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Improved molecular diagnostics and characterization of *Theileria parva* isolates from cattle and buffalo in South Africa

By

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A thesis submitted to the Faculty of Veterinary Science, University of Pretoria, South Africa, in fulfillment of the requirements for the degree

Philosophiae Doctor

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DEDICATION

I dedicate this thesis to the memory of my late grandmother,

Mrs Ethel Magaret Sibeko,

who has been a source of inspiration and a pillar of strength,

without whose unlimited support I would not have realized this dream;

I will be forever grateful for everything she has been to me.

DECLARATION

I hereby declare that this thesis is my own work. It is submitted in fulfillment of the degree, **Philosophiae Doctor**, in the University of Pretoria, South Africa. It has not been submitted before for any degree or examination in any other University, nor has it been prepared under the aegis or with the assistance of any other body or organization or person outside the University of Pretoria, South Africa, other than as indicated in the acknowledgements which follow.

Kgomotso P. Sibeko

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THESIS SUMMARY

The aim of this study was to improve the official diagnostic test package in South Africa for detection of *Theileria parva* infections in cattle and Cape buffalo (*Syncerus caffer*) and to investigate the presence of cattle-type *T. parva* parasites in buffalo and cattle in South Africa. To improve diagnosis of *T. parva* infections, a *T. parva*-specific real-time polymerase chain reaction (PCR) assay based on hybridization probe technology was developed. Oligonucleotide primers and hybridization probes used in the assay were designed based on the 18S ribosomal RNA (rRNA) gene. The primers amplify *T. parva* and *Theileria* sp. (buffalo) DNA but the hybridization probes specifically detect *T. parva* amplicons. Because of the high sequence similarity between the *T. parva* and *Theileria* sp. (buffalo) 18S rRNA genes, amplification of *Theileria* sp. (buffalo) DNA could not be avoided; no other bovine blood pathogens tested were amplified by these primers. The real-time PCR assay demonstrated superior sensitivity compared to other molecular tests used in detection of *T. parva* infections, reliably detecting the parasite in carrier animals with a piroplasm parasitaemia as low as $8.79x10^{-4}$% with minute template DNA input. The assay requires less time to perform with a low risk of contamination because of the closed-tube system that does not require handling of amplicons for post-PCR analysis.

The presence of cattle-type *T. parva* parasites in buffalo and cattle was investigated using restriction fragment length polymorphism (RFLP) profiles of PCR products and sequences of the parasite genes which code for the antigenic proteins p67, p104, and the polymorphic immunodominant molecule (PIM). Cattle-type p67, p104 and PIM alleles were identified from three *T. parva* samples obtained from cattle from a farm near Ladysmith in the KwaZulu-Natal Province. These cattle-type alleles were identical to those previously identified from a cattle-derived *T. parva* stock, *T. parva* Muguga, a parasite stock that causes East Coast fever (ECF) in Kenya; however, ECF was not diagnosed in animals in this farm. Cattle-type alleles identical to those previously reported were not identified from *T. parva* buffalo samples, but variants of p67 allele 1 as well as p104 allele 1, both previously obtained from *T. parva* Muguga, were identified. It is not known if parasites that possess these variants can cause disease, and the risk of their adapting to cattle as in the case of ECF and January disease needs to be evaluated. Furthermore, these findings suggest that cattle-like alleles may not be exclusively associated with cattle-derived *T. parva* parasites. Most of the p67, p104 and PIM gene sequences obtained in this study were not identical to known sequences; furthermore, novel alleles were identified, demonstrating extensive genetic diversity in the

South African *T. parva* parasite population in buffalo. The significance of the parasites that possess 'novel' alleles in the epidemiology of theileriosis in South Africa still needs to be determined. The identification of variants and novel alleles reveals that p67, p104 and PIM gene PCR-RFLP profiles are more complex than previously thought and the classification of buffalo- and cattle-derived *T. parva* parasites in South Africa based on p67, p104 and PIM gene profiles would not be possible. Identification of more reliable markers that can be directly associated with the theilerial disease syndromes remains a challenge.

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).

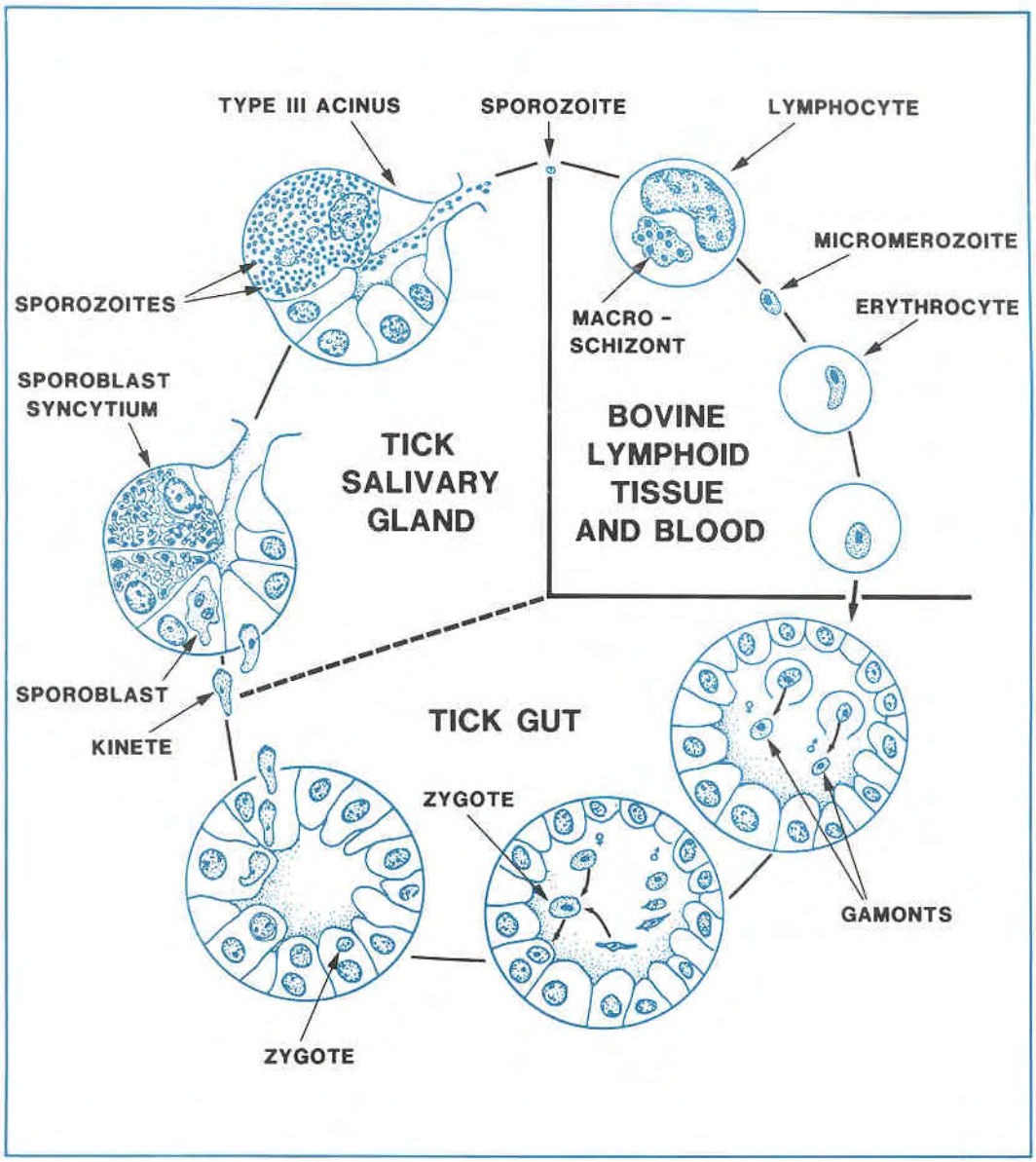

Figure 2.1 The life cycle of *Theileria parva* [from International Labotoratory for Research on Animal Diseases (ILRAD) 1983. Annual report 1982. Nairobi, ILRAD].

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

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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.

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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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CHAPTER 3

Development and evaluation of a real-time PCR test for detection of *Theileria parva* infections in Cape buffalo (*Syncerus caffer*) and cattle

Whatsoever thy hand findeth to do, do it with thy might; for there is no work, nor device, nor knowledge, nor wisdom, in the grave, whither thou goest. ***Ecclesiastes 9:10***

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"The journey of a thousand miles begins with one step." ***Lao-Tse***
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3.1 Abstract

Corridor disease, caused by the tick-borne protozoan parasite *Theileria parva*, is a controlled disease in South Africa. The Cape buffalo (*Syncerus caffer*) is the reservoir host and uninfected buffalo have become sought-after by the game industry in South Africa, particularly for introduction into Corridor disease-free areas. A real-time polymerase chain reaction (PCR) test for detection of *T. parva* DNA in buffalo and cattle was developed to improve the sensitivity and specificity of the official diagnostic test package in South Africa. Oligonucleotide primers and hybridization probes were designed based on the 18S ribosomal RNA (rRNA) gene. Amplification of control DNA using *Theileria* genus-specific primers resulted in detection of *T. taurotragi* and *T. annulata,* in addition to *T. parva.* A *T. parva*-specific forward primer was designed which eliminated amplification of all other *Theileria* species, except for *Theileria* sp. (buffalo); however only the *T. parva* product was detected by the *T. parva*-specific hybridization probe set. The real-time PCR assay requires less time to perform, is more sensitive than the other molecular assays previously used in *T. parva* diagnostics and can reliably detect the parasite in carrier animals with a piroplasm parasitaemia as low as 8.79×10^{-4}%.

3.2 Introduction

Theileria parva is a tick-borne protozoan parasite which occurs in eastern, central and southern Africa, and causes East Coast fever (ECF), Corridor disease and January disease (Uilenberg *et al.*, 1982; Perry *et al.*, 1991). The Cape buffalo (*Syncerus caffer*) is the natural reservoir of the parasite. Buffalo are also carriers of several other *Theileria* species which can infect cattle, including the relatively benign *T. mutans* and the apathogenic *T. velifera*. Under certain circumstances another group of relatively benign organisms, belonging to the *T. buffeli* / *T. orientalis* / *T. sergenti* complex can also cause disease in cattle and loss of production (Norval *et al.*, 1992). *Theileria buffeli* and *Theileria* sp. (buffalo) have been identified in some buffalo populations in South Africa (Stoltsz, 1996). Very little is known about *Theileria* sp. (buffalo) which was first recognised in an isolate from a buffalo in Kenya (Allsopp *et al.*, 1993). The eland (*Taurotragus oryx*) is the reservoir host of *T. taurotragi*, which can also infect cattle and has been implicated in bovine cerebral theileriosis in South Africa (De Vos *et al.*, 1981). *Theileria annulata,* which causes tropical theileriosis in cattle in other parts of the world, does not occur in South Africa.

Theileria parva infection of cattle is a controlled disease in South Africa. Transmission of the parasite to cattle by three species of ticks, *Rhipicephalus appendiculatus, R. zambeziensis* and *R. duttoni,* causes a form of theileriosis known as Corridor disease (Neitz, 1955; Neitz, 1957; Blouin and Stoltsz, 1989). Corridor disease is an acute, usually fatal disease of cattle resembling ECF. 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. Contrary to ECF, the course of the disease is usually shorter, death occurring only three to four days after the onset of first clinical signs (Lawrence *et al*., 1994). 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. The South African cattle population is completely naïve to *T. parva* infection and should be protected against exposure. Therefore, contact between infected buffalo in game reserves and cattle, is strictly controlled by the veterinary authorities (Stoltsz, 1989).

In recent years, there has been an increased risk of theileriosis in South Africa through the breeding and translocation of so-called “disease-free” buffalo, i.e. buffalo that test negative for foot-and-mouth disease (FMD), bovine brucellosis, bovine tuberculosis and Corridor disease. Buffalo must be tested for these diseases before they are allowed to be relocated throughout the country. In the case of Corridor disease, depending on the origin and history of buffalo, buffalo must undergo one to five tests, the indirect immunofluorescent antibody (IFA) test and PCR/probe assay each time, before they are allowed to be moved or certified disease free as determined by the Veterinary authorities. With the expansion of the game industry in South Africa in the 1990s, “disease-free” buffalo have become a sought-after commodity, particularly for introduction into Corridor disease-free areas. The movement of buffalo from the large, genetically diverse herds in the Kruger National Park is prohibited as FMD, bovine tuberculosis and Corridor disease are endemic there. The relocation of buffalo from the KwaZulu-Natal parks is also prohibited as Corridor disease is endemic there. The major source of animals free from these diseases has previously been a relatively small herd at the Addo Park in the eastern Cape (Stoltsz, 1989). However, there are not enough animals at Addo to meet the increased demand and the stock is limited in genetic diversity. This led to the establishment of buffalo breeding projects from infected parent stock, some of them in areas where the vector ticks for Corridor disease occur.

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A further area of concern is the possibility of the creation of *T. parva* carrier cattle. If *T. parva* infected cattle are treated or recover spontaneously, they may become carriers of the parasite (Potgieter *et al*., 1985; Dolan, 1986; Maritim *et al*., 1989; Kariuki *et al*., 1995; Marcotty *et al*., 2002). Ticks can acquire infections from carrier cattle and 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). Accurate diagnostic tests are therefore required in South Africa to identify infected buffalo and cattle and to assist the veterinary regulatory authorities to control the movement of buffalo.

Conventional diagnosis of *T. parva* is based on the microscopic demonstration of schizonts in lymphocytes, piroplasms in erythrocytes, clinical signs and pathology as well as detection of serum antibodies to schizont antigens, using the (IFA) test (Brocklesby and Barnett, 1966; Burridge *et al*., 1973; Burridge *et al*., 1974; Radley *et al*., 1974; Goddeeris *et al*., 1982). It is impossible to differentiate *T. parva* schizonts and piroplasms from most other *Theileria* spp. using light microscopy. Disadvantages of the IFA test include cross-reactivity between certain species, difficulty in standardization and subjectivity in interpretation of the results (Norval *et al*., 1992). In addition, antibodies may not be detected if the animal is not subject to a continuous tick challenge (Burridge and Kimber, 1972). Several molecular techniques for diagnosing *Theileria* infections have therefore been developed involving the use of the polymerase chain reaction (PCR) and DNA probes (Bishop *et al*., 1992; Allsopp *et al*., 1993; Bishop *et al*., 1995; Gubbels *et al*., 1999; Collins *et al*., 2002; Ogden *et al*., 2003). These techniques have improved the sensitivity and specificity that previous diagnostic tests lacked. However, PCR and probing assays are relatively time-consuming and labour intensive, particularly when separate hybridization steps are required to confirm test outcomes. There is therefore a need for a rapid, more sensitive and specific diagnostic test to accurately detect *T. parva* infections in buffalo and cattle.

Recently, 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). This technique enables the accurate detection and quantification of specific DNA in various biological samples and also allows differentiation of species or strains of important pathogenic organisms. The use of the LightCycler® (Roche Diagnostics, Mannheim, Germany) allows fast real-time monitoring of a

PCR, where amplification and detection can be accomplished in a closed capillary tube, minimizing contamination problems. Therefore real-time PCR technology was chosen for the development of a rapid, sensitive and specific assay for detection of *T. parva*.

3.3 Materials and methods

3.3.1 Sample collection

Cattle and buffalo blood samples from different areas in South Africa were investigated. These included three known *T. parva* positive and 55 negative samples as well as 309 field samples of unknown status (Table 3.1). Gold standard positive samples included a naturally infected buffalo (KNP102 donated by South African National Parks) and two experimentally infected cattle, 9288 (splenectomized) and 9445 (intact). Both cattle (9288 and 9445) were infected with the Welgevonden *T. parva* isolate, which originated from two buffalo (welg23/04 and welg24/04) from the Welgevonden Private Game Reserve, located in the Limpopo Province of South Africa. The buffalo tested positive for *T. parva* using standard PCR (Allsopp *et al*., 1993) and reverse line blot (RLB) hybridization (Gubbels *et al*., 1999) tests. Bovine 9288 was infected with *T. parva* using *R. appendiculatus* adults of which the nymphal stage fed on buffalo welg23/04 and welg24/04 to pick-up the infection (xenodiagnosis). The animal reacted severely but recovered without treatment. Subsequently laboratory-reared *R. appendiculatus* nymphs were fed on it and the ensuing adult ticks were placed on Bovine 9445, which developed classical Corridor disease and died. Fifty-five fully susceptible cattle bred, reared and maintained under tick-free conditions for the purpose of live-blood vaccine production, at the Agricultural Research Council-Onderstepoort Veterinary Institute (ARC-OVI), South Africa, were used as gold standard negative samples.

Table 3.1 Origin and number of samples used for the evaluation of the *T. parva* real-time PCR test

Sample type	Origin of samples	Number of blood samples and animal of origin
Gold standard positive	Kruger National Park (KNP Welgevonden Game Reserve	1 buffalo (KNP102) 2 cattle [9288 (splenectomized), 9445 (intact)]*
Gold standard negative	ARC-OVI	55 cattle
Field	KNP Hluhluwe-iMfolozi Game Reserve Ladysmith farm Mabalingwe Game Reserve Marekele National Park Bloemfontein Kaalplaas farm ARC-OVI	65 buffalo 41 buffalo 34 cattle 6 buffalo and 6 cattle 15 buffalo 1 bovine 34 cattle 107 buffalo and cattle

*Experimentally infected cattle

3.3.2 DNA extraction

All DNA extractions were performed using the High Pure PCR template preparation kit (Roche Diagnostics, Mannheim, Germany) from 200 µl of EDTA blood samples. Extracted DNA was eluted in 100 µl elution buffer and stored at 4°C until further analysis.

3.3.3 Design of primers and hybridization probes

Theileria genus-specific forward [5'-GGT AAT TCC AGC TCC AAT AG-3'] and reverse [5'-ACC AAC AAA ATA GAA CCA AAG TC-3'] primers were designed for amplification of a 230 bp fragment of the V4 variable region of the 18S rRNA gene from all *Theileria* species (Figure 3.1). In addition, a forward primer [5'-CTG CAT CGC TGT GTC CCT T-3'] for specific amplification of *T. parva* was designed. For the specific detection of *T. parva* amplicons, a pair of hybridization probes [*T. parva* anchor: 5'-GGG TCT CTG CAT GTG GCT TAT--FL; *T. parva* sensor: 5'-LCRed640-TCG GAC GGA GTT CGC T—PH] was designed complementary to a *T. parva*-specific region within the amplicon (Figure 3.1). For the detection of the presence of any *Theileria* species in a sample, a pair of hybridization probes was selected complementary to a region conserved between nine *Theileria* species for which 18S rRNA gene sequence data is known (*T. annulata*_AY150056, *T. annulata*_M64243, *T. lestoquardi*_AF081135, *T. parva*_L28999, *T. parva*_L02366, *T. parva*_AF013418, *Theileria* sp. (buffalo) (Allsopp *et al.*, 1993), *T. taurotragi*_L19082, *T. buffeli*_AF236094, *T. buffeli*_Warwick-Australia_AB000272, *T. sergenti*_AY661514, *T. buffeli*_DQ104611, *T. buffeli*_AF236097, *T. buffeli*_Z15106, *T. sergenti*_AB016074,

*T. sergenti*_AF081137, *T. velifera*_AF097993, *T. mutans*_AF078815) [*Theileria* genus anchor: 5'-AGA AAA TTA GAG TGC TCA AAG CAG GCT TT--FL; *Theileria* genus sensor: 5'-LCRed705-GCC TTG AAT AGT TTA GCA TGG AAT—PH] (Figure 3.1). All primers and fluorescently-labelled hybridization probes were synthesized by TIB Molbiol (Berlin, Germany).

3.3.4 Optimized real-time PCR conditions

Amplification mixtures consisted of 4 µl of 10x LightCycler-FastStart DNA Master^{Plus} Hybridization Probes mix, yielding a final concentration of 2x in 20 µl total volume (Roche Diagnostics, Mannheim, Germany), 0.5 µM of each primer, 0.1 µM of each hybridization probe, 1U uracil deoxy-glycosylase (UDG) (Roche Diagnostics, Mannheim, Germany) and 1 to 2.5 µl (~15 ng to ~37.5 ng) of template DNA in a final volume of 20 µl. Temperature cycling was performed in a LightCycler® v2 (Roche Diagnostics, Mannheim, Germany). The UDG was activated at 40°C for 10 min before the FastStart *Taq* DNA polymerase activation step of 10 min at 95°C. The amplification programme included 45 cycles of three steps each, comprising denaturing at 95°C for 10 sec, primer annealing at 58°C for 10 sec, and product extension at 72°C for 15 sec. Following amplification, a melting curve analysis was performed by heating the samples from 40°C to 95°C with a heating rate of 0.2°C/sec. Fluorescence values were measured at 640 and 705 nm.

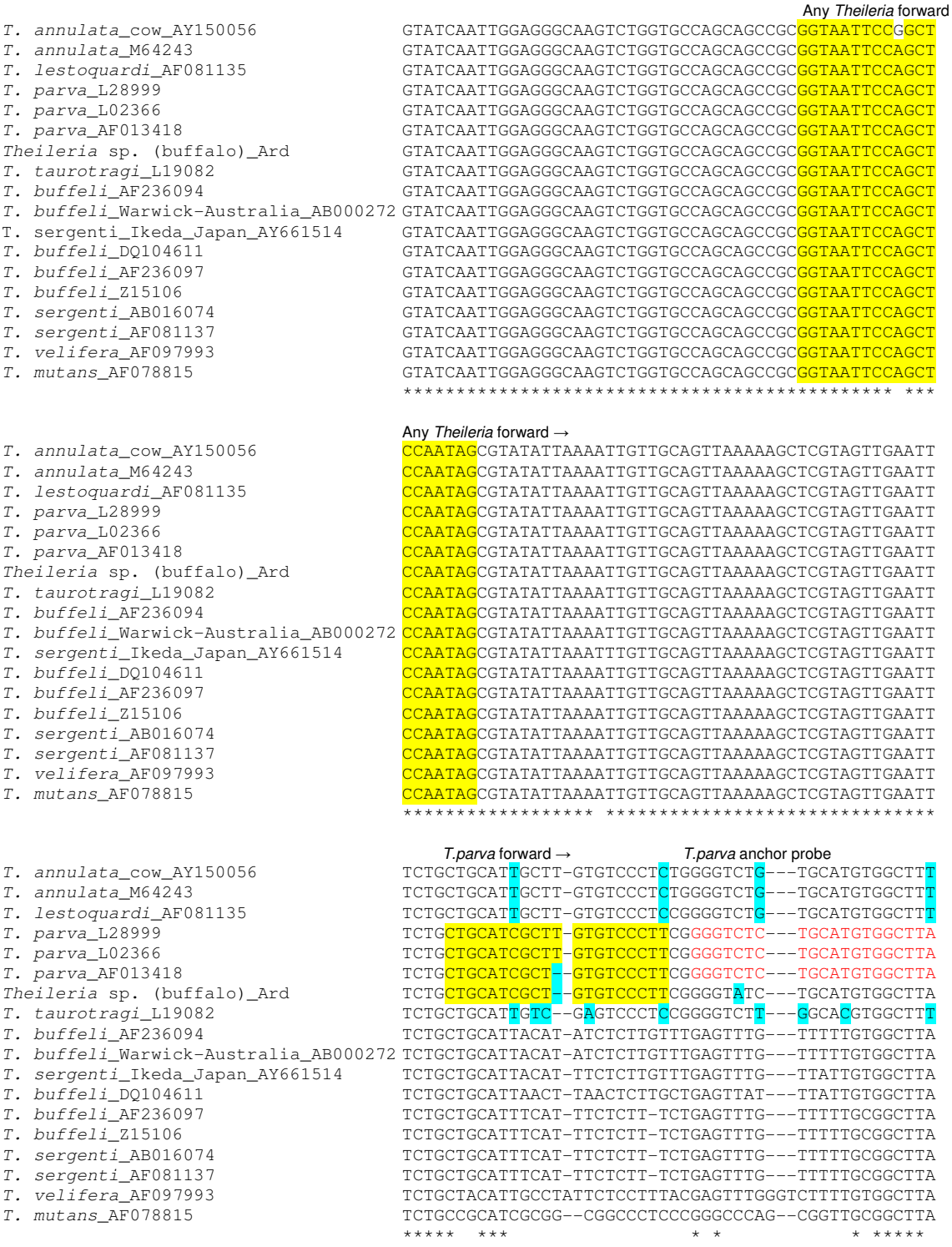

Figure 3.1 CLUSTAL X (1.81) multiple sequence alignment of the V4 variable region of published *Theileria* 18S rRNA gene sequences. Accession numbers for each sequence are provided. Amplification primers are highlighted in yellow. *T. parva*-specific hybridization probes are coloured in red, and hybridization probes designed to detect the presence of any *Theileria* species are in blue. Differences between the *T. parva* sequence and the four most closely related species (*T. annulata*, *T. lestoquardi*, *Theileria* sp. (buffalo), and *T. taurotragi*) are highlighted in cyan. This figure continues on page 49.

T.parva sensor probe

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T. annulata_cow_AY150056                TTTCGGACGGAGTTT-CTTTGTCTGAATGTTTACTTAGAGAAAAT-AGAG
T. annulata_M64243                      TTTCGGACGGAGTTT-CTTTGTCTGAATGTTTACTTTGAGAAAATTAGAG
T. lestoquardi_AF081135                 TTTCGGACGGAGTTT-CTTTGTCTGAATGTTTACTTTGAGAAAATTAGAG
T. parva_L28999                         TTTCGGACGGAGTTCGCTTTGTCTGGATGTTTACTTTGAGAAAATTAGAG
T. parva_L02366                         TTTCGGACGGAGTTCGCTTTGTCTGGATGTTTACTTTGAGAAAATTAGAG
T. parva_AF013418                       TTTCRGACGGAGTTCGCTTTGTCTGGATGTTTACTTTGAGAAAATTAGAG
Theileria sp. (buffalo)_Ard             TTTCAGACGGAGTTTACTTTGTCTGGATGTTTACTTTGAGAAAATTAGAG
T. taurotragi_L19082                    TTTCGGACGG--TTCGCT--GTCTGGATGTTTACTTTGAGAAAATTAGAG
T. buffeli_AF236094                     TTTCGGTTTGATTTTT-TCTTTCCGGATGATTACTTTGAGAAAATTAGAG
T. buffeli_Warwick-Australia_AB000272   TTTCGGTTTGATTTTT-TCTTTCCGGATGATTACTTTGAGAAAATTAGAG
T. sergenti_Ikeda_Japan_AY661514        TTTCGGATTGATTTTTATCATTCCGGATGATTACTTTGAGAAAATTAGAG
T. buffeli_DQ104611                     TTTCGGATTGATTTTT-TCTTTCCGGATGATTACTTTGAGAAAATTAGAG
T. buffeli_AF236097                     TTTCGGTTTGATTTTT-TCTTTCCGGATGATTACTTTGAGAAAATTAGAG
T. buffeli_Z15106                       TTTCGGTTTGATTTTT-TCTTTCCGGATGATTACTTTGAGAAAATTAGAG
T. sergenti_AB016074                    TTTCGGTTTGATTTTT-TCTTTCCGGATGATTACTTTGAGAAAATTAGAG
T. sergenti_AF081137                    TTTCGGTTTGATTTTT-TCTTTCCGGATGATTACTTTGAGAAAATTAGAG
T. velifera_AF097993                    TCTGGGTTCGCTTGCT----TCCCGGTGTTTTACTTTGAGAAAATTAGAG
T. mutans_AF078815                      TTTCGGACTCGCTTGC--GTCTCCGAATGTTTACTTTGAGAAAATTAGAG
                                        * *  *                * *     ****** ******** ****
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Any *Theileria* anchor probe Any Theileria sensor probe

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T. annulata_cow_AY150056                TGCTCAAAGCAGGCTTTCGCCTTGAATAGTTTAGCATGGAATAATAAAGT
T. annulata_M64243                      TGCTCAAAGCAGGCTTTCGCCTTGAATAGTTTAGCATGGAATAATAAAGT
T. lestoquardi_AF081135                 TGCTCAAAGCAGGCTTTTGCCTTGAATAGTTTAGCATGGAATAATAGAGT
T. parva_L28999                         TGCTCAAAGCAGGCTTTTGCCTTGAATAGTTTAGCATGGAATAATAAAGT
T. parva_L02366                         TGCTCAAAGCAGGCTTTTGCCTTGAATAGTTTAGCATGGAATAATAAAGT
T. parva_AF013418                       TGCTCAAAGCAGGCTTTTGCCTTGAATAGTTTAGCATGGAATAATAAAGT
Theileria sp. (buffalo)_Ard             TGCTCAAAGCAGGCTTTTGCCTTGAATAGTTTAGCATGGAATAATAAAGT
T. taurotragi_L19082                    TGCTCAAAGCAGGCTTTTGCCTTGAATAGTTTAGCATGGAATAATAAAGT
T. buffeli_AF236094                     TGCTCAAAGCAGGCTTTTGCCTTGAATAGTTTAGCATGGAATAATAAAGT
T. buffeli_Warwick-Australia_AB000272   TGCTCAAAGCAGGCTTTTGCCTTGAATAGTTTAGCATGGAATAATAAAGT
T. sergenti_Ikeda_Japan_AY661514        TGCTCAAAGCAGGCTTTTGCCTTGAATAGTTTAGCATGGAATAATAGAGT
T. buffeli_DQ104611                     TGCTCAAAGCAGGCTTTTGCCTTGAATAGTTTAGCATGGAATAATAAAGT
T. buffeli_AF236097                     TGCTCAAAGCAGGCTTTTGCCTTGAATAGTTTAGCATGGAATAATAAAGT
T. buffeli_Z15106                       TGCTCAAAGCAGGCTTTTGCCTTGAATAGTTTAGCATGGAATAATAAAGT
T. sergenti_AB016074                    TGCTCAAAGCAGGCTTTTGCCTTGAATAGTTTAGCATGGAATAATAAAGT
T. sergenti_AF081137                    TGCTCAAAGCAGGCTTTTGCCTTGAATAGTTTAGCATGGAATAATAAAGT
T. velifera_AF097993                    TGCTCAAAGCAGGCTTTTGCCTTGAATAGTTTAGCATGGAATAATAAAGT
T. mutans_AF078815                      TGCTCAAAGCAGGCCTTGCCTTGAATACTTTAGCATGGAATAATAAAGT
                                        ************** * ********** ***************** ***
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← Any *Theileria* reverse

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T. annulata_cow_AY150056                AGGACTTTGGTTCTATTTTGTTGGTT
T. annulata_M64243                      AGGACTTTGGTTCTATTTTGTTGGTT
T. lestoquardi_AF081135                 AGGACTTTGGTTCTATTTTGTTGGTT
T. parva_L28999                         AGGACTTTGGTTCTATTTTGTTGGTT
T. parva_L02366                         AGGACTTTGGTTCTATTTTGTTGGTT
T. parva_AF013418                       AGGACTTTGGTTCTATTTTGTTGGTT
Theileria sp. (buffalo)_Ard             AGGACTTTGGTTCTATTTTGTTGGTT
T. taurotragi_L19082                    AGGACTTTGGTTCTATTTTGTTGGTT
T. buffeli_AF236094                     AGGACTTTGGTTCTATTTTGTTGGTT
T. buffeli_Warwick-Australia_AB000272   AGGACTTTGGTTCTATTTTGTTGGTT
T. sergenti_Ikeda_Japan_AY661514        AGGACTTTGGTTCTATTTTGTTGGTT
T. buffeli_DQ104611                     AGGACTTTGGTTCTATTTTGTTGGTT
T. buffeli_AF236097                     AGGACTTTGGTTCTATTTTGTTGGTT
T. buffeli_Z15106                       AGGACTTTGGTTCTATTTTGTTGGTT
T. sergenti_AB016074                    AGGACTTTGGTTCTATTTTGTTGGTT
T. sergenti_AF081137                    AGGACTTTGGTTCTATTTTGTTGGTT
T. velifera_AF097993                    AGGACTTTGGTTCTATTTTGTTGGTT
T. mutans_AF078815                      AGGACTTTGGTTCTATTTTGTTGGTT
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3.3.5 Specificity of the real-time PCR assay

To determine the analytical specificity of the real-time PCR assay, 2.5 µl (~37.5 ng) DNA of several different *Theileria* species, including *T. annulata, T. taurotragi, T. velifera, T. buffeli, T. mutans*, *Theileria* sp. (buffalo) and *T. parva* was subjected to the assay. In addition, DNA from other blood parasites including *Babesia* spp., *Ehrlichia* spp., *Anaplasma* spp., *Trypanosoma* spp. and bacteria commonly found in cattle and buffalo were tested for this purpose (Table 3.2). DNA from the 55 gold standard negative cattle (Table 3.1) was also tested. Both sets of primers (the *T. parva*-specific forward primer together with the *Theileria* genus-specific reverse primer and the *Theileria* genus-specific forward primer together with the *Theileria* genus-specific reverse primer) were used in separate amplification reactions and both probe sets were included in the reactions for detection of PCR products.

