Aus dem Institut für Parasitologie und Tropenveterinärmedizin
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Delimiting *Theileria parva* strain diversity towards targeted and unified approaches in the control of *T. parva* cattle infections

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zur Erlangung des Grades eines
Doctor of Philosophy (PhD)
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Micky Mwananje Mwamuye
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In loving memory of

Clarkson M. Jirra (1959-2014)

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List of abbreviations

AIC	Akaike Information Criteria
AU-IBAR	African Union Inter African Bureau for Animal Resources
BEB	Bayes Empirical Bayes
BMGF	Bill and Melinda Gates Foundation
CD163	Cluster of Differentiation 163
CD8	Cluster of Differentiation 8
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CTL	Cytotoxic T cell Lymphocytes
DFG	Deutsche Forschungsgemeinschaft
DFID	Department for International Development, UK
ECF	East Coast Fever
ELISA	Enzyme-Linked Immunosorbent Assay
ELISPOT	Enzyme-Linked ImmunoSpot
GMP	Good Manufacturing Practice
GTR + I + G	General Time Reversible model, Proportion of Invariable Sites and Gamma Distribution
GUTS	Ground Up Tick Supernatant
ICTTBD	International Centre for Ticks and Tick-borne Diseases
ILRI	International Livestock Research Institute
ITM	Infection and Treatment Method
MHC	Major Histocompatibility Complex
PAML	Phylogenetic Analysis by Maximum Likelihood
PAUP	Phylogenetic Analysis Using Parsimony
PIM	Polymorphic Immunodominant Molecule
SG	Salivary glands
SNPs	Single Nucleotide Polymorphisms

SRA	Sequence Reads Archive
TAWIRI	Tanzania Wildlife Research Institute
TVM + G	Transversion Model and Gamma Distribution
VNTR	Variable Number Tandem Repeats

Preface

More than a century since the recognition of the first East Coast Fever (ECF) outbreak in cattle, the control of *Theileria parva* infections remains an urgent concern in sub-Saharan Africa. *T. parva* infections in the Cape buffalo (*Syncerus caffer*) are largely asymptomatic despite the buffalo harbouring heterogeneous *T. parva* populations. In contrast, *T. parva* parasites transmitted by the vector ticks from buffalo are frequently more virulent and can 'breakthrough' a live vaccine immunity in cattle to cause a severely acute form of infection. Consequently, the existence of heterologous parasite populations, each of which has different dynamics, and the Cape buffalo's involvement are among critical factors that complicate the control of *Theileria parva* infections in cattle – especially in wild-livestock interface areas. Insufficient or lack of parasite genotype data in such interface areas hampers development of effective control strategies. Moreover, differentiating strains responsible for different clinical outcomes caused by the same parasite is still forthcoming. Identifying definitive markers to distinguish cattle- and buffalo-derived parasites, and knowledge of parasite genotypes in interface areas will have practical applications for targeted *T. parva* infections control.

This thesis centres on the challenges posed by *T. parva* strains transmitted from buffalo in the control of *T. parva* theileriosis. More importantly, a new approach to delineate *T. parva* strains based on their mitochondrial genomes is herein suggested. A major highlight is that a further understanding of *T. parva* strain diversity and its field population dynamics is key to targeted control of East Coast Fever (ECF) and the related *T. parva* infections. The synthesis consists of five chapters that include three published peer-reviewed articles. The first chapter introduces the transmission complexities and the challenge of *T. parva* theileriosis control. The objectives of the thesis research are specified.

The second chapter reviews current research in the understanding of *T. parva* genetic and antigenic diversity. The implication of the diversity for the infection and treatment method for *T. parva* infections is briefly reviewed. In addition, as the spread of the parasite is regarded to be limited by its tick vector, *Rhipicephalus appendiculatus,* the population genetics and distribution of the tick are discussed. A key highlight is that the anthropogenic movements of cattle homogenise tick populations as revealed by their lack of genetic structure in the field. However, the ticks' distribution in the field appears to be constrained by yet unknown biotic factors based on the tick absence in areas considered to be within the fitting climatic and ecological range of the tick.

The third chapter focuses on the limitation of the widely used live vaccine against heterologous buffalo-derived *T. parva* challenge. Notably, vaccine inefficiencies have only been reported in Kenya but not in Northern Tanzania, despite the vaccine being in use for over two decades with no 'breakthrough' infections reported - even though the Cape buffalo is present. To

understand this disparity, the chapter explores the exposure to buffalo-derived *T. parva* in pastoralist cattle from northern Tanzania compared to cattle in central Kenya, where there have been breakthrough infections. Specifically, the chapter compares the parasite gene pools in the wildlife-livestock interface area in Northern Tanzania to an interface area in Kenya using both the sporozoite surface protein (*p67*) gene and a single Cytotoxic T cell Lymphocytes (CTL) target antigen gene (*Tp2*).

The fourth chapter addresses the challenge of defining *T. parva* strains focusing on differentiating buffalo-derived strains from cattle derived strains. The chapter explores the utility of mitochondrial genomes generated from both Sanger and publicly available next-generation sequence data to delineate *T. parva* strains. Moreover, the near-complete full-length mitogenome sequences of other host leukocyte transforming *Theileria* species are compared with *T. parva* strains to identify valuable phylogenetic characters. The final chapter consolidates the findings generated from the studies conducted herein. It provides an overview of their implications for *T. parva* epidemiology and control, specifically in supporting the adoption of the existing live vaccine besides informing its improvement and the development of alternative novel subunit vaccines.

CHAPTER 1

Introduction

1.1 General Background

Theileria parva is a tick-transmitted haemoparasite whose infection in cattle ravages the livelihoods of mainly resource-poor livestock farmers in eastern, central, and southern sub-Saharan Africa endemic areas (Norval et al. 1992). The parasite is primarily transmitted by a three-host ixodid tick, *Rhipicephalus appendiculatus.* However, a closely related species, *Rhipicephalus zambeziensis,* has been implicated to be an efficient vector within the southern *T. parva* endemic range (Lawrence et al. 1983). Recent evidence indicates that *T. parva* is further spreading towards northern Africa and is emerging in western Africa (Marcellino et al. 2017; Silatsa et al. 2020). With estimated mortality rates of more than one million deaths every year and at least 28 million cattle under risk, the control of *T. parva* remains a pressing concern on poverty alleviation among resource-poor farmers (Grace et al. 2015; Spielman 2009). As the spread of the parasite is dependent on the tick vector that has an ecological range considered to be expanding under the influence of climate change (Norval et al. 1992), understanding the parasite's transmission dynamics and biology is vital to control efforts.

1.2 The taxonomical classification

Theileria parva is classified under the phylum Apicomplexa, which comprises eukaryotic organisms known to be obligate parasites of vertebrates and invertebrates (Mans et al. 2015). Members of the phylum characteristically possess an apical complex containing secretory organelles implicated in the parasite's invasion and establishment in their hosts (Bishop et al. 2004). The genus *Theileria* belongs to the family *Theileriidae*, which falls under the order *Piroplasmida* whose members are tick-borne pathogens of veterinary and medical importance (Barbosa et al. 2019). *Theileria* parasites have a wide host-range, including both domestic and wild ruminants (Nene and Morrison 2016; Mans et al. 2015). Some, notably *T. parva, T. annulata, T. lestoquardi, T. taurotragi,* and *Theileria sp.* (buffalo), are known to induce a neoplastic transformation of their vertebrate host cells. Accordingly, *Theileria* can further be categorised into "transforming" and "non-transforming" species (Sivakumar et al. 2014).

Among the transforming species, *T. parva* and *T. annulata* are by far the most important *Theileria spp.* that cause severe disease in cattle (Nene and Morrison 2016), while *T. lestoquardi* causes malignant ovine theileriosis in sheep (Brown et al. 1998). Moreover, *T. parva* and *T. annulata* infect wild bovids; the African buffalo (*Syncerus caffer*) and the Asian buffalo (*Bubalus bubalus*), respectively (Nene and Morrison, 2016). However, *T. parva* infections in the African buffalo are asymptomatic, while the Asian buffalo is susceptible to *T. annulata* infections and may suffer clinical symptoms (Morrison et al. 2020; Osman and Al-Gaabary 2007).

1.3 *Theileria parva* sexual life cycle – a primer for strain diversity

Apicomplexan parasites have a complex lifecycle involving asexual phases, usually within their vertebrate host's blood cells, and sexual reproduction within their invertebrate vectors (reviewed by Jalovecka et al. 2018; Mehlhorn and Schein, 1985). *Theileria parva* is transmitted transstadially by ticks and undergoes several differentiated stages specially adapted for the cells within the host in which it resides depending on its cycle stage (Bishop et al. 2004; Morrison et al. 2020). An illustration of the lifecycle features is shown in (Figure 1.1). The parasite is largely haploid throughout its life cycle except for a transient but obligatory diploid phase that occurs after a sexual union of *T. parva* micro- and macrogametes to form zygotes in the gut lumen of its tick vector (Mehlhorn and Schein, 1985; Morrison et al. 2020).

The zygotes develop into motile kinetes that penetrate the tick gut walls to get to the hemolymph. Subsequently, the kinetes migrate into the tick salivary glands where they produce sporoblasts. During the next tick feeding, the sporoblasts mature and divide to produce thousands of sporozoites, the stage infective to bovine hosts (Bishop et al. 2004; Mans et al. 2015; Mehlhorn and Schein, 1985).

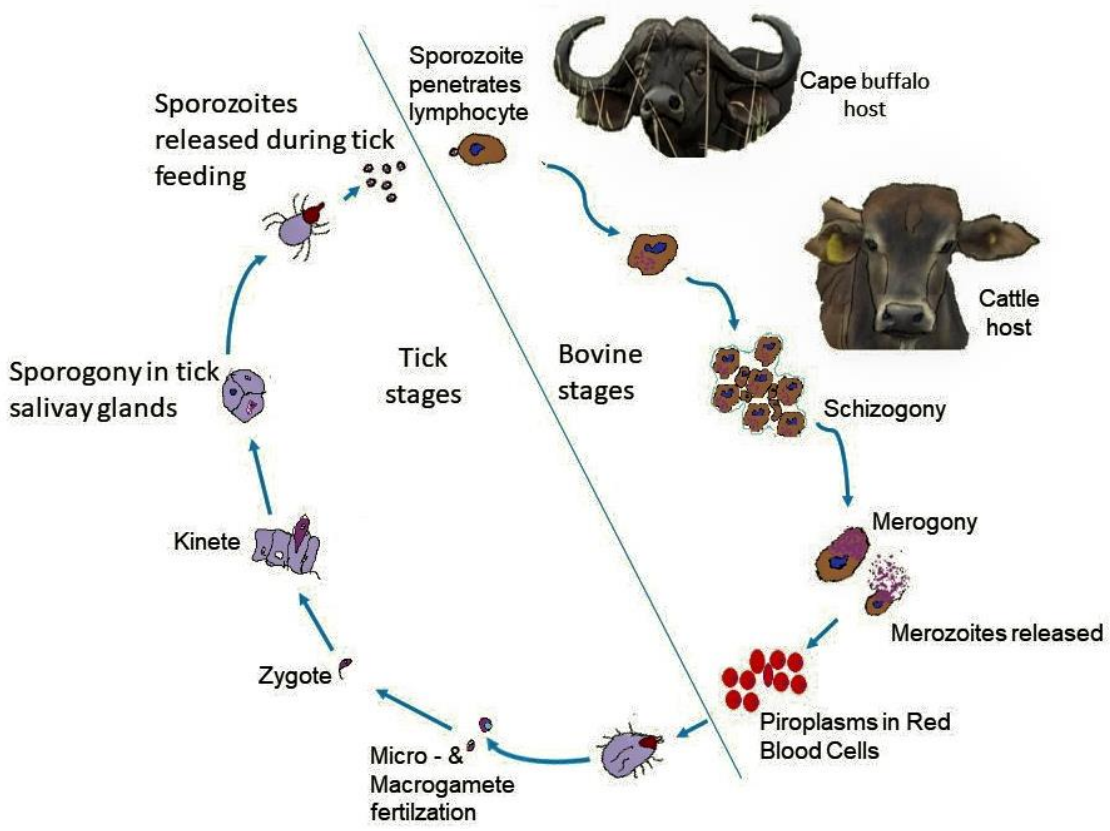

Figure 1.1 Schematic illustration of the *T. parva* life cycle (Image credit: own work).

The sporozoites are secreted with the saliva and rapidly penetrate bovine lymphocytes during tick feeding. The sporozoites develop into a multinucleate schizont stage within the invaded

cell cytoplasm and induce the transformation of host lymphocytes cells into a reversible cancer-like state characterized by uncontrolled proliferation (Spooner et al. 1989; Tretina et al. 2015). The schizonts divide synchronously with the lymphocytes by taking advantage of the invaded host's cell division machinery while disseminating and maintaining the multiplied parasite in the resultant daughter cells (von Schubert et al. 2010). This unfolding leads to a rapid clonal increase of both the parasite in the infected cells. Subsequently, a proportion of the schizonts transform into merozoites by merogony. The merozoites invade erythrocytes and develop into piroplasms, which is the tick-infective stage (Bishop et al. 2004).

The ability to undergo a sexual life cycle offers an opportunity to any organism for genetic recombination to maintain population diversity, which is beneficial to ensuing generations' survival and fitness (Butlin 2005). As such, *T. parva*'s maintenance of sexual reproduction appears to be an important process behind its extensive genotypic diversity and survival (Katzer et al. 2010). As will be discussed later in Chapter 2, the distribution and maintenance of this genetic diversity has implications in the molecular epidemiology of *T. parva* populations.

1.4 *Theileria parva* clinical manifestations

ECF, the typical *T. parva* infection, manifests clinically in susceptible animals as a lymphoproliferative disorder symptomized by fever, enlarged lymph nodes, anorexia, nasal discharge, and pulmonary oedema that may result in massive fluid outflow into the respiratory tract (Irvin and Mwamachi 1983). This pathology is driven by parasitized lymphocytes as they disseminate systematically through the lymphatic and blood circulatory systems into various tissues – with the severity of the pathology notable in the lungs considered among hallmarks of the infection (Fry et al. 2016).

Additionally, the parasite is associated with two other related syndromes exhibiting differential clinical outcomes referred to as Corridor disease and January disease (Norval et al. 1992). The former is more virulent and causes outbreaks in livestock-wildlife interface areas when susceptible cattle become exposed to *T. parva* infected *R. appendiculatus* ticks from buffalo. It has almost similar symptoms to classical ECF but with a lower schizont parasitosis and piroplasm parasitemia (Irvin and Mwamachi 1983; Jura and Losos 1980). The latter is a lesser acute infection associated with cattle-derived *T. parva* and has marked seasonality, usually coinciding with the peak activity of *R. appendiculatus* ticks in southern Africa (Norval et al. 1992).

How the same parasite can exist to cause differential disease outcomes is not only a reflection of its epidemiological complexity but also a peak illustration of its heterogeneity. As such, it was earlier thought that the three disease manifestations; ECF, Corridor disease and January disease, were caused by three related parasites species designated with a trinomial

nomenclature; *T. parva parva, T. parva lawrencei,* and *T. parva bovis,* respectively (Uilenberg et al. 1982). Since there is no evidence to date that justifies their classification as separate species (Morrison et al. 2020), this trinomial designation is now obsolete, and the parasites are referred to as either buffalo-derived or cattle-derived, based on their different epidemiological manifestations and symptomatology.

1.5 *T. parva* treatment, infection control and challenges

Chemotherapy with antiparasitic drugs such as buparvaquone can be effective with early diagnosis of *T. parva* infections but may not necessarily inhibit disease progression (Fry et al. 2016). As such, tick control by use of acaricides is the most common prevention method. However, acaricide use is unsustainable both in the short- and long-term as frequent application is expensive, may lead to acaricide resistance and could leave toxic residues in food products (George et al. 2004; Narasimhan et al. 2020). Similarly, treatment of the disease is relatively expensive, especially for smallholder farmers and pastoralists, and early detection for successful treatment is often impractical in resource-poor settings (Abbas et al. 2014; Matovelo et al. 2003).

Nevertheless, cattle that survive the infection become immune against homologous parasite strains, but immunity is variable with heterologous strain challenges (Radley et al. 1975). Subclinical levels of parasitemia can persist for several months up to 14 years in animals that recover from infection (Gwakisa et al. 2020). Although such carrier state infections may not be detectable microscopically, they remain infective to feeding ticks (Norval et al. 1992). It is possible to induce and replicate transient disease in susceptible cattle using cryopreserved live *T. parva* sporozoites with simultaneous treatment using long-acting oxytetracyclines, which controls the parasite replication. This possibility is the basis of an immunization approach, known as the infection and treatment method (ITM) (Radley et al. 1975).

Due to limited cross-reactivity among *T. parva* strains, the live vaccine was initially formulated to comprise a stock of three strains to assure broader protection in the field (Radley et al. 1975, 1979). However, the ITM vaccine production is laborious and costly, with standardization challenges on a commercial scale as there are batch to batch variations. In addition, being a live vaccine, its distribution to farmers is logistically challenging as it requires cold chain maintenance (Nene and Morrison 2016).

1.6 The African Cape buffalo (*Syncerus caffer*) in *T. parva* epidemiology

Theileria parva is considered a parasite of the Cape buffalo but has also adapted for transmission and infection in cattle (Morrison et al. 2020). As a wild reservoir host, the role of buffalo in the epidemiology of *T. parva* complicates the control of ECF as infections are asymptomatic but frequently fatal when the parasites are transmitted from buffalo to cattle.

