of the tick (Melhorn and Schein, 1984). In the tick gut lumen gametogenesis and fertilization take place resulting in the production of a zygote. The zygote invades the gut cell and remains there throughout the tick moulting cycle and develops into a single motile kinete. Kinetes escape the gut cells and invade the salivary glands. The parasites remain in the salivary gland until transmitted to another mammalian host when the resulting post-moult nymph or adult feeds. Tick feeding initiates rapid sporozoite development and infective sporozoites are released during the later stages of feeding (Norval *et al.*, 1992).

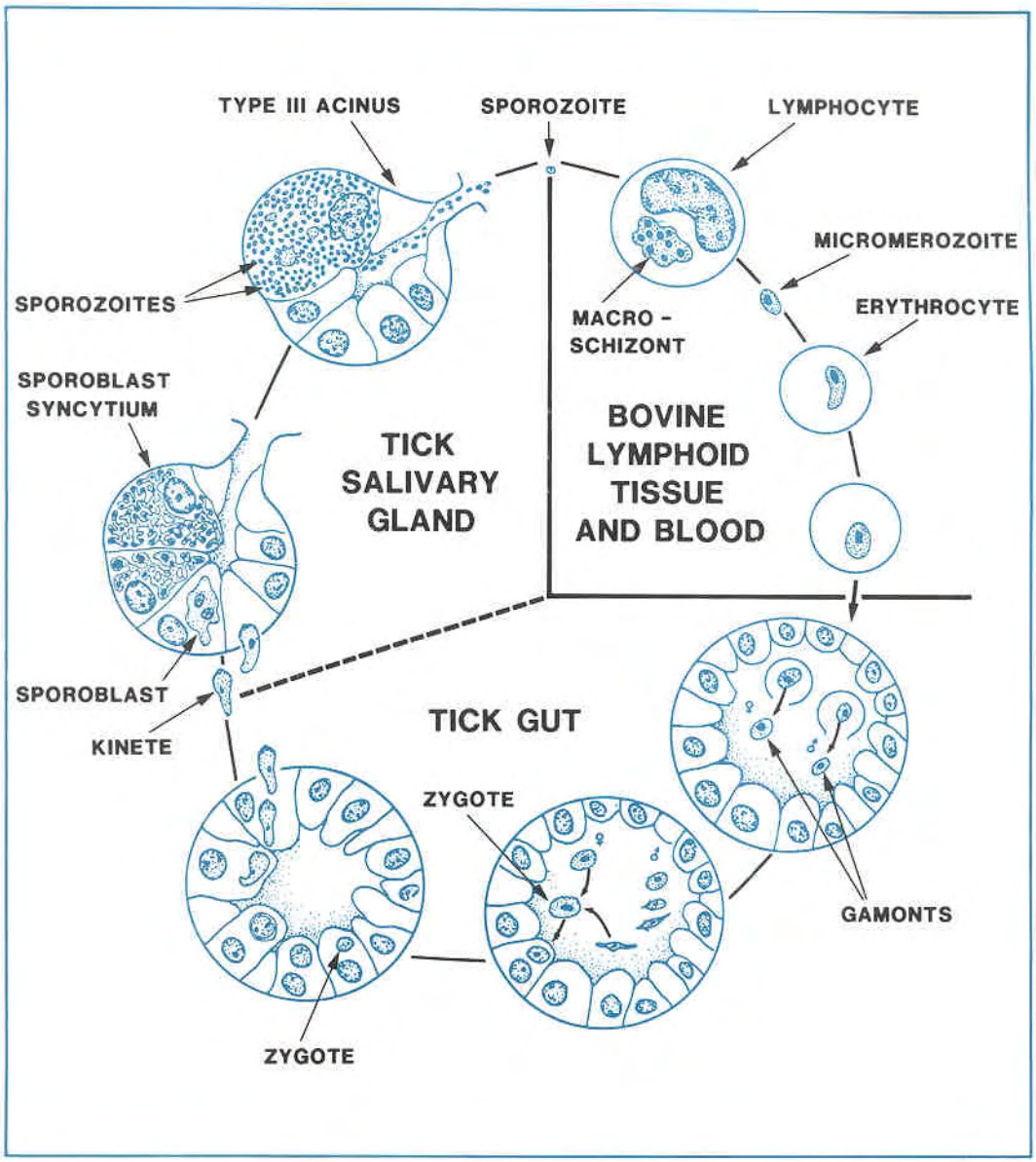

Figure 2.1 The life cycle of *Theileria parva* [from International Labotoratory for Research on Animal Diseases (ILRAD) 1983. Annual report 1982. Nairobi, ILRAD].

2.3 Disease syndromes caused by *T. parva* infections

East Coast fever, Corridor disease and January disease are disease syndromes caused by *T. parva* infections in cattle. These disease syndromes result from tick transmission of the parasite from infected cattle or buffalo to susceptible cattle. Cattle-to-cattle transmissions of *T. parva* result in ECF and January disease while buffalo-to-cattle transmissions of *T. parva* result in Corridor disease.