Table 3.2 Specificity of the *T. parva* real-time PCR test using the *T. parva*-specific forward primer, the *Theileria* genus-specific reverse primer and both probe sets

Sample	Results			
	Amplification		Melting peak (temperature)	
	***T. parva* 640 nm**	***Theileria* species 705 nm**	***T. parva* 640 nm**	***Theileria* species 705 nm**
Theileria parva (KNP102)	+	+	+	+
Theileria sp. (buffalo)	(+)	+	-	+ (63°C)
Theileria buffeli + *Theileria mutans* (14044)	-	-	-	-
Theileria mutans (14043)	-	-	-	-
Theileria taurotragi + *Theileria mutans* + *Theileria parva* (14048)	+	+	+ (63°C)	+ (62°C)
Theileria taurotragi + *Theileria mutans* + *Theileria parva* (14049)	+	+	+ (63°C)	+ (62°C)
Theileria taurotragi + *Theileria mutans* (14045)	-	-	-	-
Theileria taurotragi + *Theileria mutans* (14046)	-	-	-	-
Theileria taurotragi + *Theileria mutans* (14047)	-	-	-	-
Arcanobacterium pyogenes Bacteria 6964/1B (1)	-	-	-	-
Bacillus lactosporus Bacteria 9879/2(2)	-	-	-	-
Staphyloccocus aureus Bacteria 9351/1(3)	-	-	-	-
Escherichia coli Bacteria 097(4)	-	-	-	-
Salmonella typhimurium Bacteria1021/6(5)	-	-	-	-
Enterococcus facium Bacteria 9351/3(6)	-	-	-	-
Ehrlichia ruminantium (Ball3 vaccine strain) Heartwater-5540 (16928)	-	-	-	-
Ehrlichia ruminantium (Ball3 vaccine strain) Heartwater-5244 (16929)	-	-	-	-
Anaplasma centrale-8230 (16931)	-	-	-	-
Babesia bigemina + *A. centrale*-9456.1 (16932)	-	-	-	+ (48.8)*
Babesia bovis (16823)	-	-	-	-
Babesia bigemina (16824)	-	-	-	+ (48.8)*
Theileria equi-20 (16369)	-	-	-	-
Babesia caballi- 502 (16368)	-	-	-	-
Trypanosome-29b (16367)	-	-	-	-
Trypanosome -27b (16366)	-	-	-	-
Trypanosome -24b (16365)	-	-	-	-
Trypanosome -8a (16363)	-	-	-	-

*Although no amplification was observed in these samples, a melting peak at 48.8°C was detected at 705 nm. This probably indicates that a non-specific PCR product was obtained from *Babesia bigemina* DNA, and the *Theileria* genus-specific hybridization probes were able to anneal to this product, yielding a melting peak at 48.8°C. The *T. parva*-specific probes did not hybridize to this non-specific product.

3.3.6 Sensitivity of the real-time PCR assay

To determine the lower limit of detection of the real-time PCR assay for *T. parva*, blood from a naturally infected buffalo (KNP102) with a piroplasm parasitaemia of 0.009% was used. The parasitaemia was determined by examining approximately 34000 erythrocytes and counting the number of infected cells. The *T. parva* infection status of the buffalo was confirmed by performing a xenodiagnosis. Approximately 1200 *R. appendiculatus* nymphs were fed on buffalo KNP102 in ear-bags. After 5 days, approximately 450 ticks were collected; the engorged nymphs were washed, packed in containers and placed in an acaridarium to moult to the adult stage. The ensuing adult ticks (n=220) were used to infect Bovine 9446/6 with *T. parva*, which died from classical Corridor disease as confirmed by post-mortem examination, conventional PCR and RLB. A 10-fold dilution series of infected blood from buffalo KNP102 from 10^{0} to 10^{-6} was prepared in uninfected bovine blood. A blind experiment was performed: a set of thirty replicates of each dilution was made and the identity of the samples was concealed from the operator of the real-time PCR assay. DNA was extracted as previously described and eluted in 100 µl elution buffer. Two and a half microlitres of DNA were used in the amplification reaction with the *T. parva*-specific forward primer, *Theileria* genus-specific reverse primer and both probe sets. The estimated sensitivity and 95% confidence intervals for the true sensitivity for each group of dilutions prepared from KNP102 *T. parva*-infected blood were calculated using the standard error of the estimated sensitivity of each dilution group.

3.3.7 Comparison of the real-time PCR assay with other molecular tests

Three other molecular tests used for detection of *T. parva,* the conventional PCR/probe assay (PP) as described by Allsopp *et al*. (1993), the RLB (Gubbels *et al*., 1999) and the *cox*III PCR-based restriction fragment length polymorphism (RFLP) assay, described below, were selected for comparison with the real-time PCR assay. DNA samples from three gold standard positive animals (two cattle and one buffalo), 55 gold standard negative cattle and 309 field samples including both cattle and buffalo (Table 3.1) were subjected to the three tests and the real-time PCR assay using the *T. parva*-specific primer set. The sensitivity of the three tests in detecting *T. parva* was compared.

Cox*III PCR-based RFLP assay:* Primers were designed based on the *T. parva* cytochrome oxidase subunit III gene sequence (accession number: Z23263): Cox F [5'-CAA CAT TGT TAA AGC TAT CCA A-3'], Cox R [5'-ATG CGA AAC AGC GTA CAA TCA TA-3'] and Cox nR [5'-TTA TAG TAC AGG ATT AGA TAC CC-3']. A nested PCR was performed to amplify a *Theileria* genus-specific region in the *cox*III gene using primer sets Cox F and Cox R for the primary PCR and Cox F and Cox nR for the secondary PCR. The amplification mixture consisted of 1 µl yellow sub (GENEO BioProductions, Hamburg, Germany), 12.5 µl PCR buffer [KTT buffer: 150mM KCl, 30 mM Tris-HCl pH 8.6, 3% Triton X-100 and 3.3 mM MgCl2], 0.2 mM dNTPs, 0.4 µM of each primer, 0.5 U *Taq* polymerase and 5 µl DNA (~75 ng) in a final volume of 25 µl. Half a microlitre of the primary PCR product from a 25 µl PCR mixture was used as a template for the secondary PCR. A Hot start PCR programme was followed with the temperature of the thermocycler (BIOMETRA) (Whatman Biometra, Göttingeng, Germany) increased and held at 84°C until all the samples were loaded into the machine. For the primary PCR, the amplification programme included an initial denaturation step at 94°C for 4 min followed by 35 cycles of three steps each, comprising denaturing at 94°C for 45 sec, primer annealing at 59°C for 45 sec, and product extension at 72°C for 1 min. The PCR conditions for the secondary PCR were the same as described above except for the annealing temperature and amplification cycles which were reduced to 56°C and 25, respectively. The amplicons were digested with *Mbo*I overnight and separated on a 10% polyacrylamide gel before DNA detection by silver staining.

3.3.8 Proficiency testing

To determine the reproducibility of the real-time PCR assay, the test was performed in two different laboratories; at the Department of Veterinary Tropical Diseases (DVTD), Faculty of Veterinary Science, University of Pretoria and the Parasitology Division, ARC-OVI. A total of 115 blood samples (different from those used in the comparison of the real-time PCR assay with other molecular tests) including 20 cattle samples from known negative animals (vaccine animals), 41 buffalo samples from a *T. parva* endemic area, Hluhluwe-iMfolozi Game Reserve (expected to be positive) and 54 samples of unknown *T. parva* infection status, including cattle and buffalo received by the ARC-OVI for routine diagnostics, were used. A blind sample set was prepared and the DNA was extracted using the MagNA Pure LC System (Roche Diagnostics, Mannheim, Germany). The DNA samples were equally divided and subjected to the real-time PCR assay at the two laboratories. The data were analysed independently and forwarded to an independent analyst to determine the agreement of results.

3.4 Results

3.4.1 Specific detection of *T. parva* using the real-time PCR assay with hybridization probes

Initially, a primer set was designed for amplification of the V4 variable region of the 18S rRNA gene from all *Theileria* species. These *Theileria* genus-specific forward and reverse primers yielded a PCR product of approximately 230 bp from any *Theileria* species tested. The *T. parva*-specific hybridization probes were used for detection of *T. parva* amplicons generated using these primers. In *T. parva* positive samples, an increase in fluorescence was detected at 640 nm and melting curve analysis indicated that the *T. parva*-specific melting peak was at 63 ±0.62°C (Figure 3.2a). A smaller shoulder peak at 51 ±0.55°C was observed in all *T. parva* isolates. In addition to *T. parva* amplicons, the *T. parva*-specific hybridization probes recognised *T. taurotragi* and *T. annulata* PCR products generated by the *Theileria*-genus specific primers. As has been demonstrated even with single base differences in heterozygotes (Bollhalder *et al*., 1999), the nucleotide base differences in the amplicons of *T. taurotragi, T. annulata* and *T. parva* resulted in different melting peaks when used with the *T. parva*-specific hybridization probes, allowing discrimination between the different amplicons, with the *T. annulata* Tm at 48 ±0.09°C and *T. taurotragi* at 45 ±0.19°C (Figure 3.2b).

For the detection of the presence of any *Theileria* species in a sample, the *Theileria* genus-specific probe set was used to detect amplicons generated using the *Theileria* genus-specific primers. An increase in fluorescence at 705 nm was detected when all control *Theileria* DNA samples were tested, but it should be noted that this result gives no indication of which *Theileria* species is present.

To increase the specificity and sensitivity of the test, a *T. parva*-specific forward primer was designed for specific amplification of *T. parva* from a mixed infection, since competition between different templates could result in preferential amplification of the template with the highest starting concentration (Contamin *et al*., 1995). However, the *Theileria* sp. (buffalo) 18S rRNA sequence (accession number: DQ641260) is very similar to that of *T. parva* (accession number: L02366) and it was not possible to design an amplification primer that will not also amplify DNA from the former species. The *T. parva*-"specific" forward primer, used together with the *Theileria* genus-specific reverse primer, yielded a product of 167 bp

from both *T. parva* and *Theileria* sp. (buffalo) control samples. However, when these primers were used to amplify the *Theileria* control DNA samples in the presence of the *T. parva* probe set, a *T. parva*-specific melting peak at 63 ±0.62°C was only observed in the reaction containing *T. parva* positive control DNA (Figure 3.3). Except for *Theileria* sp. (buffalo) (see results below), no amplification or melting peaks at 640 nm were observed from any of the other *Theileria* species (Figure 3.3) or from any of the other blood parasites including *Babesia* spp., *Ehrlichia* spp., *Anaplasma* spp. and *Trypanosoma* spp. and bacterial DNA samples tested (Table 3.2). No increase in fluorescence in either the 640 nm or 705 nm channels was detected from any of the 55 negative bovine samples tested for *T. parva* (results not shown).

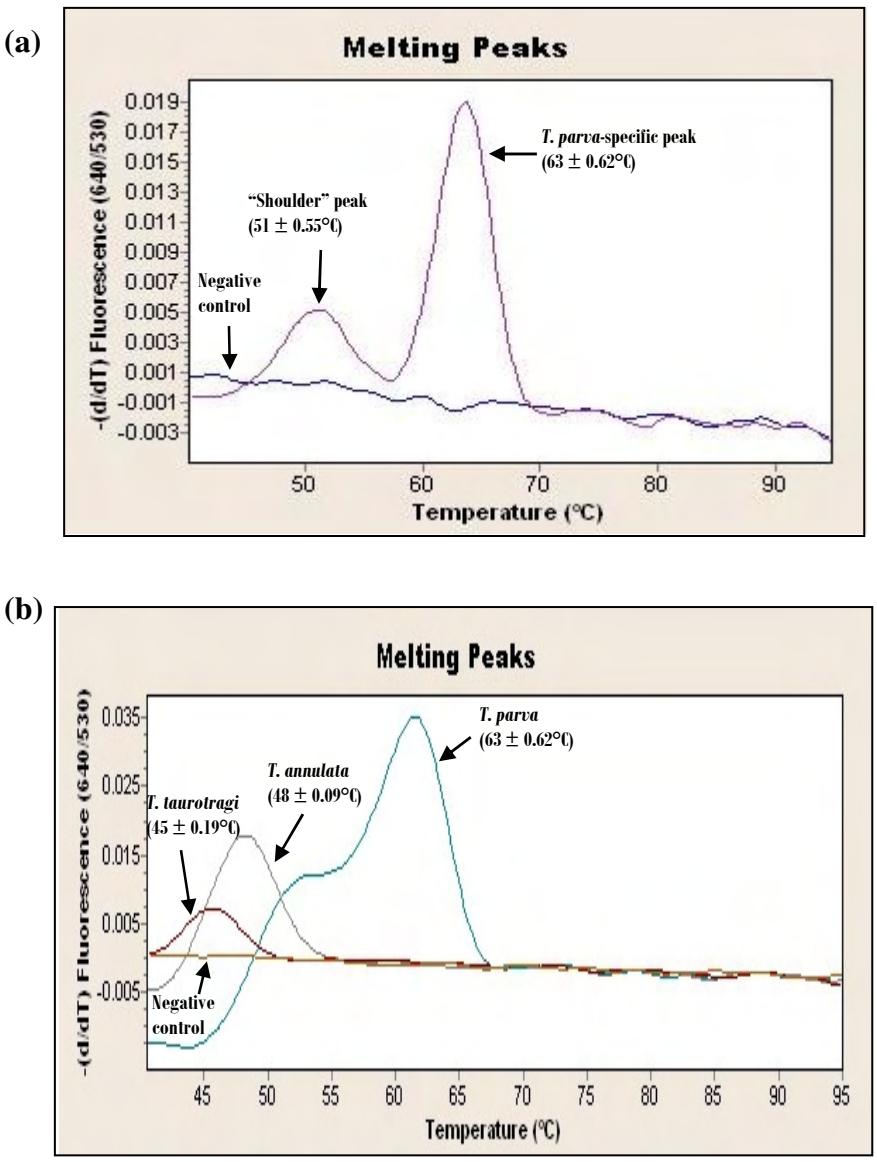

Figure 3.2 Detection of *T. parva* positive control DNA using the real-time PCR assay with *Theileria* genus-specific forward and reverse primers together with *T. parva*-specific hybridization probes. (a): Melting curve analysis showing *T. parva*-specific melting peak at 63 ±0.62°C and no fluorescence in the negative controls. (b): Discrimination between *T. parva, T. annulata* and *T. taurotragi* using melting curve analysis, with the melting peak for *T. parva* at 63 ±0.62°C, for *T. annulata* at 48 ±0.09°C and for *T. taurotragi* at 45 ±0.19°C.

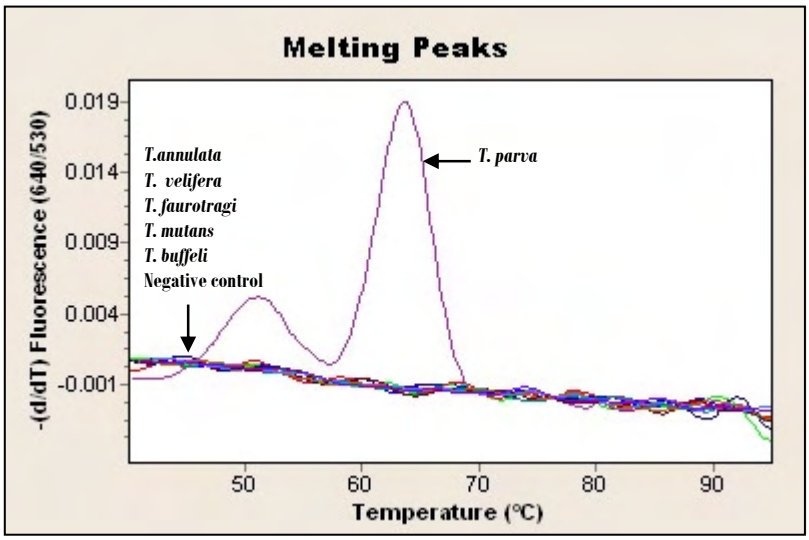

Figure 3.3 Specific detection of *T. parva* DNA. Amplicons were generated with the *T. parva*-specific primer together with the *Theileria* genus-specific reverse primer and detected with the *T. parva*-specific hybridization probe set. The *T. parva*-specific melting peak at 63 ±0.62°C was only observed in the *T. parva* positive control DNA samples, with no indication of amplification from any of the other *Theileria* species tested.

Theileria sp. (buffalo) control samples showed a slight increase in fluorescence at 640 nm (Figure 3.4a). An amplicon was obtained from *Theileria* sp. (buffalo) DNA as evidenced by an increase in fluorescence at 705 nm (Figure 3.4b), but no melting curve was produced in the 640 nm channel (Figure 3.4c). Therefore when both probe sets are included in a reaction with the *T. parva*-specific forward primer and the *Theileria* genus-specific reverse primer, an increase in fluorescence detected at 705 nm, but no melting curve at 640 nm indicates the presence of *Theileria* sp. (buffalo).

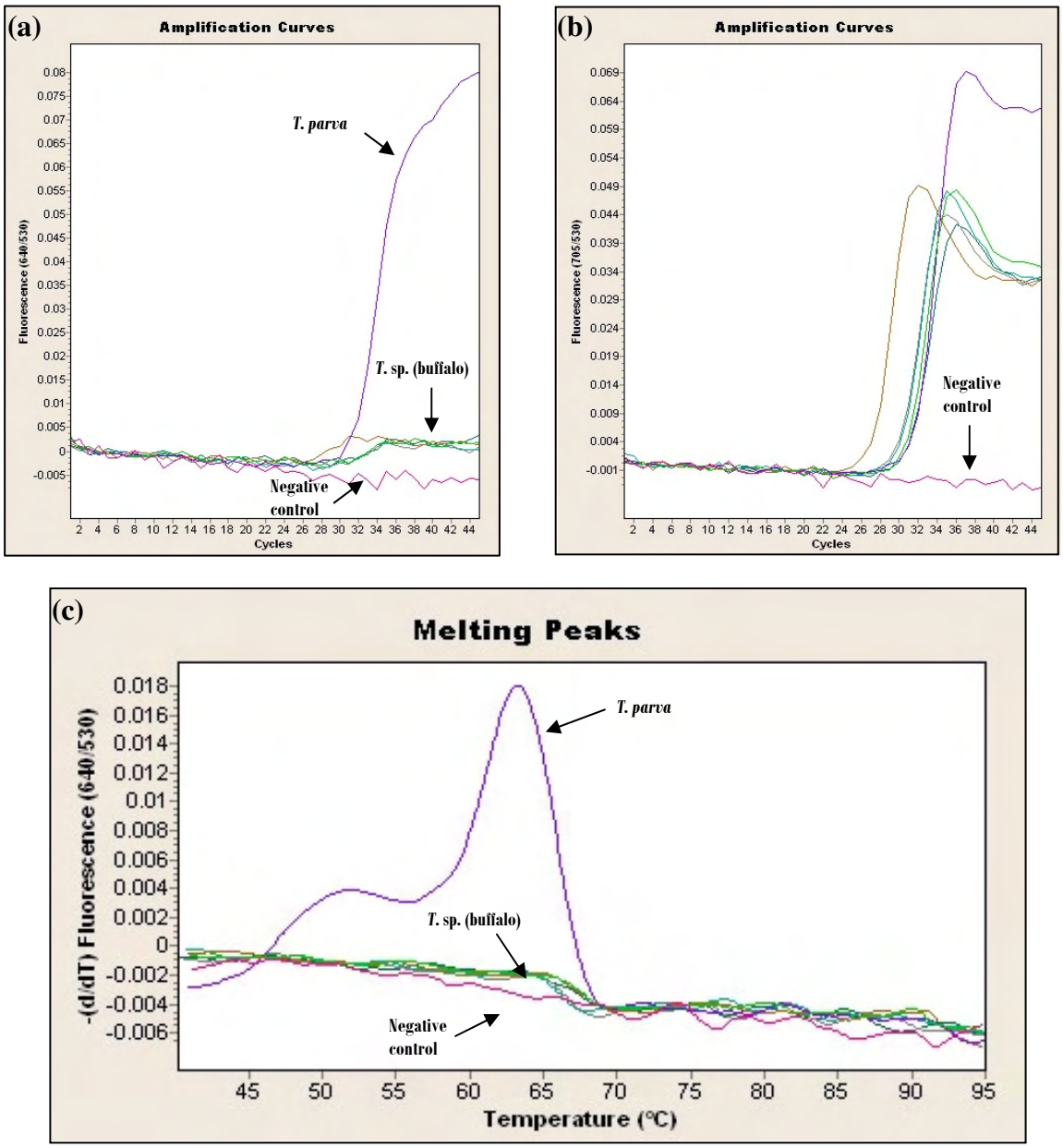

Figure 3.4 Discrimination between *T. parva* (blue lines), and *Theileria* sp. (buffalo) (green lines) using melting curve analysis. Amplicons were generated using the *T. parva*-specific forward primer and the *Theileria* genus-specific reverse primer, and detected with the *T. parva*-specific hybridization probe set. Amplification curves showing increase in fluorescence at (a) 640 and (b) 705 nm in *T. parva* and *Theileria* sp. (buffalo) DNA samples. (c): Melting curve analysis at 640 nm, showing the *T. parva*-specific melting peak at 63 ±0.62°C only for *T. parva* DNA.

3.4.2 Analytical sensitivity

From the set of dilutions prepared from *T. parva* positive buffalo KNP102, *T. parva* DNA was detected in all 30 replicates of the undiluted (3870 parasites/reaction) and the 10^{-1} dilution (387 parasites/reaction). As the dilutions increased, fewer of the 30 replicates tested positive until only one tested positive from each of the 10^{-5} (0.0387 parasites/reaction) and 10^{-6} (0.00387 parasites/reaction) dilutions (Figure 3.5).

At 3870 and 387 parasites/reaction (equivalent to $8.79x10^{-3}$ and $8.79x10^{-4}$% parasitaemia respectively) the sensitivity of the test was 100%; this decreased to 96.7% at 38.7 parasites/reaction ($8.79x10^{-5}$% parasitaemia) with a 95% confidence interval of 90.2%-100%. At 3.87 parasites/reaction ($8.79x10^{-6}$% parasitaemia) the probability of a positive test result, given that the individual tested actually has the parasite, was 80% with a 95% confidence interval of 65.7%-94.3%. The sensitivity of the test decreased to 26.67% at 0.387 parasites/reaction ($8.79x10^{-7}$% parasitaemia) with a 95% confidence interval of 10.8%-42.49% (Figure 3.5).

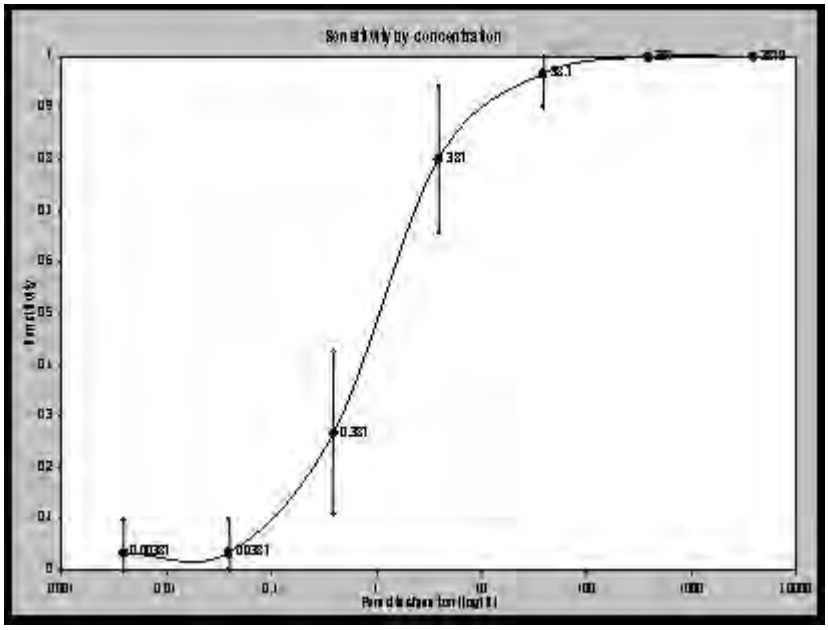

Figure 3.5 The sensitivity and 95% confidence intervals for a 10-fold dilution series from 10^{0} (3870 parasites/reaction) to 10^{-6} (0.00387 parasites/reaction) prepared from blood from a naturally infected buffalo (KNP102) with a parasitaemia of 0.009%.

3.4.3 Comparison of molecular tests

Three of the four molecular tests, namely the RLB, the real-time PCR and the *cox*III assays, detected *T. parva* DNA in all three gold standard positive samples, whereas the conventional PCR/probe assay detected *T. parva* DNA in only two of the three positive samples. *Theileria parva* was detected in 154 (49.8%) of the 309 field samples using the real-time PCR assay, 103 (33.3%) using the *cox*III assay, and 73 (23.6%) and 67 (21%) using the conventional PCR/probe assay and the RLB respectively (Figure 3.6a). All four assays detected *T. parva* DNA in the same samples. It should be noted that for the purposes of this experiment 1.0 µl of input DNA was used in the real-time PCR assay. Although the sensitivity of the real-time PCR assay was already better than the other tests using 1.0 µl of input DNA, it could be further improved by increasing the volume of input DNA. When input DNA was increased to 2.5 µl in 105 of the field samples, *T. parva* could be detected in 50 (52.4%) of the tested samples in contrast to 33 (34.3%) when 1.0 µl of input DNA was used (Figure 3.6b). Increasing the input DNA for the other three assays may have improved their sensitivities, but already their input DNA was relatively high [*cox*III assay (5 µl in a 25 µl reaction), conventional PCR/probe assay (2.5 µl in a 25 µl reaction), RLB (2.5 µl in a 25 µl reaction)] compared to that originally used for the real-time PCR assay (1.0 µl in a 20 µl reaction).

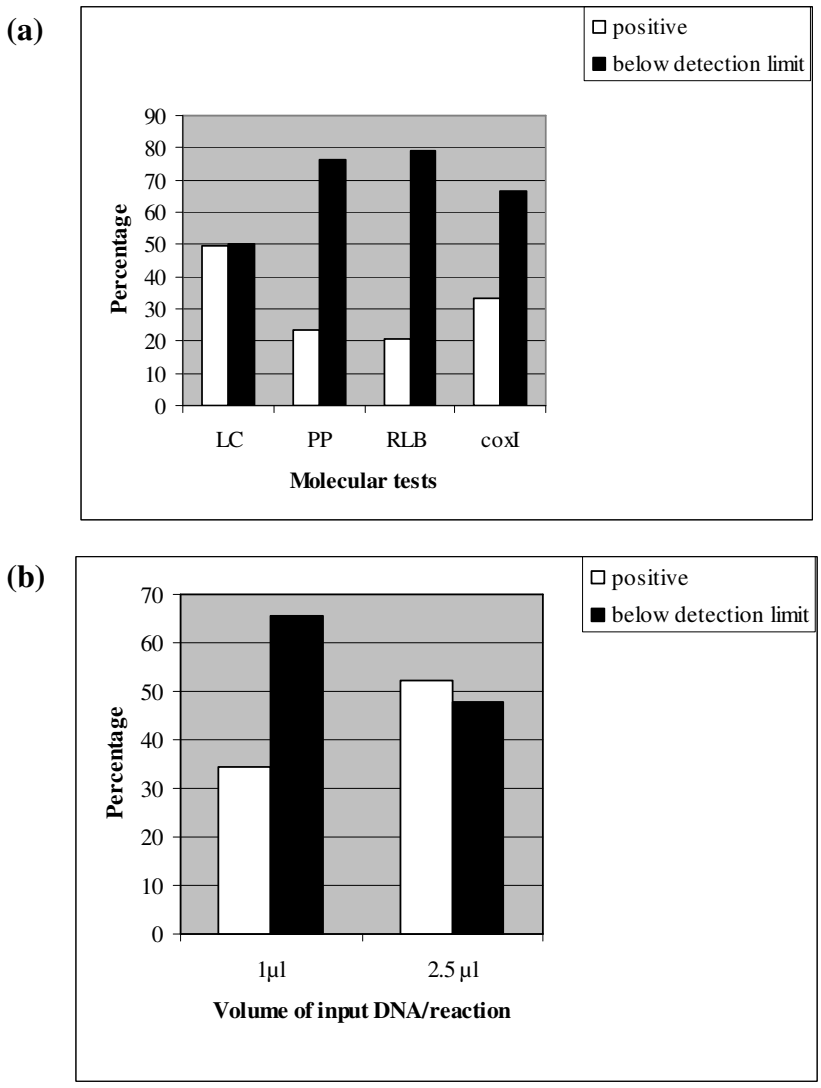

Figure 3.6 (a): Comparison of the sensitivity of the real-time PCR assay to that of other molecular assays in detecting *T. parva* from 309 field samples. One microlitre (~15 ng) of input DNA was used in the real-time PCR assay (LC), 2 µl (~30 ng) in the conventional PCR and probing test (PP), 2.5 µl (~37.5 ng) in the RLB, and 5 µl (~75 ng) in the *cox* III assay. (b): Improved ability of the real-time PCR assay to detect *T. parva* in 105 field samples when the input DNA was increased from 1 µl (~15 ng) to 2.5 µl (~ 37.5 ng)

3.4.4 Proficiency testing

A set of blood samples, including known negative animals, buffalo samples from a *T. parva* endemic area which were expected to be positive and diagnostic samples of unknown *T. parva* infection status, were subject to the real-time PCR test by different operators at the DVTD and OVI laboratories. Except for one sample, the laboratories obtained identical results which were also confirmed by an independent analyst (Figure 3.7).

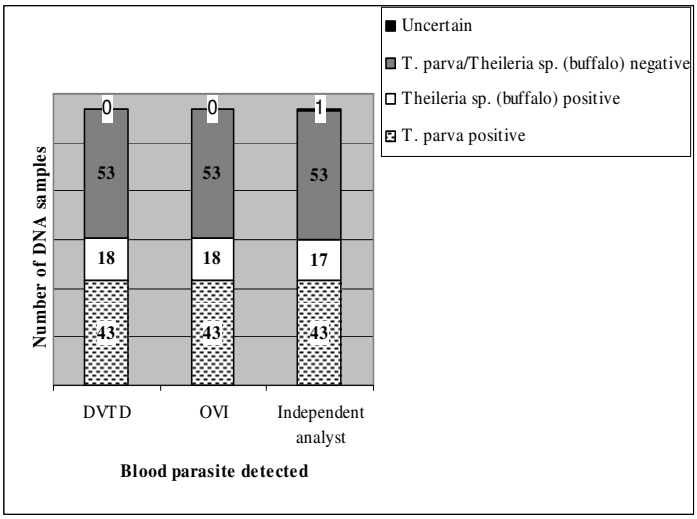

Figure 3.7 Comparison of results obtained from the DVTD and OVI laboratories when the real-time PCR assay was used to detect *T. parva*.

3.5 Discussion

A diagnostic assay for *T. parva* must be highly specific and sensitive in the presence of mixed infections, as the distribution of this pathogenic species coincides in many areas in southern Africa with that of other *Theileria* species, e.g. *T. mutans, T. velifera*, *T. buffeli*, *Theileria* sp. (buffalo) and *T. taurotragi* (Irvin, 1987; Norval *et al*., 1992). While the two sets of primers designed for the real-time PCR assay successfully amplified *T. parva* DNA under the conditions optimized for this assay, the *T. parva*-specific probe set also detected *T. taurotragi* and *T. annulata* when the *Theileria* genus-specific primers were used. Fortunately, this did not influence the specificity of the assay because the different products were easily discriminated by melting curve analysis.

The sensitivity of the real-time PCR assay may be compromised when *Theileria* genus-specific primers are used in samples containing mixed infections. In instances where *T. parva* infection is low and other *Theileria* species are present at higher levels, preliminary results indicate that competition for primers may result in a misdiagnosis of the species that is under-represented (data not shown). The specificity and sensitivity of the test were therefore improved by designing a *T. parva*-specific forward primer. However, since the *Theileria* sp. (buffalo) 18S rRNA gene sequence is very similar to that of *T. parva*, it was impossible to design an amplification primer that will not also amplify *Theileria* sp. (buffalo) DNA. Therefore, competition for primers between the different target sequences will still occur in mixed infections of *T. parva* and *Theileria* sp. (buffalo). Although an amplicon was generated from *Theileria* sp. (buffalo) DNA, the test still remained specific for *T. parva* since only a *T. parva*-specific melting curve was generated. It is possible that the *Theileria* sp. (buffalo) template-*T. parva* probe complex has a lower Tm than the starting temperature (40°C) used in the melting curve analysis. If this is the case, all *Theileria* sp. (buffalo) template-*T. parva* probe helices would have separated into single strands before the melting curve analysis began and no melting peak would have been observed. It is interesting that melting peaks were observed for *T. taurotragi* and *T. annulata* when the *Theileria*-genus specific primers were used. This may be explained by the fact that these sequences differ from the *T. parva* sensor probe sequence at two positions while there are three nucleotide differences in *Theileria* sp. (buffalo). Therefore the *T. taurotragi* and *T. annulata* template-probe complexes would have a slightly higher Tm and would still have been double-stranded at the beginning of the melt, explaining why melting curves were observed for these species but not for *Theileria* sp. (buffalo).

In addition to the peak at 63 ±0.62°C specific for *T. parva*, a shoulder peak was observed at 51 ±0.55°C. Such peaks can be due to mismatched bases in the probe region, but this was not the case in this study, since cloning and sequencing results from several *T. parva* isolates revealed no sequence variations between the two copies of the 18S rRNA gene. In addition, the same peak was observed when plasmids containing cloned *T. parva* 18S rRNA genes were subjected to the real-time PCR assay (results not shown). Such peaks are thought to result from back-folding of the amplicon on itself downstream of the sensor probe (Simpson *et al.*, 2007). This back-folding of the amplicon competes with the FRET probes binding to the amplicon and thus creates a lowered melting peak.