Although ECF is reported to have been eradicated from South Africa since the mid-20th century, Corridor disease caused by buffalo-derived *T. parva* parasites persists to date (Lawrence 1992; Nene et al. 2016).

The ECF eradication strategy in South African countries was a raft of measures crafted into legislation grounded on movement control and quarantine, slaughter with compensation and compulsory short-interval dipping in acaricides for tick control (Norval et al. 1992; Perry and Young 1995). However, different epidemiological contexts exist between South Africa and East Africa, which have rendered the measures applied in South Africa to be impractical to East Africa (Perry and Young 1995). Among the possible reasons for this discrepancy is that buffalos are widely distributed and free-ranging in East Africa, resulting in instances of pasture sharing with livestock (Walker et al. 2014). As buffalo remain a constant source of both ticks and *T. parva* infection in shared pasture lands in East Africa, the control of ECF requires advanced infection prevention strategies (Walker et al. 2014).

Unfortunately, the live vaccine is ineffective against Corridor disease as buffalo-derived *T. parva* are more heterogeneous than cattle-derived strains and are likely to 'breakthrough' the strain-restricted immunity afforded by the vaccine strains (Bishop et al. 2015; Pelle et al. 2011; Sitt et al. 2015). The phenomenon of 'breakthrough' infections is the focus of the third chapter in this thesis. Further, given the genetic basis of buffalo- and cattle-derived *T. parva* remains an open question, it has been considered that identification of markers to distinguish cattle- and buffalo-derived parasites in areas co-grazed by both livestock and buffalo would have practical control applications (Morrison et al. 2020). This theme is explored in the fourth chapter of this thesis.

1.7 Study objectives

The aim of the research described in this thesis focuses on two broad objectives:

1. To genotype *T. parva* parasites from wildlife-livestock areas that have no record of live-vaccination 'breakthroughs' in contrast to areas with reported vaccine 'breakthrough'.

   Under this goal, the objective is specified as follows:

   i. To assess the level of exposure of Tanzanian pastoralist cattle to buffalo type *T. parva* based on the sporozoite surface protein antigen (*p67*) gene polymorphism,

   ii. To identify the similarities and differences of the *p67* alleles between the interface areas in Tanzania and the buffalo-derived *T. parva* strains in Kenya.

iii. To compare the variability of the *T. parva* candidate subunit vaccine schizont antigen (*Tp2*) gene between *T. parva* parasites in northern Tanzania and central Kenya wildlife-livestock interface areas.

2. To identify molecular characters for a finer resolution of *T. parva* strain genotypes based on full length *T. parva* mitochondrial genomes. This objective is specified with the following targets.
   i. To generate and annotate the full-length mitochondrial genomes of characterized *T. parva* parasites from widely separated *T. parva* endemic regions used as live strains.
   ii. To characterize the mitogenome heterogeneity patterns among different *T. parva* strains in comparison to other host-leukocyte transforming *Theileria* species.
   iii. To identify synonymous and parsimony-informative mitochondrial SNPs that can define useful haplotypes for *T. parva* high-resolution genotyping.

CHAPTER 2

A review of recent research on *Theileria parva*: implications for the infection and treatment vaccination method for control of East Coast fever

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2.1 Abstract

The infection and treatment (ITM) live vaccination method for control of *Theileria parva* infection in cattle is increasingly being adopted, particularly in Maasai pastoralist systems. Several studies indicate positive impacts on human livelihoods. Importantly, the first detailed protocol for live vaccine production at scale has recently been published. However, quality control and delivery issues constrain vaccination sustainability and deployment. There is evidence that the distribution of *T. parva* is spreading from endemic areas in East Africa, North into Southern Sudan and West into Cameroon, probably as a result of anthropogenic movement of cattle. It has also recently been demonstrated that in Kenya, *T. parva* derived from Cape buffalo can 'breakthrough' the immunity induced by ITM. However, in Tanzania, breakthrough has not been reported in areas where cattle co-graze with buffalo. It has been confirmed that buffalo in North Uganda national parks are not infected with *T. parva* and *R. appendiculatus* appears to be absent, raising issues regarding vector distribution. Recently there have been multiple field population genetics studies using variable number tandem repeat (VNTR) sequences and sequencing of antigen genes encoding targets of CD8+ T cell responses. The VNTR markers generally reveal high levels of diversity. The antigen gene sequences present within the trivalent Muguga cocktail are relatively conserved among cattle transmissible *T. parva* populations. By contrast, greater genetic diversity is present in antigen genes from *T. parva* of buffalo origin. There is also evidence from several studies for transmission of components of stocks present within the Muguga cocktail, into field ticks and cattle following induction of a carrier state by immunization. In the short term this may increase live vaccine effectiveness, through a more homogeneous challenge, but the long term consequences are unknown.

2.2 Introduction

Theileria parva is a tick transmitted apicomplexan parasite that causes East Coast fever (ECF) resulting in frequently lethal infections in cattle, with serious economic consequences in eastern, central and southern Africa (Norval et al. 1992). *Theileria parva* infection was the most prevalent tick-borne disease in cattle in Uganda according to reverse line blot assay (RLB) data (Oura et al. 2004). A recent longitudinal study in an endemic area in Kenya revealed that *T. parva* was the single most important pathogen inducing calf mortality in a cohort of African zebu calves kept by poor livestock keepers, and monitored for health and infection status during their first year (Thumbi et al. 2013).

There have been a number of reviews of *Theileria parva* biology and genomics (Bishop et al. 2009; Dobbelaere and McKeever 2002; Weir et al. 2009) and numerous reviews relating to approaches to live vaccination and attempts to develop recombinant vaccines, most recently by Nene and Morrison (2016). There have also been several reviews of the infection and treatment (ITM) vaccination method, notably that by Di Giulio et al. (2009) which overviews the methodology and focuses on the first large scale delivery of the live vaccine to pastoralists in Tanzania, and more recently by Perry (2016) which documents the historical and institutional context of the development of the live vaccine and the current status of commercial deployment and production. A recent important addition to the ITM literature was the first publication of a detailed protocol for large scale production of a million doses of the live vaccine for field use (Patel et al. 2016). Infection and treatment, as applied to *T. parva* is not new and was first conceptualized as long ago as the 1920s. However, two key innovations, approximately 40 years ago, were crucial to making this a practical technology: (i) the ability to cryopreserve sporozoites from whole ground up ticks (Cunningham et al. 1973a) and (ii) the commercial availability of an effective long acting formulation of oxytetracycline (Radley et al. 1975; Reveiwed by Radley, 1981). Subsequently, a variety of logistical issues and concerns among veterinary policy makers and regulatory bodies delayed widespread adoption of the ITM vaccination method until the mid-1990s.

This article is not intended to duplicate the above reviews, to which the reader is referred for further details and primary references, but is intended to provide an update on recent research on *T. parva* population genetics and genomics, parasite and vector distribution, and the challenge posed by the Cape buffalo (*Syncerus caffer*) wildlife reservoir for live vaccine effectiveness in areas where buffalo interface closely with cattle. Improvements to genotyping for quality control of ITM stabilates and the feasibility of improving the composition of the trivalent vaccine are also covered. Potential priority areas for future research designed to support and improve understanding of *T. parva* population genomics and the biological impact of ITM, against a background of increased deployment are highlighted.

2.3 The immunopathology of *Theileria parva* infection and live vaccination

It used to be assumed that the mortality and pathology associated with ECF was due to the capacity of the *T. parva* schizont to induce immortalization of host lymphocytes, a phenomenon which has been intensively studied *in vitro* (Dobbelaere and McKeever 2002). The schizonts initially divide in synchrony with host lymphocytes, which are thought to ultimately invade multiple host tissues in an uncontrolled fashion *in vivo*, in a process with similarities to the metastasis of tumours. Although schizont-infected lymphocytes are found in many tissues during acute infection (Kessy and Maotvelo 2011), recent immunohistochemical studies suggest that mortality, which is the result of respiratory failure (Irvin and Mwamachi, 1983), in fact involves activation and dysregulation of macrophages and resultant severe vasculitis (Fry et al. 2016). In this, macrophages activated during the acute disease course infiltrate and destroy vessels within the lungs, lymph nodes, spleen, and liver. The macrophages are strongly positive for the scavenger receptor, CD163, and for the pro-inflammatory cytokine IL-17. Macrophage-mediated vessel destruction in turn causes significant fluid leakage into surrounding tissues. Within the lung, this results in obstruction of airways by pulmonary edema, and restriction of lung expansion due to pleural effusion. These changes culminate in respiratory failure and death of the affected bovine, a hallmark of severe ECF (Fry et al. 2016). In addition to pulmonary changes, animals that succumb to ECF also exhibit marked lymphocyte death within all lymphoid tissues (Mbassa et al. 2006). These findings, coupled with the observation that acute ECF leads to severe peripheral blood lymphocyte decline, rather than expansion (Fry et al. 2016; Irvin and Mwamachi, 1983) as one would expect in a lymphocytic tumor-like disease, support the assertion that non-lymphoid components of the immune response play a significant role in ECF mortality.

This recent paradigm shift in ECF pathogenesis has given way to the discovery of potential correlates of ECF progression and severity in cattle. Such correlates will allow objective assessment and comparison of new ITM formulations and/or dosages, and of next-generation vaccines for *T. parva.* Although the development of a CD8+ cytotoxic T-lymphocyte response to *T. parva* schizont infected cells is required for protection against *T. parva* (McKeever et al. 1994), this response develops late in the disease course, and is thus not a useful measure of early ECF progression. Recently, a subset of the authors (Fry, Knowles, Bishop) found that cattle undergoing lethal *T. parva* infection, but not non-lethal infection, developed significant changes in peripheral blood monocyte phenotype and function, and that these changes could be used to predict development of severe ECF (Bastos et al. 2019). Moving forward, the use of these disease correlates, and the development of additional correlates representing the entire breadth of the bovine immune response to *T. parva*, is paramount to the development of improved pre-immunization strategies for ECF.

2.4 Deployment and impact of live vaccination

To date in excess of 1.6 million cattle, at a minimum estimate, have been immunized using the Muguga cocktail trivalent version of ITM (Perry 2016). The vaccine was initially produced at ILRI Nairobi Kenya in the mid-1990s using FAO funding, and again in 2008 at the request of the African Union Inter African Bureau for Animal Resources (AU-IBAR). Subsequently production has been transferred to the AU-IBAR International Centre for Ticks and Tick-borne Diseases (ICTTBD) in Malawi, which was formally opened in December 2014, with support from GalvMed, a public-private partnership facilitation organization based in Edinburgh UK. Funding has been provided by the Department for International Development (DFID) UK and the Bill and Melinda Gates Foundation (BMGF) USA.

Deployment of the vaccine to date has been primarily in pastoralist systems in Tanzania and Southern Kenya, but it has also been successfully tested in a field trial in cross-bred cattle in central Tanzania (Lynen et al. 2012). On farm trials of the antigenically diverse (Hemmink et al. 2016) Marikebuni stock have also been performed in Western Kenya (Wanjohi et al. 2001). Pilot trials of the cocktail have been made in other countries, particularly Uganda, which is currently hosting a larger scale trial, which will likely influence regulatory approval in that country. In addition, 'local parasite stocks' have been used for vaccination in Zambia and Zimbabwe, in the latter country, sometimes without the use of oxytetracycline, which is possible due the low abundance of infection of the Boleni (Zimbabwe) stock in ticks (Di Giulio et al. 2009; Latif and Hove, 2011).

Vaccination campaigns in Zambia, using the locally isolated Katete stock were the result of a Belgian initiative that provided a successful alternative to the use of the trivalent Muguga cocktail. By 1997, 130 000 cattle had been immunized in the eastern province of Zambia (Manangi 1999). Economic analysis demonstrated that it was a cost effective strategy for control of ECF in the traditionally managed Sanga cattle (Minjauw and McLeod 2003). Production of the Katete stock has now been transferred to the ICTTD vaccine factory in Malawi. One factor potentially contributing to the success of the local stock is that, as indicated by genotyping using Southern blotting with repetitive DNA probes, including Tpr and minisatellite 221, combined with PCR RFLP of antigen genes, there appears to be a relatively homogeneous parasite population and therefore presumably a homologous challenge, in much of eastern Zambia (Geysen et al. 1999). However, there may be additional *T. parva* epidemiological situations within East Africa where a local stock may be an appropriate alternative to the Muguga cocktail.

As mentioned above, an alternative to the infection and treatment approach is the use of the Boleni stock of *T. parva* which has been delivered in Zimbabwe, without concurrent

administration of oxytetracycline, thereby greatly reducing the cost of vaccination. The pros and cons of this approach are discussed in detail by Latif and Hove (2011). Although the stock was virulent in cattle when first isolated, after experimental passage through ticks it produced stabilates with low numbers of sporozoites relative to other stocks, 8-9 time less than Muguga and Serengeti from East Africa, and 14 times less than Kasoba from Malawi (Latif and Hove, 2011). This allowed use of the stock without antibiotics with considerable reductions in the cost of vaccination. Boleni also protected well against infected tick challenge within Zimbabwe and thus represented a very attractive option for less wealthy farmers, and was used for ECF control in the country for at least ten years. However, one problem was that the number of sporozoites within a stabilate batch was highly variable, making vaccine standardization difficult. In addition, in other laboratories the virulence was found to vary, for example a cloned Boleni stabilate generated at ILRI (Morzaria et al. 1995)was highly virulent. Thus wider scale out of the Boleni vaccine would be unlikely to meet with regulatory approval. However, the goal of creating a whole-sporozoite-based vaccine that does not require simultaneous administration of oxytetracycline remains a desirable option.

2.5 The socio-economic impact of live vaccination

There have now been three different herd and homestead level impact studies of the trivalent Muguga cocktail version of ITM delivered on a cost recovery, or fully commercial basis to pastoralists in northern Tanzania and southern Kenya (Homewood et al. 2006; Martins et al. 2010; Marsh et al. 2016). All three concluded that the intervention was profitable and resulted in increased yields of meat and milk and disposable household income that translated into increased human capital, including female education (Marsh et al. 2016). The Homewood study concluded that wealthier pastoralists were more likely to adopt ITM initially, which is a likely scenario, given that the resource poor are typically least able to afford implementation of new technologies. However, significantly, although ITM is generally regarded as a private good, the Marsh study indicated that the subsequent use of acaricides for tick control and antibiotics for treatment of animals was reduced as result of vaccination, suggesting the accrual of broader societal benefits.

The cost of ITM is relatively high at an estimated US$ 7 per animal (Lynen et al. 2012). However even in cross-bred dairy systems, where the economies of scale due to the smaller numbers of animals (3-7) kept are less than in pastoralist systems, the intervention is potentially profitable, since the cost of treatment is much higher at approximately US$40 per animal. Field trials in two ecologically distinct small dairy production systems in Hanang (highland) and Handeni (coastal) districts in Tanzania resulted in annual savings of US$4.77 per animal due to increased acaricide application intervals (Lynen et al. 2012). Despite the

potential benefits highlighted by this pilot study, adoption of ITM has to date been relatively limited in the small dairy sector.

Large scale deployment of the ITM vaccination method and formal analysis of socio economic impact, has primarily been in pastoralist systems in Tanzania and southern Kenya. In Tanzania which has a cattle population of 18 million in total, with large numbers in the pastoralist sector, an analysis of 100 cattle (50 immunized and 50 unimmunized) from a Maasai homestead revealed a profit estimated to be US$ 5.58 per immunized calf (Martins et al. 2010). This was based on an increase in calf trade, an increased value of immunized calves in the market, and higher milk production stimulated by increased letdown due to improved calf survival. It should be noted that in this study, control animals with symptoms of ECF were immediately treated, which is not a realistic scenario in normal production situations, given the requirement for early diagnosis and expense of treatment. A more recent study in southern Kenya which encompassed a range of production systems from pastoralist to agropastoral, used a conceptual framework that went beyond the direct benefits of vaccination including increased meat and milk yields and reduced expenditure on acaricides, to examine indirect benefits on human education, health and nutrition (Marsh et al. 2016). The conclusions of the Marsh et al. study were that for the average household of 15 people in southern Kenya with a mean herd size of 66 cattle, the net benefit of ITM was US$ 35.80 at a vaccination cost of US$6.5 per head, with an average increase on educational expenditure of US$ 38.95 over a four month period.

Despite the benefits, the cost of vaccination of US$ 7 per animal may still be beyond the reach of poorest households as pointed out by Homewood et al (2006). One solution to this constraint is that production and delivery (which is currently a commercial enterprise) should be subsidized by major development agencies and donors such as FAO, as was the case in the eradication of rinderpest through live vaccination (Njeumi et al. 2012).

2.6 Vaccine stabilate composition

The Muguga cocktail trivalent ITM vaccine was designed following experiments performed over 40 years ago (Radley et al. 1975) that indicated that a panel of three stocks exhibited broader protection against heterologous *T. parva* challenge than any single constituent stock. A variety of recent studies using 'deep' NGS sequencing of variable number tandem repeats, antigen genes and complete genomes have indicated that: (i) this cocktail contains only a very small proportion of the diversity within the *T. parva* gene pool (Hemmink et al. 2016; Norling et al. 2015; Pelle et al. 2011), (ii) the Serengeti-transformed and Muguga *T. parva* stocks are very similar to one another, whereas the third stock Kiambu V is very 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). Virtually all known *T. parva* antigen encoding genes are identical between Muguga and Serengeti including the polymorphic immunodominant molecule (PIM). Interestingly, the Serengeti stock does exhibit some non-synonymous substitutions relative to the Muguga reference genome, primarily in polymorphic multicopy gene families, but also in ATP binding cassette transporter genes that are located as single copies immediately interior to the two major arrays of sub-telomerically encoded multicopy gene families.

