Population genetic structure, genotypic and antigenic diversity of *Theileria parva* field strains from eastern and southern Africa

By

Donald Lubembe Mukolwe

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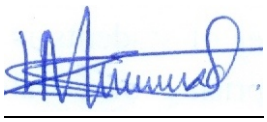
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

DEDICATION

This thesis is dedicated to my lovely wife Janesally A. Lubembe, son Nathan J. M. Lubembe and daughter Olive G. M. Lubembe. I will be forever grateful for your love, prayers, moral support and patience during my studies.

DECLARATION

I hereby declare that this thesis is my original work and has not been presented for any degree at any other university or for any other award. It is submitted in fulfillment for the award of degree Doctor of Philosophy Veterinary Science, at the University of Pretoria, South Africa.



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THESIS SUMMARY

Population genetic structure, genotypic and antigenic diversity of *Theileria parva* field strains from eastern and southern Africa

By

Dr. Donald Lubembe Mukolwe (BVetMed, MSc.)

Supervisor:

Prof. Kgomotso P. Sibeko-Matjila (Ph.D.)
Vectors and Vector-borne Diseases Research Programme
Department of Veterinary Tropical Diseases
Faculty of Veterinary Science
University of Pretoria, South Africa.

Co-Supervisor:

Dr. David O. Odongo (Ph.D.)
School of Biological Sciences
College of Biological and Physical Sciences
University of Nairobi, Kenya.

Department: Veterinary Tropical Diseases

Degree: Ph.D.

Theileria parva utilizes genetic diversity as a survival strategy in evasion of the host's immune system. Hence, effective control of *T. parva* infections is highly reliant on understanding the extent of genotypic and antigenic diversity of *T. parva* in cattle-derived and buffalo-derived isolates. Thus, the aim of this study was to identify differences between cattle- and buffalo-derived *T. parva* field parasites from eastern and southern Africa based on antigenic and genotypic diversity, and define the population genetic structure of *T. parva* parasites from the two regions.

Sequence analysis of the central variable region of the sporozoite antigen gene, p67, revealed two subtypes of p67 allele type 1, an allele type previously exclusively associated with East Coast fever. Each subtype was unique to parasites from eastern and southern Africa, thus differentiating the p67 allele type 1 population responsible for Corridor disease in South Africa from that which occurs in East Africa. The other three p67 allele types (2, 3 and 4) were detected only from buffalo-derived *T. parva* parasites from buffalo and Corridor disease cases. Sequences of regions containing CD8+ T-cell epitopes in ten schizont antigens, designated Tp1 to Tp10, showed epitope variants in Tp1, Tp2, Tp4, Tp5 and Tp9, where Tp2 and Tp5 had the most and least variants respectively. Tp1, Tp2 and Tp9 had variants that were common in cattle- and buffalo-derived parasites from the two regions investigated. Variants on the immunodominant $Tp2_{49\text{-}59}$ and $Tp2_{50\text{-}59}$ epitopes were only identified in buffalo-derived parasites from South Africa, while one variant of $Tp1_{214\text{-}224}$ was common in parasites from the two regions. The significance of Tp4 and Tp5 in immunity is not known and the effects of natural variants of Tp9 epitope on CTL recognition have not been reported. MS19 and ms5 loci were the most and least diverse respectively, and buffalo-derived *T. parva* parasites showed high levels of genetic diversity. Parasites associated with Corridor disease in South Africa and East Coast fever in eastern Africa had distinguishing allelic profiles on three loci (MS8, MS19 and MS33). Individual populations from the two regions were in linkage equilibrium (V_D<L), however, when considered as one combined population, linkage disequilibrium (V_D>L) was observed. The population structure of *T. parva* depicts a non-structured population with limited genetic intermixing between than within subpopulations, but with a close genetic relationship among cattle- and buffalo-derived *T. parva* parasites.

Collectively, findings in this study show two subpopulations of *T. parva* that have adapted and are circulating in the two regions of Africa. Although some genotypes or alleles were exclusively detected in specific regions, it is possible that they could be underrepresented in

the other region; hence, were not detected. The findings from analysis of the schizont antigens suggest that Tp1 and Tp2 could be better vaccine candidates compared to p67, in protection against a challenge with both cattle- and buffalo-derived *T. parva* parasites. The unique MS8, MS19 and MS33 profiles can be explored as possible markers for differentiation of ECF and Corridor disease parasites.

CHAPTER 1

General Introduction

1.1 Background

Theileriosis is a tick-transmitted disease caused by a protozoan parasite of the genus *Theileria*, which affects both wildlife and domestic animals (Norval *et al.*, 1992; Bishop *et al.*, 2004). The genus *Theileria* comprises of sporozoan protozoa that cause disease syndromes in domestic and wild ruminants, with some species having the capability to transform hosts' lymphocytes (Bishop *et al.*, 2004). In cattle, *Theileria parva* causes Corridor disease that is common in South and East Africa where there is contact with infected buffalo, East Coast fever (ECF) common in eastern, and parts of central and southern Africa, and January disease in Zimbabwe (Norval *et al.*, 1992). Cattle theilerioses still remain the most important *T. parva* infections in the affected countries due to high levels of mortality in cattle resulting in vast economic losses (Mukhebi *et al.*, 1992). Currently, immunization of cattle using live *T. parva* sporozoites, chemotherapy and tick control are strategies which have been adopted for the control of East Coast fever in eastern Africa (Di Giulio *et al.*, 2009). Compulsory short-interval dipping of cattle for the control of ticks has been enforced in controlling January disease and other tick-borne diseases in Zimbabwe (Latif *et al.*, 2001; Latif and Hove, 2011). Although Corridor disease is a controlled disease in South Africa, sporadic outbreaks occur when there is contact between infected buffalo and susceptible cattle in the presence of the tick vector during relocation of buffalo (reviewed in Mbizeni *et al.*, 2013). Chemotherapy and use of live vaccine are prohibited in South Africa due to the risk of development of carrier status in cattle, which can act as sources of infection to other susceptible cattle (reviewed in Mbizeni *et al.*, 2013). However, transmission from suspected carrier cases of Corridor disease to other susceptible cattle has not been reported in South Africa, suggesting that Corridor disease may not have a 'true' carrier status (Thompson *et al.*, 2008; Mbizeni *et al.*, 2013).

In an effort to understand the biology of *T. parva* infections in cattle for effective control, genes that encode antigens involved in *T. parva* immunity which include the sporozoite antigen p67 (Musoke *et al.*, 1984; Nene *et al.*, 1996) and the schizont antigens designated Tp1 -Tp10 (Graham *et al.*, 2006) have been identified. In addition, a panel of polymorphic micro- and minisatellite markers have been identified (Oura *et al.*, 2003; Katzer *et al.*, 2010) and used to characterize *T. parva* parasites from eastern Africa (Oura *et al.*, 2005; Katzer *et al.*, 2010; Elisa *et al.*, 2015; Muwanika *et al.*, 2016; Rukambile *et al.*, 2016; Salih *et al.*, 2018) and parts of southern Africa (Muleya *et al.*, 2012), revealing extensive genotypic diversity in field parasites from Uganda (Oura *et al.*, 2005; Oura *et al.*, 2011; Muwanika *et al.*, 2016), Tanzania (Elisa *et al.*, 2015; Rukambile *et al.*, 2016), Sudan (Salih *et al.*, 2018) and Zambia (Muleya *et al.*, 2012),

and in components of the live vaccine from Kenya (Katzer *et al.*, 2006; Katzer *et al.*, 2010; Patel *et al.*, 2011). Up to date, there is no official report on the genotypic diversity of *T. parva* parasites from South Africa.

Initial characterization of the central variable region of the gene encoding the sporozoite antigen, p67, revealed conservation and polymorphism in cattle- and buffalo-derived *T. parva* parasites respectively (Nene *et al.*, 1996). The p67 sequences identified in cattle-derived parasites which were identical to the Muguga *T. parva* stock sequence led to the conclusion that such sequences are exclusively associated with ECF in eastern Africa (Nene *et al.*, 1996; 1999). However, subsequent characterization of buffalo-derived *T. parva* parasites from South Africa revealed similarities with East African parasites on p67, where the p67 sequences thought to be solely associated with ECF were also identified in these parasites (Sibeko *et al.*, 2010) raising concerns on whether these sequences are exclusive to ECF or could be an indication of the possibility of re-emergence of ECF in South Africa. Although a recent study has demonstrated limited diversity of p67 in *T. parva* parasites transmitted from buffalo to cattle in Kenya (Sitt *et al.*, 2019), the need to establish what the differences are between parasites from eastern and southern Africa based on p67 still remain.

Characterization of schizont antigen genes in few field parasites and *T. parva* cell lines derived from cattle- and buffalo-derived *T. parva* parasites has revealed diversity on these genes, especially in Tp1, Tp2 and Tp9 (Pelle *et al.*, 2011; Hemmink *et al.*, 2016; Salih *et al.*, 2017; Sitt *et al.*, 2018). Polymorphism of *T. parva* antigen-encoding genes is thought to result from interchromosomal recombination in the tick vector (Graham *et al.*, 2006) where the resultant antigenic peptides expressed by the parasite are not recognized by the protective elements of the host. It is hypothesized that due to this phenomenon, *T. parva* and other apicomplexan parasites manipulate the host cell and evade the immune responses elicited by their vertebrate hosts (Seeber and Steinfelder, 2016). Immunity to *T. parva* infections is mainly cell-mediated involving cytotoxic T lymphocytes (CTLs) (McKeever *et al.*, 1994; Taracha *et al.*, 1995) which recognize peptides presented by MHC class I phenotype. However, antibody-mediated immunity involving the sporozoite surface protein encoded by the p67 gene has also been demonstrated (Musoke *et al.*, 1982; Musoke *et al.*, 1984; Nene *et al.*, 1999). *Theileria parva* peptides are encoded by antigenic epitope regions on the schizont antigen genes and have been characterized (Gardner *et al.*, 2005; Akoolo *et al.*, 2008; Graham *et al.*, 2008; Nene *et al.*, 2012). Some epitopes on Tp1 and Tp2 antigens have been identified to be immunodominant

in *T. parva* immunity (MacHugh *et al.*, 2009; Connelley *et al.*, 2011; Connelley *et al.*, 2016). The diversity of antigenic epitopes in Tp antigens in *T. parva* cell lines and few field parasites from cattle and buffalo from East Africa (Pelle *et al.*, 2011; Hemmink *et al.*, 2016; Salih *et al.*, 2017; Hemmink *et al.*, 2018; Sitt *et al.*, 2018) and a few from buffalo in South Africa (Hemmink *et al.*, 2018) has been reported.

Up to date, information on the genotypic diversity and variations within the schizont antigen genes of *T. parva* parasites from cattle in southern Africa is scanty. The African buffalo (*Syncerus caffer*) being the natural reservoir of *T. parva*, contains heterogenous parasites (Sibeko *et al.*, 2011), further complicating the epidemiology and control of *T. parva* infections. Hence, for a better understanding of the biology and epidemiology of *T. parva*, an evaluation of parasites from the buffalo is significant. Therefore, the need for conducting an expansive comparative study involving *T. parva* field parasites from cattle and buffalo in eastern and southern Africa where the most important *T. parva* disease syndromes occur still remain. Thus, the aim of this study was to assess the population genetic structure, genotypic and antigenic diversity of *T. parva* field parasites from cattle and buffalo in eastern and southern Africa.

1.2 Thesis Rationale

Following the eradication of ECF in South Africa in the 1950s, Corridor disease caused by the buffalo-derived *T. parva* is the form of cattle theileriosis that is of concern. East Coast fever caused by the cattle-derived *T. parva* is the most common and important tick-borne disease in cattle in eastern, and parts of central and southern Africa where it causes vast economic losses in the livestock industry, estimated at ~USD300 million annually (reviewed in Nene *et al.*, 2016). Recent concerns of re-emergence of ECF in South Africa following the identification of p67 sequences similar to the typical cattle-derived *T. parva* Muguga stock from Kenya still remain (Sibeko *et al.*, 2010). Several control methods of *T. parva* infections targeting both the vector and the parasite have been developed, including the current use of a live vaccine in eastern Africa which has been shown to confer protection against cattle-derived but not buffalo-derived *T. parva* parasites. The success of control methods targeting the vector and the parasite have been limited by resistance of the tick vector to the available acaricides, and diversity of the parasite respectively. *Theileria parva* employs diversity, especially of the antigen genes that are recognized by the immune cells of the bovine host, as a survival strategy where genetic changes involving the antigenic epitope regions on the antigens may lead to

the parasite evading the host's immune system (Connelley *et al.*, 2011). Therefore, understanding the antigenic and genotypic diversity of the parasite in both the bovine and reservoir host is paramount in designing appropriate control methods against the parasite. It is for these reasons that one sporozoite and ten schizont antigen genes of *T. parva* were analyzed to establish the diversity of *T. parva* in buffalo- and cattle-derived field parasites from East and southern Africa. In addition, the genotypic diversity and population structure of *T. parva* parasites in East and southern Africa was assessed.

1.3 Thesis Objectives

1.3.1 Aim

This study aimed at assessing the population genetic structure, genotypic and antigenic diversity of cattle- and buffalo-derived *T. parva* field parasites from East and southern Africa.

1.3.2 Specific Objectives

The following are the specific objectives aimed to be achieved:

i. Identify the differences between cattle- and buffalo-derived *T. parva* parasites based on the sporozoite antigen gene p67.
ii. Investigate the diversity of schizont antigen genes in cattle- and buffalo-derived *T. parva* parasites from East and southern Africa.
iii. Determine the genotypic diversity of *T. parva* parasites obtained from cattle and buffalo from East and southern Africa.
iv. Determine the population genetic structure of *T. parva* parasites obtained from cattle and buffalo from East and southern Africa.

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CHAPTER 2

Literature Review

2.1 The genus *Theileria*

Theileria parasites are classified under subclass *Piroplasmidia*, class *Sporozoa* and phylum apicomplexa (Levine *et al.*, 1980). Members of the phylum apicomplexa possess an apical complex which contains secretory organelles involved in the invasion and survival of the parasites in the mononuclear cells of the mammalian host and in the gut epithelial cells of the invertebrate host (Fawcett *et al.*, 1982). In *Theileria*, the apical complex is thought to play a role in the survival of the parasite within the host cell (reviewed in Nene *et al.*, 2016). *Theileria* is an obligate intracellular protozoan parasite that infects both domestic and wild ruminants. The genus *Theileria* comprises tick-transmitted intracellular protozoa that cause a spectrum of disease syndromes in domestic livestock including large and small ruminants in most parts of the world (Mukhebi *et al.*, 1992). *Theileria parva* which occurs in eastern, central and southern Africa, and *T. annulata* that is common in southern Europe, northern Africa, Middle East and Central Asia are the most pathogenic species of *Theileria* (Norval *et al.*, 1992). *Theileria taurotragi* and *T. mutans* found in Africa cause mild disease, whereas *T. velifera* is nonpathogenic. *Theileria orientalis/buffeli* complex consisting of *T. orientalis* found in the far east and *T. buffeli* distributed globally (Gubbels *et al.*, 2000; Jeong *et al.*, 2010) also cause disease and loss of production (Norval *et al.*, 1992). *Theileria lestoquardi* found in north Africa and Asia is the most important species that infects sheep and goats (Norval *et al.*, 1992). *Theileria equi* found in Asia, Europe, South America and Africa is an important species that infects horses (Uilenberg, 2006), whereas *T. sergenti* is the common cause of Japanese bovine theileriosis (Uilenberg, 2011).

2.2 *Theileria parva*

Among the *Theileria* parasites, *T. parva* which causes classical East Coast fever (Norval *et al.*, 1992), Corridor disease and Zimbabwean theileriosis (Uilenberg *et al.*, 1982), is the most pathogenic and common in eastern, central and parts of southern Africa. The former nomenclature used for the causative agent of ECF (*Theileria parva parva*), Corridor disease (*Theileria parva lawrencei*) and January disease (*Theileria parva bovis*) has been abandoned and *T. parva* parasites are now referred to as cattle- or buffalo-derived (reviewed in Nene *et al.*, 2016). *Theileria parva* is distantly related to the malaria parasite *Plasmodium* with their life cycles and the immune responses by the mammalian hosts to their infections being similar (Morrison, 2009). The geographical distribution of *T. parva* and the associated disease syndromes are associated with the distribution of the main tick vector *Rhipicephalus*

appendiculatus (Norval *et al.*, 1992), although *R. zambeziensis* and *R. duttoni* are possible vectors (Lawrence *et al.*, 1983). However, the range of *T. parva* is less than that of the principal vector where some *T. parva*-free *R. appendiculatus* populations have been found to occur in Kenya, Zambia and South Africa (Norval *et al.*, 1992). The African buffalo (*Syncerus caffer*) is the natural reservoir host of *T. parva* (Barnett and Brocklesby, 1966) although the waterbuck (*Kobus defassa*) is thought to be a possible reservoir host (Stagg *et al.*, 1994).

2.3 Life cycle of *Theileria parva*

The life cycle of *T. parva* is similar to other apicomplexan parasites and involves obligate asexual reproduction and sexual reproduction phases in the mammalian host and the arthropod vector respectively (Figure 2.1). The life cycle is haploid except for the brief diploid phase which occurs in the tick vector. During feeding, the infective sporozoites are introduced into the host by the vector. The infective sporozoites invade bovine lymphocytes by a process similar to receptor-mediated endocytosis (Fawcett *et al.*, 1982; Toye *et al.*, 2014). Receptor-mediated endocytosis is a process whereby a cell takes in only specific molecules as determined by the receptors on the cell plasma membrane. Lysis of the enveloping host cell membrane enables the parasite to access the cytosol where it undergoes an initial schizogonous division resulting in a multinucleate schizont stage that causes transformation of the infected lymphocytes (reviewed in McKeever, 2006). The parasite divides simultaneously with the lymphocytes and by associating with the cellular spindle apparatus, the infection spreads to the daughter cells (McKeever, 2006; McKeever, 2007). This division results in a state of uncontrolled lymphoid proliferation leading to a lymphoproliferative disease.

The parasitized cells invade lymphoid and non-lymphoid tissues throughout the body (Katzer *et al.*, 2006) causing a clinically fatal disease in cattle characterized by generalized lymphadenopathy. Other symptoms characterizing the disease include loss of body condition, petechial hemorrhages on the buccal and lingual mucosa, fever, enlargement of superficial lymph nodes and anemia (Raddostits *et al.*, 2000). Proliferation of the parasite ceases in some infected cells and undergoes further differentiation to form uninucleate merozoites that are subsequently released to invade erythrocytes and develop into piroplasms. As a result, piroplasms multiply in the erythrocytes causing anemia and cell death (Nene *et al.*, 2000). Susceptible animals often die within 2-3 weeks of infection. Ingestion of infected erythrocytes by feeding ticks completes the asexual phase of the life cycle in the mammalian

host. These erythrocytes are lysed in the tick gut to release piroplasms into the lumen, where they differentiate into both micro- and macrogametes.

The two undergo syngamy to form diploid zygotes, which invade gut epithelial cells to differentiate to motile kinete forms. Once formed, the kinetes are released into the haemocoel through which they migrate to the salivary glands, where they invade type III acini cells (reviewed in McKeever, 2006). Further development of the kinetes within the infected type III acini cells results in large syncytial structures known as the sporoblasts which contain the sporozoites that are infective to the next mammalian host during a blood meal. Each sporoblast has been estimated to give rise to 10^4 - 10^5 uninucleate haploid sporozoites (McKeever, 2006).

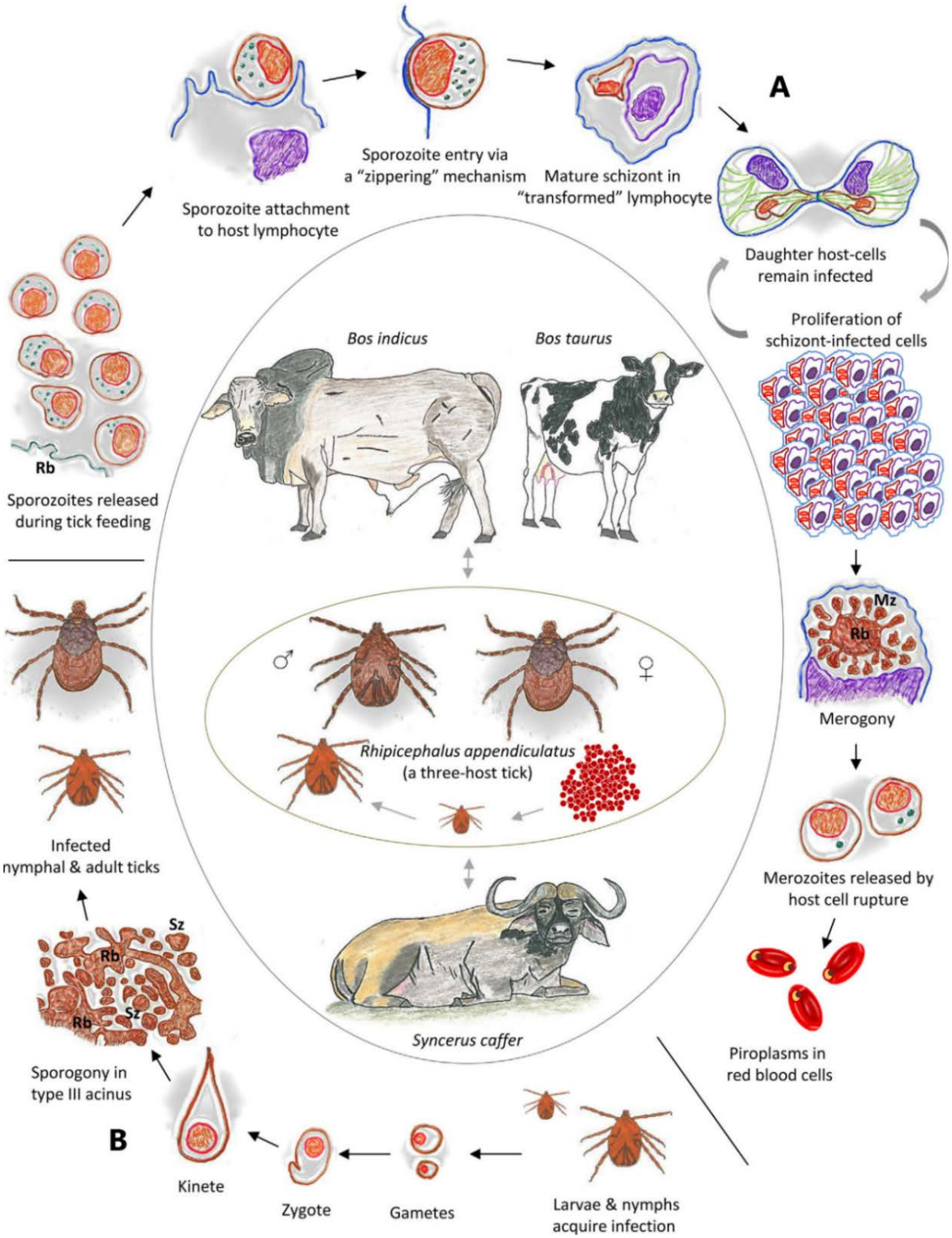

Figure 2.1: Life cycle of *Theileria parva* in the mammalian host (A) and arthropod vector (B). Larval and nymphal stages of the tick vector acquire an infection by feeding on infected cattle or buffalo, and transmit the parasite as nymphs or adults. This figure was obtained from Nene *et al*. (2016).

2.4 *Theileria parva* disease syndromes

2.4.1 East Coast fever (ECF)

East Coast fever (ECF) is a fatal bovine disease caused by tick-transmitted cattle-derived *T. parva*, previously known as *T. parva parva* (Lawrence *et al.*, 1994b). It is a lymphoproliferative disease with high mortality in the exotic cattle breeds (Lawrence *et al.*, 1994b) resulting in vast economic losses in eastern, central and southern Africa (Mukhebi *et al.*, 1992). The natural vector is the brown ear tick, *R. appendiculatus* (Uilenberg *et al.*, 1982) and transmission is usually from cattle to cattle by the nymph and adult stages of the tick vector. Acute cases of ECF with a course of three weeks are common in exotic cattle breeds introduced in enzootic areas, and are usually characterized with high mortalities (Norval *et al.*, 1992; Lawrence *et al.*, 1994b). However, for Zebu cattle and other indigenous breeds of cattle raised in endemically stable areas, mortality is relatively low (Norval *et al.*, 1992; Lawrence *et al.*, 1994b). The early phase of the disease is characterized by pyrexia, lymphadenopathy, restlessness, anorexia and reduction in production (Norval *et al.*, 1992). Terminally there occurs severe pulmonary edema characterized by respiratory distress, and wasting. Major pathology in ECF occurs as a result of invasion of tissues with parasitized cells followed by rapid lymphoproliferation and subsequent destruction of infected lymphoid cells by cytotoxic T-lymphocytes (CD8+) (Ivan Morrison *et al.*, 1995). There also occurs nonspecific natural killer cells effect resulting in an extensive MHC-unrestricted lymphocytolysis (Ahmed and Mehlhorn, 1999; McKeever *et al.*, 1999). Control strategies currently used include regular tick control, chemotherapy, and immunization of cattle with live *T. parva* sporozoites in East Africa. ECF is prevalent in 11 countries in eastern, central and southern Africa including Kenya, Uganda, Tanzania, Burundi, Rwanda, South Sudan, Democratic Republic of Congo, Malawi, Zambia, Zimbabwe and Mozambique (reviewed in Nene *et al.*, 2016) and was recently detected in Comoros Islands (De Deken *et al.*, 2007). The distribution of ECF in the affected countries coincides with that of the vector, although clinical cases are less prevalent in some areas due to the occurrence *T. parva* free *R. appendiculatus* or endemic stability (Kivaria *et al.*, 2004; Gachohi *et al.*, 2012; Kabi *et al.*, 2014).

2.4.1.1 The live vaccine for ECF

The Infection and Treatment Method (ITM) is a vaccination procedure used to protect cattle against ECF. It involves inoculation of cattle with live *T. parva* sporozoites and simultaneous administration of a long-acting oxytetracycline which result in prolonged immunity (Radley

et al., 1975). The Muguga cocktail ITM vaccine is composed of the three (trivalent) *T. parva* stock components comprising of two cattle-derived isolates namely, Muguga, Kiambu 5, and the buffalo-derived Serengeti-transformed whose names were derived from the sites they were isolated. The Muguga cocktail vaccine was first produced in the1970s at the East Africa Veterinary Research Organization based in Muguga, Kenya. Subsequently, two batches of the vaccine were produced by the International Livestock Research Institute (LIRI) in mid-1990s funded by the Food and Agriculture Organization (FAO) of the United Nations, and were referred to as FAO 1 and FAO 2 (Patel *et al.*, 2016). In 2008, another batch was produced at ILRI and named ITM ECF MC ILRI08, which has been widely commercialized for ECF vaccination in eastern Africa (Patel *et al.*, 2016). The live vaccine has been shown to confer protection against a wide range of geographically distinct cattle-derived parasite (Di Giulio *et al.*, 2009) but not against buffalo-derived parasites (Sitt *et al.*, 2015)

2.4.2 Corridor disease

Corridor disease is a buffalo associated form of cattle theileriosis originally identified in South Africa (Neitz *et al.*, 1955). It is an acute and fatal disease caused by a buffalo-derived *T. parva*, previously known as *T. parva lawrencei*. Its clinical presentation and pathology resemble ECF, with Corridor disease being acute and with low parasitemia compared to ECF (Lawrence *et al.*, 1994a). Buffalo-derived *T. parva* is endemic in buffalo populations in eastern and southern Africa. In South Africa, it is endemic in buffalo populations in the northern parts of the Provinces of Mpumalanga and Limpopo (at the Kruger National Park interface), and KwaZulu-Natal (reviewed in Mbizeni *et al.*, 2013). Transmission occurs when cattle co-graze with infected buffalo in the presence of the tick vectors *R. appendiculatus* and *R. zambeziensis* (Uilenberg *et al.*, 1982), or when contact between buffalo and cattle occurs in the presence of the vector during relocation of buffalo. Corridor disease is believed to be self-limiting as infected cattle die before the parasite can develop to the tick infective stage. Control strategies involve regular tick control, and limiting contact between buffalo and cattle by fencing game parks and restriction of relocation of buffalo. Chemotherapy and immunization of cattle with live *T. parva* sporozoites is prohibited in South Africa due to development of a carrier state which would be a source of infection to susceptible cattle.

2.4.3 January disease

January disease (also known as Zimbabwean theileriosis) is an acute and fatal type of theileriosis that regularly occurs in Zimbabwe with clinical and pathological features similar to ECF (Lawrence *et al.*, 1994c). It emerged following eradication of ECF in Zimbabwe in 1954 (Lawrence, 1991), and is caused by a cattle-derived *T. parva*, previously known as *T. parva bovis* (Lawrence, 1991), whose natural vectors are *Rhipicephalus appendiculatus* and *Rhipicephalus zambeziensis* (Uilenberg *et al.*, 1982). The name "January disease" is attributed to its seasonality with occurrences being between January and March (Matson, 1967; Lawrence, 1991) coinciding with the seasonal occurrence of *R. appendiculatus*. The main control strategy for January disease has been compulsory dipping to control the tick vector (Latif *et al.*, 2001).

2.5 Theileriosis in South Africa

Theileria parasites affect cattle and wildlife with several *Theileria* species having been isolated in cattle and buffalo. *Rhipicephalus appendiculatus* and *R. zambeziensis* are the natural vectors (Lawrence *et al.*, 1983; Lawrence, 1991) of *T. parva* that causes Corridor disease in cattle in South Africa. Recent studies have indicated that other than *T. velifera* and *T. mutans*, there is also *Theileria sp.* (strain MSD) circulating in some buffalo and cattle populations in southern Africa (Chaisi *et al.*, 2013). Following the eradication of ECF in South Africa in 1950s, Corridor disease remains as the important form of theileriosis whose natural reservoir is the Cape buffalo (*Syncerus caffer*).

Cattle contract Corridor disease by sharing grazing ground with *T. parva*-infected buffalo, in the presence of the tick vector *R. appendiculatus*. Since the relocation of buffalo in South Africa is controlled, unprecedented contact of *T. parva*-infected buffalo with cattle contributes to the sporadic outbreaks of Corridor disease (Sibeko *et al.*, 2010). Although it was initially established that buffalo-derived *T. parva* cannot adapt to cattle (Neitz *et al.*, 1955), and cattle that naturally recover from the infection or after treatment can become *T. parva* carriers (Potgieter *et al.*, 1988), Mbizeni *et al.* (2013) established that some *T. parva* seropositive cattle thought to be carriers were not true carriers since they had lost their circulating *T. parva* parasites.

Theileria parva carrier state is defined as the ability of animals recovered from *T. parva* infection to infect tick vectors which subsequently transmit the parasite to susceptible bovine hosts

(Maritim *et al.*, 1989). Although it has been established that non-clinical *T. parva* positive cases may not be sources of infection to susceptible cattle in South Africa (Thompson *et al.*, 2008; Mbizeni *et al.*, 2013), the possibility of transmission cannot be completely overruled and hence, carrier state remains a threat to the control of theileriosis in South Africa. Subsequently, to avoid development of carrier state, immunization of cattle using the live vaccine, and treatment of theileriosis using the common anti-*Theileria*l drugs (buparvaquone and parvaquone) are prohibited in South Africa. In adherence to "The Buffalo Veterinary Procedural Notice of South Africa", cattle infected with *T. parva* should be slaughtered and owners compensated.

Corridor disease has not been of concern since it is a controlled disease in South Africa (Chaisi *et al.*, 2013). However, the recent characterization of buffalo-derived *T. parva* field parasites from South Africa which revealed the presence of p67 allele type 1 sequences similar to that of Muguga *T. parva* stock (an ECF isolate from Kenya) raises concerns of the possibility of re-emergence of ECF in South Africa (Sibeko *et al.*, 2010). In addition, should the ECF-like *T. parva* populations circulating in the South African buffalo become adapted to cattle, cattle-to-cattle transmission as in the case of ECF may be possible thus, the re-emergence of this disease in South Africa cannot be ignored.

2.6 Detection of *T. parva* in blood samples

Control of theileriosis in cattle is dependent on reliable diagnostic tools for differentiating *T. parva* infections. Conventional diagnostic methods based on microscopic demonstration of schizonts and piroplasms, pathological lesions, and detection of serum antibodies using Immunofluorescence Antibody Test (IFAT) and Enzyme-linked Immunosorbent Assay (ELISA) tests have been used (reviewed in Sibeko *et al.*, 2008). Molecular techniques based on PCR and DNA probing assays have also been developed (Bishop *et al.*, 1992; Bishop *et al.*, 1995; Sibeko *et al.*, 2008) and shown to be reliable, more sensitive and faster compared to the conventional methods.

2.6.1 Conventional and serological methods

Conventional diagnosis of *T. parva* is based on the microscopic examination of peripheral blood smears to identify piroplasms in erythrocytes, lymph node biopsies to identify schizonts in lymphocytes, clinical signs and pathological changes in tissues and organs following *T. parva* infection (Raddostits *et al.*, 2000). Serological assays such as IFAT and ELISA which

detect specific antibodies to *T. parva* schizonts or sporozoites (Katende *et al.*, 1998) have been applied. Xenodiagniosis has also been used to determine *T. parva* carrier state (Young *et al.*, 1981). Microscopic examinations only detect the presence of *T. parva* schizonts or piroplasms but cannot be used to differentiate *Theileria* species in a mixed infection (Norval *et al.*, 1992). IFAT only detects *T. parva* antibodies using piroplasm and schizont antigens (Burridge *et al.*, 1973), and has limitations of cross-reactivity between antigens, difficulty in standardization and subjectivity in interpretation of the results (Norval *et al.*, 1992). ELISA is a high throughput, cheap and fast method for screening and diagnosing large numbers of *T. parva* samples (Mans *et al.*, 2015). Nonetheless, it does not detect *Theileria* parasite itself hence cases that are seropositive may be negative for the parasite (Bishop *et al.*, 1992).

Clinical manifestations of cattle- and buffalo-derived *T. parva* infections are similar and characterized by common clinical signs including; generalized lymphadenopathy, loss of body condition, petechial hemorrhages on the buccal and lingual mucosa and enlargement of superficial lymph nodes (Raddostits *et al.*, 2000). Common post mortem findings include severe pulmonary edema associated with frothing in the upper respiratory tract, petechiations and ulceration in the gastrointestinal tract (Norval *et al.*, 1992). Collectively, considering the above limitations, conventional methods are not ideal for differential diagnosis of infections due to cattle- and buffalo-derived *T. parva*.

2.6.2 Molecular methods

Molecular methods are used to confirm for the presence of parasite genomic material following an infection. A PCR-probing assay involving amplification of the variable region of the 18S rRNA gene of *Theileria* followed by hybridization with radioactively labeled species-specific oligonucleotide probes (Allsopp *et al.*, 1993) was developed for detection of different *Theileria* parasites. Despite being effective in discriminating closely related *Theileria* species and detection of other related piroplasmids, the assay was noted to be laborious and time-consuming due to separate hybridization steps required to confirm the outcomes (Norval *et al.*, 1992; Collins *et al.*, 2002). Semi-nested PCR-RFLP assays based on the single copy p104 gene and 18S rRNA gene of *T. parva* have also been used for detection of *T. parva* infections (Geysen *et al.*, 1999; De Deken *et al.*, 2007). However, just like the PCR-based hybridization assays, these assays are time consuming and not ideal for distinguishing buffalo- and cattle - derived *T. parva* parasites.

The recently developed hybridization real-time PCR assay based on the 18S rRNA gene (Sibeko *et al.*, 2008) is specific, reliable, more sensitive and faster for detection of *T. parva*. However, the sensitivity of the assay especially in cases of mixed infections is still a problem due to competition for primers especially in samples from buffalo and cattle containing *Theileria sp.* (buffalo) and *T. parva* (Sibeko *et al.*, 2008), which have similarities in the 18S rRNA gene (Sibeko *et al.*, 2008; Chaisi *et al.*, 2011). A highly sensitive nested PCR based on the *T. parva*-specific 104-kDa (p104) gene was developed for detection of asymptomatic carrier state *T. parva* infections (Odongo *et al.*, 2010) and has been widely used for screening field samples for *T. parva* infections. A quantitative real-time PCR assay based on the cox III gene has also been evaluated for detection of *T. parva* from buffalo and cattle (reviewed in Chaisi *et al.*, 2013). Nonetheless, due to the extensive sequence variation within the cox III gene of the mild pathogenic *Theileria* species of cattle and buffalo, such could not be detected by the same assay (Chaisi *et al.*, 2013). Up to date there is no official report of a tool that can be used to differentiate between cattle- and buffalo-derived *T. parva*.

2.7 Characterization of *T. parva* parasites

Several genes have been identified and characterized in *T. parva* parasites. The single copy genes encoding immunogenic proteins; the p67 sporozoite surface antigen (Nene *et al.*, 1992), the polymorphic immunodominant molecule (PIM) expressed by the infective sporozoite and the intracellular schizont stages (Toye *et al.*, 1995), the 150 kDa microsphere protein (Skilton *et al.*, 1998), the p104 protein (Iams *et al.*, 1990), and the TpR1 gene (Bishop *et al.*, 1993) have been identified. *Theileria parva* schizont antigen genes which play a key role in the induction of immune responses in theileriosis have also been described and designated Tp1, Tp2, Tp3, Tp4, Tp5, Tp6, Tp7, Tp8, Tp9 and Tp10 (Gardner *et al.*, 2005; Graham *et al.*, 2006). The general findings revealed that buffalo-derived parasites are more genetically diverse compared to cattle-derived *T. parva*.

The initial analysis of the gene encoding p67 revealed the presence of a 129 bp deletion in the central region of the gene in cattle-derived *T. parva* parasites but not in buffalo-derived parasites (Nene *et al.*, 1996; Nene *et al.*, 1999). These were subsequently named p67 allele type 1 (with 129 bp deletion) and type 2 (without the 129 bp deletion). Subsequent analysis of buffalo-derived parasites from South Africa revealed two additional p67 allele types (types 3 and 4) (Sibeko *et al.*, 2010) where allele type 3 has a 174 bp deletion but the same deletion is absent in allele type 4. Successive analysis of buffalo-derived parasites from Kenya (Obara

et al., 2015; Sitt *et al.*, 2019) further supported the previous reports on p67. Initially, it had been hypothesized that p67 allele type 1 is exclusively found in cattle-derived parasites from East Africa (Nene *et al.*, 1996), but following the detection of similar p67 sequences in buffalo-derived parasites from South Africa (Sibeko *et al.*, 2010), and recently in Kenya (Sitt *et al.*, 2019), the suitability of p67 allele type 1 in distinguishing cattle- and buffalo-derived parasites remains in question.

As indicated earlier, *T. parva* genes encoding the schizont antigens have been described and designated as Tp1 - Tp10 (Gardner *et al.*, 2005; Graham *et al.*, 2006), and their antigenic epitope regions identified (Akoolo *et al.*, 2008; Graham *et al.*, 2008; Nene *et al.*, 2012). Characterization of these genes and their epitope regions in *T. parva* cell lines, vaccine components, and field parasites in East Africa (Pelle *et al.*, 2011; Hemmink *et al.*, 2016; Salih *et al.*, 2017; Hemmink *et al.*, 2018; Sitt *et al.*, 2018), and parasites from buffalo in South Africa (Hemmink *et al.*, 2018) has revealed buffalo-derived parasites to be more diverse than cattle-derived parasites. Genetic diversity is a survival strategy deployed by *T. parva* in adapting to the changing environment. In *T. parva*, genetic diversity results from sexual recombination which occurs during the sexual phase of *T. parva* life cycle in the tick vector (reviewed in Nene *et al.*, 2016). Field populations of *T. parva* are antigenically heterogeneous as a result of re-assortment of cytotoxic T lymphocyte (CTL) determinants in the tick vector (Katzer *et al.*, 2006). Hence, the current live vaccine widely deployed in eastern Africa induces cell-mediated immunity which is protective against cattle-derived parasites (Radley *et al.*, 1975; Di Giulio *et al.*, 2009; Magulu *et al.*, 2019) but not buffalo-derived parasites (Sitt *et al.*, 2015). Considering the role of schizont antigen genes in immunity to *T. parva* (McKeever *et al.*, 1994; Taracha *et al.*, 1995; Graham *et al.*, 2006), the effects of their diversity in recognition by the host's memory CTLs cannot be underestimated. Therefore, the need for establishing differences in the diversity of these genes in cattle- and buffalo-derived *T. parva* parasites from East and southern Africa, and the possible effects on host's immune response still remain.

2.8 Genotypic diversity among *T. parva* parasites

A panel of polymorphic mini- and microsatellites distributed on the entire *T. parva* genome have been identified (Oura *et al.*, 2003; Katzer *et al.*, 2010). Microsatellites are short tandem repeats (STRs) of 2-8 bps while minisatellites have longer repeat units (8-100 bps) (Oura *et al.*, 2003). They are distributed throughout the *T. parva* genome and are effective in assessing the extent of allelic polymorphism in *T. parva* parasites (Oura *et al.*, 2003; Oura *et al.*, 2005; Katzer

et al., 2010). They have been used to assess the genotypic diversity of *T. parva* field populations in Uganda (Oura *et al.*, 2005; Oura *et al.*, 2007; Oura *et al.*, 2011; Muwanika *et al.*, 2016), Tanzania (Elisa *et al.*, 2015; Rukambile *et al.*, 2016), Kenya (Katzer *et al.*, 2010), Sudan (Salih *et al.*, 2018), and Zambia (Muleya *et al.*, 2012), and components of the live vaccine (Katzer *et al.*, 2006; Patel *et al.*, 2011; Hemmink *et al.*, 2016) and revealed extensive diversity in field parasites. With the exception of Zambia, there is no official report on the genotypic diversity of *T. parva* parasites in southern Africa, especially in areas where Corridor disease has been reported. It is argued that in cases where parasites originate from complex mixtures comprising multiple *T. parva* genotypes especially in field parasites from the buffalo, these markers may not discriminate genotypes of individual parasites (Sibeko *et al.*, 2011).