2.3.1 East Coast fever (ECF)

East Coast fever is a fatal disease of cattle caused by the cattle-derived strains of *T. parva* (formerly known as *T. parva parva*). The parasite is transmitted through tick infection from infected cattle to susceptible cattle (cattle-to-cattle transmission). The disease is characterized by the proliferation of lymphoblasts infected with theilerial schizonts throughout the body, particularly in the lymph nodes, lymphoid aggregates, spleen, kidneys, liver and lungs (Lawrence *et al*., 1994a). There is a marked variation in the susceptibility of cattle to infection; a proportion of animals may recover, however the recovery process might be prolonged. Recovered animals may remain emaciated and unproductive for months.

Epidemic ECF occurs when infection is introduced into a previously disease-free area infested with the tick vector, *R. appendiculatus*, with a fully susceptible cattle population. When the disease progresses without intervention by treatment, mortality may exceed 90%. East Coast fever is primarily controlled through treatment with acaricidal tick control, however treatment is expensive and the diagnosis must be made early for treatment to be effective (Potgieter *et al*., 1988). The economical implications associated with the control measures required are enormous for both livestock owners and veterinary authorities (Dolan and Young, 1981). East Coast fever was eradicated in South Africa between 1946 and 1955 (Anonymous, 1981) and since then there have been no reports of ECF infections in cattle.

2.3.2 Corridor disease

Corridor disease is an acute, usually fatal disease of cattle resembling ECF. The disease is caused by infection with buffalo-derived strains of *T. parva* (formerly known as *T. parva lawrencei*). The pathogenesis and pathology of Corridor disease are very similar to those of ECF, although Corridor disease is characterized by low schizont parasitosis and piroplasm

parasitaemia. Clinical features exhibited are also the same as ECF except that the course is usually shorter, death occurring only three to four days after the onset of first signs (Lawrence *et al*., 1994b).

Transmission of the disease occurs in cattle sharing grazing grounds with infected buffalo in the presence of the tick vector, resulting in buffalo-to-cattle transmission. It has long been believed that the parasite cannot adapt to cattle and usually fails to complete its development as most cattle die before the parasite develops to the tick-infective stage, the piroplasm (Neitz, 1955; Neitz *et al*., 1955). The disease was thus considered self-limiting. However, Potgieter *et al.* (1988) demonstrated that cattle infected by buffalo-derived *T. parva* can recover from the infection after treatment by chemotherapy and can become carriers of the parasite. When ticks were fed on the carrier animals and used to infect susceptible cattle, these animals died from classical Corridor disease suggesting that natural recovery might be possible and the fatal disease is possibly dose-dependent.

Buffalo-derived *T. parva* remains endemic in the buffalo populations in East and southern Africa. Corridor disease is still a serious threat in areas where there are common grazing grounds between cattle and infected buffalo and where the tick vectors, *R. appendiculatus, R. zambeziensis* and *R. duttoni* occur.

2.3.3 January disease (Zimbabwean theileriosis)

After the eradication of ECF in Zimbabwe, another type of theileriosis known as January disease emerged. The name January disease is attributed to the strict seasonality of the disease occurrence which is between December and March, coinciding with the seasonal activity of adult *R. appendiculatus*, the natural tick vector of cattle-derived *T. parva* parasites (Matson, 1967). January disease is an acute, frequently fatal disease caused by the cattle-derived *T. parva* parasite formerly known as *T. parva bovis*.

January disease exhibits the same clinical features as ECF; the pathogenesis and pathology of the disease are also very similar to those of ECF (Lawrence *et al*., 1994c). The disease occurs regularly in Zimbabwe, causing a significant number of deaths each year and necessitating the implementation of intensive dipping regulations to control its vector. There is no evidence that the infection occurs in South Africa where the clinical disease is currently not recognized (Lawrence *et al*., 1994c).

2.4 Epidemiology of theileriosis in southern Africa

2.4.1 Introduction and eradication of cattle theileriosis, East Coast fever, in southern Africa

In southern Africa, ECF was first recognized south of the Zambezi River in 1901/02 in cattle (Lawrence, 1992; Norval *et al*., 1992). It was named East Coast fever as its origin was traced back to the importation of cattle from the eastern African coast, following the devastation of the cattle population in southern Africa by the rinderpest pandemic less than a decade earlier and by the Anglo-Boer War. After a long struggle, with intensive dipping, quarantine and compensated slaughtering, ECF was finally eradicated between 1946 and 1955 in most southern African countries and Swaziland was the last country to eradicate ECF in 1960 (Anonymous, 1981). Since ECF was eradicated from South Africa, cattle-derived *T. parva* has not become re-established in cattle, possibly because strict regulations have prevented the establishment of conducive conditions. Between 1912 and 1922 ECF appeared in Malawi, Northern Province of Zambia and the Tete Province in Mozambique. In these countries the disease still persists to date and is a major constraint on the development of cattle production (Lawrence *et al.*, 1994a).