The real-time PCR test is extremely sensitive and can detect *T. parva* with 100% certainty in carrier animals with a piroplasm parasitaemia as low as $8.79x10^{-4}$%. However, in animals with lower parasitaemia, the test will be less reliable. It is not known whether a parasitaemia lower than $8.79x10^{-4}$% occurs in buffalo. In endemic areas where buffalo are constantly exposed to the parasite, the parasite load is likely to be within the detection limit of the real-time PCR test. However, in buffalo reared under tick-free conditions, the parasitaemia in infected animals, although fluctuating, is likely to remain extremely low, as observed in the naturally infected buffalo KNP102, since they are not exposed to constant re-infection. The ability of the real-time PCR assay to detect *T. parva* in such animals needs to be assessed.

The sensitivity of the real-time PCR assay was improved by increasing the amount of input DNA from 1.0 µl to 2.5 µl (~15 ng to ~37.5 ng) which increased the number of positive field samples by approximately 20%. Increasing the total volume of the real-time PCR reaction could further increase the sensitivity of the test. This would allow an even larger volume of input DNA, thereby increasing the chance of including parasite rDNA in the reaction. However, this would result in a fivefold increase in the cost of the test. Alternatively, DNA could be extracted from a larger volume of blood and eluted in a smaller volume, effectively concentrating the parasite DNA. Again this might increase the chance of including parasite rDNA in the reaction.

Several molecular tools have been developed for detection and differentiation of *Theileria* species (Morzaria *et al*., 1999). Most of these assays are based on conventional PCR and probing techniques (Bishop *et al*., 1992, Allsopp *et al*., 1993, Gubbels *et al*., 1999) and are relatively sensitive. However, they are laborious and time consuming. Real-time PCR tests have been developed for *Theileria* and *Babesia* parasites including *Theileria sergenti*, *Theileria equi, Babesia bovis and Babesia bigemina*, (Jeong *et al*. 2003; Kim *et al*., 2007, 2008). In most cases the sensitivity and specificity of real-time PCR tests not only compare well with those of conventional PCR-based methods, but significantly improve the sensitivity and specificity of the detection of these parasites, as in the case of the *T. parva* real-time test reported here. However, the *T. parva* real-time PCR test (based on hybridization probe chemistry) has an additional benefit over most real-time PCR tests developed for other *Theileria* and *Babesia* parasites (which are based on hydrolysis probe chemistry), as it is coupled with melting peak analysis, which confirms the identity of the amplified product.

Recently, loop-mediated isothermal amplification (LAMP) technology has been applied to the detection of *Theileria* and *Babesia* parasites (Iseki *et al*., 2007; Thekisoe *et al*., 2007). This technology allows amplification of as little as 1fg DNA in sixty minutes and is very cost effective as it does not require specialized equipment for amplification or analysis of amplicons. However, specific detection of *T. parva* has not yet been achieved using this technology and differentiation between *T. annulata*, *T. mutans*, *T. taurotragi* and *T. parva* amplicons is not possible (Thekisoe *et al*., 2007). In the case of mixed infections, a restriction enzyme analysis is required subsequent to amplification to allow differentiation of different parasite species, thus compromising the rapidity of the test (Iseki *et al*., 2007). The *T. parva* real-time PCR test reported here is currently the most rapid and reliable test available for specific detection of *T. parva* in cattle and buffalo in South Africa. This test is also more sensitive than other molecular assays currently used in the diagnosis of *T. parva* with its increased sensitivity accounted for by the fact that real-time PCR technology allows the measurement of the total amplification product in a reaction, in contrast to an aliquot that is analysed when using conventional PCR assays.

In May 2006, the newly developed real-time PCR test was adopted as a diagnostic test by the ARC-OVI, the only institution in South Africa authorized to test for *T. parva* infections in buffalo as part of the Corridor disease control strategy. To date, the assay has been used to test approximately 7420 field samples and 4% of these tested positive for *T. parva*. In the field, low piroplasm parasitaemias are a problem and continue to pose a challenge when interpreting the results. Melting curves are not well defined in some cases, which may be the result of low piroplasm parasitaemias and /or mixed infections with *Theileria* sp. (buffalo). Hence, buffalo from breeding projects are required to undergo five consecutive negative tests before they can be released on registered and approved properties only.

3.6 Summary

In summary, the real-time PCR assay reported here is specific for *T. parva* and more sensitive and faster than other molecular assays previously used in *T. parva* diagnostics. The assay is highly reproducible and has been shown to be reliable in the detection of *T. parva* piroplasm levels as low as $8.79x10^{-4}$%. However, sensitivity may still be a problem at infection levels lower than $8.79x10^{-4}$%.

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CHAPTER 4

Four p67 alleles identified in South African *Theileria parva* field samples

Trust in the LORD with all thine heart; and lean not unto thine own understanding. In all thy ways acknowledge Him, and He shall direct thy paths. ***Proverbs 3:5-6.***

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"I'm a slow walker, but I never walk back." ***Abraham Lincoln***
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4.1 Abstract

Previous studies characterizing the *Theileria parva* p67 gene in East Africa revealed two genotypes. Cattle-derived isolates associated with East Coast fever (ECF) have a 129 bp deletion in the central region of the p67 gene (allele 1), compared to buffalo-derived isolates with no deletion (allele 2). In South Africa, Corridor disease outbreaks occur if there is contact between infected buffalo and susceptible cattle in the presence of vector ticks. Although ECF was introduced into South Africa in the early 20th century, it has been eradicated and it is thought that there has been no cattle-to-cattle transmission of *T. parva* since. The variable region of the p67 gene was amplified and the gene sequences analyzed to characterize South African *T. parva* parasites that occur in buffalo, in cattle from farms where Corridor disease outbreaks were diagnosed and in experimentally infected cattle. Four p67 genotypes were identified, including alleles 1 and 2 previously detected in East African cattle and buffalo, respectively, as well as two novel genotypes, one with a different 174 bp deletion (allele 3), the other with a similar sequence to allele 3 but with no deletion (allele 4). Sequence variants of allele 1 were obtained from field samples originating from both cattle and buffalo. Allele 1 was also obtained from a bovine that tested *T. parva* positive from a farm near Ladysmith in the KwaZulu-Natal Province. East Coast fever was not diagnosed on this farm, but the p67 sequence was identical to that of *T. parva* Muguga, an isolate that causes ECF in Kenya. Variants of allele 2 were obtained from all *T. parva* samples from both buffalo and cattle, except Lad 10 and Zam 5. Phylogenetic analysis revealed that alleles 3 and 4 are monophyletic and diverged early from the other genotypes. These novel genotypes were not identified from South African field samples collected from cattle; however allele 3, with a p67 sequence identical to those obtained in South African field samples from buffalo, was obtained from a Zambian field isolate of a naturally infected bovine diagnosed with ECF. The p67 genetic profiles appear to be more complex than previously thought and cannot be used to distinguish between cattle- and buffalo-derived *T. parva* isolates in South Africa. The significance of the different p67 alleles, particularly the novel variants, in the epidemiology of theileriosis in South Africa still needs to be determined.

4.2 Introduction

Infections by *Theileria parva*, a tick-borne protozoan parasite, are responsible for classical East Coast fever (ECF), Corridor disease and January disease in cattle and normally inapparent infections in buffalo in eastern and southern Africa (Theiler, 1904; Neitz, 1955; Lawrence, 1992). The Cape buffalo (*Syncerus caffer*) is the natural reservoir host of *T. parva* and the parasite is transmitted by *Rhipicephalus appendiculatus, R. zambeziensis* and *R. duttoni* (Lawrence *et al*., 1983; Uilenberg, 1999).

East Coast fever was introduced into southern Africa in the early 1900s through the importation of cattle from East Africa, and was finally eradicated between 1946 and 1955 (Anonymous, 1981). In 1953, another form of cattle theileriosis, Corridor disease, was diagnosed after infected buffalo came into contact with cattle in the corridor of land between the then separate Hluhluwe and iMfolozi game reserves in South Africa (Neitz, 1955). The clinical symptoms and pathology of the disease were distinct from ECF. Schizont and piroplasm parasitoses were very low and it was thought that the parasite could not be transmitted between cattle, as affected animals usually died before piroplasms appeared. Corridor disease was thus considered to be caused by a different parasite, which was named *Theileria lawrencei* (Neitz, 1955). After the eradication of ECF in Zimbabwe, another form of theileriosis, known as January disease, emerged in that country, and the causative agent was named *Theileria bovis* (Lawrence, 1979; Uilenberg *et al*., 1982). Although the parasites causing ECF, Corridor Disease and January Disease were originally thought to be three different pathogenic species, they are now all considered to be *T. parva*, and *T. parva* isolates are now classified as cattle-derived or buffalo-derived (Perry and Young, 1993).

In southern Africa today, susceptible cattle sharing the same grazing as infected buffalo in the presence of vector ticks, can contract Corridor disease. The original buffalo-derived *T. parva* (the causative agent of Corridor disease) remains endemic in some parts of South Africa, hence the persistence of sporadic outbreaks of Corridor disease in South Africa. There is a concern that ECF could re-emerge in South Africa since cattle which recover from *T. parva* infections may become carriers of the parasite (Barnett and Brocklesby, 1966). A carrier state of ECF has also been shown to develop in cattle after immunization and treatment (Dolan *et al*., 1984; Maritim *et al*., 1989). Carrier state can develop in cattle that recover from Corridor disease following chemotherapy (Potgieter *et al*., 1985) and if this can also happen following natural infection, the disease may not be self-limiting as previously thought. Ticks

can be infected by feeding on carrier cattle and a situation may eventually develop where the parasite becomes adapted to cattle, resulting in cattle-to-cattle transmission. The South African cattle population would be highly susceptible should the parasite be introduced from an endemic area as the principal vector, *R. appendiculatus,* is still widespread. It is not known whether the parasite that caused ECF was transmitted to buffalo during the ECF epidemic, or whether South African strains of *T. parva* could eventually become adapted to cattle and cause ECF. There is therefore a need to establish the current status of *T. parva* parasites that are circulating in South African buffalo and possibly in cattle which may have recovered from buffalo-derived Corridor disease outbreaks.

In the past decade, several genes have been investigated in search of discriminatory sequence differences between *T. parva* isolates. Among these is the sporozoite antigen gene, p67 (Iams *et al*., 1990; Nene *et al*., 1996). Characterization of the p67 gene sequence in East Africa has revealed the presence of a 129 bp deletion in the central region in cattle-derived *T. parva* isolates, while there is no deletion in buffalo-derived isolates (Nene *et al*., 1992; 1996). Since the p67 sequences obtained from cattle-derived parasite stocks characterized in studies in East Africa were identical, it was assumed that the presence or the absence of the 129 bp deletion in the p67 gene could be used to differentiate between cattle- and buffalo-derived *T. parva* isolates (Nene *et al*., 1996). In South Africa, however, Collins (1997) obtained both p67 alleles in South African buffalo from the Kruger National Park. Although the p67 allele with a deletion was obtained from this isolate it was not established whether this particular strain could cause ECF.

The aim of this study was to characterize *T. parva* parasites that occur in buffalo and cattle in South Africa using sequence analysis of the p67 gene in an attempt to establish whether classical ECF-like parasites are present in South Africa.

4.3 Materials and methods

4.3.1 Sample collection

Cattle and buffalo blood samples were collected from different areas in South Africa. *Theileria parva* positive samples were selected using a *T. parva*-specific real-time PCR assay (Chapter 3; Sibeko *et al*., 2008). A total of 66 South African *T. parva* positive samples were characterized, including 62 field samples from cattle and buffalo and four experimentally

infected cattle (Table 4.1). One sample from a bovine (Zam 5) from Zambia was also analyzed.

4.3.2 DNA isolation

The High Pure PCR template preparation kit (Roche Diagnostics, Mannheim, Germany) was used to extract DNA from 200 µl of EDTA blood samples, according to the manufacturer's instructions. DNA was eluted in 100 µl elution buffer, rather than the recommended 200 µl, to increase the concentration of extracted DNA. The DNA was stored at 4°C until further analysis.

4.3.3 PCR amplification of the p67 gene from *T. parva*

Primers 613 (p67 forward primer) and 792 (p67 reverse primer) (Nene *et al*., 1996) were used to amplify the variable region of the p67 gene. Five microlitres of extracted DNA was used in a 25 µl PCR reaction and the amplification conditions applied were as described by Nene *et al*. (1996). For samples with low parasitaemia, 0.5 µl of the primary PCR product was used as a template for a secondary PCR using the same protocol but with the number of amplification cycles reduced from 40 to 25. The PCR products were analyzed by agarose gel electrophoresis.

Table 4.1 Source of samples used for characterization of *T. parva* parasites and results obtained from PCR amplification of the p67 gene. Table 4.1 continues in page 79.

Geographical origin of parasites	Province	Sample designation	Band size(s) obtained from PCR amplification (Kb)	Reference
Kruger National Park (KNP) (n=20)	Mpumalanga	KNP W8#	0.8, 0.9, 1.0, 1.1	
		KNP V5	0.9, 1.1	
		KNP 102#	0.8, 0.9, 1.0, 1.1	
		KNP 9446*#	0.9, 1.1	Sibeko *et al.* (2008)
		KNP B2	0.8, 0.9, 1.1	
		KNP M12	0.8, 0.9, 1.0, 1.1	
		KNP U3	0.9	
		KNP H12	1.1	
		KNP M2	0.8, 0.9, 1.0	
		KNP B1	1.0, 1.1	
		KNP B2	0.8, 1.1	
		KNP B15	0.9, 1.0, 1.1	
		KNP C5	0.9, 1.1	
		KNP O10	0.9, 1.0	
		KNP O11	1.1	
		KNP O14	1.0	
		KNP O17	0.8, 0.9, 1.0	
		KNP AA4	0.9, 1.0, 1.1	
		KNP A18	1.0	
		KNP A20	0.8, 0.9, 1.0, 1.1	
Ithala Game Reserve (n=9)	KwaZulu-Natal	Itha 2	0.8, 0.9, 1.0, 1.1	
		Itha 3#	0.8, 0.9, 1.0, 1.1	
		Itha 4	0.8, 0.9, 1.0, 1.1	
		Itha 5	0.8, 0.9, 1.0, 1.1	
		Itha 6	0.8, 0.9, 1.0, 1.1	
		Itha 7	0.8, 0.9, 1.0, 1.1	
		Itha 8#	0.8, 0.9, 1.0, 1.1	
		Itha 9	0.8, 0.9, 1.0, 1.1	
		Itha 10	0.8, 0.9, 1.0, 1.1	
Marakele National Park (n=11)	Limpopo	Mar 1.1	1.1	
		Mar 4	0.8, 0.9, 1.1	
		Mar 5	0.8, 0.9, 1.1	
		Mar 6	0.8, 0.9, 1.1	
		Mar 7	0.8, 0.9, 1.1	
		Mar 8	0.8, 0.9, 1.1	
		Mar 9	0.8, 0.9, 1.1	
		Mar 10	0.8, 0.9, 1.1	
		Mar 11	0.8, 0.9, 1.1	
		Mar 1#	0.8, 0.9, 1.1	
		Mar 75#	1.1	
Welgevonden Game Reserve (n=4)	Limpopo	Wel 23/04#	0.8, 0.9, 1.1	
		Wel 24/04#	0.8, 1.1	
		Wel 9288*#	1.1	Sibeko *et al.* (2008)
		Wel 9445*#	1.1	Sibeko *et al.* (2008)

Geographical origin of parasites	Province	Sample designation	Band size(s) obtained from PCR amplification (Kb)	Reference
Hluhluwe-iMfolozi Park (n=10)	KwaZulu-Natal	HIP 5#	0.8, 0.9, 1.0, 1.1	
		HIP 19	0.9	
		HIP 22	0.8, 0.9, 1.1	
		HIP 32	0.8, 0.9, 1.1	
		HIP 34	0.9	
		HIP 36	0.8, 0.9, 1.1	
		HIP 39	0.8, 0.9, 1.1	
		HIP 40	0.9	
		HIP 41	0.8, 0.9, 1.1	
		HIP 42	0.8, 0.9, 1.1	
Hluhluwe (n=1)	KwaZulu-Natal	**Hlu 9433*#**	1.1	Potgieter *et al.* (1988)
Ladysmith (n=4)	KwaZulu-Natal	**Lad 10#**	0.9	
		Lad 17#	1.1	
		Lad I238#	1.1	Thompson *et al.* (2008)
		Lad M119#	1.1	Thompson *et al.* (2008)
Bloemfontein (n=1)	Free-State	**Bloe B#**	1.1	
Mabalingwe Game Reserve (n=6)	Limpopo	Mab A13	0.9, 1.1	
		Mab B21	0.9, 1.1	
		Mab A22	0.9, 1.1	
		Mab BB37	0.9, 1.1	
		Mab BB38#	0.9, 1.1	
		Mab BB43#	1.0, 1.1	
Zambia (n=1)	East	**Zam 5#**	0.8	Geysen (2000)

All the samples in bold were obtained from cattle.
*Experimentally infected cattle.
PCR products obtained from these isolates were selected for sequencing.

4.3.4 Cloning and sequencing of p67 amplicons

Amplicons obtained from 21 selected samples (Table 4.1) were purified using the MinElute™ PCR Purification Kit (Qiagen, Venlo, the Netherlands). The p67 PCR products were cloned into the pGEM®-T Easy cloning vector (Promega, Madison, USA). Recombinant plasmid DNA was isolated using the High Pure Plasmid isolation kit (Roche Diagnostics, Mannheim, Germany). The presence of inserts was confirmed by colony PCR following the PCR protocol described by Nene *et al.* (1996). Three hundred to 450 ng of plasmid DNA was used in sequencing reactions prepared using the ABI Big Dye Terminator Cycle Sequencing kit version 3.1 (Applied Biosystems, Foster City, CA, USA). Sequencing was performed using a

SpectruMedix model SCE 2410 automated sequencer (SpectruMedix, State College, PA, USA) at INQABA Biotechnologies (South Africa).

4.3.5 Sequence analysis

4.3.5.1 Sequence editing

One hundred and forty sequences were obtained from clones produced from the 21 selected *T. parva* samples. Sequences were assembled and edited using GAP4 of the Staden package (version 1.6.0 for Windows) (Bonfield *et al*., 1995; Staden, 1996; Staden *et al*., 2000).

4.3.5.2 Sequence alignment

The p67 sequences obtained in this study were aligned with other published p67 sequences (Table 4.2). Sequences were initially aligned with the multiple sequence alignment program, MAFFT version 6 (Katoh *et al*., 2002) (http://align.bmr.kyushu-u.ac.jp/mafft/software/) and the final alignment was performed by eye using MacClade v4.0 (Maddison and Maddison, 1992). Alignment of nucleotide sequences was optimised using the amino acid reading frame. The lengths of the p67 sequence fragments varied between 751 bp and 967 bp. The first 167 bp and last 313 bp could be aligned without any difficulty. The middle region of the fragment was, however, highly divergent among strains and could not be reliably aligned across the entire data set. However, several strains shared similar insertions or deletions and these were coded as present or absent to represent a total of 13 unique synapomorphic characters. These characters can contribute phylogenetic signal, and therefore, to limit homoplasy, they were only scored if they comprised at least 9 base pairs in length (Matthee *et al*., 2001; 2007). The nucleotides comprising these inserts were deleted from further analyses.

Table 4.2 p67 reference sequences used for analysis of data obtained in this study

Geographical origin of parasites	Province	Isolate or sequence designation	Accession number	References
South Africa, Schoonspruit	Mpumalanga (former Transvaal)	Schoonspruit		Neitz (1948), Collins (1997)
South Africa, Kruger National Park (KNP)	Mpumalanga	KNP 1_S		Collins (1997)
		KNP 1_L1		Collins (1997)
		KNP 1_L2		Collins (1997)
		KNP 1_M		Collins (1997)
		KNP 2	AF079177	Nene *et al.* (1999)
South Africa, Hluhluwe	KwaZulu-Natal	Hluhluwe3	AF079176	Nene *et al.* (1999)
Uganda	North-west	Uganda		Minami *et al.* (1983), Morzaria *et al.* (1995), Collins (1997)
Kenya	Kilifi District	Muguga	M67476	Nene *et al.* (1996)
		Marikebuni		Irvin *et al.* (1983)
		7014	U40703	Nene *et al.* (1996)
		Waterbuck (Waterbuck experimentally infected with *T. parva* derived from buffalo 7014 which transmitted a subpopulation of parasites causing ECF in cattle.)		Stagg *et al.* (1994), Collins (1997)
		7344 (Tissue culture-clone generated from stock 7014.)		Morzaria *et al.* (1995), Collins (1997)

4.3.6 Phylogenetic analysis

Duplicate sequences were excluded from phylogenetic analyses and a total of 53 p67 sequences from nine representative samples were used. Parsimony analyses were performed in PAUP v4.0b10 (Swofford, 2003) based on heuristic searches with 100 random additions of taxa and tree bisection and reconnection (TBR) branch swapping. As a large number of equally parsimonious trees were found during each search the maximum number of trees saved during each replicate was constrained to 500. Data were analyzed using the 480 homologous nucleotides only and also in a combined fashion by adding the 13 unique characters that originated from length differences among the fragments. Substitutions were unordered and the 13 unique length differences among strains were analyzed unweighted (the insertion or deletion of each unique stretch of DNA contributed the same weight as a single nucleotide change), and weighted (3 times and 10 times heavier than a single nucleotide

change). One thousand parsimony bootstrap replicates were performed to obtain confidence values for the nodes.

4.4 Results

4.4.1 Amplicon analysis by agarose gel electrophoresis

Two p67 alleles have previously been reported in East Africa, the p67 gene with a 129 bp deletion (designated allele 1 in this study) and the p67 gene with no deletion (designated allele 2) (Nene *et al*., 1996). In this study, up to four p67 PCR products of sizes ~ 0.8, 0.9, 1.0 and 1.1 kb, were obtained (Figure 4.1 and Table 4.1). It is more than likely that the multiple bands obtained in this study are authentic and representative of several different *T. parva* parasites present in a single sample, because p67 is a single copy gene.

A single amplicon was obtained from all *T. parva* positive samples obtained from cattle, from both experimentally infected and field samples, except for one, bovine KNP 9446, from which a double band was obtained (Table 4.1). The PCR product sizes obtained from KNP 9446 consisted of the 1.1 and 0.9 kb fragments. A 0.9 kb PCR product was obtained from Lad 10, a 0.8 kb PCR product from Zam 5 and 1.1 kb amplicons were obtained from all other cattle samples.

Single amplicons of band sizes 0.9, 1.0 or 1.1 kb, were obtained from 10 buffalo samples; otherwise, two to four amplicons were obtained from *T. parva* field samples originating from buffalo. The 0.8, 0.9, 1.0 and 1.1 kb bands were present in, respectively, 61%, 77%, 39% and 82% of the 57 buffalo samples analyzed.

Heterogeneous p67 PCR product profiles were obtained from *T. parva* samples from buffalo from the Kruger National Park and Hluhluwe-iMfolozi, while the p67 PCR products obtained from Ithala, Marakele and Mabalingwe samples appeared to be homogeneous within each game reserve. PCR products of the same sizes were obtained from *T. parva* samples from buffalo from Ithala (with 100% of samples producing all four PCR products), Marakele (with 81 % of samples producing the 0.8, 0.9 and 1.1 kb PCR products) and Mabalingwe (with 83% of samples producing the 0.9 and 1.1 kb PCR products).

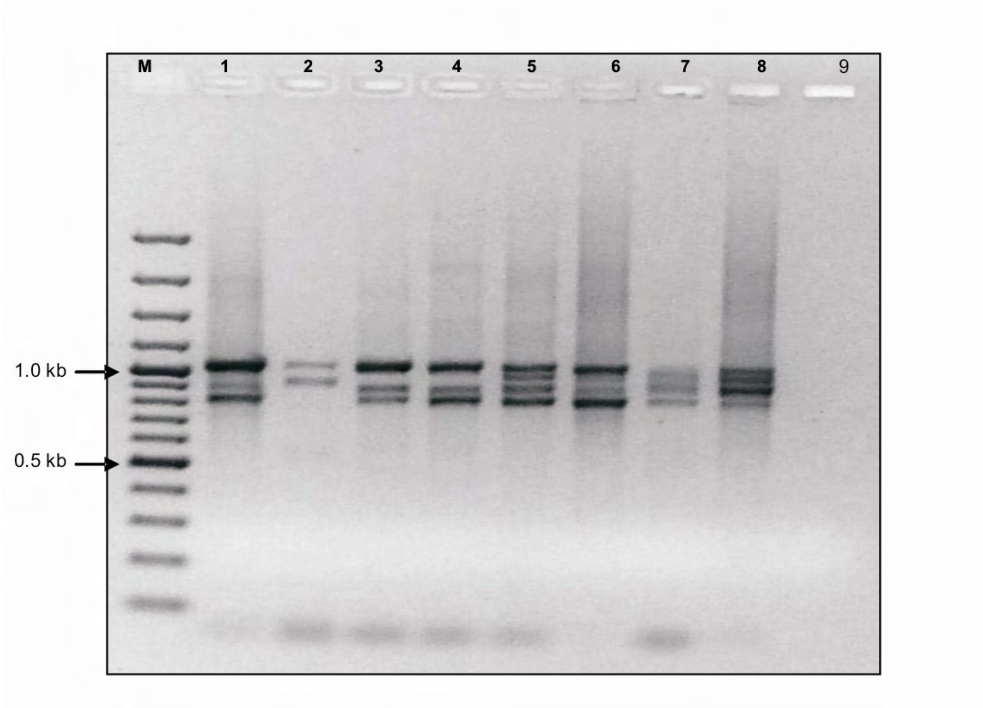

Figure 4.1 Amplicon profiles obtained from amplification of the central region of the p67 gene from buffalo-derived *T. parva* isolates collected from different geographical areas in South Africa. Lanes: M=1 kb plus DNA marker (Fermentas Life Sciences), 1= Wel 23/04, 2= Mab 43, 3=Mar 1, 4= Itha 3, 5= Itha 8, 6= HIP 5, 7 = KNP W8, 8= KNP 102, 9 = negative control. See Table 4.1 for geographical origin of isolates.

4.4.2 Sequence analysis

The p67 sequences obtained in this study were aligned with the published sequences shown in Table 4.2. Four groups of p67 sequences were identified (Figure 4.2), including the previously identified alleles 1 and 2, as well as two novel alleles, one with a different 174 bp deletion (allele 3), the other with no deletion (allele 4). The deletion in allele 3 occurs ~ 20 bp upstream of the position where the deletion occurs in allele 1 (Figure 4.2).

Sequences characteristic of alleles 1 and 2 were obtained from the 0.9 and 1.1 kb amplicons, respectively, and allele 3 and 4 sequences were obtained from the 0.8 and 1.0 kb PCR products, respectively (Table 4.3). Although it was possible to sequence all bands from all samples, at least two p67 allele sequences were obtained from *T. parva* samples originating from buffalo (Table 4.3), except for Mar 75 which had one p67 allele. Sequences of all four alleles were present in two individual isolates, KNP W8 and KNP 102, and three in Itha 8 and

HIP 5. Allele 2 sequences were obtained from all *T. parva* samples originating from cattle except two, Lad 10 and Zam 5 (Table 4.3), which had alleles 1 and 3, respectively. In addition to allele 2 sequences, KNP 9446, a bovine experimentally infected with *T. parva* parasites from buffalo KNP102 (Chapter 3; Sibeko *et al*., 2008) also contained parasites with p67 allele 1 sequences. Allele 1 was also obtained from a naturally infected *T. parva*-carrier bovine, Lad 10, originating from a farm near Ladysmith, and the sequence was identical to that of *T. parva* Muguga. An allele 3 p67 sequence, similar to allele 3 sequences obtained from South African *T. parva* field samples originating from buffalo, was obtained from a Zambian isolate, Zam5, originating from a naturally infected bovine. Sequence variants of alleles 1 and 2, similar to p67 sequences from a sample obtained from a buffalo at KNP in 1994 reported by Collins (1997), namely KNP1_M and KNP1_L1, were also obtained. These p67 variants had sequences similar but not identical to previously reported alleles 1 and 2 (Nene *et al*., 1996). The novel sequences, alleles 3 and 4, obtained in this study were similar to KNP1_S and KNP1_L2 p67 sequences, respectively, reported by Collins (1997). Among allele 2 sequences, sequence variations within the 129 bp region specific to buffalo-derived parasites were observed (Figure 4.2).

Cloning and sequence analysis also revealed the presence of more than one p67 sequence variant from what appeared to be a single PCR product (or single band on an agarose gel). For example, two different sequence variants of allele 2 were obtained from DNA clones prepared from the amplicons obtained from experimentally infected bovine, Hlu 9433, and a naturally infected bovine, Lad M119 (results not shown).

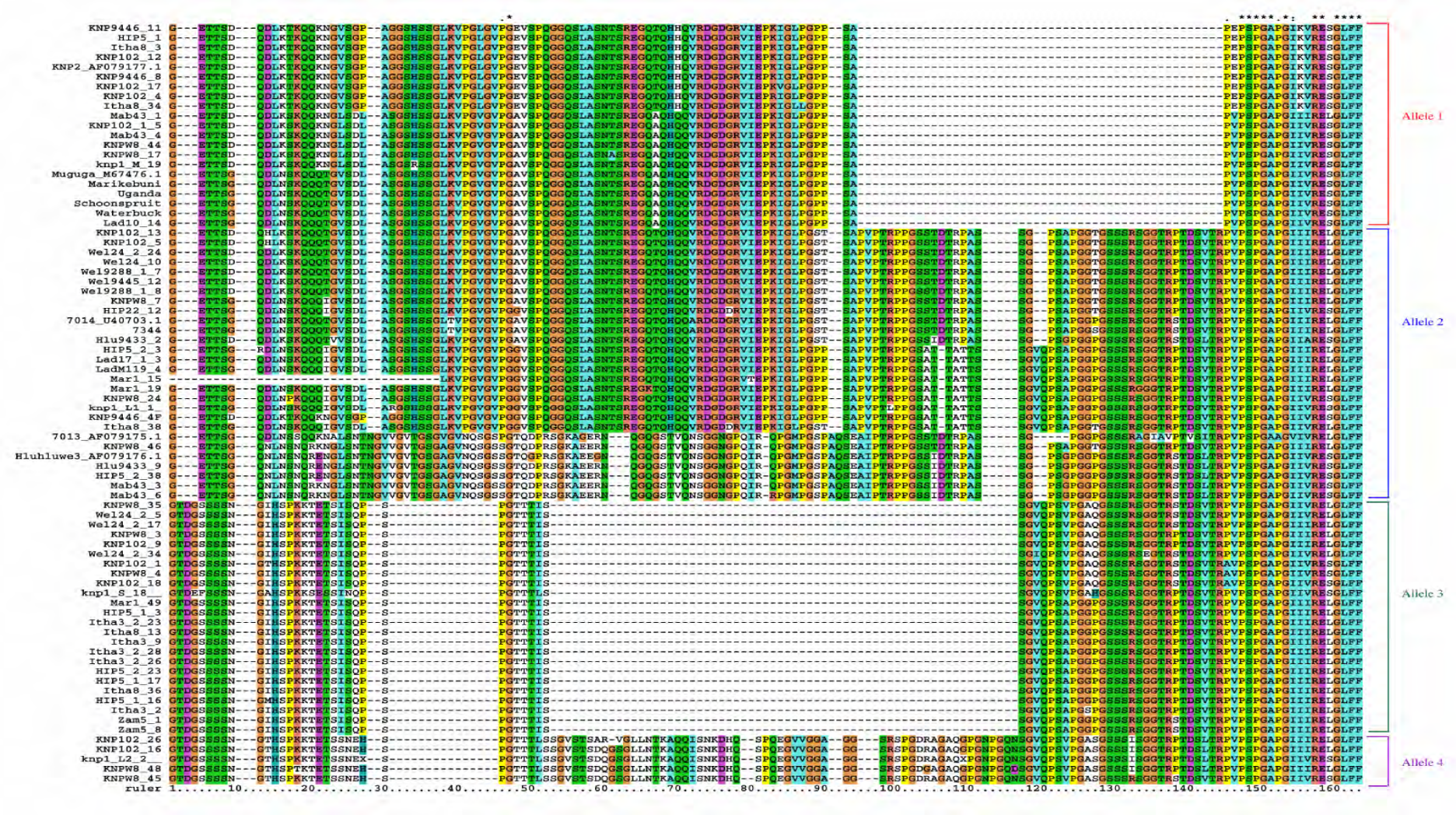

Figure 4.2 Alignment of the inferred amino acid acid sequences of a portion of the ~600 bp variable region of the p67 gene amplified from representative *T. parva* strains. The alignment was generated using the multiple sequence alignment program Mafft version 6 (Katoh *et al*., 2002) (**http://align.bmr.kyushu-u.ac.jp/mafft/software/**).

Table 4.3 Number and type of p67 sequences obtained from 21 selected *T. parva* samples collected from both cattle and buffalo

Sample name	Total number of clones sequenced [140]	Number of p67 sequences			
		Allele 1 (0.9 kb)	Allele 2 (1.1 kb)	Allele 3 (0.8 kb)	Allele 4 (1.0 kb)
KNP W8	10	2	3	3	2
KNP 102	16	6	4	3	3
Itha 3#	8	-	3	5	-
Itha 8#	16	7	4	5	-
HIP 5#	15	6	3	6	-
Mab BB38	6	3	3	-	-
Mab BB43	9	6	3	-	-
Wel 23/04#	3	-	3	-	-
Wel 24/04	9	-	5	4	-
Mar 1#	11	-	6	5	-
Mar 75	3	-	3	-	-
KNP 9446*	4	2	2	-	-
Zam 5	2	-	-	2	-
Wel 9288*	6	-	6	-	-
Wel 9445*	2	-	2	-	-
Hlu 9433*	3	-	3	-	-
Lad 17	5	-	5	-	-
Lad M119	4	-	4	-	-
Lad I438	4	-	4	-	-
Lad 10	1	1	-	-	-
Bloe B	3	-	3	-	-

All the samples in bold were obtained from cattle.
Note that not all bands were sequenced from these samples.
*Experimentally infected cattle.