The two main techniques for stabilate characterization are the use of a panel of micro and minisatellites (variable tandem number variable repeats, acronym VNTRs) originally developed from the draft *T. parva* Muguga reference sequence (Oura et al. 2003). They are informative when applied to characterization of vaccine stabilates, especially when next generation sequencing technologies are applied to specific VNTR loci to generate high coverage data that reveals the presence of 'minor' alleles (Hemmink et al. 2016).

CD8+ T cell responses induced by ITM live vaccination have been shown to be important in mediating protection following adoptive transfer of a highly enriched population of CD8+ T cells between two identical twin calves generated by embryo splitting technology (McKeever et al. 1994). Genes encoding proteins recognized by CD8+ T cells induced by ITM, known as the '*Tp*' (*Tp1-Tp10*) antigens were cloned by screening libraries transfected into cell lines using an ELISPOT assay that measures production of gamma interferon by T cell lines stimulated by autologous *T. parva* infected T cells as the readout for positivity (Graham et al. 2006). To date, convincing protection induced by a defined *Tp* antigen has only been demonstrated for *Tp1* (Svitek et al. 2018)and amounted to 36% protection in a group of cattle whose class I MHC loci were selected to bind the single currently known epitope encoded by the Tp1 gene. Following ITM vaccination, responses detected in an individual animal using assays designed to detect and selectively enrich for CD8+ T cells typically identify an 'immunodominant' antigen that is strongly associated with the MHC haplotype of the individual animal. However, it is possible that an aggregation of weaker T cell responses to minor antigens, contributes to the protection mediated by a CD8+ T cell population such as those used in the adoptive transfer study (McKeever et al. 1994).

To date a limited number of target populations have been immunized by infection and treatment. A recent study of an Ankole (an African taurine cattle breed) herd revealed that their class I MHC molecules were functionally distinct to those characterized previously, in terms of predicted peptide binding specificities, and none of the *T. parva*-infected cattle were positive using ELISPOT when their PBMC were sensitized with overlapping peptides derived from *Tp1, Tp2, Tp3, Tp4, Tp5, Tp6, Tp7* and *Tp8* (Obara et al. 2016). There are likely thousands of as yet uncharacterized bovine class I MHC loci in Africa, for example a recent project using next

generation sequencing which genotyped predominantly Holstein-Friesian cattle, but also included some African cattle identified 140 novel alleles (Vasoya et al. 2016). Furthermore, there are 211 cattle breeds documented in the FAO database (http://dad.fao.org) for the region of Africa where ECF occurs. It therefore seems likely that there could be many more *T. parva* CTL target antigens, which potentially contribute to the protection mediated by ITM, awaiting discovery.

A recent experiment using MHC matched animals demonstrated that *T. parva* Muguga alone provides an equivalent level of protection to challenge with the trivalent Muguga cocktail, as animals immunized with complete cocktail (Steinaa et al. 2018). These authors also note that the original experiment of Radley used Kiambu 1, (presumably representing a distinct stock that has not yet been genotyped or characterized phenotypically) as the heterologous challenge parasite (Radley et al. 1975). Collectively these data suggest that it would be worthwhile to perform additional experiments to revisit whether a single stock, either *T. parva* Muguga, Serengeti, or Kiambu V (which induces a long term carrier state [Oura et al. 2007]), unlike Muguga (Skilton et al. 2002a) could replace the trivalent cocktail. Kiambu V is the most clonal of the three vaccine components according to deep sequencing (Hemmink et al. 2016) and might therefore be the best candidate. This would simplify the production process and in addition reduce the risk of recombination between vaccine components.

2.7 Stabilate production and delivery: potential improvements

Major avenues for improving production and delivery of ITM were discussed at a GalvMed convened meeting of all major stakeholders held at the International Livestock research Institute (ILRI) and the recommendations are summarized by Toye and Ballantyne, (2015). These included relatively easily implementable measures such as simplification of the composition and stabilization at room temperature of the diluent. An additional goal was increasing post reconstitution stability of the cryopreserved sporozoites from four to 12 hours. More challenging would be an attempt to thermostabilize the sporozoites so that they remain viable at room temperature, using lyophilization, a strategy which is effective for live attenuated viral vaccines, but has proved challenging for eukaryotic pathogens. Another recommended modification was packaging containing smaller doses than the current 40 dose straw, for dairy systems with smaller numbers of more valuable taurine or cross-bred animals. In this context, an innovation has very recently been described that would allow delivery of 4-8 doses per straw (Patel et al. 2019).

A longer term option would be to try to attenuate the parasite with the goal of removing the requirement for the use of oxytetracycline in live vaccination. Irradiation of sporozoites has proved successful *for Plasmodium falciparum* and these provide protection in humans

(Hoffman et al. 2002). Irradiation has been attempted for purified *T. parva* sporozoites using a ^{60}Co source at doses between 4.0-10 kilorads, without any apparent attenuation *in vivo* in cattle (Cunningham et al. 1973b). Chemical attenuation involving treating *Plasmodium* sporozoites with the DNA alkylating agent centanamycin has also worked well in rodent malaria models (Purcell et al. 2008), but has not yet been attempted for *Theileria.* Parasite transfection and genetic modification is a further option and has worked for *Plasmodium*. However, this has proved technically difficult in *Theileria*. Transient transfection systems have been reported for *T. parva* (De Goeyse et al. 2015), with reporter genes being expressed in sporozoites, driven from parasite promoters following nucleofection to introduce the constructs into the parasite. There is also an earlier protocol published for the related *Theileria annulata* involving transient expression of green fluorescent protein from several parasite promoters in sporozoites treated with a lipofectin reagent (Adamson et al. 2001). There have not been any further publications in this area to date. However, with the development of more sensitive reporter molecules such as nanoluciferase, and CRISPR CAS9 technology for precise genome editing (reviewed by Kim 2016), which has been applied successfully to protozoan parasites including *Toxoplasma* and *Plasmodium* (reviewed by Suarez et al. 2017), this approach may now be worth revisiting. One recent initiative which may increase the sustainability of delivery, by ultimately reducing the dependence on liquid nitrogen involves simple modifications to the structure and handling of liquid nitrogen dewars that are designed to minimize temperature fluctuation in straws that are briefly exposed to ambient temperatures, when the flask is opened . This has worked well for improving artificial insemination by ensuring that the straws, although their contents are not thawed, do to reach the critical transition temperature of water of -137 ^{O}C, which results in reorganization of water molecules and damage to membranes when the contents are returned to the liquid nitrogen (Lieberman et al. 2016). As an extension of this work, the Seattle based company Intellectual Ventures is exploring the potential of keeping sporozoite stabilates on dry ice at -80, for various periods of time with support from the Intellectual Ventures Global Good Fund, (funded by the Bill and Melinda Gates foundation). The rationale for this is that many countries in the endemic ECF region, including Tanzania and Uganda, do not currently have sustainable liquid nitrogen production industries but do produce dry ice, which has a wide range of industrial applications.

2.8 The spread of *Theileria parva* within sub-Saharan Africa

The distribution of *T. parva* is not as wide as that predicted for the vector according to climate-based models (Norval et al. 1992), so there is theoretically potential for spread of *T. parva*, especially given the largely un-regulated transboundary cattle movements between certain countries within the East and central African region. *Theileria parva* is moving North in the Sudan (Malak et al. 2012; Marcellino et al. 2017) and has recently been discovered for the first

time in Cameroon (Silatsa et al. 2020). *T. parva* can therefore be considered an emerging pathogen.

It is likely that anthropogenic cattle movement has played a major role in the spread of the parasite in both instances, but some aspects of the epidemiology are distinct between Cameroon and South Sudan. In South Sudan, the tick vector *Rhipicephalus appendiculatus* is present in all of four cattle populations surveyed and 25% were infected with *T. parva* according to a sensitive PCR assay based on the *p104* antigen (Marcellino et al. 2017; Odongo et al. 2010). The PCR assay revealed a 13% infection rate in cattle blood and the PIM ELISA (Katende et al. 1998) demonstrated 23% seroprevalence. Genotyping using 14 micro and minisatellites (Salih et al. 2018) revealed very high levels of diversity with an average of 9.57 alleles per locus and a range of between 6-15 alleles per locus. Principal components analysis indicated a degree of substructure suggesting that the populations were partially distinct with limited gene flow between them. This might suggest that there have been multiple migrations of *T. parva*-infected cattle, together with the tick vector, into different regions of South Sudan. The fact that the vector is present suggests that ECF is likely to be a serious problem for cattle keepers in South Sudan. In a participatory survey, farmers from one of two districts regarded ECF as their most serious animal health problem (Malak et al. 2012). Live vaccination should therefore seriously be considered as a control option in this country, especially given that sequencing of the *Tp1* and *Tp2* genes from Sudanese isolates revealed close genetic similarity of these antigens to stocks within the Muguga cocktail (Salih et al. 2017).

By contrast, in Cameroon, all animals surveyed were asymptomatic and a parallel comprehensive countrywide tick survey did not reveal the presence of the major vector *R. appendiculatus*, although the other expected ixodid tick species associated with livestock were present (Silatsa et al. 2019). Among animals surveyed using ELISA, 23.76 % were serologically positive and of 1,324 DNA samples from blood assessed by PCR using the p104 nested assay, 1.86% representing 25 animals were positive. Sequencing of the *p104* gene has generally received limited use as a polymorphic marker to date, perhaps because it is the standard diagnostic PCR gene (Odongo et al. 2010; Skilton et al. 2002). The *p104* sequences of *T. parva* from Cameroon revealed seven genotypes, some of the rarer ones not having been described previously. There were only two *p104* alleles present in the complete genome sequences of the Muguga cocktail component stocks, while the most frequent genotype was identical to that of the Kenyan coastal stock *T. parva* Marikebuni (Morzaria et al. 1995) that was field tested as an ITM vaccine on farms near Kitale in western Kenya (Wanjohi et al. 2001). The *Tp1* alleles present were identical to those in the three stocks comprising the ITM stabilate. This scenario may indicate spread of *T. parva* by vaccination as suggested by McKeever, (2007). However, the distances involved for cattle migration would be very

substantial and additional analyses of VNTRS and Tp antigens using high resolution next generation sequencing (NGS) will be required to determine the origin of the *T. parva* parasites in Cameroon. More detailed monitoring of transboundary cattle movements will also contribute to our understanding of how *T. parva* has reached Cameroon.

What is clear from the data is that *T. parva* is present in asymptomatic cattle in Cameroon and that there is currently no proven tick vector in the country, although tick species that have been shown to be susceptible to *T. parva* infection in the laboratory (Norval et al. 1992) do occur. This situation could change either by migration of ticks into the country on cattle, or evolution of a novel vector. The data also raises the question of how long the parasite has been present in Cameroon and, whether there are other countries in the region where cattle may harbor cryptic *T. parva* infections.

2.9 *Theileria parva* diversity in the field

Theileria parva diversity in field populations has been assessed in multiple recent studies using *Tp* CD8+ target antigen gene polymorphism and analysis of VNTRs. Whole genome sequencing is emerging as a tool for the future (Hayashida et al. 2013; Henson et al. 2012). The initial and most comprehensive study on variation within the *Tp* antigens, in which 82 cattle and buffalo field isolates were examined, focused on *Tp1* and *Tp2* which contain one and six mapped epitopes, respectively (Pelle et al. 2011). Both antigens were highly variable, but much of this diversity was either in buffalo isolates, or in cattle isolates that were obtained from animals that had been challenged by ticks that fed on co-grazing buffalo and succumbed to ECF (Bishop et al. 2015). Diversity among cattle isolates was limited. Interestingly, although analysis of the ratio of synonymous to nonsynonymous substitution was consistent with selection in some domains in these two antigens, there was no evidence for positive selection within the mapped epitopes (Pelle et al. 2011).

Over the last five years, there have been a number of studies examining *T. parva* diversity in field populations (Figure 2.1). These have used between 12-30 VNTRS, often in combination with the *Tp1* and *Tp2* antigens. The VNTR data typically reveals very high levels of diversity when applied to field populations from endemic areas in Kenya, Tanzania, Uganda, and South Sudan (Mwega et al. 2015; Nanteza et al. 2020; Odongo et al. 2006; Oura et al. 2005; Salih et al. 2018). For example 5-11 alleles per locus and a total of 183 alleles at 30 loci were identified among schizont-infected lymphocyte isolates from three regions in Kenya (Odongo et al. 2006) and 82 multilocus genotypes were identified from three distinct regions in Uganda (Oura et al. 2005). In studies involving multiple geographically separated regions, analysis of the total datasets often reveals linkage equilibrium and linkage disequilibrium, which is presumably at least in some instances a consequence of epidemic structures, apparent when

samples from smaller regions are examined individually. Analysis of molecular variance has revealed that typically the majority of the variation appears to be within rather than between populations. Additionally, the genotypes of isolates separated by large (between countries or regions) and medium scale distances (within countries), show little correlation with the geographical origin of the parasite.

The high levels of diversity in VNTRs and some antigen genes including *p104* (Skilton et al. 2002) and *PIM* (Toye et al. 1995) can be attributed to the obligate sexual cycle of *T. parva* in the tick vector. Two experimental crosses have been performed involving co-infections of cattle with parasite clones and tick feeding on cattle and subsequent isolation of clones (Henson et al. 2012; Katzer et al. 2011). The first of these studies revealed a high frequency of recombination in the 35 progeny clones, with specific 'hotspots' of recombination identified (Katzer et al. 2011). The second study used 454 Roche pyrosequencing to analyze the progeny clones and identified a total of 64,000 SNPs when progeny and parents were compared to the *T. parva* Muguga reference strain. 50 % of the crossovers were accompanied by gene conversion events. Such a high frequency of gene conversions means that meiotic recombination plays a significant role in the evolutionary process by not only re-distributing genetic material but also altering allelic frequencies (Henson et al. 2012).

By contrast the *Tp1* and *Tp2* antigen genes, exhibit rather limited variation with the alleles observed frequently being the same as those present in the Muguga cocktail, with occasional variants within *Tp2* (Nanteza et al. 2020; Mwega et al. 2015; Salih et al. 2017). Since the class I MHC haplotype of the animals involved in these field studies is largely unknown, it is unclear whether *Tp1* and *Tp2* are actually CD8+ T cell targets in the *T. parva* populations sampled in these studies.

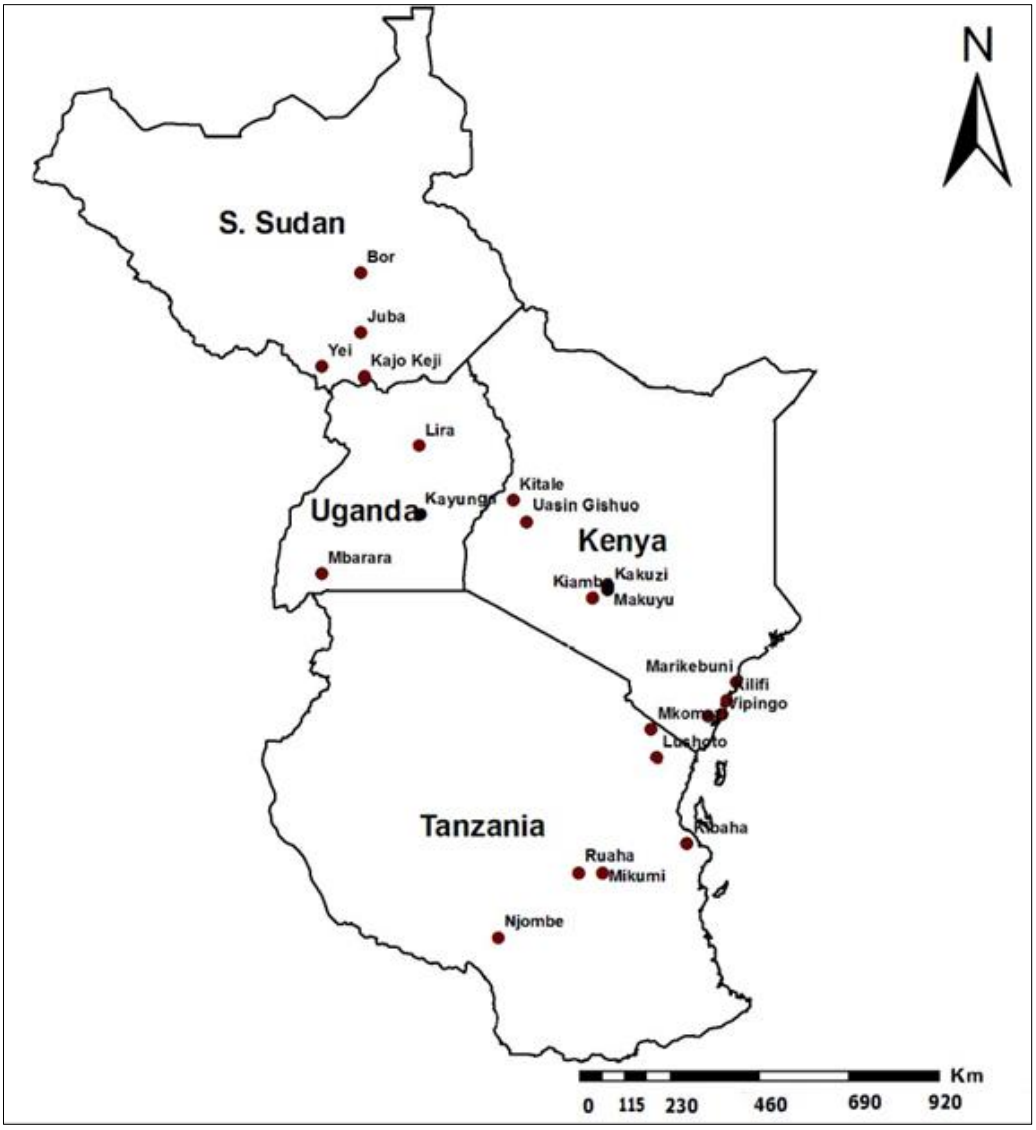

Figure 2.1 Map showing location of VNTR genotyping studies in Kenya (Odongo et al. 2006), Uganda (Oura et al. 2005), Tanzania (Mwega et al. 2015) and South Sudan (Salih et al. 2018)

2.10 *Theileria parva* diversity in the wildlife reservoir host Cape buffalo (*Syncerus caffer)*

The Cape buffalo wildlife reservoir of *T. parva*, as already mentioned harbors a much greater diversity of *T. parva* genotypes according to analysis of *Tp* antigen gene sequences (Pelle et al. 2011) and also *p67* gene sequences (Obara et al. 2015). The greater genetic diversity in buffalo has been confirmed by whole genome sequencing of DNA isolated from partially purified schizonts from infected T cell lines (Hayashida et al. 2013). Additionally buffalo are almost invariably infected with multiple parasite genotypes (Oura et al. 2011).

