GENETICS DIVERSITY OF *THEILERIA PARVA* AND MUGUGA COCKTAIL VACCINE EFFICACY IN CATTLE IN KABASHA VILLAGE IN EASTERN DEMOCRATIC REPUBLIC OF CONGO

By

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Copyright Declaration

Declaration

I, Mulonga Simbuwa, declare that this research report is my own work. It is being submitted for the Masters of Science Tropical infectious diseases and zoonosis at the University of Zambia. It has not been submitted either wholly or partially for any degree at this or other universities.

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This dissertation of Mulonga Simbuwa has been approved as having fulfilled the requirement for the award of the degree of Master of Science in Tropical Infectious Diseases and Zoonosis at the University of Zambia.

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Abstract

East Coast Fever (ECF), caused by the protozoan *Theileria parva*, is a major constraint to cattle health and productivity in Eastern, Central, and Southern Africa. In Eastern Democratic Republic of Congo (DRC), where the disease is endemic, control efforts are challenged by limited knowledge on the genetic diversity of circulating *T. parva* strains and their relationship to vaccine stocks. The Muguga Cocktail (MC), the most widely used live vaccine, may offer only partial protection in this region due to potential genetic divergence between local field strains and vaccine strains. This study was therefore conducted to characterize the genetic diversity and antigenic similarity of *T. parva* strains in Eastern DRC, to inform immunization strategies and guide effective deployment of the Infection and Treatment Method (ITM).

The main objective was to determine the population structure and diversity of *T. parva* in vaccinated, unvaccinated, and sentinel cattle. Blood samples from Kabasha village were analysed using PCR targeting the *Tp1* and *Tp2* antigen coding genes. Sequences were aligned and compared with those of the Muguga Cocktail using phylogenetic trees, haplotype networks, and similarity scoring. Microsatellite and minisatellite genotyping covering six loci provided data on population structure assessed using allele diversity, Principal Component Analysis (PCA), and Fst statistics.

The *Tp1* gene was found to be relatively conserved. Out of 43 sequences analysed, 33 had 100% amino acid homology with the Muguga Cocktail epitope. Phylogenetic and haplotype analyses grouped most sequences closely with MC strains, indicating potential coverage by the current vaccine. In contrast, the *Tp2* gene was highly polymorphic, with 235 of 258 epitopes being unique. These sequences formed distinct clusters, most of which were divergent from the vaccine strains. Microsatellite analysis revealed 91 unique alleles and 38 shared alleles among the three cattle groups. PCA showed partial clustering of field samples with MC stocks, while some samples formed distinct sub-populations. Fst values ranging from 0.096 to 0.119 indicated moderate genetic differentiation.

These findings reveal the presence of multiple *T. parva* populations in Eastern DRC, some closely related to the Muguga Cocktail and others genetically distinct. The

conserved nature of *Tp1* epitopes suggests partial vaccine coverage, while the high diversity in *Tp2* epitopes and microsatellite loci suggests the existence of local strains not fully protected by the current vaccine.

Dedication

I dedicate this thesis to my father and mother, whose invaluable life teachings have continued to shape the person I am becoming. Their wisdom, guidance, and unwavering support inspire me daily to work hard and pursue excellence.

To my lovely daughter, Tabo Namasiku Simbuwa may this be a springboard for you to believe that with hard work and discipline, you can achieve even greater heights.

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List of Abbreviation

ABI	Applied Biosystems
AMOVA	Analysis of Molecular Variance
BLAST	Basic Local Alignment Search Tool
Bp	Base pair
CD8 T	Cytotoxic T cells type 8
CTL	Cytotoxic T Lymphocyte
DDBJ	DNA data base of Japan
DNA	Deoxyribonucleic Acid
DNTPs	Deoxynucleoside triphosphates
DR Congo	Democratic Republic of Congo
E2F	A cellular signaling pathway involved in cell cycle regulation and proliferation
ECF	East Coast Fever
EDTA	Ethylenediaminetetraacetic acid
FTA	Flinders Technology Associates
GL Sciences	A scientific equipment and services company based in Japan
ITM	Infection and Treatment Method
MC	Muguga Cocktail
NCBI	National Center for Biotechnology Information
Ng	Nanogram
OD	Optical density
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
Pp	Percent Positive
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
Tp1	Theileria parva strain 1
USA	United States of America
µL	Microliter
µM	Micromolar

CHAPTER ONE

INTRODUCTION

1.0 Background

Theileria parva (T. parva), a protozoan parasite of the phylum Apicomplexa, is the causative agent of East Coast fever (ECF) in cattle across eastern, central, and southern Africa (Norval et al., 1991). This parasite relies on the brown ear tick (*Rhipicephalus appendiculatus*) for transmission (Konnai et al., 2006). The geographical distribution of *T. parva* is influenced by factors such as climate variations, host density, and land use patterns, affecting parasite prevalence and infection intensity (Njiiri et al., 2015). The complex life cycle of *T. parva* involves both vertebrate and invertebrate hosts, with cattle as the primary vertebrate host and the brown ear tick as the vector (Konnai et al., 2006). Within the tick, the parasite develops in the gut before migrating to the salivary glands for transmission during subsequent feeding (Konnai et al., 2006).

Interactions between *T. parva*, its hosts, and other organisms in the ecological community are crucial for the parasite's transmission and persistence. Molecular studies have revealed insights into the genetic diversity and population structure of *T. parva,* aiding in understanding its evolution and transmission dynamics (Tretina et al., 2020). Immunity against *T. parva* is primarily mediated by $CD8^+$ T cells (Graham et al., 2008). Despite advances in control measures, challenges in managing ECF persist due to drug-resistant tick populations, changes in land use and climate, and the introduction of new parasite strains (Palmateer et al., 2020).

Theileria parva's evolutionary history and patterns of transmission within and between host populations are essential for developing effective control strategies to mitigate ECF spread (Tretina et al., 2020). The parasite's ability to subvert host signalling pathways, such as E2F signalling, contributes to leukocyte proliferation and pathogenesis (Tindih et al., 2012). The genetic diversity of *T. parva* has been studied in cattle and wildlife populations, emphasizing the importance of understanding parasite prevalence in different hosts (Oura et al., 2011).

To combat ECF, control measures primarily focus on tick control using acaricides and immunization through the infection and treatment method which involves

administering a lethal dose of *T. parva* alongside a long-acting oxytetracycline formulation (Amzati et al., 2019). Additionally, chemotherapy, specifically using halofuginone lactate, has been studied as an effective strategy for managing ECF (Naciri et al., 1993).

The intricate interactions between *T. parva*, its hosts, and the environment shape the transmission dynamics of this parasite, highlighting the need for continued research to address challenges in controlling ECF and to enhance our understanding of the parasite's biology and epidemiology.

1.1 Statement of the Problem

In Eastern DR Congo, conducting urgent research on ECF is imperative due to its substantial threat to cattle, resulting in significant economic losses over USD 300 million annually across the region in agriculture sector (Kiara et al., 2014; Norval et al., 1991). The primary mode of transmission is through brown ear ticks during tick feeding (Nejash, 2016; Norval et al., 1991), and ECF has been reported in 11 African countries (Amzati et al., 2018), presenting formidable challenges for farmers and local economies. While control methods include tick control and immunization (Graham et al., 2006), it's worth noting that tick control through acaricides can be costly up to USD 20-30 per animal annually for local farmers. Consequently, immunization stands out as a more cost-effective approach. However, the parasite's genetic diversity raises concerns about the efficacy of current vaccines (Atuhaire et al., 2020). To address this issue, it is crucial to characterize regional *T. parva* strains and validate vaccine assumptions through comprehensive field trials.

1.2 Rationale of the Study

The need for a comprehensive characterization study of ECF in Eastern DR Congo is evident due to the substantial threat posed by this disease to cattle, resulting in significant economic losses in the agricultural sector. Though control measures, such as tick control exist, it's worth noting that tick control through acaricides can be financially burdensome for local farmers. Thus, immunization emerges as a more cost-effective approach. However, concerns have arisen about the introduction of new parasite strains in naive areas as well as the effectiveness of current vaccines due to the parasite's substantial genetic diversity. To provide accurate information and guide the introduction of Infection and Treatment Method in eastern DRC,

characterizing the parasite strains in the DRC is imperative. The outcomes of this study will provide policymakers with crucial insights for selecting the most effective vaccines to control East Coast Fever in Eastern DRC and this, in turn, will lead to enhanced livestock health, improved productivity, and an overall increase in the region's well-being.

1.3 Significance of the Study

This study will provide valuable information to policymakers in the eastern region of DRC regarding which immunization stocks to use for ITM-based control of ECF. The results obtained from the genetic diversity analysis of *T. parva* in vaccinated and unvaccinated cattle will be significant for improving the selection and development of effective vaccines tailored to the specific strains prevalent in the region. Moreover, the data generated from this research will contribute to the broader understanding of *T. parva* population dynamics and diversity in Eastern DRC.