4.4.3 Phylogenetic analysis

Analyses of the 480 flanking nucleotides (60 parsimony informative characters) resulted in more than 500 equally parsimonious trees which were largely unresolved after bootstrap analyses (<50%). Although 13 nodes were supported by ≥50% bootstrap support these were mostly restricted to the terminal associations among strains. Inclusion of the 13 unique insertions or deletions significantly increased the phylogenetic resolution and bootstrap support was obtained for an additional 15 nodes when the analyses included 73 parsimony informative characters (60 nucleotides and 13 insertions/deletions). The majority of the additional signal resolved the more basal associations in the topology (among alleles).

Two major clades, A and B, were identified from the most parsimonious tree (Figure 4.3). Alleles 3 and 4 clustered together in a single clade (A) suggesting that they diverged early and evolved separately from other p67 alleles. Clade B included known alleles 1 and 2 and their sequence variants. Each major clade consisted of subgroups that could also be divided based on the presence or absence of the deletion (Figure 4.3). Subgroup A2 consisted of novel p67 sequences with a deletion (allele 3) and A1 consisted of novel sequences with no deletion (allele 4). Similarly in clade B, subgroup B1 consisted of p67 sequences with a different deletion (allele 1) while B2 consisted of sequences without the deletion (allele 2). B2 formed a basal clade for B1 suggesting that allele 1 is derived from allele 2. The Muguga p67 sequence together with other cattle-derived isolates shared a common ancestor, clade C, with groups D1, D2 and E comprising p67 allele 1 variants. Cattle-derived sequences [Muguga, Schoonspruit, Marikebuni, *T. parva* (waterbuck-passaged) and Uganda (Table 4.2)] and sequence variants grouping with the KNP2 p67 sequence (Table 4.2) in subgroup B1 appeared to be monophyletic. p67 sequences from a Zambian isolate obtained from a naturally infected bovine diagnosed with ECF (Geysen, 2000) grouped with South African novel allele 3 sequences, in clade A subgroup A2.

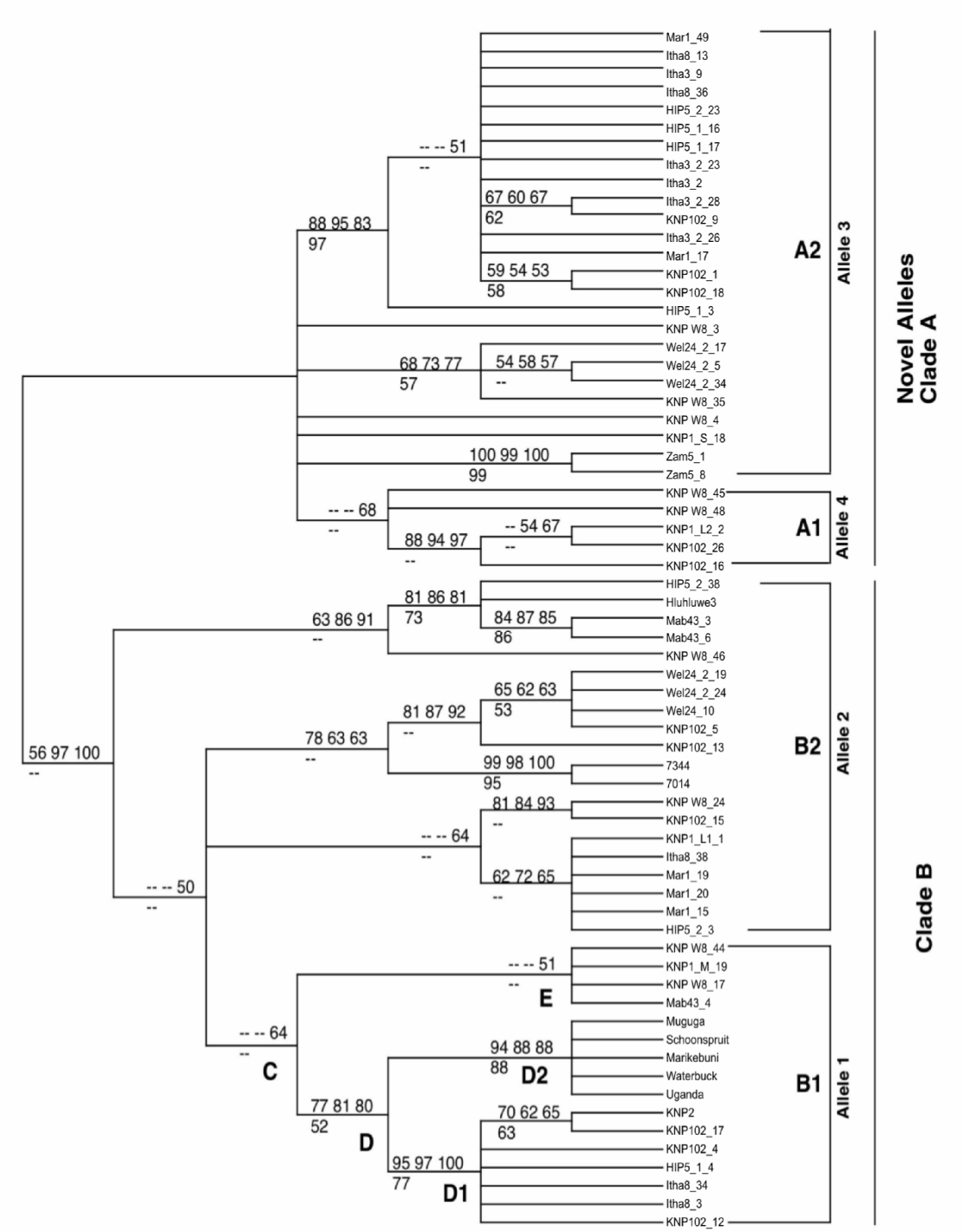

Figure 4.3 Phylogenetic relationship of *T. parva* strains as revealed by p67 gene sequences. The phylogenetic tree was calculated by maximum parsimony analysis using TBR swapping in PAUP*4.0b10 (Swofford, 2003) and the tree where unique insertions are weighted 10:1 is shown. Nodal support was assessed with 1000 bootstrap replicates and indicated above for 1:1; 1:3; 1:10 weighting of indels while values below represent bootstrap support for the nucleotide analyses only (see text for details).

4.5 Discussion

According to data obtained in East Africa, the cattle-derived *T. parva* isolates, Boleni, Muguga, Marikebuni, Mariakana and Uganda, have an identical p67 gene sequence which contains a 129 bp deletion (allele 1) and this deletion is not present in the p67 gene sequence from buffalo-derived isolates (allele 2) (Nene *et al.*, 1996). Consequently, it has been speculated that all *T. parva* stocks which can be maintained by passage between cattle and the tick vector, have the same p67 gene sequence containing the 129 bp deletion (Nene *et al.*, 1996). In this study both alleles were obtained from many of the *T. parva* field samples obtained from buffalo and cattle. In addition to alleles 1 and 2, two novel alleles (alleles 3 and 4) were also identified by PCR and sequence analysis. Although it is possible that PCR artifacts, such as overlap extension, could result in sequence variants, there was no evidence in the data obtained in this study to suggest that overlap extension may have occurred. A non-PCR based method such as Southern blot could have been used to confirm that the variants characterized by deletions were authentic; however, the parasite DNA in field samples is often too low to detect even by PCR. The sporozoite antigen gene, p67, codes for a stage-specific protein involved in the process of entry of the *T. parva* sporozoite into the host lymphocytes (Webster *et al.*, 1985; Shaw, 2003). It is possible that the p67 allele type might be associated with the ability of the parasite to infect a specific host, which could explain the apparent selection of parasites with p67 allele 1 in cattle in East Africa.

Analysis of p67 PCR product profiles indicated that four p67 alleles are present in *T. parva* parasites in buffalo in South Africa. Alleles 1 and 2 occurred more frequently than alleles 3 and 4. Relatively uniform p67 profiles were obtained from *T. parva* samples from buffalo from Ithala, Marakele and Mabalingwe game reserves, suggesting that homogeneous populations of parasites could be circulating among buffalo on these properties. On the contrary, more heterogeneous p67 PCR product profiles were obtained from samples from Kruger National Park and Hluhluwe-iMfolozi Park suggesting an extensive diversity of the parasite population occurring in larger populations of buffalo.

In this study, a 0.9 kb PCR product, representative of p67 allele 1, was found in 77% of the *T. parva* samples obtained from buffalo. All of the p67 allele 1 sequences from samples from buffalo were variants of the previously reported allele 1 from isolates obtained from cattle, with a number of amino acid substitutions which distinguished them from the known cattle-derived p67 allele 1. One such variant has previously been obtained from KNP2, a stock

originating from a naturally infected buffalo cow captured in the southern part of the Kruger National Park (Collins, 1997; Nene *et al*., 1999). *Rhipicephalus zambeziensis* nymphs were fed on this buffalo, and adult ticks reared from these nymphs transmitted Corridor disease to an adult bovine cow, B9678-2. Schizont-infected lymphoblastoid cells were established *in vitro* from lymph node aspirates obtained from this animal (H. Stoltsz, pers. comm.). It is possible that this cell culture is representative of a sub-population of the *T. parva* parasites that were present in the original buffalo from which the *T. parva* KNP2 isolate was prepared. Selection of a subpopulation of parasites may have occurred in the *T. parva* KNP2 stock, either *in vivo* or in cell culture. However, evidence is required to establish if this *T. parva* stock can be maintained in cattle. In a separate tick transmission study, parasites with a similar variant of allele 1 and also allele 2 were transmitted from buffalo KNP 102, which had a multiple infection of *T. parva* parasites possessing all four p67 alleles, into bovine KNP 9446/6. However, this animal died from classical Corridor disease (Chapter 3; Sibeko *et al*., 2008).

Only one p67 sequence obtained from a naturally infected bovine, Lad 10, from a farm near Ladysmith, KwaZulu-Natal, was identical to the typical cattle-derived p67 sequence, allele 1 (Nene *et al*., 1999). It is not known if the parasites that infected bovine Lad 10 originated from buffalo or cattle; however, this animal did not exhibit any disease symptoms associated with *T. parva* infection, suggesting that it was a carrier.

Except for Lad 10, all p67 gene sequences obtained from samples originating from cattle from the Ladysmith farm were typical of buffalo-derived *T. parva* parasites (allele 2). In fact, three of the Ladysmith samples (Lad 17, Lad I438 and Lad M119) had p67 sequences that were similar to sequences identified in *T. parva* field samples HIP 5, HIP 32 and HIP 39, obtained from buffalo from Hluhluwe-iMfolozi, a game park in KwaZulu-Natal. While this suggests that the *T. parva* parasites present in cattle on this farm could have been derived from buffalo, it is not clear if these cattle had any contact with infected buffalo (Thompson *et al*., 2008).

Together, these results indicate that the additional determinants which result in ECF, including the ability to produce microschizonts and high piroplasm parasitaemias, were probably not present in the parasites sampled in this study. It is not known what these determinants are or whether they occur in *T. parva* parasites in South Africa. However, it is apparent from these findings that *T. parva*-carrier cattle containing parasites possessing p67 allele 1 are present, at least on one farm, in South Africa. This finding is of concern to the

cattle industry in South Africa, since Potgieter *et al.* (1988) showed that buffalo-derived *T. parva* parasites causing Corridor disease can be maintained by passage between cattle and the tick vector. The persistence of *T. parva*-carrier cattle in South Africa could eventually result in the selection of *T. parva* parasites adapted to cattle.

In addition to p67 alleles 1 and 2, two novel variants were obtained, allele 3, with a deletion and allele 4, with no deletion. Allele 3 was obtained from samples originating from both buffalo and cattle (Zam 5) whereas allele 4 was only obtained from *T. parva* field samples originating from naturally infected buffalo. Zam 5 is an isolate obtained from a naturally infected bovine diagnosed with ECF symptoms in the Southern Province of Zambia (Geysen, 2000). The p67 sequence obtained from this isolate was identical to the South African allele 3 sequences. This result suggests that *T. parva* parasites carrying the novel allele 3 can be transmitted to cattle and indicates that parasites that cause ECF do not exclusively contain p67 allele 1.

A majority of *T. parva* field samples from which novel p67 variants were obtained also contained *T. parva* parasites with p67 alleles 1 and 2. This was not surprising as it is expected that buffalo, as reservoir hosts, will harbour more *T. parva* strains than exist in cattle as a result of recombination occurring in the tick vector. The phylogenetic analysis presented here indicates that parasites containing p67 alleles 3 and 4 seem to have evolved separately from cattle- and buffalo-derived parasites carrying p67 alleles 1 and 2, which is surprising, given the extensive recombination known to occur between *T. parva* parasites in the tick vector (Nene *et al.*, 1998). It is possible that these parasites were introduced during the ECF epidemic along with ECF-causing parasites from East Africa, but from the results obtained in this study, it is not possible to tell whether this is the case or whether these parasites have always existed in buffalo. In addition, there are no reports of *T. parva* parasites with novel p67 alleles (alleles 3 and 4) in other East African countries such as Tanzania or Kenya. However, it is very likely that these parasites also occur there given that there was historically great connectivity between buffalo populations in East and southern Africa (Van Hooft *et al.*, 2000), and it has been shown in this study that these novel variants are also present in Zambia. In the same manner that ECF was introduced into South Africa, ECF was introduced to the Northern Province of Zambia by importation of cattle from Tanzania in 1922 (Nambota *et al.*, 1995). The disease spread to Southern Province in the early 1970s. Interestingly, the novel variant was obtained in the Southern Province from an animal with ECF (Geysen, 2000). It is of interest that it was obtained from an area known to be frequented by buffalo. If novel p67

variants do exist in countries in East Africa, it will be interesting to establish whether parasites characterized by the novel p67 alleles are implicated in ECF cases there.

Sequence analysis revealed that there are more *T. parva* p67 alleles in South African buffalo than have previously been recognized. This confirms the extensive diversity in buffalo-derived *T. parva* parasites that has previously been reported (Conrad *et al*., 1987; 1989; Morzaria *et al*., 1995; Nene *et al*., 1996; Collins and Allsopp, 1999). Not only were alleles 1 and 2 identified in this study, but also many variants of these sequences. Variants of p67 allele 1 were obtained from some *T. parva* field samples originating from buffalo; from phylogenetic analysis, these sequences group together with the Muguga p67 sequence (allele 1) in clade C. The phylogenetic analysis further suggests that allele 1 associated with ECF (clade D2) is closely related to variants of allele 1 in clades D1 and E from field samples originating from South African buffalo. As indicated earlier, parasites that occur in the South African buffalo population probably occur in the East African buffalo population as a result of historical buffalo migration; it will therefore be interesting to establish whether parasites in clades D1 and E are implicated in ECF cases in East Africa and if so, why this is not the case in South Africa. The phylogenetic analysis presented here suggests that the cattle-derived p67 *T. parva* alleles evolved from buffalo-derived p67 alleles, supporting the belief that *T. parva* is originally a buffalo parasite (Uilenberg, 1981; Young, 1981; Norval *et al*., 1992) and the hypothesis that selection of a subpopulation of *T. parva* parasites resulted in ECF (Young, 1981; Conrad *et al*., 1989).

In South Africa, cattle are kept separate from buffalo to prevent infection of susceptible cattle. Recently cattle have been observed grazing around the borders of game reserves even in Corridor disease endemic areas. This situation might result in transmission of the parasite from infected buffalo to susceptible cattle and could result in the circulation of the parasite in the cattle population. As a result, there would be a higher risk of genetic exchange (Nene *et al*., 1998) that might eventually result in a parasite population that could cause ECF. It should be noted, however, that in South Africa, attempts to demonstrate transformation of buffalo-derived *T. parva* parasites to the cattle-type have proven futile (Neitz, 1957; Potgieter *et al*., 1988) although the same experiments in East Africa were successful (Barnett and Brocklesby, 1966; 1969; Young and Purnell, 1973; Maritim *et al*., 1992). Nevertheless, should transformation or DNA recombination occur, resulting in emergence of a parasite population that can cause ECF, the cattle population in South Africa would be vulnerable. Therefore it is imperative that markers are identified which can be directly linked to the

disease syndrome of *T. parva* parasites in order to provide informative molecular epidemiological data which might help the South African veterinary authorities to make informed decisions in the control of theileriosis.

4.6 Conclusion

Theileria parva p67 gene profiles appear to be more complex than previously thought. It is apparent from the results obtained in this study that the typical buffalo- and cattle-derived p67 profile as established in East Africa cannot be used to distinguish between cattle- and buffalo-derived *T. parva* parasites in South Africa and that parasites with p67 genes that have the 129 bp deletion (allele 1), as in cattle-derived isolates, cannot be associated with a specific disease syndrome. Therefore, it is still necessary to identify markers which could be directly associated with the different disease syndromes. The significance of the different p67 alleles, particularly the novel variants, in the epidemiology of theileriosis in South Africa still needs to be determined.

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CHAPTER 5

Characterization of *Theileria parva* parasites occurring in buffalo (*Syncerus caffer)* in South Africa: In search of cattle-type p104 alleles

For the LORD giveth wisdom: out of his mouth cometh knowledge and understanding. ***Proverbs 2:6***

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"All truths are easy to understand once they are discovered; the point is to discover them." ***Galileo Galilei***
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5.1 Abstract

A recent study on characterization of South African *T. parva* field samples, based on the p67 gene, revealed the presence of an allele associated with parasites responsible for East Coast fever (ECF) in East Africa. This finding has increased concerns about the possibility of the recurrence of ECF in South Africa, and necessitated further characterization of *T. parva* parasites occurring in South African buffalo in search of cattle-type alleles. PCR-RFLP and sequencing of the variable region of the p104 gene was performed on samples obtained from South African *T. parva* parasites originating from cattle on farms with suspected theileriosis and from buffalo. RFLP profile analysis using BioNumerics revealed two major cluster groups correlating broadly with geographic origin; group A consisted primarily of samples from Hluhluwe-iMfolozi Game Park while group B consisted largely of samples from the Kruger National Park. Three subgroups were identified within each main group. In subgroup A2 within group A, PCR-RFLP profiles from cattle samples which came from a farm in Ladysmith grouped with *T. parva* Muguga, a cattle-derived stock responsible for ECF in East Africa. p104 nucleotide sequences and inferred amino acid sequences obtained from these samples were identical to that of Muguga in the region analyzed, confirming the cluster analysis results. While these results suggest the presence of a cattle-derived *T. parva* parasite, reports of cattle-to-cattle transmission could not be substantiated and ECF was not diagnosed on this farm. p104 sequences from other cattle samples grouped with samples obtained from buffalo suggesting that these parasite populations may have originated from buffalo. A p104 sequence similar to that of allele 1 (*T. parva* Muguga) was obtained from a buffalo sample from KNP. Although alleles 2 (from the cattle-derived *T. parva* Marikebuni) and 3 (from *T. parva* Boleni) grouped in sub-clade A2, none of the p104 sequences obtained in this study grouped closely with either of these alleles. Sequences obtained from three buffalo *T. parva* samples from KNP grouped closely with the p104 allele 4 sequence from the buffalo-derived isolate *T. parva* 7014. Since its eradication, ECF has not been reported in South Africa; the significance of the different p104 alleles in the epidemiology of cattle theileriosis in South Africa is not clear and therefore still needs to be established.

5.2 Introduction

Cattle theileriosis caused by *Theileria parva* is a disease of major economic importance in eastern, central and southern Africa (Young *et al*., 1988). Infections of cattle by *T. parva* parasites result in three recognized disease syndromes, East Coast fever (ECF), January disease and Corridor disease. The natural tick vectors of *T. parva* in southern Africa include *Rhipicephalus appendiculatus*, *R. zambeziensis* and *R. duttoni* (Lawrence *et al*., 1983; Lessard *et al*., 1990). *Rhipicephalus appendiculatus* is widespread in South Africa, and, in the presence of *T. parva*-infected buffalo, the vector ticks can transmit the parasite to naïve cattle resulting in Corridor disease. Corridor disease remains an important form of theileriosis in South Africa where it is a controlled disease. No cases of ECF have been reported in South Africa since its eradication between 1946 and 1955 (Anonymous, 1981). It is not known whether ECF-causing *T. parva* parasites were transmitted to South African buffalo during the ECF epidemic or if there exists in buffalo an ancestral subpopulation of *T. parva* parasites that could become adapted to cattle.

In a recent study, size differentiation and sequence variation of the central region of the *T. parva* antigen gene, p67, were used to characterize South African *T. parva* field samples (Chapter 4; Sibeko *et al*., 2010). A p67 allele (allele 1) identical to that of Muguga, a *T. parva* stock from Kenya which causes ECF, was obtained from cattle samples from Ladysmith. This finding is of concern to the cattle industry in South Africa, since Potgieter *et al*. (1988) showed that buffalo-derived *T. parva* parasites causing Corridor disease can be maintained by passage between cattle and the tick vector. Presumably the persistence of *T. parva* carrier cattle in South Africa could eventually result in the selection of *T. parva* parasites adapted to cattle. Therefore there is continuous concern that ECF could re-emerge and a serious need to establish if there are cattle-type *T. parva* parasites in buffalo in South Africa.

A range of assays has been developed and used in several studies to differentiate between cattle- and buffalo-derived *T. parva* stocks (Minami *et al.*, 1983; Allsopp *et al*., 1989; Conrad *et al*., 1989; Bishop *et al.*, 1993; Collins and Allsopp, 1999). Size polymorphisms displayed by *T. parva* antigen genes, PIM, p104, p150 and p67, have been used to develop several molecular tools for characterization of *T. parva* stocks, exploiting the variable regions of these genes (Geysen *et al*., 1999; Bishop *et al*., 2001). PCR-RFLP analysis using these antigen genes demonstrated polymorphism in field stocks of *T. parva* in Kenya, although the majority of field stocks isolated from two regions of Zambia were relatively homogeneous (Geysen

et al., 1999). Recently, mini- and micro-satellite markers have been developed for characterizing *T. parva* stocks which enable detection of higher levels of polymorphism than PCR-RFLP methods (Oura *et al.,* 2003; 2005). 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.

The gene coding for the p104 antigen was selected for PCR-RFLP analysis since there is limited polymorphism in this gene, especially amongst cattle-type alleles, thus allowing distinction of buffalo-type from cattle-type alleles (Geysen *et al*., 1999). *Theileria parva* field samples obtained from buffalo often comprise multiple strains of *T. parva* parasites, which complicate characterization by PCR-RFLP. To overcome this challenge, p104 PCR products were cloned and sequenced.

5.3 Materials and methods

5.3.1 Sample collection

Blood samples were collected in EDTA vaccutainer tubes from buffalo from three game parks, and cattle from farms where cattle theileriosis was suspected, in South Africa. The *T. parva*-specific real-time PCR assay was used to screen for *T. parva*-positive samples using the reaction and cycling conditions described previously (Chapter 3; Sibeko *et al*., 2008). A total of 100 *T. parva*-positive samples, including 91 buffalo and nine cattle samples (Table 5.1), were investigated. In addition to these, two DNA samples from cattle-derived *T. parva* stocks, Muguga (Brocklesby *et al*., 1961) and Katete (Geysen, 2000), from Kenya and Zambia, respectively, were also analysed as reference samples.

5.3.2 DNA isolation

The High Pure PCR Template Preparation kit (Roche Diagnostics, Mannheim, Germany) was used for extraction of DNA from 200 µl of EDTA blood samples. DNA extractions were performed according to the method described by the kit manufacturers, except that DNA was eluted in 100 µl elution buffer. DNA was stored at 4°C until further analysis.

5.3.3 Analysis of the p104 gene from *T. parva* samples using PCR-RFLP

The *Theileria* semi-nested p104 PCR-RFLP was performed as described by De Deken *et al.* (2007). Briefly, the variable region of the p104 gene was amplified using primers p104F2 (5'-CCA CCA TCT CCT AAA CCA CCG TT-3') and p104R (5'-TAA GAT GCC GAC TAT TAA TGA CAC CAC AA-3') for the primary PCR, and primers p104nF (5'-ACC ACC GTT TGA TCC ATC ATT CA-3') and p104R for the secondary PCR. Five microlitres of genomic DNA was used in a 25 µl amplification reaction for the primary PCR, and 0.5 µl of the primary PCR product was used as a template for the secondary PCR, using the reaction and cycling conditions previously described (De Deken *et al.*, 2007). Amplicons were digested overnight with the restriction enzyme *Alu*I and the digested products were separated on a 10% polyacrylamide gel before DNA detection by SYBR® green (SIGMA-ALDRICH, USA) for RFLP analysis.

Table 5.1 Geographic origin and source of blood samples (n=100) used for characterization of *T. parva* parasites

Geographical location	Province	Sample Name*	Source of blood sample	Year of collection/ Reference
Hluhluwe-iMfolozi Park (n=39)	KwaZulu-Natal	HIP 1, HIP 3, HIP 4, **HIP 5**, HIP 6, HIP 7, HIP 8, HIP 9, HIP 10, HIP 11, HIP 12, HIP 13, HIP 14, HIP 15, HIP 16, HIP 18, **HIP 19**, HIP 20, HIP 21, HIP 22, HIP 23, HIP 24, HIP 25, HIP 26, HIP 27, HIP 28, HIP 29, HIP 30, HIP 31, HIP 32, HIP 33, HIP 34, HIP 35, HIP 36, HIP 37, HIP 39, HIP 40, HIP 41, HIP 42	Buffalo	2004
Kruger National Park (n=46)	Mpumalanga	KNP 47, KNP 48, KNP 49, KNP 50, KNP 61, KNP 62, **KNP 63,** KNP 66, KNP 67, KNP 68, **KNP 102,** KNP AA5, **KNP AB47**, KNP AD3, KNP B2, **KNP B10**, KNP B22, KNP D11, KNP D24, KNP E7, KNP E18, KNP E20, KNP F9, KNP G2, KNP G11, KNP H8, KNP H15, KNP I23, **KNP L6**, KNP L27, KNP M2, KNP M12, KNP N1, KNP N8, KNP O1, KNP O11, **KNP P7,** KNP S17, KNP U3, KNP U20, KNP V5, **KNP W8**, KNP X4**, KNP Y4**, KNP Y19, KNP Z4	Buffalo	2003
Ladysmith (n=6)	KwaZulu-Natal	Lad 2, Lad 6, **Lad 10**, Lad 11, **Lad 15, Lad 17**	Bovines	2003
Mabalingwe Game Reserve (n=6)	Limpopo	**Mab A13**, **Mab BB43**, Mab B21**, Mab BB37**, Mab A22, **Mab BB38**	Buffalo	2004
Lydenburg (n=1)	Mpumalanga	Lyd N254	Bovine	2004
Bloemfontein (n=1)	Free-State	**Bloe B**	Bovine	2004
Schoonspruit (n=1)	Mpumalanga (former Transvaal)	**Schoonspruit**	Bovine	Neitz (1948), Collins (1997)

Samples shown in bold were used for sequencing

5.3.4 PCR-RFLP profile analysis

PCR-RFLP patterns were analysed using BioNumerics software, version 5.1 (Applied Maths, Kortrijk, Belgium). Normalization of the RFLP profiles was done using the molecular weight marker 100 bp DNA Ladder (Fermentas Life Sciences, Germany), which was run in two lanes per gel of fifteen wells. DNA fragments of less than 100 bp were excluded from the analysis as their size could not be estimated accurately using the 100 bp DNA ladder; moreover, in some instances these fragment had run out of the gel. The software was used to calculate Dice coefficients of similarity, to cluster the RFLP profiles and to generate dendrograms by the unweighted-pair group method using average linkages (UPGMA). The most appropriate settings for optimization and tolerance, as determined by the software, were calculated. Samples with similar RFLP profiles obtained from different animals were defined as clusters.

5.3.5 Cloning and sequencing of p104 PCR products

PCR products from 19 *T. parva* samples, representative of each cluster group, were selected for cloning and sequencing (shown in bold in Table 5.1). PCR products were cloned into the pCR®2.1-TOPO® cloning vector (Invitrogen, Carlsbad, USA). The presence of inserts in recombinants was confirmed by colony PCR following the secondary PCR protocol used above. At least four clones were sequenced from each sample; sequencing was performed by the Genetic Service Facility of the University of Antwerp in Belgium.

5.3.6 Sequence analysis

Sequences were assembled and edited using the GAP4 program of the Staden package (version 1.6.0 for Windows) (Bonfield *et al*., 1995; Staden, 1996; Staden *et al*., 2000). A multiple sequence alignment of p104 amino acid sequences was performed using MAFFT version 6 (Katoh *et al*., 2002) (http://align.bmr.kyushu-u.ac.jp/mafft/software), and maximum parsimony and Bayesian analyses were used to produce phylogenetic trees. The maximum parsimony analysis was performed in PAUP*4.0b10 (Swofford, 2003) with 1000 random addition sequence followed by bisection-reconnection (TBR) branch swapping and branch support was assessed with 100 bootstrap replicates. The Bayesian analysis was performed using MrBayes v3.1.1 (Ronquist and Huelsenbeck, 2003). A Markov chain Monte Carlo run of five million generations consisting of four parallel MCMC chains was performed.

5.4 Results

5.4.1 p104 PCR-RFLP profile analysis

A p104 PCR product of ~800 bp in size was obtained from all samples analyzed in this study (results not shown). From visual analysis, cattle samples, Lad 02, Lad 06, Lad 10 (from Ladysmith) and *T. parva* Schoonspruit had an identical PCR-RFLP profile to that of *T. parva* Muguga, a stock from Kenya responsible for ECF (Figure 5.1).

The cluster analysis using BioNumerics revealed two major groups, A and B, and the clustering correlated broadly with geographic origin of the samples (Figures 5.2 and 5.3). Group A consisted mainly of samples obtained from Hluhluwe-iMfolozi Game Park (39/49, 79.6%) and group B consisted largely of samples from Kruger National Park (KNP) (42/51, 82.4%), although KNP samples were also present in group A. Three subgroups were identified in each of the two major groups. Subgroup A1 consisted mainly of samples from Hluhluwe-iMfolozi while samples from Ladysmith and KNP appeared in subgroups A2 and A3. The majority of samples (42/50, 84%) in subgroups B1, B2 and B3 came from KNP. Samples from Hluhluwe-iMfolozi produced a relatively homogeneous fingerprint, with most (32/39, 82%) appearing in subgroup A1, while those from KNP buffalo samples were heterogeneous and appeared in all six fingerprint groups (Figures 5.2 and 5.3). The p104 RFLP profiles from four cattle samples, Lad 02, Lad 06, Lad 10 and *T. parva* Schoonspruit, with profiles similar to that of *T. parva* Muguga from visual inspection, grouped with *T. parva* Muguga on the dendrogram (Figures 5.2 and 5.3). These cattle samples clustered in group A amongst p104 profiles from buffalo samples from Hluhluwe-iMfolozi (Figures 5.2 and 5.3). Other cattle samples, Lad 11, Lad 15, Lad 17, Bloe B and Lyd N254 grouped with samples in group B which contained mainly buffalo samples from KNP. The *T. parva* Muguga-like cattle-type p104 fingerprints were not obtained from any of the buffalo samples analyzed in this study.

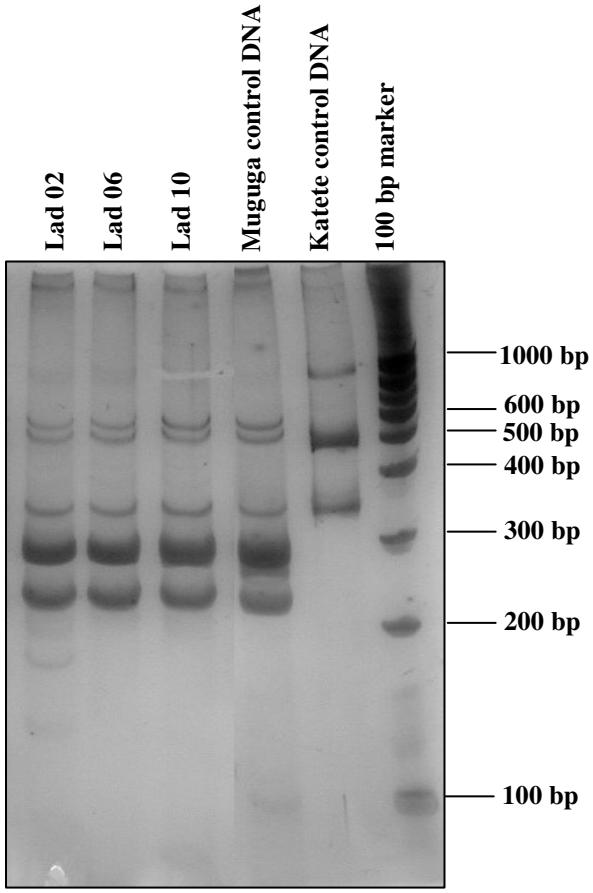


Figure 5.1 p104 gene *Alu*I RFLP profiles showing the *T. parva* Muguga RFLP profile and identical profiles obtained from cattle samples from a farm near Ladysmith.