2.4.2 Emergence of other theilerial disease syndromes

Although ECF was eradicated in southern Africa, its principal vector *R. appendiculatus* still occurs and *T. parva* remained endemic in South African buffalo (Dolan, 1999). Consequently, another form of theileriosis called Corridor disease emerged. It was first diagnosed in 1953 in a corridor of land between the then separate Hluhluwe and iMfolozi Game Reserves in South Africa, hence the name Corridor disease (Neitz *et al.,* 1955). It occurs throughout southern and eastern Africa, wherever there is contact between cattle and infected buffalo in the presence of the ticks, *R. appendiculatus, R. zambeziensis* or *R. duttoni* (Neitz *et al.*, 1955; Blouin and Stoltsz, 1989). Corridor disease has been generally regarded as self-limiting as cattle usually die in the acute stage before the parasite develops into the erythrocytic piroplasm stage which might be picked up by the tick (Norval *et al.*, 1992). Potgieter *et al.* (1988) demonstrated that cattle infected with *T. parva lawrencei* can recover after receiving low doses of sporozoites stabilate and become carriers of the parasite. The carrier state phenomenon has been accepted as valid among *T. parva* stocks derived from both buffalo and cattle (Young, 1981). Buffalo, the natural reservoirs of the parasite, pose a threat to cattle

grazing on the same areas infested by *R. appendiculatus* and related ticks, hence the persistence of Corridor disease. In South Africa, of diseases transmitted from buffalo to cattle, Corridor disease is currently the second most important after foot-and-mouth disease.

After the eradication of ECF in Zimbabwe, a virulent form of theileriosis was identified as January disease which occurs during the rainy season each year (Matson, 1967; Koch, 1990). The disease occurs mainly in the rainy season from December to March coinciding with the seasonal activity of adult *R. appendiculatus*. This form of theileriosis is also suspected to occur in the southern and central province of Zambia (Lawrence *et al.*, 1994c). No convincing evidence has been found on the occurrence of this disease in South Africa (De Vos, 1982; Thompson *et al*., 2008).

2.4.3 Transformation of buffalo-derived *T. parva* into cattle-derived *T. parva*

Barnett and Brocklesby (1966b) demonstrated in East Africa for the first time that after a number of tick transmissions, buffalo-derived *T. parva* [*T. parva lawrencei* (Kenya)] could "transform" into cattle-derived *T. parva*. However, in South Africa, attempts to demonstrate transformation of buffalo-derived *T. parva* parasites to cattle-derived have proven futile (Neitz, 1957; De Vos, 1982; Potgieter *et al.*, 1988) although the same experiments were successful in East Africa (Barnett and Brocklesby, 1966b; Young and Purnell, 1973; Maritim *et al*., 1992). It is uncertain whether true transformation was demonstrated in East Africa since there is a possibility that the researchers may have started with a mixed population of parasites and selected a subpopulation of those parasites by transmission through cattle (Collins *et al*., 2002).

2.4.4 Carrier state of *T. parva*

A carrier state of *T. parva* is defined as the persistence of a tick-transmissible infection over prolonged periods of time among host mammals including both cattle and buffalo which survive *T. parva* infection (Barnett and Brocklesby, 1966b; Young *et al*., 1978, 1986). Although this phenomenon was originally an area of controversy in the biology of *T. parva* it has become accepted widely with stocks derived from both buffalo and cattle (Dolan, 1999). Immunization and treatment by chemotherapy have been shown to result in development of carrier state (Dolan *et al*., 1984; Dolan, 1986; Maritim *et al.*, 1989; Bishop *et al*., 1992). Carriers resulting from immunization with a cocktail may result in the establishment of new

strains. Carrier animals are important contributors to the infection within vector ticks. Since carrier cattle can infect ticks, a situation could eventually develop where the parasite becomes adapted to cattle as hosts, resulting in cattle-to-cattle transmission, as appears to have happened with East Coast fever and January disease (Potgieter *et al.*, 1988).

Until some two decades ago, tick experimental transmission of the parasite from an infected animal to susceptible animals was the definitive method for determining a carrier state. This method is too expensive and not viable for epidemiological studies (Dolan, 1986). Polymerase chain reaction (PCR) methods have demonstrated reasonable sensitiviy in detecting experimentally infected carrier animals (Bishop *et al.*, 1992) and cattle in the field (Watt *et al.*, 1998; Skilton *et al.*, 2002). However, the detection of carrier state remains a challenge because the piroplasm parasitaemia in these animals can be extremely low, especially in buffalo. Carrier animals may go unnoticed as they do not display clinical symptoms. This may result in outbreaks in tick-vector areas that were previously free from the parasite. Therefore, to detect carrier buffalo and cattle, a sensitive assay is required.