Early studies involving ITM immunized cattle kept in paddocks together with buffalo indicated that *T. parva* parasites from ticks fed on buffalo could 'breakthrough' the immunity induced by live vaccination (Radley et al. 1979). Recently, two studies in ranches in the Central and Rift valley provinces of Kenya where buffalo grazed adjacent to cattle immunized with both the trivalent cocktail and the Marikebuni ITM vaccine, showed that most vaccinated cattle were not immune to challenge from ticks that fed on buffalo and, contracted severe disease (Bishop et al. 2015; Sitt et al. 2015).

By contrast in Tanzania, there are no documented cases of live vaccine 'breakthrough' in the Maasai areas where buffalo graze adjacent to ITM vaccinated cattle (Homewood et al. 2006). The only study so far conducted of the genetic diversity of *T. parva* in buffalo in Tanzania was that of Rukambile et al. (2016) using VNTR sequences applied to a limited number of buffalo (30) from several geographically separated national parks. The mean number of alleles at 3.37 was relatively lower than would be expected for *T. parva* populations in buffalo in Kenya or Southern Uganda. However, the animals were from national parks and not in pastoralist areas where buffalo co-graze adjacent to vaccinated cattle. The comparative analysis of parasites in buffalo and cattle in vaccination areas awaits a definitive study.

There have been several studies on the population genetics of the parasite in buffalo. It was shown using VNTRs that in the area adjacent to, and within, Lake Mburo National Park in South West Uganda *T. parva* populations in cattle and buffalo were distinct and presumably reproductively isolated (Oura at al., 2011a). This is consistent with infrequent transmission of parasites from buffalo to cattle that subsequently become tick transmissible between cattle. A longitudinal study is required in order to ascertain how frequent transmission from buffalo to cattle is in this area, and whether the typical buffalo-derived *T. parva* parasite 'corridor disease' syndrome of rapid death, with low schizont parasitosis and piroplasm parasitaemia in the blood is the typical outcome in infected cattle.

A recent comparison of *Tp* antigen gene diversity, using six *Tp* antigens, including *Tp1* and *Tp2* between parasite populations present in buffalo from Central Kenya and South Africa

revealed that there was between 10%-69% nucleotide variation between different genes. However, although there was extensive allelic variation within individual cattle, analysis of molecular variance suggested that much of the underlying genetic variation was ancient and preceded divergence of the two populations (Hemmink et al. 2018). The selective pressures driving divergence of the *Tp* antigens remains unknown, but is not necessarily due to the immune response of the mammalian host. A study of *Tp* antigen gene sequence variation in a buffalo population in central Kenya (Sitt et al. 2018) was notable in that certain *Tp* antigens revealed very little polymorphism indicating that this is not an invariable property of CD8+ T cell target antigens.

It is interesting to note that Cape buffalo are capable of mounting CD8+ T cell responses to *T. parva* infected lymphocytes (Baldwin et al. 1988). However, it has not yet been demonstrated whether these responses contribute to the *T. parva* tolerance phenotype exhibited by buffalo. Class I MHC haplotypes have yet to be analyzed in buffalo and the extent to which peptide binding specificity overlaps that in cattle is unknown.

2.11 The carrier state and dissemination of parasites used for infection vaccination into field ticks and cattle

Because the ITM vaccination or natural infection of cattle typically induces a tick transmissible carrier state (Bishop et al. 1992; Kariuki et al. 1995; Oura et al. 2004; Skilton et al. 2002), it is likely that immunizing parasite genotypes may transfer into field ticks and cattle. The presence of a vaccine component in unvaccinated cattle was first definitively described using molecular markers specific for the Kiambu V stock using primers derived from the PIM gene (Oura et al. 2007). This work was performed at a Muguga cocktail ITM vaccination trial site near Iganga in Eastern Uganda. The re-sampling was done 3 years after of the initial sampling that demonstrated the Kiambu V carrier status in vaccinated animals (Oura et al. 2004). Based on this result, it was suggested that live vaccination could spread, as well as control, the disease (McKeever 2007). This is a possibility, particularly in the case of vaccination in areas on the margins of the current *T. parva* distribution. However, the data from South Sudan and Cameroon suggest that the parasite may already be moving as a result of anthropogenic movement of immune carrier cattle from endemic areas. Further investigation will be required to test whether ITM may be involved in disseminating the parasite in some situations.

An additional study for which the primary data was collected approximately 20 years ago involved a 100 animal field trial of the Marikebuni live vaccine by the Kenya Agricultural Research Institute (Wanjohi et al. 2001). In the collaborative study involving ILRI and funded by DFID, 20 cattle were selected from the experimental group (10 vaccinated and 10 unvaccinated) and monitored using PCR-based assays. One set of PCR primers was derived

from the hypervariable N-terminal end of the *TpR* locus (Bishop et al. 1997) and designed to be specific for the cloned Marikebuni stock (Morzaria et al. 1995). In addition, a *p104* based nested PCR assay that detects all *T. parva* but not other *Theileria* species (Odongo et al. 2010; Skilton et al. 2002) was also used to monitor the cattle. The animals were monitored for two years and eight months post vaccination. With one exception, all of the vaccinated animals were carriers of the clonal Marikebuni component and, 4 of the unvaccinated animals were positive between 14-24 months with the Marikebuni-specific markers (Bishop et al. 2020). A panel of 24 of the 60 VNTR markers described by (Oura et al. 2003) was applied to 19 clonal *T. parva* infected T cell cultures, prepared either directly from the cattle at the vaccination site, or from ticks collected and subsequently applied to cattle for schizont infected cell line isolation. The study revealed two Marikebuni clonal component profiles and two Kiambu V profiles in *T. parva* infected cell lines isolated directly from the cattle. The seven cattle cell lines isolated following tick on cattle feeding all exhibited a profile that according to the MS7 VNTR locus could be either Serengeti or Muguga.

It is known that Muguga does not generate a long term PCR detectable carrier state, only being detectable experimentally by PCR of blood for 90 to 130 days, and being apparently unable to transmit to ticks (Skilton et al. 2002). The ability of Serengeti to induce a carrier state has never been investigated. Although, as already mentioned, very similar to *T. parva* Muguga, Serengeti does contain 53 loci with non-synonymous SNPS relative to the *T. parva* Muguga reference genome at certain highly polymorphic loci (Norling et al. 2015). There had been no application of Muguga cocktail ITM vaccination anywhere in Kenya at the time this study was performed. It therefore seems possible that Muguga cocktail parasites originated from small-scale pilot trials across the border in Uganda, Tororo being the closest live vaccine trial site. Alternatively, these genotypes could have been circulating naturally in western Kenya. A recent longitudinal study (Gwakisa et al. 2020) of cattle in Northern Tanzania also demonstrates the transfer of Serengeti/Muguga ITM components into unvaccinated cattle, providing a third example of Muguga cocktail genotypes being transmitted to un-vaccinated animals. This longitudinal study also demonstrates that although the number of PCR-detectable animals declines with age, one animal was still positive by *p104* PCR 14 years post vaccination. It has never been investigated how important the carrier state is in enhancing protection by modulating the kinetics of recall of protective T cells in cattle upon infected tick challenge. If the carrier state is important, a carrier state of this longevity would suggest that induction of life time immunity is a real possibility at least for some ITM vaccinated cattle.

2.12 Population genetics and distribution of the tick vector *Rhipicephalus appendiculatus*

A multilocus VNTR genotyping study of *Rhipicephalus appendiculatus* from 10 populations within Kenya revealed a lack of genetic structure in the field populations (Kanduma et al. 2016a,b). This is consistent with *T. parva* population analyses that revealed a similar situation for *T. parva* in Kenya (Odongo et al. 2006). The data suggests that anthropogenic cattle movement rapidly homogenizes tick populations. Another illustration of this is the rapid spread North of *T. parva*-infected *R. appendiculatus* in South Sudan.

It was recently reported that there are two major haplotypes of *R. appendiculatus* that are sympatric within Kenya (Kanduma et al. 2016b). A similar phenomenon was subsequently described in the Great Lakes region (Amzati et al. 2018). We do not yet know if there is any functional significance in these two distinct haplotypes. However, what is known is that there are major differences between *R. appendiculatus* lines in their ability to acquire and transmit *T. parva* (Ochanda et al. 1998; Odongo et al. 2009).

It is generally considered that distribution of *T. parva* is primarily limited by the tick vector which is in turn determined by climate (Norval et al. 1992). Oura et al (2011b) performed a reverse line blot (RLB) survey of buffalo in the two northern Uganda National Parks, Murchison falls and Kidepo valley and, surprisingly no *T. parva* was detected using this method although the parasite was readily detectable in the south western Ugandan National parks. Recent work (Obara et al. 2020), confirms this result using the more sensitive nested p104 PCR assay. A team of Ugandan and ILRI scientists (including the ILRI tick unit manager), twice visited Kidepo and Murchison parks to sample ticks in buffalo adjacent areas and found that, although other expected livestock ticks were present, *R. appendiculatus* was absent. This is surprising because the both these parks superficially appear climatically and ecologically suitable for the tick. It does however parallel the result of the countrywide Cameroon tick survey (Silatsa et al. 2019), where *R. appendiculatus* was also absent. This data suggests that there may be some biotic factor constraining the distribution of the tick. On a practical note, these results also indicate that buffalo derived *T. parva* challenge of ITM vaccinated cattle in northern Uganda should not pose serious problems for ITM effectiveness.

On a different theme, but likely relevant to the success of ITM in North Tanzania, a study of vaccinated cattle and unvaccinated control cattle adjacent to Tarangire National Park (Kazungu et al. 2015) revealed an incremental increase in seropositivity, assessed by PIM ELISA, the closer cattle were to the park. *Theileria parva* prevalence using p104 nested PCR, was also higher closer to the park. This suggests that challenge by ticks that have fed on wildlife (including buffalo) can have a positive effect in boosting immunity.

2.13 Future focus areas for research relevant to ITM deployment and efficacy

Suggestions for priority future research include: (i) a comparative survey of *T. parva* prevalence and diversity in buffalo, ticks and cattle in Tanzania, (ii) a meta-analysis combining data from the major *T. parva* VNTR population genetics studies performed to date in order to gain a broader overall overview of parasite population genetics, (iii) establishment of whole genome sequencing as a tool for genotyping *T. parva* field isolates, (iv) application of population genomics using both deep sequencing of individual loci and whole genome sequencing to provide insight into parasite diversity, (v) application of population genomics would be particularly informative at sites adjacent to previous vaccine trials where baseline monitoring has already been performed, (vi) surveys for *T. parva* in additional countries that are potentially exposed to trans-boundary cattle movement from endemic (or transiently affected) countries. These might include, Central African Republic, South West Ethiopia, and Nigeria, (vii) more in depth analysis of Cameroonian *T. parva* parasites and cattle migration patterns to provide insight into the origin of *T. parva* in central Africa, (viii) analysis of the limits of distribution of *T. parva* infection in Cape buffalo in countries including South Sudan, Rwanda and Democratic Republic of Congo, (ix) sampling of carrier cattle to include lymph node biopsies to determine whether apparent loss of the carrier state based on PCR detection in blood is reflected in lymphoid tissues, (x) assessment of whether induction of a long term infection (carrier state) is required to sustain immunity against field challenge, and (xi) re-assessment of the possibility of replacing the trivalent cocktail with one of the component stocks. One issue this could affect positively would be ease of vaccine registration. Given the nature of the production of ITM stabilates, good manufacturing practice (GMP) as strictly defined, is almost impossible to achieve. However, a vaccine based on a single stock would potentially be easier to standardize between different production runs.

CHAPTER 3

Variant analysis of the sporozoite surface antigen gene reveals that asymptomatic cattle from wildlife-livestock interface areas in northern Tanzania harbour buffalo-derived *T. parva.*

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CHAPTER 4

Unique Mitochondrial Single Nucleotide Polymorphisms Demonstrate Resolution Potential to Discriminate *Theileria parva* Vaccine and Buffalo-Derived Strains

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4.1 Abstract

Distinct pathogenic and epidemiological features underlie different *Theileria parva* strains resulting in different clinical manifestations of East Coast Fever and Corridor Disease in susceptible cattle. Unclear delineation of these strains limits the control of these diseases in endemic areas. Hence, an accurate characterization of strains can improve the treatment and prevention approaches as well as investigate their origin. Here, we describe a set of single nucleotide polymorphisms (SNPs) based on 13 near-complete mitogenomes of *T. parva* strains originating from East and Southern Africa, including the live vaccine stock strains. We identified 11 SNPs that are non-preferentially distributed within the coding and non-coding regions, all of which are synonymous except for two within the *cytochrome b* gene of buffalo-derived strains. Our analysis ascertains haplotype-specific mutations that segregate the different vaccine and the buffalo-derived strains except *T. parva*-Muguga and Serengeti-transformed strains suggesting a shared lineage between the latter two vaccine strains. Phylogenetic analyses including the mitogenomes of other *Theileria* species: *T. annulata*, *T. taurotragi*, and *T. lestoquardi*, with the latter two sequenced in this study for the first time, were congruent with nuclear-encoded genes. Importantly, we describe seven *T. parva* haplotypes characterized by synonymous SNPs and parsimony-informative characters with the other three transforming species mitogenomes. We anticipate that tracking *T. parva* mitochondrial haplotypes from this study will provide insight into the parasite's epidemiological dynamics and underpin current control efforts.

4.2 Introduction

The protozoan parasite *Theileria parva* that causes East Coast fever (ECF) and Corridor Disease (CD) is considered among the most debilitating tick-borne pathogens in cattle over its endemic range in East, Central, and Southern Africa (Norval et al. 1992). In typical ECF symptoms, the disease severity is mainly due to the parasites' ability to transform host lymphocytes (Tretina et al. 2015). Parasitized lymphocytes proliferate uncontrollably and disseminate the dividing parasite into multiple host tissues. Their accumulation in the lungs triggers severe vasculitis, eventually resulting in respiratory failure with death occurring within three to four weeks of infection (Fry et al. 2016; Irvin and Mwamachi, 1983). With mortalities of up to 100% in susceptible animals, an estimated one million die per year from an estimated risk population of 28 million cattle mainly belonging to livestock farmers with economically constrained livelihoods (Gachohi et al. 2012).

Thus, control of the parasite is urgent to livelihood improvement efforts among resource-poor farmers in sub-Saharan Africa, as highlighted by the World Organization for Animal Health (OIE) (Grace et al. 2015). Current control methods include strict tick control measures to curtail pathogen transmission. However, this approach relies heavily on acaricide use, which is unsustainable in the long-run due to acaricide resistance challenges, and toxicity concerns in food and the environment (Abbas et al. 2014). Anti-theilerial chemotherapy is effective but only with early detection of the disease, which is impractical under field conditions (D'haese et al. 1999; Mbwambo et al. 2002).

Early observations that cattle acquire long-term immunity when challenged with infected ticks under a long-acting antibiotic treatment opened avenues for the development of an alternative control method based on live parasite stocks, which is called the infection and treatment method (ITM) (Neitz 1953; Radley et al. 1975). ITM consists of inoculating cattle with cryopreserved *T. parva* sporozoites combined with simultaneous treatment with long-acting oxytetracyclines (Morzaria et al. 2006). Early experiments revealed that there were varying cross-reactivities between geographical strains (Cunningham et al. 1974; Young et al. 1973). Due to this limitation, a cocktail of three immunizing parasite stocks known as the 'Muguga cocktail', comprising Serengeti-transformed, Kiambu 5, and Muguga strains, were combined to achieve broad protection against diverse field isolates (Radley et al. 1975). Several other strains have been immunologically profiled to identify an isolate that cross-reacts to diverse field strains in ECF endemic areas. Among the identified strains was a Marikebuni stock isolated from the Kenyan Coast that showed cross-protection against several eastern African strains and, a Boleni strain from Zimbabwe which, apart from a cross-reactivity against Eastern and Central African strains, induced mild infections, hence eliminating the need for antibiotic use in ITM protocol (Morzaria et al. 2006).