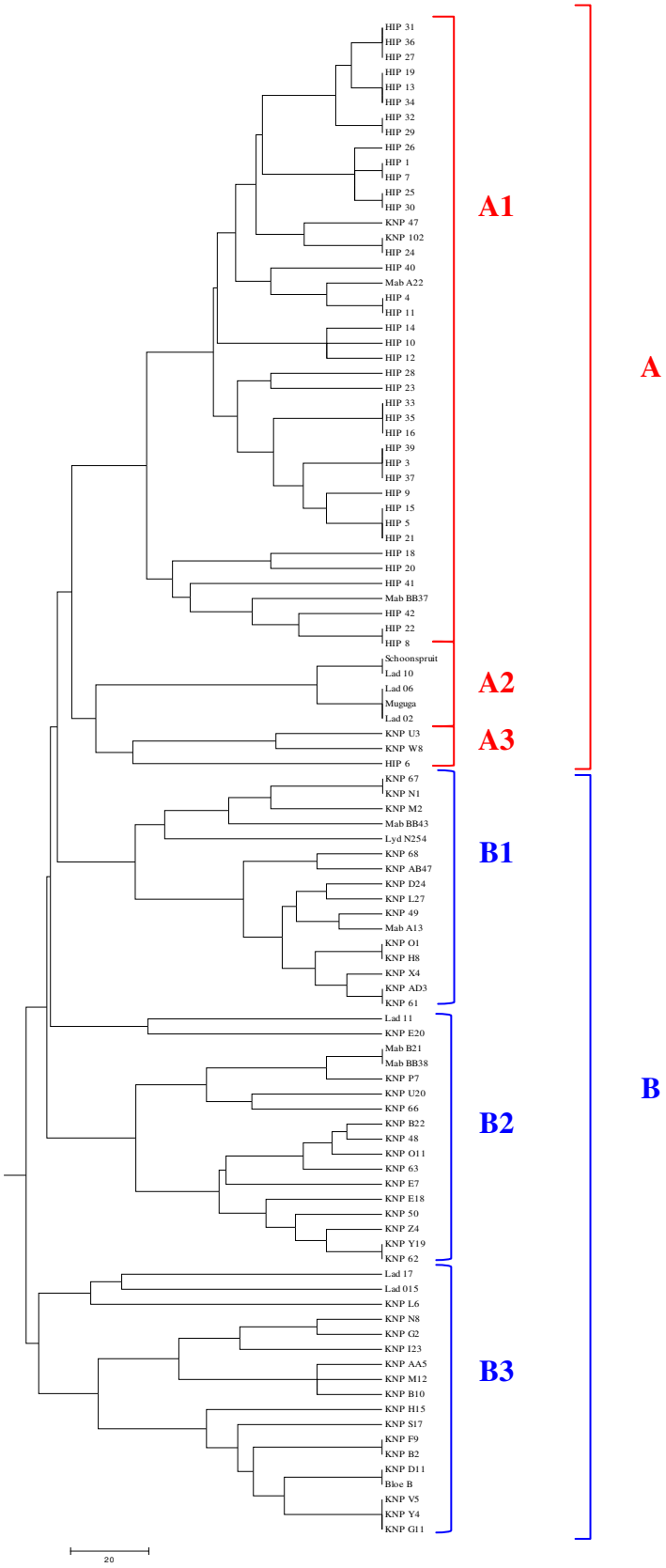


Figure 5.2 A simplified similarity dendrogram for p104 PCR-RFLP profiles cluster analysis based on the Dice Coefficient analysis produced using BioNumerics v5.1.

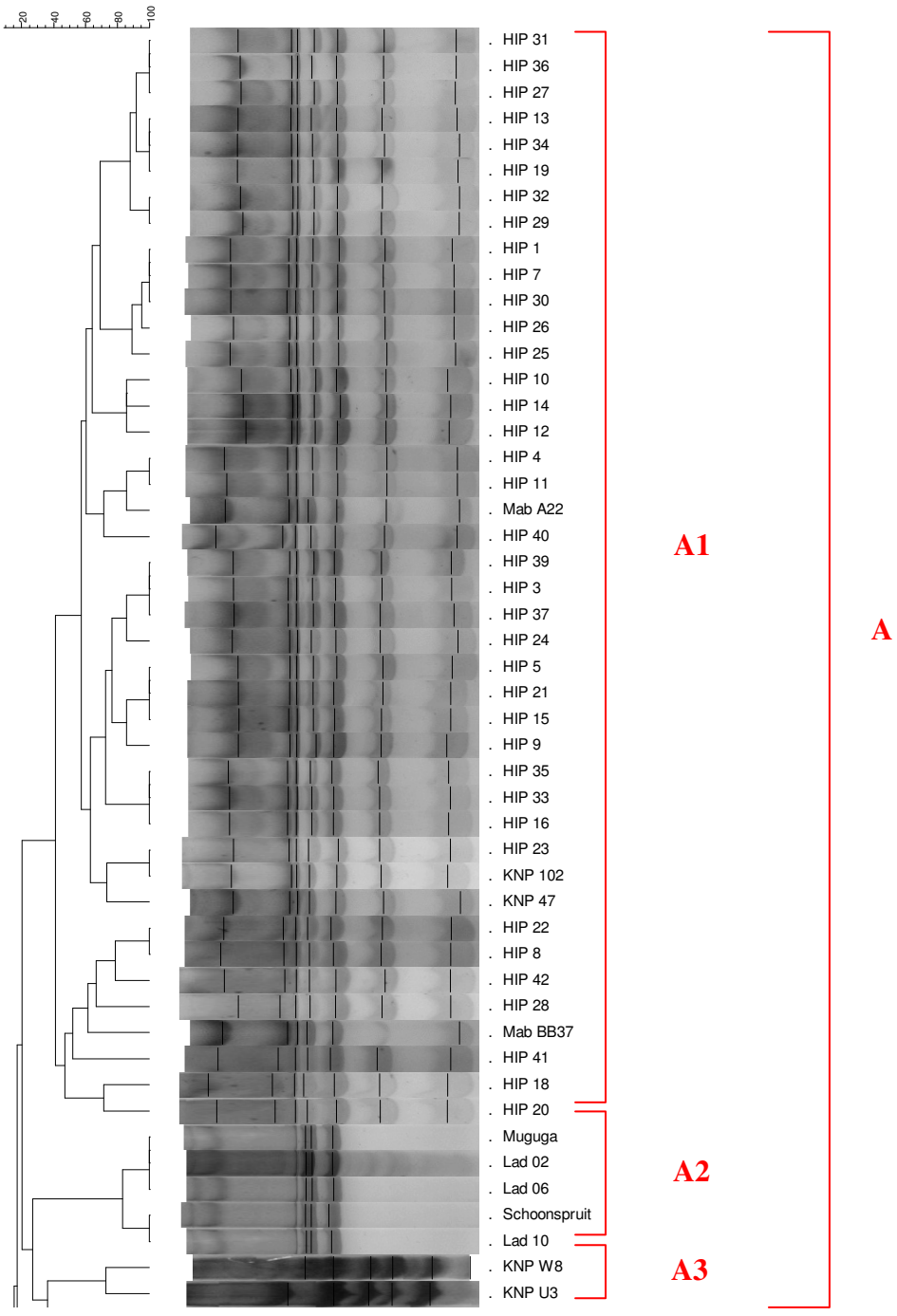
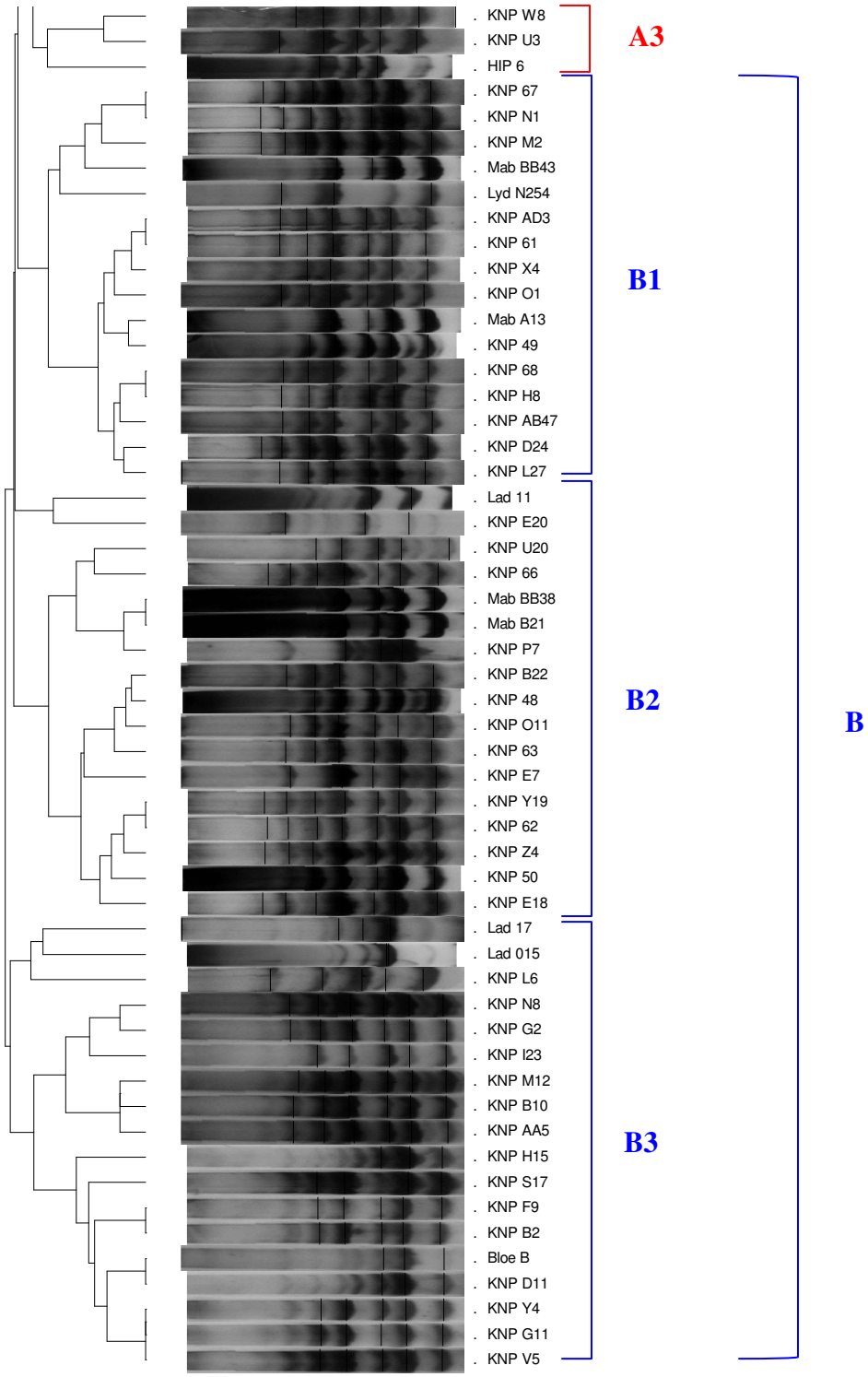


Figure 5.3 A detailed similarity dendrogram for p104 PCR-RFLP profiles cluster analysis based on the Dice Coefficient analysis produced using BioNumerics v5.1 showing actual profiles used to create the dendrogram. Figure 5.3 continues on page 110.


KNP W8
KNP U3
HIP 6
KNP 67
KNP N1
KNP M2
Mab BB43
Lyd N254
KNP AD3
KNP 61
KNP X4
KNP O1
Mab A13
KNP 49
KNP 68
KNP H8
KNP AB47
KNP D24
KNP L27
Lad 11
KNP E20
KNP U20
KNP 66
Mab BB38
Mab B21
KNP P7
KNP B22
KNP 48
KNP O11
KNP 63
KNP E7
KNP Y19
KNP 62
KNP Z4
KNP 50
KNP E18
Lad 17
Lad 015
KNP L6
KNP N8
KNP G2
KNP I23
KNP M12
KNP B10
KNP AA5
KNP H15
KNP S17
KNP F9
KNP B2
Bloe B
KNP D11
KNP Y4
KNP G11
KNP V5
A3
B1
B2
B3
B

5.4.2 p104 gene sequence analysis

To confirm results obtained by RFLP profile cluster analysis and to establish p104 alleles that occur in the different subgroups, PCR products from representatives of each cluster group were cloned and sequenced. Fifty-three good quality p104 sequences were obtained from clones produced from amplicons of the 19 selected *T. parva* samples. Except for two, the p104 amino acid sequences obtained from *T. parva* field samples analyzed in this study were not identical to the p104 sequences previously reported by Skilton *et al*. (2002) although they were very similar with sequence identities raging from 92-99% in the region analyzed. Skilton *et al*. (2002) identified four p104 alleles representing p104 amino acid sequences obtained from different *T. parva* stocks of cattle and buffalo in East Africa. According to Skilton *et al*. (2002) allele 1 (accession number: M2954) represents the *T. parva* Muguga p104 amino acid sequence (Iams *et al*., 1990); allele 2 (accession number: AY034069) is found in the Marikebuni and Uganda *T. parva* stocks and alleles 3 (accession number: AY034070) and 4 (accession number: AY034071) represent the *T. parva* Boleni and 7014 p104 amino acid sequences, respectively. Muguga, Marikebuni, Uganda and Boleni are cattle-derived *T. parva* stocks whereas 7014 is a buffalo-derived *T. parva* stock.

Both maximum parsimony and Bayesian analyses produced similar groupings of p104 sequences; however, the maximum parsimony analysis did not resolve the differences within groups containing alleles 2 and 3. Therefore only the phylogenetic tree based on Bayesian analysis is presented (Figure 5.4). Of the 53 South African p104 sequences, one sequence from the buffalo *T. parva* sample MabA13 (clone 6) had a deletion of 17 amino acids in the region analysed. The p104 sequences obtained in this study could be grouped into two main clades, A and B, and each of the major clades could be divided into two sub-clades, A1 and A2, and B1 and B2 (Figure 5.4). Alleles 2 and 3 grouped in sub-clade A2 and alleles 1 and 4 in sub-clade B1. Two p104 amino acid sequences obtained from samples collected from cattle grouped closely to the *T. parva* Muguga p104 sequence and both had sequence identity of 100% (Lad10 and *T. parva* Schoonspruit) to that of the *T. parva* Muguga p104 amino acid sequence. In addition to these, a p104 sequence obtained from a buffalo *T. parva* sample, KNP B10_3.0.2, had 97% sequence identity to that of *T. parva* Muguga and grouped closely to p104 sequences from Lad10, *T. parva* Schoonspruit and *T. parva* Muguga in sub-clade B1. Apart from *T. parva* Katete, no sequences grouped closely to allele 2; similarly no sequences grouped closely with allele 3. Sequence identities of 96-97% were obtained from sequences that grouped closely with p104 allele 4 (KNP102 8 3, KNPW8 8.0.2, KNPAB47 8.0.1).

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The groups obtained from PCR-RFLP cluster analysis did not correlate with the clades identified by phylogenetic analysis of p104 amino acid sequences (Table 5.2). Samples with sub-clade B1 p104 sequences were found in almost all RFLP subgroups. Unfortunately, DNA from samples of reference sequences was not available for PCR-RFLP analysis and it was therefore not possible to determine where the p104 profiles from these samples would fit in the cluster analysis.

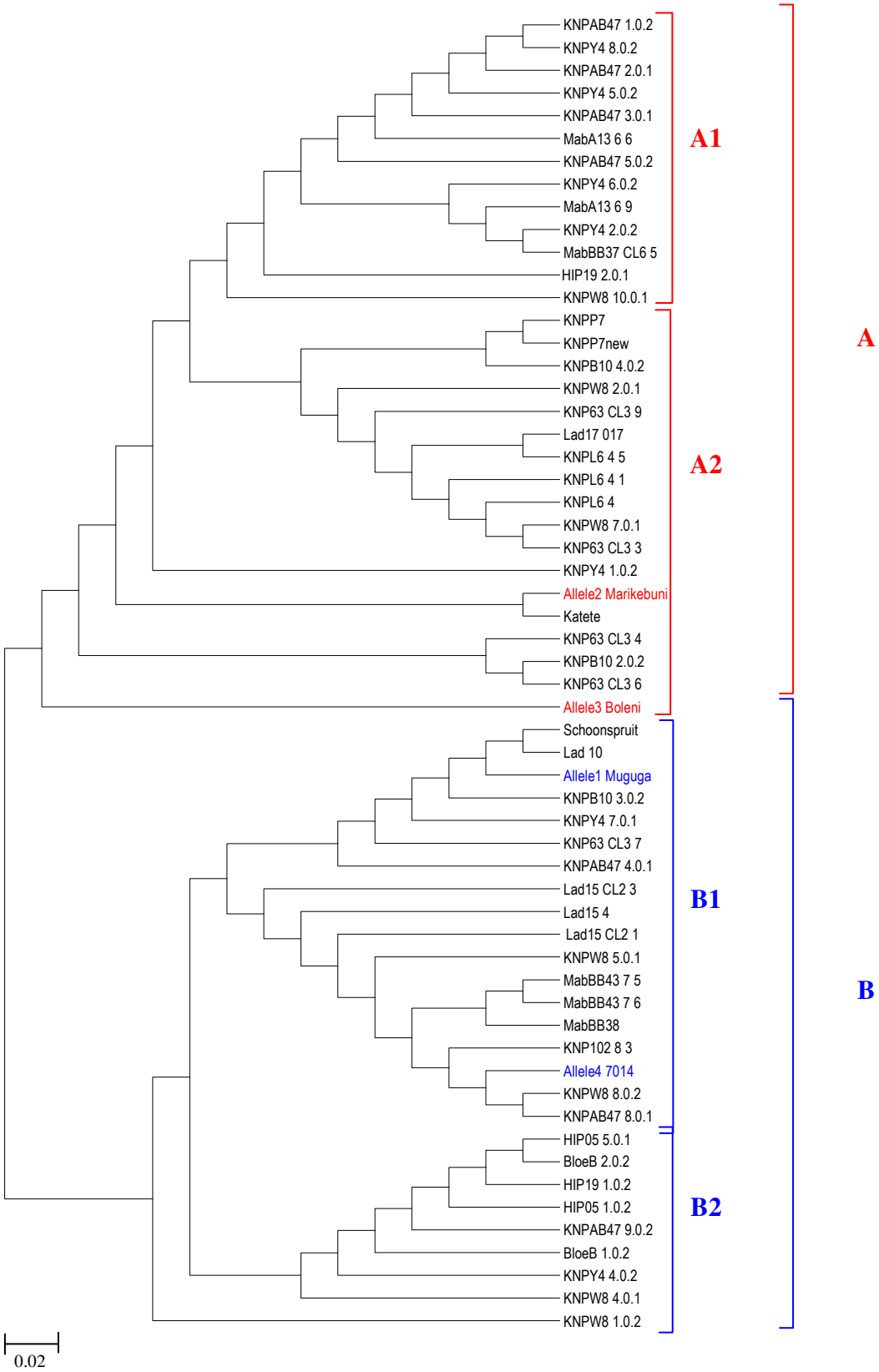

Figure 5.4 Phylogenetic relationship of *T. parva* strains as revealed by p104 amino acid sequence analysis. The phylogenetic tree was calculated by Bayesian analysis; the first 10,000 trees were discarded as burnin and the majority-rule consensus tree was generated.

Table 5.2 Comparison of p104 PCR-RFLP cluster analysis and phylogenetic analysis of p104 amino acid sequences from 19 selected *T. parva* samples

Major PCR-RFLP cluster group	PCR-RFLP cluster subgroup	Sample name	p104 phylogenetic analysis sub-clade
A	A1	HIP 5	B2
		HIP 19	A1, B2
		KNP 102	B1
		Mab BB37	A1
	A2	**Lad 10**	B1
		Schoonspruit	B1
	A3	KNP W8	A1, A2, B1, B2
B	B1	Mab BB43	B1
		KNP AB47	A1, B1, B2
		Mab A13	A1
	B2	KNP P7	A2
		Mab BB38	B1
		KNP 63	A2, B1
	B3	**Lad 15**	B1
		Lad 17	A2
		Bloe B	B2
		KNP B10	A2, B1
		KNP L6	A2
		KNP Y4	A1, A2, B1, B2

Samples shown in bold were obtained from cattle

5.5 Discussion

In South Africa, ECF, caused by the blood parasite *T. parva* was eradicated in 1954 and there have been no reports of ECF since then (Anonymous, 1981). However, the tick vector *R. appendiculatus* and the parasite that causes Corridor disease still occur and therefore this disease is a controlled disease in South Africa. It is not clear if the *T. parva* parasites that cause ECF existed in buffalo before the outbreak of ECF or if these parasites were introduced with the livestock imported from East Africa. This study was performed to establish if cattle-type *T. parva* p104 alleles exist in buffalo in South Africa.

Cluster analysis of *T. parva* p104 RFLP profiles revealed clustering correlating broadly with geographic origin. Samples from Hluhluwe-iMfolozi produced an apparently homogeneous fingerprint, and appeared in the same subgroup (A1), while those from KNP buffalo samples were heterogeneous and appeared in all six subgroups. This finding could suggest that there is a less diverse population of *T. parva* parasites circulating in the Hluhluwe-iMfolozi game park as evidenced by the relatively homogeneous RFLP profiles, while the heterogenous

profiles obtained from *T. parva* samples from buffalo from KNP might be evident of a more diverse population.

The phylogenetic analysis of p104 amino acid sequences revealed two major clades, A and B, with sub-clades A1 and A2, and B1 and B2, respectively. p104 alleles 2 (*T. parva* Marikebuni) and 3 (*T. parva* Boleni) grouped in sub-clade A2. Similarly, alleles 1 (*T. parva* Muguga) and 4 (*T. parva* 7014) grouped in sub-clade B1. From this analysis, allele 1 and allele 4 seem to have evolved from a common ancestor. A separate speciation event appears to have given rise to the cattle-type p104 allele in the cattle-derived *T. parva* isolates Marikebuni and Katete. These results support data obtained from analysis of the p67 gene (Chapter 4; Sibeko *et al*., 2010), and provide further evidence to suggest that *T. parva* was originally a buffalo parasite, and that strains that circulate in cattle are likely to originate from buffalo. Novel p104 sequences which were not similar to known p104 alleles were identified in sub-clades A1 and B2. It is not clear if sequences in these two sub-clades are variants of the known sequences; analysis of the complete p104 gene sequence from alleles 1, 2, 3 and 4 and 'new' alleles would be required for comparison studies to confirm this.

There was no significant correlation between data obtained from PCR-RFLP cluster analysis and the phylogeny obtained from sequence analysis. This is probably because field samples usually contain mixed infections. The PCR-RFLP cluster analysis therefore represents an overall p104 profile of all the *T. parva* strains in the sample, whereas each p104 sequence represents a single *T. parva* strain. Therefore, with mixed infections, several individual sequences can be obtained from clones from a single sample and these can be distributed in different clades. For example, p104 sequences obtained from clones prepared from KNPW8 (a buffalo sample) occurred in all different clades on the phylogenetic tree. Furthermore, in cases of mixed infections, PCR-RFLP cluster groups would be overwhelmed by profiles of dominant parasites, whereas profiles from less dominant parasites, which will probably not be apparent on the profile, might have grouped differently. The PCR-RFLP cluster analysis of profiles produced from clones would probably give a different picture and would be more likely to correlate better with the sequence data analysis.

Three cattle samples from a farm in Ladysmith and *T. parva* Schoonspruit had a cattle-type *T. parva* p104 RFLP profile identical to that of *T. parva* Muguga, and the p104 amino acid sequences obtained from Lad 10 and *T. parva* Schoonspruit were identical to the *T. parva* Muguga p104 sequence in the region analyzed. Since *T. parva* Schoonspruit was isolated

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during the ECF outbreak (Neitz, 1948), these results provide evidence that the *T. parva* parasites present in South Africa during the ECF epidemic were similar to those that cause ECF in East Africa. Interestingly, in a separate study, analysis of the p67 gene from Lad 10 revealed the presence of a p67 allele identical to that of *T. parva* Muguga (Chapter 4; Sibeko *et al.*, 2010). Taken together, these findings suggest that there may have been selection of cattle-type *T. parva* parasites in cattle on this farm. However, classical ECF was not diagnosed in cattle on the Ladysmith farm and reports of cattle-to-cattle transmission could not be substantiated, although *T. parva* carrier cattle were identified (Thompson *et al.*, 2008). While the presence in South African cattle of a parasite apparently similar to *T. parva* Muguga is a concern, there is no evidence to suggest that genotypes at individual loci such as p104 and p67, can be linked to the pathogenicity of the isolate.

p104 PCR-RFLP fingerprints obtained from other cattle samples from the Ladysmith farm (Lad 11, Lad 15 and Lad 17), grouped with p104 profiles from buffalo samples. The p104 amino acid sequences obtained from Lad 15 and Lad 17 grouped in sub-clades B1 and B2, respectively. The PCR-RFLP analysis and the sequence analysis therefore suggest that *T. parva* parasites circulating in some of the cattle on the Ladysmith farm may have been transmitted from buffalo to cattle, but there was no evidence of contact between these cattle and buffalo (Thompson *et al.*, 2008).

p104 PCR-RFLP fingerprints obtained from other cattle samples (Bloe B, Lyd 254) also grouped with profiles from buffalo samples suggesting that these *T. parva* parasites may have been transmitted from buffalo to cattle. These results were supported by the sequence analysis; the p104 sequence obtained from Bloe B grouped in sub-clade B2 with other p104 sequences from *T. parva* parasites obtained from buffalo. In South Africa, contact between cattle and buffalo is strictly controlled. However, in some areas cattle are grazed in pastures adjacent to game parks where buffalo are kept. If cattle break into or buffalo break out of the park where the vector tick is present, naive cattle are likely to be infected by *T. parva* infected buffalo. This may have been the case with these animals.

None of the p104 sequences obtained from buffalo *T. parva* samples in this study were identical to any of the previously reported p104 sequences (Skilton *et al.*, 2002). The sequence identities of p104 sequences obtained from buffalo in this study, when compared with published sequences, ranged from 94 to 97%. This finding suggests that *T. parva* p104

alleles in South African buffalo are more diverse and that variations in the p104 gene may not be as limited as previously thought (Geysen *et al*., 1999).

Sequences obtained from three buffalo *T. parva* samples from KNP grouped closely with the p104 allele 4 sequence. One of these sequences came from a *T. parva* sample obtained from buffalo KNP 102; this isolate has previously been used in a tick transmission experiment where the infected bovine eventually died from classical Corridor disease (Chapter 3; Sibeko *et al*., 2008). Characterization of the p67 gene from this isolate showed the presence of sequences similar to both cattle- and buffalo-type p67 alleles, and, in addition to these, two novel p67 alleles (Chapter 4; Sibeko *et al*., 2010). Although the KNP 102 *T. parva* isolate could cause Corridor disease, when a mix of parasites exists in an isolate it is impossible to determine which of these were responsible for inducing the disease.

Although alleles 2 and 3 grouped in sub-clade A2, none of the p104 sequences obtained in this study grouped closely with either of these alleles. Allele 2 was obtained from *T. parva* Marikebuni, which causes ECF in Kenya. Allele 3 was identified in *T. parva* Boleni, which was isolated in Zimbabwe and is a heterogeneous isolate that has both mild and pathogenic strains; the mild stock is used for vaccination against ECF (Irvin *et al*., 1989). Previous studies have shown that *T. parva* Boleni is genetically completely different at the p104, p150, p32 and PIM loci from the classical *T. parva* genotypes found in cattle, suggesting that it is a buffalo-type parasite, isolated from cattle (Dirk Geysen, pers. comm.). Interestingly, a sequence similar to p104 allele 1 was obtained from a buffalo sample, KNP B10. Variants of p67 allele 1 were also identified from buffalo samples from KNP (Chapter 4; Sibeko *et al*., 2010). These results could suggest that parasites with characteristics similar to cattle-derived *T. parva* might occur in *T. parva* populations circulating in buffalo in KNP. However, it is not clear if parasites that possess these alleles can cause disease and their significance in the epidemiology of theileriosis in South Africa needs to be established.

5.6 Summary

In summary, we have demonstrated that p104 amino acid sequences very similar to those of *T. parva* Muguga were present in *T. parva* parasites on a farm in South Africa where cattle theileriosis was reported. These findings, corroborated by the results obtained when p67 was used to characterize *T. parva* parasites from this farm (Chapter 4; Sibeko *et al*., 2010), remain a concern even though ECF has not been reported in South Africa since its eradication in

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1954. While the cattle-type p104 alleles 2 (*T. parva* Marikebuni) and 3 (*T. parva* Boleni) grouped with p104 sequences from buffalo in South Africa, none of the South African p104 sequences obtained in this study grouped closely with either of these alleles. However, variants of p104 allele 1 (*T. parva* Muguga) were identified in buffalo. The significance of these parasites in the epidemiology of theileriosis in South Africa will have to be determined and the risk of disease evaluated. However, from the data obtained in this study, sequence variation in the region of the p104 gene analysed cannot be used to differentiate reliably between the cattle-type and the buffalo-type alleles because of the high sequence similarity between the two. The challenge to identify more reliable and specific markers that can be directly associated with different disease syndromes caused by *T. parva* still remains. This is crucial for the design and implementation of preventative measures in South Africa to protect livestock against disease caused by *T. parva* infections.

5.7 References

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CHAPTER 6

Analysis of the gene encoding the *Theileria parva* polymorphic immunodominant molecule (PIM) reveals evidence of the presence of cattle-type alleles in South Africa

"There's two possible outcomes: if the result confirms the hypothesis, then you've made a discovery. If the result is contrary to the hypothesis, then you've made a discovery." ***Enrico Fermi***

6.1 Abstract

Analysis of two *T. parva* genes coding for the antigenic proteins p67 and p104, revealed the presence of cattle-type alleles identical to those of *T. parva* Muguga (a stock that causes ECF in Kenya) from *T. parva* samples collected from three cattle from a farm in Ladysmith. In addition, variants of p67 allele 1 and p104 allele 1, characteristic of the cattle-type alleles, were identified from *T. parva* samples obtained from buffalo from four game parks in South Africa. Consequently, polymorphic immunodominant molecule (PIM) PCR-RFLP profiles and inferred amino acid sequences were analyzed to confirm the presence of cattle-type alleles in *T. parva* samples obtained from cattle and buffalo in South Africa. PIM PCR-RFLP profiles similar to that of the *T. parva* Muguga stock were obtained from three of the six cattle samples from the Ladysmith farm and the inferred amino acid sequences of the PIM gene from two of these samples (Lad 02 and Lad 10) were almost identical to the *T. parva* Muguga PIM sequence. This finding supports recent studies in which p67 and p104 alleles similar to those of the *T. parva* Muguga stock were identified from the same Ladysmith samples. None of the PIM gene sequences obtained from *T. parva* field samples characterized in this study were identical, providing further evidence that the PIM gene evolves at an extremely high rate. Cattle-type PIM alleles were not identified from buffalo *T. parva* samples. In addition to sequences similar to known PIM alleles, for the first time, 'mixed' alleles consisting of cattle- and buffalo-type amino acid motifs were identified. The significance of *T. parva* parasites carrying 'mixed' PIM alleles will have to be established and their risk to cattle evaluated. With the extent of genetic diversity that has been demonstrated by the three characterization studies presented in this thesis, the epidemiology of theileriosis in South Africa needs to be further investigated.

6.2 Introduction

The tick transmitted apicoplexan protozoan parasite, *Theileria parva*, is by far the most pathogenic and economically significant *Theileria* sp. in eastern, central and southern Africa (Mukhebi *et al*., 1992). Infections by *T. parva* are associated with high mortality, primarily in exotic and crossbred cattle, but also in indigenous calves and adult cattle in endemically stable areas (Perry and Young, 1995). This places major constraints on cattle production and the expansion of the dairy industry. The Cape buffalo *(Syncerus caffer*) is the natural reservoir host of the parasite.

Tick transmission of the parasite from infected buffalo to susceptible cattle results in a disease syndrome called Corridor disease, while *T. parva* parasites that can circulate in cattle cause two disease syndromes, East Coast fever (ECF) and January disease (Theiler, 1904; Neitz, 1955; Lawrence, 1992). To distinguish between the different types of *T. parva* parasites, parasites that circulate in cattle and cause ECF and January disease are referred to as cattle-derived *T. parva* and parasites that originate from buffalo and cause Corridor disease are known as buffalo-derived *T. parva* (Perry and Young, 1993). East Coast fever was introduced into South Africa at the end of the 19th century and eradicated in the early 1950s (Anonymous, 1981). Although ECF was eradicated in southern Africa, its principal vector, the three-host ixodid tick *Rhipicephalus appendiculatus,* is still widespread. Corridor disease is a controlled disease in South Africa and sporadic outbreaks still occur.

Studies on two *T. parva* genes, p67 and p104, presented in the previous two chapters, revealed the presence of cattle-type alleles identical to those of *T. parva* Muguga (a stock that causes ECF in Kenya) from *T. parva* samples collected from three cattle from a farm in Ladysmith, South Africa (Chapter 4; Chapter 5; Sibeko *et al*., 2010). In addition, variants of p67 allele 1 and p104 allele 1, characteristic of the cattle-type alleles, were identified from *T. parva* samples obtained from buffalo from four game parks in South Africa, namely, Kruger National Park, Hluhluwe-iMfolozi, Ithala and Mabalingwe (Collins, 1997; Chapter 4; Sibeko *et al*., 2010). Unless these alleles can be associated with parasites that can cause fatal disease, their importance remains unclear. Consequently, another *T. parva* gene encoding an antigenic protein, the polymorphic immunodominant molecule (PIM), was investigated.