2.9 Population structure of *Theileria parva*

Determining the extent of genetic exchange in *T. parva* populations and population genetic structure is critical in its control and predicting *T. parva* evolutionary trends. Studies on the population structure of *T. parva* have been conducted using micro- and minisatellite markers in Uganda (Oura *et al.*, 2005; Muwanika *et al.*, 2016), Kenya (Odongo *et al.*, 2006), Tanzania (Elisa *et al.*, 2015; Rukambile *et al.*, 2016) and Zambia (Muleya *et al.*, 2012). These studies demonstrated sub-structuring and significant genetic diversity among cattle- and buffalo-derived *T. parva* populations. Since recent studies have demonstrated that ancient diversity occurred in *T. parva* before geographic separation of eastern and southern Africa parasites (Hemmink *et al.*, 2018), further evaluation of the population dynamics of *T. parva* parasites in the two regions is critical.

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CHAPTER 3

Diversity of the sporozoite antigen gene p67 in cattle- and buffalo-derived *Theileria parva* field strains from East and southern Africa

3.1 Abstract

East Coast fever (ECF) and Corridor disease (CD) caused by cattle- and buffalo-derived *T. parva* respectively are the most economically important Tick-borne diseases of cattle in the affected African countries. The gene encoding the *T. parva* sporozoite surface antigen, p67, has been evaluated as a recombinant subunit vaccine against ECF, and has also been used to differentiate *T. parva* parasites causing ECF and CD. Cattle-derived *T. parva* parasites (ECF parasites) are less diverse compared to the buffalo-derived parasites (CD parasites). Four p67 allele types have previously been described, with allele type 1 and types 2, 3, 4 being associated with ECF and CD respectively. However, allele type 1 has also been identified in buffalo-derived *T. parva* in South Africa, and it is not clear if parasites possessing this allele type could potentially lead to the re-emergence of ECF. In this study, we assessed the diversity of the gene encoding p67 in buffalo- and cattle-derived *T. parva* parasites from South Africa, Mozambique, Kenya, Tanzania and Uganda. Detection of *T. parva* infection was done using a *T. parva*-specific real-time PCR assay on 921 samples. A 900 bp central fragment of the p67 gene was PCR amplified and sequenced for 128 *T. parva* positive samples, followed by DNA sequence analysis. A high percentage of samples detected positive for *T. parva* was recorded from buffalo from eastern Africa (75-100%) compared to cattle, with South Africa recording the lowest detection (11.3%). Four p67 allele types previously described were identified. Although allele type 1 conserved in cattle-derived *T. parva* parasites from East Africa was not detected in parasites from buffalo in the same region, it was detected in buffalo-derived parasites from buffalo and cattle from South Africa. Analysis of two p67 B-cell epitopes (TpM12 and AR22.7) revealed several amino acid substitutions in all allele types detected in buffalo-derived *T. parva* parasites from buffalo and cattle. However, both epitopes were conserved in allele type 1 from the cattle-derived *T. parva* parasites. A subtype of allele type 1 was identified in buffalo-derived parasites from clinical cases of CD and buffalo from southern Africa. Notably, allele type 1 sequences from parasites associated with ECF in East Africa and CD in Kenya were identical. These findings reveal detection of a subtype of p67 allele type 1 associated with *T. parva* parasites transmissible between buffalo and cattle in southern Africa. The identification of p67 allele type 1 with identical sequences in ECF and CD parasites from Kenya, suggest that p67 may not be an ideal marker for specific disease syndromes caused by *T. parva*. The implication of p67 sequence polymorphism on the development of a p67 recombinant vaccine effective against a challenge with both cattle- and buffalo-derived *T. parva* parasites remains undefined.

3.2 Introduction

Theileriosis is a widespread tick-transmitted protozoal disease of wildlife and domestic animals caused by an apicomplexan parasite of the genus *Theileria* (reviewed in Nene *et al.*, 2016). In eastern, central and southern Africa, *Theileria* infections in cattle are commonly caused by *Theileria parva* which occurs naturally in the African buffalo (*Syncerus caffer*) that is an asymptomatic carrier (Lawrence, 1992). *Theileria parva* causes fatal classical East Coast fever (ECF) common in eastern Africa (Norval *et al.*, 1992; Lawrence *et al.*, 1994b), Corridor disease (CD) common in eastern and southern Africa (Neitz *et al.*, 1955; Lawrence *et al.*, 1994a) and January disease (JD) in Zimbabwe (Lawrence *et al.*, 1994c). This parasite is mainly transmitted by a three-host brown ear tick *Rhipicephalus appendiculatus*, although *R. zambeziensis* and *R. duttoni* are also possible vectors (Lawrence *et al.*, 1983; Norval *et al.*, 1992; Uilenberg, 1999).

A live trivalent sporozoite vaccine for control of ECF was developed (Radley *et al.*, 1975) and has been successfully adopted for use in eastern Africa (Di Giulio *et al.*, 2009; Perry, 2016). Although this vaccine does not confer protection against buffalo-derived *T. parva* in Kenya (Sitt *et al.*, 2015), a study in northern Tanzania suggested that the vaccine is effective in areas where cattle co-graze with buffalo (Homewood *et al.*, 2006; Magulu *et al.*, 2019). Corridor disease being a controlled disease in South Africa, vaccination and chemotherapy are prohibited due to the potential risk of development of carrier state (reviewed in Mbizeni *et al.*, 2013), although the disease is believed to be self-limiting. A carrier state is where recovered cattle maintain a low level of *T. parva* infection that is tick transmissible. However, it has been demonstrated that infection of ticks from suspected carrier cases and subsequent transmission to other cattle does not occur in South Africa due to either very low parasitemia coinciding with the tick feeding period or infected cattle lose their circulating *T. parva* parasites shortly after infection (Thompson *et al.*, 2008; Mbizeni *et al.*, 2013).

Efforts to develop a recombinant vaccine based on the antigen genes, that can confer protection against cattle- and buffalo-derived *T. parva* have been pursued for the last four decades (McKeever *et al.*, 1999). To date, this has not been successful and could be partly due to the diversity of *T. parva* involving the antigen genes targeted by the host immune system (MacHugh *et al.*, 2009; Connelley *et al.*, 2011; Pelle *et al.*, 2011). Immunity to *T. parva* infections is mainly cell-mediated involving CD8+ cytotoxic T lymphocytes, which recognize parasite peptides encoded by schizont genes, and presented by MHC Class I molecules

(McKeever *et al.*, 1994; Taracha *et al.*, 1995). Nevertheless, monoclonal antibodies have also been shown to neutralize and inhibit entry of sporozoites into the host cell hence important in antibody-mediated immune protection (Nene *et al.*, 1999).

Theileria parva schizont and sporozoite antigen genes encoding proteins recognized by the host's immune system have been identified (Toye *et al.*, 1991; Nene *et al.*, 1996; Graham *et al.*, 2006) and characterized (Toye *et al.*, 1995; Nene *et al.*, 1999; Sibeko *et al.*, 2010; Pelle *et al.*, 2011; Hemmink *et al.*, 2016; Sitt *et al.*, 2018). The gene encoding the sporozoite antigen, p67, has been widely explored for the development of a recombinant vaccine (Musoke *et al.*, 1992). Vaccination of cattle using a subunit vaccine based on recombinant versions of p67 demonstrated a reduction in disease incidence by approximately 70% in the laboratory (Musoke *et al.*, 1992; Bishop *et al.*, 2003) and about 30% under field tick challenge (Musoke *et al.*, 2005). Recent attempts have been made to improve the vaccination regimen by modifying the antigen preparation, dosage or adjuvant systems (Lacasta *et al.*, 2018). It has been demonstrated with the malaria parasite, *Plasmodium*, that the efficacy of a recombinant vaccine correlates positively with a lack of antigenic diversity in naturally acquired immunity (Neafsey *et al.*, 2015). Thus, there could be a possibility that the efficacy of the recombinant version of p67 as a vaccine candidate could be compromised by allelic diversity of the encoding gene in the population of parasites circulating in the field. However, since p67 antigen is not the target of naturally acquired immunity and the gene has not been demonstrated to be under immune selection, the scenario for the recombinant version of p67-based vaccine could be different.

Previous studies on the sequence diversity of the central variable region of the p67 gene in cattle- and buffalo-derived *T. parva* parasites from Kenya (Nene *et al.*, 1999; Obara *et al.*, 2015) and South Africa (Sibeko *et al.*, 2010) revealed four groups of related p67 alleles referred to as types 1, 2, 3 and 4. The different allele types are distinguishable based on two indels; a 129 bp insert absent in allele type 1 and present in type 2 (Nene *et al.*, 1996) and the 174 bp insert absent in allele type 3 and present in type 4 (Sibeko *et al.*, 2010). Five distinct p67 B-cell epitopes recognized by murine monoclonal antibodies have been identified (Nene *et al.*, 1999) with sequence polymorphism occurring in buffalo-derived *T. parva* parasites (Nene *et al.*, 1999; Obara *et al.*, 2015). *Theileria parva* parasites that are transmissible between cattle are known to have identical p67 allele type 1 sequences (Nene *et al.*, 1999), and the presence of this allele in *T. parva* parasites in eastern Africa is associated with classical ECF. Notably, p67

allele type 1 similar to that identified in eastern Africa has been identified in parasites implicated in Corridor disease in South Africa (Sibeko *et al.*, 2010), raising concerns on the risk of re-emergence of ECF in this country where the disease was eradicated over half a century ago. Initial characterization studies based on the central variable region of p67 gene focused on *T. parva* parasites from Kenya (Nene *et al.*, 1996). Subsequent studies with parasites from South Africa revealed more polymorphism on the p67 gene (Sibeko *et al.*, 2010), which was later supported by further analysis of buffalo-derived *T. parva* parasites from Kenya (Obara *et al.*, 2015; Sitt *et al.*, 2019) where allele types 1, 2 and 3 were identified.

A more recent study in Kenya which evaluated the levels of diversity in p67 gene in *Theileria* parasites from cattle naturally infected with parasites from buffalo revealed limited differences in the diversity of *T. parva* parasites transmitted from buffalo to cattle (Sitt *et al.*, 2019). Notably, parasites possessing p67 allele type 1 are involved in both ECF and Corridor disease in Kenya (Nene *et al.*, 1996; Sitt *et al.*, 2019), while in South Africa, there are no reports of ECF although allele type 1 has been detected in buffalo-derived parasites (Sibeko *et al.*, 2010). Since Corridor disease cases in East Africa and South Africa have a similar clinical presentation (Sitt *et al.*, 2015; Sitt *et al.*, 2019), and ECF was eradicated in South Africa, it could be possible that the parasites associated with *T. parva* infections in cattle in East and southern Africa have sequence differences on p67 allele type 1, which may be associated with the resulting disease syndromes or distinguish parasites from the two regions. Therefore, a comparative analysis of p67 sequences from cattle- and buffalo-derived *T. parva* parasites from East and southern Africa was performed.

3.3 Materials and Methods

3.3.1 Sample collection and detection of *T. parva*

Cattle and buffalo blood samples were collected in EDTA tubes from South Africa, Mozambique, Kenya and Uganda as indicated in Table 3.1. Samples obtained from previous projects (collection dates from 2013 - 2014) are indicated in Table 3.1. In addition, two DNA samples were obtained from Katete and Chitongo *T. parva* isolates from Zambia. Blood samples in EDTA tubes were aliquoted in 2 ml eppendorf tubes and stored at -20 °C. The DNeasy® Blood and Tissue kit (Qiagen, Hilden, Germany) was used to extract DNA from 200 µl of EDTA blood samples, according to the manufacturer's protocol. However, elution was done in 100 µl instead of the recommended 200 µl to increase the concentration of extracted DNA. Extracted DNA was stored at 4 °C and -20 °C for short- and long-term storage respectively, until further analysis. Detection of *T. parva* genomic DNA was done using a *T. parva*-specific hybridization probe-based real-time PCR assay targeting the 18S rRNA gene (Sibeko *et al.*, 2008). *Theileria parva*-specific forward (5'-CTGCATCGCTGTGTCCCTT-3') and *Theileria* genus-specific reverse (5'-ACCAACAAAATAGAACCAAAGTC-3') primers were used to amplify a 230 bp fragment of the variable region of the 18S rRNA gene. For detection of *Theileria parva* and *Theileria* spp. buffalo, a pair of hybridization probes (*Theileria* genus anchor: 5'-AGAAAATTAGAG TGCTCAAAGCAGGCTTT-FL; and *Theileria* genus sensor: 5'-LCRed705-GCCTTGAATAGTTTAGCA TGGAAT-PH) was used. For specific detection of *Theileria parva* another pair of hybridization probes (*Theileria parva* anchor: 5'-GGGTCTCTGCATGTGGCTTAT-FL; *Theileria parva* sensor: 5'-LCRed640-TCGGACGGAGTTCGCT-PH) was used. Samples with a melting temperature of 63 °C±0.62 °C on the melting curve were considered *T. parva* positive. A reference sample KNP102 (Sibeko *et al.*, 2008) was used as a positive control.

Table 3.1: The geographical origin of blood samples collected from East and southern Africa.

Geographical origin			Host	Date of collection (Year)	Number of samples collected
Country	**Province/Region/ sub-region**	**County/District**			
Kenya	Rift Valley Region	Nakuru County	Cattle	2017	25
		Laikipia County	Buffalo	2014	40
Uganda	Mbarara Sub-region	Kiruhura District	Cattle	2017	137
	Karamoja Sub-region	Kaabong District	Cattle	2017	99
		Nakapiripirit District	Cattle	2017	123
Tanzania	Tanga Region	Tanga District	Cattle	2014	20
	Manyara Region	Tarangire National Park	Buffalo	2014	10
		Simanjiro plains	Cattle	2014	20
South Africa	KwaZulu-Natal Province	Hluhluwe-iMfolozi Park	Buffalo	2017	100
		uMkhanyakude District	Cattle	2016	92
		Hluhluwe District	Cattle	2016	131
	Mpumalanga Province	Kruger National Park	Buffalo	2014	60
		Bushbuckridge Municipality (Mnisi)	Cattle	2013	24*
Mozambique	Sofala Province	Marromeu Game Reserve	Buffalo	2016	40
Total					**921**

*14 samples were from clinical cases of Corridor disease and 10 from non-clinical cases from a herd with previous *T. parva* infections.

3.3.2 PCR amplification of the gene encoding the p67 antigen

PCR amplification on a total of 232 *T. parva* positive DNA samples targeting the 900 bp variable region of the p67 encoding gene was done using the primer pair (IL613-5'ACAAACACAATCCCAAGTTC3' and IL792-5'CCTTTACTACGTTGGCG3') (Nene *et al.*, 1996) and the 2X Phusion™ Flash High-Fidelity PCR Master Mix (ThermoFisher Scientific™, Waltham MA, USA) containing Phusion Flash II DNA polymerase which has proof reading activity (McInerney *et al.*, 2014). At least 50 ng of extracted DNA in a total reaction volume of 12.5 µl and 10 pmol of each primer was used in the PCR reaction. The amplification conditions were as previously described (Nene *et al.*, 1996) except that the time

for each step and the denaturation condition was adjusted according to Phusion Flash High-Fidelity PCR Master Mix user guide. Thus, the initial denaturation was done at 98 °C for 10 seconds (s), followed by 30 cycles of denaturation at 98 °C for 1 s, annealing at 57 °C for 5 s and extension at 72 °C for 10 s, and then a final extension step at 72 °C for 1 minute (min). Samples that failed to amplify in the primary reaction were re-amplified in a second PCR using 0.5 µl of the primary PCR product as DNA template, and the same amplification conditions except that the amplification cycles were reduced to 20. Amplicons were resolved by gel electrophoresis using 2% agarose containing ethidium bromide and 1X Tris Acetate EDTA (TAE) as the running buffer.

3.3.3 Cloning and Sanger sequencing

The PCR products were purified using the QIAquick® PCR Purification Kit (Qiagen, Germany). At least eight microlitres of each PCR product was purified following the manufacturer's protocol with the final elution done in 25 µl instead of the recommended 50 µl, to increase the concentration of DNA in purified PCR products. Five microlitres of each purified PCR product was used to prepare a ligation reaction using pJET1.2/blunt cloning vector (ThermoFisher Scientific™, Waltham MA, USA). Five microlitres of the ligation reaction was used for transformation of Mix & Go *E. coli* competent cells strain JM109 (Zymo Research, Tustin, USA). Recombinant clones were confirmed by colony PCR performed in a 20 µl reaction volume consisting of DreamTaq DNA polymerase, 2X DreamTaq Green buffer, 0.004 µmol of each dNTP, 0.04 µmol $MgCl_2$, and forward and reverse pJET primers each at 4 pmol. The cycling conditions were as follows; an initial denaturation at 95 °C for 3 min, followed by 25 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 1 min, and then a final extension step at 72 °C for 1 min. The PCR products were analyzed by gel electrophoresis using 2% agarose containing ethidium bromide in 1X TAE running buffer. Colony PCR products were purified using the QIAquick® PCR Purification Kit (Qiagen, Hilden, Germany) following manufacturer's protocol. Bidirectional sequencing was performed using pJET primers on ABI 3500XL Genetic Analyzer, POP7™ (ThermoFisher Scientific™, Waltham MA, USA) at INQABA Biotechnologies, South Africa.

3.3.4 Sequence analysis

Forward and reverse raw p67 sequences were confirmed using the Basic Local Alignment Search Tool (BLAST), and the quality of chromatograms checked using Chromas version 2.6.5.0 (Technelysium Pty Ltd). Forward and reverse sequences were assembled against a reference sequence using the CLC Main Workbench version 8.0 (Qiagen, Hilden, Germany) where editing of conflicting regions was done manually to generate consensus sequences ranging between 740 bp to 960 bp in length. Initial alignment and where necessary, reverse complementation of consensus sequences was done using the CLC Main Workbench version 8.0 (Qiagen, Hilden, Germany). Consensus sequences were then exported in FASTA format for subsequent analysis. Multiple sequence alignment of consensus sequences, together with reference sequences (Table 3.2) was done using the online version of MAFFT (Katoh and Standley, 2013). Estimation of the effect of amino acid substitutions within the epitope regions was done using SIFT predictions (Vaser *et al.*, 2016) where a probability score >0.05 was predicted to be tolerant.

Table 3.2: *Theileria parva* p67 reference sequences used in the construction of multiple sequence alignments and phylogenetic trees.

Country	Province/ region	Host of parasite origin	Sequence ID/Name	Accession number	p67 allele type	Reference
Kenya	Kilifi	Cattle	Muguga	M67476	1	Nene *et al*. (1996)
Kenya	Kilifi	Buffalo	7014	U40703	2	Nene *et al*. (1996)
South Africa	Mpumalanga	Buffalo	KNP102_9	JX442247	3	Sibeko *et al*. (2010)
South Africa	Mpumalanga	Buffalo	KNP102_6	JX442250	4	Sibeko *et al*. (2010)
South Africa	Mpumalanga	Buffalo	KNPW8_35	JX442249	3	Sibeko *et al*. (2010)
South Africa	Mpumalanga	Buffalo	KNPW8_48	JX442251	4	Sibeko *et al*. (2010)
South Africa	Mpumalanga	Buffalo	KNPW8_44	-	1	Sibeko KP, unpublished data
South Africa	Mpumalanga	Buffalo	KNPW8_17	-	1	Sibeko KP, unpublished data

South Africa	Limpopo	Buffalo	Mab BB43_2	-	1	Sibeko KP, unpublished data
Zambia	Southern	Cattle	Zambia_L1	-	1	Sibeko KP, unpublished data
Kenya	Nakuru	Cattle	Marula_7	LK054510	3	Obara *et al.* (2015)
Kenya	Nakuru	Cattle	Marula_10	LK054513	1	Obara *et al.* (2015)
Kenya	Nakuru	Cattle	Marula_2	LK054505	2	Obara *et al.* (2015)
Kenya	Laikipia	Cattle	ILRI-1	KY912962	1	Sitt *et al.* (2019)
Kenya	Laikipia	Buffalo	ILRI-2	KY912963	1	Sitt *et al.* (2019)
Kenya	Laikipia	Cattle, Buffalo	ILRI-4	KY912965	2	Sitt *et al.* (2019)
Kenya	Laikipia	Cattle, Buffalo	ILRI-6	KY912967	2	Sitt *et al.* (2019)
Kenya	Laikipia	Cattle, Buffalo	ILRI-18	KY912979	3	Sitt *et al.* (2019)
Kenya	Laikipia	Cattle, Buffalo	ILRI-34	KY912995	3	Sitt *et al.* (2019)

3.3.5 Phylogenetic analysis

To prepare sequences for construction of phylogenetic trees, the aligned sequences and sequence matrices were viewed, edited manually and truncated using MEGA version 7 (Kumar *et al.*, 2016). The final exported matrix was used for all subsequent phylogenetic analyses. Formats of sequence files were changed for different analysis software programs i.e. from fasta to phylip relaxed-sequential and nexus sequential, using the Format Converter tool on HIV databases website (www.hiv.lanl.gov/content/index). In order to display major groupings as either monophyletic or grouping by exclusion, data-display networks (neighbor-networks) were constructed on SplitsTree4 (Huson and Bryant, 2006). Support values calculated on SplitsTree4 were based on 1000 bootstrap replicates and the networks were based on uncorrected p-distances using all characters. Bayesian Information Criterion (BIC) and Akaike Information Criterion (AIC) nucleotide substitution model selection were done using JModelTest2 (Darriba *et al.*, 2012), executed on Cipres Science Gateway platform (Miller *et al.*, 2010) to determine the best-fit model of evolution for Bayesian and Maximum Likelihood analyses respectively.

In order to inspect sample parameter stabilization before construction of a phylogenetic tree, Bayesian analysis was performed in MrBayes version 3 (Ronquist and Huelsenbeck, 2003), applying the HKY+I+G nucleotide substituion model (Table 3.3) estimated by JmodelTest2. Four Markov Chain Monte Carlo (MCMC) chains were run simultaneously for 10 million iterations and trees were sampled every 1000th iteration. Posterior probabilities were calculated from the remaining saved majority rule consensus trees (mixture of the best trees) and a value ≥0.9 (90%) was considered a statistically significant branch support. The output files (p-files) from Bayesian analysis were viewed in Tracer version 1.6 (Rambaut *et al.*, 2014) for inspection of parameter stabilization and estimation of effective sample size (ESS) where an ESS value >200 was considered significant.

The Maximum Likelihood analysis was performed in RAxML (Randomized Axelerated Maximum Likelihood) version 8 (Stamatakis, 2014), applying the default GTR+I+G model although the model of evolution based on estimation by JModelTest2 was HYK+I+G. The default model was used since RAxML does not allow alteration of the model of evolution from the complex default model (GTR+I+G) to a simple model (HKY+I+G). Bootstrap support values were calculated using the autoMRE function in RAxML and a value ≥70 was considered a significant branch support. The best-scoring ML tree was viewed and edited using FigTree version 1.4.3.

Table 3.3: The Bayesian Information Criterion and Akaike Information Criterion nucleotide substitution model selection for the best-fit model.

Model Selection method	Model	-InL	K	BIC	[a]delta	[b]Weight	[c]Cum Weight
BAYESIAN INFORMATION CRITERION (BIC)	HKY+I+G*	3433.53530	90	7488.678530	0.000000	0.691745	0.691745
	HKY+G	3439.02870	89	7492.758575	4.080045	0.089945	0.781690
	TPM1uf+I+G	3432.70295	91	7493.920585	5.242055	0.050309	0.831999
	TPM1uf+G	3436.51572	90	7494.639370	5.960840	0.035121	0.867120
	TrN+I+G	3433.37342	91	7495.261525	6.582995	0.025732	0.892852
	TrN+G	3436.82921	90	7495.266350	6.587820	0.025670	0.918522
	TPM3uf+I+G	3433.50410	91	7495.522885	6.844355	0.022579	0.941101
	TPM3uf+G	3437.20429	90	7496.016510	7.337980	0.017641	0.958742
	TPM2uf+G	3437.21855	90	7496.045030	7.366500	0.017391	0.976133
	TPM2uf+I+G	3433.97165	91	7496.457985	7.779455	0.014147	0.990280
	Model	**-InL**	**K**	**AIC**	**[a]delta**	**[b]Weight**	**[c]Cum Weight**
AKAIKE INFORMATION CRITERION (AIC)	HKY+I+G*	3433.53530	90	7047.070600	0.000000	0.227339	0.227339
	TPM1uf+I+G	3432.70295	91	7047.405900	0.335300	0.192249	0.419587
	TIM2+I+G	3432.30269	92	7048.605380	1.534780	0.105536	0.525123
	TrN+I+G	3433.37342	91	7048.746840	1.676240	0.098329	0.623452
	TIM1+I+G	3432.38929	92	7048.778580	1.707980	0.096781	0.720233
	TPM3uf+I+G	3433.50410	91	7049.008200	1.937600	0.086284	0.806517
	TPM2uf+I+G	3433.97165	91	7049.943300	2.872700	0.054060	0.860577
	TVM+I+G	3432.32990	93	7050.659800	3.589200	0.037782	0.898359
	TIM3+I+G	3433.34149	92	7050.682980	3.612380	0.037347	0.935706
	TPM1uf+G	3436.51572	90	7053.031440	5.960840	0.011542	0.947249

*the best-fit model

Parameters of the best-fit model:
Partition = 010010; Negative log likelihood (-lnL) = 3433.5353; Number of estimated parameters (K) = 90; Base frequencies (freqA = 0.3569, freqC = 0.2083, freqG = 0.2126, freqT = 0.2222); Kappa = 3.0093 (ti/tv = 1.4994); Proportion of invariable sites (p-inv) = 0.3930; gamma shape distribution = 0.5420.

[a] BIC/AIC difference

[b] BIC/AIC weight

[c] Cumulative BIC/AIC weight

3.4 Results

3.4.1 Detection of *T. parva* genomic DNA

Out of the 921 DNA samples extracted from whole blood, 321 consisting of 166 and 155 samples, from cattle and buffalo respectively tested positive for *T. parva* (Figure 3.1, Table 3.4). However, considering the number of *T. parva* positives detected vis a vis the total number of samples collected, samples from buffalo had the highest percentage of positives (62%) compared to those from cattle (24.7%) (Figure 3.1, Table 3.4). Buffalo samples from East Africa had the highest percentage of positives with Tanzania and Kenya recording 100% and 75% respectively. Whereas South African cattle had the lowest percentage of positives (11.3%) among the cattle samples investigated (Figure 3.1, Table 3.4). All positive samples were used for further analysis except for samples from Uganda cattle and South African buffalo from which 59 and 45 samples were respectively selected.

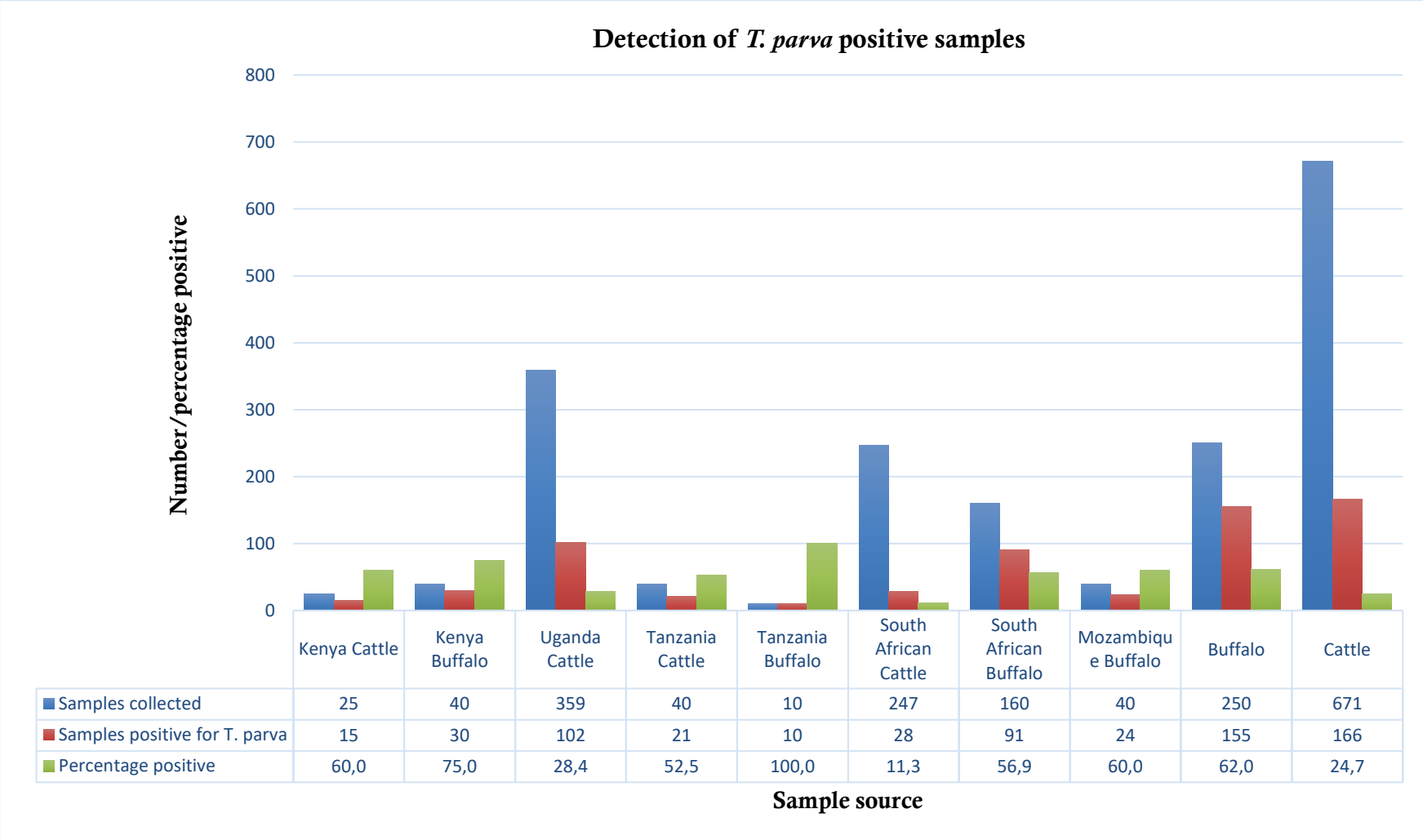

Figure 3.1: The total number of samples collected from buffalo and cattle in eastern and southern Africa, and the number detected positive for *T. parva*.

Table 3.4: *Theileria parva* positive samples detected from samples collected from East and southern Africa.

Country	**Province/District**	**Geographical origin**	**Host**	**Number collected**		**Number positive for *T. parva* (qPCR)**	
				Cattle	Buffalo	Cattle	Buffalo
Kenya	Rift Valley	Nakuru	Cattle	25	-	15	-
		Olpejeta	Buffalo	-	40	-	30
Uganda	Mbarara	Kiruhura	Cattle	137	-	73	-
	Karamoja District	Kaabong	Cattle	99	-	08	-
		Nakapiripirit	Cattle	123	-	21	-
Tanzania	Tanga Region	Tanga	Cattle	20	-	06	-
	Manyara Region	Tarangire National Park	Buffalo	-	10	-	10
		Simanjiro plains	Cattle	20	-	15	-
South Africa	KwaZulu Natal Province	Hluhluwe-iMfolozi Park	Buffalo	-	100	-	76
		uMkhanyakude District	Cattle	223	-	04	-
	Mpumalanga Province	Kruger National Park	Buffalo	-	60	-	15
		Bushbuckridge municipality (Mnisi)	Cattle	24	-	24	-
Mozambique	Sofala Province	Marromeu Game Reserve	Buffalo	-	40	-	24
Total				**671**	**250**	**166**	**155**

3.4.2 Size discrimination of p67 PCR amplicons

PCR amplification of the p67 gene targeting the central variable region was successfully performed on 128 *T. parva* positive DNA samples (Table 3.5). Amplicons of four varying fragment sizes were detected as single or multiple bands of 800 bp, 900 bp, 1000 bp and 1100 bp, representing previously identified allele types 3, 1, 4 and 2, respectively (Nene *et al.*, 1996; Sibeko *et al.*, 2010). Most of the *T. parva* positive DNA samples from buffalo as well as clinical cases of Corridor disease from South Africa generated multiple amplicon profiles representing all four p67 fragment sizes (Table 3.5, Figure 3.2a and b). *Theileria parva* positive DNA samples from cattle in East Africa (Kenya, Uganda and Tanzania) and the non-clinical case from South Africa generated single amplicon profiles consisting of the 900 bp and 1000 bp fragments respectively (Table 3.5, Figure 3.2a and c).

Table 3.5: PCR amplified p67 fragments detected in *T. parva* positive samples from cattle and buffalo.

Country	**[a]Number of samples successfully amplified**		**[b]p67 allele types fragment sizes detected in cattle**				**[b]p67 allele types fragment sizes detected in buffalo**			
			0.9Kb	**1.1Kb**	**0.8Kb**	**1Kb**	**0.9Kb**	**1.1Kb**	**0.8Kb**	**1Kb**
Kenya	Nakuru Cattle	12	n=12	ND	ND	ND				
	Olpejeta Buffalo	22					ND	n=22	n=22	ND
Uganda	Mbarara cattle	24	n=24	ND	ND	ND				
	Karamoja cattle	06	n=06	ND	ND	ND				
Tanzania	Tanga cattle	03	n=03	ND	ND	ND				
	Simanjiro cattle	06	n=06	ND	ND	ND				
	TNP Buffalo	09					ND	n=08	n=09	n=04
South Africa	HIP Buffalo	23					n=04	n=23	n=23	ND
	CD clinical cases	10	n=04	n=06	n=06	n=03				
	Non-clinical *T. parva*-positive	01	ND	ND	ND	n=01				
Mozambique	MGR Buffalo	12					n=11	n=8	n=12	n=3
Total		**128**	**n=55**	**n=6**	**n=6**	**n=4**	**n=15**	**n=61**	**n=66**	**n=7**

[a] CD - Corridor disease, TNP - Tarangire National Park, HIP - Hluhuwe-iMfolozi Park, MGR - Marromeu Game Reserve. [b] ND - "Not Detected" for the respective allele,

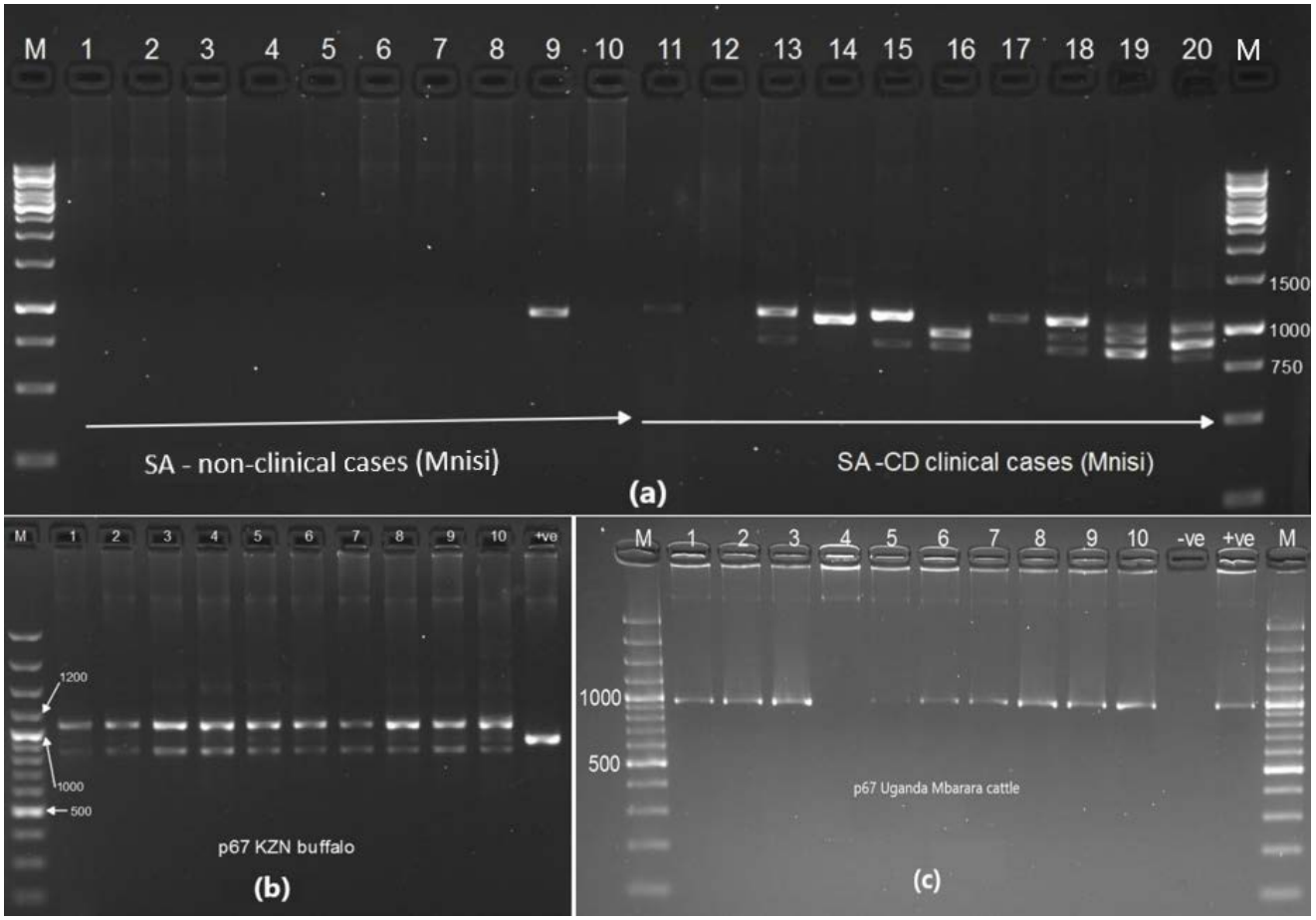

Figure 3.2: p67 PCR amplicons from cattle- and buffalo-derived *T. parva* parasites from East and southern Africa.
(a) p67 PCR amplicons from buffalo-derived *T. parva* parasites from non-clinical *T. parva* positive case and clinical cases of Corridor disease (CD) from South Africa (SA); **(b)** p67 PCR amplicons from buffalo-derived *T. parva* parasites originating from buffalo in Hluhluwe-iMfolozi Park, KwaZulu Natal; **(c)** p67 PCR amplicons from cattle-derived *T. parva* parasites originating from cattle in Mbarara district in Western Uganda. 1kb DNA ladder (#SM0311, ThermoFisher Scientific, USA) was used in (a), 100 bp plus DNA ladder (#SM0321, ThermoFisher Scientific, USA) was used in (b) and (c).

3.4.3 Detection of p67 allele types

A total of 230 p67 sequences were obtained, representing 85 samples selected for cloning (Table 3.6). All the p67 sequences were aligned to previously published and unpublished p67 sequences (n=19) (Table 3.2). Four p67 allele types were confirmed and the sequence identity was similar to the previously published p67 sequences (Figure 3.3). Allele type 1 sequences, which lack the predicted 43-amino-acid insert were common in all *T. parva* parasites from cattle from Kenya, Uganda and Tanzania, which comprised of both co-grazers (cattle that graze with buffalos) and non co-grazers (Figure 3.3). On the contrary, analysis of p67 sequences obtained from *T. parva* parasites from clinical cases of Corridor disease revealed representation of all the four allele types (Figure 3.3). However, *T. parva* parasites from the single sample from non-clinical *T. parva* positive case had only a single type sequence (allele type 4) (Figure 3.3). All the four allele types were detected in all *T. parva* parasites obtained from buffalo. Notably, allele type 1 sequences were only detected in *T. parva* parasites from buffalo from southern Africa but not in buffalo from East Africa.

Table 3.6: p67 allele types from *T. parva* positive samples from cattle and buffalo from East and southern Africa.

Country	**Samples**	**[a]Number of samples cloned**	**[b]Number of colonies sequenced**	**Number of p67 sequences**			
				Allele type 1 **(0.9Kb)**	Allele type 2 **(1.1Kb)**	Allele type 3 **(0.8Kb)**	Allele type 4 **(1Kb)**
Kenya	Nakuru Cattle	10	15	15	-	-	-
	Olpejeta Buffalo	10	38	-	13	25	-
Uganda	Mbarara cattle	10	32	32	-	-	-
	Karamoja cattle	06	15	15	-	-	-
Tanzania	Tanga cattle	03	08	08	-	-	-
	Simanjiro cattle	06	09	09	-	-	-
	TNP Buffalo	09	30	-	06	19	05
South Africa	HIP Buffalo	10	40	02	25	13	-
	CD clinical cases	10	12	03	04	04	01
	Non-clinical *T. parva*-positive	01	04	-	-	-	04
Mozambique	MGR Buffalo	10	27	12	02	11	02
Total		**85**	**230**	**96**	**50**	**72**	**12**

[a] Samples selected from those that were successfully amplified (Table 3.5) where the distribution of p67 fragment sizes was considered. [b] At least one colony was selected from each sample.