Historically, ECF is traced to have originated from East Africa and spread southwards, first being reported in present-day Zimbabwe and eventually into South Africa (Norval et al. 1992). Yet, it is notable that *T. parva* strains from different geographic regions have varying immunological profiles and epidemiological features. For example, an ability to induce a carrier state in which recovered animals remain infective to ticks has been demonstrated in some strains, enabling transmission between cattle by the vector tick, *Rhipicephalus appendiculatus* (Olds et al. 2018; Skilton et al. 2002b; Young et al. 1981). This persistence of vaccine strains raised initial concerns about spreading foreign parasite genotypes into endemic countries free of the vaccine parasite stocks, thereby possibly disrupting endemic stability (Nene et al. 2016).

By contrast, it is also known that some parasite strains, particularly of African buffalo (*Syncerus caffer*) origin, induce limited parasitosis and parasitemia, are non-persistent and not efficiently transmissible between cattle hosts (Jura and Losos, 1980). These strains are known to cause a more acute clinical syndrome called Corridor Disease in areas where susceptible cattle are exposed to vector ticks infected on buffalo, which are the primary mammalian carrier hosts (Bishop et al. 2015; Young et al. 1977). Based on its unique clinical presentation, which differs from classical ECF, these particular strains were initially recognized as *Theileria parva lawrencei* in earlier literature; however, this nomenclature was subsequently abolished with increasing molecular and antigenic data confirming similarities between the two strain populations (Allsopp et al. 1993; Maritim et al. 1992; Morrison et al. 2020). Further, these data have revealed that cattle transmissible strains are a separately maintained subset population of those found in buffalo, and to differentiate between the two populations, *T. parva* strains are arbitrarily considered to be either of buffalo or cattle-derived for epidemiological reasons (Pelle et al. 2011).

However, the genetic underpinnings of these strain differences are yet to be fully unravelled, and a precise delineation of the various genotypes is lacking (Morrison et al. 2020). This is partly because of the parasite's biology, which renders it technically unamenable for genomic studies, especially in obtaining pure parasite DNA free from host-DNA contamination (Palmateer et al. 2020). An accurate determination of the origin (buffalo or cattle derived) and geographic spread of strains will help intervention and control efforts. Additionally, accurate characterization of *T. parva* strains will help to track their frequency and distribution in specific populations, and to characterize breakthroughs in areas of live vaccine field deployments. Further, since *T. parva* has sexual reproductive phases that are associated with genetic recombination (Katzer et al. 2006), unravelling the parasite genotypes could enhance the understanding of the long-term effects of live vaccine components in the field.

Owing to limited or no recombination, uniparental inheritance patterns and a high substitution rate relative to nuclear genomes, mitochondrial genome studies on related apicomplexan parasites have provided clues of the geographical origin and variants of parasites (Joy et al. 2003; Schmedes et al. 2019). However, the utility of mitochondrial genomes in *T. parva* in delimiting the strains and their geographical origin remains unexplored. In this study, we sequenced the mitochondrial genomes of ten *T. parva* strains, found within the parasite's currently known endemic range, as well as some characterized isolates used as vaccine strains. We also included the mitogenomes of nine other *T. parva* isolates assembled from their whole-genome data that are publicly available (Hayashida et al. 2013). Further, this study assessed the divergence of *T. parva* from the closely related host-leukocyte transforming *Theileria* species; *T. annulata*, *T. taurotragi* and *T. lestoquardi* with an aim to identify phylogenetically informative mitochondrial characters.

4.3 Materials and Methods

4.3.1 Source of Isolates

The parasite material for the different strains consisted of infected frozen ground-up tick supernatants (GUTS), salivary glands (SG), cattle whole blood, or infected lymphocyte cell lines (**Table 4.1**). The GUTS and SG were collected from archived *T. parva*-infected *R. appendiculatus* specimens from early live vaccination projects. Parasite DNA from GUTS, SG, and cell culture sample sources was extracted using the NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany), whereas the NucleoSpin Blood Mini (Macherey-Nagel) was used to extract DNA from blood samples.

Table 4.1. Parasite material and origin of isolates used in the current study.

Strain/Isolate	Origin	Material Used	Year Created *	Reference
Serengeti-transformed)	Tanzania	GUTS	1981	(Young and Purnell 1973)
Boleni	Zimbabwe	GUTS	1980	(Lawrence and Mackenzie 1980)
Pugu I	Tanzania	Cell culture	1977	n.a
Lawrencei (Manyara)	Tanzania	GUTS	1980	(Schreuder et al. 1977)
Satinsyi	Rwanda	GUTS	1981	n.a
Kiambu	Kenya	Salivary glands	1980	(Irvin et al. 1974)
Marikebuni	Kenya	GUTS	1985	(Irvin et al. 1983)
Marula	Kenya	Blood	2000	(Bishop et al. 2015)
Muguga	Kenya	Salivary glands	1991	(Brocklesby et al. 1961)
Onderstepoort	South Africa	GUTS	1988	(Neitz 1948)
T. taurotragi	Tanzania	Blood	2003	(Catalano et al. 2015)
T. lestoquardi (Atbara)	Sudan	Cell culture	2001	(Bakheit et al. 2006)

* Indicates the year the material used for this study was created, which may differ from the time when the parasite was first isolated for some strains. n.a: not available.

4.3.2 Next-Generation Sequencing (NGS) *T. parva* Datasets

We additionally obtained publicly available whole-genome datasets of nine *T. parva* strains (DRR002439-46), downloaded in FASTQ from the NCBI (SRA accession number: DRA000613) for assembly of their mitogenomes sequences (Supplementary Table S4.1). The details of the parasite strains are described in a previous study (Hayashida et al. 2013). All NGS datasets comprised 36 nucleotide, single-end sequence runs performed on the Illumina GAII Analyzer (Hayashida et al. 2013).

4.3.3 Mitogenome Amplification and Sequencing

Primers were designed based on an alignment of *T. parva* and *T. annulata* mitogenomes available in the GenBank (Accession nos. AB499089 and NW_001091933, respectively). A

5808 bp fragment was amplified from all isolated DNA extracts (0.5–5 ng) in 25 µL reaction volumes comprising; 0.5 U of S7 Fusion polymerase (Biozym Scientific, Hessisch Oldendorf, Germany), 5× GC Phusion buffer (ThermoFisher Scientific GmbH, Darmstadt, Germany), 200 mM of dNTPs mix, and 0.5 µM of each primer (Supplementary Table 4.2). The cycling conditions were as follows: 98 °C for 30 s, followed by 44 cycles of 98 °C for 15 s, 60 °C for 25 s, and 72 °C for 4 min. The final extension step was maintained at 72 °C for 10 min. Amplification of expected ~5.8 kb fragments was confirmed on 1.5% agarose gels stained with GRGreen DNA stain (Excellgene, Monthey, Switzerland) under UV trans-illumination. The amplicons were purified using GeneJET PCR Purification Kit (ThermoFisher Scientific GmbH, Darmstadt, Germany) before cloning using the Strataclone blunt vector (Agilent Technologies, USA) under the manufacturer's instructions. The plasmid was purified using the GenUP™ Plasmid Kit (biotechrabbit GmbH, Berlin, Germany) and evaluated for targeted inserts based on the *Eco*RI digestion (ThermoFisher Scientific GmbH, Darmstadt, Germany). The plasmids were sequenced by Sanger technology using standard vector primers (LGC Genomics GmbH, Berlin, Germany) and 10 primers designed in this study to amplify overlapping regions of the mitogenome (Supplementary Table S4.2).

4.3.4 Assembly, Mapping, and Annotation

The Sanger generated sequences were assembled in Geneious prime 2020.2.3 (www.geneious.com) by creating consensus sequences from the approximately 1000 bp overlapping reads aligned to a reference mitogenome (GenBank Accession: AB499089), which is based on *T. parva* Muguga vaccine strain. Similarly, the Illumina NGS reads were mapped with reference to (AB499089) using the Geneious mapper under medium-low sensitivity with fine-tuning of at least five iterations. Consensus sequences were generated from contigs based on a threshold of at least 60% of the adjusted chromatogram quality of contributing bases, while ignoring reads mapped to multiple locations on the reference. The same GenBank reference was used to map and annotate protein-coding genes (PCGs) and the known rRNA genes based on nucleotide similarities.

4.3.5 Phylogenetic Analysis and Identification of Informative Single Nucleotide Polymorphisms (SNPs)

Mitogenome sequence alignments generated using MAFFT (Katoh and Standley, 2013) as well as concatenated alignments of *cox1* and *cob* sequences with additional GenBank retrieved sequences of non-transforming *Theileria* spp. and *Babesia* spp. were used to infer maximum-likelihood phylogenies. We selected best-fit models for nucleotide substitution based on the lowest Bayesian information (BIC) scores calculated using the jModel Test 2 program and tested nodal support with 100 bootstrap replicates (Darriba et al. 2012).

Phylogenetic trees were generated using PhyML implemented as a plugin within the Geneious software platform (Guindon et al. 2010).

To avoid the challenges of missing data due to incomplete read coverage of the Illumina assemblies, we used only the Sanger data to generate the multiple alignments used for the SNPs detection. We aligned the ten *T. parva* Sanger-generated mitogenome sequences together with three other host-transforming species; *T. taurotragi*, *T. lestoquardi* and *T. annulata* (retrieved from GenBank: NW_001091933). SNPs were identified in Geneious prime with reference to the *T. parva* Muguga GenBank AB499089 sequence under default settings, with analysis of the variants on protein translations based on Mold-Protozoan Mitochondrial genetic code. We determined informative SNPs for the 13 mitogenomes under the parsimony optimality criterion with equal weights for all characters using PAUP*4.0 software (Swofford, 2003).

4.3.6 *T. parva* Mitogenomes Haplotypes Definition and Network Analysis

Using a modified approach from (Hayashida et al. 2013), a second set of SNPs with consideration to the ten *T. parva* Sanger mitogenomes was extracted from the initial parsimony-informative SNPs. We used DnaSP v.6.12.03 on the second SNP data set to generate *T. parva* haplotype data (Rozas et al. 2017) and a median-joining (M-J) network was constructed using Network V. 10 software (https://www.fluxus-technology.com/) under default settings to examine relationships among the *T. parva* mitogenomes (Bandelt et al. 1999). Of the Illumina assembled mitogenomes, strains that had missing data with respect to the second SNP data set were excluded from the haplotype analysis.

4.4 Results

At least ten bidirectional overlapping Sanger reads were obtained for each strain, which were assembled into mitogenomes sequences ranging in size from 5800 to 5811 bp. The sequences are archived in NCBI's GenBank under accession numbers MW172707-MW172717; MW218514. The content and gene order for all ten *T. parva*, one *T. taurotragi*, and one *T. lestoquardi* mitogenomes were consistent with previous data, comprising three PCGs, fragmented rRNAs, and no tRNA (Hikosaka et al. 2013) (Figure 4.1).

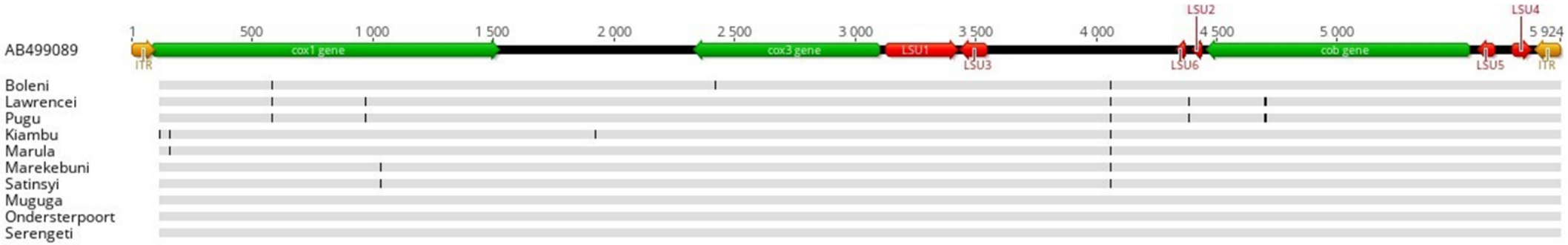

Figure 4.1. Linear map of *T. parva* mitochondrial genome and alignment showing the distribution of variants (SNPs) across the mitogenome sequences obtained by Sanger sequencing. *cox1* and *cox3*: *cytochrome oxidase* subunits; *cob*: *Cytochrome b*; LSU: large subunit; ITR: Inverted terminal repeat region; SNPs: single nucleotide polymorphisms; vertical markings indicate polymorphisms in respective nucleotide sequence relative to the reference sequence AB499089 above.

4.4.1 Divergence of *T. parva* from Other Host-Lymphocyte Transforming *Theileria* sp.

Due to length variations, we considered 5793 positions in the multiple alignment of the sanger-sequenced *T. parva* mitogenomes and the three additional host-lymphocyte transforming *Theileria* spp. Of the positions considered, there were 42 indels and 1036 SNPs. However, only 662 of the SNPs were parsimony informative across all mitogenome sequences. In terms of percentage identities of the PCG, *cob* was the most diverse gene, having 73.2–79.6% identity between *T. parva* Muguga strain and the three host-lymphocyte transforming *Theileria* (Figure 4.2). Phylogenetic analyses were congruent both using the whole mitogenomes and the concatenated gene sequences. In all instances, *T. annulata* and *T. lestoquardi* consistently formed an outgroup clade to *T. parva* and *T. taurotragi* (Figure 4.3; Supplementary Figure S4.1).

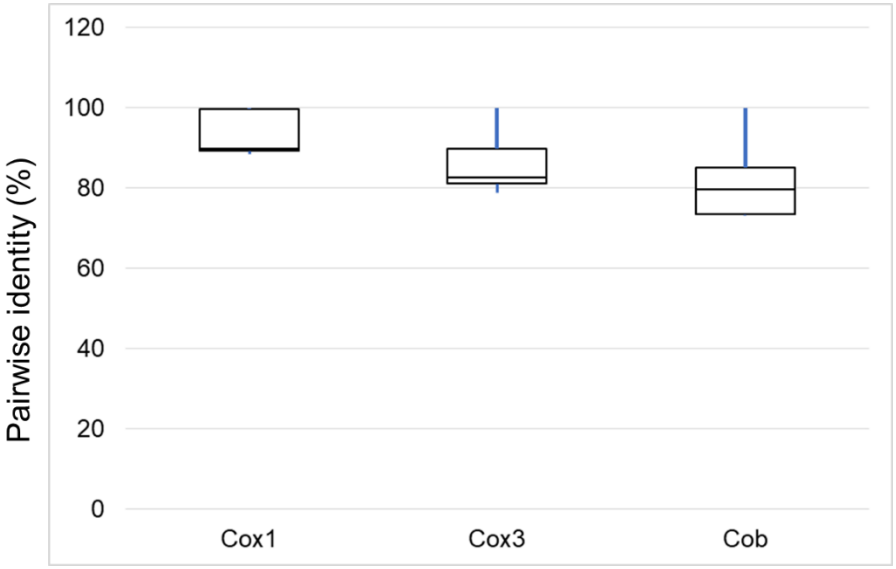

Figure 4.2. Percentage pairwise identity of the three protein-coding genes across the 14 mitogenomes analyzed in this study. The 25th and 75th percentiles are represented by the box limits; lines across the boxes indicate the median; whiskers extend to the maximum and minimum (%) identity values.

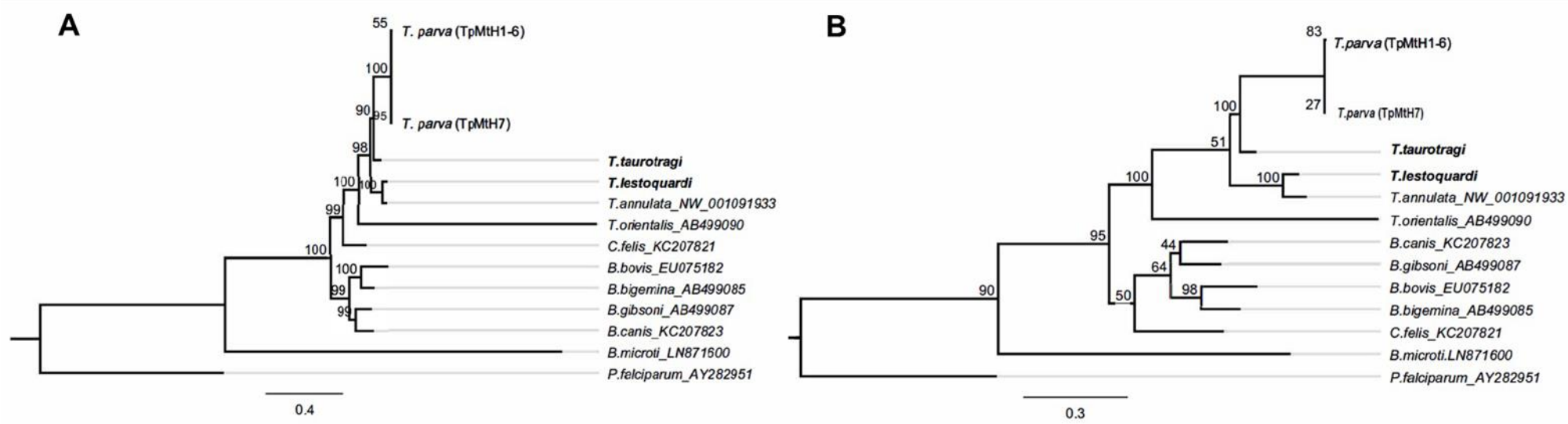

Figure 4.3. Maximum likelihood phylogeny based on (**A**) near-complete whole mitogenome sequences (~5.8 kb) and (**B**) *cob* sequences (~1.1 kb). The nucleotide substitution models for the tree constructions as determined by the lowest Bayesian information (BIC) values were TVM + G and GTR + G, respectively. Bootstrap values are based on 100 replicates. Sequences from this study are in bold.