2.5 Treatment and control of theileriosis

After the eradication of ECF in South Africa, strict measures were implemented to prevent the spread of theileriosis and these included physical separation of infected buffalo from cattle, slaughter of infected cattle with compensation and pasture spelling. Other methods used for the control of theileriosis include tick control by acaricide application, immunization and chemotherapy. In South Africa, strict physical separation of buffalo and tick control by acaricide application are applied, while immunization and chemotherapy methods are prohibited because of the risk of development of carrier state.

2.5.1 Tick control

Theileriosis has mainly been controlled by means of tick control. Tick control was first implemented in southern Africa and has been achieved through various combinations of pasture spelling, control of cattle movement and acaricide application (Dolan, 1999). Pasture spelling was abandoned as it was found impractical because of the lengthy periods this method involved (18 months). Cattle movement control and acaricide application were retained, and were made compulsory through veterinary legislation in the southern African countries to which *T. parva* had spread (Norval *et al.*, 1992). However, the rapidly rising costs

of acaricides and their application has led to the development of other control methods, including vaccines against ticks, slow-release acaricide devices, more efficient means of topical application of acaricides, manipulation of hybrid sterility between closely-related tick species and the use of pheromones to disrupt mating or to attract ticks and so improve efficiency of acaricide treatment. Other methods including biological tick control using predators and pathogens, tick-killing or -repelling plants, habitat modification and resistant hosts have been studied and, in some instances, tested but have not yet been implemented on a large scale (Norval *et al*., 1992). Development of resistance from using acaricides for maintaining effective tick control remains a major concern (Dolan, 1999).

2.5.2 Chemotherapy

Treatment was introduced as the secondary strategy in integrated tick and tick-borne disease control. Tetracylines were the first compounds to be used in the control of theileriosis (Neitz, 1953; Brocklesby and Bailey, 1962). Their effect was limited though as they have a suppressive effect only in the early stages of *Theileria* infection. Further research led to the discovery of the naphthoquinone compound, menoctone, which demonstrated theileriacidal activity but could not be further developed as it was too expensive to synthesize. Subsequently, more effective derivatives of this compound were developed: parvaquone, which was very effective and safe with a wide therapeutic index, as well as buparvaquone, which was eight times more effective than parvaquone against *T. parva* infection *in vivo* (Hudson *et al*., 1985; McHardy *et al*., 1985; Hudson *et al*., 1986; Morgan and McHardy, 1986).

Another compound used in the treatment of theileriosis is halofuginone, a quinazolinone compound. This compound was developed as a hydrobromide salt for use as an anticoccidial agent. However, the compound is also active against both *T. parva* and *T. annulata* infections in cattle (Uilenberg *et al*., 1980). In field conditions, halofuginone appears to be the most active compound against early stages of the disease as it is only active against the schizont stage (Njau *et al*., 1985), unlike parvaquone and buparvaquone, which are active against the schizont and piroplasm stages.

Successful application of chemotherapy, however, requires early diagnosis so that treatment can be given at the early stages of clinical disease. None of the new therapeutic agents is effective when given once respiratory signs are present. When effectively applied, the treatment of a significant proportion of the cattle population results in the creation of a higher number of immune animals but also contributes to an increase in the number of carrier animals. Because these animals could be potentially infective to ticks (Potgieter *et al*., 1985), in South Africa, the use of chemotherapy was banned to avoid the risk of increasing carrier state in cattle infected with buffalo-derived *T. parva*. The possibility of development of resistance though not proven remains a matter of concern considering that resistance has inevitably developed to almost every chemotherapeutic ever discovered (Schnitzer, 1963).

2.5.3 Immunization

Immunization was first attempted in South Africa early in the 20th century (Cunningham, 1977; Lawrence *et al*., 1994a). The inoculation of cattle with blood, spleen and lymph node material from infected animals, were the first methods applied for immunization. Neither of these was a success and, as a result, immunization was abandoned in favour of other methods of control in South Africa. Regardless of the failure of the initial methods, these techniques demonstrated that immunity could be acquired and Neitz (1953, 1957) showed that extended administration of tetracycline during the incubation period of ECF resulted in cattle becoming effectively immunized without adverse effect. This became the basis of the infection and treatment method of immunization. This method involves the inoculation of a live, potentially lethal dose of the parasite and simultaneous treatment with a formulation of a long-acting oxytetracycline (Radley, 1981). A severe limitation of this method, though, is that a single vaccine strain does not protect against all the different strains of *T. parva* in the field, especially infections resulting from buffalo-derived *T. parva*. The use of cocktail stabilates like the Muguga cocktail has partially overcome this problem as the combination has shown to confer broad but not universal protection even in the field (Radley *et al*., 1975; Musisi, 1990). The use of live vaccines however, is also limited by the need for cold storage, the limited shelf-life of the vaccine, the possibility of causing morbidity and mortality in vaccinates, and the risk of attenuated organisms reverting to a pathogenic state (Jenkins, 2001).