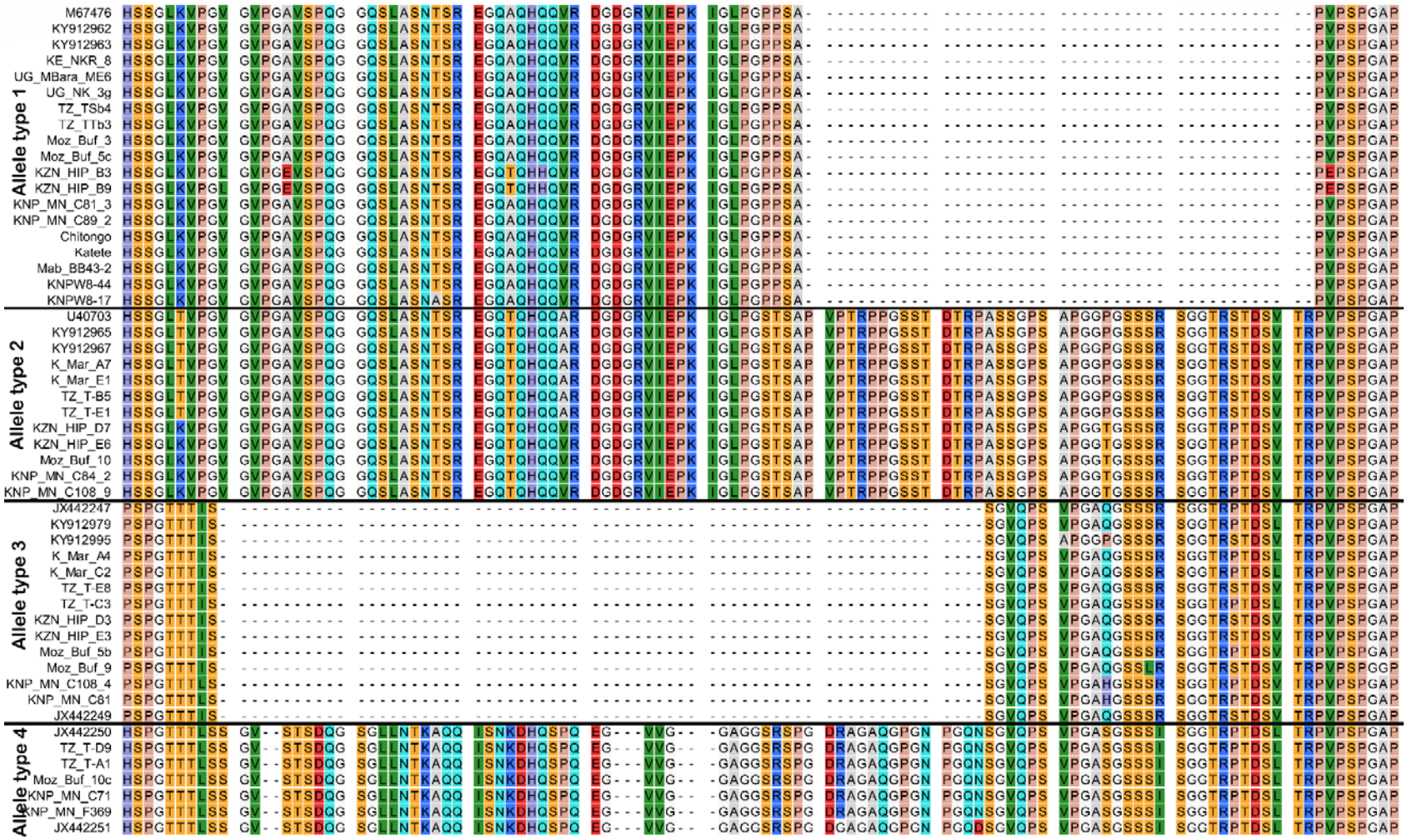

Figure 3.3: An alignment of representative p67 sequences from cattle- and buffalo-derived *T. parva* parasites from Kenya cattle (KE_NKR), Kenya buffalo (K_Mar), Uganda-Mbarara cattle (UG_Mbara), Uganda-Karamoja cattle (UG_Nk), Tanzania-Tanga cattle (TZ_TT), Tanzania-Simanjiro cattle (TZ_TS), Tanzania buffalo (TZ_T), Mozambique buffalo (Moz_buf), KZN buffalo (KZN_HIP), CD clinical cases (KNP_MN_C), Non-clinical *T. parva*-positive case (KNP_MN_F369) and reference sequences.

3.4.4 Sequence variations in the B-cell epitopes

The p67 protein has two B-cell epitopes (TpM12 and AR22.7) recognized by murine monoclonal antibodies within the central variable region (Nene *et al.*, 1999; Obara *et al.*, 2015). Analysis of the predicted protein sequences of the two epitopes in allele types 1, 2, 3 and 4 revealed amino acid substitutions in the buffalo-derived *T. parva* parasites, including those from clinical cases of Corridor disease and the non-clinical *T. parva*-positive case from South Africa. However, both epitopes were conserved in allele type 1 detected from the cattle-derived *T. parva* parasites from Kenya, Uganda and Tanzania. Fourteen amino acid substitutions were detected in TpM12 epitope in all allele types, while one substitution was detected in AR22.7 epitope in allele type 2 (Tables 3.7 - 3.10). In TpM12 epitope, allele type 1 had substitutions at positions 170 and 183 (Table 3.7), allele type 2 at position 183, allele type 3 at positions 170, 173, 176, 177, 178, 180, 181, 182 and 183, and allele type 4 at positions 170, 177, 178, 181, 182 and 183 (Tables 3.8 - 3.10). In AR22.7 epitope, only one substitution was detected on allele type 2 at position 210 (Table 3.8). A comparison of allele type 1 predicted protein sequences obtained from cattle- and buffalo-derived *T. parva* parasites from cattle revealed two subtypes (Table 3.8). Allele type 1 subtype 1 sequences with 100% sequence identity to the Muguga isolate sequence were identified in cattle-derived *T. parva* parasites from Kenya, Uganda and Tanzania, and buffalo-derived *T. parva* parasites from cattle (KY912962) and buffalo (KY912963) in Kenya (Table 3.7). Allele type 1 subtype 2 sequences with amino acid substitutions at positions 170 and 183 in TpM12 epitope, and other unique substitutions within the amplified region were identified in buffalo-derived *T. parva* parasites from clinical cases of Corridor disease and buffalo from southern Africa (Table 3.7). The probability score for the prediction of the effect on the protein function as a result of the substitutions at positions 170 and 183 was 1.00.

Table 3.7: p67 allele type 1 identified in *T. parva* parasites obtained from cattle and buffalo hosts.

Host	[a]Sequence ID/Accession number	[b]Predicted protein sequence	
		TpM12	
Cattle	**M67476**	EDSTLSTDVSPTIPTPVSEEIITPTLQAQTKEEVPPADLSDQVPSNGSDSEEEDNKS-TS	198
	KE_NKR_2	EDSTLSTDVSPTIPTPVSEEIITPTLQAQTKEEVPPADLSDQVPSNGSDSEEEDNKS-TS	198
	KE_NKR_4	EDSTLSTDVSPTIPTPVSEEIITPTLQAQTKEEVPPADLSDQVPSNGSDSEEEDNKS-TS	198
	UG_NK_2e	EDSTLSTDVSPTIPTPVSEEIITPTLQAQTKEEVPPADLSDQVPSNGSDSEEEDNKS-TS	198
	UG_NK_1e	EDSTLSTDVSPTIPTPVSEEIITPTLQAQTKEEVPPADLSDQVPSNGSDSEEEDNKS-TS	198
	UG_Mbara_ME1	EDSTLSTDVSPTIPTPVSEEIITPTLQAQTKEEVPPADLSDQVPSNGSDSEEEDNKS-TS	198
	UG_MBara_ME6	EDSTLSTDVSPTIPTPVSEEIITPTLQAQTKEEVPPADLSDQVPSNGSDSEEEDNKS-TS	198
	TZ_TSb4	EDSTLSTDVSPTIPTPVSEEIITPTLQAQTKEEVPPADLSDQVPSNGSDSEEEDNKS-TS	198
	TZ_TSd5	EDSTLSTDVSPTIPTPVSEEIITPTLQAQTKEEVPPADLSDQVPSNGSDSEEEDNKS-TS	198
	TZ_TTa3	EDSTLSTDVSPTIPTPVSEEIITPTLQAQTKEEVPPADLSDQVPSNGSDSEEEDNKS-TS	198
	TZ_TTb3	EDSTLSTDVSPTIPTPVSEEIITPTLQAQTKEEVPPADLSDQVPSNGSDSEEEDNKS-TS	198
	Chitongo	EDSTLSTDVSPTIPTPVSEEIITPTLQAQTKEEVPPADLSDQVPSNGSDSEEEDNKS-TS	198
	Katete	EDSTLSTDVSPTIPTPVSEEIITPTLQAQTKEEVPPADLSDQVPSNGSDSEEEDNKS-TS	198
	KY912962	EDSTLSTDVSPTIPTPVSEEIITPTLQAQTKEEVPPADLSDQVPSNGSDSEEEDNKS-TS	198
	KNP_MN_C81_3	EDSTLTTDLSPTIPTPVSEEIITPTLQAQTEEEVPPADLSDQVLSDGSDSEEEDNKS-TS	198
	KNP_MN_C89_2	EDSTLTTDLSPTIPTPVSEEIITPTLQAQTEEEVPPADLSDQVLSDGSDSEEEDNKS-TS	198
	KNP_MN_C108_6	EDSTLTTDLSPTIPTPVSEEIITPTLQAQTEEEVPPADLSDQVLSDGSDSEEEDNKS-TS	198
Buffalo	**KY912963**	EDSTLSTDVSPTIPTPVSEEIITPTLQAQTKEEVPPADLSDQVPSNGSDSEEEDNKS-TS	198
	Moz_Buf_2c	EDSTLTTDLSPTIPTPVSEEIITPTLQAQTEEEVPPADLSDQVLSDGSDSEEEDNKS-TS	198
	Moz_Buf_3	EDSTLTTDLSPTIPTPVSEEIITPTLQAQTEEEVPPADLSDQVLSDGSDSEEEDNKS-TS	198
	Moz_Buf_4c	EDSTLSTDVSPTIPTPVSEEIITPTLQAQTEEEVPPADLSDQVLSDGSDSEEEDNKS-TS	198
	Moz_Buf_5c	EDSTLSTDVSPTIPTPVSEEIITPTLQAQTEEEVPPADLSDQVLSDGSDSEEEDNKS-TS	198
	KNPW8-44	EDSTLTTDLSPTIPTPVSEEIITPTLQAQTEEEVPPADLSDQVLSDGSDSEEEDNKS-TS	198
	KNPW8-17	EDSTLTTDLSPTIPTPVSEEIITPTLQAQTEEEVPPADLSDQVLSDGSDSEEEDNKS-TS	198
	Mab_BB43-2	EDSTLTTDLSPTIPTPVSEEIITPTLQAQTEEEVPPADLSDQVLSDGSDSEEEDNKS-TS	198
		AR22.7	
Cattle	**M67476**	SKDEKELKKTLQPGKTSTGETTSGQDLNSKQQQTGVSDLASGSHSSGLKVPGVGVPGAVS	258
	KE_NKR_2	SKDEKELKKTLQPGKTSTGETTSGQDLNSKQQQTGVSDLASGSHSSGLKVPGVGVPGAVS	258
	KE_NKR_4	SKDEKELKKTLQPGKTSTGETTSGQDLNSKQQQTGVSDLASGSHSSGLKVPGVGVPGAVS	258
	UG_NK_2e	SKDEKELKKTLQPGKTSTGETTSGQDLNSKQQQTGVSDLASGSHSSGLKVPGVGVPGAVS	258
	UG_NK_1e	SKDEKELKKTLQPGKTSTGETTSGQDLNSKQQQTGVSDLASGSHSSGLKVPGVGVPGAVS	258
	UG_Mbara_ME1	SKDEKELKKTLQPGKTSTGETTSGQDLNSKQQQTGVSDLASGSHSSGLKVPGVGVPGAVS	258
	UG_MBara_ME6	SKDEKELKKTLQPGKTSTGETTSGQDLNSKQQQTGVSDLASGSHSSGLKVPGVGVPGAVS	258
	TZ_TSb4	SKDEKELKKTLQPGKTSTGETTSGQDLNSKQQQTGVSDLASGSHSSGLKVPGVGVPGAVS	258
	TZ_TSd5	SKDEKELKKTLQPGKTSTGETTSGQDLNSKQQQTGVSDLASGSHSSGLKVPGVGVPGAVS	258

	TZ_TTa3	SKDEKELKKTLQPGKTSTGETTSGQDLNSKQQQTGVSDLASGSHSSGLKVPGVGVPGAVS	258
	TZ_TTb3	SKDEKELKKTLQPGKTSTGETTSGQDLNSKQQQTGVSDLASGSHSSGLKVPGVGVPGAVS	258
	Chitongo	SKDEKELKKTLQPGKTSTGETTSGQDLNSKQQQTGVSDLASGSHSSGLKVPGVGVPGAVS	258
	Katete	SKDEKELKKTLQPGKTSTGETTSGQDLNSKQQQTGVSDLASGSHSSGLKVPGVGVPGAVS	258
	KY912962	SKDEKELKKTLQPGKTSTGETTSGQDLNSKQQQTGVSDLASGSHSSGLKVPGVGVPGAVS	258
	KNP_MN_C81_3	SKDEKELKNTLQPGKTSTGETTSDQDLKSKQQKNGLSDLASGSHSSGLKVPGVGVPGAVS	258
	KNP_MN_C89_2	SKDEKELKNTLQPGKTSTGETTSDQDLKSKQQKNGLSDLASGSHSSGLKVPGVGVPGAVS	258
	KNP_MN_C108_6	SKDEKELKNTLQPGKTSTGETTSDQDLKSKQQKNGLSDLASGSHSSGLKVPGVGVPGAVS	258
Buffalo	**KY912963**	SKDEKELKKTLQPGKTSTGETTSGQDLNSKQQQTGVSDLASGSHSSGLKVPGVGVPGAVS	258
	Moz_Buf_2c	SKDEKELKNTLQPGKTSTGETTSDQDLKSKQQKNGLSDLASGSHSSGLKVPGVGVPGAVS	258
	Moz_Buf_3	SKDEKELKNTLQPGKTSTGETTSDQDLKSKQQKNGLSDLASGSHSSGLKVPGVGVPGAVS	258
	Moz_Buf_4c	SKDEKELKNTLQPGKTSTGETTSDQDLKSKQQKNGLSDLASGSHSSGLKVPGVGVPGAVS	258
	Moz_Buf_5c	SKDEKELKNTLQPGKTSTGETTSDQDLKSKQQKNGLSDLASGSHSSGLKVPGVGVPGAVS	258
	KNPW8-44	SKDEKELKNTLQPGKTSTGETTSDQDLKSKQQKNGLSDLASGSHSSGLKVPGVGVPGAVS	258
	KNPW8-17	SKDEKELKNTLQPGKTSTGETTSDQDLKSKQQKNGLSDLASGSHSSGLKVPGVGVPGAVS	258
	Mab_BB43-2	SKDEKELKNTLQPGKTSTGETTSDQDLKSKQQKNGLSDLASGSHSSGLKVPGVGVPGAVS	258

Sequences identical to Muguga (M67476) form subtype 1 of p67 allele type 1, while those with amino acid substitutions (shaded cyan) form subtype 2.

[a] Reference sequences are bolded (refer to Table 3.2). Sequence IDs are as explained in Figure 3.3.

[b] Amino acid substitutions are shaded cyan. B-cell epitopes (TpM12 and AR22.7) are shaded grey.

Table 3.8: Predicted protein sequence alignment of p67 allele type 2 identified in *T. parva* parasites from cattle and buffalo.

Host	[a]Sequence ID/Accession number	[b]Predicted protein sequence	
		TpM12	
Cattle	**KY912965**	EDSTVSTDVSPTIPTPVSEEIITPTLQAQTKEEVPPADLSDQVPSNGSDSEEEDNKS-TS	198
	KY912967	EDSTVSTDVSPTIPTPVSEEIITPTLQAQTKEEVPPADLSDQVPSNGSDSEEEDNKS-TS	198
	KNP_MN_C108_9	EDSSLGTDVPQSISTPVSEEIITPTLQAQTKEEVPPADLSDQVPSNGSDSEEEDEDS-SL	198
	KNP_MN_C84_2	EDSSLGTDVPQSISTPVSEEIITPTLQAQTKEEVPPADLSDQVPSNGSDSEEEDEDS-SL	198
	KNP_MN_C133	EDSTLSTDVSPTIPTPVSEEIITPTLQAQTKEEVPPADLSDQVSSNGSDSEEEDNKS-TS	198
	KNP_MN_C3	EDSTLSTDVSPTIPTPVSEEIITPTLQAQTKEEVPPADLSDQVSSNGSDSEEEDNKS-TS	198
Buffalo	**U40703**	EDSTVSTDVSPTIPTPVSEEIITPTLQAQTKEEVPPADLSDQVPSNGSDSEEEDNKS-TS	198
	K_Mar_C6	EDSTLSTDISPTIPTPVSEEIITPTLQTQTKEEVPPADLSDQVPSNGSDSEEEDGDS-SL	198
	K_Mar_A7	EDSSLGTDVPQSIPTPVSEEIITPTLQAQTKEEVPPADLSDQVPSNGSDSEEEDGDS-SL	198
	TZ_T-B8	EDSTVSTDVSPTIPTPVSEEIITPTLQAQTKEEVPPADLSDQVPSNGSDSEEEDNKS-TS	198
	TZ_T-B5	EDSTVSTDVSPTIPTPVSEEIITPTLQAQTKEEVPPADLSDQVPSNGSDSEEEDNKS-TS	198
	KZN_HIP_D7	EDSSLGTDVPQSIPTPVSEEIITPTLQAQTKEEVPPADLSDQVPSNGSDSEEEDEDS-SL	198
	KZN_HIP_B4	EDSSLGTDVPQSISTPVSEEIITPTLQAQTKEEVPPADLSDQVPSNGSDSEEEDEDS-SL	198
	Moz_Buf_10	EDSSLGTDVPQSISTPVSEEIITPTLQAQTKEEVPPADLSDQVPSNGSDSEEEDEDS-SL	198
	KY912965	EDSTVSTDVSPTIPTPVSEEIITPTLQAQTKEEVPPADLSDQVPSNGSDSEEEDNKS-TS	198
	KY912967	EDSTVSTDVSPTIPTPVSEEIITPTLQAQTKEEVPPADLSDQVPSNGSDSEEEDNKS-TS	198
		AR22.7	
Cattle	**KY912965**	SKDEKELKKTLQPGKTSTGETTSGQDLNSKQQQTGVSDLASGSHSSGLTVPGVGVPGAVS	258
	KY912967	SKDEKELKKTLQPGKTSTGETTSGQDLNSKQQQTGVSDLASGSHSSGLTVPGVGVPGAVS	258
	KNP_MN_C108_9	GTDERNLKKTLQPGKTSTGETTSDQHLKSKQQQTGVSDLASGSHSSGLKVPGVGVPGAVS	258
	KNP_MN_C84_2	GTDERNLKKTLQPGKTSTGETTSDQDLKSKQQQTGVSDLASGSHSSGLKVPGVGVPGAVS	258
	KNP_MN_C133	SKDEKELKKTLQPGKTSTGETTSGQDLNSKQQQTGVSDLASGSHSSGLKVPGVGVPGAVS	258
	KNP_MN_C3	SKDEKELKKTLQPGKTSTGETTSGQDLNSKQQQTGVSDLASGSHSSGLKVPGVGVPGAVS	258
Buffalo	**U40703**	SKDEKELKKTLQPGKTSTGETTSGQDLNSKQQQTGVSDLASGSHSSGLTVPGVGVPGAVS	258
	K_Mar_C6	GTDERNLKKTLPPGKTSTGETTSDQDLKSKQQQTGVSDLASGSHSSGLTVPGVGVPGAVS	258
	K_Mar_A7	GTDERNLKKTLPPGKTSTGETTSDQDLKSKQQQTGVSDLASGSHSSGLTVPGVGVPGAVS	258
	TZ_T-B8	SKDEKELKKTLQPGKTSTGETTSGQDLNSKQQQTGVSDLASGSHSSGLTVPGVGVPGAVS	258
	TZ_T-B5	SKDEKELKKTLQPGKTSTGETTSGQDLNSKQQQTGVSDLASGSHSSGLTVPGVGVPGAVS	258
	KZN_HIP_D7	GTDERNLKKTLQPGKTSTGETTSDQDLKSKQQQTGVSDLASGSHSSGLKVPGVGVPGAVS	258
	KZN_HIP_B4	GTDERNLKKTLQPGKTSTGETTSDQDLKSKQQQTGVSDLASGSHSSGLKVPGVGVPGAVS	258
	Moz_Buf_10	GTDERNLKKTLQPGKTSTGETTSDQDLKSKQQQTGVSDLASGSHSSGLKVPGVGVPGAVS	258
	KY912965	SKDEKELKKTLQPGKTSTGETTSGQDLNSKQQQTGVSDLASGSHSSGLTVPGVGVPGAVS	258
	KY912967	SKDEKELKKTLQPGKTSTGETTSGQDLNSKQQQTGVSDLASGSHSSGLTVPGVGVPGAVS	258

[a] Reference sequences are bolded (refer to Table 3.2). Sequence IDs are as explained in Figure 3.3.

[b] Amino acid substitutions are shaded cyan. B-cell epitopes (TpM12 and AR22.7) are shaded grey.

Table 3.9: Predicted protein sequence alignment of p67 allele type 3 identified in *T. parva* parasites from cattle and buffalo.

Host	[a]Sequence ID/Accession number	[b]Predicted protein sequence	
		TpM12	
Cattle	**KY912979**	EDSTVSTDVSPTIPTPVSEEIITPTLQAQTKEEVPPASSSD---SEQEDSEENGDNVLKN	198
	KY912995	EDSTLSTDISPTIPTPVSEEIITPTLQTQTKEEVPPASSSD---SEQEDSEENGGDGLKN	198
	KNP_MN_C81	EDSTLSTDVSPTIPTPVSEEIITPTLQGQTKEEVPPASGSD---SEQEDSEENEDDVLKN	198
	KNP_MN_C108_4	EDSTLSTDVSPTIPTPVSEEIITPTLQGQTKEEVPPASGSD---SEQEDSEENEDDVLKN	198
	KNP_MN_C89_1	EDSTLSTDVSPTIPTPVSEEIITSTLQAQTKEEVPPASSSD---SEQEDSEENGNDGLKN	198
	KNP_MN_C91	EDSTLSTDVSPTIPTPVSEEIITSTLQAQTKEEVPPASSSD---SEQEDSEENGNDGLKN	198
Buffalo	**KY912979**	EDSTVSTDVSPTIPTPVSEEIITPTLQAQTKEEVPPASSSD---SEQEDSEENGDNVLKN	198
	KY912995	EDSTLSTDISPTIPTPVSEEIITPTLQTQTKEEVPPASSSD---SEQEDSEENGGDGLKN	198
	JX442249	EDSTVSTDVSPTIPTPVSEEIITPTLQAQTKEEVPPASSSD---SEQEDSEENGNDGLKN	198
	K_Mar_B6	EDSTVSTDVSPTIPTPVSEEIITPTLQAQTKEEVPPASSSD---SEQEDSEENGDNVLKN	198
	K_Mar_D2	EDSTVSTDVSPTIPTPVSEEIITPTLQAQTKEEVPPASSSD---SEQEDSEENGDNVLKN	198
	K_Mar_A4	EDSTVSTDVSPTIPTPVSEEIITPTLQAQTKEEVPPASSSD---SEQEDSEENGDNVLKN	198
	K_Mar_C2	EDSTVSTDVSPTIPTPVSEEIITPTLQAQTKEEVPPASSSD---SEQEDSEENGDNVLKN	198
	TZ_T-A10	EDSTLSTDVSPTIPTPVSEEIITPTLQSQTKEEVPPASSSD---SEQEDSEENGGDGLKN	198
	TZ_T-D7	EDSTVSTDVSPTIPTPVSEEIITPTLQAQTKEEVPPASSSD---SEQEDSEENKDDILKN	198
	TZ_T-E8	EDSTLSTDVSPTIPTPVSEEIITPTLQSQTKEEVPPASSSD---SEQEDSEENGGDGLKN	198
	TZ_T-C3	EDSTVSTDVSPTIPTPVSEEIITPTLQAQTKEEVPPASSSD---SEQEDSEENGDNVLKN	198
	KZN_HIP_D3	EDSTLSTDVSPTIPTPVSEEIITSTLQAQTKEEVPPASSSD---SEQEDSEENGNDGLKN	198
	KZN_HIP_B8	EDSTLSTDVSPTIPTPVSEEIITSTLQAQTKEEVPPASSSD---SEQEDSEENGNDGLKN	198
	KZN_HIP_D10	EDSTLSTDVSPTIPTPVSEEIITSTLQAQTKEEVPPASSSD---SEQEDSEENGNDGLKN	198
	KZN_HIP_E3	EDSTLSTDVSPTIPTPVSEEIITSTLQAQTKEEVPPASSSD---SEQEDSEENGNDGLKN	198
	Moz_Buf_5b	EDSTVSKDVSPTIPTPVSEEIITPTLQAQTKEEVPPASSSD---SEQEDSEENKDDILKN	198
	Moz_Buf_8b	EDSTVSKDVSPTIPTPVSEEIITPTLQAQTKEEVPPASSSD---SEQEDSEENKDDILKN	198
	Moz_Buf_7	EDSTLSTDVSPTIPTPVSEEIITPTLQTQTKEEIPPKSDSE---SEQEDSEENEDDVLKN	198
	Moz_Buf_9	EDSTLSTDVSPTIPTPVSEEIITPTLQTQTKEEIPPKSDSE---SEQEDSEENEDDVLKN	198
	Moz_Buf_1c	EDSTLSTDVSPTIPTPVSEEIITPTLQTQTEEEIPPKSDSE---SEQEDSEENEDDVLKN	198
	Moz_Buf_6c	EDSTLSTDVSPTIPTPVSEEIITPTLQTQTEEEIPPKSDSE---SEQEDSEENEDDVLKN	198
	JX442247	EDSTLSTDVSPTIPTPVSEEIIKPTLHTQTKEEIPPKSDSE---SEQEDSEENEDDVLKN	198
Cattle	**KY912979**	GRTDGKNGDAGAKGVGTDGSSSSNGTHSPKKTETSISQ-----PSPGTTTIS--------	258
	KY912995	GRTDGKNGDAGARGVGTDGSSSSNGIHSPKKTETSISQ-----PSPGTTTIS--------	258
	KNP_MN_C81	GRTDGKKGAAGARGVGTDEFSSSNGAHSPKKSESSINQ-----PSPGTTTLS--------	258
	KNP_MN_C108_4	GRTDGKKGAAGARGVGTDEFSSSNGAHSPKKSESSINQ-----PSPGTTTLS--------	258
	KNP_MN_C89_1	GRTDGKNGAAGARGVGTDGSSSSNGIHSPKKTETSISQ-----PSPGTTTIS--------	258
	KNP_MN_C91	GRTDGKNGAAGARGVGTDGSSSSNGIHSPKKTETSISQ-----PSPGTTTIS--------	258

Buffalo	**KY912979**	GRTDGKNGDAGAKGVGTDGSSSSNGTHSPKKTETSISQ-----PSPGTTTIS--------	258
	KY912995	GRTDGKNGDAGARGVGTDGSSSSNGIHSPKKTETSISQ-----PSPGTTTIS--------	258
	JX442249	GRTDGKNGAAGARGVGTDGSSSSNGIHSPKKTETSISQ-----PSPGTTTIS--------	258
	K_Mar_B6	GRTDGKNGDAGAKGVGTDGSSSSNGTHSPKKTETSISQ-----PSPGTTTIS--------	258
	K_Mar_D2	GRTDGKNGDAGAKGVGTDGSSSSNGTHSPKKTETSISQ-----PSPGTTTIS--------	258
	K_Mar_A4	GRTDGKNGDAGAKGVGTDGSSSSNGTHSPKKTETSISQ-----PSPGTTTIS--------	258
	K_Mar_C2	GRTDGKNGDAGAKGVGTDGSSSSNGTHSPKKTETSISQ-----PSPGTTTIS--------	258
	TZ_T-A10	GRTDGKNGDAGARGVGTDGSSSSNGIHSPKKTETSISQ-----PSPGTTTIS--------	258
	TZ_T-D7	GRTDGKNGDAGAKGVGTDGSSSSNGTHSPKKTETSISQ-----PSPGTTTIS--------	258
	TZ_T-E8	GRTDGKNGDAGARGVGTDGSSSSNGIHSPKKTETSISQ-----PSPGTTTIS--------	258
	TZ_T-C3	GRTDGKNGDAGAKGVGTDGSSSSNGTHSPKKTETSISQ-----PSPGTTTIS--------	258
	KZN_HIP_D3	GRTDGKNGAAGARGVGTDGSSSSNGIHSPKKTETSISQ-----PSPGTTTIS--------	258
	KZN_HIP_B8	GRTDGKNGAAGARGVGTDGSSSSNGIHSPKKTETSISQ-----PSPGTTTIS--------	258
	KZN_HIP_D10	GRTDGKNGAAGARGVGTDGSSSSNGIHSPKKTETSISQ-----PSPGTTTIS--------	258
	KZN_HIP_E3	GRTDGKNGAAGARGVGTDGSSSSNGIHSPKKTETSISQ-----PSPGTTTIS--------	258
	Moz_Buf_5b	GRTDGKNGDAGAKGVGTDGSSSSNGTHSPKKTETSISQ-----PSPGTTTIS--------	258
	Moz_Buf_8b	GRTDGKNGDAGAKGVGTDGSSSSNGTHSPKKTETSISQ-----PSPGTTTIS--------	258
	Moz_Buf_7	GRTDRKNGAAGDRGVGTDGSSSSNGIHSPKKTETSISQ-----PSPGTTTIS--------	258
	Moz_Buf_9	GRTDRKNGAAGDRGVGTDGSSSSNGIHSPKKTETSISQ-----PSPGTTTIS--------	258
	Moz_Buf_1c	GRTDRKNGAAGDRGVGTDGSSSSNGIHSPKKTETSISQ-----PSPGTTTIS--------	258
	Moz_Buf_6c	GRTDRKNGAAGDRGVGTDGSSSSNGIHSPKKTETSISQ-----PSPGTTTIS--------	258
	JX442247	GRTDRKNGTAGARGVGTDGSSSSNGIHSPKKTETSISQ-----PSPGTTTIS--------	258

[a] Reference sequences are bolded (refer to Table 3.2). Sequence IDs are as explained in Figure 3.3.

[b] Amino acid substitutions are shaded cyan. B-cell epitope (TpM12) is shaded grey. Epitope AR22.7 not found on allele type 3.

Table 3.10: Predicted protein sequence alignment of p67 allele type 4 identified in *T. parva* parasites from cattle and buffalo.

Host	[a]Sequence ID/Accession number	[b]Predicted protein sequence
		TpM12
Cattle	KNP_MN_C71	KIAPLSTDVSPTIPTPVSEEIITPTLQAQTKEEVPPASSSD---SEQEDSEENGNDGLKN 198
	KNP_MN_F369	EDSSLGTDVPHSIPTPVSEEIITPTLQAQTNEEVPPADLSD---SEQEDSEENEDDTLKN 198
	KNP_MN_F369_2	EDSSLGTDVPHSIPTPVSEEIITPTLQAQTNEEVPPADLSD---SEQEDSEENEDDTLKN 198
	KNP_MN_F369_1	EDSSLGTDVPHSIPTPVSEEIITPTLQAQTNEEVPPADLSD---SEQEDSEENEDDTLKN 198
	KNP_MN_F369_4	EDSSLGTDVPHSIPTPVSEEIITPTLQAQTNEEVPPADLSD---SEQEDSEENEDDTLKN 198
Buffalo	**JX442251**	EDSTLSTDVSPTIPTPVSEEIITPTLQAQTKEEVPPASSSD---SEQEDSEENGNDGLKN 198
	TZ_T-D9	EDSTLTTDVSPTIPTPVSEEIITPTLQAQTKEEVPPASSSD---SEQEDSEENGDNVLKN 198
	TZ_T-B1	EDSSLGTDVPQSIPTPVSEEIITPTLQAQTKEEVPPASGSD---SEQEDSEENEDDTLKN 198
	TZ_T-B9	EDSSLGTDVPQSIPTPVSEEIITPTLQAQTKEEVPPASGSD---SEQEDSEENEDDTLKN 198
	TZ_T-A1	EDSSLGTDVPQSIPTPVSEEIITPTLQAQTKEEVPPASGSD---SEQEDSEENEDDTLKN 198
	Moz_Buf_10c	EDSSLGTDVPHSIPTPVSEEIITPTLQAQTNEEVPPADLSD---SEQEDSEENEDDTLKN 198
	JX442250	EDSSLGTDVPHSIPTPVSEEIITPTLQAQTNEEVPPADLSD---SEQEDSEENEDDTLKN 198
Cattle	KNP_MN_C71	GRTDGKNGDAGARGVGTDGSSSSNGTHSPKKTETSSNE-----HSPGTTTLSSGV--STS 258
	KNP_MN_F369	GRTDGKNGDAGARGVGTDGSSSSNGTHSPKKTETSSNE-----HSPGTTTLSSGV--STS 258
	KNP_MN_F369_2	GRTDGKNGDAGARGVGTDGSSSSNGTHSPKKTETSSNE-----HSPGTTTLSSGV--STS 258
	KNP_MN_F369_1	GRTDGKNGDAGARGVGTDGSSSSNGTHSPKKTETSSNE-----HSPGTTTLSSGV--STS 258
	KNP_MN_F369_4	GRTDGKNGDAGARGVGTDGSSSSNGTHSPKKTETSSNE-----HSPGTTTLSSGV--STS 258
Buffalo	**JX442251**	GRTDGKNGDAGARGVGTDGSSSSNGTHSPTKTETSSNE-----HSPGTTTLSSGV--STS 258
	TZ_T-D9	GRTDGKNGDAGAKGVGTDGSSSSNGTHSPKKTETSSNE-----HSPGTTTLSSGV--STS 258
	TZ_T-B1	GRTDGKNGDAGARRVGTDGSSSSNGTHSPKKTETSSNE-----HSPGTTTLSSGV--STS 258
	TZ_T-B9	GRTDGKNGDAGARRVGTDGSSSSNGTHSPKKTETSSNE-----HSPGTTTLSSGV--STS 258
	TZ_T-A1	GRTDGKNGDAGARRVGTDGSSSSNGTHSPKKTETSSNE-----HSPGTTTLSSGV--STS 258
	Moz_Buf_10c	GRTDGKNGDAGARGVGTDGSSSSNGTHSPKKTETSSNE-----HSPGTTTLSSGV--STS 258
	JX442250	GRTDGKNGDAGARGVGTDGSSSSNGTHSPKKTETSSNE-----HSPGTTTLSSGV--STS 258

[a] Reference sequences are bolded (refer to Table 3.2). Sequence IDs are as explained in Figure 3.3.

[b] Amino acid substitutions are shaded cyan. B-cell epitope (TpM12) is shaded grey. Epitope AR22.7 not found on allele type 3.

3.4.5 Phylogenetic analysis

Thirty-two (32) randomly selected sequences representing the four p67 allele types and 17 reference sequences (ntax=49), and comprising of 999 characters with 263 informative parsimony sites were analyzed. Estimated proportion of invariant sites (p-inv) was 0.407 with Bayesian effective sample size value >200 (ESS=1739.3) and a PhiTest (p-value) <1 (p=0.0) indicating significant evidence of recombination. Two clades were identified from the best-scoring ML tree with bootstrap values >0.7, and the corresponding posterior probability values >0.9 (Figures 3.4), the Bayesian inference consensus tree (Figure 3.5) and the neighbor-networks (Figure 3.6). Clade 1 comprised of p67 sequences representing allele types 3 and 4, which were obtained from buffalo-derived *T. parva* parasites from buffalo from East and southern Africa, and cattle (Corridor disease cases) from South Africa. Clade 2 comprised of p67 sequences representing allele types 1 and 2 obtained from cattle- and buffalo-derived *T. parva* parasites. Allele type 1 had two separate subgroups; subgroup 2A1 formed by sequences from the cattle-derived *T. parva* parasites exclusively from East Africa, as well as the *T. parva* Muguga isolate (M67476), Zambia_L1 from Zambia, and buffalo-derived *T. parva* parasites from cattle (KY912962) and buffalo (KY912963) from Kenya, and subgroup 2A2 formed by sequences from the buffalo-derived *T. parva* parasites exclusively from clinical cases of Corridor disease in South Africa, and buffalo from Kruger National Park in South Africa and Marromeu Game Reserve in Mozambique (Figure 3.4).

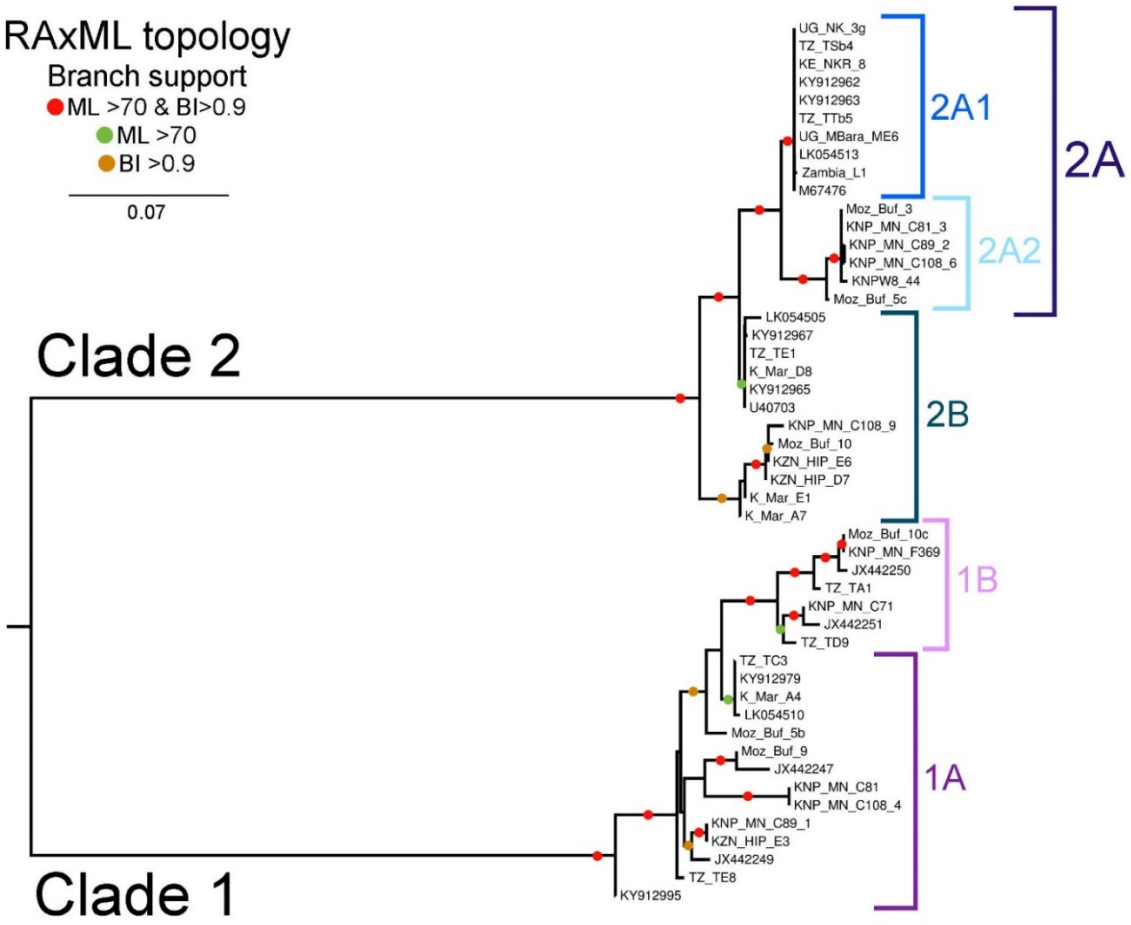

Figure 3.4: Phylogeny recovered from a RAxML analysis for assessing the relationship between *T. parva* parasites from eastern and southern Africa.

Bootstrap support from a maximum likelihood analysis and posterior probabilities from a Bayesian analysis are imposed on the phylogeny as branch support. Clade **1** (allele types 3 and 4), **1A** (allele type 3), **1B** (allele type 4). Clade **2** (allele types 1 and 2), **2A** (allele type 1), **2A1** (subtype of allele type 1 from East African parasites), **2A2** (subtype of allele type 1 from parasites from southern Africa), **2B** (allele type 2).

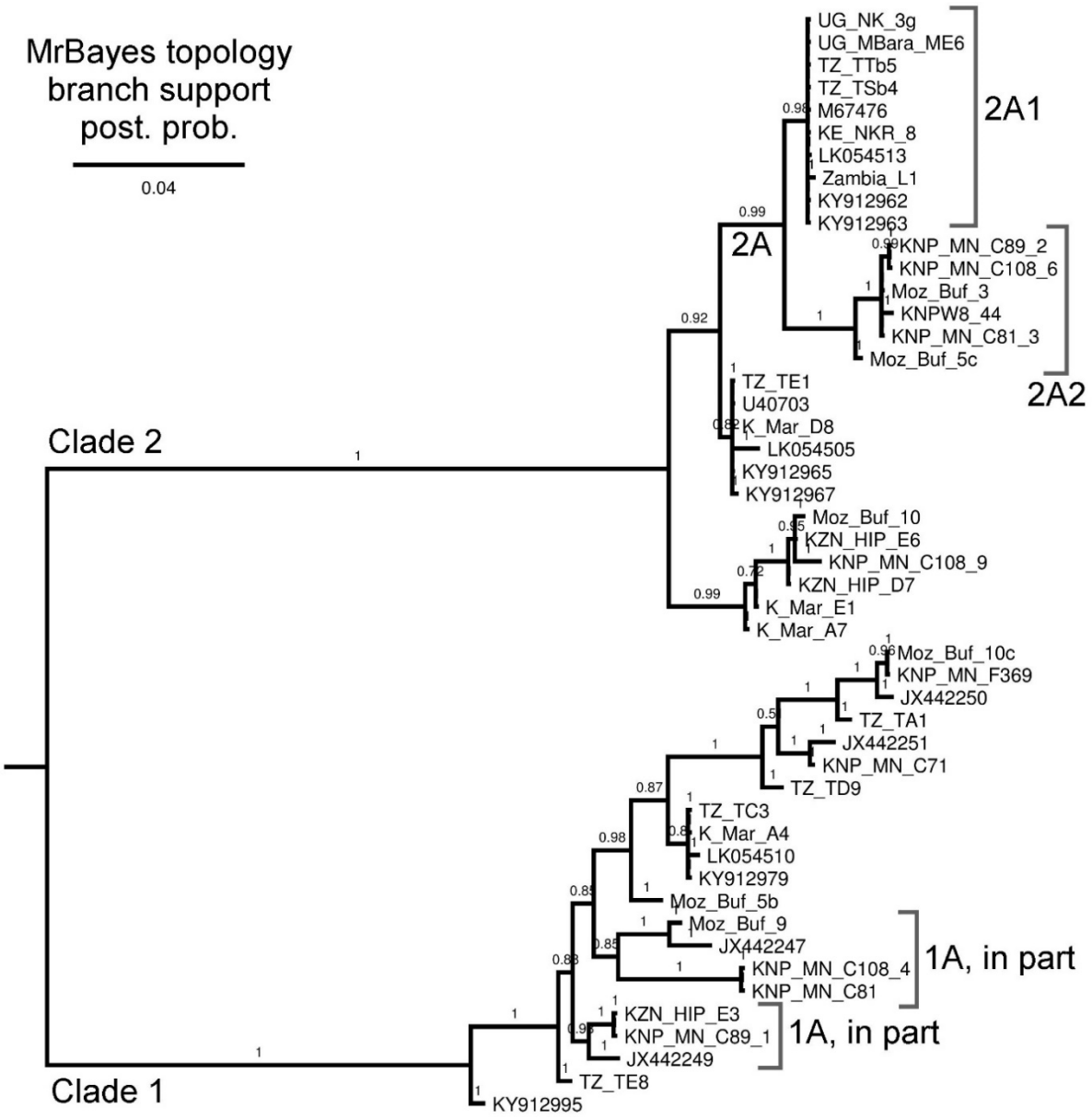

Figure 3.5: Bayesian inference consensus tree generated in MrBayes, (10 000 000 iterations, four chains, sample 1000th tree, HKY+G+I model, 15% discarded as burnin, ESS as per Tracer >200), posterior probabilities displayed on branches.