4.4.2 *T. parva* Haplotype Analysis

We used the extracted second set of SNPs, which comprised nine informative SNPs for *T. parva* haplotype analysis. As previously noted, we included three Illumina assembled mitogenomes (Nyakizu from Rwanda, MandaliZ22H10, and Buffalo Z5E5 from Zambia) that had data on all the determined nine informative SNPs, irrespective of the other missing regions lacking reads coverage. In total, 13 *T. parva* strains were considered for the haplotype analysis. The SNPs segregated the *T. parva* strains used into seven haplotypes, which we have identified in this study by assigning the TpmtH prefix numerically beginning with Muguga as a reference sequence (Figure 4.4). The Muguga strain isolate was assigned into one haplotype identical to Onderstepoort and Serengeti isolates (TpMtH1). Similarly, *T. parva lawrencei* (Manyara) isolated from an African buffalo, and Pugu I, both from Tanzania, together with a Zambian buffalo isolate (Buffalo Z5E5), formed one haplotype (TpMtH7) that was characterized by two SNPs within both the *cox* 1 and *cob* genes. We presumed Pugu I to have originated from buffalo *T. parva* based on analysis of its sporozoite surface (p67) antigen gene, which showed that it lacked the typical 129 bp deletion that is present in cattle transmissible *T. parva* (Nene et al. 2006; Obara et al. 2015). The p67 sequence generated (See supplementary information 4SI for primer information and cycling conditions) for the Pugu I isolate is archived under accession no MW183674 in the GenBank.

Further, the Marikebuni strain originating from the Kenyan coast, Mandali from Zambia, and Satinsyi strain from Rwanda formed one haplotype (TpMtH3) characterized by two SNP transitions relative to *T. parva* Muguga (Table 4.2; Figure 4.4). The Marula and Kiambu-V isolates formed independent haplotypes (TpMtH4 and 5), but differed with one SNP position (119) between them (Figure 4.5).

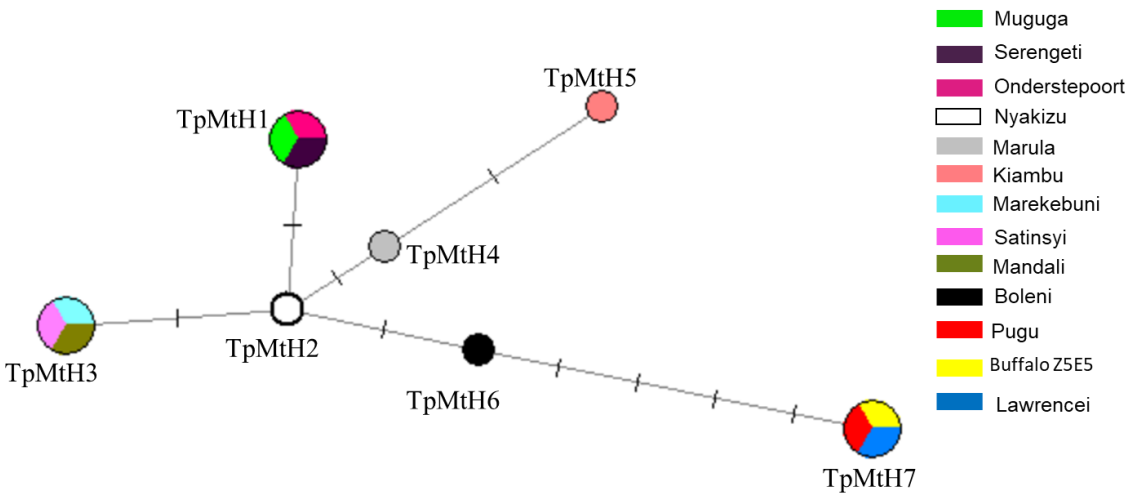

Figure 4.4. A median-joining (MJ) parsimony network for the 13 *T. parva* haplotype mitogenome sequences. Node labels TpMtH1-7 represents the unique haplotypes. Lines between nodes indicate mutation points. Larger and fractionated nodes indicate shared haplotypes with multiple strains, each marked with a different color key, as shown.

The Boleni isolate (TpMtH6) possessed a transversion mutation within the *cox1* gene (SNP 584). This transversion mutation was also notable within the buffalo haplotype (TpMtH7). Interestingly, all haplotypes deviate from TpMtH1 by a transition (A→G) at SNP position 4060, which lies in a currently functionally unknown region, but appears to be ancestral in the other transforming *Theileria* (Table 4.2; Figure 4.5). This transition is the single defining SNP of the Nyakizu (Rwanda) strain from Muguga, and makes TmMtH2 a central node from which all other haplotypes deviate. However, there was no apparent differentiation by geographic origin as the M-J network nodes associated with multiple haplotypes clustered isolates of diverse origin (Figure 4.4).

Table 4.2. SNPs among the seven haplotypes based on the GenBank reference AB499089. The reference sequence matches haplotype TpMtH1 in the present study.

Haplotype	Gene	Variant Type	Change	Codon Change	Codon Position	AA Change	Protein Effect
	Cox1						
TpMtH3		Transition	C→T	GCC→GCT	951		
TpMtH4		Transition	C→T	CTG→TTG	76		
TpMtH5		Transition	G→A	GTG→GTA	36		
		Transition	C→T	CTG→TTG	76		
TpMtH6		Transversion	A→C	GTA→GTC	501		
TpMtH7		Transversion	A→C	GTA→GTC	501		
		Transition	C→T	TAC→TAT	891		
	Cox3						
TpMtH6		Transition	T→C	CAA→CAG	555		
	Cob						
TpMtH7		Transition	A→G	GTT→GCT	848	V→A	Substitution
		Transition	A→G	GTA→GCA	851	V→A	Substitution
	Intergenic				**SNP position**		
TpMtH2		Transition	A→G		4060		
TpMtH3		Transition	A→G		4060		
TpMtH4		Transition	A→G		4060		
TpMtH5		Transition	T→C		1924		
		Transition	A→G		4060		
TpMtH6		Transition	A→G		4060		
TpMtH7		Transition	A→G		4060		
		Transversion	T→A		4382		

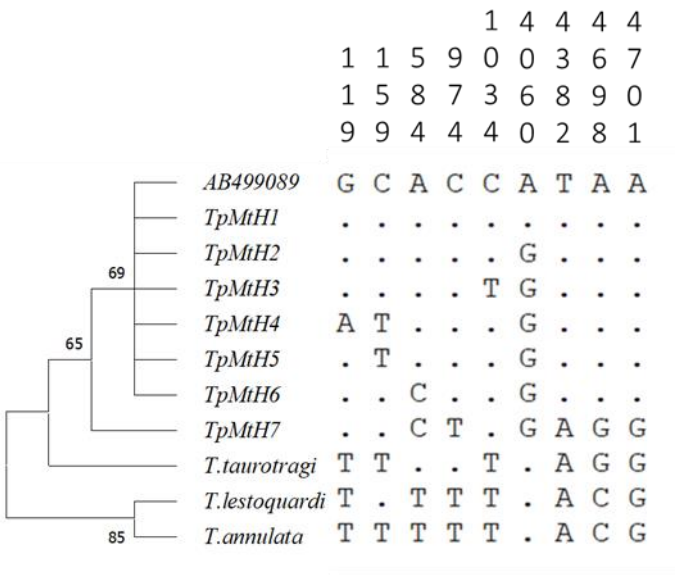

Figure 4.5. Phylogenetic grouping of the seven *T. parva* haplotypes identified in this study. The neighbor-joining tree is constructed based on the Jukes–Cantor Model using 9 *T. parva* only SNPs out of 662 informative SNPs extracted from 13-mitogenome sequences alignment that included; *T. taurotragi, T. annulata,* and *T. lestoquardi*. Numbers behind the nodes indicate bootstrap values based on 1000 replicates. The positions are relative to the AB499089 *T. parva* Muguga mitogenome sequence. Orthologous positions in the three other transforming *Theileria* are shown for comparison.

4.4.3 Intraspecific Divergence among *T. parva* Strains

Relative to the GenBank reference AB499089, an alignment of 10 Sanger-sequenced *T. parva* mitogenomes showed variation at 11 sites, all of which were SNPs with no indels observed (Figure 4.1). Of the 11 SNPs, only three were found within the intergenic region and involved one transversion within the haplotype associated with buffalo *T. parva.* In total, there were three transversion SNPs positions, two of which were observed within the *cob* gene of the buffalo-associated haplotype. Among the genes, *cox3* was most conserved with only a single SNPs position within the Boleni mitogenome, while the *cox1* gene had five mutated positions, all of which were synonymous. The remaining two SNPs that were found within the *cob* gene sequences were non-synonymous and resulted in two contiguous amino acid substitutions in their predicted proteins (Table 4.2). Both substitutions involved the valine codon, which was replaced by an alanine amino acid codon. Notably, these substitutions were only in the haplotype associated with the buffalo *T. parva* isolates.

4.5 Discussion

In this study, we describe promising mitogenome-based SNPs that demonstrate precision and convenience in characterizing *T. parva* strains. Previously identified nuclear-based markers mainly based on a panel of mini- and micro-satellites are sometimes biased due to selective amplification of predominant strain clonotypes during passages through cattle and ticks (Katzer et al. 2006; Oura et al. 2003). In addition, since the design of the initial markers relied on the genome of *T. parva* Muguga stock, some markers are possibly biased in detecting diversity within this stock (Norling et al. 2015). Although whole-genome SNPs analysis has been demonstrated to have high discriminatory power in typing vaccine strains, it is yet to find field applications (Bishop et al. 2020; Norling et al. 2015). Additionally, obtaining pure parasite DNA for whole-genome sequencing, especially for buffalo-derived *T. parva* is a hurdle due to its biology and may be complicated in the field where the parasite exists as a mixed diverse population (Katzer et al. 2006; Morrison et al. 2020; Pelle et al. 2011).

Mitochondrial genomes and their individual genes have been extensively used to study phylogeny and applied in species identification and delimitation across broad taxonomic levels (Bernt et al. 2013). The majority of apicomplexan mitochondrial genomes that have been sequenced to date exhibit an extreme size reduction, containing at most three protein genes (*cox1, cox3,* and *cob*) and fragmented rRNA genes (Hikosaka et al. 2013). The extreme mitogenomes size reduction and a faster coalescence make mtDNA attractive to study differentiated *T. parva* population strains. Indeed, our analysis revealed haplotype-defining SNPs within the *T. parva* mitogenomes, which are parsimonious with other host-leukocytes transforming *Theileria* species. Based on median joining (MJ) parsimony analysis, the *T. parva* mtDNA sequences generated were segregated into an unambiguous network, congruent with the existence of multiple linked lineages. With respect to the *T. parva* Muguga isolate haplotype, each haplogroup was defined by synonymous nucleotide changes, except for non-synonymous changes leading to amino acid substitutions within the *cob* of the buffalo-associated strains; *T. parva lawrencei*, *T. parva* Buffalo Z5E5, and one field isolate, *T. parva* Pugu I.

Our analysis identifies *T. parva* Muguga and Serengeti-transformed as belonging to the same haplotype characterized by nine defining SNPs positions from the strains used in this study. This is not surprising as previous studies have demonstrated a similar monoclonal antibody profile and conservation on their known *T. parva* antigen coding genes (Bishop et al. 2001; Norling et al. 2015). Further, the two strains are strikingly similar at the whole genome level, with only 420 non-synonymous substitutions in Serengeti-transformed relative to the Muguga reference genome reported (Norling et al. 2015). These substitutions occur in a paltry 53 genes

(out of over 4000 *T. parva* genes) mainly within polymorphic multicopy gene families and ATP-binding cassette transporter genes located in subtelomeric ends (Norling et al. 2015). With the almost similar identity of the two strains, our results, in addition to previous studies, question the necessity of both Muguga and Serengeti-transformed in the trivalent cocktail instead of a divalent cocktail containing either of the two and Kiambu-V. Interestingly, both Muguga and Serengeti-transformed *T. parva* strains also shared the same haplotype with a historical isolate *T. parva* Onderstepoort, a laboratory maintained stock isolated in 1937 on the farm Schoonspruit in the Transvaal, South Africa prior to ECF eradication in this country (Neitz 1948, 1957; Norval et al. 1992). Earlier analyses on three *T. parva* antigen proteins; the Polymorphic immunodominant molecule (*PIM*), sporozoite surface protein (*p67*), and *p104*, have shown that these nuclear-encoded antigen genes are, in fact, identical to those of the Muguga parasite (Norling et al. 2015; Sibeko et al. 2011). It is thus conceivable that the ECF-causing strains derive from a common lineage that can be inferred at the mitochondrial genome.

An important finding of this study is the clustering of buffalo-derived *T. parva* strains under one haplotype (TpMtH7) with the same nine SNPs. It is noteworthy that the buffalo strains used in this study originate from two different countries (Zambia and Tanzania), while the field isolate (Pugu I) was isolated during vaccine field trials in Tanzania. And although a Kenyan Buffalo (*T. parva* LAWR) from the NGS assembly was not included in the haplotype analysis due to missing data on SNP position (159), all its other SNPs positions also matched haplotype (TpMtH7) (data not shown). The buffalo has long been recognized as the natural reservoir of *T. parva*. The *T. parva* strains maintained in cattle are considered a subset population from that maintained in buffalo (Morrison et al. 2020; Pelle et al. 2011). However, there has not been a definitive genetic basis to differentiate what constitutes a buffalo-derived *T. parva* and a cattle-derived *T. parva* or whether their designation as a single species is justified (Morrison et al. 2020; Sibeko et al. 2011). The available approach of their differentiation based on the *p67* alleles only provides a preliminary indication of presumptive exposure of cattle to buffalo *T. parva* based on alleles-2, 3, and 4, which are considered highly probable to be of buffalo origin in contrast to allele-1 that is found in cattle transmitted *T. parva,* but does not necessarily preclude its presence in buffalo (Nene et al. 1996; Obara et al. 2015; Sitt et al. 2019). Our analysis suggests strain defining mitochondrial SNPs that are potential markers for buffalo-derived *T. parva* lineages.

Noticeably, the Boleni strain formed a separate haplotype (TpMt6) that shared a transversion mutation within the *cox 1* gene with the buffalo haplotype. This strain was isolated from Zimbabwe from a farm that had experienced a severe theileriosis outbreak in January 1978 (Lawrence and Mackenzie, 1980). Under the now obsolete trinomial nomenclature of *T. parva,* it was named *Theileria parva bovis,* which was associated to what was referred to as January

disease (Uilenberg et al. 1982). The delineation of this strain from our data is thus a significant find as it agrees with the epidemiological distinctions that have been apparent from earlier investigations on theilerioses caused by *T. parva.* Further, our analysis identifies the Kenyan-Marekebuni, Zambian-Mandali, and Rwandese Satinsyi strains as one haplotype (TpMtH3). Although the shared haplotypes from widely separated regions may suggest a lack of geographical differentiation of the haplotypes, our observations could also be because of a limited sample size as well as through spread by carrier animals.

A high level of interspecies divergence among the transforming *Theileria* is observed that is characterized by up to 42 indels with respect to *T. parva* Muguga. However*,* a limited polymorphism is observed amongst the 13 *T. parva* mitogenomes analyzed, which is also observed in other apicomplexan species such as *Plasmodium falciparum* (Preston et al. 2014)*.* Of the eleven *T. parva* SNPs observed, only nine were informative. We modestly suppose this may be convenient compared to whole-genome-based SNPs in which up to >120,000 SNPs have been observed in buffalo strains alone (Hayashida et al. 2013). Additionally, we think our approach to defining SNPs that are foremost parsimonious with other leukocyte transforming *Theileria* provides initial indications on the potential of the identified SNPs to be informative for typing of recently diverged field *T. parva* strains from common leukocyte transforming ancestor. Nonetheless, further investigation to test the utility of the SNPs is necessary with a larger field population across the *T. parva* endemic range, especially in wildlife-livestock areas where 'breakthrough' infections against the trivalent live vaccine are known to occur.

The phylogenetic analysis of both the full-length near-complete mitochondrial genomes and the concatenated *cox1* and *cob* genes place *T. parva* and *T. taurotragi* in one clade, consistent with previous analyses using nuclear genes such as the 18S RNA gene. The same phylogenetic tree topology is maintained with the sporozoite surface protein gene and its orthologues in respective leukocyte host-transforming species (Sivakumar et al. 2014). Thus, the mitogenomes data's observed congruency with nuclear-based data rules out possibilities of inheritance patterns specific to mitochondria in our analysis.