To overcome the limitations outlined above, research focus has moved towards the development of subunit vaccines derived from native antigens of the parasite or as recombinant proteins from cloned DNA. In the past decade, there have been advancements in attempts to identify *Theileria* antigens from sporozoite, schizont and piroplasm stages that are suitable for testing vaccine components. Several monoclonal antibodies against *T. parva* and *T. annulata*, which neutralize sporozoite entry into host cells *in vitro,* have been generated. These antibodies detect surface proteins such as p67 and the polymorphic immunodominant molecule (PIM) of *T. parva* and SPAG-1 of *T. annulata* (Shapiro *et al*., 1987; Williamson *et al*., 1989; Hall *et al*., 1992; Nene *et al*., 1992). SPAG-1 and p67 have both been tested in vaccination trials and each induces some degree of protection against *T. annulata* and *T. parva* sporozoite challenge, respectively (Musoke *et al*., 1992; Nene *et al*., 1995; Nene *et al*., 1996; Boulter *et al*., 1998).

2.6 Detection of *T. parva* infections

In South Africa, only buffalo that have been certified disease-free can be translocated to disease-free areas. The South African veterinary authorities require buffalo to be tested for four controlled diseases that are transmissible to cattle, namely: foot-and-mouth disease, bovine tuberculosis, bovine brucellosis and Corridor disease. Because of the carrier state and mixed infections which are common in buffalo, sensitive and specific tests are required for detection of *T. parva* infections. Diagnostic tests currently used for detection of *T. parva* infection include conventional, serological and molecular methods.

2.6.1 Conventional methods

Conventional diagnosis of *Theileria* parasites has mainly been based on microscopic examination of blood and lymph node smears for the presence of the parasites which could be differentiated from other blood parasites by morphological and staining properties. The method is routinely used for the detection of *Babesia*, *Theileria* and *Anaplasma* species in acutely infected animals (Morzaria *et al*., 1999). This method is the method of choice for early and rapid treatment of the disease. However, it has major limitations in that *T. parva* schizonts and piroplasms are difficult if not impossible to differentiate from those of other *Theileria* species which may occur simultaneously within the same blood sample (Norval *et al*., 1992). One important epidemiological limitation is that microscopic examination is less

sensitive in detecting piroplasms in carrier animals. Following recovery from ECF, the parasitaemia in carriers is often below the threshold of detection by microscopy.

2.6.2 Serological methods

There have been a variety of serological tests described for *T. parva* piroplasm antigens. These include capillary tube agglutination (CA) (Ross and Löhr, 1972), conglutination (Cawdery *et al.,* 1968), indirect immunofluorescent antibody (IFA) test (Burridge, 1971), indirect hemagglutination assay (IHA) (Duffs and Wagner, 1974) and the Enzyme-linked immunosorbent assay (ELISA) (Katende *et al.*, 1998). In a comparative study between IFA, IHA, CA, complement fixation (CF), and immunodiffusion (ID), it was suggested that IFA and IHA were the best assays for field work and CF, IFA, IHA were most suitable for experimental work, with regard to their sensitivity (Duffs and Wagner, 1980). Although the sensitivity and specificity of the ELISA surpasses that of the IFA test (Katende *et al*., 1998), the IFA test remains widely used in the diagnosis of *Theileria* parasites and has also been the main tool in many assessments of endemic stability of *T. parva* infections.

2.6.2.1 Indirect immunofluorescent antibody test

Initially, the IFA test was developed to detect *T. parva* antibody using piroplasm antigens and later adapted for use with schizont antigens because of the longer duration of the serological response against the latter (Burridge, 1971; Burridge *et al*., 1973). Although widely used, the IFA test has many disadvantages, which include cross-reactivity due to the use of crude antigens, difficulty in standardization, subjectivity in interpretation of the results, and the impracticability of processing large numbers of samples (Norval *et al.*, 1992). The detection of antibodies to *T. parva* is also unsuitable for determining carrier status since serology is unable to differentiate between previously cleared and current infections (Young *et al.*, 1986; Bishop *et al.*, 1992). Antibodies tend to disappear in long-term carriers, whereas *Theileria* piroplasms persist. Therefore, animals with a negative serological test can still infect ticks and thus IFA test results cannot be independently used to determine the *Theileria* infection status of carrier animals.

2.6.2.2 Enzyme-linked immunosorbent assay (ELISA)

Two decades after the development of the IFA test another serological assay, the ELISA, was developed for detection of *T. parva* antibody using recombinant polymorphic immunodominant molecule (PIM) (Katende *et al.*, 1998). When compared to the IFA test, this

assay has a higher degree of sensitivity (>99%) and specificity (between 94% and 98%). Despite the superior performance of the ELISA over that of the IFA test, the ELISA has similar limitations to the IFA test: it does not detect the parasite itself, the animal may have already cleared the pathogen but remained sero-positive (Dolan, 1986; Bishop *et al.*, 1992).