1.4 Research Questions

1.4.1 What are sequence similarities between field samples and vaccine stocks based on Tp1 and Tp2 genes?

1.4.2 What is the genetic diversity of *T. parva* in vaccinated and unvaccinated cattle in Kabasha Village in eastern DRC?

1.5 Objectives

1.5.1 General Objectives

1.5.1.1 To determine the population structure and diversity of *T. parva* in vaccinated and unvaccinated cattle in Kabasha Village of the Eastern DRC.

1.5.2 Specific of Objectives

1.5.2.1 To determine the sequence similarities between field samples and vaccine stocks based on Tp1 and Tp2 genes.

1.5.2.2 To determine the genetic diversity of *T. parva* in vaccinated and unvaccinated cattle in Kabasha Village in the eastern DRC.

CHAPTER TWO

LITERATURE REVIEW

2.0 Introduction

2.1 Etiology and transmission of *T. parva*

East Coast Fever (ECF) is one of the most economically significant tick-borne diseases affecting cattle in Eastern, Central, and Southern Africa. It is caused by *Theileria parva*, an intracellular protozoan parasite of the phylum Apicomplexa (Olwoch et al., 2008). The parasite is primarily transmitted through the three-host tick *Rhipicephalus appendiculatus*, which predominantly parasitizes cattle (Kalume et al., 2013). The economic burden of ECF is considerable, resulting in substantial losses for livestock producers and rural economies (Nene et al., 2016).

The transmission dynamics of *T. parva* are shaped by both ecological and host related factors. The survival and reproductive success of the tick vector are influenced by climatic conditions, vegetation, and the availability of suitable hosts (Nene et al., 2016). Seasonal changes, particularly those conducive to tick proliferation, play a key role in determining the incidence of infection, with outbreaks often coinciding with peaks in tick population densities (Simuunza et al., 2011).

Furthermore, the epidemiology of ECF is complex and can be influenced by interactions with less pathogenic *Theileria* species. Mixed infections have been shown to reduce the severity of *T. parva* infections in cattle, potentially complicating disease control and altering risk patterns across different regions (Nyabongo et al., 2021). Livestock movement further exacerbates the spread of the parasite by introducing it into previously unaffected areas, often leading to new outbreaks (Marcellino et al., 2017).

2.2 Life cycle of *T. parva*

The life cycle of *Theileria parva* is intricate, involving obligatory developmental stages in both mammalian and vector hosts (Ahmed & Mehlhorn, 1999). Initially, the parasite exists in a haploid form, with a brief diploid stage occurring in the tick vector during sexual recombination, which enhances genetic diversity through meiotic processes (Katzer et al., 2010). Cattle become infected through the inoculation of sporozoite forms present in tick saliva during feeding, and these

sporozoites rapidly differentiate into the microschizont, the primary pathogenic form, which induces proliferation in the host. Microschizonts gradually develop into macroschizonts and ultimately into merozoites, which are released from leukocytes and develop into piroplasms, the form infective to ticks (Nejash, 2016).

During the next feeding cycle, the vector tick ingests piroplasms, and the released parasites undergo syngamy in the tick gut, forming a zygote, the only diploid stage. This zygote then divides into motile kinetes that infect the tick gut epithelial cells and migrate to the hemolymph, subsequently infecting the salivary glands. After molting and commencement of feeding by the tick, sporogony results in the multiplication of sporozoites in the salivary gland acini before injection into the feeding site by nymphs or adult ticks (Nejash, 2016).

2.3 Clinical Manifestations of East Coast Fever

The incubation period varies from 4 to 14 days after the attachment of infected ticks to the host. The disease may last as little as three to four days in its acute form or may be prolonged for about 20 days (Ganaie et al., 2019). The disease significantly affects exotic and crossbred cattle, as well as indigenous calves under six months old, resulting in elevated mortality rates. The indigenous cattle breeds in Eastern DRC, such as the Ankole and the local Zebu, exhibit varying degrees of susceptibility to ECF (Tshilenge et al., 2019). Research indicates that indigenous breeds tend to have better resistance to ECF compared to exotic breeds, which are often more susceptible due to their lack of co-evolution with local pathogens. The case fatality rate for untreated East Coast fever can be as high as 100% in taurine, zebu, or sanga cattle from non-endemic areas (Nejash, 2016). In contrast, the morbidity rate approaches 100% among indigenous cattle, but the mortality rate is usually low (Nejash, 2016). In endemic areas, chronic ECF manifests with less quantifiable effects, such as reduced growth, diminished milk production, poor weight gain, low fertility rates, paralysis, and vulnerability to secondary attacks from other parasites (Graf & Weiss, n.d.).

2.4 Antigens and antigenic variation of in *T. parva*

Immune responses against *T. parva* are primarily mediated by $CD8^+$ cytotoxic T lymphocytes (CTLs), which recognize specific peptide epitopes presented by Major Histocompatibility Complex (MHC) molecules (Graham et al., 2006). The

understanding of *T. parva's* antigenic diversity is based on the identification of several CTL targeted antigens notably *Tp1*, *Tp2*, and more recently, *Tp9* and *Tp10* which are recognized by the immune systems of cattle with specific MHC haplotypes (MacHugh et al., 2009). This antigenic diversification is a key mechanism by which the parasite evades host immunity, allowing it to persist despite the host's efforts to mount an immune response (Bishop et al., 2015).

Research has shown that the nucleotide sequences encoding these antigens vary significantly among different *T. parva* strains, particularly between cattle derived and buffalo derived strains. These variations may hinder effective CTL recognition, thereby enhancing the parasite's survival (Pelle, Graham, Njahira, Osaso, Saya, Odongo, et al., 2011). While antigens such as *Tp1* are considered immunodominant and elicit robust responses in most cattle, sequence diversity especially in buffalo derived strains can lead to differential CTL responses across cattle populations (Bishop et al., 2015). This underscores the critical role of antigenic variation in parasite persistence and highlights the difficulties in designing a broadly protective vaccine.

In addition to CTLs, $CD4^+$ T cells play an essential role in shaping the immune response to *T. parva*. Studies suggest that $CD4^+$ T cells enhance the priming and memory formation of $CD8^+$ T cells specific to *T. parva* antigens, contributing to a more effective and long-lasting immune defense (Morrison et al., 2021). The search for novel vaccine candidates continues, with molecular advances identifying additional antigens that may not be prominently recognized during natural infection but could serve as promising targets for vaccine development (Connelley et al., 2022).

2.5 Economic Impact of East Coast Fever

ECF remains an acute and usually lethal disease in countries including Rwanda, South Sudan, Kenya, Zimbabwe, Tanzania, Uganda, Burundi, the Democratic Republic of Congo, Mozambique, Zambia, and Malawi (Ganaie et al., 2019). In the DRC, particularly in North Kivu Province, cattle breeders often implement tick control measures based on their experiences with the disease's incidence and severity, which directly correlates with the economic impacts they face at the farm level (Kalume et al., 2013). The economic burden of ECF is substantial, with annual

economic losses of about USD 300 million and cattle mortality amounting to approximately one million cattle per year (Ganaie et al., 2019) highlighting the need for active control measures.

2.6 Control Strategies for East Coast Fever

2.6.1 Tick Control Measures

Control of ECF consists of two main strategies: (i) control of the vector tick through one or more options, including acaricides, chemotherapy, and livestock movement control and (ii) vaccination (Nejash, 2016) through the Infection and Treatment Method (ITM). The use of acaricides remains a widespread method of tick control, despite challenges such as elevated costs, the emergence of tick resistance (Dolan, 1999), and insufficient dipping facilities. Despite these obstacles, many farmers continue to rely on acaricides for ECF control, even though the most effective preventive approach is the ITM. ITM involves the use of live attenuated vaccines, which utilize the infective sporozoite of *Theileria parva* and are administered alongside long-acting oxytetracycline (Cox, 1992).

2.6.2 Vaccination Strategies

While ITM is stock-specific, combinations of stocks, such as the widely used 'Muguga cocktail' (Dolan, 1999), comprising *T. parva* Muguga, Kiambu 5, and Serengeti-transformed stocks, provide broad protection. The Serengeti-transformed and Muguga *T. parva* stocks are very similar to one another, whereas the third stock, Kiambu 5, is distinct, with close to 40,000 single nucleotide polymorphisms (SNPs) when the whole genome is compared to that of the reference *T. parva* Muguga genome (Norling et al., 2015). Despite its efficacy, ITM adoption faces challenges due to cold chain difficulties and concerns about vaccine strains establishing in resident tick populations and intermingling with local parasite genotypes (Katzer et al., 2010). Additionally, recombinant vaccines targeting specific antigens, such as the sporozoite surface antigen p67, have been developed and show promise in inducing protective immune responses (Marcelino et al., 2021).