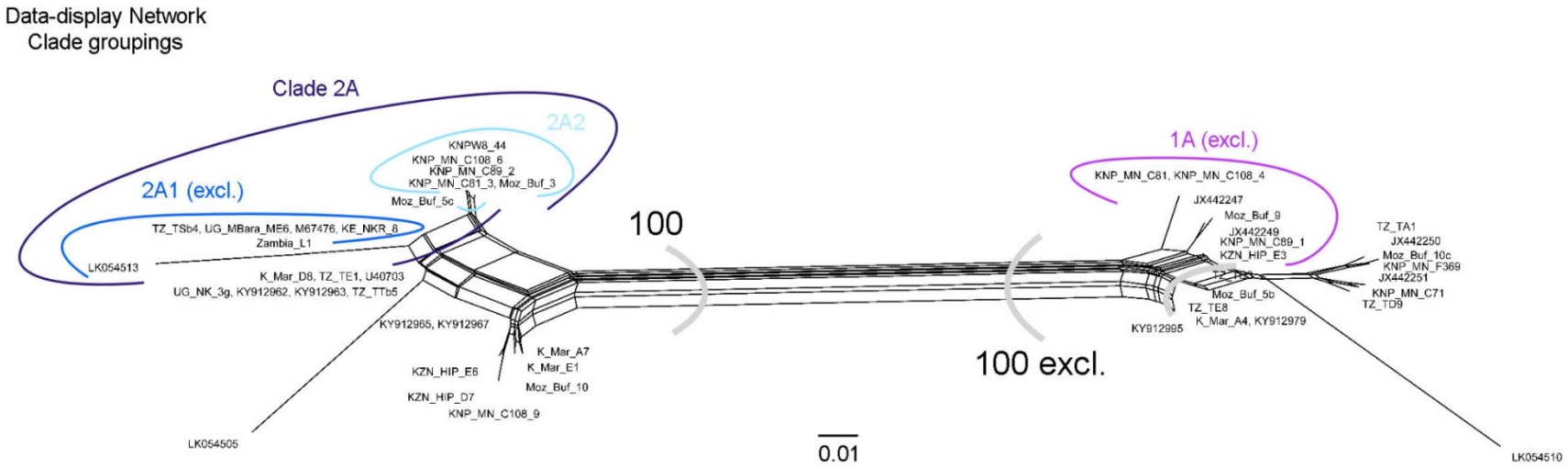

Figure 3.6: Data-display Network generated in Splitstree v. 4 using all characters and uncorrected p-distances. Bootstrap support calculated from 1000 replicates. Network display major groupings as either monophyletic or groupings by exclusion.

3.5 Discussion

The central variable region of the p67 gene has been explored for assessment of the diversity of *T. parva* parasites originating from the buffalo and cattle in East and South Africa (Nene *et al.*, 1999; Sibeko *et al.*, 2010; Obara *et al.*, 2015; Sitt *et al.*, 2019). Due to the similarities in *T. parva* parasites on p67 allele type 1 and the associated disease syndromes in cattle in East and southern Africa, we evaluated cattle- and buffalo-derived parasites from the two regions to identify possible differences. Genomic DNA extracted from 671 and 250 whole blood samples collected from cattle and buffalo respectively from East and southern Africa were screened for the presence of *T. parva* before subsequent analysis based on the p67 gene.

A higher percentage of samples from the buffalo were positive for *T. parva* compared to those from cattle. This was not peculiar since the African buffalo is the natural reservoir of *T. parva* among other haemoparasites that cause diseases in cattle. Cattle from East Africa had a higher percentage of *T. parva* positive cases than South African cattle. In East Africa, cattle-to-cattle transmission of *T. parva* occurs, indicating the possibility of maintenance of a subpopulation of cattle-adapted *T. parva* that can be transmitted to susceptible cattle in the presence of the tick vector. Although cattle-to-cattle transmission of the buffalo-derived *T. parva* was demonstrated through tick-cattle passage in East Africa (Maritim *et al.*, 1992), a similar attempt failed in South Africa (Potgieter *et al.*, 1988), and subsequent attempts for experimental transmission in South Africa have not been successful (Thompson *et al.*, 2008; Mbizeni *et al.*, 2013). Consistently, in South Africa, it has been demonstrated that cattle which recover naturally remain *T. parva*-positive for a short period (~ 1.5 months) (Mbizeni *et al.*, 2013) and do not seem to infect ticks (Thompson *et al.*, 2008; Mbizeni *et al.*, 2013). Such animals may be seropositive with parasitemia levels that are below the real-time PCR detection limit (Sibeko *et al.*, 2008; Mbizeni *et al.*, 2013).

One of the sites of sample collection in the current study was uMkhanyakude district bordering Hluhluwe-iMfolozi Park in KwaZulu Natal, which is among the designated Corridor disease-infected areas. Samples were collected in the month of October, approximately one month after the last transmission period (June-August) of *T. parva* for the year (Mbizeni *et al.*, 2013). It is therefore most likely that some of the cattle which may have been infected with *T. parva* and subsequently recovered had very low parasitemia, and only 4 (1.79%) out of the 223 cattle sampled were positive on real-time PCR. This

therefore explains the low number of *T. parva* positive samples detected in cattle from South Africa compared to East Africa.

Among the samples collected from cattle in East Africa, samples from cattle from Uganda had an overall low percentage of *T. parva* positives due to the low number of samples detected positive for *T. parva* from Kaabong and Nakapiripirit districts in Karamoja subregion of North-Eastern Uganda. Kaabong district borders Kidepo Valley National Park whose buffalo population is negative for *T. parva* (Oura *et al.*, 2011), whereas Nakapiripirit district is in the semi-arid region, and in both districts, the East African Zebu is the dominant cattle breed. Hence, the absence of *T. parva* in the reservoir host, the ecological conditions which do not favour the survival of the tick vector, and the breed factor, could account for the low prevalence of *T. parva* in Karamoja. It is probable that due to cross boundary movement of cattle, the few positive cases detected were carrier cases introduced in Kaabong district from the neighboring states of the Central Equatoria State of South Sudan, and in Nakapiripirit district from the neighboring districts of Amudat and Moroto which border Kenya.

A high percentage of *T. parva* positive cases were detected in cattle from Kiruhura district found in the high potential region of western Uganda. None of the cattle that was positive for *T. parva* showed signs of clinical ECF indicating that they were either subclinical cases or *T. parva* carriers. However, the cycle threshold (Ct) values obtained on qPCR were <30 for majority of the *T. parva* positive samples (76.7%), indicative of an active infections. These cattle co-graze with a *T. parva* positive buffalo population in Lake Mburo National Park, and the ecological conditions favor the survival of the tick vector *Rhipicephalus appendiculatus* (Oura *et al.*, 2011). Non-clinical *T. parva* infections in cattle in East Africa have been attributed to the concept of endemic stability (Kivaria *et al.*, 2004; Gachohi *et al.*, 2012; Kabi *et al.*, 2014) involving an interplay of the breed and ecological factors suitable for the tick vector. The cattle breed population in western Uganda was largely Ankole and its crosses. This breed has some level of tolerance to theileriosis (Paling *et al.*, 1991; Mwai *et al.*, 2015), thus it is possible that this trait has promoted endemic stability to *T. parva* infections in this area.

Analysis of p67 amplicons and sequences from *T. parva* parasites obtained from cattle and buffalo blood samples from East and southern Africa resulted in the identification of the four allele types previously reported (Nene *et al.*, 1996; Sibeko *et al.*, 2010). Buffalo-derived

T. parva parasites from cattle in South Africa were heterologous with all the four p67 allele types present while cattle-derived *T. parva* parasites from East Africa (Kenya, Uganda and Tanzania) were invariant with only allele type 1 identified. Collectively, the previous reports (Nene *et al.*, 1996; Obara *et al.*, 2015) and the results of the current study demonstrate a remarkable reduction in the diversity of the p67 gene in cattle-derived *T. parva* parasites. A conceivable explanation to this could be that a subpopulation of *T. parva* parasites from the buffalo probably possessing allele type 1 have undergone selection and adaptation for cattle-to-cattle transmission and are circulating in the cattle population in eastern Africa. It has been hypothesized that only *T. parva* parasites possessing p67 allele type 1 are maintained by passage between cattle and the tick vector (Nene *et al.*, 1996), further suggesting that infection by cattle-derived *T. parva*, the causative agent of ECF can be maintained in this manner.

Although it is expected that, being the natural reservoir, buffalo will harbor *T. parva* parasites representing all four p67 allele types, not all the four types were detected from buffalo-derived *T. parva* parasites from buffalo from East Africa and South Africa. It is possible that the absence of some of the p67 allele types in buffalo-derived *T. parva* from these areas could be due to limited sample size analyzed and not necessarily the true absence, especially because they have previously been identified (Sibeko *et al.*, 2010; Obara *et al.*, 2015; Sitt *et al.*, 2019), and all four allele types were detected from parasites from cattle from South Africa. On the other hand, the four p67 allele types were detected from *T. parva* parasites from a buffalo population from Mozambique. This herd has been isolated for many years in the Zambezi delta within the East coast of southern Africa. The subtype of p67 allele type 1 detected in parasites from this buffalo population was identical to that identified in clinical cases of Corridor disease from South Africa. Although there is no official report of Corridor disease from this area, the results suggest that should there be contact between the Zambezi delta buffalo herd and naïve cattle in the presence of the tick vector, there is a likelihood that an outbreak of Corridor disease could occur.

The p67 allele types 3 and 4 identified from buffalo-derived *T. parva* parasites were similar to those previously identified (Sibeko *et al.*, 2010). Allele type 3 has been reported to occur concurrently with allele type 1 in an ECF case in Zambia (Sibeko *et al.*, 2010) and therefore hypothesized to be part of the parasites that could possibly be associated with ECF. However, this may not be the case in East Africa since previous studies (Nene *et al.*, 1996; Obara *et al.*, 2015) and the current study demonstrate that cattle-derived *T. parva* strains

from East Africa contain exclusively p67 allele type 1. Other than p67 allele type 3 being identified in parasites from buffalo and Corridor disease cases in the current study, it was also previously detected from cattle challenged with field buffalo-derived *T. parva* following vaccination with a live sporozoite vaccine in Kenya (Obara *et al.*, 2015). Therefore, it appears that allele type 3 would be associated more with Corridor disease than ECF as previously hypothesized (Sibeko *et al.*, 2010). Besides allele type 4 being identified in *T. parva* parasites from buffalo, it was also found in clinical Corridor disease cases, and was the only allele identified in the single sample analyzed from the non-clinical *T. parva*-positive case. Due to the limited number of detectable *T. parva* positive samples from non-clinical *T. parva*-positive cases, the involvement of allele type 4 in *T. parva* infections remains unpredicted and therefore analysis of more samples would provide further information.

Recently, it has been established that the level of polymorphism within the p67 epitopes (TpM12 and AR22.7) is similar in buffalo-derived *T. parva* parasites from the buffalo and cattle, for parasites transmitted naturally from the buffalo to cattle in Kenya, during the early stages of infection (up to 23 days) (Sitt *et al.*, 2019). These epitopes have been shown to be reactive to murine monoclonal antibodies (Nene *et al.*, 1999), and are also a target of the host's B-cell responses (Obara *et al.*, 2015). The analysis of the two epitopes in the current study resulted in the identification of a subtype of the p67 allele type 1 from the buffalo-derived *T. parva* parasites from South Africa, including parasites from clinical cases of Corridor disease, thus differentiating them from parasites possessing allele type 1 from East Africa. Interestingly, the p67 allele type 1 sequences reported from the buffalo-derived *T. parva* parasites from cattle and buffalo from East Africa (Sitt *et al.*, 2019) were identical to the Muguga isolate sequence and all other allele type 1 sequences from cattle-derived *T. parva* parasites, suggesting that there could be a subpopulation of parasites circulating in buffalo that can establish a carrier state more efficiently in cattle. This could be the subpopulation that is circulating in cattle in eastern Africa, and probably responsible for classical ECF.

Since the clinical presentation of Corridor disease in East Africa (Sitt *et al.*, 2015) and South Africa (Mbizeni *et al.*, 2013) is similar, it could be suggested from the current findings that the differences between the parasites involved, based on p67 allele type 1, have no link to disease manifestation, but rather suggestive of restriction of the respective allele type 1 subtypes to the two regions. However, it has recently been established that

the diversity of *T. parva* parasites arose before geographic separation of eastern and South African parasites (Hemmink *et al.*, 2018), and the predominance of p67 allele types in parasites from the two regions vary (Sibeko *et al.*, 2010; Obara *et al.*, 2015; Sitt *et al.*, 2019). Therefore, it is possible that both subtypes of p67 allele type 1 are present in the two regions, but some are underrepresented in one geographical region over the other.

Nonetheless, phylogenetic trees from Maximum Likelihood and Bayesian analyses, as well as neighbor-networks, displayed clustering which separated the subtype of allele type 1 associated with Corridor disease in South Africa from the subtype associated with ECF in East Africa and Corridor disease in Kenya. These findings on the evolution of *T. parva* provide additional evidence on the difference between parasites from the two African regions investigated. Subsequently, the concern about the possibility of re-emergence of ECF in South Africa based on p67 allele type 1 (Sibeko *et al.*, 2010) may be annulled. However, should the buffalo-derived *T. parva* adapt to cattle to establish a long-lasting carrier status as in the case with ECF, then this scenario poses a high risk to the cattle population in South Africa. On the other hand, since cattle are kept separate from buffalo in South Africa, buffalo to cattle transmission of *T. parva* and other pathogens is greatly minimized. Furthermore, due to the strict control of movement of buffalo in South Africa by the South African Directorate of Animal Health (Veterinary Procedural Notice: Buffalo Disease Risk Management), Corridor disease remains a controlled disease.

When we compared the variation of *T. parva* parasites from East and southern Africa based on the two immunogenic p67 epitopes (TpM12 and AR22.7), the predicted protein sequences of the subtype of p67 allele type 1 identified from the buffalo-derived *T. parva* parasites from southern African had two amino acid substitutions at positions 2 and 15 of TpM12 epitope (T**E**EEVPPADLSDQV**L**) and none in AR22.7. Although the predicted effects of the substitutions in TpM12 were tolerant (probability scores = 1.00), further investigation will be necessary to ascertain whether these variations affect recognition by murine monoclonal antibodies, and eventually the effectiveness of the current p67 recombinant vaccine, which is dependent on the recognition of these epitopes and others (Nene *et al.*, 1999).

3.6 Conclusion

We report identification of a subtype of p67 allele type 1, unique to buffalo-derived *T. parva* parasites from southern Africa. Corridor disease samples from South Africa were obtained from the designated Corridor disease infected areas bordering Kruger National Park in Mpumalanga province and Hluhluwe-iMfolozi Park in KwaZulu Natal province. Due to very low parasitemia, we were not able to generate p67 amplicons from the four cattle samples from uMkhanyakude district that were positive for *T. parva* based on real-time PCR. Therefore, to further support the results of the current study, it would be necessary to analyze more samples from Corridor disease cases in KwaZulu-Natal where the disease is likely to occur. Nonetheless, the results of the current study form preliminary evidence of the difference between allele type 1 *T. parva* parasites from eastern and southern Africa.

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CHAPTER 4

Antigenic diversity of schizont antigen genes in cattle- and buffalo-derived *Theileria parva* from East and southern Africa

4.1 Abstract

Immunity to *T. parva* infections in cattle is mainly cell-mediated where cytotoxic T lymphocytes (CTL) play a major role. Cytotoxic T lymphocytes have T-cell receptors containing a glycoprotein (CD8) which recognize *T. parva* peptides bound to MHC Class I molecules. *Theileria parva* peptides are encoded by epitope regions within the schizont antigen (TpAg) genes designated Tp1 to Tp10 and their diversity has been shown to contribute to the evasion of the host's immune system by *T. parva* parasites. Due to limited information on the diversity of schizont antigen genes in *T. parva* field parasites, we assessed the diversity of ten schizont antigen genes in cattle- and buffalo-derived *T. parva* field parasites from eastern and southern Africa. Gene regions containing the CD8+ T-cell epitopes of TpAg genes were amplified from genomic DNA extracted from blood of *T. parva* positive samples, cloned and sequenced. Analysis of sequence data revealed a greater diversity in buffalo-derived than cattle-derived *T. parva* parasites, with parasites from southern Africa being more diverse. Missense epitope substitutions were identified in Tp1, Tp2, Tp4, Tp5 and Tp9 where Tp2 and Tp5 had the most and least variants respectively. All substitutions in Tp2 (^{40}DGFDRDALF^{48}), Tp6, Tp7 and Tp8 were synonymous. The results show that immunogenic epitopes $Tp1_{214\text{-}224,}$ $Tp2_{49\text{-}59}$ and $Tp2_{50\text{-}59}$, which dominate CTL response, are considerably conserved, hence considered promising subunit vaccine candidates. Overall, the TpAg genes variations display two subpopulations of *T. parva* where one group has adapted to the cattle host for cattle-to-cattle transmission in East Africa, while the other subpopulation comprising of buffalo-derived parasites circulates in the wildlife reservoir as well as in cattle in southern Africa.

4.2 Introduction

Theileria parva has a complex life cycle that occurs in two phases; the asexual phase involving the intra-lymphocytic transforming schizonts in the mammalian host, and the sexual phase in the tick vector (reviewed in Nene *et al.*, 2016). Several control strategies have been devised which are targeted at breaking the life cycle of the parasite. Such strategies include tick control, chemotherapy and immunization of cattle with live *T. parva* sporozoites in East Africa.

In South Africa, Corridor disease is controlled by limiting buffalo-cattle interaction through control of movement of buffalo from endemic to non-endemic areas, and separation of livestock from wildlife by fencing game parks. In addition, chemotherapy and use of live vaccines are prohibited to avoid carrier status. In East Africa, the infection and treatment method (ITM) vaccine based on live sporozoites has been widely adopted for control of ECF and has been shown to confer protection against cattle-derived *T. parva* parasites (Radley *et al.*, 1975; Di Giulio *et al.*, 2009) but not against buffalo-derived *T. parva* parasites (Sitt *et al.*, 2015). Immunity induced by the live vaccine is cell-mediated, where CD8+ cytotoxic T lymphocytes recognize *T. parva* peptides presented by the MHC Class I molecule (Morrison *et al.*, 1987; McKeever *et al.*, 1994; Taracha *et al.*, 1995). These peptides are encoded by antigen genes found on the lymphocyte-transforming schizont stage of *T. parva* and have been designated Tp1-Tp10 (for the purpose of this study, they will be referred to as TpAg) (Gardner *et al.*, 2005; Graham *et al.*, 2006), and their CD8+ T-cell epitopes defined (Akoolo *et al.*, 2008; Graham *et al.*, 2008; Nene *et al.*, 2012). Analyses of TpAg-encoding genes have revealed more sequence variations in buffalo-derived than cattle-derived *T. parva* parasites, as well as more polymorphism in the CD8+ T-cell epitopes in Tp1, Tp2 and Tp9 compared to other TpAg genes in parasites originating from the buffalo (Pelle *et al.*, 2011; Hemmink *et al.*, 2016; Sitt *et al.*, 2018; Kerario *et al.*, 2019).

Tp1 and Tp2 have been shown to dominate cytotoxic T lymphocytes responses (MacHugh *et al.*, 2009; Connelley *et al.*, 2011; Connelley *et al.*, 2016), and the role of the other TpAg genes during an immune response still remains unclear (Morrison *et al.*, 2015). Even though detailed studies on the diversity of these genes have been done, they focused on evaluation of *T. parva* cell lines and field parasites from cattle and buffalo from East Africa (Pelle *et al.*, 2011; Hemmink *et al.*, 2016, 2018; Salih *et al.*, 2017; Sitt *et al.*, 2018; Kerario *et al.*, 2019), and a single buffalo population from South Africa (Hemmink *et al.*, 2018).

Therefore, a comprehensive analysis of the diversity of these genes in *T. parva* field parasites especially from ECF and Corridor disease cases remains significant. This is important in establishing the differential variations on schizont antigen genes in cattle- and buffalo-derived *T. parva* parasites for two reasons; (1) identification of additional parasite types for possible inclusion in the live vaccine for broader protection, and (2) identification of promising TpAg candidates for development of a recombinant vaccine that can confer protection against most field strains. This study therefore sought to establish a comparative diversity of CD8+ T-cell epitopes of TpAg genes in cattle- and buffalo-derived *T. parva* field parasites from East and southern Africa.

4.3 Materials and Methods

4.3.1 Sample collection and detection of *T. parva*

Collection of cattle and buffalo blood samples and detection of *T. parva* was done as described in Chapter three of this thesis.

4.3.2 PCR amplification of the schizont antigen genes

The primers used for PCR amplification of the targeted regions of TpAg genes (Tp1, Tp2, Tp3, Tp4, Tp5, Tp6, Tp7, Tp8, Tp9 and Tp10), annealing temperatures and the expected amplicon sizes are shown in Table 4.1. A total of 185 *T. parva* positive DNA samples (Table 4.5), representing all study sites were randomly selected for PCR amplification of the 10 TpAg genes listed in Table 4.1. At least 50 ng of extracted DNA was used as a template in a PCR reaction of 12.5 µl prepared using 2X Phusion Flash High-Fidelity commercial master mix (ThermoFisher Scientific, USA). Each amplification reaction consisted of Phusion Flash II DNA polymerase, 2X Phusion Flash buffer, 0.0024 µmol of each dNTP, 0.024 µmol $MgCl_2$, and 10 pmol of each primer. The amplification conditions used were as follows; an initial denaturation at 98 °C for 10 s, followed by 30 cycles of denaturation at 98 °C for 1 s, annealing at temperatures shown in Table 4.1 for 5 s and extension at 72 °C for 10 s, and then one cycle for the final extension step at 72 °C for 1 min. Samples that failed to amplify in the primary reaction, they were re-amplified in a secondary PCR using 0.5 µl of the primary PCR product as DNA template under the same amplification conditions. However, the number of amplification cycles were reduced to 20. PCR products were analyzed by agarose gel electrophoresis using a 2% gel containing ethidium bromide in 1X TAE running buffer.

Table 4.1: Details of the targeted regions, oligonucleotide primer sequences and PCR parameters for amplification of TpAg genes.

Antigen gene	Region targeted for amplification	Primer sequences (Hemmink *et al.*, 2016)	Annealing temperature (°C)	Expected amplicon (bp)
Tp1	CD8+ T-cell epitope	F: CTGGTGTACAATTTGGTGGG R: AACTTNMCTTCTTGCGAACC	50	428
Tp2	Full-length ORF	F: ATGAAATTGGCCGCCAGATTA R: CTATGAAGTGCCGGAGGCTTC	65	525
Tp3	CD8+ T-cell epitope	F: AGCAGATTTCACTCAAGCTGC R: TCCCCCAGAACATTAAACGG	65	407
Tp4	CD8+ T-cell epitope	F: GCAACACAATACTTTGCAGG R: CCTCAAACACWCCACAAGTTCC	55	424
Tp5	CD8+ T-cell epitope	F: GTATGCTCGGTAATGGCAG R: GATTTTGGTCGCTTCAGGC	65	347
Tp6	CD8+ T-cell epitope	F: CGTCCAATAATTTACGATGTGAG R: GCTTAAGTGGGTTAAGGAGACA	55	326
Tp7	CD8+ T-cell epitope	F: TGAAGAAGGACGACTCGCAC R: TCCTCGTCAGTGACGTCGG	65	292
Tp8	CD8+ T-cell epitope	F: ATCCACAACCAAGTGCCCAG R: TGCTATTGCGAGTCAACAG	65	305
Tp9	Full-length ORF	F: ATGAATGTTCTAACTACTGG R:TTATTGTTTTGTCCATGGTTTATTACG	53	900
Tp10	CD8+ T-cell epitope	F: GGTCGTCTGACAATAACC R: CTAMCATGTAAATCCAGC	50	314

4.3.3 Cloning and sequencing of CD8+ T-cell antigen genes

Purification of the PCR products and cloning for all TpAg genes was done as described in Chapter three. Up to 10 samples (purified PCR products) were selected for cloning of each TpAg gene. The specific number of samples selected for each geographic and host group (sample group) are shown in Table 4.2 for Tp1, Tp2, Tp3, Tp4, Tp5, Tp6, Tp7, Tp8 and Tp10, and Table 4.3 for Tp9. Clones from Tp1 - Tp8, and Tp10 genes were prepared for high-throughput sequencing, and amplicons from Tp9 were prepared for Sanger sequencing.

4.3.3.1 High-throughput sequencing of Tp1 - Tp8, and Tp10 genes

Below are the three stages followed for the processing of selected TpAg genes colony PCR products for high-throughput sequencing.

4.3.3.1.1 Preparation of pools of colony PCR products

The preparation of pools of PCR products from recombinant clones is presented in a schematic diagram in Figure 4.1. Although Figure 4.1 is based on the processing of two TpAg clones for two samples from one sample group (for the sake of simplicity), all TpAg (n=9) clones for all samples in each group (n=12, formulated based on geographic location and host or origin of sample) were processed in this manner. Thus, for each sample group (Table 4.2), the first pooling resulted in nine pools where each pool represented each TpAg gene, and the second pooling resulted in one pool which included the nine TpAg gene products.

Up to 10 clones from each sample for each of the TpAg genes (Tp1, Tp2, Tp3, Tp4, Tp5, Tp6, Tp7, Tp8 and Tp10) were analyzed by colony PCR. For the first pooling, colony PCR products (hereafter referred to as samples) were purified using QIAquick® PCR Purification Kit (Qiagen, Germany) following manufacturer's protocol except that the final elution was done using low salt Tris-EDTA (TE) buffer (10mM Tris, 0.1mM EDTA, pH 8.0) (Invitrogen, Carlsbad USA). In order to create equimolar pools (containing samples with equimolar concentrations) for each TpAg gene, the samples were quantified using Qubit 2.0 fluorometer (Invitrogen, Carlsbad USA). Appropriate volumes (determined based on the DNA concentrations) of the samples were pooled, resulting in nine pooled samples representing the nine TpAg genes for all groups except four (Kruger National Park buffalo group and the three Tanzania groups) (Table 4.2 and Figure 4.1) due to inadequate quantities of DNA. Hence, seven pools were obtained from the Kruger National Park (KNP) buffalo group, and two pools from each of the three Tanzania groups (Table 4.2). The pooled samples were resolved in 2% agarose gel electrophoresis using 1X TAE as the running buffer, and the DNA concentration was determined using Qubit 2.0 fluorometer (Invitrogen, Carlsbad USA). For the second pooling, equimolar concentrations of the pooled samples (from the first pooling) were further pooled together for each sample group, resulting in one sample representing all the TpAg genes (except KNP and Tanzania groups as stated above) (Table 4.2 and Figure 4.1). Subsequently, the DNA concentrations of the final twelve samples (representing the 12 sample groups)

(Table 4.2) were determined before library preparation. The minimum recommended concentration for Ion Torrent sequencing is 10ng/µl.

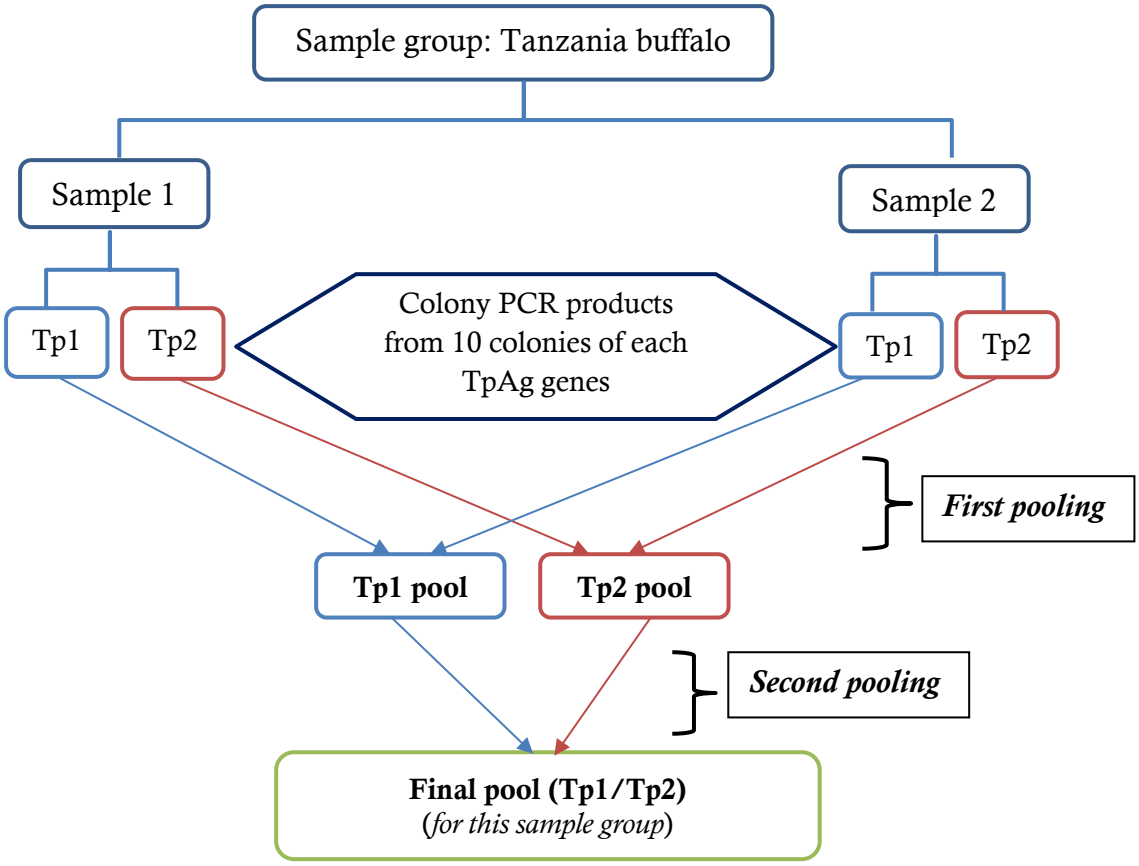


Figure 4.1: A schematic diagram showing how each of the 12 pools representing the different sample groups, each consisting of colony PCR products from clones of the nine TpAg genes were prepared.

Table 4.2: The number of PCR products selected for cloning per gene and colony PCR products selected for preparing sample pools for high-throughput sequencing.

Sample group (n=12)	Amplicons selected for cloning	1st Pooling		2nd Pooling	
		Colony PCR products selected for pooling	[c]Pools per group after 1st pooling	[d]Pools per group after 2nd pooling	Final Concentration (ng/µl)
Kenya buffalo	10	10-20	9	1	75.2
Kenya cattle	10	10-20	9	1	70.8
Uganda-Mbarara cattle	10	10-20	9	1	68.0
Uganda-Karamoja cattle	10	10-20	9	1	66.4
Tanzania buffalo[a]	08	10-20	2	1	81.0
Tanzania-Tanga cattle[a]	04	05-10	2	1	35.2
Tanzania-Simanjiro cattle[a]	10	10-20	2	1	90.8
Mozambique buffalo	10	10-20	9	1	76.8
KwaZulu Natal (KZN) buffalo	10	10-20	9	1	63.2
Corridor disease (CD) clinical cases	09	10-15	9	1	80.0
Non-clinical *T. parva*-positive case	01	05-10	9	1	66.8
Kruger National Park (KNP) buffalo[b]	08	10-20	7	1	56.4
Total	**100**		**85**	**12**	

[a] Only analyzed for Tp1 and Tp2.
[b] Not analyzed for Tp3 and Tp8.
[c] The number of pools represent the number of TpAg genes in the specific sample groups.
[d] Each pool combines all TpAg genes in the specific sample groups.

4.3.3.1.2 Library preparation

Library preparation was done at the Ion Torrent Sequencing Facility, Department of Plant and Soil Sciences, University of Pretoria, South Africa. Since Ion Torrent sequencing has a size limit of 400 bp and the sizes of the samples ranged between 450-650 bp, a fragmentation stage was included in the library preparation. The samples were fragmented using the Ion Xpress™ Plus Fragment Library kit (ThermoFisher Scientific, USA) according to the Ion Xpress™ Plus gDNA fragment library preparation protocol (Publication number MAN0009847). This was followed by an initial clean-up, ligation of adapters and barcodes (for specific sample groups), and final clean-up following the Ion Xpress™ Plus gDNA fragment library preparation protocol. Subsequently, each of the 12 samples (pools) corresponding to each sample group had a unique barcode. Size selection of the fragmented samples was done using the E-Gel™ SizeSelect™ II Agarose Gels, 2% (Invitrogen, Carlsbad USA). The libraries were then PCR amplified, cleaned-up and quantified using Qubit 2.0 fluorometer (Invitrogen, Carlsbad USA) following the manufacture's protocol.

4.3.3.1.3 Ion Proton™ and Ion S5™ Systems sequencing

Deep sequencing was done on the Ion Proton™ and Ion S5™ Systems (ThermoFisher Scientific, Carlsbad, USA) at the Central Analytical Facilities, Stellenbosch University, South Africa. On the Ion Proton™ System, template amplification was completed using the Ion PI™ Hi-Q™ Chef Kit (Part No. A27198) (ThermoFisher Scientific, Carlsbad, USA). Subsequent sequencing was done on the Ion Torrent Proton using the Ion PI™ Hi-Q™ Sequencing 200 Solutions (Part No. A26430) with the Ion PI™ Chip Kit v3 (Part No. A26770) (ThermoFisher Scientific, Carlsbad, USA). Template amplification and sequencing was performed as described in the manufacturer's protocol (MAN0010967, Rev B.0). On the Ion S5™ System, template amplification was completed using the Ion 520™ & Ion 530™ Chef-Kit (Part No. A30010) (ThermoFisher Scientific, Carlsbad, USA). Subsequent sequencing was done on the Ion GeneStudio™ S5 system using the Ion S5™ Sequencing Solutions (Part No. A27767) and Ion S5™ Sequencing Reagents (Part No. A27768) with the Ion 530™ Chip Kit (Part No. A27764) (ThermoFisher Scientific, Carlsbad, USA). Template amplification and sequencing was performed as described in the manufacturer's protocol (MAN0010846, Rev D.0).

4.3.3.2 Sanger sequencing of the Tp9 gene

Approximately 04 - 10 clones were selected per sample group (defined by geographic and/or host origin) for Tp9 sequencing (Table 4.3). The selected clones were analyzed by colony PCR to confirm recombinants, following which the colony PCR products were purified using QIAquick® PCR Purification Kit (Qiagen, Germany) as described by the manufacturer. The purified PCR products were submitted for bidirectional sequencing using pJET1.2 primers on ABI 3500XL Genetic Analyzer, POP7™ (ThermoFisher Scientific, USA) at INQABA Biotechnologies, South Africa.

Table 4.3: The number of samples amplified, PCR products selected for cloning and clones selected for Tp9 sequencing.

Country	Sample group	Number of samples successfully amplified	Number of samples selected for cloning	Number of clones selected for sequencing per group
Kenya	Cattle	10	08	10
	Buffalo	13	08	10
Uganda	Mbarara cattle	14	10	10
	Karamoja cattle	12	10	10
Tanzania	Tanga cattle	04	04	06
	Simanjiro cattle	09	09	10
	Buffalo	07	07	06
South Africa	KZN buffalo	15	10	10
	CD clinical cases	08	08	10
	Non-clinical *T. parva*-positive case	01	01	04
Mozambique	Buffalo	11	08	10
Total		**104**	**83**	**96**

4.3.4 Sequence data analysis

4.3.4.1 Analysis of Tp1 - Tp8, and Tp10 sequence data

A total of ~20 million sequence reads in FASTQ format were obtained from all sites with each site contributing ~1.7 million reads. Adapter and barcode sequences, and pJET1.2 primers were trimmed off using FLEXBAR (Dodt *et al.*, 2012) and CutPrimers (Kechin *et al.*, 2017) respectively. The quality of sequence reads was then checked using FastQC version 0.11.8 (Andrews, 2014) where ends of sequence reads with a per base sequence

quality score <20 were trimmed off using TrimGalore Version 0.5.0, a wrapper tool featured in FastQC (Andrews, 2014). Trimmed sequences were then mapped to the reference sequences (Table 4.4) using Bowtie2 version 2.3.4.3 (Langmead and Salzberg, 2012) where Binary Alignment Map (BAM) files per gene per group were generated. In order to visualize regions of sequence reads with SNPs, the alignment maps were viewed using Integrative Genomics Viewer (IGV) version 2.4.15 (Robinson *et al.*, 2011). The BAM files were then converted to FASTA files using SAMtools (Li *et al.*, 2009), for use in subsequent analyses.

For identification of nucleotide or amino acid variations within the antigenic epitopes, the sequence reads in FASTA format, for each TpAg gene, were aligned to the respective genebank files (.gb file) of the reference sequences (Table 4.4) using NextGENe version 2.4.2.3 (https://softgenetics.com/NextGENe.php). In the NextGENe analysis setup, the overall matching base percentage was set at 85% and sequence reads within 100-500 bp size range were selected for alignment. In order to align reads that contain indels, a rigorous alignment was performed and the overall mutation score set at ≥ 12.00, a value equivalent to the statistical 95% confidence, indicating that the mutation call is true. Since TP1, Tp2, Tp4, Tp5, Tp7 and Tp8 have defined antigenic epitopes (Table 4.4), the epitope regions were set as the "regions of interest" (ROI) (Table 4.4) in the mutation report settings. Tp3, Tp6 and Tp10 do not have defined antigenic epitopes and therefore, the known antigenic regions (Hemmink *et al.*, 2016) of the aligned sequence reads were set as ROI (Table 4.4). The ROI were defined by the first and last nucleotides of each epitope or antigenic region in a text file. All other alignment and mutation report settings were used as default on NextGENe or adjusted according to NextGENe user's manual. The mutation reports generated, which reflected the ROI only, were used to tabulate the mutation details of each TpAg gene. Venn diagrams (Bardou *et al.*, 2014) and bar charts were used for comparative analysis of the epitope variants identified on TpAg genes in cattle- and buffalo-derived *T. parva* parasites in East and southern Africa.

4.3.4.2 Analysis of Tp9 gene sequences

Raw nucleotide sequences were confirmed to be Tp9 sequences by sequence similarity analysis using the Basic Local Alignment Search Tool (BLAST) executed on the National Centre for Biotechnology Information (NCBI) platform. Sequence assembly, editing and translation was done using CLC Main Workbench version 8.0 (Qiagen, Germany). Multiple sequence alignment of consensus sequences together with Tp9 reference

sequence (Table 4.4) was done using MAFFT version 7 (Katoh and Standley, 2013) applying the default parameters. Aligned sequences/sequence matrices were viewed, edited manually and truncated using MEGA version 7 (Kumar *et al.*, 2016). Phylogenetic analysis of 37 selected predicted protein sequences based on the distribution of the epitope variants was performed using MEGA version 7 (Kumar *et al.*, 2016). Evolutionary history was inferred using Maximum Likelihood applying the default substitution model (General Reverse Transcriptase model), and phylogeny tested using 100 bootstrap replicates, where a bootstrap support ≥0.7 (70%) was considered significant.

Table 4.4: Details of the reference sequences used in the alignment of respective TpAg genes sequence reads.

Antigen Gene	Reference Sequence/Locus Tag	Accession number	[a]Antigen annotation	CD8+ T-cell Epitope	[b]ROI (defined by reference nucleotide positions)	Epitope/gene Reference
Tp1	*T. parva* Muguga (TP03_0849)	XM_757880	Hypothetical	$Tp1_{214-224}$VGYPKVKEEML	Incl:779-811 Excl:1-778;812-1040	Graham *et al.*, 2008
Tp2	*T. parva* Muguga (TP01_0056)	XM_760490	Hypothetical	$Tp2_{27-37}$SHEELKKLGML	Incl:207-239, Excl:1-206; 240-245	Graham *et al.*, 2008
				$Tp2_{40-48}$DGFDRDALF	Incl:246-272	Nene *et al.*, 2012
				$Tp2_{49-59}$ KSSHGMGKVGK	Incl:273-305	Graham *et al.*, 2008
				$Tp2_{50-59}$SSHGMGKVGK	Incl:276-305, Excl:306-413	Graham *et al.*, 2008
				$Tp2_{96-104}$FAQSLVCVL	Incl:414-440	Graham *et al.*, 2008
				$Tp2_{98-106}$QSLVCVLMK	Incl:420-446, Excl:447-539	Graham *et al.*, 2008
				$Tp2_{138-147}$KTSIPNPCKW	Incl:540-569 Excl:570-675	Akoolo *et al.*, 2008
Tp3	*T. parva* Muguga (TP01_0868)	XM_761296	Hypothetical	N/A	Incl:280-356;360-550;590-650, Excl:1-279;357-359;551-589;651-730	Gardner *et al.*, 2005
Tp4	*T. parva* Muguga (TP03_0210)	XM_758135	eta-TCP1	$Tp4_{328-336}$TGASIQTTL	Incl:982-1008 Excl:1-981;1009-1060	Graham *et al.*, 2008
Tp5	*T. parva* Muguga (TP02_0767)	XM_760241	eIF-1A	$Tp5_{87-95}$SKADVIAKY	Incl:318-344 Excl:1-317;345-435	Graham *et al.*, 2008
Tp6	*T. parva* Muguga (TP01_0188)	XM_760622	Prohibitin	N/A	Incl: 220-550 Excl: 1-219;551-625	Gardner *et al.*, 2005
Tp7	*T. parva* Muguga (TP02_0244)	XM_759717	Hsp90	$Tp7_{206-214}$EFISFPISL	Incl: 718-744 Excl: 1-717;745-900	Graham *et al.*, 2008

Tp8	*T. parva* (TP02_0140)	Muguga	XM_759616	Cysteine proteinase	$Tp8_{379-387}$CGAELNHFL	Incl: 1135-1161 Excl: 1-1134;1162-1300	Graham *et al.,* 2008
Tp9	*T. parva* (TP02_0895)	Muguga	XM_760370	Hypothetical	$Tp9_{67-76}$AKFPGMKKSK	N/A	Nene *et al*., 2012
Tp10	*T. parva* (TP04_0772)	Muguga	XM_759315	Coronin	N/A	Incl: 770-930, Excl: 1-769	Gardner *et al*., 2005

[a] **eta-TCP1** (eta subunit of the T-complex protein 1), **eIF-1A** (translation elongation initiation factor 1A), **Hsp90** (heat shock protein 90).