2.6.3 Molecular techniques

Detection of *T. parva* infections in carrier animals has always been a challenge when using parasitological and serological methods. Until recently, experimental tick transmission of the parasite from infected animals to susceptible animals was the definitive method of determining a carrier state. However this approach is expensive and time consuming and can also be intermittent (Dolan, 1986). The advent of molecular diagnosis has led to the discovery of molecular techniques ranging from the classical single polymerase chain reaction (PCR) to more sophisticated techniques based on the use of DNA probes. Their use in diagnosis has improved the sensitivity and specificity that previous diagnostic tests lacked over the years.

2.6.3.1 Conventional PCR assays

The use of primers derived from a repetitive sequence (TpR locus) in a PCR successfully amplified *T. parva* DNA in experimentally infected animals but not in field cattle (Bishop *et al.*, 1992; Watt *et al.*, 1998). These findings demonstrated the importance of the DNA target region for the successful use of PCR-based methods. Because the TpR locus exhibits some degree of polymorphism among *T. parva* stocks, it was not suitable for amplification of DNA from all field isolates including *T. parva* Muguga, an ECF-causing *T. parva* stock (Bishop *et al.*, 1992, 1997). TpR primers also lack specificity as they amplify other closely related non-pathogenic *Theileria* parasites (Watt *et al.*, 1997).

2.6.3.2 PCR-based hybridization assays

To improve the sensitivity of PCR-based diagnostic techniques, PCR assays are usually coupled with hybridization methods. A PCR-probing assay based on the small subunit ribosomal RNA (18S rRNA) gene was developed for detection of different *Theileria* parasites (Allsopp *et al.*, 1993). The assay involves amplification of a portion of the variable region of the parasite's 18S rRNA gene followed by hybridization with radioactively labelled species-specific oligonucleotide probes. Ribosomal RNA genes are widely used in diagnosis, since amplification primers can be designed in conserved regions of the gene to amplify the gene from all related organisms, while the variable regions can be used to differentiate between

different species. This technique has been used for discrimination of *Theileria* spp., *Babesia* spp., *Anaplasma* spp. and *Ehrlichia* spp. (Allsopp *et al.*, 1993; Bishop *et al.*, 1995; Gubbels *et al.*, 1999; Georges *et al.*, 2001). The use of PCR-based hybridization assays does not only allow discrimination of closely related species but simultaneous detection of other piroplasmids and detection of previously unrecognized species (Gubbels *et al.*, 1999; Birkenheuer *et al.*, 2004; Nijhof *et al.*, 2005; Oosthuizen *et al.*, 2008, 2009). The *T. parva* 104 kDa rhoptry antigen (p104) gene has also been successfully exploited in the detection of *T. parva* infection particularly in carrier animals (Skilton *et al.*, 2002; Kaba *et al.*, 2005; Konnai *et al.*, 2006). However, a drawback with PCR-based hybridization assays is that they are very laborious and time-consuming because of the additional hybridization step required to confirm a positive result.

2.6.3.3 PCR-based RFLP assays

Recently, semi-nested PCR-RFLP assays based on the *T. parva* p104 and 18S rRNA genes have been used for detection of *T. parva* infections (Geysen, 2000; Bazarusanga *et al.*, 2007; DeDeken *et al.*, 2007). Since p104 is a single copy gene this makes the assay less sensitive and not ideal for detection of *T. parva* infections in carrier animals. Although the 18S rRNA-based assay is effective in detecting *T. parva* DNA, the additional restriction enzyme digestion, which often requires overnight incubation makes these assays time-consuming and laborious. Therefore these assays suffer the same limitations as the PCR-based hybridization assays.

2.6.3.4 Real-time PCR assays

Real-time PCR has greatly improved molecular detection and diagnosis of organisms belonging to the same genus (Nicolas *et al.*, 2002; Kares *et al.*, 2004). This technique allows not only the accurate detection and quantification of specific DNA in various biological samples but also allows differentiation of species or strains of several medically important pathogenic organisms by melting curve analysis of fluorescent hybridization probes (Nicolas *et al.*, 2002). The use of the LightCycler® (Roche Diagnostics) allows fast real-time monitoring of a PCR, where amplification and detection can be accomplished in one closed capillary tube, which minimizes contamination problems.

Although the real-time PCR technology has greatly improved molecular detection of organisms of veterinary, medical and economic importance (Nicolas *et al.,* 2002; Moonen *et al.*, 2003; Stone *et al.*, 2004; Kares *et al.*, 2004; Orrù *et al.*, 2004; Whiley *et al.*, 2004; Bischoff *et al.*, 2005; Kim *et al.*, 2005; Ramaswamy *et al.*, 2005), until this study, this technology has not been exploited in the detection of *T. parva* infections.