2.6.3 Livestock Movement Control

Livestock movement control is also a critical component of ECF management. The movement of cattle between regions can facilitate the spread of *T. parva* and exacerbate outbreaks. Therefore, implementing stringent regulations on livestock

movements, particularly during high-risk periods, is vital (Ministry of Fisheries and Livestock et al., 2020). This includes monitoring and restricting the movement of animals from areas with known ECF outbreaks to prevent the introduction of the parasite into unaffected regions. Such measures are particularly important in areas where pastoralist communities frequently move their herds in search of grazing land, as these practices can inadvertently contribute to the transmission of ECF (Silatsa et al., 2020).

2.6.4 Integrated Approaches

Integrated control strategies that combine vaccination, tick control, and livestock management practices are increasingly advocated. For instance, using acaricides to manage tick populations alongside vaccination efforts can significantly reduce the incidence of East Coast Fever (Kiragu, 2023). Developing new vaccines and improving delivery methods could further enhance the effectiveness of these integrated approaches, making them more accessible to farmers (Maina et al., 2023; Nene et al., 2016).

2.7 Importance of Local Strain Characterization

2.7.1 Role of Genetic Characterization

The effectiveness of the Infection-and-Treatment Method (ITM) in providing protection is contingent on the selection of an appropriate immunizing stock, underscoring the need to characterize local strains in Eastern Democratic Republic of Congo (DRC). Characterization is essential to maximize cross-immunization possibilities. Although there is no direct correlation between molecular profiling and immunogenicity, characterizing local strains helps narrow down the parasite stocks or isolates for cross-immunization trials, thereby reducing the cost of introducing immunization. For comparative purposes, different and well-characterized strains are often included in a single vaccine, as demonstrated by the success of the Muguga Cocktail in immunization trials.

2.7.2 Cytotoxic T Lymphocyte (CTL) Antigens and Diversity

The sequencing of *Theileria parva* cytotoxic T lymphocyte (CTL) antigens has emerged as a valuable tool for assessing the diversity of *T. parva* in endemic areas (Atuhaire et al., 2021; Chatanga et al., 2020; MacHugh et al., 2009; Pelle, Graham, Njahira, Osaso, Saya, David, et al., 2011). Additionally, the identification of

polymorphic minisatellites and microsatellite markers in the *T. parva* genome enables direct genotyping of isolates from blood samples using specific primers (D. Odongo et al., 2006; Oura et al., 2005). These locus-specific markers, based on variable number tandem repeats (VNTRs) in non-coding regions, are valuable for studying population structure due to their technical simplicity in analyzing repeat motif copy number variation. The use of minisatellites and microsatellites in *T. parva* genotyping has revealed diverse population structures (Muleya et al., 2012, 2022; D. Odongo et al., 2006; Oura et al., 2005).

2.7.3 Population Genetics and Regional Context

2.7.3.1 Influence of Cattle Movement on Genetic Diversity

Studies focused on population genetics in the Great Lakes region of Central Africa reveal that genetic variation in *T. parva* is more influenced by cattle translocation and ecological factors than by host immune pressure. This finding underscores the importance of region-specific control strategies that consider local ecological and genetic contexts. In Rwanda, satellite marker analysis has been used to inform immunization strategies against ECF, emphasizing the necessity of understanding genetic diversity to improve vaccine efficacy.

Case Study: Malawi

A comprehensive genotyping study conducted using nine mini- and microsatellite markers on *T. parva* samples from both vaccinated and non-vaccinated cattle revealed distinct genetic structures between populations. This highlights the significance of local genetic diversity in vaccine efficacy and the potential for vaccine escape variants (Chatanga et al., 2022). Furthermore, a study by the same authors assessed the genetic diversity of *T. parva* antigens recognized by CD8+ T cells, which are crucial for vaccine design. Phylogenetic analysis indicated that haplotypes from Malawi shared similarities with those from live vaccine strains, suggesting that understanding local genetic variations is essential for effective immunization strategies (Chatanga et al., 2020).

2.8 Insights from Eastern Democratic Republic of Congo

2.8.1 Tick Species and Seroprevalence

Research in Eastern DRC has largely centered on identifying tick species and examining their link to *T. parva* seroprevalence in cattle under extensive farming

systems. Findings indicated the predominance of *Rhipicephalus appendiculatus*, suggesting potential endemicity of *T. parva* in the area (Kalume et al., 2013). Studies have also shown that ticks of the *Rhipicephalus* genus are the most abundant in DRC cattle (Kalume et al., 2013).

2.8.2 Socioeconomic Impact

Communities in Eastern DRC heavily relying on livestock for their livelihoods, theileriosis is a serious impediment to the livelihoods of these communities as well as livestock sector growth (Surve et al., 2023). Effective control, especially through immunization, is essential due to its cost-efficiency compared to chemotherapy (Rodriguez et al., n.d.) and integrating genetic data into vaccine design and disease management remains crucial to combating ECF effectively. Thus, this study specifically aimed to assess the genetic similarity between field (sentinel) samples from previously vaccinated and control animals and vaccine stocks, focusing on the *Tp1* and *Tp2* genes, and to evaluate *T. parva* diversity in both vaccinated and unvaccinated cattle in Eastern DRC.

CHAPTER THREE

MATERIAL AND METHODS

3.0 Study Design

The study utilized an analytical cross-sectional design to assess the sequence similarities of *Tp 1* and *Tp 2*, as well as the genetic diversity within vaccine stocks and field (sentinel) samples collected from Kabasha village. Additionally, it examined samples from both vaccinated and control animals that had been part of a field challenge trial conducted in Kabasha village, located in eastern DRC. This study was a small component of a broader research project focused on characterizing *T. parva* in eastern DRC, aimed at guiding the introduction of ITM in endemic areas.

3.1 Sample Size

The study utilized archived blood samples collected from Kabasha village, comprising 20 control animals, 22 immunized animals, and 5 sentinel animals, as well as vaccine stocks (Muguga, Kiambu 5, and Serengeti transformed). These samples were preserved in EDTA and spotted onto FTA cards at the University of Zambia.

3.2 Study site

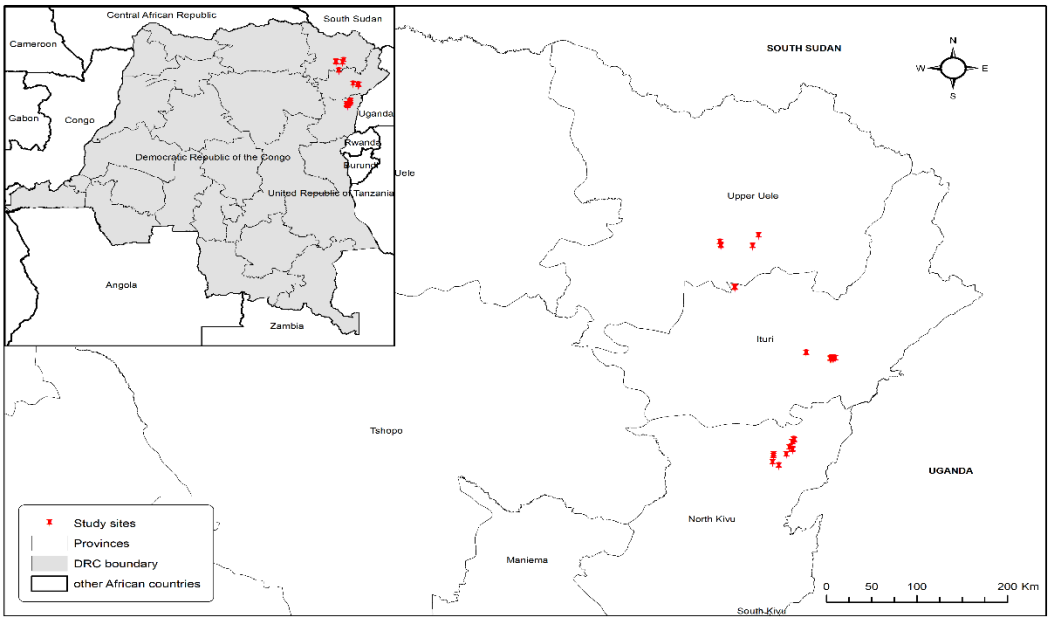

Figure 3.1: Map showing the location of Kabasha village in Eastern DRC, the study site for the genetic analysis of *Theileria parva.*

3.3 DNA Extraction

For PCR screening, DNA was purified from cattle blood spotted onto FTA filter papers (Whatman Bio-Science) (n = 47). The discs were washed twice for 15 minutes with 500 µL of FTA purification reagent (Whatman), followed by two rinses of 15 minutes with 1.0 mL of Tris-EDTA buffer (10 mM Tris–HCl, 0.1 mM EDTA, pH 8.0). Next, 50 µL of lysis buffer (10 mM Tris-HCl, pH 8.0; 0.1 mM EDTA; 0.5% SDS) was added to the tube and incubated at 56°C for 30 minutes to release DNA. After incubation, the punch was discarded, and 100 µL of 100% ethanol and 10 µL of 3 M sodium acetate (pH 5.2) were added to the DNA-containing supernatant. The mixture was gently mixed and incubated at -20°C overnight, followed by centrifugation at 12,000 x g for 15 minutes to pellet the DNA. The supernatant was carefully discarded, and the pellet was washed with 70% ethanol. Finally, the pellet was briefly air-dried to remove residual ethanol, and the DNA was resuspended in 40 µL of nuclease-free water.