Our data indicate *T. annulata* and *T. lestoquardi* form an outgroup clade among the transforming parasites, reflecting an allopatric speciation separation from *T. parva* and *T. taurotragi,* and conforms to their currently known demography. Noticeably, *T. taurotragi* was initially described as a parasite of the eland (*Taurotragus oryx*) (Brocklesby and Martin, 1960), but is also reported to cause infections in cattle in the known endemic range (Eastern, Central, and Southern Africa) of *T. parva* and its tick vector *R. appendiculatus,* alongside other tick vectors (Catalano et al. 2015). As such, co-infections of *T. taurotragi* and *T. parva* are, in fact, frequently common (Njiiri et al. 2015). While the pathogenicity of *T. taurotragi* in cattle is not

clearly understood, it has been shown to transform a wide range of host cells in in vitro studies (Stagg et al. 1983), and has been associated with cases of cerebral theileriosis (BCT) (Biasibetti et al. 2016; Catalano et al. 2015).

Similarly sympatric, *T. annulata* and *T. lestoquardi*, occur within the same currently known endemic range (N. Africa, S. Asia, and S. Europe) and are transmitted by ticks belonging to *Hyalomma* genus. Both are important parasites responsible for heavy economic losses and have an intertwined epidemiology that poses interpretation challenges in their overlap in affected countries (Brown et al. 1998). *Theileria lestoquardi* is a parasite of small ungulates and causes malignant ovine theileriosis, while *T. annulata* causes bovine tropical theileriosis but also co-infects with the former in sheep (Al-Hamidhi et al. 2016; Bishop et al. 2004; Brown et al. 1998).

In conclusion, this study catalogs SNPs based on mitogenomes of characterized *T. parva* strains and vaccine stocks that can facilitate their tracking in the field. We identify haplotypes defined by SNPs that are initially parsimonious among transforming *Theileria; T. parva, T. annulata*, *T. taurotragi,* and *T. lestoquardi* mitogenomes, the latter two reported herein for the first time. We anticipate that the knowledge of the circulating haplotypes with reference to the live vaccine strains haplotypes will be insightful in characterizing *T. parva* epidemiology with important implications for control, and have a predictive value on the success of live vaccine deployments besides characterization of breakthrough infections.

CHAPTER 5

General Discussion, Conclusion and Recommendations

The tick-transmitted *Theileria parva* infection severely affects animal health in Sub-Saharan Africa with direct devastating economic impacts on livestock-dependent poor farmers' livelihood. As such, control of *T. parva* infections will potentially promote sustainable development in Sub-Saharan Africa's burdened livestock industry, thereby reducing poverty in the region (Spielman 2009). Existing control methods such as vector control, which, as previously highlighted in Chapter 1, are expensive and unsustainable because of overreliance on acaricides. Additionally, the wild Cape buffalo poses control challenges as buffaloes — the reservoir host, freely range within the eastern Africa endemic area (Walker et al. 2014). Thus, wildlife conservation efforts and livestock production are often brought into conflict. With the buffalo being a constant source of both the vector ticks and *T. parva* parasites, the prevalent ECF management approaches become impractical (Walker et al. 2014).

Therefore, innovative approaches are necessary to control and prevent *T. parva* infections, especially in areas where buffaloes and cattle share pastures. The success of such approaches will additionally depend on a proper epidemiological understanding of the parasite population dynamics and diversity while taking into account the role of its wildlife reservoir host (Perry and Young, 1995). While increasing molecular data reveals the heterogeneity of the parasite population in the field, most of the available data largely originates in areas where the buffalo is absent. Infection 'breakthroughs' against the ITM live vaccine — the only currently known approach of inducing solid immunity in cattle against *T. parva* infections necessitates further genotypic studies in cattle-buffalo co-grazing areas (Bishop et al. 2020; Morrison et al. 2020).

The studies described in this thesis highlight two critical aspects towards control of *T. parva* infections: (i) the dearth of data on the parasite's population structure in areas co-grazed by cattle and buffalo (Bishop et al. 2020; Morrison et al. 2020), and (ii) the lack of tools to definitively distinguish buffalo- and cattle-derived *T. parva* (Morrison et al. 2020; Sibeko et al. 2011). Specifically, the data discussed in Chapter 3 addresses the challenge of limited data in cattle and wild buffalo co-grazing areas by examining *p67* and *Tp2 T. parva* gene pools. In the attempt to find out the disparity in the efficacy of the live vaccine between Kenya and Tanzania wildlife–livestock interface areas, the observation of a buffalo type *T. parva* allele - 4 in Tanzania, which has been rare in the studies conducted so far within the East African *T. parva* endemic region is among the important findings in this study (Nene et al. 1996; Obara et al. 2015; Sibeko et al. 2010; Sitt et al. 2019). Thus, it can be inferred that, whereas the live vaccine can protect against buffalo-derived *T. parva*, this may depend on the parasite infecting genotype. This finding is also relevant for the current drive towards the development of more efficacious sub-unit vaccines.

In contrast to the previously known complete conservation of the sporozoite surface protein antigen among cattle-derived *T. parva* – frequently associated with *p67* allele-1 (Nene et al. 1996), the observation of epitope polymorphisms in a strain from Kenya requires further investigations to determine the existence of more genotypes and their impacts on vaccine efficacy. Similar and further allele variations have also been recently reported in p67 allele-1 from South Africa (Mukolwe et al. 2020). As described in Chapter 3, low levels of immunity have previously been achieved in field studies relative to laboratory data in cattle vaccinated using either sporozoite needle challenges or recombinant *p67* (Nene et al. 2016). Whereas the reasons for this discrepancy are unclear, *p67* allelic and epitope variations in the field may partly explain this. The current efforts towards recombinant vaccines development based on the p67 gene should take into account such variations.

The ITM, as a live vaccine, does not prevent the entry of the parasite but rather primes the immune system and establishes an artificially induced carrier state. The generation of a carrier state has been a critical concern as vaccinated animals remain a source of infection to ticks (Nene and Morrison, 2016). As critical epidemiological questions including both short- and long-term ITM vaccine impacts in the field remain unclear, the introduction of foreign genotypes into areas they are hitherto absent has been a concern (Bishop et al. 2020; Nene et al. 2016). Ideally, an evaluation of strain diversity in an area to determine the most appropriate vaccine cocktail needs to done before adopting a live vaccine (Salih et al. 2017). This will help the prediction of target populations for vaccines, especially in wildlife-livestock areas. Moreover, strain evaluation would be useful in tracking variants and establishing a baseline upon which the impact of the live vaccine deployment on both the short- and long-term can be evaluated in an area (Nene et al. 2016).

The development of strain typing tools with a finer resolution to monitor and account for all evolving strains in a particular endemic region would be an important achievement towards this aim. As such, the data presented in the fourth chapter represents an important contribution with significant promise towards a genetic basis of differentiating *T. parva* strains. By defining haplotypes based on nine mitogenome SNPs with reference to other cell-transforming *Theileria* spp., buffalo-derived strains were clustered under one haplotype. Moreover, the Boleni strain previously designated as *T. parva bovis* in the now obsolete trinomial nomenclature was separated into its own haplotype. The separation of the strains in this study, consistent with earlier distinctions based on epidemiology and clinical manifestations, demonstrates the markers' potential utility for the differentiation of *T. parva* strains.

Further, the supposedly buffalo-derived Serengeti-transformed was found identical with the Muguga strain, which agrees with previous studies using whole-genome sequencing and

antibody profile studies (Bishop et al. 2001; Norling et al. 2015). Based on reports of vaccine breakthrough against the Muguga cocktail (Sitt et al. 2015), it can be concluded that the Serengeti-transformed strain that was isolated from buffalo and adapted to cattle by serial passages strain is not representative of buffalo-derived isolates in the cocktail. This finding reveals the possible usefulness of the haplotypes from this study in identifying suitable strains from the heterogeneous buffalo-derived *T. parva* population.

To improve the efficacy of the current Muguga cocktail vaccine, the inclusion of real buffalo-derived strains would be advantageous. However, the poor transmissibility of buffalo-derived strains in cattle is likely to impede its production. Moreover, the choice of a representative buffalo-derived isolate from the heterogeneous *T. parva* population may be an insurmountable task (Morrison et al. 2020). It has been suggested that determining the genetic basis of transmissibility in cattle could identify buffalo-derived *T. parva*-parasites that could potentially adapt to transmission between cattle (Morrison et al. 2020). Such an effort could be profitable in predicting a possible vaccine failure, identifying genotype strains from buffalo that can be included in a live vaccine or informing the development of a recombinant vaccine. Conclusively, the data presented herein is a significant step towards such an effort with important practical implications for targeted control of *T. parva* infections.

Future outlook and Recommendations

In retrospect, it is noteworthy that ECF was eliminated in South Africa, oblivious of the existence of *T. parva* strains which cause Corridor disease that persists to date. Therefore, targeted control for the different *T. parva* strains appears to be possible, and may be a practical approach given their different dynamics. With the current trends in climate change and transboundary movements of cattle potentially driving *R. appendiculatus* ecological range expansion, the emergence of *T. parva* into new areas is likely to increase along with its tick vector expansion. Knowledge of *T. parva* strains diversity and its field population dynamics is, therefore, key to targeted control of East Coast Fever (ECF) and the related *T. parva* infections.

The implication of the potential role of the infecting parasite genotype in affecting the efficacy of ITM in cattle as suggested by the data in Chapter 3, dictates a need for comprehensive investigations with an extended sample size across livestock-wildlife interface areas. That the infecting parasite genotype may potentially influence ITM efficacy conforms to the fact that different strains have different clinical manifestations in cattle. This argument contrasts the view that the parasite undergoes a 'behavioural transformation' when transmitted from buffalo followed by a series of passages in cattle (Maritim et al. 1992; Morrison et al. 2020). The mitochondrial genetic data as described in Chapter 4 suggests the existence of a genetic basis upon which the different clinical manifestation of different strains are derived. Accordingly, the

subject of cattle-derived and buffalo-derived strains needs to be re-examined to possibly arrive at a definitive basis of *T. parva* strains description.

Mutations in the *cytochrome b* gene have previously been linked to *T. annulata* resistance against the Buparvaquone drug (Mhadhbi et al. 2015). Understanding the functional implications of the two *cytochrome b* gene nonsynonymous SNPs identified in buffalo-derived strains would be interesting. More relevantly would be to understand the molecular mechanisms underlying the peculiarities of different *T. parva* strains transmission dynamics, and development of the variant clinical disease manifestations. Compared to other Apicomplexan parasites such as *Plasmodium spp*., the molecular mechanisms underlying the transmission of *T. parva* are yet to receive considerable attention. However, with renewed interest, the current rapidly advancing technological tools and resources can be exploited towards understanding the intricacies of the *T. parva* parasite biology. Such advances include:

i. Artificial tick feeding techniques (Kröber and Guerin 2007), and the ability to establish *in vitro* cell lines enriched for parasite-specific T/B cells in the laboratory (Brown 1987), open the possibilities of studying the complete *T. parva* life cycle *in vitro.* Such possibilities will be helpful in functional studies to elucidate the parasite's biology while eliminating the need for experimental animals.
ii. Emerging genome editing techniques, such as CRISPR/Cas9 and CRISPR-free base-editing systems such as Base Editors (BE) and Prime Editors (PE) (Abdullah et al. 2020), may make it possible in the future to edit both nuclear and mitochondrial genomes of *T. parva* for genomic studies with implications for parasite control. Such advances have already been used for other Apicomplexan parasites, including *Plasmodium* and *Toxoplasma* with significant success (Kim 2016; Suarez et al. 2017).

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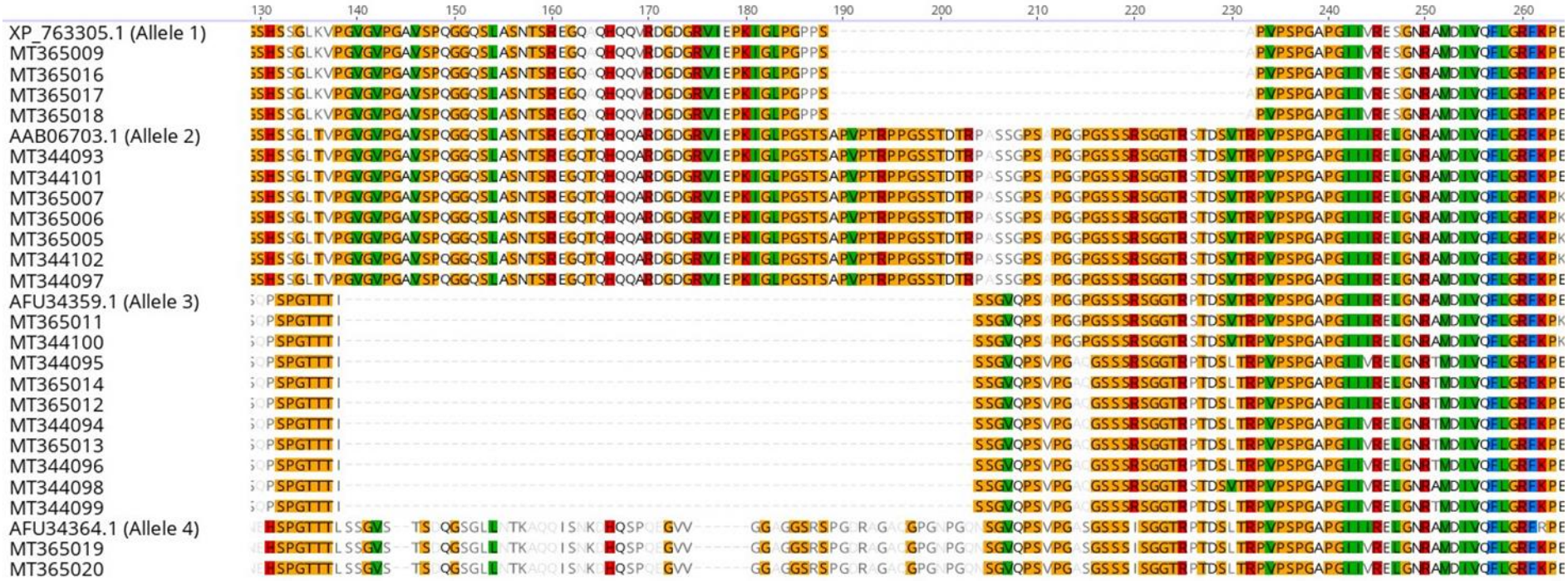
Appendices



Supplementary Figure S3.1 P67 multiple sequence alignment used to generate the classification of the alleles identified in this study

Supplementary Table S3.5 Table Pairwise identity matrix for the unique Tp2 sequences in this study

Cattle_TZ	Cattle_KE	Buffalo_TZ
94.44	56.36	78.74
92.68	57.14	78.16
98.18	56.55	65.52
	56.02	60.92
	78.18	97.13
	77.58	63.22
	78.18	60.92
	97.62	63.79
	98.19	61.49
	98.8	67.24

Supplementary Table S4.1. Summary of NGS reads (SRA accession number: DRA000613) mapped to *T. parva* muguga-mitochondrial sequence (AB499089)

Strain	Accession no.	Origin	Total reads downloaded	Mapped reads	Read coverage
Buffalo Z5E5	DRR002446	Zambia	14,821,055	2,627	96.4%
Nyakizu	DRR002443	Rwanda	31,095,446	2,574	98.9%
Buffalo LAWR	DRR002445	Kenya	17,072,361	2,021	95.0%
Entebbe	DRR002442	Uganda	10,171,313	1,826	96.8%
ChitongoZ2	DRR002438	Zambia	14,405,286	1,198	94.4%
MandaliZ22H10	DRR002441	Zambia	16,362,288	591	91.1%
Katumba	DRR002444	Tanzania	35,406,726	463	85.3%
KiambuZ464/C12	DRR002440	Kenya	15,848,448	428	84.1%
KateteB2	DRR002439	Zambia	16,558,766	426	81.1%