[b] **ROI** ("Region of Interest" defined by respective nucleotide positions on the reference sequence), **Incl** - ROI of the sequence reads included in the analysis as defined by nucleotide positions, **Excl** - region of the sequence reads excluded in the analysis as defined by nucleotide positions.

4.4 Results

4.4.1 Detection of *T. parva* positive samples

A total of 321 samples positive for *T. parva* were detected as shown in Figure 3.1 of Chapter three of this thesis. For the groups which had >20 *T. parva* positive samples, 20 were randomly selected for PCR amplification of TpAg genes. For the groups which had <20 *T. parva* positive samples, all the positive samples were used for PCR amplification.

4.4.2 Analysis of specific TpAg genes PCR products

The numbers of *T. parva* positive samples from which the targeted regions of TpAg genes were successfully amplified are shown in Table 4.5. Single PCR amplicons were obtained for each TpAg gene with the corresponding sizes indicated in Table 4.1. Due to the possibility of infection with multiple *T. parva* parasites, there was a likelihood of obtaining multiple PCR products of the same size but with sequence variations. In order to identify such sequence differences among amplicons of the same size, cloning of amplicons of interest was done. Figure 4.2 shows the quantity and quality of colony PCR products obtained and their respective sizes for the targeted regions of TpAg genes (Tp1, Tp2, Tp3, Tp4, Tp5, Tp6, Tp7, Tp8 and Tp10) after the first pooling. Similar quality products were also obtained for Tp9 antigen gene after cloning.

Table 4.5: Summary of the numbers of samples from which the targeted regions of TpAg genes were successfully amplified.

Country	Host/Source	No. of *T. parva* positive samples selected for TpAg genes PCR	[a]Number of samples successfully amplified on targeted regions of TpAg genes									
			Tp1	Tp2	Tp3	Tp4	Tp5	Tp6	Tp7	Tp8	Tp9	Tp10
Kenya	Cattle	15	12	12	13	12	12	13	11	12	10	13
	Buffalo	20	15	15	16	17	16	18	14	15	13	15
Uganda	Mbarara cattle	20	15	16	16	17	15	17	15	15	14	16
	Karamoja cattle	20	15	13	14	15	13	15	13	15	12	14
Tanzania	Tanga cattle	06	04	04	ns	ns	ns	ns	ns	ns	04	ns
	Simanjiro cattle	15	11	10	ns	ns	ns	ns	ns	ns	09	ns
	TNP Buffalo	10	08	08	ns	ns	ns	ns	ns	ns	07	ns
South Africa	KNP buffalo	15	10	09	ns	10	11	09	08	ns	ns	09
	KZN buffalo	20	15	17	18	16	15	15	16	17	15	16
	CD clinical cases	14	10	10	09	09	09	10	09	11	08	10
	Non-clinical *T. parva*-positive cases	10	01	01	01	01	01	01	01	01	01	01
Mozambique	Buffalo	20	15	14	15	15	15	15	13	12	11	15
Total		**185**	**131**	**129**	**102**	**112**	**107**	**113**	**100**	**98**	**104**	**109**

[a] ns - "no samples" were available for analysis due to low quantities.

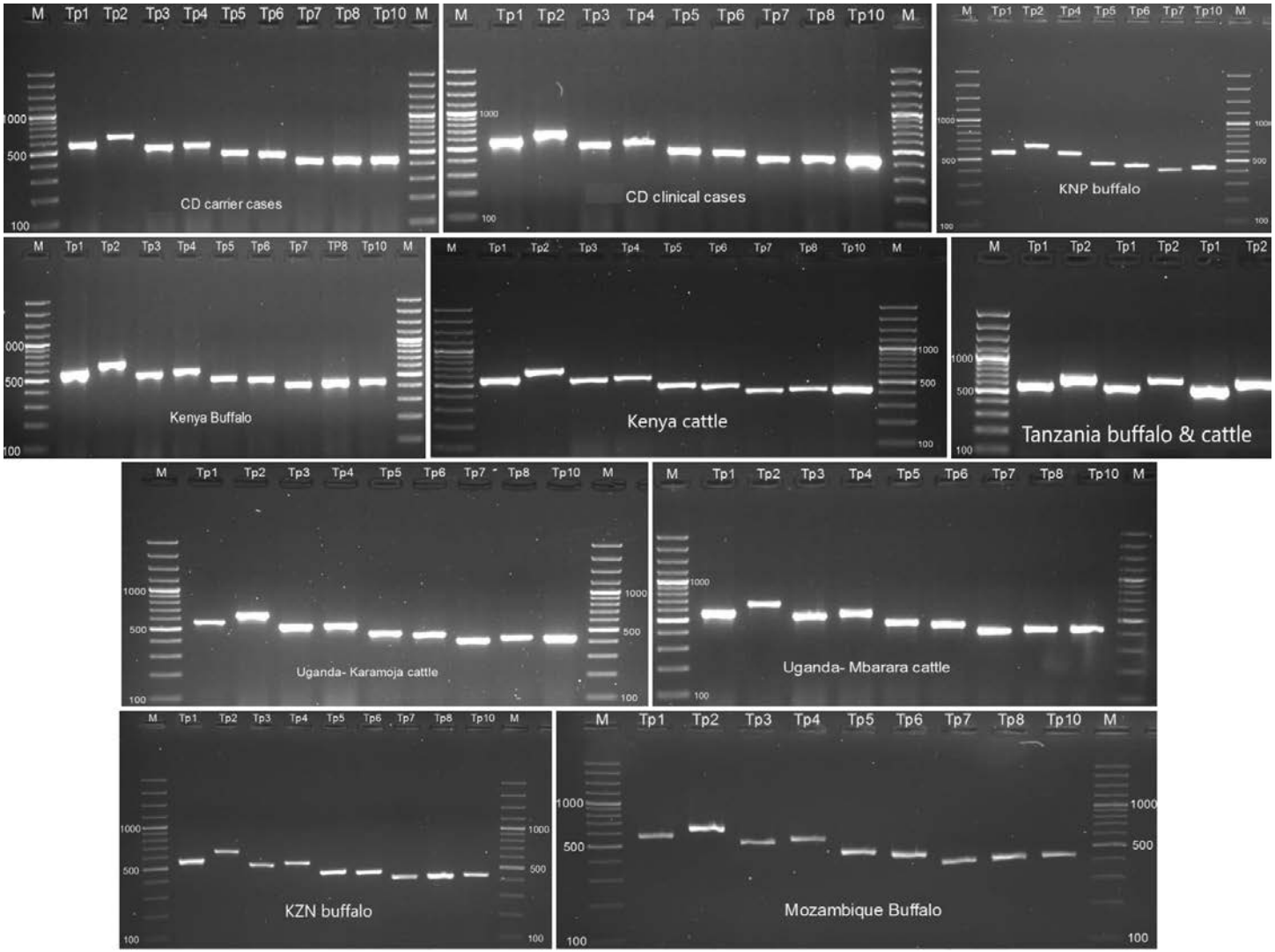


Figure 4.2: Purified colony PCR amplicons of the nine TpAg-encoding genes representing samples per group after the first pooling. 100 bp plus DNA ladder (#SM0321, ThermoFisher Scientific, USA) was used to estimate the size of the amplicons.

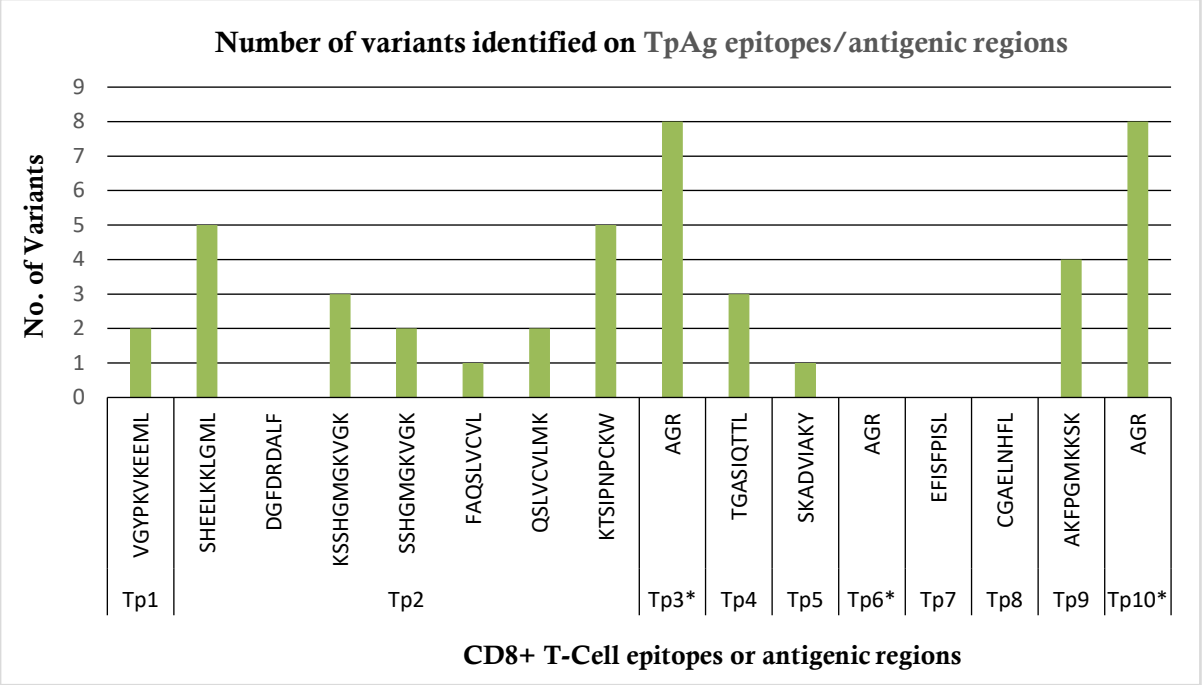
4.4.3 Detection of antigen epitope variants

The overall sequence coverage for the targeted TpAg genes epitope/antigenic regions from various sample groups ranged from 642 to 94,958 reads (Appendix 1). Details about sequence coverage, nucleotide positions where mutations were detected, and the respective types of epitope variants identified for each TpAg gene in different sample groups are shown in Appendix 1.

4.4.3.1 TpAg genes epitope variants

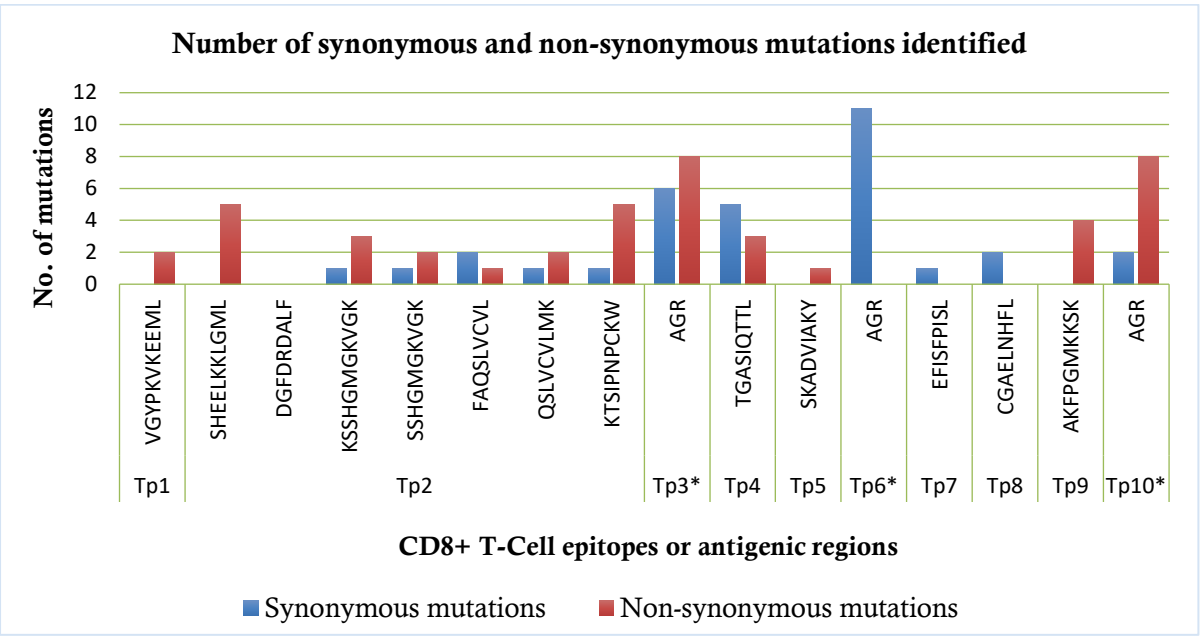
All variants identified on TpAg genes epitopes were in comparison to the reference sequences indicated in Table 4.4. As indicated earlier, the antigenic epitopes of Tp3, Tp6 and Tp10 have not been identified, therefore, antigenic regions known to be recognized by CD8+ T-cells (Hemmink *et al.*, 2016) were analyzed. For the purpose of this study, only non-synonymous mutations were considered significant since they altered the predicted protein sequences of the TpAg gene epitopes/antigenic regions resulting in variants. TpAg genes with epitopes that had synonymous mutations were considered conserved since this type of mutation do not result in substitution of amino acid residue(s), due to degeneracy. Variants were detected in all the other TpAg gene epitopes/antigenic regions except for one of the Tp2 epitopes (^{40}DGFDRDALF^{48}), Tp7 and Tp8 epitopes, and the antigenic region of Tp6 (Figure 4.3). The distribution of the variants identified based on the host and region is outlined hereinafter.

Most epitope variants were detected for Tp2, where epitopes ^{27}SHEELKKLGML^{37} and ^{138}KTSIPNPCKW^{147} of Tp2 had five variants each, followed by Tp9 (^{67}AKFPGMKKSK^{76}) with four variants (Figure 4.3). Tp2 (^{49}KSSHGMGKVGK^{59}) and Tp4 (^{328}TGASIQTTL^{336}) epitopes had three variants each, whereas Tp1 (^{214}VGYPKVKEEML^{224}) and Tp2 (^{50}SSHGMGKVGK^{59}, ^{98}QSLVCVLMK^{106}) had two variants each (Figure 4.3). Tp2 (^{96}FAQSLVCVL^{104}) and Tp5 (^{87}SKADVIAKY^{95}) were the least variable with each having one variant (Figure 4.3). Among the TpAg genes with unidentified antigenic epitopes, Tp3 and Tp10 were the most variable, with each having eight non-synonymous substitutions within the antigenic regions, whereas Tp6 was invariable with all substitutions being synonymous (Figure 4.4). Generally, the number of non-synonymous mutations was more than synonymous (Figure 4.4).



*Antigens with unidentified epitopes hence antigenic regions (AGR) were analyzed.

Figure 4.3: Number of variants/non-synonymous substitutions identified on TpAg gene epitopes/antigenic regions for *T. parva* parasites from East and southern Africa.

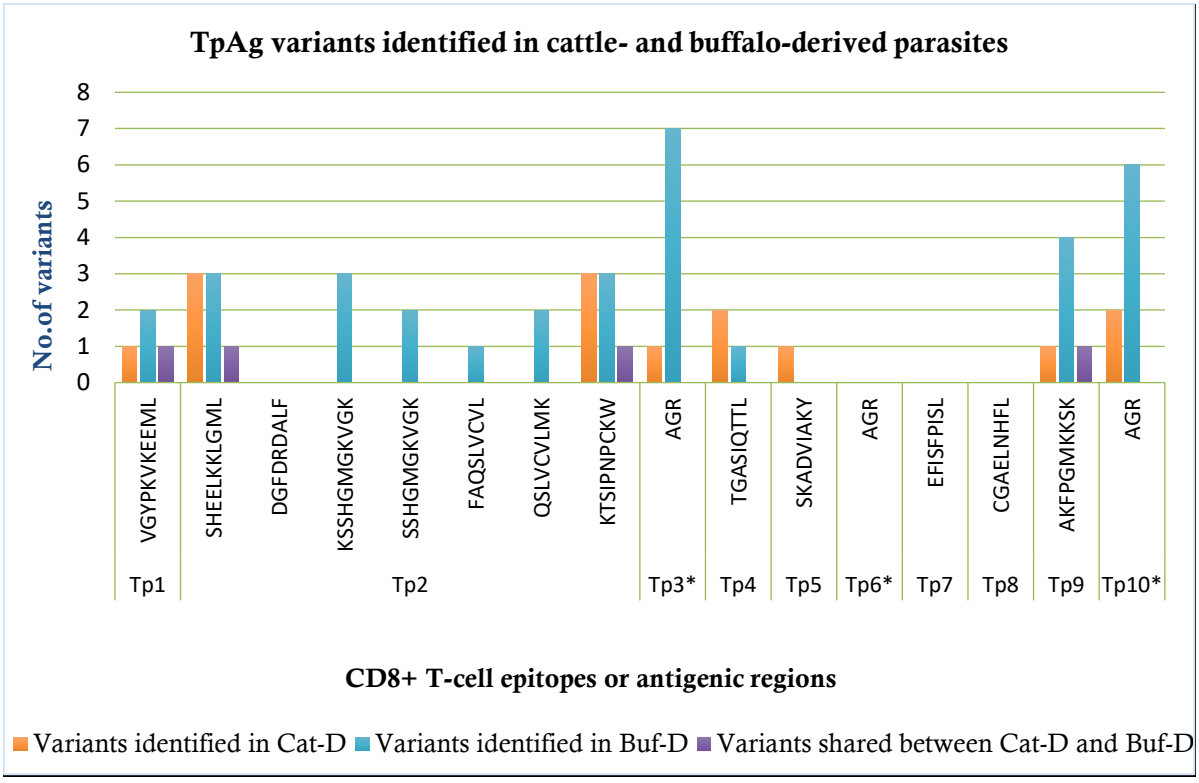


*Antigens with unidentified epitopes hence antigenic regions (AGR) were analyzed.

Figure 4.4: Number of synonymous and non-synonymous substitutions identified on TpAg gene epitopes/antigenic regions for *T. parva* parasites from East and southern Africa.

4.4.3.2 Comparison of variants identified in cattle- and buffalo-derived *T. parva* parasites

Analysis of variants from non-synonymous mutations revealed that most epitope variants occurred in the buffalo-derived *T. parva* parasites. Notably, variants on four of the six Tp2 epitopes were conserved in cattle-derived parasites (Table 4.6), with respective variants occurring only in buffalo-derived *T. parva* parasites (Figure 4.5). Although majority of the epitope variants (n=17) occurred in buffalo-derived *T. parva* parasites, epitope variants unique to cattle-derived *T. parva* parasites (n=7) were also detected, as well as those common (n=4) to both cattle- and buffalo-derived parasites (Figure 4.6). The most conserved epitope in buffalo-derived parasites was from Tp5 while epitopes from four TpAg's were the most conserved in both parasite types (Table 4.6).

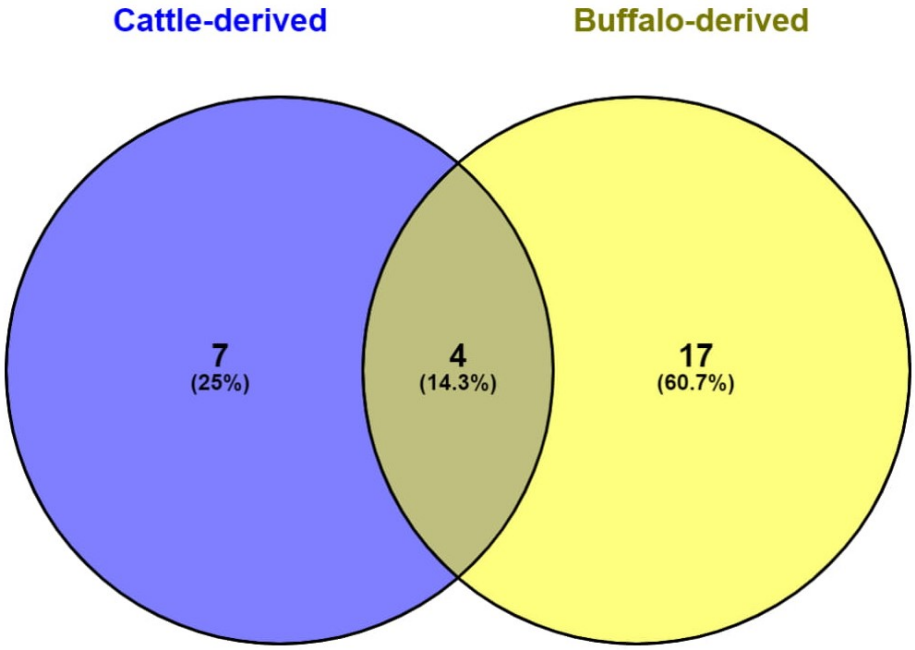


Cat-D (Cattle-derived *T. parva*); Buf-D (Buffalo-derived *T. parva*); *Antigens with unidentified epitopes hence antigenic regions (AGR) were analyzed.

Figure 4.5: Variants identified on TpAg gene epitopes/antigenic regions in cattle- and buffalo-derived *T. parva* parasites.

Table 4.6: The summary of most conserved epitopes in cattle- and buffalo-derived *T. parva* parasites, as well as in both parasite types.

Cattle-derived *T. parva*	**Buffalo-derived *T. parva***	**Both parasite types**
Tp2 (^{49}KSSHGMGKVGK^{59})	Tp5 (^{87}SKADVIAKY^{95})	Tp2 (^{40}DGFDRDALF^{48})
Tp2 (^{50}SSHGMGKVGK^{59})		Tp6
Tp2 (^{96}FAQSLVCVL^{104})		Tp7 (^{206}EFISFPISL^{214})
Tp2 (^{98}QSLVCVLMK^{106})		Tp8 (^{379}CGAELNHFL^{387})



Group	TpAg gene	Epitope variants	Number (%)
Exclusively in "Cattle-derived "	Tp2	SHEEL**N**KLGML, SH**G**ELKKLGML, K**P**SIPNPCKW, KTS**V**PNPCKW	7 (25)
	Tp4	TGASIQT**S**L, TG**D**SIQTTL	
	Tp5	**N**KADVIAKY	
Exclusively in "Buffalo-derived "	Tp1	VGYPKVKEEM**I**	17 (60.7)
	Tp2	SH**D**ELKKLGML, SHEEL**T**KLGML, K**T**SHGMGKVGK, KSSH**A**MGKVGK, **R**SSHGMGKVGK, **T**SHGMGKVGK, SSH**A**MGKVGK, FAQS**I**VCVL, QS**I**VCVLMK, **E**SLVCVLMK, KTSIPNPC**N**W, KTSIPNPC**Q**W	
	Tp4	**S**GASIQTTL	
	Tp9	**N**KFPGMKK**G**K, AKF**KH**M**GMG**K, **E**KF**KH**M**GIG**K	
Common in "Cattle-derived " and "Buffalo-derived ":	Tp1	VGYPKVKEE**I**I	4 (14.3)
	Tp2	S**D**EELKKLGML, KTSIPNPC**E**W	
	Tp9	AKFPGMKK**G**K	

Figure 4.6: The detection of exclusive and shared TpAg epitope variants in predicted protein sequences from cattle- and buffalo-derived *T. parva* parasites.

4.4.3.3 Tp1 epitope variants

Epitope variants of this TpAg gene were detected in seven of the twelve sample groups (Table 4.7 and Appendix 1: Table 1.0). Notably, no variants unique to the cattle-derived *T. parva* parasites were identified on Tp1 epitope. [214]VGYPKVKEEM**I**[224] was identified among variants exclusively detected in buffalo-derived parasites (Figure 4.6). This variant was also unique to *T. parva* parasites from South Africa, mainly from buffalo in Hluhluwe-iMfolozi Park, KwaZulu-Natal (Table 4.7). Variant [214]VGYPKVKEE**II**[224], detected among variants shared between cattle- and buffalo-derived *T. parva*, was identified in cattle-derived *T. parva* parasites from Kenya, Uganda-Mbarara and Tanzania-Tanga, and in buffalo-derived *T. parva* parasites from clinical cases of Corridor disease, KNP and Kenya buffalo (Table 4.7).

Table 4.7: Non-synonymous mutations detected within Tp1 antigenic epitope.

Sample group	Coverage	Reference	Nucleotide mutation position	Epitope variants	Function
Kenya buffalo	40,001	TP03_0849	808_809	VGYPKVKEE**II**	Missense
Kenya cattle	73,728			VGYPKVKEE**II**	Missense
Uganda-Mbarara cattle	73,226			VGYPKVKEE**II**	Missense
Tanzania Tanga cattle	21,550			VGYPKVKEE**II**	Missense
CD clinical cattle	44,813			VGYPKVKEE**II**	Missense
KNP buffalo	54,519			VGYPKVKEE**II**	Missense
KZN buffalo	35,107		809	VGYPKVKEEM**I**	Missense

4.4.3.4 Tp2 epitope variants

Tp2 was the most variable of all the TpAg genes investigated with parasites from clinical cases of Corridor disease having the highest number of variants (n=7) amongst the buffalo-derived *T. parva* parasites (Table 4.8). Epitopes of *T. parva* parasites from Tanzania and Uganda cattle were the most variable among the cattle-derived *T. parva* parasites (Table 4.8; Appendix 1: Table 1.1). From cattle-derived *T. parva* parasites, non-synonymous mutations were identified from only two of the seven epitopes [27]SHEELKKLGML[37] and [38]KTSIPNPCKW[147], which had three variants each (Table 4.8).

Four Tp2 epitope variants were exclusively detected in sequences from cattle-derived *T. parva* and 12 exclusively from buffalo-derived *T. parva* (Figure 4.6). Tp2 variants exclusive to cattle-derived *T. parva* were identified in parasites from Kenya and Tanga-Tanzania (^{27}SHEEL**N**KLGML^{37}), Simanjiro-Tanzania (^{27}SH**G**ELKKLGML^{37}), and Uganda (^{138}K**P**SIPNPCKW^{147} and ^{138}KTS**V**PNPCKW^{147}) (Figure 4.6 and Table 4.8). Most of the variants (n=10) exclusive to buffalo-derived *T. parva* were identified in parasites from South Africa, with 70% detected from clinical cases of Corridor disease (^{27}SH**D**ELKKLGML^{37}, ^{27}SHEEL**T**KLGML^{37}, ^{49}K**T**SHGMGKVGK^{59}, ^{49}KSSH**A**MGKVGK^{59}, ^{50}**T**SHGMGKVGK^{59}, ^{50}SSH**A**MGKVGK^{59} and ^{98}**E**SLVCVLMK^{106}), followed by parasites from KZN (^{138}KTSIPN**P**C**Q**W^{147}, ^{49}**R**SSHGMGKVGK^{59}) and KNP (^{49}**R**SSHGMGKVGK^{59}). Three variants (^{96}FAQS**I**VCVL^{104}, ^{98}QS**I**VCVLMK106, ^{138}KTSIPNPC**N**W^{147}) were identified in parasites from buffalo from Kenya, one variant (^{27}SH**D**ELKKLGML^{37}) from Mozambique buffalo, and one (^{138}KTSIPNPC**N**W^{147}) from Tanzania buffalo (Figure 4.6 and Table 4.8).

Variant ^{27}S**D**EELKKLGML^{37} was identified in cattle-derived parasites from Kenya and Tanga-Tanzania as well as in buffalo-derived parasites from Kenya, Tanzania and Mozambique buffalo, and clinical cases of Corridor disease. Variant ^{138}KTSIPN**P**C**E**W^{147} identified in Uganda cattle was also identified in KNP, KZN and Mozambique buffalo (Figure 4.6 and Table 4.8).

Table 4.8: Non-synonymous mutations detected within Tp2 antigenic epitopes.

Cattle-derived *T. parva* parasites			**Buffalo-derived *T. parva* parasites**		
Epitope	**Variant type**	**Sample group**	**Epitope**	**Variant type**	**Sample group**
SHEELKKLGML	S**D**EELKKLGML	Kenya and Tanzania-Tanga cattle	SHEELKKLGML	S**D**EELKKLGML	Kenya, Tanzania and Mozambique buffalo, CD clinical cases
	SHEEL**N**KLGML			SH**D**ELKKLGML	CD clinical cases, Mozambique buffalo
				SHEEL**T**KLGML	CD clinical case
	SH**G**ELKKLGML	Tanzania-Simanjiro cattle			
DGFDRDALF	None	-	DGFDRDALF	None	-
KSSHGMGKVGK	None	-	KSSHGMGKVGK	K**T**SHGMGKVGK	CD clinical cases
				KSSH**A**MGKVGK	
				RSSH**G**MGKVGK	KZN buffalo
SSHGMGKVGK	None	-	SSHGMGKVGK	**T**SHGMGKVGK	CD clinical cases
				SSH**A**MGKVGK	
FAQSLVCVL	None	-	FAQSLVCVL	FAQS**I**VCVL	Kenya buffalo
QSLVCVLMK	None	-	QSLVCVLMK	QS**I**VCVLMK	Kenya buffalo
				ESLVCVLMK	CD clinical cases
KTSIPNPCKW	K**P**SIPNPCKW	Uganda-Mbarara and Karamoja cattle	KTSIPNPCKW	KTSIPNPC**N**W	Kenya, Tanzania buffalo
	KTS**V**PNPCKW			KTSIPNPC**Q**W	KNP, KZN buffalo
	KTSIPNPC**E**W			KTSIPNPC**E**W	KNP, KZN, Mozambique buffalo

Bolded and underlined shows the variant amino acid residue

4.4.3.5 Tp3 antigenic region variations

No sequence variants were shared between cattle- and buffalo-derived parasites (Table 4.9). Almost all variants were identified in buffalo-derived parasites with only a single mutation detected from cattle-derived *T. parva* parasites from Uganda-Karamoja. No mutation was identified in the other cattle-derived *T. parva* parasite groups (Appendix 1: Table 1.2).

Table 4.9: Non-synonymous mutations detected within Tp3 antigenic region.

Sample group	Coverage	Reference	[a]Amino acid change	Function
Kenya buffalo	69475	TP01_0868	p.**M**135**TM**	Missense
	74811		p.**N**141**NT**	Missense
	65751		p.**V**172**L**	Missense
Uganda-Karamoja cattle	73632		p.**P**136**PS**	Missense
CD clinical cattle	70760		p.**M**135**T**	Missense
KZN buffalo	25469		p.**V**109**AV**	Missense
	79593		p.**L**163**IL**	Missense
Mozambique buffalo	91534		p.**N**158**NT**	Missense
	74809		p.**V**172**AV**	Missense

[a] p.**M**135**TM** - "p" refers to protein sequence. At the indicated reference amino acid position, the reference sequence has an amino acid residue "**M**" where are the alternate/variant sequence has a "**T**" or "**M**".

4.4.3.6 Tp4 epitope variants

No epitope variants were identified in *T. parva* parasites from Uganda-Karamoja and Corridor disease cases for this antigen gene (Appendix 1, Table 1.3). From the identified Tp4 epitope variants, two (^{328}TG**D**SIQTTL^{336} and ^{328}TGASIQT**S**L^{336}) were exclusively detected in sequences of cattle-derived *T. parva* parasites from Kenya and Uganda-Mbarara cattle (Figure 4.6 and Table 4.10). One variant, ^{328}**S**GASIQTTL^{336} was identified in buffalo-derived *T. parva* parasites from KZN buffalo (Figure 4.6 and Table 4.10). No common variants were detected between cattle- and buffalo-derived *T. parva* parasites.

Table 4.10: Non-synonymous mutations detected within Tp4 antigenic epitope.

Sample group	Coverage	Reference	Nucleotide mutation position	Epitope variants	Function
Kenya cattle	9,121	TP03_0210	1003	TGASIQT**S**L	Missense
Uganda-Mbarara cattle	3,981		989	TG**D**SIQTTL	Missense
KZN buffalo	11,598		981	**S**GASIQTTL	Missense

4.4.3.7 Tp5 epitope variants

This 9-mer epitope was less variable in both buffalo- and cattle-derived *T. parva* parasites. One variant, ^{87}NKADVIAKY^{95}, was identified in cattle-derived *T. parva* parasites from Mbarara-Uganda (Figure 4.6). No mutation was identified in *T. parva* parasites from the other groups when compared to the reference epitope sequence (Appendix 1: Table 1.4).

4.4.3.8 Tp10 antigenic region variations

Six and two non-synonymous mutation types were detected in buffalo- and cattle-derived *T. parva* parasites respectively (Table 4.11). No mutations common to cattle- and buffalo-derived *T. parva* parasites were identified (Table 4.11).

Table 4.11: Non-synonymous mutations detected within Tp10 antigenic region.

Sample group	Coverage	Reference	[a]Amino acid change	Function
Kenya cattle	4732	TP04_0772	p.**L**284**PL**	Missense
Uganda-Mbarara cattle	36923		p.**S**277**P**	Missense
Uganda-Karamoja cattle	32183		p.**S**277**P**	Missense
CD carrier cattle	9772		p.**Q**309**QR**	Missense
CD clinical cattle	12230		p.**S**288**SL**	Missense
KZN buffalo	19037		p.**P**304**QP**	Missense
	32198		p.**Y**279**NY**	Missense
	31682		p.**S**288**SL**	Missense
Mozambique buffalo	24549		p.**M**299**TM**	Missense
	35408		p.**Y**280**YC**	Missense

[a] Amino acid changes are as explained in the footnote of Table 4.8.

4.4.3.9 Tp9 epitope variants

The number of Tp9 amplicons (from colony PCR) sequenced and the sequence reads obtained are summarized in Table 4.12. All the 62 sequences obtained from 96 clones of 83 samples (Table 4.12) were aligned to the previously published Tp9 *T. parva* Muguga sequence (Table 4.4). Four variants; ^{67}AKFPGMKKGK^{76}, ^{67}NKFPGMKKGK^{76}, ^{67}AKFKHMGMGK^{76} and ^{67}EKFKHMGIGK^{76} were identified on Tp9 epitope (Figure 4.7 and Table 4.13), three from buffalo-derived *T. parva* parasites and one (^{67}AKFPGMKKGK^{76}) in both parasite types (Figures 4.5, 4.6 and Table 4.13). Buffalo-derived *T. parva* parasites from clinical cases of Corridor disease had two variants; one (^{67}NKFPGMKKGK^{76}) shared with parasites from non-clinical *T. parva*-positive cases and Kenya buffalo, and the other (^{67}AKFPGMKKGK^{76}) shared with parasites from Tanzania and KZN buffalo, and cattle-derived parasites (Figure 4.6 and Table 4.13). Two unique variants (^{67}AKFKHMGMGK^{76} and ^{67}EKFKHMGIGK^{76}) were detected from buffalo-derived *T. parva* parasites from Mozambique buffalo (Table 4.13). Phylogenetic analysis displayed clustering that was congruent to the variants identified, where three main clusters corresponding to ^{67}AKFPGMKKGK^{76}, ^{67}NKFPGMKKGK^{76} and ^{67}AKFPGMKKSK^{76} were identified (Figure 4.8).

Table 4.12: Summary of the number of Tp9 sequences obtained from *T. parva* positive samples from cattle and buffalo.

Country	Sample group	Number of samples selected for cloning	Number of clones selected for sequencing per group	Number of Tp9 sequence reads obtained after quality and BLAST analysis
Kenya	Cattle	08	10	06
	Buffalo	08	10	05
Uganda	Mbarara cattle	10	10	09
	Karamoja cattle	10	10	05
Tanzania	Tanga cattle	04	06	05
	Simanjiro cattle	09	10	09
	Buffalo	07	06	04
South Africa	KZN buffalo	10	10	05
	CD clinical cases	08	10	05
	Non-clinical *T. parva*-positive cases	01	04	04
Mozambique	Buffalo	08	10	05
Total		**83**	**96**	**62**

Table 4.13: Summary of Tp9 epitope variants identified in buffalo- and cattle-derived *T. parva* parasites.

Buffalo-derived *T. parva* parasites		**Cattle-derived *T. parva* parasites**	
Sample group	**Variant**	**Sample group**	**Variant**
CD clinical cases	**N**KFPGMKK**G**K	Uganda-Karamoja cattle	AKFPGMKK**G**K
	AKFPGMKK**G**K	Tanzania-Tanga cattle	AKFPGMKK**G**K
Non-clinical *T. parva*-positive cases	**N**KFPGMKK**G**K		
Tanzania buffalo	AKFPGMKK**G**K	Kenya cattle	AKFPGMKK**G**K
KZN buffalo	AKFPGMKK**G**K		
Kenya buffalo	**N**KFPGMKK**G**K		
Mozambique buffalo	AKF**KH**M**GMG**K		
	EKF**KH**M**GIG**K		

Bolded and underlined shows the variant amino acid residue.

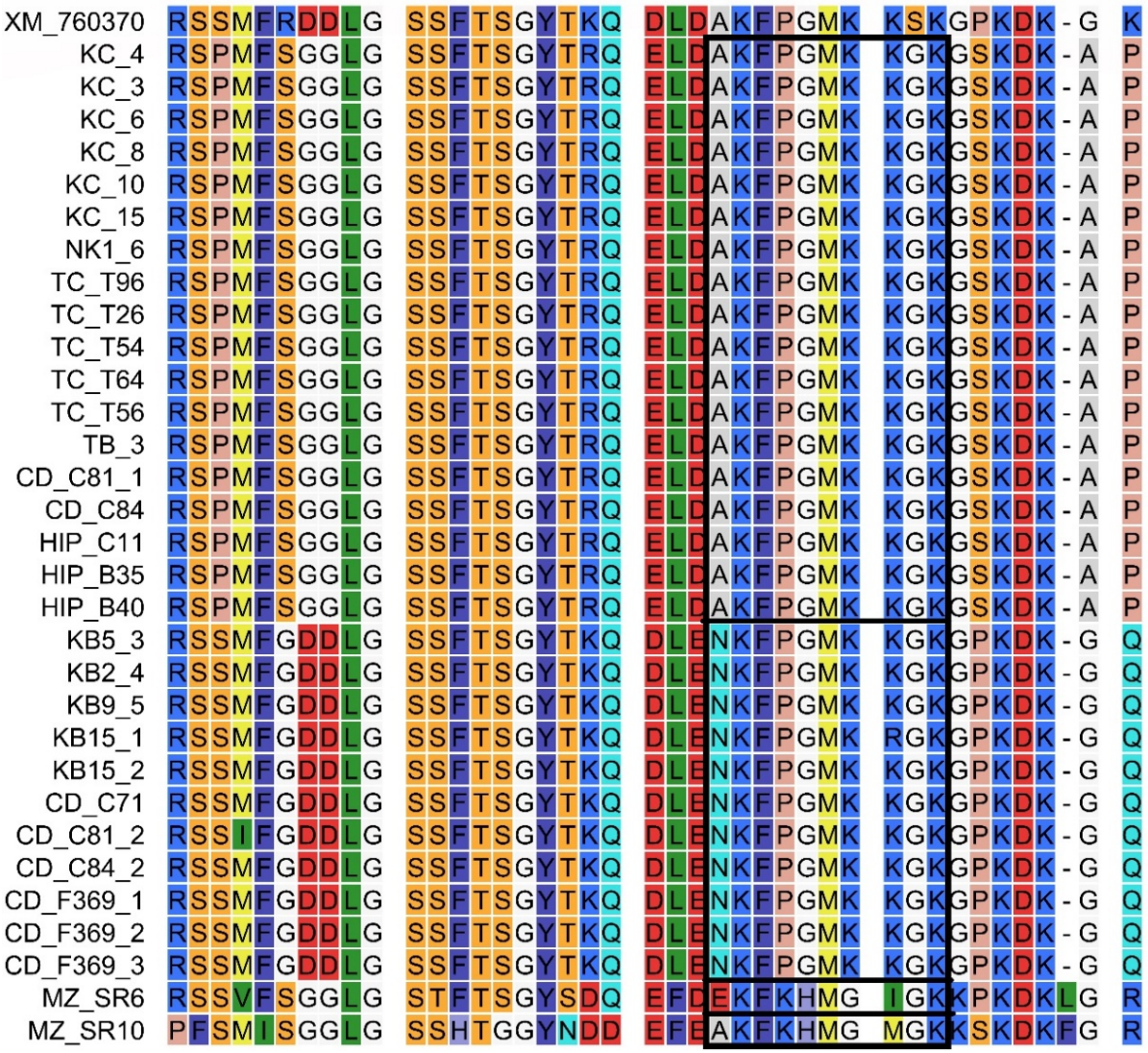


Figure 4.7: Variants of Tp9 epitope identified in buffalo- and cattle-derived *T. parva* parasites from clinical cases of Corridor disease (CD_C), non-clinical *T. parva*-positive case (CD_F), KZN buffalo (HIP), Kenya buffalo (KB), Kenya cattle (KC), Mozambique buffalo (MZ), Uganda-Karamoja cattle (NK), Tanzania buffalo (TB) and Tanzania-Tanga cattle (TC_T).