3.4 *T. parva* screening using p104 gene

47 Field samples were screened for *T. parva* DNA using *T. parva*-specific p104 gene primers, as designed by (Skilton et al., 2002) and AmpliTaq Gold PCR Master Mix (Invitrogen, CA). A nested PCR approach was employed. Each 20 µL reaction mixture contained 1.5 µL of template DNA, 10 µL of 2x AmpliTaq Gold Master Mix, 0.5 µM of each primer, and distilled water. The PCR protocol included an initial denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 96°C for 30 seconds, annealing at 50°C for 45 seconds, and extension at 68°C for 60 seconds, with a final extension at 72°C for 5 minutes. PCR products from the initial PCR served as templates for the nested PCR, which used the same reaction mixture and cycling conditions. The nested PCR products were then visualized on 1.5% agarose gels stained with ethidium bromide, with expected product sizes of 500 bp for the initial PCR and 300 bp for the nested PCR (Bishop et al., 2015; D. O. Odongo et al., 2010).

3.5 *Tp1* and *Tp2* gene PCR

33 positive samples were amplified for *T. parva* Tp1 and Tp2 genes using the AmpliTaq Gold PCR kit (Invitrogen, CA) with primers designed by (Pelle, Graham, Njahira, Osaso, Saya, David, et al., 2011). A 20 µL reaction mixture was prepared, containing 1.5 µl of template DNA, 10 µL of 2x AmpliTaq Gold master mix, 0.5 µM

of each primer, and distilled water. The amplification cycles included an initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 96°C for 30 seconds, annealing at 50°C for Tp1, and 53°C for Tp2 for 45 seconds, and extension at 68°C for 60 seconds. The process concluded with a final extension step at 72°C for 5 minutes. The PCR products were visualized on 1.5% ethidium bromide-coated agarose gels, with expected product sizes of 432 bp and 525 bp for Tp1 and Tp2, respectively.

3.6 PCR product purification, Ethanol precipitation and big dye cycle sequencing

Using the Monofas purification kit (GL Sciences, Japan), 15 µL of PCR products and 150 µL of Buffer A were loaded into the spin column and centrifuged at 9,000 × g (10,000 rpm) for 30 seconds. The column was rinsed with 500 µL of Buffer B and centrifuged again at 9,000 × g (10,000 rpm) for 30 seconds. Then, 30 µL of Buffer C was added to the column, incubated for 1 minute followed by centrifugation at 9,000 × g (10,000 rpm) for 1 minute to obtain the purified DNA fragments.

Subsequent cycle sequencing was performed using the BigDye™ Terminator v3.1 cycle sequencing kit (Life Technologies, Applied Biosystems). A sequencing reaction containing BigDye™ Terminator v3.1 Ready Reaction Mix, primers, and a purified DNA template with a total volume of 20 µL was prepared in microcentrifuge tubes. The tubes were then placed in a thermal cycler, set to run at 96°C for 1 minute, followed by 96°C for 10 seconds, and 50°C for 5 seconds, with an extension at 60°C for 4 minutes. This cycle of denaturation, annealing, and extension was repeated 25 times. After cycling, the reaction was held at 4°C until ready to proceed with purification. Upon completion of the cycle sequencing, the tubes were centrifuged.

The resultant cycle sequencing products were purified to eliminate excess labeled dNTPs, buffers, and enzymes. The sequencing plates were centrifuged at 1,000 × g for 10 seconds. Then, 20 µL of the sequencing reaction, 5 µL of 125 mM EDTA solution, and 60 µL of absolute ethanol were added. The plate was sealed and vortexed for 2–3 seconds, followed by brief centrifugation at 1,000 × g for 10 seconds. The plate was left at room temperature for 15 minutes and then centrifuged at 14000 rpm for 20 minutes at 4°C after which supernatant was removed. The pellet was then washed with 70% ethanol and centrifuged at 14000 rpm for 15 minutes and then air dried. The DNA was then resuspended in 30 µl of Hi-Di formamide (Applied

Biosystems) and immediately denatured in a thermocycler at 95 degrees for 5 minutes. The denatured products were then subjected to capillary electrophoresis on the ABI 3500 genetic analyzer (Applied Biosystems).

3.7 Microsatellite and minisatellite genotyping

Genetic markers used in this study are provided in Appendix B. Fluorescent dye-labeled forward primers were utilized along with specified annealing temperatures. 20 µL reaction mixture contained 1.5 µL of template DNA, 10 µL of 2x AmpliTaq Gold Master Mix, 0.5 µM of each primer, and distilled water. The PCR protocol included an initial denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 96°C for 30 seconds, annealing at 50°C for 45 seconds, and extension at 68°C for 60 seconds, with a final extension at 72°C for 5 minutes. PCR products from the initial PCR served as templates for the nested PCR, which used the same reaction mixture and cycling conditions. PCR products were visualized on a 1.5% agarose gel treated with ethidium bromide. The successful PCR products were then mixed with 10 µl of Hi-Di formamide and 0.5 µl of Genescan Liz 600 size standard and then denatured at 95 degrees Celsius for 6 minutes followed by capillary electrophoresis using the ABI SeqStudio Genetic Analyzer (Life Technologies). The sizes of DNA fragments from various strains, including control, immunized, sentinel, and vaccine strains (Muguga, Kiambu, Serengeti, and Chitongo vaccine isolates), were subsequently determined using GeneMapper software version 5 (Applied Biosystems, Waltham, Massachusetts, USA). The software identified peaks with the highest area as indicative of the most dominant allele, facilitating the construction of a multi-locus genotype (MLG) representing the predominant genotype within each sample.

3.8 Data Analysis

3.8.0 Sequence and Haplotype Analysis

Blast analysis was carried out on the NCBI website (https://www.ncbi.nlm.nih.gov) for nucleotide sequence validation. The sequences acquired were compiled and refined using the ATGC plug-in in Genetyx ver. 12. Multiple sequence alignment of the Muguga, Kiambu 5, Serengeti transformed, Katete stock and Chitongo vaccine strain reference sequences in addition to other references from East and Central Africa was performed using Clustal W1.6 (Chenna, 2003). A fasta file representing this alignment was produced and transformed into a MEGA file format to create

phylogenetic trees with MEGA ver. 6 (Tamura et al., 2013). The construction of phylogenetic trees involved 1000 bootstrap replicates after model selection. Analysis of CTL epitopes on both Tp1 and Tp2 was conducted through multiple sequence alignments of amino acid sequences derived from nucleotide alignments. DnaSP ver. 5 (Librado & Rozas, 2009) was used to calculate DNA polymorphisms for each gene. The mean ratio of non-synonymous substitutions to synonymous substitutions (dN/dS) per site was calculated using the single likelihood ancestor counting method with the F81 model with a 0.05 confidence level on the Data Monkey website (http://www.datamonkey.org). The examination of genetic variation distribution and population differentiation among sequences was carried out through AMOVA using GenAIEx6 (Peakall, 2006).

Haplotype similarities between Tp1 and Tp2 nucleotide sequences of vaccine stocks and field samples were evaluated using Network ver. 10 (http://fluxus-engineering.com/). All nucleotide sequences obtained in this study have been submitted to the DNA Data Bank of Japan (DDBJ).

3.9 Microsatellite analysis

Initially, microsatellite analysis was conducted utilizing the microsatellite toolkit available at (http://animalgenomics.ucd.ie/sdepark/ms-toolkit/) to evaluate the degree of similarity within the MLG. The outcomes were presented through allele frequency distribution and Principal Component Analysis (PCA) generated using GenAIEx6 (Peakall, 2006). Assessment of population sub-structuring was performed using the FSTAT computer package version 2.9.3.2 (https://www2.unil.ch/popgen/softwares/fstat.htm). The evaluation of the null hypothesis of panmixia and linkage equilibrium employed LIAN (Haubold, B and Hudson, RR, 2000), which encompassed the computation of the standardized index of association, the variance of pairwise differences (VD), the variance of differences necessary for panmixia (VE), and L, denoting the 95% confidence interval for VD. Values of the index of association that are negative or close to zero indicate panmixia (random mating), while positive values, considerably above zero denote non-panmixia (non-random mating). The rejection of the null hypothesis of panmixia is indicated when VD surpasses the L value, indicating linkage disequilibrium (LD). Conversely, the acceptance of the null hypothesis of panmixia is observed when the calculated VD is lower than the L value, suggesting linkage equilibrium (LE).

3.10 Ethical Considerations

Ethical approval was obtained from the Biomedical Research Ethics Committee at the University of Zambia (UNZABREC) under REF. 233-2019 for conducting recombinant DNA experiments in the present study.