The PIM gene has previously been successfully used to differentiate between cattle- and buffalo-derived *T. parva* stocks (Geysen *et al*., 1999; Bishop *et al*., 2001). Although PIM is expressed by both the sporozoite and the schizont stages of the parasite, it is expressed predominantly by the schizont stage and is thus believed to play a role as a target antigen in the induction of the cytotoxic T cell response (Shapiro *et al*., 1987; Toye *et al*., 1991; Katende *et al*., 1998; Shaw, 2003; Graham *et al*., 2007). It is also capable of inducing sporozoite-neutralizing antibodies and has been exploited in discriminatory assays for *T. parva* isolates because of its highly conserved termini flanking a variable region with a highly polymorphic central region (Toye *et al*., 1995a; 1995b; Bishop *et al*., 2001; Geysen *et al*., 1999; 2004; De Deken *et al*., 2007). The variations in the central region of the PIM gene produce the polymorphism that has been exploited for discrimination between *T. parva* stocks (Geysen *et al*., 1999; Bishop *et al*., 2001).

In this study, the PIM gene was characterized to confirm the presence of cattle-type alleles in *T. parva* samples obtained from cattle and buffalo in South Africa. The diversity of the parasite populations circulating in buffalo and cattle in South Africa was also demonstrated.

6.3 Materials and methods

6.3.1 Sample collection

Blood samples were collected from buffalo from different game parks, and from cattle from farms with suspected theileriosis. The samples were collected in EDTA vaccutainer tubes and stored at -20 ºC, for long term storage, or 4 ºC for short term storage, before extraction of DNA.

6.3.2 DNA isolation and selection of *T. parva*-positive samples

Total DNA was extracted from 200 µl of EDTA blood using the High Pure PCR Template Preparation kit (Roche Diagnostics, Mannheim, Germany), according to the method described by the manufacturer, except that extracted DNA was eluted in 100 µl elution buffer. Extracted DNA was stored at 4°C until further analysis. The presence of *T. parva* DNA was determined using the real-time PCR assay as previously described (Chapter 3; Sibeko *et al*., 2008). One hundred and nine *T. parva*-positive samples consisting of 101 field samples collected from buffalo from different game parks, and eight collected from cattle from farms with suspected theileriosis, were investigated (Table 6.1).

Table 6.1 Geographical origin and source of blood samples (n=109) used for characterization of *T. parva* parasites

Geographical location	Province	Sample Name*	Host of blood sample	Date of collection/ References
Hluhluwe-iMfolozi Park (n=38)	KwaZulu-Natal	HIP 1, HIP 3, HIP 4, **HIP 5**, HIP 6, HIP 7, HIP 8, HIP 9, HIP 10, HIP 11, HIP 12, HIP 13, HIP 14, HIP 15, HIP 16, HIP 18, HIP 19, HIP 20, HIP 21, **HIP 22**, HIP 23, HIP 24, HIP 25, HIP 26, HIP 27, HIP 28, HIP 30, HIP 31, **HIP 32**, HIP 33, HIP 34, HIP 35, HIP 36, HIP 37, HIP 38, **HIP 39**, HIP 42, HIP 49	Buffalo	2004
Kruger National Park (KNP) (n=47)	Mpumalanga	**KNP 39**, **KNP 42**, **KNP 43**, KNP 47, KNP 48, KNP 49, KNP 50, KNP 61, KNP 62, KNP 63, KNP 66, KNP 67, KNP 68, **KNP 102,** KNP AA5, KNP AB47, KNP AC10, KNP AD3, KNP B10, KNP B22, KNP D11, KNP D24, KNP E7, KNP E18, KNP F9, KNP G2, KNP G11, KNP H8, KNP J5, KNP L6, KNP L27, KNP M2, KNP M12, KNP M2706, KNP N1, KNP N8, KNP O1, **KNP O11**, KNP S17, KNP U3, KNP U20, **KNP V5**, **KNP W8**, KNP X4, KNP Y4, KNP Y19, KNP Z4	Buffalo	2003
Ladysmith (n=6)	KwaZulu-Natal	**Lad 2,** Lad 06, **Lad 10, Lad 17 Lad M119, Lad I438**	Bovines	2003 Thompson *et al*. (2008)
Mabalingwe Game Reserve (n=6)	Limpopo	**Mab A13, Mab A22**, **Mab B21**, **Mab BB37**, **Mab BB38, Mab BB43**	Buffalo	2004
Ithala Game Reserve (n=10)	KwaZulu-Natal	**Itha 1**, **Itha 2, Itha 3, Itha 4, Itha 5, Itha 6, Itha 7, Itha 8, Itha 9, Itha 10**	Buffalo	2005/6
Schoonspruit (n=1)	Mpumalanga	**Schoonspruit**	Bovine	Neitz (1948)
Bloemfontein (n=1)	Free-State	**Bloe B**	Bovine	2004

*Samples in bold were selected for cloning to produce RFLP profiles from individual clones and only 27 of the 35 were used for sequencing (see Table 6.2).

6.3.3 Amplification of the PIM gene from *T. parva* samples

The variable region of the *T. parva* PIM gene was amplified from *T. parva* positive DNA samples using the semi-nested PCR described by De Deken *et al*. (2007). A nested PCR was performed using primers Pim1 [5' GTG AAT GTT GTG ATC TTA ATC C 3'] and PimR4 [5' CCC ACA ACC GTG GAA TGG CGT A 3'] for the primary PCR and primers PimFm [5' ATT CCA CTG GTT CTT CCG ATS TA 3', where S = C or G] and PimR4 for the secondary PCR. Briefly, 5 µl of total DNA was used in a 25 µl amplification reaction for the primary PCR and half a microlitre of the primary PCR product was used as a template for the secondary PCR, using the reaction and cycling conditions previously described (De Deken *et al*., 2007).

6.3.4 Analysis of the PIM gene from *T. parva* samples using PCR-RFLP

Restriction fragment length polymorphism was performed as described by De Deken *et al*. (2007); briefly, PCR products were digested overnight with the restriction enzyme, *Bcl*I; the digested products were separated on a 10% polyacrylamide gel before DNA detection by SYBR® green (SIGMA-ALDRICH, USA). RFLP patterns were analysed by visual inspection and by using BioNumerics version 5.1 (Applied Maths, Kortrijk, Belgium). Normalisation of the RFLP profiles was done using the molecular weight marker 100 bp DNA Ladder (Fermentas Life Sciences, Germany), which was run in two lanes per gel. The software was used to calculate Dice coefficients of similarity, to cluster the RFLP profiles and to generate dendrograms by the unweighted-pair group method using average linkages (UPGMA). The most appropriate settings for optimization and tolerance, as determined by the software, were calculated. DNA fragments of less than 100 bp were excluded from the analysis as these could not be accurately estimated using the 100 bp DNA ladder and in some instances had run out of the gel. Samples with similar RFLP profiles obtained from different animals were defined as clusters.

6.3.5 Cloning and sequencing of PIM PCR products

The PIM PCR products from 34 selected *T. parva* samples, including 27 buffalo and seven cattle samples (shown in bold in Table 6.1), were cloned into pCR®2.1-TOPO® cloning vector (Invitrogen, Carlsbad, USA); at least 24 clones were screened for each sample. The presence of inserts in the recombinants was confirmed by colony PCR following the secondary PCR

protocol used above. Amplicons produced from colony PCR were digested with *Bcl*I to produce RFLP profiles for individual clones. Only clones that produced amplicons which successfully digested with *Bcl*I were considered for further analysis. Consequently, clones from 20 *T. parva* samples from buffalo and seven from cattle were sequenced using the ABI Big Dye Terminator Cycle Sequencing kit version 3.1 (Applied Biosystems, Foster City, CA); 300 to 450 ng of plasmid DNA were used in the sequencing reactions. Sequencing was performed by INQABA Biotechnologies in South Africa, using a SpectruMedix model SCE 2410 automated sequencer (SpectruMedix, State College, PA).

6.3.6 Sequence analysis

The PIM gene sequences were assembled and edited using the GAP4 program of the Staden package (version 1.6.0 for Windows) (Bonfield *et al*., 1995; Staden, 1996; Staden *et al*., 2000). Sequences were aligned with previously published *T. parva* PIM sequences [Muguga (accession number: L06323), Marikebuni (accession number: L41148) and 7104 (accession number: L41833)] using MacClade v4.0 (Maddison and Maddison, 1992); the alignment was adjusted manually because of the highly polymorphic structure of the PIM gene. It was impossible to perform phylogenetic analysis for the PIM sequences because of the polymorphic nature of this gene.

6.4 Results

6.4.1 PIM PCR-RFLP profile analysis

The PIM gene PCR products obtained from *T. parva* positive samples analyzed in this study ranged in size from 0.7 to 1.2 kb (results not shown). Since PIM is a single copy gene (Toye *et al*., 1995b), multiple infections were indicated by multiple PCR products in cases where the amplicon sizes obtained from a single sample varied.

From visual inspection of the PIM PCR-RFLP profiles, profiles from all 47 samples from KNP were heterogeneous (Figure 6.1a). However, the profiles obtained from 23/38 (61%) of the Hluhluwe-iMfolozi samples from buffalo were relatively homogeneous (Figure 1b). Similarly 4/6 (67%) samples from Mabalingwe and all 10 from Ithala produced relatively homogeneous profiles (Figures 6.1c and 6.1d). Further analysis of this result by cluster analysis using BioNumerics was not possible as the PCR-RFLP profiles were too complex, as

a result of mixed infections. The profiles were characterized by multiple bands from multiple PCR products; the PCR products were present at different concentrations resulting in multiple bands of different intensities. It was difficult to distinguish between bands from incompletely digested amplicons and authentic bands. Therefore, 27 samples representative of *T. parva* samples from buffalo that produced homogeneous and heterogeneous profiles as well as seven cattle samples (shown in bold in Table 6.1) were selected for cloning in order to produce RFLP profiles from individual clones.

Mixed infections were evident from 31/34 (91.2%) samples, as more than one profile was obtained from different clones of each of these samples. Three samples from Ladysmith (Lad 02, Lad 06 and Lad 10) were exceptions, as all clones from these samples produced only one profile and this profile was identical to that of *T. parva* Muguga (a *T. parva* stock causing ECF in Kenya) and *T. parva* Schoonspruit (an isolate obtained from a bovine infected during the ECF epidemic in the former Transvaal, now Gauteng Province, in South Africa) (Neitz, 1948) (Figure 6.2). When RFLP profiles obtained from clones produced from samples which had homogeneous overall profiles were visually analysed, it was observed that there were dominant profiles that were responsible for the apparently homogenous overall profile between different samples. For example, among other profiles, three profiles were found to be dominant in clones produced from the 10 samples from Ithala; the three profiles were obtained in, respectively, 20/61 (33%), 14/61 (23%) and 11/61 (18%) clones produced from four different samples.

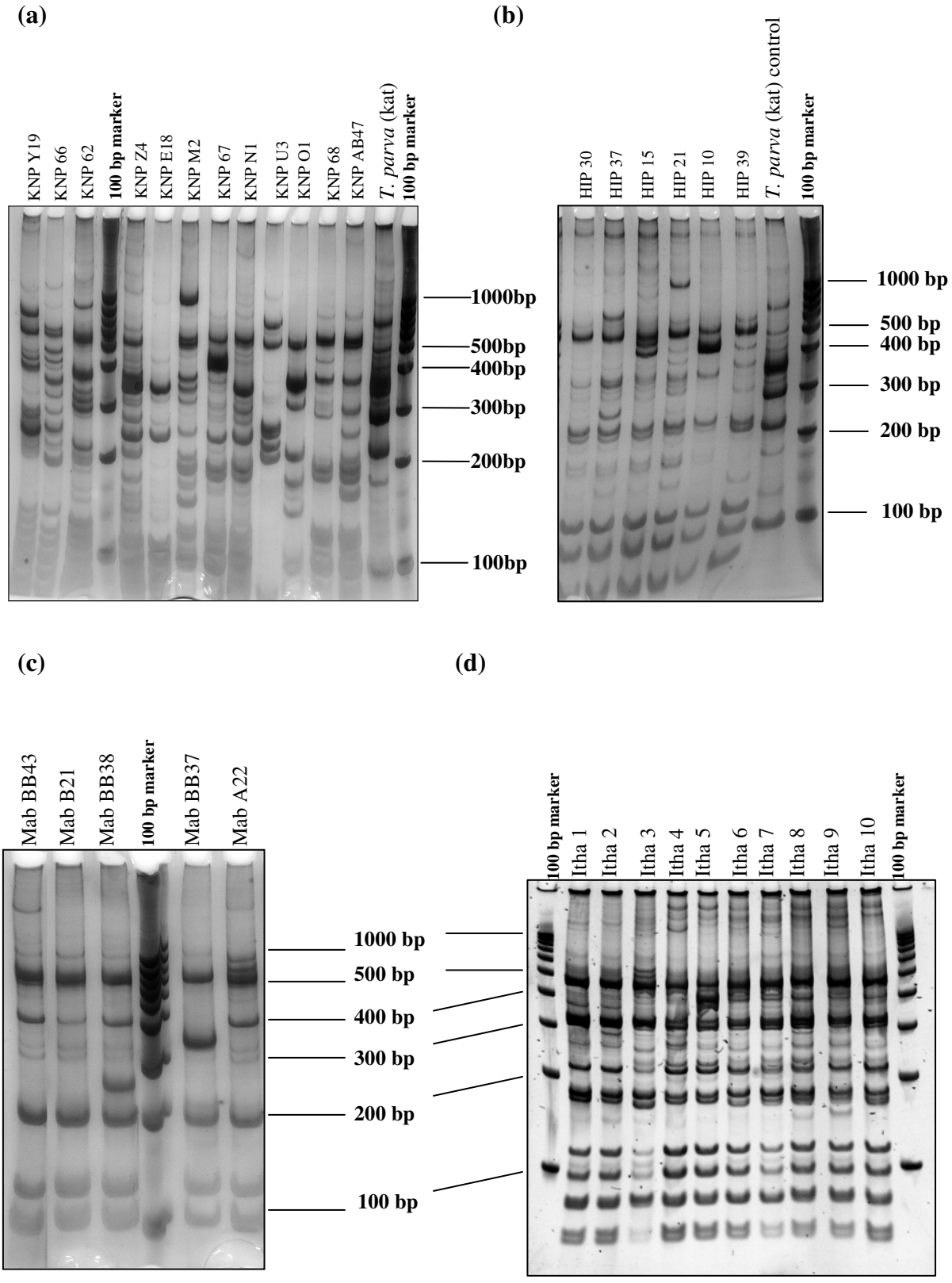


Figure 6.1 Representative PIM gene *Bcl*I PCR-RFLP profiles showing (a) heterogeneous profiles obtained from buffalo *T. parva* samples from KNP, (b), (c) and (d) homogeneous profiles obtained from buffalo samples from Hluhluwe-iMfolozi, Mabalingwe and Ithala, respectively.

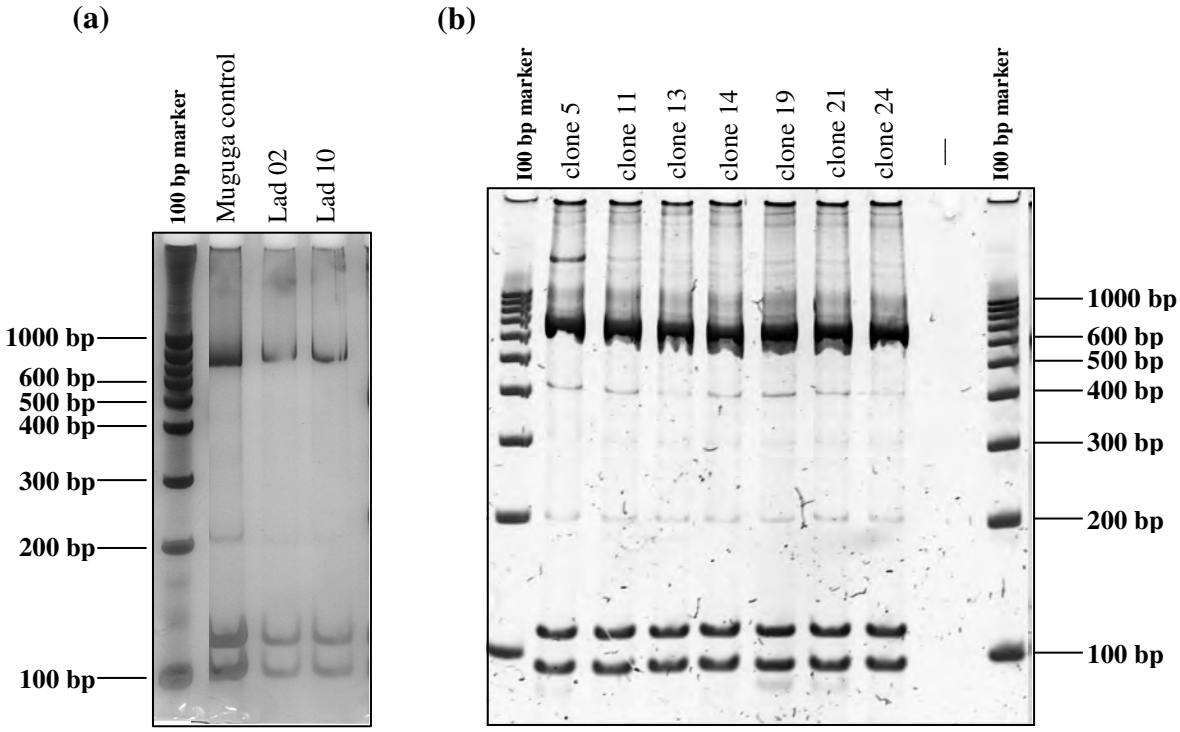


Figure 6.2 PIM gene *Bcl*I PCR-RFLP profiles obtained from (a) cattle *T. parva* samples from Ladysmith and (b) clones produced from cattle sample, Lad 10.

Cluster analysis of PCR-RFLP profiles using BioNumerics identified five cluster groups, A, B, C, D and E, from 261 clones produced from both buffalo and cattle *T. parva* samples (Figures 6.3 and 6.4). Cluster A was the largest group with 105/261 (40%) clones followed by cluster D with 73/261 (28%), then C (42/261, 16%), B (26/261, 10%) and E (15/261, 6%). No correlation with geographic distribution could be established from the major cluster groups. PIM profiles from clones obtained from KNP, Hluhluwe-iMfolozi, Mabalingwe and Ithala buffalo *T. parva* samples were distributed in all five cluster groups on the dendrogram. Profiles from clones produced from cattle samples Lad 02, Lad 06 and Lad 10 grouped with *T. parva* Muguga and *T. parva* Schoonspruit profiles in cluster A (Figures 6.3 and 6.4). Although most PIM profiles in cluster A were obtained from buffalo samples, 42/48 (88%) of the PIM profiles from clones obtained from cattle samples occurred in this group. PIM profiles from clones produced from the other cattle *T. parva* samples from Ladysmith and Bloemfontein grouped closely with profiles obtained from buffalo samples from Hluhluwe-iMfolozi and Ithala in different subgroups within cluster A (Figures 6.3 and 6.4).

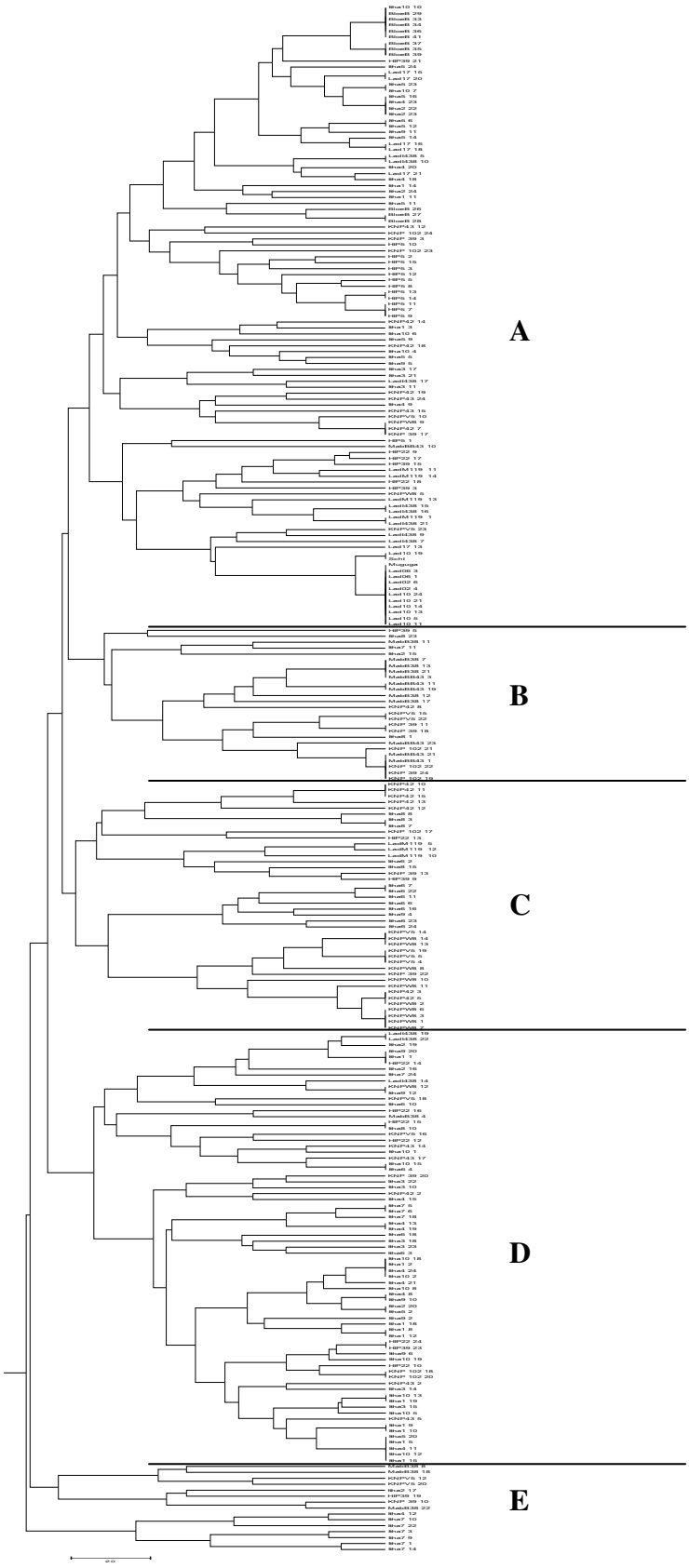
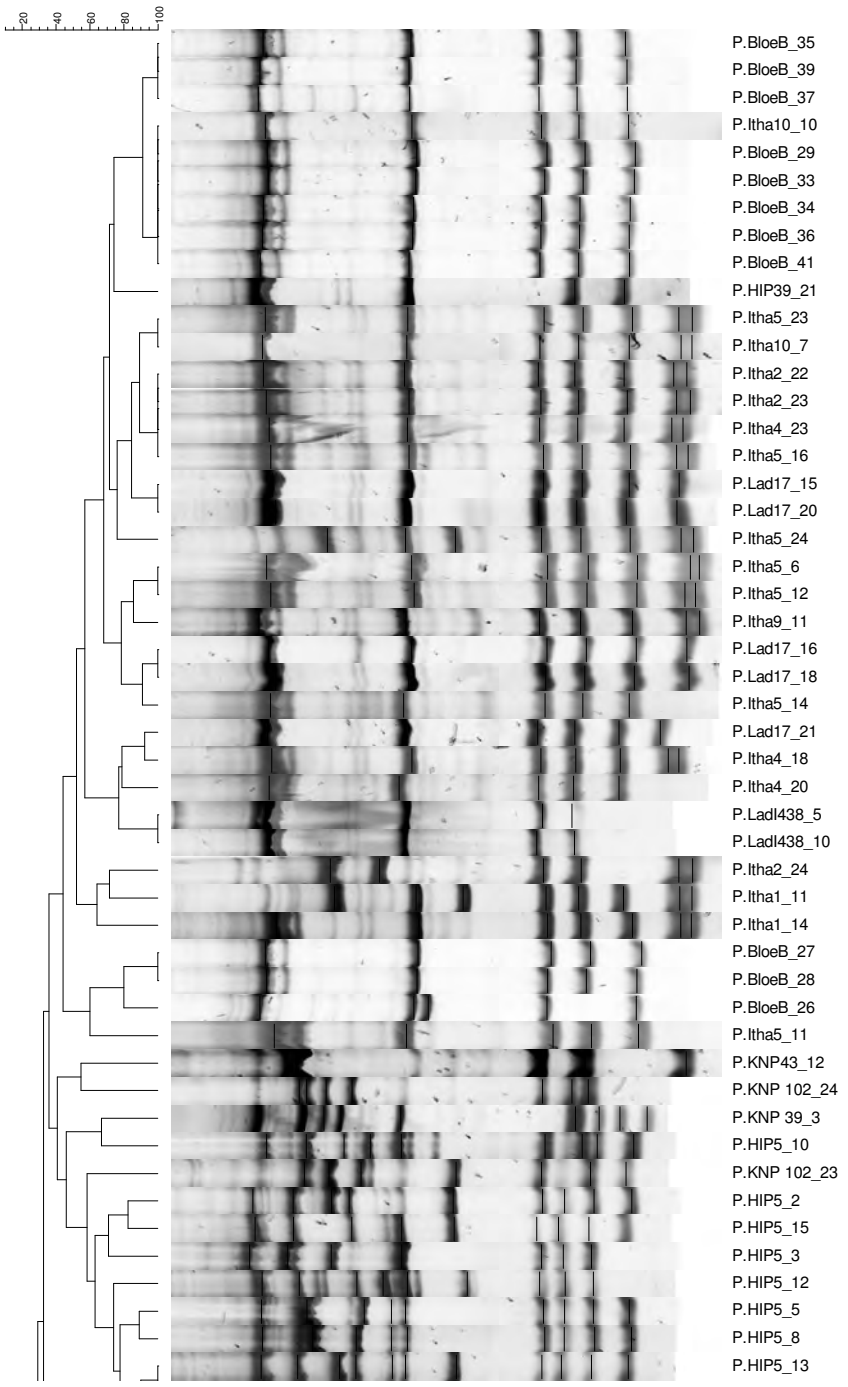
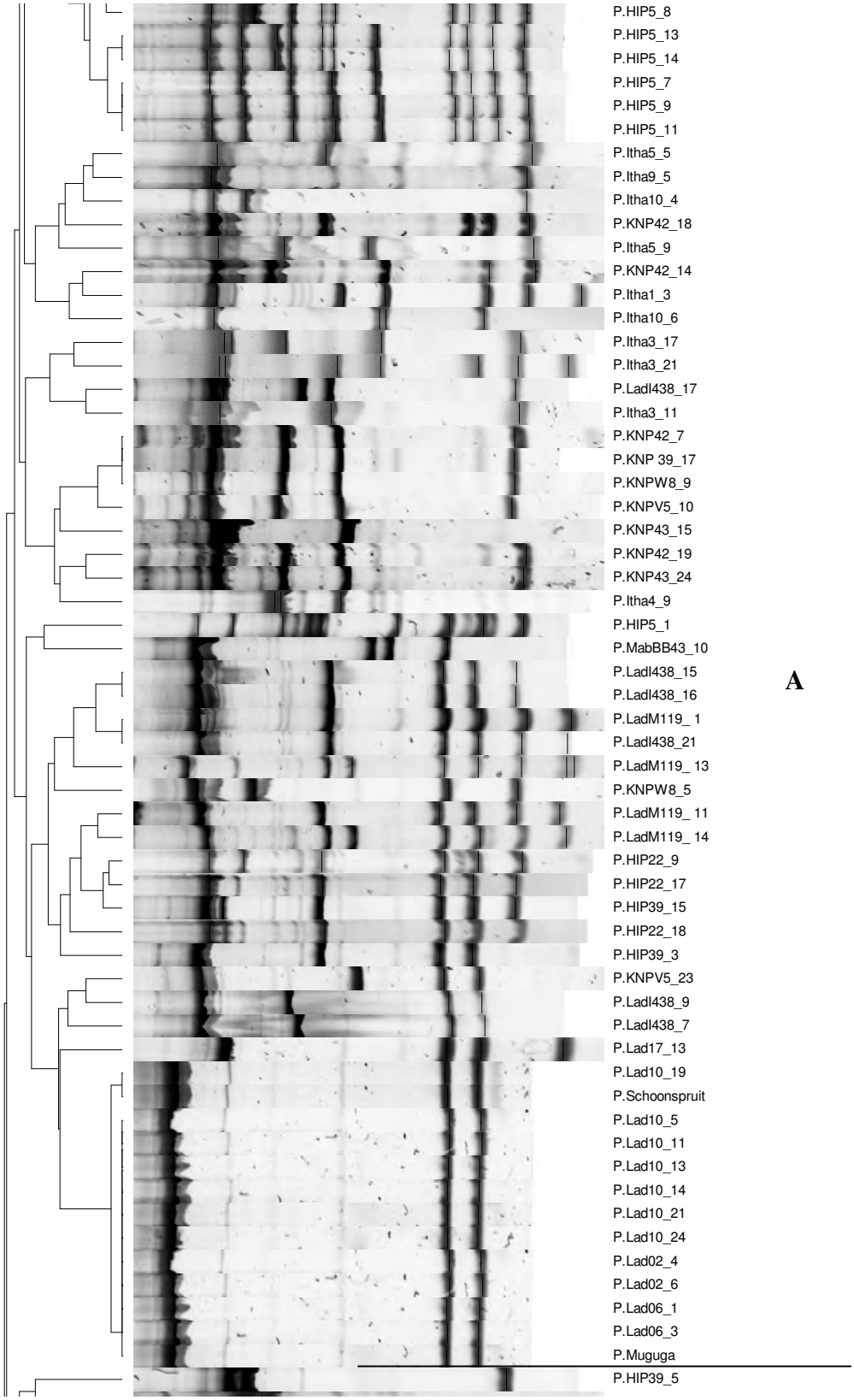


Figure 6.3 A simplified similarity dendrogram generated by BioNumerics v5.1 cluster analysis of PCR-RFLP profiles from cloned PIM amplicons using the Dice Coefficient analysis.