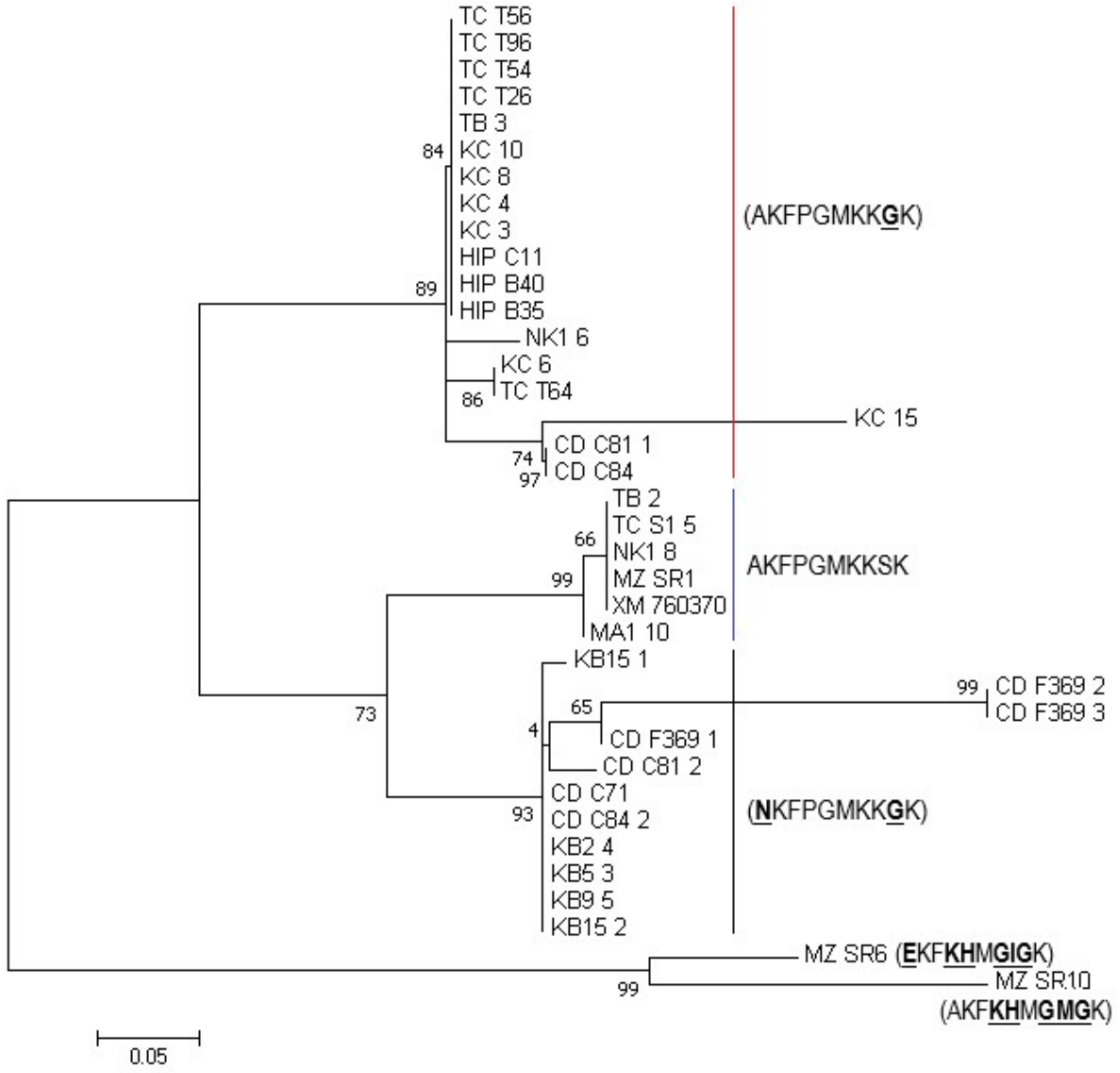


Figure 4.8: Evolutionary history of Tp9 epitope variants inferred using Maximum Likelihood based on the General Reverse Transcriptase model.

The tree topology with the highest log likelihood (-994.90) is shown, with branch lengths measured in the number of substitutions per site. The analysis involved 37 amino acid sequences, and all positions containing gaps and missing data were eliminated. There was a total of 102 positions in the final dataset, and phylogeny was tested using 100 bootstrap replicates. Evolutionary analyses were conducted in MEGA7.

4.4.3.10 Comparison of variants in parasites from East and southern Africa

The geographic distribution of the 28 epitope variants detected from all TpAg genes (except Tp3, Tp6 and Tp10 which lack characterized epitope regions) was also investigated (Figures 4.9 and 4.10). Besides the five epitope variants shared between the two regions, parasites from southern Africa had more variants (n=13) exclusive for this region compared to East Africa (n=10) (Figure 4.10). Notably, TpAg epitope variants detected exclusively in buffalo- and cattle-derived parasites were similar to those exclusive to parasites from southern and East Africa respectively. From epitope variants exclusive to East Africa, 70% were exclusively detected from cattle-derived, and 30% from buffalo-derived parasites (Figures 4.6 and 4.10), while 100% of epitope variants exclusive to southern Africa were also exclusively detected from buffalo-derived parasites. Likewise, TpAg epitope variants shared between cattle- and buffalo-derived parasites were also common to parasites from the two regions, except for Tp9 (^{67}NKFPGMKKGK^{76}) which was exclusively detected from buffalo-derived parasites. (Figures 4.6 and 4.10). Tp2 was the most variable TpAg gene with 18 epitope variants, of which two were shared between the two regions, 56% (9/16) were associated exclusively with parasites from southern Africa, and 44% (7/16) with parasites from East Africa (Figures 4.9 and 4.10). This was consistent with the general observation for all TpAg genes.

Among the TpAg genes with unidentified antigenic epitopes, Tp3 and Tp10, most sequence variations occurred in parasites from southern Africa; 57% (4/7) for Tp3 and 75% (6/8) for Tp10 (Figure 4.9). Only Tp3 had a non-synonymous mutation that was common in parasites from both East and southern Africa (Figure 4.9).

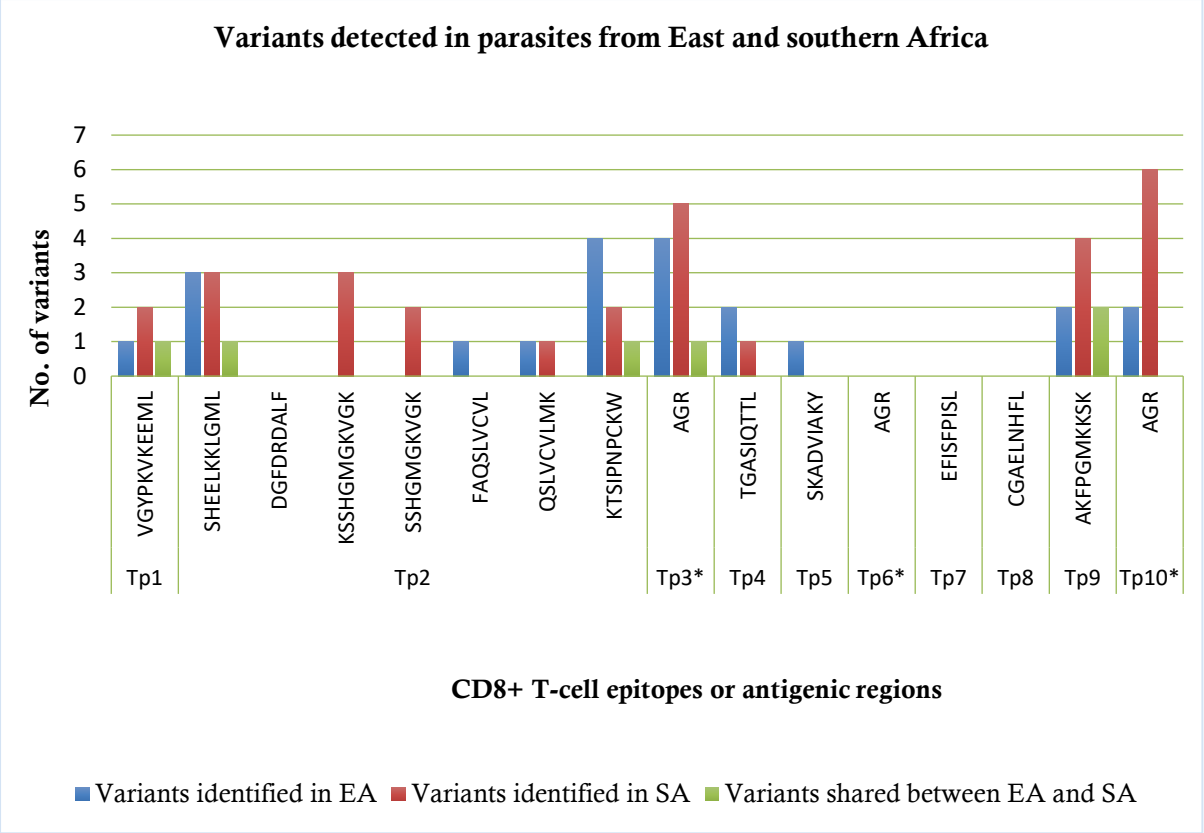
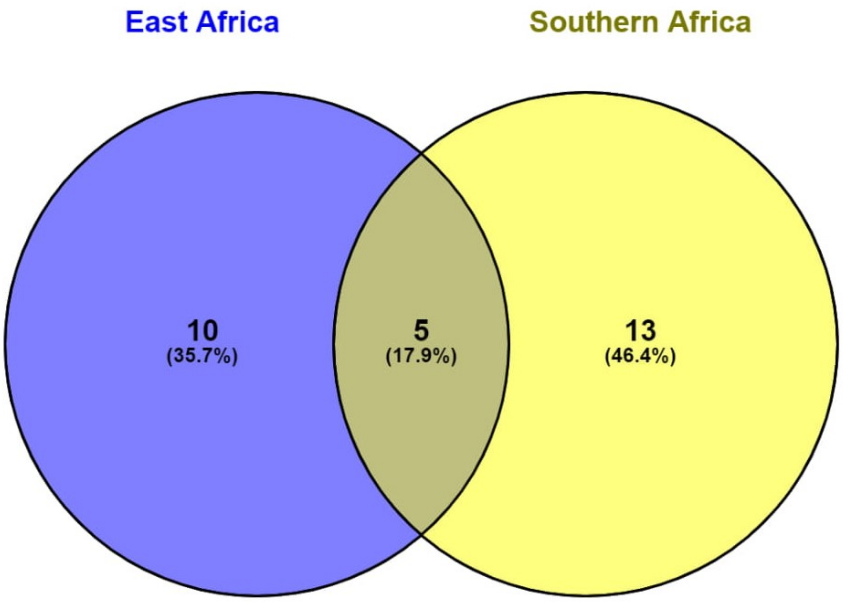


Figure 4.9: Variants identified on TpAg gene epitopes/antigenic regions in *T. parva* parasites from East Africa (EA) and southern Africa (SA).



Group	TpAg gene	Epitope variants	Number (%)
Exclusively in "East Africa"	Tp2	SHEEL**N**KLGML, SH**G**ELKKLGML, K**P**SIPNPCKW, KTS**V**PNPCKW, FAQS**I**VCVL, QS**I**VCVLMK, KTSIPNPC**N**W	10 (35.7)
	Tp4	TGASIQT**S**L, TG**D**SIQTTL	
	Tp5	**N**KADVIAKY	
Exclusively in "Southern Africa"	Tp1	VGYPKVKEEM**I**	13 (46.4)
	Tp2	SH**D**ELKKLGML, SHEEL**T**KLGML, K**T**SHGMGKVGK, KSSH**A**MGKVGK, **R**SSHGMGKVGK, **T**SHGMGKVGK, SSH**A**MGKVGK, **E**SLVCVLMK, KTSIPNPC**Q**W	
	Tp4	**S**GASIQTTL	
	Tp9	AKF**KH**M**GMG**K, **E**KF**KH**M**GIG**K	
Common in "East and southern Africa"	Tp1	VGYPKVKEE**II**	5 (17.9)
	Tp2	S**D**EELKKLGML, KTSIPNPC**E**W	
	Tp9	AKFPGMKK**G**K, **N**KFPGMKK**G**K	

Figure 4.10: Venn diagram showing the relationship between the TpAg epitope variants identified in East and southern Africa.

4.5 Discussion

The current live vaccine used to immunize cattle against *T. parva* infections in East Africa confers protection against a homologous parasite challenge but not heterologous parasites especially of buffalo origin (Sitt *et al.*, 2015). Similar to other apicomplexan parasites (Ferreira *et al.*, 2004; Blake *et al.*, 2015), *T. parva* utilizes antigenic diversity as a survival strategy in the evasion of the host's immune system (Katzer *et al.*, 2006; Katzer *et al.*, 2010). Moreover, since immunity to *T. parva* is mainly cell-mediated, the failure of the vaccine to protect against heterologous parasites could be attributed to failure of the memory CTLs to recognize variant types of peptides presented by MHC class I molecules following a challenge with *T. parva* field parasites. Therefore, this study sought to establish the diversity of TpAg genes in expansive *T. parva* field parasites from East and southern Africa, with an aim of identifying TpAg genes epitope variants that are unique to, or shared between ECF and Corridor disease parasites.

A total of 185 *T. parva* positive field samples from cattle (54.05%) and buffalo (45.95%) were used to assess the diversity of ten TpAg genes focusing on the epitope regions recognized by CD8+ cytotoxic T lymphocytes. Similar to previous studies (Pelle *et al.*, 2011; Sitt *et al.*, 2018), comparison of buffalo- and cattle-derived *T. parva* parasites demonstrated extensive antigenic diversity in the former than the latter, especially in epitope variants identified from Tp1, Tp2 and Tp9 antigens. The T-cell receptors (TCR) of CD8+ cytotoxic T lymphocytes recognize parasite peptides encoded by the epitope regions on the antigen genes and presented by MHC class I haplotype. Thus, the sequence diversity in the epitope regions may result in altered peptide sequences, consequently leading to the evasion of the host immune system by the parasite.

The CTL response to *T. parva* is further complicated by polymorphism of the host's MHC molecules cognate to the BoLA haplotype (reviewed in Steinaa *et al.*, 2018). The CD8+ T-cell response to *T. parva* infections is reported to predominantly involve recognition of $Tp1_{214\text{-}224,}$ $Tp2_{49\text{-}59}$ and $Tp2_{50\text{-}59}$ epitopes by the TCR (MacHugh *et al.*, 2009; Connelley *et al.*, 2016). Cytotoxic T lymphocytes from animals homozygous for BoLA-A18 and BoLA-A10 haplotypes have demonstrated immunodominance towards the 11-mer $Tp1_{214\text{-}224}$ epitope, and the 11- and 10-mer, $Tp2_{49\text{-}59}$ and $Tp2_{50\text{-}59}$ epitopes respectively (MacHugh *et al.*, 2009; Connelley *et al.*, 2016). Although the BoLA types of the animals sampled in this study were not determined, these three epitopes ($Tp1_{214\text{-}224,}$ $Tp2_{49\text{-}59}$ and $Tp2_{50\text{-}59}$) essential in

immune response to *T. parva* infections were affected. Furthermore, specificity of the CTL responses varies even among animals that have identical MHC class I haplotype (Steinaa *et al.*, 2018).

Two variants of the 11-mer $Tp1_{214\text{-}224}$ epitope were identified with substitutions occurring at positions 10 and 11 involving two terminal amino acids. In the first variant, ^{214}VGYPKVKEE**II**^{224}, methionine and leucine were substituted with isoleucine, whereas only leucine was substituted with isoleucine in the second variant ^{214}VGYPKVKEEM**I**^{224} exclusively identified in buffalo-derived parasites from South Africa. The Tp1 epitope variant ^{214}VGYPKVKEE**II**^{224} was common in ECF (cattle-derived) and Corridor disease (buffalo-derived) *T. parva* parasites. The three amino acid residues (methionine, leucine and isoleucine) involved on this epitope are non-polar and similar in size and shape, a feature that promotes cross-reactivity of the CTL (Frankild *et al.*, 2008; Steinaa *et al.*, 2012). Substitutions involving amino acids at positions 3, 6, 7, 8 and 9 of the epitope are reported to substantially affect CTL killing of infected lymphocytes (Macdonald *et al.*, 2010; Steinaa *et al.*, 2012) whereas position 10 and 11 substitutions fairly reduce CTL killing (Macdonald *et al.*, 2010). Taken together, previous studies (Pelle *et al.*, 2011; Hemmink *et al.*, 2018; Kerario *et al.*, 2019) and the current study demonstrate that Tp1 epitope is less variable and that the common occurring variations on this epitope in both cattle- and buffalo-derived *T. parva* parasites mostly involve the two carboxy-terminal amino acids. Considering the involvement of Tp1 epitope in the recognition of *T. parva* infected cells by the CTL, and that the variants identified are likely to be cross-reactive to CTL, and some are common to both cattle- and buffalo-derived parasites, the findings in the current study reinforce the evidence that Tp1 could be an ideal vaccine candidate. Furthermore the, the amino acid positions important for CTL killing (3, 6, 7, 8 and 9) do not seem to be prone to mutations in both cattle- and buffalo-derived *T. parva* parasites.

Seven antigenic epitopes have been identified on the Tp2 gene (Akoolo *et al.*, 2008; Graham *et al.*, 2008; Nene *et al.*, 2012), and previous studies have demonstrated that this gene is highly polymorphic (Pelle *et al.*, 2011; Hemmink *et al.*, 2016; Salih *et al.*, 2017; Sitt *et al.*, 2018) with *T. parva* parasites of buffalo origin being more polymorphic. As would be expected, due to the difference in the number of epitopes per loci, the current study confirmed that Tp2 antigen is more variable than the other antigens and that buffalo-derived *T. parva* parasites are more variable than cattle-derived *T. parva* parasites. In fact,

four of the seven Tp2 epitopes are well conserved in cattle-derived parasites; interestingly one is conserved in both *T. parva* parasite types. The three variants of $Tp2_{49\text{-}59}$ (K**T**SHGMGKVGK, KSSH**A**MGKVGK, **R**SSH**G**MGKVGK) and two of $Tp2_{50\text{-}59}$ (**T**SHGMGKVGK, SSH**A**MGKVGK) exclusively identified in the buffalo-derived *T. parva* parasites from South Africa, involve substitutions at positions 1, 2 and 5 on $Tp2_{49\text{-}59}$, and positions 1 and 4 on $Tp2_{50\text{-}59}$. Although the natural variants of these epitopes would be expected to affect the MHC-binding capacity, the magnitude of their effect depends on the number of substitutions and the epitope positions affected (Connelley *et al.*, 2011). Notwithstanding that, any change that disrupts the distinct epitope ligands for TCR recognition will result in partial or total escape from immune recognition (Connelley *et al.*, 2011).

The CTL response induced by the other Tp2 epitopes has been reported to be minimal (Morrison *et al.*, 2015). Furthermore, it has been demonstrated that substitutions resulting in the loss of lysine residue at position 8 of $Tp2_{49\text{-}59}$, which corresponds to position 7 of $Tp2_{50\text{-}59}$, result in evasion of TCR recognition (Connelley *et al.*, 2011). Although the epitope changes identified in the current study may affect MHC-binding capacity, it is evident that there could be no abrogation of TCR recognition since none of the substitutions involve the residue at position 8. The results of the current study demonstrate that Tp2 epitopes are less variable in cattle-derived parasites, and the variants identified largely represent a *T. parva* sub-population circulating in the wildlife reservoir and sporadically transmitted to cattle to cause Corridor disease in South Africa. Although similar findings on the diversity of Tp2 have been reported in Kenya from *T. parva* cell lines (Pelle *et al.*, 2011; Sitt *et al.*, 2018), field parasites from cattle in Sudan (Salih *et al.*, 2017), parasites from cattle and buffalo in Tanzania (Kerario *et al.*, 2019), and parasites from buffalo from Kenya and South Africa (Hemmink *et al.*, 2018), the current study provides the first report on the differences between field parasites from both mammalian hosts, cattle and buffalo, from East and southern Africa. While Hemmink *et al.* (2018) demonstrated that parasites from buffalo in Kenya and South Africa had similar Tp2 sequences, the current study involving an expanded host range and geographical distribution identified variants of $Tp2_{49\text{-}59}$ and $Tp2_{50\text{-}59}$ epitopes that are unique to buffalo-derived parasites from cattle from South Africa. In addition, it is worth noting that despite the limited number of samples analyzed from South African cattle, these variants that were not identified in East Africa, were detected in South Africa.

Four variants of the Tp9 epitope ([67]AKFPGMKKSK[76]) were identified; three from the buffalo-derived *T. parva* parasites, and one (AKFPGMKK**G**K) common to both cattle- and buffalo-derived parasites, suggesting that Tp9 gene is less variable in cattle-derived than buffalo-derived parasites. This can be expected since the buffalo host is known to harbor heterogenous parasites, and the presence of multiple *T. parva* parasites seems to promote genetic exchange resulting in a diverse population. Two unique Tp9 variants were identified from *T. parva* parasites from Mozambique buffalo suggesting a unique *T. parva* population among parasites circulating in this population. However, with further analysis of more samples from the other parasites of buffalo origin, the possibility of identifying similar variants cannot be overruled. It has been demonstrated that the Tp9 epitope elicits a weak CTL response, hence is not a suitable vaccine candidate (Bastos *et al.*, 2019). On the contrary, a full-length recombinant signal peptide of Tp9 has been shown to induce both humoral and cellular response, and with further immunological evaluation, it could be a potential candidate for the *T. parva* subunit vaccine (Bastos *et al.*, 2019). Since the effects of natural variants of Tp9 epitope on CTL recognition have not been reported, an evaluation of the significance of substitutions of specific epitope residues on CTL recognition is necessary, otherwise, any speculations on the same are unfounded.

The role of the other antigens with defined epitopes (Tp4, Tp5, Tp7 and Tp8) in CD8+ T-cell response has not been established (Morrison *et al.*, 2015), and similarly, the role of antigens with undefined epitopes (Tp3, Tp6 and Tp10) is not known. However, they may have other roles in *T. parva* infection and pathogenesis of theileriosis, and their sequence variations may be useful in differentiation of *T. parva* populations of cattle and buffalo origin from the two regions investigated. Among the four antigens with defined epitopes, non-synonymous amino acid substitutions were detected in Tp4 and Tp5 whereas all substitutions in Tp7 and Tp8 were conserved in both parasite types. The latter two may have a significant role in the infection and possibly survival of the parasite or maintenance of infection in the host. Tp4 epitope had the highest number of sequence variants (n=3) amongst which two were exclusively detected in cattle-derived parasites from East Africa, and one from buffalo-derived parasites from southern Africa. Likewise, Tp5 had one variant detected in the cattle-derived parasites from East Africa. Previous studies on parasites from buffalo in Kenya (Hemmink *et al.*, 2018; Sitt *et al.*, 2018) and South Africa (Hemmink *et al.*, 2018) detected limited variations on these four antigens. It was rather peculiar in the current study that cattle-derived parasites had more variants of Tp4 epitope

than buffalo-derived parasites, as the opposite would be expected. However, since diversity of TpAg genes is not a universal phenomenon (Sitt *et al.*, 2018), there is a likelihood that some TpAg genes may display limited to no diversity in both buffalo- and cattle-derived *T. parva* parasites. Collectively, Sitt *et al.* (2018), Hemmink *et al.* (2018) and the current study demonstrate limited diversity of these antigens in buffalo-derived parasites as well as in cattle-derived parasites. This could possibly suggest that either these genes are under negative selection (Hemmink *et al.*, 2018), or much of the evolution of *T. parva* involving these genes has occurred in the wildlife reservoir, and that the evolved parasites are maintained in the wildlife cycle (buffalo/ticks) as well as in cattle.

The recent study by Hemmink *et al.* (2018) was the first to report on the comparative diversity of TpAg genes in *T. parva* parasites from the buffalo in Kenya and South Africa. The current study expanded the geographic and host range to include parasites from cattle and buffalo from Kenya, Tanzania, Uganda, South Africa and Mozambique. From the current study, *T. parva* parasites from southern Africa are generally more diverse than those from East Africa, and buffalo-derived parasites are more diverse than cattle-derived parasites. One variant of $Tp1_{214\text{-}224}$ (VGYPKVKEE**II**), two of Tp2 (^{27}S**D**EELKKLGML^{37}, ^{138}KTSIPN**P**C**E**W^{147}), and one of $Tp9_{67\text{-}76}$ (AKFPGMKK**G**K) were shared between the two regions, and also between cattle- and buffalo-derived parasites. All variants of $Tp2_{49\text{-}59}$ and $Tp2_{50\text{-}59}$ were only detected in buffalo-derived parasites from clinical cases of Corridor disease and KZN buffalo from South Africa, and none of the variants detected on Tp4 and Tp5 epitopes was common between the two regions. Considering the involvement of $Tp1_{214\text{-}224,}$ $Tp2_{49\text{-}59}$ and $Tp2_{50\text{-}59}$ in *T. parva* immunity (MacHugh *et al.*, 2009; Connelley *et al.*, 2011), it could be concluded from the findings of the current study that Tp1 and Tp2 are promising vaccine candidates, since most of the variants of their dominant epitopes in cattle- and buffalo-derived parasites from cattle do not involve amino acid residues that would lead to abrogation of TCR recognition.

The recent observation that genetic diversity among *T. parva* populations from eastern and southern Africa probably arose prior to geographic separation (Hemmink *et al.*, 2018), indicates that the genetic differences observed in parasites from the two regions are possibly due to parasite adaptation though they share a common ancestral origin as displayed by similarities in TpAg. Most antigen variants in parasites from southern Africa were common for clinical Corridor disease cases and buffalo parasites, indicating that the

parasites that cause Corridor disease originate from the reservoir buffalo host, as previously observed in Kenya (Sitt *et al.*, 2019). Hence, these parasites represent a population of *T. parva* that circulates in buffalo and is occasionally transmitted to cattle in southern Africa. On the contrary, the *T. parva* population in East Africa demonstrates a sub-structured population, where one sub-population has adapted to the cattle host for cattle-to-cattle transmission, while the other sub-population circulates in the wildlife host with a possibility of transmission to cattle.

4.6 Conclusion

The current study provides the first report on the comparative diversity of *T. parva* schizont antigens in parasite from cattle and buffalo from East and southern Africa. Parasites from southern Africa are more diverse than East Africa, and Tp2 is the most diverse TpAg gene. We report identification of variants on immunodominant $Tp1_{214\text{-}224,}$ $Tp2_{49\text{-}59}$ and $Tp2_{50\text{-}59}$ epitopes common to both cattle- and buffalo-derived parasites from the two regions, and some unique to buffalo-derived parasites from cattle from South Africa. Considering that immunity to *T. parva* is mainly cell-mediated, Tp1 and Tp2 could be ideal candidates for recombinant vaccine development since their epitope variants in parasites from cattle may not lead to escape of *T. parva* infected lymphocytes from TCR recognition.

4.7 References

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CHAPTER 5

Genotypic diversity and population structure of *Theileria parva* field parasites from eastern and southern Africa

5.1 Abstract

Theileria parva employs genetic diversity as a survival strategy in the evasion of the host's immune system. A panel of microsatellite and minisatellite markers have been identified and used to assess the genotypic diversity and population structure of *T. parva* field parasites and components of the live vaccine in eastern Africa. In order to obtain insights in the genotypic diversity and population structure of *T. parva* field parasites from eastern and southern Africa, we performed a comparative analysis of parasites from cattle and buffalo from these two regions. A set of 14 polymorphic microsatellite and minisatellite markers were used to genotype *T. parva* parasites using genomic DNA prepared from cattle and buffalo blood samples from South Africa, Mozambique, Kenya and Uganda. Analysis of genotypic data revealed MS19 to be the most diverse loci and ms5 the least. *Theileria parva* parasites associated with Corridor disease and East Coast fever had distinguishing allelic profiles on three loci (MS8, MS19 and MS33). All individual populations when analyzed separately were in linkage equilibrium (V_D<L) indicating panmixia but when considered as one combined population, linkage disequilibrium (V_D>L) was observed. Genetic divergence was observed to be more within (AMOVA=74%) than between (AMOVA=26%) populations. Principal Coordinate Analysis (PCoA) displayed clustering that separated buffalo-derived from cattle-derived *T. parva* parasites, although an admixed group was formed by cattle- and buffalo-derived parasites from cattle, and parasites from Mozambique buffalo. The results demonstrate geographic sub-structuring of *T. parva* parasites based on the disease syndromes caused in cattle with limited genetic exchange between populations but high genetic diversity within populations due to panmixia. Although *T. parva* parasites associated with Corridor disease and ECF are different, the admixed group formed by these parasites indicate that they have some genotypic similarities with distinguishing allelic profiles on three loci. The findings of the current study reveal genotypic differences between buffalo- and cattle-derived *T. parva* parasites based on three loci (MS8, MS19 and MS33). These may affect the efficacy of the current live *T. parva* vaccine in areas where cattle co-graze with buffalo.

5.2 Introduction

The causative agent of cattle theileriosis in eastern and southern Africa *Theileria parva*, is known to adopt diversity as a survival strategy (Katzer *et al.*, 2010) and its presence in the buffalo further complicates its epidemiology. Consequently, the success in control of *T. parva* has been undermined by the diversity of the parasite in the buffalo and cattle hosts resulting from recombination activities in the tick vector. Therefore, establishing the diversity and population structure of *T. parva* field parasites is critical in the development of effective control strategies for this parasite. Several molecular tools including PCR based assays (Iams *et al.*, 1990; Nene *et al.*, 1992; Toye *et al.*, 1995; Skilton *et al.*, 1998; Bishop *et al.*, 2001), restriction fragment length polymorphisms (RFLP) analysis of *T. parva* DNA (Bishop *et al.*, 1993) and Southern blotting using *T. parva* repetitive DNA probes (Bishop *et al.*, 2001) were developed to characterize *T. parva* parasites. However, these tools only detect polymorphisms in limited regions of *T. parva* genome, hence they are not suitable for extensive genome wide characterization.

Subsequently, studies conducted by Oura *et al.* (2003) and Katzer *et al.* (2010) identified genome-wide polymorphic microsatellite (ms) and minisatellite (MS) markers for characterizing *T. parva* parasites. Microsatellite markers are short tandem repeats (STRs) of 2-8 bps while minisatellites have longer repeat units (8-100 bps) (Oura *et al.*, 2003). Satellite markers show high rates of mutation which result in high levels of polymorphism making them ideal for molecular profiling of *T. parva* parasites (Oura *et al.*, 2003). Hence, characterization of a panel of 11 and 68 polymorphic micro- and minisatellites, respectively, in the *T. parva* genome (Oura *et al.*, 2003; Katzer *et al.*, 2010) as well as 42 PCR-RFLP markers (Katzer *et al.*, 2010) has been a significant advancement in the analysis of genomic changes in *T. parva* parasite populations. Studies on *T. parva* field parasites using satellite markers have demonstrated extensive diversity of the parasite in Uganda (Oura *et al.*, 2005; Oura *et al.*, 2011b; Muwanika *et al.*, 2016), Tanzania (Elisa *et al.*, 2015; Rukambile *et al.*, 2016), Sudan (Salih *et al.*, 2018) and Zambia (Muleya *et al.*, 2012), cultured *T. parva* isolates from Kenya (Odongo *et al.*, 2006; Katzer *et al.*, 2010), and limited diversity within the Muguga cocktail vaccine stabilates in Kenya (Patel *et al.*, 2011; Hemmink *et al.*, 2016). Despite *T. parva* infections being of economic importance in southern Africa, reports on the diversity of the parasite in this region are limited with the only available data focusing on Zambia (Muleya *et al.*, 2012). In the absence of such information especially in South Africa where Corridor disease is important, the current

study sought to establish a comparative diversity of *T. parva* field parasites from eastern and southern Africa, and to identify markers that can differentiate cattle- and buffalo-derived *T. parva*.

5.3 Materials and Methods

5.3.1 Sample collection and detection of *T. parva*

Collection of samples and detection of *T. parva* was done as described in Chapter three of this thesis. Due to inadequate quantities of DNA samples obtained from Tanzania (cattle and buffalo) and Kruger National Park (KNP) buffalo, satellite marker analyses could not be performed on these two sample groups.

5.3.2 Selection of samples for analysis

Samples positive for *T. parva* on qPCR (n=176) from the following sample groups were selected; KZN buffalo=23, Mozambique buffalo=17, Kenya cattle=15, Kenya buffalo=28, Uganda Karamoja cattle=20, Uganda Mbarara cattle=49, CD clinical cases=14 and non-clinical *T. parva*-positive cases =10. Initially, a nested PCR was performed using ms2 satellite marker for selection of samples for subsequent analysis with the other satellite markers. Consequently, 96 samples from which ms2 was successfully amplified were selected for downstream analysis. These included KZN buffalo (n=14), Mozambique buffalo (n=12), Kenya cattle (n=10), Kenya buffalo (n=17), Uganda Karamoja cattle (n=10), Uganda Mbarara cattle (n=18), CD clinical cases (n=12) and non-clinical *T. parva*-positive cases (n=3).

5.3.3 Mini- and microsatellite PCR assay

Nested PCR was performed using nested primers previously described and designed to amplify three microsatellite and eleven minisatellite loci based on the *T. parva* genome (Oura *et al.*, 2003; Oura *et al.*, 2005; Salih *et al.*, 2018) (Table 5.1). The primers were synthesized by INQABA Biotechnologies South Africa, and the inner forward nested primers were labelled at the 5' end with one of the following fluorescent dyes; ATTO-565-red, ATTO-550-orange, ATTO-532-blue, CAL fluoro-green, Cy3-yellow, 6-FAM-blue (Sigma-Aldrich, USA) (Table 5.1). The primary PCR amplification was carried out in a 10 µl reaction volume consisting of DreamTaq DNA Polymerase, 2X DreamTaq buffer (10mM Tris–HCl, 4mM $MgCl_2$), 0.0024 µmol of each dNTP, 10 pmol (from a working stock concentration of 10 pmol/µl) of each primer. The cycling conditions employed were as follows; an initial denaturation at 95 °C for 3 minutes (mins), followed by 35 cycles of denaturation at 94 °C for 30 seconds (s), annealing at 60 °C for 30 s and extension at 72 °C for 1 min, and then a final extension step at 72 °C for 10 mins. For the secondary PCR,

0.5 µl of the primary PCR product was used as the template in a reaction similar to the primary PCR. The cycling conditions were as follows: initial denaturation at 95 °C for 5 mins, followed by 25 cycles of denaturation at 94 °C for 30 s, primer annealing at 58 °C for 1 min, primer extension at 72 °C for 1 min, and then a final extension step at 72 °C for 20 mins. The PCR products were resolved by 2% agarose gel electrophoresis in 1X TAE buffer.

Table 5.1: Details of *T. parva* minisatellite (MS) and microsatellite (ms) makers used.

[a]**Satellite Marker**	**Outer nested primers** (Oura *et al.*, 2003; Oura *et al.*, 2005; Salih *et al.*, 2018)	**Inner nested primers** (Oura *et al.*, 2003; Oura *et al.*, 2005)	**Consensus repeat sequences**	**Size range (bp)**	**CNR**	**Dyes**
ms2 (1)	Fwd: aagttagtatcaccaccaggctgg	Fwd: gcccaatgtaccgagaatcctcac	tat	176-250	20	ATTO-565 (red)
	Rev: ggctcatctaccactccaactcc	Rev: attctccgcattctccaccacctc				
ms5 (1)	Fwd: aacacagtaactaacccaggcc	Fwd: aatcttccaatcccaaccacatac	att	152-170	36	CAL fluoro (green)
	Rev: cccgaaataaaaccaaattccacc	Rev: aactccagcggaatcccgaaata				
ms7 (2)	Fwd: tgggtagagattggaatacgcgag	Fwd: ttaacttatctccctctccctccc	att	146-175	30	Cy3 (yellow)
	Rev: aataccgacgcgttccgaggaatc	Rev: acactctcaacaactcactcttcc				
MS3 (1)	Fwd: cccgatctcactcacatacaacc	Fwd: ccaccgtaaccctctataccat	tataccaaat	193-380	13	CAL fluoro (green)
	Rev: cagcaaatccaactcgtcgtcctg	Rev: gacatctccctcaaatcagactc				
MS7 (1)	Fwd: ctcctcagcatcctgctgctcattg	Fwd: gttcagtcctatggcaattcag	gtaactataactatgtaaaca	150-380	12	6-FAM (blue)
	Rev: gcgcatgactgcttttacattaaccc	Rev: caaacctcttcaaattcactctagg				
MS8 (1)	Fwd: ggcgtgacggtaatacaccttcc	Fwd: gcctcctcaagcaattcagta	ttacacagta	160-330	20	6-FAM (blue)
	Rev: cctcctagacactcccgaagatg	Rev: ctggttcaaacacatcaaggtaca				
MS16 (2)	Fwd: catggcattcctaggcatcacatc	Fwd: cctcctccatactactaacctacc	actaatatttgttattt	188-393	14	ATTO-565 (red)
	Rev: ccaagggaattaatactgttggag	Rev: cagcgctcagattcacttgtact				
MS19 (2)	Fwd: ccgcgtacagactaaactcgccg	Fwd: ccagacacctcaaatcccaagta	ataattaa	130-320	26	ATTO-532 (yellow)
	Rev: cctccaactgatccaccctcgcag	Rev: ccacactgccacctaatacaaa				
MS21 (3)	Fwd: gatgagcacaaggagttcctggtg	Fwd: ttctacccaacgccactctatgcg	atactatt	170-400	29	ATTO-550 (orange)
	Rev: gacggcgtctgagatggcgatgac	Rev: tgactcccgtcttatccaaattcg				
MS25 (3)	Fwd: ccagagatctcggacacaactcc	Fwd: acacacccatcaacgtagtaac	ttatatagttaagt	180-340	18	ATTO-565 (red)
	Rev: taaggtgccaaacggcggacac	Rev: caccatcacactcttaaccat				
MS27 (3)	Fwd: ccgccacctcagtctcgaga	Fwd: cctgcgatacatttctaatcc	taatcaaattat	130-220	10	6-FAM (blue)
	Rev: cacaactcacaccggaatctcac	Rev: gtaataccattcccacctctac				
MS33 (4)	Fwd: ctcattaccactctacccatcgc	Fwd: cttctcaaggtaccgtaaacc	atatagttaatt	150-220	11	ATTO-532 (yellow)
	Rev: catggtccatcttcatctggtcc	Rev: cctcactactccaatagttcttc				
MS34 (4)	Fwd: gtgcctagagagggaacggatatg	Fwd: gattacgccactgtcataaccacc	actatttccat	180-280	12	ATTO-565 (red)
	Rev: cctccggctgagattagtggcagg	Rev: aacactccacgctccacattcacc				
MS40 (4)	Fwd: cggtggaggctcggatgtgtgc	Fwd: catatcacctcatggtacacac	aaattaataaata	150-250	8	CAL fluoro (green)
	Rev: gcaggatcaacatcgccaaccac	Rev: ccagccctaatacacaaatc				

[a] Numbers in () indicate the chromosome on which the marker is located. CNR (Copy number of repeats)

5.3.4 Fragment Analysis

5.3.4.1 Preparation of amplicons

All secondary PCR products for each marker were subjected to fragment analysis. Size fractionation was done using Gene Scan 500 Liz internal lane size standard (Applied Biosystems, USA). Since a denaturation step was included in the preparation of amplicons for fragment analysis, highly deionized (Hi-Di) formamide (Applied Biosystems, USA) was added to stabilize single strands of denatured DNA. First, the Liz-HiDi formamide mixture was prepared by mixing Gene Scan 500 Liz size standard and Hi-Di formamide in the ratio of 12 µl to 1000 µl respectively. Nine microlitres (9 µl) of the Liz-HiDi formamide mixture was loaded in each well of the 96-well plate (compatible with ABI 3730 Genetic Analyzer) following which one microlitre (1 µl) of each PCR product was added. Samples in the 96-well plate were then denatured at 96 °C for 3 mins using GeneAmp® PCR system 9700 thermocycler, after which the plate was transferred onto an ice-ethanol bath to cool for one minute before capillary electrophoresis.

5.3.4.2 Capillary electrophoresis

The 96-well plates containing denatured amplicons and the size standard were subjected to capillary electrophoresis performed using an ABI 3730 Genetic Analyzer (Applied Biosystems, USA) at the SegoliP sequencing unit of the BecA-ILRI Hub, Nairobi, Kenya.

5.3.4.3 Genotyping of micro- and minisatellite loci`

Results were scored using the GeneMapper® Software version 5.0 (ThermoFisher Scientific, USA). The expected size range of each satellite marker (Table 5.1) was used as the reference to identify the genotypic profiles of the *T. parva* parasites in each sample. Due to poor quality of the runs of two satellite markers (MS21 and MS40) on all samples, these markers were excluded from subsequent analyses. Alleles with maximum peak heights per marker per sample were scored, and those with the largest area under the curve, and by default with the highest peak height, were considered predominant alleles, while alleles with at least one third of the predominant allele's height were considered minor (Salih *et al.*, 2018). Two types of data files were generated; the Multi-locus genotypes file (MLG file) comprising of only the predominant allele(s) at each locus, which was used to assess the diversity of populations, and the allelic profile file comprising of all alleles

(predominant and minor alleles) identified at each locus, which was used to assess multiplicity of infection (MOI) and linkage.

5.3.5 Population genetic analysis

The MLG and allelic data profile files were formatted for different analysis software packages according to the user manuals. Subsequent analysis was based on *T. parva* populations formulated to correspond to the sample groups i.e. Uganda-Mbarara, Uganda-Karamoja, non-clinical *T. parva*-positive cases, CD clinical cases, Kenya cattle, Kenya buffalo, KZN buffalo and Mozambique buffalo, hereinafter referred to as individual populations.