CHAPTER FOUR

RESULTS

4.0 Tp1 locus

The *Tp1 CTL* gene (432 bp) of *T. parva* was sequenced from the p104 gene-positive samples. A total of 33 samples exhibited 100% amino acid sequence homology with the Muguga Cocktail (MC) Epitope. Among these, two samples (UC16, UC18) were from the sentinel group, while the remaining 31 were from the immunized (n=13) and control (n=18) groups. Three variant epitopes were observed: **VGYPKVEEEML** (n=1, immunized), **VGYPKVKEEMI** (n=4, all immunized), and **VGYPKVKEEII** (n=9, 5 immunized, 3 controls, and 1 sentinel). The identified epitopes in the control, immunized, and sentinel groups were largely similar to those found in the Muguga cocktail. Collectively, the samples produced a DNA polymorphism of 0.6% and a mean ratio of dN/dS of 2.15, with one positive selection site indicating positive selection.

Tp1 gene phylogenetic analysis revealed that all study sequences clustered in major cluster A. Within this cluster, samples UC16 and UC18 from sentinel cattle formed a minor cluster together with the MC in cluster AIIa, while UC20 clustered in AIa (Fig. 3.1). Sequences from both controls and immunized animals segregated into two minor clusters AIa and AIIb. Cluster AIIb was closely related to the Muguga cocktail strains (cluster AIIa), while cluster AIa was not.

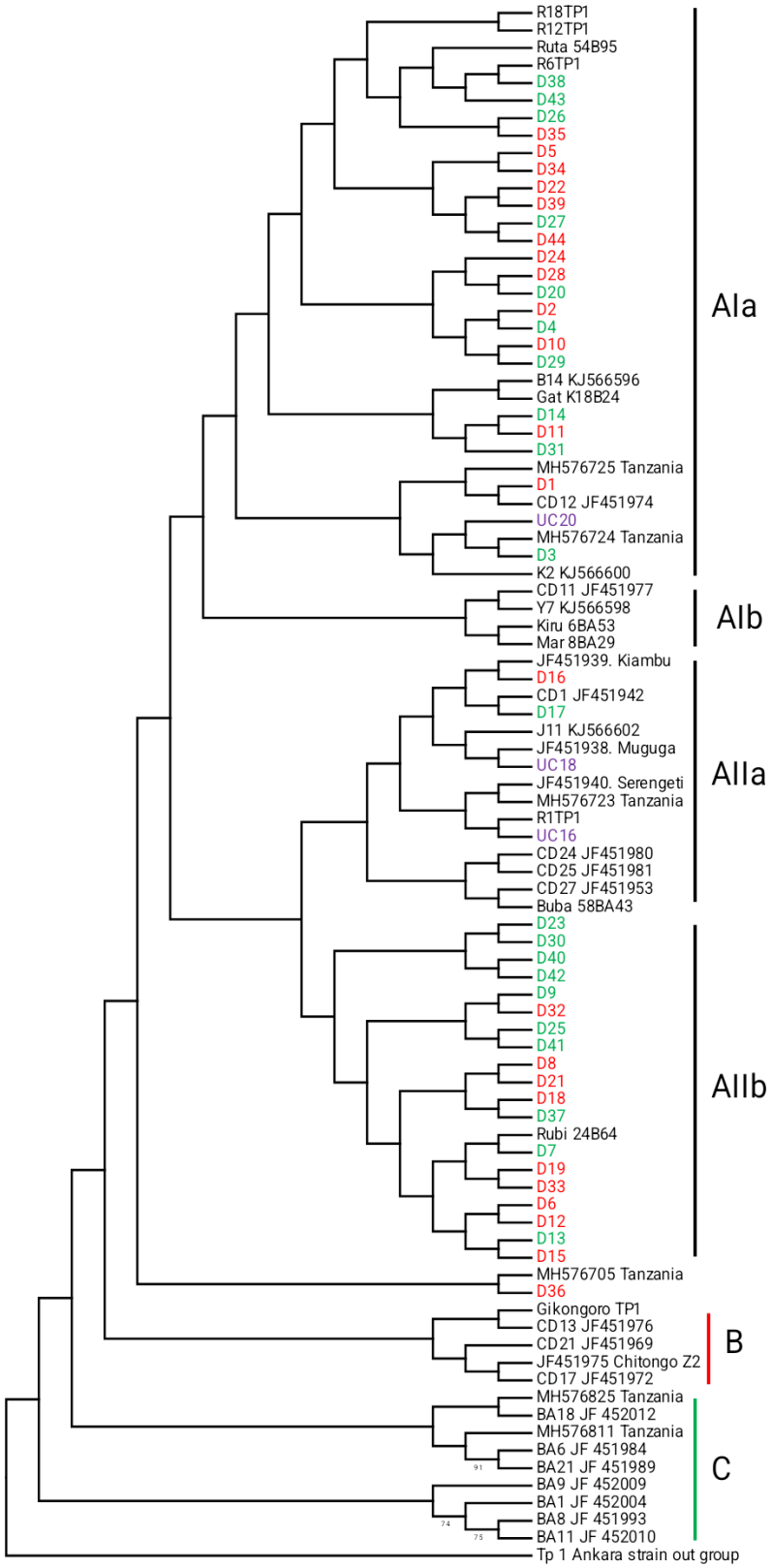

Figure 4.1: *T. Parva* Tp1 phylogenetic tree. The tree was constructed from 432 bp nucleotide sequences using MEGA version 6 1000 bootstrap replicates for

confidence. Sample from sentinel, immunized and control groups are bold and purple, green and red, respectively.

Analysis of Tp1 haplotypes using network 10 showed a star-like pattern with nine haplotypes (H11, H16, H15, H22, H18, H8, H23, H9, H8) radiating from haplotype H7 (Fig. 4.1), indicating the possibility of population expansion. H7, mainly comprising samples from the control group, was the most abundant haplotype (n=15). H1, despite being one of the minor haplotypes, included sequences from the sentinel, control, and immunized groups. All other haplotypes either comprised one group (e.g., H22 with control group samples) or included samples from both the control and immunized groups. The MC Haplotypes were least represented overall, suggesting that the majority of samples from both the control and immunized groups differed from the MC.

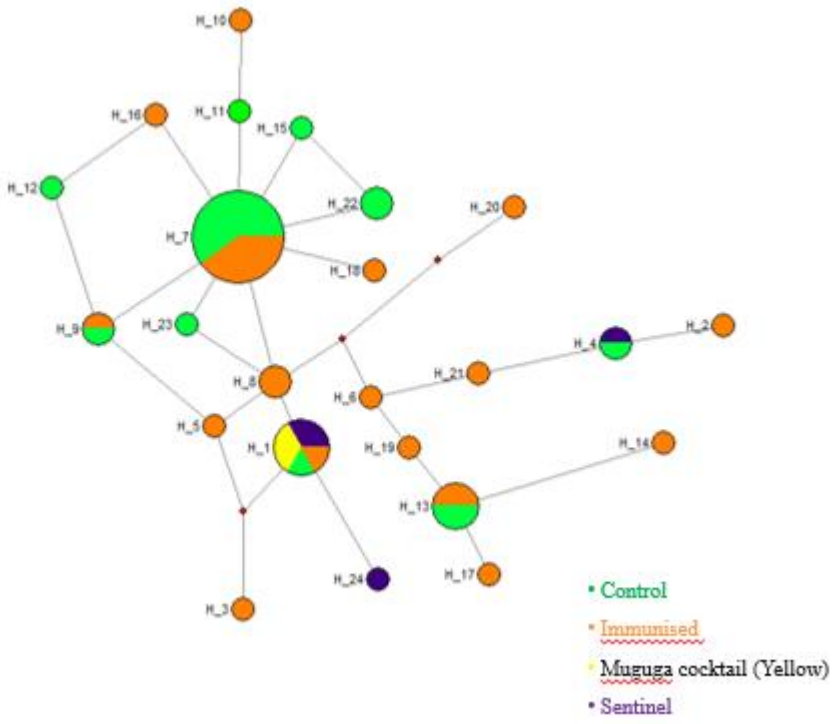

Figure 4.2: Median-joining network of the T parva Tp1 gene, constructed using Network 5 and based on the polymorphic sites of Tp1. The network displays a star-like radiation pattern, with circle sizes corresponding to haplotype frequency and colour codes indicating the origin of the samples.