A

Figure 6.4 A detailed similarity dendrogram generated by BioNumerics v5.1 cluster analysis of PCR-RFLP profiles from cloned PIM amplicons using the Dice Coefficient analysis showing actual profiles used to produce the dendrogram. Figure 6.4 continues on pages 136 to 139.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P.HIP5_13
P.HIP5_14
P.HIP5_7
P.HIP5_9
P.HIP5_11
P.Itha5_5
P.Itha9_5
P.Itha10_4
P.KNP42_18
P.Itha5_9
P.KNP42_14
P.Itha1_3
P.Itha10_6
P.Itha3_17
P.Itha3_21
P.LadI438_17
P.Itha3_11
P.KNP42_7
P.KNP 39_17
P.KNPW8_9
P.KNPV5_10
P.KNP43_15
P.KNP42_19
P.KNP43_24
P.Itha4_9
P.HIP5_1
P.MabBB43_10
P.LadI438_15
P.LadI438_16
P.LadM119_ 1
P.LadI438_21
P.LadM119_ 13
P.KNPW8_5
P.LadM119_ 11
P.LadM119_ 14
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P.HIP22_17
P.HIP39_15
P.HIP22_18
P.HIP39_3
P.KNPV5_23
P.LadI438_9
P.LadI438_7
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P.Lad10_19
P.Schoonspruit
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P.Lad10_11
P.Lad10_13
P.Lad10_14
P.Lad10_21
P.Lad10_24
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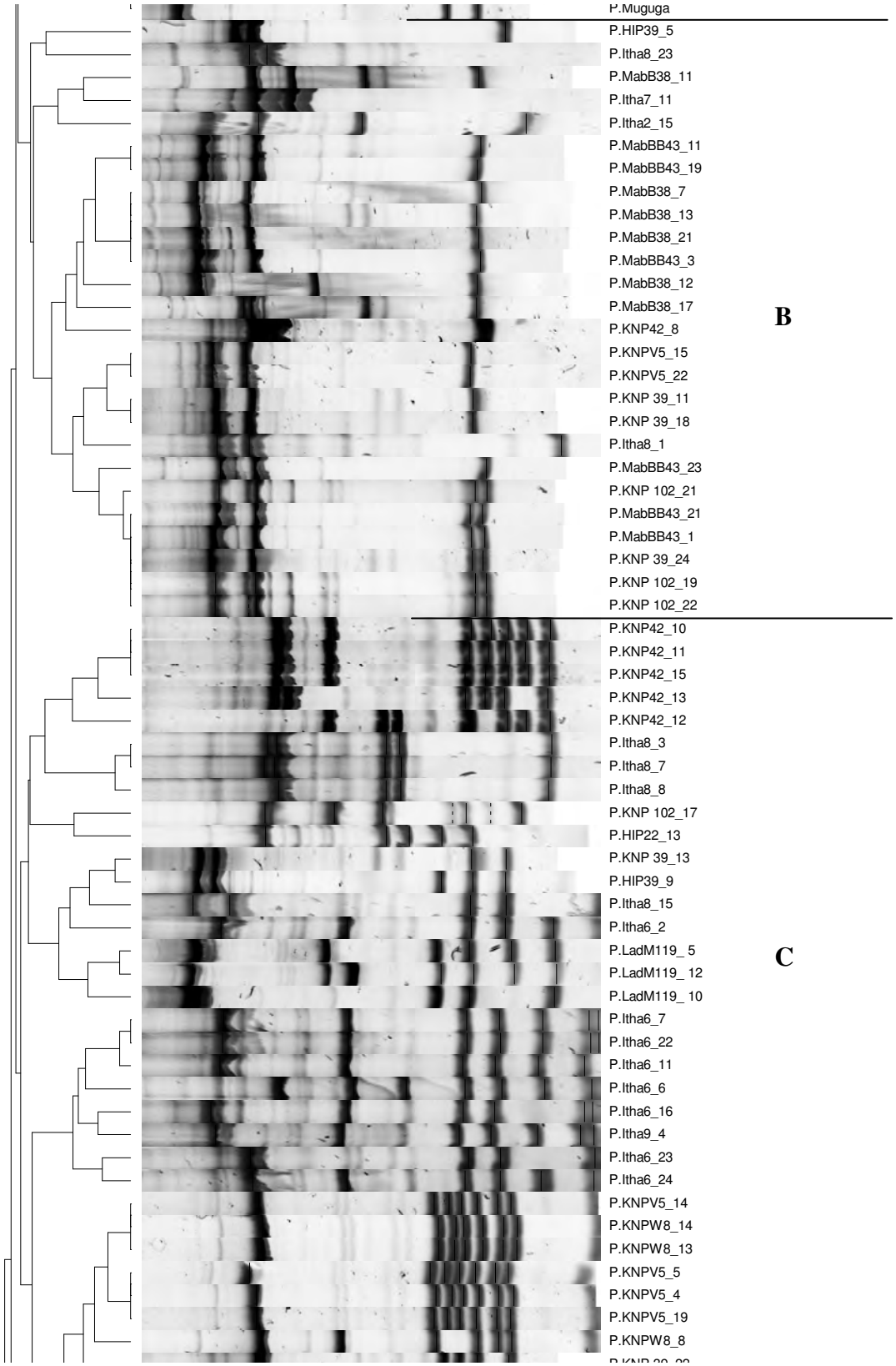
P.HIP39_5

A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P.MabB38_17
P.KNP42_8
P.KNPV5_15
P.KNPV5_22
P.KNP 39_11
P.KNP 39_18
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P.Itha8_3
P.Itha8_7
P.Itha8_8
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P.LadM119_ 12
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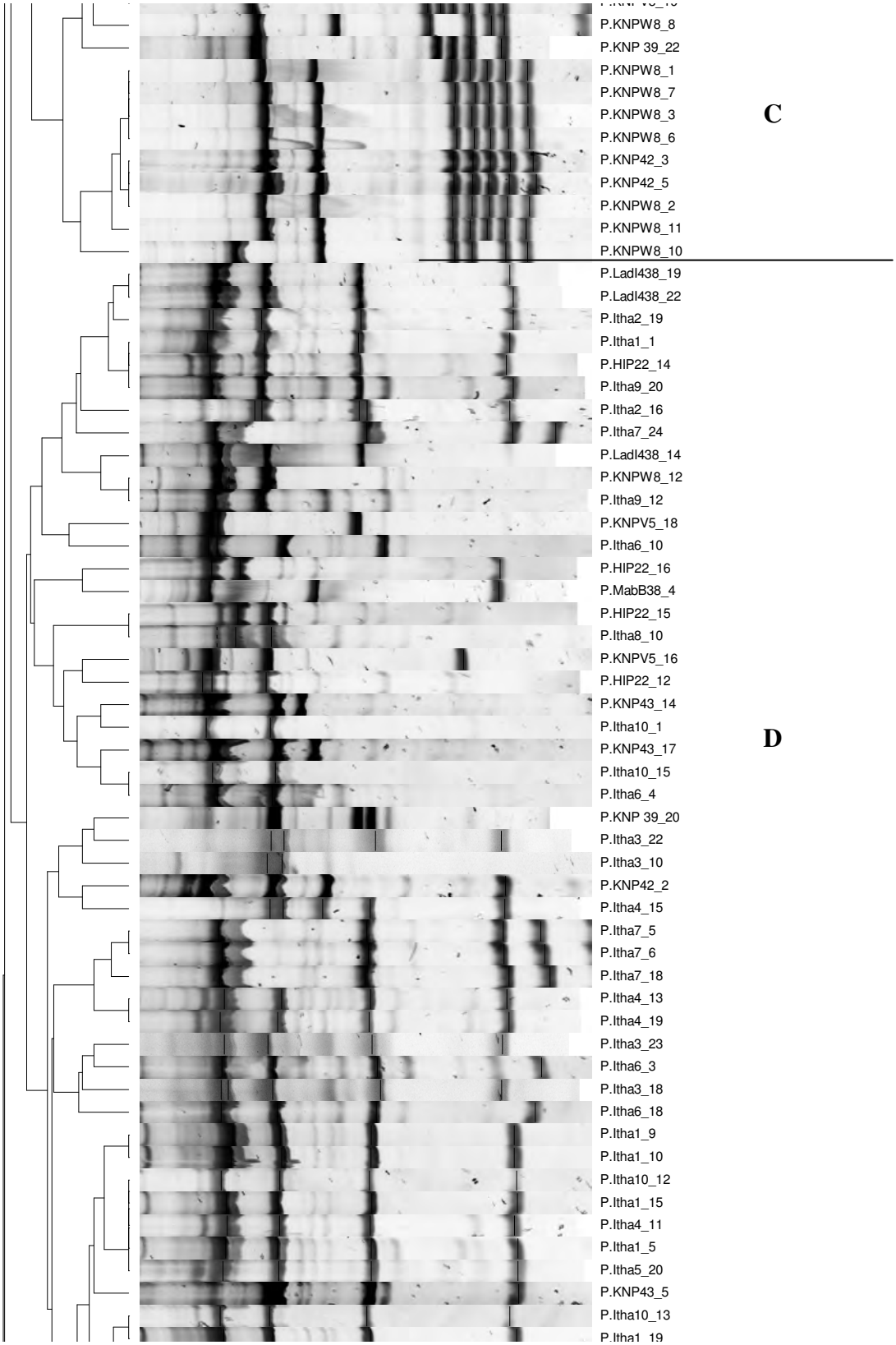
P.KNPW8_8
B
C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P.Ladl438_22
P.Itha2_19
P.Itha1_1
P.HIP22_14
P.Itha9_20
P.Itha2_16
P.Itha7_24
P.Ladl438_14
P.KNPW8_12
P.Itha9_12
P.KNPV5_18
P.Itha6_10
P.HIP22_16
P.MabB38_4
P.HIP22_15
P.Itha8_10
P.KNPV5_16
P.HIP22_12
P.KNP43_14
P.Itha10_1
P.KNP43_17
P.Itha10_15
P.Itha6_4
P.KNP 39_20
P.Itha3_22
P.Itha3_10
P.KNP42_2
P.Itha4_15
P.Itha7_5
P.Itha7_6
P.Itha7_18
P.Itha4_13
P.Itha4_19
P.Itha3_23
P.Itha6_3
P.Itha3_18
P.Itha6_18
P.Itha1_9
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P.Itha1_15
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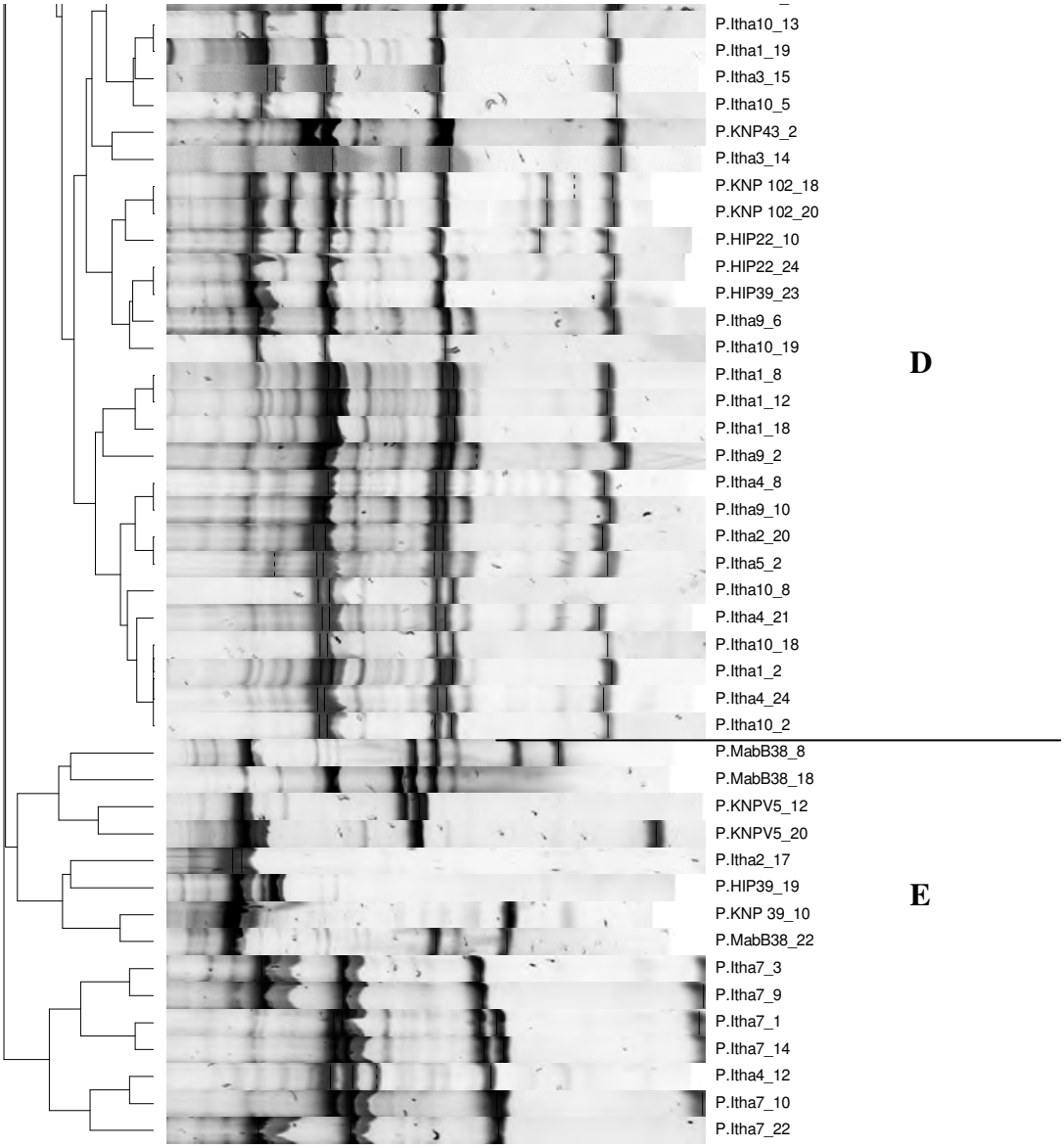
P.KNP43_5
P.Itha10_13
P.Itha1_19
C
D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P.MabB38_22
P.Itha7_3
P.Itha7_9
P.Itha7_1
P.Itha7_14
P.Itha4_12
P.Itha7_10
P.Itha7_22
D
E

Specific 'signatures' composed of several small fragments (less than 150 bp) were produced in the PIM PCR-RFLP profiles obtained from some *T. parva* field samples from KNP and Hluhluwe-iMfolozi. Two specific 'signatures' were associated with some RFLP profiles from clones from KNP samples. One of these was defined by five small DNA fragments of sizes ~50, 60, 80, 100 and 120 bp, and the other defined by six fragments of sizes ~50, 60, 80, 100, 120 and 130 bp (Figure 6.5a); both 'signatures' co-occurred with other bands of larger sizes. The Hluhluwe-iMfolozi 'signature' was characterized by four fragments of approximately 50, 80, 100 and 120 bp in size (Figure 6.5b). The KNP 'signatures' were observed in 26/52 (50%) clones from three samples, while the Hluhluwe-iMfolozi 'signature' was observed in almost all the clones (31/36, 86%) from the two Hluhluwe-iMfolozi *T. parva* samples that were analyzed. The Hluhluwe-iMfolozi 'signature' was also apparent in profiles from field samples obtained from buffalo from Ithala (Figure 6.1d), and from three bovines from Ladysmith (Lad 17, Lad I438 and Lad M119) and a bovine from Bloemfontein (results not shown). It was also found in profiles obtained from clones of PIM amplicons from these samples (results not shown).

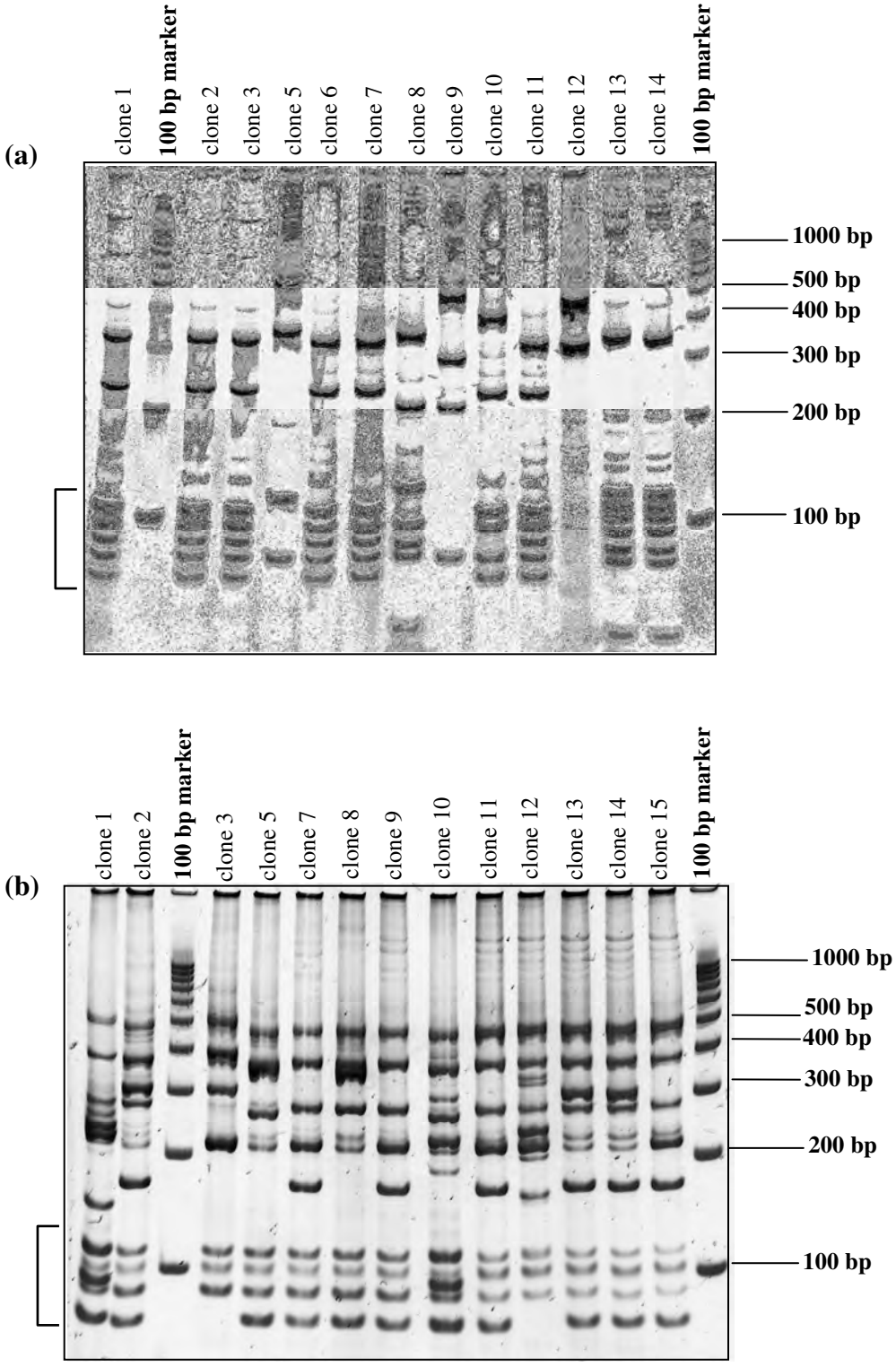


Figure 6.5 Characteristic 'signatures', indicated by brackets ([), were identified in *T. parva* PIM RFLP profiles obtained from cloned PCR products from samples collected from buffalo from Kruger National Park and Hluhluwe-iMfolozi Game Park. (a): PIM RFLP profiles with specific band 'signatures' obtained from clones from sample KNP W8 from Kruger National Park, characterized by five (clones 1, 2, 3, 6, 7 and 11) and six (clones 13 and 14) small DNA fragments of sizes ranging from ~ 50 to 130 bp. (b): PIM RFLP profiles obtained from clones from sample HIP 5 from Hluhluwe-iMfolozi with the 'four band signature' consisting of ~ 50, 80, 100 and 120 bp DNA fragments.

6.4.2 PIM gene sequence analysis

Clones from different cluster groups were selected for sequencing. A total of 97 PIM sequences were obtained from cloned amplicons produced from 27 selected *T. parva*-positive samples (Table 6.2). Analysis of the amino acid alignment of the PIM sequences revealed three groups of PIM sequences, cattle-type, buffalo-type and 'mixed'-type (Figure 6.6).

Cattle-type PIM sequences:

A tetrapeptide repeat, QPEP (position 428-447 shown in a solid-line block in Figure 6.6), in the variable region was previously identified by Toye *et al.* (1995b) as characteristic of cattle-derived *T. parva* PIM sequences. The amino acid sequences between positions 29 and 259 and positions 493 and 497 were also identified as exclusive to cattle-type PIM alleles in this study. In this way, seven PIM sequences obtained from samples investigated in this study were identified as cattle-type *T. parva* PIM sequences (Table 6.2). Six of these were obtained from two *T. parva* cattle samples from a farm in Ladysmith (Lad 02 and Lad 10) and one from *T. parva* Schoonspruit. These sequences had 98% identity to the *T. parva* Muguga PIM sequence, with one to three amino acid differences; the major difference being a deletion of eight amino acids at position 420-427 in the South African sequences (Figure 6.6). Cattle-type PIM sequences were not identified from samples obtained from buffalo in this study.

Table 6.2 Number of different types of PIM sequences obtained from clones produced from 27 selected *T. parva* samples

Origin of sample	Sample designation	Type and number of PIM sequence(s) obtained		
		Cattle-type (7 sequences obtained from 3 samples)	**Buffalo-type** (53 sequences obtained from 20 samples)'	**'Mixed' type** (37 sequences obtained from 12 samples)
Kruger National Park (n=4)	KNP V5	-	1	-
	KNP W8	-	-	4
	KNP 102	-	2	-
	KNP O11	-	2	-
Hluhluwe-iMfolozi Park (n=4)	HIP 5	-	3	-
	HIP 19	-	3	2
	HIP 22	-	1	4
	HIP 32	-	6	4
Ithala Game Reserve (n=9)	Itha 2	-	1	2
	Itha 3	-	5	-
	Itha 4	-	6	1
	Itha 5	-	3	-
	Itha 6	-	1	6
	Itha 7	-	2	-
	Itha 8	-	2	-
	Itha 9	-	4	-
	Itha 10	-	2	-
Mabalingwe Game Reserve (n=3)	Mab A13	-	5	-
	Mab BB38	-	2	-
	Mab BB43	-	-	1
Schoonspruit (n=1)	**Schoonspruit**	1	-	-
Ladysmith (n=5)	**Lad 2**	2	-	-
	Lad 10	4	-	-
	Lad 17	-	-	7
	Lad M119	-	-	1
	Lad I438	-	1	2
Bloemfontein (n=1)	**Bloe B**	-	1	3

All samples in bold were obtained from cattle

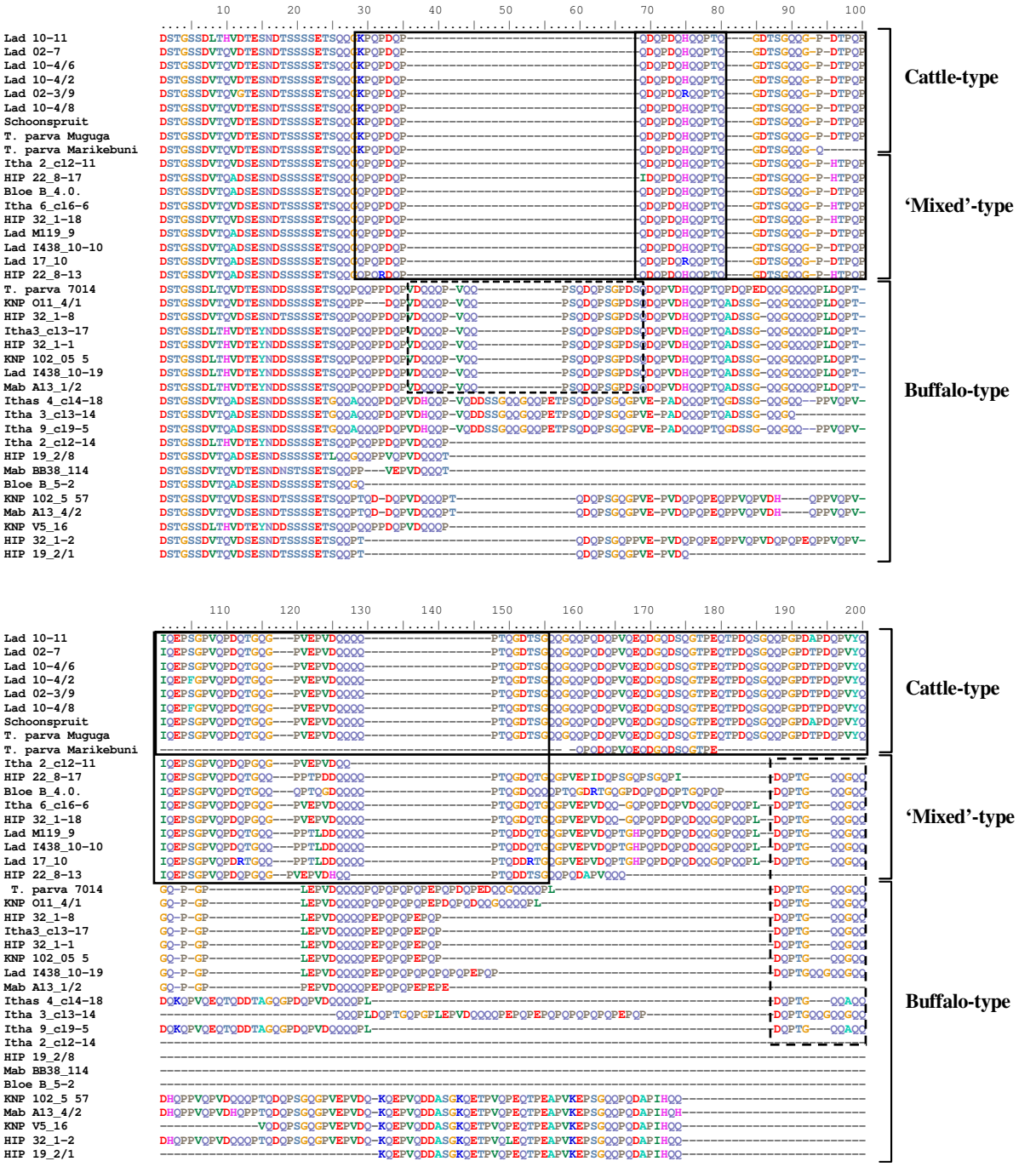
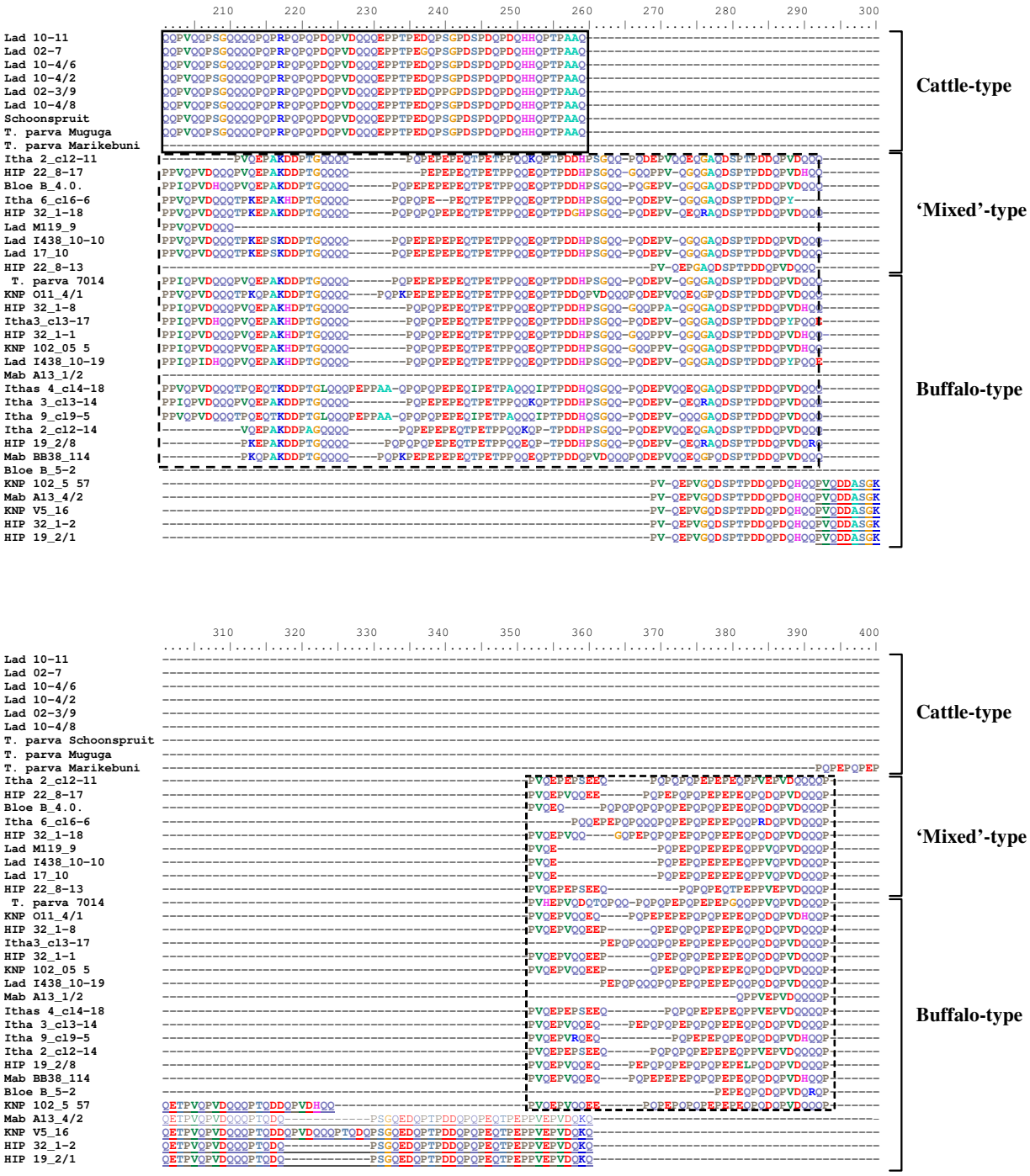
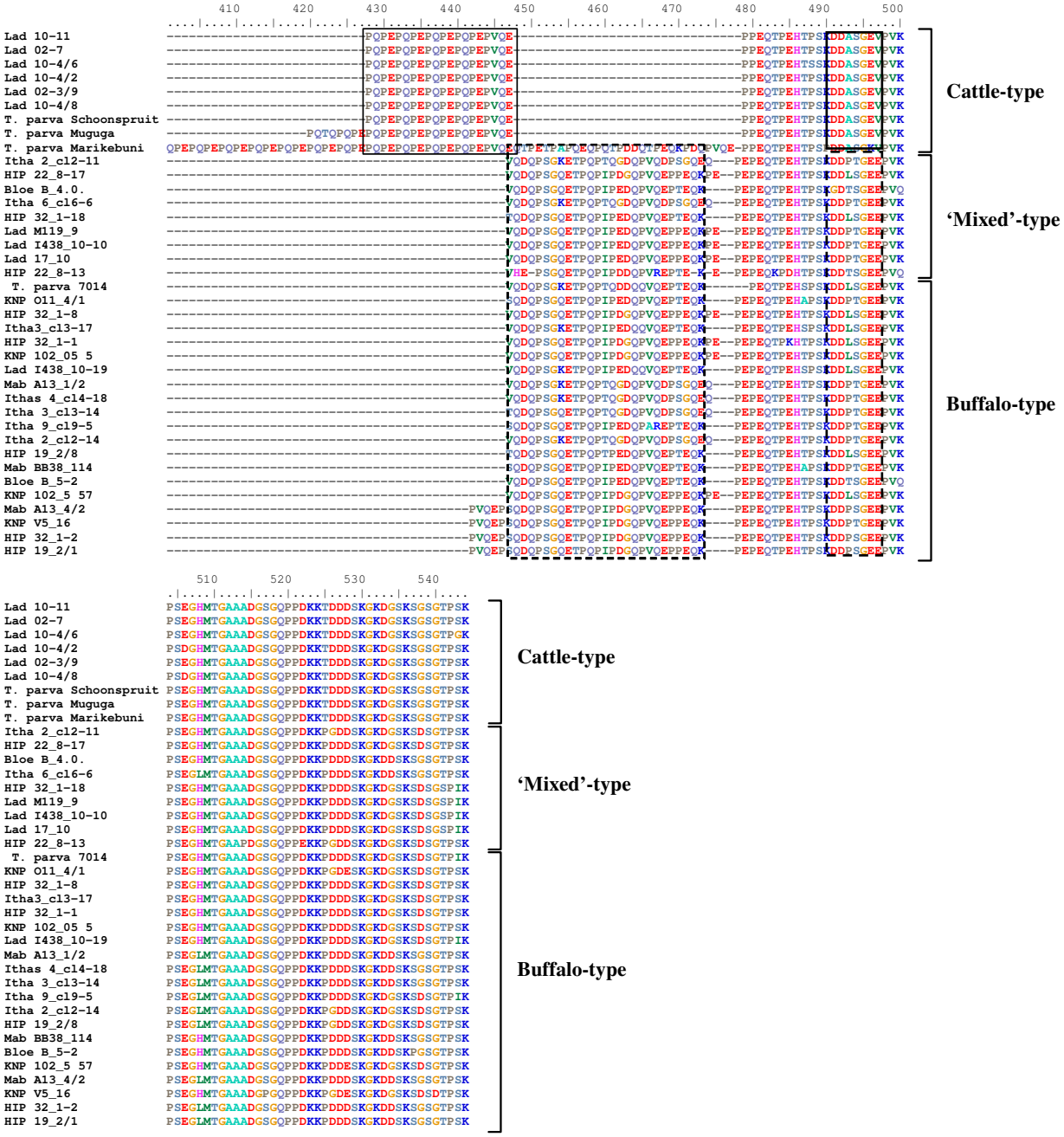


Figure 6.6 Multiple sequence alignment of the inferred PIM amino acid sequences obtained from buffalo and cattle *T. parva* samples collected from different geographical areas in South Africa (Table 6.1). PIM sequences were aligned with previously published *T. parva* PIM sequences, Muguga (accession number: L06323), Marikebuni (accession number: L41148) and 7104 (accession number: L41833). The sequence alignment was constructed manually because of the extreme polymorphism in the central region of the PIM gene. Amino acid motifs characteristic of cattle-type PIM sequence are shown in solid-line blocks including the tetrapeptide repeat characteristic of the central variable region of cattle-derived PIM sequences at positions 428-447. All amino acid motifs characteristic of buffalo-type PIM sequence are shown in broken-line blocks. Figure 6.6 continues on pages 145 and 146.


Cattle-type
'Mixed'-type
Buffalo-type
Cattle-type
'Mixed'-type
Buffalo-type


Cattle-type
'Mixed'-type
Buffalo-type
Cattle-type
'Mixed'-type
Buffalo-type

Buffalo-type PIM sequences:

Toye *et al*. (1995b) identified a 20-amino-acid insert (VDQQQPVQQPSQDQPSGPDS shown in broken-line block at position 36-68 in Figure 6.6) as characteristic to buffalo-type PIM amino acid sequences. In addition to this, two other buffalo-type amino acid motifs occurring at positions 352-393 and 447-473, were identified in this study (shown in broken-line blocks in Figure 6.6). However, some of the PIM sequences obtained from *T. parva* field samples collected from buffalo from KNP, Hluhluwe-iMfolozi, Mabalingwe and Ithala game parks lacked the 352-393 amino acid motif; instead, these sequences contained unique inserts of variable sizes between position 292-360 (underlined in Figure 6.6). The 20-amino-acid insert identified by Toye *et al*. (1995b) from PIM sequences from buffalo-derived *T. parva* 7014 and Hluhluwe stocks PIM sequences, was missing from 9/53 (17%) of the buffalo-type PIM sequences obtained in this study. Furthermore, a different insert between positions 36 and 68 was identified from buffalo-type sequences obtained from Ithala buffalo samples.

Using these motifs, 54.6% (53/97) of the PIM sequences obtained in this study were identified as buffalo-type PIM sequences and were obtained from *T. parva* samples collected from both cattle and buffalo (Table 6.2). One sequence obtained from KNP V5 had a large insert in the central region of the gene which was very different in sequence from that of other PIM sequences (results not shown). Very short PIM sequences, missing most of the variable central region, were obtained from some clones of PIM amplicons obtained from samples originating from buffalo (results not shown). However, the sequences flanking the central region from these alleles were characteristic of buffalo-type PIM sequences as defined in this study.