In order to test for the hierarchical variance of gene frequencies within and between individual populations, an analysis of molecular variance (AMOVA) was performed using the add-in excel software GenAlEx 6.5 (Peakall and Smouse, 2012) following the user's manual. A Mantel test of GenAlEx 6.5 (Peakall and Smouse, 2012) was performed to examine the genetic distance between individual populations where mean Shannon (sHua) values over loci were obtained. The lower the Shannon value, the closer the genetic distance and vice versa. The extent of linkage within and between individual populations and the standardized index of association within populations was determined using LIAN 3.7 programme (Haubold and Hudson, 2000). The standardized index of association is a measure of the association between alleles at pairs of loci where a predicted value close to zero or negative indicates panmixia, and greater than zero a non-panmictic population. LIAN 3.7 programme tests the null hypothesis of linkage equilibrium (LE) by calculating a 95% confidence limit 'L' (L_{MC}) where when the observed mismatch (pairwise) variance (V_D) is greater than L, the null hypothesis of LE is rejected. For linkage analysis, each individual population was analyzed separately to check for geographical sub-structuring, following which the individual populations were analyzed as a combined population to establish whether the combined population was in linkage equilibrium or disequilibrium. Multiplicity of infection (MOI) defined by the number of alleles (>1) per locus was determined using LIAN 3.7 programme (Haubold and Hudson, 2000) where the mean value per individual population was obtained. The genetic relationship of *T. parva* parasites between individual populations was determined by performing Principal Coordinate Analysis (PCoA) in GenAlEx 6.5 (Peakall and Smouse, 2012).

5.4 Results

5.4.1 Satellite loci diversity

Considering both cattle- and buffalo-derived *T. parva* parasites, the satellite loci were found to be highly polymorphic. MS19 showed the highest diversity (uh=0.906) while ms5 was the least diverse (uh=0.695) (Table 5.2). Diversity in the other loci ranged from 0.705 - 0.903. The number of alleles per loci ranged from six (6) in ms7 to 23 in MS3 with an average number of alleles (Na) per loci being 13 (Table 5.2). The number of alleles identified in each individual population on the 12 satellite markers ranged from 16-55 with the highest number observed in *T. parva* parasites from Mozambique buffalo (Table 5.3).

Table 5.2: Satellite marker diversity in cattle- and buffalo-derived *T. parva* parasites.

	ms2	**MS3**	**ms5**	**MS7**	**ms7**	**MS8**	**MS16**	**MS19**	**MS25**	**MS27**	**MS33**	**MS34**
N	95	86	85	83	64	92	75	81	67	78	84	88
Na	11	23	11	14	6	16	16	18	13	8	10	10
Ne	4.607	9.268	3.193	6.361	3.266	6.739	8.775	9.550	3.969	4.666	7.028	3.530
I	1.776	2.609	1.591	2.147	1.410	2.194	2.362	2.542	1.871	1.749	2.118	1.580
h	0.783	0.892	0.687	0.843	0.694	0.852	0.886	0.895	0.748	0.786	0.858	0.717
uh	0.791	0.903	0.695	0.853	0.705	0.861	0.898	0.906	0.759	0.796	0.868	0.725

N - sample size, **Na** - number of different alleles, **Ne** - number of effective alleles, **I** - Shannon's information index, **h** - diversity, **uh** - unbiased diversity.

Table 5.3: Summary of the number of alleles identified in individual populations.

Marker	[a]Number of alleles identified per population							
	Uganda Karamoja cattle (n=10)	Uganda Mbarara cattle (n=18)	CD clinical cases (n=12)	Non-clinical *T. parva*-positive cases (n=3)	Kenya cattle (n=10)	Kenya buffalo (n=17)	KZN buffalo (n=14)	Mozambique buffalo (n=12)
ms2	4	3	**5**	1	3	4	2	**5**
MS3	5	**8**	6	1	7	5	3	7
ms5	3	5	**6**	2	5	5	5	4
ms7	1	1	2	1	1	1	1	**3**
MS7	4	5	**7**	2	3	2	2	5
MS8	4	4	3	2	5	1	2	**7**
MS16	2	**6**	5	1	4	3	4	4
MS19	**5**	4	4	1	4	4	4	1
MS25	4	4	3	-	4	4	-	**6**
MS27	2	4	**5**	2	3	4	1	4
MS33	3	**4**	**4**	1	**4**	**4**	1	**4**
MS34	**5**	4	4	2	1	2	1	3
Total	42	52	54	16	44	39	26	55

[a] CD - Corridor disease, KZN - KwaZulu-Natal. Numbers in bold indicate the highest number of alleles identified per loci.

5.4.2 Predominant alleles and Multi-locus genotypes (MLGs)

The frequencies of predominant alleles identified per marker from all the samples for all individual populations, are summarized in Appendix 2. The most frequent predominant alleles were used to tabulate multi-locus genotypes as summarized in Table 5.4. The number of MLGs identified per individual population ranged from one (1) in *T. parva* parasites from KZN buffalo to four (4) in the rest of buffalo-derived parasites from Mozambique buffalo and CD clinical cases, and all cattle-derived *T. parva* parasites. Due to the limited number of samples (n=3) that were genotyped for non-clinical *T. parva*-positive cases, the MLGs from this group could not be computed. Cattle-derived *T. parva* parasites had one common MLG (ms2-179, MS3-239, ms5-172, ms7-150, MS7-152, MS8-165, MS16-273, MS19-162, MS25-199, MS27-209, MS33-175, MS34-248) but no

common MLG was identified in buffalo-derived parasites (Table 5.4). Other than a few loci (MS3, ms5, MS16, MS19 and MS27), which had >1 predominant allele with equal frequencies in cattle-derived *T. parva* parasites, each of the other loci in these parasites had one most frequent predominant allele that was common for each locus (Table 5.4). On the contrary, 75% of the loci in buffalo-derived *T. parva* parasites had predominant alleles (n=>1) with equal frequencies and there was no common predominant allele on any locus (Table 5.4). Some markers had unique common profiles of predominant alleles (MS8-165, MS16-273, MS19-162, MS33-175) in cattle-derived *T. parva* parasites, a feature that was absent in the buffalo-derived *T. parva* parasites (Table 5.4). When *T. parva* parasites from cattle associated with ECF (Uganda and Kenya cattle populations) and Corridor disease (South African CD populations) were compared, three loci with unique predominant allele profiles were identified; MS8-165, MS19-162, MS33-175 in ECF parasites, and MS8-155, MS19-127, MS33-160 in Corridor disease parasites (Table 5.4).

Table 5.4: Summary of the dominant alleles per marker and the multi-locus genotypes (MLGs) per site.

Population	[a]Dominant alleles (bp) per marker												[b]No. of MLGs
	ms2	MS3	ms5	ms7	MS7	MS8	MS16	MS19	MS25	MS27	MS33	MS34	
Uganda-Karamoja cattle	179	239 253	172	150	152	**165**	273	**162** 264	199	209	**175**	248	4
Uganda-Mbarara cattle	179	239	172	150	152	**165**	273 289	**162** 252	199	209	**175**	248	4
Kenya cattle	179	239	153 172	150	152	**165**	273	**162**	199	209 221	**175**	248	4
Kenya buffalo	202	239 253	172	142	171	191	223	165	199	185	184	182	2
CD clinical cases	202 214	219	172	150	249	**155**	176	**127**	199	149 161	**160**	248	4
Non-clinical *T. parva*-positive cases	214	248	159 162	150	152 232	155 172	189	127 179	-	149 209	160	201 195	NA
KZN buffalo	202	244	172	172	253	155	206	150	190	149	150	182	1
Mozambique buffalo	179	228	172	153	171 192	183	189	127	214	161	145 158	182	4

[a] Red coloured indicates the common MLG in cattle-derived parasites.
[a] Red bolded and underlined shows the unique profile on three loci in parasites associated with ECF.
[a] Blue bolded and underlined shows the unique profile on three loci in parasites associated with Corridor disease.
[b] NA - sample size too small to compute MLGs.

5.4.3 Population genetics analysis

5.4.3.1 Multiplicity of infection (MOI)

Theileria parva parasites from all the individual populations in eastern and southern Africa had multiple genotypes. An index value representing multiplicity of infection (MOI) was calculated from the mean number of alleles per loci. The MOIs for all the individual populations are summarized in Table 5.5. The mean values for MOI ranged from 1.03 - 2.69 with buffalo-derived *T. parva* parasites from Mozambique buffalo population recording the highest and non-clinical *T. parva*-positive cases the lowest. Parasites from Kenya and Mozambique buffalo recorded the highest maximum MOI values (3.00) among the buffalo-derived parasites, while parasites from Uganda-Mbarara recorded the highest mean (2.00) and maximum MOI value (2.67) among the cattle-derived *T. parva* parasites (Table 5.5). When all individual populations were considered as a combined population, the mean value for MOI was 2.03 with a high standard deviation (0.61) (Table 5.5). The standard deviations for each individual population was relatively high with non-clinical *T. parva*-positive cases and KZN buffalo recording the highest and the lowest standard deviations respectively.

Table 5.5: Multiplicity of infection of individual populations, and when all populations were considered as one combined population.

Population	**n**	**Multiplicity of infection (MOI)**			
		Mean	**SD**	**Min**	**Max**
Uganda-Karamoja	10	1.14	0.46	0.50	2.00
Uganda-Mbarara	18	2.00	0.53	0.58	2.67
Non-clinical *T. parva*-positive case	3	1.03	0.88	0.08	1.83
CD clinical cases	12	1.79	0.34	1.25	2.42
Kenya cattle	10	1.81	0.36	1.33	2.42
Kenya buffalo	17	2.49	0.38	1.67	3.00
KZN buffalo	14	2.14	0.26	1.75	2.75
Mozambique buffalo	12	2.69	0.29	2.00	3.00
Combined population	**96**	**2.03**	**0.61**	**0.08**	**3.00**

n: number of samples; **SD**: standard deviation

5.4.3.2 Linkage

Linkage between and within individual populations from the two regions of Africa was analyzed using the allelic profile data. When all the individual populations were analyzed as a single population, the standardized index of association (I_A^S) was greater than zero (0.0266) characteristic of a non-panmictic population, and the observed mismatch variance (V_D=3.1233) was greater than the critical value 'L' (L_{MC}=2.5460) indicating that the combined population is in a state of linkage disequilibrium (LD) (Table 5.6). When the individual populations were analyzed separately, the standardized index of association for each population was close to zero or negative indicating panmixia, and the observed mismatch variances (V_D) were less than the corresponding critical values 'L' (L_{MC}), hence, the null hypothesis of linkage equilibrium within individual populations was accepted (Table 5.6).

Table 5.6: Linkage analyses of individual populations and when combined as one population.

Population	I_A^S	V_D	L_{para}	L_{MC}	**Linkage**
Uganda-Mbarara	0.0079	2.7119	2.9863	3.1330	LE
CD clinicals cases	-0.0006	2.4552	3.1687	3.0399	LE
Kenya cattle	0.0242	3.0828	3.2534	3.3101	LE
Kenya buffalo	0.0081	2.6400	3.0099	3.1289	LE
KZN buffalo	-0.0098	2.2598	3.3262	3.6821	LE
Mozambique buffalo	0.0354	3.1655	3.2030	3.4117	LE
All population	**0.0266**	**3.1233**	**2.5324**	**2.5460**	**LD**

I_A^S = standard index of association, V_D = observed mismatch variance (linkage analysis),
LD = linkage disequilibrium, LE = linkage equilibrium,
L_{MC} and L_{para} = upper 95 % confidence limits of Monte Carlo simulation and parametric tests respectively (linkage analysis).
Note: Due to small sample sizes, linkage analysis for Uganda Karamoja and non-clinical *T. parva*-positive cases populations could not be computed.

5.4.3.3 AMOVA, PCoA and Genetic distance

An analysis of molecular variance (AMOVA) revealed that a higher percentage of variations occurred among parasites within individual populations (74%) than between populations (26%) (Figure 5.1). The Principal Coordinate Analysis (PCoA) showing the clustering of genotypes based on the genetic relationships among cattle- and buffalo-

derived *T. parva* parasites is shown in Figure 5.2. PCoA displayed four patterns of clustering; cluster 1 and 2 comprising of genotypes from the buffalo-derived parasites from buffalo, cluster 4 comprising exclusively of genotypes from cattle-derived parasites associated with ECF in eastern Africa, and cluster 3 representing an admixed group of genotypes from cattle- and buffalo-derived *T. parva* parasites from cattle in eastern and southern Africa respectively, and from buffalo in Mozambique (Figure 5.2). Parasites from Corridor disease cases from South Africa showed a close genetic relationship to those from buffalo in Mozambique. The genetic divergence between populations was determined by estimating the mean Shannon (sHua) values over loci as summarized in Table 5.7. The genetic distance between cattle- and buffalo-derived *T. parva* parasites ranged from 0.136 - 0.199 and 0.173 - 0.437, respectively (Table 5.7), and that between individual populations was in congruence to the relationship displayed by PCoA.

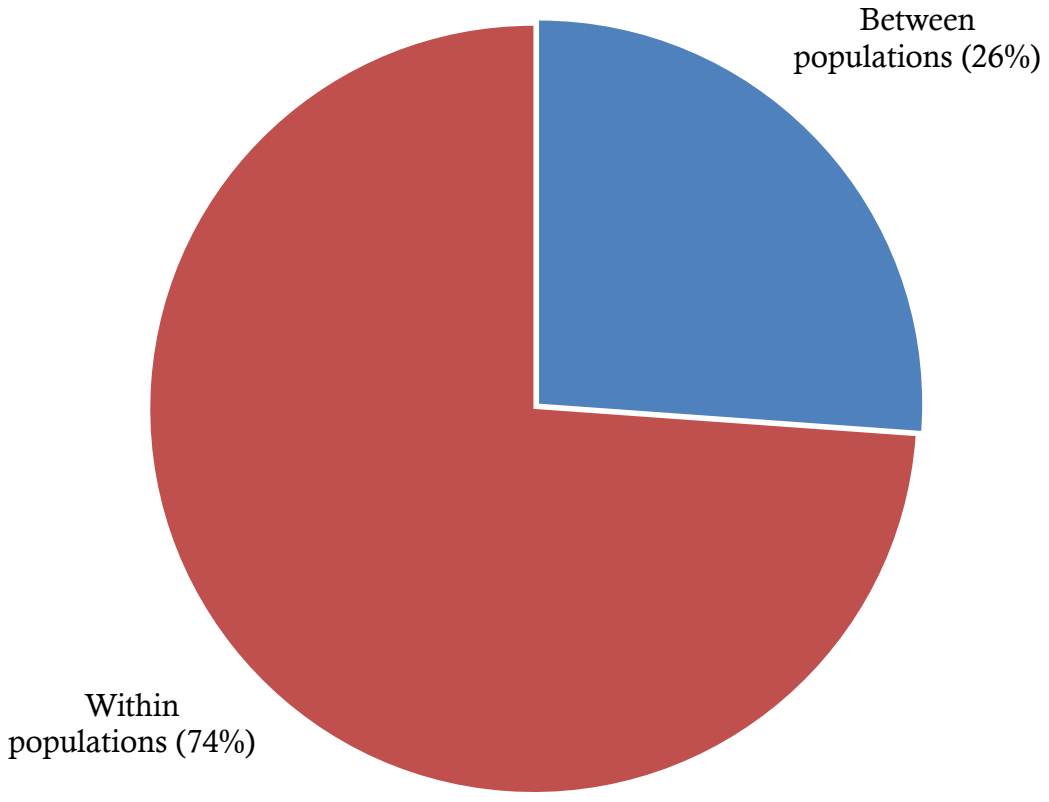

Figure 5.1: Percentage of molecular variance within and between populations.

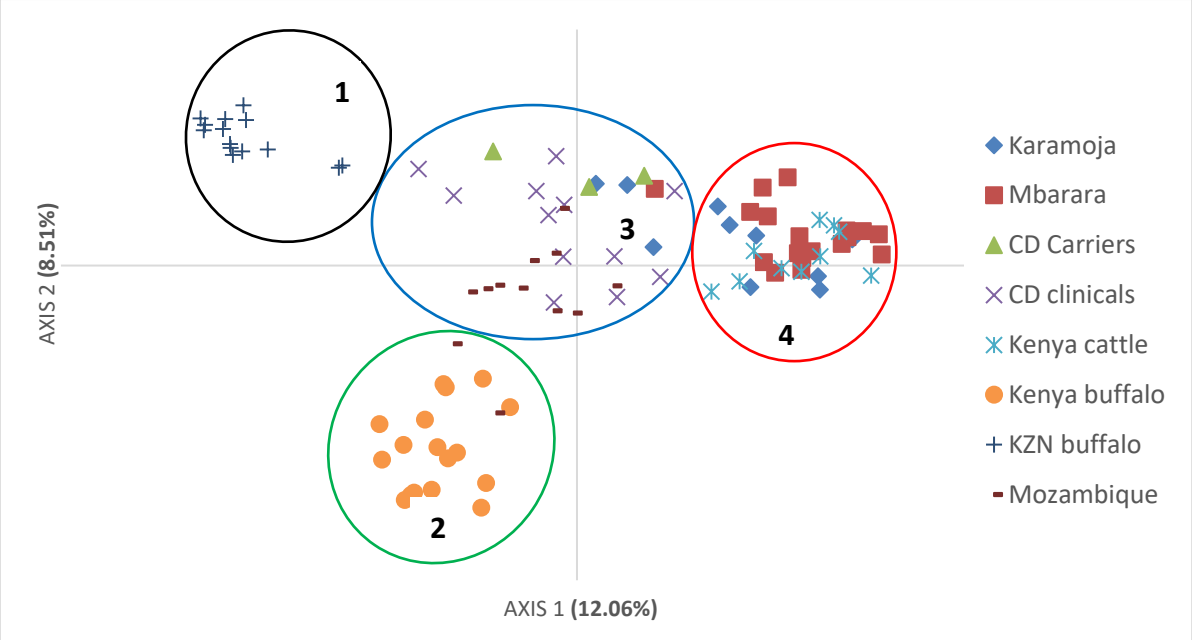

Figure 5.2: Principal Coordinate Analysis (PCoA) scatter plot showing the genetic relationship between populations.

The proportion of the variations in the dataset is indicated in each axis and the four clusters are described below:
Cluster 1 (black-bordered circle) which comprises of genotypes of parasites from KZN buffalo.
Cluster 2 (green-bordered circle) which comprises mainly of genotypes of parasites from Kenya buffalo.
Cluster 3 (blue-bordered oval) represents an admixed group comprising of genotypes of parasites from Mozambique buffalo, clinical cases of Corridor disease and non-clinical *T. parva*-positive cases from South Africa, and Uganda cattle.
Cluster 4 (red-bordered circle) which comprises of genotypes of parasites from Kenya and Uganda cattle.

Table 5.7: Pairwise Population Matrix of Mean Shannon (sHua) values over loci showing the genetic distance.

Karamoja	Mbarara	CD Carriers	CD Clinicals	Kenya cattle	Kenya buffalo	KZN buffalo	Moz. buffalo	
0.000								Karamoja
0.136	0.000							Mbarara
0.342	0.197	0.000						CD Carriers
0.430	0.408	0.173	0.000					CD Clinicals
0.199	0.182	0.305	0.456	0.000				Kenya cattle
0.468	0.515	0.255	0.437	0.493	0.000			Kenya buffalo
0.563	0.551	0.265	0.450	0.580	0.427	0.000		KZN buffalo
0.523	0.476	0.224	0.379	0.548	0.421	0.415	0.000	Moz. buffalo

5.5 Discussion

Theileria parva has a complex life cycle involving an asexual and sexual phase in the bovine host and the tick vector respectively. Diversity of *T. parva* parasites resulting from recombination activities in the tick vector has compromised the current efforts in controlling the parasite and complicated its population dynamics in the bovine and wildlife hosts. In order to establish potent control measures and evaluate their success, information on the diversity and population structure of *T. parva* field parasites is important. Thus, the current study evaluated *T. parva* parasites from bovine and wildlife hosts in eastern and southern Africa using polymorphic mini- and microsatellite markers. The satellite markers used in this study are highly informative based on their polymorphic information component (Rukambile *et al.*, 2016; Salih *et al.*, 2018). Furthermore, the markers are evenly distributed on the four chromosomes of *T. parva*, and for the pairs of loci that occur on the same chromosome, they were at least 315 kbp apart (Katzer *et al.*, 2010). Hence, the probability of the loci being physically linked is negligible.

Among the buffalo-derived *T. parva* parasites, the most diverse were from Mozambique buffalo, and the least diverse were from non-clinical *T. parva*-positive cases. Since sample size is a critical variable that influences genetic diversity in population studies (Yin *et al.*, 2018), it is highly possible that the limited diversity observed in non-clinical *T. parva*-positive cases was due to the small sample size. The African buffalo is the natural reservoir of *T. parva* which is known to harbor multiple genotypes of the parasite (Oura *et al.*, 2011b; Sibeko *et al.*, 2011), and therefore, the high levels of diversity observed in buffalo-derived parasites would be expected. Notably, there seems to be no significant differences in the diversity of *T. parva* parasites obtained from the buffalo as depicted by the MOI values. Parasites from Uganda-Mbarara were the most diverse among the cattle-derived *T. parva* parasites. The farms from which cattle were sampled are adjacent to Lake Mburo National Park (LMNP) which is not fenced, and the buffalo population in this park is *T. parva* positive (Oura *et al.*, 2011a; Oura *et al.*, 2011b). It is therefore likely that the cattle population sampled share grazing pastures with buffalo from the park, hence, the possibility of transmission of multiple *T. parva* genotypes from the buffalo to cattle. However, it is worth noting that analysis of p67 gene in parasites from Mbarara revealed limited diversity based on this gene. The possible explanation for these differences would be that p67 is a single copy gene and located on only one chromosome, and hence may not be a suitable indicator of *T. parva* diversity compared to satellite loci which are tandem

repeat sequences distributed on the entire *T. parva* genome. Nevertheless, the possibility of the large sample size analyzed for Mbarara population contributing to the high levels of diversity observed in this group compared to other cattle-derived parasites cannot be overruled. Notwithstanding that, all cattle-derived parasites from Kenya and Uganda had a common multi-locus genotype suggesting the presence of a *T. parva* population that is adapted for cattle-to-cattle transmission and circulating in eastern Africa.

When the allelic profiles of parasites associated with ECF and Corridor disease were compared, three loci (MS8, MS19 and MS33) had unique profiles, which were not shared between these parasites and other populations, suggesting a possibility of specific genetic differences between these parasites on these loci. It could be argued that the differences observed on the three loci resulted from artifacts, however, PCoA analysis of the overall data produced similar results (though not specific for the three loci) possibly indicating true genotypic differences with some similarities among parasites associated with ECF and Corridor disease. In addition, an analysis of p67 gene revealed two subtypes of allele type 1 corresponding to ECF and Corridor disease parasites, confirming that despite the similarities, these parasites have some extend of genetic differences. Considering that Corridor disease is a controlled disease in South Africa and sporadic outbreaks occur following contact between buffalo and cattle in the presence of the tick vector (Mbizeni *et al.*, 2013), the number of *T. parva* positive samples from cattle were limited for this study. This being the first diversity study on *T. parva* parasites from both cattle and buffalo from South Africa where Corridor disease is the only important *T. parva* infection, it would be essential to further confirm these findings with analysis of more *T. parva* positive field samples from cattle.

Similar to other apicomplexan parasites, *T. parva* field parasites are known to possess multiple genotypes as a result of multiplicity of infection (Oura *et al.*, 2005; Odongo *et al.*, 2006; Beck *et al.*, 2009; Muleya *et al.*, 2012; Rukambile *et al.*, 2016; Salih *et al.*, 2018) and ticks infected with a mixture of *T. parva* genotypes present an ideal prerequisite for genetic exchange (Oura *et al.*, 2005). High multiplicity of infection has been shown to positively correlate with parasite transmission intensity and host age (Weir *et al.*, 2011; Al-Hamidhi *et al.*, 2015; Roy *et al.*, 2019), where the abundance of ticks and/or the intensity of tick infections contributes to the disparities in transmission intensity (Weir *et al.*, 2011). It has also been hypothesized that due to the continuous exposure of cattle to multiple genotypes

as a result of continuous tick challenge, there is a progressive increase of co-infecting genotypes as the animals age (Weir *et al*., 2011), hence, adult animals would be expected to possess a high multiplicity of infection. All the animals sampled in the current study were adults, and the MOI values were found to be high in individual populations as well as when the populations were analyzed as one combined population indicating a likelihood of high transmission intensities within and between *T. parva* populations.

Among the buffalo-derived parasites, the highest MOI value (Max=3.00) was observed in Kenya and Mozambique buffalo parasites, while among the cattle-derived *T. parva* parasites, the population from Mbarara, adjoining LMNP, had the highest MOI value (Max=2.67). Since the wildlife host is the natural reservoir of *T. parva* and known to harbor multiple strains of the parasite (Nene *et al*., 2016), it could be possible that the observed high MOI in parasites from the buffalo is a natural phenomenon promoted by perpetual transmission by the vector. In addition, the presence of multiple strains coupled with recombination and cross-mating activities in the tick vector are likely to result in increased genetic exchange among buffalo-derived *T. parva* parasites leading to a build-up of mixed *T. parva* genotypes in the buffalo hosts. This could also happen in the bovine host especially in areas where cattle co-graze with infected buffalo in the presence of the tick vector. Hence, among the cattle-derived *T. parva* parasites, the population from Mbarara, adjoining LMNP, had the highest multiplicity of infection. Similarly, there also seems to be no significant difference in the allelic diversity in parasites obtained from cattle that co-graze with buffalo in Uganda and South Africa.

Although the current study did not establish the presence of the principal vector *Rhipicephalus appendiculatus*, in areas where samples were obtained, multi-acaricide resistance among *Rhipicephalus* ticks has been reported in Mbarara and other parts of Uganda (Vudriko *et al*., 2016) indicating the possibility of abundance of *R. appendiculatus* ticks. It is therefore likely that the abundance of *R. appendiculatus* ticks, with a likelihood of high intensity of *T. parva* infection among these ticks and the close proximity to the wildlife reservoir, could be promoting the high transmission intensities in Mbarara compared to Karamoja and Kenya. These results are concurrent with those of Oura *et al*. (2005) and Muwanika *et al*. (2016), although it would be informative to analyze samples from buffalo from LMNP to establish the possibility of transfer of buffalo genotypes to the cattle population. Parasites from non-clinical *T. parva*-positive cases had the lowest

maximum MOI value (1.83), contrary to the previous argument, since the MOI of buffalo-derived parasites would be expected to be high. The most likely justification for the low MOI value observed in these parasites could be due to the small sample size analyzed. Hence, investigation of a larger sample size would be necessary to support or invalidate this hypothesis. In general, the buffalo-derived *T. parva* parasites were more divergent compared to cattle-derived *T. parva* parasites.

Analysis of linkage equilibrium between alleles at pairs of loci within individual populations further supported the evidence of high genetic exchange among *T. parva* parasites where all individual populations were in linkage equilibrium (LE). The *T. parva* population from Mbarara was found to be in LE similar to the previous report in some populations in Mbarara (Oura *et al.*, 2005), but in contrast to linkage disequilibrium (LD) reported in the expansive western region of Uganda (Muwanika *et al.*, 2016). Parasites from cattle in Kenya were in LE contrary to LD reported in *T. parva* isolates from western, central and coastal Kenya (Odongo *et al.*, 2006). The observed LE could possibly indicate the absence of sub-structuring within populations with frequent genetic exchange promoted by panmixia. The previously reported LD in western Uganda and Kenya (Odongo *et al.*, 2006; Muwanika *et al.*, 2016) could have been due to geographical sub-structuring considering the geographical distance separating the sites from which the parasites were obtained. Although no reports are available for the parasites from southern Africa, the finding in the current study indicate that *T. parva* populations in this region are panmictic. When all individual populations were analyzed as a single metapopulation, it was found to be in LD with the standardized index of association greater than zero, indicating genetic and geographical sub-structuring of *T. parva* populations. However, a more logical explanation to the observed LD in the combined population could be that the parasites in the current study were obtained beyond geographical boundaries that could allow random genetic exchange between populations.

Analysis of molecular variance (AMOVA) revealed a higher variation of parasites within individual populations (74%) than between populations (26%) indicating that genetic divergence occurred more among parasites within specific regions (geographical areas) than between regional metapopulations. A similar phenomenon has been demonstrated in *T. parva* parasites from Kenya (Odongo *et al.*, 2006), Tanzania (Rukambile *et al.*, 2016), Uganda (Oura *et al.*, 2005; Muwanika *et al.*, 2016), Sudan (Salih *et al.*, 2018) and Zambia

(Muleya *et al.*, 2012) suggesting that cross-mating and recombination between *T. parva* parasites is restricted within populations unless there are factors that promote intermixing of cattle populations in the presence of the tick vector, which would facilitate *T. parva* spread. Genetic and geographical sub-structuring of *T. parva* parasites was observed on PCoA where parasites of buffalo origin clustered together, and parasites that have adapted to cattle for transmission among cattle formed a separate cluster. The current trivalent live *T. parva* vaccine based on cattle maintained parasites has been widely deployed in eastern Africa but does not protect against the buffalo-derived *T. parva* parasites (Sitt *et al.*, 2015). It could be hypothesized from the PCoA clustering that buffalo- and cattle-derived *T. parva* parasites are genotypically different, and hence, a possible explanation to the ineffectiveness of the live vaccine in areas where parasite sharing occurs between cattle and buffalo.

There was evidence of a very close genetic relationship between *T. parva* parasites from clinical cases of Corridor disease from South Africa and buffalo from the neighboring country, Mozambique, despite the geographical distance between these populations. Collectively, this genetic relationship together with the similarities identified on the p67 gene in buffalo-derived *T. parva* parasites from southern Africa, confirm that *T. parva* parasites responsible for Corridor disease in South Africa originate from the buffalo as a result of cross-infection. Corridor disease cases have been reported in some areas bordering Kruger National Park in Maputo province of Mozambique (Sibeko, personal communication), but none has been reported in Marromeu National Reserve (MNR) where samples in the current study were obtained. This could be due to the separation of buffalo and cattle by fencing the park. However, the genetic relationship displayed by PCoA reveal a possible risk of Corridor disease outbreak in areas bordering MNR should the buffalo population interact with cattle in the presence of the tick vector.

The genetic relationship between parasites associated with Corridor disease and East Coast fever displayed by the admixed group on PCoA analysis suggests that these parasites have similar genotypes and may have undergone geographical sub-structuring based on the disease syndromes they cause in the two African regions. However, it was recently observed that genetic diversity among *T. parva* parasites may have occurred before geographic segregation (Hemmink *et al.*, 2018). Despite the geographical distance between parasites from Kenya and Uganda, the genetic relationship between these parasites was

very close indicating a potentially homogenous *T. parva* population that is maintained in cattle and responsible for ECF in eastern Africa.

5.6 Conclusion

Evaluation of *T. parva* populations collected from cattle and buffalo in eastern and southern Africa revealed high genotypic diversity in buffalo-derived than cattle-derived parasites, with parasites associated with Corridor disease being more diverse than those associated with ECF. The *T. parva* populations analyzed were found to be panmictic, with sub-structuring based on geographical regions and the disease syndromes caused in cattle. Despite parasites associated with Corridor disease and ECF being different, their clustering on PCoA forming an admixed group suggests that they have some genotypic similarities and probably share a common ancestor. Although distinguishing allelic profiles based on MLGs on three loci were identified for Corridor disease parasites (MS8-155, MS19-127, MS33-160) and parasites associated with ECF (MS8-165, MS19-162, MS33-175), further validation by analysis of a larger sample size would be necessary.

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CHAPTER 6

General Discussion and Conclusions

6.1 General Discussion

East Coast fever and Corridor disease caused by cattle- and buffalo-derived *T. parva* respectively, are the most economically important tick-borne diseases affecting the livestock industry in sub-Saharan Africa. Although ECF was eradicated in South Africa in the 1950s, occasional outbreaks of Corridor disease still occur (reviewed in Mbizeni *et al.*, 2013). East Coast fever is prevalent and still remains a lethal bovine disease in 11 affected countries in eastern, central and southern Africa with an estimated economic loss of over USD300 million per year (reviewed in Nene *et al.*, 2016). Efforts to control *T. parva* infections in eastern Africa have been compromised by several challenges including extensive parasite diversity in the bovine and buffalo hosts (Katzer *et al.*, 2006; Oura *et al.*, 2011), and resistance of the tick-vector to the available acaricides (Ntondini *et al.*, 2008; Vudriko *et al.*, 2016; 2018). Since chemotherapy and use of live vaccine are prohibited in South Africa due to development of a carrier status (Kariuki *et al.*, 1995; Mbizeni *et al.*, 2013), consistent surveillance remains salient in containing *T. parva* infections in the country. In both cases, the presence of multiple strains of cattle- and buffalo-derived *T. parva* parasites with a likelihood of co-infections in individual animals (reviewed in Nene *et al.*, 2016), coupled with the substantial diversity of the parasite, further complicates the control efforts.

The current live vaccine deployed in eastern Africa comprises of three cattle-derived *T. parva* strains (Muguga, Kiambu V and Serengeti-transformed) which have been shown to induce an immune response that is only protective against the component strains, but not against buffalo-derived *T. parva* strains (Sitt *et al.*, 2015). One of the options that have been proposed for improvement on the scope of protection of this vaccine is inclusion of more *T. parva* strains especially from buffalo-derived parasites. Although this has been speculated to present a risk of introducing exotic *T. parva* strains in areas with endemic stability through development of carrier status in vaccinated animals, recent studies in Tanzania have shown otherwise (Magulu *et al.*, 2019). Development of a recombinant vaccine based on antigen genes that are recognized by CTLs and less diverse in both cattle- and buffalo-derived *T. parva* parasites has been envisioned to be an alternative to the live vaccine.

The identification of cattle-type parasite allele of the p67 gene from buffalo-derived parasites from South Africa (Sibeko *et al.*, 2010) similar to the allele type from cattle-

derived parasites from East Africa (Nene *et al.*, 1996; 1999) may further subvert the surveillance efforts in ensuring that ECF does not re-emerge in South Africa. It has been hypothesized that *T. parva* co-evolved with the Cape buffalo and underwent a "host jump" to become adapted to cattle where it causes ECF in eastern Africa (reviewed in Norval *et al.*, 1992). Recently, it was demonstrated that the diversity of *T. parva* in eastern and southern Africa became apparent before geographic dissociation (Hemmink *et al.*, 2018). Hence, it is possible that the parasites associated with ECF could be present in southern Africa but marginalized, and therefore, the risk of re-emergence of ECF in South Africa cannot be disregarded. Therefore, in an effort to gain more insights into the population dynamics of *T. parva* parasites, we evaluated the antigenic and genotypic diversity, and population structure of field parasites from cattle and buffalo from East and southern Africa.

We assessed the diversity of the gene encoding the surface sporozoite antigen, p67, and the antigenic epitopes of ten schizont antigen genes known to be essential in humoral and cellular immune responses respectively (McKeever *et al.*, 1994; Taracha *et al.*, 1995; Musoke *et al.*, 2005). Four allele types of the p67 encoding gene, previously described (Nene *et al.*, 1999; Sibeko *et al.*, 2010; Obara *et al.*, 2015) were identified in parasites from East and southern Africa. Of interest was p67 allele type 1 which was previously hypothesized to be exclusively found in cattle-derived parasites, hence associated with ECF in eastern Africa (Nene *et al.*, 1996). The identification of the same allele type in buffalo-derived parasites from South Africa (Sibeko *et al.*, 2010) raised concerns about the possible presence of parasites that can cause ECF in the buffalo population in that country. Thus, it became significant to determine whether this allele type is only associated with ECF parasites as previously hypothesized. The current study reveals that p67 allele type 1 has a subtype that is associated with buffalo-derived parasites from South Africa, which is different from the subtype associated with ECF and Corridor disease parasites from Kenya. It remains unknown as to whether parasites of this subtype circulating in buffalo in South Africa have the potential to adapt to cattle to eventually cause ECF as it is in East Africa. p67, a major surface sporozoite antigen, is a 67kDa protein encoded by a single copy gene that contributes to the host cell recognition and entry of the infective sporozoites into the host's lymphocytes (Musoke *et al.*, 1984), and it is the main target of sporozoite neutralizing antibodies (Dobbelaere *et al.*, 1984). Recent studies have shown that the role of p67 as a primary ligand seem to vary with the parasite type even among parasites with

p67 sequence identity (Tindih *et al.*, 2010; 2012). Subsequently, the involvement of the subtypes of p67 allele type 1 identified in the current study in *T. parva* pathogenesis require further investigation. Nonetheless, p67 has been identified as a promising vaccine candidate (Musoke *et al.*, 2005; Nene *et al.*, 2016; Lacasta *et al.*, 2018), and further improvement on the formulation of the p67-based subunit vaccine considering the differences between cattle- and buffalo-derived *T. parva* parasites from East and southern Africa identified in the current study will be necessary for enhancement of its efficacy.

The schizont antigen genes showed extensive diversity in buffalo-derived than cattle-derived parasites. The CD8+ T-cell response to *T. parva* infections predominantly involve recognition of $Tp1_{214\text{-}224,}$ $Tp2_{49\text{-}59}$ and $Tp2_{50\text{-}59}$ epitopes (MacHugh *et al.*, 2009; Connelley *et al.*, 2016). One of the $Tp1_{214\text{-}224}$ variants identified was common in both cattle- and buffalo-derived parasites from East and southern Africa, whereas $Tp2_{49\text{-}59}$ and $Tp2_{50\text{-}59}$ epitope variants were only identified in buffalo-derived *T. parva* parasites from South Africa. All variants of $Tp1_{214\text{-}224,}$ $Tp2_{49\text{-}59}$ and $Tp2_{50\text{-}59}$ epitopes resulted from amino acid residue substitutions which may not significantly affect T-cell receptor (TCR) recognition (Macdonald *et al.*, 2010; Connelley *et al.*, 2011; Steinaa *et al.*, 2012), suggesting that amino acid residues significant for immunity against *T. parva* infections may not be as prone to mutation as other residues, at least for these TpAg epitopes. Immunity to *T. parva* infections in cattle is mainly cell-mediated where CTLs recognize parasite peptides presented by MHC class I molecules (McKeever *et al.*, 1994; Taracha *et al.*, 1995). These parasite peptides are encoded by the antigenic epitope regions on the TpAg genes (Akoolo *et al.*, 2008; Graham *et al.*, 2008; Nene *et al.*, 2012). The cellular response to *T. parva* infections principally involve CTL recognition of *T. parva* peptides encoded by $Tp1_{214\text{-}224,}$ $Tp2_{49\text{-}59}$ and $Tp2_{50\text{-}59}$ epitope regions (MacHugh *et al.*, 2009; Connelley *et al.*, 2016). These results point out Tp1 and Tp2 to be favourable candidates for a recombinant vaccine against a challenge with both cattle- and buffalo-derived *T. parva* parasites, although they are not the most conserved between the cattle- and buffalo-derived parasites. Thus, it is critical to establish the role of the conserved TpAg's in the pathogenesis of cattle theileriosis caused by *T. parva*.

The population structure of *T. parva* parasites in East and southern Africa depict sub-structured populations based on the geographical region and the disease syndromes caused in cattle. There is evidence of limited genetic exchange between populations but high rate

of recombination within populations resulting in extensive genetic diversity within populations. The sub structuring illustrates two important populations of *T. parva* with one having adapted to cattle for cattle-to-cattle transmission causing ECF in eastern Africa, and the other maintained in the wildlife host with occasional transmission to the bovine host where it causes Corridor disease in South Africa. The clustering also demonstrate that the parasites associated with ECF and Corridor disease have some genotypic similarities but with differences on three satellite loci (MS8, MS19 and MS33). Collectively, the results of the current study depict a sub-structured *T. parva* population based on the two regions with distinct differences in the parasites based on p67, TpAg genes and genotypic profiles. However, it is worth noting that the differences observed may not have direct link to disease outcomes, but only serve to differentiate between the parasite types from the two regions.

6.2 Conclusions

This study aimed at assessing the population genetic structure, genotypic and antigenic diversity of cattle- and buffalo-derived *T. parva* field parasites from East and southern Africa. Conclusions drawn from the findings of this study are as follows;

1. The p67 allele type 1 which was initially thought to be exclusively found in cattle-derived parasites, and later identified in buffalo-derived parasites from South Africa, comprises of two subtypes; subtype 1 associated with ECF and Corridor disease *T. parva* parasites from Kenya, and subtype 2 associated with buffalo-derived *T. parva* parasites from southern Africa.
2. $Tp2_{49\text{-}59}$ and $Tp2_{50\text{-}59}$ epitope variants unique to buffalo-derived *T. parva* parasites from South Africa were identified. Although amino acid substitutions in both Tp1 and Tp2 immunodominant epitopes did not affect amino acid residues significant for CTL response, it is possible that residues involved in TCR recognition in Tp1 and Tp2 epitopes are less susceptible to mutation. Tp1 and Tp2 are promising vaccine candidates compared to p67 in conferring protection against cattle- and buffalo-derived *T. parva* parasites.
3. Three satellite loci (MS8, MS19 and MS33) have distinct genotypic profiles for parasites associated with ECF in East Africa, and Corridor disease in South Africa. Buffalo-derived parasites are more genotypically diverse than cattle-derived parasites.

Cattle-derived parasites have common multi-locus genotype unlike buffalo-derived parasites which lack shared genotypes.

4. The population structure of *T. parva* is sub-structured based on the geographical region and the disease syndrome caused in cattle in the two regions investigated. There is also limited genetic mixing between than within *T. parva* populations in the two regions. There is a subpopulation of *T. parva* which is adapted for cattle-to-cattle transmission and causing ECF in eastern Africa, and another subpopulation circulating in the wildlife host with sporadic transmission to cattle causing Corridor disease in South Africa.

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APPENDIX 1: Tables with mutations details of TpAg genes epitopes

Table 1.0: Details of mutations identified on Tp1 antigenic epitope.