4.2 Tp2 Locus

The Tp2 gene encodes a 174-amino acid protein that consists of six epitopes: CTL 1 **(SHEELKKLGML),** CTL 2 **(DGFDRDALF),** CTL 3 **(KSSHGMGKVGK),** CTL 4 **(FAQSLVCVL),** CTL 5 **(QSLVCVLMK),** and CTL 6 **(KTSIPNPCKW).** The complete Tp2 gene (531 bp) of *T. parva* was sequenced from samples in the sentinel, immunized, and control groups. Regarding CTL 1, no MC Epitopes were identified in both control and immunized samples; instead, ten (10) variants were identified (Table 1). MC Epitopes were only identified on CTL 4, 5, and 6 in 6, 6, and 11 post-immunization samples, respectively. The observed MC variants were 10, 10, 7, 9, 10, and 7 on epitopes 1 to 6, respectively. Out of a total of 258 epitopes identified across all loci, only 23 were similar to MC epitopes, while the majority (n=235) were variants of the MC epitope (Table 4.1). Additionally, all epitopes identified from sentinel sequences were variants of the MC Epitope. Overall, the MC Epitope was poorly represented across all six epitopes of the Tp2 protein (Table 4.1). A DNA polymorphism score of 13.2%, indicating high nucleotide polymorphism, was calculated from the sentinel, control, and immunized samples. The mean ratio of dN/dS was 0.659 with 15 purifying selection sites.

Table 4.1: Tp2 epitope variants identified in samples from eastern DRC.

#	CTL 1	CTL 2	CTL 3	CTL 4	CTL 5	CTL 6
1	SDNELDTLGLL (13)	EGFDRDALF (10)	KSSHGMGKIG K (10)	FAQSLMCV L (5)	QSLMCVLM K (5)	VNDIPNPCK W (1)
2	SDDELKKMGM V (6)	PDLDKNRL F (1)	KSSHGMGKIG R (3)	FAQSIVCVI (1)	QSIVCVIMK (1)	VSDIPNPCSG (1)
3	SDDELNNLGMV (1)	PDFDKNRL F (19)	LSSHGMGKIG R (21)	LAQSIVCVV (1)	QSIVCVVSK (1)	KPSIPNPCEW (1)
4	SDDELNKLGML (5)	EGFDKEKL F (7)	KSSHGMGIVG R (6)	LAQSIKCVL (1)	QSIKCVLHK (1)	KPSVPNPCD W (20)
5	SDDELDTLGLL (9)	PDFDRNTLF (1)	LSSHGMGKIG K (1)	LAASIKCVS (19)	ASIKCVSHH (12)	VNDIPNPCK W (7)
6	SDDELNKMGM L (5)	PDFDKNTLF (1)	LTSHGMGKIG R (1)	FVQSIMCVI (7)	ASIKCVSQY (7)	KTSVPNPCE W (1)
7	SDDELKKMGM L (1)	EGFDKDTL F (1)	KSSKSMGIVGR (1)	FAPSIKCVS (1)	QSIMCVINK (7)	KPSVPNPCE W (1)

8	SEAELRKMGMI (1)	PDFDRDALF (1)		FAQSLMCV S (1)	PSIKCVSQ Y (1)	
9	TEEELKKMGM V (1)	GNFDRELLF (1)		LAPSIKCVS (1)	QSLMCVSQN (1)	
10	SDDELDNLGLL (1)	PGFDKNTLF (1)			PSIKCVSHH (1)	
Total variant s	43	43	43	37	37	32

Furthermore, the phylogenetic analysis of the Tp2 gene (Fig. 4.2) unveiled three primary clusters, with sequences from this study grouping within clusters D and E. Cluster D further divided into two sub-clusters: DI, associated with the Muguga cocktail strains, and DII, linked with the Chitongo strain (Fig. 4.2). Cluster DI encompassed 11 study sequences (6 immunized and 5 control), while cluster DII and cluster E comprised 23 (12 immunized, 10 controls, and 1 sentinel) and 9 (5 immunized, 2 controls, and 2 sentinels) sequences, respectively. In summary, the phylogenetic analysis revealed that the majority of the study sequences did not closely cluster with the Muguga cocktail strains.

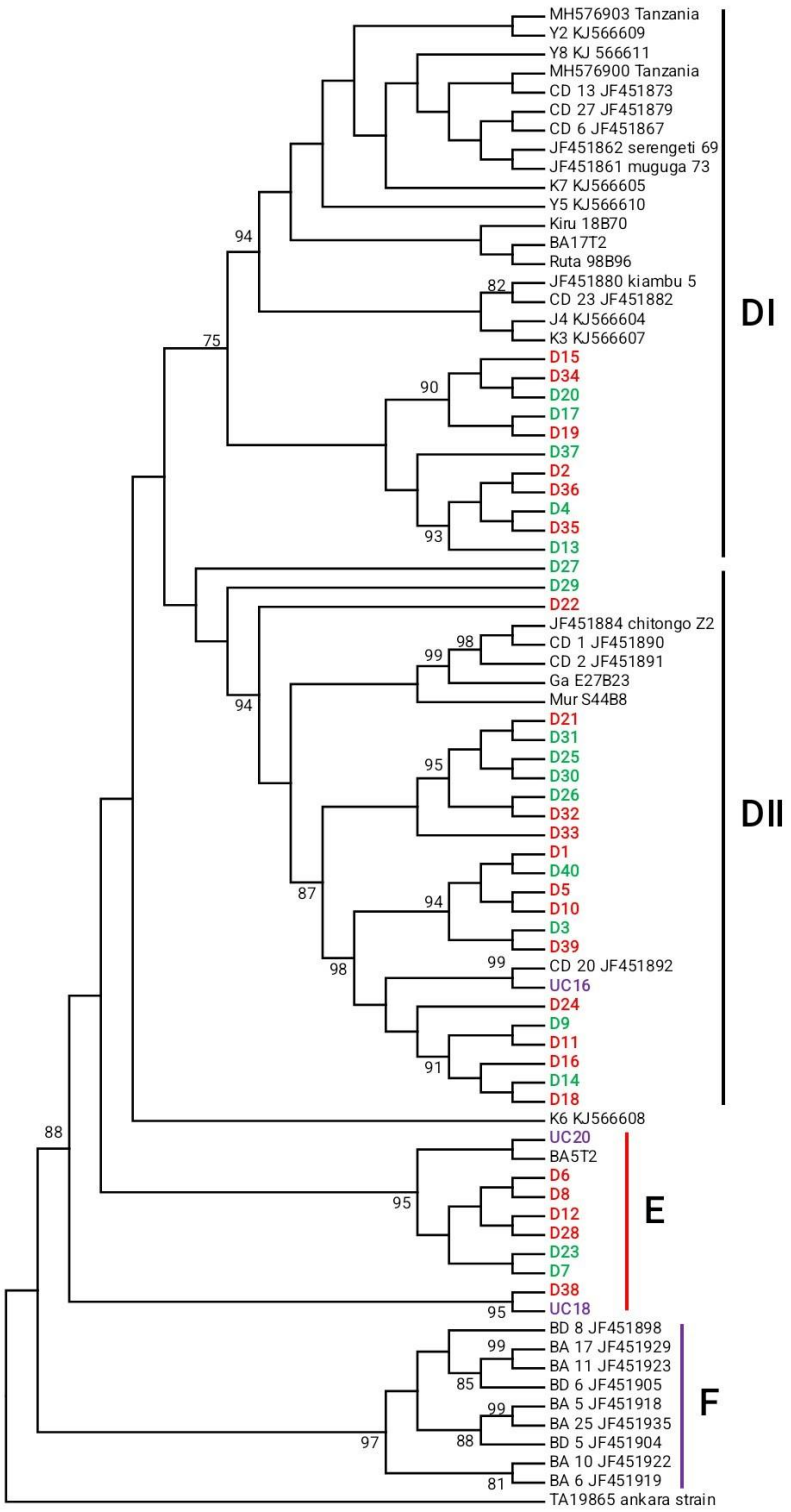

Figure 2.3: *T. parva* Tp1 phylogenetic tree. The tree was constructed from 531 bp nucleotide sequences using MEGA version 6 with 1000 bootstrap replicates as a measure of confidence. Sentinel, immunized and control sequences are given in bold and purple, green and red respectively.

The analysis of haplotype diversity revealed distinctive and non-shared haplotypes in the MC and sentinel samples, which were not shared among the immunized or control groups (Fig. 4.3). Specifically, Muguga cocktail haplotypes H1 and H2 exhibited a direct relation only to H8, encompassing both immunized and control group samples. Within the sentinel haplotypes, only haplotype H17 showed a direct connection to H13, involving both immunized and control group sequences. The remaining sentinel haplotypes were indirectly linked to other haplotypes, such as H15, through median vectors. In summary, network analysis demonstrated that sentinel haplotypes were not connected to the MC Haplotypes, nor were they associated with the haplotypes found in both the immunized and control groups.