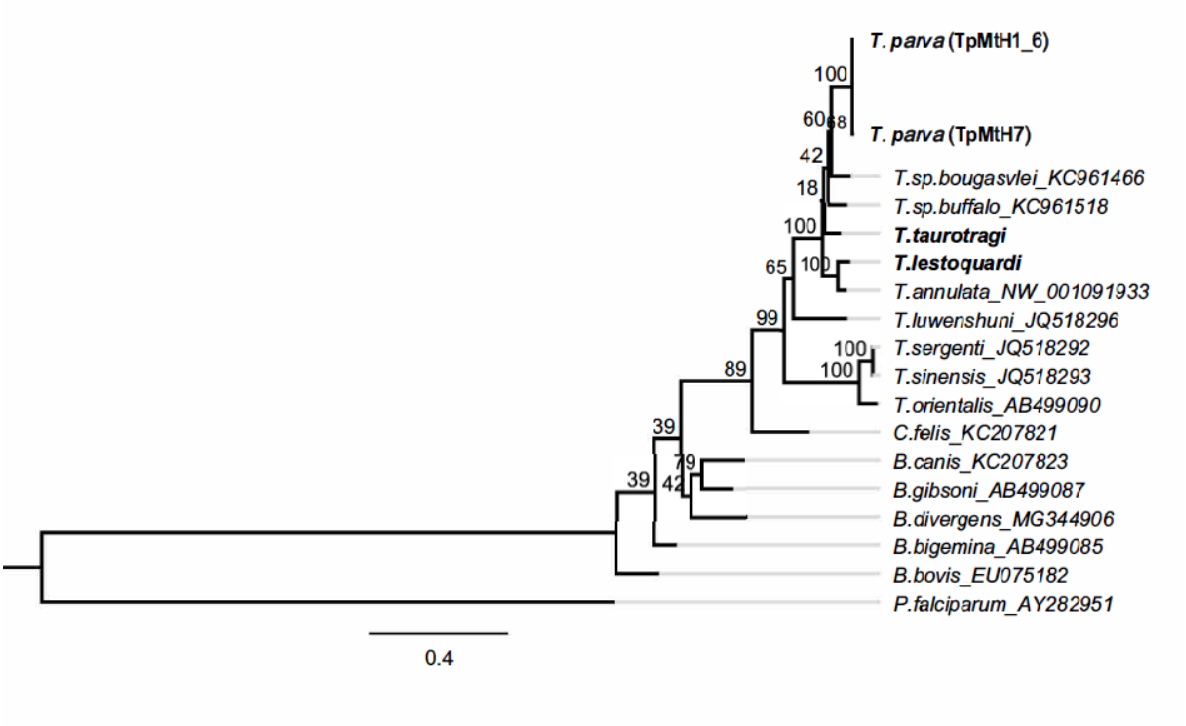
Supplementary Table S4.2 Amplification and sequencing primer sequences

Reaction	Primer name	Sequence
Amplification	Tp_mit_F79	5'-GTGTCAGGAAATCATAAAATTATTGG-'3
	Tp_mit_R5885	5'-TGAGTAAGAATAATGATACTCAAATATATGTCG-'3
Sanger sequencing		
	Tp_mit_F118	5'-GTGGCTGGCTTATTGGTTCG-'3
	Tp_mit_R965	5'-GCGAGTATCTGCTTCCAAACC-'3
	Tp_mit_F905	5'-GTTAGGTTGTTTGGTTTGGGGAC-'3
	Tp_mit_R5610	5'-TTTAGTGAAGGAACTTGACAGGTACA -'3
	Tp_mit_R2080	5'-TTTGAACACACTGCTCGACAC-'3
	Tp_mit_R1379	5'-CAGGATAATCTGGTATTCTTCTTGG-'3
	Tp_mit_F1863	5'-CAAGGTAGTTGACAGTGAACTTGTAGC-'3
	Tp_mit_F3056	5'-CGCTGTTTCGCATTTGACTAC-'3
	Tp_mit_R4186	5'-TTCTTTGCCTTGGATGTCAGTTAG-'3
	Tp_mit_F3659	5'-CAATCCTTATGTATGCTTGAATGCTG-'3
	M13-24F- Blue*	5'-GTAAAACGACGGCCAGTGAGCGCG-'3
	M13-24R- Blue*	5'-AACAGCTATGACCATGATTACGCC-'3

* pSC – B – amp/kan PCR cloning vector standard sequencing primers

Supplementary information 4SI:

The pugu *p67* gene was amplified by PCR in a 25 µL reaction using published primers [48]. Briefly, the amplification reaction contained; 5× High-Fidelity (HF) Phusion buffer (ThermoFisher Scientific), 200 µM dNTPs (Biozym Scientific GmbH), 0.5 µM forward primer (IL 6133: 5′- ACAAACACAATCCCAAGTTC-3′), 0.5 µM reverse primers; IL 7922: 5′-CCTTTACTACGTTGGCG-3′), 0.02 U/µL HF Phusion DNA polymerase, sterile PCR-grade water (Carl Roth GmbH, Karlsruhe), and 2 µL of template DNA. The amplification cycle was set as follows; 98 °C for 30 s, followed by 35 cycles of 98 °C for 30 s, an annealing step at 58 °C for 40 s, and elongation at 72 °C for 1 min. The final extension step was 10 min at 72 °C. The p67 amplicon was purified using GeneJET PCR Purification Kit (ThermoFisher Scientific), and sequenced using Sanger technology (LGC Genomics, Berlin). The obtained sequence was translated and evaluated for the presence/absence of the 43 amino-acid deletions used to categorize cattle and buffalo derived p67 sequences.



Supplementary Figure 4S1. Maximum likelihood phylogeny based on *cox1* gene sequences (~1.4 kb). The nucleotide substitution model for the tree constructions as determined by the lowest BIC values was GTR + I + G. Bootstrap values are based on 100 replicates. Sequences from this study are in bold.

Summary

Delimiting *Theileria parva* strain diversity towards targeted and unified approaches in the control of *T. parva* cattle infections

The fatality of the tick-transmitted *Theileria parva* infections in cattle heavily impacts farmers in sub-Saharan Africa who depend on livestock for subsistence. Infection prevention by tick control relying on acaricides is expensive and unsustainable, while cattle vaccination by the "infection and treatment" is marred by 'breakthrough' infections in some areas within eastern Africa - where cattle share pasture grazing lands with wild Cape buffaloes. While it is known that buffalo-maintained parasites are diversely heterogeneous than cattle-maintained parasites, there is still a scarcity of parasite genotype data from wildlife-livestock interface areas. Moreover, a definitive genetic basis for differentiating cattle- and buffalo-derived parasites from such co-grazed areas is yet forthcoming. In addressing the two foregoing aspects, this thesis describes studies in which parasite genotypes based on the *T. parva* sporozoite surface antigen (*p67*) and the CD8+ antigen *Tp2* genes were compared between Tanzanian and Kenyan wildlife-livestock interface areas. In addition, heterogeneity patterns of thirteen full-length near-complete *T. parva* mitogenomes from East and Southern Africa *T. parva* strains were examined.

In the first set of analyses, *T. parva*-parasites antigen genes amplified from buffaloes, vaccinated and unvaccinated cattle from wildlife-livestock areas of northern Tanzania where vaccine 'breakthrough' have not been reported – despite the presence of Cape buffaloes, were compared against parasites in vaccinated cattle exposed to buffalo-derived *T. parva* challenge in central Kenya – where vaccine 'breakthroughs' have been reported. The analysis revealed additional epitope variants within the Tp2 antigen genes amplified from Tanzanian buffaloes, which adds to the evidence of parasite heterogeneity within the buffalo population. Moreover, a buffalo type p67 (allele 4), an allele reported in South Africa but is rare among East African isolates studied thus far, was identified in Tanzania, while *p67* alleles (2 and 3) that are presumptive buffalo origin were observed in Kenyan cattle.

In the second set of analyses, the heterogeneity patterns in 13 near-complete mitogenomes revealed seven haplotypes defined by nine SNPs that were initially parsimonious among transforming *Theileria*; *T. parva*, *T. annulata*, *T. taurotragi*, and *T. lestoquardi* mitogenomes, with the latter two sequenced in this study for the first time. All nine SNPs were synonymous except for two that cause amino acid substitutions within the *cytochrome b* gene of buffalo-derived strains. Notably, the buffalo-derived *T. parva* strains were clustered under one haplotype defined by the same nine SNPs. Further phylogenetic analyses were congruent with nuclear-encoded genes, which rules out possibilities of mitochondria specific inheritance patterns in the analysis.

Although preliminary, the totality of the findings in the *p67* and *Tp2* analyses suggests that whereas the live vaccine can protect against buffalo-derived *T. parva*, this may be dependent, among other factors, on the parasite infecting genotype. Thus, it is emphasized that a further understanding of *T. parva* strains diversity and its field population dynamics is key to targeted control of East Coast Fever (ECF) and the related *T. parva* infections. As such, the identified *T. parva* mitochondrial haplotypes from this study will be insightful in understanding *T. parva* epidemiology with important implications for control, prediction of target populations for vaccination, and characterization of breakthrough infections by buffalo *T. parva* in ITM vaccinated cattle.

Zusammenfassung

Abgrenzung der *Theileria parva*-Stammdiversität für gezielte und einheitliche Ansätze bei der Bekämpfung von *T. parva*-Infektionen bei Rindern

Die Tödlichkeit der von Zecken übertragenen *Theileria parva*-Infektionen bei Rindern hat schwere Auswirkungen auf Landwirte in Afrika südlich der Sahara, die für ihren Lebensunterhalt auf die Viehhaltung angewiesen sind. Die Vorbeugung von Infektionen durch Zeckenbekämpfung, die sich auf Akarizide stützt, ist teuer und nicht nachhaltig, während eine Immunprophylaxe ("infection and treatment method", ITM) in einigen Gebieten Ostafrikas, wo Hausrinder die Weideflächen mit wilden afrikanischen Büffeln teilen, durch "Durchbruchsinfektionen" beeinträchtigt wird. Obwohl bekannt ist, dass Parasiten, die von Büffeln übertragen werden, heterogener sind als Parasiten, die von Rindern übertragen werden, gibt es immer noch kaum Daten über den Genotyp von Parasiten aus Gebieten, die an der Schnittstelle zwischen Wildtieren und Nutztieren liegen. Eine eindeutige genetische Grundlage zur Unterscheidung von Rinder- und Büffelparasiten aus solchen Gebieten steht noch aus. In Hinblick auf die beiden vorgenannten Aspekte werden in dieser Dissertation Studien beschrieben, in denen Parasitengenotypen basierend auf den *T. parva*-Sporozoiten-Oberflächenantigen- (p67) und den CD8+-Antigen-*Tp2*-Genen zwischen tansanischen und kenianischen Wildtier-Vieh-Schnittstellengebieten verglichen wurden. Zusätzlich wurden die Heterogenitätsmuster von dreizehn nahezu vollständigen *T. parva*-Mitogenomen in voller Länge aus *T. parva*-Stämmen aus dem östlichen und südlichen Afrika untersucht.

In der ersten Analysenreihe wurden die *p67*- und *Tp2*-Gensequenzen von Büffeln, geimpften und ungeimpften Rindern aus Wild- und Viehbestandsgebieten im Norden Tansanias, wo trotz des Vorhandenseins von Kap-Büffeln von keinem "Impfdurchbruch" berichtet wurde, mit Gensequenzen von geimpften Rindern verglichen, die an von Büffeln stammenden *T. parva* in Zentralkenia exponiert waren, wo von einem "Impfdurchbruch" berichtet wurde. Zusätzliche Epitopvarianten innerhalb des *Tp2*-Antigen-Gens wurden bei tansanischen Büffeln identifiziert, was die Parasitenheterogenität innerhalb der Büffelpopulation weiter ergänzt. Außerdem wurde in Tansania ein Büffeltyp *p67* (Allel 4) identifiziert, was vorher in Südafrika gefunden wurde, das aber unter den bisher untersuchten ostafrikanischen Isolaten selten ist, während bei kenianischen Rindern nur *p67*-Allele (2 und 3) beobachtet wurden, die vermutlich von Büffeln stammen.

Bei der zweiten Analysenreihe ergaben die Heterogenitätsmuster in 13 nahezu vollständigen Mitogenomen sieben Haplotypen, die durch neun SNPs definiert wurden, die zunächst unter den transformierenden *Theileria* parsimonisch waren; *T. parva, T. annulata, T. taurotragi* und *T. lestoquardi* Mitogenome, wobei die beiden letzteren in dieser Studie zum ersten Mal

sequenziert wurden. Alle neun SNPs waren synonym, mit Ausnahme von zwei, die Aminosäure-Substitutionen innerhalb des cytochrom *b*-Gens der von Büffeln abstammenden Stämme verursachen. Bemerkenswert ist, dass die von Büffeln stammenden *T. parva*-Stämme unter einem Haplotyp geclustert wurden, der durch dieselben neun SNPs definiert ist. Weitere phylogenetische Analysen waren kongruent mit nuklearkodierten Genen, was die Möglichkeit von mitochondrienspezifischen Vererbungsmustern in der Analyse ausschließt.

Obwohl vorläufig, deutet die Gesamtheit der Befunde in den *p67*- und *Tp2*-Analysen darauf hin, dass der Lebendimpfstoff zwar gegen *T. parva* vom Büffel schützen kann, dies aber neben anderen Faktoren vom Genotyp des Parasiten abhängt, der ihn infiziert. Es wird daher betont, dass ein weiteres Verständnis der Diversität der *T. parva*-Stämme und ihrer Populationsdynamik im Feld der Schlüssel für eine gezielte Kontrolle des Ostküstenfiebers (ECF) und der damit verbundenen *T. parva*-Infektionen ist. Die in dieser Studie identifizierten mitochondrialen Haplotypen von *T. parva* sind aufschlussreich für das Verständnis der *T. parva*-Epidemiologie mit wichtigen Auswirkungen auf die Kontrolle, die Vorhersage von Zielpopulationen für die Impfung und die Charakterisierung von Durchbruchsinfektionen mit *T. parva* bei ITM-geimpften Rindern.

List of publications

1. **Mwamuye M M**, Obara I, Elati K, Odongo D, Bakheit M A, Jongejan F Nijhof A M (2020): Unique Mitochondrial Single Nucleotide Polymorphisms Demonstrate Resolution Potential to Discriminate *Theileria parva* Vaccine and Buffalo-Derived Strains. Life*,* 10(12):334.

2. **Mwamuye M M**, Odongo D, Kazungu Y, Kindoro F, Gwakisa P, Bishop R P, Nijhof A M, Obara I (2020): Variant analysis of the sporozoite surface antigen gene reveals that asymptomatic cattle from wildlife-livestock interface areas in northern Tanzania harbour buffalo-derived *T. parva*. Parasitol Res*, 119,* 3817–3828.

3. Bishop R P, Odongo D, Ahmed J, **Mwamuye M**, Fry L M, Knowles D P, Nanteza A, Lubega G, Gwakisa P, Clausen P H, Obara I (2020): A review of recent research on *Theileria parva*: Implications for the infection and treatment vaccination method for control of East Coast fever. Transbound Emerg Dis, 67 (S1): 56– 67.

4. **Mwamuye M M**, Kariuki E, Omondi D, Kabii J, Masiga D Villinger J (2017): Novel Rickettsia and emergent tick-borne pathogens: A molecular survey of ticks and tick-borne pathogens in Shimba Hills National Reserve, Kenya. Ticks Tick Borne Dis, 8(2) 208-218

5. Omondi D, Masiga D, Fielding B C, Kariuki E, Ajamma Y, **Mwamuye M M**, Ouso D O Villinger J (2017): Molecular detection of tick-borne pathogens in ticks from livestock and reptiles along the shores and adjacent islands of Lakes Baringo and Victoria, Kenya. Front vet sci, 4*,* 73.

Abstract presentations

1. **Mwamuye M M**, Obara I, Elati K, Odongo D, Bakheit M A, Jongejan F Nijhof A M (2020): Mitogenome Single Nucleotide Polymorphisms distinguish between buffalo and cattle transmissible *Theileria parva* strains. 14th International Symposium on Ticks and Tick-borne Diseases, 24–26 March 2021. Oral presentation abstract SK2-2.

2. **Mwamuye M M**, Odongo D, Kazungu Y, Kindoro F, Gwakisa P, Bishop R P, Nijhof AM, Obara I (2021): Sporozoite surface antigen (*p67*) gene analysis reveals a rare buffalo-derived *T. parva* allele in asymptomatic cattle from wildlife-livestock interface areas in northern Tanzania. 29th Annual Meeting of the German Society for Parasitology, 15-17 March 2021. Oral presentation abstract 0234.

3. **Mwamuye M M**, I Obara, Odongo D, Gwakisa P, Clausen P H, Nijhof A M, (2020): Evidence for diversifying selection within CD8+ T cell epitopes of the *Theileria parva* Tp2 antigen gene: Implications for the development of *T. parva* subunit vaccines. Süddeutscher Zeckenkongress, 02-04 März 2020. Oral presentation abstract 19.

Intellectual content disclosure

Prof. Dr. Ard Nijhof, Prof. Dr. Peter-Henning Clausen and Dr. Isaiah Obara were all involved in the study's conceptualisation. Micky M. Mwamuye designed the experiments and was responsible for the generation, curation, analysis and interpretation of the data that formed the basis for the peer-reviewed publications that constitute Chapters 3 & 4. Prof. Dr. Ard Nijhof and Dr. Isaiah Obara contributed to data analysis and interpretation. Micky M. Mwamuye drafted both manuscripts independently. All co-authors revised and approved the publication of the articles. The review article that appears in Chapter 2 was a synthesis of multiple authors on *T. parva* molecular epidemiology and its implication for control. The authors were part of a Deutsche Forshungsgemeinschaft (DFG) funded International Infectiology Network supporting live vaccination. The thesis research was embedded within this network framework, titled "Molecular epidemiology network for promotion and support of delivery of live vaccines against *Theileria parva* and *Theileria annulata* infection in Eastern and Northern Africa". Micky M. Mwamuye contributed to the review of literature and discussions relating to the spread and diversity of *T. parva* in the field, besides generating the map that appears in Fig. 2.1. The co-authors of the review article unanimously consented to its inclusion in this thesis.

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Statement of authorship

Unless otherwise stated in the text and acknowledgements, this thesis is my original work researched under the supervision and mentorship of Prof. Dr. Ard Nijhof, Prof. Dr. Peter-Henning Clausen and Dr. Isaiah Otieno Obara. All information sources have been duly cited, and no material in this thesis has been submitted for award of any academic qualification at any other university.

Micky Mwananje Mwamuye

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