'Mixed'-type PIM sequences:

The use of the amino acid motifs identified in this study allowed identification of 37 'mixed' PIM sequences (Figure 6.6). It is possible that hybrid PCR products could arise during PCR amplification in samples containing mixed infections, as a result of template switching. These PCR artifacts are identifiable by sequence identities in hybrid sequences when compared with sequences of other amplicons produced in the same amplification reaction. To confirm whether the 'mixed' sequences obtained in this study were authentic, the PIM nucleic acid sequences in the more conserved regions, i.e. sequences flanking the central variable region, were compared to those of other sequences obtained from the same sample. None of the 'mixed' sequences were identical to any of the other PIM sequences obtained from the same

sample in these conserved regions, suggesting that these sequences were therefore genuine and could not have resulted from PCR artifacts.

Two subtypes of 'mixed' PIM sequences were identified, M-I (32/37) and M-II (5/37) (Figure 6.7). Subtype M-I consisted of sequences characteristic of cattle-type *T. parva* PIM sequences at the amino-terminus and buffalo-type sequences at the carboxy-terminus (Figure 6.7). Subtype M-II comprised sequences characteristic of buffalo-type *T. parva* PIM sequences at the amino-terminus and cattle-type sequences at the carboxy-terminus (Figure 6.7).

The 37 'mixed' PIM sequences were obtained from samples collected from both cattle and buffalo (Table 6.2). Of the 22 *T. parva* PIM sequences obtained from seven cattle samples in this study, 31.8% (7/22) were cattle-type, 59.1% (13/22) were 'mixed'-type, and only 9.1% (2/22) were buffalo-type (Table 2). In comparison, no cattle-type PIM sequences were identified from 20 buffalo samples, 32% (24/75) of the PIM sequences obtained from buffalo samples were mixed-type, and 68% (51/75) were buffalo-type.

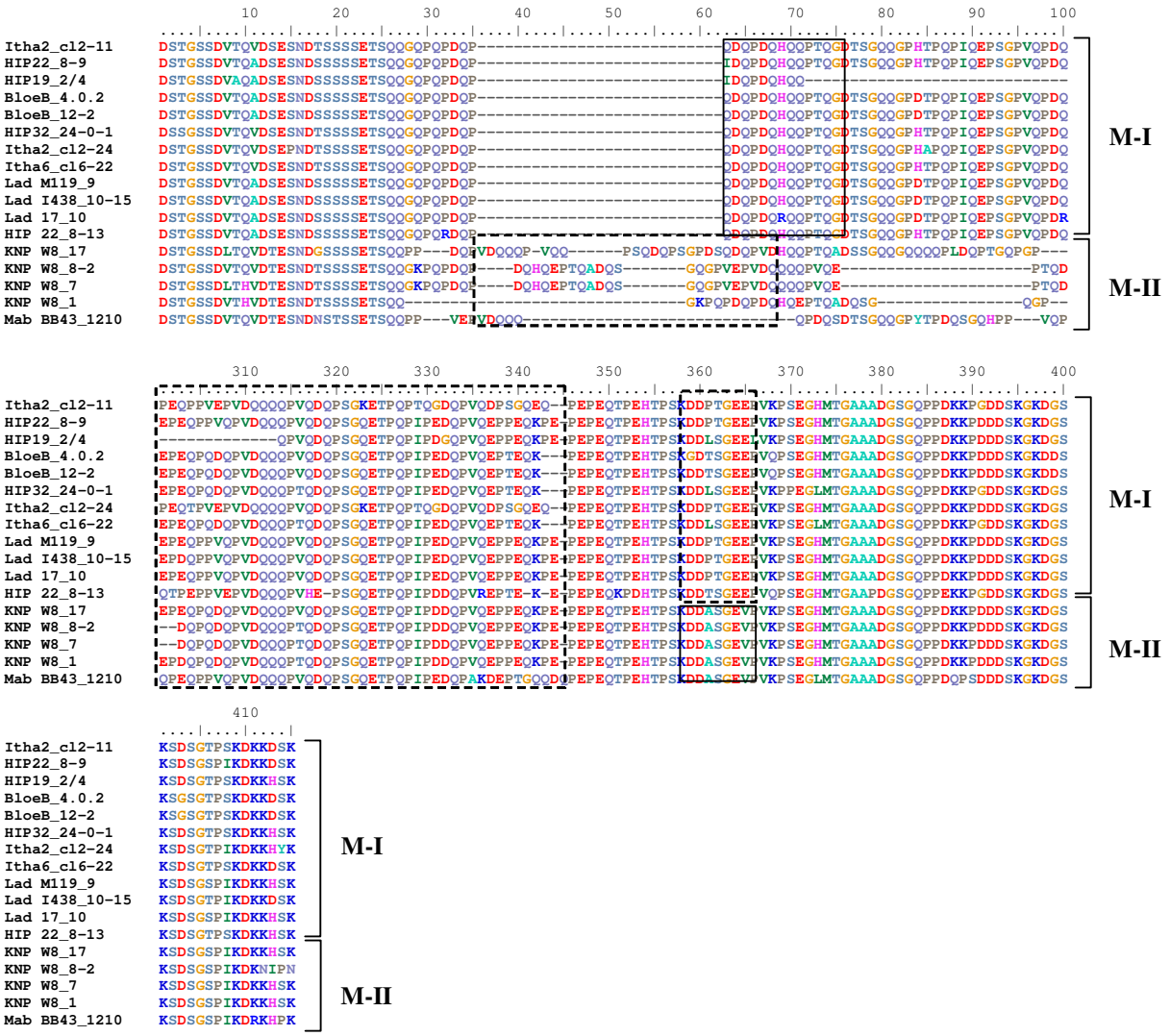


Figure 6.7 Multiple sequence alignment of representative amino acid sequences of 'mixed'-type *T. parva* PIM sequences, showing subtypes M-I and M-II. Regions typical of buffalo-type and cattle-type PIM sequences are indicated in broken-line and solid-line blocks, respectively. This alignment excludes the region between positions 101 and 300, which has buffalo-type PIM sequences in all subtypes.

6.5 Discussion

Previous studies on characterization of South African *T. parva* field samples revealed the presence of cattle-type p67 and p104 alleles (Chapter 4; Chapter 5; Sibeko *et al*., 2010), suggesting the presence of parasites with characteristics of cattle-derived *T. parva* in South Africa. Although ECF has not been reported in South Africa since its eradication in the early 1950s, these findings are a concern. In this chapter, a third gene was investigated to further characterize *T. parva* samples obtained from cattle and buffalo in South Africa to confirm results obtained by analysis of p67 and p104 genes.

Polymorphic immunodominant molecule PCR-RFLP profiles for the majority of *T. parva* field samples obtained from buffalo in this study were complex. Buffalo-derived *T. parva* isolates are expected to be heterogeneous and have previously been shown to display widely variable RFLP profiles because of multiplicity of infections in buffalo compared to single infections in cattle (Geysen, 2000; Geysen *et al*., 2004). Five PIM RFLP cluster groups were identified from BioNumerics cluster analysis of RFLP profiles obtained from cloned PIM amplicons but these showed no correlation to geographic origin of the samples within each group, as was shown for p104.

Surprisingly, relatively homogeneous PIM profiles were observed from samples originating from buffalo from Hluhluwe-iMfolozi, Mabalingwe and Ithala game parks. These findings could suggest that there is limited genetic diversity in *T. parva* parasites in these relatively small buffalo populations. However, RFLP profiles obtained from clones of PIM amplicons indicated that genetic diversity does exist in the Hluhluwe-iMfolozi, Mabalingwe and Ithala *T. parva* populations, as profiles from clones produced from these samples clustered in different groups. Although multiple PIM profiles were identified in cloned PIM amplicons from samples with homogeneous profiles, some profiles occurred more frequently than others, explaining the apparently homogeneous profiles obtained from these *T. parva* populations. In addition, in the Hluhluwe-iMfolozi samples the presence of a common ‘signature’ contributed to the apparent homogeneity of the profiles.

The extensively characterized *T. parva* PIM (Baylis *et al*., 1993; Toye *et al*., 1996) is encoded by a single copy gene and its structure consists of a central variable region, flanked by conserved 5’ and 3’ termini (Toye *et al*., 1995a; 1995b; Geysen *et al*., 2004). Previous studies have identified two groups of PIM sequences, and a number of characteristics could be used

to distinguish PIM sequences from cattle-derived *T. parva* isolates from those from buffalo-derived isolates (Toye *et al*., 1995b; Geysen *et al*., 2004). None of the PIM gene sequences obtained from *T. parva* field samples characterized in this study were identical, providing further evidence that the PIM gene evolves at an extremely high rate (Toye *et al*., 1995b). Extensive sequence heterogeneity among PIM sequences was demonstrated in the South African *T. parva* samples from buffalo examined in this study, confirming the extensive genetic diversity reported previously in *T. parva* parasites in buffalo (Chapter 4; Chapter 5; Collins and Allsopp, 1999; Sibeko *et al*., 2010). Furthermore, sequence variants of buffalo-type PIM sequences were identified which have never been reported before. The extensive polymorphism found in the coding region of the PIM gene is associated with selective pressure from the protective immune response and may confer selective advantage to the parasite (Toye *et al*., 1995a). Geysen *et al*. (2004) reported a high Ns/S (non-synonymous / synonymous substitutions) ratio in the PIM sequence which is an indication of the influence of selective forces on the sequence and the evasion of the host immune responses. Mechanisms responsible for the extensive diversity of the PIM gene and which influence its mosaic structure are not known, although X-like recombination motifs responsible for initiation of gene conversion events have been identified in PIM sequences (Geysen *et al*., 2004) and it is thought that this mechanism is likely to be responsible for the mosaic nature of the PIM gene. Novel alleles have been reported to arise from re-shuffling of important epitopes as a result of gene conversion and reciprocal intergenic exchanges (Dormoy *et al*., 1997). The discriminative characteristics of the PIM gene render it a good candidate for exploitation in discriminatory assays used for *T. parva* isolates (Bishop *et al*., 2001; De Deken *et al*., 2007). Nevertheless, it is advisable that assays based on this gene should be continuously evaluated because of the rapid evolution of the PIM gene.

In this study, for the first time, 'mixed' PIM sequences with characteristics of both PIM sequence types were identified. The combinations of different "blocks" of sequence observed in the PIM gene are reminiscent of the structure that has previously been shown for the precursor to the major merozoite surface antigens (PMMSA) in *Plasmodium falciparum* (Peterson *et al*., 1988). Recombination within the conserved blocks in the PMMSA is thought to result in reassembling of the variable blocks and accounts for much of the antigenic variation in this molecule. Studies of the population structure of *T. parva* parasites in Uganda showed that genetic exchange occurs frequently between isolates of *T. parva*, confirming the existence of a sexual cycle (Oura *et al*., 2005). Evidence for recombination between *T. parva* parasites, in a form of mosaic segments, has previously been observed in the internal

transcribed spacer (ITS) region (Collins and Allsopp, 1999) and sexual recombination between different *T. parva* stocks has been demonstrated in the laboratory (Morzaria *et al*., 1993; Bishop *et al*., 2002). It seems likely that the 'mixed' PIM sequences have arisen through recombination between cattle-type and buffalo-type PIM sequences.

While the 'mixed' PIM sequences identified in this study may well represent recombination events, we do not know the full extent of the recombination in the rest of the genome in these parasites. Recombination between buffalo-type *T. parva* parasites and cattle-type parasites can only occur where there has been contact between buffalo and cattle, and it has been reported that it is unlikely for recombinant parasites to become established in the cattle population (Geysen, 2000; Geysen *et al*., 2004). Nonetheless, 'mixed' PIM sequences were obtained from samples originating from both buffalo and cattle in this study, and the results indicate that 'mixed' PIM sequences are more prevalent in *T. parva* samples from cattle than in *T. parva* samples from buffalo. This could suggest that *T. parva* parasites with the 'mixed' PIM allele might be more likely to establish in cattle. In fact, very few buffalo-type PIM sequences were identified in cattle, suggesting that there may have been selection for both 'mixed'-type and cattle-type PIM sequences in cattle

Interestingly, no cattle-type PIM sequences were obtained from *T. parva* samples collected from buffalo. Buffalo are believed to carry a heterogeneous population of parasites and, as original hosts of the parasite, cattle-derived *T. parva* parasites are thought to have originated in buffalo, so we might have expected to see cattle-type PIM sequences in *T. parva* samples from buffalo. However, given the rapid nature of the evolution of the PIM gene, it is probable that cattle-type alleles are present at a low frequency in *T. parva* parasites in buffalo. It would therefore be necessary to clone the PIM genes from a larger number of buffalo samples in order to identify cattle-type PIM sequences. Findings obtained in a study on characterization of another *T. parva* antigenic gene, p67, revealed the presence of variants of the p67 allele similar to those of cattle-derived parasites in *T. parva* samples collected from buffalo in South Africa (Collins, 1997; Chapter 4; Sibeko *et al*., 2010), although no p67 sequence identical to the cattle-type allele was identified in buffalo samples. The PIM sequences obtained from samples with variants of cattle-type p67 were either buffalo-type (6/25, 24%) or 'mixed'-type (19/25, 76%) showing that other genes in parasites with variants of the cattle-type p67 allele are not necessarily cattle-type alleles. There is therefore a need to establish the significance of these alleles in the epidemiology of theileriosis and the risk they pose to the naïve cattle population in South Africa.

PCR-RFLP profiles similar to that of the *T. parva* Muguga stock were obtained from three of the six cattle samples from the Ladysmith farm and the inferred amino acid sequences of the PIM gene from two of these samples (Lad 02 and Lad 10) were almost identical to the *T. parva* Muguga PIM sequence. This finding supports recent studies in which p67 and p104 alleles similar to those of the *T. parva* Muguga stock were identified from the same Ladysmith samples (Chapter 4; Chapter 5; Sibeko *et al*., 2010). While it is not known if the Muguga-like RFLP profiles or sequences can be associated with the pathogenicity of *T. parva* isolates, findings in these studies strengthen the evidence for the presence of a subpopulation of *T. parva* parasites similar to ECF-causing East African strains in South Africa, at least on one farm. Our results might provide evidence for the selection of a subpoplution of *T. parva* parasites through cattle-to-cattle transmission of *T. parva* on the Ladysmith farm (resulting in parasites with cattle-type p67, p104 and PIM alleles). However, similarity between sequences is not necessarily an indicator of the association of the cattle-type PIM sequence with the disease syndrome, as ECF was not diagnosed on the Ladysmith farm.

Polymorphic immunodominant molecule PCR-RFLP profiles obtained from the remaining three samples from cattle from the Ladysmith farm occurred in three cluster groups, A, C and D. All three had the specific 'signature' which was observed from most of the *T. parva* samples collected from buffalo from Hluhluwe-iMfolozi Park, suggesting that some of the *T. parva* parasites on this farm may have originated from the Hluhluwe-iMfolozi buffalo. Furthermore, buffalo-type p67, p104 and PIM sequences were obtained from these three Ladysmith samples, further supporting the possibility of buffalo-to-cattle transmission on this farm. Unfortunately it was not possible to confirm whether the cattle on this farm had contact with infected buffalo (Thompson *et al*., 2008), but circumstantial evidence and our results indicate that this might have occurred.

The PIM profile obtained from a bovine sample from a farm in Bloemfontein had the 'signature' characteristic of most Hluhluwe-iMfolozi profiles also observed in some of the Ladysmith samples. The RFLP profiles from this sample occurred in cluster A where 28% and 18% of Ladysmith and Hluhluwe-iMfolozi profiles, respectively, also grouped. Moreover, buffalo-type and 'mixed'-type PIM sequences were obtained from the Bloemfontein sample. These results suggest that the *T. parva* parasite characterized from the Bloemfontein bovine sample was similar to *T. parva* parasites from Hluhluwe-iMfolozi, and in fact, the infected buffalo breeding herd on the Bloemfontein farm originated from Hluhluwe-iMfolozi. Therefore, it might be possible to use PIM RFLP profiles to track the

origin of *T. parva* infections, especially when the profiles have a specific signature that characterizes a particular parasite population. This case presents something of a puzzle: it is not known how the bovine on the Bloemfontein farm was infected as the tick vector, *R. appendiculatus*, has not been known to occur in the Bloemfontein area. It is possible that the vector tick was introduced onto the property along with the infected buffalo but did not become established, as intensive tick surveys failed to identify the presence of vector ticks (FT Potgieter, unpublished results).

6.6 Summary

In summary, the findings in this study confirm the presence of a cattle-type PIM allele in the same cattle *T. parva* samples from which p67 and p104 alleles similar to that of *T. parva* Muguga were obtained. Results presented here suggest that there may have been both cattle-to-cattle and buffalo-to-cattle transmission of *T. parva* on the Ladysmith farm. No cattle-type PIM alleles were identified from samples obtained from buffalo in this study. However, if the cattle-type alleles are present at a low frequency in *T. parva* parasites in buffalo, it is possible that insufficient samples were examined in this study and a larger number of samples would have to be investigated before any conclusions could be made. The extensive genetic diversity of *T. parva* parasite populations in South Africa was demonstrated in the identification of novel and 'mixed' PIM sequences. The significance of *T. parva* parasites carrying 'mixed' PIM alleles will have to be established and their risk to cattle evaluated. With the extent of genetic diversity that has been demonstrated by the three characterization studies presented in this thesis, it is clear that the population genetic structure of *T. parva* parasites in South Africa needs to be studied in detail, possibly by using other molecular tools such as mini- and micro-satellite markers (Oura *et al.*, 2003; 2005), to fully establish the parasite populations circulating in the country, and their threat and significance in the epidemiology of theileriosis in South Africa.

6.7 References

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CHAPTER 7

General Discussion and Conclusion

For as the heavens are higher than the earth, so are my ways higher than your ways, and my thoughts than your thoughts. ***Isaiah 55:9***

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"As long as you are going to be thinking anyway, think big." ***Donald Trump***
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7.1 Improvement of molecular diagnosis of *T. parva* infections

Real-time PCR technology has revolutionized molecular diagnostics allowing analysis of total PCR product accumulated in a reaction. This, combined with the use of minute amounts of input DNA, confers superior sensitivity when compared to conventional PCR techniques. In addition, real-time PCR ensures accurate detection of specific targeted nucleic acid sequences by allowing differentiation of PCR products of the same size without the need for post-amplification analysis, which usually risks contamination and is time consuming. These qualities of real-time PCR technology are essential for diagnosis of *T. parva* infections, especially when *T. parva* DNA has to be detected from carrier animals. Hence real-time PCR was exploited in this study to improve the molecular diagnosis of *T. parva* in South Africa. While other molecular tests evaluated in this study, namely RLB, PCR/probe and *cox*III PCR-RFLP, required 2.5 (~37.5 ng) to 5.0 (~75 ng) µl of input DNA, the real-time PCR assay showed superior sensitivity and could detect target DNA using only 1.0 µl (~15 ng) of input DNA. Increasing the input DNA to 2.5 µl improved the sensitivity by 20% making the real-time PCR assay the most sensitive molecular assay developed for *T. parva* DNA detection to date, detecting a piroplasm parasitaemia as low as 8.79×10^{-4} % with 100% certainty.

Using melting curve analysis, real-time PCR technology can be used to differentiate between specific and non-specific PCR products, because of differences in the PCR products' melting temperature (T_m). In this way, it is even possible to distinguish between amplicons with a single nucleotide difference. Achieving this form of discrimination is impossible with other molecular assays, especially when there is high sequence similarity between the target product and the non-specific PCR product and even more so when the non-specific PCR product is the same size as the target product. This was the challenge with the real-time PCR assay developed in this study, which amplified *Theileria* sp. (buffalo) DNA in addition to the targeted *T. parva* DNA. Amplification of *Theileria* sp. (buffalo) DNA could not be avoided as the 18S rRNA gene sequence of this species is very similar to that of *T. parva.* However, the assay successfully discriminated between the PCR products of the two *Theileria* species because they have different T_ms at the region where the probe is designed. Only *T. parva* amplicons were detected by the *T. parva*-specific probe subsequent to melting curve analysis; the real-time PCR assay was therefore specific for detection of *T. parva* infections. To avoid the amplification of *Theileria* sp. (buffalo) DNA the use of a different target gene with significant differences between *Theileria* sp. (buffalo) and *T. parva* will have to be explored

as differences in the 18S rDNA variable region are not sufficient to improve the assay based on this gene.

Contamination remains a major problem in diagnostics; most molecular methods require post amplification handling of PCR products for confirmation of a positive result which increases the risk of contamination that can result in the reporting of false positive results. This is a serious challenge because it is almost impossible to differentiate between a true positive and a false positive result. In South Africa, one of the most important applications of tests for detection of *T. parva* infections is in the regulation of "disease-free" buffalo breeding projects, and false positive results can have an enormous financial implication. "Disease-free" buffalo have become a sought-after commodity, but, once reported positive for *T. parva* infection, these animals lose their value and the game farmer's investment in the animals is threatened. Thus, inaccurate results are to be avoided wherever possible. The contamination-controlled system offered by the real-time PCR assay greatly reduces incidents of contamination while allowing rapid processing of samples and increases the number of samples that can be analyzed in a day by three fold or more; these are much desired qualities of a diagnostic assay, especially when large numbers of samples are to be processed as is more often the case with the "disease-free" buffalo breeding projects in South Africa.

The reproducibility of the real-time PCR assay and the consistency in interpretation of test results by different personnel has resulted in the successful transfer of the technology to the ARC-OVI laboratory, a national laboratory with a mandate from the South African Department of Agriculture, Forestry and Fisheries (DAFF) to perform tests for *T. parva* infections in cattle and buffalo blood samples.

7.2 Molecular characterization of South African *T. parva* parasites

7.2.1 Evidence of cattle-type p67, p104 and PIM alleles in *T. parva* parasite populations in South Africa

The cattle-derived *T. parva* stocks, Muguga, Marikebuni and Boleni, characterized by Nene *et al*. (1996), all had the same p67 allele with a 129 bp deletion, while there was no deletion in the central region of the gene in the buffalo-derived stock 7014. The PIM gene from these same stocks was relatively homogenous for the cattle-derived *T. parva* stocks and heterogeneous for buffalo-derived stocks (Toye *et al*., 1995). p104 sequences were assigned as alleles 1, 2, 3 and 4 for the sequence obtained from *T. parva* Muguga, *T. parva* Marikebuni, *T. parva* Boleni and *T. parva* 7014, respectively (Skilton *et al*., 2002). It is believed that *T. parva* parasites are originally buffalo parasites and that there is a heterogeneous population of parasites circulating among buffalo, only some of which can be transmitted within the cattle population (Toye *et al*., 1995; Nene *et al*., 1996). In an attempt to identify cattle-type p67, p104 and PIM alleles in South African *T. parva* parasite populations, both samples from cattle and buffalo were investigated in this study.

7.2.1.1 Identification of cattle-type alleles from cattle T. parva *samples*

In this study, cattle-type p67, p104 and PIM alleles were identified from three *T. parva* samples obtained from cattle from a farm near Ladysmith in the KwaZulu-Natal Province. These cattle-type alleles were almost identical to those previously identified in a cattle-derived *T. parva* stock, *T. parva* Muguga, which causes ECF in Kenya, East Africa. It was an unexpected finding to identify Muguga-like cattle-type alleles from these animals, since ECF was not diagnosed in animals on this farm, and there have not been reports of ECF in South Africa since its eradication in the early 1950s. Between 2002 and 2004, there were seasonal outbreaks of what was initially suspected to be babesiosis on the Ladysmith farm, as the farm is located outside the designated *T. parva*-infected area (Thompson *et al*., 2008). Thompson *et al*. (2008) reported the presence of *T. parva* infections on this farm and attributed the source of infection to possible contact with infected buffalo or *T. parva*-infected ticks, but such contact could not be confirmed. The epidemiology of the disease on this farm was complicated by the fact that during the 2003 and 2004 outbreaks, the entire herd was treated with tetracyclines, and regularly and intensively dipped against ticks. Apparently healthy,

T. parva-positive animals were identified both in the study by Thompson *et al.* (2008) and in the present study. It is a known fact that cattle that survive *T. parva* infections can become carriers of the parasite, and furthermore, carriers may arise from being immunised and after treatment with anti-theilerial drugs (Dolan, 1986; Irvin *et al.*, 1989; Bishop *et al.*, 1992). Our results might provide evidence for the selection of a subpoplution of *T. parva* parasites through cattle-to-cattle transmission of *T. parva* on the Ladysmith farm, resulting in parasites with cattle-type p67, p104 and PIM alleles.

p67, p104 and PIM alleles obtained from *T. parva* samples from several other animals from this farm were either buffalo-type or 'novel' (or mixed-type in the case of PIM) suggesting that the parasite population circulating on this farm may have originated from buffalo. Although contact between these animals and *T. parva*-infected buffalo could not be established, findings in this study suggest that this event may have occurred. The Hluhluwe-iMfolozi 'signature' obtained in PIM PCR-RFLP profiles from cattle samples from the Ladysmith farm, similar to that obtained from buffalo samples from Hluhluwe-iMfolozi game park, provides support for the hypothesis that there may have been contact between cattle on this farm and *T. parva*-infected buffalo.

While our results suggest that there may have been transmission of *T. parva* from buffalo to cattle on the Ladysmith farm, possibly followed by cattle-to-cattle transmission, the data obtained in this study cannot be used to establish the route of infection, i.e. if the parasite was transmitted from buffalo-to-cattle, cattle-to-cattle or cattle-to-buffalo.

7.2.1.2 Identification of cattle-type alleles from buffalo T. parva *samples*

No alleles identical to *T. parva* cattle-type alleles were obtained from buffalo samples analyzed in this study. It is possible that the pool of buffalo that were investigated was not large enough to allow detection of all possible *T. parva* alleles that occur in buffalo. Therefore, a larger pool of samples will have to be characterized in order to determine if there are cattle-type alleles in buffalo. Although cattle-type alleles identical to those previously reported (Toye *et al.,* 1995; Nene *et al.*, 1996; Skilton *et al.*, 2002) were not identified from buffalo samples that were characterized in this study, variants of p67 allele 1 as well as variants of p104 allele 1 (both cattle-type alleles associated with cattle-derived *T. parva* stocks), initially identified from the cattle-derived *T. parva* Muguga stock (a cattle stock that can cause fatal disease) were obtained from *T. parva* samples collected from buffalo. This finding suggests that parasites possessing cattle-type p67 and p104 alleles may not be

exclusively associated with cattle-derived *T. parva* parasites. While these results could suggest that there may have been transmission of cattle-derived *T. parva* parasites to buffalo, they could also be indicative of an ancestral buffalo-derived *T. parva* subpopulation with characteristics that might aid the parasite to establish in cattle. The importance of these parasites in the epidemiology of theileriosis in South Africa needs to established. Furthermore, it will be important to determine whether parasites that possess these variants can cause disease in cattle; this could be achieved by means of tick transmission experiments.

It is still not clear if the parasite that caused ECF in South Africa in the early part of the 20th century was transmitted to buffalo during the ECF epidemic, or whether South African strains of *T. parva* could eventually become adapted to cattle and cause ECF. To date, there are no reports where the source of infection was determined in cases where both cattle- and buffalo-derived *T. parva* parasites occur, and more reliable markers to distinguish between cattle-derived and buffalo-derived parasites would be needed in order to perform such experiments. There are also no reports indicating whether cattle-derived parasites can be transmitted to buffalo. Such transmisson, if it did occur, could play an important role in the evolution of genetic polymorphism in *T. parva* parasites and in the selection for genetically variant *T. parva* strains. It would be necessary to perform tick transmission studies to show whether *T. parva* parasites that are adapted to cattle could be transmitted to buffalo.

7.2.2 Extensive genetic diversity among South African *T. parva* parasites

Most sequences obtained from the three antigen genes, p67, p104 and PIM, analysed in this study were not identical to the previously reported sequences (Toye *et al.*, 1995; Nene *et al.*, 1996; Skilton *et al.*, 2002), demonstrating extensive genetic diversity among South African *T. parva* parasite populations. Diversity can be either generated by random mutation of nucleotides during asexual reproduction and selection of changes that confer a biological advantage or sexual reproduction through intra- and inter-genetic recombination and the random assortment of alleles (Gubbels *et al.*, 2000). There is no evidence from this study to conclusively implicate any of these mechanisms in the diversity that was observed and the generation of novel alleles. Two different novel p67 alleles, 3 and 4, were identified in addition to the previously reported alleles (Nene *et al.*, 1996). Although the novel alleles were only identified from samples that were obtained from buffalo in South Africa, allele 3 was obtained from a Zambian field isolate (Zam 5) from a naturally infected bovine diagnosed with ECF (Geysen, 2000). This result suggests that cattle-derived *T. parva* parasites,

especially those that can cause ECF, do not exclusively contain p67 allele 1 as previously believed.

Sequence stability may differ in different domains in the genome, which implies that some genes may be reasonably conserved while others are more prone to variation (Bishop *et al*., 1997). While p67 and p104 gene sequences examined in this study were relatively conserved, and most variation appeared to be due to single nucleotide polymorphisms, the PIM gene was highly polymorphic as evident from the multiple deletions and insertions in the central variable region. Furthermore, in addition to the known PIM sequence types (Toye *et al*., 1995), 'mixed'-type PIM sequences, suggestive of recombination between cattle-type and buffalo-type PIM alleles, were identified. There was no evidence from data obtained in this study to suggest that mixed-type PIM sequences were due to PCR artifacts generated by *in vitro* cross-over events. In the tick vector, sexual reproduction between different strains of *T. parva* could result in recombinant progeny. The PIM gene is known to evolve very rapidly (Toye *et al*., 1995). Shared sequences between genes may arise as a result of common ancestry, convergent evolution, recombination or gene conversion (McDevitt, 1995; Klein and O'hUigin, 1995; Parham *et al*., 1995). According to Geysen *et al*. (2004), recombination and gene conversion may be involved in the generation of shared sequences in the PIM gene. The 'mixed' PIM sequences identified in this study appeared to be recombinants of cattle- and buffalo-type sequences. Whatever mechanism is involved, the alteration of amino acid sequences is perceived as an evolutionary advantage (Bishop *et al*., 1997); there is evidence that frequent genetic exchange has the potential to generate parasites that have high antigenic diversity (Morrison, 2007). Findings from this study have shown that the sequence heterogeneity within the *T. parva* antigen genes, p67, p104 and PIM, is greater than previously thought. It will be interesting to establish the significance of this genetic diversity in relation to the antigenic diversity of the parasite and the epidemiology of the disease.

While an integrated phylogenetic study of the sequence data obtained from all three genes might be used to further elucidate the evolution of *T. parva* strains in cattle and buffalo, the data from the present study was unfortunately not suitable for this purpose because of mixed infections with different *T. parva* strains in a single sample. More than one sequence was obtained for each of the three genes from most field samples examined in this study. It was therefore not possible to match sequences obtained from each of the three genes to a single parasite. Such a study could be done in future by making parasite clones from field isolates

and characterizing all three genes from individual clones to obtain data that could then be integrated and used to establish the evolution of different parasite strains.

7.3 Conclusion

Similar to other diagnostic tests, the real-time PCR assay developed in this study has challenges that still need to be addressed to improve on the performance of the assay. The sensitivity of the *T. parva*-specific real-time PCR assay is still compromised in cases of mixed infections with *Theileria* sp. (buffalo), especially when the ratio of *Theileria* sp. (buffalo) to *T. parva* is high. Increasing input DNA or extracting total DNA from a larger volume of infected blood may increase the probability of detecting the relevant parasite DNA template in the real-time PCR assay. The specificity of the real-time PCR assay could not be further improved using the 18S rRNA gene because of the high similarities between *Theileria* sp. (buffalo) and *T. parva* in this gene sequence. For a more specific test it is advisable that a different gene with enough variation at species level should be targeted. Until then, the real-time PCR test reported here remains the most sensitive and specific molecular test for detection of *T. parva* infection.

Findings from this study have shown that *T. parva* parasites with cattle-type p67, p104 and PIM alleles were present on a farm near Ladysmith in South Africa. However, ECF pathology was not diagnosed in these animals. The PIM PCR-RFLP profiles and amino acid sequence data provided evidence that the source of *T. parva* infections in other cattle on the Ladysmith farm was infected buffalo originating from Hluhluwe-iMfolozi. This supports the suspicion of the involvement of *T. parva*-infected buffalo in the events that resulted in an outbreak in the Ladysmith farm (Thompson *et al*., 2008). No gene sequences identical to cattle-type p67, p104 and PIM were identified from *T. parva* samples obtained from buffalo; however, variants of cattle-type p67 and p104 alleles were identified.

Extensive genetic diversity amongst South African *T. parva* populations in buffalo was evident as shown by identification of novel p67 alleles and PIM 'mixed'-type alleles. The existence of these 'new' alleles will make it difficult to use these antigen genes to characterize South African *T. parva* isolates because it has not yet been established if these isolates are associated with cattle- or buffalo-derived parasites. Although p67, p104 and PIM gene profiles and sequences have been previously used successfully to characterize *T. parva* parasites in East Africa (Bishop *et al*., 2001), identification of markers that are differentially

expressed between cattle- and buffalo-derived *T. parva* parasites will be required for successful characterization of *T. parva* parasites in South Africa. The presence in South African *T. parva* field samples of alleles associated with *T. parva* parasites responsible for classical ECF remains a concern and the significance of the different alleles, particularly the novel variants, in the epidemiology of theileriosis in South Africa should be established.

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For my thoughts are not your thoughts, neither are your ways my ways, saith the LORD. ***Isaiah 55:8***

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For I know the thoughts that I think toward you, saith the LORD, thoughts of peace, and not of evil, ***to give you an expected end****.* ***Jeremiah 29:11***
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