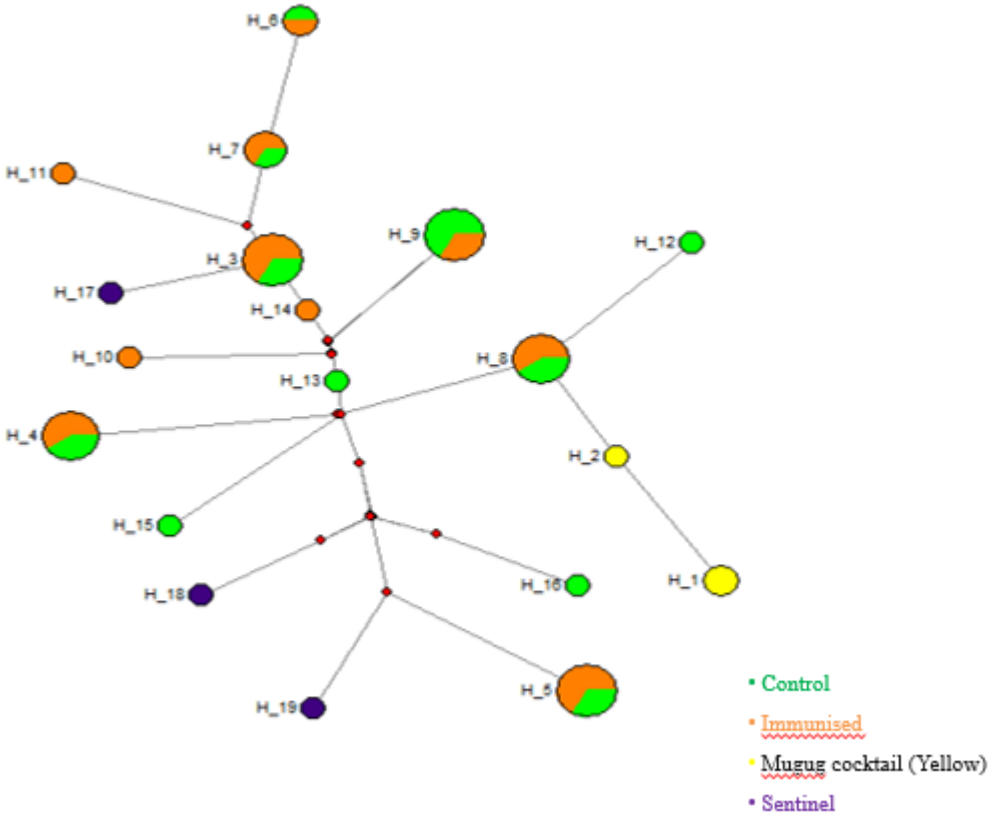

Figure 4.4: Median-joining network of the *T parva* Tp2 gene constructed using Network 5 and based on the polymorphic sites of Tp2. The size of the circle corresponds to haplotype frequency and the color codes indicate the origin of the samples.

4.3 Marker diversity and allelic variation

Samples from control, immunized, and sentinel cattle from the DRC were genotyped using a panel of five microsatellites (MS39, MS25, MS7, MS33, and MS19) and one mini-satellite marker (ms9), covering the four chromosomes of *T. parva*. Among the markers, MS39 was the most polymorphic, producing 25 alleles, while MS19 was the least polymorphic, identifying only 13 alleles (Table 4.2). High genetic diversities were also observed in sentinel, control and immunized populations.

Table 4.2: Alleles identified and genetic diversity in sentinel, immunized and control groups

	Population	**N**	**MS39**	**MS25**	**MS7**	**ms9**	**MS33**	**MS19**
Number of Alleles	Control	20	15	10	11	14	13	9
	Immunized	22	12	10	10	14	16	8
	Sentinel	5	4	4	3	5	3	4
	Muguga Cocktail	3	3	3	3	3	3	3
	Total		25	20	16	24	24	13
Gene Diversity	Control	20	0.968	0.863	0.895	0.958	0.958	0.858
	Immunized	22	0.887	0.879	0.866	0.944	0.961	0.853
	Sentinel	5	0.900	0.900	0.800	1.0	0.700	0.900
	Muguga	3	1.0	1.0	1.0	1.0	1.0	1.0

Across the six loci, there were both shared and unique alleles. The MC exhibited a total of 18 alleles, with 14 being unique to MC and 4 shared with the control, immunized, and sentinel populations. The control and immunized populations each possessed a total of 35 and 30 unique alleles, respectively. When considering all populations together, the total number of unique alleles across the six loci was 91, while the number of shared alleles was 38. The greater proportion of unique alleles indicated genetic sub-structuring among the populations.

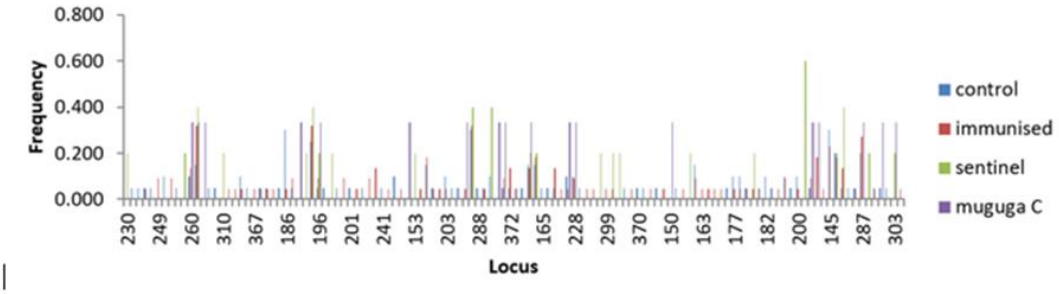

Figure 4.5: Overall frequency of alleles from sentinel, immunized, control and vaccine groups. The dataset presents shared and unique alleles determined by calculating the proportions of the predominant alleles relative to the total of each satellite marker. Histograms were generated using multi-locus genotype data.

To evaluate the observed genetic sub-structuring depicted in Fig. 4.5, Principal Component Analysis (PCA) was employed. The PCA results revealed that samples from the Kabasha village in Democratic Republic of the Congo (DRC) occupied all four quadrants, whereas the Muguga cocktail (MC) samples were confined to a single quadrant (Fig. 4.5). Notably, thirteen control (n=13), immunized (n=18) and sentinel (n=5) samples closely clustered with the MC in cluster A, while the remaining eleven (11) were divided between cluster B (n=6) and C (n=5), comprising of both control and immunized samples. This cluster pattern indicates a degree of sub-structuring. The degree of sub-structuring, as illustrated in Fig. 4.4 and Fig. 4.5, was further assessed using Wright's F index. Fst values of 0.119 and 0.096 were obtained when cluster population A, (MC, control, sentinel and immunized samples), was compared to cluster populations B and C, respectively. When all populations were treated as one, an Fst value of 0.118 was obtained.

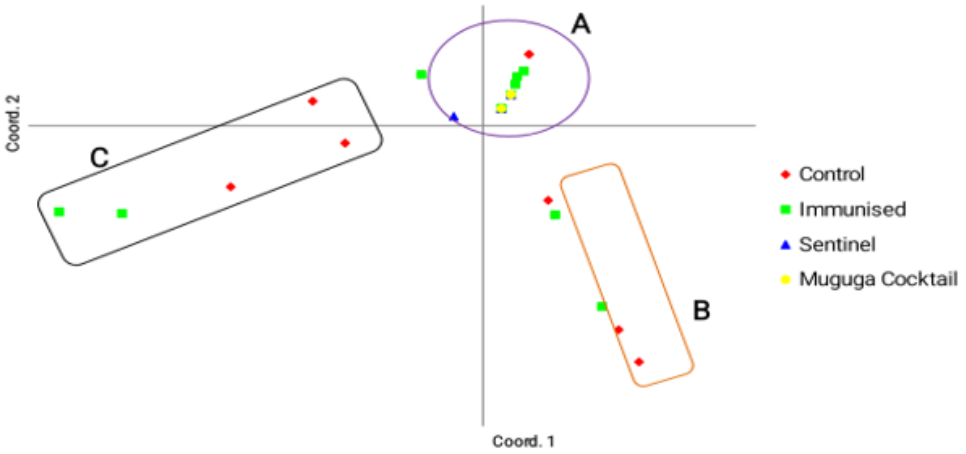

Figure 4.6: Principal Component Analysis (PCA) for sentinel, immunized, control and muguga cocktail vaccine samples revealing sub-structuring among three populations: Population A includes muguga cocktail, immunized, control and sentinel samples; Population B and C comprises of immunized and control samples.

CHAPTER FIVE

DISCUSSION

5.0 Discussion

Molecular techniques, such as Polymerase Chain Reaction (PCR) and Deoxyribonucleic Acid (DNA) sequencing, have significantly transformed the identification of previously undiscovered pathogens in regions that were previously considered free of pathogens. To establish effective vaccination programs and ensure the maximal protection of the local cattle population, it is crucial to pinpoint appropriately matched vaccine strains of *Theileria parva* for use in new areas. Molecular-oriented studies on existing *Theileria parva* populations are essential for this objective, given that the method of infection and treatment exclusively provides immunity specific to particular strains (Taracha et al., 1995). Positive outcomes from similar studies conducted in Rwanda (Atuhaire et al., 2020) and Burundi (Atuhaire et al., 2021) highlights the potential success of this approach. To evaluate similarities between *T. parva* populations of eastern region of DRC, and the Muguga cocktail vaccine stocks, this study employed an analytical cross-sectional design to to assess the sequence similarities of *Tp1* and *Tp2* and evaluate the genetic diversity among field (sentinel) samples, vaccine stocks, and animals (both vaccinated and control) used in a field challenge trial in Kabasha village, eastern DRC. Notably, this is the first study to utilize the sequence diversity of *T. parva* CTL antigens *Tp1* and *Tp2*, along with mini- and microsatellite analysis to characterize and compare parasite populations with known vaccine isolates in the DRC.