2.7 Characterization of *T. parva* stocks

Initially, the causative agents for ECF, Corridor disease and January disease were called *T. parva*, *T. lawrencei* and *T. bovis*, respectively. However, upon realization that *T. bovis* is morphologically and serologically indistinguishable from *T. parva* the causative agent of ECF (Lawrence *et al*., 1994b), a trinomial system was proposed to define the three types of disease, based on the names for the formerly separate species. The original buffalo parasite causing Corridor disease was designated as *T. parva lawrencei* and *T. parva* parasites responsible for classical ECF and January disease were called *T. parva parva* and *T. parva bovis*, respectively (Lawrence, 1979; Uilenberg, 1976). However, this system was later discarded as a result of lack of biological evidence for discrimination of the subspecies (Perry and Young, 1993). *Theileria parva* parasites that cause Corridor disease are now referred to as buffalo-derived parasites because the transmission of the parasite occurs from buffalo to cattle and *T. parva* parasites that cause ECF and January disease are referred to as cattle-derived parasites because transmission occurs from cattle to cattle. Serological and molecular evidence suggests that, while *T. parva bovis* and *T. parva parva* parasites are antigenically and genetically similar, there is a great deal of antigenic and genetic variation between *T. parva lawrencei* isolates (Irvin *et al.,* 1983; Minami *et al.,* 1983; Conrad *et al.,* 1987; 1989; Collins and Allsopp, 1999).

Differentiation of the three *T. parva* types was traditionally based on the differences in the numbers of schizonts and piroplasms present in the infected animal and the epidemiology of the diseases they cause (Norval *et al.*, 1992). A range of assays have subsequently been developed for the differentiation of buffalo-derived and cattle-derived *T. parva* isolates.

2.7.1 Monoclonal antibody screening assays

Immunofluorescence assays employing a panel of anti-schizont monoclonal antibodies (Mabs) and schizont-infected lymphoblastoid cell cultures have been used to define the Mab reactivity profile of schizonts from different *T. parva* isolates (Minami *et al*., 1983; Irvin *et al*., 1983; Conrad *et al*., 1987; 1989). Variations in Mab profiles amongst *T. parva* isolates have been observed and are attributed to epitope variation in single antigens, although cross-reaction might appear with other antigens.

In vitro characterization of *T. parva* isolates is based mainly on the PIM which has been shown to be abundant and localized on the surface of the schizont stage (Shapiro *et al*., 1987). The PIM molecule has been extensively characterized (Baylis *et al*., 1993; Toye *et al*., 1996; Geysen *et al*., 2004) and is utilized in recombinant form for diagnosis (Katende *et al*., 1998). Serological characterization is based on the use of a battery of Mabs raised against PIM that has revealed extensive diversity in buffalo-derived isolates and a more limited diversity in cattle-derived parasites (Irvin *et al*., 1983; Minami *et al*., 1983; Conrad *et al*., 1987; 1989). This variation is presumed to be due to the variable central region of the molecule.

Several Mabs, which neutralize sporozoite entry into host cells *in vitro,* have been generated. These antibodies detected other parasite surface proteins including, p67, p104 and p150 (Shapiro *et al*., 1987; Iams *et al*., 1990; Nene *et al*., 1992; Skilton *et al*., 1998). These surface proteins are capable of inducing sporozoite-neutralizing antibodies and have also been exploited in discriminatory assays for *T. parva* isolates (Shapiro *et al*., 1987; Iams *et al*., 1990; Nene *et al*., 1992; Skilton *et al*., 1998). These surface proteins are usually encoded by single copy genes and contain a polymorphic central region of amino acid sequence flanked by a conserved amino and carboxyl terminus (Toye *et al*., 1995; Skilton *et al*., 1998). While PIM and p150 are expressed in both the sporozoite and the schizont stages of the parasite, p67 and p104 are only expressed in the sporozoite stage (Shapiro *et al*., 1987; Katende *et al*., 1998). Mabs raised against p150, an immunodominant antigen of *T. parva*, have shown cross-reactivity with PIM as a result of sequence homology (Skilton *et al*., 1998). Five variants of p150 were revealed in an analysis of different stocks of *T. parva* and the variation is attributed to the variable proline-rich central region of repeated amino acid motifs of the molecule (Skilton *et al*., 1998).

2.7.2 Molecular characterization

Theileria parva genes coding for antigenic proteins (PIM, p67, p104, p150) and ribosomal RNA gene [internal transcribed spacers (ITS), small subunit rRNA (SSUrRNA) and large subunit rRNA (LSUrRNA)] sequences have been analysed in search of discriminatory differences between *T. parva* isolates (Baylis *et al*., 1993; Toye *et al*., 1996; Nene *et al*., 1996; Collins *et al*., 1999; Geysen *et al.*, 2004).