Sample group	11-mer $Tp1_{214-224}$ (VGYPKVKEEML) epitope mutation details								
	Coverage	**Alt#**	**Alt# (F; R)**	**Reference**	**Mutation call**	**Reference nucleotide position**	**Overall mutation score**	**Epitope variants**	**Function**
Kenya buffalo	40,001	2444	1433;1011	TP03_0849	delGCinsTA	807_808	35.00	VGYPKVKEE**II**	Missense
Kenya cattle	73,728	71367	39841;31526		delGCinsTA	807_808	35.30	VGYPKVKEE**II**	Missense
Uganda-Mbarara cattle	73,226	31938	18046;13892		delGCinsTA	807_808	35.10	VGYPKVKEE**II**	Missense
Uganda-Karamoja cattle	17,972	-	-		None	-	-	VGYPKVKEEML	-
Tanzania buffalo	70,578	-	-		None	-	-	VGYPKVKEEML	-
Tanzania Simanjiro cattle	73,820	-	-		None	-	-	VGYPKVKEEML	-
Tanzania Tanga cattle	21,550	20821	11061;9760		delGCinsTA	807_808	39.50	VGYPKVKEE**II**	Missense
Non-clinical *T. parva*-positive cases	13,052	-	-		None	-	-	VGYPKVKEEML	-
CD clinical cases	44,813	12424	6347;6077		delGCinsTA	807_808	30.10	VGYPKVKEE**II**	Missense
KNP buffalo	54,519	26026	15812;10214		delGCinsTA	807_808	35.20	VGYPKVKEE**II**	Missense
KZN buffalo	35,107	9686	6000;3686		C>AC	809	33.00	VGYPKVKEEM**I**	Missense
Mozambique buffalo	13,506	-	-		None	-	-	VGYPKVKEEML	-

Coverage - the total number of reads that aligned at the variant location.

Alt # - the total number of reads that contained the alternate (variant) nucleotide at the variant position.

Alt # (F; R) - the number of reads that showed the alternate (variant) nucleotide in the forward (F) direction and the number of reads that showed the alternate (variant) nucleotide in the reverse (R) direction.

Overall mutation score - an empirical estimation of the likelihood that a given variant/mutation call is real and not an artifact of sequencing or alignment errors. A score ≥12.00 (95% confidence) is considered statistically significant.

delGCinsTA - deletion of GC and insertion of TA at the indicated reference nucleotide position(s).

C>AC - at the indicated reference nucleotide position, the reference sequence has a "C" whereas the alternate/variant sequence has either an "A" or "C".

NB: Bolded and underlined shows the variant amino acid residue.

Table 1.1: Details of mutations identified Tp2 antigenic epitopes.

Source of samples	**Tp2 epitopes** (^{27}SHEELKKLGML^{37}, ^{40}DGFDRDALF^{48}, ^{49}KSSHGMGKVGK^{59}, ^{50}SSHGMGKVGK^{59}, ^{96}FAQSLVCVL^{104}, ^{98}QSLVCVLMK^{106}, ^{138}KTSIPNPCKW^{147}) **mutation details**								
	Coverage	**Alt#**	**Alt# (F;R)**	**Reference**	**Mutation call**	**Reference nucleotide position**	**Overall mutation score**	**Epitope variants**	**Function**
Kenya buffalo	10340	10170	6153;4017	TP01_0056	C>G	210	27.50	S**D**EELKKLGML	Missense
	17657	922	129;793		A>AG	419	25.60	FAQSLVCVL	Synonymous
	18012	586	59;527		C>AC	426	25.30	FAQS**I**VCVL	Missense
								QS**I**VCVLMK	
	17761	473	51;422		C>CT	434	27.80	FAQSLVCVL	Synonymous
								QSLVCVLMK	
	8490	197	136;61		A>AT	566	27.10	KTSIPNPC**N**W	Missense
Kenya cattle	31195	30693	11433;19260		C>G	210	34.60	S**D**EELKKLGML	Missense
	28425	27154	5066;22088		A>AT	224	34.90	SHEEL**N**KLGML	Missense
Uganda-Mbarara cattle	1671	1662	1383;279		A>C	543	20.90	K**P**SIPNPCKW	Missense
	1840	1832	1501;331		A>G	549	26.40	KTS**V**PNPCKW	Missense
	2061	2025	1609;416		A>T	560	21.10	KTSIPN**P**CKW	Synonymous
	2153	2143	1706;437		A>G	564	20.60	KTSIPN**P**C**E**W	Missense
Uganda-Karamoja cattle	817	740	3;737		A>AG	302	18.80	KSSHGMGKV**G**K	Synonymous
								SSHGMGKV**G**K	
	3223	3061	2323;738		A>C	543	16.80	K**P**SIPNPCKW	Missense
	3616	3593	2625;968		A>G	549	25.40	KTS**V**PNPCKW	Missense
	4195	4131	2785;1346		A>T	560	23.20	KTSIPN**P**CKW	Synonymous
	4396	4381	2961;1420		A>G	564	23.30	KTSIPN**P**C**E**W	Missense
Tanzania buffalo	28837	28463	9908;18555		C>G	210	32.40	S**D**EELKKLGML	Missense
	31803	745	522;223		A>AT	566	26.20	KTSIPNPC**N**W	Missense
Tanzania Simanjiro cattle	66074	13469	3027;10442		A>AG	214	28.30	SH**G**ELKKLGML	Missense
Tanzania Tanga cattle	94312	92999	23797;69202		C>G	210	37.20	S**D**EELKKLGML	Missense
	94958	90300	12573;77727		A>AT	224	39.00	SHEEL**N**KLGML	Missense
Non-clinical *T. parva*-positive cases	-	-	-		-	-	-	-	-
CD clinical cases	7629	7397	2455;4942		C>CG	210	23.00	S**D**EELKKLGML	Missense
	7256	6941	1801;5140		A>AT	215	22.50	SH**D**ELKKLGML	Missense
	8362	1216	272;944		A>AC	223	22.60	SHEEL**T**KLGML	Missense

	11160	861	220;641		T>AT	276	21.80	K**T**SHGMGKVGK **T**SHGMGKVGK	Missense
	10843	507	140;367		G>CG	286	23.80	KSSH**A**MGKVGK SSH**A**MGKVGK	Missense
	10838	10107	3920;6187		T>AT	287	28.30	KSSH**G**MGKVGK SSH**G**MGKVGK	Synonymous
	6021	5865	2814;3051		C>G	420	17.00	**E**SLVCVLMK	Missense
KNP buffalo	642	636	13;623	TP01_0056	T>A	287	21.80	KSSH**G**MGKVGK SSH**G**MGKVGK	Synonymous
	2825	2754	1423;1331		A>T	560	26.20	KTSIPN**P**CKW	Synonymous
	2928	822, 2082	479;343, 1031;1051		A>C A>G	564	16.60	KTSIPN**P**C**Q**W KTSIPN**P**C**E**W	Missense
KZN buffalo	942	925	338;587		A>G	274	20.80	**R**SSH**G**MGKVGK	Missense
	1027	960	366;594		T>AT	287	22.30	KSSH**G**MGKVGK SSH**G**MGKVGK	Synonymous
	3571	868, 2617	703;165, 2038;579		A>C A>G	564	19.60	KTSIPN**P**C**Q**W KTSIPN**P**C**E**W	Missense
Mozambique buffalo	1704	1696	633;1063		C>G	210	25.40	S**D**EELKKLGML	Missense
	1270	1247	175;1072		A>T	215	16.60	SH**D**ELKKLGML	Missense
	3623	3586	1723;1863		A>G	564	26.50	KTSIPN**P**C**E**W	Missense

Table 1.2: Details of mutations identified on Tp3 antigenic region.

Sample group	Tp3 gene mutation details								
	Coverage	**Alt#**	**Alt# (F;R)**	**Reference**	**Mutation call**	**Reference Nucleotide position**	**Overall mutation score**	**Amino acid change**	**Function**
Kenya buffalo	69475	12788	4973;7815	TP01_0868	T>CT	404	31.30	p.**M**135**TM**	Missense
	74811	14160	6222;7938		A>AC	422	32.30	p.**N**141**NT**	Missense
	90193	58586	30879;27707		C>CT	465	38.40	p.**T**155**TT**	Synonymous
	71348	25256	14712;10544		C>CT	507	36.10	p.**P**169**PP**	Synonymous
	65751	12677	7935;4742		G>C	514	35.80	p.**V**172**L**	Missense
Kenya cattle	30020	-	-		-	-	-	-	-
UG-Mbarara cattle	43604	-	-		-	-	-	-	-
UG-Karamoja cattle	73632	16050	8809;7241		C>CT	406	35.80	p.**P**136**PS**	Missense
Non-clinical *T. parva*-positive cases	84113	43563	20645;22918		A>AG	435	33.00	p.**T**145**TT**	Synonymous
	84980	44231	21138;23093		G>AG	438	33.10	p.**P**146**PP**	Synonymous
	87673	45412	22510;22902		T>GT	450	33.60	p.**V**150**VV**	Synonymous
	90533	89766	43153;46613		C>T	465	38.10	p.**T**155**T**	Synonymous
	78134	76886	42398;34488		C>T	507	38.40	p.**P**169**P**	Synonymous
	32169	12397	9984;2413		C>AC	600	33.00	p.**T**200**TT**	Synonymous
CD clinical cases	70760	70498	34476;36022		T>C	404	38.00	p.**M**135**T**	Missense
	78344	77903	39793;38110		G>A	438	37.60	p.**P**146**P**	Synonymous
	72587	71190	41225;29965		C>T	507	38.10	p.**P**169**P**	Synonymous
KZN buffalo	25469	15434	4638;10796		T>CT	326	30.10	p.**V**109**AV**	Missense
	86991	72348	27379;44969		G>AG	438	37.40	p.**P**146**PP**	Synonymous
	89386	88597	37619;50978		C>T	465	39.20	p.**T**155**T**	Synonymous
	79593	13062	6262;6800		C>AC	487	36.40	p.**L**163**IL**	Missense
	71358	70153	33939;36214		C>T	507	38.50	p.**P**169**P**	Synonymous
Mozambique buffalo	89368	38730	15531;23199		A>AG	435	32.00	p.**T**145**TT**	Synonymous
	90111	88996	41013;47983		G>A	438	37.40	p.**P**146**P**	Synonymous
	92462	91780	43915;47865		T>G	450	38.40	p.**V**150**V**	Synonymous
	91534	49765	26818;22947		A>AC	473	33.30	p.**N**158**NT**	Missense
	80725	79300	47168;32132		C>T	507	38.50	p.**P**169**P**	Synonymous
	74809	40529	27141;13388		T>CT	515	31.90	p.**V**172**AV**	Missense

p.**M**135**TM** - "p" refers to protein sequence. At the indicated reference amino acid position, the reference sequence has an amino acid residue "**M**" where are the alternate/variant sequence has a "**T**" or "**M**". The resulting non-synonymous substitution (with amino acid residue "**T**") is due to the corresponding nucleotide substitution (T>CT) at the indicated reference nucleotide position.

p.**T**155**TT -** the nucleotide substitution (C>CT) at the indicated reference nucleotide position results in synonymous amino acid residue substitution with a "**T**" due to degeneracy.

p.**V**172**L** - at the indicated reference amino acid position, the reference sequence has an amino acid residue "**V**" whereas the alternate/variant sequence has a "**L**".

Table 1.3: Details of mutations identified on Tp4 antigenic epitope.

Sample group	9-mer Tp4$_{328-336}$ (TGASIQTTL) epitope mutation details								
	Coverage	**Alt#**	**Alt# (F;R)**	**Reference**	**Mutation call**	**Reference Nucleotide position**	**Overall mutation score**	**Epitope variants**	**Function**
Kenya buffalo	14,245	307	43;264	TP03_0210	T>CT	1005	21.70	TGASIQT**T**L	Synonymous
Kenya cattle	9,121	191	160;31		delCinsT	1003	17.30	TGASIQT**S**L	Missense
Uganda-Mbarara cattle	3,981	211	172;39		delCinsA	989	24.50	TG**D**SIQTTL	Missense
Uganda-Karamoja cattle	7,525	-	-		None	-	-	TGASIQTTL	-
Non-clinical *T. parva*-positive cases	9,525	-	-		None	-	-	TGASIQTTL	-
CD clinical cases	3,823	-	-		None	-	-	TGASIQTTL	-
KNP buffalo	7,417	2838	1023;1815		T>CT	984	23.50	**T**GASIQTTL	Synonymous
	7,161	6850	2557;4293		T>CT	993	28.30	TGA**S**IQTTL	Synonymous
	6,727	6448	2301;4147		C>CT	1002	29.40	TGASIQ**T**TL	Synonymous
KZN buffalo	11,598	2263	1488;775		A>T	981	31.50	**S**GASIQTTL	Missense
	18,062	2592	830;1762		T>AT	993	27.80	TGA**S**IQTTL	Synonymous
	16,948	2426	713;1713		T>CT	1005	27.30	TGASIQT**T**L	Synonymous
Mozambique buffalo	4,946	1082	560;522		T>CT	984	21.88	**T**GASIQTTL	Synonymous
	9,446	1555	511;1044		T>CT	996	16.46	TGAS**I**QTTL	Synonymous
	9,233	1505	469;1036		C>CT	1002	16.30	TGASIQ**T**TL	Synonymous

Table 1.4: Details of mutations identified on Tp5 antigenic epitope.

Sample group	9-mer $Tp5_{87-95}$ (SKADVIAKY) epitope mutation details								
	Coverage	**Alt#**	**Alt# (F;R)**	**Reference**	**Mutation call**	**Reference nucleotide position**	**Overall mutation score**	**Epitope variants**	**Function**
Kenya buffalo	10,834	-	-	TP02_0767	None	-	-	SKADVIAKY	-
Kenya cattle	9,816	-	-		None	-	-	SKADVIAKY	-
UG-Mbarara cattle	29,503	3766	3352;414		G>AG	319	32.10	**N**KADVIAKY	Missense
UG-Karamoja cattle	12,004	-	-		None	-	-	SKADVIAKY	-
Non-clinical *T. parva*-positive cases	8,269	-	-		None	-	-	SKADVIAKY	-
CD clinical cases	6,195	-	-		None	--		SKADVIAKY	-
KNP buffalo	9,328	-	-		None	-	-	SKADVIAKY	-
KZN buffalo	15,047	-	-		None	-	-	SKADVIAKY	-
Mozambique buffalo	11,042	-	-		None	-	-	SKADVIAKY	-

Table 1.5: Details of mutations identified on Tp6 antigenic region.

Sample group	Tp6 gene mutation details								
	Coverage	**Alt#**	**Alt# (F; R)**	**Reference**	**Mutation call**	**Reference Nucleotide position**	**Overall mutation score**	**Amino acid change**	**Function**
Kenya buffalo	67287	64941	37478;27463	TP01_0188	C>G	364	29.60	p.**G**118**GG**	Synonymous
	67873	67030	40838;26192		T>C	370	28.00	p.**D**120**D**	Synonymous
	67542	66818	41223;25595		C>T	373	27.70	p.**Y**121**Y**	Synonymous
	52827	27222	24374;2848		T>AT	421	33.10	p.**I**137**II**	Synonymous
Kenya cattle	75105	31700	17826;13874		C>CG	364	30.70	p.**G**118**GG**	Synonymous
	76033	32626	19176;13450		T>CT	370	31.00	p.**D**120**DD**	Synonymous
	75778	32608	19298;13310		C>CT	373	31.00	p.**Y**121**YY**	Synonymous
	66367	27924	20703;7221		T>AT	421	29.00	p.**I**137**II**	Synonymous
	65586	26512	19702;6810		G>GT	424	28.00	p.**V**138**VV**	Synonymous
	62749	26296	20147;6149		C>CT	436	35.20	p.**N**142**NN**	Synonymous
	54461	22243	17934;4309		A>AG	466	35.80	p.**R**152**RR**	Synonymous
UG-Mbarara cattle	69451	13851	7722;6129		C>CG	364	30.10	p.**G**118**GG**	Synonymous
	70866	14419	8634;5785		T>CT	370	30.50	p.**D**120**DD**	Synonymous
	70471	14364	8686;5678		C>CT	373	30.50	p.**Y**121**YY**	Synonymous
	60177	12083	10214;1869		T>AT	421	29.10	p.**I**137**II**	Synonymous
	59599	11499	9818;1681		G>GT	424	28.40	p.**V**138**VV**	Synonymous
	58022	11641	10110;1531		C>CT	436	35.40	p.**N**142**NN**	Synonymous
	53325	10489	9303;1186		A>AG	466	35.40	p.**R**152**RR**	Synonymous
UG-Karamoja cattle	72644	58176	33959;24217		T>CT	370	31.30	p.**D**120**DD**	Synonymous
	72193	57917	34207;23710		C>CT	373	31.30	p.**Y**121**YY**	Synonymous
	60396	48618	40511;8107		T>AT	421	28.40	p.**I**137**II**	Synonymous
	59945	46555	39113;7442		G>GT	424	26.90	p.**V**138**VV**	Synonymous
	58196	35672	30608;5064		C>CT	436	36.50	p.**N**142**NN**	Synonymous
	52569	31616	27640;3976		A>AG	466	36.00	p.**R**152**RR**	Synonymous
Non-clinical *T. parva*-positive cases	49928	16404	4983;11421		C>CT	307	31.90	p.**C**99**CC**	Synonymous
	58357	19361	7334;12027		C>CT	325	33.90	p.**P**105**PP**	Synonymous
	69081	66523	35071;31452		C>G	364	28.70	p.**G**118**GG**	Synonymous
	69923	69060	38767;30293		T>C	370	28.10	p.**D**120**D**	Synonymous
	69562	68811	39008;29803		C>T	373	28.70	p.**Y**121**Y**	Synonymous
	58823	37841	29030;8811		T>AT	421	37.30	p.**I**137**II**	Synonymous
	58218	35939	27678;8261		G>GT	424	36.90	p.**V**138**VV**	Synonymous

	36951	12990	10787;2203		G>GT	496	35.60	p.**V**162**VV**	Synonymous
CD clinical cases	38195	37588	17296;20292		C>T	325	33.80	p.**P**105**P**	Synonymous
	44551	42664	23239;19425		C>G	364	29.00	p.**G**118**GG**	Synonymous
	45315	44625	25515;19110		T>C	370	27.30	p.**D**120**D**	Synonymous
	45265	44699	25723;18976		C>T	373	27.30	p.**Y**121**Y**	Synonymous
	39304	7656	5321;2335		T>AT	421	34.50	p.**I**137**II**	Synonymous
	39037	7324	5105;2219		G>GT	424	34.10	p.**V**138**VV**	Synonymous
	24028	18235	14544;3691		G>GT	496	34.00	p.**V**162**VV**	Synonymous
KNP buffalo	49495	5486	1761;3725		C>CT	307	34.10	p.**C**99**CC**	Synonymous
	57370	36827	17730;19097		C>CT	325	32.80	p.**P**105**PP**	Synonymous
	67978	58544	34534;24010		T>CT	370	28.60	p.**D**120**DD**	Synonymous
	67679	66887	40761;26126		C>T	373	28.00	p.**Y**121**Y**	Synonymous
	67970	8485	6020;2465		C>CT	376	26.90	p.**D**122**DD**	Synonymous
	36282	12907	10910;1997		G>T	495	29.70	p.**V**162**V**	Synonymous
KZN buffalo	52388	20615	8228;12387		C>CT	325	37.00	p.**P**105**PP**	Synonymous
	55032	9954	4557;5397		A>AG	337	36.00	p.**R**109**RR**	Synonymous
	61697	48149	25064;23085		C>CG	364	28.10	p.**G**118**GG**	Synonymous
	62008	61133	34981;26152		T>C	370	27.50	p.**D**120**D**	Synonymous
	61320	60548	35156;25392		C>T	373	27.50	p.**Y**121**Y**	Synonymous
	51194	10787	9049;1738		T>AT	421	37.50	p.**I**137**II**	Synonymous
	50867	10196	8642;1554		G>GT	424	35.00	p.**V**138**VV**	Synonymous
	44625	8356	7385;971		A>AG	466	34.50	p.**R**152**RR**	Synonymous
Mozambique buffalo	38103	7519	2299;5220		C>CT	307	32.60	p.**C**99**CC**	Synonymous
	44241	19146	9279;9867		C>CT	325	31.20	p.**P**105**PP**	Synonymous
	53050	50796	28468;22328		C>G	364	27.70	p.**G**118**GG**	Synonymous
	53816	53090	31924;21166		T>C	370	26.80	p.**D**120**D**	Synonymous
	53451	52809	32115;20694		C>T	373	27.40	p.**Y**121**Y**	Synonymous
	35635	7229	6354;875		A>AG	475	35.80	p.**K**155**KK**	Synonymous
	27950	5849	5049;800		G>T	495	30.40	p.**V**162**V**	Synonymous

Table 1.6: Details of mutations identified on Tp7 antigenic epitope.

Sample group	9-mer $Tp7_{206-214}$ (EFISFPISL) epitope mutation details								
	Coverage	**Alt#**	**Alt# (F; R)**	**Reference**	**Mutation call**	**Reference nucleotide position**	**Overall mutation score**	**Epitope variants**	**Function**
Kenya buffalo	15,822	-	-	TP02_0244	None	-	-	EFISFPISL	-
Kenya cattle	17,797	-	-		None	-	-	EFISFPISL	-
UG-Mbarara cattle	20,665	-	-		None	-	-	EFISFPISL	-
UG-Karamoja cattle	17,846	-	-		None	-	-	EFISFPISL	-
Non-clinical *T. parva*-positive cases	17,214	-	-		None	-	-	EFISFPISL	-
CD clinical cases	32,689	32,504	3515;28989		G>A	741	34.30	EFISFPI**S**L	Synonymous
KNP buffalo	44,998	44,750	1488;43262		G>A	741	35.90	EFISFPI**S**L	Synonymous
KZN buffalo	32,736	32,536	1270;31266		G>A	741	35.80	EFISFPI**S**L	Synonymous
Mozambique buffalo	20,602	-	-		None	-	-	EFISFPISL	-

Table 1.7: Details of mutations identified on Tp8 antigenic epitope.

Sample group	9-mer $Tp8_{379-387}$ (CGAELNHFL) epitope mutation details								
	Coverage	**Alt#**	**Alt# (F, R)**	**Reference**	**Mutation call**	**Reference nucleotide position**	**Overall mutation score**	**Epitope variants**	**Function**
Kenya buffalo	32003	-	-	TP02_0140	None	-	-	CGAELNHFL	-
Kenya cattle	34176	-	-		None	-	-	CGAELNHFL	-
UG-Mbarara cattle	43687	-	-		None	-	-	CGAELNHFL	-
UG-Karamoja cattle	39679	-	-		None	-	-	CGAELNHFL	-
Non-clinical *T. parva*-positive cases	47627	-	-		None	-	-	CGAELNHFL	-
CD clinical cases	31782	-	-		None	-	-	CGAELNHFL	-
KNP buffalo	-	-	-		-	-	-	-	-
KZN buffalo	30563	-	-		None	-	-	CGAELNHFL	-
Mozambique buffalo	87624	39990	27189;12801		A>AG	1146	33.60	CGA**E**LNHFL	Synonymous
	86787	16247	10227;6020		G>AG	1149	25.90	CGAE**L**NHFL	Synonymous

Table 1.8: Details of mutations identified on Tp10 antigenic region.

Sample group	Tp10 gene mutation details								
	Coverage	**Alt#**	**Alt# (F; R)**	**Reference**	**Mutation call**	**Reference Nucleotide position**	**Overall mutation score**	**Amino acid change**	**Function**
Kenya buffalo	17209	-	-	TP04_0772	None	-	-	-	-
Kenya cattle	4732	2086	226;1860		T>CT	851	28.50	p.**L**284**PL**	Missense
UG-Mbarara cattle	36923	36114	2207;33907		delTCTinsCCG	829	36.10	p.**S**277**P**	Missense
UG-Karamoja cattle	32183	31512	2447;29065		delTCTinsCCG	829	34.90	p.**S**277**P**	Missense
Non-clinical *T. parva*-positive cases	9772	299	38;261		A>AG	926	29.70	p.**Q**309**QR**	Missense
CD clinical cases	12230	2640	648;1992		C>CT	863	28.70	p.**S**288**SL**	Missense
KNP buffalo	14605	-	-		None	-	-	-	-
KZN buffalo	31941	25458	2869;22589		T>AG	831	33.40	p.**S**277**SS**	Synonymous
	32198	6226	652;5574		T>AT	835	34.20	p.**Y**279**NY**	Missense
	31682	20530	3718;16812		C>CT	863	35.00	p.**S**288**SL**	Missense
	19037	2505	571;1934		C>AC	911	33.50	p.**P**304**QP**	Missense
Mozambique buffalo	35432	6422	1203;5219		T>CT	832	33.80	p.L278LL	Synonymous
	35408	6985	1172;5813		A>AG	839	36.40	p.Y280YC	Missense
	24549	4749	1032;3717		T>CT	896	34.80	p.M299TM	Missense

APPENDIX 2: Summary of the frequencies of predominant alleles for every marker per population

Marker	Uganda-Karamoja cattle			Uganda-Mbarara cattle			CD clinical cases			Non-clinical *T. parva*-positive cases		
	Allele (bp)	Allele freq.	Most frequent allele (bp)	Allele (bp)	Allele freq.	Most frequent allele (bp)	Allele (bp)	Allele freq.	Most frequent allele (bp)	Allele (bp)	Allele freq.	Most frequent allele (bp)
ms2	179	0.40	179	179	0.5	179	179	0.2	202, 214	214	1	214
	188	0.30		188	0.3		202	0.3				
	204	0.10		214	0.2		209	0.1				
	214	0.20					214	0.3				
							250	0.1				
MS3	209	0.11	239, 253	228	0.06	239	190	0.11	219	248	1	248
	239	0.33		239	0.24		219	0.33				
	253	0.33		248	0.06		259	0.22				
	278	0.11		253	0.06		268	0.11				
	377	0.11		259	0.06		327	0.11				
				263	0.06		358	0.11				
				278	0.12							
				286	0.06							
				309	0.06							
				358	0.06							
				367	0.12							
				377	0.06							
ms5	153	0.13	172	149	0.07	172	147	0.08	172	159	0.5	159, 162
	172	0.75		153	0.14		153	0.08		162	0.5	
	185	0.13		162	0.14		159	0.08				
				172	0.57		162	0.25				
				179	0.07		172	0.42				
							177	0.08				
ms7	150	1	150	150	1	150	142	0.25	150	150	1	150
							150	0.75				
MS7	152	0.5	152	152	0.38	152	152	0.10	249	152	0.5	152, 232
	249	0.13		208	0.08		171	0.10		232	0.5	
	291	0.13		291	0.31		208	0.10				
	334	0.25		312	0.08		232	0.20				
				334	0.15		249	0.30				
							317	0.10				
							359	0.10				

Marker	Uganda-Karamoja cattle			Uganda-Mbarara cattle			CD clinical cases			Non-clinical *T. parva*-positive case		
	Allele (bp)	Allele freq.	Most frequent allele (bp)	Allele (bp)	Allele freq.	Most frequent allele (bp)	Allele (bp)	Allele freq.	Most frequent allele (bp)	Allele (bp)	Allele freq.	Most frequent allele (bp)
MS8	165	0.38	165	165	0.72	165	155	0.5	155	172	0.50	155, 172
	191	0.13		243	0.06		172	0.25		155	0.50	
	252	0.38		271	0.11		191	0.25				
	291	0.13		281	0.11							
MS16	273	0.80	273	257	0.07	273, 289	176	0.50	176	189	1	189
	289	0.20		273	0.33		184	0.10				
				289	0.33		189	0.10				
				309	0.13		206	0.20				
				324	0.07		363	0.10				
				389	0.07							
MS19	162	0.33	162, 264	162	0.30	162, 252	127	0.40	127	127	0.50	127, 179
	264	0.33		252	0.30		131	0.20		179	0.50	
	271	0.11		264	0.20		139	0.30				
	324	0.11		312	0.20		179	0.10				
	338	0.11										
MS25	199	0.40	199	199	0.74	199	199	0.50	199	-		-
	205	0.20		205	0.07		187	0.33				
	219	0.20		214	0.40		269	0.17				
	269	0.20		219	0.07							
MS27	124	0.33	209	124	0.06	209	149	0.30	149, 161	149	0.50	149, 209
	209	0.67		149	0.13		161	0.30		209	0.50	
				209	0.69		173	0.10				
				221	0.13		185	0.20				
							209	0.10				
MS33	175	0.60	175	145	0.12	175	145	0.20	160	160	1	160
	216	0.40		160	0.06		160	0.60				
				175	0.71		175	0.10				
				216	0.12		216	0.10				
MS34	182	0.11	248	182	0.06	248	178	0.18	248	201	0.50	201, 195
	233	0.11		195	0.06		192	0.18		195	0.50	
	248	0.44		248	0.56		248	0.55				
	279	0.33		279	0.31		257	0.09				

Marker	Kenya cattle			Kenya buffalo			KZN buffalo			Mozambique buffalo		
	Allele (bp)	Allele freq.	Most frequent allele (bp)	Allele (bp)	Allele freq.	Most frequent allele (bp)	Allele (bp)	Allele freq.	Most frequent allele (bp)	Allele (bp)	Allele freq.	Most frequent allele (bp)
ms2	179	0.50	179	188	0.06	202	190	0.08	202	179	0.33	179
	188	0.10		190	0.06		202	0.92		188	0.17	
	217	0.40		202	0.82					190	0.25	
				224	0.06					202	0.17	
										211	0.08	
MS3	190	0.11	239	196	0.07	239, 253	239	0.07	244	228	0.36	228
	228	0.22		239	0.40		244	0.86		239	0.09	
	239	0.33		248	0.07		253	0.07		244	0.18	
	248	0.11		253	0.40					268	0.09	
	253	0.11		263	0.07					273	0.09	
	339	0.11								294	0.09	
										334	0.09	
ms5	149	0.11	153, 172	149	0.13	172	149	0.21	172	159	0.25	172
	153	0.33		153	0.07		153	0.14		162	0.25	
	157	0.11		162	0.07		162	0.21		170	0.13	
	159	0.11		172	0.73		170	0.07		172	0.38	
	172	0.33					172	0.36				
ms7	150	1	150	142	1	142	172	1	172	146	0.25	153
										153	0.75	
MS7	152	0.75	152	152	0.29	171	253	0.92	253	152	0.08	171, 192
	291	0.13		171	0.71		274	0.08		171	0.33	
	374	0.13								192	0.33	
										208	0.17	
										232	0.08	

Marker	Kenya cattle			Kenya buffalo			KZN buffalo			Mozambique buffalo		
	Allele (bp)	Allele freq.	Most frequent allele (bp)	Allele (bp)	Allele freq.	Most frequent allele (bp)	Allele (bp)	Allele freq.	Most frequent allele (bp)	Allele (bp)	Allele freq.	Most frequent allele (bp)
MS8	165	0.44	165	191	1	191	155	0.64	155	152	0.08	
	168	0.11					172	0.36		155	0.08	183
	243	0.11								172	0.17	
	252	0.22								183	0.42	
	260	0.11								211	0.08	
										220	0.08	
										243	0.08	
MS16	273	0.40	273	189	0.17	223	176	0.20	206	176	0.33	189
	289	0.30		223	0.67		189	0.10		189	0.42	
	309	0.10		244	0.17		194	0.10		206	0.17	
	389	0.20					206	0.60		223	0.08	
MS19	162	0.40	162	128	0.31	165	150	0.42	150	127	0.08	127
	264	0.10		150	0.06		156	0.25				
	297	0.30		159	0.06		162	0.08				
	324	0.20		165	0.56		175	0.25				
MS25	187	0.10	199	184	0.18	199	190	1	190	187	0.08	214
	194	0.10		199	0.65					190	0.08	
	199	0.60		203	0.06					199	0.08	
	275	0.20		214	0.12					205	0.08	
										214	0.42	
										251	0.25	
MS27	124	0.25	209, 221	135	0.09	185	149	1	149	149	0.25	161
	209	0.36		149	0.18					161	0.33	
	221	0.36		161	0.18					173	0.25	
				185	0.55					209	0.17	
MS33	175	0.50	175	148	0.23	184	150	1	150	145	0.33	145, 158
	181	0.10		150	0.07					158	0.33	
	184	0.20		181	0.23					160	0.08	
	216	0.20		184	0.40					208	0.25	
MS34	248	1	248	182	0.88	182	182	1	182	178	0.10	182
				232	0.12					182	0.60	
										195	0.30	

APPENDIX 3: Research project approvals and permits

AEC approval:

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Population genetic structure and antigenic diversity of Theileria parva in cattle within the wildlife-livestock interface and in peri-urban areas**
PROJECT NUMBER	**V080-16**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. DL Mukolwe**

STUDENT NUMBER (where applicable)	**UP_15282962**
DISSERTATION/THESIS SUBMITTED FOR	**PhD**

ANIMAL SPECIES	**Bovine**	
NUMBER OF ANIMALS	**384**	
Approval period to use animals for research/testing purposes		July 2016 – July 2017
SUPERVISOR	**Dr. KP Sibeko-Matjila**	

KINDLY NOTE:

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

APPROVED	Date 27 July 2016
CHAIRMAN: UP Animal Ethics Committee	Signature

Section 20:

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture,, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/4/1

Dr Donald Lubembe Mukolwe
Department of Veterinary Tropical Diseases
Paraclinical Building 2-13
Faculty of Veterinary Science

Email:dlubembe@gmail.com

Dear Dr Mokulwe

RE: Permission to do research in terms of Section 20 of the ANIMAL DISEASES ACT, 1984 (ACT NO. 35 of 1984)

Your fax / memo / letter/ Email dated 10 October 2017, requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

I am pleased to inform you that permission is hereby granted to perform the following research/study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. All potentially infectious material utilised or collected during the study is to be destroyed at the completion of the study. Records must be kept for five years for audit purposes. A dispensation application may be made to the Director Animal Health in the event that any of the above is to be stored or distributed;
3. A veterinary import permit will be required prior to the importation of DNA samples from Kenya and Mozambique;

– 1 –

4. No sampling may take place on farms with active outbreaks of any controlled/notifiable diseases. Blood samples may only be collected in the region for which Provincial State Veterinary permission has been granted;
5. Only bovine blood samples on the attached list from the Han Hoheisen biobank, may utilised;
6. Only extracted DNA may be transported to the Department of Veterinary Tropical Diseases.

Title of research/study: Population genetic structure and antigenic diversity of Theileria parva in cattle at the wildlife-livestock interface and in peri-urban areas
Researcher (s): Dr Donald Lubembe Mukolwe
Institution: Department of Veterinary Tropical Diseases, Faculty of Veterinary Science
Your Ref./ Project Number: V080-16
Our Ref. Number: 12/11/1/1/8
Expiry Date: 31 December 2018

Kind regards

Maja.

DR MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2018 -01- 17

CLASSIFICATION: CONFIDENTIAL

SUBJECT: THE PREVALENCE, PERCEPTIONS AND MITIGATIONS OF ANTIMICROBIAL RESISTANCE IN PIG FARMS OF GAUTENG PROVINCE AND SURROUNDINGS IN SOUTH AFRICA

Import permits:

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

DEPARTMENT OF AGRICULTURE
DIRECTORATE: ANIMAL HEALTH
PRIVATE BAG X 138
2018 -02- 01
PRETORIA 0001
FORESTRY & FISHERIES

Directorate of Animal Health
Import-Export Policy Unit
Private Bag X138
Pretoria, 0001
Republic of South Africa
Tel: (27)-012-319 7514
Fax: (27)-012-329 8292
PERMIT NO: 13/1/1/30/2/0-201802000021
Valid from: 2018-02-01
Expiry date: 2018-05-01

IMPORTER:
DR KGOMOTSO P.SIBEKO-MATJILA
DEPARTMENT VETERINARY TROPICAL DISEASES
FACULTY OF VETERINARY SCIENCE
OLD SOUTPAN ROAD
ONDERSTEPOORT

VETERINARY IMPORT PERMIT FOR PATHOLOGY SPECIMENS
[Issued in terms of the Animal Diseases Act, 1984 (Act No. 35 of 1984)]

Authority is hereby granted for you to import 300 TUBES DNA SAMPLES FROM CATTLE AND BUFFALO BLOOD into Republic of South Africa:

From: KENYA
subject to the following conditions:

1. The consignment must be accompanied by this original permit and an original veterinary health certificate, complying with the conditions stipulated overleaf (IMP.PATH.CE.10/2013), duly completed and signed by an official veterinarian, authorised thereto by the Veterinary Authority of KENYA.
2. The specimens are to be securely packed and transported in leakproof containers, sealed by an authorised official of the Veterinary Authority of the exporting country;
3. The specimens must be kept and used for purposes of testing/research at the laboratories of MOLECULAR BIOLOGY LABORATORY , FACULTY OF VETERINARY SCIENCE under the personal supervision of DR KGOMOTSO SIBEKO-MATJILA / DR DONALD L .MUKOLWE;
4. On completion of tests/research the specimens, including all contaminated/infectious things or animal products (as defined by the Animal Diseases Act, 1984 [Act No. 35 of 1984]) derived/produced from or that came into contact with the above-mentioned specimens, must be destroyed by incineration. Records of the incinerations must be maintained for a period of 5 years, and made available for auditing to the Veterinary Authority upon request.
5. The consignment must be airfreighted through port of entry OR TAMBO INTERNATIONAL AIRPORT. **Samples may only be imported as manifest cargo under an airwaybill number and may not be imported as personal luggage.**
6. The consignment must be accompanied by this permit and its arrival reported immediately to the inspecting veterinary official: KEMPTON PARK Tel: 011 393 7980, and may not be released without his/her written permission.
7. Upon arrival the inspecting veterinary official will inspect the consignment and release it to the importer only after he/she is satisfied that all the import conditions have been complied with in full.
8. **This permit does not absolve the importer from compliance with the provisions of any other legislation relating to this import.**
9. This permit is subject to amendment or cancellation by the Director Animal Health at any time and without prior notice being given.
10. This permit is valid for three (3) months from date of issue and FOR ONE CONSIGNMENT ONLY.

SPECIAL CONDITIONS:
1.IN ADDITION , THE VETERINARY HEALTH CERTIFICATE (DESCRIBED IN CONDITION 1 ABOVE) ISSUED BY A VETERINARIAN AUTHORIZED HERETHO BY THE VETERINARY AUTHORITIES OF KENYA , MUST STATE THAT THE SAMPLES ARE EXTRACTED DNA SAMPLES FROM CATTLE AND BUFFALO BLOOD ONLY
2.EXTRACTED DNA SAMPLES MAY BE STRORED AT THE DEPARTMENT OF VETERINARY TROPICAL DISEASES

DIRECTOR: ANIMAL HEALTH
NOTE:
- All imports for research purposes require Section 20 permission in compliance with the Animal Diseases Act.

(IMP.PATH.CE.10/2013)

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

DEPARTMENT OF AGRICULTURE
DIRECTORATE: ANIMAL HEALTH
PRIVATE BAG X 138
2018 -02- 01 11
PRETORIA 0001
FORESTRY & FISHERIES

Directorate of Animal Health
Import-Export Policy Unit
Private Bag X138
Pretoria, 0001
Republic of South Africa
Tel: (27)-012-319 7514
Fax: (27)-012-329 8292
PERMIT NO: 13/1/1/30/2/0-201802000016
Valid from: 2018-02-01
Expiry date: 2018-05-01

IMPORTER:
DR KGOMOTSO P.SIBEKO-MATJILA
DEPARTMENT VETERINARY TROPICAL DISEASES
FACULTY OF VETERINARY SCIENCE
OLD SOUTPAN ROAD
ONDERSTEPOORT

VETERINARY IMPORT PERMIT FOR PATHOLOGY SPECIMENS

[Issued in terms of the Animal Diseases Act, 1984 (Act No. 35 of 1984)]

Authority is hereby granted for you to import 150 TUBES DNA SAMPLES FROM CATTLE AND BUFFALO BLOOD into Republic of South Africa:

From: MOZAMBIQUE
subject to the following conditions:

1. The consignment must be accompanied by this original permit and an original veterinary health certificate, complying with the conditions stipulated overleaf (IMP.PATH.CE.10/2013), duly completed and signed by an official veterinarian, authorised thereto by the Veterinary Authority of MAZAMBIQUE.
2. The specimens are to be securely packed and transported in leakproof containers, sealed by an authorised official of the Veterinary Authority of the exporting country;
3. The specimens must be kept and used for purposes of testing/research at the laboratories of MOLECULAR BIOLOGY LABORATORY , FACULTY OF VETERINARY SCIENCE under the personal supervision of DR KGOMOTSO SIBEKO-MATJILA / DR DONALD L .MUKOLWIE;
4. On completion of tests/research the specimens, including all contaminated/infectious things or animal products (as defined by the Animal Diseases Act, 1984 [Act No. 35 of 1984]) derived/produced from or that came into contact with the above-mentioned specimens, must be destroyed by incineration. Records of the incinerations must be maintained for a period of 5 years, and made available for auditing to the Veterinary Authority upon request.
5. The consignment must be airfreighted through port of entry OR TAMBO INTERNATIONAL AIRPORT. **Samples may only be imported as manifest cargo under an airwaybill number and may not be imported as personal luggage.**
6. The consignment must be accompanied by this permit and its arrival reported immediately to the inspecting veterinary official: KEMPTON PARK Tel: 011 393 7980, and may not be released without his/her written permission.
7. Upon arrival the inspecting veterinary official will inspect the consignment and release it to the importer only after he/she is satisfied that all the import conditions have been complied with in full.
8. **This permit does not absolve the importer from compliance with the provisions of any other legislation relating to this import.**
9. This permit is subject to amendment or cancellation by the Director Animal Health at any time and without prior notice being given.
10. This permit is valid for three (3) months from date of issue and FOR ONE CONSIGNMENT ONLY.

SPECIAL CONDITIONS:
1.IN ADDITION , THE VETERINARY HEALTH CERTIFICATE (DESCRIBED IN CONDITION 1 ABOVE) ISSUED BY A VETERINARIAN AUTHORIZED HERETHO BY THE VETERINARY AUTHORITIES OF MOZAMBIQUE , MUST STATE THAT THE SAMPLES ARE EXTRACTED DNA SAMPLES FROM CATTLE AND BUFFALO BLOOD ONLY
2.EXTRACTED DNA SAMPLES MAY BE STRORED AT THE DEPARTMENT OF VETERINARY TROPICAL DISEASES

pp
DIRECTOR: ANIMAL HEALTH

NOTE:
- All imports for research purposes require Section 20 permission in compliance with the Animal Diseases Act.

(IMP.PATH.CE.10/2013)