The molecular analysis revealed three distinct phylogenetic clusters for both *Tp1* (fig 4.1) and *Tp2* CTL antigens (fig 4.2), differing from clustering patterns reported in earlier studies (Atuhaire et al., 2020, 2021; Chatanga et al., 2020; MacHugh et al., 2009; Pelle, Graham, Njahira, Osaso, Saya, Odongo, et al., 2011). This divergence may reflect the unique evolutionary dynamics or transmission ecology of *T. parva* in Eastern DRC. The finding that two-thirds of sentinel samples had high genetic similarity with the Muguga Cocktail for *Tp1* suggests that this gene is relatively conserved in the local *T. parva* population. This conservation likely contributes to partial vaccine coverage, as *Tp1* encodes an epitope recognized by CD8+ T cells, and

close similarity to the vaccine strain would promote effective immune responses in immunized cattle.

In contrast, *Tp2* displayed considerable divergence, forming more genetically distinct clusters. This indicates that the *Tp2* region is under stronger selective pressure, potentially driven by host immune responses or recombination events within the tick vector. The diversity observed in *Tp2* is particularly significant because it encodes multiple epitopes that are key to protective cytotoxic T cell responses. The presence of unique *Tp2* variants implies that some circulating strains may not be effectively targeted by immune responses primed by the Muguga Cocktail, thus limiting the breadth of protection conferred by the vaccine.

The contrast between the conserved nature of *Tp1* and the high variability of *Tp2* suggests that different evolutionary forces may be acting on these genes. *Tp1* may be more functionally constrained or less exposed to host immune surveillance, while *Tp2*'s greater polymorphism could reflect antigenic escape mechanisms. These findings underscore the importance of including multiple, region-specific *Tp2* variants in vaccine formulations, or at least monitoring *Tp2* diversity over time to adjust immunization strategies accordingly.

In practical terms, the clustering and divergence patterns support the need for localized *T. parva* surveillance to guide vaccine policy. The high *Tp1* similarity to the Muguga strain offers some optimism for existing vaccine coverage in Eastern DRC, but the significant divergence in *Tp2* highlights a potential vulnerability in the current immunization strategy

The distinct phylogenetic clustering observed between immunized and control samples, despite sharing some similar strains, suggests that vaccination may be exerting selective pressure on circulating *T. parva* populations. This could lead to the emergence or maintenance of antigenically distinct sub-populations within the broader parasite community. The unique cluster formed by sentinel, control, and immunized animals in the *Tp2* phylogeny likely represents a divergent *T. parva* subpopulation that is not derived from the Muguga Cocktail and may reflect either local transmission cycles or introduction of new strains. The fact that both vaccinated and unvaccinated animals carried these distinct variants raises concerns about incomplete cross-protection by the current vaccine.

The antigenic divergence seen in *Tp2,* especially in samples that did not cluster with Muguga, suggests that these strains express epitopes that differ significantly from those included in the vaccine. Since *Tp2* is more polymorphic and encodes a CTL antigen critical for immune recognition, the presence of novel *Tp2* epitope variants may result in immune evasion, thereby reducing the effectiveness of immunization. In contrast, the conserved nature of *Tp1* epitopes across samples may contribute to partial protection, as more field strains share antigenic similarity with the vaccine.

These patterns of clustering imply that *T. parva* populations in the study area are not homogenous and consist of at least two sub-structured populations with varying antigenic profiles. This highlights the potential for immune mismatch between field strains and vaccine strains, particularly in antigens like *Tp2* that are more diverse. Therefore, ongoing genetic monitoring and antigenic characterization are crucial to ensure that vaccine formulations remain relevant and effective in providing broad protection.

Low nucleotide diversity was observed in *Tp1*, indicating gene conservation, while *Tp2* showed high diversity and polymorphism, as supported by previous studies (Amzati et al., 2019; Atuhaire et al., 2020; Chatanga et al., 2020) and further evidenced by the *Tp2* MJ network (Fig. 4.3). These results highlight potential genetic differences between the field *T. parva* population and the vaccine strains, particularly in the *Tp2* antigen. This further suggests that while field *T. parva* shares some genetic features with the Muguga cocktail, it may also possess unique traits in the *Tp2* region, which could have implications for vaccine efficacy and pathogen evolution in the region.

Ultimately, the implication is that most *T. parva* field populations, while appearing Muguga cocktail-like, might not share the same immunological response and could constitute a different population from the Muguga cocktail. This is further supported by the few haplotypes that were shared between the sentinel, immunized, and control populations and the Muguga cocktail vaccine stocks (Fig. 4.2 and 4.3). This observation aligns with previous studies in different regions (Atuhaire et al., 2020, 2021; Muleya et al., 2022). Additionally, evidence of expanding populations with similar haplotypes across the region suggests the possibility of open grazing, coupled with free trade in animals or movement among local farmers, driving the spread of *T.*

parva infection. This practice hampers the implementation of adequate control strategies, such as frequent dipping and livestock movement bans, crucial in mitigating the effects of theileriosis.

It is important to note that at the time of sampling, part of this region was experiencing armed conflict; therefore, the samples used for molecular analysis were obtained from the same village and as such, samples that appeared different from the Muguga cocktail may represent breakaway populations that did not succumb to ITM using Muguga cocktail. Nevertheless, sequence analysis alone is insufficient to determine the most appropriate vaccine strain for field challenge trials with respect to *T. parva*. Furthermore, the highly conserved nature of *Tp1* and diversity of *Tp2* genes, warranty determination of the similarity of the proposed vaccine isolates to field populations challenging. Therefore, in order to enhance result resolution, population genetic analysis involving six (6) loci on the *T. parva* genome was conducted. Population genetic analysis revealed high gene diversities in all populations across all loci (Table 4.2), indicating the presence of a diverse population. Despite the lack of close clustering with MC vaccine isolates on phylogenetic and network analysis, sentinel, immunized, and control samples also did not share the majority of alleles on microsatellite analysis (Fig. 4.4). In contrast, PCA (Fig 4.5) showed close clustering of samples comprising sentinel, immunized, and control populations, with a few clustering independently. This could be attributed to detecting Muguga cocktail vaccine strains, along with control samples infected with Muguga cocktail-like parasites. Furthermore, based on sequence diversity and population genetic data, it is likely that more than one population similar to the Muguga cocktail but genetically sub-structured from it is present at the study site. This is evidenced by the breakaway populations observed in clusters B and C on the PCA (Fig. 4.5) and the Fst values of 0.119 and 0.096 for comparisons of A vs. B and A vs. C, respectively. An Fst value of 0.118 when all populations were treated as a single population indicated moderate genetic differentiation among the populations.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion

This study revealed significant differences in antigenic profiles and indicated the presence of distinct populations with some population appearing muguga cocktail-like. Despite the armed conflict in the region during sampling, the study provided valuable insights into the genetic diversity of *T. parva* populations, suggesting the presence of diverse populations. The immunization and field challenge trial indicated a low survival rate, underscoring the limitations of the current vaccine against field strains in Kabasha Village, eastern DRC. The study concludes that more than one population similar to the Muguga cocktail may be present in the study site. Nevertheless, this study contributes significantly to the understanding of *T. parva* dynamics in the region, emphasizing the complexities of vaccine strain selection and the importance of continuous monitoring and adaptation of control strategies in the face of evolving parasite populations. This work was of a larger research initiative aimed at characterizing *T. parva* in eastern DRC, with the goal of informing the introduction of ITM in regions where the disease is endemic.

6.2 Recommendations

- Conducting additional field trials to ensure that the muguga cocktail vaccine provide effective immunity against local strains.
- Routine molecular surveillance of *T. parva* strains in the East DRC to detect potential new or emerging variants.

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Appendices

Appendix A: S1 Table

S1 Table: Microsatellites and minisatellite specific annealing temperature

Marker	Specific annealing temperature
MS39	55
MS25	58
MS7	60
MS33	57
MS19	56
ms9	60

Appendix B: National Health Research Authority Certificate of Registration

MINISTRY OF HEALTH

NATIONAL HEALTH RESEARCH AUTHORITY

The Health Research Act (Act No. 2 of 2013)

CERTIFICATE OF REGISTRATION

THIS IS TO CERTIFY THAT

Simbuwa Mulonga

has been registered as a Health Researcher Dated this 20th August 2024

Registration number NHRAR-R-1883/16/08/2024

A/DIRECTOR AND CHIEF EXECUTIVE OFFICER PROF. VICTOR CHALWE