Characterization of the p67 gene sequence has revealed the presence of a 130 bp deletion in the central region in cattle-derived *T. parva* isolates and this deletion is not present in buffalo-derived parasites in East Africa (Nene *et al.,* 1996; Nene *et al.,* 1999). Consequently it has been assumed that all cattle-derived isolates have the deletion while buffalo-derived *T. parva* isolates lack the deletion. However, Collins (1997) found that several South African isolates from the Kruger National Park, one of which caused classical Corridor disease in cattle, also had the deletion in p67.

DNA markers, DNA probes and oligonucleotide primers for *T. parva* characterization have been developed and selectively used to genotype *T. parva* stocks from buffalo and cattle in several studies (Conrad *et al*., 1987; 1989; Allsopp *et al*., 1989; Bishop *et al*., 1993; Oura *et al*., 2003). Recently, restriction fragment length polymorphism profiles exploiting the variable region of the parasite antigen genes have been used to discriminate between buffalo- and cattle-derived *T. parva* isolates (Geysen *et al*., 1999; Bishop *et al*., 2001), including the PIM-based and p104-based semi-nested PCR-RFLP assays (Geysen, 2000). Polymorphic immunodominant molecule and p104 RFLP profiles from buffalo-derived *T. parva* stocks are more polymorphic than those from cattle-derived stocks (Geysen *et al*., 1999). Profiles obtained from buffalo stocks are also heterogeneous whereas cattle-derived *T. parva* stocks are often homogeneous. This form of distinction has been established using *T. parva* stocks from East Africa (Geysen *et al*., 1999; Bishop *et al*., 2001).

Recently, micro- and mini-satellite markers have been developed for characterizing *T. parva* stocks and enable detection of higher levels of polymorphism than PCR-RFLP methods (Oura *et al.*, 2003; 2005). Micro- and mini-satellite markers are regarded as powerful tools for investigating population structure because they allow simple analysis of variation in the copy number of repeat motifs present within such loci, and are frequently located in non-coding regions, therefore believed to be representative of population history (Oura *et al*., 2003;

Odongo *et al*., 2006). However, the use of microsatellite markers is limited to parasite clones and cannot be directly used to characterize field samples, which usually contain complex mixtures of multiple *T. parva* strains.

2.8 Aim

Effective control of cattle theileriosis depends on the effective tools for detection of *T. parva*, particularly in carrier animals, and understanding the epidemiology of the parasite. Therefore, the aim of this study was to improve the sensitivity and specificity of the official diagnostic test package in South Africa by developing a real-time PCR test for detection of *T. parva* infections in buffalo (*Syncerus caffer*) and cattle and to characterize South African *T. parva* isolates using PCR-RFLP profiles and sequences of the parasite genes coding for p67, p104, and PIM to identify cattle-type parasites.

2.9 Thesis overview

In view of the above aim, the following is the overview of this thesis:

Chapter 3: Seeking to improve the current diagnosis of *T. parva* infections in cattle and buffalo, this chapter describes the development and evaluation of a *'T. parva*-specific' real-time PCR assay. The specificity and the sensitivity of this assay is evaluated and compared to that of other molecular assays currently used in the detection of *T. parva*. The applicability and reproducibility of the real-time PCR assay for routine diagnostics is also assessed.

Chapter 4: This chapter focuses on the characterization of *T. parva* parasites that occur in cattle and buffalo in South Africa using analysis of the *T. parva* p67 gene. The application of PCR product profiles and sequences in the identification and differentiation of cattle- and buffalo-type p67 alleles is also reviewed.

Chapter 5: In this chapter, p104 PCR-RFLP profiles and inferred amino acid sequences obtained from cattle and buffalo *T. parva* samples are analysed in an attempt to identify cattle-type alleles. The findings from the analysis of the p104 gene are compared with those obtained in the previous chapter where the p67 gene was analyzed.

Chapter 6: Analysis of the p67 and p104 gene sequences in the previous two chapters revealed the presence of parasites possessing cattle-type alleles in cattle from a farm in Ladysmith. Furthermore, cattle-like alleles were identified from buffalo *T. parva* samples. This chapter discusses the characterization of the *T. parva* gene encoding the PIM, to confirm these findings. Data from analysis of all three genes is compared.

Chapter 7: The overall findings and conclusions on the thesis outcome are discussed in this chapter. The successes and challenges in the development of a specific, sensitive and rapid diagnostic test are reviewed. Recommendations to be considered for improving the assay are outlined. The identification of cattle-type alleles, in both cattle and buffalo samples and their implication on genetic diversity and in the epidemiology of theileriosis in South Africa are discussed. Reflections on the concerns and challenges in the application of individual gene profiles in trying to distinguish between cattle- and buffalo-derived *T. parva* parasites in South Africa are also included in this chapter.

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